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# Differences in membrane acyl phospholipid composition between an endothermic mammal and an ectothermic reptile are not limited to any phospholipid class

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## Summary

This study examined questions concerning differences in the acyl composition of membrane phospholipids that have been linked to the faster rates of metabolic processes in endotherms *versus* ectotherms. In liver, kidney, heart and brain of the ectothermic reptile, *Trachydosaurus rugosus*, and the endothermic mammal, *Rattus norvegicus*, previous findings of fewer unsaturates but a greater unsaturation index (UI) in membranes of the mammal *versus* those of the reptile were confirmed. Moreover, the study showed that the distribution of phospholipid head-group classes was similar in the same tissues of the reptile and mammal and that the differences in acyl composition were present in all phospholipid classes analysed, suggesting a role for the physical over the chemical properties of membranes in determining the faster rates of metabolic processes in endotherms. The most common phosphatidylcholine (PC) molecules present in all tissues (except brain) of the reptile were 16:0/18:1, 16:0/18:2, 18:0/18:2, 18:1/18:1 and 18:1/18:2, whereas arachidonic acid (20:4), containing PCs 16:0/20:4, 18:0/20:4, were the common molecules in the mammal. The most abundant phosphatidylethanolamines (PE) used in the tissue of the reptile were 18:0/18:2, 18:0/20:4, 18:1/18:1, 18:1/18:2 and 18:1/20:4, compared to 16:0/18:2, 16:0/20:4, 16:0/22:6, 18:0/20:4, 18:0/22:6 and 18:1/20:4 in the mammal. UI differences were primarily due to arachidonic acid found in both PC and PEs, whereas docosahexaenoic acid (22:6) was a lesser contributor mainly within PEs and essentially absent in the kidney. The phospholipid composition of brain was more similar in the reptile and mammal compared to those of other tissues.

Key words: fatty acid, metabolism, lipid, mass spectrometry, glycerophospholipid, reptile, mammal, endothermy, ectothermy.

## Introduction

The resting metabolic rates of endothermic vertebrates, such as mammals, are several times greater than those of similar-sized ectothermic vertebrates, such as reptiles, when measured at the same body temperature. These differences in resting metabolic rates have been associated with faster membrane-associated processes, such as 'leakier' cells (Else and Hulbert, 1987; Hulbert and Else, 1990), and faster turnover rates of membrane-associated proteins, such as the plasmalemmal sodium pump (Else et al., 1996; Else and Wu, 1999; Wu et al., 2004) and mitochondrial cytochromes (Hulbert et al., 2006) in the tissues of endotherms compared to those of ectotherms. Differences in the rate of activity of the membrane-linked processes, in turn, have been causally related to the lipid composition of the bilayer, and specifically to the fatty acid or acyl composition of the membrane phospholipids (Hulbert and Else, 1999; Hulbert and Else, 2000). For example, phospholipids from rat liver and kidney have a lower percentage of unsaturated fatty acids but a greater degree of unsaturation (as measured by unsaturation index, i.e. the number of double bonds per 100 fatty acids) than phospholipids from liver and kidney of the similar-sized bearded dragon lizard *Pogona vitticeps* (Hulbert and Else, 1989). This is because the acyl chains that constitute membrane bilayers in the mammalian tissues are more polyunsaturated than those making up the reptilian membranes, especially the greater concentrations of the long-chain polyunsaturates such as arachidonic acid (AA; 20:4 n-6) and docosahexaenoic acid (DHA; 22:6 n-3) in the mammalian phospholipids. Similar differences have also been observed for mitochondrial phospholipids from liver, kidney and skeletal muscle from these two species (Brand et al., 1991; Hulbert et al., 2006).

All previous comparisons of reptile *versus* mammal membrane composition have measured acyl composition by methanolysis of total phospholipids and analysis of the fatty acid methyl ester mixture thus produced by gas chromatography (Brand et al., 1991; Brookes et al., 1997; Else and Wu, 1999; Hulbert and Else, 1989; Turner et al., 2005). Although this work has produced valuable information on the fundamental differences in the membrane composition between reptiles and mammals, and more generally between ectotherms and endotherms, many important questions remain unanswered. Shotgun lipidomics is a technique whereby the individual phospholipid molecules that make up complex phospholipid

mixtures can be identified and quantified by electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS) (Ekroos et al., 2002; Han and Gross, 2005; Pulfer and Murphy, 2003). Here we have exploited the capabilities of a highly automated technique (Ejsing et al., 2006) to answer the important questions relevant to the compositional differences at the molecular level associated with the differences in membrane acyl composition between reptiles and mammals.

In this study we compare the molecular composition of phospholipids isolated from liver, kidney, heart and brain of the rat and the similar-sized shingleback lizard. The shingleback lizard (*Trachydosaurus rugosus*) is a skink from the arid zone of Australia, and has a preferred body temperature of ~34°C (Licht, 1965). Measurement of the acyl composition of phospholipids isolated from the inner membrane of liver mitochondria from this lizard species show it to differ from the rat in a similar manner as the bearded dragon, i.e. it has a lower concentration of polyunsaturates (especially 20:4n-6 and 22:6n-3) and higher levels of monounsaturates (Brookes et al., 1997). This analysis is restricted to some of the main lipids that predominate in membrane bilayers, the phospholipids (or more precisely the glycerophospholipids), and does not include the sphingomyelins.

In this study we will examine if the reptile–mammal differences in acyl composition are general for all phospholipid classes or restricted to one or a few membrane phospholipid classes. This has important implications in determining if the physical or chemical properties of the membrane are important in changing the activities of membrane-linked metabolic processes. We will examine if the different classes of phospholipid are similarly distributed in the same tissues in both the reptile and the mammal, and if the most common acyl combinations within each phospholipid class are common to all or are different between the tissues of each species. This has importance in determining if common principles apply in the use of phospholipids in the construction of membrane bilayers of species and between different animal groups such as endothermic and ectothermic vertebrates.

## Materials and methods

### Materials

All solvents were of HPLC grade and purchased from either Crown Scientific (Moorebank, Australia), Rathburn Chemicals Ltd (Walkerburn, Scotland) or Merck (Darmstadt, Germany). Butylated hydroxytoluene was purchased from Sigma Aldrich (Castle Hill, Australia). Ammonium acetate was purchased from Sigma Chemicals (St Louis, MO, USA). All lipid standards were synthesized by Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and purchased from Auspep (Parkville, Australia).

### Animals

Tissues used in this study were excised from rats (*Rattus norvegicus* Berkenhout 1769*;* male) and shingleback lizards (*Trachydosaurus rugosus* Gray; mixed gender) housed under 12:12 h light:dark cycle with free access to food and water at 20–25°C. The lizards were also provided with access to radiant heat lamps during the lights-on period in order to allow them to thermoregulate and maintain their preferred body temperature

range, which is reported to be ~34°C under laboratory conditions (Licht, 1965). Both animal species were killed by cardiac removal following anaesthesia (sodium pentobarbital 60 mg kg$^{-1}$) and the tissues were stored either in liquid nitrogen or at –80°C until extraction of lipids. All experimental procedures were approved by the University of Wollongong Animal Ethics Committee.

### Lipid extraction

Lipids were extracted by standard methods (Folch et al., 1957) using ultra-pure grade chloroform:methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene as an antioxidant. Prior to extraction, 4 ml g tissue$^{-1}$ of a mixture of internal standards (PC19:0/19:0, 250 μmol l$^{-1}$; PE17:0/17:0, 187.5 μmol l$^{-1}$; PS17:0/17:0, 125 μmol l$^{-1}$; PA17:0/17:0, 25 μmol l$^{-1}$; PG17:0/17:0, 25 μmol l$^{-1}$ in chloroform:methanol) was added to the homogenate. These specific phospholipid molecules used as standards were not naturally present in the tissue phospholipids.

### Mass spectrometry

All analysis was performed in negative ion mode by multiple precursor ion scanning (MPIS) on a QSTAR XL QqTOF mass spectrometer with ion trapping capabilities (MDS Sciex, Concord, ON, Canada) as described previously (Ekroos et al., 2002). The mass spectrometer was equipped with an automated chip-based nanoelectrospray system (TriVersa NanoMate; Advion BioSciences, Ithaca, NY, USA) allowing automated sample application (Linden et al., 2006). Briefly, samples suspended in chloroform:methanol (1:2, v/v) containing 5 mmol l$^{-1}$ ammonium acetate were loaded onto 96-well microtiter plates (Eppendorf AG, Hamburg, Germany) and 5 μl aliquots aspirated and delivered to the nanoESI chip. The electrospray process was initiated by applying 1.3 kV and 0.3 p.s.i. nitrogen head pressure to ensure constant sample flow. For fatty acid scanning analysis of infused lipid extracts, precursor ion spectra were simultaneously acquired for 30–50 FA anions, containing 12–22 carbon atoms and 0–6 double bonds. Collision energy was set at 40 eV and fragment ions selected within an *m/z* window of 0.15 Da. The scanning quadrupole (Q1) was set at unit resolution and scanned from *m/z* 400 to *m/z* 900 with a step size of 0.2 Da and a dwell time of 30 ms. Peak enhancement, i.e. trapping of target FA fragment ions, was applied according to the manufacturer's specifications (Chernushevich, 2000). Fatty acid scanning spectra were interpreted using a prototype of LipidProfiler 1.0 software (MDS Sciex, Concord, ON, Canada) (Ejsing et al., 2006). Individual molecules were quantified by comparison to the internal standard with the same head group, after correction for isotope contributions, as recently described (Ejsing et al., 2006). The phospholipid fatty acid composition was calculated directly from the molecular phospholipid data. Specifically, the mol% of each FA moiety was calculated as the sum of molar concentrations of phospholipids containing the respective FA moiety, followed by normalization to the total molar concentration of all FA moieties. The FA concentration corresponding to symmetric phospholipids was multiplied by a factor of 2 to account for two identical FA moieties.

Table 1. *Acyl composition of phospholipid of rat and lizard tissues*

| | Liver | | Kidney | | Heart | | Brain | |
|---|---|---|---|---|---|---|---|---|
| | Lizard | Rat | Lizard | Rat | Lizard | Rat | Lizard | Rat |
| 14:0 | – | – | – | – | – | – | 2.0±0.2 | –‡ |
| 16:0 | 16.1±1.3 | 20.3±1.0* | 20.1±0.4 | 29.3±1.4† | 11.6±0.3 | 18.4±0.4‡ | 34.7±0.1 | 34.9±1.1 |
| 18:0 | 17.4±1.4 | 23.8±1.4* | 15.4±0.5 | 23.1±0.4† | 22.5±0.3 | 26.3±0.5‡ | 16.4±0.4 | 24.7±0.7‡ |
| 16:1 n-7 | – | – | 1.7±0.2 | –* | – | – | 8.0±0.3 | –‡ |
| 18:1 n-9[1] | 24.6±2.5 | 9.0±0.8† | 29.1±1.6 | 7.2±0.4† | 30.9±0.3 | 14.0±0.8‡ | 19.7±0.9 | 21.7±0.6 |
| 18:2 n-6 | 38.1±1.9 | 13.6±0.5‡ | 27.0±2.4 | 8.4±0.1* | 25.6±0.2 | 13.1±0.8‡ | – | – |
| 20:3 n-6 | – | 1.0±0.3* | – | – | – | – | – | – |
| 20:4 n-6 | 3.1±0.2 | 28.0±0.7‡ | 5.7±1.0 | 31.7±0.5‡ | 9.3±0.7 | 19.7±0.8‡ | 14.1±0.8 | 8.6±0.3‡ |
| 22:4 n-6 | – | – | – | – | – | – | 1.5±0.0 | 1.4±0.1 |
| 22:5 n-3[1] | – | – | – | – | – | 1.9±0.1‡ | – | – |
| 22:6 n-3 | – | 4.0±0.2‡ | – | – | – | 6.2±0.2‡ | 2.9±0.1 | 8.2±0.3‡ |
| ΣSFA | 33.5±0.8 | 44.1±0.5‡ | 35.5±0.2 | 52.4±1.0‡ | 34.1±0.5 | 44.7±0.5‡ | 53.1±0.6 | 59.6±0.4‡ |
| ΣMUFA | 25.0±2.7 | 9.0±0.8† | 31.6±1.5 | 7.2±0.4† | 30.9±0.3 | 14.2±0.9‡ | 27.7±1.1 | 21.9±0.5† |
| ΣPUFA | 41.5±1.9 | 46.9±0.5* | 32.9±1.4 | 40.4±0.6* | 34.9±0.6 | 41.1±0.7† | 19.2±0.6 | 18.5±0.3 |
| ΣPUFA n-3 | –‡ | 4.3±0.3 | – | 0.3±0.0‡ | – | 8.0±0.2‡ | 3.1±0.1 | 8.6±0.3‡ |
| ΣPUFA n-6 | 41.5±1.9 | 42.6±0.9 | 32.9±1.4 | 40.1±0.6* | 34.9±0.6 | 33.0±0.8 | 16.1±0.6 | 10.0±0.3‡ |
| n-3 % PUFA | – | 9.3±0.7‡ | –‡ | 0.8±0.1 | – | 19.6±0.7‡ | 16.2±0.9 | 46.2±1.4‡ |
| C20-22 | 3.3±0.3 | 33.4±0.3‡ | 6.7±1.1 | 32.0±0.5‡ | 9.3±0.7 | 28.0±0.9‡ | 18.6±0.7 | 18.5±0.3 |
| UI | 115±1.2‡ | 179±0.4 | 109±0.8 | 153±2.7‡ | 120±2.5 | 167±2.2‡ | 109±1.5 | 113±1.6 |
| UI (20:4) | 12.4±1.0 | 111.9±2.7‡ | 22.7±4.1 | 126.9±2.1‡ | 37.3±3.0 | 78.8±3.3‡ | 56.2±3.1 | 34.5±1.3‡ |
| UI (22:6) | 0.0±0.0 | 23.7±1.3‡ | 0.0±0.0 | 1.8±0.0‡ | 0.0±0.0 | 37.0±1.3‡ | 17.3±0.6 | 49.5±2.0‡ |

FA, fatty acid; ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids; UI, unsaturation index being the number of double bonds per 100 fatty acid molecules. Fatty acids either not detected or less than 1% of total FA detected in any tissue are not listed but have been included in all calculations.

Values are mean % ± s.e.m. ($N$=4). Superscripts indicate significant difference from the corresponding rat tissue; *$P<0.05$, †$P<0.005$, ‡$P<0.001$.

[1]Since MS cannot readily determine the position of bonds in isobaric species and therefore double isomers may be present and cannot be distinguished by the MS method used, the omega designation of molecules is based on the dominant isomers previously identified using GC (for example, 18:1 can exist as 18:1 n-9 or as 18:1 n-7).

## Statistical analysis

Data analysis was performed using a one-way analysis of variance (ANOVA) with animal species as the fixed factor. Significance was accepted at the level of $P<0.05$ and results are presented as means ± s.e.m. Statistical analyses were performed using JMP 3.2.6 (SAS Institute, Cary, NC, USA).

## Results

### Phospholipid acyl composition

The calculated acyl composition of the total phospholipids analysed by mass spectrometry for each of the four tissues (liver, kidney, heart and brain) are shown in Table 1. These results show the phospholipids of all the rat tissues to have a significantly lower percent of total unsaturated fatty acids (UFA) than those of the lizard. The phospholipids of liver, kidney and heart in the lizard were composed of ~66% UFA compared to 48–56% UFA in the same rat tissues ($P<0.001$). Phospholipids from the lizard brain had 47% UFA compared to 40% UFA in the rat brain ($P<0.001$). In most tissues both palmitic acid (16:0) and stearic acid (18:0) contributed almost equally to the greater saturated fatty acid content of phospholipids from the rat tissues compared to the lizard (Table 1).

Although the liver, kidney and heart phospholipids of the rat had a lower %UFA, they had significantly more polyunsaturates (PUFA) than the phospholipids from the respective lizard tissues. The phospholipids from these tissues in the rat had 40–47% PUFA compared to 33–42% PUFA in the lizard tissues. In the liver and heart, this difference was due to the presence of significant quantities of n-3 PUFA in the rat phospholipids compared to their absence in the lizard, while there was little difference in the % n-6 PUFA between the two species. Docosahexaenoic acid was the predominant n-3 PUFA found in the rat phospholipids. For the kidney phospholipids, there was significantly greater percentage of n-6 PUFA in the rat compared to the lizard and an almost complete absence of n-3 PUFA in both species. The predominant n-6 PUFA in liver, kidney and heart phospholipids was linoleic acid (18:2 n-6) in the lizard whilst for the rat it was arachidonic acid (20:4 n-6). The dominance of 20- and 22-carbon PUFA in the rat phospholipids meant that for the phospholipids from these three tissues, the % long-chain PUFA (i.e. C20-22) was 28–33% in the rat compared to 3–9% in the lizard.

In all tissues, phospholipids from the rat had significantly lower content of monounsaturates (MUFA) than the lizard. For the liver, kidney and heart, the rat phospholipids contained 7–14% MUFA compared to 25–32% in the lizard. The primary driving force behind these differences was oleic acid (18:1 n-9), which was the main MUFA present and was 2–3-fold higher in the lizard compared to the respective rat tissue. For these three

tissues, the ratios of PUFA:MUFA were, respectively, 5.2, 5.6 and 2.9 in the rat compared to 1.7, 1.0 and 1.1, respectively, in the lizard. These differences meant that although there were less % UFA in phospholipids from the rat liver, kidney and brain compared to the lizard, the rat phospholipids actually exhibited a greater degree of relative unsaturation than the respective lizard phospholipids. This is demonstrated by the significantly greater unsaturation index (UI) in the rat liver, kidney and brain phospholipids.

The brain phospholipids differed from the liver, kidney and heart phospholipids in that they had a more similar composition in both the reptile and mammal than did phospholipids from the other three tissues. Although brain phospholipids from the rat had significantly lower %UFA and %MUFA than the lizard, there was no difference in their relative PUFA content. The brain was the only tissue from the lizard whose phospholipids contained n-3 PUFA and in both species, the PUFA were all long-chain PUFA with the complete absence of 18:2 n-6. In essence, the membranes of the lizard brain are n-6 PUFA dominated whereas the brain membranes of the rat exhibited a balance between n-6 and n-3 PUFA.

*Phospholipid class distribution*

Six phospholipid classes were analysed in the two species. These were phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG) and phosphatidylinositol (PI). The relative abundance of each class of phospholipid in each tissue of each species is shown in Fig. 1. A major feature of the phospholipid class distribution was the strong similarity between the two species for each tissue. This similarity in phospholipid class distribution was in distinct contrast to the difference between the lizard and rat tissues in the acyl composition of the phospholipids. The second obvious feature was the dominant presence of two phospholipids (i..e. PC and PE), which together comprised ~96% of total phospholipids in the liver, ~88% in the kidney, ~95% in the heart and ~86% in the brain of the rat and lizard. PC was the major class of phospholipids present, representing approximately 68%, 57%, 68% and 64% of total phospholipids in the liver, kidney, heart and brain, respectively, in both lizard and rat. Phosphatidylserine (PS) was the third most abundant class of phospholipids, being present as 1.7–3.6% of total phospholipids in the liver and heart, 4.9–8.4% in kidney and 10.9–17% in the brain. PS was present at consistently higher concentration in the rat compared to the lizard, with the rat value being 56%, 71%, 112% and 58% higher than the lizard value for the liver, kidney, heart and brain, respectively. Of the remaining three classes of phospholipids, PA was found only in the kidney at ~4%, PI was found in trace amounts (0–1%) in each of the four tissues, PG was not present in the brain of either species but was present in the liver (2.7%) and kidney (2.6%) of the lizard but not the rat, while both species had small amounts (~1.3%) in their hearts.

The overall acyl composition of phosphatidylcholines and phosphatidylethanolamines are compared in Table 2. These values can be compared to the same values for total acyl compositions for phospholipid presented in Table 1. It is notable, comparing the unsaturation index (UI) of PC and PE, that in the tissues of both the lizard and rat, the UI of PE is much
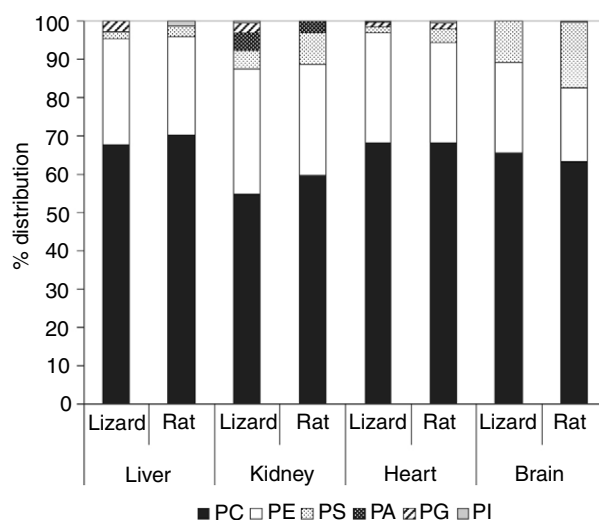


Fig. 1. Distribution of rat and lizard phospholipid classes in liver, kidney, heart and brain of the lizard (*Trachydosaurus rugosus*) and rat (*Rattus norvegicus*). Values are presented as mean percentages of total phospholipids. PC, phosphatidylcholine; PE, phosphatidyl-ethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidyic acid; PG, phosphatidylglycerol.

higher than that of PC. Table 2 also shows the contributions of both arachidonic acid (AA) and docosahexaenoic acid (DHA) to UI of both PC and PE. For PC, AA is the major contributor to UI in all the rat tissues whereas in the lizard it contributes nothing except a little in brain. Similarly DHA contributes nothing to lizard PC UI in any tissue whereas in rat it contributes at a low level, ~12%, to all tissues except the n-6 dominated kidney. For PE, AA is again a major contributor to UI in all the rat tissues. A major change in PE is that AA is also used as a major contributor to UI in all the lizard tissues whereas in PC it is virtually absent. DHA in PE has an increased role in contributing to UI, particularly in the heart and brain of the rat, whereas it is again absent in the lizard tissue with the exception of the brain.

*Phospholipid molecules*

The distribution of the major molecules of PC, PE and PS for liver, kidney, heart and brain are presented in Figs 2–5, respectively, while the significant differences between the lizard and rat for these molecules for all tissues are presented in Table 3. As can be seen from Table 3 there were a large number of reptile–mammal differences that were statistically significant. In the rat tissues there were 7–15 different types of PC molecules, 8–14 different types of PE molecules and 1–7 different types of PS molecules compared to 5–9 different types of PC, 8–10 different types of PE and 1–5 different types of PS molecules in the lizard tissues. In the liver and heart, the rat has approximately twice the number of different types of phospholipid molecules as the lizard, while in both the kidney and the brain the two animal species have an approximately equal number of different types of phospholipid molecules.

In the liver (see Fig. 2) of the lizard PC-16:0/18:2 and PC-18:0/18:2 are the two most abundant molecular species of PC.

Table 2. *Fatty acid class distribution as phosphatidylcholine and phosphatidylethanolamine*

| | Liver | | Kidney | | Heart | | Brain | |
|---|---|---|---|---|---|---|---|---|
| | Lizard | Rat | Lizard | Rat | Lizard | Rat | Lizard | Rat |
| **Phosphatidylcholine** | | | | | | | | |
| ΣSFA | 36.5±0.6 | 42.5±0.6‡ | 44.5±0.9 | 58. 5±1.6† | 33.3±0.7 | 43.8±0.6‡ | 58.6±1.2 | 65.6±0.3‡ |
| ΣMUFA | 23.2±2.8 | 10.5±1.1§ | 27.3±1.6 | 8.0±0.3† | 37.1±0.4 | 16.2±1.1‡ | 35.4±1.0 | 26.3±0.5‡ |
| ΣPUFA n-6 | 40.3±2.2 | 43.7±0.6 | 28.2±1.3 | 33.5±1.3 | 29.6±0.5 | 36.4±1.0‡ | 5.7±0.1 | 6.7±0.3 |
| ΣPUFA n-3 | 0.0±0.0 | 3.2±0.1‡ | 0.0±0.0 | 0.0±0.0 | 0.0±0.0‡ | 3.6±0.2 | 0.0±0.0 | 1.4±0.0‡ |
| UI | 103.8±1.7 | 171.3±0.9‡ | 83.6±1.6 | 121.7±4.5† | 96.3±1.2 | 151.5±2.8‡ | 56.9±1.2 | 61.6±0.9* |
| UI (20:4 n-6) | 0.0±0.0 | 105.5±2.7‡ | 0.0±0.0 | 93.3±3.3‡ | 0.0±0.0 | 84.1±5.2‡ | 19.1±0.7 | 27.0±1.3§ |
| UI (22:6 n-3) | 0.0±0.0 | 19.4±0.6‡ | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 15.2±0.7‡ | 0.0±0.0 | 8.4±0.3‡ |
| **Phosphatidylethanolamine** | | | | | | | | |
| ΣSFA | 19.2±1.8 | 45.5±0.3‡ | 22.0±0.7 | 42.9±0.6‡ | 36.1±0.3 | 44.1±0.4‡ | 38.9±0.8 | 45.7±0.3‡ |
| ΣMUFA | 40.1±4.1 | 6.4±0.6‡ | 38.0±0.1 | 7.4±0.7‡ | 16.2±0.4 | 7.1±0.4‡ | 15.2±0.8 | 15.3±1.0 |
| ΣPUFA n-6 | 40.7±2.4 | 40.1±0.9 | 40.0±0.8 | 48.6±0.0† | 47.7±0.6 | 27.4±1.1‡ | 42.8±0.2 | 23.3±0.6‡ |
| ΣPUFA n-3 | 0.0±0.0 | 8.0±0.7‡ | 0.0±0.0 | 1.1±0.0‡ | 0.0±0.0 | 21.4±1.0‡ | 3.1±0.2 | 15.7±0.4‡ |
| UI | 147.1±2.1 | 195.4±1.6‡ | 146.1±4.8 | 198.9±0.6† | 152.5±5.1 | 215.8±2.3‡ | 205.0±0.8 | 201.8±2.5 |
| UI (20:4 n-6) | 47.4±3.8 | 123.4±2.8‡ | 59.9±11.7 | 175.7±1.9† | 114.4±8.1 | 70.4±2.4† | 160.4±1.5 | 76.1±2.2‡ |
| UI (22:6 n-3) | 0.0±0.0 | 38.9±3.1‡ | 0.0±0.0 | 6.4±0.2‡ | 0.0±0.0 | 101.4±4.9‡ | 12.8±0.8 | 88.7±2.7‡ |

FA, fatty acid; ΣSFA, total saturated fatty acids; ΣMUFA, total monounsaturated fatty acids; ΣPUFA n-6, total polyunsaturated n-6 fatty acids; ΣPUFA n-3, total polyunsaturated n-3 fatty acids; UI, unsaturation index (number double bonds per 100 fatty acid molecules); UI (20:4 n-4), contribution of 20:4 (AA, arachidonic acid) to unsaturation index; UI (22:6 n-3), contribution of 22:6 (DHA, docosahexaenoic acid) to unsaturation index.

Values are mean % ± s.e.m. ($N$=4). Superscripts indicate significant difference from the corresponding lizard tissue; *$P$<0.05, §$P$<0.01, †$P$<0.005, ‡$P$<0.001.

Table 3. *Significant differences between an endotherm (the rat) and an ectotherm (a lizard) in the level of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) molecules present in tissue phospholipids*

| Predominant FA | | Liver | | | Kidney | | | Heart | | | Brain | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sn-1 | sn-2 | PC | PE | PS | PC | PE | PS | PC | PE | PS | PC | PE | PS |
| 16:0 | 16:0 | | | | NS | | | * | | | * | | |
| | 16:1 | | | | | | | | | | * | | |
| | 18:0 | * | | * | | | | * | | * | * | | * |
| | 18:1 | NS | * | | NS | * | | NS | * | | NS | * | |
| | 18:2 | * | * | | * | NS | | * | * | | | | |
| | 20:4 | * | * | | * | * | | * | | | | * | |
| | 22:4 | | | | | | | | | | | * | |
| | 22:6 | * | * | | | * | | * | * | | * | * | |
| 16:1 | 18:1 | | | | * | | | * | | | | | |
| 18:0 | 18:0 | | | | | | | | | | | | * |
| | 18:1 | * | * | | * | * | * | * | NS | * | * | * | * |
| | 18:2 | * | * | * | * | NS | * | * | * | * | | | |
| | 20:4 | * | * | * | * | * | * | * | * | * | * | * | * |
| | 22:4 | | | | | | | | * | | | * | * |
| | 22:5 | | * | | | | | | * | | | * | * |
| | 22:6 | * | * | | | | | * | * | | * | NS |
| 18:1 | 18:1 | * | * | | * | * | * | * | * | | NS | * |
| | 18:2 | * | * | | * | * | | * | * | | | |
| | 20:4 | * | NS | | NS | | | * | * | | NS | |
| 18:2 | 18:2 | * | * | | * | | | * | NS | | | |

Significant differences are indicated, with $P$<0.05 (asterisk) as a minimum; NS, non-significant difference.
Values are shown in Figs 2–5.

Together these constitute 60.5% of all PC molecules, while PE-18:0/18:2 and PE-18:1/18:2 are the two most abundant PE molecules. Together these make up 46.1% of all PE molecules and PS-18:0/18:2 is the only molecule of PS present. However, in the rat liver membranes the most abundant phospholipids contain 20:4 instead of 18:2. For example, PC-16:0/20:4 and PC-18:0/20:4 are the two most abundant PC molecules, together making up 47.1% of all PC, while PE-16:0/20:4 and PE-18:0/20:4 are the two most abundant PE molecules representing 55.2% of all PE, and PS-18:0/20:4 is the most abundant PS
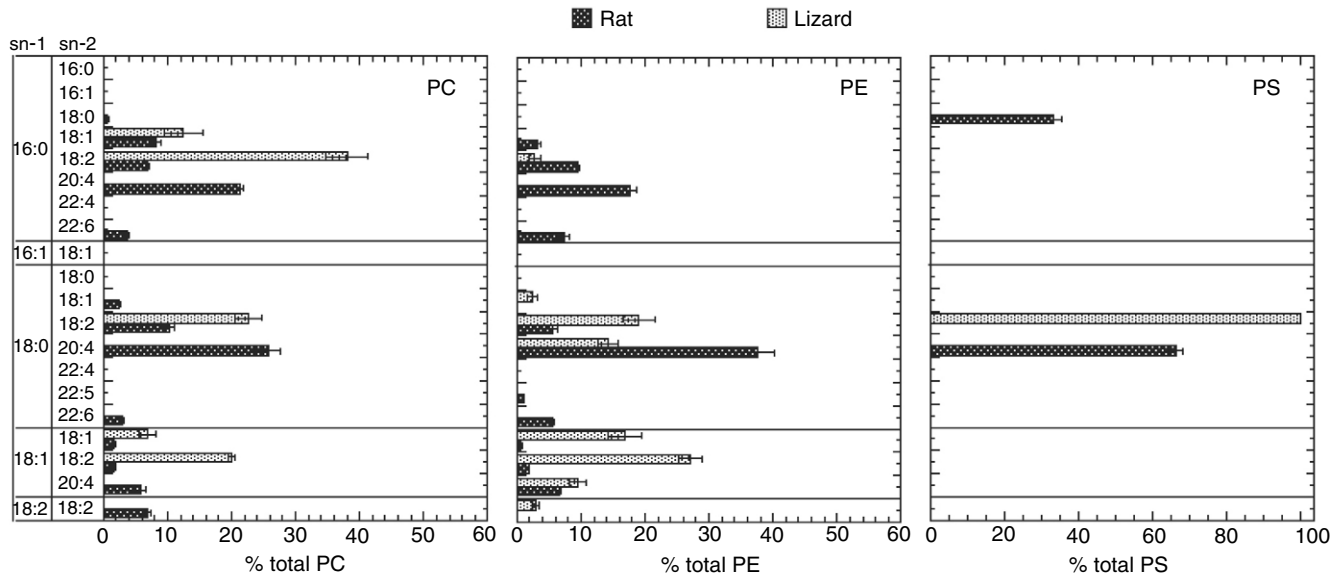
Figs 2. The distribution of the major phospholipid molecules of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in liver of the lizard (*Trachydosaurus rugosus*) and rat (*Rattus norvegicus*). Significant differences between the lizard and rat for these molecules in all these tissues are presented in Table 3. Values are mean % ± s.e.m. of total phospholipid (nmol g⁻¹ wet tissue). The FA shown in the left-most box is the predominant sn-1 FA with the predominant sn-2 FA shown in the right hand box. Molecules that contribute to less than 1.0% in each tissue are not shown but are included in any calculations.

molecule. There are three highly unsaturated molecules of PC (i.e. 16:0/20:4, 18:0/20:4, 16:0/22:6) and two highly unsaturated molecules of PE (i.e. 16:0/20:4, 16:0/22:6) that were only found in rat liver and were absent in the liver membranes of the lizard.

In the kidney (see Fig. 3), there was a similar emphasis on 18:2-containing molecules in the lizard and 20:4-containing phospholipids in the rat. In the lizard kidney, PC-16:0/18:2 and PC-18:0/18:2 constituted 45.6% of all PC molecules, while PE-18:0/18:2 and PE-18:1/18:2 made up 36.4% of PE molecules; PS-18:0/18:2 was the most abundant PS molecule (representing 44.9% of all PS). In the rat kidney, PC-16:0/20:4 and PC-

18:0/20:4 together constituted 46.1% of PC, PE-16:0/20:4 and PE-18:0/20:4 made up 74.3% of PE, while PS-18:0/20:4 was the only PS molecule present. There were no PC molecules containing 20:4 in the lizard kidney. There were almost no phospholipids molecules containing omega-3 PUFA in the kidney of either species.

The heart (see Fig. 4) was similar to both the liver and kidney with respect to PC molecules but differed from liver and kidney with respect to PE molecules. In the lizard heart, PC-16:0/18:2, PC-18:0/18:2 and PC-18:1/18:2 were the three most abundant PC molecules, together making up 59.1% of PC molecules.
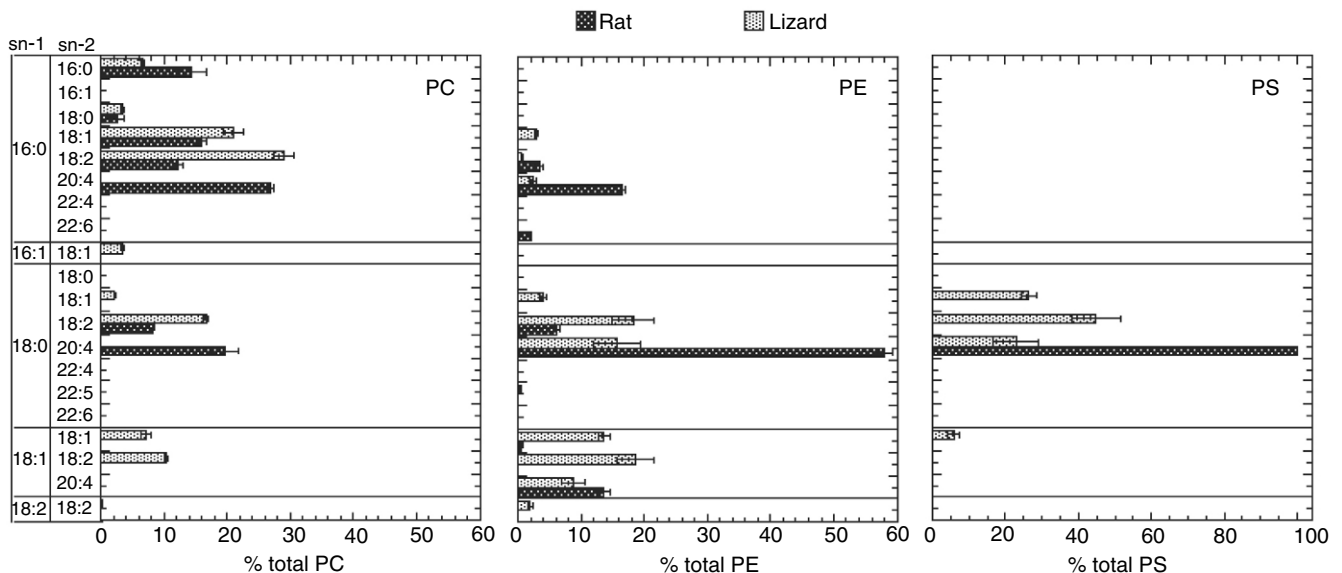


Fig. 3. The distribution of the major phospholipid molecules of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in kidney of the lizard (*Trachydosaurus rugosus*) and rat (*Rattus norvegicus*). For further details, see legend to Fig. 2.
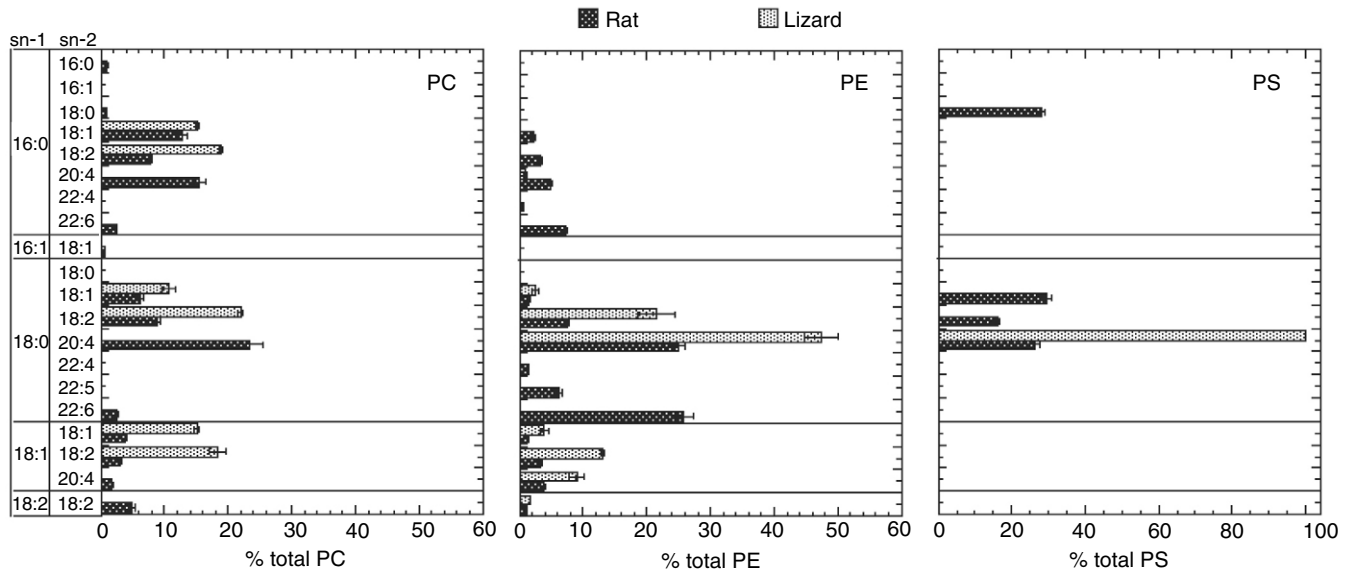
Fig. 4. The distribution of the major phospholipid molecules of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in heart of the lizard (*Trachydosaurus rugosus*) and rat (*Rattus norvegicus*). For further details, see legend to Fig. 2.

There were no 20:4-containing PC molecules in the lizard heart, while in the rat heart PC-18:0/20:4 and PC-16:0/20:4 were the two most abundant molecules, together being responsible for 38.9% of all PC molecules. However, unlike the situation in the liver and kidney, the most abundant PE molecule in the lizard heart was PE-18:0/20:4 (47.4% of all PE), with all PE molecules containing 18:2 (i.e. PE-18:0/18:2, PE-18:1/18:2 and PE-18:2/18:2) being 45.3% and thus less than the total of those PE molecules containing 20:4 (i.e. PE-16:0/20:4, PE-18:0/20:4 and PE-18:1/20:4), which made up 57.2% of all PE molecules. In

the rat heart the two most abundant molecules were PE-18:0/20:4 and PE-18:0/22:6, which together made up about half (50.8%) of all PE molecules. While both PC and PE had 22:6-containing molecules in the rat heart, there were no omega-3 PUFA containing phospholipids in the lizard heart. The only PS molecule in the lizard heart was PS-18:0/20:4, while this PS molecule was only 26.4% of all PS molecules in the rat heart.

For the brain (see Fig. 5) there was a more similar profile in the rat and lizard than observed for the other three tissues. In both the lizard brain and the rat brain there were essentially no
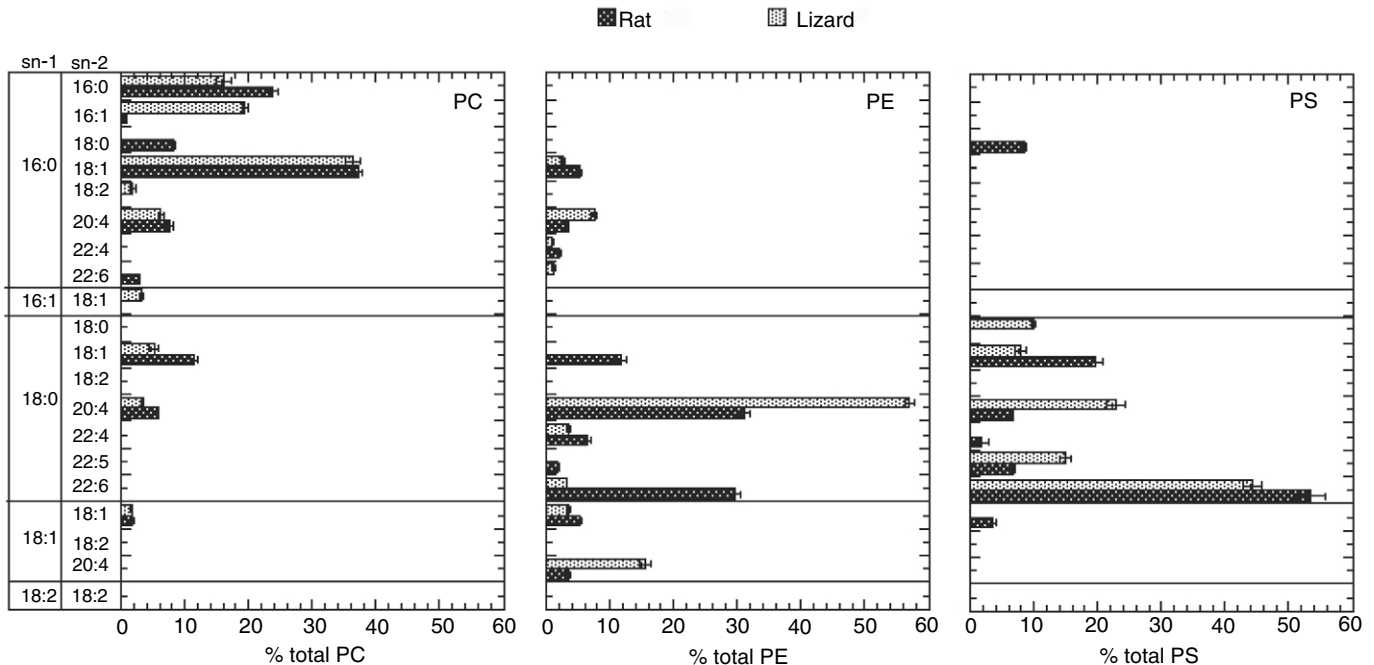


Fig. 5. The distribution of the major phospholipid molecules of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) in brain of the lizard (*Trachydosaurus rugosus*) and rat (*Rattus norvegicus*). For further details, see legend to Fig. 2.

18:2-containing phospholipids, and in both animal species the dominant PC molecules contained monounsaturates, with PC-16:0/18:1 and PC-16:0/16:1 together being responsible for 55.8% of all PC molecules in the lizard brain, while in the rat PC-16:0/18:1 and PC-18:0/18:1 made up 48.5% of all PC molecules. The dominant PE molecules in the lizard brain were PE-18:0/20:4 (57% of all PE) while in the rat brain PE-18:0/20:4 and PE18:0/22:6 were equally abundant, being 31.1% and 29.6% of all PE molecules respectively. In both the lizard and the rat, PS-18:0/22:6 were the most abundant PS molecules in the brain. As can be seen from Table 2, although there was a slight tendency for PE to be more polyunsaturated than PC molecules in liver, kidney and heart, this was very pronounced in the brain and especially so with respect to the omega-3 PUFA.

## Discussion

This study reveals that the difference in phospholipid acyl composition between an ectothermic reptile (*Trachydosaurus rugosus*) and an endothermic mammal (*Rattus norvegicus*), of similar body size and body temperature but with very different (~sevenfold) resting rates of metabolism, was not restricted to any particular phospholipid class. It showed that the reptile–mammal difference in acyl composition (Brand et al., 1991; Brookes et al., 1997; Else and Wu, 1999; Hulbert and Else, 1989; Turner et al., 2005) was not limited to a single phospholipid class, nor to a few specific molecular species of phospholipid, but instead was widespread across a broad range of phospholipid molecules and was observed in all classes of phospholipid. The functional significance of this finding is that it implies that the physical properties of the bulk, non-raft portions of membrane lipids (raft portions primarily composed of cholesterol and sphingomyelins) is a key factor in determining the higher molecular activity of membrane proteins and subsequently the higher metabolic rate of endotherms compared to ectotherms (at the same temperature).

One of the fundamental differences in phospholipid acyl composition between the mammal and the reptile was the higher level of unsaturation present in the mammal. The highly unsaturated PUFA molecules that contributed most to this increased level of membrane unsaturation in the mammal included both an omega-6 and an omega-3 molecule in the form of 20:4 n-6 and 22:6 n-3. Precisely how the presence of these highly unsaturated PUFA molecules, together with other variations in phospholipid composition that change the physical properties of membranes, translates into changes in the molecular activity of membrane proteins is currently unknown. What we do know is that the highly unsaturated PUFA molecules (such as 22:6 n-3 and 20:4 n-6) are both flexible and very physically active (Feller et al., 2002; Mitchell et al., 1998; Stillwell and Wassall, 2003). The reason for this increased dynamic motion and flexibility seems to reside on either side of the multiple double bonds of these fats. Although there is no rotation around the *cis*-double bonds of unsaturated fatty acid chains (and the double bonds themselves are rigid), there are extremely low potential barriers to rotation around the carbon–carbon single bonds (the allylic bonds) that occur on either side of the doubled bonded carbon units in fatty acid chains. The result is that when these highly polyunsaturated

molecules are present in the membrane these dynamic components are likely to exert high lateral pressure on the surrounding components within the membrane bilayers containing them. In this respect it is of interest that in a study comparing the molecular activity of $Na^+,K^+$-ATPase from the kidney and brain of the rat and toad showed that the molecular activity was more strongly correlated with the lateral pressure measured than with the individual fatty acid composition of the surrounding membrane lipid (Wu et al., 2001).

A further finding of the present study was that the distribution of phospholipid head groups (classes) within the same tissues of both the reptile and the mammal were essentially the same. Therefore the difference in phospholipid acyl composition was due to differences in the diversity of phospholipid molecules that exist within the same phospholipid classes that were similarly distributed in the two animal species. This suggests that the distribution of phospholipid classes may be a property of membranes that is well preserved among vertebrates. Considering the known asymmetry of phospholipid classes (where PC is commonly exofacial and PE and PS cytofacial) and the common requirements of membranes shared by all animals in fulfilling both general (e.g. facilitation of transport; maintenance of concentration gradients) and specific (e.g. in cell signalling; apoptosis) functions, the conservatism in the distribution of phospholipid class may not be too surprising. For example, in the present study it was observed that 22:6 was relatively more abundant in PE and PS than it was in PC. As both PE and PS are preferentially associated with the cytoplasmic leaflet of the membrane bilayer (Balasubramanian and Schroit, 2003), this suggests that 22:6 acyl chains may also be preferentially associated with the cytofacial leaflet of cellular membranes. The physical and functional implications of such an asymmetric distribution of 22:6 within membrane bilayers is, however, unknown.

The different acyl composition of membranes previous reported between reptiles and mammals in comparisons using gas chromatography (Brand et al., 1991; Brookes et al., 1997; Else and Wu, 1999; Hulbert and Else, 1989; Turner et al., 2005), and confirmed in the present study using mass spectrometry, have shown that the level of polyunsaturation, as indicated by the unsaturation index, was much lower in the tissues of the lizard compared to those of the rat. This was due to the preponderance of short-chain, less-unsaturated molecules that included a higher level of monounsaturates and a slightly lower percentage of PUFA (~6% on average) in the phospholipids of the lizard compared to the rat. These differences were not due to the effects of body size or body temperature as the reptile and mammal selected in the present study, and those animals selected in previous studies, were of similar size with high 'preferred' body temperatures. Furthermore, the greater amount of polyunsaturated acyl chains (especially 22:6) and reduced amount of monounsaturated acyl chains (primarily 18:1) in the mammalian compared to reptilian membranes was not due to the simple replacement of one for the other. For example, the much higher level of 22:6 in mammalian compared to reptilian phospholipids was due to the presence of molecules of 22:6 combined with either 16:0 or 18:0 in the rat coupled to the absence of such molecules in phospholipids from the reptilian tissues. However, the converse was not true for the high levels

of monounsaturates in the reptilian compared to mammalian phospholipids. In this second situation, the very high level of monounsaturated acyl chains in the lizard phospholipids was overwhelmingly due to the much greater abundance of molecules with 18:1 coupled with another unsaturated acyl chain (either MUFA or PUFA) in the lizard phospholipids compared to those from mammalian tissues.

This study also revealed a number of reptile–mammal differences not previously obvious. In the present study it was obvious that whereas 22:6 is relatively more abundant in PE than PC and therefore the reptile–mammal difference in 22:6 is emphasised in PE compared to PC molecules, the reptile–mammal difference in di-unsaturated molecules with 18:1 is equally evident in the PC and PE phospholipid classes. One clear reptile–mammal difference was the complete lack of PC molecules containing arachidonic acid in all the reptile tissues except the brain, instead using the less unsaturated linoleic acid 18:2. A further observation from the present study was that the two saturated fatty acids (16:0 and 18:0) found in phospholipids were not equally distributed between PC and PE. Palmitic acid chains (16:0) were more abundant in PC than in PE, while stearic acid chains (18:0) were preferentially found in PE than in PC phospholipids. This tendency is most pronounced in brain but was also observed in phospholipids from the other tissues. There did not seem to be any reptile–mammal difference in this trend.

An exception to many of the generalisations associated with the differences in acyl composition of tissue phospholipids in ectotherms and endotherms was the brain. Although the lizard brain had low levels of saturated and n-3 fatty acids indicative of an ectotherm, it was more similar in its acyl composition to the mammalian brain phospholipids than any other tissue. Examples were the similar high levels of long-chain PUFA, similar unsaturation indices and total PUFA content of the lizard and rat brain phospholipids. Differences did, however, exist: the primary long-chain fatty acid in the rat brain was DHA (22:6 n-3), whereas in the lizard, although some DHA was present (in contrast to all other lizard tissues where it was virtually absent), the primary long-chain PUFA present was arachidonic acid (20:4 n-6). The high level of acyl chain unsaturation in brain membranes is a feature commonly observed across a wide variety of species (Hulbert et al., 2002; Turner et al., 2005).

Previous studies examining the membranes of cultured cells have shown that polyunsaturated fatty acids are not randomly distributed within membranes, especially in the plasmalemma. Firstly, the exofacial and cytofacial leaflets of the plasmalemma differ in phospholipid headgroup composition, cholesterol content as well as fatty acid composition, and this non-symmetric composition results in a transbilayer fluidity gradient regulated largely by unsaturated fatty acids (Kier et al., 1986; Sweet and Schroeder, 1988b). Secondly, the plasmalemma also contains lateral domains (lipid rafts/caveolae as well as non-raft domains) that differ in their lipid composition and protein distribution (for reviews, see Pike, 2003; Pike et al., 2002; Schroeder et al., 2005). For example, the $Na^+,K^+$-ATPase appears to be distributed primarily in the non-raft domains of the plasmalemma, at least in some cells (Atshaves et al., 2003; Gallegos et al., 2006) and $Na^+,K^+$-ATPase activity is influenced by the transbilayer fluidity gradient (Schroeder and Sweet, 1988; Sweet and Schroeder, 1986a; Sweet and Schroeder, 1986b; Sweet and Schroeder, 1988a). Lipid bilayers with a high content of cis-polyunsaturated fatty acid chains are both thinner and more flexible than bilayers with predominantly monounsaturated fatty acids (Rawicz et al., 2000).

A major benefit of analysing membrane phospholipid composition using mass spectrometry is the detail provided about the molecules within each class of phospholipid. This can likely provide more insight into differences in membrane structure and function between species (and tissues, cells etc). Our results for the rat were essentially similar to those recently reported by Hicks et al. for a large number of rat tissues (Hicks et al., 2006). Our study differs slightly from Hicks et al.'s report in that our techniques were able to separate and quantify isobaric molecules (those with the same mass-to-charge ratio; for example, we were able to differentiate between PC-16:0/20:4 and PC-18:2/18:2), and because of our use of internal standards we were able to quantify more accurately the abundance of particular molecules between different classes of phospholipid (i.e. we could take into account the different ionisation efficiencies and thus combine data from the classes of phospholipids, such as PC and PE). The most abundant phospholipids identified in each tissue were essentially the same in both studies on the rat. To our knowledge, there are no data available on any other reptile with which to compare our findings on the molecular composition of tissue phospholipids. One difference noted between the present study (using mass spectrometry) and previous ones (using gas chromatography) is the slightly lower level of n-3 PUFA (especially 22:6) reported here. This discrepancy may be related to the fact that the present study was restricted to the major glycerophospholipids while the other studies analysing total phospholipids included other classes of phospholipids (such as the sphingomyelins and cardiolipins), which may have contributed to the higher n-3 PUFA levels.

Very little is known about how membrane acyl composition is regulated and thus how species differences are maintained. In rats, the relative content of saturated, monounsaturated and polyunsaturated fatty acids of membrane phospholipids is fairly constant, irrespective of changes in the fatty acid composition of the diet, and this is indicative of a degree of homeostatic regulation (see Hulbert et al., 2005). The mechanisms involved in such regulation will include synthesis of de novo phospholipids followed by membrane remodelling via enzyme-mediated deacylation–reacylation of membrane phospholipids. To date, this has been studied in only a limited number of cell types and species. Work on rat hepatocytes (Schmid et al., 1995) suggests that only four molecular species (16:0/18:2, 16:0/18:1, 16:0/22:6 and 18:1/18:2) of both PE and PC are synthesized de novo and that all other PC and PE molecular species are produced by remodelling via deacylation–reacylation at either or both the sn-1 and sn-2 positions. The enzymes responsible for remodelling (by deacylation–reacylation) appear to be especially important, as it can be seen that the predominant molecules of both PC and PE in rat liver (see Fig. 2) do not correspond to those produced by de novo synthesis. Notably, 18:0 and 20:4 acyl chains appear only to be incorporated into phospholipids via deacylation–reacylation and not during de

*novo* synthesis. In this respect, although we do not know if the same *de novo* synthesis of specific molecules occurs in lizard liver cells as has been documented for the rat, it is of interest that the predominant molecules of PC and PE in the lizard liver more closely reflect the *de novo* synthesised group than is the situation for the rat. This suggests that membrane remodelling pathways maybe especially important in determining the reptile–mammal differences we report here.

The role of headgroup interconversion (i.e. PC↔PE↔PS) in determining the specific molecular profiles described here is unknown. A study using rat hepatocytes (De Long et al., 1999) indicates that the production of PC (*via* methylation of PE) yields PCs with a high level of unsaturation (e.g. PC-18:0/20:4 and PC-18:0/22:6). The high level of unsaturated PC molecules in the rat (in particular those containing 20:4) suggests a greater contribution of the PE methylation pathway to PC synthesis in the rat liver than in the lizard liver. The fact that this pathway is absent in heart and kidney (Arthur and Page, 1991), however, combined with our findings that the same differences are observed between animals for both these tissues, suggest this may not necessarily be the case.

In summary, the present study reports the first comparison of the distribution of fatty acid composition among different phospholipid classes between an ectothermic and endothermic vertebrate of the same body size and body temperature. It confirmed the presence of differences in acyl composition of membrane phospholipids between a reptile and a mammal. It showed that these differences occurred across a broad range of phospholipid molecules and were observed in all classes of phospholipid. Furthermore it showed that the distribution of membrane phospholipid classes (head groups) in the same tissue of the reptile and mammal were essentially the same.

### List of abbreviations

| | |
|---|---|
| AA | arachidonic acid |
| DHA | docosahexaenoic acid |
| FA | fatty acid |
| MUFA | monounsaturated fatty acids |
| PA | phosphatidic acid |
| PC | phosphatidylcholine |
| PE | phosphatidylethanolamine |
| PG | phosphatidylglycerol |
| PI | phosphatidylinositol |
| PS | phosphatidylserine |
| PUFA | polyunsaturated fatty acids |
| SFA | saturated fatty acids |
| UFA | unsaturated fatty acids |
| UI | unsaturation index |

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