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Cutaneous water loss and sphingolipids covalently bound to corneocytes in the stratum corneum of house sparrows *Passer domesticus*

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SUMMARY

The barrier to water loss from the skin of birds and mammals is localized in the stratum corneum (SC), the outer layer of the epidermis. The SC consists of corneocytes, each surrounded by a protein envelope, and a lipid compartment, formed by an extracellular matrix of lipids and by lipids covalently bound to the protein envelope. In mammals, covalently bound lipids in the SC consist of ω-hydroxyceramides attached to the outer surface of corneocytes. Evidence suggests that covalently bound lipids in the SC might be crucial for the establishment of a competent permeability barrier. In this study we assessed the composition of covalently bound lipids of the avian SC and their relationship to cutaneous water loss (CWL) in two populations of house sparrows, one living in the deserts of Saudi Arabia and the other in mesic Ohio. Previously, we showed that CWL of adult desert sparrows was 25% lower than that of mesic birds. In the present study we characterize covalently bound lipids of the SC using thin layer chromatography and high performance liquid chromatography coupled with atmospheric pressure Photospray® ionization mass spectrometry. Our study is the first to demonstrate the existence of sphingolipids covalently bound to corneocytes in the SC of birds. Although ω-hydroxyceramides occurred in the lipid envelope surrounding corneocytes, the major constituent of the covalently bound lipid envelope in house sparrows was ω-hydroxycerebrosides, ceramides with a hexose molecule attached. Sparrows from Saudi Arabia had more covalently bound cerebrosides, fewer covalently bound ceramides and a lower ceramide to cerebroside ratio than sparrows living in Ohio; these differences were associated with CWL.

Key words: covalently bound lipid, house sparrows, desert, cutaneous water loss.

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. In birds, evaporative water losses are the major avenue of water efflux, often five times greater than urinary and fecal water losses (Dawson, 1982). Given that cutaneous water loss (CWL) is 50–70% of total evaporative water loss at moderate air temperatures (Tieleman and Williams, 2002), it becomes apparent that the role played by skin as a barrier to water vapor diffusion is important. In both birds and mammals, resistance to CWL is conferred by 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 is composed 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. In mammals, intercellular lipids of the SC consist of nearly equimolar proportions of ceramides, cholesterol and free fatty acids (Bouwstra et al., 2003; Madison, 2003). These lipid molecules are structured in bilayers called lamellae (Swartzendruber et al., 1989; Bouwstra et al., 2003; Hill and Wertz, 2006). In the SC of birds, these same three classes of extracellular lipids occur with the addition of cerebrosides, which are ceramide molecules with an attached hexose (Wertz et al., 1986; Muñoz-Garcia and Williams, 2005).

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, which were 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 structures of the avian protein and the covalently bound lipid of the SC have received less attention than their counterparts in 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. In this report we test the idea that corneocytes of avian SC have covalently bound ω-hydroxyceramides, as found in the SC of mammals. Further, we explore the idea that birds from two radically different environments have different lipids attached to their corneocytes, which might lead to a different organization of lipids in the extracellular spaces. Our results indicate 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 lipid components covalently attached to corneocytes of a vertebrate.

MATERIALS AND METHODS

Capture of house sparrows and measurement of cutaneous water loss

We mist netted 12 house sparrows *Passer domesticus* L. at the National Wildlife Research Center (22°15′N, 41°50′E) in Taif, Saudi Arabia, and 8 sparrows in Columbus (Ohio, USA, 40°00′N, 83°10′W), during October–November 2003. Average ambient temperature at the time of the study was 20.7°C at Taif and 12.0°C at Columbus, Ohio. Sparrows at the Research Center where we captured them have water continuously available. Prior to measurements, sparrows were held in captivity for 1–2 days; they were fed with a mixture of seeds, mealworms and egg yolk, and provided with water *ad libitum*.

We measured cutaneous water loss (CWL) using an open flow mask respirometry system (Tieleman and Williams, 2002). Data for CWL of desert and mesic house sparrows are reported elsewhere (see Muñoz-Garcia and Williams, 2005).

Extraction of covalently bound lipids

After measuring CWL, we sacrificed birds, and removed their skin. We then isolated the stratum corneum (SC) and extracted intercellular lipids following published procedures (Haugen et al., 2003; Muñoz-Garcia and Williams, 2005). The SC was stored in glass test tubes at –20°C under an atmosphere of nitrogen.

To confirm that all extracellular lipids had been extracted, we soaked the SC for each bird for 2 h in chloroform:methanol 1:2 (v/v). We then examined extracts for lipids using thin layer chromatography (TLC). No lipid bands were detected in our plates, indicating that all the intercellular lipids had been removed.

Next we searched for covalently bound lipids (CBL) on corneocytes by immersing the SC in 2 ml of 1 mol l^{-1} NaOH in 90% methanol at 60°C for 2 h (Wertz and Downing, 1987). This mild alkaline hydrolysis breaks the ester bonds of lipids attached by an ester linkage to proteins (Wertz and Downing, 1987). We then adjusted the to pH 6 by adding 3 mol l^{-1} HCl, and added 2.5 ml of chloroform. The solution was then passed through a sintered glass filter, and centrifuged at 3000 ***g*** 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 recentrifuged at 3000 ***g*** for 10 min. We combined the organic fractions, and removed any remaining small particles by passing the solution through a PTFE filter, 0.45 μm pore size (Millex, Millipore Corp., Bedford, MA, USA). We dried the filtrates with a stream of nitrogen and stored them at –20°C. Prior to analysis of lipids, the extracts were re-constituted in 50 μl of chloroform:methanol (2:1, v/v) containing 50 mg l^{-1} of the antioxidant butylate hydroxytoluene, BHT.

Identification and quantitation of covalently bound lipids

We tested for CBL in the stratum corneum by using analytical thin layer chromatography (TLC) on 20 cm×20 cm glass plates covered with silicic acid (0.25 mm thick, Adsorbosil-Plus 1, Alltech, Deerfield, IL, USA). Plates were prepared by developing them with chloroform:methanol (2:1, v/v) to the top, air drying them, and activating them for 30 min at 110°C. Then, we divided each plate in 10 mm wide lanes. We prepared standards with known concentrations of nonhydroxy fatty acid ceramides, galactocerebrosides, cholesterol and a mixture of free fatty acids, all purchased from Sigma (St Louis, MO, USA). The concentration of the standards varied from 0.3 to 10 μg μl^{-1}, a range that spanned the concentration of lipids in our extracts. We loaded 5 μl of both standards and samples on to plates, both in duplicate, with a Teflon tipped Hamilton syringe. More polar lipids, such as ceramides and cerebrosides, were separated using a development of chloroform:methanol:water (40:10:1, v/v/v) to 8 cm 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. For neutral lipids, such as free fatty acids, we developed plates with hexane to the top, followed by a development with toluene to the top, and a final development with hexane:diethyl ether:acetic acid (70:30:1, v/v/v) to half. After development, we sprayed plates with a solution of 3% cupric acetate in 8% phosphoric acid, placed them on aluminum hotplates and slowly raised the temperature to 160°C over a period of 30 min. The procedure charred the lipids, allowing their visualization.

Some of the lipids in our extracts migrated on TLC plates at a rate consistent with cerebroside standards. To confirm that these lipids were cerebrosides, we tested these bands for the presence of sugars by spraying plates with a mixture of 100 g of 2,4-dinitrophenylhydrazine in 100 ml phosphoric acid/ethanol (1:1, v/v). Then, we heated the plate at 110°C for 10 min. In the presence of sugars, 2,4-dinitrophenylhydrazine yields an orange color (Wall, 2005).

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. For this procedure we used 10×20 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. Plates were developed with chloroform:methanol:water (40:10:1, v/v/v) to the top of the plate and bands of lipid visualized as above.

To quantify the concentrations of the CBL classes, we scanned carbonized plates with a Hewlett Packard scanner, and measured lipid amounts with the software TN Image (Nelson, 2003). Validation of our methods indicates that we can routinely measure lipid amounts within ±2% (Muñoz-Garcia and Williams, 2005).

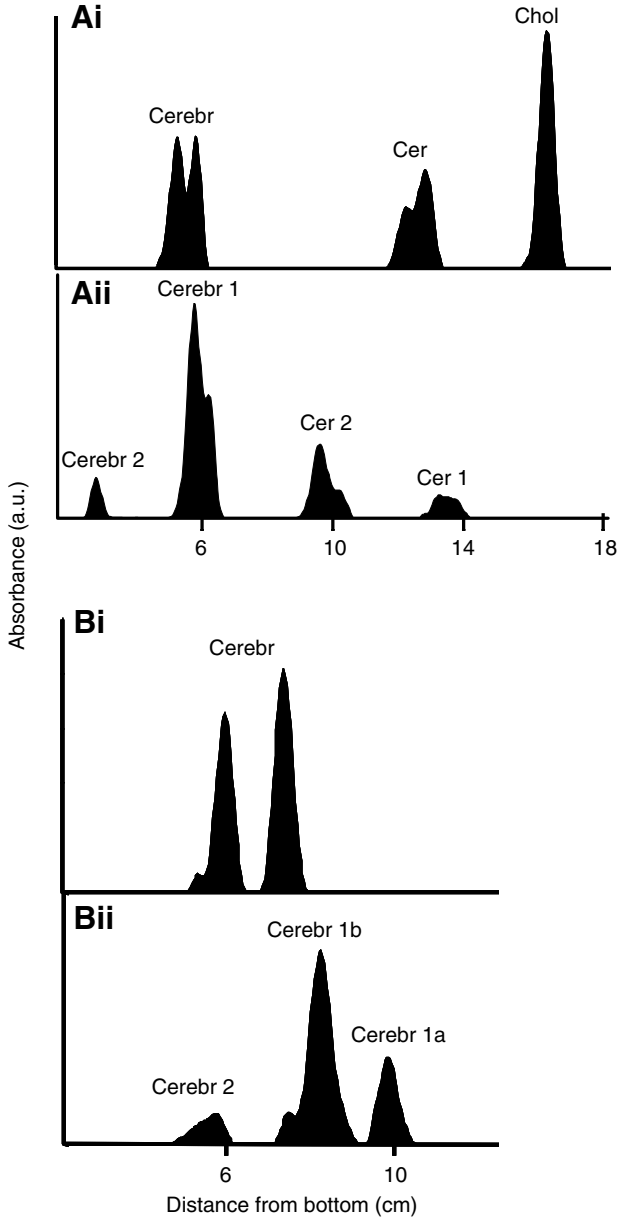

Fig. 1. Carbon density profile using TLC (A) and HPTLC (B) of lipid standards (Ai, Bi) and sphingolipids (Aii, Bii) from the SC of house sparrows. Cerebr, cerebroside; Cer, ceramide; Chol, cholesterol; a.u., arbitrary units.

Preparative thin layer chromatography

To fractionate lipids in our samples before analysis by mass spectrometry, we used preparative TLC on 20 cm×20 cm glass plates covered with silica gel (0.5 mm thick, Adsorbosil-Plus 1, Alltech, Deerfield, IL, USA). 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 8 cm 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 used for analytical TLC.

Table 1. Quantities of covalently bound ceramides and cerebrosides in the stratum corneum of house sparrows from Saudi Arabia and Ohio as determined by thin layer chromatography

	Concentration in SC (mg lipid g^{-1} dry SC mass)	
	Saudi Arabia	Ohio
Dry SC mass (mg)	11.27±2.71	14.18±7.35
Total lipid	14.22±5.42	11.15±3.79
Total ceramides[a]	2.27±0.65	3.17±0.85
Ceramide 1[b]	0.50±0.46	0.98±0.20
Ceramide 2[b]	1.63±0.64	2.18±0.71
Total cerebrosides[a]	12.88±5.05	7.99±2.99
Cerebroside 1	11.31±6.23	6.85±2.73
Cerebroside 2[b]	1.19±0.31	1.14±0.31
Ceramide:cerebroside ratio[b]	0.21±0.08	0.41±0.05

Values are means ± s.d., N=11 house sparrows (from Saudi Arabia) and N=8 (from Ohio).
Superscripts indicate statistical significance; [a]P<0.05; [b]P<0.003.

To isolate classes of lipids on preparative plates without changing their chemical structure, we sprayed plates with 0.2% 2,7-dichlorofluorescein 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 N_2 at –20°C until analyses with HPLC-APPI-MS.

Confirmation of covalently bound cerebrosides using HPLC coupled to atmospheric pressure photo ionization mass spectrometry (APPI-MS)

Because cerebrosides covalently bonded to corneocytes have not previously been found to be in SC of any vertebrate, we wanted to confirm our identification of these lipids. Extracts of putative cerebrosides from preparative TLC were re-dissolved in chloroform:methanol 2:1. We injected 10 µl of this sample onto a HPLC column (Phenomenex Luna C18, spherical 5 µm particle size, 150×2.0 mm i.d., 100 Å pore size, Phenomenex, Torrance, CA, USA), thermostatically controlled at 48°C, and eluted lipids using a reverse phase solvent system (Muñoz-Garcia et al., 2006). We used a gradient solvent system with methanol:water 95:5 (v/v) as the initial solvent, changed in steps to 100% ethyl acetate, with flow rate programmed from 180 µl min^{-1} to 350 µl min^{-1} over 30 min.

After passing through our HPLC system, sphingolipids were routed into an Applied Biosystems Q TRAP® hybrid triple quadrupole linear ion trap SCIEX mass spectrometer equipped with a PhotoSpray® ion source (Applied Biosystems, Ontario, Canada) operated in positive ion mode. Toluene, a dopant for our system, was delivered at a flow rate of 20 µl min^{-1}.

Parameters used on the Q TRAP were: collision gas set to High, curtain gas to 27, Ion Transfer voltage set at 2000 V, nebulizer temperature of 460°C, declustering potential set at 40 V and interface heater on.

Using Enhanced MS with signals collected by dynamic fill time, we surveyed lipid molecules between 450 and 1450 amu (±0.4 amu) at a scan rate of 4000 amu s^{-1}. We confirmed the

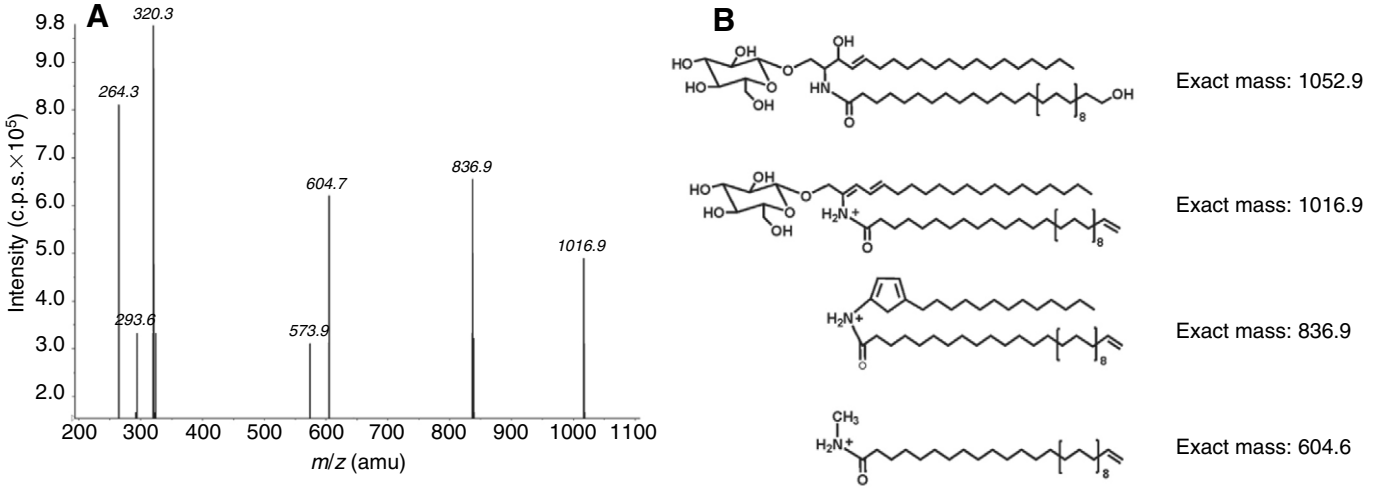

Fig. 2. (A) Molecular spectrum obtained by HPLC-APPI-MS of a representative covalently bound cerebroside. This spectrometric profile might correspond to cerebroside OS, a cerebroside with a terminal hydroxyl group at the omega position of the fatty acid moiety. (B) Interpretation of the molecular structure of the fragments of cerebroside OS 40:0. See text for explanation.

presence of cerebroside molecules by MS/MS by searching for a hexose fragment.

Analyses of data from mass spectrometry were performed using Analyst 1.4.2 software (Applied Biosystems). For each sample, we generated contour plots, two-dimensional graphs of all molecules found during the scan with mass/charge (m/z) as the y-axis, and retention time as the x-axis.

Statistics

All statistical tests were performed with SPSS 14.0 (SPSS, 2007) with the null hypothesis being rejected when $P<0.05$. Values are reported as means ± s.d. We tested for significant differences between means using two-tailed t-test for independent samples.

RESULTS

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. 1A). To further separate cerebrosides, we used HPTLC, which allowed us to resolve the less polar band of cerebrosides into two separate bands (Fig. 1B). After spraying these plates with 2,4-dinitrophenylhydrazine and heating 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 the presence of a sugar molecule in the band was confirmed by chemical tests. We did not detect any neutral lipids such as free fatty acids on the 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). 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 cerebroside molecules by photospray ionization mass spectrometry

Using HPLC-APPI-MS we confirmed the presence of cerebroside molecules covalently bound to corneocytes in SC of sparrows from Ohio. A representative spectrum of one of the cerebrosides in our extract is shown in Fig. 2A. The molecular ion $[M+H^+–2H_2O]$ had a mass of 1016.9, which is consistent with a cerebroside having a hydroxyl group at the omega position (Fig. 2B). Hydroxyl ions in sphingolipids are typically lost as water in the photospray ionization process. The ion 836.9 is consistent with $[M+H^+–Hexose]$ and indicated that the molecular ion contained a sugar moiety. The fragment at 604.6 further supports the view that the molecule had a terminal hydroxyl group. The fragment at 264.3 indicates the presence of sphingosine (Muñoz-Garcia et al., 2006).

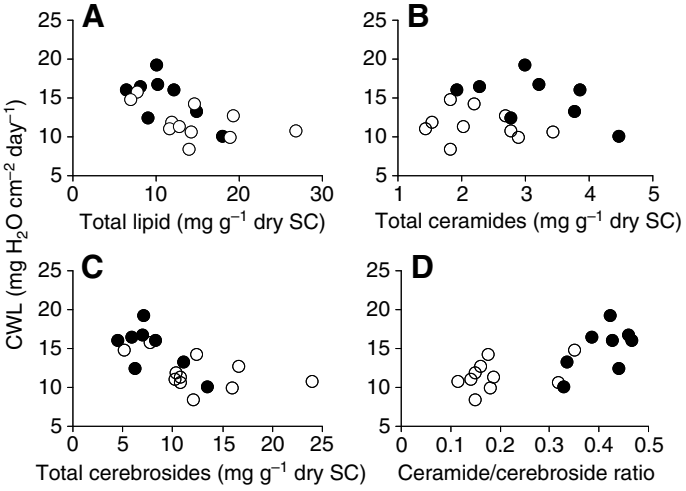

Fig. 3. Cutaneous water loss (CWL) of sparrows from Saudi Arabia (open circles) and Ohio (filled circles) as a function of concentrations of (A) covalently bound total lipid (B) covalently bound ceramides (C) covalently bound cerebrosides, and (D) ceramide to cerebroside ratio in the SC as determined by TLC.

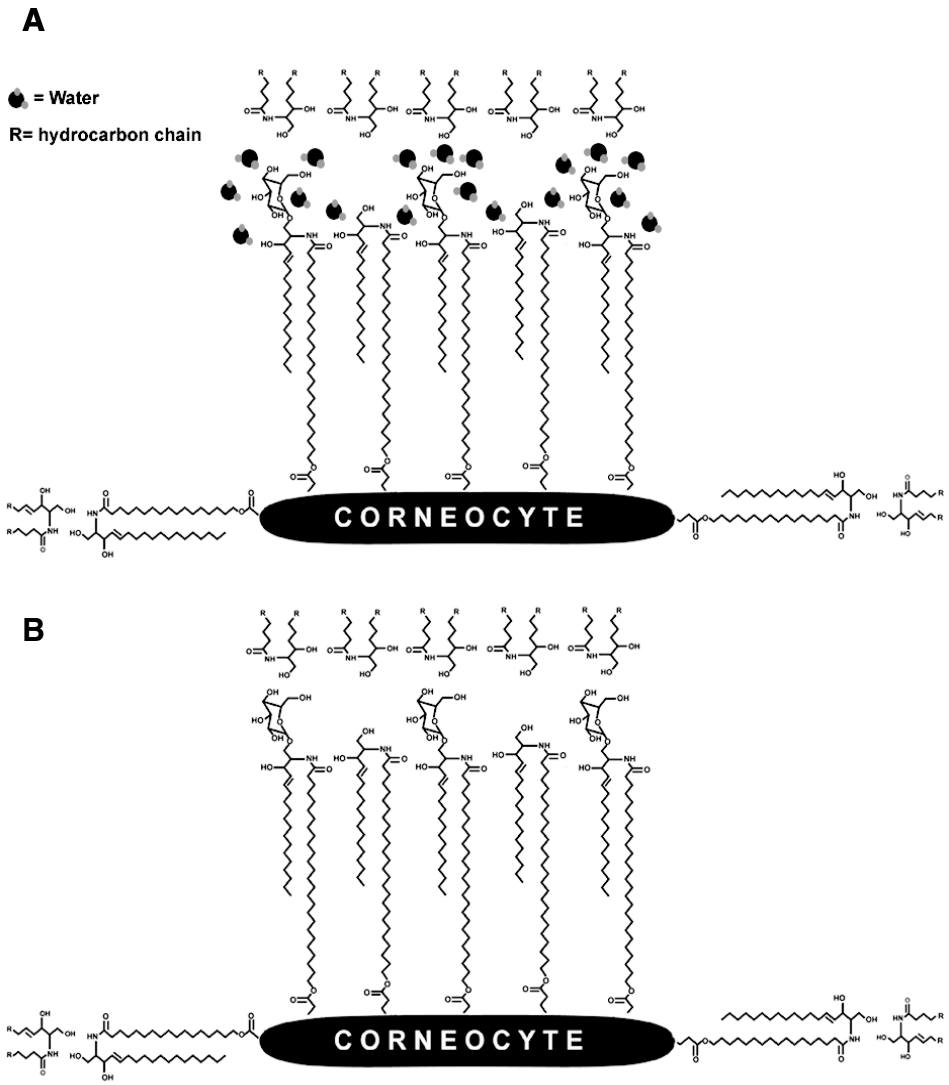

Fig. 4. Hypothesized models for the organization of the covalently bound sphingolipids in the SC in house sparrows from mesic and desert environments. (A) Water shell model. The hexose group of cerebrosides would sequester water molecules. Desert sparrows, with more cerebrosides, could hold a higher 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.

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. 3). CWL of mesic sparrows was $14.98\pm2.92\,mg\,H_2O\,cm^{-2}\,day^{-1}$, whereas sparrows from Saudi Arabia lost $11.87\pm2.22\,mg\,H_2O\,cm^{-2}\,day^{-1}$ 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.

DISCUSSION

Our study showed that, like mammals, sparrows have ceramides covalently bound to corneocytes of the SC, but unlike mammals, sparrows also have 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., 2008), a finding that is consistent for over 20 species of birds (J.R., A.M.-G., J.C.B. and J.B.W., unpublished 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.

Previously we showed that rates of CWL for adult sparrows from deserts in Saudi Arabia were 25% lower than for their mesic counterparts (Munoz-Garcia and Williams, 2005). 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 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., 2008) 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 (Fig. 4A). 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. 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.

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.

LIST OF ABBREVIATIONS

APPI	atmospheric pressure photo ionization
BHT	butylate hydroxytoluene
CBL	covalently bound lipids
CWL	cutaneous water loss
HPTLC	high performance thin layer chromatography
MS	mass spectrometry
SC	stratum corneum
TLC	thin layer chromatography

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REFERENCES

Alibardi, L. and Toni, M. (2004). Localization and characterization of specific cornification proteins in avian epidermis. *Cells Tissues Organs* **178**, 204-215.
Bouwstra, J. A., Honeywell-Nguyen, P. L., Gooris, G. S. and Ponec, M. (2003). Structure of the skin barrier and its modulation by vesicular formulations. *Prog. Lipid Res.* **42**, 1-36.
Coderch, L., López, O., de la Maza, A. and Parra, J. L. (2003). Ceramides and skin function. *Am. J. Clin. Dermatol.* **4**, 107-129.
Dawson, W. R. (1982). Evaporative losses of water by birds. *Comp. Biochem. Physiol.* **71A**, 495-509.
Downing, D. T. (1992). Lipid and protein structures in the permeability barrier of mammalian epidermis. *J. Lipid Res.* **33**, 301-313.
Farwanah, H., Pierstoff, B., Schmelzer, C. E. H., Raith, K., Neubert, R. H. H., Kolter, T. and Sandhoff, K. (2007). Separation and mass spectrometric characterization of covalently bound skin ceramides using LC/APCI-MS and Nano-ESI-MS/MS. *J. Chromatogr. B.* **852**, 562-570.
Haugen, M., Williams, J. B., Wertz, P. W. and Tieleman, B. I. (2003). Lipids of the stratum corneum vary with cutaneous water loss among larks along a temperature-moisture gradient. *Physiol. Biochem. Zool.* **76**, 907-917.
Hill, J. R. and Wertz, P. W. (2003). Molecular models of the intercellular lipid lamellae from epidermal stratum corneum. *Biochim. Biophys. Acta* **1616**, 121-126.
Landmann, L. (1980). Lamellar granules in mammalian, avian, and reptilian epidermis. *J. Ultrastruct. Res.* **72**, 245-263.
Lillywhite, H. B. (2006). Water relations of tetrapod integument. *J. Exp. Biol.* **209**, 202-226.
Madison, K. C. (2003). Barrier function of the skin: 'La raison d'être' of the epidermis. *J. Invest. Dermatol.* **121**, 231-253.
Marekov, L. N. and Steinert, P. M. (1998). Ceramides are bound to structural proteins of the human foreskin epidermal cornified cell envelope. *J. Biol. Chem.* **273**, 17763-17770.
Menon, G. K., Brown, B. E. and Elias, P. M. (1986). Avian epidermal differentiation: role of lipids in permeability barrier formation. *Tissue Cell* **18**, 71-82.
Muñoz-Garcia, A. and Williams, J. B. (2005). Cutaneous water loss and lipids of the stratum corneum in house sparrows *Passer domesticus* from arid and mesic environments. *J. Exp. Biol.* **208**, 3689-3700.
Muñoz-Garcia, A., Ro, J., Brown, J. C. and Williams, J. B. (2006). Identification of complex mixtures of sphingolipids in the stratum corneum by reversed-phase high-performance liquid chromatography and atmospheric pressure photospray ionization mass spectrometry. *J. Chromatogr. A* **1133**, 58-68.
Muñoz-Garcia, A., Ro, J., Brown, J. C. and Williams, J. B. (2008). Cutaneous water loss and sphingolipids in the stratum corneum of house sparrows, *Passer domesticus* L., from desert and mesic environments as determined by reversed-phase high-performance liquid chromatography coupled with atmospheric pressure photospray ionization mass spectrometry. *J. Exp. Biol.* **211**, 447-458.
Nelson, T. J. (2003). TN-Image. http://brneurosci.org/tnimage.html.
SPSS (2007). *SPSS 14.0 for Windows*. Chicago: SPSS.
Stewart, M. E. and Downing, D. T. (2001). The ω-hydroxyceramides of pig epidermis are attached to corneocytes solely through ω-hydroxyl groups. *J. Lipid Res.* **42**, 1105-1110.
Swartzendruber, D. C., Wertz, P. W., Kitko, D. J., Madison, K. C. and Downing, D. T. (1989). Molecular models of the intercellular lipid lamellae in mammalian stratum corneum. *J. Invest. Dermatol.* **92**, 251-257.
Tieleman, B. I. and Williams, J. B. (2002). Cutaneous and respiratory water loss in larks from arid and mesic environments. *Physiol. Biochem. Zool.* **75**, 590-599.
Wall, P. E. (2005). *Thin-layer Chromatography: A Modern Practical Approach*. Cambridge: The Royal Society of Chemistry.
Wertz, P. W. (2000). Lipids and barrier function of the skin. *Acta Derm. Venereol. Suppl.* **208**, 7-11.
Wertz, P. W. and Downing, D. T. (1987). Covalently bound ω-hydroxyacylsphingosine in the stratum corneum. *Biochim. Biophys. Acta* **917**, 108-111.
Wertz, P. W., Stover, P. M., Abraham, W. and Downing, D. T. (1986). Lipids of chicken epidermis. *J. Lipid Res.* **27**, 427-435.
Wertz, P. W., Swartzendruber, D. C., Kitko, D. J., Madison, K. C. and Downing, D. T. (1989). The role of the corneocyte lipid envelopes in cohesion of the stratum corneum. *J. Invest. Dermatol.* **93**, 172.