Cutaneous Water Loss and Covalently Bound Lipids of the Stratum Corneum in Adult and Nestling House Sparrows (*Passer domesticus*) from Desert and Mesic Habitats

THESIS

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By

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Abstract

Lipids of the stratum corneum (SC), the outer layer of the epidermis of birds and mammals, provide a barrier to water vapor diffusion through the skin. The SC of birds consists of flat dead cells, called corneocytes, and two lipid compartments: an intercellular matrix and a monolayer of covalently bound lipids (CBL) attached to the outer surface of corneocytes. We previously found two classes of sphingolipids, ceramides and cerebrosides, covalently bound to corneocytes in the SC of house sparrows and that these were associated with cutaneous water loss (CWL). In this study, we collected adult and nestling house sparrows from Ohio and nestlings from Saudi Arabia, acclimated them to either a high or low humidity environment, and measured their rates of CWL. We also collected natural populations of nestlings from Ohio and Saudi Arabia from 2 days after hatching until they fledged, and measured their CWL rates. We then evaluated the composition of the CBL of the SC using thin layer chromatography. We found that CBL development differed between habitats, but that lipid density generally increased with age. CBL profile did not exhibit phenotypic plasticity with acclimation and was mostly the same between habitats. CWL appears to be functionally related with the interactions of CBL classes as a whole, and this may be associated with age. Finally, we found that house sparrows have a very diverse range of CBLs in their SC, including free fatty acids and cholesteryl esters, and we propose a new model for CBL organization.
Dedication

This document is dedicated to my Grandma and Grandpa Baker for always giving me a real answer when I was a child and repeatedly asked "why?"
I thank Joe Williams for first introducing me to research as an undergraduate, inspiring me to go to graduate school, and advising me as a graduate student; Dave Denlinger, Mitch Masters, and Carmi Korine for their helpful conversations and comments on this manuscript; John Harder for recommendations and lending equipment; Jenny Ro for assistance in the lab when I was first learning techniques; Cindy Bronson for giving me the pleasure of being able learn about physiology while teaching physiology; Agus Muñoz-Garcia for his collaboration and outstanding mentorship (I have no doubt that he will be an excellent advisor to graduate students of his own in the near future), and he and Alex Champagne, Liz Calhoon, and Leslie Sadowski for support, assistance, and friendship, both in the lab and out, and for comments on earlier drafts of this manuscript.

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# Table of Contents

Abstract ........................................................................................................................................ ii

Dedication ................................................................................................................................... iii

Acknowledgments ..................................................................................................................... iv

Vita .............................................................................................................................................. v

List of Tables ............................................................................................................................ vii

List of Figures .......................................................................................................................... viii

Chapter 1: Introduction ............................................................................................................. 1

Chapter 2: Materials and Methods ......................................................................................... 7

Chapter 3: Results ..................................................................................................................... 13

Chapter 4: Discussion ............................................................................................................... 17

References ................................................................................................................................. 24

Appendix A: Tables ................................................................................................................. 28

Appendix B: Figures ................................................................................................................ 29
List of Tables

Table 1: Sample sizes of natural populations of nestlings and adults by age (in days) and habitat.................................................................28

Table 2: Principal component analysis of quantities (mg/g dry SC) of covalently bound lipid classes of the SC of adult and nestling house sparrows from desert and mesic habitats. ..........................................................................................................................28
List of Figures

Figure 1: Mean cutaneous water loss of natural populations of Ohio and Saudi Arabia nestlings from age 2 days to adult................................................................. 29

Figure 2: Mean cutaneous water loss of Ohio and Saudi Arabia fledglings after acclimation to high or low humidity................................................................. 30

Figure 3: Mean cutaneous water loss of Ohio fledglings and adults after acclimation to high or low humidity................................................................. 31

Figure 4: Mean quantity of covalently bound cerebroside in natural populations of Ohio and Saudi Arabia nestlings by age................................................................. 32

Figure 5: Mean quantity of covalently bound cholesterol in natural populations of Ohio and Saudi Arabia nestlings by age................................................................. 33

Figure 6: Mean quantity of covalently bound ceramide in natural populations of Ohio and Saudi Arabia nestlings by age................................................................. 34

Figure 7: Mean ratio of covalently bound free fatty acid:ceramide in natural populations of Ohio and Saudi Arabia nestlings by age................................................................. 35

Figure 8: Mean ratio of covalently bound ceramide:cerebroside in natural populations of Ohio and Saudi Arabia nestlings by age................................................................. 36

Figure 9: Means of covalently bound lipid quantities and ratios of Ohio and Saudi Arabia fledglings after acclimation to high or low humidity................................................................. 37
Figure 10: Means of covalently bound lipid quantities of Ohio fledglings and adults after acclimation to high or low humidity .......................................................... 38

Figure 11: Means of covalently bound lipid ratios of Ohio fledglings and adults after acclimation to high or low humidity .......................................................... 39

Figure 12: Eigenvector plot of covalently bound lipid loadings for principal components 1 and 3 .......................................................... 40

Figure 13: Principal component analysis score plot of mean scores of sparrow groups for principal components 1 and 3 .......................................................... 41

Figure 14: A new model for CBL arrangement .......................................................... 42
Chapter 1: Introduction

Being the largest organ of the vertebrate body, the skin is involved in a number of complex physiological processes, including protection from invading pathogens, vitamin D production, lipid synthesis, protection from UV radiation, and formation of the lipid barrier that prevents excessive water loss from the skin (Elias and Feingold, 2006). This last function is performed by the outer layer of the epidermis of the skin, called the stratum corneum (SC), which is composed of layers of corneocytes embedded in a lipid matrix. The SC regulates cutaneous water loss (CWL), thereby assuring the hydration of internal tissues, which is essential for normal chemical reactions within the cell (Lillywhite, 2006). CWL is the most important avenue of water loss, exceeding fecal water loss 5 fold in small avian species (Bartholomew, 1972). CWL accounts for more than 65% of total evaporative water loss, the remainder being respiratory water loss (Tieleman and Williams, 2002; Ro and Williams, 2010). In desert environments, where high ambient temperatures and low relative humidity create conditions which result in high evaporative water demand (Tieleman and Williams, 2002), birds likely experience intense selection to minimize CWL (Williams and Tieleman, 2005).

In birds, the skin is comprised of the lower vascular dermis and the avascular epidermis, which receives all nutrients and oxygen by diffusion from the dermis. The epidermis of birds consists of a basal layer of mitotic cells and several transitional layers of cells called
the stratum transitivum, and the outer SC which consists of layers of flattened corneocytes embedded in a matrix of lipids (Lucas and Stettenheim, 1972). In the lower layers of the stratum transitivum, cells contain organelles known as multigranular bodies (similar to lamellar granules in mammals; Landmann, 1988), the structures involved in lipid synthesis (Menon and Menon, 2000). Multigranular bodies are thought to coalesce into lipid droplets at the stratum transitivum/corneum interface, and these lipids are thought to escape into the extracellular domain via porosities or breaks in the cell membrane (Menon and Menon, 2000). Prior to the extrusion of the lipids, some are enzymatically converted into other lipid moieties. In mammals, the first lipids to be exocytosed fuse to the protein envelope of the corneocyte, forming a monolayer of lipids ester-linked to glutamate residues of involucrin, known as the lipid envelope (Wertz and Downing, 1987; Menon and Menon, 2000; Wertz, 2000; Gu et al., 2008). These covalently bound lipids are thought to provide scaffolding for the organization of the intercellular lipid layers, but the exact mechanism of how they accomplish this feat remains unknown. The rest of the lipids are exocytosed into the intercellular space where they form the intercellular lipid lamellar compartment (Bouwstra et al., 2003; Menon and Menon, 2000). These two lipid compartments, the intercellular lipids (ICLs) and covalently bound lipids (CBLs), together with the corneocytes of the SC, constitute the epidermal permeability barrier (Elias, 1983; Blank et al., 1984; Elias and Menon, 1991; Menon and Menon, 2000; Bouwstra, 2003; Madison, 2003). In this study, we focus on the permeability barrier function of the CBLs in birds.
The ICL compartment in birds consists of cholesterol esters, methyl esters, triacylglycerides, cholesterol, free fatty acids, and two classes of amphipathic sphingolipids: ceramides and cerebrosides, the latter are ceramide molecules with a hexose sugar attached to the sphingosine head group (Muñoz-Garcia and Williams, 2005; Ro and Williams, 2010). CBLs in house sparrows (*Passer domesticus*) consist of ω-hydroxyceramides and ω-hydroxycerebrosides (Gu et al., 2008). In mammals, all cerebrosides are converted to ceramides at the stratum granulosum/corneum interface by removal of the hexose sugar by acid hydrolases, specifically the enzyme β-glucocerebrosidase (Holleran et al., 1994; Madison, 2003). The presence of cerebrosides in the SC of mammals is diagnostic for Gaucher's disease, a pathology involving drastically increased CWL rates (Holleran et al., 1994). In birds, however, both ceramides and cerebrosides are found in the intercellular and covalently bound SC lipid compartments, and these molecules function together to form the barrier (Muñoz-Garcia and Williams, 2005; Gu et al., 2008). The reason why the presence of cerebrosides in the SC of mammals is pathological but in birds seems to reduce CWL remains unknown.

It may be that CBLs contribute to the permeability barrier in birds by forming hydrogen bonds with water molecules and providing cohesion between corneocytes (Munoz-Garcia et al, 2008). The large sugar moieties of the covalently bound cerebroside head groups could be sequestering water molecules in the SC, which would aid internal hydration (Gu et al., 2008). The polar heads of covalently bound ceramides of adjacent corneocytes may interact with one another to bind corneocytes together at their end plates (Gu et al, 2008;
However, the role of CBLs in barrier formation, or even the exact nature of the lipids that form this layer, remains largely unknown in birds.

CBLs may also indirectly influence CWL by acting as a template for the ICLs during the exocytosis of lipid droplets from the corneocytes at the stratum transitivum/corneum interface, with the ICLs in turn directly influencing CWL (Wertz, 2000; Gu et al., 2008). CBLs are formed when the terminal hydroxyl group of the fatty acid moiety is enzymatically linked to a hydroxyl group on the protein envelope (Wertz and Downing, 1987; Downing, 1992; Stewart and Downing, 2001). If the ceramides are organized in this way in birds, as they are in mammals, with the fatty acid "tails" attached to corneocytes, then the sphingosine "heads" of ceramides and possibly cerebrosides would project into the intercellular space and interact with the ICLs, which have been shown to play a major role in adjusting CWL (Muñoz-Garcia and Williams, 2005; Muñoz-Garcia and Williams, 2008; Muñoz-Garcia et al., 2008), possibly by converting ICL cerebrosides to ceramides with β-glucocerebrosidase (Cox et al., 2008). It is unknown whether CBLs influence CWL, but house sparrows living in environments with different aridity levels have been shown to have different compositions of CBLs. Adult desert house sparrows have a lower ratio of covalently bound ceramides to cerebrosides than mesic adult house sparrows, which correlated with lower CWL rates (Gu et al, 2008). However, we do not know if sparrows exposed to humidity regimes different from their natural environment will show plasticity for CBL composition. If the CBLs do orchestrate the arrangement of
ICLs, it is possible that they may also exhibit phenotypic plasticity and provide different scaffolding for the formation of ICL lamellae in response to changing CWL needs.

The differences observed in CBLs between desert and mesic house sparrows could be the result of natural selection, phenotypic flexibility of adults, developmental plasticity of young, or a combination of these factors. In our experiment, we took desert and mesic nestling house sparrows and split each population into two groups, then exposed the groups to different humidity acclimation treatments until they fledged. We established that each population experienced different CWL levels at high and low humidity, and then quantified their CBLs to test for plasticity. We repeated this experiment with adult mesic house sparrows to test for differences with ontogeny within that population. Based on what we know about CBLs and what has been observed in the ICL compartment in prior studies (Muñoz-Garcia and Williams, 2005; Muñoz-Garcia and Williams, 2008; Muñoz-Garcia et al., 2008; Gu et al., 2008), we hypothesized that mesic adult house sparrows would exhibit phenotypic plasticity of CBLs in response to humidity acclimation treatment, that desert and mesic fledgling house sparrows would show some genetic divergence in their CBL levels, but also exhibit plasticity of CBLs in response to humidity acclimation treatment, and that CBL quantities would increase with ontogeny within the population of mesic house sparrows.

Little is known about the ontogeny of CWL in nestling birds, or the development of the lipids of their SC that influence this parameter. The selection pressure for CWL on
nestling birds may be different than that of adults, because adults can seek shade during hot parts of the day, but nestlings are confined to the nest and therefore have limited opportunity to behaviorally thermoregulate. Nestlings cannot search for drinking water but rather must rely on parents to supply it, usually in the foods given them (Williams and Tieleman, 2001). Menon et al. (1988) found that nestling zebra finches (*Poephilia guttata*) increased CWL with age and that only dehydrated finches extruded lipid droplets into the intercellular matrix, whereas Groff et al. (2007) found that surface-specific CWL decreased with age in nestling house sparrows (*Passer domesticus*). They found that ICLs in the epidermis were low at hatching, increased around day four, coincident with the eruption of feathers, and then steadily decreased to day 12 in young sparrows, regardless of hydration status. This change in the ICL compartment may be related to the non-linear change in CWL seen in desert house sparrow nestlings. The ontogeny of CBLs and their influence on CWL is unknown, as well as how the ontogeny of CBLs may differ between desert and mesic habitats. To explore this, we tracked the CWL and CBLs of natural populations of desert and mesic house sparrows, one population in Saudi Arabia, the other in Ohio. Based on the results of a study that tracked ICLs of desert and mesic house sparrow nestlings (Muñoz-Garcia and Williams, in-press), we hypothesized that nestling house sparrows from Saudi Arabia and Ohio would have different developmental trajectories for their CBLs and exhibit genetic divergence in CBL profiles due to natural selection acting on CBLs to minimize CWL in desert birds.
Chapter 2: Materials and Methods

Nestling house sparrows from Ohio and Saudi Arabia

We placed nest boxes around the National Wildlife Research Center, near Taif, Saudi Arabia (22°15'N, 41°50'E), between March and April, 2006, and Columbus, OH (40°00'N, 83°10'W), between mid April and late August, 2006. After pairs built nests and laid eggs, we checked boxes daily until eggs hatched; we designated the day of hatch as day 0. We collected two 3- to 4-day-old chicks from each nest, which we assumed were full-siblings. Thereafter, we randomly assigned one of the siblings from each pair to a low humidity regime (6.5g H₂O/m³ at 30°C) and the other to a high humidity regime (31 g H₂O/m³ at 30°C). Details of the experimental design are described in Muñoz-Garcia and Williams (2008). In total, we had four groups of nestlings in our experiment; dry-acclimated and humid-acclimated individuals from Saudi Arabia (n = 7 and n = 5, respectively) and from Ohio (n = 11 and n = 12, respectively). CWL was measured as described in Muñoz-Garcia and Williams (2008; see also Tieleman and Williams, 2002). We later compared the CBLs of fledglings from Saudi Arabia and Ohio to each other to examine the effects of variables "treatment" and "habitat" on CBL profile. Experiments were approved by the Institutional Laboratory Animal Care and Use Committee of Ohio State University (Protocol 2006-A0085) and the National Commission for Wildlife Conservation and Development, Riyadh, Saudi Arabia.
In addition to the nestlings assigned to acclimation groups, we randomly selected nestlings of known age from their nest box, beginning with day 0, the day of hatch, to fledgling, at day 14-16, for measurements of CWL and CBL composition without acclimation. See Table 1 for sample sizes. In this group of birds, we later compared CBLs of nestling house sparrows from Saudi Arabia and Ohio to each other to examine the effects of the variables "age" and "habitat" on CBL profile. CWL of sparrows was measured as described in Muñoz-Garcia and Williams (in-press; see also Tieleman and Williams, 2002).

**Adult house sparrows from Ohio**

We captured adult house sparrows (*Passer domesticus*) with mist nets and Potter Traps in Columbus, OH (40°00'N, 83°10'W) during February to March 2006. After taking initial measurements of CWL, respiratory water loss, and oxygen consumption, the sparrows were randomly assigned to one of three acclimation treatment groups: high humidity (31 g H₂O/m³ at 30°C, \( n = 9 \)), low humidity (6.5 g H₂O/m³ at 30°C, \( n = 13 \)), and non-acclimated (\( n = 9 \)). CWL was measured as described in Muñoz-Garcia et al. (2008; see also Tieleman and Williams, 2002). We later compared the CBLs of adult sparrows from Ohio with the CBLs of the fledgling sparrows from Ohio acclimated to high or low humidity, to examine the effects of the variables "treatment" and "age" on CBL profile. Experiments were approved by the Institutional Laboratory Animal Care Use Committee of Ohio State University (protocol 2003-A0072).
Extraction of covalently bound lipids

After measuring CWL, we sacrificed the birds, plucked their feathers, and removed their skin. The SC was isolated and the ICL compartment was removed following the protocol in Muñoz-Garcia et al. (2008).

To confirm that all intercellular lipids had been extracted, we selected 8 samples at random and soaked the SC for each bird for 2h in chloroform:methanol 1:2 (v/v) containing the antioxidant butylated hydroxytoluene (BHT). We examined the extracts for the presence of lipids using thin layer chromatography. No lipids were detected, indicating complete extraction of ICL.

After ICL were extracted, we freeze-dried the SC samples for 12 hours and stored them at -20°C in an atmosphere of nitrogen. Thereafter, we thawed samples, weighed the dry SC, and extracted the CBL (Gu et al., 2008). We washed the CBL extracts again via Folch extraction (Folch et al., 1957) to remove any inorganic solutes, and removed any remaining particulate matter by passing the solution through a 0.45 µm PTFE filter (Millex, Millipore Corp., Bedford, MA, USA). We dried the CBL extracts under a stream of nitrogen and stored them at -20°C.

Prior to thin layer chromatography, we re-constituted the CBL extracts in 40-130 µl of chloroform:methanol 2:1 (v/v) containing 50 mg/l of BHT, with the volume of solvent
chosen based on the original mass of the dry SC for each sample (about 10 µl of solvent per 1.5 mg of dry SC).

Isolation and quantification of lipid classes

We separated lipid classes of the SC using analytical thin layer chromatography on 20 x 20-cm glass plates (0.25 mm thick; Adsorbosil-Plus 1; Altech). We developed the plates with chloroform:methanol 2:1 (v/v) to the top to remove contaminants, activated them in an oven for 30 minutes at 110°C, and divided them into 10mm lanes.

We prepared a series of five lipid standards of known concentration via serial dilution. These standards contained ceramides, cerebrosides, cholesterol, free fatty acids, and cholesteryl oleate. We dissolved the standards in chloroform:methanol 2:1 (v/v) with BHT. We loaded 5 µl of each standard and sample onto the preadsorbant area of the plates in duplicate with a Teflon-tipped Hamilton syringe.

We used two solvent systems for development. We developed more polar lipids, such as ceramides, cerebrosides, and cholesterol esters, with chloroform:methanol:water (40:10:1 v/v/v) to 5cm from bottom, chlofoform:methanol:acetic acid (190:9:1 v/v/v) to the top twice, and hexane:ethyl ether:acetic acid (70:30:1 v/v/v) to 15 cm from bottom. For more non-polar lipids (such as free fatty acids and cholesterol), we developed the plates with hexane:ethyl ether:acetic acid (70:30:1 v/v/v) to the top once. After development, we
sprayed the plates with 3% cupric acetate in 8% phosphoric acid and visualized the lipids by charring them on an aluminum hotplate for 30 min at 160°C.

To quantify the concentration of lipid classes, we scanned the plates with a Hewlett-Packard scanner and measured absorbance of lipid bands using IMAL TN-Image 3.5.10c (T.J. Nelson, 2008: Shared software available at http://brneurosci.org/imal.html). We calculated standard curves of each lipid class and compared the absorbance of lipids in our samples with that of the standards of known concentration to determine the sample lipid concentration. To validate our method of calculating lipids, we followed our protocol using known concentrations of cholesterol as our unknown and compared that to our cholesterol standards. The average error, calculated as [(observed - actual) / actual] x 100 was 3.97% ± 2.82% (n = 30).

Statistics
Lipid quantities were log transformed if they did not meet the assumption of normality after examining them with a normal probability plot. We performed all statistical tests with SPSS 16.0 (SPSS Inc.; Chicago, IL) and Minitab 15.0 (Minitab Inc.; State College, PA) with the null hypothesis being rejected when \( p \leq 0.05 \).

To compare CWL and lipid quantities in adults and fledglings from Ohio after acclimation to high or low humidity, we used a two-way ANOVA with age and treatment as fixed factors. We also used two-way ANOVA, with habitat and treatment as fixed
factors, to compare CWL with lipid quantities in fledglings from Saudi Arabia and Ohio after acclimation to high or low humidity. Finally, we used two-way ANOVA to compare the effects of age and habitat on CWL with lipid quantities in natural populations of nestlings from Ohio and Saudi Arabia. We did not do three-way ANOVAs because while we had three variables, the three groups differed in which variables they contained. When differences amongst groups were significant, we performed a Tukey test to determine which groups were different.

We interpreted a significant interaction term as differences in the degree of phenotypic plasticity between groups. If the interaction term was not significant, but treatment was significant, we interpreted this result to mean that experimental groups had the same degree of phenotypic plasticity. If neither the interaction term nor treatment were significant, we concluded that our experimental groups were not plastic.

To evaluate the functional relationship between CBL composition and CWL, we used PCA on the quantities (in milligrams of lipid per gram of dry SC) of each family of CBLs (Shaw, 2003). This analysis yielded uncorrelated composite variables, the principal components. We extracted components with eigenvalues greater than one as our selection criterion. We determined associations between CWL and principal component scores using linear regression models with principal component scores as our independent variables and CWL as our dependent variable.
Chapter 3: Results

Cutaneous water loss

Natural populations of sparrow nestlings from Ohio and Saudi Arabia

The interaction term for age and habitat was significant for CWL ($F = 6.2, p < 0.001$; Fig 1). Nestlings from Ohio and Saudi Arabia showed different developmental trajectories for CWL.

Saudi Arabia fledglings vs. Ohio fledglings after humidity acclimation

The interaction term for treatment and habitat was significant for CWL ($F = 7.6, p = 0.009$; Fig 2). Fledglings from Ohio showed plasticity for CWL, whereas fledglings from Saudi Arabia did not.

Ohio adults vs. Ohio fledglings after humidity acclimation

Treatment: Adults and fledglings from Ohio showed equal plasticity for CWL. Treatment was a significant factor, with birds acclimated to high humidity regimes had significantly higher CWL than birds acclimated to low humidity regimes ($F = 29.5, p < 0.001$; Fig 3).

Age: There were differences in CWL with ontogeny for birds in Ohio. Age was a significant factor, with fledglings from Ohio had a significantly higher CWL than adults from Ohio ($F = 94.2, p < 0.001$; Fig 3).
Covalently bound lipid quantities and ratios

Lipid classes detected in the CBL compartment of the stratum corneum

In our thin layer chromatography plates, we detected cholesteryl esters, free fatty acids, ceramides (five separate classes), cerebrosides, and cholesterol, all covalently bound to corneocytes of adult and nestling house sparrows. We also detected several neutral lipid classes, which appeared to be triglycerides based on Rf values. We combined the quantities of cholesteryl esters and cholesterol for statistical analysis because we think that the free cholesterol we observed was likely cholesteryl esters that had their fatty acid moiety hydrolyzed during the extraction process. We quantified the lipids as milligrams of lipid per gram of dry SC for analysis. In our ANOVA calculations, we used total ceramide quantities. In our PCA calculations, we kept the five ceramide classes separate.

Natural populations of Ohio nestlings vs. Saudi Arabia nestlings

The interaction term for habitat and age was significant in cerebroside quantities \( (F = 4.8, p = 0.002; \text{Fig 4}) \). Natural populations of nestlings from Ohio and Saudi Arabia showed different developmental trajectories for covalently bound cerebrosides.

Age: There were similar trajectories in lipids with ontogeny across habitats. Age was a significant factor \( (p < 0.001 \text{ in all cases}) \) in cholesterol \( (F = 6.37; \text{Fig 5}) \) and ceramide quantities \( (F = 15.8; \text{Fig 6}) \), and in the ratio of free fatty acids to ceramides \( (F = 5.5; \text{Fig 7}) \) and ceramides to cerebrosides \( (F = 8.19; \text{Fig 8}) \).

Habitat: There were similar developmental trajectories of different magnitudes between Ohio and Saudi Arabian populations. The quantity of cholesterol \( (F = 6.9, p = 0.012; \text{Fig} \)
5) and the ratio of free fatty acids to ceramides ($F = 9.1, \ p = 0.004$; Fig 7) were higher in Saudi Arabian nestlings than Ohio nestlings. The quantity of ceramides ($F = 10.4, \ p = 0.002$; Fig 6) and ceramides to cerebrosides ($F = 69.9, \ p < 0.001$; Fig 8) were higher in Ohio nestlings than Saudi Arabian nestlings.

**Saudi Arabia fledglings vs. Ohio fledglings after humidity acclimation**

**Treatment:** Treatment was not a significant factor for any lipid quantity.

**Habitat:** There was some genetic divergence between Ohio and Saudi Arabian populations. Ohio fledglings had a higher quantity of ceramides ($F = 10.5, \ p = 0.003$; Fig 9) and a lower free fatty acid to ceramide ratio than Saudi Arabia fledglings ($F = 11.96, \ p = 0.002$; Fig 9).

**Ohio adults vs. Ohio fledglings after humidity acclimation**

**Treatment:** Treatment was not a significant factor for any lipid quantity.

**Age:** There were differences in lipids with ontogeny for Ohio birds. Ohio adults had a significantly higher quantity of cholesterol ($F = 5.5, \ p = 0.023$; Fig 10), ceramides ($F = 19.3, \ p < 0.001$; Fig 10), and cerebrosides ($F = 110.4, \ p < 0.001$; Fig 10) than fledglings. Ohio fledglings had a significantly higher ratio of free fatty acids to ceramides ($F = 15.9, \ p < 0.001$; Fig 11) and ceramides to cerebrosides ($F = 21.99, \ p < 0.001$; Fig 11) than adults.

*Relationship between cutaneous water loss and covalently bound lipids*
To reduce the number of variables in our data, we used PCA on the quantity (in milligrams of lipid per gram of dry SC) of each class of CBLs. Three axes accounted for 76.6% of the variance (Table 2). We regressed the principal component scores against CWL and found PC1 and PC3 to be significant \( \text{CWL} \ [\text{mg H}_2\text{O}/(\text{cm}^2\cdot\text{d})] = 18.376 - 1.356 \ (\text{PC1 score}) - 1.852 \ (\text{PC3 score}); \ R^2 = 0.23, \ p < 0.001 \).

We plotted our eigenvector loadings (Fig. 12), and PC1 appears to separate ceramide 1 and, to a lesser extent, free fatty acids from the rest of the lipid classes. PC3 further separates the lipids roughly according to polarity: free fatty acids, the least polar, are at the bottom, and the lipids increase in polarity towards the top.

A plot of average scores for the five groups of sparrows shows clear separation between age groups along PC1 (Fig. 13). When this plot is overlaid with the eigenvector plot, the younger sparrows appear to cluster near the eigenvector for ceramide 1, while the adults cluster with the rest of the polar lipids. The adult sparrows had greater variance in principle component scores than nestlings, with the fledglings somewhat in-between the other two.
Chapter 4: Discussion

This is the first study to examine the experimental effects of changing environmental factors on phenotypic plasticity in CBLs, and one of the first studies of CBL function in birds. Overall, we found that young sparrows from Ohio and Saudi Arabia have different developmental trajectories for several CBL classes; however there is a general trend of increasing lipid density with ontogeny. CBL profile does not appear to be a plastic trait in sparrows in response to short-term change in environmental humidity, but that there is still some degree of genetic divergence between Ohio and Saudi Arabia populations of house sparrows with respect to their CBL profiles. We found that CWL appears to be functionally related with the interactions of CBL classes as a whole, and that this may be associated with age. Finally, we found that house sparrows have a very diverse range of CBLs in their SC, particularly when compared with mammals. It is already known that sparrows have covalently bound cerebrosides, which mammals do not. In addition, we also found that sparrows have cholesteryl esters in their CBL compartment, which has not been seen in any other animal, as well as neutral lipids in abundance, which are generally considered to be present only in trace amounts in mammals.

*Development of covalently bound lipids in natural populations*

The developmental trajectories of covalently bound ceramides and cholesteryl esters from hatch to fledgling were similar in nestling house sparrows from Ohio and Saudi Arabia,
but Saudi Arabian nestlings had higher quantities of cholesteryl esters at each step of
development than Ohio nestlings, and the reverse was true for ceramides. This trend of
Ohio nestlings having more CBL ceramides than Saudi Arabian nestlings is the opposite
of ICL ceramide development trends in Saudi Arabian and Ohio nestling sparrows,
where Saudi Arabian nestlings have more ICL ceramides than Ohio nestlings at each step
of development (Muñoz-Garcia and Williams, in-press). Interestingly, while Ohio
nestlings had a higher quantity of CBL ceramides in adulthood than as nestlings, Saudi
Arabian sparrows maintained a similar level of CBL ceramides into adulthood (data on
ceramide quantities in Saudi Arabian adults from Gu et al., 2008, see Fig 6). Saudi
Arabian nestlings had a higher quantity of covalently bound cerebrosides than Ohio
nestlings at hatch, but their trajectories converged around day 8 and followed similar
upward trajectories from days 8 to 16. Adult sparrows had more CBL cerebrosides than
nestlings, and Ohio adults had more CBL cerebrosides than Saudi Arabian adults,
however, suggesting that the trajectories diverge sometime after day 16.

*Effects of humidity acclimation, habitat aridity, and ontogeny on covalently bound lipids*

None of the CBL classes in the SC of sparrows were sensitive to humidity acclimation
treatment in any of our treatment groups, indicating that CBLs do not show phenotypic
plasticity in response to short term changes in water loss. We chose an acclimation period
of three weeks because mammals are known to renew their SC within that amount of
time (Rawlings, 2005), but there is not sufficient data to ascertain how long it takes for
birds to turn over the cells of their SC. The concentration of CBLs do not change with
respect to SC depth as ICLs do (Popa et al., 2010) because they are covalently attached to the protein envelope, therefore it is possible that the SC must be completely renewed before changes in CBL are observable.

Covalently bound ceramides were found to be significantly different between desert and mesic sparrows, indicating a genetic divergence between the two populations, presumably based on some kind of selective pressure over evolutionary time. It is possible that there may be indirect selection pressure on covalently bound ceramides based on habitat aridity. ICLs do show phenotypic plasticity to humidity treatment and evidence indicates that they are susceptible to selection pressure based on habitat aridity (Muñoz-Garcia et al., 2008; Muñoz-Garcia and Williams 2005; Muñoz-Garcia and Williams, 2008). If CBLs play a role in orchestrating the ICLs (Wertz and Downing, 1987; Wertz, 2000; Gu et al., 2008), they may have an indirect effect on CWL, and could then be under selection pressure due to habitat aridity. This could be the reason why we see a genetic difference in covalently bound ceramides between desert and mesic populations.

All of the lipid classes we found in the CBL compartment except free fatty acids showed an increase in quantity with age. Adult house sparrows from Ohio had more covalently bound cholesteryl esters, cerebrosides, and ceramides than fledgling house sparrows from Ohio. This is consistent with trends in the ICL compartment, with adult birds generally
having a higher quantity of skin lipids per gram SC than younger birds (Muñoz-García and Williams, 2008; Muñoz-García and Williams, in-press).

**Relationship between covalently bound lipids and cutaneous water loss**

Two of our principal components were correlated with CWL in all sparrows, indicating a functional relationship between CBL composition and CWL. Our first principle component appeared to separate ceramide 1, the least polar ceramide, and free fatty acids from the other lipid classes. Ceramide 1 was only found in nestlings and fledglings, and the nestling and fledgling sparrows tended to cluster in the same quadrant on the score plot as the eigenvector for ceramide 1 (Figs 12 and 13). Nestlings and fledglings also had higher CWL rates than adult sparrows, so ceramide 1, and to some degree free fatty acids, may be an important part of the CBL barrier in young sparrows that is lost as the sparrows age. Nestling birds have an abundance of neutral lipids in their plasma for energy during growth that is not seen in adult birds (Dobado-Berrios et al., 1998; Quillfeldt et al., 2004; Williams, personal communication), and this may translate into nestlings having more free fatty acids incorporated into their epidermal lipids just by virtue of their abundance in the body. An increased level of free fatty acids in the CBL compartment would result in a series of "gaps" in the polar head groups that interact with water molecules and provide cohesion between the CBL and ICL compartments (Gu et al., 2008). If the CBLs orchestrate the organization of the ICL lamellae, the gaps would result in disruptions in proper ICL organization. This would permit more water to flow
between the CBL and ICL, as well as between the layers of the ICL, and would result in higher CWL rates in nestlings.

Adult sparrows tend to cluster in the same quadrant on the score plot as the eigenvectors for cerebrosides and many ceramides. Adult house sparrows from Saudi Arabia and Ohio have different covalently bound ceramide to cerebroside ratios, and this is correlated with different CWL rates (Gu et al., 2008). Clearly ceramides and cerebrosides are influencing CWL in adult house sparrows. It may be the case that the lipid classes of primary influence on CWL in the CBL compartment shift during ontogeny. Ceramide 1 and free fatty acids are more abundant in younger birds, whereas adult birds do not have ceramide 1 and have greater control over their free fatty acid levels, resulting in fewer free fatty acids being incorporated into the CBL compartment. This would allow adult birds to have greater control over their CWL via the covalently bound ceramides and cerebrosides.

A new model for covalently bound lipids

Previous studies have indicated the existence of sphingolipids and free fatty acids in the CBL compartment of birds and mammals (Wertz and Downing, 1987; Gu et al., 2008); however this study is the first to show the existence of covalently bound cholesteryl esters and significant amounts of covalently bound free fatty acids in avian SC. Cholesteryl esters are likely attached to the protein envelope of corneocytes via the terminal hydroxyl group of the fatty acid moiety, similar to the mechanism of attachment
of covalently bound sphingolipids in skin (Wertz and Downing, 1987; Downing, 1992; Stewart and Downing, 2001).

Gu et al. (2008) proposed a water shell model, in which the hexose moieties of covalently bound cerebrosides sequester water molecules to reduce CWL. This sequestration may take place via an ordering of water molecules around the hydroxyl groups of the hexose moieties, forming an aggregate of water molecules. The ordered water molecules will exhibit strong hydrogen bonding, thus individual water molecules will require more energy to break away from the aggregate and percolate out of the SC through the intercellular lipids. Gu et al. (2008) also determined that covalently bound ceramides and cerebrosides in house sparrows have fatty acid moieties that are 26 hydrocarbons long. Our new model predicts that the hydrocarbon chains of the fatty acid moiety for cholesteryl esters would be either significantly shorter or significantly longer than 26 hydrocarbons, so that the non-polar cholesteryl groups would sit between the fatty acid moieties of either the covalently bound sphingolipids or the intercellular sphingolipids, possibly interdigitating the two lipid compartments (Fig 14). Our prediction is based on the arrangement of cholesterol in the plasma membrane, where free cholesterol sits mainly within the nonpolar interior, associating with the fatty acid chains of phospholipids or glycosphingolipids (Simons and Ikonen, 1997; Brown, 2002). Free fatty acids would likely not have hydrocarbon chain lengths longer than 26 hydrocarbons, so as to avoid the water molecules sequestered by cerebrosides. The arrangement of these
CBLs would be constricted by the size of the lipids and the positioning of the glutamate residues on involucrin.

**Implications and avenues for further research**

In light of human-induced environmental change, such as global warming, it is crucial to understand the mechanisms that produce phenotypic variation during the lifetime of an individual (Helmuth et al., 2005). From this study, we suggest that future research focus on understanding the mechanism by which covalently bound ceramides and cerebrosides influence CWL. It may be that covalently bound ceramides, particularly ceramide 1, play a bigger role in orchestrating ICL arrangement and regulating CWL during ontogeny, with cerebrosides and the more polar ceramides (ceramides 2-5) becoming involved in the process later in life. Additionally, the role of cholesteryl esters in the structure of the corneocyte lipid envelope, which we have detected for the first time, should be explored to better understand their impact on CWL and membrane fluidity. Studying the effect of skin lipids on CWL can give us a broader perspective on how populations of birds and mammals will be able to weather climate change, as well as allowing us to study skin lipids such as cerebrosides in non-pathological states in order to better understand certain human diseases (see Gaucher's disease; Holleran et al., 1994).
References


### Appendix A: Tables

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<th>Age</th>
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<th>6</th>
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<th>10</th>
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<td>1</td>
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Table 1: Sample sizes of natural populations of nestlings and adults by age (in days) and habitat.

- Data from this study, fledglings acclimated to humidity treatment.
- Data taken from Gu et al, 2008.

These groups are included in the figures for reference but were not included for statistical analysis.

<table>
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<th>Correlation PC and original variable</th>
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<th>PC3</th>
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Table 2: Principal component analysis of quantities (mg/g dry SC) of covalently bound lipid classes of the SC of adult and nestling house sparrows from desert and mesic habitats.
Figure 1: Mean (± S.E.) cutaneous water loss of natural populations of Ohio and Saudi Arabia nestlings from age 2 days to adult. The interaction term for age and habitat was significant.

Values for Saudi Arabia nestlings at day 16 are taken from acclimation experiments in this study. Values for Saudi Arabia adults are taken from Gu et al, 2008. These groups were not included in the statistical analysis but are included here for reference.
Figure 2: Mean (± S.E.) cutaneous water loss of Ohio and Saudi Arabia fledglings after acclimation to high or low humidity. The interaction term for humidity treatment and habitat was significant. Letter groupings show statistical significance.
Figure 3: Mean (± S.E.) cutaneous water loss of Ohio fledglings and adults after acclimation to high or low humidity. Age and humidity treatment were both significant factors. All four groups are statistically significant.
Figure 4: Mean (± S.E.) quantity of covalently bound cerebroside in natural populations of Ohio and Saudi Arabia nestlings by age (in days). The interaction term for age and habitat was significant.

Values for Saudi Arabia nestlings at day 16 are taken from acclimation experiments in this study. Values for Saudi Arabia adults are taken from Gu et al, 2008. These groups were not included in the statistical analysis but are included here for reference.
Figure 5: Mean (± S.E.) quantity of covalently bound cholesterol in natural populations of Ohio and Saudi Arabia nestlings by age (in days). Both age and habitat are significant factors.

Values for Saudi Arabia nestlings at day 16 are taken from acclimation experiments in this study. This group was not included in the statistical analysis but has been included here for reference. No data was available for cholesterol in Saudi Arabia adults.
Figure 6: Mean (± S.E.) quantity of covalently bound ceramide in natural populations of Ohio and Saudi Arabia nestlings by age (in days). Both age and habitat are significant factors.

Values for Saudi Arabia nestlings at day 16 are taken from acclimation experiments in this study. Values for Saudi Arabia adults are taken from Gu et al, 2008. These groups were not included in the statistical analysis but are included here for reference.
Figure 7: Mean (± S.E.) ratio of covalently bound free fatty acid:ceramide in natural populations of Ohio and Saudi Arabia nestlings by age (in days). Both age and habitat are significant factors.

Values for Saudi Arabia nestlings at day 16 are taken from acclimation experiments in this study. This group was not included in the statistical analysis but has been included here for reference. No data was available for free fatty acids in Saudi Arabia adults.
Figure 8: Mean (± S.E.) ratio of covalently bound ceramide:cerebroside in natural populations of Ohio and Saudi Arabia nestlings by age (in days). Both age and habitat are significant factors.

Values for Saudi Arabia nestlings at day 16 are taken from acclimation experiments in this study. Values for Saudi Arabia adults are taken from Gu et al, 2008. These groups were not included in the statistical analysis but are included here for reference.
OH Fledglings SA Fledglings

Figure 9: Means (± S.E.) of covalently bound lipid quantities and ratios of Ohio and Saudi Arabia fledglings after acclimation to high or low humidity. Habitat was a significant factor for the lipids listed here. Treatment groups have been combined since it was not a significant factor.
Figure 10: Means (± S.E.) of covalently bound lipid quantities of Ohio fledglings and adults after acclimation to high or low humidity. Age was a significant factor for the lipids listed here. Treatment groups have been combined since it was not a significant factor.
Figure 11: Means (± S.E.) of covalently bound lipid ratios of Ohio fledglings and adults after acclimation to high or low humidity. Age was a significant factor for both ratios. Treatment groups have been combined since it was not a significant factor.
Figure 12: Eigenvector plot of covalently bound lipid loadings for principal components 1 and 3. See Table 2 for abbreviations. Based on principal component analysis of quantity of covalently bound lipid classes (mg/g dry SC).
Figure 13: Principal component analysis score plot of mean scores (± S.E.) of sparrow groups for principal components 1 and 3. Based on principal component analysis of quantity of covalently bound lipid classes (mg/g dry SC).
Figure 14: A new model for CBL arrangement. We predict that the hydrocarbon chains of the covalently bound free fatty acids and the fatty acid groups of covalently bound cholesteryl ester would need to be significantly shorter than the fatty acid moiety of covalently bound sphingolipids in order to avoid water molecules sequestered by cerebroside hexose groups.