Lipid class and phospholipid species composition associated with life history variation in north temperate and neotropical birds

DISSEETATION

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By

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Abstract

Life-history traits are often linked, generating a spectrum where organisms that have long lifespans usually have low metabolic rates and low reproductive effort, whereas organisms with short lifespans usually have high metabolic rates and high reproductive effort. Physiological mechanisms likely underlie these variations in life history. As such, a recent focus in the field of physiological ecology has been connecting life-history traits to physiological attributes. An emerging study system for research on these connections is tropical and temperate bird species. Temperate bird species tend have low annual survival, high metabolic rates, and high reproductive effort, whereas tropical birds tend to have high annual survival, low metabolic rates, and low reproductive effort. Also emerging as a tool in physiological ecology, primary cell culture allows researchers to take samples from individuals in minimally invasive ways and compare cells grown under the same environmental and nutritive conditions.

In this dissertation, I first ascertained what kinds of differences there are between cultured fibroblast cells and their progenitor cells extracted from individual organisms. Fibroblasts are likely to change in culture due to differences in the environment around them and because the cells switch from a mostly quiescent state to an actively proliferating state. Nonetheless, the extent to which these changes could affect the results of comparative studies is not well understood. I compared five bird species across passages 0, 2, and 4 in terms of cell size and lipid class composition. I found that most
variables I compared did not change, changed in the same way across species, or changed
in a way that made cells of each species become more similar to those of other species. In
a limited number of variables, however, the cells of some species changed differently
from other species over time. These data suggest that fibroblasts are generally a useful
tool for comparative studies, but some caution should be applied using fibroblasts,
including using the earliest possible passage of cells where possible.

I then compared lipid class composition of mitochondria in fibroblasts from ten
pairs of tropical and temperate bird species. I found that temperate bird species had
significantly more mitochondrial lipid per million cells than did tropical birds and that
this difference was particularly pronounced in the amount of cardiolipin per million cells.
These data indicate that temperate bird species likely have more or larger mitochondria
than do tropical species. Additionally, plasmalogen, an antioxidant phospholipid class,
made up a larger percentage of lipid in tropical species than in temperate ones.

Lastly, I reviewed the literature on the connections among fatty acyl composition
of membrane phospholipids, metabolism, and longevity. Weaknesses of the current
literature on this topic include lack of distinction among phospholipid classes in fatty acyl
composition, a lack of distinction among cellular organelles, and a lack of broad
comparative studies. To remedy this, I compared the fatty acyl composition of different
phospholipid classes in mitochondrial and non-mitochondrial membranes between
tropical and temperate bird species. I found that tropical and temperate species were not
different at broad scale metrics of fatty acyl composition, but did differ when I compared
specific phospholipid classes in the mitochondria. These fine scale differences in fatty
acyl composition were also correlated with differences in cellular ability to resist stress and cellular metabolic rate. These data support connections among fatty acyl composition, metabolism, and longevity, but suggest that the connections are more complicated than previously hypothesized.
Dedication

This document is dedicated to my friends and family.
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Linkages between mitochondrial lipids and life-history in temperate and tropical


Fields of Study

Major Field: Evolution, Ecology and Organizmal Biology
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Chapter 1: Changes in cultured dermal fibroblasts during early passages across five wild bird species

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Abstract

With the advent of the usage of primary fibroblasts in comparative and evolutionary biology, it is important for researchers to know the extent to which cells might be altered during the culturing process and how much species might differ in response to cell culture. I compared early changes in cell size and lipid composition of primary dermal fibroblasts grown at physiologically relevant oxygen concentrations (5% O₂) from wild-caught species of birds. Fibroblasts from American Robins (Turdus migratorius) and Bobwhite Quails (Colinus virginianus) increased in size early in the culture process and cells from all five species of wild-caught birds exhibited changes in lipid-class composition. The two most common phospholipids, phosphatidylcholine and phosphatidylethanolamine, increased in concentration in all species between early passages and later passages of fibroblasts. Some less abundant lipid species, such as cardiolipin and sphingomyelin, exhibited similar concentrations in all three passages that I measured. Other lipid classes, such as cholesterol, increased in some species in later
passages and decreased in others. Although results may vary with cell-culture conditions, this study points to a need for researchers comparing multiple species to take precautions when using cell culture, such as experimenting on the earliest possible passage of cells.

**Introduction**

Cell culture has traditionally been used by the research community to study a host of basic questions in cell physiology, often with direct medical applications (De Boni, 1985; Cruz et al., 2009), but recently it has also been employed in other research fields such as animal conservation, physiological ecology, and evolution (León-Quinto et al., 2011; Harper et al., 2011; Gomes et al., 2011; Alper et al., 2015). Primary cell culture involves removing tissue from an animal, dispersing cells enzymatically, and then explanting cells to a cell-culture flask. Often these cells are fibroblast cells, but other types of cells, such as myoblasts, can be cultured (Nehlin et al., 2011). One advantage of using primary fibroblasts in cell culture is that a harmless biopsy of tissue can be taken to establish cultures, which is especially beneficial for work on endangered species (León-Quinto et al., 2011). Furthermore, cell culture is advantageous in comparative research because cultured cells experience a uniform environment. Physiological measurements of tissues taken directly from animals in the wild can be heavily affected by diet or reproductive status (Miller et al., 2011). Consequently, primary fibroblast cell culture has been utilized to create cell banks for endangered species (León-Quinto et al., 2011), for investigating the toxic effects of environmental contaminants (Marsili et al., 2008), in comparative studies for understanding of the course of mammalian evolution (Gomes et
al., 2011), and for exploring differences in cells among organisms with differing life histories (Harper et al., 2011; Jimenez et al., 2013; Alper et al., 2015).

Whereas many studies look at the differences in cultured cells across time as a model for understanding the aging process (Stanulis-Praeger, 1987; Van Gansen and Van Lerberghe, 1988; Rubin, 1997), far fewer studies have documented changes in cells during the initial stages of cell culture from their original state when first extracted from the organism and put in the cell-culture environment (Levinthal and Rubin, 1968). Thus, investigators that explore attributes of cells in culture as a model for those same cells within an organism assume that cells in culture are representative of cells within the animal. Information on if and how cells change will aid in interpreting experimental results.

A standard way of marking how long cells are in culture is by passage number. When cells are nearly confluent in a culture flask, they are detached enzymatically, and transferred at a specific density to new culture flasks, a process called passaging. The higher the passage number, designated as $P_x$, where $x$ is the number of times the cells have been subcultured, the more likely cells will go through changes in ploidy and karyotype (Holeckova and Cristofalo, 1970), increases in size (Rubin, 1997), increases in susceptibility to oxidative stress (Yuan et al., 1996), and other changes in cellular function (Sahm and Seifert, 2000; Nehlin et al., 2011).

Even with a number of studies done on the effects of increasing passage number on cellular attributes (Stanulis-Praeger, 1987; Van Gansen and Van Lerberghe, 1988), some gaps remain in our knowledge of how cells change in vitro, especially during early
passage numbers, as most studies are done on $P_8$ or later (Polgar et al., 1978). The majority of studies that have examined changes in cells during culture have been done on cells from humans, rats, and mice, with a few on domestic chickens (Stanulis-Praeger, 1987; Van Gansen and Van Lerberghe, 1988). However, no studies have examined what changes in cellular properties may occur during the culture of cells derived from wild species, ones under the influence of natural selection, and few have examined whether cells from different species might respond differently to cell-culture conditions, both of which are particularly important for scientists doing research comparing multiple species of organisms.

The aim of this study was to compare the responses of fibroblasts from different species to cell-culture conditions and to discover whether cell culture may create any artificial differences between species that are not present in the original tissue. I compared changes in cell size and lipid composition after 24 h of cell growth, or $P_0$, to $P_2$ and $P_4$ of primary dermal fibroblasts grown at physiologically relevant oxygen concentrations (5% O$_2$) from five species of wild-caught birds. My findings provide insights into how cells change in specific cell-culture conditions and show that researchers need to be aware that using primary cell culture may affect the outcome of comparative studies.

**Methods**

*Capture and handling of birds*

Three Mourning Doves (*Zenaida macroura*) and three American Robins (*Turdus migratorius*) were mist netted in and around central Ohio (39°59=N, 82°59=W) and skin
samples of four Mallards (*Anas platyrhynchos*), two Northern Shovelers (*Anas clypeata*), and four Bobwhite Quails (*Colinus virginianus*) were also collected in late spring of 2009 and 2011. All birds were adults. This research was done under Public Health Service Animal Welfare Assurance No. A3261-01 from the Institutional Animal Care and Use Committee of Ohio State University.

**Cell culture**

After birds were sacrificed, their feathers were plucked, exposed skin was washed twice with antimicrobial soap, and a 5 mm × 5 mm skin sample was taken and placed into cold transfer medium, which contains “complete medium” with the addition of 10 mmol/L culture-grade HEPES. Complete medium consists of Dulbecco’s modified Eagle medium (DMEM), high-glucose variant (4.5 mg/mL), with sodium pyruvate (110 mg/L) and L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum, and antibiotics (100 U/mL penicillin and streptomycin, where 1 U = 1 µmol/min ≈ 16.67 nkat). Skin samples were shipped in transfer medium on ice overnight to the University of Michigan in 2009 or processed at Ohio State University in 2011.

Fibroblast cultures were established following Harper et al. (2011) and Jimenez et al. (2013). Skin was submerged in 70% EtOH for 30 s, minced, and placed in collagenase B overnight at 37 °C. The following day, the mixture of cells was filtered through a 20 µm cell strainer, centrifuged to pack cells, and plated in a culture flask. Every 3–4 days, 75% of the fresh complete medium was replaced in the culture flasks. Cells were subcultured when they reached confluence into new 75 cm² flasks at a density of 10^4
cells/cm². The cells were harvested by trypsinization (0.25%) for subculturing when they reached 90% confluence and cultured at 5% CO₂ and 5% O₂. I used 5% O₂ because it is a physiologically relevant level (Alaluf et al., 2000).

At passages P₀ and P₂, half of the cells were removed from the flask, counted with a hemocytometer, and cryopreserved in 10% glycerol in culture media under nitrogen gas to avoid lipid oxidation. The other half of the cells were cultured further. At P₄, all cells were counted and frozen using the same conditions as P₀ and P₂. I then extracted lipids from the cells and quantified their lipid classes using thin-layer chromatography (TLC) and an iodine-addition assay.

Cell size

For robins and quails, I took images of rounded cells at each passage after trypsinization using a CCD digital camera through a 10× power objective and measured cell diameter using Labkit version 1.1 software (Phenix).

Lipid extraction

After washing cells and suspending them in ultrapure water, I extracted lipids from cells using a modified Bligh and Dyer method (Bligh and Dyer, 1959). The modifications I used to increase lipid yield were substituting 1 mol/L NaCl for water and doing a second extraction of the samples (Jean-Louis et al., 2006). I analyzed the bottom layer, containing the lipids, using TLC and a spectrophotometric assay for plasmalogens.

Thin-layer chromatography

Classes of phospholipids were separated using TLC on 20 cm × 20 cm Silica Gel H plates (Analtech, Newark, New Jersey, USA; Kupke and Zeugner, 1978) using the
technique from Calhoon et al. (2014). Briefly, plates were developed to 5 cm from the top with chloroform:methanol:acetic acid:acetone:water (35:25:4:14:2 v/v), let dry, then to 3 cm from the top with chloroform:methanol:acetic acid (190:10:1 v/v), let dry, then to 1 cm from the top with hexane:diethyl ether:acetic acid (80:20:2 v/v), and dried. I visualized bands of lipids by spraying the plates with a solution of 3% cupric acetate in 8% phosphoric acid and then charring them in an oven for 40 min at 160 °C. The mean error of the control samples in my plates was <3%. I used the following lipids as standards: triglyceride, cholesterol, free fatty acid, cardiolipin, ceramide, cerebroside, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin. All chemicals and standards were procured through either Sigma–Aldrich or Avanti Polar Lipids.

Iodine-addition assay

To quantify plasmalogens, I used the colorimetric iodine-addition method (Huque et al., 1987; Calhoon et al., 2014). I validated my ability to detect plasmalogens by running known concentrations of plasmalogens and found a mean error rate of 2%.

Statistics

All statistical tests were performed using SPSS version 19.0 (SPSS Inc., Chicago, Illinois, USA), with the null hypothesis rejected at $p \leq 0.05$. I calculated the total mass of lipids by adding up all of the lipids detected by TLC. I tested for normality and homogeneity of variance with Shapiro–Wilk and Levene’s test of homogeneity, respectively, prior to analysis and I transformed data that differed from these assumptions by using logarithms. Values are reported as means ± 1SE.
I ran ANOVAs with passage and species as independent variables for each dependent variable, first with an interaction between passage number and species and, then, if the interaction was not significant, without the interaction term. These ANOVAs had repeated measures for individual cell lines because each cell line was used three times, once for each passage. Whether or not there was an interaction, I ran post hoc tests using the Bonferroni correction to test which passages were significantly different from each other. Additionally, I ran Levene’s test of homogeneity on the mean values for each species between passages to see if there was more homogeneity among species in the later passages.

Results

Changes in cell size across passages

For cells from robins and quail, cell diameter increased from $P_0$ to $P_4$ by about 200% (Table A.1, Appendix A). The interaction term was not significant, indicating that the relative change in cell size was the same among species (Table 1.1). When the interaction term was excluded in the analysis, there were significant differences among passages and between species. Post hoc analysis indicated that cells at $P_0$ were smaller than cells at $P_2$ and $P_4$, but that the latter cells were not significantly different from each other in size ($p < 0.001$, for both).

Changes in lipid-class concentrations across passages

The concentration of cardiolipin, ceramide, and sphingomyelin did not differ among passages, but was significantly different among species (Figure 1.1A, Table 1.1).
The concentration of phosphatidylcholine and phosphatidylethanolamine as a proportion of total lipid amount increased in the later passages. These changes were consistent across species (Figure 1.1B, Table 1.1). Post hoc analysis indicated that phosphatidylcholine as a proportion of the total amount of lipid increased significantly in later passages relative to its concentration at $P_0$ ($p < 0.001$, in both cases) and phosphatidylethanolamine as a proportion of the total lipid increased significantly between $P_0$ and $P_2$ ($p = 0.002$). I also found that the ratio of nonphospholipids to phospholipids decreased with increasing passage number in all species, but there was not a significant interaction term between species and passage, indicating that the ratio changed similarly across species. The ratio of nonphospholipids to phospholipids provides a broad view of how the lipids are changing in cells (Holeckova and Cristofalo, 1970). Post hoc analysis indicated that the ratio of nonphospholipids to phospholipids was significantly decreased between $P_0$ and $P_2$ and between $P_0$ and $P_4$ ($p = 0.002$, for both cases).

The concentrations of phosphatidylinositol, plasmalogen, and triglyceride were altered in a species-specific manner as passage number increased, as indicated by the significant interaction term in each of the models, but also increased in homogeneity between species (Figure 1.1C, Table 1.1). In triglyceride and plasmalogens, this was mostly driven by a decrease in these lipids in those species that had the highest amounts to a more uniform low value at the later passages, whereas in phosphatidylinositol, despite large variation in starting points, all species ended up with uniformly moderate concentrations by $P_4$. 
Lastly, I found that the concentrations of free fatty acids, cholesterol, and phosphatidylserine changed differently among species across passages. In contrast to results for phosphatidylinositol, plasmalogen, and triglycerides, concentrations of free fatty acids, cholesterol, and phosphatidylserine did not become more similar in later passages (Figure 1.1D, Table 1.1).

Discussion

I have examined how cells derived from wild species of birds change in culture during early passages. Cells of robins and quail increased in diameter, in quail by more than 200%. Changes in cell size appeared to be generally consistent across species, as did changes in the two most common phospholipids, phosphatidylcholine and phosphatidylethanolamine. Two of the less common phospholipids, phosphatidylinositol and phosphatidylserine, several neutral lipids, and plasmalogens did not change consistently across species with increasing passage number; however, plasmalogen, phosphatidylinositol, and triglyceride tended to become more homogeneous among species with increasing passage number.

Changes in cell size have also been documented for cells from domestic bird species (Rubin and Hatié, 1968). The environment that cells are exposed to in cell culture is more enriched than that within an organism and this difference in nutrients could result in an increase in nutrient uptake and cell growth (Rubin and Hatié, 1968). Within birds, an increase in cell size, even at the earliest passages, has been recorded in chick fibroblasts maintained under ambient air (Rubin and Hatié, 1968; Levinthal and Rubin, 1968); here I extend this finding to five avian species maintained under physiologically
relevant oxygen tension. Whereas during the first 24 h the increase in cell size is due to actual increase in size of the cells (Rubin and Hatié, 1968), it may be that later increases in size are due to selection of larger cells. Either way, cell size is correlated with a host of other traits, including metabolic rate and genome size (Gregory, 2002), making it important in comparative studies to understand how cells change during early passages.

In studies that examined changes in lipid composition of fibroblasts between passages, researchers have reported no differences (Holeckova and Cristofalo, 1970; Polgar et al., 1978), but these studies were done on $P_{18}$–$P_{43}$. Studies using early passages of myoblasts and endothelial cells showed changes in lipid composition (Sahm and Seifert, 2000; Nehlin et al., 2011), especially triglyceride content. Changes in lipid content create downstream effects because the proportion of lipid classes affect properties as diverse as membrane fluidity (Ramstedt and Slotte, 2002), enzyme function (Huang et al., 2008), and cell death (Rosivatz and Woscholski, 2011).

Proportions of several lipid classes either did not change between passages, as in cardiolipin (Figure 1.1A), or changed in similar ways across all species, as in phosphatidylcholine (Figure 1.1B). Comparative studies on concentrations of these lipids in fibroblasts, even in passages as late as $P_4$, will show a relatively accurate reflection of actual differences between species, rather than artifacts of cell culture. Moreover, the lipids that show this pattern are important to the functioning of the cell. For instance, the amount of cardiolipin is important for the functioning of mitochondrial complexes and oxidative stress (Huang et al., 2008) and sphingomyelin influences lipid-raft formation (Ramstedt and Slotte, 2002).
I also found multiple lipid classes that changed differently among species. Some of these lipids, such as triglyceride and plasmalogen, became more homogeneous in amount among species over the course of serial passaging (Figure 1.1C). It would appear that, in these cases, as cells are maintained progressively longer in culture, they converge toward a less specified cell type owing to a common extracellular environment (Bailey et al., 1959) and their removal from exogenous sources of lipids, such as diet (Rubin, 1997). Given that plasmalogens are thought to function as an antioxidant (Brosche and Platt, 1998), another possible mechanism for the uniformly low levels of plasmalogens in later passages is that being exposed to chronic oxidative stress, as a result of cell-culture conditions, may lead to the irreplaceable destruction of individual plasmalogen molecules as happens with some other antioxidant molecules throughout serial passaging (Yuan et al., 1996; Alaluf et al., 2000). Research on cultured fibroblast lipids that become more homogenous in amount over the course of passaging would potentially understate rather than exaggerate differences between species, though an alternative explanation is that the differences were due to diet or environment and culturing in a common environment eliminated those differences.

An understanding of how different species respond to cell culture is crucial to interpreting the results of comparative studies using fibroblasts. It would be incorrect to say that cultured fibroblasts are the same as their progenitors in the original animal tissue, as I saw changes among passages. Nevertheless, if cells of different species are changing in the same way or are becoming more similar to each other, differences in cultured fibroblasts among species could be attributed to actual differences among the species.
themselves, rather than artifacts of cell culture. This study provides evidence that the large-scale changes among passages, such as cell size, ratio of nonphospholipids to phospholipids, and the concentrations of the two most common phospholipid classes, tend to be similar among species. Out of the changes that differ among species, some change the cells in such a way that species are becoming more similar to each other. Thus, for the most part, this cell-culture method will keep cell lines from different species at similar levels of difference or even decrease differences among species, rather than creating differences where none exist in the original organisms, but there are exceptions.

The specific changes in lipid composition that I have found may be a result of the culture conditions used in this study. Nonetheless, this study points to a likely need for researchers doing comparative research using fibroblasts to either use the earliest possible passage or, preferably, do a study comparing changes in the variable of interest across passages to see if species change differently.
### ANOVA results

<table>
<thead>
<tr>
<th></th>
<th>Passage x Species</th>
<th>Passage</th>
<th>Species</th>
<th>Levene’s test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No statistically significant differences</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of cerebroside (mg) to total lipids (mg)</td>
<td>$p=0.202$</td>
<td>$p=0.301$</td>
<td>$p=0.818$</td>
<td>$p=0.246$</td>
</tr>
<tr>
<td><strong>Only differences between species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ratio of cardiolipin (mg) to total lipids (mg)</td>
<td>$p=0.853$</td>
<td>$p=0.117$</td>
<td>$p&lt;0.001^*$</td>
<td>$p=0.627$</td>
</tr>
<tr>
<td>Ratio of ceramide (mg) to total lipids (mg)</td>
<td>$p=0.347$</td>
<td>$p=0.514$</td>
<td>$p=0.034^*$</td>
<td>$p=0.921$</td>
</tr>
<tr>
<td>Ratio of sphingomyelin (mg) to total lipids (mg)</td>
<td>$p=0.078$</td>
<td>$p=0.128$</td>
<td>$p=0.001^*$</td>
<td>$p=0.319$</td>
</tr>
<tr>
<td><strong>Differences between passages, but no interaction with species</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell diameter (µm)</td>
<td>$p=0.364$</td>
<td>$p&lt;0.001^*$</td>
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<tr>
<td>Ratio of non-phospholipids (mg) to phospholipids (mg)</td>
<td>$p=0.071$</td>
<td>$p&lt;0.001^*$</td>
<td>$p=0.042^*$</td>
<td>$p=0.115$</td>
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<tr>
<td>Ratio of phosphatidylethanolamine (mg) to total lipids (mg)</td>
<td>$p=0.187$</td>
<td>$p=0.005^*$</td>
<td>$p=0.002^*$</td>
<td>$p=0.693$</td>
</tr>
<tr>
<td>Ratio of phosphatidylcholine (mg) to total lipids (mg)</td>
<td>$p=0.952$</td>
<td>$p&lt;0.001^*$</td>
<td>$p=0.191$</td>
<td>$p=0.818$</td>
</tr>
<tr>
<td><strong>Homogeneity increasing</strong></td>
<td></td>
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</tr>
<tr>
<td>Ratio of phosphatidylinositol (mg) to total lipids (mg)</td>
<td>$p=0.002^*$</td>
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<td>-</td>
<td>$p=0.022^*$</td>
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<tr>
<td>Ratio of plasmalogen (µmoles) to total lipid (mg)</td>
<td>$p&lt;0.001^*$</td>
<td>-</td>
<td>-</td>
<td>$p=0.001^*$</td>
</tr>
<tr>
<td>Ratio of triglyceride (mg) to total lipids (mg)</td>
<td>$p&lt;0.001^*$</td>
<td>-</td>
<td>-</td>
<td>$p=0.037^*$</td>
</tr>
<tr>
<td><strong>Interaction between passage and species with no increase in homogeneity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ratio of cholesterol (mg) to total lipids (mg)</td>
<td>$p=0.001^*$</td>
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<td>-</td>
<td>$p=0.263$</td>
</tr>
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<td>Ratio of free fatty acid (mg) to total lipids (mg)</td>
<td>$p=0.007^*$</td>
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<td>-</td>
<td>$p=0.654$</td>
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<tr>
<td>Ratio of phosphatidylserine (mg) to total lipids (mg)</td>
<td>$p=0.027^*$</td>
<td>-</td>
<td>-</td>
<td>$p=0.489$</td>
</tr>
</tbody>
</table>

* Significant difference

Table 1.1. Statistical results of repeated measure ANOVAs and Levene’s test for homogeneity on cellular characteristics during the course of serial passaging in five wild-caught bird species.
Figure 1.1. Representative examples of different types of changes in concentration of lipid classes that occurred during serial passaging of primary dermal fibroblasts. The four types of changes that I noted were: (A) significant differences only between species, (B) significant differences between passages, (C) significant interactions between species and passage where homogeneity between species was increasing during passaging, and (D) significant interactions between species and passage where homogeneity between species was not increasing. $P_0$ is indicated by a black bar, $P_2$ by a white bar, and $P_4$ by a hashed bar. The species included in this analysis were: Mallards ($Anas platyrhynchos$), Northern Shovelers ($Anas clypeata$), Bobwhite Quails ($Colinus virginianus$), Mourning Doves ($Zenaida macroura$), and American Robins ($Turdus migratorius$). Values are means ± 1 S.E.
Abstract

Temperate birds tend to have a fast pace of life, having short life-spans with high reproductive output, whereas tropical birds tend to have a slower pace of life, investing fewer resources in reproduction and having higher adult survival rates. How these differences in life history at the organismal level are rooted in differences at the cellular level is a major focus of current research. Here, I cultured fibroblasts from phylogenetically-paired tropical and temperate species, isolated mitochondria from each, and compared their mitochondrial membrane lipids. I also correlated the amounts of these lipids with an important life history parameter, clutch size. I found that tropical birds tended to have less mitochondrial lipid per cell, especially less cardiolipin per cell, suggesting that cells from tropical birds have fewer mitochondria or less inner mitochondrial membrane per cell. I also found that the mitochondria of tropical birds and the species with the smallest clutch sizes had higher amounts of plasmalogens, a lipid that could serve as an antioxidant. Overall, my findings are consistent with the idea that there
are underlying molecular and cellular physiological traits which could account for the differences in whole animal physiology between animals with different life histories.

**Introduction**

Life history is defined as a set of evolved strategies that directly influence rates of survival and reproduction. Attributes of an animal’s life history, such as clutch size, nestling growth rate, rate of metabolism, and longevity typically fall along a “slow-fast” continuum (Promislow and Harvey, 1990; Ricklefs, 2000; Dobson and Oli, 2008). Animals at the fast end of this continuum are thought to invest resources in reproduction early in life and have relatively short lives, whereas animals at the slow end have fewer offspring and invest more resources into self-maintenance. This continuum is thought to be created by trade-offs, as an animal investing large amounts of resources in reproduction will devote less to self-maintenance (Kirkwood and Rose, 1991; Wiersma et al., 2004; van de Crommenacker et al., 2011). Birds that breed in North America tend to have a fast pace of life with relatively large clutch sizes, high rates of metabolism, and low annual survival, whereas lowland tropical birds tend to have a slower pace of life with smaller clutch sizes, lower metabolic rates, and higher annual survival (Johnston et al., 1997; Ricklefs, 1997; Tieleman et al., 2006; Wiersma et al., 2007a; Wiersma et al., 2007b; Jimenez et al., 2014b).

A major challenge in physiological ecology is to connect patterns of life history traits, embodied in a slow or fast pace of life, to physiological attributes at the organ, tissue, and cellular level (Ricklefs and Wikelski, 2002; Monaghan et al., 2009; Williams et al., 2010; Selman et al., 2012; Sutherland et al., 2013). Although whole-organism
metabolism has been shown to be reduced in lowland tropical birds (Wiersma et al., 2007a; Wiersma et al., 2007b), the physiological mechanisms underpinning this reduction in metabolic rate remain unclear. Part of the explanation may lie in tropical birds having somewhat smaller central organs than do temperate birds of similar body size (Wiersma et al., 2012). However, there may be other physiological mechanisms that contribute to lower metabolic rate in tropical birds. Here, I explore whether cells from tropical birds have fundamental differences from cells in temperate birds, attributes that might influence metabolism and longevity. Cells from tropical birds are more resistant to chemical stressors than cells from temperate birds (Jimenez et al., 2013), supporting the hypothesis that cells of tropical and temperate birds differ in their chemical make-up.

Differences at the cellular level between long- and short-lived species could be especially pronounced in the mitochondria (Kowald, 2001). Mitochondria are responsible for most of the ATP produced in the cell (Samuels, 2005), are important in the lifespan of cells through the regulation of apoptosis (Green and Reed, 1998), and are the source and the target of many reactive oxygen species (ROS) (Wallace, 2005). This connection between mitochondria and longevity is embodied in the Mitochondrial Theory of Aging, a corollary to the Free Radical Theory of Aging (Harman, 1972; Miguel et al., 1980; Shigenaga et al., 1994; Pak et al., 2003). The Mitochondrial Theory of Aging posits that animals age as a result of the long-term damage from ROS to mitochondrial lipids, proteins, and DNA (Harman, 1972; Miguel et al., 1980; Shigenaga et al., 1994). Given that phospholipids are a common structural component in membranes of mitochondria and are a major target of ROS molecules (Jeon et al., 2001), comparison of these lipids
among animals with different life history strategies may lead to insights about how these lipids may be connected to particular phenotypes. Higher amounts of polyunsaturated acyl chains (PUFA) in the phospholipids of mitochondria and other membrane bilayers have been implicated in increasing metabolism and decreasing longevity, suggesting a possible link between the rate of metabolism and longevity (Hulbert et al., 2002a; Hulbert, 2003; Hulbert et al., 2006; Buttemer et al., 2008; Hulbert, 2008a) and decreases in PUFA in phospholipids of the mitochondria may be more correlated with increases in longevity than PUFA in the phospholipids of other membranes (Munro and Blier, 2012). However, the connection between PUFA and metabolism has mixed support (Valencak and Ruf, 2007; Haggerty et al., 2008; Konarzewski and Książek, 2013; Wone et al., 2013), so there may be other explanations for the connection between physiology and life-history traits. Because temperate birds have higher rates of basal metabolism and shorter life spans (Wiersma et al., 2007b), I hypothesized that mitochondria of temperate species may have greater amounts of lipid classes associated with increased metabolism and lower amounts of those that protect against oxidative damage compared with lipids from mitochondria of tropical species. For additional insight into the connections between life history and mitochondrial lipids, I also correlated the amount of each lipid class with clutch size, in order to see if trends in mitochondrial lipids could be related to a specific life history parameter. I predicted that clutch size would be positively correlated with amounts of mitochondrial lipid classes that increase metabolism and negatively correlated with amounts of those that protect against oxidative damage.
Two phospholipid classes were targeted as potential candidates for differences in membranes in the mitochondria of birds with different life histories: cardiolipin and plasmalogen. Cardiolipin is located in the inner mitochondrial membrane and binds with a variety of proteins, including most of the complexes in the electron transport chain, stabilizing their conformation or modulating their activities (Chicco and Sparagna, 2006; Petrosillo et al., 2008; Paradies et al., 2010; Dumas et al., 2013; Pöyry et al., 2013). Cardiolipin is required for maintenance of the electrical potential across the inner membrane and decreases in cardiolipin content can result in a decrease in the activity of Complex I, the rate-limiting complex of the electron transport chain (Petrosillo et al., 2008; Joshi et al., 2009; Dumas et al., 2013). Because of its close association with Complexes I and III and its high content of unsaturated fatty acids, cardiolipin is a target of ROS (Petrosillo et al., 2008). Reduction in cardiolipin content in mitochondria can result in a decrease in cardiolipin peroxidation and an increase in the ability of cells to resist oxidative stress (Huang et al., 2008). Therefore, I reasoned that the amount of cardiolipin in the mitochondria of an organism may be the result of an evolutionary trade-off between maximizing the efficiency of the metabolic machinery and minimizing oxidative damage. Based on this, I hypothesized that mitochondria in cells of tropical birds will have less cardiolipin than mitochondria in cells of temperate birds and also that cardiolipin content would be positively correlated with clutch size.

Plasmalogens are glycerophospholipids that contain a vinyl ether moiety at the sn-1 position of the glycerol backbone. Several studies have suggested that plasmalogens function as antioxidants (Zoeller et al., 1988; Engelmann et al., 1994; Murphy, 2001;
Leray et al., 2002; Maeba and Ueta, 2003; Morandat et al., 2003; Braverman and Moser, 2012). It is thought that the vinyl ether linkage is part of the key to its antioxidant function, as it attracts radicals instead of allowing unsaturated fatty acids to be attacked (Brosche and Platt, 1998), but the exact mechanism remains unclear (Felde and Spiteller, 1995; Lessig and Fuchs, 2009). Murphy (2001) posited plasmalogens reduce or slow down the propagation of the free radicals by keeping them near the polar region of the membrane, away from the hydrophobic interior where double bonds, which are susceptible to oxidative damage, are more abundant. Further, some long-lived species have more plasmalogens in their mitochondrial membranes than their shorter-lived relatives (Mitchell et al., 2007; Munro and Blier, 2012), though this research has been done on a relatively small subset of species. Plasmalogens may also have a link to metabolic rate given that they impede passive ion leakage across membranes (Hazel and Williams, 1990; Chen and Gross, 1994), which would theoretically slow metabolism, because increasing ion leak through the inner mitochondrial membrane, or uncoupling, increases metabolism (Mookerjee et al., 2010; Seebacher et al., 2010). Taken together, these findings led us to predict that mitochondria from tropical bird species would have a higher percentage of plasmalogens than mitochondria from temperate species and that the mitochondria of species with small clutch sizes would have greater amounts of plasmalogens than the mitochondria of species with large clutch sizes.

I compared differences in the lipids of mitochondrial membranes of cells from tropical and temperate birds and also correlated the amounts of these lipids to clutch size to search for connections between life history and mitochondrial lipid composition. I used
primary cultured cells exposed to a common nutrient environment to search for intrinsic differences in mitochondrial lipids among cells. My data showed that cells from temperate species had a greater amount of total mitochondrial lipid and more cardiolipin per cell. Mitochondria of cells from tropical bird species and species with smaller clutch sizes had a higher proportion of plasmalogens compared with temperate species and species with larger clutch sizes.

**Methods**

*Capture and handling of birds*

Birds were captured by mist-netting in and around Gamboa, Panama (9°7’N, 79°42’W), a lowland tropical environment, in December 2009 and May 2011, and around Central Ohio (39°59’N, 82°59’W) in late spring and summer of 2009 and 2011. Birds were kept for 1-2 days in small wire cages and provided with food and water ad libitum. The food provided was based on the natural food of the species: insectivores were given mealworms and grasshoppers, frugivores fruit, nectivorous birds sugar water, and seed eaters were fed seeds and bread. In accordance with my animal care protocol, Public Health Service Animal Welfare Assurance Number A3261-01 from the Institutional Animal Care and Use Committee of Ohio State University, cervical dislocation was used to humanely sacrifice the birds.

In comparative biology, one needs to take into consideration the phylogenetic relatedness among species in order to avoid statistical issues of the lack of independence of data (Felsenstein, 1985; Harvey and Pagel, 1991; Garland and Adolph, 1994). Many researchers use various forms of phylogenetically informed statistics to account for this
problem, but these methods contain a variety of assumptions that may or may not be true (Westoby et al., 1995, Garland et al., 2005). As an alternative, I have elected to work on cells from pairs of species, one tropical and one temperate, which are closely related phylogenetically (Table 2.1). My approach resolves many of the ambiguities associated with phylogenetic statistics, reduces complications associated with variation in body mass, and elevates statistical power (Garland et al., 2005, Williams et al., 2010).

*Cell culture*

Fibroblasts are easily cultured and ubiquitous in the body and, when they are proliferating in cell culture, fibroblasts have a higher metabolic rate than proliferating myoblasts (Jimenez et al. 2014a), so they are not the quiescent cells that they are sometimes painted to be.

After birds were sacrificed and weighed, their feathers were plucked, exposed skin was washed twice with anti-microbial soap, and a 5 x 5 mm² skin biopsy was taken and was placed into cold transfer media, which contained “complete media” with the addition of 10 mM culture-grade HEPES. Complete media (CM) had a pH of 7.4 and consisted of Dulbecco’s modified Eagle medium (DMEM), high-glucose variant (4.5 mg/ml), with sodium pyruvate (110 mg/L) and L-glutamine, supplemented with 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum, and antibiotics (50 µg/ml each of penicillin and streptomycin). Skin samples were shipped in transfer media on ice overnight to Ohio State University or University of Michigan.

Fibroblast cultures were established following Harper et al. (2007). Skin was submerged in 70% EtOH for 30 sec, and then immediately minced and placed in
complete media containing collagenase B for an overnight digestion at 37 °C. The following day, the mixture of cells was filtered through a 20 µm cell strainer, centrifuged to pack cells, and plated in a culture flask. Every 3-4 days, 75% of the media was replaced. Cells were sub-cultured when they reached confluence into new 75 cm² flasks at a density of 10⁴ cells/cm². The cells were harvested by trypsinization (0.25%) for sub-culturing when they reached 90% confluence. Cells were cultured at 5% O₂, which is a physiologically relevant level of oxygen, to avoid oxidative stress and premature senescence (Chen et al., 1995; Alaluf et al., 2000). At passage 3, cells were harvested, counted with a hemocytometer, suspended in culture media with 10% glycerol as a cryoprotectant, and cryopreserved at 80°C under nitrogen gas to avoid lipid oxidation.

Whereas during cell culture, cells can change, resulting in different properties than when functioning in an animal, I have previously shown that neither of the lipids that I targeted in this study change in a manner that would introduce artificial differences between species, at least during the first three passages (Calhoon et al., 2013).

Cell size

Differences in cell size between tropical and temperate birds could confound my analyses. Cells from 7 species pairs were harvested and placed on a hemocytometer slide. Images of the rounded cells were recorded using a CCD digital camera through a 10x power objective, and diameter was measured using Labkit 1.1 software (Phenix).

I compared cells from passages 1, 2, and 3 to see if there were any differences in the way that the species from different environments changed in cell size. I tested for normality and homogeneity of variance with Shapiro-Wilk and Levene’s test of
homogeneity, respectively, prior to analysis and I corrected data that differed from these assumptions by transforming using logarithms. I ran a repeated-measures mixed model using environment and passage as covariates. Cells increased in size with passage number ($F_{\text{passage}}=4.6$, $P<0.02$), as is typical of primary cells in culture (Rubin and Hatie, 1968; Calhoon et al., 2013), but there were neither differences between species from different environments in the cell growth over the three passages ($F_{\text{environment x passage}}=1.108$, $P>0.33$), nor differences in cell size between environments ($F_{\text{environment}}=2.6$, $P>0.11$).

**Mitochondrial isolation**

Frozen cells were thawed and, following washing with 50 µl of PBS, suspended in 1.5 ml Eppendorf tubes with 750 µl of Isolation Buffer (IB) pH 7.4: 0.3 M mannitol, 0.1% BSA, 0.2 mM EDTA, 20 mM HEPES, and 0.01 mM leupeptin hydrochloride. I disrupted the plasma membrane of the cells by means of nitrogen cavitation using a Parr Cell Disruption Vessel (Parr Instrument Company Moline, Illinois). This system breaks up the plasma membrane without damaging the organelles (Hunter and Commerford, 1961; Simpson, 2010). I loaded the cell solution into the bottom of the cooled disruption chamber and allowed it to equilibrate for 20 min at a pressure of 800 psi (Graham, 1993a). The pressure was released and cell lysate was collected for mitochondrial isolation using differential centrifugation with an Eppendorf 5415 centrifuge. I used spins of 700xg for 10 min to pellet nuclei and spins of 7,000xg for 10 min to separate the mitochondria from the plasma membrane and endoplasmic reticulum. I carried out all mitochondrial isolation procedures, including differential centrifugation, at 2-4°C. I used
enzyme assays (Graham, 1993b) to determine that my mitochondrial isolation procedure concentrates >95% of the mitochondria from the cells into my mitochondrial fraction, while >93% of the plasma membrane and endoplasmic reticulum are in a separate fraction (Figure 2.1; see also Figure B.1, Appendix B). Finally, I re-suspended the mitochondria in 100 µl water to prepare for lipid extraction.

Lipid extraction

Lipids were extracted from the mitochondria using a modified Bligh and Dyer method (Bligh and Dyer, 1959). For each 0.1 ml of mitochondria, I added 0.375 ml chloroform:methanol (1:2 v/v) and vortexed for 10 min. Then, I added another 0.125 ml of chloroform per 0.1 ml of sample and vortexed for 1 min., followed by 0.125 ml of 1M NaCl per 0.1 ml of sample and vortexing (Jean-Louis et al., 2006). Then, the samples were centrifuged for 15 min. at 1000xg, which resulted in two distinct layers. The lower layer, containing the lipids, was collected and the upper, aqueous layer extracted again by adding an additional 0.188 ml of chloroform per 0.1 ml original sample. The samples were centrifuged again and the new lower layer added to the first bottom layer. I analyzed this mixture, containing the mitochondrial lipids, using thin layer chromatography (TLC) and a spectrophotometric assay for plasmalogens. To assure that the upper, aqueous phase contained no lipid, I evaporated this layer, added chloroform:methanol 2:1, and ran a TLC plate, but found no discernible amount of lipid. To prevent lipid oxidation, I used chloroform and methanol that contained 50mg/L butylated hydroxytoluene for all steps (Muñoz-Garcia and Williams, 2005).

Thin layer chromatography
Classes of lipids were separated on 20x20 cm Silica Gel H plates using TLC (Analtech Newark, NJ; Kupke and Zeugner, 1978; Vecchini et al., 1995; Xu et al. 1996; Calhoon et al., 2013). Contaminants were removed from the plates by developing them to the top with chloroform:methanol (2:1 v/v). Then the plates were dried and activated in an oven for 30 min at 110°C. After that, I scored the plates into 13 lanes. I used five lanes for a mixture of lipid standards, two for controls of known lipid concentration, and the remaining lanes for samples, each in duplicate. The plates were developed to 5 centimeters from the top with chloroform:methanol:acetic acid:acetone:water (35:25:4:14:2 v/v/v/v/v), let dry, then developed to 3 cm from the top with chloroform:methanol:acetic acid (190:10:1 v/v/v). After air-drying, they were developed to one centimeter from the top with hexane:diethyl ether:acetic acid (80:20:2 v/v/v), and dried. I visualized bands of lipids by spraying the plates with a solution of 3% cupric acetate in 8% phosphoric acid and then charring them in an oven for 40 min at 160°C. Immediately after, I scanned plates using a Hewlett Packard flatbed scanner. The quantity of lipids in each band was determined by photodensitometry using the LINUX version of IMAL (T.J. Nelson, http://laplace.ucv.cl/AnalisisImagenes/tnimage/tnimage-manual.html). If the error between standards and the controls of known concentration on a plate were larger than 5%, then I ran a new plate. The mean error of the control samples in my plates was < 3%. I used the following lipids as standards: triglyceride, cholesterol, free fatty acid, cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, phosphatidylcholine, and sphingomyelin, run in serial dilution from
a concentration of 2.5 mg/ml to 0.16 mg/ml. All of the chemicals and standards were purchased from either Sigma-Aldrich or Avanti Polar Lipids.

*Iodine addition assay*

To quantify plasmalogens, I used an iodine addition method, which relies on the addition of I$_2$ in methanol, a solution with a yellow color, to the mixture of plasmalogens (Huque et al., 1987). I$_2$ complexes specifically with the vinyl ether double bond of plasmalogens and the complex depreciates the yellow color of the mixture in a quantitative manner (Gottfried and Rapport, 1962; Huque et al., 1987). The moles of I$_2$ lost from solution are equal to the moles of vinyl ether bonds in the mixture and, thus, to the moles of plasmalogens.

I evaporated duplicate aliquots of lipid sample under nitrogen gas to remove chloroform and then re-dissolved samples in 75 μl of methanol. To one of the samples, 75 μl of 3% aqueous KI was added, as a blank, and to the other sample, 75 μl of iodine solution (3x10$^{-4}$ M I$_2$ in 3% KI) was added. I mixed this vigorously and then incubated it at room temperature for 20 minutes. Lastly, I added 600 μl of 95% ethanol and recorded the absorbance of each sample at 355 nm. A control sample was included in which the lipid sample was omitted. The molar extinction coefficient for this assay was 27,500 M$^{-1}$ cm$^{-1}$.

*Clutch sizes*

Clutch sizes were obtained from the literature (Table 2.1; Tieleman et al., 2004; Auer et al. 2007; Jetz et al. 2008). Tropical birds had significantly smaller clutch sizes than temperate birds in a paired t-test (t=3.5, P=0.007).
Statistics

I compared the total amount of mitochondrial lipid per million cells, the amount of each individual mitochondrial lipid per million cells, and the amount of each mitochondrial lipid as a proportion of total mitochondrial lipid. The number or size of mitochondria may differ between cells of different species, so examining the amounts of each lipid per million cells allowed us to look at mitochondrial lipid classes in the context of the entire cell, whereas examining lipids as a proportion of total mitochondrial lipid allowed us to look at the lipid classes in the context of the mitochondria.

I performed all statistical tests using SPSS 19.0, with the null hypothesis rejected at P≤0.05. I calculated the total amount of lipids by summing all lipids in the mitochondrial fraction detected by TLC. I tested for normality and homogeneity of variance with Shapiro-Wilk and Levene’s test of homogeneity, respectively, and I corrected data if necessary by transforming to logarithms. I tested for homogeneity of variance on the means for species before analysis and also on the residual values for the models and if either test showed that the data lacked homogeneous variance, I transformed the data. If the data were not normally distributed or did not have homogeneous variance after transformation, I used the means for each species in a Related-Samples Wilcoxon Signed Rank Test. Means were reported ± 1 S.E.

If the data were normally distributed and had homogeneous variance, I ran repeated-measures ANCOVAs for each variable, with individuals as the repeated-measure for each species and using body mass of the individual from which the cells were collected as a covariate. If mass was significantly correlated with the variable, then I
used it as a covariate in two more tests, to first determine whether there was a difference between the slopes in tropical and temperate bird species and, if there was no difference between the slopes, to determine if there was a difference between the intercepts. If mass was not significantly correlated, I used the same model, except I eliminated body mass as a variable, to analyze whether any differences in mean between each species pair was consistently positive or negative.

In addition to the analyses above, I also wanted to analyze whether clutch size was an important variable, so I ran stepwise regressions with the species averages of each variable using clutch size, environment, and body mass as predictors. The stepwise regression had an $F$ of 0.05 to enter and an $F$ of 0.1 to remove. Though this analysis did not employ the same paired species approach as the ANCOVA models, it would appear that phylogeny does not have a large effect on the results of these regression models. When regressions taking phylogeny into account were run using the ape package of the statistical software R (R Core Team, 2015), the p-values that were produced were within ±0.01 of p-values produced by stepwise regressions using the same variables.

**Results**

*Total mitochondrial lipid*

Compared with tropical birds, temperate birds had significantly more mitochondrial lipid and phospholipid per million cells (Wilcoxon test; $P<0.04$ and $P<0.03$, respectively; Figure 2.3A).

In a stepwise regression, mitochondrial lipid per million cells was not related to clutch size ($R^2=0.07$, $P=0.26$). Because the amount of mitochondrial lipid per million
cells was different between tropical and temperate birds, there are likely to be cases where the amount of a specific lipid per million cells may be significantly different between tropical and temperate birds, but the amount of that lipid as a proportion of total lipids will not be significantly different and vice versa. For lipid percentage averages, see Figure 2.2.

**Cardiolipin**

Mitochondrial cardiolipin per million cells was higher in temperate birds than tropical birds (Wilcoxon test; \( P<0.04 \); Figure 2.3B).

In addition, in a stepwise regression, neither body mass nor clutch size was significant, but environment was significantly related to cardiolipin per million cells \( (F=4.46, P<0.05) \), suggesting that cardiolipin per million cells was more related to the difference between tropical and temperate birds than to body mass or clutch size.

Cardiolipin content relative to total mitochondrial lipid was not significantly different between tropical and temperate bird mitochondria \( (F_{\text{environment}}=2.12, P=0.15) \), nor was it related to clutch size \( (R^2=0.03, P=0.81) \).

**Plasmalogen**

The plasmalogen content relative to the total mitochondrial lipid was not related to body mass \( (F_{\text{interaction}}=2.49, P>0.09) \), but was significantly higher in mitochondria of tropical bird species than in temperate ones, once body mass was excluded \( (F_{\text{environment}}=4.17, P=0.002; \text{Figure 2.4A}) \).

In a stepwise regression, plasmalogen content relative to total mitochondrial lipid had a significant negative association with clutch size \( (R^2=0.40, P=0.003; \text{Figure 2.4B}) \).
Plasmalogen content per million cells was not significantly different between tropical and temperate bird mitochondria ($F_{\text{environment}}=2.48$, $P=0.12$), nor was it related to clutch size ($R^2=0.10$, $P=0.19$).

*Other lipids*

Mitochondrial phosphatidylethanolamine and phosphatidylinositol per million cells were higher in temperate birds than tropical ones (Wilcoxon test; $P<0.05$, for both).

The amount of phosphatidylcholine as a proportion of total mitochondrial lipids increased slightly with body mass in tropical species, but decreased in temperate ones ($F_{\text{interaction}}=8.13$, $P<0.01$).

The proportion of sphingomyelin in total mitochondrial lipids decreased with body mass in tropical species, but increased in temperate ones ($F_{\text{interaction}}=5.78$, $P<0.03$). Clutch size was significantly related to a number of lipids in stepwise regression. The ratio of phosphatidylserine and sphingomyelin to total mitochondrial lipids had a negative association with clutch size but phosphatidylcholine and phosphatidylethanolamine had positive ones ($R^2=0.24$, $P<0.03$, $R^2=0.35$, $P=0.006$, $R^2=0.28$, $P<0.02$, and $R^2=0.26$, $P<0.03$, respectively).

**Discussion**

I have tested the idea that differences in life history are related to changes in the cellular machinery of birds. I found that cells from temperate species had a greater amount of mitochondrial lipid per million cells and higher amount of cardiolipin per million cells than did tropical species. Additionally, the mitochondria of tropical bird
species had higher amounts of plasmalogens as a proportion of total mitochondrial lipids than temperate species and this proportion was also related to clutch size.

Though cell size was not different between tropical and temperate bird species, the amount of mitochondrial lipid per million cells was higher in temperate species than in tropical ones. If mitochondrial lipid could be used as a proxy for number or size of mitochondria, this could imply that there are more or larger mitochondria in cells of temperate birds compared with cells from tropical species. There is currently no literature to support whether or not temperate birds have more or larger mitochondria than do tropical birds. An interesting next step would be to confirm that cells from tropical birds have fewer mitochondria than do cells from temperate birds, using electron microscopy or immunohistochemistry. One caveat to mention is that my conclusions about the amount of mitochondrial lipid within cells are dependent on the assumption that my methods of mitochondria isolation and lipid extraction from cells yields the same extraction efficiency for cells from both tropical and temperate birds. Whereas I think that this is highly likely, given that I used same cell type, on the same day, and the same methods, I cannot completely rule out the possibility of differential extraction efficiency between cells of different species.

The amount of mitochondrial cardiolipin per million cells was also consistently higher in temperate species than tropical ones. Given that cardiolipin is highly localized to the inner mitochondrial membrane (Paradies et al., 2010), an increase in this lipid could imply that their mitochondria may have more inner mitochondrial membrane than that of mitochondria of tropical birds, which is consistent with a higher rate of
metabolism (Hulbert and Else, 2000). Further, I found that there was more mitochondrial phosphatidylethanolamine per million cells in temperate birds. Given that phosphatidylethanolamine is more abundant in the inner mitochondrial membrane than the outer one (Daum, 1985), this finding provides additional evidence of greater inner mitochondrial membrane in cells from temperate birds. Together, the above suggest that cells from temperate birds likely have either more mitochondria or an increased amount of inner mitochondrial membrane. There could be different implications for metabolism and oxidative stress depending on which is true, but these findings are generally consistent with the idea that cells from tropical birds have a lower rate of basal metabolism than would cells from temperate birds, and this appears to be the case (Jimenez et al., 2014c). However, though an increase in either would likely increase metabolic rate, many other factors are involved, such as the concentration of various enzymes, cell size, and the unsaturation of the membranes that would modulate how and how much metabolic rate would be affected by increased mitochondria or increased inner mitochondrial membrane.

Mitochondria of tropical species had greater amounts of plasmalogens per total mitochondrial lipid than did mitochondria of temperate birds. Additionally, clutch size was negatively related to the amount of plasmalogens. Because clutch size and environment are interrelated it is unclear which is driving this relationship, though it is interesting to note that the two pairs which did not follow the tendency for tropical bird mitochondria to have more plasmalogens than temperate were also pairs that had clutch sizes that were either identical or the difference between them was less than 1. The
greater amounts of plasmalogen in the mitochondria of tropical birds compared with the mitochondria of temperate birds is consistent with the result that there are more plasmalogens in the membranes of long-lived naked mole rats compared with the short-lived laboratory mice (Mitchell et al., 2007) and also with the fact that the longest-living metazoan species, the mud clam *Artica islandica*, has higher amounts of mitochondrial plasmalogen than its shorter-lived relatives (Munro and Blier, 2012). The higher levels of plasmalogen in naked mole rats, *A. islandica*, and tropical bird species may be a molecular mechanism underlying their longer lifespans. Additionally, plasmalogens decrease membrane fluidity (Hermetter et al., 1989) and impede passive ion leak (Hazel and Williams, 1990), so their increased presence in tropical bird mitochondria could partially explain the difference in metabolic rate between tropical and temperate bird species.

In summary, my data offer compelling evidence of a connection between life history and lipids of the mitochondria in tropical and temperate birds, given that most of my results can be related to metabolism or oxidative stress. I have shown that cells of tropical birds have less total mitochondrial lipid and less cardiolipin per cell, indications that these cells have fewer mitochondria or less inner mitochondrial membrane than cells from temperate birds. It seems reasonable to suggest a connection between these findings and a slow pace of life in tropical species. The antioxidant plasmalogens were more abundant in mitochondria of tropical birds and species with smaller clutch size, a finding consistent the idea that species with a slow pace of life invest in lipids that thwart ROS. Whereas looking at the intrinsic differences between tropical and temperate species is an
important first step, one of the next steps will be to look at how environment interacts with these intrinsic differences to get the phenotypes I see in the wild and also to expand this research into other cell types and organelles. Another important next step will be to examine whether there are any differences in the types of fatty acids attached to the phospholipids in tropical and temperate birds given that increases in the saturation of fatty acids attached to phospholipids is also thought to increase oxidative stress and metabolic rate (Hulbert, 2003; Hulbert, 2008).
<table>
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<th>Clutch Size</th>
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</table>

**SOURCES.** – Clutch size data from Jetz et al. (2008) except for <sup>a</sup>Tieleman et al. (2004) and <sup>b</sup>Auer et al. (2007).

Table 2.1. Sample sizes, body masses, and clutch sizes of phylogenetically-matched pairs of temperate and tropical bird species.
Figure 2.1. For each enzyme, the percentage of enzyme activity found in the mitochondrial fraction (black bars) out of the total cellular activity of that enzyme and the percentage of activity found in other fractions (white bars). Succinate dehydrogenase is a mitochondria-specific enzyme, ouabain-sensitive Na+/K+-ATPase is specific for plasma membrane, and NADPH-Cytochrome c Reductase is specific for endoplasmic reticulum.
Figure 2.2. (A) Each individual lipid class as a proportion of the total mitochondrial lipid and (B) individual lipid classes (µg) per million cells from temperate (black bars) and tropical (white bars) birds. Total mitochondrial lipid is the total amount of lipid extracted from mitochondria and measured on TLC plates. Values are mean of all temperate species and the mean of all tropical species ± SEM of all temperate species and the SEM of all tropical species.

* Plasmalogen weight (mg) approximated by converting mol to mg using the molecular weight of 1-O-1’-(Z)-octadecenyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine.
Figure 2.3. (A) Total mitochondrial lipid (mg) per million cells and (B) mitochondrial cardiolipin (µg) per million cells from temperate (black bars) and tropical (white bars) birds. Total mitochondrial lipid is the total amount of lipid extracted from mitochondria and measured on TLC plates. Values are means ± SEM.
Figure 2.4. The ratio of plasmalogens (µmoles) to total mitochondrial lipids (mg) in (A) the mitochondria of temperate (black bars) and tropical (white bars) bird species and (B) its relationship to clutch size. The relationship between clutch size and the ratio of plasmalogens to total lipids in the mitochondria of temperate (black circles) and tropical (white circles) bird species with the regression shown in a dotted line. Mitochondrial lipid is the total amount of lipid extracted from mitochondria and measured on TLC plates. Values are means ± SEM.
Chapter 3: Perspectives on the membrane fatty acid unsaturation/pacemaker hypotheses of metabolism and aging.

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Abstract

The membrane pacemaker hypotheses of metabolism and aging are distinct, but interrelated hypotheses positing that increases in unsaturation of lipids within membranes are correlated with increasing basal metabolic rate and decreasing longevity, respectively. The two hypotheses each have evidence that either supports or contradicts them, but consensus has failed to emerge. In this review, I identify sources of weakness of previous studies supporting and contradicting these hypotheses and suggest different methods and lines of inquiry. The link between fatty acyl composition of membranes and membrane-bound protein activity is a central tenet of the membrane pacemaker hypothesis of metabolism, but the mechanism by which unsaturation would change protein activity is not well defined and, whereas fatty acid desaturases have been put forward by some as the mechanism behind evolutionary differences in fatty acyl composition of phospholipids among organisms, there have been no studies to differentiate whether desaturases have been more affected by natural selection on aging and metabolic rate
than have elongases or acyltransferases. Past analyses have been hampered by potentially incorrect estimates of the peroxidizability of lipids and longevity of study animals, and by the confounding effect of phylogeny. According to some authors, body mass may also be a confounding effect that should be taken into account, though this is not universally accepted. Further research on this subject should focus more on mechanisms and take weaknesses of past studies into account.

**Introduction**

One of the first hints that the fatty acyl composition of phospholipids could be systematically related to other important physiological parameters came with the observation that the amount of docosahexaenoic acid (DHA) attached to phospholipids in the heart was positively correlated with heart rate (Gudbjarnason, 1989). This observation motivated a series of studies that found that polyunsaturated fatty acid (PUFA) concentration in liver and muscle membranes was positively correlated with body mass in mammals and birds (Brand et al., 2003; Couture and Hulbert, 1995; Hulbert et al., 2002b, 2002c). The amount of PUFA in membranes was also found to be positively correlated with the activity of certain membrane-bound proteins, such as Na+/K+-ATPase, and correlations between unsaturation index and proton leak were likely to be mediated through proteins, because the effects of changes in PUFA concentration on proton leak disappear when there are no proteins in a membrane (Brookes et al., 1998, 1997). It was hypothesized that these increases in protein activity may increase metabolic rate as well, thus leading to the membrane pacemaker hypothesis of metabolism (Brookes et al., 1998; Else and Wu, 1999; Hulbert and Else, 1999; Porter et al., 1996). This seminal series of
studies spawned decades of research as it was a striking contrast to previous research on lipid-protein interactions, which studied specific lipid-protein interactions, rather than viewing lipids in a more generalized context (Hulbert and Else, 1999). Shortly thereafter, investigators showed that the level of unsaturation in mitochondrial membranes was negatively correlated with longevity in heart and liver from mammals (Pamplona et al., 2002a, 1999c, 1996; Portero-Otín et al., 2001), which led to the so-called degree of fatty acid unsaturation (Pamplona et al. 2002a) or membrane pacemaker (Hulbert, 2005, 2003) hypotheses of aging. The two membrane pacemaker hypotheses, one connecting phospholipid fatty acyl composition to metabolic rate and the other connecting fatty acyl composition to longevity, are linked because metabolism and longevity are correlated with body mass and with each other (Hulbert et al., 2007). Unfortunately, these intercorrelations make it difficult to test the validity of each hypothesis separately. The correlations among level of unsaturation in membranes, longevity, and metabolic rate could be causally linked to one another or one or more of them could be separately related to body mass and not to each other.

Here, I discuss current evidence for and against the membrane pacemaker hypotheses of metabolism and aging and suggest changes that could be made to the lines of inquiry used to test these hypotheses. The mechanism by which the unsaturation of membranes could be connected to protein activity remains poorly defined in the literature on the membrane pacemaker hypothesis of metabolism. Additionally, it is unclear in species in which natural selection has brought about differences in the unsaturation of membranes and, subsequently, in ageing and metabolic rate, whether natural selection
was operating on desaturase activity, elongase activity, acyltransferase activity, or some combination of these enzymes. Experiments shedding light on these mechanisms would lead to a better understanding of these two hypotheses. Understanding would be further aided by adopting changes in the approaches used to study these hypotheses. For instance, the practice of combining fatty acid data from all phospholipid classes and all subcellular membranes could be obscuring differences that would help create a more nuanced understanding of mechanisms that influence metabolism and longevity, given that the fatty acyl composition of certain phospholipids, such as cardiolipin, appears to have a greater effect on oxidative stress and the activity of membrane proteins in the mitochondria than do other phospholipids (Aoun et al., 2012; Chicco and Sparagna, 2007; Hoch, 1992; Paradies et al., 2010a, 2010b; Watkins et al., 1998). One method that would help solve this issue would be to use high-performance liquid chromatography coupled to mass spectrometry to quantify phospholipid species separately. Other difficulties with interpreting the conclusions of the current literature include potentially inaccurate measures of maximum lifespan and peroxidizability of lipids as well as a lack of consideration of the confounding factor of phylogeny. Body mass may also be a confounding factor, but correcting for body mass should be treated with some caution (Barja, 2014). Resolving mechanisms and being aware of these potential problems in future studies would help in determining validity of these two hypotheses.

**Membrane pacemaker hypothesis of metabolism**

Centered on several tenets, the membrane pacemaker hypothesis of metabolism suggests that a significant portion of basal metabolic rate is dictated by membrane-
associated protein activity, such as the maintenance of chemical gradients in Na+ across the plasma membrane and protons across the inner mitochondrial membrane (Rolfe and Brown, 1997). Furthermore, the proportion of basal metabolic rate that these activities utilize is approximately the same in all organisms (Brand, 2000; Brand et al., 1991).

Thirdly, unsaturated fatty acyl groups attached to phospholipids have physical properties that increase the activity of membrane-bound proteins and thus increase the metabolic rate of the whole organism (Turner et al., 2003). Therefore, species with more PUFA in their membranes will have higher mass-specific metabolic rates than those with a lower concentration of PUFA (Hulbert and Else, 2000; Sprecher, 2000).

Most of the evidence supporting this hypothesis comes from two sources: (i) correlations among the number of double bonds in a membrane, protein activity, and basal metabolic rate and (ii) experiments introducing proteins from one species into the membrane environment of a second species and vice versa. The unsaturation index, or mean number of double bonds in the fatty acyl groups of phospholipids in a membrane, and the percentage of DHA were positively correlated with metabolic rate, proton leak, and Na+/K+ ATPase activity within birds, within mammals, and among several ectotherms in liver, kidney, heart, and skeletal muscle (Brand et al., 2003; Brookes et al., 1998; Hulbert et al., 2002c; Porter et al., 1996; Turner et al., 2005c, 2003). Differences in unsaturation index and DHA concentration in the muscle mitochondria of rats, toads, and lizards were also correlated with differences in their mitochondrial oxidative capacities (Guderley et al., 2005). Furthermore, dietary manipulations in trout and genetic manipulations in mammals that increased the amount of PUFA in membranes increased
mitochondrial activity or increased the activities of individual complexes in the electron
transport chain (Guderley et al., 2008; Hagopian et al., 2010; Jaureguiberry et al., 2014).
Experiments where Na+/K+ ATPase from rats and cattle was put into the less unsaturated
membranes of toads and crocodiles exhibited a decrease in Na+/K+ ATPase activity.
When the reverse experiments were done, Na+/K+ ATPase from toads and crocodiles
exhibited increased activity in the more unsaturated membranes of rats and cattle,
implying that the membrane environment of the Na+/K+ ATPase is at least partially
responsible for changes in its activity levels (Else and Wu, 1999; Wu et al., 2004).

The membrane pacemaker hypothesis of metabolism, however, is not consistent
with some data from intraspecific comparisons, with analysis that address the potentially
confounding effect of body mass, and with comparisons of birds and mammals
(Konarzewski and Książek, 2013). There was no correlation between metabolic rate and
unsaturation index among individuals of the MF1 strain of rats, a strain where longevity
and metabolic rate are positively correlated (Haggerty et al., 2008). Mice selected for
differences in basal metabolic rate or maximal metabolic rate did not exhibit the expected
correlations between basal metabolic rate and unsaturation index or DHA concentration,
though different organs appeared to have different relationships between metabolic rate
and fatty acid composition (Brzęk et al., 2007; Wone et al., 2013). In one of these studies,
the selection for increased metabolic rate led to an increase in relative internal organ size
(Brzek et al., 2007). Given that metabolic rate and relative internal organ size are
positively correlated (Holliday et al., 1967; Wiersma et al., 2012), it seems likely in this
case that selection for increased metabolic rate led to an increase in organ size, rather
than changes in fatty acyl composition. Hamsters fed diets high in different PUFA did not have differences in resting metabolic rate, though differences in tissue unsaturation index were not confirmed and dietary manipulations of PUFA concentration are notorious for not changing unsaturation index without extreme and often unhealthy dietary changes (Hulbert et al., 2014; Pannorfi et al., 2012). When the potentially confounding variables of phylogeny and body mass were taken into account, the correlations between basal metabolic rate and unsaturation index or DHA concentration in skeletal muscle were no longer significant in mammals (Valencak and Ruf, 2007). There were potential problems with the methods used in Valencak and Ruf (2007), including lack of information about diet and about time elapsed between the death of zoo/roadkill animals and the freezing of tissue. Diet and the storage conditions of lipids should be considered in research on these hypotheses because they affect the fatty acid composition of membranes (Hodson et al., 2002; Pamplona et al., 1998; Turner et al., 2004).

Although basal metabolic rate and unsaturation index were correlated within mammals and within birds, when rats and pigeons were compared with each other, rats had a consistently higher unsaturation index in multiple tissues, despite rats having a lower mass-specific metabolic rate (Montgomery et al., 2011; Pamplona et al., 1996). Similarly, canaries and parakeets had a lower unsaturation index than did mice, despite having a higher metabolic rate (Pamplona et al., 1999). Birds usually have higher metabolic rates and longer lifespans than do mammals of the same body mass (Hickey et al., 2012), so the idea that birds may mostly have lower unsaturation indexes than do mammals (Hulbert, 2003) is seen by some as support for the membrane pacemaker
hypothesis of aging being more generally applicable than is the membrane pacemaker hypothesis of metabolism (Pamplona et al., 2002a). Nevertheless, the few birds that do not follow the pattern of having higher metabolic rate and longer lives than similarly-sized mammals, where fatty acyl composition has been measured, have had lower unsaturation indexes. That is, emus, which are similar to sheep in size (both approximately 35,000 g; Turner et al., 2005a) and longevity (both approximately 20 years; Tacutu et al., 2013), had a lower basal metabolic rate than sheep (0.017 and 0.028 kcal g\(^{-1}\) day\(^{-1}\), respectively; Turner et al., 2005a), but still had a lower unsaturation index than did sheep (135 and 206, respectively; Brand et al., 2003; Porter et al., 1996).

Similarly, king quail have similar longevity to mice (both about 5 years; Tacutu et al., 2013) but had a lower basal metabolic rate (1.35 and 1.51 ml O\(_2\) g\(^{-1}\) h\(^{-1}\), respectively; Montgomery et al., 2012; Turner et al., 2005a) and lower peroxidation index (138 and 195, respectively; Montgomery et al., 2012; Porter et al., 1996) than did mice. Thus, the low unsaturation index of birds in comparison with mammals may be a more general characteristic of birds and mammals, rather than specifically being related to metabolism or aging. More measurements of fatty acid composition in membranes of birds that do not follow the pattern of higher metabolic rate and longer lifespan would be required to clarify whether that is the case.

**Membrane pacemaker hypothesis of aging**

The membrane pacemaker hypothesis of aging, also referred to as the homeoviscous longevity adaptation, postulates that PUFA are more easily oxidized than are saturated or monounsaturated fatty acids because probability of lipid peroxidation
increases as a function of the number of double bonds and that this difference in PUFA should be more important in mitochondrial membranes than in other membranes (Holman, 1954; Hulbert, 2008a; Pamplona et al., 2002a). Lipid peroxidation could be especially important in aging because reactive oxygen species are often lipophilic and tend to partition themselves into the middle of phospholipid bilayers rather than aqueous environments, making lipids a common target of oxidative damage (Gamliel et al., 2008; Möller et al., 2005; Naudí et al., 2013). In addition, lipid peroxidation products are also particularly harmful in that they are stable enough to attack macromolecules far from the site where the lipid was oxidized (Pamplona, 2008). Peroxidized lipids often oxidize other macromolecules, most notably mitochondrial DNA, and accumulation of oxidative damage to mitochondrial DNA can lead to progressive decline in the function of electron transport chain complexes and, subsequently, decline in cellular and tissue function (Paradies et al. 2010b). Further supporting the idea that the peroxidation of lipids leads to aging, replacement of oxidatively damaged lipids with undamaged lipids can restore mitochondrial function and reduce fatigue, a symptom of several age-related conditions (Nicolson and Ash, 2014).

In most naturally-occurring fatty acids, if there are two or more double bonds, the double bonds are separated by a single carbon with two hydrogens attached, called bis-allylic hydrogens. Bis-allylic hydrogens are prone to oxidative attack, more so than any other hydrogens on the fatty acid, such that DHA, which has ten bis-allylic hydrogens, is by some estimates 320 times more susceptible to lipid peroxidation than is oleic acid, which has one double bond and no bis-allylic hydrogens (Hulbert, 2005). Some authors
have attempted to estimate the susceptibility of entire membranes using a peroxidation index (Pamplona et al., 1998), which is based on a calculation that includes multiplying the percentages of each fatty acid by their peroxidizability, as estimated by Holman (1954). If oxidative stress leads to aging, as proposed by the oxidative stress theory of aging, then this information suggests that increases in the number of double bonds in an organism’s membranes should result in an increase in oxidative stress and a decrease in longevity (Hulbert, 2008a; Naudí et al., 2013).

The membrane pacemaker hypothesis of aging has gained more support than has the membrane pacemaker hypothesis of metabolism, though this may be in part because more groups are studying the connection between levels of unsaturation in membranes and longevity (Hulbert, 2010; Pamplona, 2008). Similar to the membrane pacemaker hypothesis of metabolism, the membrane hypothesis of aging has been tested across species within specific vertebrate classes. Within mammals, maximum lifespan is inversely correlated with peroxidation index and DHA content in membranes from heart, liver mitochondria, kidney, and skeletal muscle (Hulbert et al., 2002c; Pamplona et al., 1999c; Portero-Otín et al., 2001). Within birds, the same is true in membranes from liver mitochondria and skeletal muscle (Hulbert, 2005). Similarly, in bivalves, peroxidation index and DHA content of mitochondrial membranes decreased with increasing longevity (Munro and Blier, 2012). There have also been multiple comparisons of the peroxidation index of long and short-lived species: pigeons, naked mole rats, and humans have a lower peroxidation index and DHA content in the mitochondrial membranes of their livers and hearts than do rats, which have a shorter lifespan (Hulbert, 2006; Mitchell et al., 2007;
Pamplona et al., 1999a, 1996). Short-beaked echidnas had a lower peroxidation index and DHA content in membranes of their liver mitochondria and skeletal muscle than would be expected for their body size, but their peroxidation index was close to what would be expected based on their maximum longevity (Hulbert et al., 2008). Species of birds within the order of Procellariiformes, which tend to have long lives, have consistently lower peroxidation indexes in membranes of their heart mitochondria than do species from Galliformes, an order of birds with short lifespans (Buttemer et al., 2008). Multiple long-lived strains of mice had a lower peroxidation index and DHA content in membranes from their liver and skeletal muscle tissue than did control strains of mice (Hulbert et al., 2006a; Valencak and Ruf, 2013). Peroxidation index was inversely correlated with lifespan in six strains of Caenorhabditis elegans (Shmookler Reis et al., 2011). Queen bees, which live an order of magnitude longer than workers, had a lower peroxidation index than did worker bees (Haddad et al., 2007). Finally, in many cases, higher peroxidation index and shorter lifespan were predictive of higher oxidative damage to lipids, DNA, and proteins (Aguilar-Toral et al., 2014; Gómez et al., 2014; Jeon et al., 2001; Pamplona et al., 1999a, 1999c, 1996).

There is also evidence against the membrane pacemaker hypothesis of aging. Long-lived parrot species did not have lower peroxidation indexes in membranes from heart, skeletal muscle, or liver mitochondria than did short-lived quail species (Montgomery et al., 2012). Fatty acid profile and longevity did not correlate with each other in three lines of Drosophila melanogaster that were selected for increased longevity (Moghadam et al., 2013). Cold-acclimation in killifish and bluegill changed the
unsaturation index of muscle mitochondria, but did not produce changes in the susceptibility of lipids to peroxidation, as measured using C11-BODIPY (Grim et al., 2010). Rats fed different diets had differences in the peroxidation index and DHA content in heart and liver tissues, but not in median lifespan (Valencak and Ruf, 2011). Correcting for phylogeny and body mass eliminated the significance of correlations between peroxidation index and longevity in mammals, though the study that found this result (Valencak and Ruf, 2007) did have several methodological problems as noted in other sections of this review. Finally, treatment with atenolol, a β1-adrenergic receptor blocker, decreased unsaturation index in mice, but did not lead to a significant difference in longevity (Gómez et al., 2014). Atenolol treatment did, however, decrease oxidative damage and improved age-related behavioral functions, such as motor coordination, so the lack of longevity increase was likely related to decreases in heart rate and increases in arterial pressure variation, similar to results from an analogous study (Spindler et al., 2013; Gómez et al., 2014). In addition to the problems listed above with specific studies, some studies may not have found correlations between peroxidation index and longevity because of individuals that were not of equivalent ages, as fatty acyl composition changes with age (Hulbert, 2005). Additionally, measurement of reactive oxygen species and oxidative damage should be treated with a degree of caution as there is no single biomarker of oxidative damage and many of the methods used to measure reactive oxygen species and oxidative damage are easily misinterpreted (Halliwell and Whiteman, 2004).

Mechanisms
Mechanism connecting the unsaturation of membranes, protein activity, and metabolic rate

Whereas increases in the activity of specific membrane-bound proteins correlate with increasing unsaturation index, the underlying mechanisms of this phenomenon remain unclear (Else and Hulbert, 2003; Else and Wu, 1999; Else et al., 2003; Hulbert et al., 2006b; Hulbert, 2008b; Turner et al., 2006a, 2005c, 2003; Wu et al., 2004). Although it could be assumed that differences in protein activity were a result of differences in membrane fluidity caused by the greater number of double bonds in fatty acids, overall membrane fluidity is only strongly increased by the addition of the first two double bonds to a saturated acyl chain; each subsequent double bond has less effect on membrane fluidity (Brenner, 1984; Pamplona et al., 2002a). Thus, the pronounced influence that DHA seems to have on protein activity would make little sense if membrane fluidity was driving differences in protein activity. The mechanism that is often invoked in research on the membrane pacemaker hypothesis of metabolism accounting for how differences in fatty acyl composition affect protein activity is that there is some form of energy transfer between the polyunsaturated fatty acyl groups and the membrane-bound proteins because of collisions between the flexible PUFA and the membrane-bound proteins (Else and Hulbert, 2003; Else et al., 2003; Hulbert and Else, 1999; Hulbert, 2008b; Wu et al., 2001). Evidence for this hypothesis comes from a study which found that the activity of Na+/K+-ATPase was correlated with lateral pressure of membrane phospholipids, a property correlated with the unsaturation of the membrane and also presumed to be related to the number of collisions between molecules in the membrane (Wu et al., 2001).
Whereas this mechanism may make sense intuitively, it also relies somewhat on an assumption of a homogeneous membrane environment, where proteins are equally likely to collide with any lipid molecule in the membrane. It has, however, become increasingly clear that membranes are not homogeneous environments and that they are composed of a variety of lipid/protein domains, including lipid rafts (Quinn, 2010; Wassall and Stillwell, 2009). Lipid rafts have specific lipid compositions, mainly being composed of cholesterol, sphingolipids, and phospholipids with saturated fatty acyl chains, and they have some resident proteins and some proteins which move between the rafts and non-raft areas of the membrane, often with effects on the function or activity of the protein (Lucero and Robbins, 2004). One of the proteins that is sometimes associated with lipid rafts and sometimes with non-raft areas of the membrane is Na+/K+-ATPase (Krivoi, 2014; Lingwood et al., 2005; Welker et al., 2007), so Na+/K+-ATPase may not be continuously exposed to PUFAs in the membrane, despite the fact that most of the evidence linking increasing protein activity with increasing PUFA concentration comes from Na+/K+-ATPase (Else and Hulbert, 2003; Else and Wu, 1999; Else et al., 2003; Hulbert, 2008b; Turner et al., 2006a, 2005b, 2003; Wu et al., 2004).

The addition of phospholipids with highly polyunsaturated fatty acyl chains, such as DHA, to membranes increases the formation and stability of lipid raft domains, suggesting a possible alternative mechanism to explain the correlation between the unsaturation of membranes and protein activity (Shaikh et al., 2015; Wassall and Stillwell, 2009; Wassall et al., 2004). DHA and cholesterol are sterically incompatible, so the inclusion of phospholipids containing DHA into less unsaturated membranes drives
cholesterol into lipid rafts, making the rafts larger and more stable, while simultaneously driving phospholipids with DHA into more disordered domains (Shaikh et al., 2015; Wassall and Stillwell, 2009; Wassall et al., 2004). Other highly polyunsaturated fatty acyl chains, such as those with 5 double bonds, have a similar, but less marked effect (Shaikh et al., 2015). This differentiation into ordered and disordered domains could allow for better localization of raft proteins within the larger, more stable raft domains, as well as allowing proteins that require more disordered environments to be recruited into the regions enriched with DHA (Figure 3.1, Wassall and Stillwell, 2009). If the function of some proteins is improved by being in a raft and the function of other proteins is improved by being in a disordered and PUFA-rich environment, then an increase in the segregation into raft and non-raft domains could increase the activity of multiple types of proteins. Incorporation of Na+/K+-ATPase into lipid raft domains appears to increase its activity in a variety of contexts (Fujii et al., 2008; Lingwood et al., 2005; Welker et al., 2007). If increasing PUFA concentration is found to increase the incorporation of Na+/K+-ATPase into lipid rafts, it would provide evidence towards PUFA-mediated lipid domain formation being the mechanism connecting fatty acyl composition and protein activity. It would also provide evidence against the current accepted mechanism, as the Na+/K+-ATPase would not be exposed to PUFAs in lipid rafts. This idea has not been tested.

A related mechanism that could account for the connection between unsaturation index and metabolic rate is that increases in the unsaturation of membranes affect membrane structure and phase behavior which, in turn, affect the activity of proteins.
Increases in the unsaturation of membranes change the phase behavior of membranes, increasing the propensity of membranes to form hexagonal (H$_{II}$) phases (Epand et al., 1991; Furse et al., 2015; Yang et al., 2005). Increases in H$_{II}$ phase propensity increase the activity of multiple classes of signaling proteins due to increasing recruitment of these signaling proteins into membranes (Escribá et al., 1995; Martinez et al., 2005; Vögler et al., 2004; Yang et al., 2005). Increases in the activity of these signaling proteins, G protein-coupled receptors and protein kinase C, have far-reaching effects on transcription and cell death or survival (Escribá et al., 1995; Martinez et al., 2005; Vögler et al., 2004; Yang et al., 2005) that could, in and of itself, drive differences in metabolic rate. If other metabolically important proteins, such as Na$^+$/K$^+$-ATPase, also increase activity due to changes in lipid phase behavior, then this would further strengthen this as a potential mechanism explaining the connection between unsaturation of membranes and protein activity. Given that lateral surface pressure, which is related to lipid phase behavior (Seddon, 1990), is proportional to Na$^+$/K$^+$-ATPase activity (Wu et al., 2001), this could be the case. One potential caveat to this mechanism is that the relationship between unsaturation of fatty acids and H$_{II}$ phase propensity is not linear and increasing amounts of DHA produce less of an increase in H$_{II}$ phase propensity than would be expected for DHA’s level of unsaturation, whereas oleic acid has a disproportionally large effect (Epand et al., 1991). If oleic acid, and not DHA, is shown to have a disproportionately large effect on basal metabolic rate as well, then this mechanism would be supported.

*Enzymes mediating phospholipid acyl composition*
Several groups of enzymes are involved in changing phospholipid fatty acyl composition. Desaturases and elongases introduce new double bonds into fatty acids and elongate fatty acids, respectively (Guillou et al., 2010; Miyazaki and Ntambi, 2008). Once a specific type of fatty acid is produced or has been ingested by an organism, it can be primed for addition to a phospholipid using an acyl-CoA synthetase and then added to a phospholipid using an acyltransferase or it can be directly transferred between two phospholipids using a transacylase (Yamashita et al., 2014).

There is little agreement as to which genes and enzymes have been selected upon in the evolution of differences in the unsaturation of membranes among species. Discovering the mechanisms behind differences among organisms, brought about by natural selection, in the aging process is important because the differences in longevity that have been produced in the laboratory with mutation are orders of magnitude lower than the differences in longevity that have been produced by natural selection. Furthermore, many of the candidate genes identified in mutation experiments have been shown to be unlikely to have had a role in the evolution of mammal longevity (Hulbert et al., 2014; Jobson et al., 2010). Desaturases appear to be rate-limiting enzymes in the synthesis of most polyunsaturated fatty acids and they are also 10 times less active in rats than are acyltransferases, implying that desaturases could be more rate-limiting than acyltransferases as well (Ivanetich et al., 1996; Pamplona et al., 2002a, 1996). Some authors have argued that differences in these rate-limiting enzymes, especially in $\Delta_5$ and $\Delta_6$ desaturases, which are involved in forming double bonds in PUFA, are the primary factor responsible for evolutionary differences in the unsaturation of membranes.
(Pamplona and Costantini, 2011; Pamplona et al., 2002a, 1998, 1996). Presumably, this would be the case because smaller changes in the amount of rate-limiting enzymes would be required to produce changes in fatty acid composition than changes in the amounts of enzymes that are not rate-limiting.

Nevertheless, there may be a separate control point for the formation of one of the most unsaturated fatty acids, DHA, and thus for some of the largest effects of the unsaturation of membranes on metabolism and aging (Gregory et al., 2011). This 22-carbon fatty acid is produced when a 20-carbon fatty acid goes through two rounds of elongation to become 24 carbons long, followed by desaturation and partial β-oxidation (Guillou et al., 2010; Sprecher, 2000). The extra elongation and β-oxidation steps are required because Δ4 desaturases, which are the type of desaturase that would be needed to produce these fatty acids in fewer steps, are not present in the majority of vertebrate species (Li et al., 2010). In the pathway producing phospholipids with DHA chains, elongase-2 appears to be a control point for the synthesis, making it likely that, at least in the case of DHA, more than just desaturase activity would need to be changed to affect fatty acid composition of phospholipids (Gregory and Geier, 2013; Gregory et al., 2011).

Little has been done to systematically test whether there are consistent differences in desaturases, elongases, or acyltransferases among long-lived and short-lived organisms or among organisms with higher and lower metabolic rates. Some authors have sought to estimate the activity of desaturases in organisms by dividing the amounts fatty acid products of desaturation/elongation reactions by their substrates. Often desaturation and elongation are not separated from each other, as seen in dividing the amount of dihomo-
gamma-linoleic acid (20:3) by the amount of linoleic acid (18:2) to estimate the amount of Δ6 desaturase, such that it is difficult to determine if desaturase activity or elongase activity is really being estimated (Jeon et al., 2001; Pamplona et al., 1996; Porter et al., 1996; Turner et al., 2005a, 2004). Additionally, these estimates do not take into account fatty acids in the diet of the organism or the activities of specific acyltransferases or transacylases, many of which have preferences for different fatty acids (Yamashita et al., 2014).

The fatty acid unsaturation or membrane pacemaker hypotheses of aging and metabolism would be significantly strengthened by a better understanding of the mechanism behind evolutionary differences in the fatty acyl composition of phospholipids in membranes. A clarifying first step could be comparing the transcriptomes or proteomes of organisms of varying metabolic rates and varying longevities to see whether certain desaturases, elongases, or acyltransferases are upregulated or have different isoforms in organisms with short lives or high metabolic rates. In a genomic scan of mammals with a wide range of longevities, several genes involving fatty acid synthesis were identified as being associated with differences in longevity (Jobson, 2010). These genes included an elongase, elongase-5, and a desaturase, Δ9 desaturase (Jobson, 2010). Creating mutants, especially mutant cell lines, that modify the amount of each of these enzymes and then quantifying the effects of these mutations on fatty acid composition, metabolic rate, and oxidative stress would also be an effective test of the membrane pacemaker hypotheses. For instance, it would be interesting to see if modifying the expression of one or two genes could cause cells from
a mouse to have a lower metabolic rate, closer to that of human cells, and *vice versa.* With newer genome modification tools, such as the CRISPR-Cas system, these modifications could be done easily and cheaply (Sander and Joung, 2014). Interestingly, a cell line altered to overexpress Δ5 and Δ6 desaturases was found to have increased mitochondrial activity and RNAi knockdown of Δ5 desaturase, elongase-1, and elongase-2 in *Caenorhabditis elegans* increased lifespan and oxidative stress resistance (Hulbert, 2011; Jaureguiiberry et al., 2014; Shmookler Reis et al., 2011). These studies need to be expanded upon in ways that inform understanding of the membrane pacemaker hypotheses of metabolism and aging. For instance, if changes in some enzymes produce differences in metabolic rate, whereas changes in other enzymes produce changes in longevity, this could change the way the membrane pacemaker hypotheses are understood and help explain why longevity and metabolic rate are not always strongly coupled. This research could also be done at the level of transcription factors affecting the expression of these enzymes, affecting several enzymes at once (Miyazaki and Ntambi, 2008).

**Other potential weaknesses**

*Phospholipid class differences*

The fatty acyl composition of different phospholipid classes is regulated separately and could have different effects on metabolic rate and oxidative stress, yet the origin of fatty acyl moieties from different phospholipid classes is rarely specified and often completely dismissed from experimental designs. Phospholipid classes start out with different fatty acyl compositions in their *de novo* synthesis pathways; for example,
phosphatidylserine is initially synthesized with more PUFA than is phosphatidylcholine (Schenkel and Bakovic, 2014). These differences in initial PUFA composition can be further amplified by acyltransferases and transacylases that affect only some phospholipid classes and have preferences for different fatty acids (Yamashita et al., 2014). Thus, differences in unsaturation index could be driven mostly by differences in only one or two phospholipid classes. Delineating the fatty acyl composition of different phospholipid classes could be important in determining the mechanisms underlying evolutionary differences in the unsaturation of membranes, given that differences in the expression of some enzymes would have effects on specific phospholipid classes whereas other enzymes have more global effects.

Furthermore, these differences could have consequences for metabolic rate and oxidative stress. For instance, phosphatidylethanolamine and cardiolipin tend to be closely associated with the protein complexes of the electron transport chain, so they are often close to the site of the formation of reactive oxygen species, making them potentially greater targets of lipid peroxidation than are other phospholipids (Böttinger et al., 2012; Chicco and Sparagna, 2007). There is also evidence to suggest that phosphatidylethanolamine is more susceptible to oxidation than is phosphatidylcholine, even given the same fatty acyl composition, though that may be dependent on the oxidative agent (Kawakatsu et al., 1984; Wang and Shibata, 1995; Wang et al., 1994). Lastly, which phospholipid head group a fatty acyl group is attached to can affect important physical properties of membranes such as the partitioning of phospholipid species in the inner and outer leaflet of a membrane and the partitioning of phospholipid

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species within lipid rafts (Kubo et al., 2005; Shaikh et al., 2015). Phosphatidylcholine species with DHA as an acyl group displace cholesterol from lipid rafts, the opposite of the effect of phosphatidylethanolamine species with DHA in them, which tend to be more common phospholipid species (Shaikh et al., 2015).

Phospholipid head groups impact the susceptibility of their fatty acyl moiety to change by experimental methods, such as diet or genetic manipulation. For instance, dietary restriction in rats had an effect on the fatty acyl composition of phosphatidylethanolamine and cardiolipin in heart mitochondria, but not on the fatty acyl composition of phosphatidylcholine (Merry, 2000). Alternatively, diets with different lipid sources had greater impact on the fatty acyl composition of phosphatidylcholine than on phosphatidylethanolamine or cardiolipin in the muscle mitochondria of trout (Martin et al., 2015). Fat-1 transgenic mice, which express a desaturase allowing the synthesis of n-3 fatty acids from n-6 fatty acids, had significant increases in the DHA content of phosphatidylethanolamine and phosphatidylcholine, but not in cardiolipin or phosphatidylserine, in comparison with control mice (Hagopian et al., 2010). It is reasonable to assume that, had the acyl composition of different phospholipid classes been separated out in more studies, other differences in the way phospholipid class acyl composition react to experimental treatments would have been found, potentially explaining why differences in PUFA composition of phospholipids do not always correlate with changes in metabolic rate and longevity.

Because examining fatty acyl composition in association to phospholipid classes could bring greater biochemical insights, usage of mass spectrometry is a powerful tool to
study phospholipid composition (Yamashita et al., 2014). This method allows for the precise analysis of individual phospholipid species with smaller samples, making it more simple to analyze the fatty acyl composition of phospholipid classes separately than past methods (Yamashita et al., 2014). It improves the analysis of phospholipid species by eliminating the need for the hydrolysis of phospholipids and subsequent derivatization of fatty acids, thereby limiting sample handling and the potential for incomplete hydrolysis or derivatization (Blank and Robinson, 1984; Houjou et al., 2005; Patton et al., 1982). In addition, mass spectrometry is a flexible method that allows non-targeted analyses. For instance, the peroxidation index of free fatty acids, not just acyl chains, in plasma was recently found to be correlated with longevity (Jové et al., 2013); this finding would not have been possible using the techniques that have been traditionally used to study the membrane pacemaker hypotheses. Some authors have started to use mass spectrometry to analyze phospholipid species (Mitchell et al., 2004, 2007), but multiple recent studies still have used gas chromatography to analyze fatty acyl composition (Aguilar-Toral et al., 2014; Almada-Pagán et al., 2014; Gómez et al., 2014). By employing mass spectrometers, we could have a more complete understanding of patterns of fatty acyl composition in phospholipids and of the mechanisms behind the membrane pacemaker hypotheses of metabolism and aging.

*Subcellular membranes*

The PUFA content in membranes of different organelles could have different effects on metabolic rate and aging and is also differentially regulated. This has been recognized in the literature on the membrane pacemaker hypothesis of aging, as many
studies have analyzed mitochondrial fatty acyl composition, but few authors of studies on the membrane pacemaker hypothesis of metabolism have separated out any of the subcellular membranes in their analyses. The particular importance of PUFA in mitochondrial membranes to the membrane pacemaker hypothesis of aging is because mitochondria are the source and target of most reactive oxygen species (Gruber et al., 2008; Pamplona et al., 1999a). In the one study that separated mitochondria from other membranes and then used those other membranes, rather than whole tissue, to connect peroxidation index to longevity, the peroxidation index of mitochondrial membranes and the peroxidation index of non-mitochondrial membranes were both correlated with longevity in bivalves, though the correlation between mitochondrial peroxidation index and longevity was stronger (Munro and Blier, 2012).

The predictions of the membrane pacemaker hypothesis of metabolism are less specific to one organelle than are the predictions of the membrane pacemaker hypothesis of aging. The maintenance of chemical gradients across the plasma membrane and the inner mitochondrial membrane are together responsible for about half of the energy consumed at rest (Hulbert, 2007). This could lead to the prediction that unsaturation index would be higher in the plasma membrane and also in the mitochondria in organisms with higher metabolic rate than in those with lower metabolic rates. The fatty acid composition of mitochondrial and non-mitochondrial membranes have not been compared in terms of their effects on basal metabolic rates, however. Non-mitochondrial PUFA composition was related to Na+/K+ ATPase activity of birds and mammals in a variety of organs, but there has been no comparison of how PUFA concentration in
different sub-cellular membranes affects basal metabolic rate (Turner et al., 2006a, 2005a, 2005b).

The differences in predictions of which subcellular membranes have an effect on aging versus metabolic rate could be especially important in cases where longevity and metabolic rate of organisms are positively correlated, rather than the more common negative correlation between longevity and metabolic rate. For instance, in MF1 rats, which have a positive correlation between longevity and metabolic rate, whole tissue peroxidation index did not correlate with longevity (Haggerty et al., 2008). This lack of correlation could be due to increases in PUFA in the plasma membrane which were compensated for by decreases in PUFA in the mitochondrial membranes. If subcellular membranes had been separated out in (Haggerty et al., 2008) and if there were compensation between increases in PUFA in one membrane and decreases in PUFA in a different organelle, then this study would have been evidence for both of the hypotheses, rather than evidence against one of them.

There are well-established differences in the regulation of fatty acyl composition of phospholipids in different subcellular membranes, in particular between mitochondria and other membranes in the cell (Schenkel and Bakovic, 2014). For instance, phosphatidylethanolamine starts out with more double bonds in the mitochondria because it is mostly synthesized from phosphatidylserine within the mitochondria and from phosphatidylcholine when it is synthesized outside of the mitochondria (Schenkel and Bakovic, 2014). From there, there are isoforms of acyltransferases that are found only in the mitochondria, so different fatty acyl compositions can be established within the
mitochondria even in phospholipids such as phosphatidylcholine, which is synthesized in the endoplasmic reticulum and transported to the mitochondria (Schenkel and Bakovic, 2014; Yamashita et al., 2014). Therefore, there are not only reasons that differences in fatty acid composition among different subcellular membranes should interest researchers investigating the membrane pacemaker hypotheses, but there are also mechanisms that separately regulate different subcellular membranes.

*Potentially inaccurate estimates: maximum lifespan and peroxidation index*

Maximum lifespan can be problematic to use as a variable in comparative research for a variety of reasons. The largest problem with the use of maximum lifespan as a correlate for the speed of aging is that records of maximum lifespan are dependent upon sample size (Carey, 2003; Speakman, 2005). For instance, the maximum lifespan for humans of 120 years comes from hundreds of thousands of records; only maximum lifespans of domesticated animals have nearly as large sample sizes (Speakman, 2005). For many wild animals and organisms that do poorly in captivity, estimates of maximal lifespan are inaccurate and can be biased by multiple factors, such as the capture methods used to study these organisms or the husbandry techniques used in rearing captive animals (Baylis et al., 2014). Therefore, studies using maximal longevity as a correlate for the speed of aging should be limited to organisms that have birth and death records with large sample sizes and where husbandry techniques are well-established. This limits the potential for research on the evolution of lifespan considerably when using maximum lifespan as a variable. Furthermore, differences in measurements of maximum longevity can be large, even in organisms where the maximum longevity is thought to be
reasonably well-established. For instance, when maximum longevities in a relatively recent paper (Valencak and Ruf, 2007) are compared with maximum longevities of the same species on AnAge, an extensive online database of maximum lifespan data (Tacutu et al., 2013), they differ by a mean of 21%, with one of the pairs differing by 67% (Figure 3.2). There is no widely accepted variable to replace maximum lifespan, despite the acknowledgment by multiple authors that maximum lifespan is a problematic variable (Baylis et al., 2014; Munro and Blier, 2012; Speakman, 2005). Potential alternatives include using “shared maximum longevity,” a variable composed of the highest longevity achieved in at least two populations of a species, or using death curve models to estimate maximum longevity (Baylis et al., 2014; Munro and Blier, 2012).

The use of peroxidation index, or peroxidizability index, as a variable should also be treated with caution. The peroxidation index has been used by many authors to estimate the susceptibility of a membrane to peroxidation. It is based on multiplying the percentage of fatty acids with a particular number of double bonds by a number proportional to the peroxidation rate of a fatty acid with that number of double bonds (Almaida-Pagán et al., 2014; Hulbert et al., 2007; Montgomery et al., 2012; Pamplona et al., 2005, 2002b, 1998; Valencak and Ruf, 2007):

\[
(0.025x\sum\text{mol}\% \text{monoenoic}) + (\sum\text{mol}\% \text{dienoic}) + (2x\sum\text{mol}\% \text{trienoic}) + \\
(4x\sum\text{mol}\% \text{tetranoic}) + (6x\sum\text{mol}\% \text{pentaenoic}) + (8x\sum\text{mol}\% \text{hexaenoic})
\]

The first use of this equation appears to be in a fifty-year-old paper and the numbers used in it are based on a study done ten years before that (Holman, 1954; Witting and Horwitt, 1964). Unfortunately, newer estimates of the peroxidizability of
fatty acids estimate differences in peroxidizability of fatty acids with different numbers of
double bonds to be lower than originally estimated (Bielski et al., 1983; Cosgrove et al.,
1987; Xu et al., 2009). These estimates put the proportional peroxidizability of fatty acids
with 2, 3, 4, and 6 double bonds at about 1, 2, 3.4, and 5.4, rather than the 1, 2, 4, and 8
originally estimated (Cosgrove et al., 1987; Xu et al., 2009). Even this estimate may be
higher than the actual relative peroxidizabilities, depending on the oxidizing agent used
(Bielski et al., 1983). Moreover, the relative peroxidizabilities of free fatty acids may not
always be relevant to how they act as acyl groups when attached to larger molecules
(Cosgrove et al., 1987; Kubo et al., 2005; Norris et al., 2012; Wijesundera et al., 2008;
Zerouga et al., 1995). For instance, phospholipids containing two DHA groups were
peroxidized at the similar rates or sometimes a lower rate than phospholipids containing a
DHA and a palmitic acyl group when put into liposomes composed of phospholipids with
saturated fatty acyl groups, contrary to what would be predicted based on the
peroxidizability indexes (Norris et al., 2012). Additionally, phosphatidylethanolamine
with DHA appears to have protective effects against peroxidative damage when it is
positioned in the outer leaflet of liposomes (Kubo et al., 2005). If the peroxidation index
is potentially neither a good estimate of the actual peroxidizability of free fatty acids, nor
of the peroxidizability of fatty acyl groups on phospholipids, it is unclear why it is often
correlated with maximum lifespan, as well as actual measures of oxidative damage
(Aguilar-Toral et al., 2014; Hulbert et al., 2014; Jeon et al., 2001; Naudi et al., 2013;
Pamplona et al., 2004, 2000, 1999a, 1998, 1996). It could be that the peroxidation index
is a close enough estimate of peroxidizability to be picked up as statistically significant

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or, perhaps, it is indirectly related to oxidative stress and longevity in some currently unknown way. Given the number of studies that have correlated longevity and oxidative damage to peroxidation index (Hulbert et al. 2014; Naudi et al., 2013; Pamplona et al. 2004, 2000, 1999a, 1999c, 1996), peroxidation cannot be completely discounted, but it should be used with more caution and should not be used at the exclusion of double bond index, as it has been recently (Aguilar-Toral et al., 2014; Hulbert et al. 2014; Montgomery et al., 2012; Naudi et al., 2013).

Complications of phylogeny and body mass

The potentially confounding effects of phylogeny and body mass have mostly been ignored throughout the study of the membrane pacemaker hypotheses of aging and metabolism. Phylogenetic relationships should be taken into account when comparing characteristics of species because closely related species should not be considered completely independent data points, violating the assumption of independence of data of many statistical tests (Speakman, 2005). For instance, comparing three species of parrot to two species of quail, as was done in (Montgomery et al., 2012), should be treated statistically more similarly to one species of parrot and one species of quail, which is a weak comparison for drawing solid conclusions (Garland and Adolph, 1994). Other studies have failed to take phylogeny into account (Brand et al., 2003; Brookes et al., 1998; Buttemer et al., 2008; Hulbert et al., 2002b; Pamplona et al., 2005, 1999a, 1996). Body mass is correlated with longevity and metabolic rate, making all of the intercorrelations hard to tease apart from each other and from other traits associated with body mass. In order to resolve the issue of these intercorrelations, some authors suggest
correcting for the body mass of organisms (Speakman, 2005), but this practice is controversial, as it may sometimes lead to a lack of attention to causal relationships between variables that are separately correlated to body mass (Barja, 2014; Harper et al., 2007). Correcting for phylogenetic relationships allows us to be more certain that statistics are being applied in a correct manner and correcting for body mass allows us to determine whether there are correlations between two traits when body mass is taken out of the equation (Speakman, 2005).

In some studies that have paid attention to phylogeny or body mass, the predictions of the membrane pacemaker hypotheses have held up, whereas in other cases they have not. Marine bivalves that have similar body masses and similar relatedness to each other have correlations between the peroxidation index of their membranes and their longevities (Munro and Blier, 2012). Correlations between unsaturation index and Na+/K+ ATPase activity in the hearts of birds and mammals continue to be significant after phylogeny is taken into account (Turner et al., 2006a). Conversely, correlations between peroxidation index of skeletal muscle and longevity in mammals disappeared when body mass and phylogeny were taken into account, as did correlations between the unsaturation index and metabolic rate (Valencak and Ruf, 2007). If the body masses from that study are regressed on metabolic rate, the R-squared value is 0.96, so it is not entirely surprising that no correlations were found between unsaturation index and metabolic rate after that variation was removed. The unsaturation index could still explain the fraction of basal metabolic rate (96%) that is correlated with body mass, a fact that the authors make note of, but mostly dismiss as unimportant (Valencak and Ruf, 2007). To get around
issues of phylogeny and body mass, it would be wise to look for closely related species and species of similar body mass that vary widely in longevity or metabolic rate, so that accounting for phylogeny and body mass do not create similar problems in the future. Unfortunately, for a variety of reasons, including lack of reliable maximum lifespan estimates for many species, these types of comparative studies are rare.

*Using one organ to estimate whole organism effects*

Another consistent problem, especially in the evidence against the membrane pacemaker hypotheses, has been the assumption that analysis of differences, or lack of differences, in the fatty acyl composition of one organ is enough to resolve the validity, or lack of validity, of the membrane pacemaker hypotheses. There are several issues with this assumption, not the least of which is that it has been acknowledged from the beginning that not all organs have predictable correlations between fatty acyl composition and metabolism or aging, the brain being an example of an organ that has similar fatty acyl composition across a range of mammalian species (Couture and Hulbert, 1995; Hulbert et al., 2002c). Even in organs which have been established as having correlations among fatty acyl composition, metabolism, and longevity, there are variations in the strength of these correlations (Couture and Hulbert, 1995; Montgomery et al., 2011). Experimental manipulations also seem to have different degrees of effects on the fatty acyl composition of membranes that are dependent on the organ examined (Brzek et al., 2007; Stroud et al., 2009). For example, mice which were genetically manipulated to be unable to express Δ6 desaturase had significantly smaller decreases in the amount of DHA and other PUFA in their brain and testes tissues than in liver tissue,
when compared with control mice (Stroud et al., 2009). Furthermore, some organs contribute more to basal metabolic rate and aging than do others. For instance, energy use in heart, brain, liver, and kidneys make up the majority of basal metabolic rate (Krebs, 1950; McKechnie and Swanson, 2010). Other organs, such as the brain are particularly prone to damage from oxidative stress (Ohtsuki et al., 1995). Thus, a lack of correlation in one organ between the unsaturation index and metabolic rate, as in (Haggerty et al., 2008; Valencak and Ruf, 2007), is not strong evidence against the membrane pacemaker hypothesis of metabolism because there could be correlations between unsaturation index and metabolic rate in other organs.

Methods that would get partially around the problem of studying one organ and making generalizations to the entire organism include studying oxidative stress or respiration rate in mitochondria or whole tissue of the same organ that fatty acyl composition is measured in. Some studies have done this (Brand et al., 1991; Hulbert et al., 2002a; Pamplona et al., 1999a, 1999c, 1996), but the methods used in these studies have their own problems, including a lack of reliability in measures of lipid peroxidation used and disruption of mitochondrial structure in isolated mitochondria (Halliwell and Whiteman, 2004; Perry et al., 2013; Zhang et al., 2012).

Use of cultured cells, such as fibroblasts, could circumvent some of these and other problems with past methodologies. The use of primary cell cultures has multiple advantages including the ability to culture cells from relatively harmless biopsies, elimination of the effects of diet by culturing in a common environment, and the ability to measure several physiological parameters in the same primary cell lines, such as
metabolic rate of whole cells, phospholipid composition, and in situ measures of lipid peroxidizability (Calhoon et al., 2014, 2013; Jimenez et al., 2014b, 2014c). Furthermore, genetic manipulations, such as CRISPR-Cas, could be done in cultures from a variety of species, not just in the model species that have been used so far to test these hypotheses (Hagopian et al., 2010; Sander and Joung, 2014; Shmookler Reis et al., 2011). Use of cell culture would also help eliminate problems with conflicting effects of experimental treatments on different organs (Price and Guglielmo, 2009; Stroud et al., 2009; Wone et al., 2013), as well as the confounding factor of relative internal organ size (Brzęk et al., 2007). Fibroblasts are ubiquitous throughout the body, are one of the most common cell types, and have similar metabolic profiles to other cell types in culture, such as myoblasts, all of which make them a suitable cell model for relating fibroblast physiology to the physiology of the whole organisms from which they were derived (Jimenez et al., 2014a). Also, there appear to be correlations among the fatty acid composition of fibroblast membranes, longevity, and metabolic rate in birds (Figure 3.3, Ro and Williams, Unpublished results). Although these data are preliminary, if this correlation remains sound in a larger number of species and across multiple taxa, it would suggest that primary cell culture is an untapped resource that could be used to answer a variety of questions related to the membrane pacemaker hypotheses of metabolism and aging.

Conclusions

The membrane pacemaker hypotheses of metabolism and aging have been promoted by some authors (Hulbert, 2010; Kelly et al., 2014; Naudí et al., 2013) and dismissed by others (Konarzewski and Książek, 2013; Valencak and Ruf, 2007), but
there are still basic gaps in the understanding of the mechanisms behind these hypotheses, as well as consistent problems with the way that they are studied. A more concrete understanding of how the amount of PUFA in a membrane affects protein activity would significantly strengthen the membrane pacemaker hypothesis of metabolism. The mechanism leading to evolutionary differences in fatty acid composition has also not been well established. Understanding of these mechanisms would be aided by the use of methods that separate out the fatty acyl composition of different phospholipids, such as mass spectrometry, and by analyzing sub-cellular membranes separately. Acknowledging problems with the use of maximum lifespan and peroxidation index as variables and including phylogeny, and potentially body mass, in analyses would strengthen conclusions based on these analyses. Lastly, fibroblast cell culture could be a useful tool for studying these hypotheses as it allows multiple physiological parameters to be studied in the same cell lines while eliminating potential confounding problems of dietary differences and internal organ size.

The mechanisms that lead to differences in metabolic rate and aging are complex and multifaceted, with no one mechanism or hypothesis explaining all differences in metabolic rate or aging (Konarzewski and Książek, 2013; McKechnie and Swanson, 2010; Tosato et al., 2007; Turner et al., 2006b). Therefore, even with these changes, determining the validity of the membrane pacemaker hypotheses will be difficult, but instituting these changes will substantially aid us in detangling if one or both of these hypotheses are valid or if some element of fatty acyl composition besides number of double bonds, such as n-3/n-6 ratio or carbon length (Buttemer et al., 2010; Paula et al.,
1996; Valencak and Ruf, 2007), is responsible for correlations among fatty acyl composition, metabolic rate, and longevity.
Figure 3.1. A schematic of how the organization of membrane lipids and lipid rafts is changed based on the addition of phospholipids that contain DHA to a membrane. This addition increases the size of lipid rafts, pushing sphingomyelin and cholesterol into rafts, while simultaneously creating disordered and DHA-rich domains. These DHA-rich domains can then recruit proteins that require disordered membrane environments in order to function (Wassall and Stillwell, 2009).
Figure 3.2. Comparison of estimates of maximum lifespan between a relatively recent paper (Valencak and Ruf, 2007) and AnAge, an extensive database of maximum lifespan data (Tacutu et al., 2013). Solid line indicates where points would fall if estimates were the same. N=37.
Figure 3.3. (A) Correlations between maximum lifespan (years) and peroxidation index in avian fibroblasts (*black circles, solid line*) and in two commonly studied organs: skeletal muscle (*grey triangles, dashed line*) and liver mitochondria (*white diamonds, dotted line*) of birds. (B) Correlations between mass-corrected basal metabolic rate (W/kg) and unsaturation index, also in fibroblasts, skeletal muscle, and liver mitochondria from birds. The data on skeletal muscles and liver mitochondria are from (Brand et al., 2003; Hulbert, 2005; Hulbert et al., 2002b) and the data on fibroblast fatty acid composition are from unpublished data (Ro and Williams) with maximum lifespan and metabolic rate data from (Tacetu et al., 2013; Wiersma et al., 2007b).
Chapter 4: Fatty acid compositions of mitochondrial and non-mitochondrial membranes are influenced by life-history traits in neotropical and north temperate bird species

Abstract

Neotropical bird species generally have life-history traits consistent with a slower pace of life than do north temperate bird species, including having lower metabolic rates and longer lifespans than temperate species do. This, along with the fact that a variety of life-history parameters have been measured in this system, makes tropical and temperate bird species a useful system to study the interactions among membrane fatty acyl composition, metabolic rate, and longevity. I compared fatty acyl composition of common phospholipid classes in mitochondrial and non-mitochondrial membranes of primary dermal fibroblasts from tropical and temperate bird species. I found that the membranes of tropical and temperate birds were not different at broad-scale levels, such as mean number of double bonds and peroxidation index. Furthermore, these coarse variables were not usually correlated with life-history variables, such as cellular and whole-organismal metabolic rates, cellular stress resistance, and maximum lifespan. Nevertheless, fatty acyl composition compared at a finer scale, looking at specific fatty acids and specific phospholipid classes, was different between tropical and temperate species and was correlated with life-history variables. For instance, in mitochondrial phosphatidylcholine, increases in 22-carbon fatty acids, arachidonic acid (20:4), and
saturated fatty acids as well as decreases in oleic acid (18:1) and linoleic acid (18:2) were greater in temperate species than in tropical ones and these changes in fatty acyl composition were negatively related to cellular stress resistance. Multiple fatty acyl composition variables correlated with measures related to metabolic rate and longevity, but the relationships were more complicated than proposed by the membrane pacemaker hypotheses of metabolism and aging. This evidence suggests that these hypotheses need to be refined farther than the current consideration of overall number of double bonds and peroxidation index to include more attention to individual fatty acids and specific phospholipid classes.

Introduction

The fatty acyl composition of membranes is thought to be related to metabolic rate and longevity, with organisms that have highly unsaturated fatty acyl compositions having higher metabolic rates and shorter lifespans than organisms with more saturated fatty acyl compositions (Hulbert, 2003; Hulbert et al., 2007; Pamplona, 2008). The membrane pacemaker hypothesis of metabolism predicts that increases in membrane unsaturation lead to increases in metabolic rate due to interactions between unsaturated fatty acyl groups and membrane-associated proteins, leading to increases in the activity of these proteins and an overall increase in basal metabolic rate (Else and Hulbert, 2003; Hulbert and Else, 2000). Unsaturated fatty acids are also likely more prone to peroxidation, leading to the inverse correlation between membrane unsaturation and longevity that is hypothesized in the membrane pacemaker hypothesis of aging (Hulbert, 2008a; Pamplona et al., 2002a). There is evidence both supporting and contradicting
these relationships, however, and it has become increasingly clear that more research needs to be done to elucidate the relationships among fatty acyl composition, metabolic rate, and longevity (Calhoon et al., 2015).

Several problems exist with the methodology and lines of inquiry used in previous studies that make it difficult to parse whether the evidence supporting the membrane pacemaker hypotheses is stronger or weaker than the evidence contradicting them (Calhoon et al., 2015). In most studies supporting and contradicting these hypotheses, the fatty acyl composition of all phospholipids was combined (Hulbert, 2005; Hulbert et al., 2007; Pamplona, 2008). Given that fatty acyl composition of particular phospholipid classes, such as cardiolipin, could have greater effects on the activity of membrane proteins and susceptibility to oxidative stress than the fatty acyl composition of other phospholipid classes (Aoun et al., 2012; Chicco and Sparagna, 2007; Hoch, 1992; Paradies et al., 2010a), grouping all phospholipids together may hide nuances in the relationships among fatty acyl composition, metabolism, and longevity (Calhoon et al., 2015). Not only are phospholipid classes usually combined, but the fatty acyl composition of all organelles in a cell are also usually combined. Some studies separated mitochondria from other organelles and examined the fatty acyl composition of mitochondria, but few separated mitochondria out and compared the compositions of mitochondrial and non-mitochondrial membranes in relation to metabolism or longevity (Calhoon et al., 2015; Munro and Blier, 2012). Finally, research on the membrane pacemaker hypotheses has often focused on fatty acyl composition of membranes of one organ, such as liver or muscle, and has related this membrane composition back to the
whole organism. Given that there is variation in fatty acyl composition and variation in the strength of the connection between fatty acyl composition and whole-organism metabolic rate among organs (Couture and Hulbert, 1995; Montgomery et al., 2011), relating one organ to whole-organism effects may, at times, lead to incorrect conclusions.

One study system that has promise for examining the relationships among membrane fatty acyl composition, metabolic rate, and longevity is comparison of tropical and temperate bird species. Tropical bird species tend to have a slower pace of life than do birds species that breed in north temperate areas (Wiersma et al., 2007b). Life history differences between tropical and temperate bird species include higher metabolic rates, lower annual survival, larger clutch sizes, and faster growth rates in temperate species in comparison with tropical species (Jimenez et al., 2014b; Londoño et al., 2014; Ricklefs, 1997; Tieleman et al., 2006; Wiersma et al., 2007a, 2007b). Furthermore, lipid class composition is different between tropical and temperate bird species, though it is yet unknown whether there are also differences in fatty acyl composition (Calhoon et al., 2014). Lastly, a variety of data has been collected on cellular characteristics, such as fibroblast metabolic rate, fibroblast resistance to cellular stress, and fibroblast lipid peroxidation rates (Jimenez et al., 2014b, 2014c, 2013), that could be valuable in getting around the problem of extending results from one organ to whole organisms, as long as the fatty acyl composition in fibroblasts is measured (Calhoon et al., 2015).

In this study, I used high performance liquid chromatography-mass spectrometry (HPLC/MS) to compare the fatty acyl composition of common phospholipid classes in mitochondrial and non-mitochondrial membranes between tropical and temperate bird
species. I further correlated fatty acyl composition variables with previously measured
variables relating to metabolism and longevity at the cellular and organismal levels.
These variables included organismal basal metabolic rate, fibroblast basal metabolic rate,
fibroblast non-mitochondrial respiration, maximum lifespan, annual survival rate, cellular
lipid peroxidation rates, and measures of the resistance of fibroblasts to cellular stressors,
in the form of concentration of a cellular stressor that it takes to kill 50% of the fibroblast
population (LD$_{50}$). My results show that broad scale measures of fatty acyl composition,
such as number of double bonds and peroxidation index, are not consistently different
between tropical and temperate birds, nor are they generally correlated with cellular or
organismal measures of metabolic rate and longevity. Nevertheless, specific fatty acyl
groups attached to particular phospholipid classes have correlations with these life-
history measures. This evidence supports there being connections among fatty acyl
composition, metabolism, and longevity, but contradicts the simple connections between
number of double bonds, metabolism, and longevity predicted by the membrane
pacemaker hypotheses of metabolism and aging.

**Methods**

*Collection and care of birds*

Birds were mist-netted in central Ohio (39°59’N, 82°59’W) in late spring and
early summer and in Gamboa, Panama (9°7’N, 79°42’W) before and after the rainy
season. Collection of birds was permitted by Ohio Division of Wildlife permit number
15-29 and by the Panamanian Autoridad Nacional del Ambiente (no. SEX/A-22-12).
Birds were humanely killed using cervical dislocation, in accordance with procedures
approved by the Institutional Animal Care and Use Committee of the The Ohio State University (Public Health Service and Animal Welfare Assurance A3261-01).

I chose closely-related pairs of species, one tropical and one temperate (Table 4.1, Figure 4.1). This powerful approach helps reduce statistical problems with phylogenetic relatedness and also with body mass, as these closely-related species are usually similar in size (Calhoon et al., 2014; Jimenez et al., 2014c).

Establishment of cell lines

Primary fibroblast cultures were established from skin samples (Calhoon et al., 2014; Jimenez et al., 2014c). Immediately after the birds were sacrificed, their feathers were plucked and their skin was washed twice with anti-microbial soap. A 5 x 5 mm² biopsy of skin was taken and placed into cold cell culture transfer media containing: Dulbecco’s modified Eagle medium, high-glucose variant (4.5 mg/mL), sodium pyruvate (110 mg/L), 10% heat-inactivated fetal bovine serum, 2% heat-inactivated chicken serum, antibiotics (50 µg/mL each of penicillin and streptomycin), and 10 mM HEPES. The skin samples were transported in this cold transfer media. In the case of the tropical samples, samples were shipped on ice overnight to Ohio, where they were cultured using the same procedures as used on the temperate samples.

The skin biopsies were taken out of transfer media and submerged in 70% EtOH for 30 s. Samples were minced and placed into a similar media to the transfer media, except that it did not contain HEPES and 0.5% collagenase B was added. This solution was incubated overnight at 37°C and then the cells were filtered through 20-µm cell strainer and plated in cell culture flasks in complete media, which had all the same
components as transfer media, except that it did not contain HEPES. The cells were
grown in the culture flasks at 37°C in an atmosphere of 5% O₂, a physiologically relevant oxygen level (Alaluf et al., 2000), and 75% of the media was replaced every 3-4 days. When cells reached 90% confluence, they were harvested using trypsanization (0.25%) and subcultured. Once the cells reached passage 3, they were harvested, suspended in cell culture media with 10% glycerol as a cryoprotectant, and stored at -80°C under nitrogen gas.

Cellular fractionation

I isolated the mitochondria from the nuclei, plasma membrane, and endoplasmic reticulum by disrupting the cell membrane using nitrogen cavitation and separating components using differential centrifugation, as in Calhoon et al. (2014). Briefly, I suspended the cells in isolation buffer composed of 0.3 M mannitol, 0.1% BSA, 0.2 mM EDTA, 20 mM HEPES, and 0.01 mM leupeptin hydrochloride, at pH 7.4. I loaded the cell solution into a cooled Parr cell disruption vessel and let the solution rest for 20 minutes at 800 psi. Once pressure was released, I used an Eppendorf 5415 centrifuge to spin down nuclei at 700 g for 10 min and then pellet mitochondria using spins of 7,000 g for 10 minutes. This method resulted in three different fractions, one containing a pellet of nuclei, one with a pellet containing >95% of mitochondria in the cell, and a last fraction containing >93% of the plasma membrane and endoplasmic reticulum (Calhoon et al., 2014).
Lipid extraction

I extracted lipids in the mitochondrial and plasma membrane/endoplasmic reticulum fractions using a modified Bligh and Dyer (Bligh and Dyer, 1959; Calhoon et al., 2013). For every 0.1 mL of each fraction, I added 0.375 mL of HPLC-grade chloroform:methanol (1:2 v/v) and vortexed for 10 min. After that, I added 0.125 mL of chloroform and vortexed for 1 min and then 0.125 mL of 1 M NaCl and vortexed for another 1 min. I then centrifuged the samples at 1,000 g for 15 min, resulting in two layers: a lower layer, which contained lipids, and an upper, aqueous layer. I extracted further lipids from the aqueous layer by adding 0.188 mL of chloroform and centrifuging at 1,000 g for another 15 min. I added the resulting bottom layer to the original bottom layer and used these extracted lipids in the following HPLC/MS methods. As previously described, this lipid extraction method results in no easily detectable lipids left in the aqueous layer (Calhoon et al., 2014). All chloroform and methanol used contained 50 mg/L butylated hydroxytoluene to prevent lipid oxidation (Calhoon et al., 2013). I filtered lipid extracts through a Millex syringe filter (0.45µm pore size) compatible with organic solvents before HPLC/MS analysis.

Reversed phase HPLC and mass spectrometry of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol species

Subsamples of the lipid extracts from mitochondrial and plasma membrane/endoplasmic reticulum fractions were each spiked with internal standards, dried under nitrogen gas, and re-dissolved in a mixture of first ¼ part chloroform:methanol (2:1 v/v) and then ¾ part water:methanol:acetonitrile (13:53:35...
The internal standards that I used were deuterated versions of 16:0/18:1 phosphatidylcholine, phosphatidylserine, and phosphatidylinositol, as well as non-deuterated 18:3/18:3 phosphatidylethanolamine, a phosphatidylethanolamine species not originally in the samples.

To separate out the lipids, I preformed HPLC using a Shimadzu system of two pumps, an in-line degasser, autosampler, and column heater (Shimadzu, Columbia, MD). For each run, I injected a 5 µl sample onto a Phenomenex Synergi Max-RP column that was 150 x 2.1mm with 4 µm particle size and 80 Å pore size (Phenomenex, Torrence, CA). I used two eluents: eluent A contained water:methanol:acetonitrile (13:53:35 v/v/v) with 3 µM serine and 2.5 mM ammonium acetate and eluent B contained methanol:acetonitrile:ethyl acetate (5:85:10 v/v/v) with 3 µM serine and 2.5 mM ammonium acetate. I varied the gradient as follows: 0% eluent B to 35% B in 8 min, 35% B to 48% B in the next 12 min, 48% B to 100% B in 10 min, and then I held the concentration of B at 100% for 10 min. The initial flow rate of the mobile phases was 0.2 mL/min and I increased the flow rate linearly to a final concentration of 0.4 mL/min at 40 min. I maintained the column temperature at 35 °C.

To detect and quantify the phospholipid species, I used an Applied Biosystems QTRAP® 2000 equipped with a TurboIonSpray ion source (Applied Biosystems/AB SCIEX, Foster City, CA). The instrument parameters I used included: curtain gas set to 35, ion spray voltage set to 4500, temperature set to 450 °C, nebulizer gas set to 45, heater gas set to 25, ion source heater turned on, and collision gas set at Medium. I first used HPLC/MS/MS to determine which phospholipid species of phosphatidylcholine,
phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol were common enough in my samples to be detectable and quantifiable. I determined which retention times and mass/charge ratios were associated with certain phospholipid fatty acid compositions of phospholipid classes using head-group specific neutral loss and precursor ion scans along with HPLC/MS/MS/MS of fatty acid fragments (Pulfer and Murphy, 2003). Once I had determined what set of phospholipid species were in my samples, I used a targeted approach, only searching for and quantifying those specific lipid species. For examples of common lipid species, see Figure D.1 (Appendix D).

I ran positive ion MRM scans to quantify phosphatidylcholine and phosphatidylethanolamine. I used neutral loss of $m/z$ 141 to detect and quantify specific phosphatidylethanolamine species and the product ion $m/z$ 184 to detect and quantify specific phosphatidylcholine species, having already eliminated sphingomyelin species, which also have the product ion $m/z$ 184, from consideration.

Negative ion MRM scans to were used to quantify phosphatidylinositol and phosphatidylserine. I used neutral loss of $m/z$ 241 to detect and quantify specific phosphatidylinositol species and the product ion $m/z$ 87 to detect and quantify specific phosphatidylserine species.

As relative intensities of phospholipid species were proportional to concentration, I quantified phospholipids using standard curves of each of phospholipid class. The phospholipid species I used for the standard curves were: 16:0/18:1 phosphatidylcholine, 18:1/18:1 phosphatidylserine, 16:0/18:1 phosphatidylinositol, and 18:1/18:1 phosphatidylethanolamine. In addition, because plasmalogens ionize at different rates
than do regular ester-linked phospholipids (Mitchell et al., 2007), I quantified these with separate standard curves of 18:0p/18:1 phosphatidylcholine and phosphatidylethanolamine. Other ether-linked non-plasmalogen phosphatidylcholine species were also quantified using these plasmalogen standard curves. I purchased all phospholipid internal and regular standards used in these experiments from Avanti Polar Lipids (Alabaster, AL).

*Reversed phase HPLC and mass spectrometry of cardiolipin species*

Subsamples of the lipid extracts from the mitochondrial fraction were each spiked with an internal standard, dried under nitrogen gas, and re-dissolved in acetonitrile:2-propanol:water:triethylamine:glacial acetic acid (225:225:50:2.5:2.5 v/v/v/v/v). Cardiolipin was only quantified in mitochondrial fractions, as cardiolipin is specific to mitochondria (Chicco and Sparagna, 2007). I used 14:0/14:0/14:0/14:0 cardiolipin as an internal standard.

The same HPLC and MS equipment as in the previous section was also used for the separation and detection of cardiolipin species, with the exception that I used a Phenomenex Luna® column that was 150 x 2.1mm with 5 µm particle size and 100 Å pore size (Phenomenex, Torrence, CA). The method I used for cardiolipin detection and quantification was based on (Minkler and Hoppel, 2010). I used two eluents to separate the cardiolipin species: eluent A consisted of acetonitrile:water:triethylamine:glacial acetic acid (450:50:2.5:2.5 v/v/v/v) and eluent B consisted of 2-propanol:water:triethylamine:glacial acetic acid (450:50:2.5:2.5 v/v/v/v). I varied the gradient as follows: 50% eluent B for 5 min, 50% B to 80% B in the next 10 min, 80% B
to 100% B in 15 min, and I then held the concentration of B at 100% for 10 min. The
flow rate was 0.08 mL/min and the column was kept at 35 °C.

The MS instrument parameters included: curtain gas set to 25, ion spray voltage
set to -4500, temperature set to 500 °C, nebulizer gas set to 60, heater gas set to 50, ion
source heater turned on, and collision gas set to Low. I confirmed the identity of
cardiolipin species in peaks at various retention times and mass/charge ratios using
HPLC-MS/MS and HPLC-MS/MS/MS and then ran my samples in enhanced MS (EMS)
mode. Cardiolipin concentration was proportional to intensity, so I quantified cardiolipin

Statistics

For each phospholipid class in each fraction, I calculated the mean number of
double bonds per fatty acid, the mean number of carbons per fatty acid, and the
peroxidation index. Mean number of double bonds and carbons per fatty acid for each
phospholipid class were calculated by multiplying the percentage of each fatty acid in
that phospholipid class by the number of double bonds or number of carbons the fatty
acid had. I calculated peroxidation index by multiplying the percentage of each fatty acid
by a number proportional to the hypothesized peroxidation rate of the number of double
bonds that fatty acid has (Almada-Pagán et al., 2014; Calhoon et al., 2015; Hulbert,
2007; Pamplona et al., 1998). In order to also have comparable data to what is in the
literature, I calculated mean number of double bonds per fatty acid of all phospholipids
combined in each organelle fraction by multiplying the mole percentage of each
phospholipid class in the fraction by mean number of double bonds in that fraction. I ran
similar calculations to find the mean number of carbons per fatty acid and peroxidation index so that I had the combined means of all phospholipids for each fraction in number of double bonds, number of carbons, and peroxidation index.

I tested data for normality and homogeneity of variance with Shhpiro-Wilk tests and Levene’s test of homogeneity, respectively. If necessary, I corrected by log-transforming the data. If the data did not have normality or homogeneity of variance after a log transformation, I analyzed whether there were significant differences between tropical and temperate birds in that variable by inputting the means for each species into a Wilcoxon signed-rank test. If the data were normally distributed and had homogeneity of variance with or without a logarithmic transformation, I ran ANCOVAs for that variable. In these ANCOVAs, the body masses of individuals from which the cells were collected were a covariate and individuals within each species were a repeated measure. If mass was significantly correlated with a variable, then I used it as a covariate to determine if there was a difference between slopes or if tropical and temperate bird species had the same slope, with different intercepts. If body mass was not significantly correlated with the variable, then body mass was eliminated as a covariate and I ran the same model to determine if there was a consistent positive or negative difference in the mean between the species pairs. These analyses were all carried out in SPSS 19.0, with the null hypothesis rejected at $p \leq 0.05$.

Many of the fatty acid percentage and phospholipid species percentage variables were related to one another, so also I performed principal component analyses (PCA) to create uncorrelated composite variables. I log-transformed percentages of fatty acids or
phospholipid species and ran “Factor Analysis” in SPSS, with eigenvectors less than one excluded. In order to not bias the eigenvectors towards species with higher samples sizes, I used the mean for each species as a selection variable. I performed ANCOVAs as previously described comparing the principal component scores from these PCAs between tropical and temperate and temperate birds.

Lastly, I ran regressions among previously measured life-history traits (Table E.1, Appendix E) and fatty acid composition variables. The life history traits included maximum lifespan (Tacutu et al., 2013), residual basal metabolic rate (Brunton, 1988; Tacutu et al., 2013; Wiersma et al., 2007b; Yarbrough, 1971), fibroblast metabolic rates (Jimenez et al., 2014c), cellular resistance to stress (Jimenez et al., 2013), cellular lipid peroxidation rates based on C_{11}-BODIPY^{581/591} (Jimenez et al., 2014b), and annual survival rates (Jimenez et al., 2014b). Given that these comparisons were not between tropical and temperate species, phylogenetic relatedness among species had to be taken into account, so I used phylogenetic regressions (Felsenstein, 1985). I constructed a phylogenetic tree (Figure 4.1) based on Sibley and Alquist (Sibley and Alquist, 1990) and Jetz et al. (Jetz et al., 2012) with branch lengths from Sibley and Alquist (Sibley and Alquist, 1990). Phylogenetic regressions were carried out in the ape package (Paradis et al., 2004) of the statistical program R (R Core Team, 2015).

**Results**

*Double bonds, peroxidation index, and number of carbons*

There were no significant differences between mean number of double bonds per fatty acid of the combined phospholipids of the plasma membrane/endoplasmic reticulum
or of the combined phospholipids of mitochondrial membranes between tropical and temperate birds (Wilcoxon test, \( P>0.6 \) and \( F_{\text{environment}}=0.09, \ P>0.7 \), respectively; Figure F.1, Appendix F). The mean number of double bonds per fatty acid differed among the phospholipid classes and between the different organelle membranes, but there were no consistent and significant differences between tropical and temperate birds (Figure 4.2). Similarly, there were no differences in peroxidation index of combined phospholipids or of individual phospholipid classes in the two membrane fractions between tropical and temperate birds (Figure F.2, Appendix F).

Whereas there were no differences between tropical and temperate birds in the mean number of carbons per fatty acid for all phospholipids combined and for most phospholipid classes in the two fractions (Figure F.3, Appendix F), there were significant differences in one phospholipid class, phosphatidylcholine. In the mitochondrial membrane, the mean number of carbons per fatty acid was significantly higher in temperate bird species than in tropical ones (Wilcoxon test, \( P=0.02 \); Figure 4.3A).

The number of carbons per fatty acid in mitochondrial phosphatidylcholine was also related to life-history variables in phylogenetic regressions. For instance, it was significantly positively correlated with fibroblast non-mitochondrial respiration (\( R^2=0.42, \ P=0.005 \); Figure 4.3B). Similarly, scores of a PCA of that corresponded to high LD\(_{50}\)s for fibroblasts in response to several cellular stressors (Figure 4.3C) had a significantly negative relationship with the number of carbons per fatty acid in mitochondrial phosphatidylcholine (\( R^2=0.39, \ P=0.007 \); Figure 4.3D). The principal component from Figure 4.3C accounted for >43% of the variation in LD\(_{50}\) for cellular stressors.
Phylogenetic regressions revealed several other relationships among life history variables, number of double bonds, number of carbons, and peroxidation indexes of various phospholipids. In particular, scores for the PCA of the LD50s from Figure 4.3C correlated with several variables. They were negatively correlated with the overall mean number of double bonds per fatty acid in the mitochondria ($R^2=0.28$, $P<0.03$; Figure 4.4A), the mean number of double bonds per fatty acid of phosphatidylcholine in the plasma membrane/endoplasmic reticulum membranes ($R^2=0.29$, $P=0.02$; Figure 4.4B), the mean number of double bonds per fatty acid of mitochondrial phosphatidylcholine ($R^2=0.47$, $P<0.003$; Figure 4.4C), and the mean number of double bonds per fatty acid of mitochondrial phosphatidylethanolamine plasmalogen ($R^2=0.27$, $P<0.03$; Figure 4.4D). Although this is not shown, scores for the PCA of LD50s were also negatively correlated with the overall mean number of carbons per fatty acid in mitochondria ($R^2=0.31$, $P<0.02$) and the mean number of carbons per fatty acid of mitochondrial phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, and phosphatidylcholine ether ($R^2=0.23$, $P=0.04$, $R^2=0.42$, $P=0.005$, $R^2=0.26$, $P=0.03$, and $R^2=0.25$, $P=0.03$, respectively). Finally, scores for this PCA were negatively correlated with peroxidation indexes of phosphatidylcholine in the plasma membrane/endoplasmic reticulum and mitochondrial membranes ($R^2=0.38$, $P=0.01$ and $R^2=0.52$, $P=0.001$, respectively) and with the peroxidation indexes of mitochondrial phosphatidylinositol, phosphatidylethanolamine plasmalogen and phosphatidylcholine ether ($R^2=0.37$, $P<0.001$, $R^2=0.25$, $P<0.04$, and $R^2=0.35$, $P<0.02$, respectively).
Fibroblast non-mitochondrial respiration was positively correlated with several variables including: mean number of double bonds per fatty acid of mitochondrial phosphatidylcholine ($R^2=0.31$, $P<0.02$; Figure 4.4E), mean number of carbons per fatty acid of mitochondrial phosphatidylethanolamine plasmalogen ($R^2=0.23$, $P=0.04$), mean number of carbons per fatty acid of phosphatidylcholine in plasma membrane/endoplasmic reticulum ($R^2=0.33$, $P<0.01$), and peroxidation index of phosphatidylcholine in mitochondria ($R^2=0.23$, $P<0.05$).

Lastly, change in fluorescent intensity over time in C$_{11}$-BODIPY$^{581/591}$ was negatively related to mean number of carbons per fatty acid in mitochondrial cardiolipin ($R^2=0.49$, $P=0.002$; Figure 4.4F).

**Fatty acid composition of plasma membrane/endoplasmic reticulum and mitochondrial membranes**

I ran a PCA on the percentage of each fatty acid in each phospholipid in the plasma membrane/endoplasmic reticulum and mitochondrial fractions in order to observe patterns across the two membrane fractions. The first principal component explained 34% of the variance. This principal component showed that across both fractions, as the fatty acids 18:0, 20:0, 20:4, 22:4, 22:5, and 22:6 went up in percentage, the fatty acids 18:1 and 18:2 went down (Figure 4.5A). This principal component had significantly higher scores in temperate bird species than in tropical bird species ($F_{\text{environment}}=6.3$, $P<0.02$; Figure 4.5B). Additionally, in phylogenetic regression, the scores were negatively associated with the cellular stress resistance composite variable based on principal component analysis of LD$_{50}$s from Figure 4.3C ($R^2=0.45$, $P=0.003$; Figure 4.5C).
The fourth principal component of this analysis had scores that were negatively correlated with cellular metabolic rate and non-mitochondrial respiration ($R^2=0.38$, $P=0.009$ and $R^2=0.39$, $P=0.008$, respectively). This principal component accounted for 7% of variance in fatty acid composition. Principal component 4 appeared related to increases in minor fatty acids in phosphatidylcholine, each making up less than 1% of the fatty acids in phosphatidylcholine, and also to the fatty acid composition of phosphatidylethanolamine plasmalogen.

Fatty acid composition of mitochondrial phosphatidylcholine

I also ran a PCA on fatty acid composition of mitochondrial phosphatidylcholine because it appeared to have particular significance based on previous analyses. The first principal component in this analysis explained 41% of variance in fatty acyl composition of mitochondrial phosphatidylcholine. It had a similar pattern in eigenvector loadings to the analysis on all phospholipids (Figure 4.6A). Increases in fatty acids 18:0, 20:0, 20:4, 22:4, 22:5, and 22:6 were offset by decreases in 16:1, 18:1, 18:2, and 20:3. Also similarly, scores from this principal component were significantly higher in temperate birds species than in tropical ones ($F_{\text{environment}}=10.3$, $P=0.003$; Figure 4.6B) and the scores were negatively correlated with the principal component scores for LD$_{50}$ ($R^2=0.54$, $P=0.001$; Figure 4.6C).

Principal components 2, 4, and 5 of this analysis, similar to principal component 4 of the full phospholipid PCA, appeared related to increases in minor fatty acids in phosphatidylcholine. These principal components explained 17%, 8%, and 7% of variation in fatty acyl composition of mitochondrial phosphatidylcholine, respectively.
Scores of principal component 2 were negatively correlated with cellular basal metabolic rate ($R^2=0.30$, $P=0.02$). Scores of principal component 4 were significantly negatively related to residual basal metabolic rate and annual survival rates ($R^2=0.20$, $P<0.05$ and $R^2=0.21$, $P=0.04$, respectively). Lastly, principal component 5 scores were positively correlated with cellular basal metabolic rate and PCA scores for LD$_{50}$ ($R^2=0.27$, $P=0.03$ and $R^2=0.20$, $P<0.05$, respectively).

**Cardiolipin species composition**

Molecular symmetry of cardiolipin species is thought to be important in its ability to bind to proteins in the mitochondrial membrane and to other cardiolipin molecules (Schlame et al., 2005), so I ran a PCA on the percentages of the different cardiolipin species. None of the resulting principal components were significantly different between tropical and temperate bird species.

Principal component 3 of this analysis appeared to go from high amounts of cardiolipin species that are more diverse in numbers of carbons and double bonds to cardiolipin species that all had more similar numbers of carbons and double bonds (Figure 4.7A). The scores of this principal component were positively related to scores of the PCA of LD$_{50}$ measurements and positively correlated with annual survival rate ($R^2=0.44$, $P=0.004$ and $R^2=0.74$, $P<0.0002$, respectively; Figure 4.7B and C). Principal component 3 explained 12% of the variation in cardiolipin species composition.

The sixth principal component, accounting for 7% of the variation in cardiolipin species composition, had high eigenvector loadings for cardiolipin species with three fatty acids that were 18 carbons long and had low eigenvector loadings for cardiolipin
species with four 18-carbon fatty acids (Figure 4.7D). This principal component, opposite to principal component 3, was associated with cardiolipin species becoming less similar in number of carbons and number of double bonds. The scores of this principal component were negatively correlated with scores of the PCA of LD50 measurements, but positively correlated with fluorescent intensity over time of C11-BODIPY581/591 ($R^2=0.42$, $P=0.005$ and $R^2=0.22$, $P<0.05$, respectively; Figure 4.7E and F).

Fatty acid ratios indicative of specific desaturases and elongases

Desaturases and elongases are thought to affect longevity and metabolic rate (Calhoon et al., 2015; Hulbert, 2011; Jobson et al., 2010; Porter et al., 1996), so I ran a PCA on several ratios of fatty acids that could be indicative of specific enzymes. The ratios that I put into the analysis were: 16:1/16:0 and 18:1/18:0, which are indicative of several stearoyl-coA desaturases, and 18:0/16:0, 20:0/18:0, 20:3/18:3, 22:4/20:4 and 22:5/20:5, which are indicative of several elongases in the ELOVL family. None of the principal components from this analysis yielded scores that differed significantly between tropical and temperate bird species.

The first principal component, accounting for 31% of the variance, had eigenvector loadings that were consistently high for ratios that indicate stearoyl-coA desaturases and mixed loadings for ratios that indicate elongases (Figure 4.8A). The scores for this principal component were positively correlated with the scores of the PCA for LD50 measurements ($R^2=0.41$, $P=0.006$; Figure 4.8B).

The second principal component, explaining 20% of the variance in these fatty acid ratios, showed a shift in the eigenvector loadings from ratios indicative of stearoyl-
coA desaturases towards ones indicative of elongases (Figure 4.8C). This shift mostly appears to have occurred in the plasma membrane. The scores for principal component 2 were positively correlated with cellular basal metabolic rate ($R^2=0.25$, $P=0.03$; Figure 4.8D).

The fifth principal component, which accounted for 9% of the variance, had positive eigenvector loadings for ratios indicative of desaturases and elongases (Figure 4.8E). This principal component was positively correlated with cellular basal metabolic rate ($R^2=0.36$, $P=0.01$; Figure 4.8F).

**Discussion**

I found that there were no differences in the number of double bonds, number of carbons, or peroxidation index in the plasma membrane/endoplasmic reticulum or mitochondrial membranes between tropical and temperate bird species. There were, however, higher numbers of carbons in the fatty acids of mitochondrial phosphatidylcholine in temperate species than in tropical ones. Based on PCA analysis of the fatty acids in mitochondrial phosphatidylcholine, the difference in number of carbons was due to increases in 20- and 22-carbon fatty acids at the same time as decreases in 16- and 18-carbon fatty acids. This increase in number of carbons was not associated with significant increases in number of double bonds in mitochondrial phosphatidylcholine between tropical and temperate species because increases in highly unsaturated fatty acids were offset by increases in saturated fatty acids and decreases in fatty acids with 1, 2, and 3 double bonds (Figure 4.6A). A PCA analysis of fatty acyl composition of all phospholipids in both fractions revealed that differences between tropical and temperate
species in phosphatidylcholine fatty acids were echoed in other phospholipid classes and in plasma membrane/endoplasmic reticulum, though to a lesser extent (Figure 4.5). Furthermore, increases in number of carbons in mitochondrial phosphatidylcholine and the fatty acid changes exhibited in Figures 4.5 and 4.6 were correlated with low ability to resist cellular stress. Lower ability to resist cellular stress positively correlated with number of double bonds, number of carbons, or peroxidation index in all phospholipid classes of mitochondria except phosphatidylcholine plasmalogen and cardiolipin. Cellular non-mitochondrial respiration was also correlated with variables associated with high numbers of double bonds and carbons, such as the number of carbons per fatty acid in mitochondrial phosphatidylcholine.

Increases in ability of fibroblasts to resist cellular stress correlated with decreases in the number of double bonds, number of carbons, and peroxidation index of the combined phospholipids in the mitochondria as well as being correlated to decreases in the number of carbons in most individual phospholipid classes in the mitochondria. Given that long-lived species tend to have a superior ability to resist cellular stress than do shorter lived species (Harper et al., 2011, 2007; Jimenez et al., 2013; Kapahi et al., 1999; Ogburn et al., 1998), this evidence supports the membrane pacemaker hypothesis of aging. There are some caveats, however. The fatty acyl composition of mitochondria appears more important in determining resistance to cellular stress than does fatty acyl composition of the plasma membrane/endoplasmic reticulum. This is unsurprising given that mitochondria are the source and target of most reactive oxidative species (Wallace, 2005), but had yet to be tested. Furthermore, the fatty acid composition of some
phospholipid classes, particularly phosphatidylcholine, appears to have more effect on the ability to resist cellular stress than does the fatty acid composition of other phospholipid classes, such as phosphatidylethanolamine plasmalogens. Surprisingly, the number of carbons in fatty acids was more often related to cellular stress resistance than was the number of double bonds or peroxidation index. This may have something to do with the fact that number of carbons and number of double bonds is correlated. In the mitochondria, for instance, out of all phospholipid classes, only cardiolipin and phosphatidylethanolamine plasmalogen have a correlation coefficient below 0.85 between number of carbons and number of double bonds. It is also possible that the number of carbons has some hitherto unrecognized effect on cellular resistance to stress that has previously been interpreted as an effect of the number of double bonds, because of this correlation.

Maximum lifespan was not correlated with any fatty acid composition variables. This was probably due to the fact that maximum lifespan estimates for tropical bird species, where they exist, are likely underestimates of true maximum lifespan. For instance, white-tipped doves (Leptotila verreauxi) and clay-colored thrushes (Turdus grayi) have estimated maximum lifespans that are about half that of their temperate pair species, despite having annual survival rates that were 1.5 times as high (Table A.1, Appendix A). Phylogenetic corrections magnified these differences, such that these two species pairs created outliers in regressions, drowning out other signals in the data. Underestimation of maximum lifespan has been recognized in other places (Baylis et al.,
2014; Calhoon et al., 2015; Carey, 2003; Speakman, 2005), but appears to be a particular problem for this analysis.

Fatty acyl composition parameters, such as carbon chain length in phosphatidylcholine, were also correlated with cellular metabolic parameters, especially non-mitochondrial respiration. Non-mitochondrial respiration, or oxygen consumption other than that of the electron transport chain, has been mostly discounted as an important contributor to the membrane pacemaker hypothesis of metabolism, as most of the focus has been on proton leak and ion pumps (Hulbert and Else, 2000; Hulbert, 2007). A variety of processes throughout the cell consume oxygen, besides the electron transport chain (Andronis et al., 2014; Behrouzian and Buist, 2003; Sprecher, 2000). Of potential importance to this study, oxygen is consumed in fatty acid desaturation (Behrouzian and Buist, 2003) and in the process of building docosahexaenoic acid (22:6)(Guillou et al., 2010; Sprecher, 2000). This brings up the possibility that, in some cases, instead of high membrane unsaturation leading to high basal metabolic rate, it could be high basal metabolic rate that leads to high membrane unsaturation, due to the contribution of non-mitochondrial respiration to basal metabolic rate. Additionally, non-mitochondrial oxygen consumption from oxidases, such as NADH oxidase and polyamine oxidase, is a key source of reactive oxygen species outside of the mitochondria (Andronis et al., 2014; Kleniewska et al., 2012; Labat-Robert and Robert, 2014), providing another crosslink between metabolic rate and longevity.

Most correlations in this study were among fatty acid composition and cellular measures, rather than organismal-level measures. This highlights the fact that the current
practice of taking fatty acyl composition at the organ level and applying it to the organismal level may lead to false conclusions. Studies that refute the membrane pacemaker hypotheses of aging and metabolism, but only study one organ (Haggerty et al., 2008; Valencak and Ruf, 2013), are not strong evidence against the hypotheses because they do not provide a picture of the metabolic rate at the organ level or acknowledge whether other organs may be contributing more to differences in metabolic rate or longevity in a specific study system. This also highlights primary fibroblasts as a potentially important tool in comparative physiology and in studying the membrane pacemaker hypothesis. Primary fibroblasts can be cultured from species where metabolic rate and maximum longevity may not be well established and a variety of measurements related to metabolism and cellular stress resistance can be taken, all on the same cell lines (Alper et al., 2015; Jimenez et al., 2014b).

Cardiolipin species are almost exclusively composed of three or fewer different types of fatty acyl groups and fatty acyl symmetry is thought to be important in their ability to interact efficiently with membrane proteins and with each other (Chicco and Sparagna, 2007; Schlame et al., 2005). Though the number of double bonds and carbons in cardiolipin appeared to have little effect on cellular stress resistance or metabolic rate in my analyses, cardiolipin species composition was related to cellular stress resistance. In particular, as the species composition became more similar in terms of similar fatty acyl groups attached to cardiolipin, cellular stress resistance and annual survival rates increased (Figure 4.7). Counterintuitively, intensity of C_{11}-BODIPY\textsuperscript{581/591}, a measure inversely proportional to lipid peroxidation rates, was negatively correlated with the same
variable (Figure 4.7F). This may be related to the fact that the total number of carbons increased with the shift towards all 18-carbon fatty acyl chains in eigenvector loadings of Figure 4.7D, as the only other variable significantly correlated with intensity of C\textsubscript{11}-BODIPY\textsuperscript{581/591} was number of carbons in cardiolipin (Figure 4.4F). Therefore, the relationship between the fatty acyl composition of cardiolipin and longevity appears to be complex. The combination of fatty acids in cardiolipin may be as, or more, important than mean number of double bonds in determining cardiolipin’s contribution to the membrane pacemaker hypothesis of aging.

There is currently little agreement as to which enzymes primarily lead to the differences in fatty acyl composition that are correlated with metabolism and aging. Several different classes of enzymes affect fatty acyl composition of phospholipids. Elongases and desaturases affect the fatty acids themselves by adding carbons and double bonds, respectively (Guillou et al., 2010). Acyl transferases and transacylases are enzymes that transfer fatty acids to phospholipids (Yamashita et al., 2014). The PCA with high loadings of ratios of 16:1/16:0 and 18:1/18:0, the reaction of which is catalyzed by stearoyl-coA desaturases (Guillou et al., 2010), had scores that were positively correlated with the ability to resist cellular stress. This is consistent with the finding that Scd5, a stearoyl-coA desaturase, was related to longevity in a genomic scan of mammal species (Jobson et al., 2010). I also found that, generally, ratios associated with elongases were positively correlated with cellular basal metabolic rate. This was particularly true of elongases in the pathways to making long and highly unsaturated fatty acids, such as the elongases that correspond with 20:3/18:3, 22:4/20:4, and 22:5/20:5, which belong to the
ELOVL family of desaturases, specifically ELOVL 2 and 5 (Guillou et al., 2010). Nevertheless, acyltransferases and transacylases must also play an important role in developing the patterns that led to the membrane pacemaker hypotheses, given that some phospholipid classes, such as phosphatidylcholine, were more associated than other classes with differences in metabolic rate and cellular stress resistance.

In conclusion, I found evidence that supports connections among fatty acyl composition of phospholipids, metabolism, and aging, but the connections appear to be more complicated than the simple relationship of increased unsaturation leading to high metabolic rate and short lifespan predicted by the membrane pacemaker hypotheses of metabolism and aging. If cellular stress resistance is a reliable correlate of longevity, my evidence more strongly supports the membrane pacemaker hypothesis of aging than it supports the membrane pacemaker hypothesis of metabolism. Additionally, I raise the possibility that correlations between fatty acyl composition and basal metabolic rate, may at times, be increases in non-mitochondrial oxygen consumption causing increases in the unsaturation of membranes, rather than unsaturation of membranes causing increases in basal metabolic rate.
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Table 4.1. Sample sizes and body masses of phylogenetically-matched pairs of temperate and tropical bird species.
Figure 4.1. Phylogenetic tree of tropical and temperate species in this study. Temperate species are labeled in red and tropical species are labeled in black. The units are $\Delta T_{50}H$, which is a measure of relatedness based on the difference in temperature at which 50% dissociation occurs between DNA formed solely from one species and DNA heteroduplexes.
Figure 4.2. The mean number of double bonds per fatty acid in each phospholipid class in plasma membrane/endoplasmic reticulum and mitochondrial membranes. All individuals within a species were averaged, so each box represents the lipid compositions of 10 temperate (no pattern) or tropical (hashed pattern) species in the plasma membrane/endoplasmic reticulum (white) or mitochondrial (red) membranes.
Figure 4.3. (A) The mean number of carbons per fatty acid in mitochondrial phosphatidylcholine from temperate (black bars) and tropical (white bars) bird species. Values are means ± SEM. (B) Phylogenetic regression between mean number of carbons per fatty acid in mitochondrial phosphatidylcholine and non-mitochondrial respiration (pMole/min/50K cells). (C) Eigenvector loadings for LD$_{50}$s of fibroblasts in response to peroxide, paraquat, cadmium, UV radiation, methane methylsulfonate, and tunicamycin (Jimenez et al., 2013). (D) Phylogenetic regression between mean number of carbons per fatty acid in mitochondrial phosphatidylcholine and principal component scores for the PCA of LD$_{50}$. 
Figure 4.3.

A. Mean number of carbons per fatty acid in mitochondrial phosphatidylcholine.

B. Phylogenetically corrected non-mitochondrial cellular respiration.

C. Eigenvector loadings for LD$_{50}$ principal components analysis.

D. Phylogenetically corrected principal component score of LD$_{50}$. 

Phylogenetically corrected mean number of carbons per fatty acid in mitochondrial phosphatidylcholine.
Figure 4.4. Phylogenetic regressions among principal component scores for LD\textsubscript{50}, (A) mean number of double bonds per fatty acid in mitochondrial membranes for all phospholipids, (B) mean number of double bonds per fatty acid in plasma membrane/endoplasmic reticulum membranes for phosphatidylcholine, (C) mean number of double bonds per fatty acid in mitochondrial phosphatidylcholine, and (D) mean number of double bonds per fatty acid in mitochondrial phosphatidylethanolamine plasmalogen. (E) Phylogenetic regression between non-mitochondrial respiration (pMole/min/50K cells, Jimenez et al., 2014c) and mean number of double bonds per fatty acid in mitochondrial phosphatidylcholine. (F) Phylogenetic regression between C\textsubscript{11}-BODIPY\textsuperscript{581/591} (ΔFluorescent Intensity/min/100K cells, Jimenez et al., 2014b) and mean number of carbons per fatty acid in mitochondrial cardiolipin.
Figure 4.4.

A. Phylogenetically corrected double bonds per fatty acid in the mitochondria

B. Phylogenetically corrected double bonds in fatty acids of phosphatidylcholine in the plasma membrane/endoplasmic reticulum

C. Phylogenetically corrected double bonds per fatty acids of phosphatidylcholine in the mitochondria

D. Phylogenetically corrected carbons per fatty acid in mitochondrial phosphatidylethanolamine plasmalogen

E. Phylogenetically corrected double bonds per fatty acid of phosphatidylcholine in the mitochondria

F. Phylogenetically corrected carbons per fatty acid in mitochondrial cardiolipin
Figure 4.5. (A) Eigenvector loadings for principal component 1 of the fatty acids from all phospholipids in plasma membrane/endoplasmic reticulum and mitochondrial membranes. For ease of understanding, only loadings where the absolute values were equal to or greater than 0.5 are shown. Eigenvector loadings are organized here by fatty acid on the y-axis and separated out into plasma membrane/endoplasmic reticulum (no pattern) or mitochondrial (checkered pattern) membranes and phosphatidylcholine (white), phosphatidylcholine - ether (red), phosphatidylcholine - plasmalogen (orange), phosphatidylethanolamine (yellow), phosphatidylethanolamine - plasmalogen (green), phosphatidylinositol (blue), or phosphatidylserine (purple). Note: cardiolipin fatty acid composition was included in this PCA but did not make significant contributions to eigenvector loadings. * indicates the following fatty acids in order from left to right: 16:1, 20:2, 22:3. (B) Principal component 1 scores of the fatty acids from all phospholipids in plasma membrane/endoplasmic reticulum and mitochondrial membranes compared between temperate (black bars) and tropical (white bars) birds. Values are means ± SEM. (C) Phylogenetic regression between principal component scores for LD$_{50}$ and principal component 1 scores of the fatty acyl composition of all phospholipids in plasma membrane/endoplasmic reticulum and mitochondrial membranes.
Figure 4.5.
Figure 4.6. (A) Eigenvector loadings for principal component 1 of the fatty acyl composition of mitochondrial phosphatidylcholine. For ease of understanding, only loadings where absolute values were equal to or greater than 0.5 are shown. (B) Principal component 1 scores compared between temperate (black bars) and tropical (white bars) birds. Values are means ± SEM. (C) Phylogenetic regression between principal component scores for LD₅₀ and principal component 1 scores.
Figure 4.6.

A

B

C

Principal components analysis score 1 of fatty acids of mitochondrial phosphatidylcholine.

Phylogenetically corrected principal components analysis score 1 of fatty acids of mitochondrial phosphatidylcholine.

Eigenvector loadings for principal components analysis 1 of the fatty acids of mitochondrial phosphatidylcholine.

Phylogenetically corrected principal component score of LD50.
Figure 4.7. (A) Eigenvector loadings for principal component 3 of cardiolipin species. For ease of understanding, only loadings where absolute values were equal to or greater than 0.3 are shown. (B) Phylogenetic regression between principal component scores for LD$_{50}$ and principal component 3 scores of cardiolipin species. (C) Phylogenetic regression between annual survival rate (S, Jimenez et al., 2014b) and principal component 3 scores of cardiolipin species. (D) Eigenvector loadings for principal component 6 of cardiolipin species. For ease of understanding, only loadings where absolute values were equal to or greater than 0.3 are shown. (E) Phylogenetic regression between principal component scores for LD$_{50}$ and principal component 6 scores of cardiolipin species. (F) Phylogenetic regression between C$_{11}$-BODIPY$_{581/591}$ (ΔFluorescent Intensity/min/100K cells, Jimenez et al., 2014b) and principal component 6 scores.
Figure 4.7.

A. Eigenvector loadings for principal components analysis of cardiolipin species.
B. Phylogenetically corrected principal components analysis score 3 of cardiolipin species.
C. Phylogenetically corrected survival rate (S).
D. Eigenvector loadings for principal components analysis 6 of cardiolipin species.
E. Phylogenetically corrected principal components analysis score 6 of cardiolipin species.
F. Phylogenetically corrected BODIPY 581/591.
Figure 4.8. (A) Eigenvector loadings for principal component 1 of ratios of fatty acids that may be indicative of specific desaturases and elongases. For ease of understanding, only loadings where absolute values were equal to or greater than 0.5 are shown. Eigenvectors are divided into ratios from plasma membrane/endoplasmic reticulum (*no pattern*) and mitochondrial (*checkered pattern*) membranes, a convention also used in (C) and (E). (B) Phylogenetic regression between principal component scores for LD<sub>50</sub> and principal component 1 scores of ratios of fatty acids. (C) Eigenvector loadings for principal component 2 of ratios of fatty acids that may be indicative of specific desaturases and elongases. For ease of understanding, only loadings where absolute values were equal to or greater than 0.5 are shown. (D) Phylogenetic regression between cellular basal metabolic rate (pMole/min/50K cells, Jimenez et al., 2014c) and principal component 2 scores of ratios of fatty acids. (E) Eigenvector loadings for principal component 5 for ratios of fatty acids that may be indicative of specific desaturases and elongases. For ease of understanding, only loadings where absolute values were equal to or greater than 0.3 are shown. (F) Phylogenetic regression between cellular basal metabolic rate (pMole/min/50K cells, Jimenez et al., 2014c) and principal component 5 scores for ratios of fatty acids.
Figure 4.8.
References


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Skin-derived


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fatty acid unsaturation protects against lipid peroxidation in liver mitochondria
66. doi:10.1016/0047-6374(95)01673-2


Appendix A: Data averaged by species for each passage from Chapter 1
No statistically significant differences
Ratio of cerebroside (mg) to total lipids (mg) 0.021±0.008 0.055±0.032 0.011±0.003

Only differences between species
Ratio of cardiolipin (mg) to total lipids (mg) 0.031±0.007 0.021±0.002 0.031±0.005
Ratio of ceramide (mg) to total lipids (mg) 0.016±0.002 0.017±0.006 0.016±0.004
Ratio of sphingomyelin (mg) to total lipids (mg) 0.156±0.014 0.110±0.007 0.134±0.015

Differences between passages, but no interaction with species

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<tr>
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<th>Mallard (Anas platyrhynchos)</th>
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<tr>
<td></td>
<td>$P_0$</td>
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<tr>
<td>Cell diameter (µm)</td>
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<tr>
<td>Ratio of non-phospholipids (mg) to phospholipids (mg)</td>
<td>0.450±0.052</td>
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<tr>
<td>Ratio of phosphatidylethanolamine (mg) to total lipids (mg)</td>
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<tr>
<td>Ratio of phosphatidylcholine (mg) to total lipids (mg)</td>
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Homogeneity increasing
Ratio of phosphatidylinositol (mg) to total lipids (mg) 0.035±0.008 0.047±0.016 0.044±0.014
Ratio of plasmalogen (µmoles) to total lipid (mg) 0.054±0.012 0.058±0.017 0.052±0.010
Ratio of triglyceride (mg) to total lipids (mg) 0.072±0.014 0.045±0.005 0.082±0.015

Interaction between passage and species with no increase in homogeneity
Ratio of cholesterol (mg) to total lipids (mg) 0.120±0.009 0.091±0.014 0.133±0.027
Ratio of free fatty acid (mg) to total lipids (mg) 0.077±0.016 0.046±0.006 0.033±0.006
Ratio of phosphatidylserine (mg) to total lipids (mg) 0.075±0.010 0.064±0.002 0.079±0.007

Table A.1. Changes in lipid composition and size among cells from five bird species over passaging. Lipid quantities were measured using thin layer chromatography and an iodine addition assay for plasmalogens with the total lipids being the sum of all lipid quantities measured by thin layer chromatography. Cell diameter was measured using CCD digital camera and Labkit 1.1 software (Phenix). Values are means ± 1 S.E.
Table A.1 Continued

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<th>Northern Shovelers (Anas clypeata)</th>
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<td>0.176±0.006</td>
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<td>0.022±0.012</td>
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<td>0.072±0.018</td>
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<td>0.134±0.044</td>
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<td>0.117±0.020</td>
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<td>0.040±0.012</td>
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Continued
Appendix B: Western blot secondary confirmation of mitochondrial isolation
Figure B.1. Changes in lipid composition and size among cells from five bird species over passaging. Lipid quantities were measured using thin layer chromatography and an iodine addition assay for plasmalogens with the total lipids being the sum of all lipid quantities measured by thin layer chromatography. Cell diameter was measured using CCD digital camera and Labkit 1.1 software (Phenix). Values are means ± 1 S.E.
Appendix C: Unused protein data from the samples used in Chapters 1 and 2
I used acetone to precipitate the proteins out of the Bligh and Dyer aqueous layer in all samples from Chapters 1 (Figure C.1) and 2 (Figure C.2). I analyzed protein using a commercially available Modified Lowry Protein Assay Kit (Pierce Product #23240). These data were never used in the publications related to this work.

Additionally, I took a rough measure of protein amount right after I isolated the mitochondria from the nucleus and other fractions (Figure C.3). I measured the absorbance of 2 µl of each fraction using a NanoDrop 2000 in Protein A280 mode.
Figure C.1. Differences in protein (mg) per million cells across different species and passages of fibroblasts. $P_0$ is indicated by a black bar, $P_2$ by a white bar, and $P_4$ by a hashed bar. The species included in this analysis were: Mallards (*Anas platyrhynchos*), Northern Shovelers (*Anas clypeata*), Bobwhite Quails (*Colinus virginianus*), Mourning Doves (*Zenaida macroura*), and American Robins (*Turdus migratorius*). Values are means ± 1 S.E.
Figure C.2. Differences in the amount of mitochondrial protein (mg) per million cells between temperate (*black bars*) and tropical (*white bars*) birds. Values are means ± SEM.
Figure C.3. A rough measure of percentage protein in each fraction for temperate (white) and tropical birds (grey). In both cases, the fractions are represented by nucleus (no pattern), mitochondria (diamond pattern), and plasma membrane/endoplasmic reticulum (hashed pattern).
Appendix D: Representative structures of all phospholipid classes
Figure D.1. Structures of the most common species of each phospholipid class, in order of how common that phospholipid class was. (A) Stearoyl-oleoyl-\textit{sn}-phosphatidylcholine (PC (18:0)(18:1)), (B) stearoyl-arachidonoyl-\textit{sn}-phosphatidylethanolamine (PE (18:0)(20:4)), (C) octadecenyl-arachidonoyl-\textit{sn}-phosphatidylethanolamine (PE plasmalogen (18:0p)(20:4)), (D) stearoyl-oleoyl-\textit{sn}-phosphatidylserine (PS (18:0)(18:1)), (E) stearoyl-arachidonoyl-\textit{sn}-phosphatidylinositol (PI (18:0)(20:4)), (F) hexadecyl-oleoyl-\textit{sn}-phosphatidylcholine (PC ether (16:0e)(18:1)), (G) octadecenyl-eicosatrienoyl-\textit{sn}-phosphatidylcholine (PC plasmalogen (18:0p)(20:3)), and (H) dioleoyl-dilinoleoyl-\textit{sn}-cardiolipin (CL (18:1)\textsubscript{2}(18:2)\textsubscript{2}). Note: except for cardiolipin, this is based on most common phospholipid species in the plasma membrane/endoplasmic reticulum, though most of the common phospholipid species were common in both fractions.
Figure D.1. Continued

Continued
Appendix E: Life history variables used in Chapter 4
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<th>Species</th>
<th>Maximum lifespan$^a$</th>
<th>Residuals of basal metabolic rate (W)$^b$</th>
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Table E.1. Life history variables used in phylogenetic regressions
SOURCES. – $^a$ Tacutu et al. 2013
$^b$ Residuals based on basal metabolic rates and body masses from Wiersma et al. 2007b, except where numbered
$^1$ Tacutu et al. 2013
$^2$ Yarbrough 1971
$^3$ Brunton 1988
$^c$ Jimenez et al. 2014c
$^d$ Jimenez et al 2013
$^e$ Jimenez et al. 2014b
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<th>Species</th>
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<th>Peroxide LD$_{50}^d$</th>
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Table E.1. Continued

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Continued
Appendix F: Additional figures depicting fatty acid composition in tropical and temperate bird fibroblasts
Figure F.1. Mean number of double bonds per phospholipid between temperate (turquoise) and tropical (white) bird species in plasma membrane/endoplasmic reticulum (no pattern) and mitochondria (checkered pattern).
Figure F.2. Peroxidation index of each phospholipid class in plasma membrane/endoplasmic reticulum and mitochondrial membranes. All individuals within a species were averaged, so each box represents the lipid compositions of 10 temperate (no pattern) or tropical (hashed pattern) species in the plasma membrane/endoplasmic reticulum (white) or mitochondrial (red) membranes.
Figure F.3. The mean number of carbons per fatty acid in each phospholipid class in plasma membrane/endoplasmic reticulum and mitochondrial membranes. All individuals within a species were averaged, so each box represents the lipid compositions of 10 temperate (no pattern) or tropical (hashed pattern) species in the plasma membrane/endoplasmic reticulum (white) or mitochondrial (red) membranes.