EPIDERMAL LIPIDS AND THEIR RELATIONSHIP TO CUTANEOUS WATER LOSS IN HOUSE SPARROWS (PASSER DOMESTICUS) FROM DESERT AND MESIC ENVIRONMENTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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The Ohio State University
2008

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The outer layer of the integument of birds, called the stratum corneum (SC), serves as a barrier to water vapor diffusion through the skin, but also a thermoregulatory function; high CWL keeps body temperatures under lethal limits in episodes of heat stress. The SC consists of corneocytes and an extracellular matrix of lipids. We measured cutaneous water loss (CWL) in two populations of house sparrow, *Passer domesticus*, one living in the deserts of Saudi Arabia, another living in mesic Ohio. We found that CWL was lower in desert birds than in mesic birds. We analyzed intercellular lipids in the SC, sphingolipids, cholesterol, and free fatty acids (FFA), by thin layer chromatography and high performance liquid chromatography coupled with atmospheric pressure Photospray® ionization mass spectrometry. Compared with mesic sparrows, desert birds had a higher amount of sphingolipids, which had longer carbon chains in the fatty acid moiety and higher polarity than in mesic sparrows, alterations that resulted in modifications of the interactions of the lipid molecules within the SC.

We tested the effect of humidity on CWL and lipid composition of SC by acclimating adult and nestling sparrows from both populations to a dry or a humid environment. Dry-acclimated adult sparrows reduced CWL by 36% compared with those humid-acclimated. The FFA:ceramide ratio was significantly lower in dry-acclimated birds. Dry-acclimated nestlings had lower CWL than humid-acclimated birds, a reduction
associated with the modification of FFA of the SC in mesic nestlings, and with the
modification of sphingolipids in desert nestlings. We showed that nestling house
sparrows from both localities had higher CWL than adults in their natural environment, a
result of major modifications of the lipid composition of the SC. Desert nestlings were
more plastic for CWL and lipids of the SC than mesic nestlings, likely a response to
opposed selection pressures, thermoregulation and water conservation, at different life
stages.

Our results suggest that differences in CWL and the lipid composition of SC
between desert and mesic sparrows were the result of a combination of genetic
differences between populations, phenotypic flexibility of adults, and developmental
plasticity of nestlings.
Dedicated to my father
ACKNOWLEDGMENTS

I want to thank Joe Williams, the best possible adviser for me. I learnt tons of things from you. You taught me in the field, in the lab, and most important, how pivotal is to think critically in science. I think we have made a great team over the years, and I hope you agree on that.

I thank my committee members, W.M. Masters, D.L. Stetson, and P.W. Wertz, for their patience and their support all the time I have taken to finish my work. Thanks, Mitch, for being a great instructor when I was your teaching assistant.

My name appears in some papers next to that of colleagues I greatly admire at the professional and personal level; thanks to J.C. Brown, B. Cox, B. Groff, Y. Gu, M. Jurkowitz, S. Owstrowski, J. Ro, B.I. Tieleman, A. Walker, P. Wiersma, and M. Yamaguchi; it was fun and easy to work with all of you.

I thank all my lab mates these six years: Mike Haugen, Valerie White, Emily Blosser, Brandon Groff, Amy Walker, Bob Cox, Popko Wiersma, Yu Gu, Jenny Ro, Jen Olson, Helen Fan and Alex Champagne. They have all helped me in one way or another with my research, and they have all contributed to create a very stimulating environment, not only at work. I particularly thank Mike Haugen because he rescued me my first weekend in Columbus, when an unfortunate series of events made me consider the idea of coming back to Barcelona.
G. Menon, D. Crumrine, P. Elias, G. Menon, W. Holleran, Y. Uchida, W. Erdahl, C. Chapman, M. Schmittgen, R. Bluel, B.K. McNab and M. C. Sullards helped me in one way or another to have my research done. I thank Tom Grubb for his help in some of my experiments and for serving in my candidacy exam committee. I also thank Brad Ackerman, Frank Belas, Dale Duckworth, Tom Lindsay, Kenneth Roth, Kenneth Ruterbories and Steve Swanson, all of Eli Lilly, for stimulating discussions about lipids, chromatography and mass spectrometry, and for their kindness and patience when I stayed in Indianapolis learning a completely new technique for me.

I was lucky enough to travel for my research to very different and interesting parts of the world, and, fortunately, I always found good people wherever I went. In Saudi Arabia, I thank Patrice Paillat, Abdulrahman Khoja, and the people of the NWRC. I especially thank Stephane Ostrowski and Catherine Tsagarakis, not only for their assistance in various aspects of my research but also their support and hospitality while I stayed at the center. In Panama, Rainaldo Urriola, scientific coordinator of the Smithsonian Tropical Research Institute, made our work much less difficult. I also thank David Bradley and Mike Libsch, but particularly Betzi Pérez and Ruby Zambrano for their help in my research and their friendship.

I want to thank people that had a great influence on my early steps of my career. Amongst them, my previous adviser at the Universitat Autònoma de Barcelona, J.P. Hervás, and my lab mates there, Lluís Rodriguez-Dopazo, Jacques de la Flor and
Joaquim Martí-Clua. I also want to thank Josep Graells, Mari Carmen Santa-Cruz and Josep Pons. I want to thank particularly Jesús Matallanas and Lluís Tort, which helped and supported me through my stay at the UAB and beyond. They are a very important reason of why I started this journey.

Funding for my dissertation project was received from the Ohio State University, the National Wildlife Research Center (NWRC) and the National Science Foundation (IBN-0212092 to Joseph B. Williams). I express my appreciation to the National Commission for Wildlife Conservation and Development, Riyadh, for support during our research. Wildlife research programs at the NWRC have been made possible through the support of His Royal Highness Prince Saud Al Faisal and under the guidance of Dr. Abdulaziz H. Abuzinada.

The Department of EEOB has been a very nice place to work. I want to thank all the people I met here, especially Christine Johnson; we had a valuable friendship, and I enjoyed our great discussions about science and politics.

Thanks to all my friends in Columbus who made my stay here easier, particularly Hiram J. Irizarry, Reni Ayachitula, Carla Giai and her family, and Deniz Yucel for their friendship. My friends in Barcelona provided invaluable support at different stages of my dissertation; thanks to Xavier, Mariangeles and Pili.

Thanks to all the members of my family, my brother Bernat, my aunts Carmen and Balbina, my uncle Antonio and my cousins Xesca, Oriol, Alex, Bea and Bernardo,
and Carlota. I missed you all but I felt you all were with me here. This dissertation would
not have been possible without the unconditional support of my parents. I knew I wanted
to be a Biologist since I was just a kid, and my parents actively encouraged me to pursue
whatever path I chose. I want to thank especially my father; I know he would have been
very proud of me now.

I met Susana Rodriguez a week after my arrival in Columbus and all this time she
has been there for me whenever I needed it. Thanks to Maria Jose Roa, Maria Gonzalez
and Estela Quintero for all the moments we have shared here. I am really fortunate to
have enjoyed your friendship and support. You made me understand what true friendship
means all these years. Thanks for making my choice of coming to Columbus the best
decision I have ever made in my life.
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CHAPTER 1

CUTANEOUS WATER LOSS AND LIPIDS OF THE STRATUM CORNEUM IN HOUSE SPARROWS FROM ARID AND MESIC ENVIRONMENTS

INTRODUCTION

Terrestrial animals face the challenge of maintaining an adequate state of hydration of internal tissues while being exposed to a desiccating external environment. Among important evolutionary innovations of animals that became terrestrial were mechanisms that reduced overall water loss, thereby promoting water homeostasis. Given that water is the sine qua non of life, it is intriguing that animals can live in deserts, environments with little drinking water, high ambient temperatures ($T_a$), intense solar radiation, low humidity and desiccating winds. Because desert birds are typically diurnal, they at times experience high ambient temperatures, requiring them to evaporate water from their respiratory passages and skin to keep body temperature below lethal limits (Williams and Tieleman 2005). Birds also have high mass-specific rates of metabolism, which drives respiratory water loss (RWL), further exacerbating problems of water balance in birds living in deserts (Dawson and Bartholomew 1968, Noy-Meir 1973, Williams and Tieleman 2002). One might envision that natural selection has and
continues to favor those phenotypes that can minimize rates of evaporative water loss under normal circumstances. But when $T_a$s exceed body temperatures, phenotypes will be favored if evaporation from skin and respiratory passages successfully regulates body temperature.

In birds, evaporative water losses are the major avenue of water efflux, often five times greater than urinary and fecal water losses (Dawson 1982). Total evaporative water loss (TEWL), the sum of cutaneous water loss (CWL) and RWL, is reduced in birds living in deserts compared to those from mesic environments (Williams 1996, Williams and Tieleman 2000). Evidence thus far indicates that this reduction in TEWL cannot be explained by a decrease in RWL (Tieleman et al. 1999, Tieleman and Williams 1999). Exploring the idea that natural selection has influenced CWL in desert birds, Tieleman and Williams (2002) measured CWL of four species of larks, two from mesic and two from arid environments, and found that CWL was reduced in larks from arid environments.

CWL is a function of the water vapor gradient between skin and air, and the total resistance to vapor diffusion through skin, feathers, and boundary layer (Webster and King 1987, Wolf and Walsberg 1996, Williams and Tieleman 2001). Resistance across the skin contributes 70-95% of the total resistance (Webster et al. 1985). Although early studies posited that CWL was not an important source of water loss in birds (Mount 1979), later investigations showed that CWL represented more than 50% of total water
efflux (Bernstein 1971, Dawson 1982, Webster and King 1987, Wolf and Walsberg 1996, Williams and Tieleman 2005). Mechanisms that decrease CWL will therefore contribute to a reduction in total water loss in birds that live in deserts. Tieleman and Williams (2002) suggested that reduced CWL observed in desert larks could be achieved by increasing resistance through the skin, and they proposed that changes in the lipid content of the stratum corneum (SC) would serve this purpose. However, the skin of some species of birds also plays a role in thermoregulation, at least when Tₐs are high (Bernstein 1971, Marder and Ben-Asher 1983). So, when Tₐs are favorable, desert birds should have minimal water loss through skin, but when Tₐs exceed body temperature, CWL should be elevated. This dual role of skin might have implications for the lipid organization within the SC.

**The epidermis of birds**

As the largest and one of the most important, yet underappreciated, organs of the vertebrate body, the skin serves a number of functions important in survival including defense against invading pathogens, thermoregulation, and as a barrier to CWL (Chuong et al. 2002, Elias 2004).

The epidermis of the skin of birds consists of four layers, all derived mitotically from the cells in the basal layer. Cells of the stratum basale, the innermost layer, have a large Golgi apparatus that apparently synthesizes lipids (Menon et al. 1986). Cells of the
stratum intermedium, the layer above basal cells, form multigranular bodies (MGB), homologous to the lamellar bodies of mammals (Landmann 1980, Menon et al. 1986). MGB are membrane-bounded organelles about 0.5 µm in diameter that contain lipids, mainly glycosphingolipids, cholesterol, and phospholipids, which are thought to be stacked in layers called lamellae (Elias and Menon 1991). In the stratum transitivum, it is thought that lamellae inside the MGB deteriorate and MGB coalesce to form membrane-free neutral lipid droplets, at least under normal circumstances (Landmann 1986, Elias and Menon 1991). At the stratum transitivum-stratum corneum interface, lipid droplets are apparently extruded into the intercellular spaces of the SC creating the barrier to water vapor diffusion (Scheuplein and Blank 1971, Elias et al. 1981, Blank et al. 1984, Grubauer et al. 1989, Elias and Menon 1991, Menon and Menon 2000).

In mammals and birds, CWL is mediated primarily by the SC, formed by multiple layers of flattened dead cells, called corneocytes, and two compartments of lipids; one fills the intercellular spaces of the SC and the other consists of covalently bound lipids to corneocytes, called the lipid envelope (Wertz and Downing 1987, Menon and Menon 2000, Lillywhite 2006); these lipids determine the rate of water permeation through the skin (Potts and Francoeur 1991, Madison 2003).
**Intercellular lipids of the stratum corneum**

In mammals, lipids in the intercellular spaces of the SC are primarily a nearly equimolar mixture of cholesterol, free fatty acids, and ceramides, sphingolipids that consist of a sphingosine molecule attached to a fatty acid by an ester bond (Gray and Yardley 1975, Bowstra et al. 2003, Lillywhite 2006). In birds, these same lipid classes are also found in the SC, but cerebrosides, formed by a ceramide covalently linked to a hexose, also constitute a large fraction of the intercellular lipid mixture (Muñoz-Garcia and Williams 2005). Lipids within the SC are organized in layers called lamellae, but the details of how individual lipid molecules are involved in the organization of the SC remain obscure (Kitson et al. 1994, Bouwstra et al. 2003, Hill and Wertz 2003). It is thought that ceramides form the structural backbone of the lamellae, and those containing linoleic acid serve to rivet bilayers together (Bouwstra et al. 2003, Lillywhite 2006). Free fatty acids form hydrogen bonds with ceramides, maintaining the cohesion of the lamellae (Bouwstra et al. 2003). At the concentrations found in the SC of mammals, cholesterol promotes the stability of the lamellae (Norlén 2001). In mammalian epidermis, cerebrosides are initially extruded into the extracellular spaces, but thereafter enzymatically converted to ceramides and therefore they do not occur in the SC. In contrast, birds have a high proportion of cerebrosides in the SC. In humans, accumulation of cerebrosides in the SC results in a pathological state characterized by an increase in CWL and dry scaly skin, but in birds normal SC function involves cerebrosides (Holleran
et al. 1993, Muñoz-Garcia and Williams 2005). The association between cerebrosides and CWL in birds remains to be explored in detail.

If the SC is the barrier to water vapor diffusion, and if the layer of dead corneocytes and lipids forms this barrier, it would seem that the barrier properties would be the same even when skin is removed from the bird. CWL is thought to be a passive diffusion process influenced by the type and arrangement of lipids in the SC (Pinnagoda 1994, Wilson and Maibach 1994, Hoffman and Walsberg 1999). If evaporation through the skin is a passive process, then rates of water loss through the skin of a living bird, and rates of water loss through the same skin removed from the bird ought to be nearly the same. Although the physical properties of the SC do influence CWL, biological mechanisms may also operate in the live animal enhancing the water barrier (Elias 2004). Most of these processes involve the creation of gradients of ions across the SC that influence water permeation through lipid layers. For example, changes in the pH and calcium gradient across the SC alter the permeability of the skin, and affect barrier recovery after disruption (Menon et al. 1994, Bernard et al. 2003, Fluhr et al. 2004a, b). Despite the variety of biological mechanisms known to modify the performance of the permeability barrier in the skin, no studies have attempted to separate the contribution of physical and biological factors in the formation of the epidermal water barrier.

Few studies have explored the association between the lipid composition of the SC and CWL in birds. Working with 8 species of larks along an environmental gradient
from mesic to arid, Haugen et al. (2003a) found that larks living in arid environments have a reduced CWL, a higher proportion of ceramides, and a smaller proportion of free fatty acids in their SC. They concluded that reduced CWL observed in desert larks was associated with changes in ratios of lipid classes in the SC.

The biochemical properties of lipid molecules in the SC also seem to be important in determining water loss through the skin (Lillywhite 2006). Longer carbon chains in ceramides and cerebrosides presumably form a more tightly packed SC, and therefore a greater barrier to water vapor diffusion (Schaefer and Rodelmaier 1996). More polar ceramides tend to form a tighter barrier to water loss because they will create stronger molecular interactions (Haugen et al. 2003a). Cerebrosides contain a sugar molecule, and therefore can potentially bind molecules of water, in contrast to ceramides, cholesterol, and free fatty acids (Norlén 2001).

Thin layer chromatography (TLC) has been used to a useful tool to separate and quantify lipid classes in the SC of vertebrates. In theory, the use of different TLC systems in combination with other techniques, such as gas chromatography, allows the assessment of many different molecules of lipid in the SC (Wertz et al. 1985, Karlsson and Pascher 1971). However, it is not possible to resolve each individual molecule of sphingolipids unambiguously (Raith et al. 2000). Therefore, each band of lipids on a chromatography plate represents a number of different molecules that might differ in chain length, or in head groups that yield similar polarity (Ponec et al. 2003, Ro et al. unpublished data).
These uncertainties constitute an important limitation of TLC because knowledge about individual lipid molecules within the SC is crucial to our understanding of molecular underpinnings of barrier function.

To overcome the limitations of TLC in discriminating individual molecules of lipid, we developed a method to identify individual ceramides and cerebrosides using reversed phase high performance liquid chromatography (HPLC) coupled with atmospheric pressure photoionization mass spectrometry (APPI-MS) (Muñoz-Garcia et al. 2006). Our method uses retention time, molecular weight and fragmentation patterns to identify sphingolipids and, in most cases, unambiguously elucidate their biochemical structure. Using this information permits more detailed insights into the possible arrangement of the lipid molecules within the SC.

**Covalently bound lipids of the stratum corneum**

Corneocytes of the SC are encapsulated by several structural proteins, notably involucrin and loricrin (Downing 1992, Marekov and Steinert 1998). In electron micrographs of SC from which all intercellular lipids have been extracted, one can observe, on the exterior surface of corneocytes, a translucent layer of lipids shown to be ω-hydroxyceramides covalently bound to the protein envelope (Wertz and Downing 1987, Wertz et al. 1989, Downing 1992, Stewart and Downing 2001). An important protein involved in the formation of covalent bonds with lipids is involucrin, a protein
structured as a beta-sheet along the surface of the corneocyte (Downing 1992, Marekov and Steinert 1998). Non-polar amino acids face the surface of the corneocyte, whereas polar amino acids with negative charges, such as glutamate, are positioned on the external surface of the cell. Lipids covalently bound to corneocytes are thought to be ester-linked to glutamate residues of involucrin molecules and can be liberated only after mild alkaline hydrolysis (Wertz and Downing 1987, Downing 1992, Madison 2003). Hydroxyl groups of glutamate form ester bonds with the terminal hydroxyl group of the fatty acid moiety of ceramides and the sphingosine head interacts with lamellae of lipid in the extracellular spaces (Wertz et al. 1989, Downing 1992, Stewart and Downing 2001). Covalently bound lipids are thought to serve as a cohesive force binding corneocytes together at their end plates and to act as a template that orchestrates the lamellar organization of the intercellular lipids of the SC. Therefore, in mammals, these lipids appear to play a fundamental role in the formation of a barrier to water vapor diffusion (Wertz et al. 1989, Madison 2003, Farwanah et al. 2007).

The chemical structure of the avian protein and the covalently bound lipid of the SC has received less attention than that devoted to mammalian skin. Recent studies on corneocytes of avian SC hint that they are composed of proteins similar to those of mammals (Alibardi and Toni 2004). Whether corneocytes in the skin of birds also have covalently bound lipids attached to them, and if they do, what the nature of these lipids might be, remains unknown.
Hypotheses

In this study we compared CWL of populations of house sparrows, one living in deserts (Saudi Arabia) and one living in a mesic environment (Ohio), and related CWL to lipid composition of the SC. We hypothesized that desert sparrows would have a lower CWL and that this reduction would be associated with changes in the lipids of the SC. We found that desert sparrows had a reduced CWL compared with sparrows from Ohio. Further, desert sparrows had larger concentrations of ceramides and cerebrosides in their SC and the proportion of cholesterol was lower in their SC.

We attempted to separate the physical properties of the barrier to water vapor diffusion from biological properties active in a living bird. Despite variation in lipid composition of the SC, when we measured water permeation through the dead skin in both groups of sparrows, we detected no differences between desert and mesic individuals. We concluded that water loss through the skin is not simply a passive process but rather an interaction between living cells of the epidermis and the non-living layers of the stratum corneum.

Finally, we tested the idea that corneocytes of avian SC have covalently bound ω-hydroxyceramides as found in the SC of mammals. We explored the idea that birds from two radically different environments have different lipids attached to their corneocytes, which might lead of a different organization of lipids in the extracellular spaces. Our results indicated that corneocytes of house sparrows have ω-hydroxyceramides and ω-
hydroxycerebrosides attached to their corneocytes in the SC. This is the first time that cerebrosides have been found as components of lipids covalently attached to corneocytes of a vertebrate.

**MATERIALS AND METHODS**

**Natural history of house sparrows**

House sparrows in North America, members of the subspecies *P. d. domesticus*, were introduced at the end of the 19th century from England (Summers-Smith 1988). House sparrows in Saudi Arabia, *P. d. indicus*, are apparently native to the area, although they depend on humans for access to water (Cramp and Perrins 1994). The population of House Sparrows that we studied in west-central Saudi Arabia had daily access to water, and probably would not be able to survive without it. These two subspecies have been isolated from one another for at least 5,000 years (Summers-Smith 1988). Nestling sparrows in Saudi Arabia experience heat stress at some times during the day, because $T_a$ exceeds body temperature and because of intense solar radiation impinging on structures in which they nest (pers. obs).
**Collection of house sparrows**

We mist netted adult house sparrows (*Passer domesticus, L.* ) near Taif, Saudi Arabia, at the National Wildlife Research Center (22°15' N, 41°50' E) and in Columbus (Ohio, USA, 40°00' N, 83°10' W), during October-November 2003. Prior to measurements, sparrows were held in captivity for 1-3 weeks; they were provided with a mixture of seeds, mealworms and egg yolk, and had unrestricted access to water. Experiments were approved by ILACUC of Ohio State University (protocol 2003-A0072) and the National Commission for Wildlife Conservation and Development, Riyadh, Saudi Arabia.

**Measurement of metabolic rate and evaporative water loss**

We measured oxygen consumption, RWL and CWL using standard flow-through respirometry methods (Gessaman 1987, Tieleman and Williams 2002). Birds were fasted 2-3 hours prior to measurements to ensure postabsorptive conditions, which we later confirmed by examining their digestive tract. All measurements were made during the inactive phase.

We quantified CWL and RWL separately using a plastic mask system (Tieleman and Williams 2002). The mask captured all the respiratory gases, but did not cover the eyes or most of the head of the bird, so evaporation from these areas contributed to CWL. For measurements, birds were placed in a water-jacketed stainless-steel metabolic
chamber with a Plexiglas lid rendered airtight by a rubber gasket. We used a circulating water bath (Isotemp, model 900, Fisher Scientific) to control $T_a$ in the chamber, set at 30°C for adults and 35°C for nestlings and fledglings. Sparrows stood on a wire mesh platform that allowed feces to fall into a layer of mineral oil, eliminating them as a source of water in our measurements.

We routed atmospheric air through a column of Drierite to remove water, then into the metabolism chamber. Air exited the chamber through two different ports. RWL was measured from air drawn through the mask by a vacuum pump, and then routed to a dew point hygrometer (EdgeTech, model 2001-C1-S3 in Ohio, General Eastern, model M4, in Saudi Arabia), columns of Drierite and ascarite, and a mass-flow controller (Brooks, model 5850) set at 200-600 ml/min, calibrated with a 1-L bubble meter (Levy 1964). Oxygen concentration of exiting air was measured with an Applied Electrochemistry S3A-II oxygen analyzer. We also directed dry atmospheric air through a different line at a flow rate of 100 ml/min to the oxygen analyzer and used it to calculate delta $O_2$. CWL was measured from air drawn from the chamber from a second port, and directed through another dew-point hygrometer, a column of Drierite, and a calibrated mass-flow controller (Brooks, model 5850) set at 100-400 ml/min. During each measurement we directed air from the CWL port to the oxygen analyzer to verify that the mask captured all respiratory gases; the fraction of $O_2$ in chamber air was always identical to inlet air (20.95%).
After 1-3 hours, when oxygen consumption and dew-point temperatures were stable, we recorded concentration of oxygen in the inlet and outlet air, dew-point temperatures, and $T_a$ inside the dew-point hygrometers and the chamber. We averaged data that remained stable for at least ten minutes. Oxygen consumption was calculated with equation 4a of Withers (1977), and was converted to kJ/d using 20.08 J/ml O$_2$ (Schmidt-Nielsen 1997). To estimate respiratory water loss, we used the equation $\text{RWL} = (\rho_{\text{mask}} - \rho_{\text{chamber}}) (V'_{e1})$, where $\rho_{\text{mask}}$ is absolute humidity (g/m$^3$) of air leaving the mask corrected to standard temperature and pressure (STP), $\rho_{\text{chamber}}$ is the absolute humidity of air in the chamber (g/m$^3$, STP), and $V'_{e1}$ is the flow rate at the dew-point hygrometer of the first port (Tieleman and Williams 2002), assuming a respiratory quotient of 0.71 (King and Farner 1961). CWL was determined as $\text{CWL} = (\rho_{\text{chamber}} - \rho_{\text{in}}) (V'_{e1} + V'_{e2})$, where $\rho_{\text{in}}$ is the absolute humidity of the air entering the chamber (STP), and $V'_{e2}$ is the flow rate at the dew-point hygrometer of the second port, calculated as in Tieleman and Williams (2002).

Dew point hygrometers were factory calibrated against a primary standard traceable to the National Institute of Standards and Technology, less than 1 year prior to measurements. However, to confirm the accuracy of dew point hygrometers at the time of measurements, compressed air was routed through a column of Drierite, to remove water, then through a Brooks mass-flow controller (model 5850E), calibrated with a bubble meter, at a rate of 1000 mL/min. To saturate this air stream with water, we bubbled air
through a 25 cm high column of distilled water at 20°C. Next we bubbled air through a water jacketed chamber filled with distilled water controlled at 12°C with a Neslab circulating water bath. Wet saturated air exiting the chamber was directed to a dew point hygrometer. Air temperature into the chamber, measured with a thermocouple, was 12.0°C, whereas dewpoint temperatures were 11.7°C (General Eastern) and 11.3°C (EdgeTech), a deviation of 1-3% for General Eastern and 3-6% for our EdgeTech dewpoint hygrometer. These measurements were made in August 2005.

We also validated our ability to predict water loss from a bird using dew point hygrometry. Dry air was pushed through a mass-flow controller set at 1000 mL/min. Air was then directed into a 125 mL sealed flask partially filled with about 75 mL of distilled water. Air exited the flask to a dew point hygrometer. We wanted to estimate the error using our system to calculate evaporative water loss. To do so, we calculated water loss gravimetrically weighing the water in the flask when the system reached equilibrium and 2-3 hours after, and compared the mass difference with the total evaporative water loss obtained from the dew point temperature using the equations given above. The average error was 0.86 ± 1.96 % (n = 5 trials).

**Determination of passive water loss through skin removed from sparrows**

To estimate the passive permeability barrier of the skin attributable to the non-living SC, apart from active processes maintained by the living bird, we affixed skin of
the ventral apterium to a glass vial (surface area of skin = 2.13 cm²) filled with a solution of phosphate buffered saline (PBS; Na₂HPO₄·7H₂O, NaH₂PO₄·H₂O monobasic, and NaCl in distilled water, pH = 7.4, 370 mOsm). Skin was glued to the edges of the vial with cyanoacrylate glue. We assured a complete seal of our preparation by turning the vial upside down and examining for leaks. We then placed the vial in a sealed container over a layer of Drierite to ensure a low and constant water vapor pressure. The container and contents were then set in an incubator at 36°C, a temperature selected because it is near skin temperature of a desert bird. An uncovered vial filled with PBS was also placed in the same container and used as a control. After eight hours, a period previously determined for rates of water loss from the vial to stabilize, we recorded the weight of the vials with a Metler balance (model AB204, ± 0.1 mg). We re-weighed the vials 2 - 3 hours after the initial weighing.

**Extraction of intercellular lipids of the SC**

After measuring RWL, CWL and oxygen consumption, we weighed birds, sacrificed them, plucked their feathers, and removed their skin. We pinned the skin to a thin sheet of Teflon, immersed it in a distilled water bath at 65°C for three minutes, and then gently peeled the epidermis from the dermis (Wertz et al. 1986, Haugen et al. 2003a, b). We incubated the epidermis at 4°C overnight in a solution of 0.5% trypsin in phosphate buffer saline (PBS, pH = 7.4, 370 mOsm). The following day, we re-immersed
the tissue in fresh 0.5% trypsin solution for 3 hours at 38ºC, a procedure that separates the SC from remaining epidermal cells (Wertz and Downing 1987). We then rinsed the SC with distilled water over fine mesh of polyester cloth to remove any remaining feathers and epidermal tissue, freeze-dried the SC for 12 hours, and stored it at -20ºC in an atmosphere of nitrogen.

After determining dry mass of the SC (± 0.01 mg), we extracted lipids using a series of chloroform:methanol mixtures, 2:1, 1:1, and 1:2 v/v for 2h each step. The mixtures contained 50 mg/l of the antioxidant butylated hydroxytoluene (BHT) (Law et al. 1995). We then dried the mixtures using a stream of nitrogen with an evaporimeter (N-EVAP, model 11155-O, Organomation Associates, Inc., Berlin, MA). To prepare our samples for analytical thin layer chromatography (TLC), we re-dissolved the extracted lipids in 200 or 300 μl, depending on the absolute lipid amount extracted from each sample, of chloroform:methanol 2:1 containing BHT.

**Extraction of covalently bound lipids of the SC**

To confirm that all extracellular lipids had been extracted from the SC, the remaining SC for each bird was soaked for 2 hours in chloroform/methanol 1:2. We then examined extracts for lipids using TLC. Results showed no lipid bands in our plates, indicating that all the intercellular lipids were removed.
Next we searched for covalently bound lipids (CBL) on corneocytes by
immersing the SC in 2 ml of 1M NaOH in 90% methanol at 60°C for 2 hr (Wertz and
Downing 1987). This mild alkaline hydrolysis breaks the ester bonds of lipids attached by
an ester linkage to proteins (Hill et al. 2006). We then adjusted the pH of the extract to 6
by adding 3M HCl, and added 2.5 ml of chloroform. The solution was then passed
through a sintered glass filter, and centrifuged at 3000g for 15 min. After a few minutes,
the solution separated into two layers, an aqueous layer and an organic layer that
contained any lipids. The organic phase was washed twice with distilled water to remove
contaminants. The aqueous phase was mixed with 1 mL of chloroform to extract any
lipids that might be in this phase, and centrifuged again at 3000g for 10 min. We
combined organic fractions, and removed any remaining small particles by passing the
solution through a PTFE filter, 0.45µm pore size (Millex, Millipore Corp., Bedford, MA).
We dried the filtrates with a stream of nitrogen and stored them at -20°C. Prior to analysis
of lipids, we re-constituted the extracts in 50µl of chloroform:methanol (2:1, v/v)
containing 50mg/L of the antioxidant BHT.

**Analytical thin layer chromatography**

We performed analytical TLC to separate lipid classes on 20x20-cm glass plates
coated with silicic acid (0.25 mm thick; Adsorbosil-Plus 1, Altech, Deerfield, Ill.). We
developed plates with a mixture of chloroform:methanol (2:1) to the top to remove
contaminants, and thereafter activated them in an oven at 110ºC for 30 minutes. Then, we divided each plate into 6-mm or 10-mm-wide lanes. We prepared a series of 5 standards, of known concentration, each containing nonhydroxy fatty acid ceramides (a sphingosine base with a mixture of octodecanoic and cis-15-tetracosenoic acids as the N-acyl fatty acid group), galactocerebrosides, cholesterol, and a mixture of free fatty acids. We dissolved standards in chloroform:methanol (2:1) in concentrations ranging from 0.30 mg/mL to 50 mg/mL, a range that bracketed the concentrations of lipids found in the SC of house sparrows. A duplicate series of standards were run on each plate. We pipetted 5 μl of each lipid extract in duplicate or triplicate in the pre-adsorbent area of the plates using a Teflon-tipped Hamilton syringe. Two solvent systems were used, one for relatively more polar lipids, such as ceramides and cerebrosides, and another for the relatively non-polar lipids, free fatty acids and cholesterol. To separate ceramides and cerebrosides, we developed plates with chloroform:methanol:water (60:40:5) run 5 cm from the bottom, then twice with a mixture of chloroform:methanol:acetic acid (190:9:1) to the top, and a final development with hexane:ethyl ether:acetic acid (70:30:1) run to 12 cm above the preadsorbent zone. Cholesterol and free fatty acids were separated using development with hexane to the top of the plate, followed by toluene to the top, and finally a development with hexane:ethyl ether:acetic acid (70:30:1) run to 12 cm from the bottom. We visualized bands of lipids by spraying the plates with a solution of 3% cupric
acetate in 8% phosphoric acid, and then placing the plates on a 20x20 cm aluminum hotplate slowly raised to 160°C over the course of 1-2 hours.

To quantify the concentration of lipid classes, we scanned plates with a Hewlett Packard scanner and measured the amount of each class by photodensitometry using TN-Image (Nelson 2003). We calculated percentages of lipids as the amount of a given lipid class divided by the sum of the total amount of the four lipid classes analyzed. To validate our ability to quantify lipids, we followed our protocol but used known concentrations of cholesterol as our unknown. The average error, calculated as \[
\frac{(\text{observed} - \text{actual})}{\text{actual}} \times 100
\], was routinely below 5%.

**Preparative thin layer chromatography**

To fractionate lipids in our samples before analysis with mass spectrometry, we used preparative TLC on 20cm x 20cm glass plates covered with silica gel (0.5mm thick, Adsorbosil-Plus 1, Alltech, Deerfield, Il.) We combined the extracted lipids from the SC of 6 sparrows, and loaded 20 µL of this extract along with 30 µL of a mixture of standards onto the plate. Because we only detected ceramides and cerebrosides in our samples, we used a development of chloroform:methanol:water (40:10:1, v/v/v) to 8cm from the bottom, followed by two developments with chloroform:methanol:acetic acid (190:9:1, v/v/v) to the top, and a final development with hexane:diethyl ether:acetic acid
(70:30:1, v/v/v) to a half. The standard mixture for the polar lipids was the same as we used for analytical TLC.

To isolate classes of lipids on preparative plates without changing their chemical structure, we sprayed plates with 0.2% 2,7-dichloroflorescein in 95% ethanol and visualized bands under UV light. Comparing bands of standards with unknown bands allowed us to designate unknown bands as ceramides or cerebrosides. We marked their location under UV light, and scraped the silica gel from that area. Cerebrosides were recovered from the silica gel by extraction with chloroform:methanol:water (50:50:1, v/v/v), and filtration through a sintered glass filter. Fluorescein was precipitated from this mixture by washing with 2.5% potassium carbonate. Samples were dried in a stream of nitrogen gas and stored in an atmosphere of N2 at -20°C until analyses with HPLC-APPI/MS.

**High performance thin layer chromatography**

We also used high performance thin layer chromatography (HPTLC) to search for classes of covalently bound cerebrosides in the SC because this method may provide greater resolution of cerebroside classes. In this procedure we used 10 x 20 cm plates coated with a 0.20 mm thick layer of silica gel (Si 60, Merck, Darmstadt, Germany). We used the same protocol as for analytical TLC, except that we loaded 3 μL of lipid extract
and standards on plates. We developed plates with chloroform:methanol:water (40:10:1, v/v/v) to the top of the plate and visualized bands of lipid as above.

**Identification of Complex Mixtures of Sphingolipids in the Stratum Corneum by Reversed-Phase High-Performance Liquid Chromatography and Atmospheric Pressure Photospray Ionization Mass Spectrometry**

Lipid extracts from sparrows were dried with nitrogen gas and analyzed by reversed-phase HPLC-APPI/MS. Samples were re-dissolved in a mixture of isopropyl alcohol, toluene and ethyl acetate, 7.4:2:1 (v/v/v). To this solution we added 72.6 ng/mL C17:0 ceramide as an internal standard, and 43.5 µg/mL BHT as an antioxidant. For each run of each sample, we loaded 3µL onto our HPLC column.

Cerebrosides tend to lose sugar moieties when ionized in mass spectrometry, so separation of these lipids by HPLC was a crucial part of our protocol. Trials exploring methods of separation of lipids in our mixtures suggested that reversed-phase HPLC provided maximum separation of cerebrosides and ceramides, which could then be analyzed by mass spectrometry. Reversed-phase HPLC was performed on a PE Series 200 micro binary-gradient system comprising two pumps, an autosampler and a column oven, the latter maintained at 48°C (Perkin Elmer Analytical Instruments, Wellesley, MA). Separation was accomplished using a Phenomenex Luna® C18 column 150 x 2.0 mm i.d., spherical 5 µm particle size, 100 Å pore size (Phenomenex, Torrance, CA). The
HPLC run was timed beginning with the initial loading of sample onto the column. The syringe used for loading our sample on the HPLC column was washed between injections with 250 μl of ethyl acetate.

In our reversed-phase HPLC, we used a gradient solvent system with the initial solvent being methanol:isopropanol:water, 85.5:10:4.5 (v/v/v), changed in steps to 100% ethyl acetate (Table 1.1). We varied flow rates along the gradient to assure the peaks of later eluting compounds to have the same width as that of earlier eluting compounds. Steps five and six were used to precondition the column between runs.

In the common usage of reversed-phase HPLC, compounds are loaded onto the HPLC column using a polar mobile phase, typically a mixture of water and methanol or acetonitrile. Compounds are then eluted using a gradient towards an increasingly non-polar mobile phase. However, since lipids are insoluble in water, we have run our reversed-phase chromatogram using a gradient from methanol, isopropanol and a low proportion of water, to the less polar ethyl acetate (Table 1.1). Hence compounds are separated based on their hydrophobic character with more polar lipid molecules eluting first, beginning around a retention time of 3 minutes, followed by more non-polar molecules; after about 20 min, all lipid molecules had eluted from our column (Fig. 1.1).
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</tbody>
</table>

Table 1.1. Gradient used to separate lipids by reverse phase HPLC prior to mass spectrometry. Mobile Phase A was methanol:isopropanol:water 85.5:10:4.5 and mobile Phase B was 100% ethyl acetate. All flow and composition gradients were linear within the time range.

If retention time is to be used as information in identification of molecules, it must be repeatable (Ardrey 2003). Hence, we evaluated the repeatability of retention time by running a standard, ceramide 17:0, 27 times through our system. Retention time varied by less than ± 0.05% (Fig. 1.2).
Figure 1.1. A representative Total Ion Chromatogram (TIC) for House sparrows. Each point in the chromatogram represents the sum of the intensities of the ions eluted at a given time using reverse phase HPLC.
Figure 1.2. Repeatability of retention time of ceramide 10:0 for 27 runs through our HPLC-MS system. The solid line represents average retention time, 5.22 min ± 0.019 (SD).

To detect sphingolipids in our samples we used an Applied Biosystems Q-TRAP® hybrid quadrupole Linear Ion Trap mass spectrometer system (Applied Biosystems, Ontario, Canada) fitted with a PhotoSpray® ion source operated in both positive and negative ion mode. We used the PhotoSpray® ion source with toluene as the photo-ionization reagent, or dopant.

Prior to APPI-MS analyses, we ran our samples using electrospray ionization-mass spectrometry (ESI-MS), both in negative and positive mode. Our settings included
electrospray voltage set to 10 volts, declustering potential to 45 volts, curtain gas to 27, and vaporizer temperature to 460 °C. Although ESI-MS has been a commonly employed technique for the analysis of sphingolipids (Raith and Neubert 1998, Camera et al. 2004), most sphingolipids have a low proton affinity (Kauppila 2004). In positive ion mode we saw relatively weak ion signals with the signal divided among protonated molecular ions, sodium or potassium adducts and source fragments originating from the loss of sugars and water. Negative ion ESI on the other hand has been reported to provide strong signals for most sphingolipids (Merrill et al. 2005). However, when we employed negative ion ESI on our lipid extracts, we could not detect late eluting compounds; APPI appeared to eliminate this problem. APPI also overcomes some of the problems that investigators have found when trying to analyze non-polar compounds. APPI is able to ionize non-polar compounds, provides better sensitivity, lower detection limits and wider linear ranges than ESI or APCI, and matrix effects are negligible (Syage et al. 2000, Rafaelli and Saba 2003, Kauppila 2004, Cai and Syage 2006). Moreover, APPI requires less heat for desolvation, allowing the analysis of thermally labile compounds (Syage et al. 2000). In our samples, APPI also yielded a more reliable quantitative response over a wider range of concentrations, and produced less ion competition when compounds co-eluted (data not shown).

The first phase of the APPI process ionized toluene molecules, which have an ionization potential higher than that of most target molecules (Kauppila 2004), but lower
than that of all constituents of air as well as most common solvents (Syage et al. 2000). The lipid ions that we observed are formed by the gas phase transfer of charges from the more energetic ions in the photo-ionized vapor that are produced in the nebulization process. Liquid toluene was continuously delivered at 15µL/min into the sheath gas (GS2) of the heated nebulizer. Our settings on the Q TRAP included collision gas set to High, curtain gas to 27, nebulizer gas (GS1) to 40, GS2 to 12, and lamp gas to 1.0L/min. High purity nitrogen was used for all gases on the Q TRAP system. The nebulizer temperature was set to 460°C, transfer voltage to 2100 volts, decluster potential to 45 volts, and the interface heater was turned on.

Gas phase ionization of APPI produced equally strong signals in positive and negative ion modes. In negative ion mode of APPI, we observed de-protonated molecular ions. Positive ion mode APPI has a tendency to produce protonated dehydrated molecular ions (Cai and Syage 2006). The free hydroxyl group on the sphingosine was easily lost to form a pair of conjugated double bonds on the remainder of the sphingosine moiety. All positive ion APPI molecular ions from sphingolipids showed this water loss, a dehydration event that appeared to increase the molecule’s affinity for protons in the gas phase. So, for this study, we exaggerated the process by using elevated heated nebulizer temperatures (460°C), a procedure which probably caused a more efficient evaporation and ionization by decreasing the ionization potential of the compounds, but, at the same time, did not seem to affect the characteristic structure of cerebrosides and ceramides. In
summary, positive and negative ion photo-ionization were the complementary modalities of choice for detection of sphingolipids in the SC of sparrows because they gave intense signals, and simple and informative spectra.

We used the linear ion trap on our Q TRAP system to acquire HPLC-MS data by selecting the trap scan mode set at 4000 amu/s from 450amu to 1600amu (±0.4 amu). We used a collision energy setting of 10 volts to conduct the ions to the trap resulting in a minimum of source fragmentation, and a maximum signal-to-noise ratio for the parent ions. Dynamic fill time was used with a total intensity chromatogram (TIC) target of 500,000 counts and a maximum fill time of 200msec.

Analyses of data from mass spectrometry of our lipid mixtures were performed with Analyst™ 1.4.1 software (Applied Biosystems, Ontario, Canada). This software generated a contour plot for each sample consisting of a two dimensional graph with mass/charge (m/z) as the y-axis and retention time as the x-axis, each band representing a single lipid molecule. At the exact retention time at which the compound eluted, some bands corresponded to molecular ions and other bands were fragments of the parent ions. Patterns of fragmentation allowed us to distinguish molecular ions eluting at a given time.

To identify sphingolipids, we first created a table of expected molecular weights, calculated from standard isotopic masses, for all sphingolipids that we could potentially find in our samples, taking into account the number of carbons and degree of unsaturation. Then, for each band in our contour plots, we determined the mass of the
band corresponding to the parent ion for each molecule and matched this value with
expected masses in our table. This typically did not uniquely identify every molecule,
rather limited the number of possibilities. If the source fragmentation showed an
indication of a sugar loss, a mass loss of 162.0 units, the molecule was assigned as a
cerebroside (Fig. 1.3). If we did not see a sugar loss, the molecule was considered a
ceramide, either a fragment of a cerebroside, or a parent ion of a ceramide. Fragments of
cerebrosides could be distinguished by the presence of a peak in the spectrum 162.0 mass
units greater than that of the ceramide.

Next, we ascertained the identity of the sphingoid base. In positive ion mode, we
could distinguish 6-hydroxysphingosine from sphingosine or phytosphingosine because
loss of 6-hydroxysphingosine produced a fragment with a molecular weight of 261.5
units, whereas spingosine and phytosphingosine each yielded fragments having a
molecular weight of 263.5 mass units. Sphingosine or phytosphingosine could be
recognized in negative ion mode with the former producing a loss of 296.0 units, whereas
presence of the latter resulted in a molecular weight loss of 314.0 mass units.

The presence of linoleate, an integral component of ceramides EOS, EOH and
EOP, was indicated by a loss of 280.5 mass units from the parent ion in both positive and
negative ion mode.

For colleagues who wish to follow our protocol, we provide representative spectra
of ceramides and cerebrosides in Fig. 1.3A-D and detail our process of identification. In
the spectrum in Fig. 1.3A, loss of 263.5 mass units from the parent ion in positive mode indicated the presence of either sphingosine or phytosphingosine. Absence of a linoleate fragment from the parent ion combined with a known molecular weight narrowed our possibilities to two types of ceramide, NS and NP, ceramides with non-hydroxy acids and with sphingosine or phytosphingosine, respectively. We recognize that our suggestion for a sphingosine fragment with a terminal aromatic ring is unlike fragments described for MS using electrospray. Yet the marked stability to temperature of this fragment coupled with MS/MS data indicated that this structure, as we represent it, was the most likely. The loss of a fragment with a mass of 296.0 units in negative ion mode supported the assignment of this molecule to CER NS (Fig. 1.3B). In the spectrum in Fig. 1.3C, loss of a sugar molecule, 162 mass units, indicate that the parent ion is a cerebroside. Taking mass into account, this molecule could correspond to cerebroside EOH, NH, AP, or AS. Loss of 423 mass units shows that 6-hydrosphingosine is the sphingoid base (162 mass units of the sugar plus 261 mass units for 6-hydrosphingosine). Because there were no fragments suggesting the presence of linoleate, this molecule was designated as cerebroside NH. The cerebroside in Fig. 1.3D shows a fragment showing the loss of linoleate and another fragment indicating the presence of sphingosine. Therefore, this molecule was identified as cerebroside EOS.
Figure 1.3. Representative APPI-MS spectra of ceramides and cerebrosides in the SC of House sparrows. A: Spectrum of ceramide NS 39:0 in positive ion mode. The loss of 263.5 mass units indicates the presence of sphingosine or phytosphingosine. The unusual stability of the fragment along with MS/MS spectra indicated a fragment with a terminal ring structure B: Spectra of the same molecule in negative ion mode. Loss of 296.0 mass units confirms that this ceramide has sphingosine and not phytosphingosine. C: Spectrum of cerebroside NH in positive ion mode. Loss of 162.0 mass units indicates that the parent ion is a cerebroside. The sphingoid base is 6-hydrosphingosine. D: Spectrum of cerebroside EOS in negative ion mode. Fragments showing the loss of linoleate and sphingosine are depicted.
To confirm identification of sphingolipids, we analyzed MS/MS spectra for 40 representative molecules belonging to seven different sphingolipid classes using our HPLC system, APPI in positive mode, but a Q-STAR mass spectrometer (Applied Biosystems, Ontario, Canada). We provide an example of our confirmation process using MS/MS in Fig. 1.4. The spectrum in Fig. 1.4A corresponds to cerebroside EOS 34:0. The peak at 772.8 indicates a loss of 162 mass units, showing that this molecule is a cerebroside. The peak at 264.3 indicates the presence of sphingosine, whereas the peak at 280.3 shows that a linoleate residue is present in this molecule. In Fig. 1.4B we present the spectrum of a molecule that we could identify as ceramide NS 22:0. There is no evidence of a sugar loss or linoleate, and the peak at 264.3 indicates the presence of sphingosine.

One of the problems that might arise when trying to identify compounds present in complex lipid mixtures is that of co-elution from HPLC (Ardrey 2003). Co-elution results from the inability to separate two different compounds before detection by MS and can lead to inappropriate interpretations of spectra. We did find different sphingolipid molecules eluting at the same time, some were molecular ions, some their fragments. Source fragmentation analyses combined with MS/MS confirmation allowed us to unambiguously determine which molecules were parent ions. Thus, although co-elution does occur in our analyses, it does not confound our identification of each lipid molecule.
Figure 1.4. Representative MS/MS spectra in positive ion mode of cerebrosides and ceramides in the SC of House sparrows. A: Spectrum of cerebroside EOS 34:0. B: Spectrum of ceramide NS 22:0.
Because relative intensities measured for every compound are proportional to concentration, we generated calibration curves that were used for quantification of the different lipid molecules detected by mass spectrometry. Standard curves were constructed by measuring relative intensity at known concentrations of ceramide 17:0. Because our initial calibration curves did not cover the entire range of concentrations found in our samples, we repeated these curves to cover a range of concentrations of 2.5 ng/mL to 16,700 ng/mL. The relationship between intensity and concentration over the range that we used was given by log Intensity (counts per second) = 5.795 + 0.878 log Concentration (ng/mL) ($R^2 = 0.99$, $F = 1280.2$, $P< 0.001$). These concentrations fell within the linear dynamic range of the QTRAP.

To confirm that we recovered all lipid that we loaded onto our HPLC column, we compared the amount loaded with the amount recovered of a known quantity of ceramide 17:0. Our results showed that we recovered on average 95% ($\pm 10\%$) of the lipids loaded onto the column ($n = 27$ trials), which is consistent with other investigators (Mount 1979, McNabb et al. 1999, Camera et al 2004).

To calculate the relative proportion of each compound in the sample, from each spectrum, we selected the four main carbon-isotope peaks, obtained their retention time and calculated the intensity of the peak, all using the software Analyst. Intensity corresponded to the area under the extracted ion chromatogram for each compound.
Every ceramide or cerebroside molecule was then uniquely characterized by its retention time, molecular weight, source fragmentation and intensity.

We are aware that our chromatographic separation may be insufficient to provide accurate estimations of the quantities of particular compounds. We are currently working towards optimal protocols to achieve less ambiguous quantitative results with our system. We also recognize that use of a single internal standard to quantify numerous sphingolipid species might yield inaccurate results. Proton affinity for a ceramide may or may not be markedly different than that of a cerebroside. However, when we compared quantification results based on TLC, a proven method for quantification (Haugen et al. 2003a, b, Muñoz-Garcia and Williams 2005), with that of our use of MS, we found remarkable concordance (see results) suggesting that our use of a single internal standard produced errors of a small magnitude. Nonetheless, until further verification, our quantification results remain inconclusive.

Statistics

All statistical tests were performed with SPSS 12.0 or 14.0. We rejected the null hypothesis at $\alpha > 0.05$. Averages are reported ±1 SD. We tested for differences between means using a two-tailed t-test for independent samples. When multiple comparisons were performed, we used the Bonferroni correction (Zar, 1996).
Concentrations of cholesterol and free fatty acids were not normally distributed (Kolmogorov-Smirnov test, KS = 0.18, P < 0.04, and KS = 0.20, P < 0.01, respectively). We log-transformed these variables to normalize the data (KS = 0.10, P > 0.15, and KS = 0.09, P > 0.15). Percentages were logit transformed [Ln (Y/1 - Y), Zar, 1996] prior to analyses to normalize data. Differences in distributions of data were assessed using the Kolmogorov-Smirnov test.

We performed regressions using a general linear model. To test for differences between regressions for lipids from desert and mesic sparrows, we first tested for the significance of the interaction term. If the interaction was not significant, we removed it from the model, and tested for differences in intercepts assuming a common slope.

To explore underlying themes in our data, we used principal components analysis (PCA) on the quantities (in millimoles or mg lipid/g dry SC) of each family of sphingolipids (Shaw 2003). This analysis yielded uncorrelated composite variables, the principal components. We used the program “Factor analysis” in SPSS without rotation to extract components with eigenvalues greater than one as our selection criterion. Interpretation of the principal components led to the generation of hypotheses that involved chain length of the fatty acid residues and polarity of the sphingolipids in the SC. We used the two-tailed t-test for independent variables to test for differences in chain length of the fatty acid moiety and polarity of the sphingolipids between desert and mesic
sparrows. We also determined associations between CWL and the scores of the principal components for each individual bird using linear regression.

RESULTS

Body mass and surface area

For 6 males and 5 females in Saudi Arabia, and 8 males and 5 females in Ohio, average mass did not differ between sexes (p > 0.7 Saudi Arabia, p > 0.09 Ohio). Combining data for sexes, mean mass of sparrows in Saudi Arabia was 20.1 ± 1.6 g (n = 11) and in Ohio was 23.2 ± 2.8 g (n = 15), a difference that was significant (t = -3.25, df = 24, p < 0.01). Using Meeh’s equation (Walsberg and King, 1978), surface area of sparrows from Saudi Arabia equaled 74.0 ± 4.0 cm², whereas from Ohio it was 81.3 ± 6.6 cm².

Oxygen consumption

Desert sparrows consumed oxygen at a lower rate than did Ohio sparrows, 56.9 ± 5.9 mL/hr for sparrows in Saudi Arabia, 64.8 ± 6.0 mL/hr for birds in Ohio (t = -2.7, df = 17, p < 0.02). Heat production was 27.5 ± 3.0 kJ/d for sparrows from Saudi Arabia, and 31.3 ± 3.3 kJ/d for birds from Ohio. Mass-specific metabolic rates were not significantly
different between groups, \([1.37 \pm 0.14 \text{ kJ/(g } \cdot \text{d})]\) Saudi Arabia, \([1.32 \pm 0.18 \text{ kJ/(g } \cdot \text{d})]\) Ohio \((t = 0.65, \text{df} = 17, p > 0.5)\).

**Respiratory water loss**

Respiratory water loss equaled \(0.97 \pm 0.23\) and \(1.25 \pm 0.32\) g H\(_2\)O/d for sparrows from Saudi Arabia and from Ohio, respectively, values that differed significantly \((t = -2.5, \text{df} = 20, p < 0.02)\). When these values were expressed per unit body mass, we did not detect significant differences: \(48 \pm 12\) mg H\(_2\)O/(g \cdot d) (Saudi Arabia), \(54 \pm 16\) g H\(_2\)O/(cm\(^2\) \cdot d) (Ohio); \(t = -0.997, \text{df} = 20, p > 0.3\) (Fig. 1.5A).

**Cutaneous water loss of live sparrows**

With a CWL of \(0.88 \pm 0.18\) g H\(_2\)O/d, desert sparrows lost significantly less water through their skin than did sparrows from Ohio, \(1.32 \pm 0.14\) g H\(_2\)O/d \((t = -5.2, \text{df} = 23, p < 0.001)\). When CWL was adjusted for differences in surface area, CWL remained significantly different between desert and mesic sparrows: \(11.9 \pm 2.2\) mg H\(_2\)O/(cm\(^2\) \cdot d), Saudi Arabia; \(16.0 \pm 2.6\) mg H\(_2\)O/(cm\(^2\) \cdot d), Ohio \((t = -2.8, \text{df} = 23, p < 0.01; \text{Fig. 1.5B})\).
Figure 1.5. Mass-specific RWL (A) and surface-specific CWL (B) in the sparrows from Saudi Arabia and Ohio. Asterisks represent significant differences between groups (P < 0.05).
Passive water loss through non-living skin

In vials covered with skin, water loss equaled 46.0 ± 15.7 mg H₂O/(cm² · d) for sparrows from Saudi Arabia (n = 12), and 45.8 ± 27.2 mg H₂O/(cm² · d) for sparrows from Ohio (n = 11), values that were not significantly different (d.f. = 21, \( P > 0.9 \)). The rate of water loss in the uncovered vial was 456.4 ± 37.1 mg/(cm² · d) in Saudi Arabia, and 440.5 ± 59.0 mg/(cm² · d) in Ohio (\( t = 0.77 \), d.f. = 16; \( P > 0.45 \)). When compared with an open vial, a free water surface, skin-covered vials lost 10.1% and 10.4% as much water in Saudi Arabia and Ohio, respectively.

Intercellular lipids in the stratum corneum

For both groups of sparrows, our procedure using TLC revealed distinct bands of lipids corresponding to standards of cholesterol, free fatty acids, ceramides, and cerebrosides. The ceramides that separated into bands were named in order of increasing polarity, ceramides 1-4, as were the cerebrosides, 1-3 (Fig. 1.6).

Using photodensitometry to quantify lipids (mg lipid/g dry mass SC), we did not find significant differences in the quantity of total lipids, of cholesterol or of free-fatty acids between desert and mesic sparrows, but desert House Sparrows had a larger concentration of total ceramides and total cerebrosides per g dry mass SC than did sparrows from Ohio (Table 1.2). For specific sphingolipid classes, desert sparrows had significantly more ceramide 3 and cerebroside 1 in their SC. In general, cerebrosides
were more abundant in the SC of sparrows than has been previously reported for either birds or mammals.

Because it is thought that subtle changes in the proportions of lipid classes in the SC can influence the fluidity of the lipid layer and therefore water permeation (Haugen et al., 2003a, b; Bouwstra et al., 2003), we also expressed lipid classes as a percentage of the total lipids extracted. Desert sparrows had a significantly lower proportion of cholesterol and ceramide 2 than mesic birds (Table 1.2).

**Correlations among lipids in the SC**

Given that adjustments in the relative proportions of lipids in the SC may have important consequences for barrier function, we explored co-variation between the percentages of the various lipids in the SC as quantified by TLC; proportions of some classes of lipids were significantly correlated in both groups (Table 1.3). Interestingly, variation in lipids among individuals was low for desert sparrows. In general, free fatty acids are negatively correlated with the rest of the lipid classes in the SC.
Figure 1.6. A. Thin layer chromatograph of the polar lipids in the SC of House sparrows. B. Profile of lipid standards (upper graph) and cerebrosides, ceramides, and cholesterol from the extracted lipids in the SC of House sparrows obtained using densitometry (bottom graph). CHOL = Cholesterol; CER = Ceramide; CEREBR = Cerebroside; C1 to C3 = Cerebrosides 1 to 3.
<table>
<thead>
<tr>
<th>Lipid concentration (mg lipid/g dry mass SC)</th>
<th>Mean proportion lipid class</th>
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<tbody>
<tr>
<td></td>
<td>Saudi Arabia</td>
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<tr>
<td>Cholesterol</td>
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<tr>
<td>FFA</td>
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<td>Total ceramides</td>
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</tr>
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<tr>
<td>Cerebroside 3</td>
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</table>

Table 1.2. Quantities and mean proportion of each lipid class in the SC of house sparrows in Saudi Arabia and Ohio. Proportions were calculated as the amount of a given lipid class divided by the sum of the amounts of all four lipid classes. Values are means ± SD. FFA = Free fatty acids. Asterisks indicate significant differences between populations (P < 0.05).
Table 1.3. Correlations between the proportions of the different lipid classes in the SC of the House sparrows from Saudi Arabia and Ohio. Equations are of the form $Y = b \cdot X + a$. Asterisks indicate significance between populations ($P < 0.05$).
Figure 1.7. A. Contour plot showing the lipid molecules found in the SC of House sparrows. The higher mass group at earlier retention times consisted of cerebrosides. The lower mass group, eluting later, was formed by ceramides. The last group, with longer retention times and relatively low molecular weights, was presumably composed of triglycerides, and cholesterol and cholesterol esters. B. Enlarged image of a region of the contour plot showing the identities of the different families of cerebrosides and ceramides.
Identification and quantification of sphingolipids in the SC of house sparrows by HPLC-APPI-MS.

In each contour plot, we could distinguish several groups of molecules, corresponding to cerebrosides, ceramides and perhaps triglycerides and cholesterol, although we did not identify the latter compounds (Fig. 1.7). Increased polarity attributable to the sugar moiety caused cerebrosides to elute before the less polar ceramides.

In the SC of desert sparrows, we detected 79 and 107 molecular species of ceramides and cerebrosides, respectively, whereas in mesic sparrows, we found 90 molecules of ceramides and 134 cerebrosides (Appendix A). On average, we identified 181.1 ± 3.1 different molecules of ceramides and cerebrosides combined in SC of house sparrows from Saudi Arabia, and 191.7 ± 16.5, in SC of sparrows from Ohio, values that differed significantly (t = 2.39, P < 0.03). To denominate families of sphingolipids, we followed Motta et al. (1993) who designated the three types of fatty acids in sphingolipids, non-hydroxy acids, α-hydroxy acids and ω-hydroxy acids ester linked to linoleate, as N, A, and EO, respectively. The three types of sphingoid bases, sphingosine, phytosphingosine, and 6-hydroxysphingosine found in ceramides and cerebrosides were indicated as S, P, and H. Thus, a ceramide consisting of a ω-hydroxyacid ester-linked to a molecule of 6-hydroxysphingosine would be designated as CER EOH. Sphingolipids within SC of desert sparrows belonged to four families of ceramides, EOS, NS, EOH and
AH, and six families of cerebrosides, NS, NP, EOH, AS-NH, AH, and NH unsaturated, both groups in order of increasing polarity (Fig. 1.7, 1.8, 1.9). We found the same lipid families in SC of sparrows from Ohio, with an additional family consisting of diosylceramides, formed by a ceramide with two hexoses attached. Diosylceramides have not been previously identified in the SC of any vertebrate.

Within each family of sphingolipid, we identified between 11 and 40 molecules differing in carbon chain length of the fatty acid (Fig. 1.10, 1.11). The molar ratio of cerebrosides and ceramides, calculated as moles of total cerebrosides divided by moles of total ceramides, was 1.89 and 1.28 in SC of sparrows from Saudi Arabia and from Ohio.

The distribution of moles of lipid within each sphingolipid family could be the result of selective pressures that would favor the occurrence of some molecules over others, which in turn, could influence the structure and properties of the permeability barrier. Distributions of molecules within ceramide families were unimodal with the main peak located towards short-chain free fatty acids (Fig. 1.10, 1.11). The shape of the distributions of ceramides did not differ significantly within each family between sparrows from Saudi Arabia and Ohio (Kolmogorov-Smirnov test, Z < 0.926, P > 0.36). There were significant differences between desert and mesic sparrows in the distributions of the number of moles of cerebrosides NP, EOH, AS-NH, AH and NH (Kolmogorov-Smirnov, Z > 1.423, P < 0.035) (Fig. 1.10, 1.11); distributions in these families of cerebrosides were in general more flat in mesic than in desert sparrows.
Figure 1.8. Chemical structure of ceramide families found in the SC of house sparrows.

Ceramides are ordered from the least to the most polar.
Figure 1.9. Chemical structure of cerebroside families diosylceramides (DIOS) found in the SC of house sparrows. Cerebrosides are ordered from the least to the most polar.
Figure 1.10. Distributions of the amount of lipid in millimoles per g of dry SC within ceramide families in the SC of desert (white bars) and mesic (black bars) house sparrows.
Figure 1.11. Distributions of the amount of lipid in millimoles per g of dry SC within cerebroside families and diosylceramides (DIOS) in the SC of desert (white bars) and mesic (black bars) house sparrows. There were significant differences between desert and mesic sparrows in the distributions of cerebrosides NP, EOH, AS-NH, AH and NH (Kolmogorov-Smirnov, Z > 1.423, P < 0.035).
Figure 1.11. Continued
Quantification of lipid classes by thin layer chromatography and mass spectrometry

Using TLC and photodensitometry, we estimated that, on average, each g of dry SC of sparrows in Saudi Arabia contained 52.0 ± 6.1 mg of ceramides and 110.7 ± 40.6 mg of cerebrosides (Table 1.2).

Using APPI/MS we found that the relative intensity for ceramides in the SC of House sparrows from Saudi Arabia was $7.701 \cdot 10^9$ units on average, whereas for cerebrosides total relative intensity was $1.451 \cdot 10^{10}$. These values translated into a concentration of 45.70 µg/mL and 94.02 µg/mL, respectively. From these concentrations, the amount of ceramides and cerebrosides per g of dry SC obtained using APPI-MS were 52.5 ± 2.4 mg and 108.0 ± 70.6 mg, respectively.

When we compared the amounts obtained for our samples with our calibration curves with those obtained from TLC, the deviation, calculated as $[(\text{MS amount} - \text{TLC amount}) / \text{TLC amount}] \times 100$, was +0.95% for ceramides and –2.5% for cerebrosides. Despite our simplifying assumption of using one internal standard, it would appear that quantification of sphingolipids using our protocol is reasonable.

Principal component analysis on sphingolipid families

To reduce the number of variables in our data, we used PCA on the number of moles per g dry SC of each family of sphingolipids. Three axes accounted for 82.7% of the variance (Table 1.4). A plot of scores for individual birds along these 3 axes provided
clear separation between mesic and desert sparrows (Fig 1.12A). When we added to this plot the eigenvector loadings of the sphingolipid families, we were able to sort our variables in three groups (Fig. 1.12B). Diosylceramides, the only sphingolipid type with negative values for principal component 1 (PC 1), were isolated in the coordinate plane. PC 2 separated ceramides with negative scores, and cerebrosides with positive scores. PC 3 separated ceramides containing long or short fatty acid chains, and polar from non-polar cerebrosides (Fig 1.12A). These two plots combined suggest that PC 1 was related to the presence or absence of diosylceramides, and that this variable separated mesic from desert sparrows. When we repeated the analysis excluding diosylceramides, we also found that PC 1 discriminated between desert and mesic sparrows and the eigenvector loadings of the remaining variables for PC 1 were also high (>0.5). Taken together these results suggest that PC 1 is related to the interaction of sphingolipid molecules in the SC of house sparrows, and that there are some characteristic combinations of lipids in desert birds compared to mesic sparrows that yield the distinct scores in this component. The scores of all desert birds were positive for PC2 suggesting that modification of cerebrosides is more important for these birds than for mesic sparrows. PC 3 scores of the sparrows from Saudi Arabia tend to cluster close to the loadings of polar cerebrosides and long ceramides, whereas scores of sparrows from Ohio are scattered throughout the entire range of values of PC3.
<table>
<thead>
<tr>
<th>Correlation PC and original variable</th>
<th>Principal component</th>
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<tbody>
<tr>
<td></td>
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</tr>
<tr>
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<tr>
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<tr>
<td>Cerebrosides NS</td>
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<td>Cerebrosides AH</td>
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<td><strong>Eigenvalue</strong></td>
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<td><strong>Percent of variance</strong></td>
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Table 1.4. Principal component analysis of millimoles/g SC dry mass of sphingolipid families in the SC of house sparrows from desert and mesic environments.
Figure 1.12. Principal component analysis based on the number of moles per g dry SC of each sphingolipid family in the SC of house sparrows. A. Scores for individual house sparrows from desert (unfilled circles) and mesic environments (filled circles). B. Eigenvectors for each sphingolipid family. Abbreviations: CEOS = Ceramide EOS; CNS = Ceramide NS; CEOH = Ceramide EOH; CAH = Ceramide AH; NS = Cerebroside NS; NP = Cerebroside NP; EOH = Cerebroside EOH; AS-NH = Cerebroside AS-NH; AH = Cerebroside AH; NH = Cerebroside NH; DIOS = Diosyleramides. O = Origin of coordinates.
We also performed PCA on the amounts in mg lipid/g dry SC of each sphingolipid family present in the SC of sparrows. We extracted two components, accounting for 74.8% of the variance (Table 1.5). The scores for each individual bird provided a good separation between sparrows from Saudi Arabia and Ohio and the scores of desert sparrows tended to cluster closer to the loadings of longer ceramides and more polar cerebrosides (Fig. 1.13).

PCA suggests, then, that long chain length and sphingolipids with greater polarity distinguish desert sparrows from those living in Ohio leading to the idea that the decrease in CWL observed in desert sparrows is in some way related to chain length of the fatty acid moiety and to polarity of sphingolipid molecules.

**Biochemical properties of the sphingolipids in the SC of desert and mesic sparrows**

To test the hypothesis that desert sparrows have longer chain lengths in the fatty acid moieties of sphingolipids, we grouped sphingolipids that we extracted from the SC into decades, based on carbon chain length of the fatty acid (Fig 1.14). We found that desert sparrows had more sphingolipids in their SC with fatty acid tails ranging between 51 and 60 carbons than did sparrows from Ohio (t = 2.62, P < 0.02), whereas Ohio individuals had a significantly higher proportion of sphingolipids with fatty acid tails 21 to 30 carbons long (t = 4.09, P < 0.001). Therefore, desert sparrows had a larger
proportion of long sphingolipids, whereas mesic birds had proportionally more sphingolipids with short chain lengths in support of the hypothesis.

To test the hypothesis that desert sparrows had more polar sphingolipids in their SC than did mesic birds, we assigned ceramides EOS, NS, EOH and AH a value for polarity of 1 to 4, respectively; similarly, cerebrosides NS, NP, EOH, AS-NH, AH, NH, and diosylceramides ranked in our polarity scale from 1 to 7, respectively. Then we calculated the average polarity index of the sphingolipids. In support of the hypothesis, we found that desert birds had a higher polarity index, 2.68, and therefore more polar ceramides and cerebrosides than mesic sparrows, with a polarity index of 2.50 (t > 2.84, P < 0.01). However, ceramide AH, the most polar ceramide class that we found, was significantly more abundant in sparrows from Ohio (P < 0.01). The proportion of cerebrosides AS-NH and NH unsaturated was significantly higher in sparrows from Saudi Arabia but cerebroside AH was more abundant in mesic birds (t > 2.84, P < 0.01 in all cases). We only found diosylceramides, the most polar group of sphingolipids in our samples, in Ohio birds.
Table 1.5. Principal component analysis of amounts (mg lipid/g SC dry mass) of sphingolipid families in the SC of house sparrows from desert and mesic environments.

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<td>Cerebrosides NP</td>
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<td>Cerebrosides AH</td>
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<tr>
<td>Cerebrosides NH unsaturated</td>
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<td>Percent of variance</td>
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</table>
Figure 1.13. Principal component analysis based on amounts in mg of lipid per g dry SC for each sphingolipid family in the SC of house sparrows. (A) Scores for individual house sparrows from desert (unfilled circles) and mesic environments (filled circles). (B) Eigenvectors for each sphingolipid family. Abbreviations as in Figure 1.12.
Figure 1.14. Distribution of lipid amounts (millimoles of lipid per g dry SC) of sphingolipids according to the chain length of the fatty acid moiety.

**CWL and intercellular lipids as determined by TLC**

CWL did not vary with the quantity of total lipids in the SC for either group of sparrows ($R^2 < 0.06, p > 0.27$, for all cases). However, among sparrows from Ohio, CWL was positively correlated with the percentage of free fatty acids ($R^2 = 0.50, P < 0.01$), and it varied negatively with the percentage of total ceramides, ceramide 3, and total cerebrosides ($R^2 = 0.46, P < 0.02$; $R^2 = 0.36, P < 0.04$; $R^2 = 0.39, P < 0.04$, respectively) (Fig. 3). There were no significant correlations between CWL and percentages of the
different lipid classes in sparrows from Saudi Arabia, but this may not be surprising since variation tended to be lower in desert sparrows.

**Relationship between PC scores and CWL**

Our data suggested that a decrease in CWL in desert sparrows could be the result of longer free-fatty acid moieties and more polar sphingolipids in their SC. To test this idea, we explored the association between principal components, dominated by chain length and polarity of sphingolipids, and CWL in each population of sparrows. Using PCA on both the number of moles and the amounts in mg/g dry SC of each sphingolipid family, we found a negative association between PC 1 and CWL, with sparrows from both populations combined (P < 0.05).

In some cases, the relationship between CWL and PC scores differed between desert and mesic sparrows. Using PCA on the number of millimoles of each sphingolipid family, we found that CWL was significantly correlated with PC 2 in desert birds (P < 0.015), and with PC 3 in mesic sparrows (P < 0.03) (Fig. 1.16). After performing PCA on the amounts (mg lipid/g dry SC) of each sphingolipid class in SC, we found that CWL was positively associated with PC 1 (P < 0.05), and negatively correlated with PC 2 (P < 0.005), but only in desert individuals in both cases (Fig. 1.17).
Figure 1.15. Cutaneous water loss (CWL) in desert (unfilled circles) and mesic sparrows (filled circles, solid lines) as a function of the percentage of free fatty acids (FFA), ceramides and cerebrosides.
Figure 1.16. Relationship between CWL and PC 1, PC2 and PC 3 extracted from PCA based on the number of moles per g dry SC in each sphingolipid family from the SC of house sparrows from desert (unfilled circles) and mesic environments (filled circles). Plots with regression lines indicate statistical significance (P < 0.03).
Figure 1.17. Relationship between CWL and PC 1 and PC 2 extracted from PCA based on the amount in mg per g dry SC in each sphingolipid family from the SC of house sparrows from desert (unfilled circles) and mesic environments (filled circles). Plots with regression lines indicate statistical significance (P < 0.05).
Identification and quantification of covalently bound lipids by thin layer chromatography

Using TLC, we found two classes of ceramides and two classes of cerebrosides covalently bound to corneocytes in the SC of sparrows (Fig. 1.18A). To further separate cerebrosides, we used HPTLC, which allowed us to resolve the less polar band of cerebrosides into two separate bands (Fig. 1.18B). After we sprayed these plates with 2,4-dinitrophenylhydrazine and heated them, we observed the orange coloration characteristic of sugar molecules. Hence not only was the migration pattern on these plates consistent with cerebrosides, but also chemical tests confirmed the presence of a sugar molecule in the band. We did not detect neutral lipids such as free-fatty acids on plates. Therefore, ceramides and cerebrosides were the only covalently bound lipids we found in the SC of house sparrows.

To explore differences that might occur in covalently bound lipids between sparrows that inhabit markedly different environments, we compared bound lipids in the SC of sparrows from Saudi Arabia with those in the SC of sparrows from Ohio. We found that sparrows from Saudi Arabia had significantly less ceramides and more cerebrosides covalently bound to their corneocytes than did sparrows from Ohio (t = 2.48, P < 0.03; t = 2.10, P < 0.05, respectively) (Table 1.6). The ceramide:cerebroside
ratio was 0.21 in desert sparrows and 0.41 in Ohio birds, a difference that was significant (t = 5.95, P < 0.001).

**Identification of covalently bound cerebrosides by photospray ionization mass spectrometry**

Using HPLC-APPI-MS we confirmed the presence of molecules of cerebrosides covalently bound to keratinocytes in SC of sparrows from Ohio. We present a representative spectrum of one of the cerebrosides in our extract (Fig. 1.19A). The molecular ion \([M + H^+ – 2H_2O]\) had a mass of 1016.9, which is consistent with a cerebroside with a hydroxyl group at the omega position (Fig. 1.19B). Hydroxyl ions in sphingolipids are typically lost as water in the photospray ionization process. The ion 837.12 is consistent with \([M + H^+ - \text{Hexose}]\) and indicated that the molecular ion contained a sugar moiety. The fragment at 604.92 further supports the view that the molecule had a terminal hydroxyl group. The fragment at 264.36 indicates the presence of sphingosine (Muñoz-Garcia et al. 2006).
Figure 1.18 Carbon density profile of lipid standards (upper graph) and sphingolipids from the SC of house sparrows (bottom graph) using TLC (A) and HPTLC (B). Cerebr, cerebroside; Cer, ceramide, u.a., arbitrary units.
Table 1.6. Quantities of covalently bound ceramides and cerebrosides (in mg lipid/g of dry SC mass) in the stratum corneum of house sparrows from Saudi Arabia (n = 11) and Ohio (n = 8) as determined by thin layer chromatography. Superscripts indicate statistical significance; a, P < 0.05; b, P < 0.003.
Relationship between CWL and covalently bound lipid

We explored the relationship between covalently bound lipids on corneocytes and physiological function by examining these lipids in relation to water loss through the skin. Our plots showed that differences in the composition of the lipid envelope were associated with CWL (Fig. 1.20). CWL of mesic sparrows was 14.98 ± 2.92 mg H₂O/(cm² · d), whereas sparrows from Saudi Arabia lost 11.87 ± 2.22 mg H₂O/(cm² · d) through the skin, a difference that was significant (t = 2.65, P < 0.02; Munoz-Garcia and Williams 2005). Sparrows from Ohio had a lower total content of covalently bound lipids, a higher concentration of covalently bound ceramides, a lower content of covalently bound cerebrosides than desert sparrows, and a higher ratio of ceramides to cerebrosides than did desert sparrows.
Fig. 1.19. A. Molecular spectrum obtained by MS/MS of a representative covalently bound cerebroside. This spectrometric profile might correspond to cerebroside OS, a cerebroside with a terminal hydroxyl group at the omega position of the fatty acid moiety, although assignment to other cerebroside families is possible. B. Interpretation of the molecular structure of the fragments of cerebroside OS 40:0. See text for explanation.
Figure 1.20. Cutaneous water loss (CWL) of sparrows from Saudi Arabia (open circles) and Ohio (filled circles) as a function of concentrations of (A) covalently bound total lipid (B) covalently bound ceramides (C) covalently bound cerebrosides, and (D) ceramide to cerebroside ratio in the SC as determined by TLC.
DISCUSSION

For a single species of free-living house sparrow, our study provides evidence that total evaporative water loss is lower in populations living in desert environments, a diminution correlated with a reduction in CWL. Reduction in CWL was coincident with sparrows in Saudi Arabia having larger concentrations of ceramides and cerebrosides in their SC. Variation in CWL among individuals was related to changes in the lipids within the SC for sparrows from Ohio, but not those from Saudi Arabia which tended to have little variation in CWL. For birds in Ohio, free fatty acids were positively associated with CWL, whereas ceramides and cerebrosides were negatively correlated with CWL. In both populations of sparrows, we have found high concentrations of cerebrosides in their SC, a lipid class that seems to be unimportant in the SC of mammals, but may serve a prominent role in integument barrier formation in birds. Although we have detected changes in the lipids of the SC in desert sparrows compared to mesic individuals, these alterations do not seem to significantly affect passive diffusion of water through skin removed from the bird. Thus, we think that biological processes in concert with the physical lipid barrier operate to reduce CWL.
Cutaneous water loss in desert and mesic house sparrows

In order to compare house sparrows with other birds, we compiled data for CWL from the literature (Table 1.7). As one might expect, amongst 21 species of birds, CWL (g H$_2$O/d) varied positively with body mass (g) as described by CWL = 0.011 · Body Mass + 11.946 ($R^2 = 0.98$, $P < 0.01$). However, we found that surface-specific CWL also varied with body mass: CWL = 11.44 + 7.93 · log Body Mass ($R^2 = 0.45$, $p < 0.001$) (Fig. 1.21). Larger birds tend to lose more water per unit surface area than do small birds. Therefore, expressing CWL on a surface-specific basis does not standardize comparisons for interspecific data sets. Using ANCOVA with body mass as a covariate, we could not find any significant difference between desert and non-desert birds ($P > 0.45$). These data suggest that large birds, having a smaller surface to volume ratio, need higher rates of water loss for thermoregulation than birds of smaller size, a hypothesis in need of testing.

Desert house sparrows had significantly lower rates of CWL than did sparrows from Ohio, about 33% less. This reduction can be attributed to acclimation, genetic differences, or both. Using our allometric equation for surface specific CWL, sparrows in Saudi Arabia and Ohio should lose 21.8 and 22.3 mg H$_2$O/cm$^2$ · d, respectively. Sparrows from Saudi Arabia lost 11.9 mg H$_2$O/cm$^2$ · d, whereas in Ohio House sparrows evaporated 16.0 mg H$_2$O/cm$^2$ · d through their skin. The reduction in CWL among sparrows in Arabia can be attributed in part to a smaller body size, but also to changes in the rates of CWL through skin. Our calculations from allometric curves indicate that
reduction in body size only accounts for a difference of 0.17 g H₂O/d, with the remainder being an alteration in CWL. Changes in the lipid composition of the skin associated with factors operating in the live animal may explain these different water loss rates in sparrows from different environments.

Although we found that the lipid composition of the SC differs in desert house sparrows, this alteration by itself does not seem to make the physical permeability barrier more effective when compared to their mesic counterparts. If only passive diffusion through the skin were acting on water loss rates in House sparrows, we would predict differences in water loss rates through the dead skin of the sparrows from desert and mesic populations. However, water evaporation rate through the non-living epidermis attached to vials was not significantly different between the two populations. If passive diffusion were the only factor accounting for evaporation through the skin of the sparrows, birds in Saudi Arabia would lose 3.24 g/d, whereas sparrows in Ohio would evaporate 3.73 g/d. Live animals lose 28.7% and 35.4% of these values, respectively.
<table>
<thead>
<tr>
<th>Species</th>
<th>Body mass (g)</th>
<th>CWL (mg/d·cm²)</th>
<th>% of TEWL</th>
<th>Deviation from prediction</th>
<th>Source</th>
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Continued

Table 1.7. Body mass and cutaneous water loss for 21 species of birds. Predicted values for CWL were calculated using the allometric equation reported in Fig. 6. Deviation from prediction was computed as [(observed CWL – predicted CWL) x 100] / predicted CWL. All the species were measured at 30°C, except when noted.
Table 1.7. Continued

<table>
<thead>
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<th>Species</th>
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Table 1.7. Continued

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Figure 1.21. Relationship between surface-specific CWL, obtained by Meeh’s equation (Walsberg and King 1978), and log Body Mass for 21 species of birds.
Therefore, biological factors operating in the live animal influence CWL significantly. Activation of the metabolic machinery would produce a reduction of water loss through the skin. For example, pH is lower in the upper layers of the SC than in the lower SC (Elias 2004). Most of the enzymes that convert lipids in the multigranular bodies to those lipid classes that form the permeability barrier have a maximum activity within a pH of 4-6. It is likely that active processes are responsible of creating and maintaining this pH gradient allowing regulation of the biochemical properties of the SC. Consistent with the idea that there is a metabolic cost in maintaining the barrier to vapor diffusion, we found a negative correlation between surface-specific CWL and oxygen consumption in desert house sparrows (Fig 1.22).
Figure 1.22. Relationship between surface-specific CWL and mass-specific oxygen consumption in the sparrows from Saudi Arabia (open circles) and Ohio (solid circles). Regression was only significant in the sparrows from Saudi Arabia, Mass-specific oxygen consumption (kJ/g · d) = -48.1 · Surface-specific CWL (g/ cm² · d) + 1.94 (solid line, R² = 0.56, p < 0.01).
Intercellular lipids in the stratum corneum of desert and mesic house sparrows as determined by thin layer chromatography

The lipid composition of the SC of House sparrows differed from that found in other species of birds. In pigeons and chickens, free fatty acids comprised about 20-30% of the dry weight of the SC (Menon et al. 1986; Wertz et al. 1986). The percentage of free fatty acids in our samples exceeded 55% in both desert and mesic house sparrows. Sphingolipids accounted for about 25% of the dry weight of the SC in pigeons, with ceramides and cerebrosides accounting for equal proportions (Menon et al. 1986). The percentage of sphingolipids in the SC in both populations of sparrows was around 40%. Moreover, in our samples almost two thirds of the sphingolipids are cerebrosides. A high proportion of glucosylceramides apparently precludes the formation of lamellae in the intercellular spaces of the SC in mammals (Holleran et al. 1993, Proksch et al. 1993). This effect is not caused by a decrease in ceramides, so it does not imply a negative association between these two lipid classes. In fact, we found a positive relationship between ceramides and cerebrosides in House sparrows. Cerebrosides can be cleaved to form ceramides by glycosidases (Wertz and Downing 1989). Therefore, accumulation of cerebrosides could lead to a mobilization of a higher amount of ceramides whenever it is necessary by increasing the activity of this enzyme.
APPI-MS as a method to identify and quantify lipids in the stratum corneum

In this study, we used a new method of reversed-phase HPLC and APPI mass spectrometry to identify and quantify sphingolipid molecules that exist in SC of desert and mesic house sparrows. We identified over 200 different molecules of ceramides and cerebrosides, an unattainable task with other methods. Our method characterizes each lipid molecule by its retention time, molecular weight, source fragmentation and intensity. We detected 97 molecular species of cerebrosides and 79 of ceramides in the SC of House sparrows, ranging from 10 to 31 molecules in each family. Sphingolipids in the SC of sparrows differed from those of mammals in that we found a wide variety of cerebrosides. Amounts calculated from TLC and from APPI/MS differed by +0.39 % for ceramides and –2.4% for cerebrosides, close concordance despite our use of a single ceramide internal standard. APPI-MS provides a valuable tool to study sphingolipids in the SC and their role in regulating water loss through the skin. As accurate as TLC for quantification of lipids, APPI-MS goes beyond TLC offering information about the individual molecules of sphingolipids in the SC.

Biochemical properties of intercellular lipids of the stratum corneum of desert and mesic house sparrows as determined by HPLC-APPI/MS

Using HPLC-APPI/MS we identified over 200 different molecules of ceramides and cerebrosides in the SC of house sparrows, an unattainable task with other methods.
PCA reduced our variables to a few principal components that suggested that the biochemical properties of the sphingolipids of SC play an important role in the adjustment of CWL to the environment. Specifically, chain length of the fatty acid residues and polarity of the sphingolipids may establish molecular interactions that in turn seem to be important in determining CWL. Carbon chain lengths of fatty acids of sphingolipids were longer than those reported to occur in mammalian SC, which typically range between 24 and 30 carbons (Wertz 2000); in sparrows, chain lengths over 40 carbons were common. We do not know if chain lengths over 40 carbons are also common in mammals, but go undetected using current methodologies, like gas chromatography. Results indicated that desert house sparrows had longer chain lengths in the fatty acid residues and more polar sphingolipids than sparrows from Ohio, and that these differences were associated with the reduction in CWL observed in the population living in a xeric environment. Long chain lengths create more hydrogen bonds between molecules, and contribute to a tighter packing of the sphingolipids. Lipids of SC of desert sparrows were more polar than those of mesic birds, in agreement with results on larks (Haugen et al. 2003a, b). However, the most polar sphingolipid classes in sparrows were more abundant in individuals from Ohio, but their overall abundance was low compared to other lipids. Diosylceramides were found only in the SC of mesic sparrows, but the significance of this finding is unclear; diosylceramides may have the potential to attract
more molecules of water than other cerebroside classes, which might influence water permeation.

**Thermoregulation and water conservation**

The high concentration of glycosphingolipids within the SC of desert sparrows seems counterintuitive because in mammals an increase in glycosphingolipids decreases barrier function of skin (Holleran et al. 1993). Birds in general are thought to have a less competent barrier than mammals, and they typically have higher concentrations of glycosphingolipids in their SC (Elias and Menon 1991). So how can we reconcile desert sparrows having lower water loss, yet at the same time higher concentrations of cerebrosides in their SC? We suggest that desert house sparrows possess a SC designed for both thermoregulation and water conservation (Muñoz-Garcia and Williams 2005; Muñoz-Garcia et al. 2008a).

The SC serves multiple purposes in vertebrates and is not used solely for reduction in water permeation through the skin. Another important aspect of CWL is that it may be a significant component of the thermoregulatory apparatus of a living bird. House sparrows in deserts of Saudi Arabia have access to water because they are associated with Bedu camps where water is provided to livestock. At the same time, these sparrows are subjected to high $T_a$s, especially during summer when $T_a$ can reach 45°C each day. Thus selection for their ability to maintain $T_h$ may be important.
In the sandwich model for the organization of the SC of mammals (Bouwstra et al. 2000), the polar heads of ceramides line up facing each other in the lamellae, whereas non-polar tails orient inward. This creates a highly ordered lattice of lipids in crystalline phase, much like the membrane of a cell, which does not allow movement of water through the lamellae. However, in spaces between lamellae, fatty acid residues of the ceramides interact with cholesterol creating a fluid state between lamellae. Ceramide EOS, with its long carbon chain consisting of two fatty acids, one of them linoleic acid, spans the bilayers affixing them one to another and contributes to the fluidity of the central region between lamellae.

In our model, the more polar regions of ceramides line up together whereas non-polar tails orient inwardly forming a bilayer in crystalline phase, like the “sandwich model” (Fig. 1.23). However, we think that in the central fluid layer cholesterol, as found in mammals, will be replaced by cerebrosides in SC of birds, which will form a gel phase. This central layer then would contain cerebrosides, free fatty acids, small amounts of cholesterol and ceramides with short fatty acid chains. Because of the scarcity of the appropriate lipid species able to form a fluid phase in the mammalian SC, the fluid phase of the central layer of the lamellae is probably discontinuous in this taxon (Bouwstra et al. 2000). However, in birds this phase would be continuous within lamellae in the SC and would allow higher rates of water diffusion, explaining why birds have higher CWL rates than mammals. It is also likely that the polar heads of cerebrosides would interact
with water molecules, creating a pathway of diffusion of water through the skin; every cerebroside molecule can interact with 5-10 water molecules (Bach and Miller 1998).

Relative proportions of different classes of lipids in SC seem to be important in the formation of lamellar structures that are responsible for reduced CWL rates (Bouwstra et al. 2003; Muñoz-Garcia et al. 2008a). In mesic species of larks and mesic populations of house sparrows, increases in free fatty acid content may alter the free fatty acid to ceramide ratio, and affect the formation of the lamellae in the intercellular spaces of the SC (Haugen et al. 2003a, Muñoz-Garcia and Williams 2005). If we assume that the average free fatty acid molecule in the SC is 26 carbons long, free fatty acids, ceramides and cerebrosides are present in roughly equimolar amounts in sparrows from Ohio, but cholesterol is present in far lower quantities. The molar ratio between cholesterol and ceramides was 0.05-0.1. This cholesterol to ceramide ratio prevents the formation of a lamellar phase in mixtures of cholesterol, ceramides and free fatty acids (Bouwstra et al. 2000). In desert house sparrows the molar ratio of free fatty acids, ceramides and cerebrosides is approximately 1:1:2, and cholesterol still contributes little to the total, with a ratio 0.08:1 to ceramides. This result suggests that cerebrosides, and not ceramides, might be the key lipid class to explain differences in CWL between desert and mesic sparrows. Carbon chains of free fatty acids will be longer in mesic sparrows in our model, which will explain why amounts of free fatty acids are higher in mesic birds, but molar ratios with ceramides do not change, at least within species. In this case, the
periodicity between lamellae should be the same in desert and mesic birds. On the other hand, if free fatty acids are on average the same length in both populations, then periodicities should be longer in desert birds.

Also, changes in temperature might be associated with changes in phase behavior of the mixture of lipids in the SC. Therefore, the lipid composition of the SC of desert sparrows could be such that it prevents water evaporation at thermoneutral temperatures, but allows higher rates of CWL at high ambient temperatures to allow thermoregulation by evaporation, only because the same mixture of lipid can have different permeability properties at different temperatures.
Figure 1.23. Hypothesized model for the organization of lipids in the intercellular spaces of the SC in house sparrows from mesic and desert environments. In sparrows, lamellae would be formed by three layers of lipids; two outer layers consisting of ceramides, and an inner layer formed by cerebrosides and free fatty acids. Ceramides would form a highly ordered structure, whereas the inner layer would be more fluid. In this model, we assume that chain lengths of free fatty acids would be the same between desert and mesic individuals.
**Covalently bound lipids**

Our study showed that, like mammals, birds have ceramides covalently bound to corneocytes of the SC, but unlike mammals, sparrows also had large concentrations of cerebrosides attached to corneocytes. Previously, we reported the presence of cerebrosides in the intercellular lipid fraction in the SC of house sparrows (Muñoz-Garcia and Williams 2005; Munoz-Garcia et al. 2008a), a finding that is consistent for over 20 species of birds (Ro et al. unpubl. data). Thus, cerebrosides seem to constitute a major lipid class in the avian SC, in both the intercellular compartment and among lipids covalently bound to corneocytes. Because other lipid classes found in the SC are the same for birds and mammals, we suspect that the presence of cerebrosides in the SC is associated with differences in CWL rates between these taxa. However, the specific role of cerebrosides on the formation of the avian permeability barrier is not well understood.

When we compared the composition of the lipid envelope of the SC of desert and mesic house sparrows, we found that desert sparrows had a lower concentration of ceramides and a higher concentration of cerebrosides covalently bound to the corneocytes. Hence lower CWL of desert sparrows was associated with increase in cerebrosides covalently bound to the corneocytes. This result is in contradistinction to what we expected because glycosylceramides have an array of hydroxyl groups attached that we thought should interact with water and therefore increase water permeation.
This finding has prompted us to consider how covalently bound lipids might be organized on the surface of the corneocytes. We identified sphingolipids of the lipid envelope of house sparrows as ω-hydroxyceramides and ω-hydroxycerebrosides. Thus, covalently bound sphingolipids of house sparrows had a hydroxyl group at the omega position of the fatty acid residue, the same molecular structure as found in mammals (Wertz and Downing 1987, Farwanah et al. 2007). If hydroxyl groups of the acyl chains of the sphingolipids are covalently attached to the proteins of the protein envelope (Stewart and Downing 2001), the hexose moiety of the cerebrosides and the sphingosine heads of the ceramides will face the outer surface of the corneocyte. Why an increase in cerebrosides covalently bound to corneocytes reduced water vapor diffusion remains an enigma. We envision two models to stimulate thinking about the organization of these lipids in the SC of birds. Both models assume that ceramides align along the outer surface of the intercellular lamellae (Bouwstra et al. 2003, Muñoz-Garcia et al. 2008a) and that adjacent corneocytes are bound together by interactions of covalently bound ceramides.

The “water shell” model suggests that hexose moieties from cerebrosides will form hydrogen bonds with molecules of water forming a water shell around each corneocyte (Figure 1.24A). In this model, strong interactions between water molecules and hydroxyl groups of sugar residues reduce water vapor diffusion through the skin. Desert birds had a high concentration of cerebrosides in the lipid envelope, which binds with higher amounts of water, resulting in lower rates of CWL. If this model is correct,
we predict that the level of hydration of SC from desert sparrows from which all intercellular lipids have been removed, would be higher than that of SC from sparrows from Ohio treated in the same manner.

The “hexose link” model, envisions that the hexose moiety of covalently-linked cerebrosides forms molecular interactions with sphingosine heads of the intercellular ceramides. In this model, covalently bound cerebrosides of desert sparrows form tighter chemical linkages with the intercellular lipids making water permeation slower (Figure 1.24B). Desert sparrows had more cerebrosides in the layer of covalently bound lipids, implying that a higher number of sugar molecules will interact with intercellular lipid layers. These molecular interactions will promote the formation of a more ordered structure leading to lower rates of CWL. Both models are compatible with our model for the organization of the intercellular lipids of the SC (see above).
Figure 1 24. Hypothesized models for the organization of the covalently bound sphingolipids in the SC in house sparrows from mesic and desert environments. (A) Water shell model. The hexose group of cerebrosides would sequester water molecules. Desert sparrows, with more cerebrosides, could hold a higher amount of water, therefore reducing rates of CWL. (B) Hexose link model. Hexose molecules from cerebrosides would establish molecular interactions with the sphingosine heads of the ceramides that form the outer layer of the intercellular lamellae. In both models, covalently bound ceramides connect adjacent corneocytes.
Conclusions

In conclusion, CWL in house sparrows living in a desert environment was reduced compared to mesic house sparrows, and this decrease in CWL was responsible for the reduction of TEWL in desert house sparrows when compared to the mesic population. We found an alteration of the lipid composition in the SC, yet we did not find any significant difference in the properties of the physical barrier in both populations. Thus, biological control mechanisms must play a crucial role enhancing the permeability barrier.

The picture that has emerged from studies on mammals and now birds is that lipids of the SC are synthesized in the Golgi apparatus of the basal cells of the epidermis (Landmann 1980). As these cells progress towards the exterior of the epidermis, the Golgi apparatus transforms into multigranular bodies (Landmann 1980): some lipids in these organelles, mainly glycolipids and phospholipids, are stacked in lamellae, whereas others are thought to be bound to the membrane. When multigranular bodies fuse with the cell membrane of the corneocytes, lipids are extruded to the exterior. Some form intercellular lamellae while others covalently bind to proteins of the outer surface of the corneocytes, creating a monolayer of lipids that coats the cell (Wertz 2000). In mammals, the sugar moiety of covalently bound cerebrosides is cleaved enzymatically to produce ceramides, the major component of the mammalian lipid envelope (Wertz and Downing 1987). Apparently, this enzymatic transformation occurs only partially in avian SC
because we have found ceramides and cerebrosides covalently bound to corneocytes.

With their protruding sugar molecules, cerebrosides bound to corneocytes would have a profound effect on the formation of incipient lipid lamellae of the intercellular spaces in the SC. Hence covalently bound lipids affect the organization of the intercellular lipids of the SC, which in turn influences rates of CWL.

From an evolutionary perspective, the balance between requirements for thermoregulation by evaporative means and water conservation might have played an important role in the evolution of the composition of the skin in desert species of birds. Birds have higher CWL rates than mammals, a feature presumably related to thermoregulation, and the substitution of cholesterol by cerebrosides would provide a less tight permeability barrier. At the same time, adjustments in the lipid ratios in the SC will make the barrier more competent in species that live in xeric environments. It is worth noting, though, that CWL of desert birds are still higher than those of the average mammal. On the other hand, the thermoregulatory needs of mammals are satisfied in different ways than those of birds, and CWL is not an important process in this context. Free from the thermoregulatory function, the mammalian SC has evolved towards the creation of a highly efficient barrier, where cerebrosides have no part, except as ceramide precursors. However, few species of free-living mammals have been studied and therefore conclusions for this taxon are tentative.
At the population level, the lipid composition of the SC and the interactions among lipid classes are important to reduce CWL in desert house sparrows. Consistent with this idea, the coefficient of variation of the amounts of all the lipid classes that we identified in this study is larger in mesic sparrows than in desert sparrows, suggesting that selection pressures have been stronger towards the occurrence of particular combinations of lipids in the SC in birds that live in the desert. Moreover, the association between CWL and PC scores was stronger in desert individuals, a sign of tighter regulation of the composition and interactions of the lipids in the SC in sparrows from desert environments. We cannot exclude, though, the role of phenotypic plasticity. The next two chapters address the relative importance of natural selection and phenotypic plasticity in the formation of the permeability barrier in birds.
CHAPTER 2

PHENOTYPIC FLEXIBILITY IN CUTANEOUS WATER LOSS AND LIPIDS OF THE STRATUM CORNEUM IN HOUSE SPARROWS FOLLOWING ACCLIMATION TO DRY AND HUMID ENVIRONMENTS

INTRODUCTION

Balancing water intake with losses can determine survival and reproduction, and thus fitness, for many animals. Although it was once thought that cutaneous water loss (CWL) represented a small fraction of total water loss in mammals and birds (Bartholomew and Cade 1963, Schmidt-Nielsen et al. 1970, Mount 1979), it is now appreciated that CWL represents a substantial contribution to total water efflux, comprising as much as 50–70% of total evaporative water loss (TEWL) at normothermic temperatures in many species (Bernstein 1971, Wolf and Walsberg 1996, Tieleman and Williams 2002, Muñoz-Garcia and Williams 2005). Comparisons of species from arid versus humid environments support the idea that desert birds have lower TEWL than mesic counterparts (Williams 1996, Williams and Tieleman 2000), and data from larks indicate that this pattern is driven by underlying differences in CWL, rather than respiratory water loss (RWL) (Tieleman et al. 1999, Tieleman and Williams 1999, 2002).
While these differences in CWL presumably reflect some degree of underlying genetic divergence, several species can also alter water loss in response to short-term humidity or temperature acclimation (Tieleman and Williams 2002, Haugen et al. 2003b, Tieleman et al. 2003). However, the cellular and molecular mechanisms that enable such physiological plasticity in CWL are not well understood.

In birds and mammals, skin resistance to CWL is conferred by a barrier consisting of lipid molecules arranged in an extracellular matrix located in the stratum corneum (SC), the outer layer of the epidermis (Menon et al. 1986, Wertz 2000, Bouwstra et al. 2003, Coderch et al. 2003, Lillywhite 2006). The SC of mammals is composed of corneocytes, flattened dead cells embedded in a matrix of lipids, primarily ceramides, cholesterol and free-fatty acids, in near equimolar amounts (Bouwstra et al. 2003). Lipids in the mammalian SC are organized in bilayers called lamellae that retard water permeation through the skin (Menon and Ghadially 1997, Wertz 2000, Bouwstra et al. 2003). Although how each lipid class is involved in the formation of lamellae is controversial, there is general agreement that ceramides, a molecule of sphingosine amide-linked to a long-chain free-fatty acid, are the structural backbone of the bilayers, and are thus essential for the formation of a tight barrier to water vapor diffusion through the skin (Forslind et al. 1997, Norlen 2001, Hill and Wertz 2003, Bouwstra et al. 2003). Gaucher’s disease is characterized by a deficiency in the enzyme β-glucocerebrosidase (β-GlcCer’ase), which converts cerebrosides to ceramides. In the absence of functional β-
GlcCer’ase, mammalian SC is characterized by an abundance of cerebrosides, a reduction in ceramides, and impaired barrier function (Holleran et al. 1994a, Liu et al. 1998). Thus, β-GlcCer’ase is essential to the formation and maintenance of a competent lipid barrier to water loss in mammals (Glew et al. 1988, Holleran et al. 1992, 1993, 1994a, b, Liu et al. 1998, Takagi et al. 1999).

Whereas cerebrosides are present in only trace amounts in mammalian SC (Wertz 1992), the proportion of cerebrosides often equals or exceeds that of ceramides in avian SC (Menon et al. 1986, Wertz et al. 1986, Muñoz-Garcia and Williams 2005). This condition would be pathological in terrestrial mammals (e.g., Liu et al. 1998), raising the question of how and why birds differ from mammals in SC lipid composition. A lack of cerebrosides is associated with the absence of intercellular lipid lamellae in birds that are normally hydrated, a feature thought to result in a less competent permeability barrier (Menon and Menon 2000). Cerebrosides may increase water permeability of the SC because hydroxyl groups of the sugar would tend to attract water molecules rather than repel them, potentially creating a water channel, an analogous process to what appears to happen in cell membranes (Carruthers and Melchior 1983).

Unlike mammals, the skin in birds is an important component of the thermoregulatory apparatus in some species (Bernstein 1971, Peltonen et al. 2000, Webster et al. 1985, Webster and King 1987). At thermoneutral temperatures, average surface-specific CWL for 21 species of birds was 28.2 mg H₂O/ (cm² · d) (Muñoz-Garcia
and Williams 2005), 2-3 times higher than rates characteristic of mammals. At temperatures above 40°C, birds increase rates of CWL by a factor of 1.5 up to 20 fold, depending on the species (Bernstein 1971, Marder and Ben-Asher 1983, Wolf and Walsberg 1996, Tieleman and Williams 2002). Many species, however, rely on an increase in RWL for evaporative cooling under acute heat stress. In the verdin (*Auriparus flaviceps*) and four species of larks, CWL represented 65% of total evaporative water loss at 25-30°C, but the contribution of CWL decreased to 13% at temperatures above 45°C (Wolf and Walsberg 1996, Tieleman and Williams 2002). On the other hand, pigeons, *Columba livia*, chronically exposed to air temperatures (*T_a*) between 40 and 60°C, seem to use CWL to thermoregulate at high temperatures, increasing CWL rates 20 times at *T_a*s above 50°C (Marder and Ben-Asher 1983). It is not clear if the differences observed between columbiforms and Passerines are the result of phylogenetic divergence or the consequence of short-term versus long-term exposures at high *T_a*. In any case, it appears that the skin of birds serves an important thermoregulatory role by facilitating evaporative water loss when *T_a* is chronically high and maintenance of body temperature below lethal limits is important.

Changes in lipid biosynthesis and metabolism may provide a mechanism for regulation of CWL in response to environmental demands on water homeostasis. Indeed, correlated differences in CWL and SC lipid composition in birds are associated with habitat aridity (Haugen et al. 2003a, Muñoz-Garcia and Williams 2005), thermal
acclimation (Haugen et al. 2003b), and water deprivation (Menon et al. 1989), but the underlying mechanisms for these differences have not been investigated.

Phenotypic plasticity may account for some variation in CWL among and within species of birds. However, studies exploring the role of phenotypic flexibility (sensu Piersma and Lindstrom 1997) in the origin and maintenance of variation in CWL are few (Kobayashi et al. 1983, Kattan and Lillywhite 1989, Williams and Tieleman 2000, Haugen et al. 2003b, Moen et al. 2005, Tracy and Walsberg 2001). After acclimation to different temperatures (Haugen et al. 2003b), larks increased the proportion of ceramides and decreased the proportion of free fatty acids in their SC, changes presumably correlated with a decrease in CWL. Zebra finches that were water deprived for five days (Menon et al. 1989) and pigeons acclimated to high temperatures (Peltonen et al. 1998, 2000), formed intercellular lamellae in the SC, presumably to decrease CWL. Mechanisms that allow individuals to change the structure and lipid composition in the SC as a result of an environmental alteration are, however, not understood. We hypothesized that water stress will change the enzymatic activity in the SC and convert cerebrosides into ceramides, resulting in a tighter permeability barrier and reduced CWL (Muñoz-Garcia and Williams 2005, Muñoz-Garcia and Williams 2007, Cox et al. 2008).

Muñoz-Garcia and Williams (2005) reported that House Sparrows (Passer domesticus L.) from the deserts of Saudi Arabia have lower CWL and greater quantities of ceramides and cerebrosides in their SC than conspecifics from mesic Ohio (USA).
Among Ohio sparrows, CWL was negatively associated with proportions of both ceramides and cerebrosides. In this study, we experimentally tested the extent to which phenotypic plasticity was responsible for the functional relationship between the lipid composition of the SC and CWL. Humidity has been proposed as one of the factors that may determine CWL in vertebrates, by initiating a cascade of enzymatic activity that may result in a change in the SC permeability (Elias 2004). Therefore, we chose to manipulate this variable in our study.

We exposed house sparrows to two environments, one dry and the other humid, at constant $T_a$ of 30°C, a temperature within their thermoneutral zone (Hudson and Kimzey 1966). We predicted that (1) humid-acclimated sparrows would not change their CWL with respect to non-acclimated birds (2) dry-acclimated sparrows would reduce their CWL, compared with non- and humid-acclimated birds (3) reduction in CWL in dry-acclimated birds would be associated with an increase in the amounts of ceramides and cerebrosides in the SC and a decrease in the amount of cholesterol (4) the free fatty acid:ceramide ratio would decrease in the SC of dry-acclimated birds. We also hypothesize that changes in the activity of enzymes, such as $\beta$-GlcCer’ase, allow sparrows to adjust the relative proportions of ceramides and cerebrosides in the SC, thereby regulating CWL in response to environmental stimuli. Thus, desiccating conditions that favor water conservation should stimulate $\beta$-GlcCer’ase activity, leading to increased SC ceramide content and reduced CWL. We predict that sparrows
acclimated to low ambient humidity should increase β-GlcCer’ase activity, increase SC ceramide content, and reduce CWL relative to humid-acclimated sparrows and non-acclimated controls.

MATERIALS AND METHODS

Humidity acclimation

We captured adult house sparrows (*Passer domesticus*) using mist nets and Potter traps in Columbus (Ohio, USA, 40°00' N, 83°10' W), during February-March 2006. Birds were held in captivity for 1-4 days before measurements. Sparrows were fed a mixture of seeds, mealworms and boiled egg yolk, and had unrestricted access to water. Experiments were approved by ILACUC of Ohio State University (Protocol 2003-A0072).

We measured cutaneous water loss (CWL), respiratory water loss (RWL) and oxygen consumption for all house sparrows shortly after capture (see below). After measurements, we randomly assigned each bird to one of two groups: dry-acclimated (n = 14) and humid-acclimated (n = 9). We also captured 11 additional sparrows to measure initial lipid composition of the SC prior to acclimation.

We placed sparrows assigned to humid and dry acclimation groups in wire cages in separate environmental chambers (Percival, models E-30B and I-30BLL), each
containing 5 or 6 sparrows, at a constant temperature of 30ºC (± 1ºC) with a photoperiod of 12L:12D. A vacuum pump pulled atmospheric air into the chambers at a rate of 75 ml/min. Dry-acclimated sparrows experienced an average relative humidity of 15-20%, corresponding to a mean absolute humidity of 6.5 g H₂O/m³. To maintain a low humidity in these chambers, we dried inlet air with two columns of drierite prior to entry into the chamber. Inlet air of these chambers had a dewpoint of –38ºC. Humid-acclimated sparrows experienced a relative humidity of 90-95%, corresponding to a mean absolute humidity of 31 g H₂O/m³. Dewpoint of the air entering those chambers was 28ºC, near saturation. To maintain a high humidity in these chambers, prior to entry into the environmental chamber we bubbled air through water contained in a sealed stainless steel canister at 25ºC. We also placed two pans filled with water beneath the birds’ cages inside the environmental chambers. Humidity and temperature were monitored continuously in all chambers using HOBO ProSeries data loggers.

Sparrows in the non-acclimated group were sacrificed by cervical dislocation after measurements of CWL, RWL and oxygen consumption, and their SC was isolated for identification and quantification of lipids. Humid- and dry-acclimated birds were held for either 5 d (short acclimation, n = 6 per treatment) or 21 d (long acclimation, n = 9 humid, 14 dry) in environmental chambers. After the 21d acclimation period, we again measured CWL, RWL and oxygen consumption, for birds in the humid and dry acclimated groups,
sacrificed them and determined the lipid composition of their SC. We also collected samples of the SC for enzyme essays.

**Measurement of metabolic rate and evaporative water losses**

We measured oxygen consumption, RWL and CWL using standard open-flow respirometry methods, as detailed in Chapter 1, section “Measurement of metabolic rate and evaporative water loss”, pages 12-15.

**Separation and identification of skin lipids**

We analyzed the lipid composition of the SC of sparrows using analytical thin layer chromatography, using the protocol detailed in Chapter 1, sections “Extraction of intercellular lipids of the SC”, pages 16-17, and “Analytical thin layer chromatography”, pages 18-20.

**Preparation of enzyme homogenates**

We excised 4-cm² tissue samples from apteria on the dorsal and ventral surfaces of each bird. Excised portions were pinned, dermis down, on filter paper that was impregnated with 0.5% trypsin in phosphate-buffered saline (PBS; pH 7.4), and stored overnight at 4°C (Menon et al. 1989). After 24 h, we removed any remaining feathers, peeled off the SC, and manually homogenized it using ground glass vials and pestles.
Samples were homogenized in extraction buffer consisting of 300 μl PBS with 0.05% Tween 20, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mg ml⁻¹ sodium taurocholate, each included to improve extraction of soluble enzyme (Pentchev et al. 1973, Holleran et al. 1992). Samples were held on ice during homogenization and subsequent incubation for 30 min in extraction buffer. We then centrifuged samples at 10,000 g and used the supernatant for assays.

**β-Glucosidase assay**

Our assay for β-glucosidase was modified from Holleran et al. (1992). Except where noted, assays were carried out in citrate-phosphate buffer (pH 5.6; McIlvaine 1921) containing 5 mM sodium taurocholate. We added 10 μl of enzyme sample to 40 μl of citrate-phosphate buffer and pre-heated this mixture to 37°C. We initiated reactions by adding 50 μl of citrate-phosphate buffer containing 10 mM 4-methylumbelliferyl-β-D-glucopyranoside (4MUG, Acros Organics). Reactions were run at 37°C for 60 min and terminated by adding 1.25 ml of 0.2 M carbonate-bicarbonate buffer (pH 10.5; Delory 1945). We measured enzyme activity as the production of fluorescent 4-methylumbelliferone (4MU) from the 4MUG substrate using an SLM 8100 DS spectrofluorometer (excitation λ = 360 nm, emission λ = 450 nm) calibrated with a standard dilution series of 0–300 nM 4MU (Sigma-Aldrich) in carbonate-bicarbonate buffer. We used a modified Bradford protein assay kit (Bio-Rad Laboratories) to
determine the protein concentration of each sample on the basis of a bovine serum albumin standard. We report all measures of enzyme activity in units of product formed per minute per mg protein.

We initially verified that production of 4MU was linear with time (0-120 min) beyond the duration of our assay (60 min) to ensure constant reaction velocity. We also diluted samples in PBS to verify that production of 4MU was linear over the range of protein concentrations obtained from SC homogenates. We confirmed linearity with time and enzyme concentration using both sparrow SC homogenates and a purified solution of modified human β-GlcCer’ase (Ceradase® alglucerase injection, Genzyme Corp., Cambridge, MA). We varied 4MUG substrate concentration (0.01–5 mM) and determined $K_M$ and $V_{max}$ using nonlinear Michaelis-Menten curve fits implemented in GraphPad Prism (version 3.02, GraphPad Software, San Diego, CA). We tested for nonspecific product formation by assaying samples in the presence of the inhibitor conduritol B epoxide (CBE, Alexis Biochemicals, 0.4–4,000 μM) and used GraphPad Prism to fit sigmoid competitive binding curves to these data.

Formation of 4MU in our assay could reflect either specific β-GlcCer’ase or nonspecific β-glucosidase activity, since both enzymes can occur in the epidermis and both hydrolyze the 4MUG substrate. However, only β-GlcCer’ase is involved in the conversion of cerebrosides to ceramides (Glew et al. 1988). Specific β-GlcCer’ase activity can be demonstrated unambiguously by using labeled glucocerebrosidase as a
substrate or by including the selective β-GlcCer’ase inhibitor bromocondutitol B epoxide, but these compounds are impractical because of their restricted availability (Glew et al. 1988, Holleran et al. 1993). A practical solution is to include sodium taurocholate, which stimulates β-GlcCer’ase while inhibiting β-glucosidase (Glew et al. 1988, Holleran et al. 1992, Peters et al. 1976). Additionally, β-GlcCer’ase activity is typically maximal at pH values ranging from 4.8–5.8, whereas β-glucosidase activity is maximal in a lower pH range of 4.0 and below (Peters et al. 1976, Glew et al. 1988, Holleran et al. 1992). To determine whether 4MUG hydrolysis in our assay reflects specific β-GlcCer’ase activity, we conducted assays across a pH gradient (3.2–7.2) in the presence (5 mM) or absence of sodium taurocholate.

Statistics

All statistical tests were performed with SPSS 14.0 or SAS (version 8.2, SAS Institute, Cary, NC) and GraphPad Prism (version 3.02, GraphPad Software, San Diego, CA). Averages are reported ±1 SD. We rejected the null hypothesis at P > 0.05. All data were examined for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett’s test) prior to analysis.

We used repeated measures ANOVA to compare mass, metabolic rates, CWL and RWL between humid- and dry-acclimated sparrows, with time as a within-subjects effect, treatment as a between-subjects effect, and a time-by-treatment interaction. Non-
acclimated sparrows could not be included in our repeated-measures analyses because they were only measured once for each variable. Therefore, we also used one-way ANOVA to compare initial values of each variable, followed by Tukey’s post hoc test.

We expressed the composition of each lipid class as both an absolute quantity per unit SC and as a percentage of total quantity of lipids in the SC, because the structure and function of the SC permeability barrier may depend upon both the amount and the proportion of lipid classes (Haugen et al. 2003a, b, Muñoz-Garcia and Williams 2005, Muñoz-Garcia and Williams 2007). Percentages were logit transformed [Ln (Y/1 – Y); Zar 1996) prior to analyses.

Analysis of lipids in the SC is complicated by covariation among lipid classes, likely derived from metabolic sequences within lipids in the SC (Wertz 2000). To detect these interactions, we regressed different lipid classes against each other using general linear models.

We also calculated the ratio of free fatty acids to ceramides, because these lipids interact to form the highly ordered lattice that impedes water movement in mammals, and because we have found that this ratio correlates with CWL in other avian species (Muñoz-Garcia and Williams 2007). We calculated the ratio of ceramides to cerebrosides, since we predicted that an increase in the hydrolysis of cerebrosides to ceramides would occur in response to dry acclimation.
For each of these measures of SC lipid composition, we compared groups using ANOVA with treatment (non-, dry-, and humid-acclimated) as the main effect and subsequently employed a Tukey’s post-hoc test. To investigate the functional consequences of variation in SC lipid composition, we tested for correlations between individual measures of CWL and these measures of SC lipid composition, both within and among treatments.

Comparisons of enzyme activity between non-acclimated and 21-d humid- or dry-acclimated groups were performed using one-way ANOVA with three treatment effects and Student-Newman-Keuls post hoc tests. We used t-tests to compare enzyme activity for long versus short acclimation periods within humid and dry groups, and also to compare humid versus dry groups within either acclimation period. We used Welch’s t-tests to compare long and short acclimation periods, since variances were significantly ($P < 0.05$) different between groups. We correlated individual measures of β-GlcCer’ase activity and CWL or SC lipid composition using general linear models with treatment group included as a categorical effect. This allowed us to test for associations between enzyme activity and CWL or lipid composition after controlling for overall treatment effects on each variable. Because interactions with treatment were not significant ($P > 0.2$), we dropped them from our final statistical models.
RESULTS

Body Mass

Prior to acclimation, we did not detect significant differences in mass, oxygen consumption, respiratory water loss, cutaneous water loss, or total evaporative water loss among the three groups of sparrows (P > 0.19 for all comparisons). Our analyses revealed a slight decrease in mass during acclimation among groups (F = 5.57, P < 0.03), but the magnitude of this decrease did not differ between humid (2%) and dry (4%) acclimated groups (F = 0.09, P > 0.77).

Metabolic Rate

We did not find any difference in pre- and post-acclimation measures of either whole organism or mass-specific oxygen consumption (F < 0.07, P > 0.20). We also found no effect of the treatment-by-time interaction on oxygen consumption (F < 0.07, P > 0.80), indicating that humid- and dry-acclimated sparrows did not significantly differ in post-acclimation metabolic rate (F < 1.12, P > 0.47, Fig. 2.1A).

Respiratory Water Loss

Whole organism RWL increased significantly following acclimation (F = 10.45, P = 0.004), a finding that held true when we corrected for body mass (F = 12.58, P =
The average magnitude of this increase was 37% in dry-acclimated birds and 16% in humid-acclimated birds. However, we did not find a significant time-by-treatment interaction (\(F = 1.52, P = 0.23\)), indicating that neither group differed in post-acclimation RWL (\(F = 0.82, P = 0.38\), Fig. 2.1B). Because we found no differences in oxygen consumption, apparently oxygen extraction efficiency changed, a phenomenon also observed in four species of larks acclimated to different \(T_a\) (Tieleman and Williams 2002).

**Cutaneous Water Loss**

Post-acclimation CWL was significantly lower in dry- than in humid-acclimated sparrows by 36.1\% (\(F = 6.73; P = 0.02\)), even when corrected for surface area (\(F = 8.36, P = 0.01\), Fig. 2.1C), although the time-by-treatment interaction was not significant (\(F = 3.46, P = 0.08\)). However, we did find a significant time-by-treatment interaction on whole-organism CWL (\(F = 4.71, P = 0.04\)). Both whole-organism and surface-specific CWL decreased significantly during acclimation (\(F = 54.50, P < 0.001; F = 53.15; P < 0.001\), respectively, Fig. 2.1C). Compared with initial values, the average magnitude of the decrease in CWL was 45\% in the dry group and 23\% in the humid group.
Figure 2.1. Mean values (± 1 SD) for measurements of (A) metabolic rate (B) RWL, and (C) CWL prior to acclimation (solid bars) and after 21-d acclimation (light bars) to humid and dry conditions.
Total Evaporative Water Loss

Although RWL increased following acclimation in both treatment groups, this increase was offset by concomitant reductions in CWL, such that whole-animal TEWL decreased significantly following acclimation (F = 7.78, P < 0.02). However, this effect was not significant when we corrected TEWL for body mass (F = 3.47, P > 0.07). On average, TEWL decreased by 18% in dry-acclimated and 4% in wet-acclimated sparrows, but we did not find a significant time-by-treatment interaction (F = 0.47, P > 0.50). At the end of the acclimation period, whole-animal TEWL was not significantly different between dry- and wet-acclimated sparrows (F = 3.01, P > 0.09), but mass-specific TEWL was 15% lower in dry- than in humid-acclimated sparrows (F = 5.09, P < 0.04).

Lipids in the stratum corneum

Our chromatographs revealed distinct bands of lipids corresponding to standards of cholesterol, free fatty acids, ceramides, and cerebrosides (Fig. 2.2). We found significant differences among treatments in the quantity of total lipids (F = 3.56, P = 0.04), cholesterol (F = 5.29, P = 0.01) and total cerebrosides (F = 4.59, P = 0.02) (Fig. 2.3). Tukey’s post-hoc tests showed that non-acclimated birds had significantly higher amounts of cholesterol and cerebrosides than acclimated birds, and that non-acclimated birds had higher quantities of total lipids than humid-acclimated sparrows, with dry-acclimated sparrows exhibiting intermediate values that were not statistically distinct
from either group (Fig. 2.3). Although the amount of free fatty acids followed the same
trend among treatments as cholesterol and cerebrosides, differences among groups were
not significant (P > 0.20).

We also expressed lipid classes as a percentage of the total amount of lipid
extracted, because proportions of some lipid classes in the SC are associated with
changes in CWL (Haugen et al. 2003a, b, Muñoz-Garcia and Williams 2005). We found
significant differences among our experimental groups in proportions of total ceramides
(F = 4.4, P = 0.02). Tukey’s post-hoc test revealed that non-acclimated sparrows had
lower proportions of total ceramides than humid-acclimated birds, with intermediate
values in the dry-acclimated group (Fig. 2.3).

**Correlations among lipids in the SC**

Quantities of some lipid classes were significantly intercorrelated only in dry-
acclimated sparrows (Table 2.1). In this group, ceramides were positively associated with
all the other lipid classes in the SC, and cholesterol was positively correlated with total
cerebrosides.
Figure 2.2. Profile of lipid standards (upper graph) and cerebrosides, ceramides, and cholesterol from the extracted lipids in the SC of House sparrows obtained using photodensitometry (bottom graph). Ceramides that separated into bands were named in order of increasing polarity, ceramides 1-5, as were the cerebrosides, 1-3. CHOL = Cholesterol; CER = Ceramide; CEREBR = Cerebroside; C1 to C3 = Cerebrosides 1 to 3. a.u., arbitrary units.
Figure 2.3. Mean values (± 1 SD) of lipid amounts (left panels) and percentages (right panels) in the SC of house sparrows after 21-d acclimation. Letters indicate significant differences between treatments (P < 0.05).
Table 2.1. Significant correlations between quantities of the different lipid classes in the SC of dry-acclimated house sparrows. Equations are of the form $Y = a + b \cdot X$. There were no significant correlations in non- or humid-acclimated sparrows.
Ratios of lipids in the SC

The ratio FFA:Total ceramides was higher in non-acclimated sparrows than in humid-acclimated or dry-acclimated birds (F = 6.52, P < 0.01). The ratio of ceramides to cerebrosides was not different among treatments (F = 0.99, P = 0.38). However, one datum from each of the three groups was an outlier, defined as points that differed more than two standard deviations from the mean; when we removed these three outliers, the ratio was significantly lower in non-acclimated sparrows than in acclimated birds (F = 4.3, P < 0.03).

Assay validation and enzyme characterization

Using purified human β-GlcCer’ase, production of 4MUG increased linearly with reaction time (0-120 min; R² = 0.967) and sample concentration (R² = 0.978). This verified that that our assay detects pure β-GlcCer’ase. In sparrow samples, formation of 4MUG was also linear with reaction time (0–120 min; R² = 0.999), indicating that reaction velocity is constant over our assay duration of 60 min. Formation of 4MUG was also linear with sample concentration (0–0.4 mg protein per ml sample; R² = 0.988), indicating that enzyme activity is proportional to protein concentration within the range of our experimental sample concentrations (0.13–0.42 mg/ml; mean ± SD = 0.23 ± 0.06 mg/ml).
Enzyme velocity increased curvilinearly with substrate concentration ($R^2 = 0.990$) with an apparent $K_M$ of $1.51 \pm 0.16$ mM (Fig. 2.4A). Therefore, our substrate concentration for experimental samples (5 mM) was 3.3 times $K_M$, yielding enzyme activity at 80% $V_{max}$. Enzyme activity followed a negative sigmoid relationship with inhibitor (CBE) concentration (Fig. 2.4B). The estimated inhibitor concentration for half-maximal activity (IC$\text{_{50}}$) was $1.11 \pm 0.07$ mM at substrate concentration of 5 mM, with a reduction in IC$\text{_{50}}$ at lower substrate concentrations (Fig. 2.4B).

Enzyme activity doubled in the presence of 5 mM sodium taurocholate at pH 5.6 (Fig. 2.5), a stimulatory effect characteristic of mammalian $\beta$-GlcCer’ase. In the absence of sodium taurocholate, enzyme activity decreased from maximal values at pH 3.2 to minimal values at pH 7.2 (Fig. 2.5). In the presence of taurocholate, enzyme activity was maximal at pH 4.0, with formation of 4MUG reduced by 37% at pH 5.6 (Fig. 2.5). Similar pH dependence was evident across individual experimental samples, but overall treatment effects on enzyme activity were qualitatively equivalent at pH 4.0 and 5.6, so we report only the results of assays conducted at pH 5.6.
Figure 2.4 (A) β-Glucosidase velocity of sparrow SC homogenates, expressed as a function of substrate (4MUG) concentration. Dashed lines indicate maximal velocity ($V_{max}$) and the substrate concentration yielding half-maximal velocity ($K_M$). (B) β-Glucosidase activity as a function of inhibitor (CBE) concentration at two substrate concentrations. Values are expressed as percent activity relative to controls without CBE. Dashed lines indicate 50% inhibition of enzyme activity and associated CBE concentrations (IC$_{50}$). Values in each panel are means of triplicate determinations.
Figure 2.5. β-Glucosidase activity of sparrow SC homogenates, expressed as a function of pH in the presence or absence of 0.5 mM sodium taurocholate. Values are means of triplicate determinations, expressed as percentage of maximal activity at pH = 4.0. Dashed line indicates the approximate pH of the stratum corneum, at which experimental samples were assayed.

**Treatment effects on β-GlcCer’ase activity**

Measures of β-GlcCer’ase activity from dorsal and ventral SC samples of the same individuals were correlated ($R^2 = 0.80; P < 0.001$; data log$_{10}$-transformed for normality), with no systematic bias toward greater activity from either surface. Hereafter, we report results of analyses using the mean of dorsal and ventral measures. β-GlcCer’ase activity was low prior to acclimation or after only 5 d acclimation (Fig. 2.6). By contrast, β-GlcCer’ase activity was elevated after 21 d acclimation in both humid and dry groups.
Comparisons among humid, dry and non-acclimated birds revealed a significant increase in β-GlcCer’ase activity following acclimation to either humidity regime ($F_{2,33} = 6.26; P = 0.005; \text{Fig. 2.6}$). Contrary to our predictions, we found no difference in β-GlcCer’ase activity between humid and dry treatments at either 5 d ($t = 0.38; P = 0.712$) or 21 d post-acclimation ($t = 0.24; P = 0.817$). However, β-GlcCer’ase activity was greater at 21 d than at 5 d post-acclimation within both humid (Welch’s $t = 3.27; P = 0.008$) and dry groups (Welch’s $t = 4.94; P < 0.001$).

**Figure 2.6.** Mean (±1 SD) β-GlcCer’ase activity for non-acclimated and dry or humid groups after 5 or 21 d acclimation. Values for each individual are means from dorsal and ventral surfaces. Sample size is indicated at the base of each bar.
CWL and lipids

There was a significant negative association between CWL and the quantity of cerebrosides in the SC of dry-acclimated sparrows ($R^2 = 0.70, P < 0.001$). Also in dry-acclimated birds, CWL was positively correlated with the percentage of cholesterol ($R^2 = 0.33, P < 0.05$; Fig. 2.7). In non-acclimated sparrows, CWL was positively associated with quantity of cerebrosides ($R^2 = 0.44$) and percentage of cholesterol ($R^2 = 0.20$), but these correlations were not significant ($P > 0.07$). There were no significant associations between CWL and quantities or percentages of any lipid class in humid-acclimated sparrows.

We also investigated the relationship of the difference in CWL between birds before and after acclimation and the change of the lipid composition in their SC. We could not find any significant correlation between the difference in CWL, expressed as percent change from initial measurements, and the difference in quantities or proportions of any lipid class in humid-acclimated birds. However, in dry-acclimated birds we found a significant negative association between the difference in CWL and the quantities of total lipid, total ceramides and total cerebrosides ($R^2 = 0.47, P < 0.02$; $R^2 = 0.44, P < 0.02$; $R^2 = 0.33, P < 0.04$; respectively; Fig. 2.8).
Figure 2.7. Cutaneous water loss in humid- (filled circles) and dry-acclimated (open circles) sparrows as a function of the amount of cerebrosides and the percentage of cholesterol in the SC. Only correlations for dry-acclimated sparrows were significant.
Figure 2.8. Percent change in cutaneous water loss in acclimated sparrows, calculated as \((\text{initial} - \text{final}) \times 100\), as a function of the amounts of total ceramides, total cerebrosides and total lipid in the SC. Only correlations for dry-acclimated sparrows were significant.
β-GlcCer’ase and cutaneous water loss

To investigate the functional consequences of β-GlcCer’ase activity, we examined among-individual correlations between enzyme activity and whole-animal rates of CWL. Across treatment groups, we found a negative relationship between β-GlcCer’ase activity and CWL (F3,28 = 5.86; P = 0.022; Fig. 2.9A). This correlation is driven by the low enzyme activity and high CWL of non-acclimated birds, as well as the weak negative relationship between CWL and β-GlcCer’ase activity within the humid-acclimated group (R² = 0.43; P = 0.055). CWL was not significantly correlated with β-GlcCer’ase activity within either the dry- or the non-acclimated groups (P > 0.78), or across groups once we removed variance attributable to treatment (F3,28 = 2.85; P = 0.102). Further, we found no relationship between β-GlcCer’ase activity and the magnitude of change in CWL following acclimation in either the humid (R² = 0.24; P = 0.186) or dry treatment (R² = 0.05; P = 0.443), or across both groups combined (F2,20 = 1.73; P = 0.376; Fig. 2.10).
Figure 2.9. Cutaneous water loss (A) and SC concentration of ceramides (B), and cerebrosides (C), expressed as a function of β-GlcCer’ase activity for non-acclimated and 21-d humid- or dry-acclimated sparrows.
Figure 2.10. Acclimation response of cutaneous water loss (CWL) as a function of β-GlcCer’ase activity for humid or dry groups after acclimation for 21 d. CWL is expressed as percent change from initial measurements, such that negative values indicate a decrease in CWL. Percentages were arcsine transformed prior to analysis.
**β-GlcCer’ase and skin lipids**

We also examined among-individual correlations between β-GlcCer’ase activity and lipid content of the SC. After removing variance attributable to overall treatment effects, we found a weak negative relationship between β-GlcCer’ase activity and the total concentration of SC ceramides ($F_{3,28} = 4.37; P = 0.046$; Fig. 2.9B). We observed a similar trend after expressing ceramide concentration as a percentage of total SC lipid composition, but the relationship was not significant ($F_{3,28} = 3.69; P = 0.065$; percentages arcsine-transformed for analysis; Zar 1996). We also conducted separate analyses for each of the five major classes of ceramide molecules that could be distinguished on the basis of TLC. Although these classes differ in polarity and may therefore confer different permeability properties, we observed similarly weak negative relationships between SC lipid concentration and β-GlcCer’ase activity within each ceramide class ($0.03 < P < 0.2$). We did not find any correlations between β-GlcCer’ase activity and the concentration of cerebrosides within the SC (Fig. 2.9C).

**DISCUSSION**

Whereas values for CWL in sparrows before acclimation were comparable to those observed previously in the mesic Ohio populations, dry-acclimated birds reduced
CWL to values typical of house sparrows from the desert in Saudi Arabia, 12 mg/(cm^2 \cdot d) (Muñoz-García and Williams 2005), where humidity is similar to that in our dry experimental chambers. Differences in CWL and the lipid composition of the SC observed between Saudi Arabia and Ohio populations of House sparrows could be a product of acclimation, but we cannot exclude genetic differences or the role of developmental plasticity (Muñoz-García and Williams, 2008). In either case, it is clear that sparrows from mesic environments can reduce CWL substantially in response to environmental conditions that favor water conservation.

We could not find any significant difference between dry- and humid-acclimated sparrows in either the amounts or relative proportions of lipid classes in the SC, although CWL was significantly lower in the group acclimated to low humidity. However, restricting our analyses to amounts and proportions of isolated lipid classes may not properly address the complexity of the SC. Therefore, we also examined interactions among the different lipid classes in the SC. We can recognize two kinds of relationships, not necessarily mutually exclusive, among lipids in the SC: (1) functional relationships that make the SC more efficient as a regulator of water vapor diffusion through the skin, and (2) potentially non-functional relationships resulting from shared metabolic pathways, like that existing between ceramides and cerebrosides, or ceramides and some free fatty acids. Creation or degradation of a particular lipid in the SC in response to an environmental stimulus will, therefore, form byproducts that are themselves components
of the SC, either aiding or interfering as a barrier to water vapor diffusion. In the SC of
dry-acclimated birds, four out of the six possible pairs of lipids are significantly
intercorrelated. This suggests that the SC of dry-acclimated sparrows has a higher level of
organization and a more tightly regulated structure than in humid-acclimated birds, in
which we were unable to find any significant correlations. Consistent with this
interpretation, we found a negative association between CWL and the quantities of
ceramides and cerebrosides in the dry-acclimated group, but not in either humid- or non-
acclimated sparrows.

Our results also suggest that some lipid ratios are important to control water loss
through the skin. We found weak evidence that the ceramide: cerebroside ratio is
different among treatments, suggesting that more cerebrosides are metabolized into
ceramides in the dry-acclimated sparrows. The ratio of free fatty acids to ceramides was
also higher in non-acclimated birds. Dry-acclimated sparrows showed a ratio closer to
that of mammals (Wertz 2000), which might be functionally related to their more
competent barrier to water loss.

Our results provide the first documentation of β-GlcCer’ase activity in the SC of
any non-mammalian vertebrate. Overall, the specific activities that we observed (range
0.2–2.8 nmol/(min·mg) are comparable to values reported for hydrolysis of 4MUG under
similar conditions by tissue homogenate supernatants from mice (Holleran et al. 1992,
1993, 1994b). The mean β-GlcCer’ase activity that we observed in non-acclimated
sparrows [0.67 nmol/(min·mg)] is lower than typical values for homogenate supernatants from murine epidermis [2.60 nmol/(min·mg)] and SC [4.09 nmol/(min·mg); Holleran et al. 1992]. However, our data from acclimated sparrows demonstrate that comparable levels of β-GlcCer’ase activity are possible in avian SC. Unfortunately, it is difficult to assess the true magnitude of these differences between mammals and birds because of discrepancies in assay conditions. For example, we increased substrate concentration to 5 mM in order to substantially exceed our estimated $K_M$ of 1.51 mM, whereas Holleran et al. (1992) assayed murine β-GlcCer’ase activity below their estimated $K_M$ of 0.93 mM. Given that we obtained significantly lower estimates of β-GlcCer’ase activity at their preferred substrate concentration (data not shown), it is reasonable to suggest that β-GlcCer’ase activity is greater in murine than avian SC, even if the precise magnitude of this difference is uncertain. This difference may explain why cerebrosides, which occur in trace amounts in mammalian SC (Wertz 1992), are abundant in avian SC (Menon et al. 1986a, Wertz et al. 1986, Muñoz-Garcia and Williams 2005).

An unexpected result in our study was the reduction in CWL and concomitant changes in SC lipid composition observed in humid-acclimated birds compared to non-acclimated sparrows. We deliberately exposed house sparrows to 30°C to maintain an environment within their thermoneutral zone and avoid any potential confounding effects of heat or cold stress. At the time of capture, however, sparrows experienced natural environmental temperatures around 0°C, a difference of 30°C with the $T_a$ in our
chambers. It is likely, then, that acclimation to a higher temperature may account for the decrease in CWL observed in both dry and humid-acclimated birds compared to non-acclimated sparrows. Changes in the quantities and proportions of the different lipid classes in the SC between non-acclimated and acclimated sparrows may also indicate an effect of temperature. Haugen et al. (2003b) found that proportion of total ceramides in Hoopoe larks (*Alaemon alaudipes*) increased in response to temperature, although proportions did not change in three other species of larks. Therefore, temperature acclimation may have had an overriding effect on CWL and SC lipid composition in our study. However, we also found that dry-acclimated sparrows had lower CWL rates than humid-acclimated birds, suggesting that this additional reduction can be ascribed to humidity.

Given the role of β-GlcCer’ase in maintaining SC barrier function in mammals, we predicted that this enzyme is also involved in the regulation of skin resistance to CWL in birds. In the present study, we found that these same dry-acclimated sparrows exhibited a significant increase in β-GlcCer’ase activity relative to initial levels in non-acclimated birds (Fig. 2.6). However, contrary to our initial prediction, we found no difference in β-GlcCer’ase activity between humid- and dry-acclimated birds at either 5 or 21 d post-acclimation (Fig. 2.6). Although we predicted greater β-GlcCer’ase activity in dry than in humid-acclimated sparrows, this prediction was based on the expectation that CWL would only be reduced in response to low humidity. Contrary to this
expectation, we observed a significant decrease in CWL among humid-acclimated sparrows, although the average magnitude of this reduction (24%) was significantly less than in dry-acclimated birds (Muñoz-Garcia et al. 2008b) Further, both dry- and humid-acclimated sparrows exhibited increases in the proportion of SC ceramides relative to non-acclimated birds (Muñoz-Garcia et al. 2008b). This may indicate that thermal acclimation had an overriding effect on enzyme activity, SC lipid composition, and CWL. However, given that both humidity treatments decreased CWL and increased SC ceramide content (Muñoz-Garcia et al. 2008b), it is perhaps not surprising that we found increased \(\beta\)-GlcCer’ase activity in both humidity treatments. Thus, our overall treatment effects are consistent with the hypothesis that increased \(\beta\)-GlcCer’ase activity leads to increased SC ceramide content and reduced CWL, but ambiguous with respect to whether humidity, temperature, or their interaction mediates this acclimation.

To address the functional significance of \(\beta\)-GlcCer’ase activity, we examined correlations between \(\beta\)-GlcCer’ase activity, CWL, and SC lipid composition among individuals. We found a negative relationship between CWL and \(\beta\)-GlcCer’ase activity across treatments (Fig. 2.9A), but relationships within individual treatment groups were weak (humid group) or absent (non-acclimated and dry groups). Although we found a negative relationship between SC ceramide content and \(\beta\)-GlcCer’ase activity (Fig. 2.9B), this is contrary to the positive association predicted from the role of \(\beta\)-GlcCer’ase in ceramide formation. These results provide little support for a functional relationship
between β-GlcCer’ase activity and either CWL or SC lipid composition, although several factors complicate this interpretation.

First, CWL and SC lipid composition reflect the cumulative effects of lipid synthesis and metabolism throughout the acclimation period, whereas β-GlcCer’ase activity provides an instantaneous measure of this process. If sustained elevation in β-GlcCer’ase activity is not required to maintain increased skin resistance, then it may be unreasonable to expect instantaneous measures of β-GlcCer’ase activity to correlate strongly with CWL or SC lipid composition. For example, mammalian β-GlcCer’ase activity is maximal following disruption of the skin, but returns to baseline levels once ceramide content of the SC has been restored (Holleran et al. 1994a, b). By analogy, avian β-GlcCer’ase activity may only be elevated when the lipid composition of the SC is insufficient for environmental demands on thermoregulation or water conservation, perhaps explaining the negative correlation between SC ceramide concentration and β-GlcCer’ase activity that we observed (Fig. 2.9B).

Second, β-GlcCer’ase activity is maximal in the basal layers of the SC, but is also evident throughout the epidermis (Holleran et al. 1992, Takagi et al. 1999). On the basis of these data and our hypothesized functional model of avian SC (Muñoz-Garcia and Williams 2005), we assayed β-GlcCer’ase activity specifically in the SC, rather than the entire epidermis. However, our assumption that avian β-GlcCer’ase activity is primarily restricted to the SC remains to be verified (e.g., Takagi et al. 1999). We cannot discount
the possibility that β-GlcCer’ase activity in underlying layers of the epidermis is also important for the regulation of SC lipid composition.

Third, ceramides are only one of several lipid classes that may contribute to skin permeability. Likewise, β-GlcCer’ase is only one of several putative enzymes, such as transferases and phospholipases, that regulate SC lipid composition (Coderch et al. 2003). In mammals, both free fatty acid and cholesterol composition of the SC can influence skin permeability (Brod 1991, Coderch et al. 2003). Comparisons of sparrows and larks from environments along a temperature-aridity gradient indicate that proportions of free fatty acids and cholesterol may also influence CWL (Haugen et al. 2003a, Muñoz-Garcia and Williams 2005). Further, short-term temperature or humidity acclimation increases the proportion of SC cholesterol in both house sparrows and hoopoe larks (Haugen et al. 2003b, Muñoz-Garcia et al. 2008b). Interactions between the absolute quantity and relative proportions of these various lipid classes, and between various lipid molecules within each class, are likely to mediate CWL, and this complexity may confound simple attempts to correlate gross measures of enzyme activity, lipid proportions, and CWL.

Finally, lipid composition of the SC and associated skin resistance are not the only factors that determine CWL. Our rationale for focusing on structural changes to the lipid barrier of the SC is based on previous work demonstrating physiological plasticity of SC lipids in response to thermal acclimation (Haugen et al. 2003a) and natural variation in SC lipids with respect to habitat aridity (Haugen et al. 2003b, Muñoz-Garcia
and Williams 2005). However, CWL is also influenced by the water vapor gradient between the skin and air. For example, heat-acclimated rock pigeons (*Columba livia*) increase CWL in response to heat stress as an adaptive cooling mechanism (Arieli et al. 2002). Although long-term heat acclimation induces structural modifications to the epidermis that presumably reduce its resistance to water diffusion (Peltonen et al. 1998), transient increases in CWL in response to heat stress are mediated primarily by changes in dermal blood flow and hydrostatic pressure within capillaries that increase water efflux from the capillaries to the epidermis (Arieli et al. 2002). However, we do not know the extent to which CWL in house sparrows is influenced by SC lipid composition, as opposed to other structural and physiological properties of the skin.

In conclusion, we have shown that adult house sparrows acclimated to low humidity reduced rates of CWL by 36.1% compared with birds acclimated to high humidity. We observed that the lipid composition of the SC was correlated with CWL only in dry-acclimated birds, suggesting that the interactions between lipid classes and the organization of the SC are more tightly regulated in birds in response to desiccation. Therefore, structural changes in SC lipid composition may have functional implications for the regulation of CWL after acclimation to low humidity. We found evidence of a high degree of plasticity in both CWL and the lipid composition of the SC in house sparrows from a mesic environment. In addition to decreased CWL, acclimated sparrows exhibited increased SC β-GlcCer’ase activity and ceramide content, suggesting a
physiological mechanism by which sparrows can adjust SC lipid composition and, ultimately, CWL. To directly address the importance of β-GlcCer’ase activity to avian SC function, it may be necessary to manipulate enzyme activity in vivo. This could be accomplished via topical application of specific inhibitors (Holleran et al. 1993) and observation of the resultant effects on CWL and SC lipid composition. The interpretation of our results is also complicated by the similar responses that we observed in both humid- and dry-acclimated sparrows. These results indicate that ambient humidity per se may not be the primary environmental stimulus influencing SC physiology and CWL. On the basis of short-term physiological responses to thermal acclimation in other birds (e.g., Haugen et al. 2003a), we suspect that changes in ambient temperature may also promote changes in skin permeability and CWL.
CHAPTER 3

DEVELOPMENT OF CUTANEOUS WATER LOSS AND LIPIDS OF THE STRATUM CORNEUM IN NESTLING HOUSE SPARROWS FROM DRY AND HUMID ENVIRONMENTS

INTRODUCTION

One of the most important functions of the integument of terrestrial vertebrates is to prevent desiccation, a requirement to maintain adequate hydration of the internal milieu and to support metabolic reactions that allow survival and reproduction. The stratum corneum (SC), the outer layer of the epidermis, constitutes the main barrier to water vapor diffusion in mammals and birds (Scheuplein and Blank 1971, Lillywhite 2006). In birds, the function of the SC as a permeability barrier is not trivial, because over 50% of total water efflux can be attributable to cutaneous water loss (CWL) (Bernstein 1971, Webster and King 1987, Wolf and Walsberg 1996, Williams and Tieleman 2005).

The skin of adult birds consists of an inner vascularized dermis, and an outer non-vascular epidermis, the latter composed of a deeper germinative layer, consisting of the mitotically-active stratum basale, stratum intermedium, stratum transitivum, and a
superficial layer of cornified non-living cells embedded in a lipid matrix, the SC (Lucas and Stettenheim 1972). Cells of the stratum basale contain rough endoplasmic reticulum, a Golgi apparatus, mitochondria, keratin filaments, and lipid droplets. Specialized organelles, found in most layers of the epidermis, called multi-granular bodies (MGBs) appear to contain lipid droplets and lipids that organize into layers called lamellae in the SC (Menon and Menon 2000). Contents of the MGBs are apparently secreted into the extracellular domains of the SC as cells progress towards the SC (Wertz 2000, Lillywhite 2006). Thus the SC is an array of terminally differentiated, keratin-filled cells embedded in a lipid matrix (Matoltsy 1976). Thinner in birds than mammals, the SC of adult birds is composed of 5-20 layers of flattened cells called corneocytes surrounded by intercellular lipids. Lipids within the SC in mammals consist primarily of cholesterol, ceramides, and free-fatty acids (FFA) (Wertz et al. 1986, Landmann 1980, Menon and Menon 2000). Ceramides, formed by a molecule of sphingosine bonded to a fatty acid, constitute the backbone of layers of lipid in the intercellular spaces of the SC, called lamellae, whose presence decrease water permeation through the SC (Wertz 2000, Bouwstra et al. 2003). In adult birds, principal lipids of the SC include those found in the SC of mammals and also cerebrosides, a class of lipids consisting of a ceramide with a sugar attached (Menon et al. 1986, Wertz et al. 1986, Muñoz-Garcia and Williams 2005).

Minimizing water loss is especially important in deserts, environments characterized by high ambient temperatures (Tₐ), and low availability of water. Birds that
live in deserts have lower CWL than mesic species (Williams 1996), a reduction associated with changes in the lipid composition of the SC. The SC of desert birds has higher proportions of sphingolipids and lower proportions of FFA than that of birds from mesic environments (Haugen et al. 2003a, b, Muñoz-Garcia and Williams 2005).

However, in deserts, $T_a$ can become extreme during summer with the result that body temperature of some desert birds can reach 45°C during periods of activity in the hottest part of the day (Williams and Tieleman, unpubl.). At such high body temperatures, desert birds rapidly increase rates of respiratory water loss, and to a small extent CWL, to maintain body temperature below lethal limits (Tieleman and Williams 2002). Changes in the ratios of lipid classes in the SC might influence CWL in birds (Muñoz-Garcia et al. 2008a). Apparently, appropriate ratios of ceramides, cerebrosides and FFA are necessary for the formation of intracellular lipid bilayers, called lamellae, and the molecular organization of these lamellae affects CWL (Silva et al. 2007, Muñoz-Garcia et al. 2008a).

Therefore, the avian SC has to serve antagonistic roles and be structured in such a way that allows minimum rates of CWL at normothermic temperatures, but high rates of CWL when $T_a$ exceeds body temperature. The presence of cerebrosides in the SC of birds might be related with this dual function of the avian SC (Muñoz-Garcia et al. 2008a). We suggested that, besides ceramides, cerebrosides will play an important role in the organization of lamellae in the SC of birds, but at the same time cerebrosides will attract
water molecules and form a fluid intercellular lipid compartment, resulting in higher rates of CWL at high $T_{as}$ (Muñoz-Garcia et al. 2008a).

The lipid composition of the SC of birds also varies during ontogeny. However, few studies have examined the development of structure and function of the skin in birds, and with contradictory results. Menon et al. (1988) measured CWL of captive-reared nestling, fledgling and adult zebra finches (*Poephilia guttata*) and found that CWL increased with age. Compared with nestlings, fledglings increased CWL four times and adults increased CWL more than 20 times. Changes in CWL during development of finches were associated with ultrastructural modifications of the skin. Light microscopy of epidermis of nestling Zebra Finches showed an abundance of intracellular lipid droplets, which decreased in number as chicks aged. Studies using histochemistry suggested that nestlings may deposit more triglycerides in the SC than do adults (Menon et al. 1988, Muñoz-Garcia and Williams 2008). Electron micrographs revealed that the epidermis below the SC was 6 cell layers thick and the SC 20-25 layers thick in nestlings 3-7 days old, both invariant during development. Menon et al. (1988) noted that stratum intermedium cells accumulated translucent lipid droplets and electron dense lipid lamellae in MGBs. In the upper transition layers between stratum transitivum and SC, extra cellular spaces became narrower, and were filled with lamellar material and amorphous material, likely lipids presumably derived from MGB. The SC of adult zebra finches did not possess large extra cellular spaces as did that of nestlings suggesting that
contents of extra cellular space changes as nestlings develop, which the authors suggested explained why nestlings have such a low CWL compared with adults.

McNabb and McNabb (1977) showed, as opposed to Menon et al. (1988), that CWL decreased with age in nestlings of Japanese quail (*Coturnix coturnix*). Their method of choice to measure CWL, desiccant filled capsules, create an un-natural gradient of water vapor across the skin and therefore measurements of CWL by this method are abnormally high. Reduction in CWL with age was associated with an increase in the number of layers of the SC (McNabb and McNabb 1977, Groff et al. 2007). Mass-specific CWL also declined with age in the Chinese painted quail (*Excalfactoria chinensis*) but no data on skin structure was presented (Bernstein 1973). It seems, therefore, that modifications of the lipid composition of the SC of nestlings and fledglings obey in part to the process of maturation of a competent permeability barrier, resulting in a decrease of the rates of CWL to adulthood. However, nestlings might be capable of regulating CWL to some extent, through changes in the lipid composition of the SC.

Differences in physiological phenotype of organisms living in different environments are thought to be the result of natural selection across generations (Bennet and Lensky 1997, Williams and Tieleman 2005), or through the expression of phenotypic plasticity within individuals, including reversible phenotypic flexibility of adults and developmental plasticity of young. Of course phenotypic plasticity *per se* can also be
under the influence of natural selection (Tracy and Walsberg 2001, West-Eberhard 2003, Piersma and Drent 2003). CWL and its regulation by the lipid composition of the SC constitutes a useful model for studying phenotypic plasticity and its consequences on fitness.

Previously, we studied how lipids of the SC affect CWL in adult house sparrows, *Passer domesticus*, from two natural populations, one living in the deserts of Saudi Arabia, and another living in mesic Ohio. Desert individuals had a lower CWL, a lower proportion of FFA, and a higher proportion of ceramides and cerebrosides in the SC than birds from Ohio (Muñoz-Garcia and Williams 2005). These changes could be the result of genetic differences between populations, of phenotypic flexibility of adults, of developmental plasticity of young, or a combination of these. We have previously shown that adult sparrows from Ohio reduced their CWL by 36.1% when acclimated to a dry environment compared with birds acclimated to a humid environment (Muñoz-Garcia et al. 2008b). Attendant to this physiological adjustment, dry-acclimated sparrows had a significantly lower FFA:ceramide ratio than humid-acclimated sparrows (Muñoz-Garcia et al. 2008b).

The expression of phenotypic plasticity might have adaptive value in environments that are temporally heterogeneous, such as deserts (Schlichting and Smith 2002). Therefore, one might predict that individuals from deserts should alter their physiological traits more when exposed to different environments than individuals from
mesic habitats. However, Tieleman et al. (2003) found no evidence that adult desert birds were more flexible in total evaporative water loss than mesic species when exposed to different $T_a$s and Tracy and Walsberg (2001) showed that desert and mesic individuals of Merriam’s kangaroo rat ($Dipodomys merriami$) reared in different humidity regimes were equally flexible in CWL.

Hence, predictions about the occurrence and the magnitude of phenotypic plasticity in different environments do not consistently align with empirical evidence. The role that phenotypic flexibility of adults and developmental plasticity of young plays in the evolutionary process is at the forefront of modern thinking in evolutionary physiology (West-Eberhard 2003, Terblanche and Chown 2006, Pigliucci 2005, Price et al. 2003, Hoffmann et al. 2005, Gibert et al. 2001, Wilson and Franklin 2002). Few studies have sought to examine the relative importance of non-genetic effects and their adaptive value at different life stages (but see Fisher et al. 2003). One might envision that selection pressures for plasticity might be markedly different for young birds in nests and for adults. Under these circumstances, phenotypic plasticity could be a mechanism that, through the production of phenotypic variation, allows individuals to survive different environments during their lifetime.

In this study, we characterized the natural development of CWL and the lipid composition of the skin in nestlings from two populations of house sparrows ($Passer domesticus$), one living in the deserts of Saudi Arabia, another living in mesic
environments of Ohio. We found that nestlings had higher rates of CWL than adults from the same environment. The developmental trajectory for CWL was different between nestlings from desert and mesic habitats. We characterized the development of epidermis of nestlings using electron microscopy and found that thickness and number of layers of the SC are negatively related to water permeation through the skin. We also found an association between CWL and the lipid composition of the SC of nestlings. Nestlings from Saudi Arabia mainly regulated the sphingolipid content of their SC to modify rates of CWL, whereas in nestlings from Ohio proportions of sphingolipids did not vary during ontogeny and changes in neutral lipids in the SC contributed to adjust CWL. We conclude that differences in CWL between desert and mesic nestlings are the result of the different selection pressures for thermoregulation and water conservation that nestlings experience in their habitats.

We also investigated developmental plasticity of CWL and lipid composition of the SC in desert and mesic nestling house sparrows reared in low and high humidity, and compared our results with previous work on adults. We chose to study the effect of humidity on the SC given that the properties of the permeability barrier, at least in mammals, change in response to the level of hydration of the SC (Elias 2004, Silva et al. 2007, Muñoz-Garcia et al. 2008a). Our study showed that, regardless of environmental origin, nestling house sparrows had a more permeable skin than that of adults exposed to their natural environment. During the maturation process sparrows acquired a SC that is
less permeable to water vapor, a result of major modifications of the lipid composition of the extracellular matrix of the SC. Nestlings from Saudi Arabia showed a greater degree of plasticity in CWL and in lipid composition of the SC than did nestlings from Ohio, a finding consistent with the idea that organisms exposed to more environmental stress ought to be more plastic than individuals living in more benign environments.

**MATERIALS AND METHODS**

**Collection of house sparrow nestlings**

In Saudi Arabia, we placed nest boxes around the National Wildlife Research Center, near Taif, (22°15' N, 41°50' E) between March and April, whereas we set them up in Columbus, OH (40°00' N, 83°10' W) between mid April and late August. Average maximum $T_a$ during the breeding season in Taif, Saudi Arabia was 32-35°C and it was 23.4°C in Columbus (Fisher and Membery 1998, http:www.worldclimate.com). After pairs built nests and laid eggs, we checked boxes regularly until eggs hatched, designated as day 0. We removed chicks of known ages from their nest box, selected at random, from days 0, the day of hatch, to fledgling, at day 16, at intervals of two days, for measurements of CWL and lipid composition of the SC. We also selected 2 chicks from separate nest boxes in Saudi Arabia on days 0, 4, 8 and 12, for collection of skin samples.
for electron microscopy. For our humidity acclimation experiment, we collected two 3-4 day old chicks from each nest, which we assumed were full-siblings. We fed nestlings Friskies cat food (78% water, 11% protein, 2.5% fat), crickets and mealworms. Experiments were approved by ILACUC of Ohio State University (Protocol 2006-A0085) and the National Commission for Wildlife Conservation and Development, Riyadh, Saudi Arabia.

**Humidity acclimation.**

In our experimental design, we had two variables, “habitat” and “treatment”, with two levels each, dry or humid. For “habitat”, we classified nestlings as desert or mesic. For “treatment”, we randomly assigned one of the siblings from each pair to a dry treatment, and the other to a humid treatment. Therefore we had four groups of nestlings in our experiment; dry-acclimated and humid-acclimated individuals from Saudi Arabia (N=9 and N=7, respectively) and from Ohio (N=12 for each group).

We placed nestlings in separate environmental chambers at a constant T<sub>a</sub> of 35ºC (± 1ºC) and a photoperiod of 12L:12D. A vacuum pump brought atmospheric air into the chambers at approximately 75 ml/min. Humidity and T<sub>a</sub> were monitored continuously using HOBO ProSeries data loggers. To maintain low humidity for the dry treatment, we routed inlet air through two columns of Drierite prior to entry into the chambers. To maintain a high humidity in the humid treatment, we bubbled air through water in a
sealed stainless steel chamber prior to entry into the chamber, and placed pans filled with water on the bottom of the chamber. Average relative humidity was 15-20% (mean absolute humidity of 6.5 g H₂O/m³) in the dry chambers, and 90-95% (mean absolute humidity of 31 g H₂O/m³) in the humid chambers. Nestlings remained in the dry or humid environments until they fledged (day 16-17).

**Measurement of metabolic rate and evaporative water losses**

We measured oxygen consumption, RWL and CWL using standard open-flow respirometry methods, as detailed in Chapter 1, section “Measurement of metabolic rate and evaporative water loss”, pages 12-15. The only modifications were that flow rates were set at 300-600 ml/min for the mask port, and at 150-400 ml/min for the chamber port. Measurements were made at a temperature of 35°C inside the chamber. For nestlings on day 0, because they were too small for our mask system, we measured CWL of dorsal and ventral apteria using a Delphin® evaporimeter, previously calibrated in our laboratory. The evaporimeter had an error of ± 10%, slightly higher than advertised by the company. Estimates of CWL as provided by an evaporimeter and converted to the total area of a chick are not equivalent to those from a metabolic chamber, which yields an integrated estimate of the entire nestling over a protracted period. At this point, we do not know the magnitude of the differences in these estimates of CWL. Hence, in our
statistical tests relating morphology of the SC to CWL, we have run analyses with and without data for day 0 nestlings obtained with the evaporometer.

**Electron microscopy**

After measuring CWL, we excised pieces of skin, 1 cm$^2$, from both dorsal and ventral surfaces of house sparrow nestlings. Pieces of skin were immediately pinned to blocks of paraffin in a glass Petri dish and immersed in modified Karnovsky’s fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.1M cacodylate buffer pH 7.3, 0.06% CaCl$_2$) at 4°C overnight. Then samples were gently rinsed with fresh 0.1M cacodylate buffer, and stored in new buffer at 4°C. We postfix samples with 1 % osmium tetroxide overnight. We dehydrated our samples using a series of ethanol and water solutions, from 30 to 100%, followed with four rapid washes in 100% propylene oxide. We embedded samples using a mixture of propylene oxide and a low viscosity Epon Resin (Ted Pella Inc., 49% Epon 812; 12% Dodecenyl Succinic Anhydride; 37% Nadic Methyl Anhydride; and 2% 2,4,6-Tri(dimethylaminomethyl) phenol as an activator 2:1, 1:1, 1:2, and 0:1, each step at least 20 minutes. Finally, samples were placed in casts at 4°C overnight with fresh Epon, and then at 60°C for 3 days. Thick sections (1-2μm) were cut and stained with Toludine Blue to select appropriate areas for thin sectioning. Thin sections (gold-color, 70 nm) were cut using an Ultratome Nova (LKB, Bromma, Sweden) with a Diatome diamond knife, and mounted on 300-mesh copper grids with a freshly
prepared carbon film. Grids were stained with uranyl acetate then lead citrate, and allowed to dry. Samples were then observed using a Philips 300 transmission electron microscope at 60 kV.

**Morphological Analysis**

Due to the labor-intensive nature of electron microscopy, we limited the number of nestlings that we measured to 2 individuals per age. We examined numerous micrographs of epidermis, and selected from those representatives at each age, a dorsal and ventral picture for each, making a total of 2 dorsal and 2 ventral micrographs for each age. On these pictures, we drew three equally spaced perpendicular lines across the SC and counted the number of corneocytes along each line, measured total SC thickness, measured the thickness of each corneocyte, and calculated total extracellular thickness within the SC, a proxy for extracellular space where lipids that are involved in water permeation are extruded. Values were averaged for each picture.

**Separation and identification of skin lipids**

We analyzed the lipid composition of the SC of sparrows using analytical thin layer chromatography, using the protocol detailed in Chapter 1, sections “Extraction of intercellular lipids of the SC”, page 16-17 and “Analytical thin layer chromatography”, pages 18-20.
Statistics

The quantity of some classes of lipids as determined by TLC were not normally distributed, so we log-transformed them (Zar 1996). Percentages were logit transformed \([\ln (Y/1 – Y)]\) prior to analyses (Zar 1996). We tested for differences between means using a two-tailed t-test for independent samples (Zar 1996). We performed regressions using a general linear model. To test for differences between regressions, we first tested for the significance of the interaction term, and if this was insignificant, we compared elevations assuming a common slope (Zar 1996).

To study the natural development of nestlings from Saudi Arabia and Ohio at different ages, we used a two-way ANOVA to compare body mass, CWL, and amounts, percentages and ratios of lipid in the SC, with habitat and age as fixed factors. When the interaction term was significant, we interpreted occurrence of changes of the dependent variable with age and habitat, i.e., the developmental trajectories of nestlings from Saudi Arabia and Ohio were different. When only age was significant, we concluded that the dependent variable changed with age in a similar way in nestlings from both habitats. Significance of habitat indicated that desert and mesic nestlings had the same developmental trajectory but a different magnitude of the dependent variable.

To test for significance of effects of habitat and humidity acclimation on CWL and the lipid composition of the SC, we used a two-way ANOVA with habitat and
treatment as fixed factors. If the interaction was not significant, we dropped it from the analysis and tested for significance of factors. We interpreted results from our ANOVA following the rationale of reaction norms (Via 1993, Pigliucci 2001). A trait lacked developmental plasticity when neither the interaction term nor “treatment” was significant. If “habitat” was significant, we inferred the existence of genetic differences between fledglings from Saudi Arabia and Ohio in that trait. If we found an insignificant interaction term and significant differences in “treatment”, the trait was developmentally plastic to the same degree for fledglings from both habitats. When “habitat” was significant, we inferred the existence of genetic differences between fledglings from both populations in addition to an acclimation effect. A significant interaction term suggested occurrence of developmental plasticity in one habitat, or existence of developmental plasticity in both habitats to different degrees.

We used multiple regression to simultaneously investigate the relationship of habitat and treatment, included in the regression model as dummy independent variables, with our response variables. To assess the contribution of habitat and treatment to the variability of our response variables, we calculated semi-partial correlation coefficients, which evaluate the contribution of one of the independent variables to the variance of the dependent variable, not explained by the other independent variables (Field 2005).

To explore functional consequences of variation in lipid composition of the SC of fledglings, we performed stepwise multiple regression with CWL as the dependent
variable and lipid amounts and lipid percentages as independent variables (Muñoz-Garcia et al. 2008a). We also regressed CWL against total lipid and ratios of lipid classes in the SC of fledglings.

We conducted comparisons between CWL and the lipid composition of the SC of siblings assigned to different acclimation regimes using paired t-test. All statistical tests were conducted with SPSS 16.0. Averages are reported ±1 SD. We rejected the null hypothesis at P > 0.05.

RESULTS

NATURAL DEVELOPMENT OF NESTLINGS

Body mass and surface area

Average mass of nestlings from Saudi Arabia and Ohio increased with age, as did average surface area, calculated using Meeh’s equation (Walsberg and King 1978) (F = 17.3, P < 0.001). Nestlings from Saudi Arabia weighed significantly less than chicks from Ohio at all ages (F = 53.7, P < 0.001). Adults from Saudi Arabia weighed on average 20.1± 1.6 g and had a surface area of 74.0 ± 4.0 cm². Adult house sparrows from
Ohio had a mean mass of 23.2 ± 2.8 g and an average surface area of 81.3 ± 6.6 cm² (Muñoz-Garcia and Williams 2005).

**Cutaneous water loss**

Nestling sparrows from Saudi Arabia and Ohio followed a different developmental trajectory for CWL, as indicated by the significant interaction term in our ANOVA (F = 8.7, P < 0.001). CWL changed in a non-linear way with age in both nestlings from Saudi Arabia and Ohio (Fig. 3.1). Two-day-old nestlings had low rates of CWL in both habitats. CWL reached a peak at 4 and 12 days in Saudi Arabia and 8 and 14 days in Ohio, and it decreased to a minimum at 8 days of age in Saudi Arabia and 12 days in Ohio. For all ages averaged, surface-specific CWL was 15.2% lower in nestlings from deserts than in nestlings from mesic environments.

Rates of CWL of adults were lower than those of nestlings (Fig. 3.1). Adult house sparrows from Saudi Arabia and Ohio lost 11.9 ± 2.2 and 16.0 ± 2.6 mg H₂O/ (cm² • d) through their skin, respectively (Muñoz-Garcia and Williams 2005).
Figure 3.1. Developmental trajectory of cutaneous water loss (CWL) in nestling and adult sparrows from desert (open circles) and mesic (filled circles) environments. “i” indicates significance for the interaction habitat-by-age.
Ultrastructure of the epidermis

At all ages we observed in electron micrographs four strata in the epidermis, stratum basale, stratum intermedium, stratum transitivum, and SC, a pattern consistent with previous researchers (Lucas and Stettenheim 1972, Menon et al. 1988). Fig. 3.2 depicts the ventral epidermis of a nestling at day 0. Here cells of the stratum basale contained a nucleus, mitochondria, and relatively few lipid droplets and were connected to other basal cells by desmosomes. In the stratum intermedium, cells began to elongate, and contained more lipid droplets. Layers of the SC at this age varied in number between 5 and 7 and seemed to be more appressed with less lipids than older ages, consistent with high CWL at this age.

Epidermal structure below the SC varied with age (Fig. 3.3). On day 0, we noted few lipid droplets in epidermal cells, but droplets seemed to increase rapidly in number by day 4 and then declined again by day 12, suggesting marked changes in lipids during development. Also on day 4, we observed large quantities of intracellular lipids especially in the upper stratum transitivum and lower SC, but the number of droplets was variable depending on the individual.
We identified an array of types of multigranular bodies in the epidermis of nestlings at all ages (Fig. 3.4). We chose a picture of ventral epidermis of a nestling on day 8 as representative. Note that that some multigranular bodies contained lamellar disks, whereas others contained lamellar disks juxtaposed to what appeared to be amorphous lipid material, and still others did not have lipid lamellae, but consisted entirely of amorphous lipid material.
Figure 3.3. Electromicrographs of ventral epidermis for House Sparrows at day 0 (upper left), 4 (upper right), 8 (lower left), and 12 (lower right). Black bar equals 1μm.
The SC of nestlings at day 4 and after appeared to be divided into two regions, a lower region of the SC, corresponding to the stratum compactum, and an upper region of the SC, or stratum disjunctum (Fig. 3.5). Cells in the stratum compactum contained some amorphous material, apparently lipids, and sometimes lamellae. Cells in the stratum disjunctum were flat, devoid of intracellular material, with an outer cornified layer assumed to be keratin and other proteins. Desmosomes were absent in the stratum disjunctum. Extracellular space, presumably filled with lipids, increased in the stratum disjunctum.
The SC of nestling House Sparrows contained intracellular lamellar structures in the lower layers of the SC, an observation that Menon et al. (1988) also made for nestling zebra finches (Fig. 3.6). If water transport is to some degree transcellular, then lipids within these cells would contribute to reduction in water loss. As corneocytes continue to develop, these lamellae apparently are extruded to extracellular spaces of the stratum disjunctum.
Figure 3.6. Electromicrograph of ventral SC of a day 8 nestling. Note lamellae within cells of the stratum compactum. Black bar equal 0.25 μm.

**Morphological measurements of SC and age**

In order to relate CWL to morphological variables of the SC, we measured thickness of the SC, extracellular space of SC, and total corneocyte thickness at ages 0, 4, 8, and 12 days. Our measurements did not statistically differ between dorsal and ventral epidermis (ANCOVA; P > 0.3 all cases), so we combined data for each chick. We found no linear association between thickness of the SC ($R^2 = 0.42$, $F = 3.6$, $P = 0.11$), extracellular space of SC ($R^2 = 0.21$, $F = 3.2$, $P = 0.09$, $n = 14$), or total corneocyte
thickness ($R^2 = 0.14$, $F = 1.8$, $P = 0.20$, $n = 14$) and age. Thickness of the SC, extracellular space, and total cell thickness averaged $4.18 \pm 3.2 \mu m$, $1.55 \pm 1.6 \mu m$, and $2.63 \pm 1.6 \mu m$, respectively. The number of corneocyte layers increased until day 8 and then declined at day 12 (Fig. 3.7a). The amount of extracellular space expressed as a proportion of SC thickness increased as chicks aged ($R^2 = 0.75$, $F = 12.1$, $P < 0.004$, $n = 14$; Fig. 3.7b).

**Morphology of the SC and CWL**

Because we estimated CWL for day 0 chicks using an evaporometer, we performed all analyses on CWL and morphology with these data included and then re-ran them with data for day 0 excluded. For CWL vs SC thickness and CWL vs total corneocyte thickness, analyses showed identical results with data for day 0 included or excluded. CWL declined as SC thickness increased ($R^2 = 0.76$, $F = 15.6$, $P < 0.01$, $n = 7$; Fig. 3.8a), and it also declined with increasing total corneocyte thickness ($R^2 = 0.67$, $F = 10.1$, $P < 0.03$, $n = 7$; Fig. 3.8b).
Figure 3.7. Number of corneocytes layers in the SC (A) and proportion of the SC that is extracellular space (B) as a function of age (days).
Figure 3.8. Cutaneous water loss in nestling House Sparrows as a function of (A) stratum corneum thickness and, (B) total corneocyte thickness. Numbers in circles represent ages of nestlings.
Figure 3.9. Cutaneous water loss of nestling House Sparrows as a function of (A) thickness of extracellular space (B) number of corneocyte layers and (C) the proportion of SC that was extracellular space. Numbers within circles represent ages of nestlings.
For measurements of thickness of extracellular space, corneocyte layers, and the proportion of the SC that was extracellular space, inclusion of data for day 0 yielded significant results in all cases, but because deletion of these data yielded insignificant trends, they are less compelling. With data for nestlings on day 0 included, CWL declined with the thickness of extracellular space ($R^2 = 0.73$, $F = 13.5$, $P < 0.01$, $n = 7$; Fig. 3.9a), with number of corneocyte layers ($R^2 = 0.62$, $F = 8.0$, $P < 0.04$, $n = 7$; Fig. 3.9b), and with the proportion of the SC that was extracellular space ($R^2 = 0.67$, $F = 10.4$, $P < 0.03$, $n = 7$; Fig. 3.9c).

**Lipid quantities in the stratum corneum of nestlings**

The interaction habitat by age was significant for the amounts of ceramides ($F = 6.9$, $P < 0.001$), cerebrosides ($F = 4.3$, $P < 0.02$) and total lipid ($F = 2.7$, $P < 0.04$) in the SC of nestling sparrows, suggesting different developmental trajectories for these lipid classes.

The concentration of ceramides in the SC of nestling sparrows from Saudi Arabia was high at 2 and 4 days, then decreased and remained constant from 6 to 10 days, and then increased again. In nestlings from Ohio, however, the amount of ceramides did not vary with age. Nestlings from Saudi Arabia had always a higher amount of ceramides in their SC than nestlings from Ohio (Fig. 3.10a).
Two-year-old nestlings from Saudi Arabia had a high concentration of cerebrosides in their SC, which decreased at 4 days to remain constant until nestlings fledged. The concentration of cerebrosides in the SC of mesic nestlings, on the other hand, did not vary with age. The amount of cerebrosides in the SC was always higher in nestlings from Saudi Arabia compared with nestlings from Ohio (Fig. 3.10b).

Nestlings from Saudi Arabia had a lower amount of lipid in the SC than nestlings from Ohio up to 8 days of age. After that age, nestlings from Saudi Arabia had a higher amount of lipid in the SC than nestlings from Ohio (Fig. 3.10c).

Nestlings changed markedly the lipid composition of their SC during ontogeny. Adult house sparrows from natural populations in Saudi Arabia had more FFA and more cerebrosides than desert nestlings. Adult sparrows from natural environments in Ohio had more FFA, more ceramides and more cerebrosides in the SC than mesic chicks (Fig. 3.10).

Lipid percentages in the stratum corneum of nestlings

The percentage of FFA, ceramides and cerebrosides in the SC varied significantly with age in both desert and mesic nestlings (Age: F > 2.3, P < 0.05) (Fig. 3.11). For all ages, nestlings from Saudi Arabia had a lower percentage of FFA in their SC but a higher percentage of ceramides than nestlings from Ohio (Habitat: F = 12.0, P < 0.002; F = 42.4, P < 0.001, respectively) (Fig. 3.11).
Figure 3.10. Amounts of (A) ceramides, (B) cerebrosides, and (C) total lipid in the SC of nestling and adult house sparrows from desert (white bars) and mesic (black bars) environments. “i” indicates significance for the interaction habitat-by-age.
Figure 3.11. Percentages of (A) free fatty acids (FFA), (B) ceramides, and (C) cerebrosides in the SC of nestling and adult house sparrows from desert (white bars) and mesic (black bars) environments. “a” and “h” indicate significance for age and habitat, respectively.
Compared with desert nestlings, adults from Saudi Arabia had a higher percentage of FFA and a lower percentage of ceramides in the SC. Percentages of lipid classes in the SC of adults from Ohio were similar to those of mesic nestlings (Fig. 3.11).

**Ratios of lipids in the stratum corneum of nestlings**

We found that the developmental trajectory of the ratio of FFA to ceramides was similar for both nestlings from Saudi Arabia and Ohio (Interaction: $P > 0.78$). However, the FFA:ceramide ratio was significantly higher in mesic than in desert nestlings at all ages (Habitat: $F = 15.3$, $P < 0.001$) (Fig. 3.12a).

We found a significant interaction age-by-habitat for the ceramide:cerebroside ratio in nestling sparrows ($F = 3.1$, $P < 0.03$), indicating a different developmental trajectory for this ratio in the SC between nestlings from Saudi Arabia and Ohio. The ceramide:cerebroside ratio reached a peak at 4 days in desert nestlings to decrease thereafter. In mesic nestlings, on the other hand, the ceramide:cerebroside ratio remained constant at all ages (Fig. 3.12b).

Compared with desert nestlings, adults from Saudi Arabia had a higher FFA:ceramide ratio and a lower ceramide:cerebroside ratio (Fig. 3.12). In adults from Ohio, the FFA:ceramide ratio was high compared with that of nestlings, but the ceramide:cerebroside ratio was similar between mesic adults and mesic nestlings (Fig. 3.12).
Figure 3.12. (A) Free fatty acid (FFA):ceramide ratio, and (B) ceramide:cerebroside ratio in the SC of nestling and adult house sparrows from desert (white bars) and mesic (black bars) environments. “i” and “h” indicate significance for the interaction habitat-by-age and habitat, respectively.
CWL and lipids

We investigated the relationship between quantities, percentages and ratios of lipid classes in the SC and CWL of nestlings using linear regression. We found a significant negative association between CWL and the percentage of cholesterol and triglycerides in the SC of mesic nestlings ($R^2 = 0.66$, $P < 0.02$; $R^2 = 0.69$, $P < 0.02$, respectively). Percentage of FFA was positively correlated with CWL, although the association was not significant ($P < 0.10$). These results indicate that CWL in mesic nestlings tended to be correlated with percentages of neutral lipids of the SC.

Regression analysis showed that CWL was significantly related to the ceramide:cerebroside ratio in the SC of desert nestlings ($R^2 = 0.77$, $P < 0.03$). It seems, therefore, that CWL is correlated with sphingolipids of the SC in desert nestlings.

HUMIDITY ACCLIMATION OF NESTLINGS

Body mass and surface area

Fledglings from Saudi Arabia weighed significantly less than fledglings from Ohio ($14.9 \pm 2.1$ g and $22.2 \pm 1.3$ g, respectively) (habitat: $F = 173.69$, $P < 0.001$). However, with individuals from both habitats combined, body mass was not significantly different between dry- and humid-acclimated fledglings (treatment: $F = 0.01$, $P > 0.96$). Using Meeh’s equation as modified by Walsberg and King (1978), we estimated skin
surface area for fledglings from Saudi Arabia and Ohio as 60.3 ± 6.0 cm² and 79.0 ± 3.1 cm², respectively.

**Cutaneous Water Loss.**

*Treatment.* Nestling sparrows were developmentally plastic for CWL (Fig. 3.13). Whole-organism and surface-specific CWL of fledglings following acclimation was lower in dry- than in humid-acclimated fledglings, by 15.7% and 11.2%, respectively (F = 9.18; P = 0.004; F = 7.49, P = 0.01) (Fig. 3.13A, B).

*Habitat.* Part of the difference in CWL between desert and mesic nestlings could be attributed to a genetic component. Whole-organism and surface-specific CWL of fledglings from Ohio was significantly lower than CWL of birds from Saudi Arabia, in sharp contrast to findings for adults (F = 7.74, P < 0.01; F = 47.01; P < 0.001, respectively). The difference in CWL for nestlings exposed to a dry compared with a humid environment was 14.8% for whole-organism CWL and 52.9% for surface-specific CWL (Fig. 3.13).

Developmental plasticity and genetic differences between populations of fledglings contributed almost equally to variability of whole organism CWL; however, habitat explained most of the variation in surface-specific CWL (Table 3.1).
Figure 3.13. Cutaneous water loss (CWL) in fledgling and adult house sparrows from desert (unfilled circles) and mesic (filled circles) environments after acclimation to humidity. A. Whole-organism CWL. B. Surface-specific CWL. “h”, “t”, indicate significance for habitat and treatment, respectively. Error bars show ±1 SD.
<table>
<thead>
<tr>
<th>Response variable</th>
<th>Habitat R²</th>
<th>Treatment R²</th>
<th>Interaction R²</th>
</tr>
</thead>
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<tr>
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<td>0.16**</td>
<td>0.17**</td>
<td>NS</td>
</tr>
<tr>
<td>CWL</td>
<td>0.55**</td>
<td>0.05*</td>
<td>NS</td>
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<tr>
<td><strong>Lipid amounts</strong></td>
<td></td>
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<tr>
<td>FFA</td>
<td>0.49**</td>
<td>0.10NS</td>
<td>0.12*</td>
</tr>
<tr>
<td>Cer</td>
<td>0.48**</td>
<td>0.01NS</td>
<td>0.06*</td>
</tr>
<tr>
<td>Cerebr</td>
<td>0.10*</td>
<td>0.01NS</td>
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<tr>
<td><strong>Lipid percentages</strong></td>
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<td>0.11*</td>
</tr>
<tr>
<td>% FFA</td>
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<td>0.06*</td>
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<tr>
<td>% Cer</td>
<td>0.66**</td>
<td>0.06**</td>
<td>NS</td>
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<tr>
<td><strong>Lipid ratios</strong></td>
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<tr>
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<td>0.52**</td>
<td>0.12**</td>
<td>NS</td>
</tr>
<tr>
<td>Cer:Cerebr</td>
<td>0.54**</td>
<td>0.00NS</td>
<td>0.03*</td>
</tr>
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</table>

Table 3.1. Semi-partial correlation coefficients after multiple regression on CWL, lipid amounts, lipid percentages and lipid ratios of the SC using “Habitat” and “Treatment” as independent variables (see text for details). When the interaction term was significant, we included it in our multiple regression model. We only show variables for which at least one factor was significant. NS = Factor not significant, * = P < 0.05, ** = P < 0.01.
Lipid quantities in the stratum corneum of fledglings

**Treatment.** Fledglings that developed in different humidity treatments varied in the amounts of FFA and ceramides in their SC. Fledglings from desert and mesic populations had different degrees of plasticity for FFA and ceramides (interaction: $F > 4.89$, $P < 0.04$, Fig. 3.14). The magnitude of change in the concentration of FFA after acclimation was higher in fledglings from Ohio than in fledglings from Saudi Arabia. Fledglings from Saudi Arabia showed plasticity for the quantity of ceramides with higher concentrations of ceramides in their SC when reared in a dry environment than in a humid environment.

**Habitat.** Fledglings from Ohio had significantly higher amounts of FFA, but lower amounts of ceramides in their SC than did fledglings from Saudi Arabia ($F > 4.34$, $P < 0.04$, Fig. 3.14). Habitat explained a higher proportion of the variance in the amounts of FFA, ceramides and cerebrosides in the SC of fledglings (Table 3.1).
Figure 3.14. Lipid quantities in fledgling and adult house sparrows from desert (unfilled circles) and mesic (filled circles) environments after acclimation to humidity. FFA = Free fatty acids. “h”, “i”, indicate significance for habitat and interaction term, respectively. Error bars show ±1 SD.
Lipid percentages in the stratum corneum of fledglings.

Treatment. The interaction habitat-by-treatment was significant for percentage of cholesterol (F = 5.0, P = 0.03) and FFA (F = 6.3, P < 0.02) in the SC, indicating that fledglings expressed developmental plasticity for these variables when reared in different humidity, and that the degree of plasticity was different between desert and mesic populations. The magnitude of change in percentage of cholesterol in the SC after acclimation was higher in fledglings from Ohio, whereas the magnitude of change in percentage of FFA was higher in fledglings from Saudi Arabia. Fledglings also exhibited developmental plasticity for percentage of ceramides, although the degree of plasticity was the same in both populations (F = 9.0, P = 0.005).

Habitat. Fledglings from Saudi Arabia had a lower percentage of cholesterol and FFA in their SC, but a higher percentage of ceramides than fledglings from Ohio (F > 4.9, P < 0.04).

Developmental plasticity explained a higher proportion of the variance in percentage of cholesterol in SC than habitat; on the other hand, variation in percentage of FFA and ceramides in the SC of fledglings was explained by habitat (Table 3.1).

Ratios of lipids in the stratum corneum of fledglings.

Treatment. We found that fledglings exhibited developmental plasticity for the ratio of FFA to ceramides (F = 7.39, P = 0.01; Fig. 3.15A). The ceramide:cerebroside
ratio was also plastic, but only in fledglings from Saudi Arabia (interaction: $F = 6.0$, $P < 0.02$) (Fig. 3.15B).

**Habitat.** The FFA:ceramide ratio was higher in mesic fledglings than in those from the desert ($F = 28.75$, $P < 0.001$, Fig. 3.15A), whereas the ceramide:cerebroside ratio was higher in fledglings from Saudi Arabia ($F = 64.7$, $P < 0.001$, Fig. 3.15B). Habitat accounted for most of the variance in both lipid ratios in the SC of fledglings; developmental plasticity explained a significant part of the variance only for the FFA:ceramide ratio (Table 3.1).

**Comparisons of pairs of siblings**

When we evaluated differences between pairs of siblings reared in different humidity, results showed that fledglings raised in the dry environment had lower CWL, significantly less FFA, a higher percentage of FFA, a lower percentage of ceramides, and a higher FFA:ceramide ratio than their humid-acclimated siblings ($t > 2.55$, $P < 0.03$). These results suggest that fledglings showed developmental plasticity for CWL and the concentration of FFA and ceramides, but not for the concentrations of cholesterol, triglycerides and cerebrosides, in agreement with results from the comparison between acclimation groups.
Figure 3.15. FFA:ceramide ratio (A) and ceramide:cerebroside ratio (B) in fledgling and adult house sparrows from desert (unfilled circles) and mesic (filled circles) habitats after humidity acclimation. “h”, “t”, “i”, indicate significance for habitat, treatment and interaction term, respectively. Error bars show ±1 SD.
CWL and lipids

We investigated the relationship between quantities of lipid classes in the SC and CWL of fledglings using stepwise multiple regression. With all fledglings combined, we found a significant association between CWL and amounts of ceramides and cholesterol in the SC ($R^2 = 0.46, P < 0.001$). When we restricted analyses to dry-acclimated fledglings, we found that CWL was correlated with amounts of ceramides and cerebrosides ($R^2 = 0.62, P < 0.001$). In humid-acclimated fledglings, CWL was negatively associated with quantity of FFA ($R^2 = 0.21, P < 0.05$).

Again with data for all fledglings combined, multiple regression analysis showed that CWL was significantly related to percentage of ceramides and percentage of cholesterol ($R^2 = 0.44, P < 0.001$). When we considered dry-acclimated and humid-acclimated fledglings separately, we found that CWL was significantly correlated with percentage of ceramides in both cases ($R^2 = 0.61, P < 0.001$; $R^2 = 0.39, P < 0.01$, respectively).

Regressions of FFA:ceramide and ceramide:cerebroside ratios on CWL were significant when we combined all fledglings and when we separated dry- from humid-acclimated sparrows ($R^2 > 0.25, P < 0.05$).
Transition of CWL and lipids of the SC from nestlings to adults

To understand changes that occur in CWL and lipids of the skin between nestlings and adults, we compared adults from natural environments with fledglings reared in a similar humidity environment to that they experience as adults; therefore, we compared nestlings from Saudi Arabia reared in a dry environment with adults from Saudi Arabia, and we compared nestlings from Ohio raised in a humid environment with adults from Ohio.

Fledglings from Saudi Arabia and Ohio reduced CWL when adults. Adult sparrows from Saudi Arabia reduced their CWL by 57.0% compared with desert fledglings reared in the dry environment. Adults from Ohio decreased their CWL by 25.8% compared with mesic fledglings raised in the humid environment (Fig. 3.13).

Nestlings altered lipid composition of their SC during ontogeny. Adult house sparrows from natural populations in Saudi Arabia had more FFA and more cerebrosides, but less ceramides in the SC than desert fledglings acclimated to a dry environment. Adult sparrows from natural habitats in Ohio had more FFA, more ceramides, and more cerebrosides, in their SC than did fledglings raised in a humid environment (Fig. 3.14).

Compared with desert fledglings reared in the dry environment, adults from Saudi Arabia had a higher percentage of FFA and cerebrosides in the SC and a lower percentage of ceramides. Percentage of FFA was higher in adults from Ohio than in mesic fledglings acclimated to the humid environment. However, percentages of
ceramides and cerebrosides did not vary with ontogeny in mesic birds. The FFA:ceramide ratio was higher in adults from both desert and mesic populations (Fig. 3.15A). For Saudi Arabia sparrows, the ceramide:cerebroside ratio of adults was lower than that of fledglings acclimated to the dry environment, but that ratio remained constant in mesic birds (Fig. 3.15B).

**DISCUSSION**

We have characterized the development of the epidermis in nestling house sparrows from Saudi Arabia and evaluated their CWL relative to development of the epidermis, the first study to do so in nestlings of free-living birds. We found that nestlings from desert and mesic environments followed different developmental trajectories for CWL. During the first 10 days of age, CWL of nestlings from deserts was, on average, lower than that for mesic nestlings. However, there was a trend for nestlings of Saudi Arabia to increase CWL since day 8, so that at 12 days of age CWL rates were similar for nestlings from both habitats. Although we do not have data for nestlings from Saudi Arabia older than 12 days, we can estimate CWL of desert fledglings (16-day-old). We can compare rates of CWL of fledglings from Ohio reared in the humid environment and those of fledglings raised in their natural environments in Ohio (21.2 and 21.6 mg
H$_2$O/(cm$^2$·d), respectively). We can assume, then, that CWL of fledglings raised in natural conditions in Saudi Arabia will be comparable to that of desert fledglings raised in a dry environment, i.e. 27.7 mg H$_2$O/(cm$^2$·d). Thus, CWL of desert fledglings would be higher than those of mesic fledglings. The sustained increase in CWL in desert nestlings can be explained by thermoregulatory needs of the birds confined in nests (see below). Surface-specific CWL was higher in chicks than adults, a finding consistent with measurements on chicks of painted quail (Bernstein 1973) and of Japanese quail (McNabb and McNabb 1977). Our data do not support the idea of McNabb and McNabb that surface-specific CWL decreases linearly with age or with a contrasting idea that CWL increases during the nestling period though layers of the SC remain constant (Menon et al. 1988).

We found a number of similarities in the structure of the epidermis of nestling sparrows compared with the work of Menon et al. (1988) on nestling zebra finches. In both species, basal cells contained a large nucleus, rough endoplasmic reticulum, Golgi bodies, and mitochondria. As cells progressed into stratum intermedium, lipid droplets and multigranular bodies appeared. Closer to the SC, lipid droplets and perhaps multigranular bodies became larger as if to coalesce within cells. Multigranular bodies varied in form throughout the lower layers of the epidermis, some with lipid lamellae, some with lamellae and amorphous lipid material, and some with amorphous material only. Lamellar layers were observed in the cells of the lower SC, in most of the stratum
compactum, but we did not see these layers of lipid in the stratum disjunctum. The mechanism of release of lipids into the extracellular spaces has not be elucidated but we suspect that changes in hydration of these cells along with increases in osmolality may exert pressure forcing intracellular lipids into the extracellular spaces of the SC along with a battery of hydrolytic enzymes (Freinkel and Traczyk 1985). A major difference in the structure of the SC between sparrows and finches was that finches had 20-25 corneocyte layers at all ages, whereas we found that layers of the SC varied from 5-16 depending on developmental stage.

The hypothesis of McNabb and McNabb (1977) suggests that, in nestling birds, surface-specific CWL decreases with age, a response to increased thickness of the SC, the primary barrier to water vapor diffusion. Although we did not find a relationship between CWL and age or between morphological variables of the SC with age in house sparrows, our data do show significant trends with structure of the SC and function. The thickness of the SC was negatively associated with CWL in nestling house sparrows, as was corneocyte thickness. Apparently CWL varies among stages of development of the skin in nestling house sparrows, as feathers and other structures develop within the skin, and as the lipid composition of the SC changes. Moreover it appears that morphological features of the SC in nestlings at the same age also vary, perhaps as a result of different planes of nutrition.
McNabb and McNabb (1977) argued that skin permeability, as measured by a non-ventilated capsule, declined as chicks of Japanese quail aged (their Fig. 5). However, these authors presented no statistics to support this view, nor did they attempt to directly relate skin permeability to number of layers of corneocytes. We have re-analyzed their data, as obtained from their Fig. 4 and 5, and found that the number of layers in the SC significantly increased with age ($R^2 = 0.194$, $F = 13.2$, $P = 0.001$, $n = 57$), and that CWL significantly declined with age ($R^2 = 0.15$, $F = 10.9$, $P = 0.002$, $n = 66$). Next we averaged measurements for each age, and attempted to relate average CWL to average number of layers in the SC from hatching to day 14, the terminal age for which they had counts of layers in the SC. Results showed that CWL declined as the number of layers of the SC increased ($R^2 = 0.46$, $F = 9.3$, $P = 0.01$, $N = 13$). The variation in their data at each age emphasizes that chicks of the same age vary in skin development.

Menon et al. (1988) claimed that nestlings have a relatively impermeable skin, which increases in rate of water permeation as birds mature, despite that fact that layers of the SC remain constant. These authors argued that CWL of nestling zebra finches was low, around 3 ppm H$_2$O/(0.5cm$^2$·hr), near that of adult hairless mice, 4-10 ppm H$_2$O/(0.5cm$^2$·hr). They used a Meeco electrolytic moisture analyzer to determine the moisture in a stream of dry nitrogen gas passed over the skin surface. This system apparently produces abnormally high values of CWL because dry nitrogen gas increases the gradient driving moisture loss from skin. However, passing dry nitrogen gas at 25°C
over the skin of an ectothermic nestling may reduce $T_b$ and thus skin temperature, which would reduce CWL. A reduction in skin temperature negatively affects CWL (Grice and Bettley 1967). Attempts to compare values of water loss of nestling House Sparrows in this study with those of nestling zebra finches have proven problematic. Menon et al. (1988) reported that nestlings 1-10 days old lost water through their skin at a rate of 2-4 ppm/0.5cm²/min (their Fig. 1), but in the text of this same article, values were given as 2-4 ppm/0.5cm²/hr. We re-calculated water loss from nestling zebra finches 1-10 days old assuming a flow rate of 100 ml/min of dry nitrogen gas flowing through the Meeco Analyzer and a concentration of water vapor in the nitrogen gas of 2-4 ppm/0.5cm². Results showed that nestling zebra finches lost 0.29 g H₂O/(m²·hr) from their skin, a value markedly lower than what we have found for nestling sparrows.

We found that CWL varied significantly with the two components that contribute to SC thickness, corneocyte thickness and extracellular space thickness, implying the possibility of a functional role of both variables on CWL in chicks. CWL was related with the lipid composition of the intercellular spaces in the SC in adult birds (Haugen et al. 2003a, b, Muñoz-Garcia and Williams 2005). Assuming that extracellular space thickness is a proxy for the amount of lipid in the intercellular spaces, it is not surprising to find a negative association between CWL and extracellular space thickness. However, the total amount of lipid in the SC in the same nestlings for which we have ultrastructural measurements is negatively associated with thickness of the ECS of the SC, the opposite
of what we expected. Different reasons, not mutually exclusive, can explain this discrepancy. First, our assumption that the ECS of the SC is mostly filled with lipid could be wrong. Second, sample size may compromise our conclusions, because we only have morphological measurements for five nestlings of three different ages. Finally, our results may indicate that complex interactions between lipid classes in the SC influence the competence of the permeability barrier, rather than simply the accumulation of lipids in the SC. It seems that the appropriate ratios of lipid classes determine the molecular organization of the SC, which influences CWL (Muñoz-Garcia et al. 2008a). We did not expect to find a negative relationship between CWL and corneocyte thickness. The presence of intracellular lamellae in the corneocytes suggests that these lamellae can act as a barrier for water following a transcellular pathway in the SC. CWL might be regulated, then, both by extra- and intracellular lipids in the SC.

We found that developmental plasticity of house sparrow fledglings in environments that differ in water vapor pressure, and thus the environment-skin humidity gradient, is an important process modifying CWL, through changes of the lipid composition of the SC. Dry-acclimated fledglings from both Saudi Arabia and Ohio had a lower proportion of FFA in the SC when compared with humid-acclimated birds. Fledglings from Saudi Arabia decreased the proportion of ceramides in the SC in response to high humidity, whereas fledglings from Ohio kept a constant proportion of ceramides. Comparisons between siblings also indicated that ceramides and FFA were
the more flexible lipid classes in the SC. These results suggest that desert and mesic fledglings modified the lipid composition of the SC in different ways in response to acclimation. In both cases, however, the functional significance of these changes was the same: a reduction in CWL in dry-acclimated fledglings. This is consistent with our results from the natural development of CWL and the lipid composition of the SC of nestlings from both environments. We found that CWL in mesic nestlings was associated with changes in neutral lipids (FFA and triglycerides), whereas adjustment of CWL in desert nestlings was induced by modifications of the sphingolipids (ceramides and cerebrosides).

Adult house sparrows had lower CWL rates than fledglings in both Saudi Arabia and Ohio (Muñoz-García and Williams 2005, Groff et al. 2007). To achieve a more efficient skin permeability barrier, adult sparrows from Saudi Arabia increased the ratio of FFA to ceramides and decreased the ratio of ceramides to cerebrosides in the SC compared with nestlings. Adult house sparrows from Ohio, on the other hand, had a higher FFA:ceramide ratio than fledglings from Ohio, but they had the same ceramide:cerebroside ratio as fledglings, suggesting that this ratio is not a plastic trait in house sparrows from Ohio. Hence, adjustment of CWL rates in environments that differ in humidity seems to depend on two key lipid molecules, sphingolipids (ceramides and cerebrosides) and FFA in the SC; desert sparrows regulate mainly the former, whereas mesic sparrows modify the latter.
Comparisons of adults with fledglings from both Saudi Arabia and Ohio suggest that birds at different life stages face different selection pressures with regard to water conservation and thermoregulation. As one might expect, CWL in desert adult sparrows was lower than in mesic adult birds (Muñoz-Garcia and Williams 2005). Most likely, natural selection has acted on adult sparrows to minimize CWL in desert environments, where a frugal water economy is crucial for survival and reproduction. Adults can avoid heat gain using behavioral strategies, such as finding shade or restricting foraging time to cooler parts of the day. However, the intensity of selection for thermoregulation in desert and mesic habitats would be higher in nestlings, because, confined to a nest, chicks will need to rely on physiological mechanisms to regulate their body temperatures. Rates of CWL were higher in desert than in mesic fledglings in both dry and humid acclimation environments. In the light of differential selection pressures at different life stages, we suggest that nestlings in Saudi Arabia have higher rates of water loss through their skin because they are subjected to episodes of heat stress more often than mesic nestlings.

Desert and mesic fledglings regulated rates of CWL in response to environmental conditions through modifications of lipid composition of the SC. However, adjustments of lipid concentrations of the SC were different between populations of fledglings. Both desert and mesic fledglings changed concentrations of FFA in their SC, but only desert fledglings altered concentrations of ceramides. In an evolutionary context, the regulation of the sphingolipid content of the SC is more important for desert birds than for mesic
ones. Ceramides of the SC form the structural backbone of intercellular lipid lamellae, thus playing a major role preventing high water vapor diffusion rates through the skin (Bouwstra et al. 2003). The role of cerebrosides in the SC is not well understood, but we suggested that cerebrosides might be involved in the formation of lamellae, creating an ordered lattice in the central layers of the lamellae, contributing to reduce CWL. However, lipids can also change their phase behavior in response to humidity (Silva et al. 2007). High levels of hydration of the SC lead to increased fluidity of intercellular lipids. Because permeability in fluid phases is higher than in solid phases (Schaefer and Redelmaier 1996), an increase in fluidity will be associated with higher CWL without the need to modify the lipid composition of the SC. Therefore, the proper combination of ceramides and cerebrosides in the SC of desert birds appears to be crucial for the dual function of the SC of water conservation and thermoregulation in this environment (Muñoz-Garcia and Williams 2005, Muñoz-Garcia et al. 2008a). We cannot exclude the possibility that both mechanisms, changes in lipid composition of the SC, and changes in phase behavior of lipids in response to humidity, act together to influence CWL through the skin in house sparrows.

Conclusions

In conclusion, our data show that altricial nestlings do not have watertight skin as was previously envisioned: house sparrow nestlings had surface-specific CWL rates that
were higher than adults. Secondly, we show that the SC is composed of two distinct layers, a stratum compactum and stratum disjunctum, layers of which together influence water permeation through the skin. We found that morphology of the SC did not change linearly with age, but seemed to vary with developmental stage. CWL decreased as the number of layers of the SC increased, and as the total thickness of the corneocytes increased. Further, we found that CWL decreased as the thickness of the extracellular space increased, number of corneocytes increased, and total corneocyte thickness increased.

The picture emerges that variation in CWL between desert and mesic populations of house sparrows in response to humidity is attributable to developmental plasticity of nestlings and natural selection on both nestlings and adults. The expression of plasticity in CWL may be a response to opposite selection pressures, for attributes that influence their ability to thermoregulate and for those that influence water conservation. These selective forces occur with different intensities during the lifetime of house sparrows in different environments. House sparrow nestlings from deserts show larger developmental plasticity because the intensity of selection is larger in xeric environments. This result supports the idea that in stressful environments, such as deserts, organisms will show a higher degree of plasticity than those inhabiting more benign habitats (Schlichting and Smith 2002, Parson 1987). Sparrows from different habitats modify different classes of lipids in the SC to regulate CWL. Desert house sparrows seemed to control more closely
the concentration of sphingolipids, whereas mesic birds prioritized the regulation of the concentration of FFA. Modification of sphingolipids, FFA, or both, led to changes in interactions of different lipid classes in the SC, hence altering CWL.

Results from this work combined with data of previous studies on nestling house sparrows from desert and mesic populations allow us to incorporate the ontogeny of the SC in our thermoregulation-water conservation (TWC) model, outlined in Muñoz-García and Williams (2005) and Muñoz-García et al. (2008a). In desert environments, adult sparrows are subjected to strong selective pressures for water conservation at normothermic temperatures and for thermoregulation at high $T_a$s. For desert nestlings, however, the intensity of selection for water conservation will be lower, but the intensity of selection for thermoregulation will be higher, than for adults (Muñoz-García and Williams, 2008). Adult sparrows living in Ohio do not experience such extreme conditions of desiccation and temperature. Moreover, the intensity of selection for water conservation and thermoregulation will be similar for nestling and adult sparrows in mesic environments. Therefore, birds from different environments and at different life stages will adjust CWL to satisfy the opposite needs of thermoregulation and water conservation in response to the environmental conditions of aridity and temperature. Changes in CWL are mediated by the regulation of the lipid composition of the SC.

According to the TWC model, CWL of desert birds has to be lower than that of mesic birds at moderate temperatures, but higher at high $T_a$s. We showed that CWL is
lower in adult sparrows from desert environments at $T_a$ within the thermoneutral zone, as expected (Muñoz-Garcia and Williams 2005). The idea that at high $T_a$ rates of CWL will be higher in desert than in mesic birds remains to be tested. We found that CWL of adults is lower than that of nestlings in both desert and mesic habitats. Whereas adults can use behavioral strategies to avoid heat gain besides evaporative cooling, nestlings are confined to their nests and they must thermoregulate by evaporative means.

The TWC model also predicts that the SC of desert birds has to be structured in such a way that it satisfies the demands for water conservation and thermoregulation at different $T_a$. The appropriate ratio of ceramides to cerebrosides seems to determine the properties of the permeability barrier. At moderate $T_a$, cerebrosides will form the central layer of the intercellular lamellae of the SC and they will create a highly ordered structure with ceramides, preventing excessive rates of CWL (Muñoz-Garcia et al. 2008a). However, the same lipid matrix in the SC may have different properties as temperature increases (Silva et al. 2007, Schaefer and Redelmaier 1996). At high $T_a$, the central layer of the lamellae may experience a phase transition and change to a more permeable fluid phase, a hypothesis in need to be tested.

Because nestling and adult sparrows from deserts might experience different selection pressures, but nestling and adult birds from Ohio face similar conditions for humidity and temperature, we expect the lipid composition of the SC to change more dramatically during ontogeny in birds from deserts. In agreement with that idea, we
found that desert nestlings changed both the ratios of FFA to ceramides and of ceramides to cerebrosides, but mesic nestlings only changed the FFA:ceramide ratio during development. The ceramide:cerebroside ratio was constant in mesic birds at all ages.

Differences in CWL and the lipid composition of the SC between desert and mesic nestlings could be the result of genetic differences between populations or phenotypic plasticity within the lifetime of individuals. Because the intensity of selection for thermoregulation is higher in nestlings than in adults living in deserts, the ability to modify concentrations of sphingolipids in the SC in response to environmental stimuli during development of the individual will be advantageous in desert nestlings over mesic chicks. Consistent with our hypothesis, we found that nestling sparrows from desert environments showed a higher degree of plasticity than mesic nestlings in response to humidity for the concentration of sphingolipids in the SC (Muñoz-Garcia and Williams 2008). We also found plasticity for CWL and lipids of the SC in adult sparrows. We found that adult sparrows from Ohio acclimated to different humidity regimes were plastic for the FFA:ceramide ratio, but not for the ceramide:cerebroside ratio (Muñoz-Garcia et al. 2008b). Under the TWC model, we hypothesize that adult house sparrows from desert environments will be plastic for the ceramide:cerebroside ratio, instead of the FFA:ceramide ratio, in response to humidity. Accordingly, the activity of the enzyme β-glucocerebrosidase, which cleaves cerebrosides into ceramides, will be higher in the SC of desert individuals than in the SC of mesic birds (Cox et al. 2008).
LIST OF REFERENCES


200


208


210


APPENDIX A

SPHINGOLIPID MOLECULES IDENTIFIED IN THE SC OF HOUSE SPARROWS BY HPLC-APPI/MS
Table A.1. Molecules of sphingolipids identified in the SC of sparrows by HPLC-APPI-MS. Reported mass to charge ratio (m/z) correspond to (M + H – H₂O)+ species; + = presence of molecule “x” in all birds; - = absence of molecule “x” in all birds. Numbers that identify compounds correspond to the number of carbons of the fatty acid residue of the sphingolipid, followed by the number of double bonds of the acyl chain. In parentheses, percentage of birds that showed the molecule “x”, in those cases in which percentage was different from 100%. *These molecules are not distinguishable using APPI; therefore they could not be quantified separately.
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