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Membrane lipid composition and its effect on sodium pump molecular activity: a comparative study

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Membrane Lipid Composition and its Effect on Sodium Pump Molecular Activity: A Comparative Study

A thesis submitted in fulfilment of the requirements for the degree of

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by

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Department of Biomedical Science (Metabolic Research Centre)

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Certification

I, Nigel Turner, declare that this thesis, submitted in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD), in the Department of Biomedical Science, University of Wollongong, is wholly my original work unless otherwise referenced or acknowledged. All work contained in this thesis has not been submitted previously for any other degree or qualifications at any other academic institution.

Nigel Turner

23rd December 2003

Dedication

This thesis is dedicated to my parents and my beautiful wife Joanne. Their endless love and support has provided me with the strength and determination to fulfil my ambitions and achieve my very best.

Abstract

The basal metabolic rate (BMR) or energy turnover of animals varies dramatically, being several fold higher in endotherms compared to ectotherms, and much greater, on a mass-specific basis, in smaller vertebrates compared to larger vertebrates. Despite this large variation in metabolic rate between vertebrate species, a significant and relatively constant proportion of metabolism is associated with membrane-linked energy consuming processes, regardless of the absolute level of BMR. The majority of these membrane-linked processes are mediated by membrane-bound enzymes, and in general, membranes with increased levels of polyunsaturation are associated with elevated activity in these processes and subsequently increased metabolism. It has therefore been suggested that membranes, specifically their amount and composition, may be playing a role in determining the pace (or rate) of metabolism, via an effect on the molecular activity of membrane-bound proteins.

In this thesis, the relationship between membrane lipid composition and molecular activity of the sodium pump (Na^+K^+ATPase) has been assessed via comparisons of a large range of different animals. The sodium pump was chosen as a representative protein, as it is ubiquitous, has been well characterised, and accounts for a major proportion of resting energy metabolism (20%). Furthermore recent comparisons of endotherms and ectotherms, have suggested that the molecular activity of the sodium pump is highly dependent on the lipid composition of the surrounding membrane bilayer.

The aims of this study were firstly to examine sodium pump molecular activity and membrane lipid composition in tissues of mammals and birds of different body size. The mammalian species examined ranged in body mass 7500-fold and had an 11-fold difference in mass-specific BMR, while the avian species ranged 3000-fold and 22-fold for body mass and mass-specific BMR respectively. These species were chosen to try and maximise metabolic differences, and represented an ideal model to examine whether variations in membrane lipid composition may have been determining the

metabolic activity of these vertebrates, through an effect on membrane-bound proteins (i.e. sodium pump).

The second major aim of this study was to examine sodium pump molecular activity and membrane lipid composition in tissues from two ectothermic species, the bearded dragon lizard, and the octopus. These species were chosen as the bearded dragon has been shown to have very monounsaturated membranes, which are typical of ectotherms, while membranes from the octopus tend to be very polyunsaturated. Sodium pump molecular activity has been shown to be low in a large range of ectotherms and this study was designed to examine whether the high level of polyunsaturation in octopus membranes was associated with an increased molecular activity in their sodium pumps.

In the mammals and birds membrane fatty acid composition showed substantial variation, with higher unsaturation index (number of double bonds per 100 fatty acid chains) observed in heart and kidney phospholipids from the smaller species. In these tissues the most important finding was the significant and substantial allometric decline observed in the content of the highly polyunsaturated docosahexaenoic acid (22:6 *n*-3). Brain phospholipids from the mammals and birds however, showed no allometric trends and were highly polyunsaturated (especially 22:6 *n*-3) in all species. Sodium pump molecular activity was generally higher in all three tissues from the smaller mammals, while in the birds higher molecular activity was observed in the hearts of smaller birds, while no allometric trends were seen in the kidney and brain.

There were no differences observed in sodium pump molecular activity between the tissues of the bearded dragon and the octopus, in spite of the fact that the octopus had very polyunsaturated membranes. A significant contributing factor however, may have been that the octopus membranes contained high levels of cholesterol, which has been shown to have an inhibitory effect on the sodium pump.

To determine the relationship between sodium pump molecular activity and membrane lipid composition, linear correlation coefficients were determined between lipid

parameters and sodium pump molecular activity, using a combined data set from all fifteen species and six tissues examined in the current study, plus literature values for tissues from the ectothermic cane toad. While several lipid parameters were correlated with molecular activity, the most significant relationship was observed for docosahexaenoic acid (22:6 *n*-3), with high concentrations of this fatty acid associated with high sodium pump molecular activity. This fatty acid provided not only the strongest correlation for the combined data set, but was also significantly correlated within the mammals, birds and ectotherms, indicating that it may potentially play an important role in metabolism. Further research is required to determine the mechanistic basis of this relationship, however the physical properties of this fatty acid may be a major contributing factor.

Acknowledgements

I would like to begin by thanking my two supervisors Assoc. Prof. Paul Else and Assoc. Prof. Tony Hulbert. The research documented in this thesis, has only been possible because of their guidance and financial support. I would like to thank them for their critical appraisal and insightful suggestions during the writing of this thesis, and I would also like to extend my gratitude to them for the learning opportunities they have provided for me, in particular encouraging me to attend a number of conferences. Their open-minded approach to science has not only taught me a great deal in the last few years, but has inspired me to pursue a career in research. Finally on a personal note I would like to say that it has been an honour to be their student and I am now very proud to call them my friends.

I would like to extend my thanks to all the general and academic staff in the Department of Biomedical Science. They have provided a very pleasant environment for me to complete my studies and also provided the opportunity for me to gain valuable teaching experience. In particular I would like to thank Nola Hurt, Marion Harvey and Roza Dimeska, who were always there for me when I required administrative assistance, and whose help was greatly appreciated.

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List of Commonly Used Abbreviations

ATP	Adenosine Triphosphate
BMR	Basal Metabolic Rate
Chol	Cholesterol
CL	Average Fatty Acid Chain Length
DOC	Sodium Deoxycholate
EDTA	Ethylenediamine-tetracetic Acid
MUFA	Monounsaturated Fatty Acids
Pi	Inorganic Phosphate
PL	Phospholipid
PUFA	Polyunsaturated Fatty Acids
SEM	Standard Error of Mean
UI	Unsaturation Index
Wet wt	Wet Weight

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Table of Contents

Chapter I "Introduction"

Chapter II "Methods"

Chapter VI "Sodium Pump Molecular Activity and Membrane Lipid Composition in Tissues of the Octopus and Bearded Dragon Lizard"

Chapter VII "Summary and Conclusions"

Chapter VIII "Cited References"

List of Tables

List of Figures

Chapter I

Introduction

1.1 INTRODUCTION

The metabolic rate or energy turnover of animals varies considerably. Substantial metabolic variation has been demonstrated in comparisons of endotherms and ectotherms (Hemmingsen, 1960; Hulbert, 1980), during developmental periods (Adolph, 1983; Bastin et al., 1988; Else, 1991), and in comparisons of animals of different body size, where mass-specific metabolic rates can vary greater than 100-fold (Brody, 1945; Kleiber, 1961; Lasiewski and Dawson, 1967; Lasiewski and Calder, 1971). Although this large variability in metabolic rate between vertebrate species exists, a significant and relatively constant proportion of metabolism is associated with membrane-linked energy consuming processes, such as the maintenance of transmembrane ion gradients (eg H^+ and Na^+) (Rolfe and Brown, 1997). In general, membranes with increased levels of polyunsaturation are associated with elevated activity in these processes and subsequently increased metabolism (Hulbert and Else, 1989; Couture and Hulbert, 1995a; Porter et al., 1996). Hulbert and Else (1999) therefore recently suggested that membranes, specifically their amount and composition, may play a role in determining the pace (or rate) of metabolism, via an effect on the molecular activity (or molar activity, i.e. rate of substrate turnover) of membrane proteins.

The 'membrane pacemaker' hypothesis was the major stimulus for the experimental work conducted in the current thesis. Using a comparative approach, sodium pump molecular activity and membrane lipid composition were examined in animals with a diverse range of metabolic rates. The sodium pump (Na^+K^+ATPase) was chosen as a representative protein as it is ubiquitous, underpins many important cellular functions (Jørgensen, 1980; Skou, 1988), and accounts for a significant component of basal metabolism (Clausen et al., 1991). Enzyme activity was measured as the molecular activity of individual proteins, as the advantage of using this measurement, is that it represents the turnover rate of substrate by individual enzymes, and therefore allows comparison of intrinsic enzyme activity between different tissues and species where the concentration of enzyme may vary considerably, thus maximising the comparative

approach. This chapter begins with a brief background on metabolic rate and describes how interactions between membrane lipids and membrane proteins may be an important factor in the determination of the rate of metabolic activity.

1.2 Metabolic Rate

1.2.1 BMR and Other Metabolic Rates

The metabolic rate of an animal can be considered the rate of energy use associated with the maintenance of body processes and the performance of work. Metabolic rate is often measured as basal or standard metabolic rate (BMR or SMR), which is the energy expenditure of a resting, postabsorptive adult in a thermoneutral environment or at a fixed normal preferred body temperature. This measurement represents the minimal energy required to maintain normal function, and in endotherms it is the energy turnover at normal body temperature, while in ectotherms the temperature at which measurements were conducted needs to be specified. While BMR varies substantially between different animals (Hemmingsen, 1960; Kleiber, 1961), two of its major determinants appear to be the body mass and the phylogenetic group to which an animal belongs. It is possible to mathematically predict the BMR of an animal if these two factors are known.

The variation of BMR with body size has been most extensively studied in mammals, where it is well established that BMR is proportional to a power function of body mass (i.e. M^b) (Brody, 1945; Kleiber, 1961). Numerous studies have examined this relationship with the value of the allometric exponent 'b', generally approximating 0.75 (Withers, 1992). An exponent of 0.75 means that for every 100% increase in body mass BMR will only increase 68%, however when considered on a mass-specific basis (i.e. $M^{-0.25}$), metabolic intensity is higher in smaller mammals. Perhaps this relationship can best be appreciated by comparing a 30g mouse and a 300kg cow. In absolute terms the BMR of the cow is 1,000 times that of the mouse, but when considered on a mass specific basis the mouse has a BMR that is 10 times greater than that of the cow. The mechanistic basis for this allometric variation in BMR is not fully understood, however

it appears to be a general phenomenon in most animal groups (for a compilation see Peters, 1983).

As well as the large differences observed in BMR of different sized animals, quantitatively BMR can vary greatly between different phylogenetic groups. For example although they have the same allometric exponents, the BMR of mammals and birds is four to ten times higher than that of ectothermic vertebrates (reptiles, amphibians, fish) of the same size and body temperature (Hemmingsen, 1960; Hulbert, 1980).

BMR represents the energy cost at rest, while the energy cost of normal activity is represented by the field metabolic rate (FMR), which is generally derived using doubly-labelled water turnover. FMR has been determined in a large range of free-living mammals, birds, and reptiles and is also allometrically related to body mass (Nagy et al., 1999). The allometric exponents for FMR determined in the review by Nagy et al. (1999) are 0.73 for mammals, 0.68 for birds and 0.79 for Iguanid lizards. The similarity of the FMR and BMR exponents indicates that in a number of groups, BMR represents a fairly constant fraction (25-40%) of FMR regardless of species size.

1.2.2 Composition of BMR at the Cellular Level

An interesting finding that comes from the literature, is that despite there being such large variation in BMR, the percentage contribution of various processes appears to be relatively constant regardless of the actual level of BMR. This is evidenced by the fact that a large number of physiological processes in mammals, including cardiac output and glomerular filtration rate, display similar allometric variation to BMR (Peters, 1983). Urinary excretion of endogenous nitrogen and neutral sulfur by mammals have allometric exponents of 0.72 and 0.74 respectively (Brody, 1945). Whole body turnover rates of r-RNA, t-RNA, m-RNA all have exponents close to 0.75 (Schoch et al., 1990), while an exponent of 0.72 has been measured for whole body turnover rates

of protein (Waterlow, 1984). These studies therefore indicate that many cellular processes represent a constant proportion of BMR at the whole body level.

Whole body BMR represents the sum of the energy expenditure of the metabolically active organs. During resting conditions the internal organs (kidney, splanchnic organs, heart, lungs, and brain), account for a large proportion of BMR (~70%), despite the fact that they represent less than 10% of total body mass (Aschoff et al., 1971; Rolfe and Brown, 1997). Part of the scaling of BMR can be accounted for by the allometric changes in the size of these organs. Some tissues represent a relatively constant proportion of body mass (eg lungs and skeletal muscle), while other tissues (eg heart, liver, kidney and brain) are relatively larger in smaller mammals and birds and thus contribute to their higher mass-specific BMR (Grubb, 1983; Peters, 1983; Else and Hulbert, 1985).

The scaling of BMR however cannot be fully explained by allometric changes in organ size, with differences in the mass-specific metabolic rate of tissues accounting for the remaining proportion. Tissue slices (liver, kidney, brain, spleen, and lung) from smaller mammals have an increased mass-specific metabolic rate compared to slices from larger species (Krebs, 1950; Couture and Hulbert, 1995b). Porter and Brand (1995) also showed similar allometric trends in a comparison of the mass-specific metabolic rate of isolated hepatocytes in mammals ranging from mice to horses. As well as measuring mass-specific metabolic rate in mammalian hepatocytes, Porter and Brand (1995) also determined the oxygen consumption associated with (a) non-mitochondrial processes, (b) that used to counteract mitochondrial proton leak, and (c) that used to drive the phosphorylation system. They found that all of these processes varied with allometric exponents similar to that for total oxygen consumption. The contribution of these processes was relatively similar regardless of the total hepatocyte metabolic rate, and averaged 13%, 19%, and 68% for non-mitochondrial processes, mitochondrial proton leak, and mitochondrial phosphorylation respectively. These same processes have recently been measured in isolated hepatocytes from the other major endothermic

group, namely birds, with the same allometric trends observed (Else, P.L., Brand, M.D., Turner, N. & Hulbert, A.J. unpublished observations).

Similar to the allometric variation of BMR, the approximately eightfold difference in BMR between mammals and ectothermic reptiles, also appears to result from differences in both organ size and mass-specific tissue metabolic rate (Else and Hulbert, 1981; Hulbert and Else, 1981; Else and Hulbert, 1987; Hulbert and Else, 1989). Comparison of hepatocytes in mammals and reptiles indicates that despite a 4-fold difference in total oxygen consumption, a similar percentage of hepatocyte respiration is used to drive non-mitochondrial processes, mitochondrial proton leak, and mitochondrial phosphorylation (Brand et al., 1991). Furthermore the percentage contribution of these processes is similar to those noted above for mammals of different body size. Collectively these results indicate that irrespective of the absolute level of BMR, there is a consistent relative contribution from the major energy-consuming processes.

In a recent review, Rolfe and Brown (1997) determined the quantitative contribution of various processes to the BMR of mammals (see Fig. 1.1). When the contributions from various tissues were summed, it was estimated that ~10% of mammalian BMR is non-mitochondrial oxygen consumption. Of the 90% of mammalian oxygen consumption that is used by the mitochondria, ~20% is uncoupled by the mitochondrial proton leak, while the other 80% is coupled to ATP synthesis. Of this ATP consumption the estimated contribution of various processes is as follows: ~28% is used by protein synthesis, 19-28% by the Na^+/K^+-ATPase, 4-8% by the Ca^{2+}-ATPase, 2-8% by actinomyosin ATPase, 7-10% by gluconeogenesis, 3% by ureagenesis, with other processes including mRNA synthesis and substrate cycling contributing the remainder. While these values have been estimated for mammals they may be applicable to most other species. For example, the sodium pump accounts for a similar proportion of tissue metabolism in mammals, reptiles, fish, amphibians and birds (Hulbert and Else, 1981; Hulbert and Else, 1990).

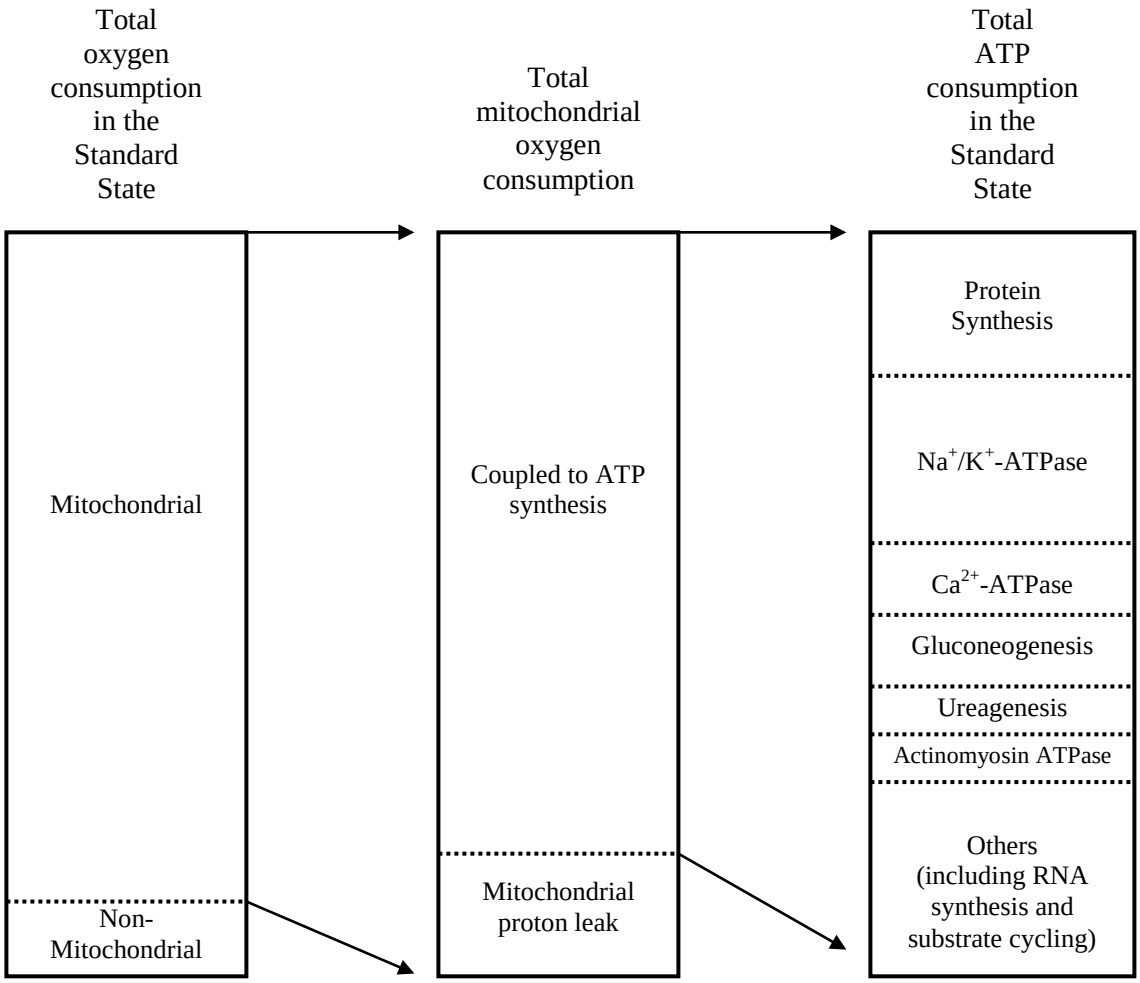


Fig. 1.1 *Estimated contribution of processes to energy utilisation in the standard state. First column shows the contribution of mitochondrial (90%) and non-mitochondrial (10%) oxygen consumption to total respiration rate. Second column shows the proportion of mitochondrial respiration used to drive ATP synthesis (80%) and proton leak (20%) in the standard state. Third column represents contribution of ATP-consuming processes to ATP consumption in the standard state. Of this ATP production, ~28% is used by protein synthesis, 19-28% by the Na^+/K^+-ATPase, 4-8% by the Ca^{2+}-ATPase, 2-8% by actinomyosin ATPase, 7-10% by gluconeogenesis, 3% by ureagenesis, with other processes including mRNA synthesis and substrate cycling contributing the remainder. Adapted from Rolfe & Brown (1997).*

1.2.3 Membrane Pacemaker Theory

Overall, these allometric and phylogenetic comparisons suggest that BMR is composed of a series of linked processes, and when variation in BMR occurs, it is not the result of

changes in a single (or a few) process, but represents the proportional variation of all processes. This potentially indicates that there may be a common factor that influences the activity all of these processes. It is clear that a substantial portion of BMR is energy used by membrane-linked processes (i.e. mitochondrial proton leak and maintenance of transmembrane ion gradients, as well as parts of protein synthesis and non-mitochondrial oxygen consumption) and recently Hulbert and Else (1999) proposed that membranes, specifically the amount and composition of the membrane bilayer, may be the common link, acting as a pacemaker of metabolism.

1.3 Cell Membranes and BMR

Cell membranes have been described as an "eternal structure" of living organisms (Hulbert and Else, 1999) and their importance to cellular function is well established. Membranes separate cells from their extracellular environment, establish specialised compartments within cells and also provide a site for numerous important physiological processes including ion and metabolite transport, enzyme reactions, immune functions and signal transduction.

1.3.1 Membrane Lipids

Membranes are bilayer assemblies of proteins and lipids held together by non-covalent bonds. Membranes are not uniform and the relative amount of protein and lipid varies dramatically, ranging from 20% (dry weight) protein in myelin up to 80% protein in mitochondria (Gurr and Harwood, 1991). The lipids in membranes are all amphipathic, and include phospholipids, glycolipids, and sterols.

Phospholipids are the most abundant lipid present in biological membranes and are composed of a polar "headgroup" region, with two hydrophobic fatty acyl chains. Structurally they are based on a three-carbon glycerol (phosphoglycerides) or sphingosine (sphingolipids) backbone. Phosphoglycerides are the major phospholipids in membranes and have fatty acyl chains esterified at the first two carbons on the glycerol backbone, termed the *sn*-1 and *sn*-2 position respectively (Fig. 1.2). Attached

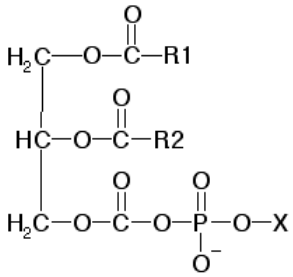


Fig. 1.2 *The general structure of a phospholipid molecule. Fatty acyl chains attach at R1 and R2, while X represents the position where organic bases, amino acids, and alcohols attach. Adapted from Stryer (1995).*

to the third carbon (*sn*-3), via a phosphate (PO_4) group, is one of a range of molecules including organic bases, amino acids, and alcohols, that comprise the headgroup portion of the phospholipid (Fig. 1.2). The major phosphoglycerides found in eukaryotic membranes include phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. Diphosphatidylglycerol (cardiolipin) and sphingomyelin are also important constituents of some membranes in animals.

The fatty acyl chains form the hydrophobic portion of the phospholipid (and glycolipid) molecules, and in vertebrate membranes are generally between 16 and 22 carbons long, with an average chain length of about 18 carbons (Gurr and Harwood, 1991). These acyl chains are referred to as saturated if there are only single bonds between carbons, with the single bond allowing rotation around the C-C unit. Many acyl chains that occur naturally in membranes however, contain methylene-interrupted double bonds in the *cis* conformation. Such acyl chains are described as monounsaturated if they contain a single C=C unit, or polyunsaturated if they contain multiple C=C units. The natural occurrence of *cis* rather than *trans* C=C bonds has important implications for the physical properties of membranes as it provides a bend or kink in the chain and thus affects several aspects of lipid behaviour. For example, phosphatidylcholine molecules with 18:0 acyl chains in both the *sn*-1 and *sn*-2 positions have a melting point of 55^{o}C and thus will be solid at mammalian body temperatures. If 18:1 (*n*-9) is substituted for

the *sn*-2 18:0 chain, a liquid crystalline state would be maintained to ~1^{o}C, and if this residue is further changed to 18:2 (*n*-6), it will maintain the liquid crystalline state to approximately -15^{o}C (Lee, 1991).

As well as the influence of C=C units on phase transition behaviour, the position and number of double bonds also have major effects on the behaviour of fatty acyl chains in the membrane. For example, 22:6(*n*-3) is the longest and most polyunsaturated acyl chain generally found in animal membranes and in recent molecular dynamics simulations it has been shown that the presence of such a large number of double bonds actually bestows incredible conformational flexibility on this molecule (Feller et al., 2002; Huber et al., 2002), such that it can be envisaged as thrashing about in the hydrocarbon core of the membrane. In terms of the effect of positional isomers, if an average fatty acid of 18 carbons is considered, an *n*-3 fatty acid will have C=C units throughout the whole length of the bilayer, while an *n*–6 fatty acid will be lacking C=C units in the middle third of the membrane. This difference in the location of double bonds has a marked effect on the behaviour of phospholipid molecules, affecting properties such as the permeability of vesicles and condensability of monolayers by cholesterol (Stillwell et al., 1994).

Other important membrane lipids are cholesterol and glycolipids. Cholesterol is the major sterol found in most animal membranes and is composed of four fused cyclohexane rings with a polar head group (the hydroxyl group) and a non-polar hydrocarbon body (Fig. 1.3). Cholesterol is concentrated in the plasma membrane (Colbeau et al., 1971; Fiehn and Peter, 1971), and orients itself perpendicular to the phospholipids in the cavities that form between phospholipid molecules due to the *cis* double bonds of the acyl chains (Bretscher and Munro, 1993). To achieve this orientation, the hydroxyl group of the cholesterol molecule is bound to the phospholipid headgroup by a hydrogen bond, while the hydrocarbon tail is located in the hydrophobic core of the bilayer (Gurr and Harwood, 1991), giving the cholesterol an appearance that has been described as “chair-like” (see Fig. 1.3). One of the

proposed reasons for this distribution is to limit the movement of small molecules across the bilayer (Bretscher and Munro, 1993). The structure of glycolipids is analogous to that of phosphoglycerides, with a sugar molecule attached to the *sn*-3 position in place of a phospholipid headgroup. These lipids are the main constituents of photosynthetic membranes in algae and plants, but only make up a small proportion of membrane lipids in animals (Gurr and Harwood, 1991).

1.3.2 The Fluid Membrane Bilayer

In 1972 the "fluid mosaic" model of membrane structure was proposed (Singer and Nicolson, 1972), and although it has been modified and extended, it still forms the basis for much of the modern understanding of cellular membranes (Fig. 1.4). In this

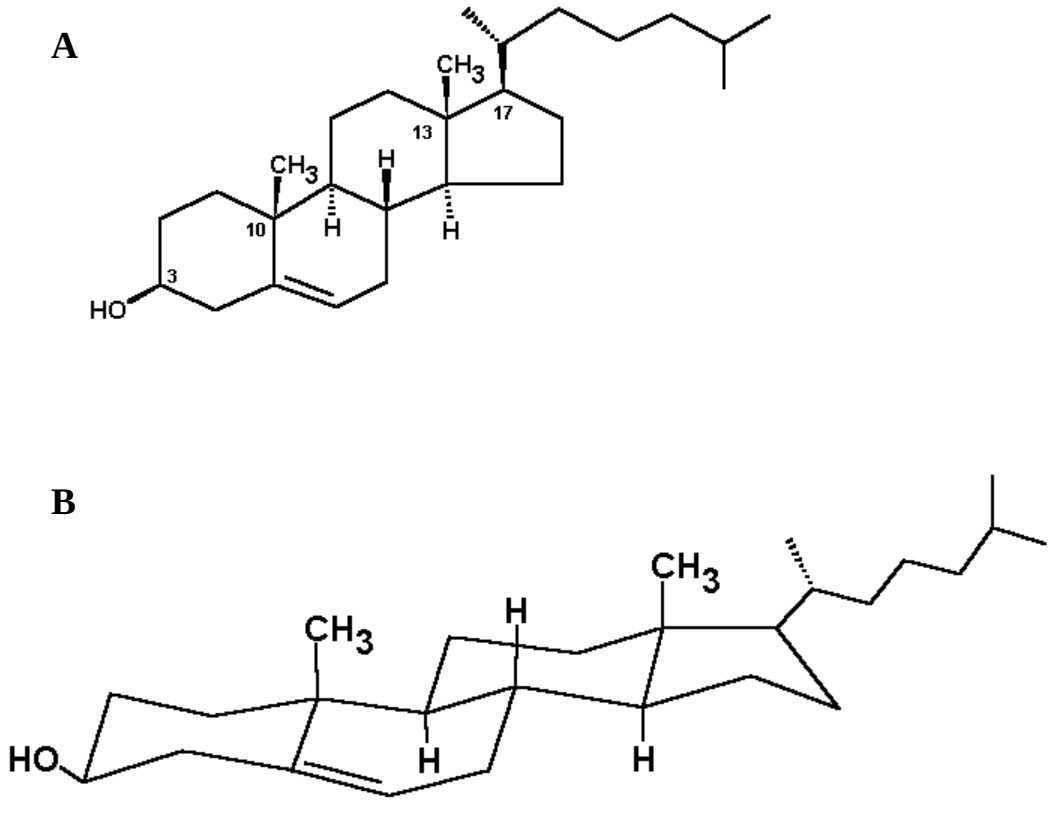


Fig. 1.3 *The molecular structure of cholesterol (A) along with the "chair-like" conformation it adopts in biological membranes (B). Adapted from Stryer (1995).*

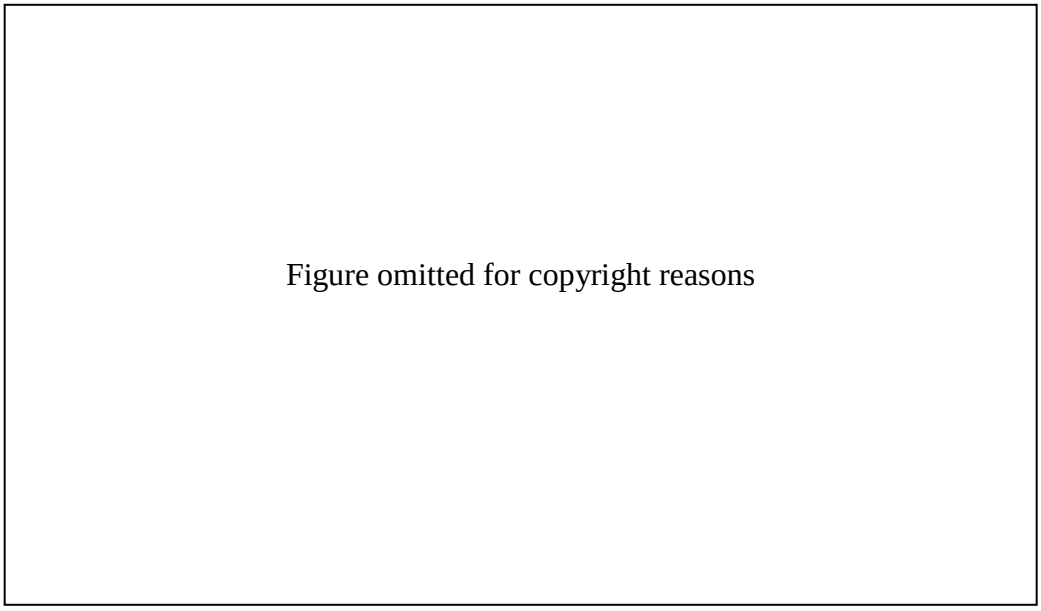


Fig 1.4 *The fluid mosaic model of membrane structure (Campbell et al., 1999).*

model, the membrane is portrayed as a fluid, asymmetric lipid bilayer with proteins either spanning the membrane or embedded into the hydrophobic core. The membrane proteins and lipids are free to diffuse laterally, however “flip-flop” movements from one side of the membrane to the other are restricted.

While the membrane is portrayed as a fluid bilayer, the term “fluidity”, which is commonly used to describe membranes, is an oversimplified term that is often misleading. Essentially fluidity refers to the reciprocal of membrane viscosity and in biological membranes, it is dependant on a number of factors, including the lateral and rotational movement of whole lipid molecules, rotation around C-C bonds, movements along the hydrocarbon chain, and oscillations of molecules between “tall and thin” and “short and wide” conformations (for discussion see Hulbert and Else, 1999). These

dynamic processes are influenced by the length and degree of unsaturation in fatty acyl chains, along with the ratio of fatty acids to cholesterol and other sterols. For example, as mentioned above, polyunsaturated fatty acids such as 22:6(*n*-3) are highly flexible (Feller et al., 2002; Huber et al., 2002), and thus will have a dramatic influence on lipid motions and in turn membrane fluidity. Quite often however, as is the case with temperature acclimation, too much emphasis is placed on the role of polyunsaturates in maintaining fluidity, as changes in monounsaturates are sufficient to maintain a fluid bilayer (see Hazel, 1995). With regards to cholesterol, it is positioned in the membrane such that its bulky fused ring structures act to sterically block the large motions of the fatty acyl chains. As a result it has a condensing or rigidifying effect, and interacts with many phospholipids to reduce the mean area per molecule (Demel et al., 1972). To achieve this condensation effect however, a saturated acyl chain is required (Demel et al., 1972; Holte et al., 1996) as cholesterol is very insoluble in polyunsaturated oils (Brzustowicz et al., 2002a; Brzustowicz et al., 2002b).

Animal membranes are mixtures of phospholipids, and as such they have the potential to "gel" out at different temperatures, resulting in phase separation in the membrane where parts of the membrane remain in the liquid crystalline phase, while other areas are more solid gel patches (Schroeder et al., 1998). Many natural membranes try to limit this situation by limiting the saturated acyl content of membranes to less than 50% and by "handcuffing" an unsaturated acyl residue to every saturated acyl residue (Hulbert and Else, 1999). Phospholipids will generally always have an unsaturated acyl chain at the *sn*-2 position, while the *sn*-1 position can be occupied by either a saturate or an unsaturate, depending on the particular membrane. For example, muscle mitochondrial phospholipids from cold-water fish can have up to ~ 90% of their fatty acyl chains as unsaturates, with 59% being the highly unsaturated 22:6(*n*-3) (Guderley et al., 1997). In many natural membranes however, the formation of distinct patches of predominantly cholesterol and sphingomyelins, termed lipid rafts, appears to be an important functional requirement (Simons and Ikonen, 1997; Tsui-Pierchala et al., 2002).

Lipids are distributed asymmetrically, such that all naturally-occurring membranes exhibit a distinct sidedness, i.e. the exofacial and cytofacial leaflets of the membrane bilayer are different. This relationship has been most extensively studied in human erythrocytes, and it has been found that phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol are primarily located in the cytoplasmic leaflet of the bilayer, while phosphatidylcholine and sphingomyelin are located in the exofacial leaflet (Rothman and Lenard, 1977). The reason for this membrane asymmetry appears to be related to factors such as differences in the radius of curvature of the bilayer leaflets (Bergelson and Barsukov, 1977), and the preferential localisation of particular phospholipids involved in intracellular metabolic pathways (eg phosphatidylinositol) (Gurr and Harwood, 1991).

1.3.3 Regulation of Membrane Lipid Composition

Biological membranes are extremely diverse and contain hundreds of phospholipid molecular species (Lee et al., 1994; Dowhan, 1997). Numerous factors, including diet (Pan and Storlien, 1993), exercise (Andersson et al., 1998), temperature (Hazel, 1979), and age (Ando et al., 2002), can influence membrane composition, however it is becoming evident that membranes are regulated in a homeostatic fashion to maintain a relatively constant physical state. This regulation is termed homeoviscous adaptation (Hazel and Williams, 1990; Hazel, 1995), and appears to be largely mediated by changes in acyl composition, as relatively smaller changes are generally observed in other membrane lipid components (eg phospholipid headgroup and cholesterol) (Hazel, 1979; Robertson and Hazel, 1995).

The regulatory systems that maintain membrane acyl composition, consist of a number of enzymes that work in concert to effect the necessary changes. In rat hepatocytes, only four phospholipid molecular species (of PC and PE) are synthesised *de novo* (Schmid et al., 1995). These are 16:0/18:2(*n*-6), 16:0/18:1(*n*-9), 16:0/22:6(*n*-3) and 18:1(*n*-9)/18:2(*n*-6), with all other molecular species resulting from remodelling of

these initial four species (Schmid et al., 1995). Such remodelling is achieved by deacylation/reacylation processes that occur through the combined action of acyltransferases, transacylases and phospholipases (Yamashita et al., 1997; Farooqui et al., 2000). *In vivo* incorporation of labelled acyl chains indicates that saturated acyl chains are incorporated from *de novo* synthesis, while polyunsaturated acyl chains are largely introduced via deacylation/reacylation systems (Valtersson et al., 1986). These changes are rapid, and in cultured cells appear within 2-10 min of labelled fatty acids being added to the culture medium (Chakravarthy et al., 1986).

The other major enzymes that modify membrane composition are desaturase and elongase enzyme systems (Stubbs and Smith, 1984). Vertebrates are only able to synthesise saturated and monounsaturated fatty acids *de novo* and must obtain *n*-6 and *n*-3 PUFAs from dietary sources or intestinal biota (assuming they are capable of synthesising them). Once obtained, the elongase and desaturase enzymes then appropriately modify the PUFA, through the addition of carbons and the introduction of double bonds at specific sites respectively. The desaturase enzymes are membrane-bound, multi-unit enzymes that consume oxygen (Pugh and Kates, 1979), and have the following preference for fatty acids: *n*-3>*n*-6>*n*-9 (Stubbs and Smith, 1984). Their activity is inversely related to fluidity and is thus increased in situations where there is a decreased fluidity (increased viscosity) in membranes (Kates et al., 1984), such as due to temperature, increased incorporation of saturated fatty acids, or increased incorporation of sterols. It is interesting that oxygen consumption by desaturases, is part of non-mitochondrial consumption by cells, and that in hepatocytes, non-mitochondrial oxygen consumption is highest in small mammals (Porter and Brand, 1995) where the level of membrane PUFA is greatest (Hulbert et al., 2002c).

Membrane acyl composition is therefore genetically determined, with the genome establishing the specific lipid composition of the membrane by determining the characteristics of the various enzymes mentioned above. Environmental factors can have some influence however, and there will be a degree of genome-diet interaction as

the enzymes can only modify the PUFAs that are obtained by the animal from their external environment.

1.3.4 Variation of Membrane Lipid Composition with BMR

In comparisons of metabolically diverse species, a consistent finding is that membrane fatty acid composition varies in a similar fashion to BMR. For example, relationships have been reported between body mass and phospholipid fatty acid composition in different tissues (skeletal muscle, liver, heart and kidney) of mammals (Couture and Hulbert, 1995a; Hulbert et al., 2002c), in mammalian mitochondria (Porter et al., 1996; Portero-Otín et al., 2001), and in bird skeletal muscle (Hulbert et al., 2002b) and liver mitochondria (Brand et al., 2003). These allometric comparisons suggest that in general, the more metabolically active smaller mammals and birds have increased levels of membrane PUFA (especially 22:6(*n*-3)), and decreased levels of MUFA compared to their larger counterparts. Such a relationship is also evident in comparisons of mammals and ectotherms, with phospholipids from the metabolically active rat being more polyunsaturated than those from the bearded dragon lizard or cane toad (Hulbert and Else, 1989; Brand et al., 1991; Else and Wu, 1999).

1.3.5 Summary

Membranes are assemblies of proteins and lipid bilayers, which perform numerous cellular functions in the body. Membrane lipid composition is regulated in a homeostatic fashion, with the phospholipid fatty acyl chains being the major site of regulation. Membranes are remodelled by a number of enzyme systems and show consistent variation with regards to BMR. In general an increased BMR is associated with more polyunsaturated membranes, while animals with a reduced BMR have greater levels of monounsaturates in their membranes.

1.4 The Sodium Pump

The sodium pump (Na^+K^+ATPase) is a membrane-bound enzyme that is member of the P-Type ATPase family, and is a ubiquitous component of all animal cells. The sodium pump hydrolyses one molecule of ATP and uses this chemical energy to couple the efflux of three Na^+ ions out of and the influx of two K^+ ions into cells against their electrochemical gradients (Skou, 1988). Physiologically the sodium pump is involved in the maintenance of the transmembrane gradient for Na^+, which is important for numerous cellular functions, such as the propagation of excitatory signals and secondary active transport via co-transport and counter-transport systems (Jørgensen, 1980; Skou, 1988).

1.4.1 Structure

The sodium pump is composed of three protein subunits, an alpha subunit, a beta subunit, and a third subunit (FXYD protein) that is a small transmembrane regulatory protein (Fig. 1.5).

The α-subunit

The α-subunit is a 112 kDa, multispanning membrane protein that contains the binding sites for the cations, ATP, and cardiac glycosides, and is responsible for the catalytic and transport properties of the enzyme (Blanco and Mercer, 1998). It is composed of 10 transmembrane helices and a large cytoplasmic domain (Hu and Kaplan, 2000), with the transport and enzymatic properties thought to be regulated in the intracellular loop between helix 4 and 5 (Kaplan, 2002). To date four isoforms of the α-subunit have been discovered (α_1, α_2, α_3, α_4) (Blanco and Mercer, 1998; Kaplan 2002). In terms of structure, there is high sequence homology amongst the different α isoforms (~85%) and an even higher homology when the same isoform is compared between different mammalian species (~93%) (Sweadner, 1989). It appears however, that as the evolutionary history of animals diverge, the homology between isoforms is reduced (Hou, X., Howard, C. and Else, P.L. unpublished observations). Functionally there are some small differences between the isoforms (eg differences in Na^+, K^+ and ouabain

affinity), however it is not known whether these are of significance (Lingrel, 1992). Subunit isoform expression is tissue-specific and also changes with age (Lingrel, 1992; Blanco and Mercer, 1998). The α_1 isoform is considered the "housekeeping" form of the sodium pump and functions in maintaining bulk Na^+ and K^+ levels. This isoform is expressed in virtually all cells, and is the major isoform in vertebrate kidney. Of the other isoforms α_2 predominates in adipocytes, heart, muscle, and brain, α_3 is abundant in the nervous system, and α_4 is present only in sperm and its precursor cells. These latter isoforms are thought to perform more specialised functions (eg Na^+/Ca^{2+} exchange) as they are localised in distinct regions of the cell, as opposed to the α_1 isoform that has a generalised distribution (Hundal et al., 1994; Juhaszova and Blaustein, 1997a; Juhaszova and Blaustein, 1997b).

The β-subunit

The β-subunit spans the membrane once and has a molecular mass of 40-60 kDa depending on the degree of glycosylation. The β-subunit is required for normal activity of the enzyme and has been described as a chaperone that facilitates the delivery and insertion of the α-subunit into the plasma membrane (Kaplan, 2002). Four isoforms of the β-subunit have also been discovered (β_1, β_2, β_3, β_4) with high homology (>90%) observed between mammalian species (Sweadner, 1989).

FXYD proteins

A family of seven proteins that share a common amino acid motif (FXYD) and include the γ-subunit, represent the third subunit of the sodium pump (Sweadner and Rael, 2000; Berribi-Bertrand et al., 2001). These proteins are differentially distributed in various tissues (Geering et al, 2003), and while their exact function is not well understood, they appear to play a regulatory role, affecting properties such as the affinity of the sodium pump for substrates and ions (Berribi-Bertrand et al., 2001; Therien et al., 2001; Geering et al., 2003)

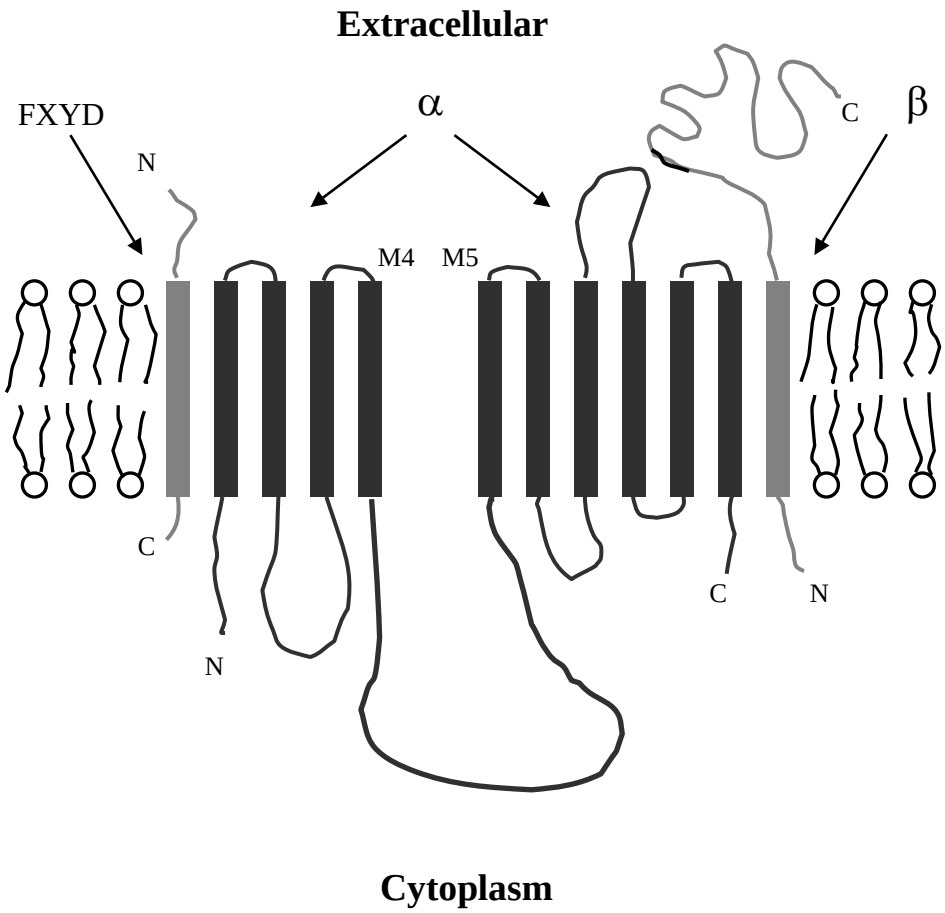


Fig. 1.5 *Diagrammatic representation of the sodium pump. The 3 subunits of the sodium pump (α, β and FXYD) are indicated along with their respective N terminus and C terminus. The large cytoplasmic loop between transmembrane segments 4 and 5 (M4 & M5), where the catalytic and transport activities of the sodium pump takes place is shown. The heavy line on the β subunit indicates the sequence involved in associations with the M7M8 loop of the α subunit.*

1.4.2 Reaction Cycle

The reaction mechanism of the sodium pump is characterised by the requirement for Na^+, ATP and Mg^{2+} on the cytoplasmic side and K^+ on the extracellular side (Skou, 1988). This catalytic cycle, shown if Fig. 1.6, involves the formation of a transient

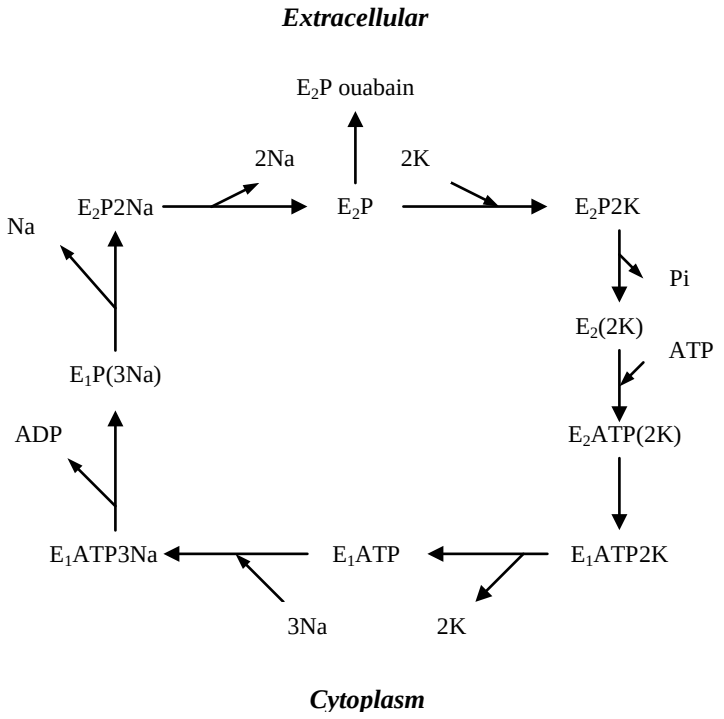


Fig. 1.6 *The Post-Albers scheme of the sodium pump reaction cycle under physiological conditions. E_1 and E_2 are the conformations of the enzyme with binding sites facing the cytoplasm and extracellular medium respectively. The occluded states, $E_1P(3Na)$, $E_2(2K)$ and $E_2ATP(2K)$ represent the conditions during which the ions are unable to bind with the aqueous phase. The state in which ouabain preferentially binds is indicated. Adapted from Kaplan (2002).*

phosphorylated intermediate at an aspartate residue, which is a common feature of all P-Type ATPases (Scarborough, 2002). The general features of the cycle are as follows: Three intracellular Na^+ ions bind to the E_1 form of the sodium pump and catalyse a phosphorylation by previously bound ATP (and Mg^{2+}). This produces the E_1P form of the protein and following the release of ADP, the Na^+ ions are now occluded (Kaplan, 2002). A conformational transition occurs, Na^+ ions leave the protein at the extracellular surface (the first one rapidly and the second and third more slowly), and

the protein is now in the E_2P form. Two K^+ ions bind at the outer surface and the ions dephosphorylate the enzyme (Pi is released at the inner surface), which occludes the K^+ ions and produces the $E_2(2K)$ form. Following this step ATP binds, which initiates a conformational transition from E_2 back to E_1, K^+ ions leave at the inner surface and the enzyme is ready to begin a new cycle.

1.4.3 Inhibitors

In 1953 it was observed that cardiac glycosides inhibited Na^+-K^+ transport (Schatzmann, 1953) and since this time they have been widely used to examine the presence and function of sodium pumps in a variety of preparations. Ouabain (Fig. 1.7) is the most water soluble of the naturally occurring glycosides and displays a near absolute specificity for Na^+K^+ATPase (Bonting, 1970). This high specificity has allowed the quantification of sodium pump number, by ^{3}H-ouabain binding, in a variety of different preparations, including intact tissue (Kjeldsen, 1986; Schmidt et al., 1990; Schmidt et al., 1992), crude homogenates (Merchant et al., 1985), microsomes (Harris et al., 1973; Chen and Lin-Shiau, 1986), and purified Na^+K^+ATPase preparations (Lane et al., 1973; Liang and Winter, 1976). Ouabain binds to the extracellular side of the Na^+K^+ATPase while it is in the E_2-P form (see Fig. 1.6). Certain ligands are required for ouabain binding to occur, with Mg^{2+} being a prerequisite (Albers et al., 1968). The binding rate is increased dramatically if the enzyme is phosphorylated by ATP or P_i, whereas internal Na^+ or K^+ or external K^+ are thought to inhibit binding (Wallick et al., 1980).

1.4.4 Sodium Pump and Metabolic Rate

As mentioned above, the sodium pump is a ubiquitous, membrane-bound enzyme, whose primary function is the maintenance of the sodium gradient. The existence of this concentration gradient is essential for normal cellular functions, providing a basis for things such as the propagation of excitatory signals in neurons and muscle cells, and

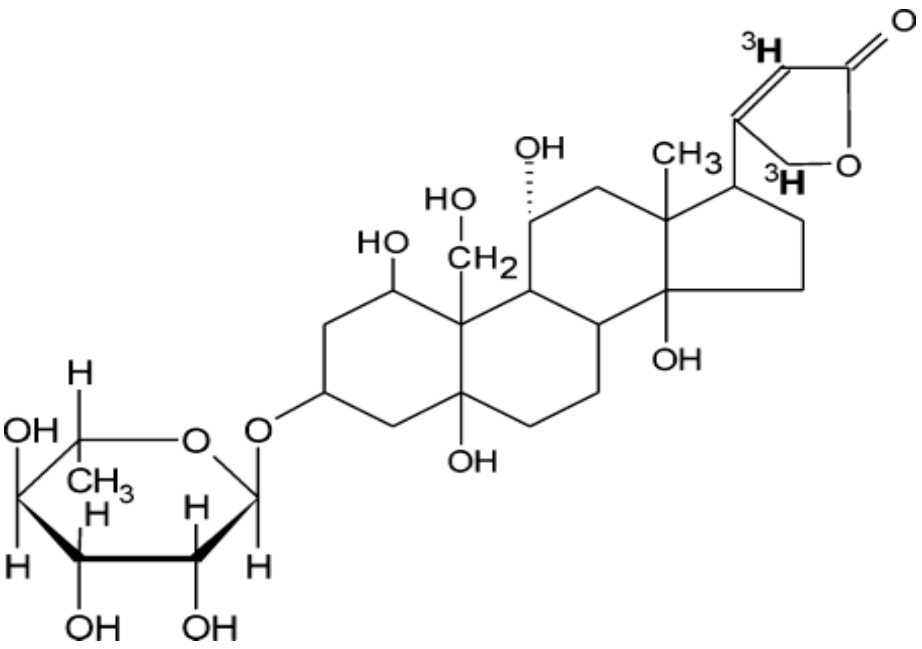


Fig. 1.7 *Molecular structure of ouabain.*

also secondary active transport of a number of substances through co-transport and counter-transport systems (Jørgensen, 1980; Skou, 1988). As a result the sodium pump is a major energy consumer, accounting for approximately 20% of overall resting metabolism (Rolfe and Brown, 1997). This energy use is not uniform in various tissues however, ranging from just a small percentage in some tissues (eg heart), up to 60-70% in the kidney and brain (Clausen et al., 1991).

The activity of the sodium pump and sodium cycling are correlated with a number of situations in which BMR is altered. For example, metabolic rate is lower in ectotherms compared to endotherms (Hemmingsen, 1960; Hulbert, 1980), and this is associated with a lower sodium leak into cells (Else and Hulbert, 1987) and a reduced sodium pump molecular activity (Else and Wu, 1999). Following birth, the post-natal peak in metabolic rate has been shown to be associated with changes in sodium pump

concentration (Kjeldsen et al., 1982; Kjeldsen et al., 1984) and sodium pump molecular activity (Wu, 2000). Allometric comparisons also suggest a similar relationship, with greater sodium pump activity (measured as Rb^+ uptake) reported in small mammals compared to large mammals (Couture and Hulbert, 1995b). The study of Couture and Hulbert (1995b) did not however address whether the increased sodium pump activity in small mammals was the result of an increased number of sodium pumps per gram of tissue, or whether there was increased intrinsic activity in individual sodium pumps (i.e. molecular activity).

1.4.5 Sodium Pump and Membrane Composition

Taken collectively the studies mentioned in the preceding sections show that animals with an increased BMR, have greater levels of PUFA in their membranes and have higher sodium pump molecular activity than less metabolically active animals. These findings were the stimulus for the experiments conducted by Else and Wu (1999), which were designed to investigate whether membranes were determining the higher sodium pump molecular activity in endotherms compared to ectotherms. In an elegant series of experiments, they used graded detergent treatments to delipidate kidney and brain microsomes from the endothermic rat (*Rattus norvegicus*) and the ectothermic cane toad (*Bufo marinus*). When the microsomes were delipidated, sodium pump molecular activity was reduced to approximately 10% of the original values. Relipidation with the original microsomal lipid restored activity to normal, however relipidation into microsomal lipids from the other species changed molecular activity levels to be similar to that of the second species. Similar results have also been demonstrated with membrane “crossover” studies between cattle and crocodile tissues, with a double reconstitution technique further accentuating the effects (Wu, 2000). These species “crossover” experiments provide direct experimental evidence that the membrane lipid environment is a major determinant of the molecular activity of the sodium pump enzyme, with polyunsaturated membranes supporting higher molecular activity levels.

1.5 Mitochondrial Proton Leak and Membranes

While this review has thus far concentrated on the sodium pump, another major energy consuming process which is associated with membranes, namely the mitochondrial proton leak, should be considered, as it has also been a major model that has contributed to the understanding of membrane lipids and their link with metabolism.

It has been known for quite some time that mitochondria consume oxygen even when they are not manufacturing ATP, and that this energy is used to pump protons across the mitochondrial membrane to counteract a proton leak in the other direction (Brand et al., 1994; Brand et al., 1999). Overall this oxygen consumption is estimated to constitute approximately 20% of resting metabolism (Rolfe and Brown, 1997), and although its identity is unknown, it has been extensively studied in liver mitochondria, and displays similar characteristics in other tissues (Rolfe et al., 1994).

In a number of studies, proton leak has been shown to vary in a similar fashion to metabolic rate. The proton leakiness of mitochondria is allometrically related to body size in mammals, with smaller species displaying a greater leak in both isolated mitochondria (Porter and Brand, 1993) and hepatocytes (Porter and Brand, 1995). These allometric trends have also recently been observed in bird mitochondria (Brand et al., 2003), and isolated hepatocytes (Else, P.L., Brand, M.D., Turner, N. & Hulbert, A.J. unpublished observations) and therefore appear to be general for endotherms. Allometric comparisons of ectotherms reveal no body-size-related variation in liver mitochondrial proton conductance (Hulbert et al., 2002a), however proton leakiness of liver mitochondria is approximately 5 times lower in the bearded dragon lizard compared to a similar sized rat (Brand et al., 1991). While proton leak can be largely explained by differences in mitochondrial membrane surface area (Porter et al, 1996), many allometric and phylogenetic studies also show correlations between proton leak and membrane fatty acid composition (Porter et al., 1996; Brookes et al., 1998; Hulbert et al., 2002a; Brand et al., 2003), with higher proton leak observed in more polyunsaturated mitochondrial membranes. Manipulative studies tend to support these

correlative findings, with increased proton leak observed in liver mitochondria from mice, when membrane 22:6(*n*-3) content is increased, both *in vivo* by feeding menhaden oil and *in vitro* by lipid fusion (Stillwell et al., 1997).

The correlations observed between membrane fatty acyl composition and mitochondrial proton leak are similar to those observed for sodium pump molecular activity, however the mechanism by which fatty acyl composition affects mitochondrial proton leak is unclear. The available evidence suggests it is not explained by proton leak directly through the mitochondrial membrane lipid bilayer, as the proton flux of liposomes prepared from mitochondrial phospholipids is only about 5% of the proton conductance of rat liver mitochondria (Brookes et al., 1997b). Proton conductance of liposomes is also independent of phospholipid fatty acyl composition (Brookes et al., 1997a), and thus the correlations between proton leak and membrane fatty acyl composition may potentially reflect an influence of the mitochondrial bilayer on mitochondrial membrane proteins, such as uncoupling proteins (Brand et al., 1999).

1.6 Membrane Lipid-Protein Interactions

The sodium pump and mitochondrial studies mentioned above tend to indicate that proteins are more active in membranes that are more polyunsaturated. Such a relationship is supported by numerous studies in the literature that report similar correlations for a variety of membrane proteins. Retinal membranes contain high levels of 22:6(*n*-3), and this is associated with high activity in visual system G-proteins (Litman and Mitchell, 1996). Similarly, protein kinase C (PKC) activity is increased in membranes with an increased amount of PUFA (Slater et al, 1996). The Ca^{2+}ATPase, which is a significant component of metabolism, particularly in muscles, has been found to be highly active in polyunsaturated membranes, especially those with high 22:6(*n*-3) content (Infante, 1987; Infante et al., 2001).

Szamel and Resch (1981) used an acyltransferase to incorporate fatty acids of varying chain length and saturation (16:0, 18:1, 18:2, 20:4) into phosphatidylcholines of

lymphocyte membranes. All fatty acids were incorporated equally with Na^+K^+ATPase activity and ouabain insensitive activity, designated as Mg^{2+}ATPase, measured. The incorporation of 16:0 had no effect on these enzymes, however there was a 10-20% increase in Na^+K^+ATPase activity and a 10% decrease in Mg^{2+}ATPase activity with the addition of 18:1. Addition of two polyunsaturated fatty acids (18:2 & 20:4) resulted in marked changes in both enzymes with the Na^+K^+ATPase activity increasing by approximately 80% and the Mg^{2+}ATPase activity decreasing by approximately 70% (Szamel and Resch, 1981).

Studies involving temperature-induced "homeoviscous adaptation" have also illustrated the effect of membrane polyunsaturation on protein function. It has been demonstrated that cold-acclimated trout, increase the PUFA content (especially 22:6(*n*-3)) of their membranes (Hazel, 1995), and this is associated with concomitant increases in sodium pump molecular activity (Raynard and Cossins, 1991). Similarly cold-acclimated goldfish have been shown to have mitochondrial membrane lipids that provide for a greater reactivation of delipidated mitochondrial enzymes, such as succinic dehydrogenase, than those from warm-acclimated goldfish (Hazel, 1972).

Further evidence for a relationship between bilayer acyl composition and protein molecular activity comes from a study examining the glucose transporter in the chick jejunum during development (Vázquez et al., 1997). In this study both glucose transporter activity and transporter density increased two-fold during early postnatal life (2 days - 14 weeks), thus there was no change in molecular activity. During the same period there were substantial changes in cholesterol content and phospholipid head group composition, while membrane fatty acyl composition remained constant. Therefore the molecular activity of glucose transporters remained constant, as did membrane acyl composition, despite large changes in other membrane lipid components.

As well as increasing the speed of active processes, a number of reports indicate that increased levels of membrane PUFA are also associated with increased passive leak in several tissues. The incorporation of PUFA into the membranes of tumor cells in culture, increases the permeability of these cells to a variety of molecules including erythritol and ^{51}Cr (Stillwell et al., 1993). Similarly, high levels of membrane polyunsaturation have been associated with increased sodium leak in erythrocytes (Ruiz-Gutierrez et al., 1993), and in vesicles prepared from retinal membrane lipids (Hendriks et al., 1976). These changes are also potentially due to an effect of the bilayer on protein function as ion channels have been shown to be directly affected by fatty acids (Ordway et al., 1989; Ordway et al., 1991).

The effect of membrane lipids on membrane proteins does not appear to be limited to the acyl chains. Cholesterol is generally thought to have an inhibitory effect on ATPases (Kimelberg and Papahadjopoulos, 1974; Yeagle, 1983; Yeagle et al., 1988; Crockett and Hazel, 1997), and a stimulatory effect on some other membrane transport proteins (Bastiaanse et al., 1997). This modulatory effect is thought to be via the influence of cholesterol on the physical properties of the membrane bilayer (Yeagle et al., 1988; Cornelius, 2001), however a recent report suggests this is potentially not always the case (Cornelius et al., 2003).

The exact nature of the effect of phospholipid headgroup on the activity of membrane proteins is less clear. Much of the evidence for specific interactions between headgroup classes and protein activity, comes from studies in which the catalytic activity of purified proteins was reduced (or completely destroyed) by delipidation, and was then subsequently restored upon reconstitution into vesicles of specific polar headgroups (Goldman and Albers, 1973; Wheeler et al., 1975; Palatini et al., 1977; De Pont et al., 1978; Mandersloot et al., 1978; Cornelius, 1991). Many of these original lipid specificities however, have been subsequently refuted or modified (Sandermann Jr., 1978). Studies have also shown that sodium pump molecular activity is very similar in the rectal gland of the shark (*Squalus acanthias*) and the electric organ of the electric

eel (*Electrophorus electricus*), despite large differences in their phospholipid headgroup composition (Hokin et al., 1973; Perrone et al., 1975). Credible evidence does exist suggesting an effect of phospholipid headgroup on some proteins (Carruthers and Melchior, 1986). For example, mitochondrial cytochrome c oxidase molecular activity is increased in hyperthyroid rats (Paradies et al., 1994), with this increase in molecular activity associated with changes in cardiolipin content of mitochondrial membranes. Thus at present, the importance of phospholipid headgroup in modulating membrane protein activity is unclear. Future research is likely to concentrate on phospholipid molecular species, as the pairing of specific fatty acids with particular phospholipid headgroups may be of greater importance (Kimelberg and Papahadjopoulos, 1974; Giorgione et al., 1995).

1.6.1 Interactions Between Lipids and Proteins

Membrane protein function thus appears to be highly dependent on the lipid composition of the surrounding bilayer. The fact that the complete removal of membrane lipids from the exterior of membrane-bound enzymes, either through phospholipase action (Tanaka and Teryua, 1973; De Pont et al., 1978), or detergent treatment (Palatini et al., 1977), results in a complete loss of activity, suggests an intimate relationship. The lipids that surround membrane proteins have been classified as either bulk lipids, which have the properties of typical membrane lipids, or annular (boundary) lipids, which are strongly immobilised by contact with the protein (Jost et al., 1973). While a specific role for annular lipids in modulating protein activity has been proposed (Nandi et al., 1983), it has been demonstrated that bulk membrane phospholipids are freely interchangeable with annular lipids (Jost et al., 1977; Cribier et al., 1993), and thus specific lipid interactions are unlikely, as is the case for bacterial solute transport proteins (In't Veld et al., 1993).

1.6.2 Mechanistic Basis of Lipid-Protein Interactions

The exact mechanism by which membrane lipids affect membrane protein activity is unknown, however may be related to the physical influence that lipids have on the

membrane bilayer. The following sections will describe the physical characteristics of membranes that may potentially influence enzyme activity.

1.6.2.1 Lateral Packing of Membrane Lipids

In membranes there is a constant lateral compression between lipids and proteins, which is dictated by the thermodynamic forces (eg *van der Waals,* dipolar, hydrogen bonding and electrostatic forces) that maintain membrane structure (Lundbæk et al., 1996). No technique is currently available that allows the direct measurement of lateral pressures in biological membranes, however monolayer studies have provided much information and are suggested to be a good model (Rebecchi et al., 1992). The lateral surface pressure in the membrane has been estimated to be between 12 and 50 mN/m (Demel, 1994), and appears to be related to the composition of membrane lipids, which dictate the molecular packing of the lipid molecules. In general, the average molecular area of isolated phospholipid molecules is determined largely by the acyl chains, with saturated fatty acyl chains occupying much smaller volumes in membranes that polyunsaturated chains (Ehringer et al., 1990; Zerouga et al., 1995; Wu et al., 2001). In biological membranes (particularly the plasma membrane) another important lipid component that influences lateral packing is cholesterol, which results in a reduced average molecular area (this has been called a “condensation” effect) (Demel et al., 1972).

The activity of several proteins, including protein kinase C (Souvignet et al., 1991), phospholipase C (Demel et al., 1975), and phospholipase A_2 (Grainger et al., 1990), have been shown to be influenced by the lateral compressive forces that result from the molecular packing of membrane lipids. A recent report by Wu et al. (2001) has also examined this relationship for the sodium pump. Using area-pressure isotherms of kidney and brain microsomes from the laboratory rat and cane toad, they found a striking relationship between the molecular packing of membrane lipids and the molecular activity of the sodium pump (Wu et al., 2001). Specifically, membranes

containing higher proportions of polyunsaturated acyl chains, occupied a larger surface area, and this was associated with an increased molecular activity. While the exact mechanism of this physical effect is unknown, a potential explanation, may lie in the fact that membrane-bound enzymes, such as the sodium pump, undergo very large conformational changes during their catalytic cycle. When surrounded by membrane lipids that occupy a smaller molecular area for a given lateral surface pressure, it is possible that conformational changes may be sterically hindered by the lipid molecules, which in turn may cause a lower turnover rate of the protein.

1.6.2.2 Molecular Movement of Lipid Molecules

Under normal conditions the membrane bilayer is in a fluid state. Membrane proteins and lipids constantly migrate within the lateral plane of the membrane, with lateral diffusion coefficients of membrane lipids being at least two orders of magnitude greater than those of proteins (Storch and Kleinfeld, 1985). This suggests that membrane proteins are being constantly bombarded by membrane lipids. Hulbert and Else (1999), suggested that these collisions may in fact represent an important means by which membrane lipids affect the molecular activity of membrane-bound enzymes. Polyunsaturated fatty acids, by virtue of the greater atomic mass associated with their double bonds, may in fact be transferring greater amounts of kinetic energy to membrane proteins with each collision. A molecule such as 22:6(*n*-3) has an even distribution of *cis* double bonds throughout the length of its chain, as well as having the greatest number of double bonds. Hulbert and Else (1999) propose that such a conformation facilitates energy transfer between itself and membrane proteins and may explain why this important polyunsaturate is associated with increased rates in many membrane associated processes (Gudbjarnarson et al., 1978; Litman and Mitchell, 1996; Stillwell et al., 1997).

Support for such a notion also comes from recent molecular dynamics simulations, which have highlighted the unique physical properties that highly polyunsaturated acyl chains such as 22:6(*n*-3) give to membrane bilayers (Feller et al., 2002; Huber et al.,

2002). Feller et al. (2002) have calculated that there are hundreds of high-probability conformations of 22:6(*n*-3) likely in a membrane bilayer. Several of these involve the methyl end of the molecule located towards the outer edge of the bilayer rather than in the middle of the membrane bilayer as it is normally drawn in static diagrams. These molecular dynamics simulations present an image of 22:6(*n*-3) as thrashing about in the hydrocarbon core of a membrane bilayer. Such dramatic molecular movement by fatty acids in the membrane, may underlie the "energising" effect of polyunsaturates such as 22:6(*n*-3).

1.6.2.3 Thickness and Curvature of Membranes

The topology of membrane proteins makes them sensitive to the thickness of the membrane. Transmembrane proteins contain a hydrophobic core, and it is necessary for the bilayer lipids to adequately solvate these hydrophobic sequences to maintain normal function (Yeagle, 1989). If a mismatch occurs, it is thermodynamically unfavourable for the protein and the resultant distortion in membrane lipids can affect protein activity. The activity of several proteins has been found to be dependent on bilayer thickness. Ca^{2+}ATPase requires phospholipids with acyl chains of 16-20 carbons (Lee et al., 1994), while the rhodopsin molecule displays optimal activity in membranes containing high proportions of 22:6(*n*-3) (Akino, 1982; Eldho et al., 2003). For the sodium pump, early studies suggested that bilayers containing acyl chains of 16-20 carbons in length were equally effective at supporting maximal activity (Johannsson et al., 1981). More recently Cornelius (2001) investigated the effect of acyl chain length (n_c 14-24) on sodium pump activity through functional reconstitution into liposomes of defined composition. The optimal length of monounsaturated phosphatidylcholine was found to be 22 in the absence of cholesterol, but was reduced to 18 in the presence of 40 mol% cholesterol, indicating that hydrophobic matching in membranes is not only affected by fatty acids but also cholesterol.

Another important property of membranes that will influence protein function is the bilayer curvature stress. In biological membranes, the equilibrium curvature is a

function of the balance between intermolecular forces among the lipid headgroups relative to those among the acyl chains (Lundbæk et al., 1997). Since many lipids in natural membranes will adopt nonbilayer structures in isolation, and phospholipids are asymmetrically distributed, bilayers exist in a state of curvature stress. Alterations in membrane lipid composition are thus able to affect protein activity through alterations in the curvature of the membrane (Epand and Lester, 1990; Brown, 1994; McCallum and Epand, 1995; Lundbæk et al., 1996).

1.6.2.4 Membrane Dipole Potential

The electrical potential of cell membranes consists of two main components: the transmembrane potential and the boundary potential (Peterson et al., 2002). The transmembrane potential is determined by differences in ion concentrations in the aqueous phase on either side of the membrane. The boundary potential consists of both a surface potential and a dipole potential existing in the region between the aqueous phase and the hydrocarbon core of the interior membrane (Peterson et al., 2002). The dipole potential therefore represents another important potential site for the regulation of membrane proteins. Clarke (1997) investigated this possible relationship by using an optical method to examine the electric field strength (dipole potential) of phosphatidylcholine vesicles with varying fatty acid compositions. In this study he found that the magnitude of membrane dipole was higher in vesicles containing short chain, saturated fatty acids, and that as the chain length and level of unsaturation was increased, there was a decrease in the dipole potential (Clarke, 1997). As the local electric field strength may affect the conformation and functioning of membrane proteins (Clarke, 1997), the reduced dipole potential seen in unsaturated bilayers, may explain why membrane protein activity is increased in such lipid environments. The reason for the decreased dipole potential in unsaturated bilayers may be related to the fact that unsaturated fatty acids occupy a larger surface area (Ehringer et al., 1990), and effectively ‘dilute’ the local electric field strength (Peterson et al., 2002).

1.6.3 Summary

In conclusion, membrane enzymes require a specific lipid environment for optimal function. This optimal physical state of the membrane appears to be influenced by a number of membrane components, but in particular fatty acids. Generally increased membrane polyunsaturation is associated with increased activity in membrane-associated processes, such as enzyme molecular activity and ion leak. The exact mechanism by which membrane acyl composition speeds up membrane processes is unknown, however physical properties such as bilayer curvature and thickness, lateral packing and movement of molecules, and intramembrane electric field strength may potentially all be important.

1.7 Thesis Outline

This thesis will use a comparative approach to examine the relationship between membrane lipid composition and sodium pump molecular activity in tissues of fifteen different animal species. Chapters three, four and five will respectively examine the kidney, heart and brain of mammals and birds of different body size. The work completed in these three chapters represents the first fully comprehensive investigation of the relationship between body mass, sodium pump molecular activity and membrane lipid composition in both the major classes of endothermic vertebrates. Chapter six will examine a number of tissues from two ectothermic species, the octopus and bearded dragon lizard. In Chapter seven, as the molecular activity measurement allows comparison between different tissues and species, the data for all of the species examined will be combined and analysed as one data set. This analysis will be correlational, and although it cannot definitively prove “cause and effect”, it will provide insight into which membrane lipid components may be having an important influence on sodium pump molecular activity.

Chapter II

Methods

2.1 Experimental Animals

All species examined in the present study were adults of either sex and are shown in Table 2.1.

Table 2.1 The Species Examined in the Current Study Along with their Respective Body Mass and Basal Metabolic Rate

Common name	Scientific name	Body Mass (g)	BMR ($Kcal.g^{-1}.day^{-1}$)
Mouse	*Mus musculus*, Qs strain	37.9 ± 1.1 (n=16)	0.171
Rat	*Rattus norvegicus*, Wistar strain	281 ± 6 (n=12)	0.100
Sheep	*Ovis aries*	38500 ± 1380 (n=8)	0.028
Pig	*Sus scrofa*	88300 ± 6990 (n=8)	0.019
Cow	*Bos taurus*	277000 ± 20800 (n=8)	0.015
Zebra Finch	*Taeniopygia guttata*	12.6 ± 0.9 (n=4)	0.370
Sparrow	*Passer domesticus*	25.9 ± 0.9 (n=4)	0.278
Starling	*Sturnus vulgaris*	75 ± 3 (n=4)	0.260
Currawong	*Strepera graculina*	283 ± 19 (n=4)	0.179
Pigeon	*Columba livia*	462 ± 35 (n=4)	0.075
Duck	*Anas platyrhynchos*	2178 ± 61 (n=4)	0.071
Goose	*Anser anser*	4444 ± 360 (n=4)	0.062
Emu	*Dromaius novaehollandiae*	34975 ± 745 (n=4)	0.017
Bearded Dragon	*Pogona vitticeps*	236 ± 37 (n=7)	0.012
Octopus	*Octopus vulgaris*	463 ± 92 (n=11)	NA

Body mass values are means ± standard errors (SEM) with (n) representing the number of animals measured. BMR values for the mammals are from Kleiber (1961). BMR values for the birds and the bearded dragon are from Hulbert et al. (2002a) and Brand et al. (1991) respectively, and were converted from ml $O_2.g^{-1}.hr^{-1}$ to $Kcal.g^{-1}.hr^{-1}$ using a conversion factor of 4.7, which assumes an RQ of 0.7. NA, not available.

The mice and rats were purchased from Gore Hill Research Laboratories (Sydney, NSW, Australia). They were housed in the animal house at the University of Wollongong, maintained at 22 ± 2°C, with 12:12 light:dark photoperiod and *ad libitum*

access to food (rodent pellets) and water. Mice were killed by cervical dislocation, while rats were killed by Nembutal® overdose (pentobarbitone sodium, 100mg.kg^{-1} body mass; intraperitoneal injection). Tissues from the sheep, pigs and cattle were obtained from Wollondilly Abbatoir (NSW, Australia) immediately following the death of the animal and transported back to the University of Wollongong on ice for immediate use in the sodium pump density experiments. The diet of the sheep, pigs and cattle before death was unknown.

The emus were purchased from Marayong Park Emu Park (Falls Creek, NSW, Australia). Zebra finches, ducks and two geese were purchased from local pet shops or the Narellan Aviary Bird Auction (NSW, Australia). The pigeons were obtained from a local pigeon breeder (T. Cooper, Corrimal, NSW, Australia) and the sparrows, starlings, currawongs and two other geese were free-living animals caught locally in the Wollongong area. Birds were either used for experiments on the day of collection or were housed short-term (2-3 days) in the animal house at the University of Wollongong at 22 ± 2°C, with 12:12 light:dark photoperiod and *ad libitum* access to food and water. For the finches and sparrows the food was mixed birdseed, and for the ducks and geese it was a commercial mixture of pellets and seeds. The diet of the other birds before purchase was generally unknown. All birds were killed by lethal overdose of either Lethabarb® or Nembutal® (pentobarbitone sodium, 100mg.kg^{-1} body mass; intraperitoneal, except in the case of the emus where the injection was intrajugular).

Octopi were caught at local seaside rockpools (Austinmer, NSW) by hand and were either used on the same day or kept overnight in an aerated seawater aquarium and used the next morning. The temperature of the rockpools, and of the aquarium where they were kept prior to use, was approximately 20-25°C. Bearded dragon lizards were caught at Fowler's Gap, western NSW and were housed in the animal house at the University of Wollongong. Lizards were housed individually in a 1m x 0.5m box, with a 12:12 light:dark photoperiod and *ad libitum* access to food (mixed vegetables and occasional mealworms) and water. The temperature of the room in which they were

housed was approximately 25°C, while above each individual box was a heating lamp that allowed them to behaviourally regulate body temperature to their preferred level. Octopi were killed by anaesthesia with MS222 (0.5% tricaine methane sulphonate; pH 7.4), while the bearded dragons were killed by lethal overdose of Nembutal® (pentobarbitone sodium, 100mg.kg^{-1} body mass; intraperitoneal injection).

Body mass and tissue weights of the mice, rats, octopi, bearded dragons and all the bird species were obtained immediately following death. For sheep and cattle, carcass weights were used to calculate the weight of the whole mammal, assuming that the carcass weight was 55% of total body weight as is routinely used commercially (Couture and Hulbert, 1995b). The carcass weight of the pigs included the skin, and since this organ accounts for around 15-20% of total body weight (Ross et al., 1995), the total body weight was calculated assuming that the carcass weight accounted for 70% of total body mass. Tissue weights for the sheep, pigs and cattle were obtained immediately prior to the commencement of experimental assays. For the mammals and birds, the kidney (cortex and medulla), heart (ventricle) and brain (cortex) were examined, while in the octopus the tissues examined were the digestive gland (hepatopancreas), kidney, gill, brain and tentacle (muscle), and in the bearded dragon the liver, kidney, brain and skeletal muscle were examined.

2.2 Materials

^{3}H-ouabain (30.0 Ci.mmol^{-1}, 37 MBq, 96.2% purity) in 1:9 toluene:ethanol was obtained from Amersham Pharmacia Biotech (Castle Hill, NSW, Australia). The solvents were removed under a light stream of nitrogen gas and the ^{3}H-ouabain was resuspended in distilled water at a concentration of 200μCi.ml^{-1}. Ouabain and Tricaine methane sulphonate (MS222) were purchased from ICN Biomedicals Inc. (OH, USA). The scintillation cocktail (Ready Safe™) was from Beckman (Gladesville, NSW, Australia). The tissue solubiliser (Soluene®-350) was obtained from Packard Biosciences (Mt. Waverley, VIC, Australia). Na_2ATP special quality was from

Boehringer Mannheim (Mannheim, Germany). Sodium deoxycholate (DOC), sodium dodecyl sulphate (SDS), ethylenediaminetetracetic acid (EDTA), ammonium molybdate, Tris salts and ferrous sulphate were BDH chemicals purchased from Merck Pty Ltd (Kilsyth, VIC, Australia). Lethabarb® and Nembutal® (pentobarbitone sodium) were from Boehringer Ingelheim Pty Ltd (Artarmon, NSW, Australia). All other chemicals and reagents were of analytical grade and were obtained from Ajax chemicals (Auburn, NSW, Australia).

Analytical grade methanol, chloroform, ethyl acetate, n-hexane, and extra pure grade diethyl ether and petroleum spirit (40-60°C) were BDH products from Merck Pty Ltd (Kilsyth, VIC, Australia). Analytical grade butylated hydroxytoluene (BHT) 14% (w/v), boron trifluoride in methanol, stannous chloride, lab reagent sodium hydrosulfite (approx. 80%), the cholesterol assay kit, the cholesterol calibrator and all fatty acid standards were from Sigma Aldrich (Castle Hill, NSW, Australia). Silane treated glass wool was purchased from Alltech associates (Baulkham Hills, NSW, Australia). Sep-Pak® Silica and Florisil® cartridges were obtained from Waters™ Division, Millipore Corporation (MA, USA). Strata® SPE SI-2 Silica and FL-PR Florisil columns were from Phenomenex (Pennant Hills, NSW, Australia). High purity hydrogen, nitrogen, instrument grade synthetic air, medical grade oxygen and medical carbogen (95% O_2: CO_2) were products of BOC gases Australia Ltd. (Chatswood, NSW, Australia).

2.3 Solutions

All general solutions used were prepared at 25°C and are listed in Table 2.2.

2.4 ^{3}H-Ouabain Binding

Sodium pump density was determined using the method described by Else et al (1996). The validation of this technique with respect to incubation time, wash profile and specific ouabain concentration, has previously been reported (Else, 1994; Else et al., 1996). During the initial stages of the current investigation, a M.Sc. (Hons) student from the same laboratory, who was examining thyroid status and the sodium pump,

Table 2.2	**General Solutions**	
Tissue homogenisation	250mM Sucrose, 5mM EDTA, 20mM Imidazole	pH 7.4
Tissue homogenisation (octopus)	750mM Sucrose, 5mM EDTA, 20mM Imidazole	pH 7.4
Na^+K^+ATPase assay medium	30mM Histidine, 4mM $MgCl_2$, 124mM NaCl, and either 1mM Ouabain or 20-40mM KCl	pH 7.4
Tris-based Na^+K^+ATPase assay medium	83.3 mM Tris Base/HCl, 5mM $MgCl_2$, 100mM NaCl, 5mM NaN_3 and either 1mM Ouabain or 15-30mM KCl	pH 7.4
Mammalian and Reptilian ^{3}H-Ouabain binding (K^+-free)	125mM NaCl, 1.2mM $MgSO_4$, 1.2mM NaH_2PO_4, 25mM $NaHCO_3$, 1.3mM $CaCl_2$, 5mM Glucose	pH 7.4
Avian ^{3}H-Ouabain binding (K^+-free)	124mM NaCl, 1.2mM $MgSO_4$, 1.1mM NaH_2PO_4, 25mM $NaHCO_3$, 2.5mM $CaCl_2$, 11.1mM Glucose	pH 7.4
Avian ^{3}H-Ouabain binding (4.5mM K^+)	125mM NaCl, 3.4mM KCl, 1.2mM $MgSO_4$, 1.1mM KH_2PO_4, 25mM $NaHCO_3$, 2.5mM $CaCl_2$, 11.1mM Glucose	pH 7.4
Octopus ^{3}H-Ouabain binding (K^+ free)	335mM NaCl, 8mM $CaSO_4$, 2.5mM NaH_2PO_4, 10mM Na_2SO_4, 10mM $NaHCO_3$, 45mM $MgCl_2$, 5mM Glucose, 20mM HEPES (N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid)	pH 7.6
Microsomal homogenisation	250mM Sucrose, 1mM EDTA, 20mM Imidazole	pH 7.4
Microsomal homogenisation (octopus)	750mM Sucrose, 1mM EDTA, 20mM Imidazole	pH 7.4
Microsomal resuspension	25mM Imidazole, 2mM EDTA	pH 7.5

was characterising ^{3}H-ouabain binding in tissues of the rat and toad. The results of his study indicated that an incubation time of 2-3 hours, with a specific ouabain concentration of 30-50μM, and 5 x 3ml washes for 8 min were the optimal conditions for this assay (Hou, 2001). These conditions were similar to those previously described (Else, 1994; Else et al., 1996), and were subsequently employed in the current study.

Incubations were conducted at 37°C for 2 hours in mammalian tissues, 40°C for 2 hours in avian tissues and 25°C for 3 hours in the tissues of the two ectothermic species. Tissues were removed from the animals immediately following death, cut into small pieces (2-10mg) and placed in ice-cold K^+-free medium. The ^{3}H-ouabain binding mediums for the mammals, birds and ectotherms closely resembled the ionic composition of the plasma of each species and are listed in Table 2.2.

Mammalian and reptilian ringer was based on a solution previously used (Else et al., 1996). Octopus ringer was based on the plasma concentrations for this species (Wells, 1978). Two different solutions were used to assess ^{3}H-ouabain binding in birds, a K^+-free medium and one containing 4.5mM K^+, which approximates the documented average plasma K^+ concentration for a large number of birds (Prosser, 1973; Altman and Dittmer, 1974). K^+-free mediums are generally used in ^{3}H-ouabain binding studies as K^+ is thought to inhibit binding of ouabain to the sodium pump (Hossler et al., 1978; Wallick et al., 1980), however increased levels of binding have been demonstrated with varying levels of K^+ (Else, 1994). Under the current experimental conditions, there was no statistical difference in the measured ^{3}H-ouabain binding sites using the different media, and therefore their average was used to estimate sodium pump density in birds.

Tissue biopsy samples were preincubated in ice-cold K^+ free medium for 2 x 10 minute periods to reduce tissue K^+. Tissue biopsies from the octopus and bearded dragon were then incubated in 2ml of K^+ free medium containing 2μCi.ml^{-1} ^{3}H-ouabain and final ouabain concentrations ranging from 5x10^{-7} M to 5x10^{-5} M to determine maximal binding (Fig. 2.1 & Fig. 2.2). For mammalian and avian tissue biopsies, incubations were conducted in 2ml of ^{3}H-ouabain binding medium containing 1μCi.ml^{-1} ^{3}H-ouabain and a final high saturating ouabain concentration of 5x10^{-5} M. For all tissues, parallel incubations at a final concentration of 10^{-2} M ouabain were also conducted to determine non-specific binding. Incubations were gassed continuously with oxygen (HEPES based medium) or carbogen (bicarbonate based medium), to maintain

physiological pH levels (7.4 - 7.6), and to circulate the incubation medium around the tissue biopsies. For the octopus and bearded dragon, liver or digestive gland incubations were performed in the presence of 1mM veratrine, according to the method described by Else et al. (1996). Veratrine is a potent inhibitor of the steroid carrier present in mammalian liver cell membranes but does not affect ouabain binding to the sodium pump (Petzinger and Fischer, 1985). The use of this inhibitor allowed an estimation of ouabain specifically bound to hepatic sodium pumps, rather than non-specifically accumulated by the steroid carrier.

After incubation, the biopsies were washed 5 times (8 min per wash) in 3 ml of ice-cold ^{3}H-ouabain binding medium to reduce ^{3}H activity associated with non-specific sites. Following the wash procedure, biopsies were blotted lightly, weighed (±0.01mg) and placed in 200µl of tissue solubiliser (Soluene®-350) overnight. Readysafe scintillation cocktail (2 ml) was added to each vial and ^{3}H activity counted on a Wallac 1409 Liquid Scintillation Counter with DPM correction.

^{3}H-ouabain binding was expressed as relative uptake, i.e. ^{3}H activity taken up per gram wet weight of tissue relative to ^{3}H activity in the incubation medium. Specific uptake was calculated following subtraction of ^{3}H activity determined in excess ouabain (10^{-2} M), which was deemed non-specific uptake. ^{3}H-ouabain binding sites per gram of tissue was determined by multiplying the specific uptake with the total ouabain concentration in the medium. Sodium pump density was calculated assuming a 1:1 stoichiometry between sodium pump units and ouabain binding sites and was expressed as picomoles of sodium pumps per gram of tissue wet weight (pmol.g wet wt^{-1}).

Examination of binding curves for the octopus (Fig. 2.1) and bearded dragon (Fig. 2.2) indicated that 50µM was the ouabain concentration at which all sodium pump sites appeared saturated. ^{3}H-ouabain binding values for this concentration were subsequently taken to represent the sodium pump density for the octopus and bearded dragon tissues.

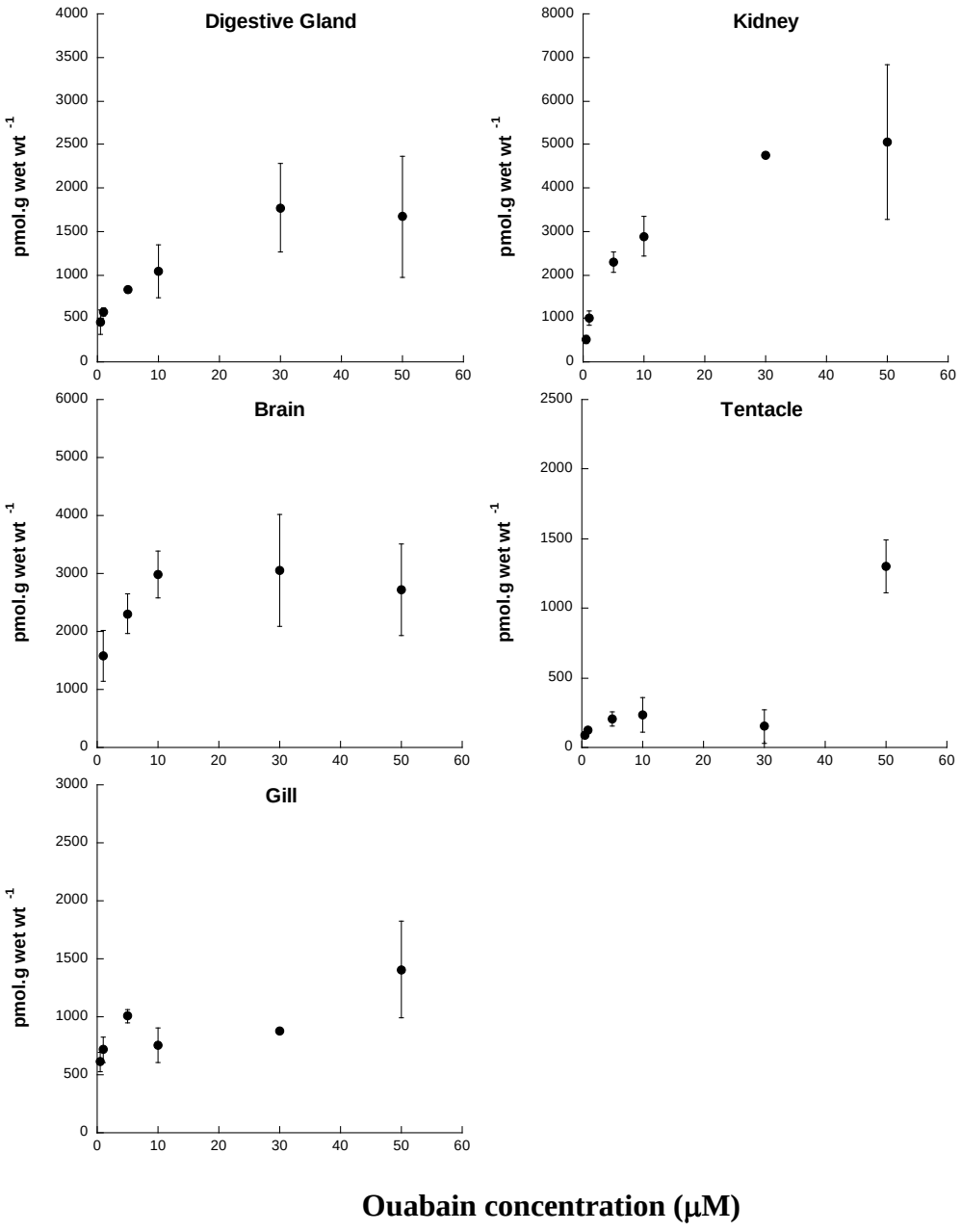


Fig. 2.1 *Binding isotherms of ^{3}H-ouabain in octopus tissues. Specific binding of ^{3}H-ouabain was determined at multiple ouabain concentrations from 0.5 - 50μM. Tissue biopsies were incubated for 3 hr followed by a washout of 5 x 8 min. A biphasic pattern, composed of a curvilinear phase followed by a plateau phase was observed in most tissues. In the tentacle and gill, there was some variability in measurements, which may have been explained by a limited number of measures at the higher concentrations, or may represent the existence of two populations of enzyme molecules with different affinities for ouabain. Values represent means ± standard errors (SEM) of 2-5 determinations.*

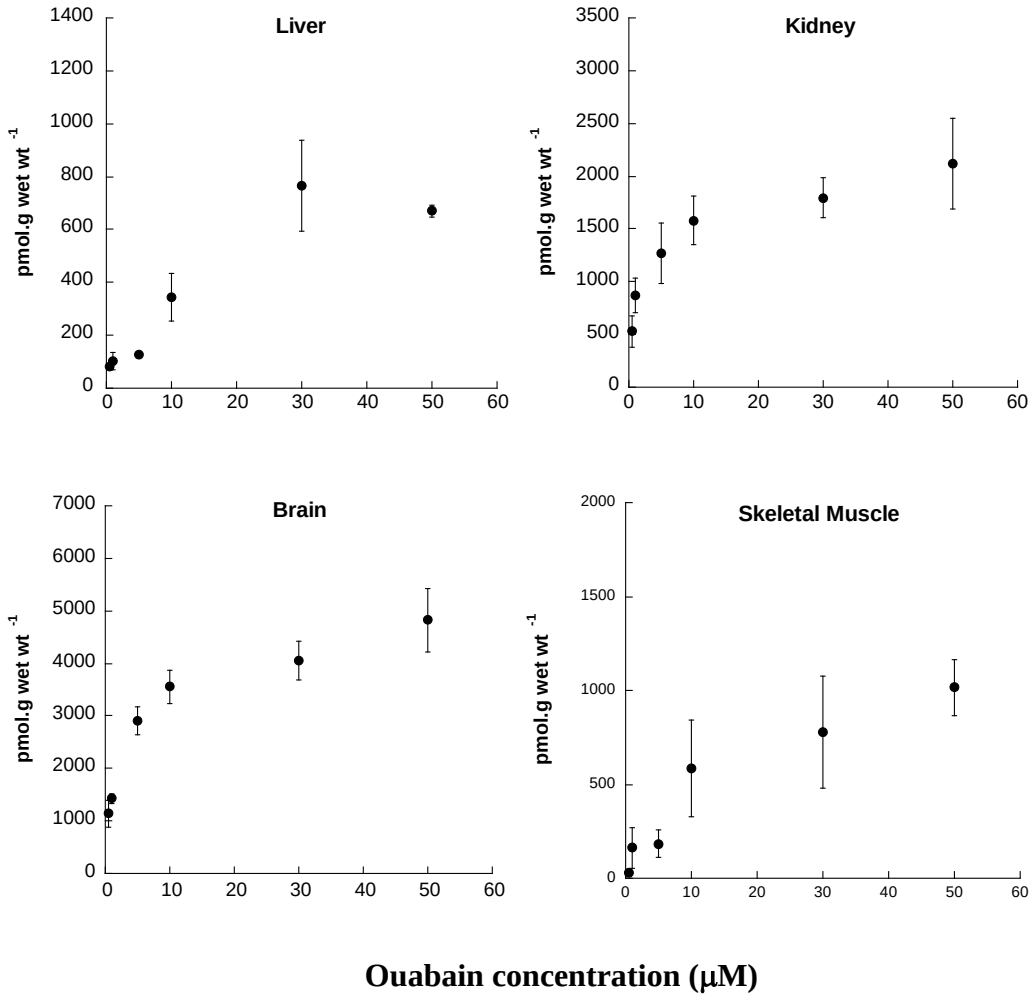


Fig. 2.2 *Binding isotherms of ^{3}H-ouabain in bearded dragon tissues. Specific binding of ^{3}H-ouabain was determined at multiple ouabain concentrations from 0.5 - 50μM. Tissue biopsies were incubated for 3 hr followed by a washout of 5 x 8 min. A biphasic pattern, composed of a curvilinear phase followed by a plateau phase was observed in the kidney and brain, while two plateau levels were potentially observed in the liver and skeletal muscle, which may represent the existence of two populations of enzyme molecules with different affinities for ouabain. Values represent means ± standard errors (SEM) of 2-4 determinations.*

The mammals and birds were only assessed at a concentration of 50μM, as it has been shown to be a saturating concentration (Hou, 2001).

2.5 Determination of Na^+K^+ATPase Activity

2.5.1 A change from the status quo

During the initial stages of this study, the assay for specific sodium pump activity (i.e. Na^+K^+ATPase activity) was altered from the method described by Akera (1984) to a modified method of that described by Esmann and Skou (1988). The Akera method employed a 15 minute incubation of 100µl of tissue homogenate in the Tris based assay solution (see Table 2.2). The modifications due to the Esmann and Skou method were decreasing the sample size to 50µl, reducing incubation time from 15 minutes to 5 minutes, and changing to a histidine based assay solution (Table 2.2). The main reasons for the alterations were firstly that cations such as Tris have been found to antagonise the binding of K^+ to the sodium pump (Esmann, 1988), hence providing competitive inhibition. Secondly, ADP has been found to inhibit the sodium pump, and since freshly solubilized ATP (3mM) was in excess in the assay, the incubation time was decreased to 5 minutes and the sample size to 50µl to limit the amount of ADP produced.

The difference in elicitable Na^+K^+ATPase activity at 37°C between the Akera method and Esmann and Skou method, measured on the same tissue homogenates from the octopus can be seen in Figure 2.3, with the magnitude of difference being 2.17, 1.43, 1.50, 2.34, and 1.72 for the digestive gland, kidney, gill, brain and tentacle respectively. Similar differences were also observed in mammalian tissues (Haga, K.L., personal communication), and subsequently all Na^+K^+ATPase activity measurements in the mammals, birds and ectotherms were conducted using the optimised method of Esmann and Skou (1988).

The ionic conditions and ATP levels employed in this modified assay are considered to be optimal for the sodium pump (Esmann, 1988). To ensure this was the case, Na^+K^+ATPase activity was determined using a range of ATP and ionic concentrations (results not shown). The only ion that showed any effect on hydrolytic activity was K^+, which was found to elicit higher activity in the octopus when 40mM KCl was used,

compared to 20mM KCl in the standard assay. Subsequently all Na^+K^+ATPase activity determinations in the octopus employed this concentration (Table 2.2).

Na^+K^+ATPase activity was determined in dilute tissue homogenates prepared (2-5%, w/v) using a glass-glass homogeniser in ice-cold tissue homogenisation medium (Table 2.2). A mild detergent treatment was applied to the samples prior to the assay to elicit maximal Na^+K^+ATPase activity. Detergent treatments improve access of substrates to 'internalised' or 'captured' sodium pumps so that all pumps can express maximal activity. Furthermore detergents have also been suggested to increase Na^+K^+ATPase activity via interaction with the FXYD protein subunit of the sodium pump (Mahmmoud et al., 2000). Sodium deoxycholate (DOC) ($C_{24}H_{39}O_4Na$) was the detergent used in the current study with an optimal concentration found to range between 0.75-1.25mg.ml^{-1}. A 150µl volume of sample was mixed under constant stirring with 150µl of DOC and was allowed to stand at room temperature for 15 minutes.

Samples (50µl) of the detergent treated homogenates were then preincubated in Na^+K^+ATPase assay medium (Table 2.2) for 10 mins at 37°C (mammals, octopus, bearded dragon) or 40°C (birds) to allow for thermal equilibration and binding of ouabain to the sodium pumps. The reaction was then initiated by the addition of 100µl of 30mM ATP, making the total volume of the medium up to 1ml and the final ATP concentration 3mM. The reaction was allowed to proceed for 5 mins and was terminated by the addition of 1ml of 0.8N perchloric acid.

The reaction mixture was then centrifuged at 1,200g for 15 mins in a Hettich Universal 16R centrifuge at 4°C. A 250µl aliquot of the supernatant was placed in a cuvette with distilled water (250µl) and assayed for inorganic phosphate, following the addition of colour reagent (500µl). The colour reagent was prepared in the following proportions: 1g of ammonium molybdate ($(NH_4)_6Mo_7O_{24}*4H_2O$) was dissolved in 90ml of distilled water under constant stirring, followed by the addition of 3.3ml of concentrated sulfuric

acid (H_2SO_4) and 4g of ferrous sulfate ($FeSO_4*7H_2O$). The solution was topped up to 100ml and used within 2 hours. After addition to the assay solution, colour was allowed to develop for 10 minutes at 25°C, and the absorbance was measured at 750nm using a Shimadzu UV 1601 spectrophotometer. A 1mM solution of potassium dihydrogen orthophosphate KH_2PO_4 (25µl-250µl) was used as the phosphate standard. All measurements were performed in duplicate or greater.

Maximal Na^+K^+ATPase activity was defined as the 'ouabain inhibitable' component of ATPase activity. This was calculated as the difference in inorganic phosphate liberated (from ATP) in the presence and absence of 1mM ouabain (minus and plus KCl respectively). Na^+K^+ATPase activity was expressed as µmoles of inorganic phosphate (specifically liberated from ATP) per gram of tissue wet weight per hour (µmol Pi.mg wet $wt^{-1}.hr^{-1}$).

Mammals and birds were assayed at different temperatures to try and approximate *in vivo* conditions. To allow comparison between the two classes, thermal quotient values (i.e. temperature sensitivity of a reaction) for Na^+K^+ATPase activity from tissues of several birds were determined. The thermal quotient values were measured as the Q_{10}, which is the change in reaction rate of a process for every 10°C change in temperature. Values were calculated according to the equation $Q_{10} = (R_2/R_1)^{(10/\ T_2\ -\ T_1)}$, where T_1 and T_2 are the temperatures (°C) that produce the rates of reaction R_1 and R_2 respectively. The average of the Q_{10} value in birds was 2.1 (range 1.6 – 2.4) and subsequently a value of 2.0, which is a thermal quotient common to many biological processes (Purves et al., 1995), was used to correct bird Na^+K^+ATPase activity to 37°C and therefore allow comparison with mammals. The Na^+K^+ATPase activity values measured at 40ºC in the birds are presented in Appendix I. To allow comparison with the mammalian and avian data, Na^+K^+ATPase assays in the octopus and bearded dragon were carried out at 37°C. To assess the thermal dependence of the sodium pumps, Na^+K^+ATPase assay was also determined at 25°C for both species. The thermal dependence is expressed as thermal quotient (Q_{10}) values (see Appendix II).

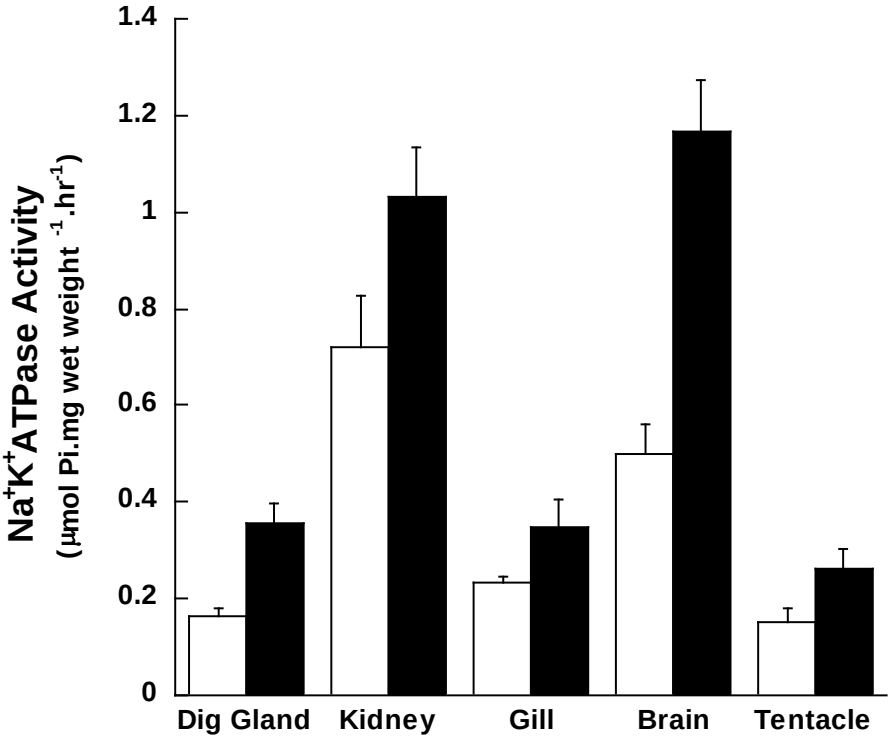


Fig. 2.3 *Comparison of the Akera (open bars) and Esmann and Skou (closed bars) methods for determining Na^+K^+ATPase activity. Values presented represent the mean ± standard error (SEM), n=3.*

2.6 Molecular Activity

Molecular activity is defined as the rate of substrate turnover by a protein, and for the sodium pump it can be expressed as the number of ATP molecules hydrolysed by each individual sodium pump per unit of time. Molecular activity of sodium pumps was calculated by dividing the maximal Na^+K^+ATPase activity (expressed as pmol Pi.g wet $wt^{-1}.hr^{-1}$) by the number of sodium pumps (in pmol.g wet wt^{-1} at 50µM) for the same preparations. The net result was expressed as the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$).

2.7 Preparation of Microsomal Membranes

Microsomal membranes were prepared from tissue homogenates (10%, w/v in the appropriate medium, see Table 2.2) that were centrifuged at 4°C in a Beckman L-80 ultracentrifuge (55.2 Ti fixed angle rotor) at 3000g for 3 minutes and a further 10 minutes at 10,000g to remove nuclei and mitochondria respectively. The supernatant was then centrifuged at 98,000g for 35 minutes and the resultant pellet, designated microsomal membranes, was resuspended in 25mM Imidazole, 2mM EDTA (pH 7.5).

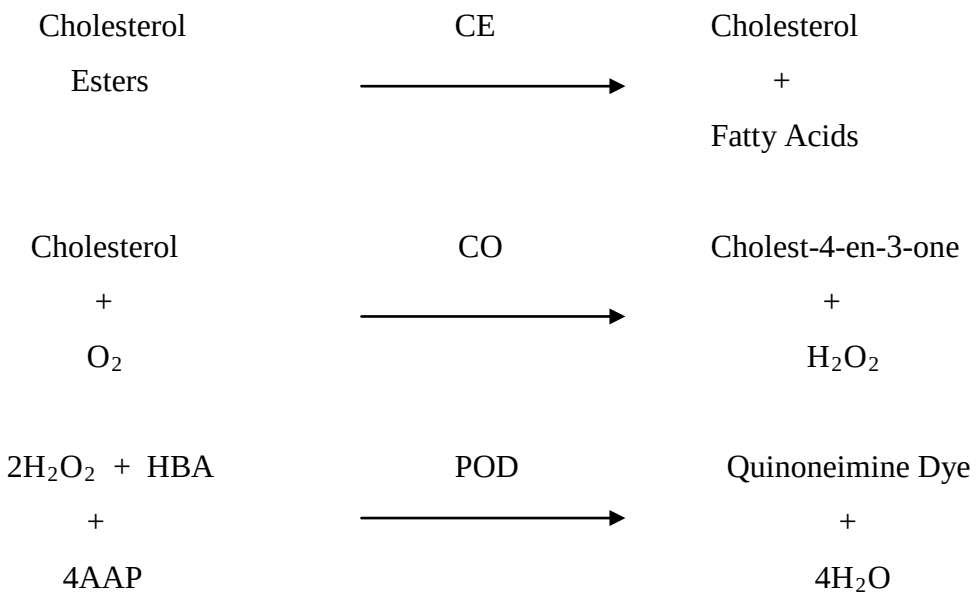
Following the sodium pump assays, most species had sufficient amounts of tissue remaining to prepare one microsomal fraction per animal for membrane lipid analysis. When there was insufficient tissue available, microsomal fractions were produced using a pooled sample of tissue from a number of individuals. Thus although there was only n=1-2 for the lipid measurements in these species, the sample was an average of a number of individuals and was thus considered a representative sample of the species. The following tissues required a pooled sample: zebra finch and sparrow kidney, one pooled microsomal fraction from 4 individuals; mouse heart, one pooled sample from 9 individuals; zebra finch and sparrow heart, two pooled microsomal fractions each containing tissue from 2 individual birds; zebra finch brain two of the three microsomal fractions examined were from individual brains, while the third was a pooled sample from two individuals.

2.8 Assay for Protein Content

The Lowry method was used to determine the protein content of tissue homogenates and microsomes (Lowry et al., 1951). Samples (20μl) were incubated with 2ml of assay solution (2% (w/v) Na_2CO_3 in 0.1M NaOH and 0.5% (w/v) $CuSO_4$ in 1% (w/v) Na-K Tartrate) for 10 mins at room temperature; 0.2ml Folins and Ciocalteus reagent was added, and absorbances were read using a SHIMADZU UV-1601 UV-visible spectrophotometer at 750nm after 30 mins. Bovine serum albumin (0-100μg) was used as the protein standard, with samples diluted to fall within the range of the standard curve.

2.9 Cholesterol Content

A standard enzymatic assay kit (Infinity™, Sigma) was used to determine microsomal membrane cholesterol content, according to the reaction outlined below.



The assay involved converting cholesterol esters to cholesterol via cholesterol esterase (CE), with subsequent conversion by cholesterol oxidase (CO) to cholest-4-en-3-one with hydrogen peroxide (H_2O_2) as a byproduct. Peroxide production was coupled with 4-aminoantipyrine (4AAP) and hydroxybenzoic acid (HBA) to yield a quinoneimine dye with an absorbance maximum at 500nm in the presence of peroxidase (POD). The cholesterol content in the microsomal sample was therefore directly proportional to the colour intensity.

The procedure involved adding 1ml of cholesterol assay reagent (Cholesterol Oxidase (microbial) >100 U/L, Cholesterol Esterase (microbial) >1250U/L, Peroxidase

(Horseradish) >800 U/L, 4-Aminoantipyrine 0.25 mmol/L, HBA 10mmol/L, pH 6.6) to 10µl of microsomal membrane (5-10 mg protein.ml^{-1}) with mixing and incubation for 25 min at 25°C. Production of the quinoneimine dye, which directly corresponded to cholesterol content was calculated by reading absorbance at 500nm in a Shimadzu UV 1601 spectrophotometer and comparing against a standard reference curve of 5, 10, 15, 20µg of cholesterol prepared concurrently.

2.10 Extraction of Total Lipid and Phospholipid Separation

During total lipid extraction and phospholipid separation all solvents used were of analytical grade or better with 0.01% (w/v) BHT added as an antioxidant. Total lipid was extracted from microsomal membrane preparations by standard methods (Folch et al., 1957). Microsomal membrane samples (5-15 mg protein) were homogenised in 12ml of chloroform/methanol (2:1, v/v) using a glass homogeniser. Samples were left on a tube rotator overnight at 4°C. The phases were separated by the addition of 1M H_2SO_4 and centrifugation at 400g for 10 minutes. The lower chloroform phase was transferred to a new tube and washed again with H_2SO_4 for further purification. The phase was then dried by the addition of sodium dithionite and filtered through silane treated glass wool. The resulting filtrate was designated total lipid, containing phospholipids, cholesterol, triglycerides and other lipid like molecules.

Phospholipids were separated using solid phase extraction on SPE® SI-2 Silica cartridges (mammals and birds) or Sep-Pak® Silica cartridges (octopus and bearded dragon). Total lipid samples were loaded onto the cartridges and neutral lipids were eluted with 30ml of ethyl acetate, while phospholipids were eluted with 20ml of methanol.

2.11 Phospholipid Content

Analysis of phospholipid content was via a phosphorus assay (Mills et al., 1984). Total lipid was extracted from microsomal membrane preparations (approx 150µg protein), dried under N_2, resuspended in 0.8ml of 72% (w/w) perchloric acid ($HClO_4$), and

heated at 190°C for 45 minutes. Tubes were then placed on ice and 5ml of water added (making sure to wash down the sides of the tubes). Following this addition, 500µl of the colour reagents ammonium molybdate (8%, w/v) and stannous chloride (0.005% dilution of 40% (w/v) $SnCl_2$ in HCl) were added and the solution was topped up to 10 ml. Colour was allowed to develop for 10 min and absorbance subsequently read at 680nm in a Shimadzu UV 1601 spectrophotometer. The µg of phosphorus associated with the microsomal membrane sample was quantified by analysis of absorbance values against a standard reference curve. The standard used was KH_2PO_4 at 20µg per ml and the curve was constructed using 1, 2, 5, 10 µg of phosphorus.

Phospholipid content was calculated using the following equation:

$$\frac{\mu g \text{ phosphorus} \times 780}{30.97}$$

Where 780 is the average mass of phospholipids and 30.97 is the molecular weight of phosphorus.

2.12 Phospholipid Fatty Acid Analysis

During extraction and derivatisation of fatty acid methyl esters, all solvents used were of high purity with 0.01% (w/v) BHT added as an antioxidant. Phospholipid fractions prepared as described earlier, were transmethylated with 14% (w/v) boron trifluoride in methanol at 85°C for 1 hour. The reaction was stopped by the addition of 3ml of ice cold water and the methyl esters extracted into 5x2ml of n-hexane, dried with sodium dithionite, filtered through silane treated glass wool, and passed through SPE® FL-PR Florisil cartridges (mammals and birds) or SPE Florisil® cartridges (octopus and bearded dragon) to remove cholesterol esters and polar contaminants. Finally fatty acid methyl esters were eluted with 7ml of 5% (v/v) diethyl ether-petroleum spirit and dried under N_2 to ~1ml for analysis.

Composition of the fatty acid methyl esters was determined by gas-liquid chromatography. A Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 automatic liquid sampler (Hewlett-Packard) was used with 1□l of sample injected on-column into a J&W DB-23 fused silica capillary column 30m x 0.25mm x 0.25μm. High purity hydrogen was used as a carrier gas at 40cm.sec^{-1} (measured at 150°C). The initial oven temperature was 170°C and a temperature program was employed: 170-200°C at 3 degrees.min^{-1}, then 200°C for 3min followed by 200-250°C at 50°C per min and finally 250°C for 2min. The fatty acid methyl esters were detected by a flame ionisation detector (FID) at 220°C with nitrogen make up gas at 30ml.min^{-1}. Fatty acid methyl esters in the sample were identified by comparing their retention times to those of external standards.

The content of individual fatty acids in microsomes was expressed as a mole percentage of total fatty acids identified. The sum of the saturates, monounsaturates, polyunsaturates and C20+22 polyunsaturates were calculated and total proportions of the n-9, n-7, n-6, and n-3 families were also determined. Unsaturation index (UI), which is the average number of double bonds per 100 fatty acid chains, and average chain length (Av CL) were calculated using the following formulas:

UI = ∑[(percentage of each unsaturated fatty acid) x (the number of double bonds therein)];
Av CL = ∑[(percentage of each fatty acid) x (its chain length)]/100

2.13 Statistical Analyses

All statistical comparisons were determined and tested for significance using the mean value for individual data points from each species (i.e. n=5 for mammals and n=8 for birds) or tissue (n=5 for the octopus and n=4 for the bearded dragon). This is a more rigorous test than using individual data points and highlights only very significant relationships. Allometric equations were determined by linear regression (least-square method) of log-transformed values using JMP® 4.0.1 software (SAS Institute Inc., NC,

USA). Linear correlation coefficients comparing lipid parameters and molecular activity were determined using JMP® 4.0.1 software. All figures were produced using KaleidaGraph™ 3.51 software (Synergy Software, USA). Allometric and linear relationships were tested for significance using the Pearson product moment correlation coefficient, with n-2 degrees of freedom. Significance for all relationships was accepted at the level of $P<0.05$ and all results are reported as means ± standard error (SEM).

2.14 Ethical Approval

All procedures were performed in conformity with the National Health and Medical Research Council Guidelines for Animal Research and were approved by the Animal Experimentation Ethics committee of the University of Wollongong. The collection of octopi was conducted with the permission of the NSW National Parks and Wildlife Service.

Chapter III

Sodium Pump Molecular Activity and Membrane Lipid Composition in the Kidney of Mammals and Birds of Different Body Size

3.1 INTRODUCTION

This chapter will examine sodium pump molecular activity and membrane lipid composition in the kidney of mammals and birds of different body size. Kidney phospholipids in small mammals have been shown to be more polyunsaturated and less monounsaturated than phospholipids from larger mammals (Couture and Hulbert, 1995a; Hulbert et al., 2002c). Similar results have been observed in bird skeletal muscle (Hulbert et al., 2002b), although whether these same allometric relationships exist in avian kidneys is unknown.

As well as having more polyunsaturated membranes, smaller mammals also display higher sodium pump activity (Rb^+ uptake) in kidney slices (Couture and Hulbert, 1995a). To date however, it has not been established whether this increased sodium pump activity is the result of changes in the number of sodium pumps per gram of tissue, or whether changes in molecular activity of individual sodium pumps are the underlying cause. Changes in the molecular activity of membrane-bound proteins and in membrane lipid composition have both been implicated as major mechanisms underpinning differences in metabolism (Hulbert and Else, 1999; Hulbert and Else, 2000). Thus the aim of the current chapter was to elucidate whether allometric changes in either membrane lipid composition or sodium pump molecular activity from the kidney of mammals and birds of different body size, may be factors contributing to the metabolic variation observed in these endothermic species.

3.2 RESULTS

The mammalian species (Table 2.1) examined represented a body mass range of approximately 7,500-fold. The two smaller species were Rodentia, while the three larger species were Artiodactyla. The bird species (Table 2.1) comprised both passerine and non-passerine species and had a body mass range of approximately 3,000-fold. The four smallest species were passerines while the four larger species were non-passerines. The species examined in both groups provided a fairly even spread across the body mass range.

The mass-specific BMR values of the mammals and birds used (Table 2.1) were considered with respect to body mass using simple power formula generation from log transformed values, as classically performed in allometric studies (Peters, 1983). The power equations generated from these analyses are presented below:

$$\textit{Mammalian BMR } (Kcal.g^{-1}.day^{-1}) = 0.47 \times \textit{Body Mass } (g)^{-0.27} \qquad (r=0.999,\ P<0.001)$$

$$\textit{Avian BMR } (Kcal.g^{-1}.day^{-1}) = 1.06 \times \textit{Body Mass } (g)^{-0.37} \qquad (r=0.967,\ P<0.001)$$

The allometric slope describing the BMR of the mammals in the current study is –0.27, which is close to the value of –0.25 generally found to describe BMR in mammals (Kleiber, 1961; Peters, 1983). The allometric slope observed in the birds (-0.37) is steeper than the –0.25 that is often ascribed for the power relationship between body mass and BMR in birds, however this is likely the result of the fact that the four smallest birds were passerines, which generally possess higher rates of basal metabolism than non-passerines (Lasiewski and Dawson, 1967).

The individual values for kidney mass and protein concentration of the respective species are presented in Appendix III. These variables were considered with respect to body mass and are presented in Fig. 3.1 & Fig. 3.2 respectively. The kidney mass of both the mammals and birds was strongly dependent on body mass. From the allometric exponents it can be calculated that a doubling in body mass would result in a

75% and 78% increase in kidney mass in the mammals and birds respectively. Thus the larger mammals and birds tended to have relatively smaller kidneys. In contrast, protein concentration expressed as milligrams of protein per gram of kidney wet weight, displayed no allometric trends in either the mammals or birds (Fig. 3.2). Mammalian kidney had an average protein content of 191 ± 8 mg.g wet wt^{-1} while the kidneys from birds contained an average protein content of 176 ± 5 mg.g wet wt^{-1}.

Na^+K^+ATPase activity, sodium pump density, and molecular activity values measured for the mammals and birds are presented in Table 3.1. Na^+K^+ATPase activity values,

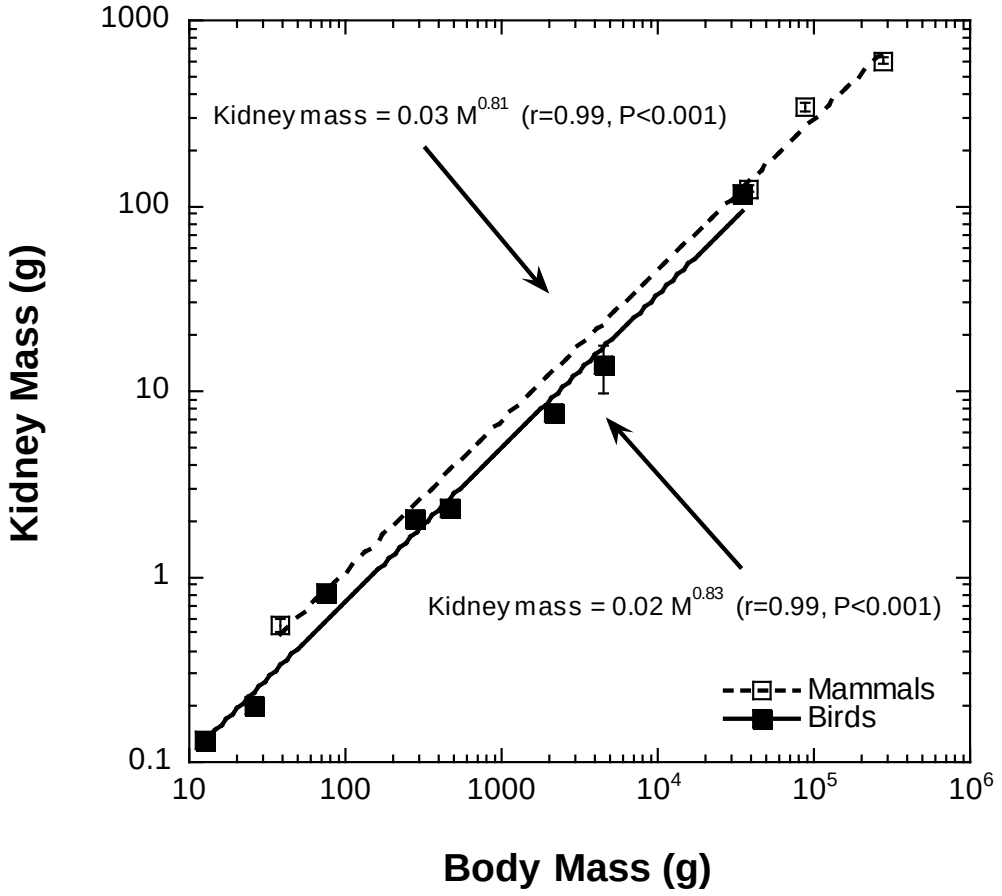


Fig. 3.1 *The relationship between the body mass of mammals and birds and the mass of their kidneys. Each point represents the mean ± standard error (SEM).*

measured at 37ºC for mammals and corrected to 37ºC for birds (see METHODS), are expressed as micromoles of inorganic phosphate liberated (from ATP) per mg of kidney wet weight per hour. When examined relative to body mass, there was a significant decrease in Na^+K^+ATPase activity in the larger birds (P<0.01), with a similar, although not quite significant (P=0.07) trend observed in mammals (Fig. 3.3). Body mass explained 85% of the variability of Na^+K^+ATPase activity in bird kidneys and calculated from the allometric exponent (–0.16) there would be a 10.4% decrease in Na^+K^+ATPase activity for every doubling in body mass.

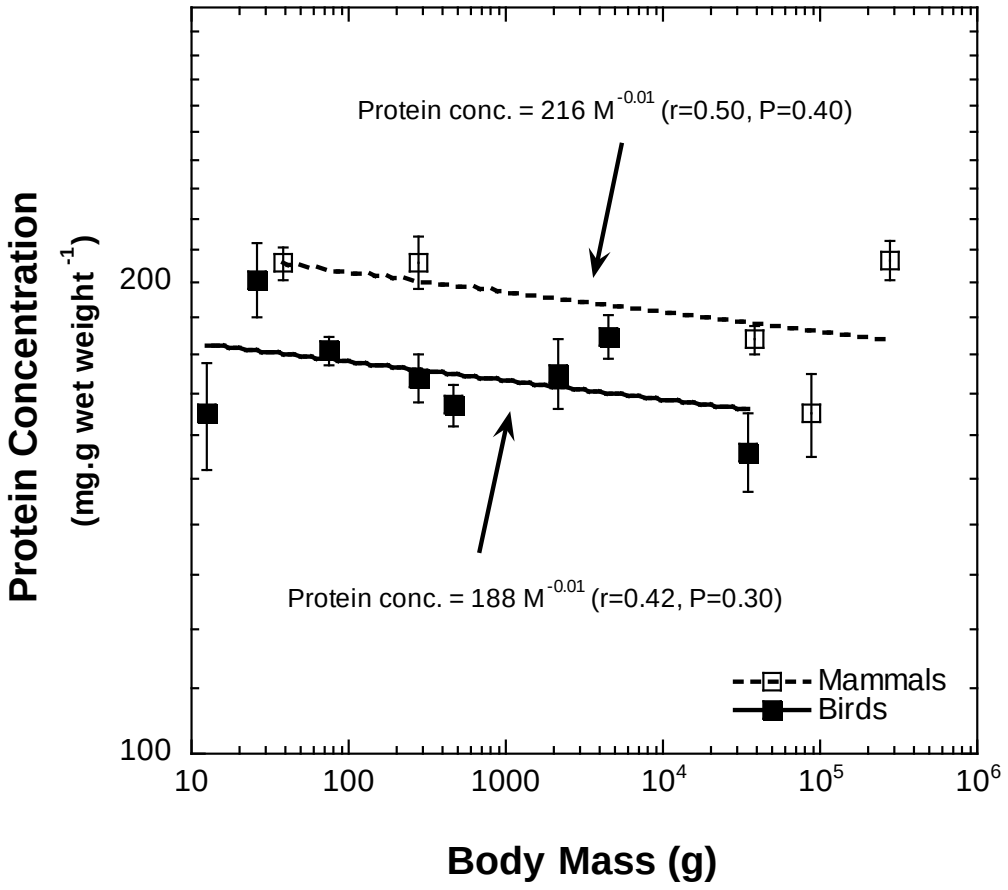


Fig. 3.2 *The relationship between the body mass of mammals and birds and kidney protein concentration. Each point represents the mean ± standard error (SEM).*

Table 3.1	**Na^+K^+ATPase Activity, Sodium Pump Density, and Molecular Activity of Sodium Pumps from the Kidney of Mammals and Birds**		
	Na^+K^+ATPase Activity (μmol Pi.mg wet $wt^{-1}.hr^{-1}$)	Sodium Pump Density (pmol.g wet wt^{-1})	Molecular Activity ($ATP.min^{-1}$)
Mammals			
Mouse	4.32 ± 0.27 (n=12)	3609 ± 404 (n=12)	24095 ± 4035 (n=12)
Rat	3.49 ± 0.16 (n=12)	5923 ± 970 (n=10)	13508 ± 2493 (n=10)
Sheep	2.90 ± 0.16 (n=8)	5651 ± 1070 (n=8)	11380 ± 2313 (n=8)
Pig	1.81 ± 0.13 (n=7)	5293 ± 1080 (n=6)	7643 ± 1523 (n=5)
Cow	2.58 ± 0.15 (n=8)	5170 ± 695 (n=8)	9487 ± 1364 (n=8)
Birds			
Zebra Finch	4.74 ± 0.22 (n=4)	9466 ± 1586 (n=4)	8908 ± 1141 (n=4)
Sparrow	3.82 ± 0.26 (n=4)	9378 ± 581 (n=4)	6941 ± 875 (n=4)
Starling	4.56 ± 0.11 (n=4)	12506 ± 633 (n=4)	6130 ± 373 (n=4)
Currawong	3.62 ± 0.09 (n=4)	7301 ± 218 (n=4)	8280 ± 311 (n=4)
Pigeon	2.28 ± 0.02 (n=4)	7889 ± 1011 (n=4)	5026 ± 563 (n=4)
Duck	2.06 ± 0.05 (n=4)	7903 ± 1036 (n=4)	4577 ± 619 (n=4)
Goose	1.87 ± 0.13 (n=4)	5150 ± 590 (n=4)	6422 ± 1168 (n=4)
Emu	1.48 ± 0.01 (n=4)	3600 ± 151 (n=4)	6888 ± 308 (n=4)

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram of wet weight each hour. Sodium pump density was measured as picomoles of sodium pumps per gram of wet weight. Molecular activity is maximal Na^+K^+ATPase activity divided by sodium pump density for the same animal, and is expressed as the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). (n) is the number of preparations used for each measurement.

Sodium pump density was measured as picomoles per gram of kidney wet weight and tended to be higher in many of the bird species compared to the mammals. Fig. 3.4 presents the sodium pump density relative to body mass. The density of sodium pumps in the kidneys of the 5 mammalian species was fairly constant and averaged 5,130

pmol.g wet wt^{-1}. In contrast sodium pump density showed a significant allometric (P<0.01) decrease in the larger birds. From the allometric exponent (-0.12) it can be calculated that for every 100% increase in body mass there would be a 7.3% decrease in the sodium pump density of bird kidneys. When sodium pump density was considered with respect to kidney mass, the number of individual sodium pumps per kidney would vary for mammals between 1.1 x 10^{15} in mice and 1,910 x 10^{15} in cattle, while for birds, the kidney of zebra finches would contain 7.1 x 10^{14} sodium pumps compared with 2,540 x 10^{14} in the emu kidney.

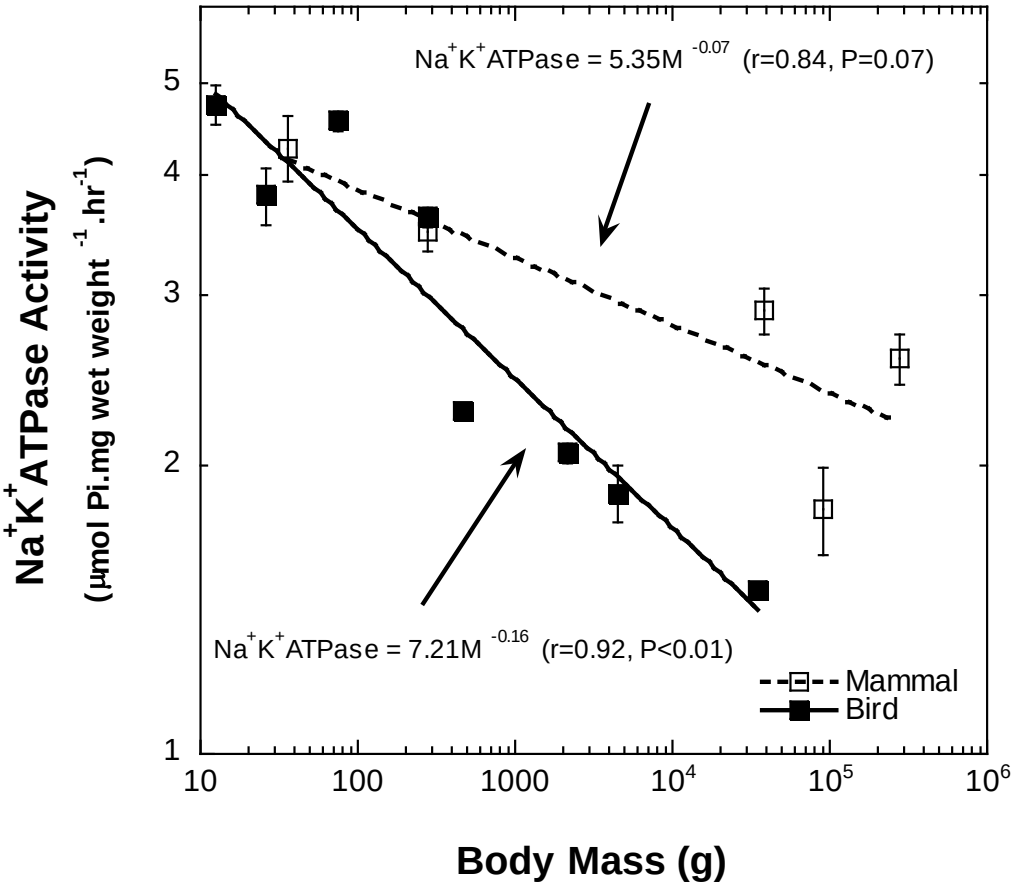


Fig. 3.3 *The relationship between the body mass of mammals and birds and the Na^+K^+ATPase activity of kidney homogenates at 37ºC. Each point represents the mean ± standard error (SEM).*

Molecular activity was calculated by dividing maximal Na^+K^+ATPase activity by the sodium pump density to give the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). Bird molecular activity values were calculated from 37°C converted Na^+K^+ATPase activity values to allow comparison with mammals. The range of molecular activity values for the mammals was 7,643 – 24,095 $ATP.min^{-1}$ while for birds the variation was modest, ranging from 4,577 – 8,908 $ATP.min^{-1}$ (Table 3.1). When examined relative to body mass (Fig. 3.5) there was a significant (P=0.04) decrease in molecular activity in the mammals with body mass explaining 81% of the

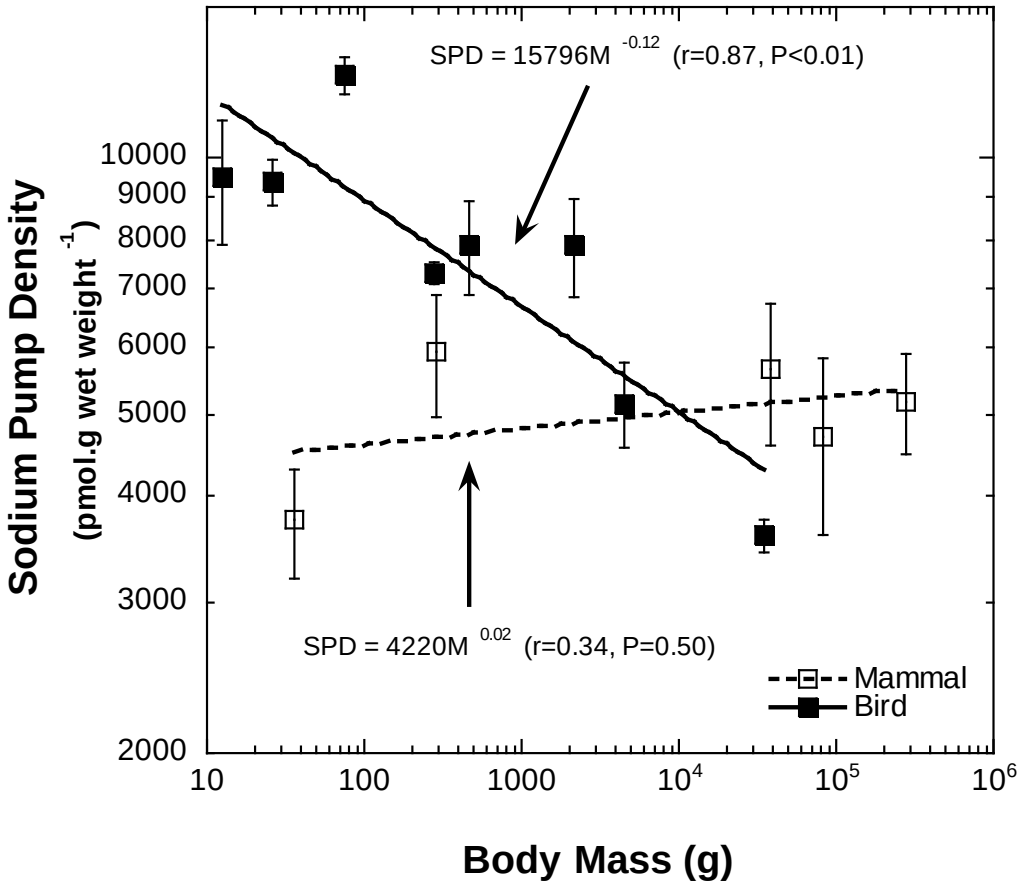


Fig. 3.4 *The relationship between the body mass of mammals and birds and the sodium pump density of kidney tissue biopsies. Each point represents the mean ± standard error (SEM).*

variability. From the allometric exponent (-0.10) it can be calculated that for every doubling in body mass there would be a 6.7% decrease in sodium pump molecular activity in mammalian kidneys. A different relationship was seen in bird kidneys, with no allometric trend observed as body mass only explained 15% of the variability seen in the molecular activity values.

The cholesterol and phospholipid content, along with their molar ratio, in microsomal membranes from the kidneys of mammals and birds are presented in Table 3.2. The

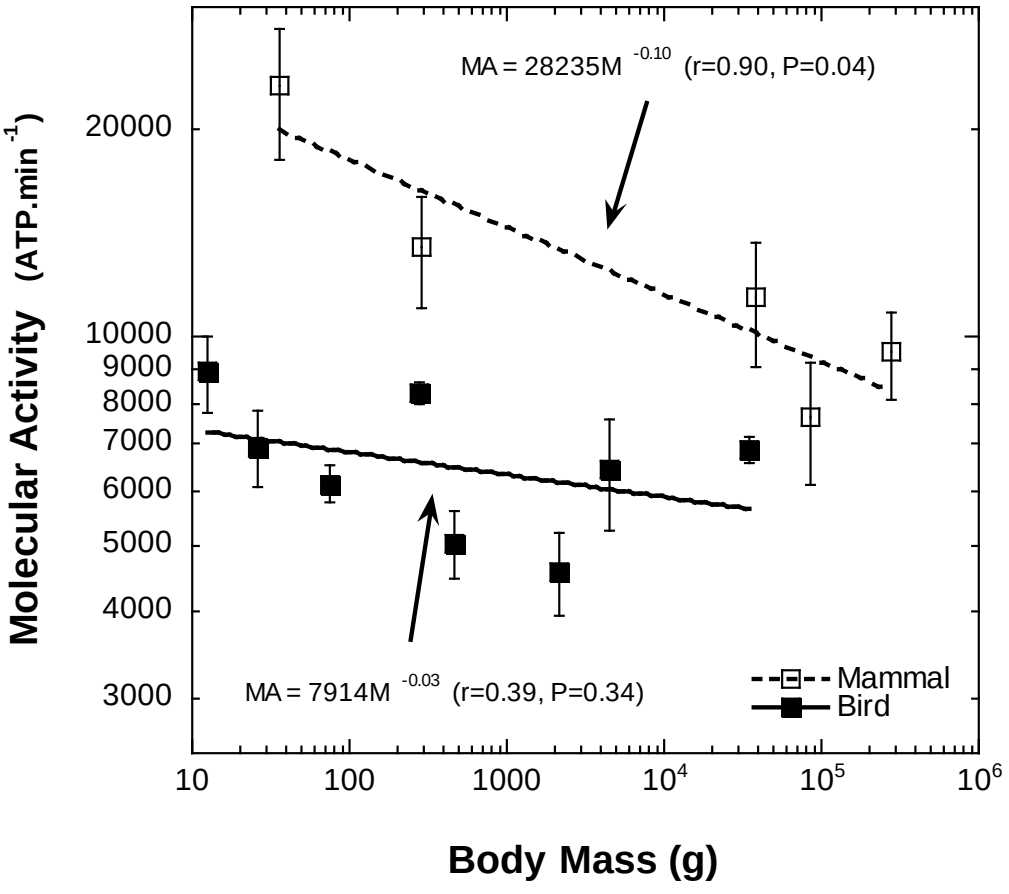


Fig. 3.5 *The relationship between the body mass of mammals and birds and the molecular activity of kidney sodium pumps. Each point represents the mean ± standard error (SEM).*

Table 3.2	**Cholesterol and Phospholipid Content of Microsomal Membranes from the Kidney of Mammals and Birds**		
	Cholesterol (μg. mg protein^{-1})	Phospholipid (μg. mg protein^{-1})	Cholesterol:Phospholipid (mole:mole)*
Mammals			
Mouse (n=4)	35.6 ± 2.1	207 ± 10	0.35 ± 0.01
Rat (n=4)	47.1 ± 1.5	220 ± 22	0.45 ± 0.06
Sheep (n=4)	46.4 ± 2.0	245 ± 12	0.38 ± 0.01
Pig (n=4)	46.9 ± 2.1	219 ± 23	0.44 ± 0.04
Cow (n=4)	44.4 ± 0.7	210 ± 8	0.43 ± 0.02
Birds			
Zebra Finch (n=1)	53.7	377	0.29
Sparrow (n=1)	55.7	404	0.28
Starling (n=4)	61.7 ± 3.5	364 ± 13	0.34 ± 0.02
Currawong (n=4)	60.7 ± 5.3	363 ± 31	0.34 ± 0.01
Pigeon (n=4)	63.3 ± 4.4	275 ± 24	0.49 ± 0.08
Duck (n=4)	55.0 ± 2.0	275 ± 24	0.41 ± 0.08
Goose (n=4)	46.7 ± 1.5	268 ± 20	0.36 ± 0.03
Emu (n=4)	43.7 ± 6.2	288 ± 26	0.30 ± 0.02

*Cholesterol and phospholipid content of microsomal membranes are expressed relative to protein content in μg.mg protein^{-1}. Cholesterol:Phospholipid ratios are the molar ratio (data from cholesterol and phospholipid content in μg.mg protein^{-1}). * moles of phospholipid calculated assuming a molecular weight of 780. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement.*

mammalian species tended to have fairly constant cholesterol content (per mg of protein), although the mouse did have cholesterol levels that were approximately 25% lower than the other mammals. Phospholipid content (per mg of protein) also displayed little variation across the different mammals and as a result the molar ratio of cholesterol:phospholipid was quite similar in all mammalian species. Thus when these variables were considered with respect to body mass (Table 3.5), no allometric trends were observed.

The cholesterol content (per mg of protein) tended to be higher in the smaller birds (Table 3.2) and when considered with respect to body mass (Table 3.5) a negative, although not significant (P=0.09), allometric slope was observed. Phospholipid content

(per mg of protein) displayed a significant (P=0.01) allometric decline in the larger birds, with body mass explaining 67% of the variability (Table 3.5). From the allometric exponent (-0.05) it can be calculated that every doubling in body mass would result in a 3.4% decrease in the phospholipid content of bird kidney microsomes. The molar ratio of cholesterol:phospholipid in birds was similar to the mammalian ratios and was not significantly related to body mass (Table 3.5). From the cholesterol:phospholipid ratios it can be seen that in kidney microsomes from mammals and birds, there are about 2-3 phospholipids per molecule of cholesterol.

The fatty acid profile of kidney microsomal phospholipids for mammals and birds are presented in Table 3.3 and Table 3.4 respectively. The major individual fatty acids, along with the composite parameters were considered with respect to body mass and the results of the analysis are presented in Table 3.5. Several parameters are also plotted allometrically in Fig. 3.6.

Phospholipids from the smaller mammals were more unsaturated (as indicated by the unsaturation index; Fig. 3.6), despite there being no allometric trend in the total percentage of unsaturated fatty acid chains (Fig. 3.6). On average, 76% of the phospholipid fatty acid chains from mammalian kidney microsomes were unsaturated. Monounsaturate content showed a significant allometric increase in the larger mammals (P=0.02; Fig. 3.6), with body mass explaining 88% of the variability in this parameter. While this allometric relationship largely reflected the significant allometric increase (P=0.02) observed in the most prominent monounsaturate, oleic acid (18:1(n-9)), contributions were also made from n-7 monounsaturates, which displayed similar allometric increases (P=0.05; Table 3.5). The total content of polyunsaturates showed a significant allometric decline in the larger mammals (P=0.03; Fig 3.6), while neither the n-6 PUFA, or the n-3 PUFA, showed any relationship with body mass. The percentage of the long-chain C20+22 PUFA was significantly reduced in the larger species (P=0.01; Table 3.5). The content of the two terminal fatty acids in the n-6 and n-3 pathway, namely 22:5(n-6) and 22:6(n-3), both declined with increasing body

Table 3.3 Microsomal Phospholipid Fatty Acid Profiles from Mammalian Kidneys

	Mouse	Rat	Sheep	Pig	Cow
Fatty Acid					
14:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
16:0	13.5 ± 0.9	12.3 ± 0.8	11.5 ± 0.4	13.1 ± 1.1	11.2 ± 0.1
17:0	0.2 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.4 ± 0.1
18:0	9.7 ± 0.9	12.5 ± 0.8	12.8 ± 0.6	11.4 ± 0.9	8.8 ± 0.6
20:0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0
16:1(*n*-7)	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
17:1(*n*-7)	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.4 ± 0.0
18:1(*n*-9)	6.1 ± 0.4	6.1 ± 0.3	15.6 ± 0.7	11.1 ± 0.1	16.7 ± 0.9
18:1(*n*-7)	1.7 ± 0.1	1.7 ± 0.1	1.7 ± 0.1	2.2 ± 0.1	2.3 ± 0.1
18:2(*n*-6)	7.5 ± 0.2	9.6 ± 0.3	9.9 ± 0.4	18.7 ± 0.9	12.0 ± 0.9
18:3(*n*-3)	0.1 ± 0.1	0.2 ± 0.0	2.3 ± 0.1	0.4 ± 0.0	2.1 ± 0.3
20:1(*n*-9)	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.0
20:2(*n*-6)	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.2	1.3 ± 0.1	0.9 ± 0.0
20:3(*n*-9)	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.5 ± 0.0	0.2 ± 0.0
20:3(*n*-6)	1.6 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	2.6 ± 0.5	2.1 ± 0.2
20:3(*n*-3)	0.1 ± 0.1	0.0 ± 0.0	0.3 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
20:4(*n*-6)	25.5 ± 2.3	46.4 ± 1.6	21.3 ± 1.4	28.4 ± 1.6	21.3 ± 1.1
20:5(*n*-3)	0.6 ± 0.0	0.9 ± 0.1	8.6 ± 0.7	1.3 ± 0.1	8.5 ± 1.2
22:4(*n*-6)	1.3 ± 0.2	1.5 ± 0.4	0.5 ± 0.1	2.2 ± 0.2	0.7 ± 0.2
22:5(*n*-6)	0.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0
22:5(*n*-3)	1.1 ± 0.1	0.7 ± 0.0	6.0 ± 0.3	1.9 ± 0.1	7.3 ± 0.6
22:6(*n*-3)	29.2 ± 2.4	5.2 ± 0.6	6.0 ± 0.7	2.2 ± 0.1	3.0 ± 0.1
% Saturates	23.7 ± 1.9	25.3 ± 1.6	25.2 ± 1.0	25.5 ± 2.2	20.9 ± 1.6
% MUFA	8.5 ± 0.4	8.3 ± 0.4	18.4 ± 0.8	14.2 ± 0.3	20.2 ± 0.9
% PUFA	67.8 ± 0.4	66.4 ± 1.7	56.4 ± 1.6	60.4 ± 2.4	58.8 ± 2.2
% *n*-9	6.3 ± 0.4	6.5 ± 0.3	15.9 ± 0.7	11.9 ± 0.2	17.2 ± 0.8
% *n*-7	2.3 ± 0.1	2.1 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	3.2 ± 0.2
% *n*-6	36.6 ± 2.1	59.2 ± 1.6	33.0 ± 1.4	53.5 ± 2.3	37.0 ± 0.5
% *n*-3	31.1 ± 2.4	6.9 ± 0.8	23.3 ± 1.2	6.3 ± 0.3	21.6 ± 2.2
% Unsaturates	76.3 ± 1.9	74.7 ± 1.6	74.8 ± 0.1	74.5 ± 2.2	79.1 ± 1.6
Unsaturation index	323 ± 12	265 ± 7	246 ± 8	219 ± 8	246 ± 11
Chain length	19.6 ± 0.1	19.0 ± 0.0	18.9 ± 0.0	18.7 ± 0.1	18.9 ± 0.1
C20+22 PUFA	60.4 ± 2.1	56.8 ± 1.4	44.6 ± 1.6	41.9 ± 1.7	45.2 ± 2.4
n-6/*n*-3	1.2 ± 0.2	8.8 ± 0.9	1.4 ± 0.1	8.4 ± 0.3	1.8 ± 0.2
20:4/18:2	3.4 ± 0.3	4.9 ± 0.1	2.1 ± 0.2	1.5 ± 0.1	1.8 ± 0.2

Microsomal phospholipid fatty acid profile of mammalian kidney expressed as mole percentage of total fatty acids. Unsaturation index is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM), n=4 for all preparations.

Table 3.4 Microsomal Phospholipid Fatty Acid Profiles from Avian Kidneys

	Zebra Finch	Sparrow	Starling	Curra-wong	Pigeon	Duck	Goose	Emu
Fatty Acid								
16:0	17.7	14.5	13.3±0.7	13.3±0.4	8.9±1.2	13.0±0.4	11.0±0.2	9.4±0.1
18:0	14.7	11.4	13.0±0.8	14.6±0.5	17.3±2.3	14.1±0.4	15.0±1.6	14.2±0.8
16:1(*n*-7)	0.5	0.4	0.4±0.0	0.4±0.0	0.5±0.1	0.4±0.0	0.7±0.1	0.8±0.1
18:1(*n*-9)	7.4	8.3	8.6±0.2	10.1±0.2	8.0±0.9	10.5±0.5	11.1±0.6	18.6±1.0
18:1(*n*-7)	1.7	1.8	2.2±0.1	1.9±0.1	2.1±0.2	2.7±0.1	1.3±0.7	0.0±0.0
18:2(*n*-6)	17.7	13.2	9.7±0.2	13.1±0.7	23.9±2.7	10.7±0.3	16.6±1.0	20.9±0.5
18:3(*n*-3)	0.2	0.0	0.2±0.0	0.3±0.0	0.3±0.1	0.2±0.0	1.3±0.7	0.1±0.0
20:2(*n*-6)	2.2	0.6	0.7±0.2	0.7±0.1	0.7±0.1	1.8±0.8	1.5±0.7	1.5±0.1
20:3(*n*-9)	0.4	0.0	0.1±0.1	0.1±0.0	0.6±0.0	0.3±0.1	0.7±0.3	1.3±0.4
20:3(*n*-6)	1.9	1.0	0.7±0.0	1.2±0.0	1.1±0.1	1.4±0.1	0.6±0.1	0.7±0.1
20:3(*n*-3)	0.0	0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.9±0.5	0.0±0.0
20:4(*n*-6)	28.3	31.5	38.0±1.1	35.0±0.9	24.7±1.8	37.7±0.4	21.3±4.1	28.5±1.1
20:5(*n*-3)	0.3	0.2	1.0±0.2	1.4±0.2	3.2±0.6	0.4±0.1	4.7±2.4	0.4±0.0
22:4(*n*-6)	1.8	2.1	1.7±0.2	1.5±0.1	1.6±0.1	3.5±0.6	1.3±0.3	1.5±0.3
22:5(*n*-6)	0.8	2.0	1.2±0.1	0.6±0.0	0.5±0.1	1.6±0.1	0.3±0.1	0.3±0.0
22:5(*n*-3)	0.6	0.5	1.1±0.1	1.0±0.0	2.0±0.3	0.1±0.0	0.7±0.2	0.2±0.0
22:6(*n*-3)	3.8	12.4	7.9±0.6	4.8±0.1	4.6±0.4	1.4±0.2	1.2±0.1	1.5±0.1
% Saturates	32.4	25.9	26.3±1.4	27.9±0.9	26.2±3.4	27.1±0.8	25.9±1.8	23.6±0.9
% MUFA	9.5	10.5	11.2±0.3	12.4±0.2	10.7±1.2	13.6±0.6	13.1±1.2	19.4±1.1
% PUFA	58.0	63.6	62.5±1.6	59.7±0.9	63.1±4.6	59.3±1.3	61.0±1.0	57.0±0.8
% *n*-9	7.8	8.3	8.7±0.2	10.2±0.2	8.6±0.9	10.8±0.4	11.8±0.4	19.9±0.8
% *n*-7	2.2	2.3	2.6±0.1	2.3±0.1	2.7±0.3	3.1±0.1	1.9±0.8	0.8±0.1
% *n*-6	52.8	50.6	52.1±1.4	52.0±0.8	52.4±3.3	56.8±1.2	51.5±4.2	53.5±0.7
% *n*-3	4.9	13.0	10.3±0.7	7.6±0.2	10.1±1.4	2.2±0.2	8.8±3.6	2.2±0.1
% Unsaturates	67.6	74.1	73.7±1.4	72.1±0.9	73.8±3.4	72.9±0.8	74.1±1.8	76.4±0.9
Unsaturation index	208	264	258±7	235±4	227±14	229±3	225±3	204±4
Chain length	18.6	19.1	19.0±0.1	18.8±0.1	18.8±0.1	18.8±0.1	18.7±0.0	18.6±0.0
C20+22 PUFA	40.1	50.4	52.5±1.6	46.3±1.1	38.9±2.3	48.5±1.5	43.1±1.3	35.9±1.2
n-6/*n*-3	10.9	3.9	5.1±0.4	6.9±0.2	5.4±0.6	27.0±3.3	13.1±6.0	25.0±0.9
20:4/18:2	1.6	2.4	3.9±0.2	2.7±0.2	1.1±0.2	3.5±0.1	1.9±0.2	1.4±0.1

Microsomal phospholipid fatty acid profile of bird kidney expressed as mole percentage of total fatty acids. Unsaturation index is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM), n=4 for all preparations except the zebra finch and sparrow where n=1.

size, although neither relationship quite reached significance (P=0.09 & P=0.08 respectively; Table 3.5). From the allometric slopes of the significant relationships in Fig. 3.6 it can be calculated that for every doubling in body mass there would be a 7.0% increase in monounsaturate content, a 1.4% decrease in polyunsaturate content and a 2.7% decrease in C20+22 PUFA content in kidney microsomal phospholipids in mammals. It is worth noting that although the allometric relationship for docosahexaenoic acid (22:6(*n*-3)) didn't quite reach significance, it showed the greatest variation of any individual fatty acid, and from the allometric exponent (-0.21) a doubling in body mass would result in a 13.5% decrease in 22:6(*n*-3) content.

In bird microsomal phospholipids there was no significant body-size-related variation observed in unsaturation index, the percentage of total unsaturates, or the percentage of total polyunsaturates (Fig. 3.6). There was however a significant allometric increase in monounsaturate content in the larger birds (P<0.01; Fig 3.6), which was similar to the trend observed in mammals and again appeared to be largely driven by changes in 18:1(*n*-9) content (Table 3.5). From the allometric exponent it can be calculated that for every 100% increase in body mass there would be a 5.0% increase in monounsaturate content. The percentage of *n*-6 PUFA was essentially constant for the birds, averaging 52.7%, while the *n*-3 PUFA content showed a non-significant but substantial decline with body size (Fig. 3.6). These changes resulted in a significant allometric elevation in the *n*-6/*n*-3 ratio with increasing body size (P=0.05; Table 3.5), such that for every doubling in body mass there would be a 14.1% increase in the content of *n*-6 PUFA relative to *n*-3 PUFA. Of the individual fatty acids palmitic acid (16:0) showed a significant allometric decrease (P=0.03), while 22:6(*n*-3) content showed a significant(P=0.01; Fig. 3.6) and substantial decrease in the larger birds. From the allometric exponent (-0.26) it can be calculated that for every doubling in body mass there would be a 16.5% reduction in 22:6(*n*-3) content.

Table 3.5 The Relationship Between Body Mass and Various Lipid Parameters in the Kidneys of Mammals and Birds

	Mammals			Birds		
	Intercept at M=1g	Exponent	Correlation coefficient	Intercept at M=1g	Exponent	Correlation coefficient
Lipid parameter						
16:0	13.8	-0.01	0.64	18.3	-0.06	0.76**
18:0	11.2	-0.00	0.09	12.9	0.02	0.35
18:1(*n*-9)	3.60	0.12	0.93**	5.54	0.10	0.89***
18:1(*n*-7)	1.52	0.03	0.71	1.95	0.00	0.03†
18:2(*n*-6)	6.29	0.06	0.73	12.2	0.03	0.30
20:3(*n*-6)	1.13	0.03	0.18	1.57	-0.07	0.51
20:4(*n*-6)	39.6	-0.04	0.52	32.3	-0.00	0.07
20:5(*n*-3)	0.21	0.27	0.81	0.33	0.14	0.32
22:4(*n*-6)	1.72	-0.05	0.34	1.98	-0.01	0.13
22:5(*n*-6)	1.20	-0.26	0.82*	2.03	-0.17	0.63*
22:5(*n*-3)	0.35	0.22	0.83*	1.28	-0.12	0.40
22:6(*n*-3)	36.7	-0.21	0.83*	16.6	-0.26	0.81**
% Saturates	25.4	-0.01	0.29	30.8	-0.02	0.69*
% MUFA	5.37	0.10	0.94**	7.84	0.07	0.91***
% PUFA	72.6	-0.02	0.91**	62.8	-0.01	0.42
% n-9	3.79	0.12	0.95**	5.56	0.10	0.91***
% n-7	1.80	0.04	0.88**	3.52	-0.08	0.57
% n-6	46.9	-0.01	0.16	51.0	0.01	0.41
% n-3	17.9	-0.02	0.12	15.3	-0.15	0.58
% Unsaturates	74.7	-0.00	0.27	69.3	0.01	0.67*
UI	340	-0.03	0.86*	254	-0.02	0.48
Chain length	19.7	-0.00	0.87*	19.0	-0.00	0.43
C20+22 PUFA	69.8	-0.04	0.96**	50.7	-0.02	0.46
n-6/n-3	2.72	0.01	0.04	2.96	0.19	0.72**
20:4/18:2	6.22	-0.10	0.86*	2.66	-0.04	0.23
Cholesterol ($\mu g.mg\ protein^{-1}$)	37.0	0.02	0.64	65.9	-0.03	0.63*
Phospholipid ($\mu g.mg\ protein^{-1}$)	210	0.01	0.32	442	-0.05	0.82**
Chol:PL (mol:mol)	0.37	0.01	0.45	0.30	0.02	0.30

*The relationship between body mass and the various lipid parameters were determined by linear regression (least-square method) of log-transformed values. The relationships were determined using the mean parameter value for each species (n=5 for mammals and n=8 for birds). *P<0.1, **P<0.05, ***P<0.01. † relationship determined using n=7 (see Table 3.4).*

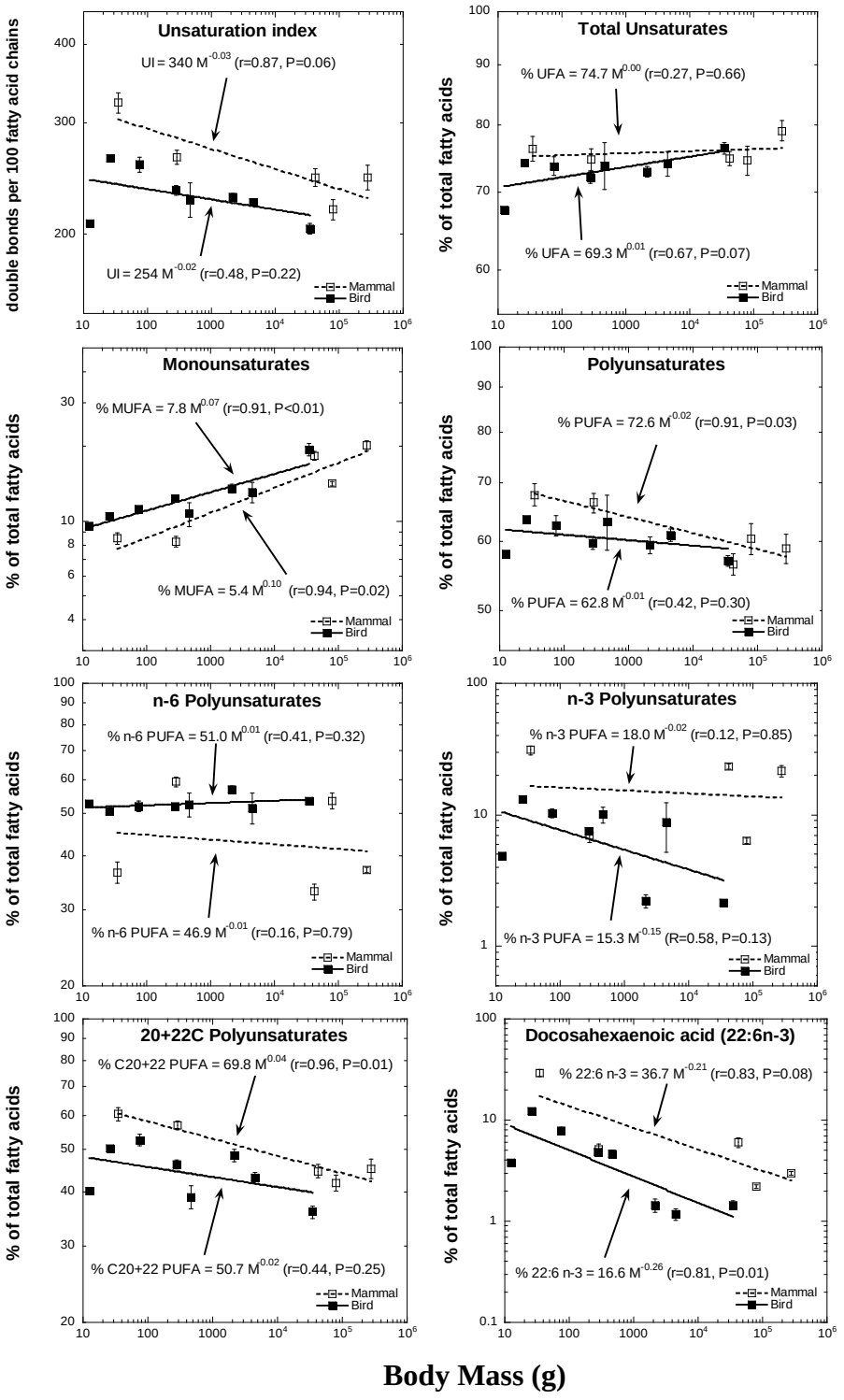


Fig. 3.6 *The relationship between body mass and the unsaturation index, content of unsaturates, monounsaturates, polyunsaturates, n-6 PUFA, n-3 PUFA, C20+22 PUFA and docosahexaenoic acid in kidney microsomal phospholipids from mammals and birds. Values are means ± standard errors (SEM).*

Table 3.6	Comparison of a 250g Mammal and Bird	
	Mammal	**Bird**
Kidney mass (g)	2.19	1.47
Protein concentration (mg.g wet weight^{-1})	201	176
Na^+K^+ATPase activity (μmol Pi.mg wet weight^{-1}.hr^{-1})	3.63	2.98
Sodium pump density (pmol.g wet weight^{-1})	4713	8143
Sodium pumps per kidney	6.1×10^{15}	7.5×10^{15}
Molecular activity (ATP.min^{-1})	16255	6706
Unsaturation index	288	227
% Unsaturates	75.5	73.2
% Monounsaturates	9.4	11.5
% Polyunsaturates	65.0	60.8
% *n*-6	44.4	52.4
% *n*-3	16.1	6.7
% 22:6(*n*-3)	11.5	4.0
Cholesterol:Phospholipid (mole:mole)	0.39	0.34

Values were calculated from the allometric equations determined for each parameter, using a body mass of 250 grams.

The allometric equations determined in the current study were used to predict the value for a number of parameters in a medium-sized (250g) mammal and bird (Table 3.6). Comparison of the mammal and bird shows that the kidney of the bird would be 33% smaller than the mammal and would contain only 88% as much protein per gram wet weight. Na^+K^+ATPase activity in the bird would be 82% that of the value for the mammal despite having 73% more sodium pumps per gram of wet weight & 23% more sodium pumps overall in the kidney. The sodium pump molecular activity of the bird would be approximately 60% lower than the mammal. Examining the microsomal phospholipids, it can be seen that the bird would possess 97% of the unsaturated fatty acids of the mammal, while having an unsaturation index that would be 78% of the value for the mammal. The bird phospholipids would have 22% more monounsaturates

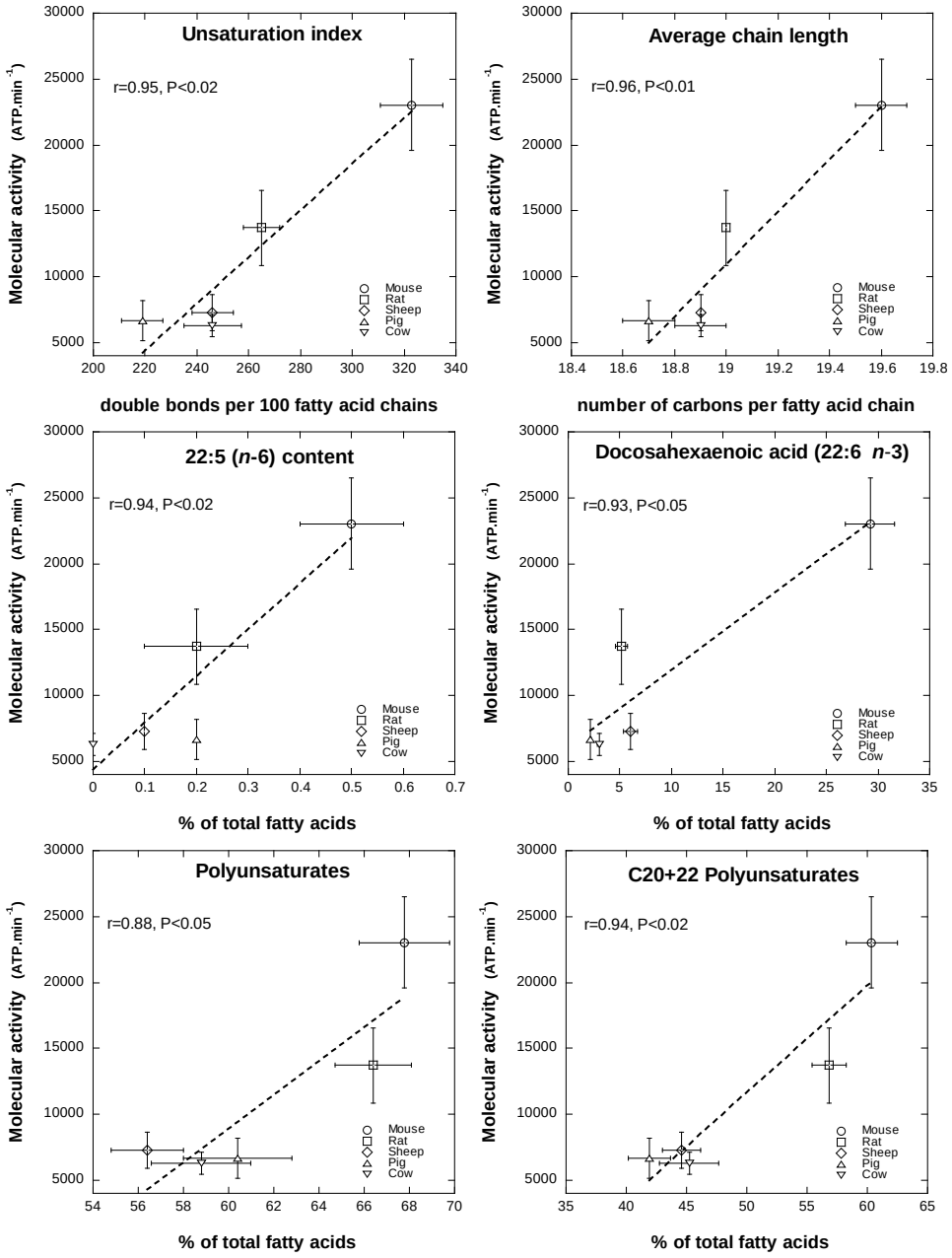


Fig. 3.7 *Linear correlations between sodium pump molecular activity in mammals and unsaturation index, average chain length and the content of 22:5(n-6), 22:6(n-3), polyunsaturates and C20+22 polyunsaturates.*

and 6% less polyunsaturates than the mammal. The *n*-6 PUFA content of the bird would be 18% higher, the content of *n*-3 PUFA would be 58% lower, and as a result

the *n*-6/*n*-3 ratio would be almost 3-fold greater in the bird. The 22:6(*n*-3) content in the phospholipids from the mammal would be approximately two-thirds greater than that of the bird. The molar ratio of cholesterol:phospholipid in bird microsomal membranes would be 87% of the value for the mammal.

To assess whether membrane lipids may have been influencing the molecular activity of the sodium pumps in the mammals and birds in the current study, linear correlation coefficients were determined between all individual lipid parameters and sodium pump molecular activity. In the mammals there were six lipid parameters that displayed significant positive correlations with molecular activity (Fig. 3.7). These parameters included the content of 22:5(*n*-6) and 22:6(*n*-3), the percentage of total polyunsaturates and C20+22 polyunsaturates, the unsaturation index and the average chain length. In birds only the molar ratio of cholesterol:phospholipid was correlated with molecular activity (Fig. 3.8), displaying an inverse relationship.

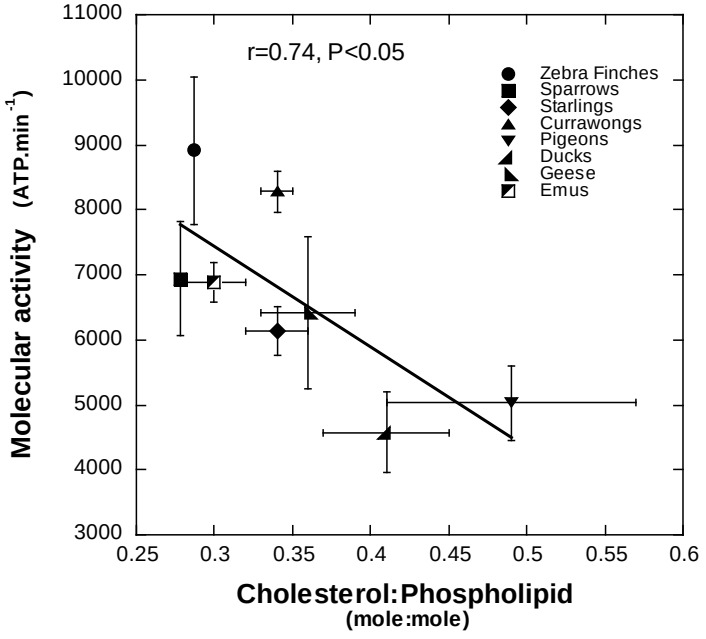


Fig. 3.8 *Linear correlation between sodium pump molecular activity in birds and the molar ratio of cholesterol to phospholipid.*

3.3 DISCUSSION

In the current chapter, mechanisms underlying differences in the metabolic rate of mammals and birds of different body size were investigated. Specifically the relationship between sodium pump molecular activity, membrane lipid composition and body mass was examined in the kidney of mammals and birds with a 9-fold and 22-fold range in BMR respectively. In both groups sodium pump (Na^+K^+ATPase) activity was higher in smaller species and declined with body mass (Fig. 3.3). These results are consistent with those reported for mammals by Couture and Hulbert (1995b) and indicate that the overall activity of the sodium pump reflects differences in metabolism. One of the major goals in this study however, was to determine whether the increased Na^+K^+ATPase activity observed in smaller mammals and birds was the result of an increased number of sodium pump molecules, an increased molecular activity of individual sodium pumps, or a combination of both. The mammals and birds differed in this respect using contrasting strategies to achieve changes in Na^+K^+ATPase activity. In mammals, the differences seen in Na^+K^+ATPase activity resulted primarily from changes in the molecular activity of their sodium pumps, which varied from 7,643 to 24,095 ATP.min^{-1} (Table 3.1). There was considerably less variation in the molecular activity determined for the birds (4,577 to 8,908 ATP.min^{-1}, Table 3.1) with Na^+K^+ATPase activity alteration achieved mainly by changing the number of sodium pumps in their kidneys (Fig. 3.4).

While there was a large range of molecular activity values determined in the mammals in the current study, most of the values are comparable to those previously reported for mammalian kidney (Bader et al., 1968; Jørgensen, 1986; Else et al., 1996). Sodium pump measurements in birds are more scarce and have primarily concentrated on salt glands, however the molecular activity in geese kidney has been reported to be 11,100 ATP.min^{-1} (Bader et al., 1968), which is almost twice the level found in the current study. Bader et al. (1968) determined molecular activity by examining the rate of appearance of the phosphorylated intermediate during the sodium pump cycle. The reason for the discrepancy between that method and the technique employed in the

current study is unknown. When comparing molecular activity values from the literature, one problem that is encountered is that the values have been determined on a variety of preparations, including homogenates, microsomes and purified pumps, that have been prepared from different sections of the kidney (cortex/medulla). Also the molecular activities have been determined using various measurement techniques for both enzyme activity and enzyme concentration. Schmidt et al. (1992) noted that during the sodium pump purification process in rat brain, there was an invariable loss of membrane material and hence the recovered sodium pumps may not be an accurate representation of the total population. A major strength of the current investigation is that all sodium pump measurements were carried out on biopsies and homogenates, and thus there was no loss of membrane material due to purification procedures. Furthermore all species were assayed using the same treatment conditions and are therefore directly comparable.

One question that arises is whether the molecular activity differences found between the various species are the result of isoform differences in the catalytic subunit? A number of different isoforms exist for both subunits of the sodium pump (Sweadner, 1989; Kaplan, 2002). Expression of these isoforms appears to be tissue-specific, with α_1 being the primary isoform found in the kidney of both mammals and birds (Sweadner, 1989; Horisberger, 1994). This isoform is common to all vertebrates, and is considered the 'housekeeping' form of the sodium pump. While α_1 may be the only isoform present in the kidney of all the species from the current study, this isoform does display some subtle kinetic differences in different animals, most notably, being considered relatively ouabain insensitive in rodents. To ensure that this had no effect on the measures from the mice and rats in the current study, saturating concentrations of ouabain (10-50μM) were used in both the Na^+K^+ATPase activity assay and the [^{3}H]-ouabain binding. This ensured that the variation observed in molecular activity was the result of differences in the intrinsic activity of the sodium pumps and not experimental error.

Many studies examining the characteristics of the sodium pump have focussed on the kidney, as it is an abundant source of sodium pump molecules (Jørgensen, 1974). The

sodium pump functions in the kidney to maintain the electrochemical gradient for sodium, which in turn acts as a driving force for secondary active transport of nutrients, metabolites and ions as well as the secretion of organic acids (Jørgensen, 1986). Furthermore the sodium pump is involved in the regulation of cell volume, the control of pH, the maintenance of ion homeostasis and is a key protein involved in reabsorptive mechanisms in kidney tubules (Jørgensen, 1980). So important is this enzyme to kidney function that up to 70% of kidney O_2 consumption has been linked to the sodium pump (Clausen et al., 1991).

In the present study all measurements were carried out on mixed sections of cortex and medulla and there were several reasons for this. Firstly it is difficult to delineate the border between the two regions in avian kidneys (Goldstein and Braun, 1989), so transverse sections were used. A similar problem was also faced in the mice where the kidneys are extremely small and trying to isolate the cortex or medulla alone would have proved difficult. Finally it has been shown that along the length of the nephron there are different concentrations of sodium pumps, that display different molecular activities depending on their function (Jørgensen, 1986). The outer medulla and cortex in particular have very high concentrations of sodium pumps and it is unclear whether these two regions account for the same proportion in kidneys of different sized vertebrates. Thus to allow direct comparisons between species mixed sections of cortex and medulla were used.

The kidney mass of the mammals and birds from the present study had allometric exponents of 0.81 and 0.83 respectively, which is similar to the findings of other researchers (Peters, 1983; Else and Hulbert, 1985). Na^+K^+ATPase activity was combined with kidney mass and expressed as the micromoles of inorganic phosphate liberated (from ATP) per kidney per hour, and assuming that this value represented the maximum rate, and using a P/O ratio of 2.0 (Rolfe and Brown, 1997), it was possible to determine the potential maximal daily energy expenditure by the sodium pumps

(Kcal.day^{-1}). When these values were compared to the BMR values for the different species (Fig. 3.9), they

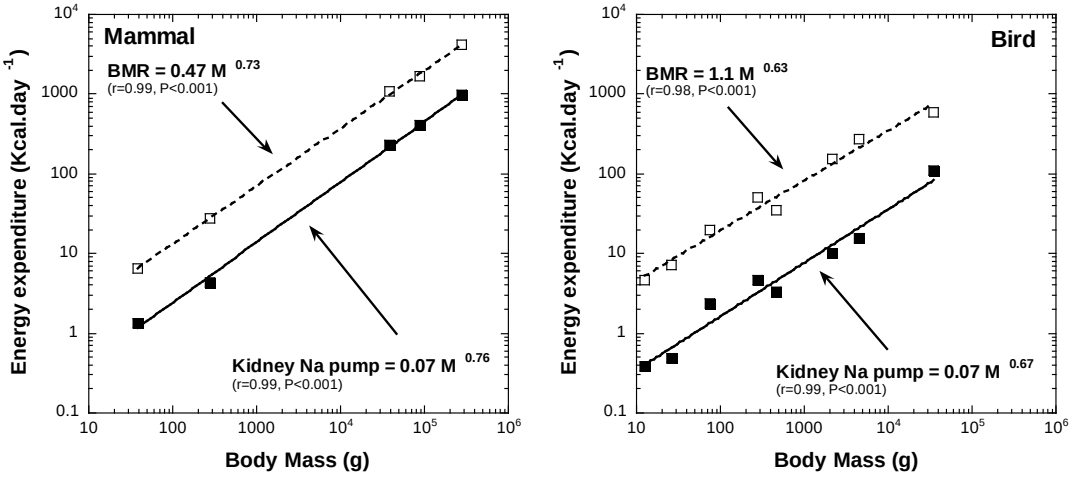


Fig. 3.9 *The relationship between the body mass of mammals and birds, basal metabolic rate and the potential maximal daily energy expenditure of kidney sodium pumps.*

represented approximately 20% and 10% of basal energy use in the mammals and birds respectively. Also of interest was the fact that in both groups the allometric exponents for sodium pump energy expenditure (0.76 and 0.67) were very close to the BMR exponents of 0.73 and 0.63 seen the mammals and birds respectively, indicating that maximal kidney Na^+K^+ATPase activity correlates well with basal metabolism and likely constitutes a constant proportion of metabolic activity irrespective of body size and metabolic intensity.

In intact mammalian kidney (rat, rabbit, dog, pig), the reabsorptive rate of sodium has been reported to be 6-24 μmol Na^+.g wet wt^{-1}.min^{-1}, with 24-54% of this sodium reabsorption linked to the sodium pump (Jørgensen, 1980). From these values *in vivo* sodium pump activity would be between 0.03 and 0.26 μmol ATP.mg wet wt^{-1}.hr^{-1}, which is approximately 10% or less than the potential maximal *in vitro* values

determined in the current study (see Table 3.1). Couture and Hulbert (1995) also measured *in vivo* sodium pump activity, as potassium uptake rate in kidney cortex slices. Their activity values ranged between 0.03 and 0.08 μmol ATP.mg wet $wt^{-1}.hr^{-1}$, which are lower than those reported for intact kidney, because in tissue slices, tubule lumens probably collapse and activity values likely solely represent the cost of self-maintenance, compared with self-maintenance and ultrafiltrate reabsorption in the case of *in vivo* kidney. Their activity values represent 1.8, 2.4, 1.8, and 1.1% of the maximal *in vitro* values for the mice, rats, sheep, and cattle respectively. Thus it appears that sodium pumps are only operating at a low percentage of their potential maximal capacity under normal conditions. It is interesting to note that during an osmotic stress, such as water deprivation, smaller mammals and birds have demonstrated a greater ability to concentrate their urine than their larger counterparts (Brownfield and Wunder, 1976; Goldstein and Braun, 1989). This may be due to superior reabsorptive mechanisms, such as increased sodium pump capacity.

A fairly constant level of protein was found in the kidney of mammals and birds of differing body size in the present study (Fig. 3.2). These results are consistent with those reported by Couture and Hulbert (1995b), who found that the mammals in their study had a relatively constant amount of protein in their kidney. In a previous investigation, the high level of metabolism in tissues of an endothermic mammal was associated with significantly more protein than an ectothermic reptile (Hulbert and Else, 1989). In the case of the kidney, the mammal contained 38% more protein than the reptile.

The rate of protein synthesis in mammals is directly proportional to BMR, scaling at $mass^{0.76}$ (Munro, 1969). While it is unknown whether all proteins in smaller mammals follow a similar pattern to the sodium pump, i.e. displaying increased molecular activity compared to larger species, the fact that the synthesis of new proteins scales negatively with body size, potentially suggests that this may be the case. A possible explanation is that since kidney protein levels are relatively constant in various mammals, the increased protein synthesis observed in smaller mammals may indicate that individual proteins

have a finite number of substrate cycles, and due to their increased molecular activity, the proteins in smaller mammals may have a reduced half-life, therefore requiring more regular replacement. The rate at which protein synthesis scales in birds is currently unknown and is an area worthy of investigation.

The increased sodium pump activity measured in smaller mammals and birds may be required to maintain the normal transmembrane concentrations of sodium and potassium as elevated metabolic rate has previously been linked to increased permeability of cell membranes. Liver slices and isolated hepatocytes from the bearded dragon lizard have reduced sodium and potassium permeability when compared to the more metabolically active rat (Else and Hulbert, 1987). Furthermore proton permeability in liver mitochondria is also reduced in the bearded dragon compared to the rat (Brand et al., 1991), while proton permeability is also increased in smaller mammals (Porter and Brand, 1993) and smaller birds (Brand et al., 2003) when compared to their larger counterparts. While passive sodium permeability of cells from mammals and birds of different body size has yet to be measured, the increased potassium ($^{86}Rb^{+}$) uptake rate measured in smaller mammals by Couture and Hulbert (1995b) indicates that permeability differences may potentially be a determining factor for the higher sodium pump activity observed in smaller species.

The second major part of this study was to investigate membrane lipid composition in the mammals and birds and to examine whether it was potentially a factor related to the molecular activity of their sodium pumps. Previous investigations have demonstrated links between membrane fatty acid composition and body mass in various tissues (heart, skeletal muscle, kidney and liver) in mammals (Couture and Hulbert, 1995a; Hulbert et al., 2002c), in skeletal muscle in birds (Hulbert et al., 2002b) and in liver mitochondria from mammals (Porter et al., 1996) and birds (Brand et al., 2003). In the current investigation microsomal membranes were examined in preference to whole tissue, as phospholipids isolated from microsomes are more representative of the lipids and fatty acids that would be directly surrounding proteins in the plasma membrane (eg sodium

pump) rather than whole tissue phospholipids that would also contain nuclear and mitochondrial membrane fractions.

The body-size-related variations in fatty acid composition observed in the mammalian microsomal phospholipids from the present study are similar to those previously observed in whole tissue phospholipids from the kidney (Couture and Hulbert, 1995a; Hulbert et al., 2002c) and also mammalian liver mitochondria (Porter et al., 1996), indicating that allometric variation in membrane fatty acid composition is a phenomenon which may occur in all subcellular membranes. The percentage of total unsaturates, *n*-6 PUFA and *n*-3 PUFA showed no significant allometric variation, while there was a significant allometric increase in monounsaturate content, a close to significant (P=0.06) decrease in unsaturation index, and significant allometric decreases in the percentage of total polyunsaturates and C20+22 PUFA (Fig. 3.6). Docosahexaenoic acid (22:6(*n*-3)) showed the greatest variation of any individual fatty acid displaying an allometric exponent of –0.21 (P=0.08), which is equal to that previously reported for whole tissue phospholipids from mammalian kidney (Hulbert et al., 2002c), and is also close to the allometric exponent describing mass-specific BMR.

In bird microsomal phospholipids there was a significant allometric increase in monounsaturate content and the ratio of *n*-6/*n*-3 fatty acids in the larger birds, as well as a significant decrease in 22:6(*n*-3) (Table 3.5). The content of palmitic acid (16:0) also showed an allometric decrease with increasing body size (Table 3.5), which is a trend that was observed in bird skeletal muscle phospholipids (Hulbert et al. 2002b). The percentage of total unsaturates, total polyunsaturates, *n*-6 PUFA, *n*-3 PUFA, C20+22 PUFA, and unsaturation index content all displayed no allometric variation with body size (Fig. 3.6). Many of these variables that displayed no significant variation were heavily influenced by the fatty acid profile of the zebra finch, which was quite anomalous compared to the other birds, and deviated substantially from the regression line (see Fig. 3.6). In particular, phospholipids from the finches contained low amounts of 22:6(*n*-3), which is generally the longest and most polyunsaturated fatty acid

occurring in membranes and therefore affects a large number of composite parameters. Omission of the zebra finch from the regression calculations (i.e. n=7), revealed significant body-size-related variation in many parameters (eg % polyunsaturates, *n*-3 PUFA, % C20+22 PUFA, unsaturation index,), with the allometric trends closely resembling those observed in mammals (see Fig. 3.6). In particular this effect was very pronounced for 22:6(*n*-3) content, whose variability, when the zebra finch was excluded, was almost completely (98%) explained by changes in body mass. Furthermore the deviation of the finches for this fatty acid could be highlighted by using the equation derived for the 7 remaining birds, which would predict the content of 22:6(*n*-3) in a zebra finch with a body mass of 12.6g to be 13.8%, which is approximately 3.5 times greater than the value actually observed (3.8%). Despite this unusual composition the allometric slope describing the variation of 22:6(*n*-3) was –0.26 (Fig. 3.6), which as was the case in mammals, is close to the allometric exponent describing mass-specific BMR. Collectively these results indicate that allometric variation in fatty acid composition may be a phenomenon that is a general trait of endotherms, despite mammals and birds having evolved endothermy independently. Furthermore these results support the notion that membrane fatty acid composition, and in particular 22:6(*n*-3) content, may be an important 'pacemaker' of animal metabolism in general (Hulbert and Else, 1999; Hulbert and Else, 2000).

The fatty acid composition of membranes is highly regulated and although the relative occurrence of various fatty acids may be influenced by their presence or absence in the diet, it is difficult to substantially alter phospholipid fatty acid composition through dietary manipulation. The main parameter that appears to be affected by diet is the relative percentage of *n*-6 and *n*-3 PUFA in the membrane (Pan and Storlien, 1993; Ayre and Hulbert, 1996). It would seem that none of the mammals and birds from the current study consumed a diet deficient in polyunsaturates as indicated by the low levels of mead acid (20:3(*n*-9); Tables 3.3 & 3.4). The presence of this unusual fatty acid is diagnostic of a dietary deficiency in essential fatty acids, as it is only synthesised in substantial amounts when there is a lack of *n*-6 & *n*-3 PUFA in the diet. The low levels

found in the current study (maximum 1.3%) suggests that the allometric variation in fatty acid composition observed in the mammals and birds was unlikely the result of dietary deficiency.

Although the diet of most of the species was unknown before the study, the phospholipid content of *n*-6 and *n*-3 PUFA provides some insight into what the food habits of some species may have been. The phospholipids from the sheep and cattle contained high proportions of a number of *n*-3 polyunsaturates (e.g. 18:3(*n*-3), 20:5(*n*-3) and 22:5(*n*-3)), as has been observed in previous studies (Couture and Hulbert, 1995a; Porter et al., 1996), which may indicate that they were pasture-fed. Pasture-fed animals tend to have higher levels of *n*-3 PUFA in their phospholipids compared to grain-fed animals of similar size whose phospholipids are dominated by *n*-6 PUFA (Cordain et al., 2002). The explanation for this probably lies in the fact that forage crops tend to contain high levels of 18:3(*n*-3), while grains tend to contain high levels of 18:2(*n*-6) (Christie, 1981). The preference of the desaturase enzymes is reported to be *n*-3>*n*-6 (Stubbs and Smith, 1984) and hence it is likely that the sheep and cattle were pasture-fed as 18:3(*n*-3) would have been the main precursor for their long chain PUFA and subsequently that is why their membranes contain high proportions of long chain *n*-3 PUFA. Similar observations were also made for the geese in the current study, two of which were purchased from a local bird auction while two were caught in the wild. The environment in which the wild geese were caught was rich in vegetation and these two geese had membrane *n*-3 PUFA levels of approx 15% which was greater than 5 times that of the purchased geese, whose diet was likely a commercial mixture, and whose membrane *n*-3 PUFA levels were 2.6%.

While it is unclear why the fatty acid profile of the zebra finch phospholipids was so disparate compared to the other birds, particularly in the content of 22:6(*n*-3), diet was possibly a contributing factor. The other passerines in the current study (sparrows, starling and currawongs) were all captured in the wild, while the zebra finches were purchased at a pet shop. The diet of the finches at the pet shop was a commercial seed

mixture and it is unlikely that any of the finches were recently captured in the wild but is more plausible that they were the progeny of many generations bred in captivity. Most seeds generally contain low levels of *n*-3 PUFA (Harwood, 1998) and if the finches were consuming a slightly *n*-3 deficient diet not only for their entire life, but also for a number of generations beforehand, it may be a potential explanation for their anomalous levels of *n*-3 PUFA. This hypothesis could be investigated by examining some wild caught finches or other similar sized wild bird species.

The rats and mice in the current study consumed exactly the same diet, however there were very large differences in their fatty acid profiles (Table 3.3). The rats were dominated by *n*-6 PUFA, particularly 20:4(*n*-6), while the mice phospholipids contained high levels of *n*-3 PUFA and in particular 22:6(*n*-3). Thus while diet has some influence on membrane fatty acid composition, other regulatory mechanisms within animals appear to be very important in providing the specific composition required by each animal. Apart from the elongase and desaturase enzyme systems, there is also constant remodelling of the membrane through deacylation-reacylation cycles (Schmid et al., 1995). The role of these regulatory processes in determining the allometric variation in membrane fatty acid composition is unknown for both mammals and birds.

Possibly the most important fatty acid was 22:6(*n*-3), which would not have been present in significant amounts in the diet of any of the species, but whose content in the membrane varied dramatically and was very strongly related to body mass and also possibly metabolic rate. The synthesis and modification of most fatty acids appears to occur in the endoplasmic reticulum, however it has been proposed that the final step in the synthesis of 22:6(*n*-3) involves a single cycle of β-oxidation of 24:6(n-3) in peroxisomes (Sprecher, 2000). The terminal fatty acid in the *n*-6 pathway, 22:5(*n*-6), is also thought to undergo a similar method of biosynthesis, and although it was present in the membrane in much lower amounts, it showed a very similar allometric variation to that observed for 22:6(*n*-3) in both the mammals and birds (Table 3.5). Thus while it is unclear why the content of 22:6(*n*-3), and to a lesser extent 22:5(*n*-6), displays such

dramatic allometric variation, it is possible that the unusual and complex synthetic pathway that produces these fatty acids may be a regulatory factor that determines the changes observed with body size.

The high molecular activity observed in the sodium pumps of smaller mammals correlated with a variety of fatty acid parameters that are all indicators of membrane PUFA levels (Fig. 3.7). These results support previous investigations of metabolically diverse species, where the high metabolism observed in endotherms compared to ectotherms was associated with increased sodium pump molecular activity (Else et al., 1996). Using 'species crossover' experiments between rats and toads, these differences have been shown to be determined by membrane lipids, with membranes containing high levels of PUFA and low levels of MUFA supporting higher molecular activity levels (Else and Wu, 1999). Similar relationships have also been observed between liver mitochondrial membrane PUFA and mitochondrial proton leak in comparisons of endotherms and ectotherms (Brand et al., 1991; Brookes et al., 1998) as well as in mammals of different body size (Porter et al., 1996). The Ca^{2+}ATPase, which is a significant component of metabolism, particularly in muscles, has also been found to be highly active in membranes that contain high levels of PUFA, especially 22:6(*n*-3) (Infante, 1987; Infante et al., 2001). Comparison of a theoretical 250g mammal and a 250g bird (Table 3.6) in the current study would also tend to suggest the existence of such a relationship. The membrane of the mammal would have increased PUFA content along with decreased MUFA levels, and this fatty acid composition would support a higher molecular activity at 37ºC.

Within the birds, the range of sodium pump molecular activity measured was quite modest (Table 3.1) and displayed no significant allometric variation. Membrane fatty acid composition however was related to body size and subsequently no correlation was observed between any fatty acid parameters and sodium pump molecular activity. The molecular activity values measured in the birds did correlate with the level of cholesterol in the membrane, measured as the molar ratio of cholesterol-to-phospholipid (Fig. 3.8).

Specifically, higher ratios of cholesterol:phospholipid were associated with decreased molecular activity levels. Reduced activity of membrane ATPases (Bastiaanse et al., 1997), including the Na^+K^+ATPase (Yeagle, 1983; Yeagle et al., 1988; Crockett and Hazel, 1997), have previously been observed in membranes containing high levels of cholesterol. Cholesterol is found predominantly in the plasma membrane of cells (Stryer, 1995), and in monolayers cholesterol interacts with phospholipids to reduce the mean area per molecule and thus condense the membrane (Stillwell et al., 1994; Zerouga et al., 1995). The effect of cholesterol on membrane protein activity is thought to be linked with changes in membrane properties such as fluidity (Bastiaanse et al., 1997), ion permeability (Bretscher and Munro, 1993) and bilayer thickness (Cornelius, 2001).

While the mammals and birds in the present study did display similar allometric variation in some aspects of membrane fatty acid composition, these changes only appeared to be linked to sodium pump molecular activity in the mammals, and not in the birds where the relatively constant sodium pump molecular activity was only related to cholesterol levels. While these differences existed within the mammals and birds, comparison of the two groups suggested that the total level of membrane unsaturation (unsaturation index) may have been the most important determinant of overall molecular activity levels as there was a striking similarity between the allometric relationships for molecular activity (Fig. 3.5) and unsaturation index (upper left panel in Fig. 3.6). Whether these relationships are specific for the kidney or are general for all tissues in mammals and birds will be investigated in the subsequent chapters that examine the heart and brain.

Chapter IV

Sodium Pump Molecular Activity and Membrane Lipid Composition in the Heart of Mammals and Birds of Different Body Size

4.1 INTRODUCTION

This chapter will examine allometric variation of sodium pump molecular activity and membrane lipid composition in the heart of mammals and birds. Previous studies have shown that tissue phospholipids from the hearts of smaller mammals are more polyunsaturated and less monounsaturated than phospholipids from larger mammals (Couture and Hulbert, 1995a; Hulbert et al., 2002c), although whether similar trends also occur in birds, has never been examined.

Sodium pump molecular activity has never been systematically examined in the heart of mammals or birds of varying body size. Molecular activity values have been measured in the heart of several mammals, with similar values found despite large differences in body mass and hence BMR (Pitts and Schwartz, 1975; Else et al., 1996). Comparisons of endotherms and ectotherms have also shown similar heart molecular activity values (Else et al., 1996) despite large differences in BMR between these two groups. This chapter therefore systematically analyses heart sodium pump molecular activity and membrane lipid composition in mammals and birds of different body size, by examining them under identical experimental conditions. The aim was to elucidate whether allometric changes in either variable may be a factor contributing to the metabolic variation observed in these endothermic species.

4.2 RESULTS

Individual values for heart mass and protein concentration from the mammals and birds are presented in Appendix III. Both variables were considered with respect to body mass and are presented in Fig. 4.1 & Fig. 4.2 respectively. Birds had larger hearts than those of similar sized mammals. The correlation coefficient for heart mass in both groups, show that this variable was almost solely determined by body mass (Fig. 4.1). For the mammals and birds used, the mass of the heart in mammals represented a fairly constant proportion of body mass, while for birds, the smaller species had relatively

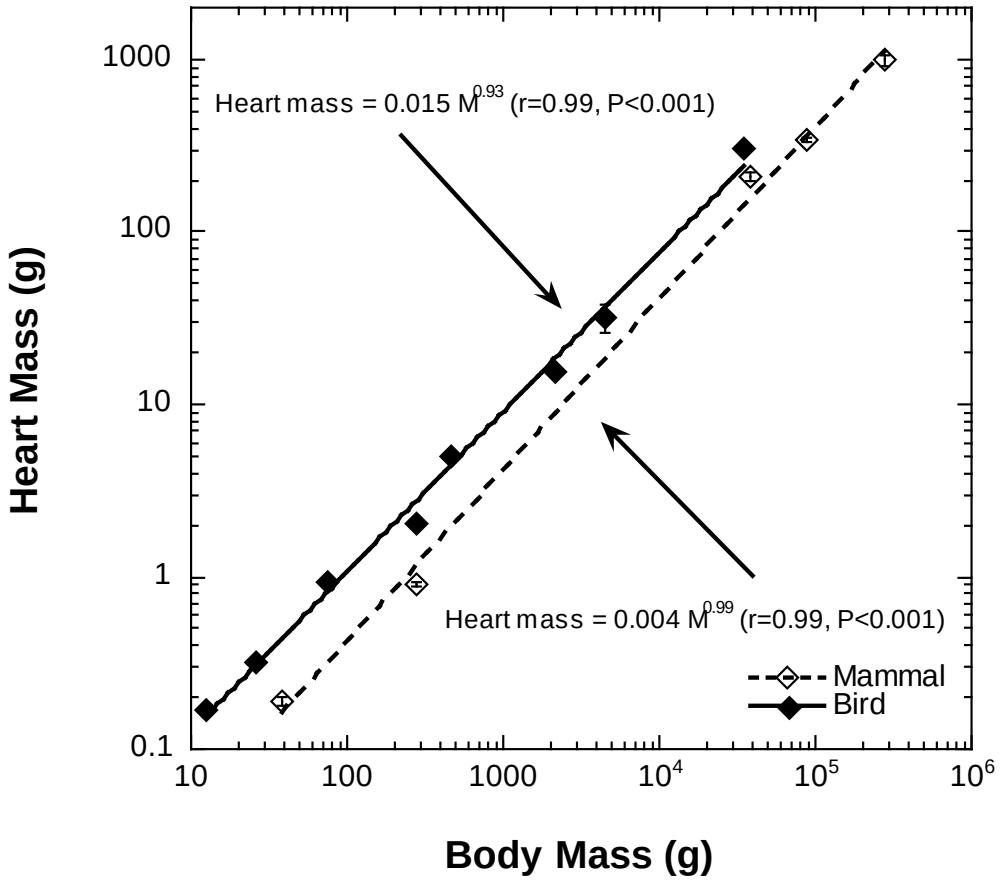


Fig. 4.1 *The relationship between the body mass of mammals and birds and the mass of their hearts. Each point represents the mean ± standard error (SEM).*

larger hearts than the bigger birds. Protein concentration, expressed as milligrams of protein per gram of heart wet weight, showed no relationship with body size in either mammals or birds (Fig. 4.2). The mean protein concentration in mammals was 212 ± 5 mg.g wet wt^{-1}, which was significantly (P<0.001) higher than the mean protein concentration (174 ± 4 mg.g wet wt^{-1}) found in birds.

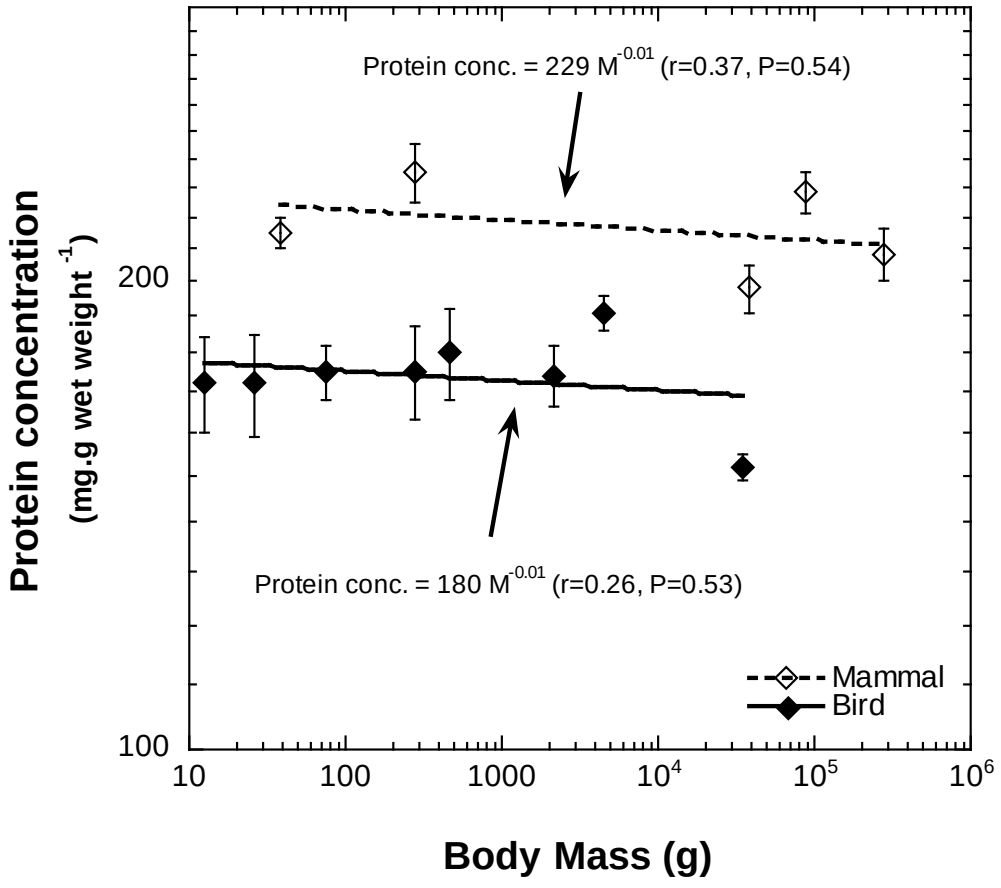


Fig. 4.2 *The relationship between the body mass of mammals and birds and heart protein concentration. Each point represents the mean ± standard error (SEM).*

Table 4.1 Na^+K^+ATPase Activity, Sodium Pump Density, and Molecular Activity of Sodium Pumps from the Heart of Mammals and Birds

	Na^+K^+ATPase Activity (μmol Pi.mg wet $wt^{-1}.hr^{-1}$)	Sodium Pump Density (pmol.g wet wt^{-1})	Molecular Activity ($ATP.min^{-1}$)
Mammals			
Mouse	0.57 ± 0.09 (n=12)	2258 ± 555 (n=9)	5518 ± 1155 (n=9)
Rat	0.50 ± 0.07 (n=12)	1761 ± 365 (n=8)	7362 ± 1892 (n=8)
Sheep	0.50 ± 0.04 (n=8)	3298 ± 618 (n=8)	3285 ± 672 (n=8)
Pig	0.38 ± 0.07 (n=7)	4217 ± 971 (n=7)	1680 ± 370 (n=7)
Cow	0.37 ± 0.06 (n=7)	2508 ± 548 (n=7)	3693 ± 1149 (n=7)
Birds			
Zebra Finch	0.65 ± 0.08 (n=4)	1066 ± 222 (n=3)	10832 ± 953 (n=3)
Sparrow	0.71 ± 0.06 (n=4)	842 ± 116 (n=4)	15316 ± 3346 (n=4)
Starling	0.54 ± 0.11 (n=4)	1762 ± 422 (n=4)	7098 ± 3486 (n=4)
Currawong	0.53 ± 0.05 (n=4)	1840 ± 382 (n=4)	5298 ± 802 (n=4)
Pigeon	0.27 ± 0.03 (n=4)	3270 ± 1174 (n=3)	1618 ± 437 (n=3)
Duck	0.24 ± 0.05 (n=4)	1454 ± 155 (n=3)	3143 ± 299 (n=3)
Goose	0.31 ± 0.05 (n=4)	1584 ± 554 (n=4)	4992 ± 1947 (n=4)
Emu	0.32 ± 0.05 (n=4)	1542 ± 129 (n=4)	3621 ± 859 (n=4)

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram of wet weight each hour. Sodium pump density was measured as picomoles of sodium pumps per gram of wet weight. Molecular activity is maximal Na^+K^+ATPase activity divided by sodium pump density for the same animal, and is expressed as the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). (n) is the number of preparations used for each measurement.

Na^+K^+ATPase activity, sodium pump density, and molecular activity values from the heart of the mammals and birds are presented in Table 4.1. Na^+K^+ATPase activity values were measured at 37ºC for mammals and corrected to 37ºC for birds (see METHODS), and are expressed as micromoles of inorganic phosphate liberated (from

ATP) per mg of heart wet weight per hour. When examined relative to body mass, there was a significant decrease in Na^+K^+ATPase activity for both the mammals (P=0.05) and birds (P=0.02) (Fig. 4.3). These allometric trends are similar to those observed in the kidney (Fig. 3.3), with the birds once again displaying a steeper relationship. From the allometric exponents (-0.04 and –0.13) it can be calculated that for every doubling in body mass there would be a 2.7% and 8.6% decrease in Na^+K^+ATPase activity for the mammals and birds respectively.

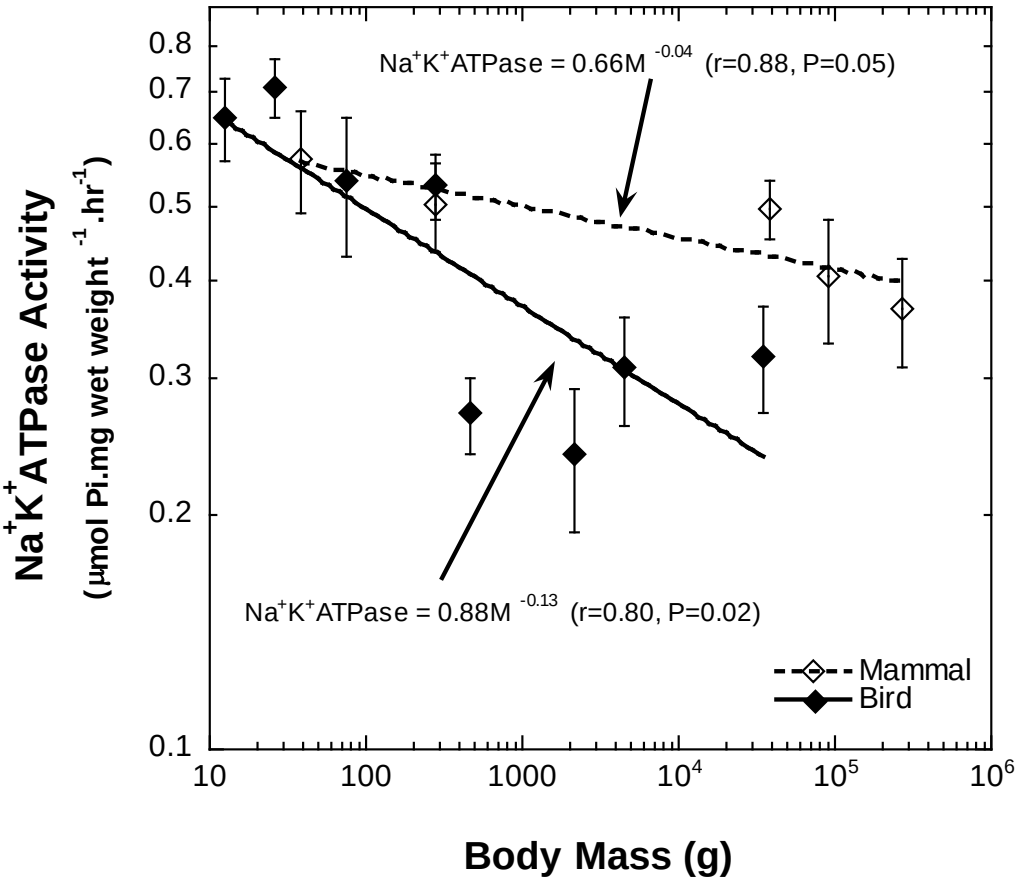


Fig. 4.3 *The relationship between the body mass of mammals and birds and the Na^+K^+ATPase activity of heart homogenates at 37ºC. Each point represents the mean ± standard error (SEM).*

Sodium pump density was measured as picomoles per gram of heart wet weight and was generally higher in the mammals compared to the birds. Sodium pump density was considered relative to body mass (Fig. 4.4), and although the relationship was not significant (P=0.22), there was a tendency for increased sodium pump density in the larger mammals. There was large variation in sodium pump density in bird hearts, with body mass only explaining approximately 4% of the variability. When sodium pump density was combined with heart mass, it can be calculated that the number of individual sodium pumps per heart would vary between 2.4 x 10^{14} in mice and 14,000 x

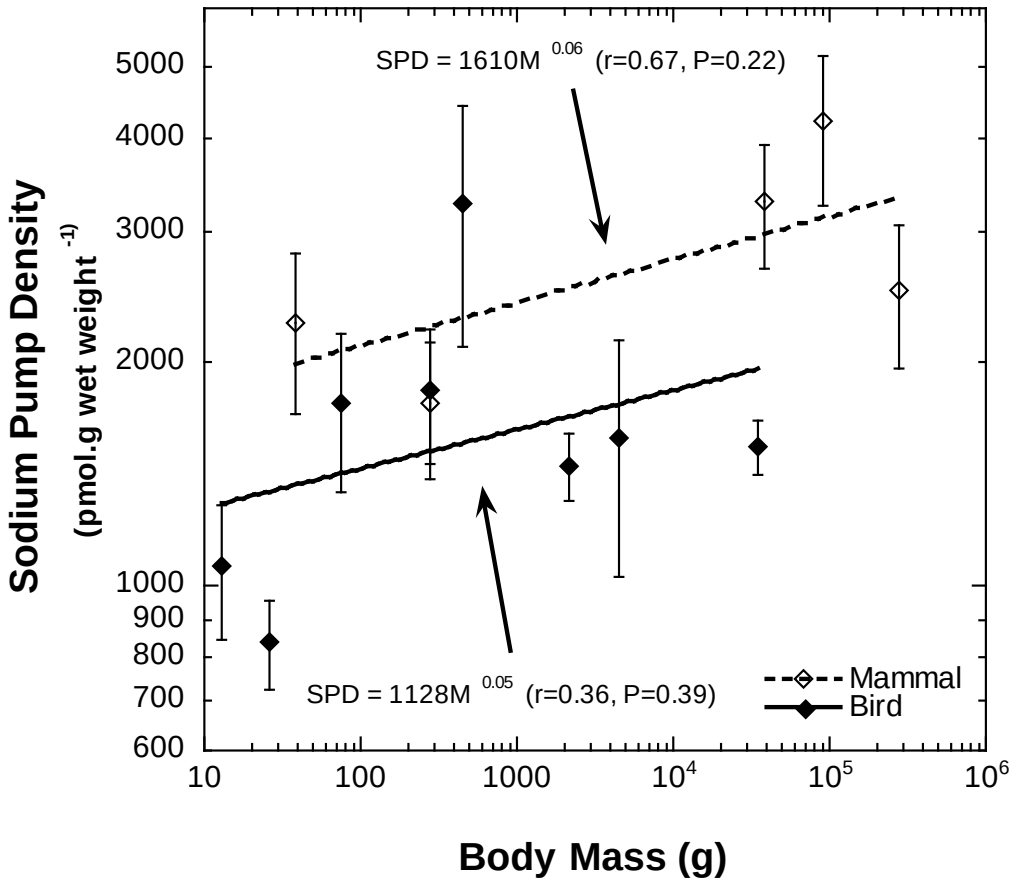


Fig. 4.4 *The relationship between the body mass of mammals and birds and the sodium pump density of heart tissue biopsies. Each point represents the mean ± standard error (SEM).*

10^{14} in cattle, while the heart of zebra finches would contain 1.1 x 10^{14} sodium pumps compared with 2,900 x 10^{14} in the emu heart.

Molecular activity, expressed as the number of ATP molecules hydrolysed by each sodium pump per minute (ATP.min^{-1}), was calculated by dividing maximal Na^+K^+ATPase activity by the sodium pump density. Bird molecular activity values were calculated from 37°C converted Na^+K^+ATPase activity values to allow for

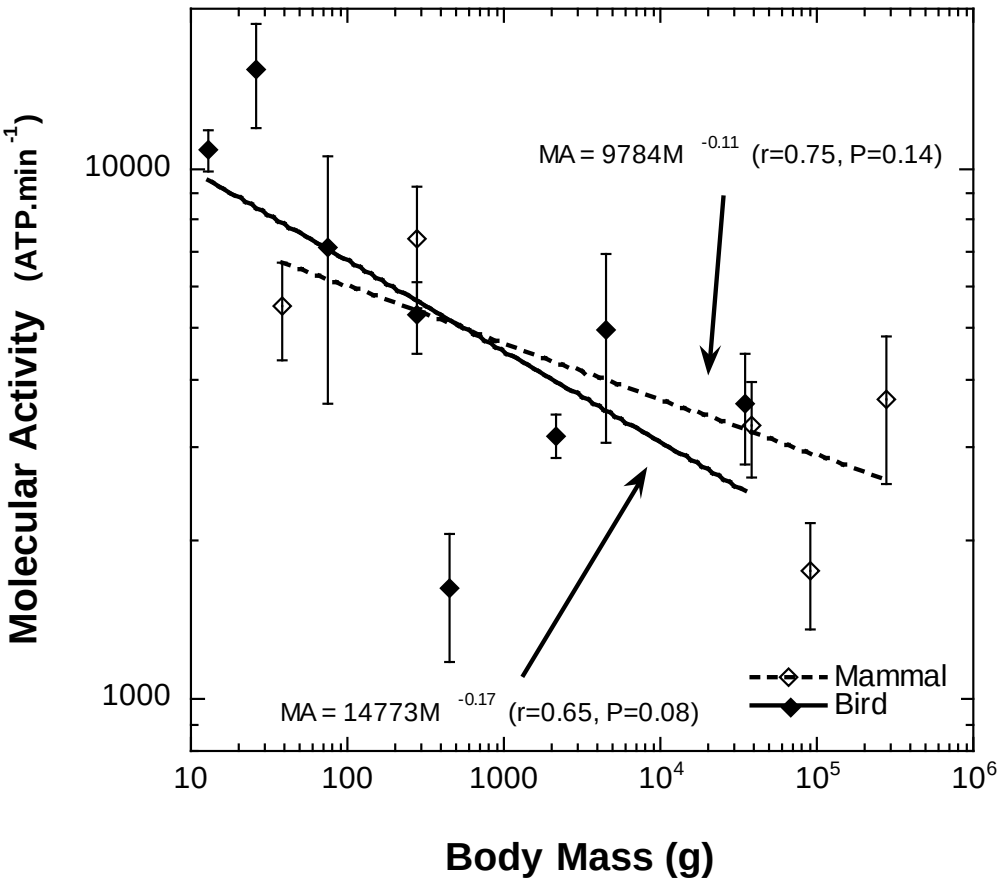


Fig. 4.5 *The relationship between the body mass of mammals and birds and the molecular activity of their sodium pumps. Each point represents the mean ± standard error (SEM).*

comparison with mammals. In the mammals molecular activity values ranged from 1,680 to 7,362 ATP.min^{-1} while for birds there was greater variation, with values ranging from 1,618 to 15,316 ATP.min^{-1} (Table 4.1). When these values were considered with respect to body mass, the trend was for higher molecular activity values in the smaller species in both groups, however neither relationship reached statistical significance (Fig. 4.5).

Table 4.2 presents the cholesterol and phospholipid content, along with their molar ratio, in heart microsomal membranes from the mammals and birds. The cholesterol content (per mg of protein) was relatively constant in both groups and averaged 23.6 μg.mg protein^{-1} in mammals and 20.8 μg.mg protein^{-1} in birds. There was a trend for phospholipid content (per mg of protein) to be greater in the larger mammals and birds, although when considered with respect to body mass (Table 4.5) these relationships were not significant. The molar ratio of cholesterol:phospholipid was constant in the mammals and averaged 0.15. In birds the trend, although not significant (P=0.16), was towards a greater cholesterol:phospholipid molar ratio in the smaller species (Table 4.5). From the cholesterol:phospholipid ratios it can be seen that heart microsomes from the mammals and birds would contain approximately 6-7 phospholipids and 7-10 phospholipids per molecule of cholesterol respectively.

The fatty acid profile of heart microsomal phospholipids for mammals and birds are presented in Table 4.3 and Table 4.4 respectively. The major individual fatty acids, along with the composite parameters were considered with respect to body mass and the results of the analysis are presented in Table 4.5. Several parameters are also plotted allometrically in Fig. 4.6.

Phospholipids from the smaller mammals contained a slightly lower percentage of unsaturated fatty acid chains (Fig. 4.6), however from the allometric slope observed in unsaturation index values, the phospholipids were actually more unsaturated (Fig. 4.6).

Table 4.2	**Cholesterol and Phospholipid Content of Microsomal Membranes from the Heart of Mammals and Birds**		
	Cholesterol (μg. mg protein^{-1})	Phospholipid (μg. mg protein^{-1})	Cholesterol:Phospholipid (mole:mole)*
Mammals			
Mouse (n=1)	23.2	308	0.15
Rat (n=4)	22.1 ± 1.5	309 ± 18	0.14 ± 0.01
Sheep (n=4)	21.9 ± 2.1	325 ± 29	0.14 ± 0.02
Pig (n=4)	23.0 ± 0.7	291 ± 15	0.16 ± 0.01
Cow (n=4)	27.9 ± 3.4	382 ± 13	0.15 ± 0.02
Birds			
Zebra Finch (n=2)	21.8 ± 0.9	313 ± 4	0.14 ± 0.01
Sparrow (n=2)	23.7 ± 2.1	334 ± 27	0.14 ± 0.00
Starling (n=4)	18.2 ± 0.8	391 ± 38	0.10 ± 0.03
Currawong (n=4)	21.1 ± 1.3	383 ± 22	0.11 ± 0.02
Pigeon (n=4)	20.0 ± 0.8	360 ± 10	0.11 ± 0.02
Duck (n=4)	20.1 ± 0.8	392 ± 11	0.10 ± 0.02
Goose (n=4)	19.2 ± 1.6	313 ± 12	0.13 ± 0.02
Emu (n=4)	22.1 ± 1.0	437 ± 25	0.10 ± 0.02

*Cholesterol and phospholipid content of microsomal membranes are expressed relative to protein content in μg.mg protein^{-1}. Cholesterol:Phospholipid ratios are the molar ratio (data from cholesterol and phospholipid content in μg.mg protein^{-1}). * moles of phospholipid calculated assuming a molecular weight of 780. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement. The mouse value is from a pooled microsomal preparation.*

Monounsaturate content showed a significant allometric increase in the larger mammals (P=0.01; Fig 4.6), with body mass explaining 90% of the variability in this parameter. The total content of polyunsaturates was fairly constant in the mammals and was on average 68.3% of all fatty acids (Table 4.3). The percentage of *n*-6 PUFA and *n*-3 PUFA showed no significant body-size-related variation, however the trend was for an increased content of *n*-6 PUFA and a concomitant decrease in *n*-3 PUFA with increasing body mass (Fig. 4.6). The percentage of C20+22 PUFA was significantly reduced in the larger mammals (P=0.03; Fig. 4.6), and the average fatty acid chain length showed a small, but significant decline with body mass (P<0.01; Table 4.5). Of the individual fatty acids, 18:2(*n*-6) and 20:3(*n*-6) both displayed an allometric increase

Table 4.3 Microsomal Phospholipid Fatty Acid Profiles from Mammalian Hearts

	Mouse	Rat	Sheep	Pig	Cow
Fatty Acid					
14:0	0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
16:0	11.8	7.5 ± 0.1	6.8 ± 0.3	11.0 ± 0.7	7.6 ± 0.9
17:0	0.3	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
18:0	13.4	14.7 ± 0.8	10.7 ± 0.6	9.5 ± 0.4	11.0 ± 1.1
14:1(*n*-7)	0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
16:1(*n*-7)	0.3	0.1 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.9 ± 0.1
17:1(*n*-7)	0.0	0.0 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.4 ± 0.1
18:1(*n*-9)	7.5	3.0 ± 0.2	8.9 ± 0.3	9.0 ± 0.3	9.8 ± 0.6
18:1(*n*-7)	0.0	3.8 ± 0.3	2.2 ± 0.1	3.4 ± 0.3	2.4 ± 0.3
18:2(*n*-6)	10.7	13.8 ± 0.6	32.6 ± 1.8	30.8 ± 1.5	26.1 ± 2.2
18:3(*n*-6)	0.3	0.1 ± 0.1	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0
18:3(*n*-3)	0.1	0.2 ± 0.0	6.3 ± 0.5	0.6 ± 0.0	5.0 ± 0.8
20:1(*n*-9)	0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:2(*n*-6)	2.3	0.6 ± 0.3	1.2 ± 0.2	2.0 ± 0.3	0.9 ± 0.1
20:3(*n*-9)	0.2	0.2 ± 0.1	0.3 ± 0.1	1.0 ± 0.2	0.3 ± 0.0
20:3(*n*-6)	0.4	0.4 ± 0.0	1.1 ± 0.1	1.0 ± 0.1	2.4 ± 0.3
20:3(*n*-3)	0.0	0.0 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.6 ± 0.1
20:4(*n*-6)	8.4	27.9 ± 0.6	14.8 ± 1.4	25.2 ± 1.3	18.1 ± 2.8
20:5(*n*-3)	0.0	0.1 ± 0.0	6.0 ± 0.4	0.6 ± 0.1	8.1 ± 1.3
22:4(*n*-6)	1.4	1.5 ± 0.3	0.3 ± 0.1	1.8 ± 0.4	0.3 ± 0.1
22:5(*n*-6)	1.8	0.9 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.0 ± 0.0
22:5(*n*-3)	1.9	3.1 ± 0.1	4.0 ± 0.7	2.4 ± 0.0	4.4 ± 0.8
22:6(*n*-3)	39.2	21.8 ± 0.7	3.0 ± 0.4	0.9 ± 0.1	1.0 ± 0.2
% Saturates	25.5	22.5 ± 0.7	18.0 ± 0.9	20.7 ± 1.1	19.0 ± 2.1
% MUFA	7.8	6.9 ± 0.5	11.8 ± 0.2	12.6 ± 0.6	13.6 ± 1.0
% PUFA	66.7	70.6 ± 0.5	70.2 ± 1.1	66.7 ± 1.6	67.4 ± 3.1
% *n*-9	7.6	3.2 ± 0.1	9.3 ± 0.4	10.0 ± 0.4	10.1 ± 0.6
% *n*-7	0.4	3.9 ± 0.3	2.8 ± 0.2	3.6 ± 0.3	3.8 ± 0.5
% *n*-6	25.3	45.2 ± 0.3	50.2 ± 1.0	61.1 ± 1.6	48.0 ± 0.9
% *n*-3	41.2	25.2 ± 0.7	19.7 ± 0.5	4.6 ± 0.1	19.1 ± 2.4
% Unsaturates	74.5	77.5 ± 0.7	82.0 ± 0.9	79.3 ± 1.1	81.0 ± 2.1
Unsaturation index	330	307 ± 4	233 ± 7	216 ± 4	235 ± 18
Chain length	19.8	19.5 ± 0.0	18.6 ± 0.1	18.6 ± 0.0	18.7 ± 0.1
C20+22 PUFA	55.6	56.5 ± 0.8	31.2 ± 2.3	35.3 ± 1.0	36.1 ± 5.2
n-6/*n*-3	0.6	1.8 ± 0.1	2.5 ± 0.1	13.4 ± 0.4	2.6 ± 0.3
20:4/18:2	0.8	2.0 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	0.7 ± 0.2

Microsomal phospholipid fatty acid profile of mammalian heart expressed as mole percentage of total fatty acids. Unsaturation index is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM), n=4 for all preparations except for mouse where a pooled preparation was used (n=1).

Table 4.4 Microsomal Phospholipid Fatty Acid Profiles from Avian Hearts

	Zebra Finch	Sparrow	Starling	Curra-wong	Pigeon	Duck	Goose	Emu
Fatty Acid								
14:0	0.1±0.1	0.0±0.0	0.2±0.0	0.1±0.1	0.0±0.0	0.1±0.1	0.2±0.2	0.4±0.2
16:0	8.1±0.6	8.4±0.4	7.2±1.0	6.1±0.3	8.8±1.0	8.0±0.4	7.9±0.5	8.0±0.8
17:0	2.2±2.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
18:0	10.5±0.7	8.8±0.6	8.2±1.2	7.8±0.3	9.1±1.2	6.4±0.3	7.9±0.8	5.9±0.5
14:1(*n*-7)	0.3±0.0	0.3±0.1	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0
16:1(*n*-7)	0.2±0.2	0.0±0.0	0.3±0.1	0.3±0.0	0.5±0.1	0.4±0.1	0.3±0.1	0.4±0.0
17:1(*n*-7)	0.3±0.3	0.2±0.2	0.7±0.1	0.8±0.1	1.2±0.0	2.8±0.0	1.1±0.4	1.5±0.6
18:1(*n*-9)	3.6±0.5	3.5±0.1	5.5±0.6	5.7±0.1	5.1±0.3	12.6±0.7	10.4±1.0	11.5±0.7
18:1(*n*-7)	0.3±0.3	0.7±0.1	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
18:2(*n*-6)	27.6±1.2	21.2±1.2	18.2±0.8	18.5±0.4	30.6±0.8	17.8±0.1	22.3±0.9	24.2±0.6
18:3(*n*-3)	0.7±0.0	0.7±0.0	1.1±0.0	1.3±0.1	0.7±0.2	1.2±0.1	5.8±2.9	0.4±0.0
20:2(*n*-6)	0.3±0.3	1.1±0.4	0.4±0.1	0.8±0.2	0.8±0.1	0.0±0.0	0.2±0.1	0.3±0.3
20:3(*n*-6)	0.7±0.2	0.6±0.2	0.5±0.1	0.6±0.0	0.5±0.1	0.5±0.0	0.3±0.0	0.0±0.0
20:3(*n*-3)	0.2±0.2	0.0±0.0	0.0±0.0	0.4±0.4	0.0±0.0	0.0±0.0	1.0±0.1	0.0±0.0
20:4(*n*-6)	30.4±1.7	20.1±1.7	29.3±1.0	42.5±0.7	28.6±1.1	42.9±0.7	32.9±3.5	44.8±2.3
20:5(*n*-3)	0.1±0.1	0.0±0.0	0.9±0.1	1.1±0.1	1.6±0.4	0.3±0.0	4.4±2.2	0.4±0.0
22:4(*n*-6)	1.8±0.1	1.4±0.1	1.1±0.1	1.6±0.1	1.5±0.1	1.9±0.1	1.0±0.3	0.9±0.0
22:5(*n*-6)	3.2±0.7	7.9±1.5	2.1±0.1	1.4±0.1	0.7±0.1	1.5±0.1	0.4±0.1	0.3±0.1
22:5(*n*-3)	0.7±0.1	1.0±0.2	2.7±0.3	2.7±0.1	6.5±0.8	0.9±0.1	2.4±0.5	0.3±0.1
22:6(*n*-3)	8.5±2.4	24.1±1.0	21.6±1.7	8.3±0.5	3.6±0.4	2.7±0.8	1.3±0.1	0.5±0.1
% Saturates	20.9±2.0	17.2±1.0	15.6±2.2	14.0±0.5	17.9±2.1	14.4±0.8	16.1±0.9	14.3±1.3
% MUFA	4.7±0.6	4.7±0.3	6.6±0.7	6.9±0.1	6.9±0.4	15.9±0.7	11.9±1.2	13.5±1.0
% PUFA	74.4±1.4	78.2±1.3	77.8±2.8	79.2±0.6	75.2±2.2	69.6±1.1	72.0±1.5	72.2±2.3
% *n*-9	3.6±0.5	3.5±0.1	5.5±0.6	5.7±0.1	5.1±0.3	12.6±0.7	10.4±1.0	11.5±0.7
% *n*-7	1.1±0.1	1.2±0.2	1.1±0.1	1.1±0.0	1.7±0.1	3.3±0.0	1.5±0.5	2.0±0.6
% *n*-6	64.1±1.2	52.3±2.5	51.5±0.9	65.5±0.9	62.7±1.9	64.6±1.0	57.1±4.3	70.6±2.2
% *n*-3	10.3±2.6	25.8±1.2	26.3±2.1	13.7±0.7	12.4±1.7	5.1±1.0	14.9±5.4	1.6±0.1
% Unsaturates	79.1±2.0	82.8±1.0	84.4±2.2	86.0±0.5	82.1±2.1	85.6±0.8	83.9±0.9	85.7±1.3
Unsaturation index	266±7	328±6	328±15	304±2	260±9	265±7	258±6	255±9
Chain length	19.0±0.1	19.6±0.1	19.5±0.1	19.3±0.0	18.9±0.1	19.0±0.1	18.8±0.0	18.8±0.1
C20+22 PUFA	46.0±0.2	56.2±2.5	58.4±3.4	59.3±0.6	43.8±2.1	50.7±1.5	43.9±1.6	47.6±2.5
n-6/*n*-3	6.7±1.8	2.0±0.2	2.0±0.1	4.8±0.3	5.4±0.9	14.1±2.4	6.7±2.7	45.5±2.6
20:4/18:2	1.1±0.1	1.0±0.1	1.6±0.1	2.3±0.0	0.9±0.0	2.4±0.2	1.5±0.1	1.9±0.1

Microsomal phospholipid fatty acid profile of bird heart expressed as mole percentage of total fatty acids. Unsaturation index is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM), n=4 for all preparations except the zebra finch and sparrow where pooled samples were used (n=2).

in the larger mammals (P=0.02; Table 4.5), while the content of 22:6(n-3) showed the greatest variation of any individual fatty acid, with an approximate 40-fold range observed in the mammals (Table 4.3). The content of this fatty acid was highly dependant on body mass, with 96% of the variability in 22:6(n-3) content explained by the size of the mammal. From the allometric slopes of the significant relationships in Fig. 4.6 it can be calculated that for every doubling in body mass there would be a 3.4% decrease in the number of double bonds per 100 fatty acid chains, a 0.7% increase in the total content of unsaturated fatty acids, a 5.0% increase in monounsaturate content, a 4.7% decrease in C20+22 PUFA content and a 26.3% decrease in 22:6(n-3) content in heart microsomal phospholipids in mammals.

In bird microsomal phospholipids there was no significant body-size-related variation observed in unsaturation index, the percentage of total unsaturates, or the percentage of polyunsaturates (Fig. 4.6). The larger birds however, did display a significant allometric increase in monounsaturate content ($P<0.05$; Fig. 4.6), which was largely the result of changes in 18:1(n-9) content (Table 4.5). From the allometric exponent (0.16) it can be calculated the there would be an 11.7% increase in MUFA content for every doubling in body mass. As was the case in bird kidney, the percentage of n-6 PUFA displayed no allometric trends, while there was a significant (P=0.04) decline in n-3 PUFA with increasing body size (Fig. 4.6). These changes resulted in a significant allometric elevation in the n-6/n-3 ratio (P=0.02; Table 4.5), such that for every 100% increase in body mass there would a 23% increase in the content of n-6 PUFA relative to n-3 PUFA. Of the individual fatty acids 18:0, 20:3(n-6) and 22:5(n-6) were all significantly reduced in the larger birds (Table 4.5). Similar to the trends observed in mammalian heart, the content of 22:6(n-3) showed the greatest variation of any individual fatty acid and was significantly ($P<0.01$) higher in the smaller species. It can be calculated from the allometric exponent (-0.45) that for every 100% increase in body size there would be a 26.8% decrease in the content of 22:6(n-3).

Table 4.6 presents the predicted values for a number of parameters in the heart of a

Table 4.5 The Relationship Between Body Mass and Various Lipid Parameters in the Hearts of Mammals and Birds

	Mammals			Birds		
	Intercept at M=1g	Exponent	Correlation coefficient	Intercept at M=1g	Exponent	Correlation coefficient
Lipid parameter						
16:0	10.7	-0.02	0.37	7.67	0.00	0.05
18:0	16.4	-0.04	0.85*	11.3	-0.06	0.84***
18:1(*n*-9)	3.61	0.08	0.62	2.28	0.17	0.92***
18:1(*n*-7)	5.01	-0.06	0.63‡	-	-	-
18:2(*n*-6)	7.11	0.12	0.94**	22.6	-0.00	0.05
20:3(*n*-6)	0.16	0.19	0.94**	1.00	-0.13	0.81†**
20:4(*n*-6)	11.1	0.05	0.41	21.6	0.07	0.69*
20:5(*n*-3)	0.01	0.54	0.82‡	0.23	0.16	0.33†
22:4(*n*-6)	2.76	-0.14	0.60	1.82	-0.05	0.47
22:5(*n*-6)	5.21	-0.31	0.89‡	10.4	-0.34	0.87***
22:5(*n*-3)	1.81	0.06	0.67	2.51	-0.09	0.24
22:6(*n*-3)	219	-0.44	0.98***	73.9	-0.45	0.92***
% Saturates	27.6	-0.03	0.89**	19.7	-0.03	0.65*
% MUFA	5.34	0.07	0.95***	3.08	0.16	0.91***
% PUFA	68.7	-0.00	0.10	79.9	-0.01	0.65*
% n-9	3.68	0.08	0.64	2.28	0.17	0.92***
% n-7	0.44	0.18	0.71	0.84	0.10	0.70
% n-6	24.1	0.07	0.81*	53.3	0.02	0.52
% n-3	61.1	-0.14	0.67	48.2	-0.25	0.74**
% Unsaturates	73.0	0.01	0.90**	80.3	0.01	0.65*
UI	391	-0.05	0.96***	327	-0.02	0.62
Chain length	20.3	-0.01	0.98***	19.6	0.00	0.69*
C20+22 PUFA	73.5	-0.07	0.91**	56.0	-0.02	0.38
n-6/n-3	0.39	0.22	0.75	1.06	0.30	0.79**
20:4/18:2	1.53	-0.07	0.48	0.97	0.07	0.52
Cholesterol ($\mu g.mg\ protein^{-1}$)	21.2	0.01	0.47	21.4	-0.01	0.18
Phospholipid ($\mu g.mg\ protein^{-1}$)	273	0.03	0.85*	315	0.02	0.53
Chol:PL (mol:mol)	0.14	0.00	0.30	0.14	-0.03	0.55

*The relationships between body mass and the various lipid parameters were determined by linear regression (least-square method) of log-transformed values. The relationships were determined using the mean parameter value for each species (n=5 for mammals and n=8 for birds). % UFA = % unsaturates, UI = Unsaturation index. *P<0.1, **P<0.05, ***P<0.01. ‡ relationship determined using n=4 (see Table 4.3). † relationship determined using n=7 (see Table 4.4)*

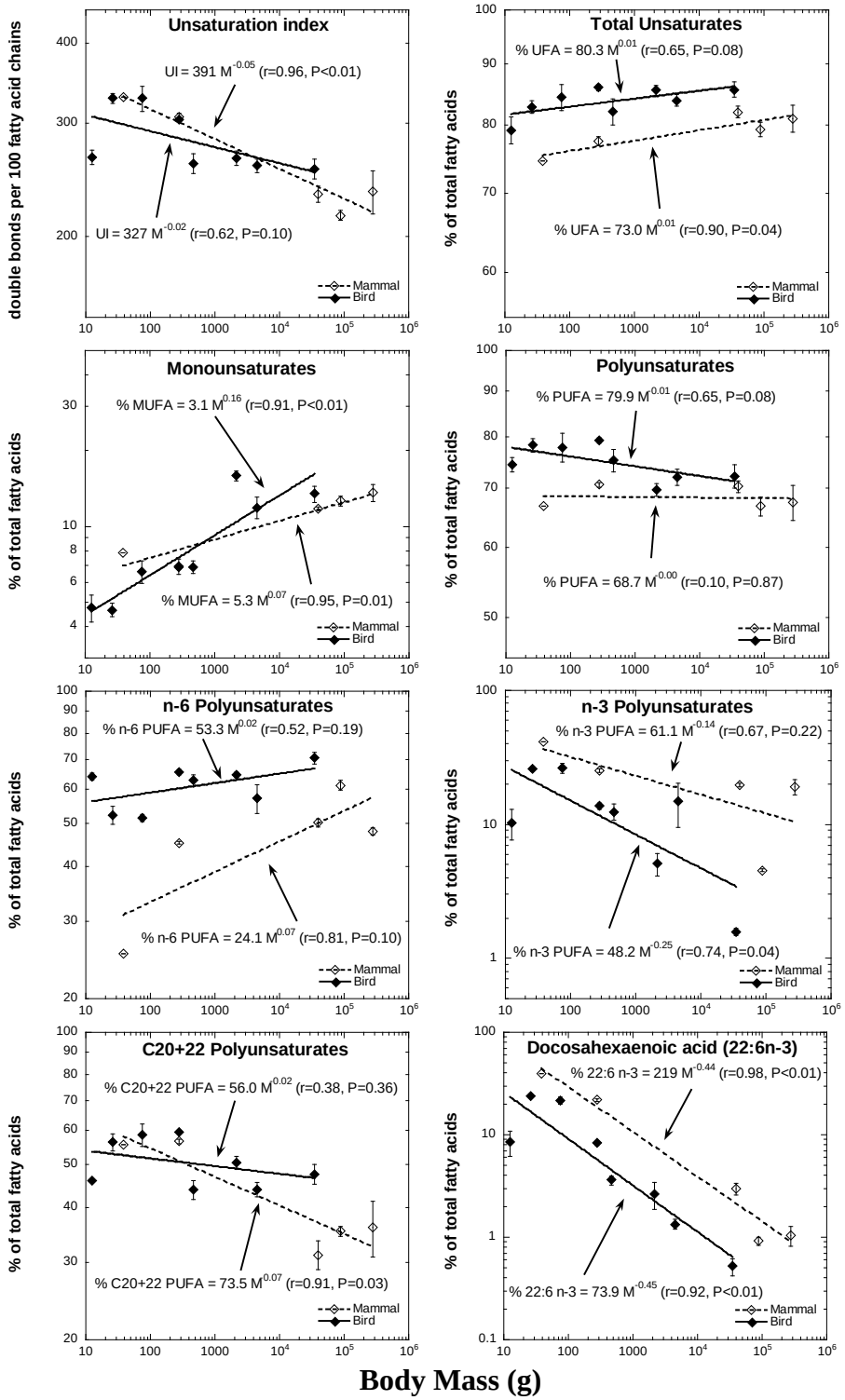


Fig. 4.6 *The relationship between body mass and the unsaturation index, content of unsaturates, monounsaturates, polyunsaturates, n-6 PUFA, n-3 PUFA, C20+22 PUFA and docosahexaenoic acid in heart microsomal phospholipids from mammals and birds. Values are means ± standard errors (SEM).*

Table 4.6 Comparison of a 250g Mammal and Bird

	Mammal	Bird
Heart mass (g)	0.95	2.55
Protein concentration (mg.g wet weight^{-1})	217	170
Na^+K^+ATPase activity (μmol Pi.mg wet weight^{-1}.hr^{-1})	0.53	0.45
Sodium pump density (pmol.g wet weight^{-1})	2242	1488
Sodium pumps per heart	1.3×10^{15}	2.3×10^{15}
Molecular activity (ATP.min^{-1})	5330	5779
Unsaturation index	297	293
% Unsaturates	77.1	84.9
% Monounsaturates	7.9	7.5
% Polyunsaturates	69.0	75.6
% n-6	35.5	59.5
% n-3	28.2	12.1
% 22:6(*n*-3)	20.4	6.13
Cholesterol:Phospholipid (mole:mole)	0.14	0.12

Values were calculated from the allometric equations determined for each parameter, using a body mass of 250 grams.

250g mammal and bird. Comparison of the mammal and bird shows that the heart of the bird would be 168% larger than the mammal and would contain only 78% as much protein per gram wet weight. Na^+K^+ATPase activity in the bird would be 85% that of the value for the mammal, the picomoles of sodium pumps per gram wet weight would be 34% lower in the birds, however the total number of sodium pumps in the bird heart would be 77% greater than the number in the mammals. The sodium pump molecular activity of the bird would be 8.4% higher than the mammal. Examining the microsomal phospholipids, it can be seen that the bird would possess approximately 10% more unsaturated fatty acids than the mammal, while having an unsaturation index that would be 99% of the value for the mammal. The bird phospholipids would have 5% less monounsaturates and 9.6% more polyunsaturates than the mammal. The *n*-6 PUFA

content of the bird would be 68% higher, the content of *n*-3 PUFA would be 57% lower, and as a result the *n*-6/*n*-3 ratio would be approximately 4-fold greater in the bird. The docosahexaenoic acid content in the phospholipids from the mammal would be approximately 70% greater than that of the bird. The molar ratio of cholesterol:phospholipid in bird microsomal membranes would be 86% of the value for the mammal.

Linear correlation coefficients were determined between all individual lipid parameters and sodium pump molecular activity values in both mammals and birds, with the significant relationships presented in Fig. 4.7 and Fig. 4.8 respectively. In mammals the content of 18:1(*n*-9) and the total content of *n*-9 fatty acids were the only lipid parameters that displayed significant relationships with sodium pump molecular activity, both being negatively correlated (Fig. 4.7). In the birds, eight lipid parameters significantly correlated with molecular activity (Fig. 4.8). Positive correlations with molecular activity were found for the content of *n*-3 PUFA, 14:1(*n*-7), 22:5(*n*-6) and 22:6(*n*-3) as well as the cholesterol:phospholipid molar ratio, while the content of *n*-6 PUFA, 16:1(*n*-7) and 17:1(*n*-7) all displayed an inverse relationship with molecular activity.

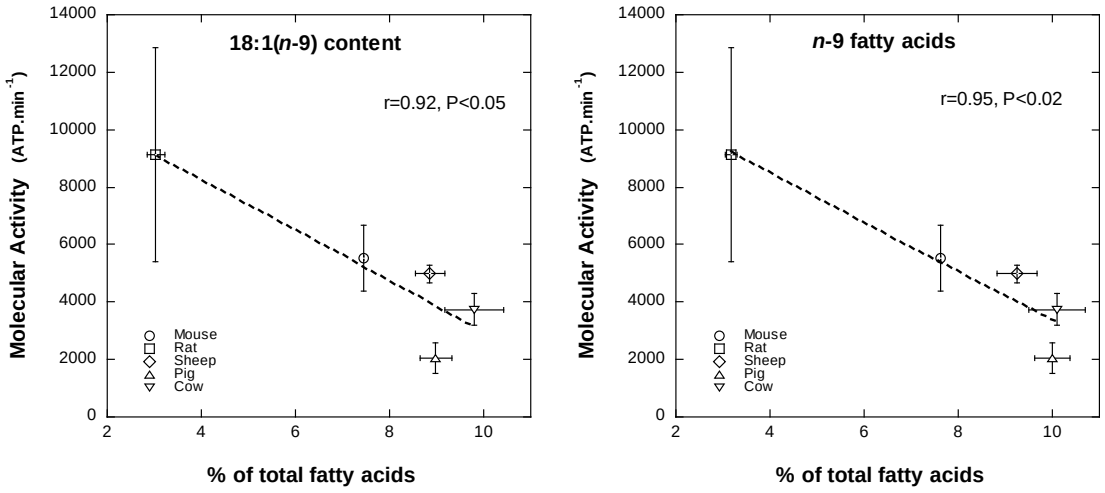


Fig. 4.7 *Linear correlations between sodium pump molecular activity in mammals and 18:1(n-9) and total n-9 content.*

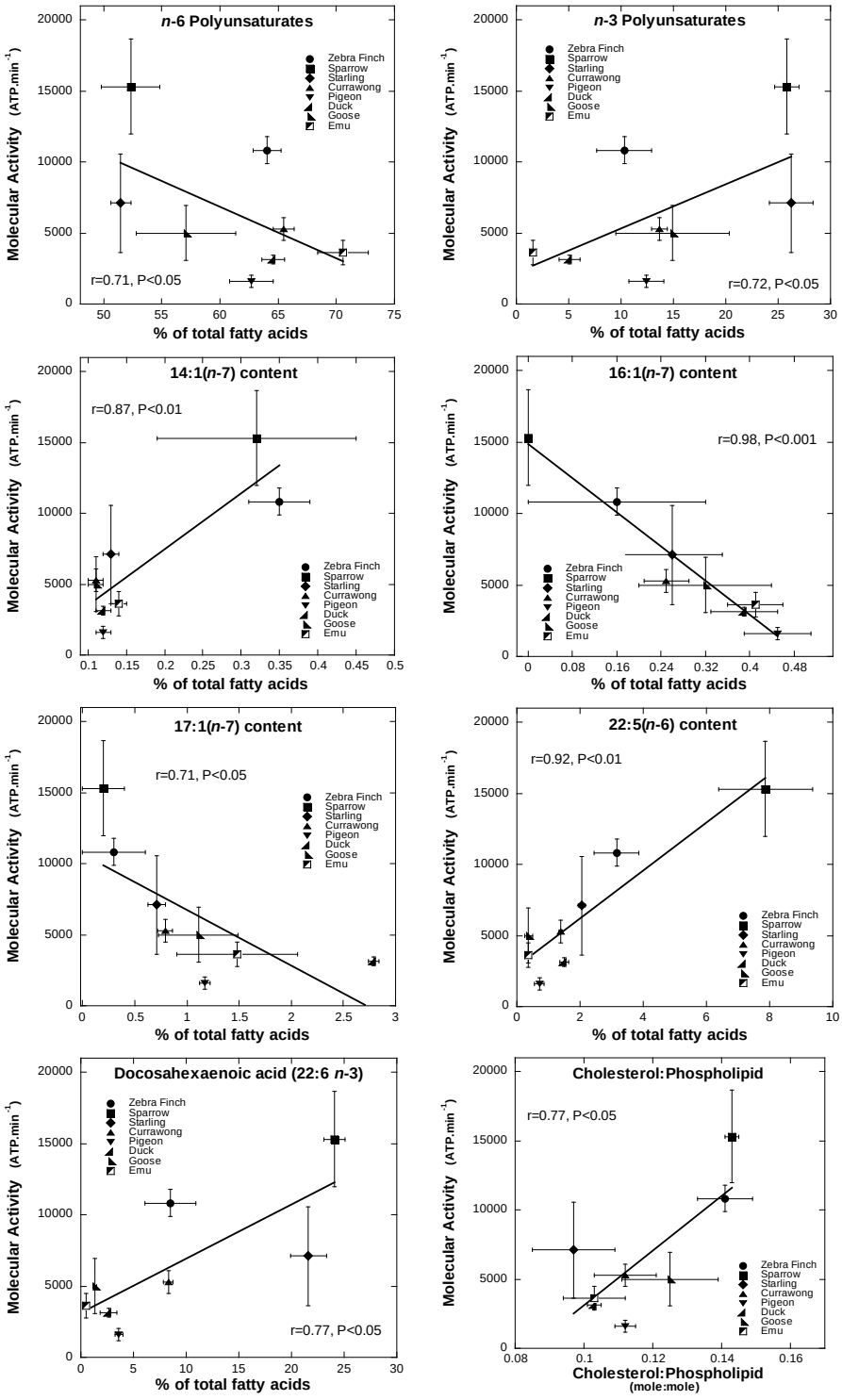


Fig. 4.8 *Linear correlations between sodium pump molecular activity in birds and the content of n-6 PUFA, n-3 PUFA, 14:1(n-7), 16:1(n-7), 17:1(n-7), 22:5(n-6) and 22:6(n-3), and the molar ratio of cholesterol:phospholipid.*

4.3 DISCUSSION

This chapter has investigated the relationship between sodium pump molecular activity and membrane lipid composition in the heart of mammals and birds of different body size. In both groups sodium pump (Na^+K^+ATPase) activity was higher in the smaller species and displayed a significant decline with body mass (Fig. 4.3). These allometric trends echo the results measured in the kidney of these species (Fig. 3.3), with the birds again showing a steeper relationship with increasing body mass, compared to the mammals. Whether the increased Na^+K^+ATPase activity in smaller mammals and birds was the result of increased sodium pump density, an increased molecular activity of their sodium pumps, or a combination of both was examined. Compared to larger mammals, the hearts of the smaller mammals tended to contain lower concentrations of sodium pumps (Fig. 4.4), and higher molecular activity values (Fig. 4.5), although neither of these allometric trends reached statistical significance. In the birds the allometric decline in Na^+K^+ATPase activity observed in the larger species primarily resulted from decrease in the molecular activity of their sodium pumps (Fig. 4.5). Power equations were determined for individual data points (results not shown), and from the allometric exponents it was calculated that the mammals used a combination of pump number changes and molecular activity changes (approx. 50% each) to vary Na^+K^+ATPase activity, while in the birds 71% of the variation in Na^+K^+ATPase activity was the result of molecular activity changes.

The molecular activity values determined for mammalian hearts in the current study varied between 1,680 – 7,362 ATP.min^{-1}, while the range in birds was 1,618 – 15,316 ATP.min^{-1} (Table 4.1). Sodium pumps in avian hearts have not been extensively studied, however sodium pumps in mammalian hearts from a variety of sources (rat, rabbit, guinea pig, cow) have shown molecular activity values that range between 4,000 – 10,000 ATP.min^{-1} (Pitts and Schwartz, 1975; Choi and Akera, 1978; Akera, 1984; Else et al., 1996). Several of these values were determined in preparations at various stages of purification (eg purified, microsomal, homogenate) and, as has been noted

several times (Mernissi, 1984; Nørgaard et al., 1986; Schmidt et al., 1992), caution should be exercised when comparing them.

In the heart the sodium pump functions to maintain the electrochemical gradient for sodium, and also plays an important role in Ca^{2+} homeostasis (Wang et al., 1996). Following the contraction cycle, the removal of intracellular Ca^{2+} is accomplished by a number of mechanisms including active transport by the Ca^{2+}ATPase of the sarcoplasmic reticulum and Na^{+}/Ca^{2+} exchange at the sarcolemma, which is in turn serviced by the sodium pump. Cardiac tissue expresses up to three isoforms of the sodium pump (α_1, α_2 and α_3), with the proportion of these isoforms varying with both age (Lingrel, 1992) and species (Sweadner, 1989). A number of characteristics clearly distinguish these isoforms from each other, including electrophoretic mobility, N-terminal amino acid sequence, antigenic determinants and affinity for ouabain (Sweadner, 1989). Recent studies have indicated that the different isoforms of the sodium pump are localised in different regions of the same cell (Hundal et al., 1994; Juhaszova and Blaustein, 1997a; Juhaszova and Blaustein, 1997b). The α_1 isoform, which undertakes the housekeeping functions (i.e. maintenance of bulk cytosolic Na^{+}) of all cells, is uniformly distributed in cells, while the α_2 and α_3 isoforms are limited to the regions of the plasma membrane that overly the endoplasmic/sarcoplasmic reticulum. The Na^{+}/Ca^{2+} exchanger appears to colocalise in the same regions (Juhaszova and Blaustein, 1997b) and it has been suggested that the specific function of the α_2 and α_3 isoforms is the regulation Ca^{2+} levels near the sarcoplasmic reticulum following the contraction cycle (Lingrel et al., 2003).

It is possible that some of the variation observed in molecular activity in the different species may have resulted from the specific expression of various isoforms. In canine heart, different molecular activity values have been observed in the high affinity (α_3) and low affinity (α_1) isoforms of the sodium pump (Maixent and Berribi-Bertrand, 1993). These molecular activity values however, only vary between 8,800 $ATP.min^{-1}$ in α_3 and 5,300 $ATP.min^{-1}$ in α_1 (Maixent and Berribi-Bertrand, 1993), and thus cannot

totally explain the 4.5-fold and 9.5-fold range observed in the mammals and birds respectively (Table 4.1).

The sodium pump density of mammalian hearts ranged between 1,761 – 4,217 pmol.g wet weight^{-1} in the current study, and appeared to be quite high when compared to the 500 – 1,400 pmol.g wet weight^{-1} generally described in the literature for mammalian myocardium (Nørgaard et al., 1986; Schmidt et al., 1990; Else et al., 1996). It is possible that experimental differences may have been responsible for this discrepancy, as mainly due to logistic reasons, there were a number of small variations between the ^{3}H-ouabain binding assay employed in the current study and the assays used in previous investigations.

To ensure complete saturation of ^{3}H-ouabain binding sites in all species in the current study, a very high specific concentration (50μM) of ouabain was used. This concentration is between 5 - 50 times higher than what has been used in previous studies (Nørgaard et al., 1986; Schmidt et al., 1990; Else et al., 1996), but was required, as it was impractical to predict the binding characteristics of each individual species, based on the amount of tissue and numbers of species available. The current study employed a phosphate-facilitated ^{3}H-ouabain binding technique, while several of the previous studies have used the vanadate-facilitated method (Nørgaard et al., 1986; Schmidt et al., 1990). Phosphate and vanadate both stabilise the sodium pump in the E2 conformation, which is the conformation required for ouabain binding. The phosphate-facilitated method requires tissue viability and thus may be affected by any necrotic changes in the tissue biopsies, however it represents the method most similar to that which occurs naturally, i.e. phosphorylation of the sodium pump. The final major difference concerned the length of the washout period, which totalled 40 minutes in the current study, but has been up to 2 hours in other investigations. The purpose of this washout period is to reduce the level of ^{3}H-ouabain bound at non-specific sites, while at the same time minimising the loss of specifically bound ligand. Despite these experimental differences compared to previous studies, the strength of the current

investigation is the examination of a large number of mammals and birds using the same assay techniques, allowing direct comparison between all species.

The increased sodium pump activity observed in the smaller mammals and birds (Fig. 4.3) may potentially be a response to increased passive permeability of tissues in these more metabolically active species. Considering the constant activity of the heart however, it is more likely that the majority of ion fluxes in this tissue would either directly or indirectly result from the contraction cycle. Heart rate is elevated in smaller mammals and birds (Grubb, 1983; Peters, 1983), and thus considering this greater frequency of contraction, the higher sodium pump activity observed in both these groups is likely to be required for the rapid reestablishment of appropriate ionic concentration following the heart beat.

The allometric exponents for heart mass in the mammals and birds were 0.99 and 0.93 respectively (Fig. 4.1). These allometric slopes agree well with previous investigations (Lasiewski and Calder, 1971; Grubb, 1983; Peters, 1983) and indicate that the heart represents a relatively constant proportion of body mass in mammals while in birds smaller species have relatively larger hearts. The hearts of the birds were larger than those of mammals of similar body mass, however as the size of the species increased the difference between mammals and birds became less (i.e. their regression lines converged). Na^+K^+ATPase activity was combined with heart mass and expressed as the micromoles of inorganic phosphate liberated (from ATP) per heart per hour, and assuming that this value represented the potential maximum rate, and using a P/O ratio of 2.0 (Rolfe and Brown, 1997), it was possible to determine the potential maximal daily energy expenditure by the sodium pumps of the heart ($Kcal.day^{-1}$). These values represented 1 – 6% and 1 – 2.5% of basal energy expenditure for the mammals and birds respectively, and when plotted allometrically (Fig. 4.9), mammals displayed an exponent of 0.96, while in birds the exponent was 0.80. Considering that BMR scales with exponents of 0.73 and 0.63 in the mammals and birds respectively (Fig. 4.9), maximal heart Na^+K^+ATPase activity in both groups accounts for a relatively higher

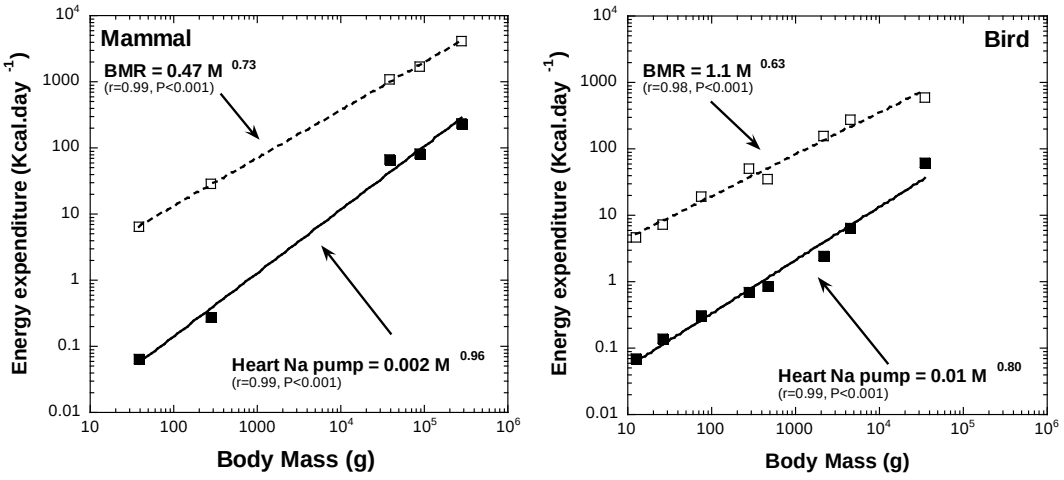


Fig. 4.9 *The relationship between the body mass of mammals and birds, basal metabolic rate and the potential maximal daily energy expenditure of heart sodium pumps.*

proportion of BMR in larger species. It should be noted however that these are potential maximal *in vitro* activities and it is currently unknown whether all of these species work at a similar percentage of their maximum values under basal conditions.

Attempts to quantify the basal energy use of the sodium pump in the heart, have been complicated by the fact that addition of ouabain, which inhibits the sodium pump, has been found to stimulate respiration (Lee et al., 1960; Gibbs and Gibson, 1969). This is thought to be a secondary effect due to alterations in free cytosolic Ca^{2+}, and subsequently ion transport rates in cardiac tissue have been used to estimate energy turnover by the sodium pump (Clausen et al., 1991). In intact mammalian heart, ouabain-suppressible K^+ influx has been reported to be between 400-2,000 nmol.g wet weight^{-1}.min^{-1} (Clausen et al., 1991), which corresponds to an *in vivo* sodium pump activity of 0.012-0.06 μmol ATP.mg wet weight^{-1}.hr^{-1}. These values are approximately 4-5% of total energy turnover in the heart (Clausen et al., 1991) and represent approximately 15% or less of the potential maximal *in vitro* values determined for the mammals in the current study (see Table 4.1).

There was a fairly constant level of protein in the hearts of the mammals and birds (Fig. 4.2). On average the mammalian hearts contained 212 milligrams of protein per gram of heart wet weight, which was significantly higher than the mean protein concentration of 174 milligrams of protein per gram of heart wet weight observed in avian hearts. Similar results were observed in the kidney of these species (Fig. 3.2), where both groups contained relatively constant amounts of protein, with higher absolute levels found in the mammals. Hulbert and Else (1989) found that the high metabolism of the rat compared to a reptile was associated with 34% more protein in the rat heart.

Membrane lipid composition was determined in both groups and in the mammals a variety of parameters displayed significant body-size-related variation (Table 4.5). Smaller mammals, despite having significantly less total unsaturates than the larger mammals (Fig. 4.6), had greater levels of unsaturation in their microsomal membranes (as indicated by unsaturation index; Fig. 4.6). The total percentage of polyunsaturates was relatively constant in all mammals, while there was a significant allometric increase in monounsaturate content in the larger species (Fig. 4.6). The percentage of *n*-6 PUFA and *n*-3 PUFA were not significantly related to body size although the trend was for smaller mammals to have greater *n*-3 PUFA and less *n*-6 PUFA (P=0.1). There were significant allometric decreases in the larger mammals observed for average chain length and the percentage of C20+22 PUFA. The two terminal fatty acids in the *n*-6 and *n*-3 families, namely 22:5(*n*-6) and 22:6(*n*-3), also both declined with increasing body mass (Table 4.5). The content of 22:6(*n*-3) once again displayed the greatest range of any individual fatty acid varying between 39% in mice and ~1% in pigs and cattle (Table 4.3). The allometric slope describing this fatty acid was –0.44, which is greater than the slope of –0.34 described for heart phospholipids by Hulbert et al. (2002c) and represents the greatest variation measured in any parameter in the current range of mammals. It should be noted that due to the small number of species used in the present study (n=5) there may have been an exaggeration of some of the allometric

relationships. For example, heart phospholipids from the very metabolically active shrew (S*orex araneus*), have been shown to contain ~28% 22:6(*n*-3) (Käkelä and Hyvärinen, 1995). Using the current allometric equation, and the body mass of the shrew from the above study (7.7g), the predicted content of 22:6(*n*-3) is 91%, which is obviously an unrealistic value.

Many of the allometric relationships determined in the heart microsomal phospholipids of the mammals were similar to those observed in the kidney (see Table 3.5), however since the relationships were steeper it indicates a greater difference between small and large mammals in this tissue. The heart and skeletal muscle tend to manifest the greatest allometric variation in fatty acid composition of all tissues (Couture and Hulbert, 1995a; Hulbert et al., 2002c), and it is currently unknown how this dramatic variation in composition is regulated.

In the microsomal phospholipids from avian hearts there was a significant allometric increase in monounsaturate content and the ratio of *n*-6/*n*-3 PUFA in the larger birds, with significant allometric decreases observed in the content of *n*-3 PUFA and 22:6(*n*-3) (Table 4.5). The allometric relationships describing the percentage of total unsaturates, total polyunsaturates, *n*-6 PUFA, C20+22 PUFA and unsaturation index were all not significantly different from zero (Fig. 4.6). As was the case in the kidney the content of 22:6(*n*-3) was reduced in the heart phospholipids from the zebra finch compared to the other small birds, and regression analysis of the other 7 birds predicted a content of 42.3% 22:6(*n*-3) in the zebra finch, which is five times higher than the value that was actually observed (8.5%). Despite this anomalous composition, the allometric slope describing the relationship for 22:6(*n*-3) was –0.45, which represents the greatest variation of any parameter in the birds. The allometric relationships for several other parameters (eg unsaturation index and average chain length) would again attain statistical significance if the zebra finch was removed from calculations, however considering the large influence that 22:6(*n*-3) content has on these measures, all of these relationships are basically describing the same phenomenon.

Muscles with high respiration rates have elevated membrane levels of 22:6(*n*-3) (Infante et al., 2001) and there is a strong positive correlation between the content of 22:6(*n*-3) in cardiac phospholipids and heart rate in mammals (Gudbjarnarson et al., 1978). Resting heart rate is an indicator of mass-specific BMR in different mammals (Brody, 1945), and such evidence coupled with the large allometric variation of 22:6(*n*-3) observed in the mammals and birds in the current study, suggests a potentially important role for this fatty acid in metabolic variation among species (Hulbert and Else, 1999; Hulbert and Else, 2000).

Many of the characteristic fatty acid profiles that were observed in the kidney of various species in the previous chapter, were also evident in the heart of these animals. As mentioned there was a reduced level of 22:6(*n*-3) in the kidney of the zebra finch, with this compositional trait also evident in the heart. The heart of the sheep and cattle contained high levels of *n*-3 PUFA, as was the case in the kidney, which is again suggestive of a diet predominantly pasture-based (Gurr and Harwood, 1991; Cordain et al., 2002). Furthermore the higher *n*-3 PUFA content (at the expense of *n*-6 PUFA) observed in the kidney of 'wild' geese compared to 'captive' geese was also evident in heart microsomes with 24.1% and 5.7% *n*-3 PUFA present in these birds respectively. Thus it appears that whatever regulatory mechanisms are responsible for these fatty acid trends (eg diet, enzyme activity), they may be having a general effect on the membranes of most tissues in these species.

There was a non-significant trend for higher sodium pump MA in the smaller mammals, which was in contrast to the very significant body-size-related variation seen in microsomal lipid composition. When correlation coefficients were determined between lipid parameters and molecular activity values only two significant relationships were revealed (Fig. 4.7). There was an inverse relationship between the 18:1(*n*-9) content of microsomal phospholipids and sodium pump molecular activity ($P<0.05$; Fig. 4.7). Wu et al. (2001) found similar results in comparisons of kidney and

brain microsomes in rats and toads. The second lipid parameter that correlated with molecular activity was total content of *n*-9 fatty acids, which is really a reflection of the above relationship as 18:1(*n*-9) accounted for greater than 90% of total *n*-9 fatty acids.

There were a number of lipid parameters that correlated with sodium pump molecular activity in the bird hearts (Fig. 4.8). The total percentage of *n*-6 PUFA showed a negative correlation with molecular activity, despite the strong positive relationship observed for 22:5(*n*-6). Increased content of 22:6(*n*-3) and total *n*-3 PUFA were associated with increased molecular activity. In the previous chapter the significant allometric variation observed in mammalian kidney molecular activity was also correlated with the content of both 22:5(*n*-6) and 22:6(*n*-3) (Fig. 3.7), which suggests that these long chain fatty acids may be important modulators of enzyme activity. Negative correlations were observed between molecular activity and 16:1(*n*-7) and 17:1(*n*-7), while 14:1(*n*-7) showed a positive correlation. These fatty acids accounted for only a small percentage of the total fatty acids and hence it is unclear what physiological role they may have been playing. Interestingly, higher cholesterol:phospholipid ratios were associated increased sodium pump molecular activity in the heart, which is the opposite to what was seen in the kidney of these birds (Fig. 3.8). It should be noted however, that microsomal preparations are a mixture of membranes from the plasmalemma, endoplasmic reticulum and golgi apparatus and it is unclear whether these subcellular membranes account for similar proportions in cells from different organs and also in cells from different species. Thus since it is considered that cholesterol is predominately found in the plasma membrane (Colbeau et al., 1971; Stryer, 1995), varying levels of cholesterol may be found depending on the proportion of the other subcellular membranes present in the microsomal preparation.

While a number of correlations existed between molecular activity and lipid parameters within the mammals and birds, when comparing the two groups it appears that the total level of membrane unsaturation may be important in determining the overall level of molecular activity. In the previous chapter, examining the kidney, it was noted that the

allometric plots of molecular activity and unsaturation index were quite similar, with the higher unsaturation levels in the mammals associated with higher molecular activity values. In the heart the allometric plots again showed similarities, with a high degree of overlap between mammalian and avian values for both molecular activity (Fig. 4.5) and unsaturation index (top left panel in Fig. 4.6). In fact when a variety of parameters were calculated in a 250g mammal and bird, it can be seen that the molecular activity values were almost equal, and while there were differences in many fatty acid parameters, the unsaturation index values were also basically equal (Table 4.6).

In this chapter the body-size-related changes in the fatty acid composition of heart microsomes in mammals and birds were very similar. These allometric trends are more pronounced than those observed in kidney microsomes from the same species, and conform to a general pattern that is evident for fatty acid variation in animals of varying body size (Couture and Hulbert, 1995a; Hulbert et al., 2002b; Hulbert et al, 2002c). In contrast to the results observed in the kidney, birds displayed an allometric decline in sodium pump molecular activity with body size, with a similar relationship seen in the mammals. When correlations were examined between lipid parameters and molecular activity, there were many significant relationships observed in the birds while only two were observed in the mammals. Thus it appears that while characteristic changes in fatty acid composition appears to be similar in various tissues of endotherms, other cellular changes (eg sodium pump molecular activity) appear to be both tissue and species specific. The next chapter will investigate these relationships in the brain of the mammals and birds.

Chapter V

Sodium Pump Molecular Activity and Membrane Lipid Composition in the Brain of Mammals and Birds of Different Body Size

5.1 INTRODUCTION

This chapter examines sodium pump molecular activity and membrane lipid composition in the brain of mammals and birds of different body size. The brain is quite a distinct tissue compared to the kidney and heart. Firstly, it is comprised of a very mixed population of cells, including neurons and a variety of supporting glial cells (astrocytes, oligodendrocytes and microglia) (Ross et al., 1995). The brain relies almost exclusively on glucose as an energy source and is essentially isolated from the general circulation via the blood brain barrier, which maintains a very controlled interstitial environment. Finally, the brain contains a much greater proportion of phospholipids than other tissues, with phospholipids constituting approximately 45% of the total brain dry weight (Purdon et al., 2002). Because of this large amount of phospholipid, and the dynamic turnover of membrane components that occurs during synaptic transmission (Ando et al., 2002), a substantial proportion (20%) of brain metabolism is devoted to the maintenance of phospholipid composition (Purdon et al., 2002).

Another major energy consumer in the brain is the sodium pump, to which approximately 50% of metabolism is linked (Clausen et al., 1991). The major function of the sodium pump in the brain is the maintenance of the sodium gradient (Clausen et al., 1991), which is particularly important considering the excitable nature of brain tissue and the continual Na^+ influx during action potentials. Many other functions in the brain are also underpinned by the sodium pump, such as the co-transport of various compounds including amino acids, and also Na^+/Ca^{2+} exchange. Sodium pump molecular activity has never been systematically examined in the brain of mammals and birds of different body size, although it has been demonstrated that molecular activity in the rat brain is 3-4 times greater than that in the toad brain (Else et al., 1996; Else and Wu, 1999), indicating that molecular activity changes in the brain may possibly reflect differences in metabolism.

In contrast to this, the fatty acid composition of the brain appears to be relatively consistent in a wide range of vertebrates with varying metabolic rates (Farkas et al.,

2000; Surai et al, 2000; Hulbert et al, 2002c). In general, brain phospholipids contain high levels of docosahexaenoic acid (22:6(*n*-3)), and unlike many other tissues, contain very low levels of linoleic acid (18:2(*n*-6)). Thus, this chapter examines sodium pump molecular activity and membrane lipid composition in brains of mammals and birds of different body size, to investigate the relationship between these two variables, and to see whether they are related to overall metabolic variation in these species.

5.2 RESULTS

The individual values for brain mass and protein concentration of the respective species are presented in Appendix III. These variables were considered with respect to body mass and are presented in Fig. 5.1 and Fig. 5.2 respectively. Body mass explained 98% and 92% of the variability in brain mass for the mammals and birds respectively (Fig. 5.1). From the allometric exponents it can be calculated that for every doubling in body mass an increase in brain mass of 65% in mammals and 38% in birds would be observed.

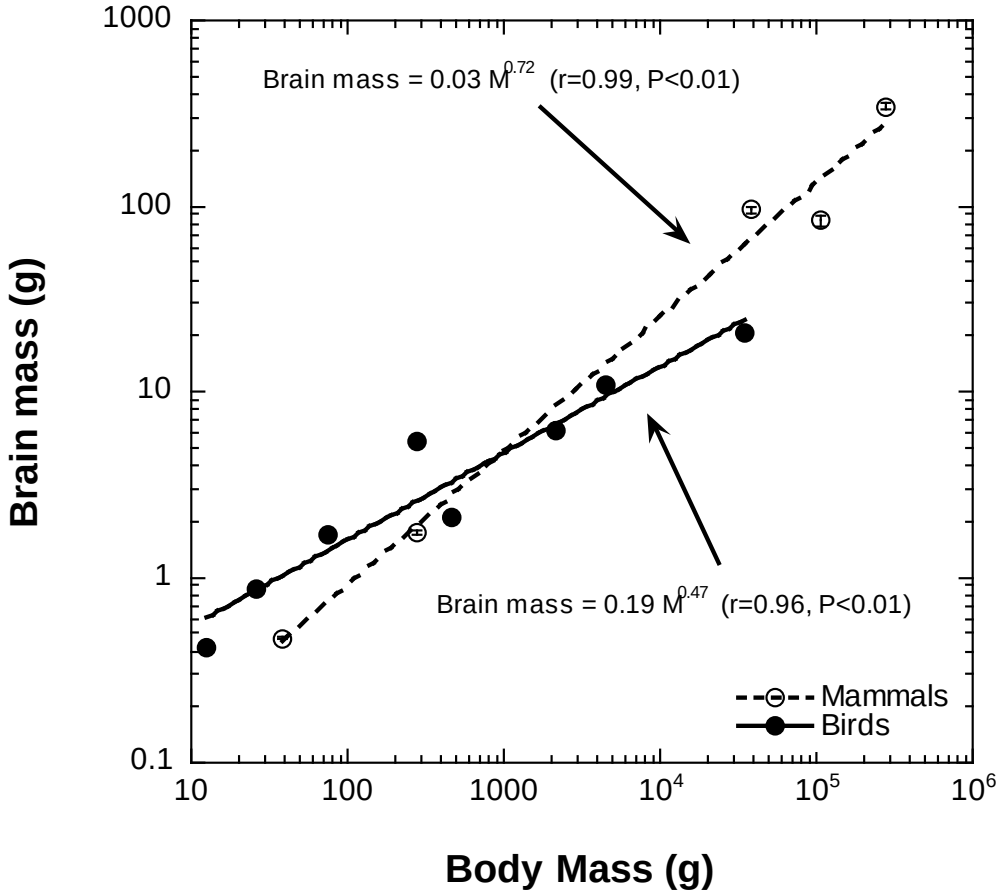


Fig. 5.1 *The relationship between the body mass of mammals and birds and the mass of their brains. Each point represents the mean ± standard error (SEM).*

Smaller species thus tended to have relatively larger brains, particularly in the birds where the brain represented 3.3% of body mass in the zebra finch, but only 0.05% in the emu. Protein concentration expressed as milligrams of protein per gram of brain wet weight (Fig. 5.2) displayed significant negative allometric trends in both the mammals (P=0.01) and birds (P=0.02). For every 100% increase in body size it can be calculated that mammalian brains would contain 1.4% less protein and avian brains would contain 2.1% less protein.

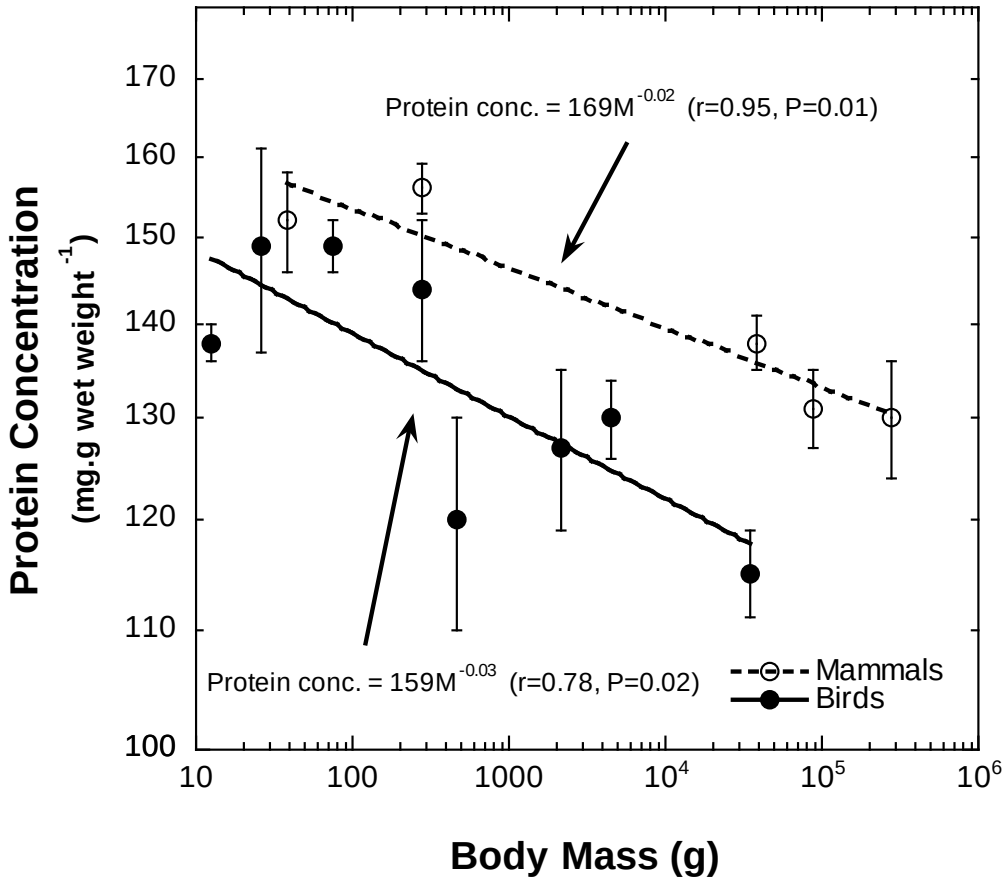


Fig. 5.2 *The relationship between the body mass of mammals and birds and brain protein concentration. Each point represents the mean ± standard error (SEM).*

Table 5.1 **Na^+K^+ATPase Activity, Sodium Pump Density, and Molecular Activity of Sodium Pumps from the Brain of Mammals and Birds**

	Na^+K^+ATPase Activity (μmol Pi.mg wet $wt^{-1}.hr^{-1}$)	Sodium Pump Density (pmol.g wet wt^{-1})	Molecular Activity ($ATP.min^{-1}$)
Mammals			
Mouse	5.12 ± 0.22 (n=16)	2804 ± 365 (n=12)	36206 ± 6362 (n=12)
Rat	5.25 ± 0.12 (n=12)	2254 ± 285 (n=10)	46901 ± 6542 (n=10)
Sheep	2.97 ± 0.16 (n=7)	4058 ± 1019 (n=6)	24238 ± 10893 (n=6)
Pig	2.63 ± 0.15 (n=8)	2744 ± 727 (n=8)	23890 ± 5819 (n=8)
Cow	3.40 ± 0.14 (n=7)	3709 ± 685 (n=7)	15716 ± 2621 (n=7)
Birds			
Zebra Finch	4.57 ± 0.15 (n=4)	5262 ± 984 (n=4)	15848 ± 2551 (n=4)
Sparrow	3.80 ± 0.31 (n=4)	3251 ± 430 (n=4)	21537 ± 5164 (n=4)
Starling	3.77 ± 0.15 (n=4)	7498 ± 995 (n=4)	8759 ± 1040 (n=4)
Currawong	3.95 ± 0.31 (n=4)	3841 ± 313 (n=4)	17502 ± 1873 (n=4)
Pigeon	3.27 ± 0.12 (n=4)	6720 ± 1476 (n=4)	9435 ± 2077 (n=4)
Duck	3.60 ± 0.28 (n=4)	4437 ± 761 (n=4)	15145 ± 3386 (n=4)
Goose	3.04 ± 0.17 (n=4)	3559 ± 860 (n=3)	16559 ± 3426 (n=3)
Emu	2.17 ± 0.10 (n=4)	2627 ± 287 (n=4)	14357 ± 1842 (n=4)

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram of wet weight each hour. Sodium pump density was measured as picomoles of sodium pumps per gram of wet weight. Molecular activity is maximal Na^+K^+ATPase activity divided by sodium pump density for the same animal, and is expressed as the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). (n) is the number of preparations used for each measurement.

The Na^+K^+ATPase activity, sodium pump density, and molecular activity measured for the mammals and birds are presented in Table 5.1. Na^+K^+ATPase activity values, measured at 37ºC for mammals and corrected to 37ºC for birds (see METHODS), are expressed as micromoles of inorganic phosphate liberated (from ATP) per mg of brain

wet weight per hour. When examined relative to body mass, there was a significant decrease in Na^+K^+ATPase activity for both the mammals (P=0.02) and birds (P<0.01) (Fig. 5.3). Body mass explained 83% and 79% of the variability of Na^+K^+ATPase activity in mammals and birds respectively. Calculated from the allometric exponents (-0.08 and –0.07) for every doubling in body mass there would be a 5.4% and 4.7% decrease in Na^+K^+ATPase activity in the mammals and birds respectively.

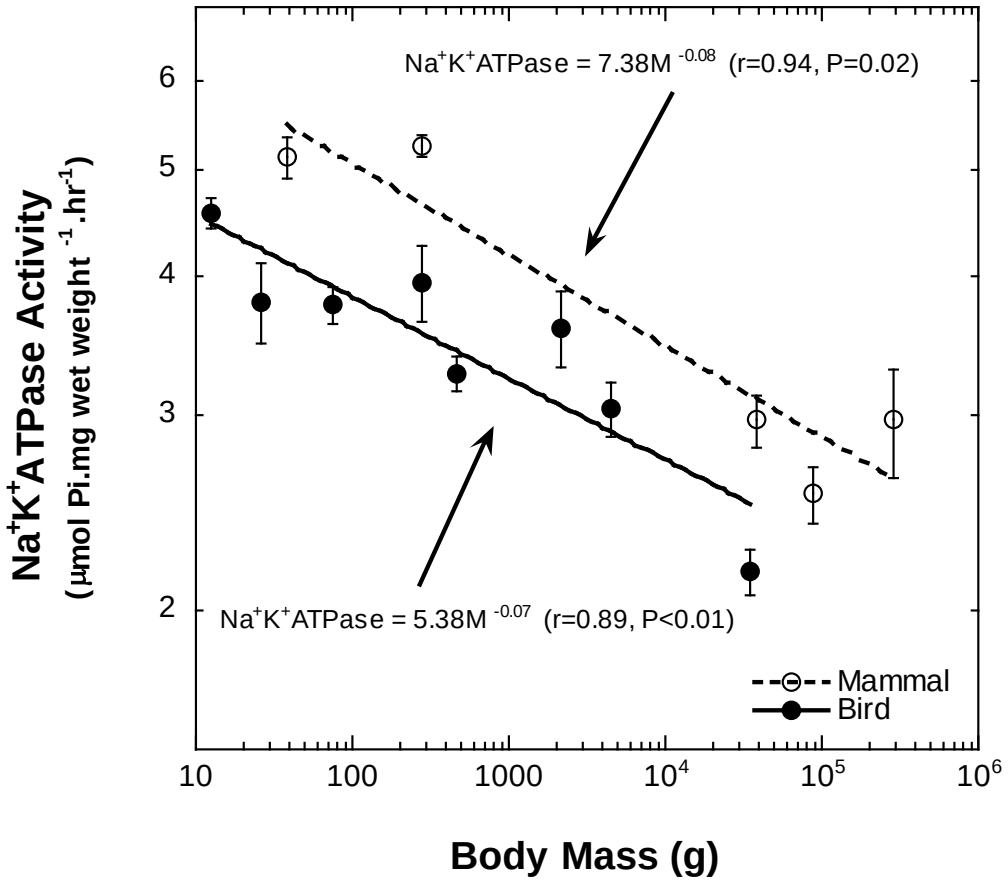


Fig. 5.3 *The relationship between the body mass of mammals and birds and the Na^+K^+ATPase activity of brain homogenates at 37ºC. Each point represents the mean ± standard error (SEM).*

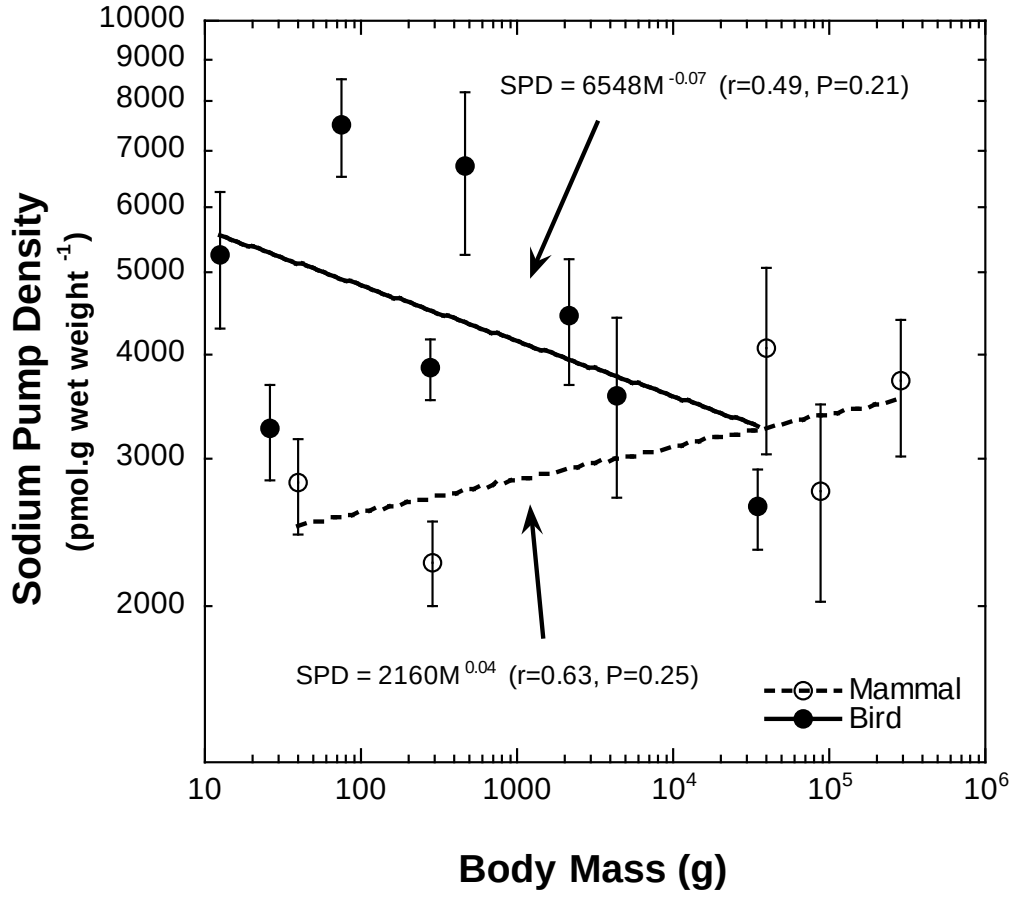


Fig. 5.4 *The relationship between the body mass of mammals and birds and the sodium pump density of brain tissue biopsies. Each point represents the mean ± standard error (SEM).*

Sodium pump density was measured as picomoles per gram of brain wet weight, and was generally higher in the bird species compared to the mammals. Sodium pump density relative to body mass is presented in Fig. 5.4, with neither the mammals or birds showing significant allometric relationships in this variable. While the relationships were not significant, the trends were for larger mammals to have higher sodium pump density and larger birds to have a reduced sodium pump density.

Molecular activity was calculated by dividing maximal Na^+K^+ATPase activity by the sodium pump density to give the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). Bird molecular activity values were calculated from Na^+K^+ATPase activity values corrected to 37°C to allow comparison with mammals. The range of molecular activity values for the mammals was 15,716–46,901 $ATP.min^{-1}$ while for birds the range was 8,759–21,537 $ATP.min^{-1}$ (Table 5.1). There was quite high variation within individual species for the molecular activity values. When examined relative to body mass (Fig. 5.5) there was a significant (P=0.04) decrease in molecular activity in the mammals with body mass explaining 79% of the variability.

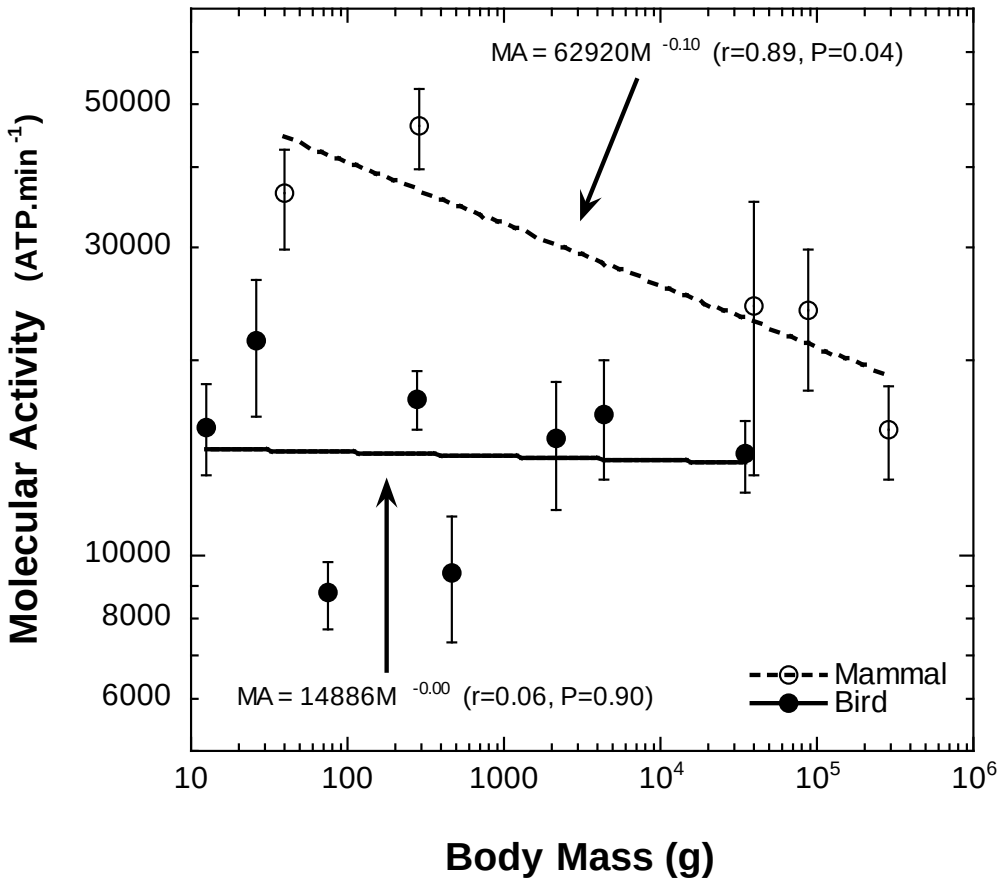


Fig. 5.5 *The relationship between the body mass of mammals and birds and the molecular activity of brain sodium pumps. Each point represents the mean ± standard error (SEM).*

Table 5.2	**Cholesterol and Phospholipid Content of Microsomal Membranes from the Brain of Mammals and Birds**		
	Cholesterol (μg. mg protein^{-1})	Phospholipid (μg. mg protein^{-1})	Cholesterol:Phospholipid (mole:mole)*
Mammals			
Mouse (n=4)	72.9 ± 3.4	271 ± 25	0.55 ± 0.05
Rat (n=3)	93.0 ± 9.3	405 ± 27	0.47 ± 0.06
Sheep (n=4)	102 ± 7.1	388 ± 26	0.53 ± 0.03
Pig (n=4)	107 ± 5.8	575 ± 51	0.38 ± 0.02
Cow (n=4)	91.0 ± 2.5	470 ± 16	0.39 ± 0.01
Birds			
Zebra Finch (n=3)	84.4 ± 10.7	227 ± 42	0.78 ± 0.14
Sparrow (n=4)	87.4 ± 3.8	167 ± 8	1.06 ± 0.05
Starling (n=4)	102 ± 6.7	264 ± 32	0.80 ± 0.08
Currawong (n=4)	96.4 ± 4.5	310 ± 17	0.63 ± 0.04
Pigeon (n=4)	95.2 ± 7.8	234 ± 39	0.86 ± 0.09
Duck (n=4)	92.8 ± 2.8	238 ± 3	0.79 ± 0.03
Goose (n=4)	84.8 ± 2.8	210 ± 23	0.85 ± 0.10
Emu (n=4)	83.3 ± 5.3	281 ± 62	0.65 ± 0.08

*Cholesterol and phospholipid content of microsomal membranes are expressed relative to protein content in μg.mg protein^{-1}. Cholesterol:Phospholipid ratios are the molar ratio (data from cholesterol and phospholipid content in μg.mg protein^{-1}). * moles of phospholipid calculated assuming a molecular weight of 780. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement.*

Calculated from the allometric exponent (-0.10), a doubling in body mass would result in a 6.7% decrease in brain sodium pump molecular activity. In the brains of the birds, less than 1% of the variability in molecular activity values could be explained by body mass.

Table 5.2 presents the cholesterol and phospholipid content, along with their molar ratio, of brain microsomal membranes from the mammals and birds. The cholesterol content (per mg of protein) was quite similar in the mammals and birds and showed no significant allometric variation in either group. Phospholipid content (per mg of protein) displayed quite a range in the mammals (271-575 μg.mg protein^{-1}) and while

the allometric exponent of 0.06 (Table 5.5) suggests a higher amount in larger species it primarily reflected the low content in the mouse. In birds the phospholipid content per mg of protein was generally lower than in the mammals (range 167 - 310 μg.mg protein^{-1}) and showed no significant body-size-related variation. The molar ratio of cholesterol:phospholipid tended to be higher in the smaller mammals and birds although this trend was not significant in either group (Table 5.5). From the cholesterol:phospholipid ratios it can be seen that brain microsomes from mammals would contain approximately 2-3 phospholipids per molecule of cholesterol, while in brain microsomes from birds there would be 1-1.5 phospholipids per molecule of cholesterol.

The fatty acid profile of brain microsomal phospholipids for mammals and birds are presented in Table 5.3 and Table 5.4 respectively. The major individual fatty acids, along with the composite parameters were considered with respect to body mass and the results of the analysis are presented in Table 5.5. Several major parameters are also plotted allometrically in Fig. 5.6.

The fatty acid profile of brain microsomal phospholipids from the mammals displayed no statistically significant allometric trends (Table 5.5). This contrasts with the kidney and heart where smaller mammals had a higher unsaturation index and 22:6(*n*-3) content than their larger counterparts. In the brain of all the mammals there was a relatively high content of 22:6(*n*-3), with the highest levels observed in the cattle and a considerably lower amount in the pigs (Table 5.3). On average 68% of the fatty acid chains were unsaturated, with an unsaturation index of 265. The mean values for the other major parameters were 20.8% MUFA, 47.4% PUFA, 18.3% *n*-6 PUFA, 29.1% *n*-3 PUFA, 47.2% C20+22 PUFA and 27.9% 22:6(*n*-3).

In microsomal phospholipids from the avian brains there were a small number of significant allometric trends (Table 5.5). The content of 18:2(*n*-6) and 20:3(*n*-6) showed a significant allometric decrease in the larger birds while the ratio of

Table 5.3 Microsomal Phospholipid Fatty Acid Profiles from Mammalian Brains

	Mouse	Rat	Sheep	Pig	Cow
Fatty Acid					
14:0	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.4 ± 0.1
16:0	18.1 ± 0.6	17.1 ± 1.7	17.0 ± 0.3	16.9 ± 0.5	14.1 ± 0.7
17:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.1
18:0	14.1 ± 0.9	14.6 ± 1.8	15.1 ± 0.5	16.5 ± 0.5	12.8 ± 1.5
14:1(*n*-7)	0.3 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
16:1(*n*-7)	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
17:1(*n*-7)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:1(*n*-9)	13.3 ± 0.2	14.7 ± 0.4	14.6 ± 0.6	19.0 ± 2.1	12.2 ± 1.5
18:1(*n*-7)	4.6 ± 0.5	4.0 ± 0.3	4.5 ± 0.2	6.3 ± 0.3	3.9 ± 0.3
18:2(*n*-6)	1.8 ± 1.3	0.8 ± 0.1	0.5 ± 0.0	0.8 ± 0.1	0.5 ± 0.1
20:1(*n*-9)	1.0 ± 0.0	1.6 ± 0.1	0.3 ± 0.0	0.6 ± 0.1	0.2 ± 0.1
20:2(*n*-6)	1.3 ± 0.9	0.3 ± 0.2	0.8 ± 0.1	0.5 ± 0.2	0.5 ± 0.2
20:3(*n*-6)	0.4 ± 0.0	0.4 ± 0.1	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
20:4(*n*-6)	11.9 ± 0.5	13.1 ± 0.7	8.6 ± 0.3	11.9 ± 0.2	9.3 ± 0.6
20:5(*n*-3)	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
22:4(*n*-6)	3.2 ± 0.2	4.5 ± 0.2	3.6 ± 0.2	5.5 ± 0.2	3.9 ± 0.4
22:5(*n*-6)	0.5 ± 0.0	0.8 ± 0.1	0.6 ± 0.0	3.0 ± 0.4	0.9 ± 0.1
22:5(*n*-3)	0.2 ± 0.0	0.3 ± 0.1	2.2 ± 0.2	0.7 ± 0.0	1.8 ± 0.1
22:6(*n*-3)	28.6 ± 1.5	27.0 ± 2.5	30.0 ± 1.6	16.5 ± 2.1	37.5 ± 4.1
% Saturates	32.7 ± 1.3	32.1 ± 3.5	32.8 ± 0.8	33.8 ± 0.8	27.6 ± 3.3
% MUFA	19.5 ± 0.6	20.8 ± 0.7	20.0 ± 0.8	26.6 ± 2.5	17.1 ± 1.9
% PUFA	47.8 ± 0.8	47.2 ± 3.1	47.2 ± 1.5	39.5 ± 2.3	55.3 ± 5.2
% *n*-9	14.3 ± 0.2	16.4 ± 0.4	14.8 ± 0.7	19.6 ± 2.2	12.4 ± 1.6
% *n*-7	5.2 ± 0.5	4.4 ± 0.3	5.2 ± 0.2	7.1 ± 0.3	4.7 ± 0.4
% *n*-6	19.1 ± 1.6	20.0 ± 0.7	14.6 ± 0.5	22.2 ± 0.5	15.7 ± 1.1
% *n*-3	28.8 ± 1.5	27.2 ± 2.4	32.6 ± 1.8	17.4 ± 2.0	39.6 ± 4.2
% Unsaturates	67.3 ± 1.3	67.9 ± 3.5	67.2 ± 0.8	66.2 ± 0.8	72.4 ± 3.3
Unsaturation index	262 ± 6	262 ± 18	269 ± 9	219 ± 11	314 ± 27
Chain length	19.2 ± 0.0	19.3 ± 0.1	19.3 ± 0.1	18.9 ± 0.1	19.7 ± 0.2
20+22C PUFA	47.1 ± 0.9	48.0 ± 3.3	46.9 ± 1.3	39.2 ± 2.3	55.0 ± 5.1
n-6/*n*-3	0.7 ± 0.1	0.7 ± 0.0	0.5 ± 0.0	1.3 ± 0.1	0.4 ± 0.0
20:4/18:2	19.3 ± 5.8	16.5 ± 2.2	16.2 ± 0.8	14.8 ± 2.2	22.5 ± 3.3

Microsomal phospholipid fatty acid profile of mammalian brains expressed as mole percentage of total fatty acids. Unsaturation index is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM), n=4 for all preparations except the rat where n=3.

Table 5.4 Microsomal Phospholipid Fatty Acid Profiles from Avian Brains

	Zebra Finch	Sparrow	Starling	Curra-wong	Pigeon	Duck	Goose	Emu
Fatty Acid								
16:0	15.1±1.2	18.0±0.6	18.0±1.3	16.9±1.2	16.5±1.2	13.5±1.0	18.7±0.9	11.7±0.9
18:0	12.5±1.3	14.8±1.1	14.6±1.6	14.3±1.0	13.5±1.0	10.5±0.8	13.9±0.8	8.4±0.9
16:1(*n*-7)	0.5±0.0	0.4±0.0	0.4±0.0	0.6±0.0	0.6±0.0	0.4±0.0	0.7±0.1	0.7±0.0
18:1(*n*-9)	6.3±0.1	6.8±0.1	7.4±0.3	8.2±0.5	9.8±0.4	7.7±0.6	9.8±0.7	6.5±0.1
18:1(*n*-7)	4.9±0.1	6.2±0.2	5.6±0.2	4.4±0.3	6.2±0.4	3.5±0.1	4.0±0.3	3.7±0.2
18:2(*n*-6)	1.2±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.1	0.2±0.0	0.3±0.0	0.2±0.0
20:2(*n*-6)	2.5±1.9	0.7±0.5	1.4±1.2	0.6±0.1	2.7±0.7	1.7±1.0	1.0±0.8	0.8±0.3
20:3(*n*-6)	1.0±0.0	0.4±0.1	0.4±0.0	0.4±0.0	0.3±0.0	0.1±0.0	0.1±0.0	0.0±0.0
20:4(*n*-6)	12.5±0.7	10.3±0.3	10.1±0.6	12.4±0.2	13.9±0.6	14.0±0.6	10.5±0.2	13.8±0.4
22:4(*n*-6)	4.7±0.1	3.3±0.1	3.1±0.1	3.5±0.1	6.2±0.2	5.7±0.4	3.5±0.3	4.7±0.3
22:5(*n*-6)	3.7±0.4	4.6±0.7	1.5±0.2	2.7±0.3	3.6±0.5	8.2±1.1	2.2±1.0	8.4±0.5
22:5(*n*-3)	0.6±0.0	0.3±0.0	0.8±0.0	0.4±0.0	0.5±0.0	0.6±0.0	1.8±0.6	0.6±0.0
22:6(*n*-3)	34.4±3.9	33.8±3.1	36.1±2.8	35.2±2.3	25.7±1.8	33.9±2.1	33.3±1.8	40.5±1.3
% Saturates	27.6±2.6	32.7±1.7	32.6±2.9	31.2±2.1	30.0±2.2	24.0±1.8	32.6±1.7	20.1±1.7
% MUFA	11.8±0.2	13.4±0.3	13.4±0.5	13.1±0.8	16.5±0.8	11.6±0.5	14.6±0.8	10.9±0.3
% PUFA	60.7±2.5	53.8±1.9	53.9±2.6	55.6±2.9	53.5±2.8	64.4±2.1	52.8±1.0	69.0±2.0
% *n*-9	6.3±0.1	6.8±0.1	7.4±0.3	8.2±0.5	9.8±0.4	7.7±0.6	9.8±0.7	6.5±0.1
% *n*-7	5.4±0.1	6.6±0.2	6.0±0.2	5.0±0.4	6.7±0.4	3.9±0.1	4.8±0.2	4.4±0.2
% *n*-6	25.7±1.4	19.7±1.2	17.0±0.9	20.0±0.6	27.4±2.0	29.9±2.0	17.4±2.1	27.9±1.9
% *n*-3	35.0±3.9	34.1±3.1	36.9±2.9	35.6±2.3	26.2±1.8	34.5±2.1	35.4±2.6	41.1±1.3
% Unsaturates	72.4±2.6	67.3±1.7	67.4±2.9	68.8±2.1	70.0±2.2	76.0±1.8	67.4±1.7	79.9±1.7
Unsaturation index	319±21	298±14	300±18	307±16	279±13	342±13	294±9	375±11
Chain length	19.7±0.1	19.5±0.1	19.5±0.1	19.6±0.1	19.4±0.1	20.0±0.1	19.5±0.1	20.2±0.1
20+22C PUFA	59.5±2.5	53.3±2.0	53.5±2.6	55.1±3.0	52.9±2.9	64.2±2.1	52.5±1.0	68.8±2.0
n-6/*n*-3	0.8±0.1	0.6±0.1	0.5±0.1	0.6±0.0	1.1±0.1	0.9±0.1	0.5±0.1	0.7±0.0
20:4/18:2	10.3±0.5	21.3±2.3	21.0±0.4	25.1±1.9	23.6±4.4	89.5±17	38.0±2.4	68.0±9.9

Microsomal phospholipid fatty acid profile of bird brains expressed as mole percentage of total fatty acids. Unsaturation index is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM), n=4 for all preparations except the zebra finch where n=3.

20:4(*n*-6):18:2(*n*-6), which is an indication of Δ5 and Δ6 desaturase enzyme activity, was increased in the same birds. All bird species had a high content of 22:6(*n*-3) with the highest levels found in the emu and the lowest levels in the pigeon (Table 5.4). The emu and duck had the highest levels of unsaturation (as indicated by unsaturation index; Table 5.4), which appeared to result from a greater content of 22:5(*n*-6) in these species, plus the high levels of 22:6(*n*-3). The average values for the major parameters were 71% total unsaturates, 314 unsaturation index, 13.2% MUFA, 58.0% PUFA, 23.1% *n*-6 PUFA, 34.9% *n*-3 PUFA, 57.5% C20+22 PUFA and 34.1% 22:6(*n*-3).

Table 5.6 presents the calculated values for a number of parameters in the brain of a 250g mammal and bird. Comparison of the mammal and bird shows that the brain of the bird would be 59% larger than the mammal and would contain 11% less protein per gram wet weight. Na^+K^+ATPase activity would be 77% of the value for the mammal, while sodium pump density in the bird would be 65% and 148% greater than the mammal on a wet weight and whole tissue basis respectively. The sodium pump molecular activity of the bird would be 60% lower than the value in the mammal. Examining the microsomal phospholipids it can be seen that the bird would possess approximately 3% more total unsaturates, while having an unsaturation index that would be 20% greater than the value in the mammal. Bird phospholipids would contain 36% less monounsaturates and 23% more polyunsaturates than the mammal. The percentage of *n*-6 PUFA and *n*-3 PUFA would be 17% and 22% greater respectively in the bird compared to the mammal. The content of 22:6(*n*-3) in birds would be 23% greater than in the mammals, while the molar ratio of cholesterol:phospholipid in the bird would be 57% greater than the value observed in the mammal.

Linear correlation coefficients were determined between molecular activity values and individual lipid parameters and there were several significant relationships observed in the mammals (Fig. 5.7), while no significant relationships were found to exist in the birds. In mammals, negative correlations occurred between sodium pump molecular

Table 5.5 The Relationship Between Body Mass and Various Lipid Parameters in the Brains of Mammals and Birds

	Mammals			Birds		
	Intercept at M=1g	Exponent	Correlation coefficient	Intercept at M=1g	Exponent	Correlation coefficient
Lipid parameter						
16:0	19.4	-0.02	0.72	19.3	-0.03	0.53
18:0	14.5	0.00	0.03	17.0	-0.05	0.68*
18:1(*n*-9)	13.7	0.01	0.17	6.89	0.02	0.30
18:1(*n*-7)	4.18	0.01	0.22	6.82	-0.06	0.73**
18:2(*n*-6)	2.10	-0.11	0.83*	1.37	-0.20	0.82**
20:3(*n*-6)	0.36	0.04	0.84*	1.99	-0.35	0.93†***
20:4(*n*-6)	14.1	-0.03	0.66	10.6	0.02	0.42
22:4(*n*-6)	3.39	0.02	0.40	3.55	0.03	0.28
22:5(*n*-6)	0.41	0.09	0.48	2.11	0.09	0.42
22:5(*n*-3)	0.06	0.27	0.90**	0.40	0.07	0.37
22:6(*n*-3)	27.5	-0.00	0.03	31.7	0.01	0.23
% Saturates	34.0	-0.01	0.39	36.3	-0.04	0.61
% MUFA	20.1	0.00	0.07	13.8	-0.01	0.18
% PUFA	46.7	0.00	0.04	51.9	0.02	0.47
% n-9	15.5	-0.00	0.04	6.89	0.02	0.30
% n-7	4.53	0.02	0.34	7.05	-0.05	0.66*
% n-6	20.9	-0.02	0.37	19.5	0.02	0.30
% n-3	26.9	0.00	0.06	32.1	0.01	0.27
% UFA	66.3	0.00	0.37	65.4	0.01	0.57
UI	254	0.00	0.13	280	0.02	0.52
Chain length	19.1	0.00	0.27	19.4	0.00	0.53
C20+22 PUFA	46.7	0.00	0.02	50.9	0.02	0.51
n-6/n-3	0.79	-0.02	0.18	0.64	0.01	0.06
20:4/18:2	17.5	0.00	0.02	7.76	0.22	0.86***
Cholesterol ($\mu g.mg\ protein^{-1}$)	72.3	0.03	0.73	94.5	-0.01	0.26
Phospholipid ($\mu g.mg\ protein^{-1}$)	249	0.06	0.80	204	0.03	0.36
Chol:PL (mol:mol)	0.60	-0.03	0.69	0.94	-0.03	0.45

*The relationships between body mass and the various lipid parameters were determined by linear regression (least-square method) of log-transformed values. The relationships were determined using the mean parameter value for each species (n=5 for mammals and n=8 for birds). *P<0.1, **P<0.05, ***P<0.01. † relationship determined using n=7 (see Table 5.4)*

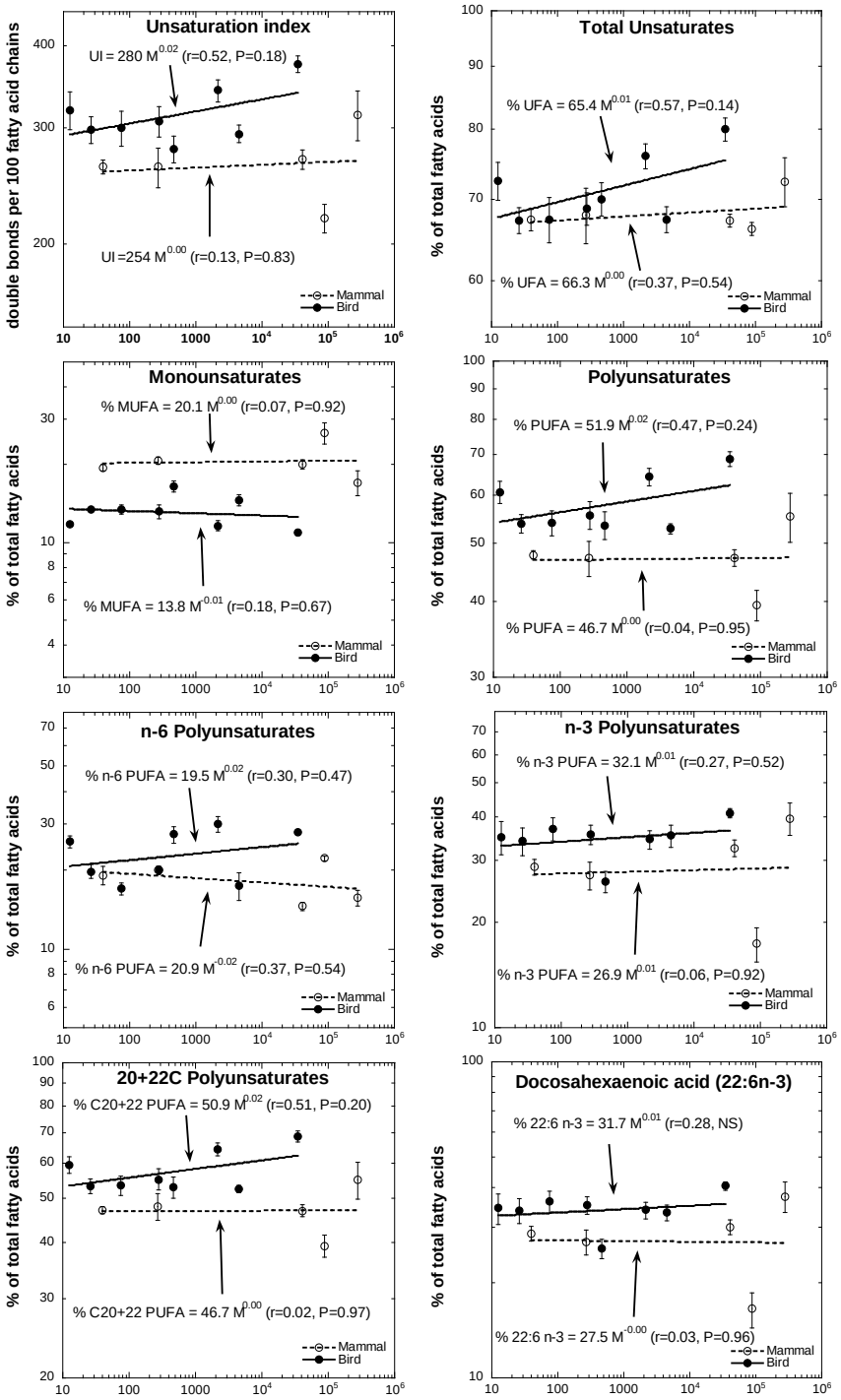


Fig. 5.6 *The relationship between body mass and the unsaturation index, content of unsaturates, monounsaturates, polyunsaturates, n-6 PUFA, n-3 PUFA, C20+22 PUFA and docosahexaenoic acid in brain microsomal phospholipids from mammals and birds. Values are means ± standard errors (SEM).*

Table 5.6	**Comparison of a 250g Mammal and Bird**	
	Mammal	**Bird**
Brain mass (g)	1.60	2.55
Protein concentration (mg.g wet weight^{-1})	151	135
Na^+K^+ATPase activity (μmol Pi.mg wet weight^{-1}.hr^{-1})	4.74	3.66
Sodium pump density (pmol.g wet weight^{-1})	2694	4449
Sodium pumps per brain	2.7×10^{15}	6.7×10^{15}
Molecular activity (ATP.min^{-1})	36224	14401
Unsaturation index	260	313
% Unsaturates	67.4	69.1
% Monounsaturates	20.4	13.1
% Polyunsaturates	47.0	58.0
% n-6	18.7	21.8
% n-3	27.7	33.9
% 22:6(*n*-3)	27.2	33.5
Cholesterol:Phospholipid (mole:mole)	0.51	0.80

Values were calculated from the allometric equations determined for each parameter, using a body mass of 250 grams.

activity and 16:1(*n*-7), 17:1(*n*-7) and 20:5(*n*-3), while the content of 20:1(*n*-9) showed a positive correlation with molecular activity.

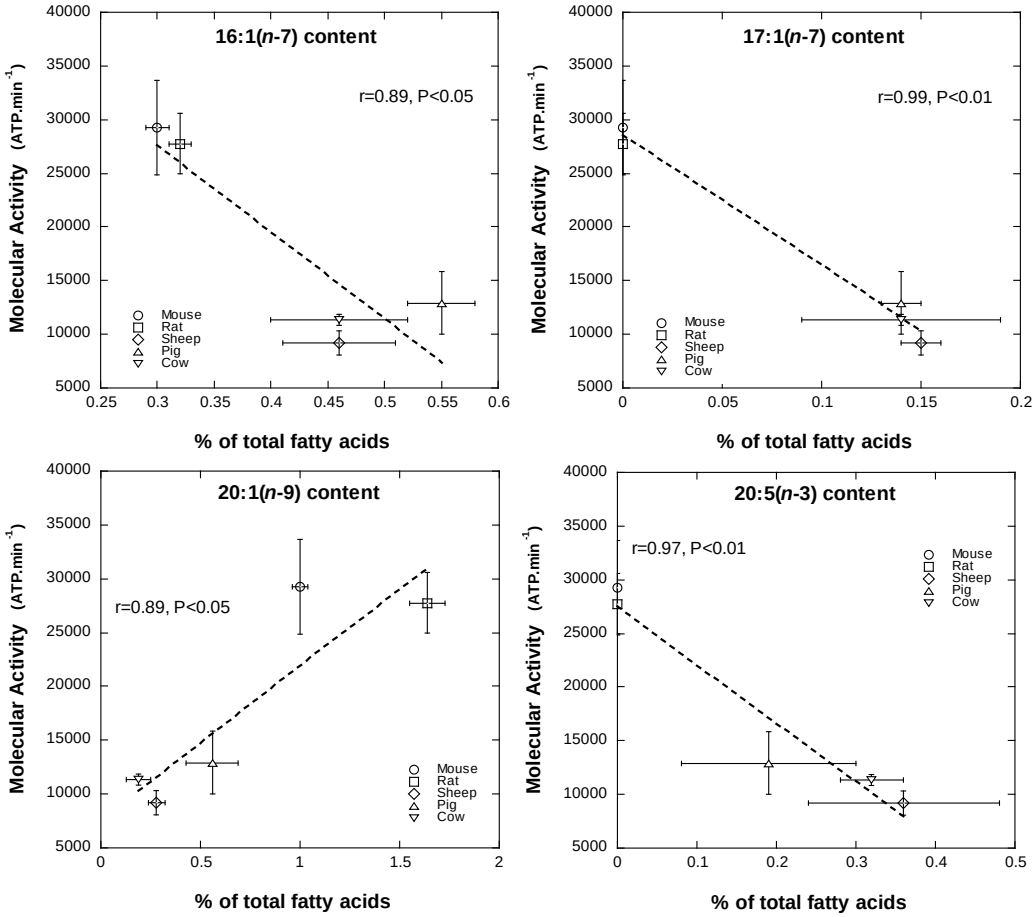


Fig. 5.7 *Linear correlations between sodium pump molecular activity in mammals and the content of 16:1(n-7), 17:1(n-7), 20:1(n-9) and 20:5(n-3).*

5.3 DISCUSSION

This chapter has examined the relationships between body size, sodium pump molecular activity and membrane lipid composition in the brain of mammals and birds. Sodium pump (Na^+K^+ATPase) enzyme activity was higher in the brain of smaller mammals and birds and showed a significant allometric decline (Fig. 5.3). Similar findings were also found for both the kidney and heart indicating that higher mass-specific metabolism in small endotherms is associated with increased enzymic activity of the sodium pump in their respective tissues. In mammals the changes in Na^+K^+ATPase activity were primarily (84%) determined by changes in the molecular activity of individual sodium pumps, where there was a significant 3-fold variation across the body size range (Fig. 5.5). In birds there was a high degree of variation amongst the species, which resulted in no significant relationships between body size and sodium pump density (Fig. 5.4) or molecular activity (Fig. 5.5). It is worth noting however, that despite this variation, the allometric exponent measured for sodium pump density (-0.07) was equal to the exponent observed for Na^+K^+ATPase activity, indicating that birds possibly vary brain Na^+K^+ATPase activity mainly through changes in the number of sodium pumps.

Molecular activity values varied between 15,716 – 46,901 ATP.min^{-1} in the mammals, while in the birds the values ranged between 8,759 – 21,537 ATP.min^{-1}. Previous measurements of brain molecular activity in mammals describe values of approximately 9,000 – 12,000 ATP.min^{-1} (Nakao et al., 1973; Chen and Lin-Shiau, 1986; Else et al., 1996), while no previous measurements of molecular activity have been conducted in avian brains. The mammalian molecular activity values in the current study were quite high compared to the literature, and this appeared to be primarily caused by the density of sodium pumps measured (Table 5.1), which were lower than the 6,000 – 10,000 pmol.g wet weight^{-1} normally described for brain tissue (Klodos et al., 1975; Schmidt et al., 1992; Else et al., 1996). As mentioned in the previous chapter the phosphate-facilitated ^{3}H-ouabain binding technique relies on tissue viability, and thus if there was any tissue necrosis during the binding assay in the

current study there may have been an underestimation of the sodium pump density and a subsequent overestimation of molecular activity. This may have been particularly prevalent in the brain where high rates of metabolism are expected. The variability encountered during the ^{3}H-ouabain binding may have also potentially contributed to the high molecular activity values observed. For example, in the mice there was little variation in the $Na^{+}K^{+}$ATPase activity measurements, while sodium pump density varied between 1,101 – 4,845 pmol.g wet weight^{-1}. As a result the calculated molecular activity values varied between 13,500 – 94,535 ATP.min^{-1}, with the higher values likely overestimating the true molecular activity, and hence increasing the mean value.

The sodium pump performs diverse functions in the brain, including the maintenance of the sodium gradient, co-transport of various compounds such as amino acids, and also providing the gradient for Na^{+}/Ca^{2+} exchange (Clausen et al., 1991). In mammals the brain expresses a number of different isoforms (α_1, α_2 and α_3) that likely mediate the various processes performed by the sodium pump (Juhaszova and Blaustein, 1997a). In intact mammalian brain, *in vivo* sodium pump activity has been estimated by isotope flux studies to be between 0.10 – 0.74 μmol ATP.mg wet weight^{-1}.hr^{-1} (Clausen et al., 1991). These values represent up to 50% of brain O_2 consumption, which is evidence of the importance of the sodium pump to brain function. Compared to the potential maximal *in vitro* activity values measured in the present study (Table 5.1), it can be estimated that *in vivo* the sodium pump is operating at less than 15% of maximum, although this may vary depending on which section of the brain is active at any particular time.

The allometric exponents for brain mass in the mammals and birds were 0.72 and 0.47 respectively. These allometric slopes are similar to previous investigations (Brody, 1945; Peters, 1983; Else and Hulbert, 1985), and demonstrate that as body size increases, relative brain size decreases in both mammals and birds. The very low allometric exponent observed in the birds was heavily influenced by the species examined, as the brain mass of all the passerines were above the mammalian regression

line, while brain mass of the non-passerines were all below the mammalian line (Fig 5.1). Na^+K^+ATPase activity was combined with brain mass and expressed as the micromoles of inorganic phosphate liberated (from ATP) per kidney per hour, and assuming that this value represented the maximum rate, and using a P/O ratio of 2.0 (Rolfe and Brown, 1997), it was possible to determine the potential maximal daily energy expenditure by the sodium pump ($Kcal.day^{-1}$). These Na^+K^+ATPase values for mammals and birds represented 8 – 23% and 5 – 28% of basal energy expenditure respectively, and when plotted allometrically (Fig. 5.8), mammals scaled with an exponent of 0.66, while the exponent for birds was 0.39. Considering the BMR exponents of 0.73 and 0.63 for the mammals and birds, maximal brain Na^+K^+ATPase activity accounted for a relatively higher proportion of BMR in smaller mammals and birds, which is the opposite to what was observed in the heart of these species. Again it should be noted that it is unknown whether all of the species are operating at a similar percentage of these potential maximal *in vitro* activities, under *in vivo* basal conditions.

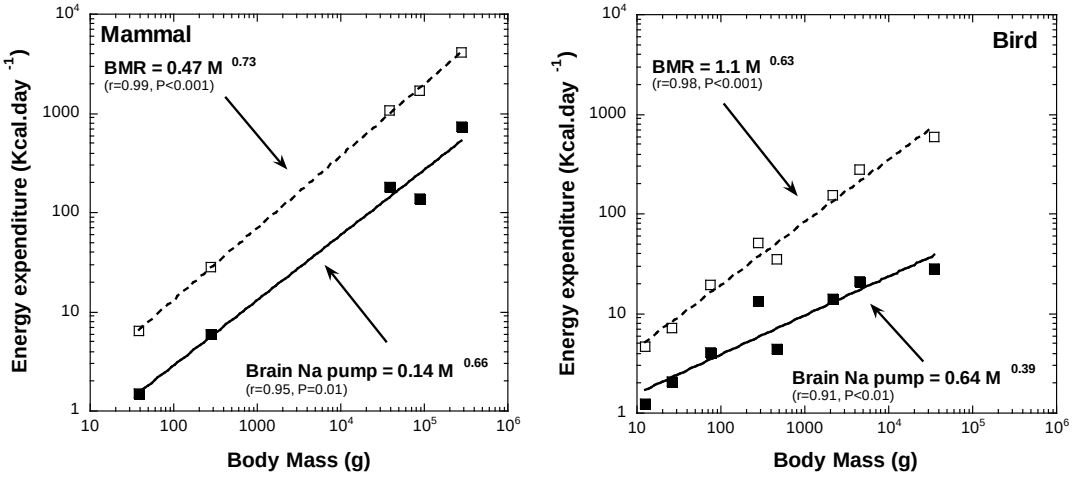


Fig. 5.8 *The relationship between the body mass of mammals and birds, basal metabolic rate and the maximal daily energy expenditure of brain sodium pumps.*

One difference between the brain compared to the kidney and heart was the protein concentration (mg.g wet weight^{-1}). The brain showed a significant allometric decrease in the larger species, with exponents of –0.02 and –0.03 determined for the mammals and birds respectively (Fig. 5.2). The brains of the mice contained 17% more protein than that of the cattle, while sparrow and starling brains contained 23% more protein than the emus. Hulbert & Else (1989) reported that when compared to a reptile, the brain of a more metabolically active mammal contained 98% more protein.

Membrane lipid composition was examined in both groups, with no significant allometric variation observed in any fatty acid parameters in the mammals, while a small number of significant relationships were observed in the birds (Table 5.5). The relative lack of any body-size-related variation is consistent with previous investigations of brain phospholipids from mammals (Couture and Hulbert, 1995a; Hulbert et al., 2002c) and birds (Hulbert, A.J., Turner, N., Brand, M.D., Else, P.L. unpublished observations). In general the fatty acid profile of both mammals and birds was dominated by 22:6(*n*-3), and unlike most other tissues, contained minimal amounts of 18:2(*n*-6). This composition appears to be common in the brain of a large number of vertebrates (Farkas et al., 2000; Surai et al., 2000; Hulbert et al., 2002c) and it is currently unknown why the fatty acid profile of this organ is so highly conserved. Perhaps the best example of the conservative nature of the brain was the fatty acid profile of the zebra finch, whose composition in heart and kidney was very anomalous compared to that of the other birds, but whose composition in the brain was very similar to the other species.

Within the birds there was a significant allometric decrease in the content of 18:2(*n*-6) and 20:3(*n*-6), along with a significant increase in the ratio of 20:4(*n*-6):18:2(*n*-6) in the larger species. In mammals, the ratio of 20:4(*n*-6):18:2(*n*-6) gives an estimate of the activity of the Δ5 and Δ6 desaturase enzymes and the increased activity observed in the larger birds, coupled with the reduced levels of both 18:2(*n*-6) and 20:3(*n*-6) indicates very active conversion of shorter chain *n*-6 PUFA to their long chain derivatives. This

was very evident in the duck and emu who had the highest ratio of 20:4(*n*-6):18:2(*n*-6) and also the highest levels of 22:5(*n*-6) (Table 5.4). Interestingly, while the estimated desaturase activity was increased in the larger birds, the elongase enzyme activity (estimated from the 18:0:16:0 ratio) was reduced in these species (results not shown). Whether elongase/desaturase enzyme systems operate in birds as they do in mammals is unknown as within the mammals there was no allometric trend seen in the ratio of 20:4(*n*-6):18:2(*n*-6), while the ratio of 18:0:16:0 was actually increased in the larger mammals.

In contrast to the general allometric trends in other tissues, the brain of several of the larger mammals and birds actually had slightly greater levels of PUFA than the smaller species. While this may just represent inter-species differences, it is also possible that the tissue selection process may have contributed to the slightly higher PUFA levels seen in the larger species. The cerebral cortex was the area of the brain used for all lipid analyses and a minimum of 0.5 - 1.0g of tissue was required for the preparation of the microsomes. In the larger species there was an excess of available tissue, while in the smaller species the cerebellum and brainstem were removed and the brain tissue remaining was subsequently used in the measurements. While this section of tissue was largely cerebral cortex, it would have also contained subcortical structures such as the hippocampus, thalamus and striatum, and also neuronal tracts (white matter). Compared to the cerebral cortex, subcortical structures (Carrié et al., 2000) and white matter (Bourre et al., 1984; Skinner et al., 1993), have reduced levels of PUFA and thus the tissue selection process may have contributed to the slightly reduced content of PUFA seen in the smaller mammals and birds.

Comparison of the mammals and birds showed that the major differences were a greater content of 18:1(*n*-9) in the mammals and higher levels of both 22:5(*n*-6) and 22:6(*n*-3) in the birds. Farkas et al. (2000) also found higher PUFA levels in birds compared to mammals and of particular interest in the current study was the high levels of 22:5(*n*-6) observed in all of the birds. Within the mammals the pig had the highest

levels of 22:5(*n*-6) along with the lowest levels of 22:6(*n*-3) (Table 5.3). These fatty acids appear to be regulated in a reciprocal manner in the brain of mammals, where 22:6(*n*-3) is the preferred long chain PUFA, but can be partially compensated for by 22:5(*n*-6), if there is a dietary deficiency of *n*-3 PUFA (Sheaff et al., 1995; Carrié et al., 2000). These fatty acids are similar in structure, however recent work suggests that the additional double bond present in 22:6(*n*-3), has a major impact on its physical properties, and as a result 22:5(*n*-6) may not compensate functionally for 22:6(*n*-3) with regards to lipid-protein interactions in the membrane (Eldho et al., 2003). Considering the high levels of *n*-6 PUFA observed in the kidney (Fig. 3.6) and heart (Fig. 4.6) of the pigs, it is possible that the dietary fat intake of these mammals was relatively *n*-3 PUFA deficient. It is unlikely that the same compensatory relationship between 22:6(*n*-3) and 22:5(*n*-6) exists in birds as the highest levels of both fatty acids were found in the emu (Table 5.4). The reason for the high levels of 22:5(*n*-6) in the birds is unclear, but may be a reflection of dietary influences or may indicate a preference for elongation and desaturation through the *n*-6 PUFA pathway, which is consistent with what was seen in the other tissues of these birds (see Fig 3.6 & Fig. 4.6).

The fact that such a large number of species maintain a relatively similar fatty acid profile in their brain, especially high concentrations of 22:6(*n*-3) (Farkas et al., 2000; Surai et al., 2000; Hulbert et al., 2002c), indicates a specific functional requirement for this fatty acid. Indeed it appears that its presence in the brain is maintained both by astrocytes, which actively desaturate and elongate *n*-3 precursors and release the 22:6(*n*-3) for uptake by the neurons (Moore, 1993), and by the neurons themselves, which preserve membrane 22:6(*n*-3) in preference to other PUFA (Kim et al., 1999). Alterations in brain fatty acid composition and in particular reductions in 22:6(*n*-3), have been linked with a number of functional deficits, both during developmental periods (Horrocks and Yeo, 1999) and during adulthood (Hibbeln, 1998; Fenton et al., 1999; Hibbeln, 2002). While the exact membrane property related to 22:6(*n*-3) that regulates function is yet to be determined, a variety of biophysical interactions have

been proposed including changes in the order and curvature of membranes (Farkas et al., 2000), interactions with G proteins (Litman and Mitchell, 1996), alterations in the permeability of membranes (Stillwell et al., 1993) and increased fusibility of 22:6(*n*-3) containing membranes (Glaser and Gross, 1994). It appears that many of these properties are not just related to the gross amount of 22:6(*n*-3) in the membrane, but may be determined by the specific pairing of 22:6(*n*-3) with other fatty acids in the phospholipid molecular species. For example, the propensity to undergo fusion is six times greater in 22:6(*n*-3)-containing plasmalogen ethanolamines compared to phosphatidylethanolamines (Farooqui et al., 2000) and furthermore the activity of protein kinase C in the brain is increased by PE (18:1(*n*-9)/22:6(*n*-3)), but not PC (18:1(*n*-9)/22:6(*n*-3)), emphasising the importance of phospholipid molecular species.

Apart from the acyl composition, another interesting finding in the current study was the high level of cholesterol observed in brain microsomal membranes from the mammals and birds (Table 5.2). These values were much higher than those observed in the kidney (Table 3.2) or heart (Table 4.2), which appears to be consistent with findings of other researchers (Yeagle, 1985; Wu et al., 2001). Cholesterol metabolism in the brain is quite complex, and the exact reason for the high levels of cholesterol is unknown. Recently it was suggested that cholesterol may be an essential factor in the formation and functioning of synapses (Pfrieger, 2003). Support for this hypothesis comes from studies which show that cholesterol turnover is much lower in the synaptic membranes of adults rats, compared to those of young rats, where presumably a large number of synapses are being established (Ando et al., 2002). Developing neurons synthesise their own cholesterol, however it is thought that neurons in adult brains derive their cholesterol from astrocytes (Pfrieger, 2002), which may in part explain the high levels of cholesterol, as astrocytes are the brain's most abundant cell type (Ross et al., 1995).

A potential functional explanation for the high level of cholesterol, is that it has been implicated as a major factor in the formation of lipid rafts. Lipid rafts represent

membrane microdomains where saturated phospholipids, sphingomyelins and cholesterol aggregate and form less mobile, gel-like areas, with PUFA rich phospholipids maintaining a very fluid environment in the remaining membrane (Simons and Ikonen, 1997). In the brain, lipid rafts are thought to be important in determining the appropriate distribution and orientation of post-synaptic receptors (Tsui-Pierchala et al., 2002). As mentioned above, brain phospholipids contain high levels of PUFA, particularly 22:6(*n*-3) and to a lesser extent 20:4(*n*-6), however cholesterol is relatively insoluble in these fatty acids (Brzustowicz et al., 2002a; Brzustowicz et al., 2002b), and requires the presence of a saturated fatty acid (Huster et al., 1998). Thus it is somewhat perplexing that there are high levels of both cholesterol and PUFA in the brain, and since cholesterol loading of cells increases PUFA content (Blom et al., 2001), an interesting question arises. Does the high level of cholesterol observed in the brain, accumulate in response to the high levels of PUFA, or does the high level of PUFA accumulate in response to the high levels of cholesterol? Since both cholesterol and PUFA appear to be supplied to neurons by astrocytes (Moore, 1993; Kim et al., 1999; Pfrieger, 2002), the exact importance of these glial cells in maintaining the appropriate neural membrane lipid composition warrants further investigation.

When correlation coefficients were determined between individual lipid parameters and molecular activity values, no correlations were observed in the birds, while in the mammals negative correlations were observed between molecular activity and 16:1(*n*-7), 17:1(*n*-7) and 20:5(*n*-3), with a positive relationship found with 20:1(*n*-9) (Fig. 5.7). The negative correlation between molecular activity and 16:1(*n*-7) and 17:1(*n*-7) are similar to those observed in avian hearts in the previous chapter (Fig. 4.8). All of the fatty acids that displayed correlations were either not present or only made up a small percentage of the lipids, and therefore further experimentation is needed to determine their physiological significance.

In conclusion, the allometric relationships observed in the brain of mammals and birds again were specific to the tissue being examined. In mammals, molecular activity was significantly higher in the smaller species and was the major determinant of changes in Na^+K^+ATPase activity, while in birds there were large inter-species differences in molecular activity and no apparent allometric trend. Membrane lipid composition was relatively consistent across the range of mammals and birds, which contrasted with the heart and kidney where smaller species maintained a higher unsaturation index and higher levels of 22:6(*n*-3). The brain of all the mammals and birds contained high levels of 22:6(*n*-3), which appears to be an essential requirement for normal brain function. A number of minor fatty acids correlated with molecular activity in the mammals, while no correlations were observed in birds. Thus it appears that while there were some allometric similarities observed in the kidney, heart and brain of the mammals and birds, many of the body size relationships were characteristic to the individual tissues. Therefore to gain a better understanding of the potential relationships between sodium pump molecular activity and membrane lipid composition, Chapter 7 will examine all three tissues together, along with values determined in tissues from two ectothermic species, the octopus and bearded dragon lizard.

Chapter VI

Sodium Pump Molecular Activity and Membrane Lipid Composition in Tissues of the Octopus and Bearded Dragon Lizard

6.1 INTRODUCTION

The previous three chapters have examined sodium pump molecular activity and membrane lipid composition in tissues from the two major endothermic groups, mammals and birds. This chapter will examine the same variables in tissues from two ectothermic species, the octopus and bearded dragon lizard.

Ectotherms have a BMR that is four to ten times lower than endotherms of the same size and body temperature (Hemmingsen, 1960; Hulbert, 1980). Part of this difference in metabolic rate is due to larger internal organs in endotherms (Else and Hulbert, 1981; Hulbert and Else, 1989), however much of the difference relates to the activity of processes occurring at the cellular level. For example, the higher metabolism observed in mammalian tissues compared to ectothermic tissues, is a reflection of differences in energy use associated with cellular processes such as the maintenance of transmembrane ion gradients for sodium and potassium (Hulbert and Else, 1981; Else and Hulbert, 1987) and also mitochondrial H^+ (Brand et al., 1991). Higher activity is required in these processes, as compared to ectotherms, the tissues of endotherms are more permeable to protons (Brand et al., 1991) and also to sodium and potassium (Else and Hulbert, 1987). This increased membrane leakiness has been proposed as one of the major mechanisms underlying the evolution of endothermy (Else and Hulbert, 1987).

One of the consequences of having cells with an increased sodium and potassium permeability, is that increased activity is required in endothermic sodium pumps. In a study of tissues from a large number of endothermic and ectothermic species, Else et al. (1996) showed that the enzymatic activity of sodium pumps in endotherms was 3-4 times higher than that in ectotherms. Of interest though, was the fact that the tissue concentration of sodium pumps was not different between the two groups, and was largely related to the tissue type, being high in the brain and kidney and low in the liver and skeletal muscle. Thus, when molecular activity was calculated by dividing enzymatic activity by sodium pump concentration, a consistent endotherm-ectotherm

difference became apparent, irrespective of the tissue. Sodium pumps from tissues of endotherms were operating at molecular activities of around 6,000–10,000 ATP.min^{-1}, while those from ectothermic tissues were operating at around 1,000–3,000 ATP.min^{-1}.

The study by Else et al. (1996) examined a wide range of ectothermic species including reptiles, amphibians, fish and even an invertebrate species, indicating that reduced sodium pump molecular activity is likely a general trend for ectotherms. A review of the literature however, indicates that there are exceptions to this general trend, where molecular activity values in some ectothermic tissues have been shown to be more similar to endothermic values. The shark (*Squalus acanthias*) rectal gland has been extensively studied as it is an abundant source of sodium pumps, and in a recent report by Cornelius (2001), sodium pump molecular activity in this organ was shown to be approximately 12,500 ATP.min^{-1}. Similarly, the electric organ of the electric eel (*Electrophorus electricus*) contains sodium pumps that display molecular activities of 7,500 ATP.min^{-1} (Perrone et al., 1975), while sodium pumps from the digestive gland and gill of the octopus turnover at a rate of 12,500 ATP.min^{-1} and 8,000 ATP.min^{-1} respectively (Bader et al., 1968).

The exact reason for the high molecular activity in these ectothermic tissues is unknown, however there are several factors that may provide a potential explanation. Firstly, these species all live in saltwater environments, and the increased molecular activity may be an adaptation to the saline environment, as has been previously observed (Towle et al., 1977). Secondly, the organs mentioned above perform specialised functions that are likely to be dependant on very active transport of sodium, thus requiring sodium pumps with a high capacity. Finally, the lipid composition of the cell membranes in these species is possibly influencing the molecular activity of the sodium pumps. Else and Wu (1999) showed that the molecular activity of ectothermic sodium pumps was increased when the surrounding membrane lipids become more polyunsaturated. Phospholipids from the shark (*Squalus acanthius*) rectal gland (Cornelius et al., 2003) and eel (*Electrophorus electricus*) electric organ (Rotstein et

al., 1987) have been shown to be highly polyunsaturated, while for the octopus, individual organs have never been measured, but phospholipids from the mantle contain a high proportion of PUFA (de Koning, 1972; Iverson et al., 2002; Passi et al., 2002). Therefore the high molecular activities may be a reflection of the influence that the surrounding membrane lipids are having on the sodium pumps.

The current study was designed to examine sodium pump molecular activity and membrane lipid composition in individual tissues of the octopus, to see whether the expected high levels of PUFA in the membrane may be associated with sodium pump molecular activities that were higher than general ectothermic levels. During the course of this study, the opportunity also arose to conduct the same measurements on the bearded dragon lizard, a reptile with a preferred body temperature of 37°C (Bartholemew and Tucker, 1963), that in contrast to the octopus, has very monounsaturated membranes (Hulbert and Else, 1989; Brand et al., 1991).

6.2 RESULTS

6.2.1 Octopus

The average body mass (in grams) of the octopi examined in the current study was 463 ± 92 (range 133 - 943g). The organ mass and protein concentration for the five tissues examined is presented in Table 6.1. The digestive gland was the largest internal organ followed by the gills, kidney and brain. Collectively these organs accounted for approximately 6% of total body mass. Protein concentration varied between tissues, ranging from 100 mg.g wet wt^{-1} in the brain up to 199 mg.g wet wt^{-1} in the digestive gland.

Na^+K^+ATPase activity, sodium pump density, and molecular activity values measured in the octopus tissues are presented in Table 6.2. Na^+K^+ATPase activities were measured at 37°C and are expressed as micromoles of inorganic phosphate liberated (from ATP) per mg wet weight per hour. The kidney and brain displayed the highest Na^+K^+ATPase, with values of around 1.2 μmol Pi.mg wet $wt^{-1}.hr^{-1}$, while the digestive gland, tentacle, and gill all displayed activities that were approximately 0.4 μmol Pi.mg wet $wt^{-1}.hr^{-1}$. The Q_{10} values determined in the octopus tissues (range 1.5 – 3.2; Appendix II) indicate that Na^+K^+ATPase activity continued to increase through to 37°C and that sodium pumps were not damaged by measurement at higher temperatures. Furthermore octopus sodium pumps were able to operate at higher rates when exposed to temperatures above those normally experienced, which is consistent with what has been observed in other ectothermic tissues, including the shark rectal gland (Esmann and Skou, 1988; Else et al., 1996).

Sodium pump density was measured as picomoles per gram wet weight and varied almost 4-fold across the different octopus tissues. The kidney contained the highest density of sodium pumps at around 5,000 pmol.g wet wt^{-1}, the brain had approximately half of this value (2,700 pmol.g wet wt^{-1}), and the other three tissues all had similar values between 1,300 – 1,700 pmol.g wet wt^{-1}. When sodium pump density was

Table 6.1	**The Mass and Protein Concentration of Tissues from the Octopus**	
	Organ mass (% body mass)	Protein Concentration (mg.g wet wt^{-1})
Digestive Gland	3.79 ± 0.47 (n=11)	199 ± 15 (n=9)
Kidney	0.42 ± 0.02 (n=11)	166 ± 6 (n=9)
Brain	0.12 ± 0.04 (n=11)	100 ± 8 (n=9)
Tentacle	ND	145 ± 7 (n=9)
Gill	1.66 ± 0.13 (n=11)	120 ± 7 (n=9)

Values are means ± standard errors (SEM). Organ mass is expressed as percentage of body mass. Protein concentration was determined in tissue homogenates and is expressed as milligrams of protein per gram of wet weight. ND, not determined. (n) is the number of preparations used for each measurement.

considered with respect to organ mass, the number of individual sodium pumps was calculated to be 1.86 X 10^{16} per digestive gland, 5.64 X 10^{15} per kidney, 4.88 X 10^{14} per brain and 6.49 X 10^{15} per gill.

Molecular activity was calculated by dividing the average maximal Na^+K^+ATPase activity by the average sodium pump density to give the number of ATP molecules hydrolysed by each sodium pump per minute (ATP.min^{-1}). Molecular activity values were approximately 4,000 ATP.min^{-1} in the digestive gland and kidney, around 5,000 ATP.min^{-1} in the tentacle and gill, and the highest molecular activity was observed in the brain with a value of 7,400 ATP.min^{-1}, which was around 50-80% higher than in the other tissues.

The cholesterol and phospholipid content, along with their molar ratio, in octopus microsomal membranes are presented in Table 6.3. The cholesterol content varied between the different tissues, being lowest in the tentacle at 45 μg.mg $protein^{-1}$, and highest in the brain at 87 μg.mg $protein^{-1}$. The digestive gland, kidney and gill all had

Table 6.2 Na^+K^+ATPase Activity, Sodium Pump Density, and Molecular Activity of Sodium Pumps from Tissues of the Octopus

	Na^+K^+ATPase Activity (μmol Pi.mg wet $wt^{-1}.hr^{-1}$)	Sodium Pump Density (pmol.g wet wt^{-1})	Molecular Activity ($ATP.min^{-1}$)*
Digestive Gland	0.37 ± 0.03 (n=4)	1668 ± 697 (n=3)	3697
Kidney	1.17 ± 0.11 (n=4)	5065 ± 1778 (n=3)	3850
Brain	1.21 ± 0.10 (n=4)	2724 ± 790 (n=4)	7403
Tentacle	0.37 ± 0.09 (n=4)	1303 ± 190 (n=3)	4732
Gill	0.43 ± 0.05 (n=4)	1408 ± 415 (n=4)	5089

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram of wet weight each hour. Sodium pump density was measured as picomoles of sodium pumps per gram of wet weight. Molecular activity is maximal Na^+K^+ATPase activity divided by sodium pump density, and is expressed as the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). SEM not given as activity and density were determined in different preparations. (n) is the number of preparations used for each measurement.*

cholesterol levels between 65 – 80 μg.mg $protein^{-1}$. Microsomal phospholipid content (per mg of protein) was highest in the digestive gland and brain, with approximately 30% less phospholipid in the kidney, gill and tentacle. The molar ratio of cholesterol:phospholipid was calculated and was found to range from 0.38 in the tentacle to 0.64 in the gill. These cholesterol:phospholipid ratios mean that for every cholesterol molecule, octopus microsomes contain approximately 1–2.5 phospholipids.

The fatty acid profile of microsomal phospholipids from the octopus tissues are presented in Table 6.4. While there was variation between the fatty acid profiles of all the tissues, there were also some similarities. All tissues were characterised by high levels of the long chain *n*-3 PUFA, 22:6(*n*-3), with the highest concentrations observed in the brain and tentacle. All tissues also contained a substantial proportion of 20:5(*n*-3), although this fatty acid appeared to have an almost reciprocal relationship with

Table 6.3 Cholesterol and Phospholipid Content of Microsomal Membranes from Tissues of the Octopus

	Cholesterol (μg. mg protein^{-1})	Phospholipid (μg. mg protein^{-1})	Cholesterol:Phospholipid (mole:mole)*
Digestive Gland	77.8±7.6 (n=6)	352±33 (n=6)	0.44±0.02 (n=5)
Kidney	64.1±7.4 (n=6)	284±8 (n=6)	0.43±0.08 (n=4)
Brain	87.1±1.9 (n=4)	355±54 (n=4)	0.53±0.08 (n=6)
Tentacle	45.2±3.7 (n=6)	256±19 (n=6)	0.38±0.04 (n=4)
Gill	79.3 ±3.8 (n=6)	252±17 (n=6)	0.64±0.03 (n=4)

*Cholesterol and phospholipid content of microsomal membranes are expressed relative to protein content in μg.mg protein^{-1}. Cholesterol:Phospholipid ratios are the molar ratio (data from cholesterol and phospholipid content in μg.mg protein^{-1}). * moles of phospholipid calculated assuming a molecular weight of 780. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement.*

20:4(*n*-6), such that the digestive gland, kidney and gill had the highest levels of 20:4(*n*-6) and lowest levels of 20:5(*n*-3), with the reverse true for the tentacle and brain. The content of 18:2(*n*-6) was low in all tissues, accounting for less than 1%, while a significant amount of 20:1(*n*-9) was observed in several tissues, accounting for nearly 9% of the total fatty acids in the kidney. For the composite parameters the octopus tissues contained on average 35% saturated, 14% monounsaturated and 51% polyunsaturated fatty acids. Unsaturation index was lowest in the digestive gland and kidney, and highest in the gill and tentacle. All tissues contained fatty acids with an average chain length of approximately 19 carbons, while the proportion of C20+22 PUFA was around 50 - 60%. The *n*-6/*n*-3 ratio was quite different between tissues, being approximately 1.0 in the digestive gland, kidney and gill, while only 0.2 and 0.3 in the brain and tentacle respectively.

Table 6.4	**Microsomal Fatty Acid Profile of Tissues from the Octopus**					
	Digestive Gland (n=6)	Kidney (n=6)	Brain (n=5)	Tentacle (n=6)	Gill (n=6)	Correlation with Molecular Activity
Fatty Acid						
14:0	3.3±0.4	1.8±0.4	1.3±0.3	1.2±0.2	2.8±1.0	-0.52
16:0	21.3±1.7	15.4±1.2	19.6±0.9	22.0±0.4	15.1±1.4	0.07
17:0	1.7±0.0	1.5±0.5	1.3±0.3	1.3±0.2	1.4±0.2	-0.73
18:0	12.2±1.0	15.7±0.6	15.3±0.5	8.0±0.4	10.3±0.8	0.22
14:1(*n*-7)	1.7±0.3	1.0±0.5	0.8±0.5	0.9±0.3	1.9±0.5	-0.41
16:1(*n*-7)	0.7±0.1	0.4±0.0	0.8±0.1	0.9±0.1	0.5±0.1	0.48
17:1(*n*-7)	1.1±0.2	0.5±0.1	0.5±0.1	0.3±0.1	0.9±0.2	-0.23
18:1(*n*-9)	7.1±1.4	5.8±1.4	5.7±0.3	5.0±0.3	5.4±1.4	-0.38
18:1(*n*-7)	1.5±0.3	1.2±0.3	0.6±0.3	1.0±0.4	0.6±0.2	-0.80
18:2(*n*-6)	0.7±0.2	0.3±0.1	0.8±0.0	0.6±0.1	0.3±0.1	0.56
20:1(*n*-9)	1.1±0.1	8.6±1.3	5.4±0.3	3.2±0.5	2.9±0.1	0.13
20:2(*n*-6)	0.5±0.1	0.5±0.1	0.7±0.1	0.5±0.0	0.3±0.0	0.56
20:3(*n*-3)	0.6±0.1	0.5±0.1	1.8±0.1	0.6±0.1	0.5±0.1	0.90**
20:4(*n*-6)	17.4±1.9	18.9±2.4	4.0±0.1	8.9±0.9	22.8±2.6	-0.69
20:5(*n*-3)	10.5±1.2	5.8±0.4	17.5±0.7	18.0±0.7	8.8±0.6	0.64
22:1(*n*-9)	0.4±0.1	0.2±0.1	0.1±0.1	0.1±0.1	0.8±0.3	-0.28
22:4(*n*-6)	1.0±0.2	2.7±0.4	0.5±0.1	1.2±0.2	3.5±0.4	-0.39
22:5(*n*-6)	0.6±0.1	0.7±0.1	0.2±0.0	0.5±0.1	0.8±0.1	-0.79
22:5(*n*-3)	1.8±0.3	1.9±0.2	1.7±0.5	2.3±0.6	2.2±0.3	-0.34
22:6(*n*-3)	12.7±1.7	15.4±1.2	20.9±0.9	22.6±1.0	17.0±1.0	0.64
% Saturates	39.2±2.5	34.7±2.1	37.6±0.7	32.8±0.6	29.9±2.3	0.05
% MUFA	14.2±1.4	17.8±1.4	14.3±0.6	11.6±0.8	13.3±1.4	-0.24
% PUFA	46.6±3.8	47.6±3.1	48.1±1.3	55.5±0.7	56.8±3.6	0.07
% *n*-9	9.2±1.4	14.9±1.3	11.5±0.3	8.7±0.6	9.5±1.4	-0.04
% *n*-7	5.0±0.6	3.1±0.3	2.8±0.5	3.0±0.3	3.9±0.6	-0.54
% *n*-6	20.6±2.2	23.5±2.9	6.2±0.2	11.8±1.1	28.0±3.1	-0.67
% *n*-3	26.0±1.8	23.9±1.4	41.9±1.3	43.7±1.1	28.7±0.8	0.68
% Unsaturates	60.8±2.5	65.3±2.1	62.4±0.7	67.2±0.6	70.1±2.3	-0.05
UI	238±17	245±12	264±7	298±3	285±14	-0.32
Chain length	18.7±0.1	19.1±0.1	19.0±0.1	19.2±0.0	19.2±0.1	0.30
C20+22 PUFA	47.6±3.8	55.9±3.6	52.8±1.5	58.2±0.4	60.1±3.6	0.10
n-6/*n*-3	0.8±0.0	1.0±0.1	0.2±0.0	0.3±0.0	1.0±0.1	-0.69

*Microsomal phospholipid fatty acid profile of octopus tissues expressed as mole percentage of total fatty acids. Unsaturation index (UI) is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement. Linear correlation coefficients were determined between individual lipid parameters and molecular activity using the mean value for each tissue. **P<0.05.*

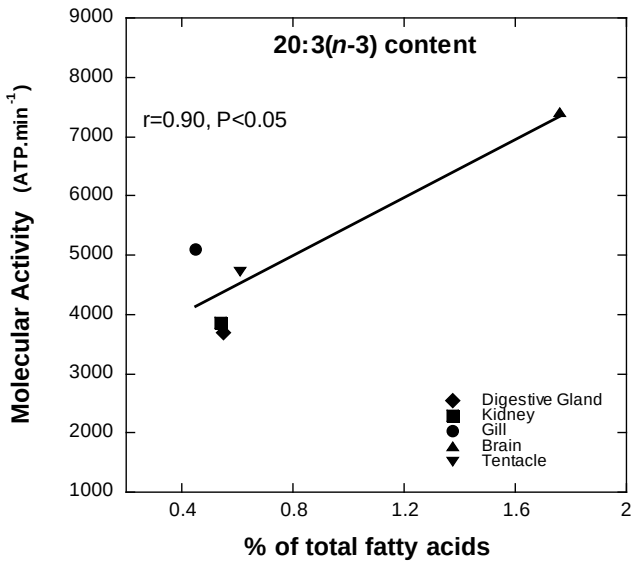


Fig. 6.1 *Linear correlation between sodium pump molecular activity in octopus tissues and the content of 20:3(n-3).*

To assess whether variation in membrane lipid composition may have been associated with the molecular activity of sodium pumps in the octopus tissues, linear correlation coefficients were determined between all individual lipid parameters and sodium pump molecular activity values. These results are presented for all fatty acid parameters in the right hand column of Table 6.4, with Fig. 6.1 showing the one significant relationship, which was a positive correlation observed between molecular activity and the content of 20:3(*n*-3).

6.2.2 Bearded Dragon

The average body mass (in grams) of the bearded dragons examined in the current study was 236 ± 37 (range 131 – 406g). Table 6.5 presents the organ mass and protein concentration for the four tissues examined. The liver was the largest internal organ and together with the kidney and brain totalled approximately 2% of body mass in the

Table 6.5 The Mass and Protein Concentration of Tissues from the Bearded Dragon

	Organ mass (% body mass)	Protein Concentration (mg.g wet wt^{-1})
Liver	1.61 ± 0.22 (n=7)	152 ± 6 (n=7)
Kidney	0.27 ± 0.20 (n=7)	138 ± 6 (n=7)
Brain	0.10 ± 0.01 (n=7)	101 ± 5 (n=6)
Skeletal Muscle	ND	108 ± 7 (n=7)

Values are means ± standard errors (SEM). Organ mass is expressed as percentage of body mass. Protein concentration was determined in tissue homogenates and is expressed as milligrams of protein per gram of wet weight. ND, not determined. (n) is the number of preparations used for each measurement.

bearded dragons. Protein concentration, expressed as milligrams of protein per gram wet weight, varied between tissues, ranging from 101 mg.g wet wt^{-1} in the brain to 152 mg.g wet wt^{-1} in the liver.

Na^+K^+ATPase activity, sodium pump density, and molecular activity values measured in bearded dragon tissues are presented in Table 6.6. Na^+K^+ATPase activity values were measured at 37°C and are expressed as micromoles of inorganic phosphate liberated (from ATP) per mg wet weight per hour. Examination of the different tissues revealed a greater than 20-fold variation in Na^+K^+ATPase activity. The brain displayed the highest activity, followed by the kidney, skeletal muscle and liver. For skeletal muscle most of the Na^+K^+ATPase activity measurements yielded negligible activity and as such, only one successful assay was recorded. The reason for this low activity is presently unknown. The Q_{10} values measured for the bearded dragon tissues were approximately 1.5 for the kidney and brain, while only 0.92 for the liver (Appendix II). The values for kidney and brain suggest that bearded dragon sodium pumps were able to operate at higher rates at 37°C, which is their preferred body temperature. The low

Table 6.6 Na^+K^+ATPase Activity, Sodium Pump Density, and Molecular Activity of Sodium Pumps from Tissues of the Bearded Dragon

	Na^+K^+ATPase Activity (μmol Pi.mg wet $wt^{-1}.hr^{-1}$)	Sodium Pump Density (pmol.g wet wt^{-1})	Molecular Activity ($ATP.min^{-1}$)*
Liver	0.09 ± 0.02 (n=5)	671 ± 23 (n=3)	2136
Kidney	0.40 ± 0.05 (n=6)	2119 ± 430 (n=4)	3178
Brain	1.89 ± 0.13 (n=6)	4823 ± 600 (n=4)	6541
Skeletal Muscle	0.13 (n=1)	762 ± 317 (n=3)	2777

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram of wet weight each hour. Sodium pump density was measured as picomoles of sodium pumps per gram of wet weight. Molecular activity is maximal Na^+K^+ATPase activity divided by sodium pump density, and is expressed as the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). SEM not given as activity and density were determined in different preparations. (n) is the number of preparations used for each measurement.*

Q_{10} value in the liver may have potentially indicated thermal damage, but also may suggest that the sodium pumps in the liver are intrinsically thermally independent, as has previously been found in the liver of the toad and carp (Else et al., 1996).

Sodium pump density was measured as picomoles per gram wet weight, and varied in a similar fashion to Na^+K^+ATPase activity. The brain contained the highest density of sodium pumps at 4,800 pmol.g wet wt^{-1}, while the values for the kidney, skeletal muscle and liver were approximately 2,200, 760, and 670 pmol.g wet wt^{-1} respectively. When sodium pump density was combined with organ mass, the number of individual sodium pumps was calculated to be 1.6×10^{15} per liver, 8.4×10^{14} per kidney and 6.7×10^{14} per brain.

Molecular activity was calculated by dividing the average maximal Na^+K^+ATPase activity by the average sodium pump density to give the number of ATP molecules hydrolysed by each sodium pump per minute ($ATP.min^{-1}$). Molecular activity values

were similar for the liver, kidney and skeletal muscle being approximately 2,000–3,000 ATP.min^{-1}. The brain however displayed a molecular activity that was 2–3 times greater than the other tissues at 6,540 ATP.min^{-1}.

The cholesterol and phospholipid content, along with their molar ratio, in microsomal membranes are presented in Table 6.7. The brain contained the greatest concentration of cholesterol (per mg of protein), having approximately two times more cholesterol than the liver and kidney and around three times more than skeletal muscle. Phospholipid content (per mg of protein) was essentially the same in the liver, kidney and skeletal muscle, whilst the brain contained approximately 70% more phospholipid. When the molar ratio of cholesterol to phospholipid was calculated it was found to range from 0.18 in skeletal muscle to 0.36 in the brain. These cholesterol:phospholipid ratios mean that microsomes from the bearded dragon contain around 3-5 phospholipids per molecule of cholesterol.

The fatty acid profile of microsomal phospholipids from the bearded dragon tissues are presented in Table 6.8. The fatty acid profile of the liver, kidney and skeletal muscle was fairly similar, with only a few percentage points difference in a couple of individual fatty acids. The brain of the bearded dragon was quite distinct from the other tissues and was characterised by high levels of 22:6(*n*-3) and low levels of 18:2(*n*-6). When the composite parameters were derived, all tissues had similar levels of MUFA (24-30%). PUFA levels were highest in skeletal muscle and lowest in brain, however the proportion of PUFA with 20+22 carbons was greatest in the brain. Unsaturation index was approximately 160 in the liver and kidney, 190 in brain, and 200 in skeletal muscle. The major difference between the tissues was the ratio of *n*-6/*n*-3 fatty acids, which was approximately 4.0 – 5.0 in the liver, kidney and skeletal muscle, while only 1.2 in the brain due to the high amount of 22:6(*n*-3) and low amount of 18:2(*n*-6).

Table 6.7 Cholesterol and Phospholipid Content of Microsomal Membranes from Tissues of the Bearded Dragon

	Cholesterol (μg. mg protein^{-1})	Phospholipid (μg. mg protein^{-1})	Cholesterol:Phospholipid (mole:mole)*
Liver	68.2±6.2 (n=6)	457±44 (n=6)	0.32±0.04 (n=6)
Kidney	61.6±2.7 (n=5)	489±38 (n=5)	0.26±0.02 (n=5)
Brain	124.0±6.1 (n=3)	702±36 (n=3)	0.36±0.02 (n=3)
Skeletal Muscle	43.2±3.3 (n=6)	499±58 (n=6)	0.18±0.01 (n=6)

*Cholesterol and phospholipid content of microsomal membranes are expressed relative to protein content in μg.mg protein^{-1}. Cholesterol:Phospholipid ratios are the molar ratio (data from cholesterol and phospholipid content in μg.mg protein^{-1}). * moles of phospholipid calculated assuming a molecular weight of 780. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement.*

To determine whether membrane lipid composition is associated with the molecular activity of sodium pumps in the bearded dragon tissues, linear correlation coefficients were determined between all individual lipid parameters and sodium pump molecular activity values. These results are presented for all fatty acid parameters in the right hand column of Table 6.8 and several of the major significant relationships are plotted in Fig. 6.2. A number of parameters displayed significant positive correlations with molecular activity, including the content of 18:1(*n*-7), 20:1(*n*-9), 20:2(*n*-6), 22:5(*n*-6), 22:6(*n*-3) and the phospholipid content (per mg of protein), while negative correlations were observed for 18:2(*n*-6), 20:3(*n*-6), 20:5(*n*-3) and 22:5(*n*-3). Examination of Fig 6.2 however, reveals that most of these significant relationships are just a reflection of the distinct fatty acid profile and high sodium pump molecular activity observed in the brain.

Table 6.8	**Microsomal Fatty Acid Profile of Tissues from the Bearded Dragon**				
	Liver (n=6)	Kidney (n=5)	Brain (n=3)	Skeletal Muscle (n=6)	Correlation with Molecular Activity
Fatty Acid					
16:0	13.1±0.6	12.6±0.3	20.4±0.4	9.4±0.8	0.90
18:0	14.6±0.9	17.9±0.6	14.0±0.3	13.2±0.3	-0.17
16:1(*n*-7)	2.5±0.3	1.7±0.2	2.7±0.2	1.8±0.3	0.52
18:1(*n*-9)	21.2±1.3	18.3±0.9	18.3±0.3	16.9±1.8	-0.29
18:1(*n*-7)	2.4±0.3	2.8±0.4	5.9±0.4	2.8±0.4	0.99***
18:2(*n*-6)	22.4±1.2	20.1±1.0	0.9±0.1	19.0±1.5	-0.99**
20:1(*n*-9)	0.4±0.0	0.7±0.2	1.4±0.0	0.7±0.1	0.99***
20:2(*n*-6)	0.5±0.0	0.6±0.0	1.6±0.3	0.7±0.1	0.98**
20:3(*n*-9)	0.9±0.1	1.3±0.0	0.2±0.0	1.7±0.2	-0.78
20:3(*n*-6)	0.8±0.1	0.6±0.0	0.3±0.0	0.7±0.0	-0.99**
20:4(*n*-6)	10.3±1.0	13.6±0.9	15.3±0.7	18.9±1.7	0.26
20:5(*n*-3)	2.7±0.3	2.3±0.2	0.5±0.1	2.5±0.2	-0.99***
22:4(*n*-6)	0.4±0.1	0.5±0.1	0.8±0.1	0.8±0.2	0.49
22:5(*n*-6)	0.7±0.0	0.9±0.1	1.7±0.1	0.6±0.0	0.97**
22:5(*n*-3)	2.8±0.2	1.8±0.1	0.4±0.1	2.5±0.2	-0.98**
22:6(*n*-3)	1.3±0.2	1.8±0.2	13.4±0.5	3.3±0.2	0.98**
% Saturates	29.7±0.9	32.0±0.4	35.4±0.3	24.4±0.7	0.73
% MUFA	27.1±1.6	24.0±0.4	29.5±0.7	23.6±2.4	0.70
% PUFA	43.2±1.6	44.0±0.3	35.1±0.5	52.1±3.0	-0.77
% *n*-9	22.6±1.3	20.2±0.8	19.9±0.3	19.3±1.8	-0.44
% *n*-7	5.4±0.6	5.1±0.4	9.4±0.7	6.0±0.6	0.95*
% *n*-6	34.5±1.2	35.4±0.3	19.4±1.1	40.4±2.5	-0.92*
% *n*-3	7.8±0.6	7.3±0.3	16.0±0.6	9.9±0.5	0.93*
% Unsaturates	70.3±0.9	68.0±0.4	64.6±0.3	75.6±0.7	-0.73
UI	159±5	163±3	190±2	200±8	0.44
Chain length	18.2±0.0	18.3±0.0	18.5±0.0	18.6±0.1	0.60
C20+22 PUFA	21.2±1.6	24.8±1.2	36.0±0.4	33.5±2.1	0.72
n-6/*n*-3	4.5±0.3	4.9±0.2	1.2±0.1	4.1±0.2	-0.94*

*Microsomal phospholipid fatty acid profile of bearded dragon tissues expressed as mole percentage of total fatty acids. Unsaturation index (UI) is the average number of double bonds per 100 fatty acid chains. Chain length is the average chain length of each fatty acid. Values are means ± standard errors (SEM). (n) is the number of preparations used for each measurement. Linear correlation coefficients were determined between individual lipid parameters and molecular activity using the mean value for each tissue. *P<0.1, **P<0.05, ***P<0.01.*

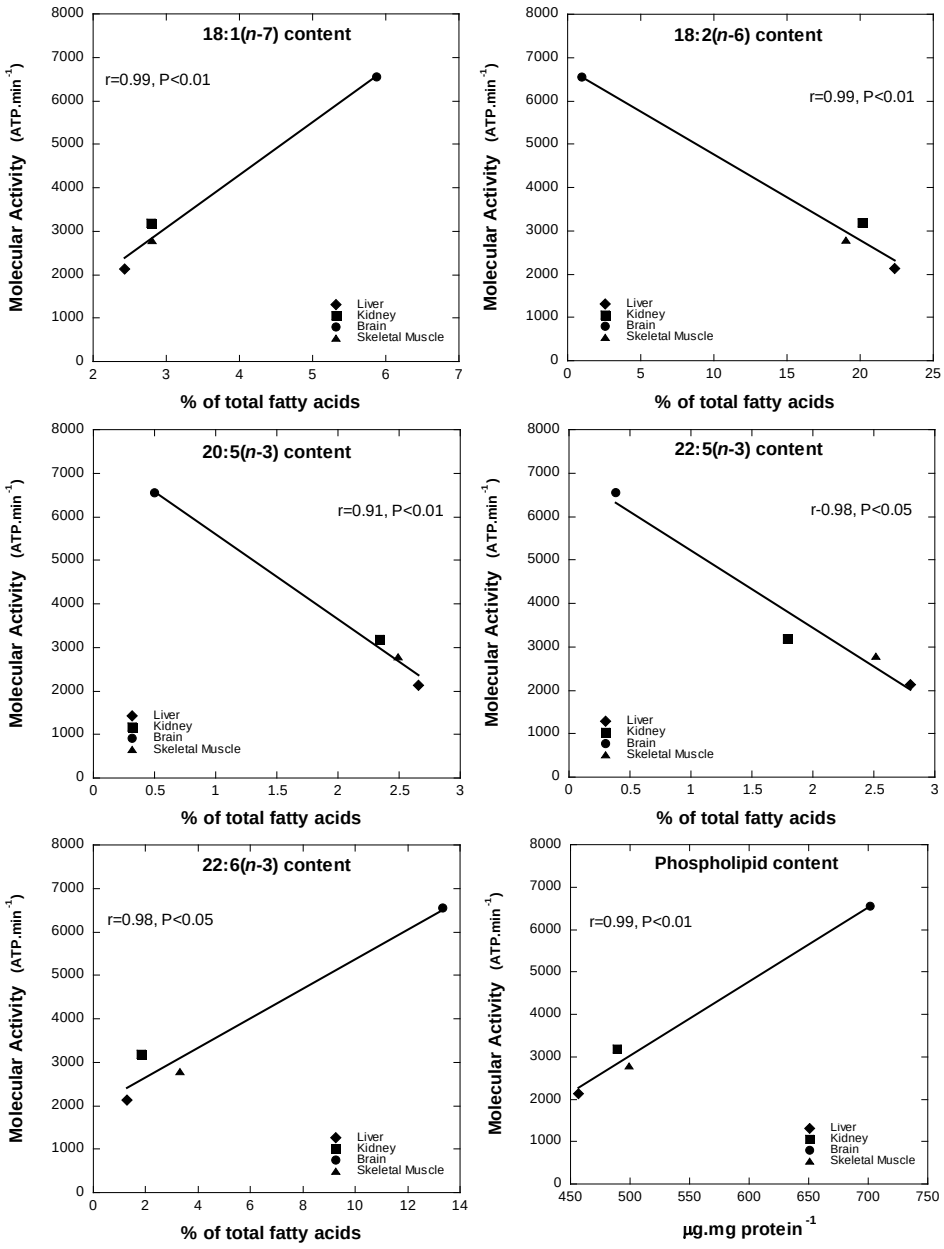


Fig. 6.2 *Linear correlations between sodium pump molecular activity in bearded dragon tissues and the content of 18:1(n-7), 18:2(n-6), 20:5(n-3), 22:5(n-3), 22:6(n-3) and microsomal phospholipid concentration.*

6.3 DISCUSSION

This chapter has examined sodium pump molecular activity and membrane lipid composition in two ectothermic species, the octopus and the bearded dragon lizard. These two species are quite disparate, the octopus being a marine invertebrate, while the bearded dragon is a desert-dwelling lizard that has a preferred body temperature of 37°C (Bartholemew and Tucker, 1963). In both species, molecular activity values in most tissues were similar to those previously reported for ectotherms (Else et al., 1996), with higher values observed in the brain (Table 6.2 & Table 6.6). Comparison of the octopus and bearded dragon indicated that there was little difference between the two, with only slightly higher molecular activity values observed in the octopus.

Else et al. (1996) determined sodium pump molecular activity (at 37°C) in a range of endotherms and ectotherms (including one invertebrate, *Charax destructor*) and found that in general, the sodium pumps of ectotherms had molecular activities of approximately 1,000 – 3,000 ATP.min^{-1}, compared with 6,000 – 10,000 ATP.min^{-1} in endotherms. In the current study, the molecular activity values for the liver, kidney and skeletal muscle of the bearded dragon (Table 6.6), would appear to be typical for an ectothermic species. The molecular activity of sodium pumps in the brain of the bearded dragon was 6,500 ATP.min^{-1}, which although it is 2 - 3 times higher than in the other tissues, is still only approximately 25 – 50% of what was measured in the current study in mammalian and avian brains under identical experimental conditions (see Chapter 5), highlighting the consistent endotherm-ectotherm difference.

While Else et al. (1996) showed that sodium pump molecular activity in a large range of ectotherms was relatively low compared to endotherms, there are several exceptions to this general trend. Sodium pumps from the shark rectal gland (Cornelius, 2001), the electric organ of the electric eel (Perrone et al., 1975), and the digestive gland and gill of the octopus (Bader et al., 1968) have all previously been reported to have molecular activities of between 7,500 – 12,500 ATP.min^{-1}. In the current study molecular activity was determined in a variety of tissues from the octopus, with values ranging between

4,000 – 7,500 ATP.min^{-1} (Table 6.2). These values, with the exception of the brain, are fairly typical for an ectotherm, and comparison of the molecular activity values determined in the present investigation with those previously determined by Bader et al (1968) for the digestive gland and gill of the octopus (12,500 & 8,000 ATP.min^{-1} respectively), shows that the values in the current study are around 2 – 3 times lower. This is similar to what was observed in the goose kidney (Chapter 3), where the molecular activity measured in the current study was lower than the value determined by Bader et al. (1968) using a phosphorylated intermediate assay, with the reason for the difference unknown.

Sodium pump density varied within the bearded dragon tissues, being approximately 670, 2,200, 4,800 and 760 pmol.g wet wt^{-1} for the liver, kidney, brain and skeletal muscle respectively (Table 6.6). A similar trend was also found in the octopus with the digestive gland, tentacle and gill having sodium pump densities of between 1,300 – 1,700 pmol.g wet wt^{-1}, while values of around 2,700 and 5,000 pmol.g wet wt^{-1} were determined for the brain and kidney respectively (Table 6.2). Else et al (1996) determined sodium pump concentration in different tissues of endotherms and ectotherms and found no difference between the two groups and concentrations (in pmol.g wet wt^{-1}) of approximately 500 for liver, 250 for skeletal muscle and 8,000 for kidney and brain. Thus, with the exception of the bearded dragon liver, sodium pump density appears to be quite different in tissues of the octopus and bearded dragon compared to other ectotherms (Else et al., 1996). For most of the tissues it would seem that the differences in sodium pump density just represent inter-species differences, and not methodological factors, as complete saturation of all ^{3}H-ouabain binding sites was observed in tissues of the octopus and bearded dragon (see Fig. 2.1 and Fig. 2.2). The higher sodium pump density observed in the skeletal muscle of the bearded dragon however, may be the result of a shorter washout period (40 min) employed in the current study as Else et al. (1996) used an extended washout period (120 min) during the ^{3}H-ouabain binding assay.

In both the octopus and bearded dragon, protein concentration varied across the different tissues. The brain contained the lowest protein concentration of all the tissues, which is likely a reflection of the fact that compared to other tissues, a greater proportion of brain dry weight is accounted for by phospholipid (~45%) (Purdon et al., 2002). In a previous investigation, Hulbert and Else (1989) found that the high level of metabolism in tissues of the rat was associated with significantly more protein than tissues of the bearded dragon. They found that the kidney and brain of the mammal contained 38% and 98% more protein respectively (Hulbert and Else, 1989). Comparison of the protein concentration in the kidney and brain of the bearded dragon, with that measured for a similar sized mammal (rat) and bird (currawong) in the current investigation (see Appendix III), also shows reduced levels in the reptile. For the kidney, the bearded dragon contained 40% and 26% less protein than the mammal and bird respectively, while protein levels in lizard brain are reduced by 46% and 43% respectively. The octopus and pigeon were also of a similar body size and comparison of the protein concentration in the kidney revealed similar levels between the two species, while the brain of the pigeon contained approximately 20% more protein than that of the octopus.

The internal organs of the octopus accounted for a relatively larger proportion of body mass than the organs of the bearded dragon, however for both species the organ mass both in absolute terms and as a percentage of body mass was lower compared to the mammals and birds (see Appendix III). When sodium pump density was combined with organ mass and the total number of sodium pumps calculated for each organ, the kidney of the bearded dragon was found to contain 8 times and 10 times less total sodium pumps than the kidneys of the rat and currawong respectively. For the brain the rat contained 4 times more sodium pumps, while the currawong, due to its unusually large brain contained almost 20 times more sodium pumps. Comparison of the octopus with the pigeon revealed a similar trend, with the kidney and brain of the pigeon containing 2 times and 17 times more sodium pumps respectively, than the corresponding tissues in the octopus.

Na^+K^+ATPase activity was also combined with organ mass and expressed as the micromoles of inorganic phosphate liberated (from ATP) per organ per hour, and assuming that this value represented the maximum rate, and using a P/O ratio of 2.0 (Rolfe and Brown, 1997), it was possible to determine the maximal daily energy expenditure by the sodium pump ($Kcal.day^{-1}$). Comparison of these values in the kidney and brain of the bearded dragon to those calculated for the rat and currawong (Chapter 3 & 5), indicates that endothermic sodium pumps would expend 20 – 50 times more energy (Kcal) per day than ectothermic sodium pumps, if operating at maximum. This very large difference is the product of reduced organ size, a reduced concentration of sodium pumps, and reduced sodium pump molecular activity in tissues of the bearded dragon. Of interest however is the fact that the maximal daily energy expenditure by sodium pumps in tissues of the bearded dragon would account for approximately 7 – 12% of the BMR reported for this species (Brand et al., 1991). Thus despite the huge difference in absolute terms, when considered relative to BMR, the maximal *in vitro* sodium pump activity in the bearded dragon accounts for a relatively similar proportion as it does in mammals and birds (see Chapters 3-5). While it is unknown whether the sodium pumps of all these species work at a similar percentage of their maximum values under basal conditions, the fact that the sodium pump accounts for a similar percentage of *in vitro* tissue metabolism in mammals, reptiles, fish, amphibians and birds (Else and Hulbert, 1981; Hulbert and Else, 1990), suggests this may be the case.

The membrane lipid composition of individual organs has never been measured in the octopus, however the fatty acid profile of the mantle has been determined in several studies (de Koning, 1972; Iverson et al., 2002; Passi et al., 2002). These studies have shown that the octopus has a fatty acid profile that is typical for a marine species, with membranes that are very unsaturated and contain high levels of the long chain *n*-3 PUFAs 20:5(*n*-3) and 22:6(*n*-3). The microsomal phospholipids from all of the individual tissues of the octopus measured in the current study showed a similar trend,

with unsaturation index varying between 240 in the digestive gland and 300 in the tentacle (Table 6.4). Very high levels of 20:5(*n*-3) and 22:6(*n*-3) were also found in the individual tissues (Table 6.4), with the highest concentrations seen in the brain and the tentacle. The reason for the high level of 22:6(*n*-3) in these two tissues is unknown, however in most animals 22:6(*n*-3) is found in the highest concentration in the brain, with the high levels found in the tentacle possibly a reflection of the fact that it also contains a significant amount of nervous tissue (Wells, 1978). The levels of 20:5(*n*-3) tended to show an almost reciprocal relationship with 20:4(*n*-6) in the different tissues. The digestive gland, kidney and gill had high levels of 20:4(*n*-6) and lower levels of 20:5(*n*-3), while the reverse was true for the brain and tentacle. Since 20:4(*n*-6) and 20:5(*n*-3) are produced from their short-chain precursors through identical enzyme steps, and since these organs were from the same animals and were thus exposed to identical dietary intake, the differences observed between tissues may represent a differential preference for elongation and desaturation through the *n*-6 or *n*-3 pathway, however this requires further investigation.

The other interesting findings from the octopus, were firstly that all tissues contained low proportions of 18:2(*n*-6), which is a fatty acid that is normally found in high concentrations in the membranes of most vertebrate tissues, with the exception of the brain (see previous chapters). Secondly the level of 20:1(*n*-9) was high in several tissues of the octopus (Table 6.4). This fatty acid generally only makes up a very small percentage of vertebrate membranes, but its high levels in the individual tissues agrees with the previous investigations in octopus mantle (de Koning, 1972; Iverson et al., 2002; Passi et al., 2002).

In contrast to the octopus, microsomal membranes from the bearded dragon tissues were much less unsaturated (as indicated by unsaturation index) and were characterised by relatively high levels of MUFA (Table 6.8). These findings are consistent with previous examinations of fatty acid composition in tissue phospholipids (Hulbert and Else, 1989) and liver mitochondria (Brand et al., 1991) from the bearded dragon, and

indicate that high levels of MUFA in reptiles are manifest in all subcellular membranes. Within the tissues, the liver, kidney and skeletal muscle were relatively similar, while the brain contained a high concentration of 22:6(*n*-3) and a low concentration of 18:2(*n*-6), which is a consistent with the fatty acid composition observed in the brain of a large range of different vertebrates (Farkas et al., 2000).

When linear correlation coefficients were determined between all individual lipid parameters and sodium pump molecular activity values, a number of significant relationships were observed in the tissues from the bearded dragon (Table 6.8), while only one significant relationship was observed in the octopus (Table 6.4). For the octopus there was a significant positive correlation between molecular activity and the content of 20:3(*n*-3) (Fig. 6.1). This fatty acid however, only constituted a small percentage (<2%) of the total fatty acids, and the relationship was driven by the fatty acid composition and sodium pump molecular activity of the brain. Similarly in the bearded dragon a large number of lipid parameters displayed significant correlations with molecular activity, with these relationships also basically a reflection of the high sodium pump molecular activity and distinct fatty acid profile observed in the brain (Fig. 6.2). Thus it is unclear at present whether these correlations are of any physiological significance.

Comparison of the two ectothermic species showed that despite the much higher levels of membrane polyunsaturation and in particular 22:6(*n*-3) observed in octopus tissues, the molecular activities were only slightly higher than those determined in the tissues of the bearded dragon. This is in contrast to other ‘high activity’ ectothermic tissues, such as the shark rectal gland and electric organ of the electric eel, where high levels of long chain PUFA (Rotstein et al., 1987; Cornelius et al., 2003) are associated with high sodium pump molecular activity (Perrone et al., 1975; Cornelius, 2001). One potential contributing factor to this lack of difference between the octopus and bearded dragon, may be the membrane cholesterol levels. All the tissues of the octopus, as well as being more polyunsaturated, also had higher cholesterol:phospholipid ratios (Table 6.3). This

elevated ratio appeared to be the result of a reduced amount of phospholipid in the microsomal membranes of the octopus, despite the same amount of cholesterol. High levels of cholesterol have been shown to be inhibitory to the sodium pump (Yeagle, 1983; Yeagle et al., 1988; Crockett and Hazel, 1997), and hence it is possible, that the stimulatory effect of high polyunsaturation on sodium pump molecular activity (Else and Wu, 1999), may have been somewhat counteracted by the high levels of cholesterol, although this requires further investigation.

Interestingly the fatty acyl profile and cholesterol concentration of the octopus membranes, are quite similar to those observed in the brain of many of the mammals and birds in Chapter 5. The molecular activities measured in the brains of the mammals and birds however, were 2 – 4 times higher, indicating that factors other than membrane lipid composition are contributing to the low molecular activities observed in the octopus. One potential factor may be isozyme differences. Recently it was observed that amino acid homology for the α_1 subunit was high within mammalian species (>90%), but when humans and rats were compared with the toad, stingray and fly, the homology was reduced to 70-80% (Hou, X., Howard, C. and Else, P.L. unpublished observations). It is therefore possible that differences in sequence homology, may be a contributing factor to the differences observed in sodium pump molecular activity in different species.

In conclusion, this chapter has shown that, with the exception of the brain, sodium pumps in the tissues of the bearded dragon and octopus, display similar molecular activities, that are typical of ectotherms, despite the fact that the lizard has very monounsaturated membranes, while the octopus has very polyunsaturated membranes. This result is somewhat surprising considering that tissues from other ectothermic species with high levels of membrane polyunsaturation, do display high molecular activities. It remains to be determined why this is not the case in the octopus.

Chapter VII

Summary and Conclusions

7.1 AN OVERVIEW

In the preceding four chapters, sodium pump molecular activity and membrane lipid composition were analysed in individual tissues of mammals, birds and ectotherms and one thing that became apparent was that there were large differences between tissues and species, and while not all relationships observed were statistically significant, there appeared to be a number of similar trends. Because the molecular activity measurement allows comparison of intrinsic enzyme activity between different tissues and species where the concentration of enzyme may vary considerably, this final summary chapter examines the relationship between sodium pump molecular activity and membrane lipid composition in the tissues of the mammals, birds and ectotherms, by analysing one data set comprised of the values from all fifteen species in the current study, plus data previously determined for the liver, kidney and brain of the ectothermic cane toad (Else et al., 1996; Wu, 2000).

Linear correlation coefficients comparing individual lipid parameters and sodium pump molecular activity were determined for the different data sets, using the mean value for each species (i.e. n=15 for mammals, n=24 for birds, n=12 for ectotherms, and n=51 for the combined data set). Table 7.1 presents the results of this analysis and it can be seen that a large number of lipid parameters showed significant correlations with molecular activity in the combined data set. The strongest correlation was observed for 22:6(*n*-3) ($r=0.65$, $n=51$, $P<0.0001$), which was one of only two fatty acids, along with 18:2(*n*-6), that also showed significant correlations within the respective data sets for the mammals, birds and ectotherms. The relationships for these two fatty acids are presented in Fig. 7.1 and Fig. 7.2, with 22:6(*n*-3) displaying positive correlations in all groups, while negative correlations were observed for 18:2(*n*-6). Fig. 7.1 shows that the relationship for 18:2(*n*-6) really just represents differences between tissues, as the data falls out into two distinct groups. Firstly there is a group of values that are all less than approximately 2%, which represent the brain and octopus measurements, and secondly there is a dispersed group of values that are greater than approximately 8% and represent the other tissues from the different species.

Table 7.1 Linear Correlation Coefficients between Molecular Activity and Lipid Parameters

	All Tissues	Mammals	Birds	Ectotherms
16:0	0.26	0.66**	0.58**	0.43
18:0	0.14	0.37	0.26	-0.08
16:1(*n*-7)	-0.30*	-0.20	0.07	-0.09
18:1(*n*-9)	-0.18	0.15	-0.32	-0.58*
18:1(*n*-7)	0.47***	0.35	0.66***	0.08
18:2(*n*-6)	-0.51***	-0.66**	-0.68***	-0.70*
20:2(*n*-6)	0.01	-0.43	0.22	0.42
20:3(*n*-6)	-0.08	-0.31	-0.23	-0.78**
20:4(*n*-6)	-0.23	-0.07	-0.80***	-0.13
22:4(*n*-6)	0.53***	0.50	0.56**	0.16
22:5(*n*-6)	0.37**	0.14	0.68***	0.00
22:5(*n*-3)	-0.37**	-0.61*	-0.40	-0.06
22:6(*n*-3)	0.65***	0.65**	0.83***	0.78**
% Saturates	0.16	0.62*	0.49*	0.33
% MUFA	-0.12	0.24	0.01	-0.36
% PUFA	0.01	-0.46	-0.37	0.07
% n-9	-0.24	0.17	-0.32	-0.54
% n-7	0.20	0.29	0.58**	0.13
% n-6	-0.41**	-0.48	-0.82***	-0.77**
% n-3	0.52***	0.30	0.80***	0.74**
% Unsaturates	-0.16	-0.62*	-0.49*	-0.32
UI	0.49***	0.27	0.56**	0.65*
Chain length	0.59***	0.40	0.70***	0.69*
C20+22 PUFA	0.46***	0.42	0.52**	0.67*
n-6/n-3	-0.33*	-0.37	-0.52**	-0.61*
20:4/18:2	0.04	0.43	0.56**	0.27
Cholesterol (μg.mg protein^{-1})	0.30*	0.48	0.63**	0.23
Phospholipid (μg.mg protein^{-1})	-0.32*	0.03	-0.64***	-0.19
Chol:PL (mol:mol)	0.50***	0.60*	0.71***	0.56

*Unsaturation index (UI) is the average number of double bonds per 100 fatty acid chains. Linear correlation coefficients were determined using one data point for each species which was the mean value for each lipid parameter and molecular activity (n=15 for mammals, n=24 for birds, n=12 for ectotherms, and n=51 for the combined data set). Fatty acids which were not present in all species were excluded from the analysis. *P<0.05, **P<0.01, ***P<0.001.*

In contrast to this, Fig. 7.2 shows that there is a spread of values for 22:6(*n*-3), with higher concentrations of this fatty acid associated with higher molecular activities. In

general, the brain for most species had the highest concentration of 22:6(*n*-3) and also the highest molecular activity, however there was substantial overlap between tissues, with a number of kidney and heart values from the smaller mammals and birds also being high for both variables. Of interest was the fact that although the mammals, birds and ectotherms all showed a fairly similar relationship with 22:6(*n*-3), there appeared to be a separation of the three groups in terms of the level of molecular activity. For example, using the equations determined for the three groups in Fig. 7.2, and a moderate value of 5% for the 22:6(*n*-3) content, it can be calculated that the molecular activity would be 8,000 $ATP.min^{-1}$, 6,500 $ATP.min^{-1}$, and 2,900 $ATP.min^{-1}$ for the mammals, birds and ectotherms respectively.

The reason for this difference between groups is unknown, but may be potentially be protein based and related to differences in sodium pump isozymes. As species diverge in their evolutionary history, their amino acid homology for similar subunit isoforms also diverges (Hou, X., Howard, C. and Else, P.L. unpublished observations). These differences in amino acids may be influencing the rate at which the sodium pump can operate and hence may explain some of the differences observed in Fig. 7.2.

Another potential reason for the molecular activity differences between the mammals, birds and ectotherms may be related to the phospholipid molecular species present. In the three groups, 22:6(*n*-3) may be associating with different fatty acids and different phospholipid headgroups, and it could be that the molecular species combinations found in mammals are more effective at “stimulating” sodium pump molecular activity, than those found in the birds or ectotherms. This is an area worthy of further investigation.

Finally, it is unknown whether part of this difference may be related to the fact that microsomal membranes were used to determine lipid composition. While microsomal membranes give a better representation of the membrane lipids that would be directly surrounding the sodium pump, as opposed to tissue phospholipids, it should also be

recognised that microsomes are a mixture of the plasma membrane, endoplasmic reticulum, and golgi apparatus. Lipid composition has been shown to be different between these subcellular membranes (Colbeau et al., 1971; Fiehn and Peter, 1971), and it is unknown whether these membranes account for similar proportions in cells from different organs and also in cells from different species. Hence differences between mammals, birds and ectotherms may also be related to the proportions of the various subcellular membranes that are present in the microsomal preparations.

Despite these differences between the mammals, birds and ectotherms, when all species were combined as one data set, a very strong correlation was evident between 22:6(*n*-3) and sodium pump molecular activity. It should be noted that one of the limitations of the current study is that it is a correlational analysis and thus does not prove "cause and

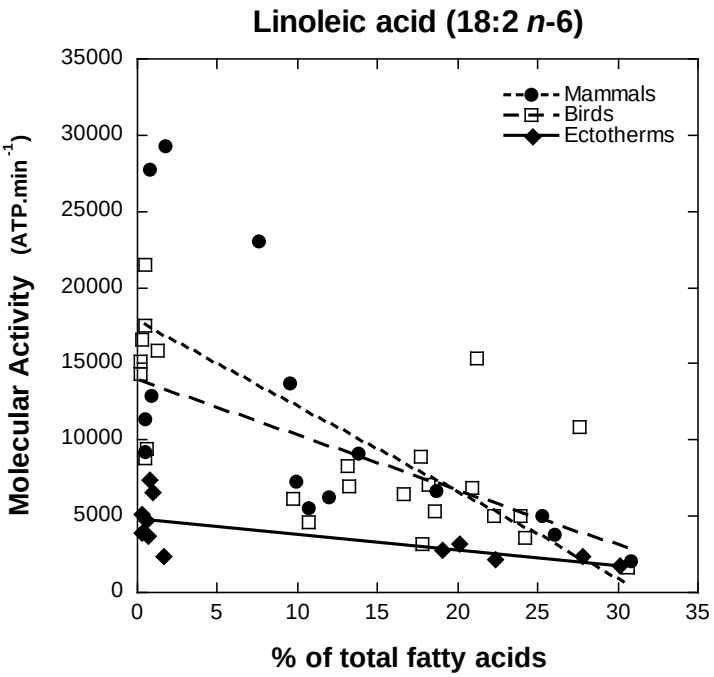


Fig. 7.1 *The relationship between sodium pump molecular activity and the content of 18:2(n-6) in microsomal phospholipids from tissues of mammals, birds and ectotherms. Each data point represents the mean of 4 - 6 determinations. n=15 for mammals, n=24 for birds and n=12 for ectotherms. Linear correlation coefficients for the relationships are indicated in Table 7.1.*

effect". However it should also be considered that "cause and effect" cannot exist without correlation, and that a lack of correlation normally precludes "cause and effect". Previous studies examining membrane crossovers between rats and toads, and also cattle and crocodiles, have provided direct experimental evidence that membrane lipid composition is a major determinant of sodium pump molecular activity (Else and Wu, 1999; Wu, 2000). Thus it would appear plausible that the relationship between 22:6(*n*-3) and sodium pump molecular activity observed in the current study, would likely indicate "cause and effect", although it requires further research.

The exact mechanism by which high levels of 22:6(*n*-3) increase sodium pump molecular activity has not been established, but possible mechanisms may relate to the

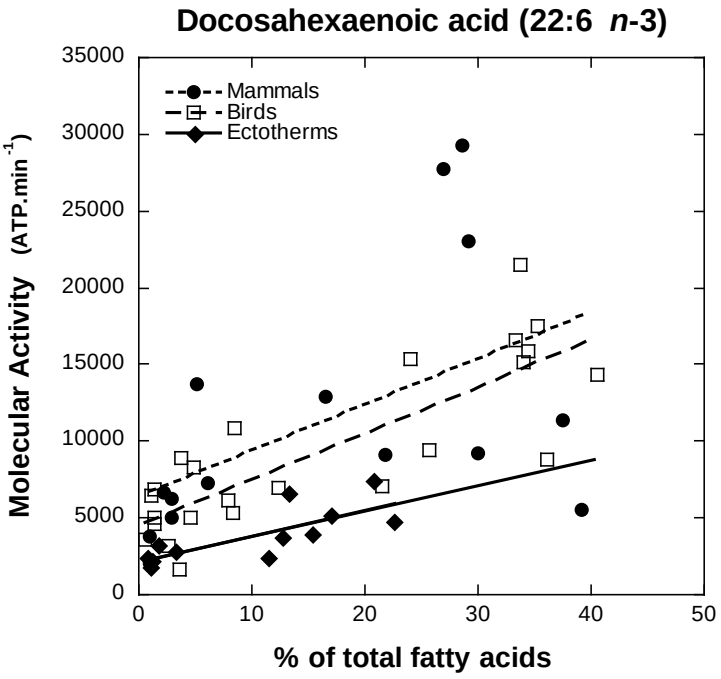


Fig. 7.2 *The relationship between sodium pump molecular activity and the content of 22:6(n-3) in microsomal phospholipids from tissues of mammals, birds and ectotherms. Each data point represents the mean of 4 - 6 determinations. n=15 for mammals, n=24 for birds and n=12 for ectotherms. Linear correlation coefficients for the relationships are indicated in Table 7.1.*

special properties of this fatty acid. Docosahexaenoic acid (22:6n-3) appears to be unusual among membrane acyl chains both with respect to its synthesis, which involves peroxisomes (Sprecher, 2000), and its physical properties. For example, 22:6(*n*-3)-containing phospholipids occupy large areas in monolayers and are not condensable by cholesterol (Zerouga et al., 1995). Recent molecular dynamics simulations have highlighted the unique physical properties that highly polyunsaturated acyl chains such as 22:6(*n*-3) give to membrane bilayers (Feller et al., 2002; Huber et al., 2002). Feller et al. (2002) calculated that there are hundreds of high-probability conformations of 22:6(*n*-3) likely in a membrane bilayer, with several of these involving the methyl end of the molecule located towards the outer edge of the bilayer rather than in the middle of the membrane bilayer as it is normally drawn in static diagrams. These molecular dynamics simulations present an image of 22:6(*n*-3) as a highly flexible molecule that is thrashing about in the hydrocarbon core of a membrane bilayer.

Such an image is compatible with the response of many animals to cold environments. Cold-water fish accumulate extremely high levels of 22:6(*n*-3) in their phospholipids (Hazel, 1995) which is associated with increases in the activity of membrane-associated enzymes such as the sodium pump (Raynard and Cossins, 1991). The increase in 22:6(*n*-3) during cold acclimation was thought to be necessary to maintain appropriate membrane fluidity for optimal protein function, however as pointed out by Hazel (1995), changes seen in monounsaturate content are sufficient to serve this function and alterations in 22:6(*n*-3) content most likely serve another purpose. Their dramatic movements in the membrane environment likely compensate for the diminishing motion of molecules associated with decreases in environmental temperature.

The molecular movement of 22:6(*n*-3) in a membrane bilayer will likely speed up many processes catalysed by membrane proteins. The retinal membranes of vertebrates and invertebrates are enriched with high levels of 22:6(*n*-3) (Hendriks et al., 1976; Akino, 1982). These high levels are essential for normal visual acuity and the physical

effects of 22:6(*n*-3) in the membrane have been suggested to enhance the interaction between rhodopsin and membrane-bound G-proteins (Litman and Mitchell, 1996).

Likely the first observation of a correlation between the speed of a membrane process and the 22:6(*n*-3) content of the membrane was the report 25 years ago that 22:6(*n*-3) content of heart phospholipids was correlated with heart rate in mammals (Gudbjarnarson et al., 1978). Mice have very high heart rates ($10.sec^{-1}$) and high 22:6(*n*-3) content in their cardiac membranes. Skeletal muscles such as the flight muscle of hummingbirds and rattlesnake tail shaker muscle reach contraction frequencies approaching 80-90 times per second, and also contain very high levels of 22:6(*n*-3) (Infante et al., 2001).

The level of 22:6(*n*-3) in membranes has also been shown to speed up the permeability of bilayers to sodium ions (Hendriks et al., 1976). Similarly, proton leak was increased when mitochondrial membrane 22:6(*n*-3) content was increased both *in vivo* by feeding menhaden oil and *in vitro* by lipid fusion (Stillwell et al., 1997). Variations in proton leak of liver mitochondria from different vertebrates have also been correlated with 22:6(*n*-3) content (Brookes et al., 1998). These studies all indicate that 22:6(*n*-3) has an "energising" effect on membrane-related processes and thus may represent an important 'pacemaker' for the determination of the normal metabolic rate of a species (Hulbert and Else, 1999; Hulbert and Else, 2000). Further evidence for such a contention also comes from studies in both rats (Pan and Storlien, 1993) and humans (Couet et al., 1997), which show that the feeding of fish oil, which is rich in 22:6(*n*-3), increases metabolic rate

As well as its role in influencing metabolism in different animals, 22:6(*n*-3) also appears to play an important role in several health disorders, including the Metabolic Syndrome and mental illness (Horrocks and Yeo, 1999). With regards to mental health, membrane 22:6(*n*-3) is essential for the normal development of the brain, and numerous studies have shown that 22:6(*n*-3) deficiency results in a variety of cognitive

disorders (Horrocks and Yeo, 1999). Furthermore it has recently been proposed that during adulthood a deficient 22:6(*n*-3) intake is associated with the development of depression and schizophrenia (Hibbeln, 1998; Horrobin, 1998; Yao et al., 2000). Indeed individuals that consume significant amounts of fish, have a reduced incidence of mental health problems (Tanskanen et al., 2001) and the national incidence of postnatal depression has been recently shown to be inversely associated with national fish and 22:6(*n*-3) intake (Hibbeln, 2002). This deleterious effect of reduced 22:6(*n*-3) may be potentially related to the sodium pump, as approximately 50% of brain metabolism is directly linked with the sodium pump (Clausen et al., 1991). Thus, considering the relationship observed in Fig. 7.2, if membrane 22:6(*n*-3) is reduced, the molecular activity of the sodium pump will also decrease, and hence function may be compromised due to a reduced enzymatic capacity in this integral protein.

Another major health benefit of fish oil (and thus 22:6(*n*-3)) consumption is the alleviation of ill health associated with the Metabolic Syndrome. This condition represents a cluster of diseases (obesity, blood dyslipidemias, hypertension and cardiovascular diseases) centred around tissue insulin resistance (Reaven, 1993). Insulin resistance has been linked with both a dietary decrease in 22:6(*n*-3) in rats and humans (Storlien et al., 1991; Vessby et al., 1994) and reduced levels of membrane 22:6(*n*-3) (Borkman et al., 1993). Fish oil diets however, have been shown to prevent dietary-induced insulin resistance in rats (Storlien et al., 1987). Thus it is likely that the intake of 22:6(*n*-3) influences the composition of membrane lipids and in turn facilitates optimal interactions between membrane lipids and proteins, maintaining the responsiveness of the membrane-bound insulin receptor.

7.2 SUMMARY AND CONCLUSIONS

The aims of this investigation were to examine sodium pump molecular activity and membrane lipid composition in tissues of metabolically diverse animals, to see whether the lipid bilayer, specifically its amount and composition, may have been associated with metabolic intensity via an effect on the molecular activity of the sodium pump (a

representative protein). The BMR of the species examined, varied over 20-fold and the experimental animals included mammals and birds of different body size, and two ectothermic species, the octopus and bearded dragon lizard.

In the mammals and birds, membrane fatty acid composition of the kidney and heart varied dramatically, with the major finding being the significant and substantial allometric decline observed in the content of the highly polyunsaturated docosahexaenoic acid (22:6 *n*-3). The allometric exponents describing the variation of this fatty acid with body size, were not only the steepest seen for any lipid parameter, but were also close to the allometric exponents describing BMR, indicating that 22:6(*n*-3) may play an important role in determining metabolic rate. Brain phospholipids were highly polyunsaturated in all species, and contained elevated levels of 22:6(*n*-3). Sodium pump molecular activity was generally higher in all three tissues from the smaller mammals, while in the birds higher molecular activity was observed in the hearts of smaller birds, while no allometric trends were seen in the kidney and brain.

There were no differences observed in sodium pump molecular activity between the tissues of the bearded dragon and the octopus, with values typical of ectotherms observed, in spite of the fact that the octopus had very polyunsaturated membranes. A significant contributing factor however, may have been that the octopus membranes contained high levels of cholesterol, which has been shown to have an inhibitory effect on the sodium pump.

To determine whether membrane lipid composition may have been affecting sodium pump molecular activity, linear correlation coefficients were determined between lipid parameters and sodium pump molecular activity, using a combined data set from all fifteen species and six tissues examined in the current study, plus literature values for tissues from the ectothermic cane toad. Several lipid parameters were correlated with molecular activity, however the most important finding was the very strong positive correlation observed for docosahexaenoic acid (22:6 *n*-3). This fatty acid provided not

only the strongest correlation for the combined data set, but was also significantly correlated within the respective data sets for the mammals, birds and ectotherms.

These results provide strong correlative evidence that 22:6(*n*-3) may play a major role in metabolic variation in animals, via an effect on the molecular activity of membrane proteins, such as the sodium pump. Future studies in this area should be directed at manipulating membrane 22:6(*n*-3) content, to examine whether predictable changes in enzyme activity and subsequently metabolic rate, can be induced. Reconstitution experiments and lipid vesicle fusions represent two potential experimental techniques by which membrane 22:6(n-3) could be specifically altered *in vitro*, while dietary manipulation and cold water acclimation studies represent possible avenues for altering 22:6(*n*-3) content *in vivo*. Future research should also be aimed at determining whether the "energising" effect of 22:6(*n*-3) is simply related to the presence of this fatty acid in the membrane, or whether it is more specifically related to its presence in particular phospholipid molecular species.

The significance of the relationship observed between 22:6(*n*-3) and molecular activity in the current study is substantial. Firstly it represents a potential link between membrane lipid composition and the metabolic rate of animals, highlighting the important role of this unique fatty acid. Secondly, it may provide some insight into the mechanisms underlying a number of metabolic conditions and mental health disorders that appear to be associated with reduced membrane levels of 22:6(*n*-3). It is possible that these conditions develop as a result of disruptions in essential lipid-protein interactions in the membrane. Furthermore, since consumption of fish oil, which is rich in 22:6(*n*-3), appears to be beneficial for these health disorders, interventions aimed at specifically increasing membrane 22:6(*n*-3) content may represent potential future therapies for these conditions.

In conclusion, while the precise mechanism by which 22:6(*n*-3) acts to increase the activity of membrane proteins such as sodium pump is not fully understood, this

polyunsaturate is consistently associated with high rates of ion transport and metabolism in animals. Furthermore dietary intake of this fatty acid alleviates symptoms of several metabolic and mental conditions that result from perturbations in membrane function. This important constituent of biological membranes may act as a 'pacemaker' of metabolic activity via its effects on membrane proteins.

Chapter VIII

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Appendix I

Na^+K^+ATPase Activity in Bird Tissues Measured at 40ºC			
	Kidney	**Heart**	**Brain**
Zebra Finch	5.83 ± 0.27 (n=4)	0.80 ± 0.10 (n=4)	5.62 ± 0.18 (n=4)
Sparrow	4.70 ± 0.32 (n=4)	0.87 ± 0.07 (n=4)	4.68 ± 0.39 (n=4)
Starling	5.62 ± 0.14 (n=4)	0.69 ± 0.13 (n=4)	4.64 ± 0.19 (n=4)
Currawong	4.45 ± 0.12 (n=4)	0.65 ± 0.06 (n=4)	4.87 ± 0.38 (n=4)
Pigeon	2.80 ± 0.03 (n=4)	0.33 ± 0.04 (n=4)	4.02 ± 0.15 (n=4)
Duck	2.54 ± 0.06 (n=4)	0.30 ± 0.06 (n=4)	4.43 ± 0.35 (n=4)
Goose	2.30 ± 0.16 (n=4)	0.38 ± 0.06 (n=4)	3.74 ± 0.21 (n=4)
Emu	1.82 ± 0.02 (n=4)	0.39 ± 0.06 (n=4)	2.67 ± 0.13 (n=4)

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram of wet weight each hour. (n) is the number of preparations used for each measurement.

Appendix II

Thermal Dependence Expressed as Q_{10} of Na^+K^+ATPase Activity Between 25 and 37°C in Tissues of the Octopus and Bearded Dragon

	37°C	25°C	Q_{10}
Octopus			
Digestive Gland	0.37 ± 0.03 (n=4)	0.23 ± 0.04 (n=5)	1.49
Kidney	1.17 ± 0.11 (n=4)	0.52 ± 0.03 (n=5)	1.97
Brain	1.21 ± 0.10 (n=4)	0.61 ± 0.05 (n=5)	1.77
Tentacle	0.37 ± 0.09 (n=4)	0.09 ± 0.03 (n=4)	3.24
Gill	0.43 ± 0.05 (n=4)	0.17 ± 0.02 (n=5)	2.17
Bearded Dragon			
Liver	0.10 ± 0.02 (n=4)	0.11 ± 0.02 (n=5)	0.92
Kidney	0.40 ± 0.05 (n=6)	0.24 ± 0.04 (n=6)	1.52
Brain	1.89 ± 0.13 (n=6)	1.19 ± 0.06 (n=6)	1.48

Values are means ± standard errors (SEM). Na^+K^+ATPase activity was measured in detergent treated homogenates as micromoles of inorganic phosphate liberated (from ATP) per gram wet weight each hour. The formula used to determine thermal quotients was $Q_{10} = (R_2/R_1)^{(10/T_2-T_1)}$ where T_1 and T_2 are the temperatures (°C) that produce the rates of reaction (R_1 and R_2). (n) is the number of preparations used for each measurement.

Appendix III

Mass and Protein Concentration of Tissues Examined in the Mammals and Birds						
	Kidney		Heart		Brain	
	Mass (g)	Protein (mg.g wet wt^{-1})	Mass (g)	Protein (mg.g wet wt^{-1})	Mass (g)	Protein (mg.g wet wt^{-1})
Mammals						
Mouse	0.55 ± 0.04 (n=16)	206 ± 5 (n=12)	0.19 ± 0.01 (n=16)	215 ± 5 (n=12)	0.47 ± 0.01 (n=16)	152 ± 6 (n=16)
Rat	2.09 ± 0.08 (n=12)	193 ± 9 (n=12)	0.90 ± 0.02 (n=12)	201 ± 12 (n=12)	1.78 ± 0.03 (n=12)	148 ± 4 (n=12)
Sheep	124 ± 5 (n=8)	184 ± 4 (n=8)	209 ± 12 (n=8)	198 ± 7 (n=8)	96 ± 4 (n=8)	138 ± 3 (n=8)
Pig	338 ± 18 (n=8)	165 ± 10 (n=7)	344 ± 9 (n=8)	228 ± 7 (n=7)	81 ± 3 (n=4)	131 ± 4 (n=8)
Cow	608 ± 25 (n=8)	207 ± 6 (n=7)	994 ± 71 (n=8)	208 ± 8 (n=7)	311 ± 17 (n=4)	126 ± 5 (n=8)
Birds						
Zebra Finch	0.13 ± 0.01 (n=4)	165 ± 13 (n=4)	0.17 ± 0.01 (n=4)	172 ± 12 (n=4)	0.42 ± 0.01 (n=4)	138 ± 2 (n=4)
Sparrow	0.20 ± 0.01 (n=4)	201 ± 11 (n=4)	0.32 ± 0.01 (n=4)	172 ± 13 (n=4)	0.86 ± 0.03 (n=4)	149 ± 2 (n=4)
Starling	0.81 ± 0.05 (n=4)	181 ± 4 (n=4)	0.92 ± 0.02 (n=4)	175 ± 7 (n=4)	1.69 ± 0.06 (n=4)	149 ± 3 (n=4)
Currawong	2.05 ± 0.06 (n=4)	174 ± 6 (n=4)	2.06 ± 0.06 (n=4)	175 ± 12 (n=4)	5.38 ± 0.15 (n=4)	144 ± 8 (n=4)
Pigeon	2.34 ± 0.2 (n=4)	167 ± 5 (n=4)	5.03 ± 0.17 (n=4)	180 ± 12 (n=4)	2.11 ± 0.05 (n=4)	120 ± 10 (n=4)
Duck	7.68 ± 0.3 (n=4)	175 ± 9 (n=4)	15.7 ± 0.6 (n=4)	174 ± 8 (n=4)	6.12 ± 0.21 (n=4)	127 ± 8 (n=4)
Goose	13.8 ± 3.9 (n=4)	185 ± 6 (n=4)	32 ± 6 (n=4)	191 ± 5 (n=4)	10.9 ± 0.17 (n=4)	130 ± 4 (n=4)
Emu	117 ± 10 (n=4)	156 ± 9 (n=4)	308 ± 8 (n=4)	152 ± 3 (n=4)	20.5 ± 0.87 (n=4)	115 ± 4 (n=4)

Values are means ± standard errors (SEM). Organ mass is in grams. Protein concentration was determined in tissue homogenates and is expressed as mg of protein per g of wet weight. (n) is the number of animals measured.