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RESEARCH ARTICLE

Cutaneous water loss and covalently bound lipids of the stratum corneum in nestling house sparrows (*Passer domesticus* L.) from desert and mesic habitats

Michelle E. Clement[1,*], Agustí Muñoz-Garcia[1,2,†] and Joseph B. Williams[1]

[1]Department of Evolution, Ecology and Organismal Biology, Aronoff Laboratory, 318 W. 12th Avenue, Columbus, OH 43210, USA and [2]Mitrani Department of Desert Ecology, The Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Sede-Boqer Campus, 84990, Midreshet Ben-Gurion, Israel

*Present address: American Chemical Society, 2450 Olentangy River Road, Columbus, OH 43210, USA

†Author for correspondence (agustimg@gmail.com)

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SUMMARY

Lipids of the stratum corneum (SC), the outer layer of the epidermis of birds and mammals, provide a barrier to water vapor diffusion through the skin. The SC of birds consists of flat dead cells, called corneocytes, and two lipid compartments: an intercellular matrix and a monolayer of covalently bound lipids (CBLs) attached to the outer surface of the corneocytes. We previously found two classes of sphingolipids, ceramides and cerebrosides, covalently bound to corneocytes in the SC of house sparrows (*Passer domesticus* L.); these lipids were associated with cutaneous water loss (CWL). In this study, we collected adult and nestling house sparrows from Ohio and nestlings from Saudi Arabia, acclimated them to either high or low humidity, and measured their rates of CWL. We also measured CWL for natural populations of nestlings from Ohio and Saudi Arabia, beginning when chicks were 2 days old until they fledged. We then evaluated the composition of the CBLs of the SC of sparrows using thin layer chromatography. We found that adult house sparrows had a greater diversity of CBLs in their SC than previously described. During ontogeny, nestling sparrows increased the amount of CBLs and developed their CBLs differently, depending on their habitat. Acclimating nestlings to different humidity regimes did not alter the ontogeny of the CBLs, suggesting that these lipids represent a fundamental component of SC organization that does not respond to short-term environmental change.

Key words: house sparrows, covalently bound lipids, stratum corneum, cutaneous water loss, ontogeny, nestlings, desert.

INTRODUCTION

Although the skin is the largest organ of the vertebrate body, its significance is underappreciated. It is involved in a number of complex physiological processes, including protection from invading pathogens, vitamin D production, lipid synthesis, protection from UV radiation, and the formation of the lipid barrier that prevents excessive water loss to the environment (Elias and Feingold, 2006). This last function is performed by the outer layer of the skin, called the stratum corneum (SC), which is composed of layers of corneocytes embedded in a lipid matrix. In birds, total evaporative water loss (TEWL), which is the sum of cutaneous water loss (CWL) and respiratory water loss, is the most important avenue of total water loss, exceeding fecal and urinary water loss 5-fold in small avian species (Bartholomew, 1972). The major contributor to TEWL is CWL, which accounts for more than 65% of the total (Tieleman and Williams, 2002; Ro and Williams, 2010). In desert environments, where high ambient temperatures and low relative humidity create conditions that result in high evaporative water demand (Tieleman and Williams, 2002), birds likely experience intense selection to minimize CWL (Williams and Tieleman, 2005).

The avian skin is composed of an inner layer, the vascular dermis, and an outer avascular epidermis. The epidermis consists of a basal layer of mitotic cells, several transitional layers of cells that form the stratum transitivum, and the outermost layer, the SC (Lucas and Stettenheim, 1972). In the lower stratum transitivum, cells contain multigranular bodies (Landmann, 1988), which are organelles that synthesize lipids for the SC (Menon and Menon, 2000). Multigranular bodies are thought to coalesce into intracellular lipid droplets at the stratum transitivum–corneum interface, where lipids are extruded into the extracellular domains and some are enzymatically converted into other lipid moieties, although the exact mechanism remains unclear (Menon and Menon, 2000). The lipids that are exocytosed form two distinct compartments; some covalently bind to the corneocytes of the SC (covalently bound lipids, CBLs) and others form the intercellular lipid (ICL) compartment, shown to be important in the formation of the barrier to water vapor diffusion through the skin (Bouwstra et al., 2003; Menon and Menon, 2000). The ICL compartment in birds consists of cholesterol esters, methyl esters, triacylglycerides, cholesterol, free fatty acids (FFA), ceramides, which are formed from a molecule of sphingosine ester-linked to a fatty acid, and cerebrosides, ceramides with a hexose sugar attached to the sphingosine head group (Muñoz-Garcia and Williams, 2005; Ro and Williams, 2010). Changes in the composition of the ICL compartment have been associated with a reduction of water loss through the skin in desert birds (Haugen et al., 2003; Muñoz-Garcia and Williams, 2005; Muñoz-Garcia and Williams, 2008; Muñoz-Garcia et al., 2008a).

In mammals, ω-hydroxyceramides bind to the protein envelope of corneocytes, forming a monolayer of lipids ester-linked to glutamate residues of involucrin (Wertz and Downing, 1987; Menon and Menon, 2000; Wertz, 2000; Gu et al., 2008). These CBLs are thought to be fundamental in the organization of the ICL layers through the provision of an important scaffold that orchestrates ICL structure (Madison, 2003).

In the first study of CBLs in avian skin, Gu and colleagues (Gu et al., 2008) identified ω-hydroxyceramides and ω-hydroxycerebrosides that were bound to the corneocytes of house sparrows (*Passer domesticus* L.). If covalently bound ceramides and cerebrosides are organized in the lipid envelope as they are in mammals, then the fatty acid 'tails' of the sphingolipids would be attached to the protein residues of the corneocytes, whereas the more polar sphingosine 'heads' would project into the intercellular space and interact with ICLs (Gu et al., 2008). In Gu and colleagues' study, desert house sparrows had a lower ratio of covalently bound ceramides to cerebrosides than did adult house sparrows from Ohio, and this was associated with a lower rate of CWL (Gu et al., 2008).

Little is known about the role of CBLs in avian skin or the mechanism(s) that potentially links CBLs with CWL. The sugar moieties of the covalently bound cerebroside head groups could form molecular interactions with water molecules in the SC, which would aid internal hydration and decrease water vapor pressure gradients between the skin and the environment (Gu et al., 2008). The polar heads of covalently bound ceramides of adjacent corneocytes may interact with one another to bind corneocytes together at their end plates, which may also impede the movement of water molecules (Gu et al., 2008; Madison, 2003).

Whether or how CBLs change during the development of nestlings remains unstudied, as does how the environment influences these lipids during ontogeny. There is evidence that the ICL composition shows phenotypic flexibility in adult birds and that modification of the ICLs influences CWL (Haugen et al., 2003; Muñoz-Garcia et al., 2008b). Alteration of the ICLs during development was associated with changes in the CWL of house sparrows from different environments (Muñoz-Garcia and Williams, 2008; Muñoz-Garcia and Williams, 2011).

In this study, we characterized for the first time the CBLs in the skin of house sparrow nestlings from arid and mesic environments during development, and we examined the degree of phenotypic plasticity of the CBLs of nestling sparrows acclimated to high or low humidity. We chose to manipulate humidity as a factor because it is thought that the movement of water through the SC alters CWL by modifying SC permeability through changes in pH, alteration of calcium gradients, or changes in the activity of enzymes that convert some lipid classes into others (Elias, 2004). Moreover, we found evidence that humidity affects the structure of the ICL compartment in nestling and adult house sparrows (Muñoz-Garcia et al., 2008b; Muñoz-Garcia and Williams, 2008). To compare results from fledglings that were acclimated to different humidity conditions with those of adults, we acclimated adult sparrows from Ohio for 3 weeks to the same humidity regimes as the nestlings. We found that nestling sparrows had more non-polar lipids, such as FFA, and fewer cerebrosides in their CBLs compared with adults, and these changes were associated with higher rates of CWL. Despite the finding that habitat and acclimation altered CWL during development, these factors did not affect CBL composition. Hence, the organization of CBLs was conserved regardless of the environment, suggesting an underlying importance of these lipids in the basic structure of the skin.

MATERIALS AND METHODS

Nestling house sparrows

We placed nest boxes around the National Wildlife Research Center, near Taif, Saudi Arabia (22°15′N, 41°50′E), in March 2006 and around a farm in Columbus, OH (40°00′N, 83°10′W), between mid-April and late August 2006. After pairs of house sparrows had built nests and laid eggs, we checked the boxes daily until the eggs hatched; we designated the day of hatching as day 0. We randomly selected nestlings of known age from their nest boxes, beginning with day 0 to just prior to fledging at day 14–16, for measurement of CWL and for determination of CBLs. CWL of nestling sparrows was measured using an open-flow respirometry mask system. Briefly, we fitted birds with a plastic mask and pulled dry, CO_2-free air into two dew-point hygrometers, to measure the dew point of the air coming from the mask and from the chamber (see Muñoz-Garcia and Williams, 2011).

In addition, in Saudi Arabia and Ohio, we also collected two siblings from each nest box when they were 3–4 days old and randomly assigned one of the siblings from each pair to a low-humidity environmental chamber (absolute humidity 6.5 g H_2O m^{-3}, relative humidity RH 15–20%, at 30°C) and the other to a high-humidity environmental chamber (absolute humidity 31 g m^{-3}, RH 90–95%, at 30°C). We reared these nestlings until they were 14–16 days old, which is the normal age of fledging (see Muñoz-Garcia and Williams, 2008). We measured CWL of fledglings using an open-flow respirometry mask system as above (see Muñoz-Garcia and Williams, 2008) then killed them by cervical dislocation for the determination of CBL. We had four groups of nestlings in our experiment: dry-acclimated and humid-acclimated individuals from Saudi Arabia (N=7 and N=5, respectively) and from Ohio (N=11 and N=12, respectively). Experiments were approved by the Institutional Laboratory Animal Care and Use Committee of Ohio State University (protocol 2006-A0085) and the National Commission for Wildlife Conservation and Development, Riyadh, Saudi Arabia.

Adult house sparrows

We mist-netted adult sparrows from Ohio and measured their CWL and CBL to compare them with those of nestlings from Ohio. To compare the CWL and CBL of adult sparrows from Saudi Arabia with those of nestlings from Saudi Arabia, we used data on adults published previously (Gu et al., 2008).

We also acclimated adult sparrows from Ohio for 3 weeks to a high- or low-humidity regime (see Muñoz-Garcia et al., 2008b). After the acclimation period, we measured their CWL using the same system (see Muñoz-Garcia et al., 2008b) and the CBL composition of their SC.

Separation and identification of CBLs

After measuring CWL, we killed the birds, plucked their feathers, if present, and removed their skin. The SC was isolated and the ICLs were extracted (see Muñoz-Garcia et al., 2008b).

To confirm that all ICLs had been extracted from the SC, we selected 8 samples at random and soaked the SC for each bird for 2 h in chloroform:methanol 1:2 (v/v) containing the antioxidant butylated hydroxytoluene (BHT). We examined the extracts for the presence of lipids using thin layer chromatography (TLC), but did not detect ICLs in any sample.

We freeze-dried the SC samples for 12 h and stored them at –20°C in an atmosphere of nitrogen. Later, we thawed the samples, weighed the dry SC, and extracted the CBLs (see Wertz and Downing, 1987). We washed the CBL extracts again *via* Folch extraction (Folch et al., 1957) to remove any inorganic solutes and removed any remaining particulate matter by passing the solution through a 0.45 μm PTFE filter (Millex, Millipore Corp., Bedford, MA, USA). We dried the CBL extracts under a stream of nitrogen and stored them at –20°C. Prior to TLC, we re-constituted the CBL extracts in 40–130 μl of chloroform:methanol 2:1 (v/v) (~10 μl of solvent per 1.5 mg of dry SC) containing 50 mg l^{-1} of BHT.

We separated the CBLs using analytical TLC on 20×20 cm glass plates (0.25 mm thick; Adsorbosil-Plus 1, Altech, Deerfield, IL, USA). We developed plates with chloroform:methanol 2:1 (v/v) to the top to remove contaminants, activated them in an oven for 30 min at 110°C, and divided them into 10 mm lanes. We prepared a series of five lipid standards of known concentration *via* serial dilution. Our standards contained ceramides, cerebrosides, cholesterol, FFA and cholesteryl oleate. We dissolved the standards in chloroform:methanol 2:1 (v/v) with BHT. We loaded 5 µl of each standard and sample onto the preadsorbent area of the plates in duplicate with a Teflon-tipped Hamilton syringe. We used two solvent systems for development: for more polar lipids (such as ceramides, cerebrosides and cholesterol esters), we ran the plates in chloroform:methanol:water (40:10:1 v/v/v) to 5 cm from the bottom, chloroform:methanol:acetic acid (190:9:1 v/v/v) to the top twice, and hexane:ethyl ether:acetic acid (70:30:1 v/v/v) to 15 cm from the bottom; for more non-polar lipids (such as FFA and cholesterol), we developed plates with hexane:ethyl ether:acetic acid (70:30:1 v/v/v) to the top once. After development, we sprayed the plates with 3% cupric acetate in 8% phosphoric acid and visualized the lipids by charring them on an aluminium hotplate for 30 min at 160°C.

To quantify the concentration of the lipid classes, we scanned the plates with a Hewlett-Packard scanner and measured the absorbance of lipid bands using IMAL TN-Image 3.5.10c (T. J. Nelson 2008: http://www.icewalkers.com/Linux/Software/5250/Tnimage.html). We calculated standard curves for each lipid class and compared the absorbance of lipids in our samples with those of the standards of known concentration to determine the concentration of lipids in the sample. To validate our method of quantifying lipids, we followed our protocol using known concentrations of cholesterol as our 'unknown' and compared them with our cholesterol standards. The mean error, calculated as [(observed–actual)/actual]×100, was 3.97±2.82% (N=30).

Statistics

We combined the quantities of cholesteryl esters and cholesterol prior to statistical analyses because we thought that the free cholesterol we identified was likely from cholesterol esters that had their fatty acid moiety hydrolyzed during the extraction process. CBL quantities (in mg g^{-1} dry SC) were log transformed if they did not meet the assumption of normality. We performed statistical tests with SPSS 16.0 (SPSS, Chicago, IL, USA) or Minitab 15.0 (Minitab Inc., State College, PA, USA), with the null hypothesis rejected when $P\leq0.05$.

We used two-way ANOVA to compare the effects of age and habitat on CWL and CBLs in natural populations of nestlings from Ohio and Saudi Arabia, and two-way ANOVA with habitat and treatment as fixed factors to compare body temperature, CWL and CBLs in fledglings from Saudi Arabia and Ohio after acclimation to high or low humidity. To compare body temperature, CWL and CBLs in adults and fledglings from Ohio after acclimation to high or low humidity, we used a two-way ANOVA with age and treatment as fixed factors.

We interpreted a significant interaction term as differences in the degree of phenotypic plasticity between groups. When interaction terms were not significant, we removed them from the analysis. After removing the interaction term, if treatment was significant, we interpreted this to mean that experimental groups had the same degree of phenotypic plasticity. If neither the interaction term nor treatment was significant, we concluded that our experimental groups were not plastic.

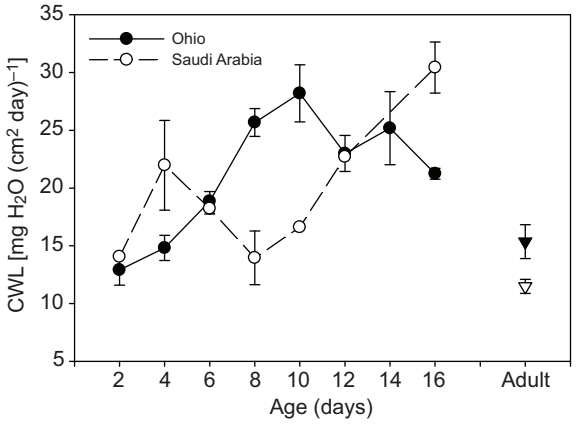

Fig. 1. Mean (±s.e.m.) cutaneous water loss (CWL) of natural nestlings from Ohio and Saudi Arabia from age 2 days to adult. The interaction term for age and habitat was significant, indicating different developmental trajectories for CWL in nestlings from desert and mesic environments. Two day old nestlings from the two habitats had similar rates of CWL, but thereafter nestlings increased their CWL, with a maximum at day 4 in desert nestlings and at day 8 in nestlings from Ohio. After day 8, CWL of nestlings from Saudi Arabia increased, whereas CWL remained constant in nestlings from Ohio. CWL of nestlings was higher than that of adults.

The influence of individual lipid classes on CWL may be less important than the interaction of the composite of all CBLs combined. To gain insight into how CBLs of the SC were collectively associated with CWL, we used principal component analysis (PCA) (Shaw, 2003). This analysis yields uncorrelated composite variables, the principal components. We extracted the components with eigenvalues greater than one. We determined associations between CWL and principal component scores using backwards multiple linear regressions, with principal component scores as our independent variable and CWL as our dependent variable. We used ANOVA to detect differences in principal component scores between age groups.

RESULTS

CWL of house sparrows

Nestlings from Ohio and Saudi Arabia showed different developmental trajectories for CWL, as judged by a significant interaction term for habitat and age (F=6.2, P<0.001; Fig. 1). In Ohio, CWL of nestlings increased until it reached a peak at day 10, and then CWL decreased through fledging and into adulthood (see Muñoz-Garcia and Williams, 2011). In Saudi Arabia, CWL of nestlings peaked at day 4, decreased until day 8, and then increased again through fledging before finally decreasing in adults.

After acclimating to different humidity conditions, fledglings from Ohio showed plasticity for CWL (F=7.6, P=0.009; Table 1), whereas fledglings from Saudi Arabia did not. The failure to detect plasticity in CWL in desert fledglings could have been the result of the high standard deviation of this group. Fledglings from Ohio that were acclimated to high humidity had a significantly higher CWL than those acclimated to low humidity. Fledglings from Ohio had a lower CWL than fledglings from Saudi Arabia under both humidity conditions. Adults and fledglings from Ohio acclimated to high humidity regimes had a significantly higher CWL than birds acclimated to low humidity regimes ($F_{treatment}$=29.5, P<0.001; Table 1). Fledglings from Ohio had a significantly higher CWL than adults from Ohio (F_{age}=94.2, P<0.001; Table 1).

Table 1. CBL quantities and CWL in adult and fledgling house sparrows from Ohio and Saudi Arabia acclimated to high or low humidity

Habitat	Age	Acclimation	Cholesteryl esters	Free fatty acids	Ceramides (total)	Cerebrosides	CWL	Mass (g)	Body temperature (°C)
Ohio	Adult	Dry	4.12±0.72	15.02±2.37	17.04±1.52	16.30±1.60	8.57±0.56	22.36±0.38	40.5±0.18
		Humid	3.44±0.36	10.24±1.20	16.02±1.50	13.53±1.24	13.73±1.62	22.04±0.39	41.28±0.23
Ohio	Fledgling	Dry	2.62±0.31	13.75±1.45	10.60±1.23	5.31±0.47	17.38±0.46	22.36±0.38	41.48±0.23
		Humid	2.81±0.23	13.39±1.41	10.09±1.14	5.50±0.44	21.76±0.88	22.04±0.39	41.28±0.22
Saudi Arabia	Fledgling	Dry	3.21±0.55	14.72±1.55	6.31±0.92	4.84±0.75	30.43±2.21	14.9±0.71	40.79±0.31
		Humid	3.14±0.58	18.13±4.79	6.72±1.97	5.46±1.60	27.43±2.35	15.38±0.76	41.34±0.19

CBL, covalently bound lipid (cholesteryl esters, free fatty acids, ceramides and cerebrosides) as mg g^{-1} dry stratum corneum (SC); CWL, cutaneous water loss as mg H_2O (cm^2 day)$^{-1}$.
Data are means ± s.e.m.

Body temperature of fledglings from Ohio acclimated to a low humidity environment (41.5°C) was significantly higher than that of adults from Ohio acclimated to low humidity (40.5°C) (ANOVA, F=3.1, P<0.02; Tukey *post hoc* test, P<0.05) (Table 1).

CBLs

We detected cholesteryl esters, FFA, five classes of ceramides and one class of cerebroside covalently bound to the corneocytes of adult and nestling house sparrows (Tables 1–4). Ceramides were named from ceramide A, the least polar, to ceramide E, the most polar on our plates. These assignments may not correspond to those of others for classes of ceramides. In addition to FFA, other bands of non-polar lipids were observed. We did not identify these lipids, although their R_f (retention factor) value was consistent with triglycerides. We did not quantify the unidentified lipid classes.

Natural populations of nestlings from Ohio and Saudi Arabia showed different developmental trajectories for covalently bound cerebrosides, as judged by a significant interaction term for habitat and age (F=4.8, P=0.002; Fig. 2). Cerebrosides increased with age for nestling sparrows in both habitats. Saudi Arabia nestlings had more cerebrosides than Ohio nestlings from hatching to day 10, after which their trajectories converged until fledging. For other lipid classes, there were similar trajectories, with lipid density increasing with ontogeny across habitats. Age was a significant factor in cholesterol (F=6.37, P<0.001; Fig. 2) and ceramide quantities (F=15.8, P<0.001; Fig. 2). The quantity of cholesterol was higher in Saudi Arabia nestlings than in Ohio nestlings (F=6.9, P=0.012; Fig. 2), whereas the quantity of ceramides was higher in Ohio nestlings than in Saudi Arabia nestlings (F=10.4, P=0.002; Fig. 2).

Treatment was not a significant factor for any lipid class after acclimation to different humidity regimes. However, Ohio fledglings had a higher quantity of ceramides than Saudi Arabia fledglings (F=10.5, P=0.003; Table 1).

There were differences in some CBL classes with ontogeny in mesic sparrows. Ohio adults had a significantly higher quantity of cholesterol (F=5.5, P=0.023; Table 1), ceramides (F=19.3, P<0.001; Table 1) and cerebrosides (F=110.4, P<0.001; Table 1) than fledglings. Ohio adults had more cerebrosides than Saudi adults.

CWL and covalently bound lipids

After using PCA on the quantity (in mg lipid g^{-1} of dry SC) of each class of CBL, we found that three principal components accounted for 76.6% of the variance (Table 5). We regressed the principal component scores against CWL and found PC1 and PC3 to be significant {CWL [mg H_2O (cm^2 day)$^{-1}$=18.376–1.356 (PC1 score)–1.852 (PC3 score); R^2=0.23, P<0.001}.

Table 2. CBL quantities and CWL in natural populations of adult and nestling house sparrows from Ohio and Saudi Arabia

Habitat	Age	N	Cholesteryl esters	Free fatty acids	Ceramides (total)	Cerebrosides	CWL	Mass (g)	Body temperature (°C)
Ohio	2 days	5	3.04±0.48	15.87±3.46	6.60±1.21	2.39±0.42	12.90±1.32	10.19±0.88	36.78±0.28
	4 days	6	1.74±0.19	6.60±1.12	4.89±0.29	1.43±0.11	14.81±1.09	15.27±1.30	37.75±0.45
	6 days	8	2.19±0.27	9.16±1.60	5.91±0.81	1.98±0.21	18.86±0.84	19.20±1.13	38.63±0.19
	8 days	3	2.81±0.28	11.22±2.60	6.94±0.63	1.94±0.07	25.67±1.20	23.77±2.68	40.47±0.09
	10 days	2	2.63±0.73	10.71±0.51	7.40±2.40	3.26±0.16	28.20±2.47	23.41±2.42	40.20±0.40
	12 days	6	1.50±0.15	13.58±2.93	7.75±0.96	3.12±0.25	22.99±1.55	24.93±1.15	40.66±0.30
	14 days	2	2.05±0.39	15.19±0.16	8.22±0.89	3.84±0.64	25.19±3.15	23.61±0.35	40.35±0.25
	16 days	2	2.08±0.49	15.44±3.66	10.62±2.94	4.48±1.06	21.24±0.47	24.87±0.63	40.64±0.45
	Adult	9	4.78±0.71	12.87±2.25	24.96±2.06	19.07±2.02	15.35±1.47	23.2±0.72	–
Saudi Arabia	2 days	1	3.83	11.46	4.17	3.61	14.05	5.6	36.3
	4 days	2	3.79±0.32	9.67±2.93	4.86±1.11	5.14±0.45	21.97±3.88	6.4±0.10	38.9±0.50
	6 days	2	2.17±0.57	12.55±2.61	3.57±1.03	2.75±0.11	18.23±0.49	8.3±0.10	37.65±0.35
	8 days	2	4.18±1.26	10.89±3.94	4.74±2.11	2.69±0.60	13.95±2.33	12.85±0.85	37.9±0.10
	10 days	2	3.84±0.26	9.69±1.46	5.37±0.98	3.09±0.08	16.61±0.03	15.55±0.95	37.6±0.80
	12 days	1	1.73	17.20	2.72	2.42	22.70	23.10	40.3
	16 days[a]	7	3.21±0.55	14.72±1.55	6.31±0.92	4.84±0.75	30.43±2.21	14.9±0.71	–
	Adult[b]	10	–	–	2.27±0.21	12.88±0.75	11.48±0.61	20.1±0.48	–

CBL, covalently bound lipid (cholesteryl esters, free fatty acids, ceramides and cerebrosides) as mg g^{-1} dry stratum corneum (SC); CWL, cutaneous water loss as mg H_2O (cm^2 day)$^{-1}$.
Data are means ± s.e.m.
[a]Data from this study, fledglings acclimated to dry treatment.
[b]Data taken from Gu et al. (2008).

Table 3. CBL quantities of ceramide classes in natural populations of adult and nestling house sparrows from Ohio and Saudi Arabia

Habitat	Age	*N*	Ceramide E	Ceramide D	Ceramide C	Ceramide B	Ceramide A
Ohio	2 days	5	1.83±0.31	1.85±0.23	1.43±0.44	1.49±0.43	0.00±0.00
	4 days	6	1.11±0.13	1.11±0.07	1.16±0.12	0.73±0.15	0.79±0.26
	6 days	8	1.41±0.22	1.44±0.13	1.24±0.14	1.13±0.21	0.69±0.29
	8 days	3	1.65±0.22	1.70±0.35	1.35±0.24	1.34±0.23	0.91±0.46
	10 days	2	1.84±0.39	1.57±0.17	1.64±0.55	1.47±0.42	0.88±0.88
	12 days	6	2.13±0.39	2.29±0.55	1.33±0.19	1.31±0.15	0.70±0.32
	14 days	2	3.25±0.60	1.55±0.10	1.71±0.21	1.70±0.02	0.00±0.00
	16 days	2	4.36±1.61	1.82±0.08	1.87±0.21	1.65±0.12	0.93±0.93
	Adult	9	11.80±1.07	4.76±0.87	4.41±0.70	3.98±1.38	0.00±0.00
Saudi Arabia	2 days	1	4.17	0.00	0.00	0.00	0.00
	4 days	2	1.91±0.15	2.02±0.04	0.93±0.93	0.00±0.00	0.00±0.00
	6 days	2	2.08±0.46	0.74±0.74	0.75±0.75	0.00±0.00	0.00±0.00
	8 days	2	1.60±0.25	1.47±0.19	0.83±0.83	0.84±0.84	0.00±0.00
	10 days	2	2.73±0.40	1.61±1.61	1.03±1.03	0.00±0.00	0.00±0.00
	12 days	1	2.72	0.00	0.00	0.00	0.00
	16 days[a]	7	2.92±0.40	1.70±0.61	1.25±0.34	0.44±0.28	0.00±0.00

CBL, covalently bound lipid (cholesteryl esters, free fatty acids, ceramides and cerebrosides) as mg g^{-1} dry stratum corneum (SC); CWL, cutaneous water loss as mg H_2O (cm^2 day)$^{-1}$.
Data are means ± s.e.m.
[a]Data from this study, fledglings acclimated to dry treatment.

PC1 separated ceramide A and, to a lesser extent, FFA from the rest of the lipid classes. PC3 further separated the lipids according to polarity: the score for PC3 increased with the polarity of the lipids (Fig. 3A).

PC1 distinguished adults, fledglings and nestlings (F=79.73, P<0.001; Fig. 3B). Tukey *post hoc* analysis indicated that the three groups were statistically distinct, with adults having the highest scores and nestlings the lowest. CBL from fledgling sparrows clustered near the vector for ceramide A, whereas those from adults aggregated with the polar lipids (ceramides and cerebrosides).

DISCUSSION

This is the first study to examine the development of CBLs in the avian SC and to determine how acclimation to humidity affects the expression of these lipids. We found that house sparrows had a more diverse range of CBLs in their SC than has been found in mammals. Previous studies have indicated the existence of sphingolipids and FFA in the CBL compartment of birds and mammals (Wertz and Downing, 1987; Gu et al., 2008); however, our study is the first to show the existence of covalently bound cholesteryl esters and significant amounts of covalently bound FFA in avian SC. Cholesteryl esters are likely attached to the protein envelope of

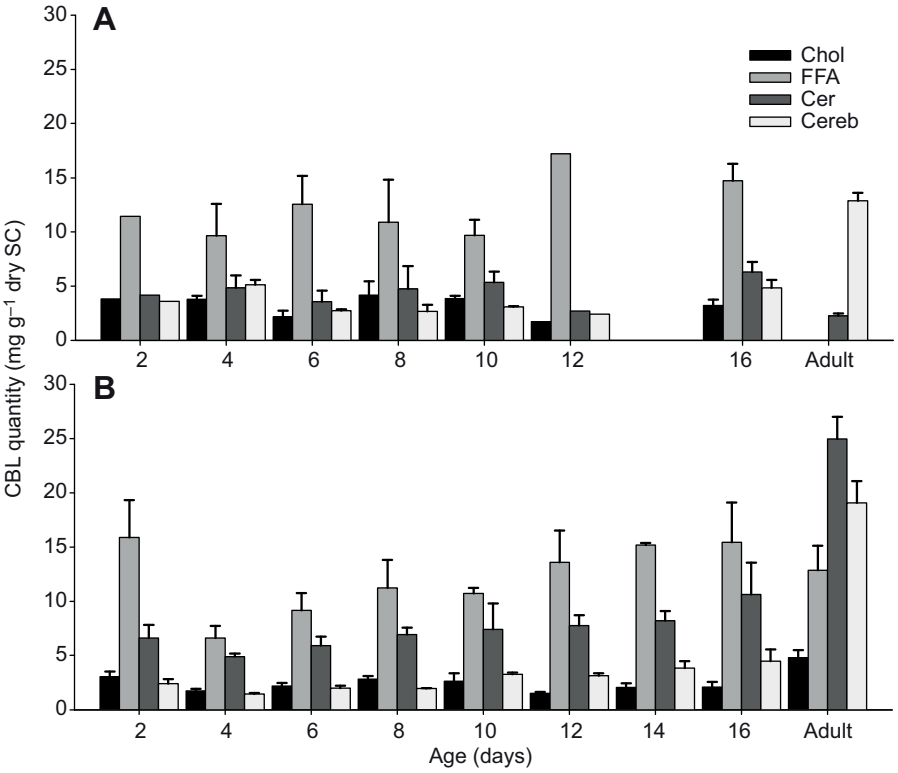

Fig. 2. Mean (±s.e.m.) covalently bound lipid (CBL) quantities of natural nestlings from (A) Saudi Arabia and (B) Ohio from age 2 days to adult. Data on covalently bound cholesteryl esters (Chol) and free fatty acids (FFA) in adults from Saudi Arabia were not available. Natural populations of nestlings from Ohio and Saudi Arabia showed different developmental trajectories for covalently bound cerebrosides: Saudi Arabia nestlings had more cerebrosides than Ohio nestlings from hatching to day 10, after which their trajectories converged until fledging. The other lipid classes showed similar trajectories, with lipid density increasing with ontogeny across habitats. Cer, ceramides; Cereb, cerebrosides.

Table 4. CBL quantities of ceramide classes in adult and fledgling house sparrows from Ohio and Saudi Arabia acclimated to high or low humidity

Habitat	Age	Acclimation	Ceramide E	Ceramide D	Ceramide C	Ceramide B	Ceramide A
Ohio	Adult	Dry	9.00±0.91	3.30±0.53	2.50±0.44	2.25±0.46	0.00±0.00
		Humid	8.82±0.79	3.08±0.43	2.25±0.45	1.87±0.61	0.00±0.00
Ohio	Fledgling	Dry	3.76±0.34	2.43±0.34	2.21±0.32	1.42±0.41	0.78±0.41
		Humid	3.91±0.29	2.33±0.38	2.25±0.37	0.95±0.36	0.65±0.35
Saudi Arabia	Fledgling	Dry	2.92±0.40	1.70±0.61	1.25±0.34	0.44±0.28	0.00±0.00
		Humid	2.98±0.70	1.98±0.70	1.76±0.63	0.00±0.00	0.00±0.00

CBL, covalently bound lipid (cholesteryl esters, free fatty acids, ceramides and cerebrosides) as mg g^{-1} dry stratum corneum (SC); CWL, cutaneous water loss as mg H_2O (cm^2 day)$^{-1}$.
Data are means ± s.e.m.

corneocytes *via* the terminal hydroxyl group of the fatty acid moiety, similar to the mechanism of attachment of covalently bound sphingolipids in the skin (Wertz and Downing, 1987; Downing, 1992; Stewart and Downing, 2001).

We found that CBLs did not show phenotypic plasticity in response to short-term changes in humidity during the nestling period. Habitat was also not a significant factor in separating CBL scores in PCA. However, the CBLs of house sparrows increased in polarity with age, and this difference was associated with changes in CWL during ontogeny.

Two of our principal components were correlated with CWL in all sparrows, indicating a functional relationship between the structure of the lipid envelope and CWL. Our first principal component separated ceramide A (the least polar ceramide) and FFA from the other lipid classes. Ceramide A was only found in nestlings and fledglings, and its presence is associated with the higher CWL rates of nestlings and fledglings when compared with those of adults, perhaps because ceramide A is the least polar ceramide class. Nestling sparrows had more FFA in their plasma than adults, probably as a source of energy during growth (Dobado-Berrios et al., 1998; Quillfeldt et al., 2004). The presence of high amounts of plasma FFA suggests that nestlings incorporate more FFA into their epidermal lipids. Adult birds have lower FFA levels in blood plasma, resulting in fewer FFA being incorporated into the CBL compartment. Adult sparrows tended to cluster in the same quadrant on the score plot as the vectors for cerebrosides and all ceramide types except ceramide A. We found that adults had a higher amount of ceramides and cerebrosides than nestlings and fledglings, which translates into a lower CWL. Adult house sparrows from Saudi Arabia and Ohio had different covalently bound ceramide to cerebroside ratios, and this was also correlated with different CWL rates, with CWL decreasing as the amount of polar sphingolipids increases (Gu et al., 2008).

Because the lipids in the SC of birds are markedly different from those in mammals, the organization of these lipids must also be different. Gu and colleagues proposed two models for the

Table 5. Principal component analysis (PCA) of quantities of CBL classes of the SC of adult and nestling house sparrows from desert and mesic habitats

	PC1	PC2	PC3
Correlation PC and original variable			
Cerebrosides	0.465	0.104	0.253
Ceramide E	0.418	0.211	0.253
Ceramide D	0.409	–0.219	–0.083
Ceramide C	0.408	–0.292	–0.149
Ceramide B	0.341	–0.414	0.153
Ceramide A	–0.081	–0.669	–0.37
Cholesteryl esters	0.358	0.302	–0.24
Free fatty acids	0.158	0.32	–0.24
Eigenvalue	3.775	1.298	1.055
Percentage variance	47.2	16.2	13.2

Quantities of CBLs (covalently bound lipids) were measured as mg g^{-1} dry stratum corneum (SC) for PCA.

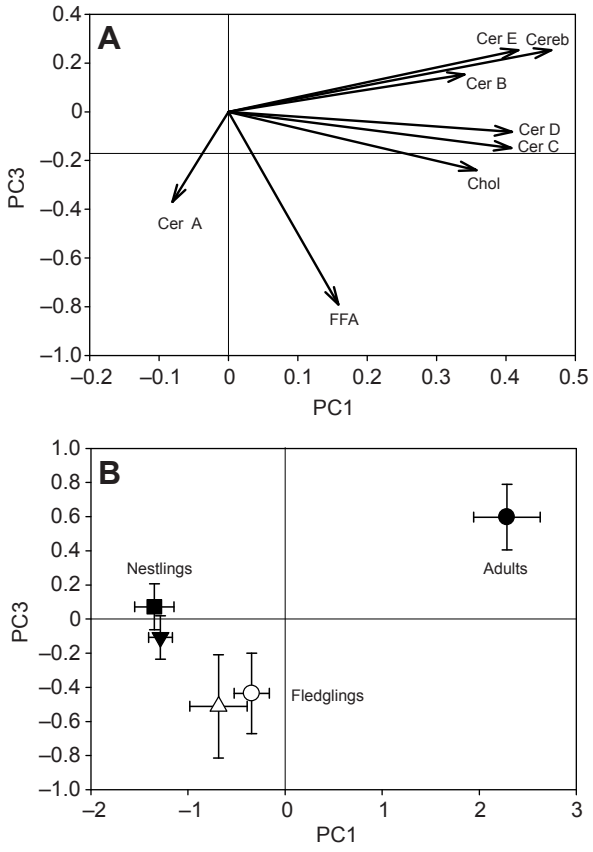

Fig. 3. Principal component analysis (PCA) of the quantity of covalently bound lipid classes (mg g^{-1} dry SC). (A) Eigenvector plot of covalently bound lipid loadings for PC1 and 3. See Fig. 2 for abbreviations. PC1 separated ceramide A and FFA from the rest of the lipid classes. PC3 separated lipid classes according to polarity. (B) PCA plot of mean (±s.e.m.) scores of sparrow groups for PC1 and 3. PC1 distinguished among age classes. CBL from fledgling sparrows clustered near the vector for ceramide A, whereas CBL from adults aggregated with the polar lipids (ceramides and cerebrosides). Filled circles, Ohio adults; open circles, Ohio fledglings; filled triangles, Ohio nestlings; open triangles, Saudi Arabia fledglings; filled squares, Saudi Arabia nestlings.

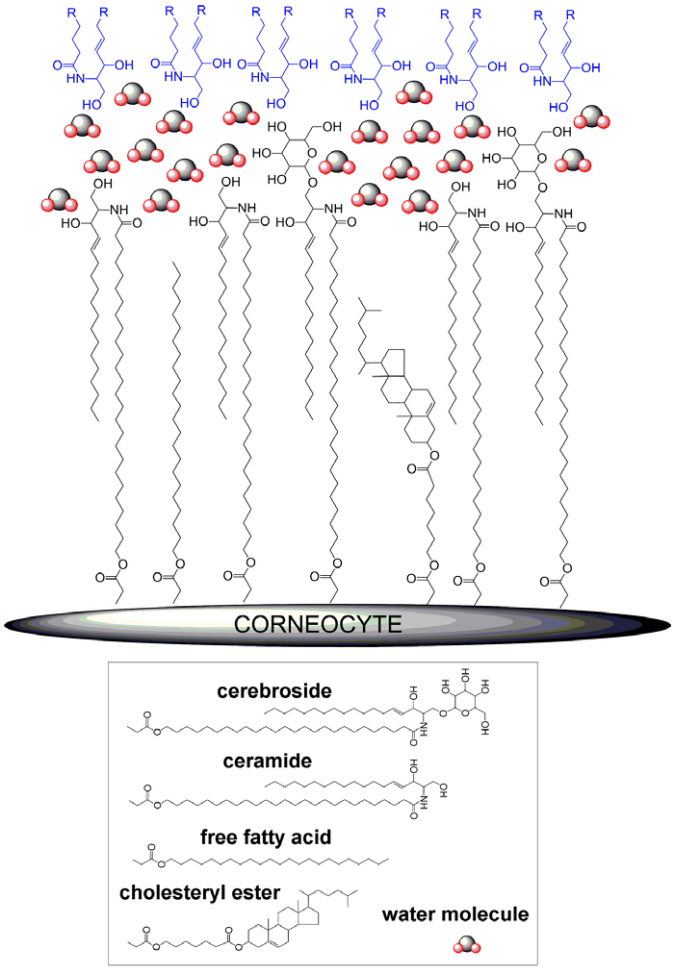

Fig. 4. A new model for the arrangement of CBLs in birds. In our model, sphingolipids and cholesteryl esters are attached to the protein envelope of corneocytes *via* the terminal hydroxyl group of the fatty acid moiety. The polar head groups of the covalently bound sphingolipids interact with intercellular lipids to reduce the evaporation of water between layers and to sequester water molecules. Cholesteryl esters and FFA would create holes in the polar shell of the CBL envelope and disrupt these molecular interactions. Our model predicts that the carbon chains of the fatty acid moiety for cholesteryl esters and the carbon chains of FFA would be short, so that both lipid classes would sit between the fatty acid moieties of the covalently bound sphingolipids and avoid the water molecules sequestered by the cerebrosides.

arrangement of CBLs, both showing the polar head groups of the covalently bound sphingolipids interacting with ICLs to reduce the evaporation of water between layers and to sequester water molecules (Gu et al., 2008). This sequestration may take place *via* an ordering of water molecules around the hydroxyl groups of the hexose moieties, forming an aggregate of water molecules. The ordered water molecules will exhibit strong hydrogen bonding; thus, individual water molecules will require more energy to break away from the aggregate and percolate out of the SC through the intercellular lipids. We propose a modified version of this model, in which we include cholesteryl esters and FFA, which would create holes in the polar shell of the CBL envelope and disrupt these molecular interactions (Fig. 4).

Gu and colleagues also determined that the covalently bound ceramides and cerebrosides in house sparrows have fatty acid moieties that are 26 hydrocarbons long (Gu et al., 2008). Our model predicts that the carbon chains of the fatty acid moiety for cholesteryl esters would be significantly shorter than 26 hydrocarbons, so that the non-polar cholesteryl groups would sit between the fatty acid moieties of the covalently bound sphingolipids (Fig. 4). This prediction is in agreement with the arrangement of cholesterol in the plasma membrane, where free cholesterol resides mainly within the non-polar interior, associating with the fatty acid chains of phospholipids or glycosphingolipids (Simons and Ikonen, 1997; Brown, 2002). FFA would likely not have carbon chain lengths longer than 26 carbons, in order to avoid the water molecules sequestered by the cerebrosides. The arrangement of these CBLs would be restricted by the size of the lipids and the positioning of the glutamate residues of involucrin.

In conclusion, the CBLs of sparrows did not exhibit phenotypic plasticity in response to short-term changes in humidity, but they did show changes in quantity and polarity during ontogeny that were functionally related to CWL. In addition to ceramides, FFA and cerebrosides, sparrows also have cholesteryl esters in their CBL compartment. Adult sparrows from Ohio and Saudi Arabia differed in their relative quantities of CBLs. In the light of human-induced environmental changes such as global warming, it is crucial to understand the mechanisms that produce the phenotypic variation of physiological traits during the lifetime of an individual (Helmuth et al., 2005). Studying the effect of skin lipids on CWL can give us a broader perspective on how populations of birds and mammals will be able to endure climate change.

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