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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

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SUMMARY

Because cutaneous water loss (CWL) represents half of total water loss in birds, selection to reduce CWL may be strong in desert birds. We previously found that CWL of house sparrows from a desert population was about 25% lower than that of individuals from a mesic environment. The stratum corneum (SC), the outer layer of the epidermis, serves as the primary barrier to water vapor diffusion through the skin. The avian SC is formed by layers of corneocytes embedded in a lipid matrix consisting of cholesterol, free fatty acids and two classes of sphingolipids, ceramides and cerebrosides. The SC of birds also serves a thermoregulatory function; high rates of CWL keep body temperatures under lethal limits in episodes of heat stress.

In this study, we used high-performance liquid chromatography coupled with atmospheric pressure photoionization-mass spectrometry (HPLC/APPI-MS) to identify and quantify over 200 sphingolipids in the SC of house sparrows from desert and mesic populations. Principal components analysis (PCA) led to the hypotheses that sphingolipids in the SC of desert sparrows have longer carbon chains in the fatty acid moiety and are more polar than those found in mesic sparrows. We also tested the association between principal components and CWL in both populations. Our study suggested that a reduction in CWL found in desert sparrows was, in part, the result of modifications in chain length and polarity of the sphingolipids, changes that apparently determine the interactions of the lipid molecules within the SC.

Key words: cutaneous water loss, house sparrows, mass spectrometry, stratum corneum.

INTRODUCTION

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 cutaneous water loss (CWL) (Chuong et al., 2002; Elias, 2004). In mammals and birds, CWL is mediated primarily by the outer layer of the epidermis, the stratum corneum (SC), formed by multiple layers of cornified cells embedded in a matrix of lipids (Menon and Menon, 2000; Lillywhite, 2006); these lipids determine the rate of water permeation through the skin (Potts and Francoeur, 1991; Madison, 2003). In mammals, lipids in the intercellular spaces of the SC are primarily a 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; Bouwstra 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 are 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.

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 Rodelmeier, 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).

Because vertebrates that live in desert environments often face high ambient air temperatures (T_a), low humidity and little surface

water for drinking, natural selection might have acted on phenotypic features of the SC that would reduce CWL, thereby promoting an efficient water economy (Williams and Tieleman, 2005). 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. For example, studies on species of larks across an aridity gradient, from Saudi Arabia to the Netherlands, revealed that desert birds had a lower CWL than did species from mesic environments (Tieleman and Williams, 2002; Williams and Tieleman, 2005). This reduction in CWL was associated with an increase in the proportion of more polar ceramides, and a decrease in the proportion of free fatty acids (Haugen et al., 2003a; Haugen et al., 2003b). However, the skin of some species of birds also plays a role in thermoregulation, at least when T_a is high (Bernstein, 1971; Marder and Ben-Asher, 1983). So, when T_a is favorable, desert birds should have minimal water loss through the skin, but when T_a exceeds body temperature, CWL should be elevated. This dual role of skin might have implications for the lipid organization within the SC.

We previously reported that CWL of house sparrows, *Passer domesticus*, living in the desert of Saudi Arabia was about 25% lower than that of sparrows living in mesic Ohio (Muñoz-Garcia and Williams, 2005). To analyze the lipid classes in the SC of sparrows, we used thin layer chromatography (TLC) with a solvent system that separated lipid classes based primarily on polarity. Sparrows from Saudi Arabia had a significantly higher concentration of ceramides and cerebrosides than did sparrows from Ohio (Muñoz-Garcia and Williams, 2005).

Although TLC has been a useful tool to separate and quantify lipid classes in the SC, it cannot unambiguously identify all individual molecules. 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) (J.R., A.M.-G., J.C.B. and J.B.W., 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 the 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 insight into the possible arrangement of the lipid molecules within the SC.

In the present study, we apply our new method of HPLC/APPI-MS to identify and quantify sphingolipids in the SC of house sparrows, one population from the desert known to have a reduced CWL and the other living in a mesic environment. We characterized the biochemical properties of the sphingolipids that we identified and grouped them into families. To search for common themes within the approximately 200 different lipid molecules that we identified in 27 sparrows, we used principal components analysis (PCA). The results of our PCA led to the hypotheses that sphingolipids in the SC of desert sparrows have longer carbon chains in the fatty acid moiety and are more polar than those found in mesic sparrows. To evaluate the functional significance of the differences in lipid composition between desert and mesic sparrows, we tested for associations between principal components and CWL in both populations. Our results suggested that the reduction in CWL found in desert sparrows was the result of modifications in chain length and polarity of the sphingolipids, changes that apparently determine the interactions of the lipid molecules within the SC with attendant modification of water permeation through the SC.

MATERIALS AND METHODS

Capture of birds

House sparrows in North America, *P. d. domesticus*, were introduced at the end of the 19th century from England (Summers-Smith, 1963). House sparrows in Saudi Arabia, *P. d. indicus*, are native to this region, 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 was subjected to episodes of extreme heat (>43°C) during the summer.

We mist netted 12 house sparrows at the National Wildlife Research Center near Taif, Saudi Arabia (22°15′N, 41°50′E) and 15 sparrows in Columbus, Ohio (40°00′N, 83°10′W), during October–November 2003. During the brief time that sparrows were held captive prior to measurements, we fed them a mixture of seeds, egg yolk and mealworms. Water was provided *ad libitum*. Experiments were approved by IACUC of Ohio State University (protocol 2003-A0072).

Isolation of the SC and separation of skin lipids

To isolate the SC we used the methods of Wertz et al. (Wertz et al., 1986) as modified by Haugen et al. (Haugen et al., 2003a). We killed birds, removed their skin, pinned it to a Teflon sheet, and immersed it in a waterbath for 3 min at 65°C. Then, we gently peeled the epidermis from the dermis. Thereafter, we incubated the epidermis in a solution of 0.5% trypsin in phosphate-buffered saline (PBS; 1.2% Na_2HPO_4 $7H_2O$, 0.06% NaH_2PO_4 H_2O monobasic, and 0.8% NaCl in distilled water, pH 7.4, 370 mosmol l^{-1}), at 4°C overnight, followed by another incubation in fresh 0.5% trypsin solution in PBS at 38°C, to separate the SC from the epidermis. The SC was freeze-dried and stored at –20°C in an atmosphere of nitrogen or argon.

We determined the dry mass of the SC and extracted the lipids with a series of chloroform–methanol (2:1, 1:1 and 1:2) for 2 h each step (Law et al., 1995). We combined the extracts and dried them under a stream of nitrogen gas.

Reversed phase HPLC

Sphingolipids of the SC of sparrows were re-dissolved in a mixture of isopropyl alcohol, toluene and ethyl acetate (7.4:2:1 v/v/v) with 72.6 ng ml^{-1} of synthetic C17:0 ceramide as an internal standard, and 43.5 μg ml^{-1} butylated hydroxytoluene (BHT) as an antioxidant. We separated sphingolipids using reversed phase HPLC with a Phenomenex Luna® C_{18} column 150 mm × 2.0 mm i.d., spherical 5 μm particle size, 100 Å pore

size (Phenomenex, Torrance, CA, USA) at 48°C. We loaded 3 μl of lipid extracts onto our HPLC column for each run of each sample. We used a gradient solvent system following Muñoz-Garcia et al. (Muñoz-Garcia et al., 2006); the initial solvent was 100% methanol:isopropanol:water (85.5:10:4.5 v/v/v) run at a flow rate of 180 μl min^{-1}, and changed gradually to a final concentration of 75% methanol:isopropanol:water (85.5:10:4.5 v/v/v) and 25% ethyl acetate during the course of 24 min. The latter solution was changed during the course of 1 min to 100% ethyl acetate at a flow rate of 300 μl min^{-1}. A final step using 100% methanol:isopropanol:water (85.5:10:4.5 v/v/v) for 5 min was used to precondition the column for the next run.

Detection and identification of sphingolipids

We detected sphingolipids coming from our HPLC system with 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 positive and negative ion mode, with toluene as a dopant. For parameters that we used on our Q-TRAP system, see Muñoz-Garcia et al. (Muñoz-Garcia et al., 2006).

Using Analyst™ 1.4.1 (Applied Biosystems, Ontario, Canada), we generated a contour plot for each sample, with mass/charge (m/z) as the y-axis and retention time as the x-axis. The contour plot yielded series of dark bands, some representing a molecular ion of a sphingolipid, whereas others corresponded to source fragments.

To denominate families of sphingolipids, we followed Motta et al. (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. We have elaborated the structure of ceramides and cerebrosides that we have found and ordered them from least to most polar (Fig. 1).

Characterization of the biochemical properties of the sphingolipids

After identification of the array of sphingolipids in the SC of both populations of sparrows, we characterized these molecules according to chain length of the free fatty acid moiety and polarity. For chain lengths, we divided sphingolipid molecules into decades, from 10 to more than 60 carbons long.

The polarity of sphingolipids is determined by the number and position of hydroxyl groups in the molecule as well as the chain length of the hydrophobic residues (Wertz and Downing, 1983). Based on Wertz and Downing (Wertz and Downing, 1983), we ranked families of ceramides and cerebrosides from the least polar, given a rank of 1, to the most polar. We assumed that a rank step in polarity was linear over the range that we considered. Because of their intrinsic differences in polarity, we ranked ceramides and cerebrosides separately.

Quantification of lipid classes in the SC of house sparrows

Previously, we explored the possibility that HPLC/APPI-MS could be used to quantify sphingolipids in the SC, and found reasonable concordance in our estimates of total ceramides and cerebrosides (mg lipid g^{-1} dry SC) using TLC and HPLC-APPI/MS, with a deviation of 0.95% for ceramides and 2.5% for cerebrosides (Muñoz-Garcia et al., 2006). The relative intensity of each individual sphingolipid was proportional to the amount of that molecule in the lipid mixture. We used calibration curves with known concentrations of ceramide 17:0 as our standard to convert intensities to absolute amounts of each lipid molecule (Muñoz-Garcia et al., 2006). We have shown an absence of matrix effects using APPI, and therefore quantification of lipids is possible (J.R., A.M.-G., J.C.B. and J.B.W., unpublished data).

Because similar masses of lipid molecules with different molecular weights yield a different number of moles, and molar ratios of the different lipids in the SC appear to be important in determining water loss through the skin (Bouwstra et al., 2003), we also calculated the number of moles of each sphingolipid molecule in our samples.

Statistics

All statistical tests were performed with SPSS 14.0 (Chicago, IL, USA), with statistical significance set at $P<0.05$. When multiple comparisons were performed, we used the Bonferroni correction (Zar, 1996). Means are reported ±1 s.d. We tested for differences between means using Student's two-tailed t-test for independent samples. Percentages were logit transformed [$\ln(Y/1-Y)$] prior to analyses to normalize data (Zar, 1996). Differences in distributions of data were assessed using the Kolmogorov–Smirnov test.

To explore underlying themes in our data, we used PCA on the quantities (in mmol or mg lipid g^{-1} 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 Student's 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

Identification and quantification of sphingolipids in the SC of house sparrows

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 molecular species of ceramides and 134 cerebrosides (see Table A1). 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 ($t=2.39$, $P<0.03$). 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). 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 previously been 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. 2). The molar ratio of cerebrosides and ceramides, calculated as moles of total cerebrosides divided by moles of total ceramides,

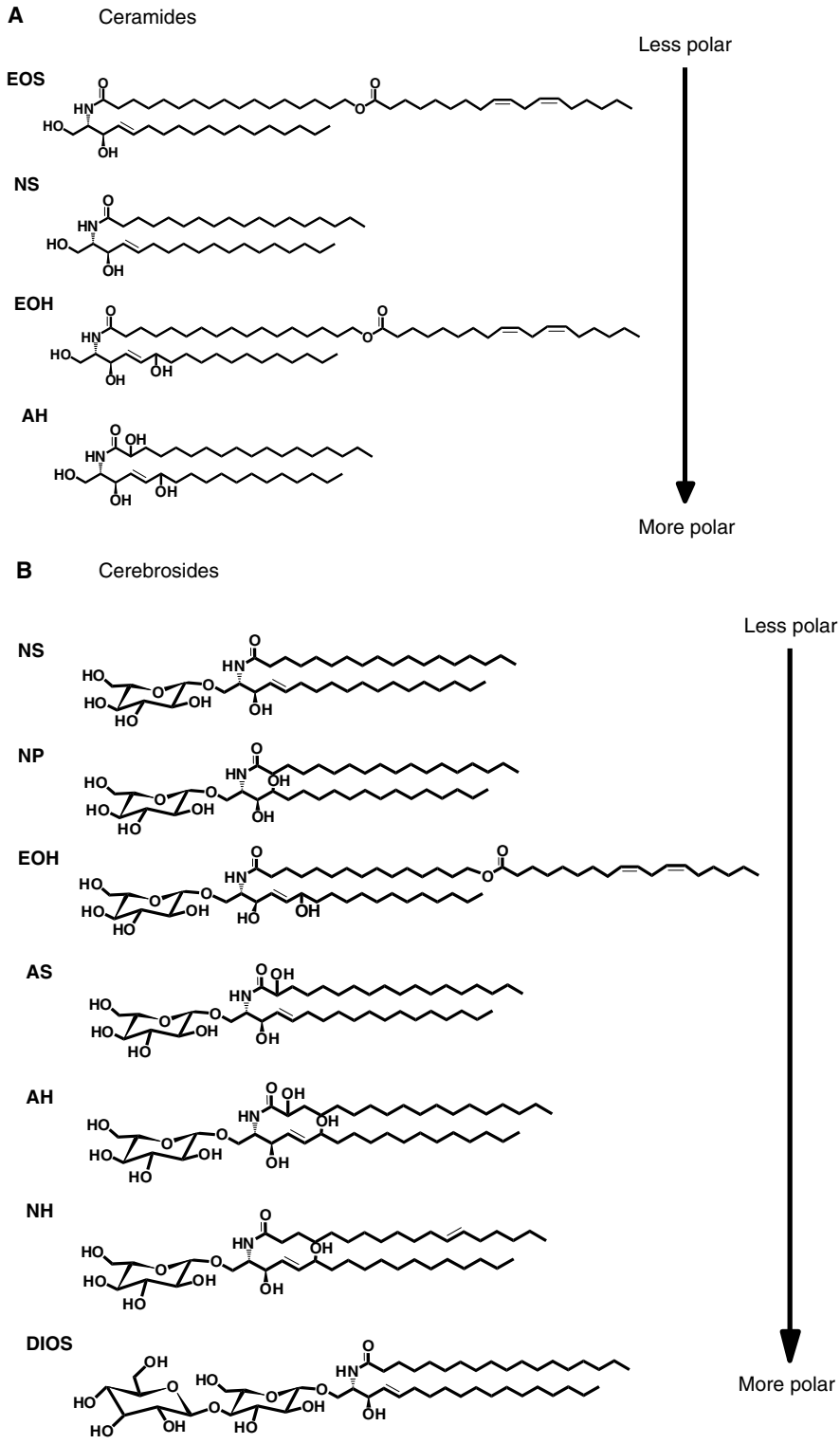

Fig. 1. Chemical structure of sphingolipid families found in the SC of house sparrows. (A) Ceramides. (B) Cerebrosides and diosylceramides (DIOS). Ceramides and cerebrosides are ordered from the least to the most polar. For definitions, see text.

was 1.89 in SC of sparrows from Saudi Arabia and 1.28 in sparrows 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. 2). 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. 2); distributions in these families of cerebrosides were in general more flat in mesic than in desert sparrows.

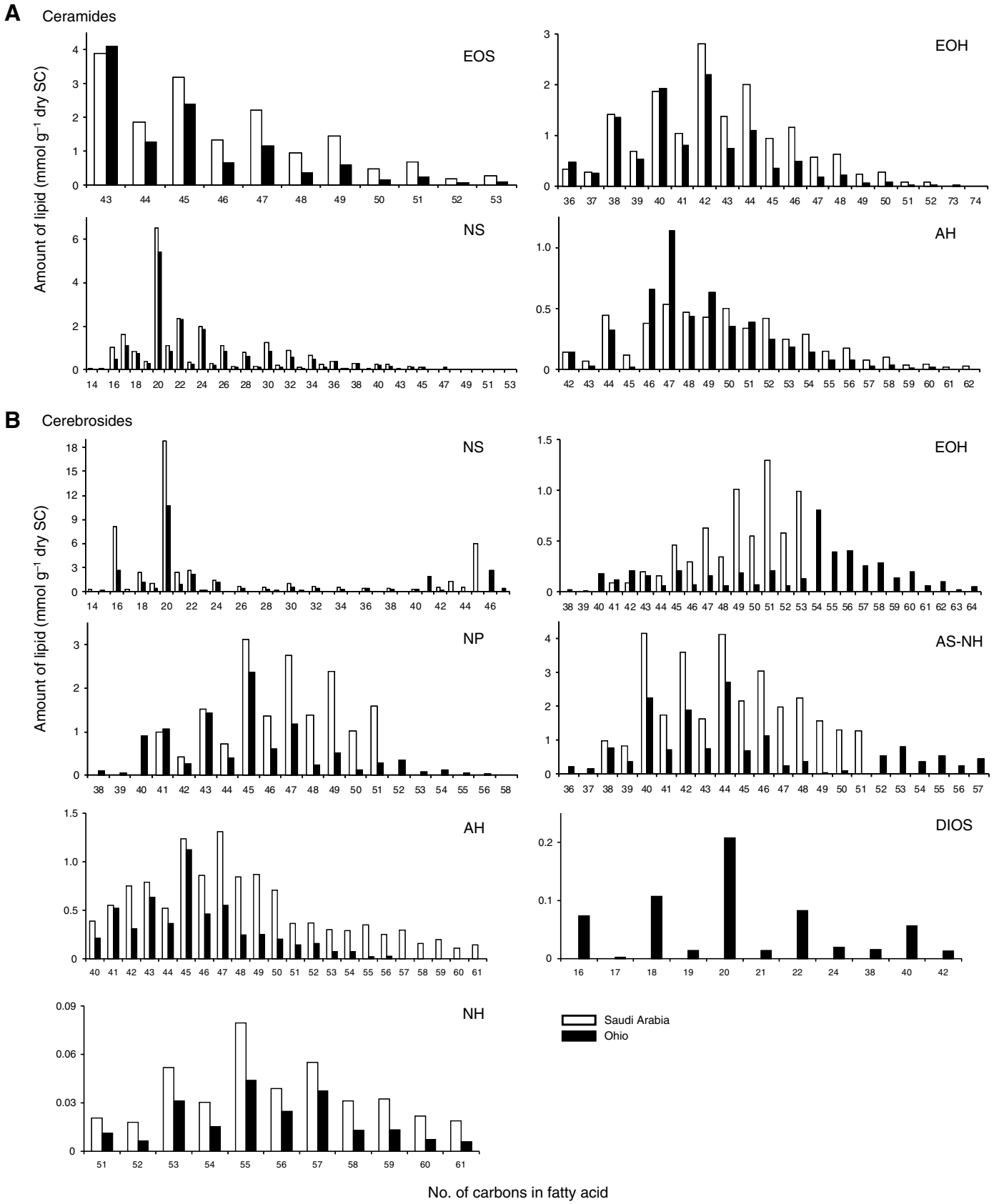

Fig. 2. Distributions of the amount of lipid in millimoles per gram of dry SC within sphingolipid families in the SC of desert (white bars) and mesic (black bars) house sparrows. (A) Ceramides. (B) Cerebrosides and diosylceramides. There were significant differences between desert and mesic sparrows in the distributions of cerebrosides NP, EOH, AS-NH, AH and NH (Kolmogorov–Smirnov, Z>1.423, P<0.035).

Table 1. Principal component analysis of millimoles per gram of SC dry mass of sphingolipid families in the SC of house sparrows from desert and mesic environments

		PC 1	PC 2	PC 3
Correlation PC and original variable				
Ceramides	NS	0.710	–0.618	–0.078
	EOH	0.864	–0.373	0.171
	AH	0.609	–0.634	–0.076
	EOS	0.733	–0.418	0.115
Cerebrosides	NS	0.819	0.087	–0.136
	AS-NH	0.881	0.353	0.242
	NP	0.799	0.314	0.476
	EOH	0.783	0.447	–0.267
	AH	0.842	0.470	0.165
	NH unsaturated	0.511	0.388	–0.663
Diosylceramides		–0.523	0.235	0.391
Eigenvalue		6.097	1.955	1.050
Percentage variance		55.4	17.8	9.6

SC, stratum corneum; PC, principal component. For definitions, see text.

PCA on sphingolipid families

To reduce the number of variables in our data, we used PCA on the number of moles per gram of dry SC of each family of sphingolipids. Three axes accounted for 82.7% of the variance (Table 1). A plot of scores for individual birds along these three axes provided clear separation between mesic and desert sparrows (Fig. 3A). When we added to this plot the eigenvector loadings of the sphingolipid families, we were able to sort our variables into three groups (Fig. 3B). 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. 3A). 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 with mesic sparrows that yield the distinct scores in this component. The scores of all desert birds were positive for PC 2 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 PC 3.

We also performed PCA on the amount in milligrams of lipid per gram of dry SC of each sphingolipid family present in the SC of sparrows. We extracted two components, accounting for 74.8% of the variance (Table 2). The results were similar to those when we used the number of moles in the analysis. The scores for each individual bird provided a good separation between sparrows from Saudi Arabia and Ohio, as when we used the number of moles in the analyses (Fig. 4A). The scores of desert sparrows tended to cluster closer to the loadings of longer ceramides and more polar cerebrosides (Fig. 4B).

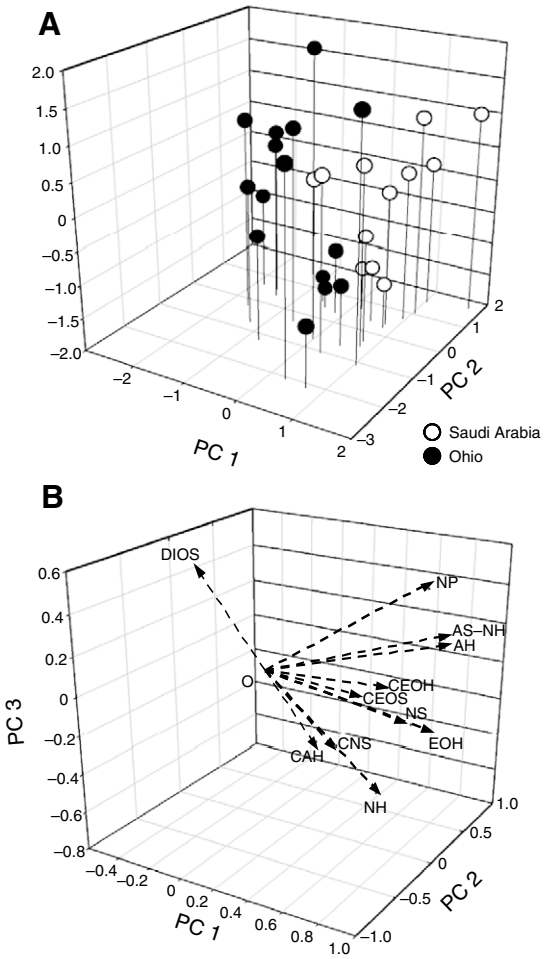

Fig. 3. Principal component analysis (PCA) based on the number of moles per gram of dry SC of each sphingolipid family in the SC of house sparrows. (A) Scores for individual house sparrows from desert (open circles) and mesic environments (filled circles). (B) Eigenvectors for each sphingolipid family. 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, diosylceramides. O, origin of coordinates.

Table 2. Principal component analysis of amounts (mg lipid g^{-1} SC dry mass) of sphingolipid families in the SC of house sparrows from desert and mesic environments

		PC 1	PC 2
Correlation PC and original variable			
Ceramides	NS	0.884	0.358
	EOH	0.818	–0.259
	AH	0.411	0.780
	EOS	0.896	–0.373
Cerebrosides	EOS	0.507	–0.347
	AS-NH	0.714	0.512
	NP	0.788	0.512
	EOH	0.848	–0.171
	AH	0.878	–0.349
	NH unsaturated	0.876	–0.282
Dyosilceramides		–0.586	–0.026
Eigenvalue		6.412	1.818
Percentage variance		58.3	16.5

Abbreviations as in Table 1.

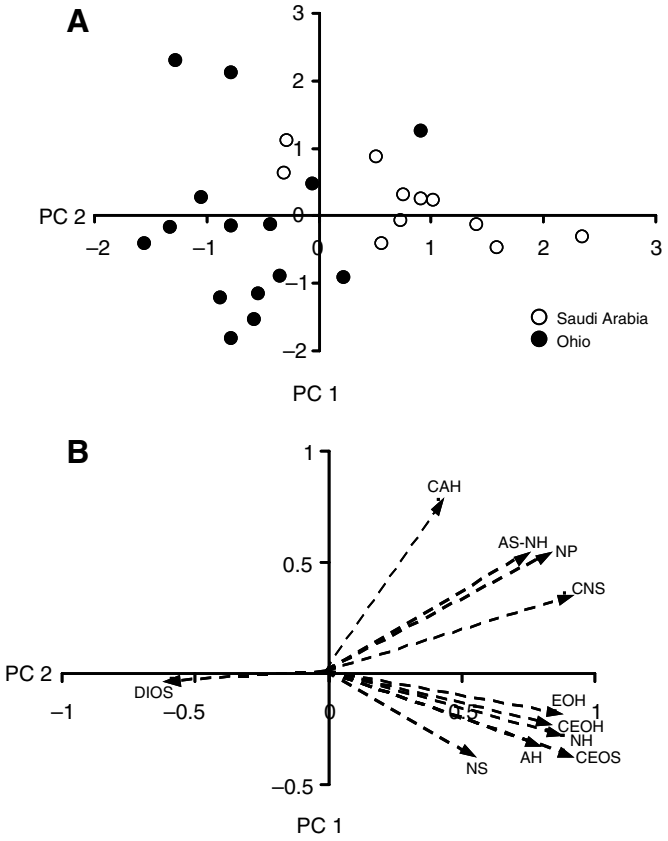

Fig. 4. PCA based on amount in milligrams of lipid per gram of dry SC of each sphingolipid family in the SC of house sparrows. (A) Scores for individual house sparrows from desert (open circles) and mesic environments (filled circles). (B) Eigenvectors for each sphingolipid family. Abbreviations as in Fig. 3.

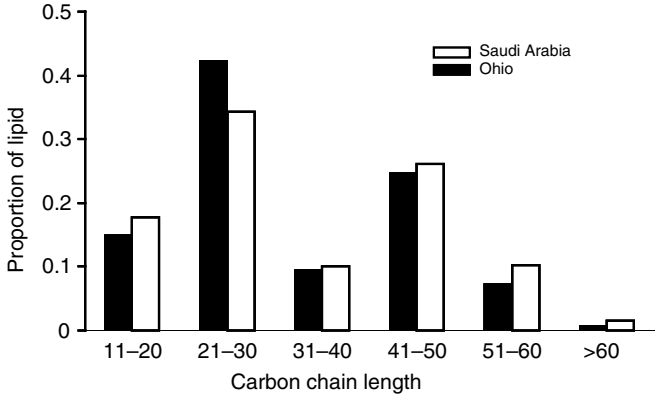

Fig. 5. Distribution of lipid amounts (millimoles of lipid per gram of dry SC) of sphingolipids according to the chain length of the fatty acid moiety.

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 the chain length of the fatty acid moiety and to the 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. 5). 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 21–30 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, monounsaturated 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, which had 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.

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 amount (mg lipid g^{-1} dry SC) of each sphingolipid family, we found a negative association between PC 1 and CWL, with sparrows from the two 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. 6). After performing PCA on the amount (mg lipid g^{-1} 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. 7).

DISCUSSION

In this study, we used a new method of HPLC-APPI/MS 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. 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

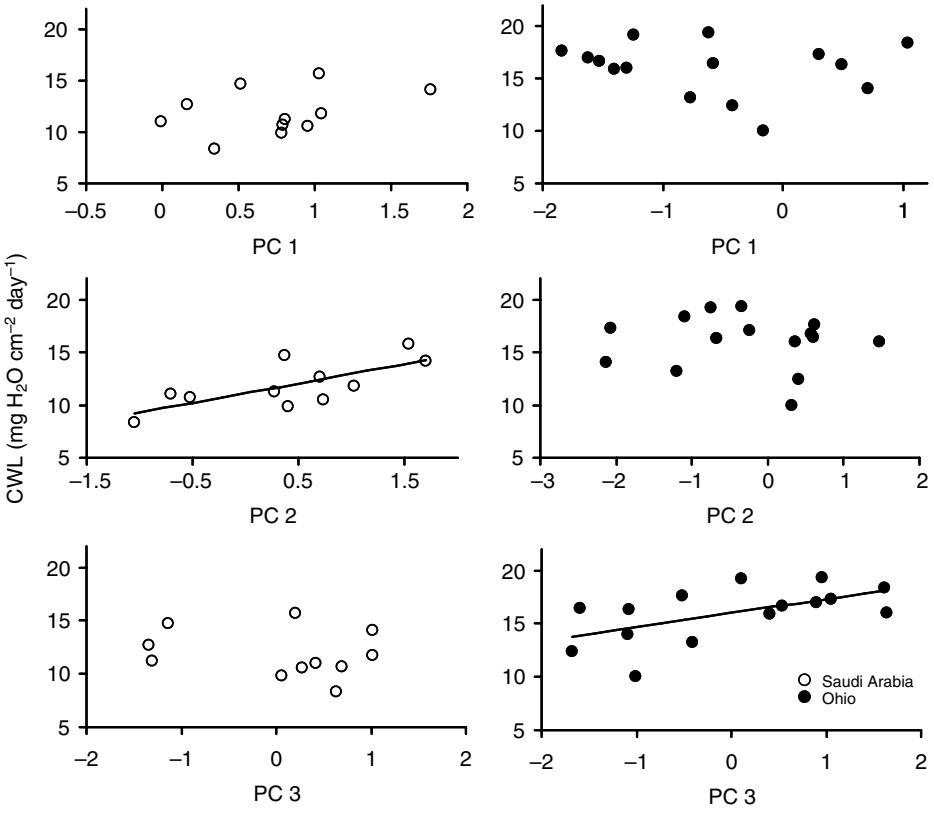

Fig. 6. Relationship between cutaneous water loss (CWL) and PC 1, PC 2 and PC 3 extracted from PCA based on the number of millimoles per gram of dry SC in each sphingolipid family from the SC of house sparrows from desert (open circles) and mesic environments (filled circles). Plots with regression lines indicate statistical significance (P<0.03).

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. The 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 stronger Van der Waals forces 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; Haugen et al., 2003b). However, the most polar sphingolipid classes in sparrows were more abundant in individuals from Ohio, but their overall abundance was low compared with 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.

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 the 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.

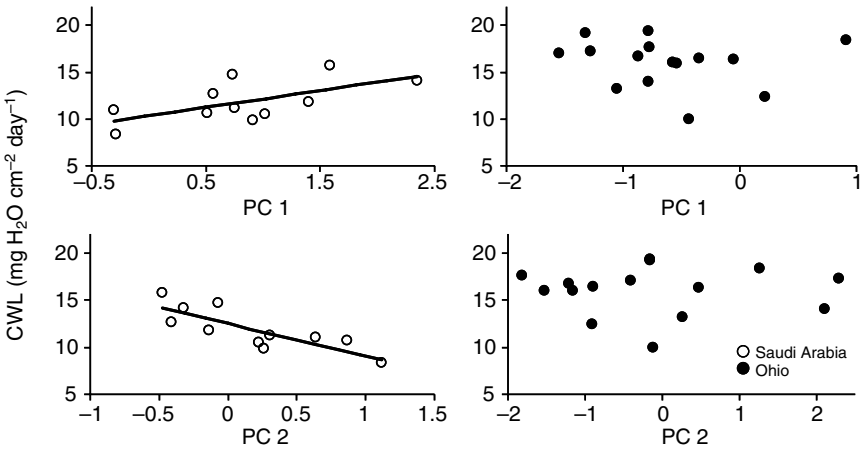

Fig. 7. Relationship between CWL and PC 1 and PC 2 extracted from PCA based on the amount in milligrams per gram of dry SC in each sphingolipid family from the SC of house sparrows from desert (open circles) and mesic environments (filled circles). Plots with regression lines indicate statistical significance (P<0.05).

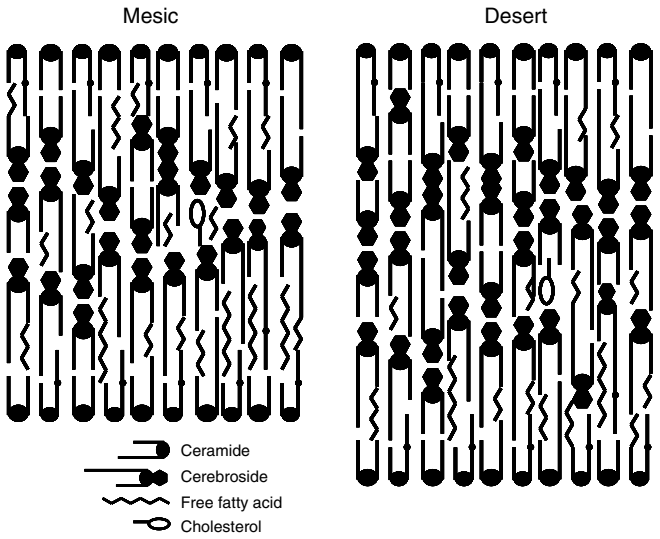

Fig. 8. Hypothesized model for the organization of lipids in the intercellular spaces of the SC in house sparrows from mesic and desert environments. In sparrows, lamellae would be formed by three layers of lipids; two outer layers consisting of ceramides, and an inner layer formed by cerebrosides and free fatty acids. Ceramides would form a highly ordered structure, whereas the inner layer would be more fluid. In this model, we assume that the chain lengths of free fatty acids would be the same between desert and mesic individuals.

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.

A salient feature of the SC of birds is the high concentration of cerebrosides, a lipid class that has been associated with high rates of CWL in mammals. In a previous paper, we outlined the thermoregulation–water conservation model for SC organization, which suggested that desert house sparrows possessed a SC designed for both thermoregulation and water conservation (Muñoz-Garcia and Williams, 2005). Here we incorporate knowledge about the molecular structure of lipids in the SC of sparrows obtained in this study. With this information, we propose a plausible model for the organization of the lipids in the intercellular spaces of the SC in house sparrows (Fig. 8). Like the 'sandwich model', our model suggests that the more polar regions of ceramides line up together whereas non-polar tails orient inwardly forming a bilayer in the crystalline phase. 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.

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., in press). 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 of 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 the two populations, then periodicities should be longer in desert birds.

From an evolutionary perspective, what is the significance of the preponderance of some lipid classes over others in organisms from different environments? 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 is still higher than that of the average mammal. On the other hand, the thermoregulatory needs of mammals are satisfied in different ways from 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; current work is addressing the relative importance of natural selection and phenotypic plasticity in the formation of the permeability barrier in birds.

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Table A1. Molecules of sphingolipids identified in the SC of sparrows by HPLC-APPI/MS

Compound	Saudi Arabia	Ohio	*m/z*
Cerebroside NS			
14:0	+	+ (93.3)	654.53
15:0	+	+	668.55
16:0	+	+	682.56
17:0	+	+ (93.3)	696.58
18:0	+	+	710.59
19:0	+	+	724.61
20:0	+	+	738.63
21:0	+	+	752.64
22:0	+	+	766.66
23:0	+	+	780.67
24:0	+	+	794.69
25:0	+ (91.7)	+	808.70
26:0	+	+	822.72
27:0	+	+ (93.3)	836.73
28:0	+	+	850.75
29:0	+	+	864.77
30:0	+	+	878.78
31:0	+	+ (93.3)	892.80
32:0	+	+	906.81
33:0	+ (41.7)	+ (93.3)	920.83
34:0	+	+	934.84
35:0	+ (33.3)	+ (86.7)	948.86
36:0	+	+	962.87
37:0	+ (8.3)	+	976.89
38:0	+	+	990.91
39:0	+ (25.0)	+	1004.92
40:0	+	+	1018.94
41:0	–	+ (93.3)	
42:0	+	+	1046.97
43:0	+ (66.7)	+ (66.7)	1060.98
44:0	+ (91.7)	+ (86.7)	1075.00
45:0	+ (25.0)	+ (60.0)	1089.02
46:0	–	+ (73.3)	
47:0	–	+ (13.3)	
Cerebroside AS-NH*			
36:0	–	+ (93.3)	960.85
37:0	–	+	974.87
38:0	+	+ (93.3)	988.89
39:0	+	+ (93.3)	1002.90
40:0	+	+ (93.3)	1016.92
41:0	+	+ (93.3)	1030.93
42:0	+	+ (93.3)	1044.95
43:0	+	+ (86.7)	1058.96
44:0	+	+ (93.3)	1072.98
45:0	+	+ (93.3)	1087.00
46:0	+	+ (93.3)	1101.01
47:0	+	+ (73.3)	1115.03
48:0	+	+ (93.3)	1129.04
49:0	+	+ (60.0)	1143.06
50:0	+	+ (93.3)	1157.07
51:0	+	+ (40.0)	1171.09
52:0	–	+ (93.3)	1185.11
53:0	–	+ (60.0)	1199.13
54:0	–	+ (66.7)	1213.15
55:0	–	+ (53.3)	1227.17
56:0	–	+ (53.3)	1241.19
57:0	–	+ (33.3)	1255.21
Cerebroside NP			
38:0	–	+ (60.0)	990.89
39:0	–	+ (33.3)	1004.91
40:0	–	+ (93.3)	1018.93
41:0	+	+	1032.95
42:0	+	+	1046.97
43:0	+	+	1060.98
Cerebroside NP			
44:0	+	+	1075.00
45:0	+	+	1089.02
46:0	+	+	1103.03
47:0	+	+	1117.05
48:0	+	+	1131.06
49:0	+	+	1145.08
50:0	+	+ (86.7)	1159.09
51:0	+	+ (93.3)	1173.11
52:0	–	+ (73.3)	1187.13
53:0	–	+ (53.3)	1201.15
54:0	–	+ (60.0)	1215.17
55:0	–	+ (40.0)	1229.19
56:0	–	+ (26.7)	1243.21
58:0	–	+ (6.7)	1271.25
Cerebroside EOH			
38:0	–	+ (6.7)	1016.90
39:0	–	+ (13.3)	1030.92
40:0	–	+ (80.0)	1044.94
41:0	+ (83.3)	+ (73.3)	1058.96
42:0	+ (91.7)	+ (13.3)	1072.98
43:0	+	+ (73.3)	1087.00
44:0	+ (91.7)	+ (46.7)	1101.01
45:0	+	+	1115.03
46:0	+	+ (86.7)	1129.04
47:0	+	+	1143.06
48:0	+	+ (66.7)	1157.07
49:0	+	+ (86.7)	1171.09
50:0	+	+	1185.10
51:0	+	+	1199.12
52:0	+	+ (80.0)	1213.14
53:0	+	+ (93.3)	1227.15
54:0	–	+ (93.3)	1241.17
55:0	–	+ (86.7)	1255.19
56:0	–	+ (93.3)	1269.21
57:0	–	+ (66.7)	1283.23
58:0	–	+ (60.0)	1297.25
59:0	–	+ (33.3)	1311.27
60:0	–	+ (26.7)	1325.29
61:0	–	+ (20.0)	1339.31
62:0	–	+ (26.7)	1353.33
63:0	–	+ (26.7)	1367.35
64:0	–	+ (26.7)	1381.37
Cerebroside AH			
40:0	+	+ (93.3)	1014.85
41:0	+	+	1028.87
42:0	+	+	1042.88
43:0	+	+	1056.90
44:0	+	+	1070.92
45:0	+	+	1084.93
46:0	+	+	1098.95
47:0	+	+	1112.96
48:0	+	+ (93.3)	1126.98
49:0	+	+ (93.3)	1140.99
50:0	+	+	1155.01
51:0	+	+	1169.03
52:0	+	+ (93.3)	1183.04
53:0	+	+ (86.7)	1197.06
54:0	+	+ (86.7)	1211.07
55:0	+	+ (60.0)	1225.09
56:0	+	+ (86.7)	1239.10
Cerebroside NH unsaturated			
51:1	+	+ (73.3)	1169.03
52:1	+	+ (86.7)	1183.05

Table A1 continues on next page.

Table A1. Continued

Compound	Saudi Arabia	Ohio	*m/z*
Cerebroside NH unsaturated			
53:1	+	+	1197.06
54:1	+	+ (80.0)	1211.08
55:1	+	+ (93.3)	1225.09
56:1	+	+ (93.3)	1239.11
57:1	+	+ (93.3)	1253.12
58:1	+	+ (80.0)	1267.14
59:1	+	+ (86.7)	1281.15
60:1	+	+ (80.0)	1295.17
61:1	+	+ (80.0)	1309.19
Diosylceramide NS			
16:0	–	+ (40.0)	844.61
17:0	–	+ (20.0)	858.63
18:0	–	+ (20.0)	872.65
19:0	–	+ (20.0)	886.67
20:0	–	+ (73.3)	900.68
21:0	–	+ (33.3)	914.70
22:0	–	+ (20.0)	928.71
24:0	–	+ (20.0)	956.74
38:1	–	+ (40.0)	1150.96
40:1	–	+ (40.0)	1178.99
42:1	–	+ (60.0)	1207.02
Ceramide NS			
14:0	+ (91.7)	+ (40.0)	492.40
15:0	+ (91.7)	+ (46.7)	506.42
16:0	+	+	520.44
17:0	+	+	534.46
18:0	+	+	548.48
19:0	+	+	562.50
20:0	+	+	576.52
21:0	+	+	590.54
22:0	+	+	604.56
23:0	+	+	618.58
24:0	+	+	632.60
25:0	+	+	646.62
26:0	+	+	660.64
27:0	+	+	674.66
28:0	+	+	688.68
29:0	+	+	702.70
30:0	+	+	716.72
31:0	+	+	730.74
32:0	+	+	744.76
33:0	+	+	758.78
34:0	+	+	772.80
35:0	+	+	786.82
36:0	+	+	800.84
37:0	+	+ (66.7)	814.86
38:0	+	+	828.88
39:0	–	+	842.90
40:0	+	+	856.92
42:0	+	+	884.96
43:0	+	+ (86.7)	898.98
44:0	+	+	913.00
45:0	+	+ (93.3)	926.96
46:0	–	+	940.98
47:0	–	+	955.00
48:0	–	+ (93.3)	969.02
49:0	–	+ (93.3)	983.04
Ceramide NS			
50:0	–	+ (40.0)	997.06
51:0	–	+ (46.7)	1011.08
52:0	–	+ (26.7)	1025.10
53:0	–	+ (20.0)	1039.12
Ceramide EOH			
36:0	+	+	826.84
37:0	+	+	840.85
38:0	+	+	854.87
39:0	+	+	868.88
40:0	+	+	882.90
41:0	+	+	896.91
42:0	+	+	910.93
43:0	+	+	924.95
44:0	+	+	938.96
45:0	+	+	952.98
46:0	+	+	966.99
47:0	+	+ (93.3)	981.01
48:0	+	+	995.02
49:0	+	+	1009.04
50:0	+	+	1023.06
51:0	+	+ (93.3)	1037.07
52:0	+	+ (93.3)	1051.09
73:0	–	+ (26.7)	1345.51
74:0	–	+ (20.0)	1359.53
Ceramide AH			
42:0	+	+	880.89
43:0	+	+ (40.0)	894.91
44:0	+	+	908.92
45:0	+	+ (13.3)	922.94
46:0	+	+	936.95
47:0	+	+	950.97
48:0	+	+	964.98
49:0	+	+	979.00
50:0	+	+	993.01
51:0	+	+	1007.03
52:0	+	+	1021.05
53:0	+	+	1035.06
54:0	+	+	1049.08
55:0	+	+	1063.09
56:0	+	+ (93.3)	1077.11
57:0	+	+ (93.3)	1091.12
58:0	+	+ (93.3)	1105.14
59:0	+	+ (66.7)	1119.16
60:0	+	+ (73.3)	1133.17
61:0	+	+ (53.3)	1147.19
62:0	+	+ (73.3)	1161.20
Ceramide EOS			
43:0	+	+	926.89
44:0	+	+	940.91
45:0	+	+	954.92
46:0	+	+	968.94
47:0	+	+	982.95
48:0	+	+	996.97
49:0	+	+	1010.98
50:0	+	+	1025.00
51:0	+	+	1039.01
52:0	+	+	1053.03
53:0	+	+	1067.05

*These molecules are not distinguishable using APPI; therefore they could not be quantified separately.

Reported mass to charge ratio (*m/z*) corresponds to $(M + H - H_2O)^+$ species, where M corresponds to molecular ion and H is an atom of hygrogen; +, 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. The percentage of birds that showed the molecule 'x' in those cases in which the percentage was different from 100% is given in parentheses.

HPLC-APPI/MS, high-performance liquid chromatography coupled with atmospheric pressure photoionization-mass spectrometry.

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