CUTANEOUS AND RESPIRATORY WATER LOSSES OF TEMPERATE BIRDS

A THESIS

Presented in Partial Fulfillment of the Requirements for
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By

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*****

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ABSTRACT

Amongst various functions of the vertebrate skin, its ability to impede water loss is critical for survival in terrestrial animals. The outer layer of skin, the stratum corneum is comprised of cornified cells surrounded by an intercellular lipid matrix. Lipids in the SC are thought to be responsible for forming a tight water barrier. In this study, I measured cutaneous water loss (CWL) and respiratory water loss (RWL) of 13 species of birds, all from a temperate environment. RWL and CWL among temperate species averaged 55.8 mg H\textsubscript{2}O/g, and 27.3 mg H\textsubscript{2}O/cm\textsuperscript{2}·d, respectively. Although I found differences in RWL among species, surface specific CWL was statistically indistinguishable across the temperate birds. I hypothesized that water vapor diffusion across the skin was subjected to biological control and tested this idea by comparing CWL of alive and dead birds. When birds were dead, CWL was significantly reduced by 13% suggesting that CWL is under biological influence. The composition of intercellular lipid matrix is influential in determining CWL. I found that ceramides, fatty acid methyl esters, sterol esters and cebrerosides were major constituents of the avian stratum corneum. Variation in CWL was positively associated with amount of ceramide 3 and cerebroside 3, but these combined represented less than 2% of the total lipids.
Dedicated to my father and mother
ACKNOWLEDGMENTS

Words would not be able to fully deliver all my thanks to Joe Williams, my advisor. You taught me what science is all about and enabled me to become an independent thinker: I learned not to trust any one and to say what I think, even when the crowd tells me no. No one had ever pushed my intellectual ability as much as my advisor did and I would not have learned what I know now, if he had not train me. I also thank my committee members, John Harder, and Dave Stetson for their support and encouragement through out of my Master’s work.

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<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>BMR</td>
<td>basal metabolic rate</td>
</tr>
<tr>
<td>( \beta )</td>
<td>beta</td>
</tr>
<tr>
<td>CWL</td>
<td>cutaneous water loss</td>
</tr>
<tr>
<td>cm(^2)</td>
<td>centimeter square</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>( \Delta )</td>
<td>delta</td>
</tr>
<tr>
<td>FAME</td>
<td>fatty acid methyl ester (s)</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>water</td>
</tr>
<tr>
<td>J</td>
<td>joule</td>
</tr>
<tr>
<td>k</td>
<td>kilo</td>
</tr>
<tr>
<td>L</td>
<td>liter(s)</td>
</tr>
<tr>
<td>m</td>
<td>milli</td>
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m³  cubic meters
min  minute(s)
O₂   oxygen
Osm  osmolality
PBS  phosphate buffer saline
PTLC preparative thin layer chromatography
pH  -\log_{10} concentration of H⁺ ions
R_f  retention factor
RMR  resting metabolic rate
RWL  respiratory water loss
ρ    rho
SA   surface area
SC   stratum corneum
SNK  Student-Newman-Kules
T_{a} ambient temperature
T_{b} body temperature
T_{dph} temperature of dew point hygrometer
TLC  thin layer chromatography
V;v  volume
ω    omega
CHAPTER 1

INTRODUCTION

As the largest, yet under appreciated, organ of the body, the vertebrate skin serves multiple roles such as protection against chemical, physical and pathogenic insults, regulation of body temperature, and more importantly, a barrier to water loss (Chuong et al., 2002). For birds and mammals, the skin consists of an underlying dermis and an outer epidermis. For these taxa, the barrier to water movement is localized in the outer most layer of the epidermis, the stratum corneum (SC) (Windsor and Burch, 1944; Menon and Menon, 2000). The SC is considered a “two-compartment system” comprised of cornified cells embedded in lipid matrix (Chandrasekaran and Shaw, 1978; Elias, 1983). Although intercellular lipids are a small portion of the SC by dry weight, experiments in which the lipids have been removed by organic solvents have convincingly shown that lipids are an integral component in impeding water loss from the skin (Sweeney and Downing, 1970; Wertz and van den Bergh, 1998; Lillywhite, 2006). In mammals, lipids that fill intercellular spaces of the SC are primarily a mixture of cholesterol, free fatty acids and ceramides, the latter a sphingosine molecule ester linked to fatty acid (Wertz and van den Bergh, 1998; Lillywhite, 2006). In the SC of birds the same lipid classes are found but cerebroside, a ceramide with a hexose attached, also makes up a significant proportion of the total lipids (Menon and Menon, 2000; Munoz-Garcia and Williams, 2005; 2007). If mammals have cerebrosides in their SC, it is considered a pathological state, and transepidermal water loss is markedly increased (Holleran et al., 1994).
Total evaporative water loss (TEWL), the sum of cutaneous and respiratory water losses (CWL, RWL), accounts for 70-80% of total water loss in birds. Evaporative water loss is 5 times greater than urinary-fecal water loss in small birds when measured at 25°C emphasizing the importance of this parameter to their survival (Bartholomew, 1972; Williams, 1996). Initially researchers surmised that CWL was insignificant- < 2%- in the overall water economy of birds (Schmidt Nielsen et al., 1969; Mount, 1979), but recent evidence suggests that CWL accounts for over 60% of TEWL in many avian species (Tieleman et al., 1999; Williams and Tieleman 2005). Thus understanding CWL and how the SC modulates water loss from birds is of fundamental importance in avian physiology.

CWL is determined by the water vapor gradient across the skin, and the resistance of the SC against water permeation, a trait subject to natural selection (Tieleman and Williams, 2002). Previous studies showed that CWL among birds living in different temperature-moisture environments were associated with lipid composition in the SC (Haugen et al. 2003a, b, Munoz-Garcia and Williams 2005). These studies suggested that natural selection has adjusted lipid composition and thus diffusional resistance in the SC to meet the requirements of thermoregulation and/or water conservation in the birds’ natural habitat. However because there are few data for CWL on wide variety of species, it is premature to infer that CWL is shaped mostly by environmental factors. Even within the same environment, different microclimates may impose different selection pressures on the skin relative to water loss, or CWL may be influenced by phylogenetic background.

Whether water transport across the skin is a passive or active process has been the subject of considerable discussion. After investigators showed that the SC forms the primary water barrier (Windsor and Burch, 1944), subsequent workers focused on the role played by the SC to understand the movement of water across the skin to the exclusion of other components of the epidermis. From experimental and theoretical work, researchers thought that water moves through the SC by passive diffusion following Fick’s diffusion theory (Kalia et al., 1996). However some argued this model failed to account for the influence of other layers of epidermis, and therefore was an inaccurate
view of CWL (Falkenberg and Georgiadis, 2008). According to these authors there is evidence that the epidermal layer below the SC actively transports ions, which influences flow of water, and thus the hypothesis that water permeation across the SC is a passive process needs to be revisited. If water movement across skin is an active process, one may expect to see changes in the rate of water loss through the skin when the animal is dead. Alternatively, if water loss is a passive process, then CWL when the animal is alive and immediately after death should be the same.

As part of the physical and chemical components of the SC, lipid composition is critical for forming the water barrier because interaction and organization of lipids are dependent on types and quantities of lipids (Bouwstra et al., 2000). This means that finding a connection between structure and function of the SC would allow us to predict how CWL will change in response to alteration in lipid composition. Haugen et al. (2003b) found that after acclimating larks to two different T_a's, the proportion of lipid classes in the SC changed and resulted in alteration in CWL.

The aim of our study was to measure CWL of a variety of temperate bird species and relate their CWL to lipid composition in the SC. I purposed to gain insight into how species from a single environment might vary lipid composition of the SC, and as a result CWL. I hypothesized that CWL will be significant proportion of TEWL in temperate birds, but that the surface specific CWL will be relatively invariant if environment is the dominant influence on this physiological variable. Our alternative hypothesis was that despite living in the same environment, temperate birds will have different CWL due to taxonomic differences, heterogeneity in habitats, and in migration patterns. Further, I tested the extent to which CWL was under biological control by comparing rates of water vapor diffusion through the skin of live and dead birds. Lastly I explored how variation in CWL is influenced by lipid composition in the SC.
CHAPTER 2

METHODS AND MATERIALS

2.1 Capture of birds

Using mist nets, I captured 65 individuals of 13 species of birds that live in Ohio, USA, from April 8 to June 20, 2008. I captured birds that reside in 5 different habitats, some year-round residents, whereas others were migrants (Table 1.1). Habitat was assigned based on information from Cornell Laboratory of Ornithology
(http://www.allaboutbirds.org/netcommunity/Page.aspx?pid=1189). For migratory distance, I assigned birds to 1 of 4 groups: residents, those that stay in same geographical area for breeding and wintering, short distance migrants, those that travel generally < 200 miles to their wintering sites, medium distance migrants, those that migrate no farther South than Mexico during winter, and long distance migrants, those that migrate to Central America or farther (Shackelfold et al., 2005).

Birds that I captured were transported to the lab, and housed in small wire cages; water and food were provided *ad libitum*. Prior to measurements of CWL, I weighed birds with a Mettler balance (± 0.01g), and measured their bill, head, head-plus-bill and tibiotarsus using dial calipers, and the length of their right wing with a ruler (Sevnsson, 1992). I used Meeh’s equation, surface area (cm²; SA) = 10* (body mass (g) ^ 0.667), to calculate SA from body mass (Walsberg and King, 1978). Sex was determined by dissection after measurements.
<table>
<thead>
<tr>
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<th>Scientific names</th>
<th>Body mass</th>
<th>Habitat</th>
<th>Migratory distance</th>
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<tr>
<td>House Wren (n=6)</td>
<td><em>Troglodytes aedon</em></td>
<td>9.39 ± 0.3</td>
<td>Forest edge</td>
<td>Long distance</td>
</tr>
<tr>
<td>Chipping Sparrow (n=5)</td>
<td><em>Spizella passerina</em></td>
<td>10.98 ± 0.5</td>
<td>Open woodland</td>
<td>Long distance</td>
</tr>
<tr>
<td>American Goldfinch (n=6)</td>
<td><em>Carduelis tristis</em></td>
<td>11.35 ± 0.1</td>
<td>Open woodland</td>
<td>Short distance</td>
</tr>
<tr>
<td>Eastern Wood-Pewee (n=1)</td>
<td><em>Contopus virens</em></td>
<td>11.58</td>
<td>Forest</td>
<td>Long distance</td>
</tr>
<tr>
<td>Red-eyed Vireo (n=6)</td>
<td><em>Vireo olivaceus</em></td>
<td>15.41 ± 0.7</td>
<td>Forest edge</td>
<td>Long distance</td>
</tr>
<tr>
<td>Tree Swallow (n=5)</td>
<td><em>Tachycineta bicolor</em></td>
<td>16.34 ± 0.6</td>
<td>Freshwater marsh</td>
<td>Medium distance</td>
</tr>
<tr>
<td>Eastern Phoebe (n=5)</td>
<td><em>Sayornis phoebe</em></td>
<td>16.60 ± 0.6</td>
<td>Freshwater marsh</td>
<td>Medium distance</td>
</tr>
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<td>Song Sparrow (n=6)</td>
<td><em>Melospiza melodia</em></td>
<td>19.26 ± 1.0</td>
<td>Open woodland</td>
<td>Short distance</td>
</tr>
<tr>
<td>Scarlet Tanager (n=4)</td>
<td><em>Piranga olivacea</em></td>
<td>27.29 ± 0.6</td>
<td>Forest</td>
<td>Long distance</td>
</tr>
<tr>
<td>Northern Cardinal (n=6)</td>
<td><em>Cardinalis cardinalis</em></td>
<td>38.84 ± 1.0</td>
<td>Forest edge</td>
<td>Resident</td>
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<td>Northern Mockingbird (n=5)</td>
<td><em>Mimus polyglottos</em></td>
<td>46.64 ± 1.8</td>
<td>Open thickets</td>
<td>Short distance</td>
</tr>
<tr>
<td>American Robin (n=5)</td>
<td><em>Turdus migratorius</em></td>
<td>73.73 ± 2.1</td>
<td>Open thickets</td>
<td>Short distance</td>
</tr>
<tr>
<td>Morning Dove (n=5)</td>
<td><em>Zenaida macroura</em></td>
<td>121.76 ± 2.3</td>
<td>Open thickets</td>
<td>Short distance</td>
</tr>
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Table 1.1: Thirteen species of temperate birds and their body mass, habitat and migratory distance. Mean body mass in grams ± 1 S.E.
2.2 Measurement of metabolic rate and evaporative water loss

Within 24 hours of capture of birds, I measured their rate of oxygen consumption, RWL, and their CWL using standard flow-through respirometry and hygrometry methods (Gessaman, 1987; Tieleman and Williams, 2002). I removed food from birds 2–3 h prior to measurements to ensure postabsorptive conditions, then placed them either in a 2L or 5.9L stainless steel chamber depending on the bird’s size, over a layer of mineral oil to trap feces, thus eliminating them as a source of water in our evaporative water loss measurements. All measurements were made during the active phase. Chambers were rendered airtight with a lid and rubber gasket. I placed chambers in an environmental cabinet in which air temperature ($T_a$) was controlled with a Peltier system (Sable Systems) at 30 ± 0.1°C.

I quantified CWL and RWL separately using a mask system (Tieleman and Williams, 2002) whose air flow was driven by two vacuum pumps. The flow rate of each air stream was governed by a separate mass flow controller (Celerity, Inc. [Allen, TX] model FC-2900VO-4S) that had been calibrated by a bubble meter (Levy 1964). Dry air was drawn into the chamber, and exited the chamber through two ports, one for the mask and the other for air within the chamber. The mask captured all respiratory gases and also drew in air from the chamber, because I set the flow rate of the second port 200 mL/min less than that of the mask. In contrast, the second port only contained water vapor that came from the skin. The dew point of each air stream was measured by a dew point hygrometer (General Eastern [Woburn, MA], model M4). The flow rate of air through the mask was set at 500 to 1050 mL/min depending on the size of a bird.

Oxygen concentration of air flowing from the mask was measured with an Applied Electrochemistry (Pittsburgh, PA) S3A-II oxygen analyzer. To measure oxygen consumption of a bird, I directed dry atmospheric air through a different line to the oxygen analyzer (inlet air) and used it to calculate $\Delta O_2$ of air from the mask. Inputs for
our dew point hygrometers were recorded each minute using a LiCor data logger (Logan, UT).

CWL of live birds was calculated from measurements of dew point of air drawn from the chamber by directing that air through our second dew point hygrometer. During each measurement I re-routed air from the chamber port to the oxygen analyzer to verify that the mask captured all respiratory gases; the fraction of O₂ in chamber air was always identical to inlet air (20.95%) confirming that the mask captured all respiratory gases.

After birds had been in the chamber 1-2 hours, oxygen consumption and dew points typically stabilized, indicating that the bird had calmed and that the system had reached equilibrium. Then I began recording ∆O₂, dew point temperatures, and Tₐ inside the chamber and inside of our dew point hygrometers (T_dph). I averaged data over 10 min and used these in our calculations. Oxygen consumption was calculated with equation (4a) of Withers (1977) and was converted to kJ/d using 20.08 J/mL O₂ (Schmidt-Nielsen 1997). To estimate RWL, I used RWL = (ρ mask - ρ chamber) * V'_e1, where ρ mask is water vapor density (g/m³) of air from the mask, corrected to standard temperature and pressure (STP), and ρ chamber is water vapor density of air in the chamber (g/m³, STP), and V'_e1 is the flow rate of air leaving the mask (Tieleman and Williams, 2002). CWL was calculated from CWL = (ρ chamber - ρ in) * (V'_e1 + V'_e2), where ρ in is the water vapor density (g/m³) of dry air entering the chamber and V'_e2 is the flow rate of the air leaving the chamber (Tieleman and Williams, 2002).

2.3 Water loss through skin from dead birds

Some have argued that water loss through skin is a passive process (Grice and Bettley 1967), whereas others have suggested that organisms exert considerable control over water permeation through skin, at least in mammals (Grice and Bettley, 1967; Elias, 2004). Part of this confusion resides in some authors thinking about water movement through the entire epidermis, whereas others have focused their attention on the SC only (Falkenberg and Georgiadis, 2008). One can envision that vertebrates could alter the
diffusion path length of water moving through the skin by means of vasodilation or constriction, and therefore exert biological control over CWL, or epidermal cells could create ion gradients that would influence water flow (Falkenberg and Georgiadis 2008). I wanted to assess the extent, if any, of the biological influence on water permeation through the skin of birds, as opposed to passive water loss. Therefore I measured CWL of dead birds (CWL$_{\text{dead}}$) and compared these data with their live CWL (CWL$_{\text{live}}$). To make these measurements, I needed to know when the system had reached equilibrium after I placed the dead bird back in the chamber, but also with the added complexity that the measurement had to be made before the bird lost a significant amount of body water. Hence, after measuring CWL of a live bird, I sacrificed it, re-attached the mask system in order to eliminate evaporation from the mouth and respiratory passages, and repeated evaporative water loss measurements using the same flow rate as for during measurements when the bird was alive. With the aid of a light bulb, I quickly adjusted $T_a$ within the chamber to 38 °C, so that skin temperature would be similar to that of live birds (Williams, unpubl.). To assure that this was so, I monitored body temperature ($T_b$) of dead birds with a 36-gauge thermocouple inserted into cloacae. Dew points, $T_b$, and $T_a$ were recorded every minute for 1.5-2 h.

To ascertain when the system had reached equilibrium after the dead bird was returned to the chamber, I needed to know when ambient air, that had been introduced when I opened the lid, was completely washed out (washout-point: Fig. 2.1a). Because I used different flow rates for each species, I needed to calculate for each one the washout-point, which is a function of flow rate and the volume of the system. This volume was the amount of dry incoming air required to flush the system (washout-volume), and was empirically determined by multiplying flow rate and time to reach the washout-point for each chamber (Fig. 2.1a). Washout-volume is independent of magnitude of the water vapor that was introduced when the lid is opened (Bartholomew, 1981). For chambers and flow rates that I used in our measurements, the minimum washout-point was 17 min and the maximum was 35 min.

Even though chamber air had reached the washout-point, it still could require added time for the skin to reach equilibrium with chamber air. To confirm that skin
would be in equilibrium shortly after the washout-point, I performed an experiment with a semi-permeable membrane, DuoDerm® hydroactive dressing (ConvaTec; Skillman, NJ), affixed to a glass Petri dish filled with water. I found that the equilibration time of the semi-permeable membrane after the washout-point was less than a minute, and thus I felt safe in assuming that dew point values after the washout-point represented water loss from a dead bird (Fig.2.1b). In practice, I waited 30min after the washout-point to assure complete equilibration, and then averaged 10min of dew point temperatures. CWL\textsubscript{dead} was calculated from the same equation as that of a live bird.

2.4 Isolation of SC and extraction of SC lipids

After measuring CWL, I plucked feathers from the bird, removed its skin, and then pinned the skin dermis side down to filter paper, impregnated with 0.5% trypsin in phosphate buttered saline (PBS; pH 7.4; 370mOsm) on a Teflon sheet. I placed the skin in a refrigerator overnight at 4\textdegree{}C, and the next morning, I peeled epidermis from the dermis, and placed the epidermis in fresh 0.5% trypsin solution for 3 hr at 38\textdegree{}C. I then rinsed the SC with distilled water over fine silk mesh to remove any remaining feathers, and stored the SC in a glass test tube filled with nitrogen gas at -20\textdegree{}C. Samples of SC subjected to light microscopy confirmed that epidermal cells were completely removed from the intact SC. Frozen SC samples were lyophilized and dry mass of SC was determined (±0.01mg) with Mettler balance (Mettler Toledo [Columbus, OH], model AB204). To extract lipids from the SC, I placed the SC in mixtures of chloroform and methanol, 2:1, 1:1, and 1:2(v/v), for 2 hr at each step, then pooled the extracted lipids into a single mixture. Each mixture of chloroform:methanol contained 50mg/L of the antioxidant butylated hydroxytoluene (Law et al., 1995; Munoz-Garcia and Williams, 2005).
Figure 2.1. Illustration of (a) washout-point and (b) post-washout equilibration in open flow-through hygrometry system.
2.5 Identification and quantification of SC lipids using thin layer chromatography

Lipid classes were identified using thin layer chromatography (TLC) following procedures modified from Munoz-Garcia and Williams (2005) and Hedberg et al. (1988). I placed 20x20cm silica gel G plates (250µm thick; Analtech Newark, NJ) in a chromatography tank that contained a mixture of chloroform: methanol (2:1 v/v) and allowed the solvent to run to the top to remove contaminants. Then plates were activated in an oven at 110ºC for 30 minutes and scored into 16 lanes. I used 6 lanes for a serial dilution of lipid standards, and the remaining lanes for our samples, each loaded in duplicate. To check accuracy of our quantification of lipids, I also loaded duplicates of a known concentration of a lipid standard mixture on each plate and quantified it to estimate error rate (average error ≤ 2%). Lipids in each sample were analyzed in two different solvent systems to quantify classes of polar and non-polar lipids (Haugen et al, 2003 a,b; Munoz-Garcia and Williams, 2005). To identify polar lipids, I used a mixture of non-hydroxy fatty acid ceramide, galactosylceramide, lactosylceramide and cholesterol sulfate for our standards. I developed each plate with chloroform: methanol: water (40:10:1 v/v/v) to 10cm from the bottom, twice, followed by chloroform:methanol:acetic acid (190:9:1 v/v/v) to 16cm from the bottom, and finally a mixture of hexanes: ethylether: acetic acid (70:30:1 v/v/v) to the top. Between each step of development, the plate was air dried in a fume hood for 10min.

To identify non-polar lipids, our standard mixture contained cholesterol oleate, methyl oleate, hexadecanoic acid, triolene and cholesterol. I developed plates with hexane:ehthyler:acetic acid (80:20:2 v/v/v) to 19cm from the bottom.

I visualized lipid classes on plates by spraying them with a solution of 3% cupric acetate in 8% phosphoric acid and charring them at 160 ºC for 30min. To quantify the amounts of lipid classes, I scanned plates immediately after charring with Hewlett Packard scanner and measured density of each chromatographic band using IMAL 3.5.10c (T.J. Nelson, 2008: Shared software available at [http://brneurosci.org/imal.html](http://brneurosci.org/imal.html)).
Amounts of lipids were expressed in units of mg/g dry SC to correct for differences in SA among birds.

If there were chromatographic bands that did not match with our polar lipid standards, I used preparative thin layer chromatography (PTLC) following Wertz et al. (1983) to isolate unknown lipids. Subsequently I injected these lipids into an Applied Biosystem QTRAP mass spectrometer to identify them (Munoz-Garcia et al., 2006). Unknown bands that did not align with non-polar standards were identified using sequence of bands from other studies which used a similar solvent system, lipid standards and the same TLC plates (Hedberg et al., 1988; Lerary, 2009).

All lipid standards were purchased either from Sigma-Aldrich (St. Louis, MO) or Matreya LLC (Pleasant Gap, PA) and organic solvents, all HPLC grade, were purchased either from Fisher scientific (Pittsburgh, PA) or Sigma-Aldrich.

2.6 Statistics

All statistical tests were performed using SPSS 17.0 with the null hypothesis rejected at \( P \leq 0.05 \). Means are reported \( \pm 1 \) S.E. Prior to analyses, data were examined for normality and homogeneity of variance with Shapiro-Wilk and Levene’s test, respectively. Percentages were logit transformed \( (\log_{10}[\frac{Y}{(1-Y)}]) \); Zar, 1996). A Red-eyed Vireo and House Wren died prior to measurement, so I only used these for the lipid analysis. I removed 5 individual measurements from our CWL data prior to analyses because their values were 2 SD above the mean, a result of their wing having a small cut, or feces sticking to the wire platform on which birds rested.

To compare means of mass specific resting metabolic rate (RMR), and RWL, surface specific CWL\textsubscript{live}, and CWL\textsubscript{dead}, as well as CWL (%TEWL) among species, I used one-way ANOVA. Effect of habitat or migration distance on surface specific CWL\textsubscript{live} was tested with one-way ANOVA as well. All analyses of variance were followed by post-hoc tests for multiple comparisons. Various Post-hoc procedures were chosen depending on homogeneity of variance and/or whether group size was equal or not (Field, 2009).
Data from Eastern Wood-Pewee were removed from all the post-hoc tests because the sample size for this species was one.

To assess the effect of biological control on CWL, I compared CWL_{live} versus CWL_{dead} using ANCOVA with body mass as a covariate. I also used mixed design ANOVA to test for effect of death on surface specific CWL. To explore relationships between body mass and RMR, RWL, CWL_{live}, and CWL_{dead}, among species, I employed conventional least square regression.

To determine the interdependency of lipid classes in the SC, I used a general liner model and regressed different lipid classes against each other. To test for difference in proportion of polar and non-polar lipids I used independent $t$-test.
CHAPTER 3

RESULTS

3.1 Body mass and surface area

For temperate birds ($N = 64$ individuals), body mass ranged from 9.4 to 121.8g where the smallest species was the House Wren and the largest the Mourning Dove (Table 1.1). Surface area of birds varied from 45 to 246 cm$^2$.

3.2 Resting metabolic rate

The rate of oxygen consumption of species varied with body mass (Fig. 3.1). The Eastern Wood-Pewee had the lowest resting metabolic rate (RMR), 18.4 kJ/d and the American robin the highest, 87.7 kJ/d (Table 3.1). Using conventional least square regression, I found a significant positive association between RMR and body mass ($F_{1,11} = 38.9, P < 0.001$; Fig. 3.1).

I calculated mass specific RMR for each individual and compared these values among species. I found that mass specific RMR varied significantly between species (ANOVA, $F_{12,47} = 21.7, P < 0.001$; Fig. 3.2).
Figure 3.1: Whole organism resting metabolic rate of temperate birds had positive association with body mass.
Figure 3.2: Mass specific RMR of temperate bird species.
Table 3.1: Whole organism resting metabolic rate and evaporative water losses of 13 temperate bird species.

<table>
<thead>
<tr>
<th>Species</th>
<th>RMR (kJ/day)</th>
<th>RWL (g/day)</th>
<th>Live CWL (g/day)</th>
<th>TEWL (g/day)</th>
<th>Dead CWL (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>House Wren</td>
<td>27.1±4.6</td>
<td>0.7±0.1</td>
<td>1.2±0.1</td>
<td>1.9±0.1</td>
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<td>0.7±0.1</td>
<td>1.1±0.1</td>
<td>1.8±0.1</td>
<td>1.1±0.1</td>
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<td>American Goldfinch</td>
<td>29.3±2.6</td>
<td>0.7±0.1</td>
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<td>1.0±0.1</td>
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<td>Eastern Wood-Pewee</td>
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<td>0.4</td>
<td>1.5</td>
<td>1.9</td>
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<td>Tree Swallow</td>
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<td>1.3±0.1</td>
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<td>Song Sparrow</td>
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<td>Scarlet Tanager</td>
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<td>3.6±0.2</td>
<td>2.4±0.2</td>
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<td>Northern Cardinal</td>
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<td>3.1±0.2</td>
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<td>Northern Mockingbird</td>
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<tr>
<td>American Robin</td>
<td>87.7±1.5</td>
<td>2.5±0.3</td>
<td>4.6±0.6</td>
<td>7.0±0.8</td>
<td>3.5±0.3</td>
</tr>
<tr>
<td>Mourning Dove</td>
<td>77.0±1.7</td>
<td>2.0±0.3</td>
<td>5.6±0.9</td>
<td>7.6±0.7</td>
<td>2.7±0.4</td>
</tr>
</tbody>
</table>
3.3 Rate of water loss from respiratory passages

Among temperate birds, RWL varied from a low of 0.4 g H₂O/d for the Eastern Wood-Pewee to a high of 3.0 g H₂O/d for the Northern Mockingbird (Table 3.1). I regressed RWL with body mass, and found a significant positive relationship ($F_{1,11} = 10.1, P < 0.01$; Fig. 3.3). To compare RWL among species I normalized values with an individual's body mass (Table 3.2). I found a significant difference in mass-specific RWL among 13 species (ANOVA, $F_{12,46} = 2.4, P < 0.02$). Games-Howell post-hoc test revealed mass-specific RWL of Mourning Dove was significantly lower than that of Northern Cardinal, Scarlet Tanager, Song Sparrow and Tree Swallow. On the other hand, Northern Cardinal had significantly higher RWL per unit body mass than American Robin and Scarlet Tanager.

3.4 Cutaneous water loss of live birds

Cutaneous water loss varied from a low of 1.1 g/d for the Chipping Sparrow to a high of 5.6 g/d for the Mourning Dove (Table 3.1). Using least square regression, I found a positive relationship between body mass and whole organism CWL ($F_{1,11} = 125.6, P < 0.001, r^2 = 0.9$; Fig. 3.4). To explore the relationship between CWL at thermoneutral temperatures and total evaporative water loss (TEWL), the sum of CWL and RWL, I expressed mean CWL of each species as a percentage of TEWL, calculated as $\text{CWL} / (\text{RWL} + \text{CWL}) \times 100$. On average CWL accounted for $64.2 \pm 1.5\%$ of TEWL. Based on ANOVA there was a significant difference between CWL (%TEWL) among the 13 species of temperate birds ($F_{12,46} = 2.1, P < 0.04$). A Student Newman Keuls (SNK) post-hoc test showed that CWL of the Northern Cardinal tended to be a smaller percentage of TEWL than Mourning Dove and Eastern Phoebes (Fig. 3.6). When I expressed CWL per unit surface area, on average, temperate birds lost $27.3 \pm 1.0 \text{ mg H}_2\text{O/cm}^2\cdot\text{day}$. I found no
significant trends in surface specific CWL though our sample size for each species was small ($F = 1.9$, $P > 0.06$; Fig 3.5). It is noteworthy that the Tree Swallow and Eastern Phoebe, both aerial foragers, had the highest CWL per unit surface area (Table 3.2).

### 3.5 Cutaneous water loss of dead birds

Water loss through skin of dead birds, CWL\textsubscript{dead}, varied from 1.0 g/d for the American Goldfinch to 3.5 g/d for the American Robin (Table 3.2). CWL\textsubscript{dead} was positively associated with body mass (g) in dead birds ($F_{1, 11} = 155.4$, $P < 0.001$, $r^2 = 0.9$; Fig. 3.4). Surface specific CWL\textsubscript{dead} varied significantly among species (ANOVA, $F_{12, 44} = 3.4$, $P = 0.001$; Table 3.2). A SNK post-hoc test revealed that passive water loss per unit surface area for Tree Swallows was higher than all other species.

I found that water loss through skin significantly depreciated after death (ANCOVA, $F_{2, 23} = 64.4$, $P < 0.001$; Fig. 3.4). To explore the disparity between CWL of alive and dead birds, I calculated % difference as (CWL\textsubscript{live} – CWL\textsubscript{dead})/CWL\textsubscript{live} * 100, a value that reflects the biological influence on CWL. The mean difference was 13.2 % (Fig. 3.7). Using surface specific data for CWL, I also found that rate of water loss per unit area of skin was significantly lower than that from live birds (mixed design ANOVA, $F_{1, 44} = 21.9$, $P < 0.001$).
Figure 3.3: Whole organism RWL varied positively with body mass.
Figure 3.4: Body mass and rate of water loss through skin from live and dead birds.
Figure 3.5: Surface specific CWL of temperate species.
Figure 3.6: CWL as a percentage of TEWL in 13 species of temperate birds. Dashed line shows 50%. Lower case letters above bars indicate significant difference among species ($P < 0.05$).
Figure 3.7: Percent difference between CWL$_{live}$ and CWL$_{dead}$. 
Table 3.2: Mass-specific RWL and surface-specific CWL\textsubscript{live} and CWL\textsubscript{dead} of 13 temperate bird species. RWL values are in mg H\textsubscript{2}O/cm\textsuperscript{2}·g and CWL values are in mg H\textsubscript{2}O/cm\textsuperscript{2}·d.

<table>
<thead>
<tr>
<th>Species</th>
<th>RWL</th>
<th>Live CWL</th>
<th>Dead CWL</th>
</tr>
</thead>
<tbody>
<tr>
<td>House Wren</td>
<td>71.6±11.4</td>
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<td>25.8±2</td>
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<td>Chipping Sparrow</td>
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<td>21.8±2.3</td>
<td>21.6±1.7</td>
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<tr>
<td>American Goldfinch</td>
<td>62.1±12.0</td>
<td>26.5±3.2</td>
<td>20.2±1.5</td>
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<td>Eastern Wood peewee</td>
<td>35.4</td>
<td>28.5</td>
<td>24.8</td>
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<td>Eastern Phoebe</td>
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<td>19.7±7.0</td>
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<td>77.0±4.3</td>
<td>24.9±3.7</td>
<td>20.1±1.5</td>
</tr>
<tr>
<td>Northern Mockingbird</td>
<td>65.4±20.4</td>
<td>25.1±3.1</td>
<td>20.9±3.5</td>
</tr>
<tr>
<td>American Robin</td>
<td>33.6±4.4</td>
<td>25.8±3.4</td>
<td>19.4±1.7</td>
</tr>
<tr>
<td>Mourning Dove</td>
<td>16.6±2.3</td>
<td>22.6±3.5</td>
<td>19.5±1.6</td>
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</tbody>
</table>
3.6 Effect of habitat and migration distance on cutaneous water loss

Habitat selection of temperate bird species significantly influenced their surface specific CWL\(_{\text{live}}\) \((F_{4, 54} = 2.8, P < 0.04; \text{Fig. 3.8})\). Because of different sample sizes among habitat groups, I used Gabriel’s post-hoc test to determine if groups were statistically different. This test showed that birds living in marshy areas near water tended to have higher surface specific CWL\(_{\text{live}}\) than ones reside in open thickets (Fig. 3.8).

Surface specific CWL\(_{\text{live}}\) varied with the distance that species traveled during migration \((\text{ANOVA, } F_{3, 55} = 5.9, P = 0.001; \text{Fig.3.9})\). Using Gabriel’s post-hoc test, I discovered that medium distance migrants had higher surface specific CWL\(_{\text{live}}\) than other groups of migrants (Fig.3.9).

3.7 Lipids in the stratum corneum

Thin layer chromatography revealed that the SC of temperate birds contained various classes of lipids. To identify these lipid classes I calculated a \(R_f\), the distance traveled by a chromatographic band divided by the distance of the solvent front, for each lipid standard and for unknown bands (Table 3.3). \(R_f\) values for each band were reproducible with little variation between plates \((\pm 0.001)\). I found 5 bands of non-polar and 6 bands of polar lipids that had \(R_f\) values identical to our standards (Table 3.3; Fig. 3.10).

However I also found additional bands that did not coincide with \(R_f\) values of our lipid standards. In our non-polar solvent system, there were 6 unknown bands that appeared close to hexadecanoic acid and cholesterol standards. Here I identified these lipids based on the relative order of bands as given by Hedberg et al. (1988) and Laura et al. (2009), who employed a similar solvent system to the one I used. In our polar solvent system, I found 5 chromatographic bands that migrated close to but not identical to our ceramide standard, and 3 bands that were close to the galactosylceramide standard. For
those unknown bands, using PTLC and mass spectrometry, I confirmed that these bands were ceramides or cerebrosides, but differing in number of hydroxyl groups or double bonds. To distinguish among subclasses of ceramides and cerebrosides, I assigned a number to each band beginning with the band that was closest to the top of the plate, hence the least polar. Presence of ceramide 1, ceramide 6, cerebroside 5, cholesterol derivatives, and cholesterol sulfate varied among species but other lipid classes were consistently found in the SC of all 13 temperate bird species (Table 3.4).

For comparison among species, I normalized the amount of lipids that I extracted by expressing them as mg lipids /g dry SC. Temperate birds had an average of 237.4 mg lipids /g dry SC. The Eastern Wood-Pewee had the smallest total amount of lipids, 129.3 mg/g dry SC, and the Red-eyed Vireo had the largest, 346.0 mg/g dry SC.

To establish the relative contribution of each lipid class to the total, I divided the amount of each class by the quantity of total lipids that I extracted. When I compared the percentage of polar and non-polar lipids of each species, I found that on average non-polar lipids were a larger constituent, 56.1% (t = -4.8, d.f. = 24, P < 0.001).
Figure 3.8: Effect of habitat on surface-specific CWL_{live} of 13 temperate species. Lower case letters above bars indicate significant difference of surface specific CWL_{live} among habitats ($P < 0.05$).
Figure 3.9: Effect of migration distance in surface-specific CWL_{live} of 13 temperate species. Lower case letters above bars indicate significant difference of surface specific CWL_{live} among migration distances ($P < 0.05$).
Figure 3.10: TLC plates after (a) non-polar and (b) polar solvent system developments, showing standard lipids and unknown bands from a sample.
<table>
<thead>
<tr>
<th>Lipids</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol oleate</td>
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<tr>
<td>Methyl oleate</td>
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<td>Hexadecanoic acid</td>
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<tr>
<td>Cholesterol Derivative 3</td>
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<tr>
<td>Diacylglycerol&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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</tr>
<tr>
<td>Cholesterol&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Ceramide 2&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>Cholesterol sulfate</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 3.3: R<sub>f</sub> values of lipid standards and unknown bands from TLC analysis.

<sup>a</sup> Diacylglycerol appeared in two separate bands due to isomerization in acidic solvents (Hedberg et al., 1988)

<sup>b</sup> Cholesterol band appeared in both non-polar and polar solvent systems. The latter number is R<sub>f</sub> from the polar solvent system.

* Lipid classes that were found in samples, but did not match standards.
We combined 4 cholesterol derivatives together.

Table 3.4: Quantity of lipid classes (mg/g dry SC) that are found in the SC of 13 temperate bird species.

|-------------|----------------|--------------------|----------------|------------------|---------------------|-----------------|-------------|------------------|-------------|--------------|---------------|
3.8 Relationship between SC lipids and body mass among species

Classes of lipids varied with mean body mass of 13 species of temperate birds. Ceramide 2 varied positively with body mass for amount and for percentage of total lipids (Fig. 3.11 a,b), whereas the amount of ceramide 3 and %ceramide 5 displayed a negative relationship with body mass (Fig. 3.11d,e). Proportion of cholesterol esters and total cerebrosides changed negatively with body mass as well (Fig. 3.11c,f).

3.9 Relationship between cutaneous water loss and SC lipids

Surface specific CWL_{live} increased as the amount of ceramide 3 and cerebroside 3 increased (Fig. 3.12 a,b). Likewise surface specific CWL_{dead} increased with the amount of cerebrosides 3 in the SC (Fig. 3.13). CWL from live and dead birds did not vary with total amount of lipids in the SC, nor did with the amounts of total polar and non-polar lipids. I did not find any relationship between % lipid classes and CWL.

3.10 Correlations among SC lipids

I found that cholesterol esters, fatty acid methyl esters (FAME) and ceramide 4 varied with total amount of lipids in the SC (Fig. 3.14 a,b,c). When I converted those three lipids into percentage of total amount of lipids, they accounted for 45.3 ± 4.7 %, a large proportion of the total.

There were significant positive associations between total amount of cholesterol and triacylglycerol, and between total amount of ceramides and cerebrosides (Fig. 3.15 a,b). Also percentage of ceramides and cerebrosides displayed the same positive trends.
between each other (Fig. 3.16b). However, I found a significant inverse relationship between % FAME and % triacylglycerol in the SC (Fig. 3.16a).

To further investigate relationships between major lipid classes, I sub-divided ceramides and cerbrosides into subclasses according to their polarity and looked for relationships among SC lipids. I found that the amount of ceramide 5 had a significant positive relationship with cerebroside 2, a parallel to the relationship I found between total amount of cereamide and cerebrosides (Fig. 3.17a). On the other hand, % ceramide 2 was inversely associated with % cholesterol esters (Fig. 3.17b), as was the amount of these two lipids (data not shown). Also % cerebroside 1 and % cerebroside 2 had a negative relationship to each other (Fig. 3.17c).
Figure 3.11: Relationship between body mass and (a) amount of ceramide2, (b) %ceramide2, (c) %cholesterol esters, (d) amount of ceramide3, (e) %ceramide5, and (f) %cerebrosides. Number denotes species by increasing order of body mass.

Number denotes species as follow: 1 = House Wren, 2 = Chipping Sparrow, 3 = American Goldfinch, 4 = Eastern Wood Pewee, 5 = Red-eyed Vireo, 6 = Tree Swallow, 7 = Eastern Phoebe, 8 = Song Sparrow, 9 = Scarlet Tanager, 10 = Northern Cardinal, 11 = Northern Mockingbird, 12 = American Robin, 13 = Mourning Dove.
Figure 3.12: Relationship between surface-specific CWL_{live} and amount of (a) ceramide3 and (b) cerebroside3. Number denotes species as in Fig. 3.11.
$r^2 = 0.76$

$P < 0.001$

Figure 3.13: Relationship between surface specific CWL$_{dead}$ and amount of cerebroside3. Number denotes species as in Fig. 3.12.
Figure 3.14: Amount of total lipid was positively associated with amount of (a) cholesterol esters, (b) FAMEs, and (c) ceramide4.
Figure 3.15: Positive correlations of (a) amount of cholesterol and triacylglycerols, and (b) amount of total ceramides and cerebrosides.
Figure 3.16: Negative association between (a) % FAMEs and % triacylglycerols. Positive associated between (b) % ceramides and % cerebrosides.
Figure 3.17: Positive correlation between (a) amount of ceramide5 and cerebroside2, (b) % ceramide 2 and % cholesterol esters, (c) %cerbroside1 and cerebroside2.
CHAPTER 4

DISCUSSION

Resting metabolism increased with body mass in the 13 species of temperate birds that I measured. When I compared these data with an allometric equation for basal metabolic rate (BMR) of passerine and non passerine birds combined (Tieleman and Williams 1999), RMR that I measured was 1.4 to 2.2 times higher than BMR (Fig. 3.1). Given that metabolic rate of resting birds during their active period is about 25% higher than BMR, which is measured during rest phase, one would expect our values for RMR to be higher than BMR (King, 1974).

Though RWL is a major component of TEWL, and therefore of seminal importance in understanding the water economy of birds, only a few researchers have made direct measurements of RWL (Tieleman and Williams, 1999). Using an indirect method, which incorporated tidal volume, breathing frequency and temperature of exhaled air, Tieleman and Williams (1999) constructed a model for RWL and body size in birds. Combing our data with data in the literature, I compared predictions of the T & W model with our direct measurements of RWL. I included data on RWL for birds that were directly measured (Bernstein, 1979; Wolf and Walsberg, 1996b; Tieleman and Williams, 2002; Mekechnie and Wolf, 2004; Munoz-Garcia and Williams, 2005) or calculated from respiratory parameters (Withers and Williams, 1990; Michaeli and Pinshow 2001; Larcombe et al., 2003). For temperate birds, RWL (g/day) as a function of body mass was described as log RWL\textsubscript{normothermic} = -0.78 + 0.62 log mass (g) ($r^2 = 0.62$, $F = 47.4$, $P < 0.001$, $N = 29$). This is the most complete data available to predict RWL from body mass. The T&W model overestimated RWL by 15% for small birds and by 33% for species that weighed 300g (Fig.4.1).
Figure 4.1: Comparison of allometry of RWL from Tieleman and Williams (1999) model and empirical data.
Desert birds are thought to have evolved a lower CWL than species living in mesic environments (Williams and Tieleman 2005). When Williams and Tieleman (2001) tested this idea using data from literature, they did not find significant differences between desert and mesic species, but data were few. Our larger data set for CWL of mesic birds afforded us an opportunity to revisit this hypothesis. In this analysis I restricted data to birds that had been measured with a mask system as in this study. I found that birds living in arid environments have significantly reduced CWL compared with species living in the mesic habitat (ANCOVA, $F_{1,16} = 13.3, P = 0.002$; Fig. 4.2).

Early on some authors argued that water loss through skin was insignificant compared with the amount of water lost in respiration (Rawles, 1960; Bartholomew and Cade, 1963; Schmidt Nielsen et al., 1969; Mount, 1979), but now it is appreciated that CWL can be a significant proportion of TEWL (Williams and Tieleman 2005). In this study, at thermoneutral temperatures, CWL of temperate birds amounted to, on average, 64.2%, of TEWL. This emphasizes that CWL is a fundamental component of the water economy of birds.

Some have argued that water permeation through the skin is a passive process (Squier and Hall, 1985; Wilson and Mailbach, 1994; Wertz and van den Bergh, 1998), whereas others have suggested that, even though the SC is often envisioned as “dead”, the process of water loss through skin is under biological control (Bernard et al., 2003; Fluhr et al., 2004a,b; Elias, 2004; Falkenberg and Georgiadis, 2008). Munoz-Garcia and Williams (2005) measured surface specific CWL of house sparrows when they were alive, and then compared this value with that obtained by measuring water loss from a water-filled vial covered with a piece of ventral skin glued to the rim. Their experiment was an attempt to isolate biological and physical factors that were influencing water vapor diffusion through skin. They found that average rate of water loss through non-living skin was 3 times higher than surface specific CWL of live birds. In the present study, I measured CWL\textsubscript{live} and CWL\textsubscript{dead} to gain an appreciation of the influence of biological factors on CWL in living birds. However in contrast to what Munoz-Garcia and Williams (2005) found, when biological factors were removed from the skin, water loss rate was depreciated by 13.2% (Fig. 3.7). Removing the skin from a bird might
eliminate some of the physiological gradients that are part of the physical components of the intact skin, and thus explain differences in results of these two studies. For example the calcium gradient in the skin, a critical element for normal barrier function, is formed and maintained without metabolic activity (Elias et al., 2002). Whether such gradients are present in avian skin needs to be determined.

Despite taxonomic and ecological differences among the 13 species, surface specific CWL was remarkably constant and averaged $27.2 \pm 1.0 \text{ mg H}_2\text{O/cm}^2 \text{ day}$ for all species combined. This result is consistent with other investigators who have suggested that environmental influences are a significant factor to determine CWL. (Tieleman and Williams, 2002; McKechnie and Wolf, 2004; Munoz-Garcia and Williams, 2005; 2007).

Previously investigators hypothesized that migratory distance is constrained by water imbalance in long distance migrants (Yapp 1956; 1962; Klaassen, 1995; Klaassen et al., 1999). More importantly Landys et al. (2000) found supporting evidence that long distant migratory birds have adapted behavioral and physiological mechanisms to reduce the water loss while traveling. Therefore I explored relationships between migration distance of temperate bird species and their surface specific CWL. One may expect to find reduced CWL in long distance migratory birds compared with residents or short-distance migrants. Counter intuitively I found birds that travel to Gulf of Mexico for wintering had highest CWL per unit surface area than other type of migrants.

Lipid composition in the intercellular matrix of the SC is a key element for forming a barrier to water vapor diffusion, a fundamental evolutionary innovation that influenced the survival of terrestrial organisms when they invaded land (Williams and Tieleman, 2005). Originally it was thought that lipids played only a minor role in impeding CWL, primarily because they accounted for only 10-15% of the dry weight of the SC (Wertz and van den Bergh, 1998). However experiments that removed intercellular lipids with organic solvents demonstrated that intercellular lipids are an integral component of the permeability barrier in the skin (Sweeney and Downing 1970). Later Golden et al. (1987) monitored thermal behavior of SC lipids via differential calorimetry and infrared spectroscopy and concluded that intercellular lipids in the SC form an impermeable barrier against water by maintaining higher activation energy for
water flux than that of free diffusion at physiological temperature. In our study I found 
that intercellular lipids in the SC of temperate bird species accounted for \( 24.5 \pm 1.5 \% \) of 
the total dry mass of the SC, as much as a 2-fold increased compared with human SC. 
Despite its higher lipid content, the skin of birds is approximately 2 times more 
permeable to water vapor than to that of mammals (Marder et al., 1987; Menon et al., 
1986; Menon and Menon, 2000; Munoz-Garcia and Williams, 2005). Together these 
findings, higher lipid content and greater water diffusion, suggest that types and 
proportions of lipids rather then the density per se of lipids in the SC determines the rate 
of water loss through the skin (But see Lampe et al., 1983).

Birds possess a unique lipid composition in their SC compared with that of 
humans and/or other tetrapod vertebrates (Fig 4.3; Menon et al. 1986; Munoz-Garcia and 
Williams, 2005; 2007; Lillywhite, 2006). In mammals and humans the major lipids in the 
SC are ceramides (ca. 38%), cholesterol (ca. 26%) and free fatty acids (ca. 16%) (Melnik 
et al., 1989; Elias et al., 1979; Law et al., 1995). Although many authors have indicated 
that the major lipid constituents of the reptilian meso layer, homologous to the SC in 
mammals and birds, are cholesterol, FFA, ceramides, and phospholipids (Robert and 
Lillywhite, 1980; Robert and Helmkamp, 1982; Burken et al., 1983; Elias and Menon, 
1991; Lillywhite, 2006), I were unable to find quantitative data to support this claim. The 
only data that I could find were from Ahern and Downing (1974) who found no polar 
lipids in Florida Indigo snake. Hence I used data from Ahern and Downing (1974) for 
comparison. I found striking differences in the proportions of lipids in the SC of birds 
compared with other taxa: the proportions of ceramides and free fatty acids are evidently 
reduced, but the proportions of triacylglycerols, FAMEs, and cerebrosides were markedly 
higher.
Figure 4.2: Whole organism CWL as a function of a body mass in desert and mesic environments.
Figure 4.3: Comparison of (a) polar and (b) non-polar lipid composition from the SC of human, mammals, birds and reptiles.
The intercellular lipid composition of avian SC and mammalian stratum granulosum (SG) shows close resemblance (Elias et al., 1979). SG is a mammalian epidermal layer where hydrolytic enzyme activities take place to transform lipids before they migrate into the SC (Elias et al., 1979). Therefore in the SG, high levels of phospholipids and cerebrosides but low levels of free fatty acids were found as a result of incomplete enzymatic modification (Lampe et al.; 1983). In bird epidermis, the layer before the SC is called stratum transitivum (ST), where multigranular bodies release precursor lipids and enzymes into the extracellular space as they move toward the SC (Wertz, 2000; Groff et al., 2007; But see Menon and Menon, 2000). Similarity between the lipid composition of avian SC and mammalian SG implies that enzymes may still be active in the SC of birds like in the SG of mammals. Cox et al. (2008) reported presence of β-glucocerebrosidase, which converts cerebrosides into ceramide in mammalian epidermis to achieve proper barrier function in the avian SC. (Holleran et al., 1994). Although the authors demonstrated that enzyme activity can be altered by acclimation in birds, they found a negative relationship between concentration of ceramide and activity of β-glucocerebrosidase. This suggests that there are more hydrolytic enzymes than β-glucocerebrosidase involved in modification of lipid molecules in the avian SC. Positive association between concentrations of ceramides and cerebrosides from this study also supports this idea because if β-glucocerebrosidase was a primary enzyme, then I should observe a negative correlation between ceramides and cerebrosides (Fig 3.15b;3.16b).

Comparisons of our results on lipids with other work is problematic because of ambiguity in identification and/or quantification of lipid classes (Haugen et al., 2002 a,b; Munoz-Garcia and Williams, 2005; 2007). Haugen et al., (2002 a,b) only used ceramide, cholesterol, and stearic acid as their lipid standards for TLC analysis. Galactosylerceramide standard was added to the analysis in Munoz-Garcia and Williams (2005; 2007), but they omitted non-polar lipid standards such as sterol esters, triacylglycerols, and FAME. When I first attempted to develop TLC plates using non-polar solvent system from Munoz-Garcia and Williams (2005), I learned that triacylglycerols, FAME, and free fatty acids were not well resolved in the chromatography (Ro, unpubl.). Therefore in this study I modified the solvent system to achieve optimal separation among unknown bands.
Insufficient thin-layer chromatographic separation in Munoz-Garcia and Williams’ (2005) lipid analysis probably led them to overestimate the concentration of free fatty acids.

Cholesterol esters are thought to be important in diffusional pathways for hydrophilic solutes across the skin (Swartzendruber et al. 1995). A broadly accepted hypothesis is that hydrophilic materials diffuse through aqueous channels or pores in the skin. Although direct evidence for such channels is yet to be found, many researchers have inferred the presence of aqueous channels by localizing lacunar domains within the SC (Swartzendruber et al. 1995; Menon and Elias, 1997; Sznitowska et al., 1998; Kushner et al., 2007). Swartsendruber et al. (1995) claimed that lacunar domains do not contain lamellae and thus they are comprised of non-lamellar phase lipids such as cholesterol esters. From our study I found that some lipid classes were positively associated with total amount of SC lipids indicating those lipids were major ingredients that can significantly influence the overall amount of lipids. Of note, one of the lipid classes was cholesterol esters, which presumably can form aqueous channels in the SC. Moreover the proportion of cholesterol esters in the SC lipids varied across species from 2% in Mourning Doves to 20% in House Wrens.

Relationships among SC lipids are thought to influence characteristics of the skin, such as fluidity in layers of the SC, and formation of lamellae (Bouwstra et al. 2003; Haugen et al., 2003a,b). Because lipids get “processed” as they move from the lower epidermal layers to the SC via hydrolytic enzymes, different lipids are interrelated to each other by being a precursor or a product before and after enzyme activity (Elias et al, 1979; Wertz and van den Bergh, 1998). In our study I found an inverse relationship between triacylglycerol and FAME, which are thought be metabolically related (Fig.3.16a). However in most cases the significance of correlations among lipid classes remains an enigma. For example ceramides and cerebrosides are thought to be metabolically related because the enzyme, β-glucocerebrosidase, cleaves a hexose from cerebrosides to produce ceramides. Hence one would expect to see an inverse relationship of between them. However, I consistently found that concentrations of ceramide and cerebroside were positively associated (Fig. 3.15b;3.16b;3.17a). This could
indicate that there are multiple enzymes that influence concentration of a single lipid class. For example sphingomyelinase can also produce ceramide molecules from altering sphingomyelins (Menon et al. 1986). On the other hand it could be that molar concentration should be considered to understand relationships among lipid classes rather than concentration by weight.

How lipids organize in the intercellular space of the SC is critical for constructing a potent water barrier. Much of information about SC lipid organization came from electron microscopy or small and wide angle X-ray diffraction studies (Madison et al., 1987; Swartzendruber, 1992; Garson et al., 1991; Bouwstra et al., 1992). X-ray diffraction research suggested that intact SC possesses approximately an equimolar ratio of cholesterol:ceramides and this ratio is responsible for a lamellar phase in the mammalian SC, complementing earlier electron microscopic observations of lamellar structure in the SC (Bouwstra et al., 2000). Although lipids in the avian SC are believed to be organized in lamellae (Peltonen et al., 2000), whether lipid composition follows the same molar ratio of mammalian SC lipids is uncertain. Because avian SC contains a small proportion of cholesterol, the molar ratio of cholesterol:ceramide was 0.05:1 in temperate House sparrows (Munoz-Garcia et al. 2008). In our study, the molar ratio of cholesterol to ceramide was also 0.05 to 1, assuming fatty acid chain length was 18 carbons long in ceramides. This supports observations from Bouwstra et al. (2000) that phase behavior of *in vitro* cholesterol and ceramide mixture was insensitive to alteration in molar ratio unless ceramide 1, a ceramide molecule containing ω-hydroxyacid ester-linked to linoleate, was absent. Therefore presence of ceramide 1 will be critical for lamellar formation in the avian SC. Munoz-Garcia and collaborators (2008) confirmed that ceramide 1 was one of the most abundant ceramide sub-classes in the SC of House sparrows.

I attempted to connect structure and function of stratum corneum within a wide array of species from temperate environment. Early work, which explored natural variation in CWL and SC lipids in larks, found that lower surface specific CWL was influenced by higher proportion of ceramides in the SC (Haugen et al., 2003a; Munoz-Garcia et al., 2005). In present study I also found the same trend between CWL and
proportion of total ceramides, but it was not statistically significant ($P > 0.4$). Although I did not find statistical difference in surface specific CWL among temperate species, I found that marginal variations were positively associated with amounts of ceramide 3 and cerebroside 3, which accounted for $1.4 \pm 0.2$ and $0.4 \pm 0.1\%$ of total lipids respectively (Fig. 3.12a,b). This result indicates that the minor constituents can have a significant effect on the overall function of the SC. To understand how minor components can alter the permeability of the SC, I would need to first investigate molecular identities of ceramide 3 and cerebroside 3.


