

# Lipids of the Stratum Corneum Vary with Cutaneous Water Loss among Larks along a Temperature-Moisture Gradient

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Accepted 7/30/03

## ABSTRACT

We explored the relationship between lipids of the stratum corneum (SC), the barrier to water-vapor diffusion of the skin, and cutaneous water loss (CWL) of species of free-living larks along a temperature-moisture gradient. Our results showed that free fatty acids, cholesterol, and ceramides were the major constituents of SC in larks from different environments including the Netherlands, a mesic environment; Iran, a semiarid region; and several areas in Saudi Arabia, a hot dry desert. We found that CWL was reduced among larks inhabiting deserts, but our data did not support the hypothesis that birds from desert environments have larger quantities of lipids per unit dry mass of the SC than larks from more mesic environments. Instead, larks in arid environments had a higher proportion of ceramides, especially the more polar fractions 4–6, and a smaller proportion of free fatty acids in their SC, an adjustment that apparently reduced their CWL. Subtle changes in the ratios of lipid classes can apparently alter the movement of water vapor through the skin. We hypothesize that desert birds have higher proportions of ceramides in their SC and lower proportions of free fatty acids because this combination allows the lipid lamellae to exist in a more highly ordered crystalline phase and consequently creates a tighter barrier to water-vapor diffusion.

## Introduction

During the Carboniferous period, vertebrates invaded land, a major evolutionary shift introducing pioneering terrestrial animals to new ecological opportunities while at the same time exposing them to a desiccating environment. To maintain cellular water homeostasis, natural selection modified the integument of these animals to reduced water loss, enabling them to successfully occupy terrestrial environments (Lillywhite and Maderson 1982). For extant vertebrates living in desert environments where ambient temperatures ( $T_a$ ) can reach 50°C, relative humidities are low and drinking water is scarce, problems of water loss can be extreme (Noy-Meir 1973; Williams and Tieleman 2001). Because birds have a higher mass-specific metabolic rate than other animals and are diurnal, and therefore experience high  $T_a$ 's, problems of water balance may be especially severe for them in desert environments (Dawson and Bartholomew 1968; Tieleman and Williams 2000). Despite this, birds thrive in most of the world's deserts, leading one to hypothesize that they have evolved unique behavioral and physiological mechanisms to reduce their water losses.

In birds, total evaporative water loss (TEWL), the sum of evaporative water loss from respiratory passages and from skin, is the major avenue of water loss to the environment, especially for small species in which TEWL is five times greater than urinary-fecal water loss (Bartholomew 1972; Dawson 1982; Williams and Tieleman 2000). Using both conventional least squares regression and phylogenetic independent contrasts, Williams (1996) showed that TEWL for 38 species from arid regions was significantly lower than for 64 species from more mesic regions. Because species within broad-scale interspecific comparisons differ not only in environment but also in diet and in phylogenetic background, the resolving power of these kinds of studies has been questioned (Harvey et al. 1991; Leroi et al. 1994). Tieleman et al. (2003) investigated TEWL of birds within a single phylogenetic clade, larks (Alaudidae), along a temperature-moisture gradient extending from the Netherlands, a mesic region, to the arid deserts of Saudi Arabia. They found that mass-adjusted TEWL correlated significantly with environment with mesic species having rates of water loss nearly double those from deserts. After calculating that differences in respiratory water loss could not account for the differences in TEWL, Tieleman et al. (1999) concluded species from deserts had a lower cutaneous water loss (CWL).

Early investigators assumed that most evaporative water loss took place across the respiratory passages and that CWL was

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insignificant in the process of thermoregulation (Bartholomew and Cade 1963; Mount 1979). Subsequent work has shown that CWL can equal or exceed evaporation from respiratory passages at moderate  $T_a$ 's (Dawson 1982; Webster and King 1987; Wolf and Walsberg 1996). In a study on larks, Tieleman and Williams (2002) found that CWL accounted for about 65% of TEWL at moderate  $T_a$ 's, and surface-specific CWL was about 30% lower in arid species compared with ones living in mesic environments. They hypothesized that natural selection has equipped desert birds with a mechanism or mechanisms that impedes water loss through the skin, at least at moderate  $T_a$ 's, as a water conservation mechanism.

The skin of birds is composed of a thicker (120  $\mu\text{m}$ ) vascularized dermis and a thin (13–22  $\mu\text{m}$ ) nonvascular epidermis, which has two layers: a viable layer composed of the stratum transitivum, stratum intermedium, and the mitotically active stratum basale, and an outer layer of cornified nonliving cells embedded in a lipid matrix called the stratum corneum (SC; Lucas and Stettenheim 1972). In mammals, lipids of the SC constitute 10%–15% of its dry mass (Gray and Yardley 1975) and primarily consist of cholesterol, ceramides (amides of fatty acids with hydroxylated long chain amines), and free fatty acids (Elias and Friend 1975; Wertz et al. 1986; Menon and Menon 2000). In the SC of mammals, ceramides account for as much as 50% of the total lipids (Raith and Neubert 2000).

From work primarily on mammals, evidence has accumulated that the SC forms the barrier to water-vapor diffusion from the animal to its environment (Blank 1953; Blank et al. 1984; Golden et al. 1987; Warner et al. 1988; Potts and Franconer 1990; Squier et al. 1991). Early work showed that topical applications of organic solvents, chemicals that disrupt the organization of the lipid matrix in the SC, increased water vapor loss (Scheuplein and Blank 1971), and that replenishment of lipids of the SC from where they had been removed restored barrier function (Elias and Brown 1978; Grubauer et al. 1989; Elias and Menon 1991). Confined to the domestic chicken (*Gallus domesticus*), zebra finch (*Taeniopygia guttata*), and pigeon (*Columba livia*), studies on the avian epidermis also suggest that lipids of the SC form the permeability barrier (Landmann 1980; Menon et al. 1986; Peltonen et al. 2000).

In birds, the lipids of the SC are synthesized in the viable layer of epidermis, most likely in the Golgi apparatus, and are assembled as membrane-bound multigranular bodies (Landmann 1980); in mammals, similar but smaller structures are called lamellar bodies (Elias and Menon 1991). Within these structures, glycosphingolipids and their derivatives, ceramides, create a framework of layers with which other lipids can be associated. Six chromatographic fractions of ceramides have been identified in the SC of mammals and of chickens, labeled in order of increasing polarity, ceramides 1–6 (Wertz and Downing 1983; Wertz et al. 1985; Wertz et al. 1986; Schaefer and Redelmeier 1996). Compared with lamellar bodies in mammals, multigranular bodies in birds are four to five times larger

and comprise a larger number of lipid layers (Menon et al. 1986). As cells differentiate to form the SC, some of the multigranular bodies extrude their contents into the intercellular matrix, creating layers of lipids that form the barrier to water-vapor diffusion (Landmann 1980; Sawyer et al. 1986; Peltonen et al. 2000), whereas others apparently degrade into droplets of neutral lipids (Menon et al. 1989). Under cold exposure or water deprivation, contents of the multigranular bodies are entirely extruded into the intercellular space (Menon et al. 1986; Menon et al. 1989; Peltonen et al. 2000).

This investigation explored the interplay between lipids of the SC and CWL of birds from the wild, species under the influence of natural selection rather than domesticated or laboratory species. We identified the major lipid classes in the SC of free-living larks and compared our results with reports on domestic species. Then we quantified the major lipids in the SC of larks from different environments: the Netherlands, a cool moist environment; Iran, a semiarid region; and several areas in Saudi Arabia, a hot dry desert. Although much research has been done on lipid composition and arrangement in the SC of mammals, little is known about lipids in the SC of wild birds. Our working hypothesis was that birds from desert environments would have larger quantities of lipids per unit dry mass of the SC than would larks from more mesic environments. We tested whether individual classes of lipids were associated with reduced CWL that had been measured in larks from deserts compared with larks in more mesic regions (Tieleman and Williams 2002). Because ceramides play a key role in resistance of the integument to water-vapor diffusion (Wertz and van den Bergh 1998), we hypothesized that the lower CWL of larks from deserts would be associated with increased ceramide content in the SC.

## Material and Methods

### Capture of Birds

Using mist nets, we captured species of larks (Alaudidae) along a temperature-moisture gradient. Extremes of the gradient were the Netherlands, where we captured skylarks (*Alauda arvensis*) and woodlarks (*Lullula arborea*), and Saudi Arabia, where we netted crested larks (*Galerida cristata*), black-crowned finch-larks (*Eremopterix nigriceps*), desert larks (*Ammomanes deserti*), hoopoe larks (*Alaemon alaudipes*), and Dunn's larks (*Eremalauda dunnii*). Christian Gross provided calandra larks (*Melanocorypha calandra*) from a semiarid area in northern Iran (35°00'N, 51°00'E; Tieleman et al. 2003). Climate data, geographical locations, and classification of species, mesic, semiarid, and arid, are reported in Tieleman et al. 2003.

### Environments

Birds in the Netherlands were captured during the breeding season in the province of Drenthe (52°52'N, 06°20'E), where

rainfall averages 750 mm per year and mean maximum  $T_a$  in July is 21.7°C (Tieleman et al. 2003). In Saudi Arabia, birds were netted in late spring in Mahazat as Sayd, a reserve in the Arabian Desert (22°15'N, 41°50'E), where average yearly rainfall is 90 mm and mean maximum  $T_a$  in July is 40.2°C (National Wildlife Research Center, unpublished data).

To characterize environment quantitatively, we used the aridity index of Emberger (1955):  $Q = P/[(T_{\max} + T_{\min})(T_{\max} - T_{\min})] \times 1,000$ , where  $P$  is average annual precipitation (mm),  $T_{\max}$  is the mean maximum temperature of the hottest month, and  $T_{\min}$  is the mean minimum temperature of the coldest month. Low in hot, dry deserts, and high in cool, moist regions,  $Q$  has been derived as a proxy for primary productivity in arid and semiarid environments (Emberger 1955; Meigs 1966). Because we noticed that  $Q$  rapidly increases when environments become more mesic, we avoided unequal weighting of data on mesic species by using  $\log Q$  in our analyses (Tieleman et al. 2003).

#### Cutaneous Water Loss

We used mean values of CWL (mg H<sub>2</sub>O [cm<sup>2</sup> d]<sup>-1</sup>) measured at 25°C for skylarks, woodlarks, hoopoe larks, and Dunn's larks (Tieleman and Williams 2002). For desert larks, we measured respiratory water loss (RWL) and CWL simultaneously by placing a bird fitted with a plastic mask in a metabolism chamber into which dry, CO<sub>2</sub>-free outside air was drawn. Steel metabolic chambers had an airtight Plexiglas lid and were water-jacketed to control  $T_a$  by a Neslab circulating water bath (RTE-140;  $\pm 0.2^\circ\text{C}$ ). Birds were placed on a wire mesh platform over a layer of mineral oil to trap excrement, excluding it as a source of water in measurements. Birds became accustomed to experimental conditions in about 15 min and generally remained inactive during the measurement period. Because air that passes through Drierite is not completely dry, we measured the water-vapor density of air entering the chamber ( $\rho_{v-in}$ ). Air pulled through the mask contained all respiratory gases and was routed through polytetrafluoroethylene tubing, a General Eastern dew point hygrometer (M4), columns of Drierite and Ascarite to remove H<sub>2</sub>O and CO<sub>2</sub>, a previously calibrated Brooks mass flow controller (model 5850E; Levy 1964), and a vacuum pump. We calculated the flow rate through the dew point hygrometer ( $V'_{el}$ ) by adjusting the value recorded at the mass flow controller for H<sub>2</sub>O and CO<sub>2</sub> added (Tieleman and Williams 2002), the latter estimated assuming a respiratory quotient of 0.71 (King and Farner 1961). In practice, these adjustments were <1%. To calculate RWL, we used the equation of Tieleman and Williams (2002). Air was also drawn from the chamber through a second exit port and passed along a second train identical to the first, except that air from the vacuum pump was vented to the room. Calculation of CWL was complicated by the fact that air within the chamber that contained water vapor from the skin was

exiting through two ports. We calculated the flow rate of air leaving the chamber by summing the flow rates from the mask ( $V'_{el}$ ) and from the chamber ( $V'_{e2}$ ) and determined CWL with the equation  $\text{CWL} = (\rho_{v-chamber} - \rho_{v-in})(V'_{el} + V'_{e2})$ . After a 2–3-h equilibration period, we recorded the dew points of inlet, chamber, and mask air, the temperature of the dew point hygrometers, and  $T_a$  in the chamber, using a Campbell Scientific data logger model 21X or CR23X. When, during the third hour of measurements, the traces for dew points were stable for at least 10 min, we noted these times and used these data for calculations. Validated by Tieleman and Williams (2002), this system measures water loss from the skin, including the head and neck along with water lost from the eyes.

In our comparison of CWL versus aridity, we had direct measurements of CWL for five species. In addition, we estimated CWL in grey-backed finchlarks (*Erenopterix verticalis*), Stark's larks (*Eremalauda starki*), black-crowned finchlarks, crested larks, calandra larks, horned larks (*Eremophila alpestris*), and spike-heeled larks (*Chersomanes albofasciata*), based on the idea that CWL is a relatively constant proportion of TEWL at 25°C ( $n = 4$  species, mean =  $0.65 \pm 0.05$ ; Tieleman and Williams 2002).

#### Separation of Stratum Corneum

In the lab, we determined the mass of birds, killed them, plucked their feathers, and removed the skin. After determining skin wet mass and pinning the skin to a thin sheet of Teflon, we dipped it in distilled water at 65°C for 3 min and then gently peeled the epidermis from the dermis (Wertz et al. 1986; Haugen 2003). Next we placed the epidermis in a vial containing 0.5% Trypsin in phosphate-buffered saline (pH 7.4, 370 mOsm) and placed it in a refrigerator overnight (4°C). The following day, we rinsed the intact SC tissue with distilled H<sub>2</sub>O to remove epithelial cells, reimmersed the remaining tissue in fresh 0.5% Trypsin solution, and allowed it to stand at  $38^\circ \pm 2^\circ\text{C}$  for 3 h. After a final rinse in distilled H<sub>2</sub>O, we separated SC tissue from the solvent by filtering and froze it at  $-80^\circ\text{C}$  under an atmosphere of argon. Thereafter we lyophilized the SC tissue to dryness (12 h), sealed it in a test tube, again under an atmosphere of argon, and then stored it at  $-80^\circ\text{C}$  pending further analysis. In preliminary trials, we confirmed that 12 h of lyophilization was adequate to completely dry the sample by repeatedly weighing samples during the drying process (Haugen 2003).

#### Identification of Lipids

After transport to the United States, we relyophilized the SC tissue, weighed it to determine dry mass, and extracted the lipids with a chloroform : methanol series of 2 : 1, 1 : 1, and

1 : 2 for 2 h at each step (Law et al. 1995). Chemicals and tissues were placed in covered glass funnels equipped with Teflon stopcocks and preextracted cotton plugs. We combined extracts and evaporated the mixture under nitrogen. To prepare the lipid residue for thin layer chromatography (TLC), we dissolved the extracted lipids in 200  $\mu$ L chloroform methanol 2 : 1 containing the antioxidant butylated hydroxytoluene (50 mg/L).

We separated classes of lipids using analytical TLC on 20  $\times$  20-cm glass plates coated with silicic acid (0.25 mm thick; Adsorbosil-Plus 1, Alltech, Deerfield, Ill.; Downing 1968). Identification of lipids was achieved by comparing their chromatographic properties with known standards. To prepare our plates, we placed them in chromatographic tanks with a mixture of chloroform methanol (2 : 1) until the solvent reached the top, removing contaminants, activated them in an oven at 110°C, and scored the adsorbent into 6-mm-wide lanes (Wertz et al. 1986). Using a Teflon-tipped Hamilton syringe (no. 80055; Hamilton, Reno, Nev.), we pipetted 5  $\mu$ L of either lipid extract from the SC or with a mixture of lipid standards onto a preabsorbent area on the lower portion of the plate. We ran each sample in triplicate and used the mean value of these determinations in our analyses. Our standard lipid mixture included nonhydroxy fatty acid ceramides (a sphingosine base with a mixture of octadecanoic and *cis*-15-tetracosenoic acids as the N-acyl fatty acid groups), cholesterol, and stearic acid, a free fatty acid, all purchased from Sigma (St. Louis, Mo.), and dissolved in chloroform methanol (2 : 1) in concentrations from 0.625 mg/mL to 10 mg/mL.

Two solvent systems were used to separate lipids of different polarities. For the more nonpolar lipids, such as cholesterol and free fatty acids, we developed plates to the top in tanks of hexane, followed by toluene, followed by a mixture of hexane/ethyl ether/acetic acid, 70 : 30 : 1, run to 12 cm from the bottom. For the more polar lipids, including the six classes of ceramides, plates were developed twice with a mixture of chloroform methanol acetic acid solution, 190 : 9 : 1, followed by development with a mixture of hexane/ethyl ether/acetic acid, 70 : 30 : 1.

We visualized bands of lipids by spraying plates with a charring agent, a solution of 3% cupric acetate in 8% phosphoric acid, then placing the plate on a 20  $\times$  20-mm polished aluminum hotplate that was slowly heated to 220°C, effectively carbonizing all lipids (Wertz et al. 1986).

Bands of lipids were quantified using photodensitometry (Downing 1968). We scanned each lane on plates with a Hewlett Packard scanner and the computer software TN-Image (Nelson 2003) to determine areas of charred regions. At the same time we scanned lanes that contained a series of known concentrations of standards to establish standard curves (Vecchini et al. 1995; Johnson 2000).

Although analytical thin layer chromatography is a standard procedure, we wanted to estimate our error in using the method to quantify concentrations of lipids. To do so, we pipetted

known concentrations of cholesterol, ranging from 4.2 mg/mL to 14.3 mg/mL, onto plates in triplicate and followed our protocol for determining lipid concentrations. We averaged values for the three lanes for each trial and compared those results with the known concentration. Calculated as  $([\text{observed} - \text{actual}]/\text{actual}] \times 100)$ , the average error was +0.82%, indicating that our method provides a reasonable estimate of quantities of lipids (Haugen 2003).

Even though bands on chromatograms coincided with known classes of lipids, we wanted to confirm our identification of lipids. Using our lipid extracts from the SC of larks, we ran preparative thin layer chromatography on 0.5-mm silica gel plates, again divided into 6-mm lanes, using the same mobile phase as described earlier. After spraying the plate with an ethanol solution of 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (10 mg/100 mL), lipid bands were visualized by viewing under ultraviolet light. Then bands were scraped off into a glass dish, mixed with a solution of chloroform methanol 2 : 1 to extract lipids, which were subsequently eluted for analysis (Wertz et al. 1986). For free fatty acids and sterols, we added a trimethylsilylation reagent (pyridine-hexamethyldisilazane-trimethylchlorosilane [9 : 3 : 1]; Supelco, Bellefonte, Pa.; Bleton et al. 2001) to prepare the lipids for gas chromatography–mass spectrometry (GC-MS). Analyses were carried out on a Finnigan Trace GC-MS with a 30-m  $\times$  0.32-mm inner diameter fused silica column and with a 0.25  $\mu$ m XTI-5 film of 5% diphenyl/95% dimethyl polysiloxane (Restek, Bellefonte, Pa.). We verified ceramide bands using electrospray ionization tandem mass spectrometry on a Bruker Esquire LC/MS-MS system in positive ion mode (Raith and Neubert 2000).

### Statistics

All statistical analyses were performed using SPSS version 11.0 with the null hypothesis rejected at  $\alpha = 0.05$ . For comparisons of lipid concentrations among species, we performed ANOVA followed by a Tukey's test to identify significant differences between species. To search for correlations, we calculated Spearman's rho for each combination of variables. All percentages were arcsine transformed for statistical analysis. Means are in units of mg lipid/g dry mass of SC, unless otherwise specified. Experiments were carried out in the Netherlands under license no. DEC2425, in Ohio under ILACUC permit no. 00A0161, and in Saudi Arabia with permission from the National Commission for Wildlife Research and Development.

## Results

### *Cutaneous Water Loss among Larks*

Mean values of CWL ( $\text{mg H}_2\text{O} [\text{cm}^2 \text{d}]^{-1}$ ), determined at 25°C, varied positively with aridity when we included five species for

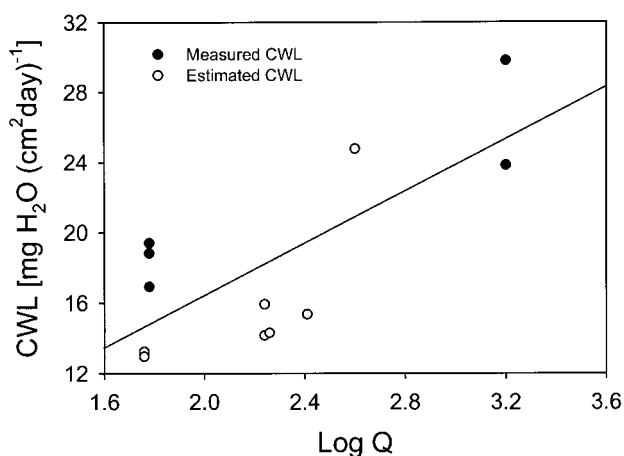


Figure 1. Mean rates of cutaneous water loss (CWL) of larks as a function of environmental aridity ( $\text{Log } Q$ ). Filled circles represent measured values; open circles are estimated values of cutaneous water loss using rates of total evaporative water loss (Tieleman and Williams 2002).

which we had measured values of CWL ( $n = 5$ ,  $r = 0.88$ ,  $P < 0.05$ ) or when we included both measured and estimated values of CWL ( $n = 12$ ,  $r = 0.74$ ,  $P < 0.006$ ; Fig. 1). Woodlarks and skylarks from mesic environments had higher CWL than species from more arid environments (Tieleman and Williams 2002).

#### Identification of Lipid Classes

Chromatographs showed distinct bands of lipids corresponding to standards of cholesterol, free fatty acids, and ceramides, supporting the idea that these are the important classes of lipids in the SC of wild birds (Fig. 2). Ceramides separated into five distinct bands on plates; ceramides 4 and 5 were indistinguishable from each other, and as a result, we have combined them in our analyses. We confirmed these bands were ceramides using electrospray ionization tandem mass spectrometry by identifying the mass spectra associated with fragmentation of the long chain bases. Using GC-MS, we identified free sterols, mostly cholesterol with small amounts of cholestanol, and an array of mostly saturated free fatty acids ranging from 16 to 30 carbons in length (Haugen 2003).

#### Comparisons of Lipids among Species

Woodlarks and skylarks tended to have larger concentrations of free fatty acids in their SC than did other species, but only significantly so for woodlarks (Fig. 3). Sterols (cholesterol) were significantly higher in woodlarks compared with other species. Total ceramides varied among species, but we did not find any trends across our temperature-moisture gradient ( $n = 8$ ,  $r =$

$-0.03$ ,  $P = 0.95$ ). Dunn's larks seemed to have lower concentrations of lipids in their SC than did other species, despite the fact that they are residents of the Arabian desert. Concentrations of ceramide 2 were similar among all species. Because our data are the first on concentrations of lipids in the SC of wild birds, we also report means along with sample size and measurement variance so that future investigators will be able to compare results (App. A).

#### Correlations of Cutaneous Water Loss, Lipids in the Stratum Corneum, and Aridity

We searched for correlations between CWL and mean concentrations (mg lipid/g dry SC) of free fatty acids, cholesterol, and ceramides, both total ceramides and ceramides 1–6, among five species of larks. Mean free fatty acids were significantly correlated with CWL ( $n = 5$ ,  $r = 0.97$ ,  $P < 0.005$ ). Hence birds with higher CWL, such as those in mesic environments, tended to have a higher concentration of free fatty acids in their SC. Concentrations of other classes of lipids were uncorrelated with CWL ( $P > 0.05$ ).

Not only concentrations of lipids in the SC influence water permeation but also the proportions of various classes of lipids. Total ceramides as a percentage of the total lipid extracted was negatively correlated with CWL ( $n = 5$ ,  $r = -0.96$ ,  $P < 0.01$ ; Fig. 4). Also ceramides 4 and 5 and ceramide 6 as a percentage of the total lipids were negatively associated with CWL ( $n = 5$ ,  $r = -0.91$ ,  $P < 0.04$ ; Fig. 4; and  $n = 5$ ,  $r = -0.98$ ,  $P = 0.002$ ; Fig. 4). Free fatty acids as a percentage of total lipid were positively correlated with CWL ( $n = 5$ ,  $r = 0.88$ ,  $P < 0.05$ ; Fig. 4).

A positive correlation existed between concentration (mg lipid/g dry SC) of free fatty acids and aridity ( $n = 8$ ,  $r =$

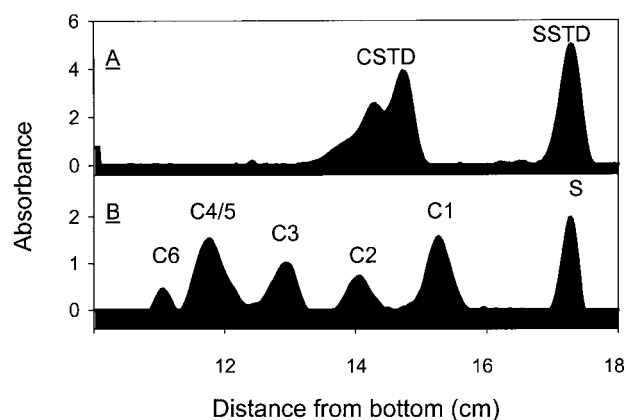


Figure 2. Densitometric profiles of (A) lipid standards and (B) ceramides and free sterols from extracted lipids. CSTD = ceramide standard; SSTD = free sterol standard; S = free sterols; and C1–C6 = ceramides 1–6.

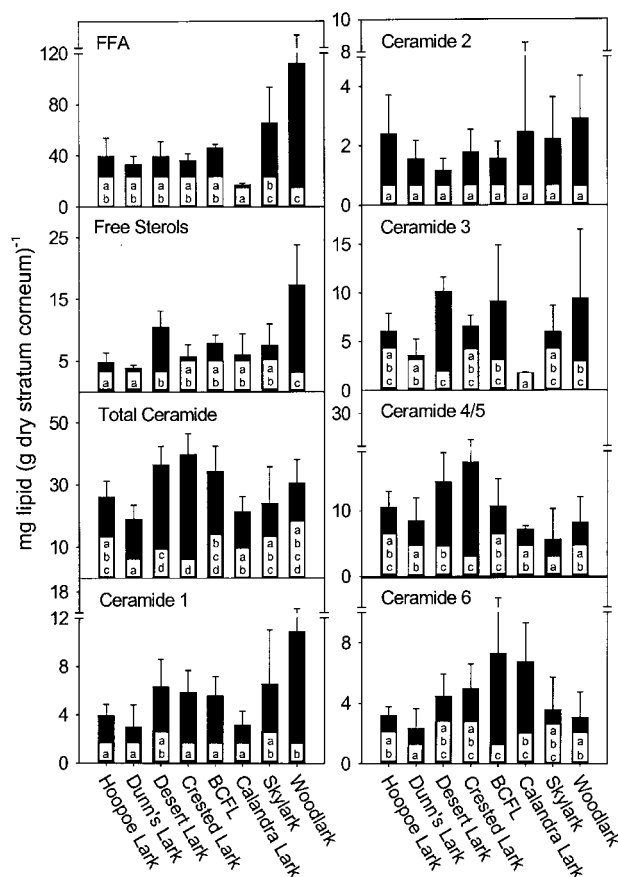


Figure 3. Comparisons of the lipid concentrations (mg/g dry stratum corneum) of species of larks from different environments. Letters represent homogenous subsets for each lipid group. Bars represent  $\pm 1$  SD. BCFL = black-crowned finchlark.

0.77,  $P < 0.03$ ; Fig. 5A) and between concentration of ceramide 1 and aridity ( $n = 8$ ,  $r = 0.72$ ,  $P < 0.05$ ; Fig. 5A).

#### Correlations among Lipid Classes

Some classes of lipids covaried among species (Fig. 5B). Mean cholesterol and mean free fatty acids had a positive correlation ( $n = 8$ ,  $r = 0.85$ ,  $P < 0.01$ ), although data for woodlarks drive this relationship. Ceramide 1 was also significantly correlated with both free sterols and free fatty acids ( $P < 0.001$  in both cases; Fig. 5B). Other combinations of variables were insignificant ( $P > 0.05$ ).

#### Discussion

Our study is the first to examine the important classes of lipids in the SC of wild birds. We have focused our work on free fatty acids, free sterols, and ceramides because these have been shown to be the major constituents of the barrier to water permeation in the SC in laboratory species of both mammals and birds (Elias and Friend 1975; Wertz et al. 1986; Menon

and Menon 2000). Our results showed that free fatty acids, cholesterol, and ceramides are also the major constituents of SC in wild larks.

Comparisons of our results with previous studies are problematic because investigators often isolated lipids from the entire epidermis, as in the chicken (Wertz et al. 1986) and in zebra finches (Menon et al. 1989). Menon et al. (1986) scraped SC cells from the epidermis in domestic pigeons and reported that free fatty acids, free sterols, and ceramides were 19.6%, 3.6%, and 25% of SC lipid, respectively. However, it is unclear whether this method isolates all of the layers of the SC. In porcine SC, isolated with methods similar to ours, free fatty acids, cholesterol, and ceramides amounted to 10%, 25%, and 50% of the total lipids (Law et al. 1995). These data indicate that the proportion of lipids in SC varies between birds and mammals, the significance of which awaits further study.

We have shown that birds in arid environments have lower CWL than species from more mesic environments. The association between reduced CWL and aridity, combined with a lowered TEWL in more arid environments (Tieleman et al. 2003), is evidence that birds in arid environments lose less water

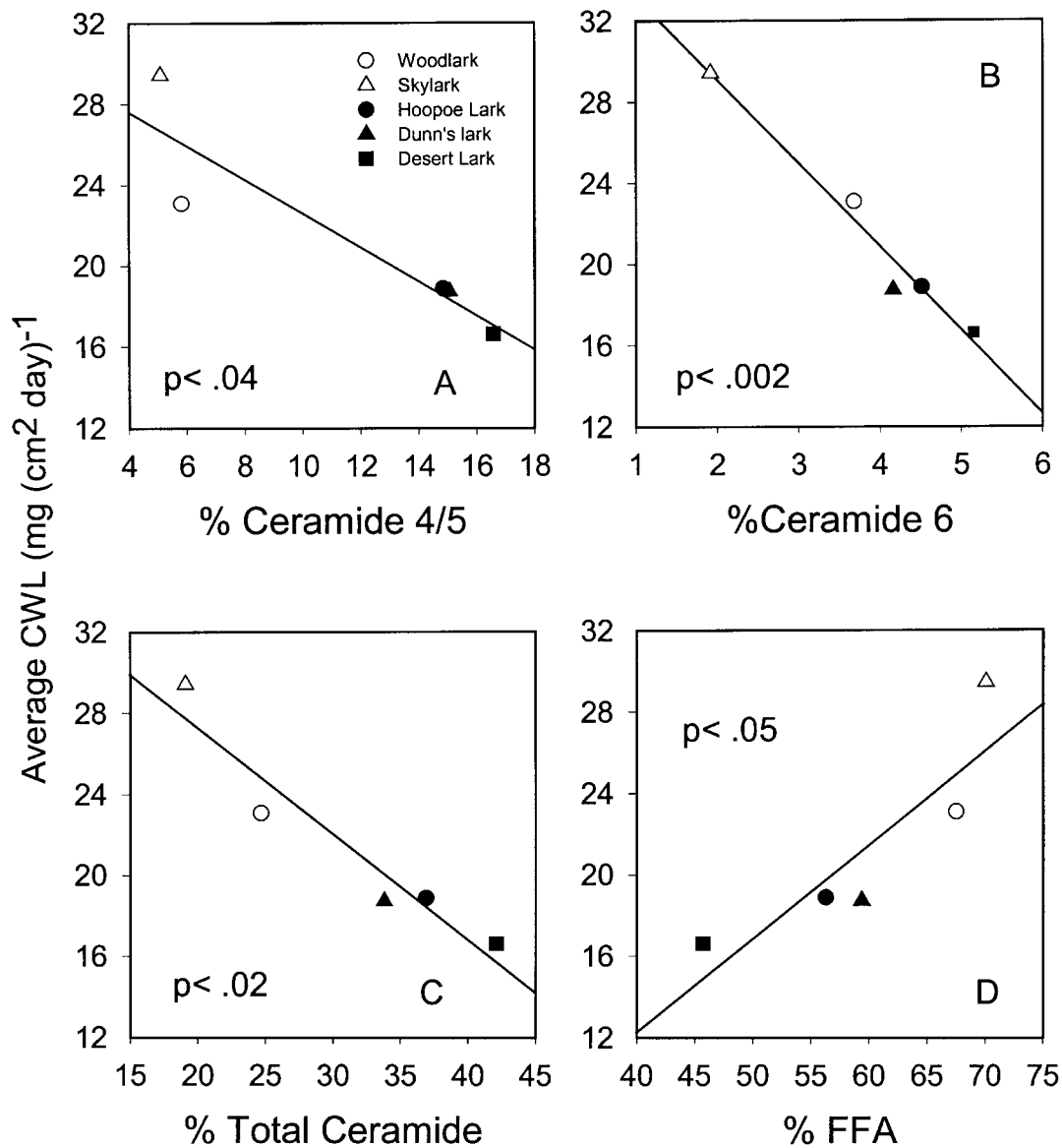


Figure 4. Mean surface-specific cutaneous water loss of hoopoe larks, Dunn's larks, desert larks, woodlarks, and skylarks as a function of the percent of total lipid quantity measured.

through their skin than those species living in more mesic environments. We hypothesized that larks from arid environments would have larger quantities of lipids per unit dry mass SC than would larks from more mesic environments. However, our results do not support this hypothesis. Larks from more mesic environments tended to have higher concentrations of free fatty acids in their SC, which would increase the fluidity of the SC and increase CWL (Wertz 2000; Bouwstra et al. 2003). Although Quay (1964) suggested that the epidermis was thicker in desert rodents compared with their more mesic counterparts, subsequent work has indicated that the thickness of the SC is

highly variable and that thickness does not seem to be related to the permeability barrier in mammals (Elias et al. 1981) or in birds (Hattingh 1972). This implies that it is not the number of cell layers of the SC that are important in barrier formation, but perhaps the combinations of lipids in the SC that are most influential in impeding water loss.

Larks in arid environments had a higher proportion of ceramides and a smaller proportion of free fatty acids in their SC, an adjustment that apparently influences CWL. This implies that subtle changes in the ratios of lipid classes can alter the movement of water vapor through the skin. We propose that

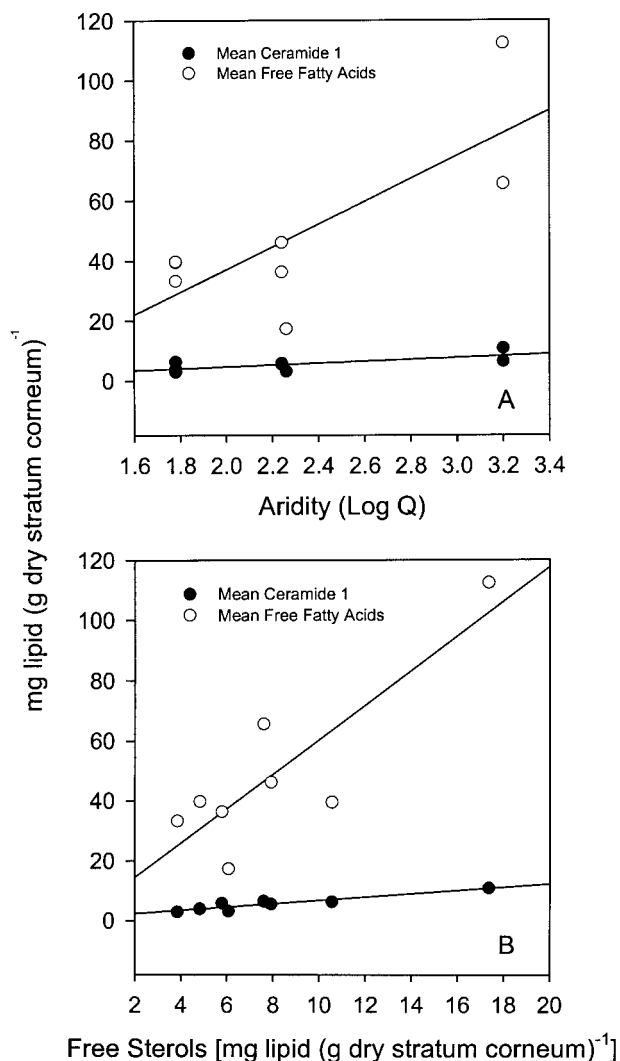


Figure 5. A, Mean ceramide 1 and mean free fatty acid amounts extracted from the stratum corneum of eight species of larks as a function of environmental aridity. B, Relationship between mean ceramide 1 and mean free fatty acid amounts with respect to free sterol amounts from the stratum corneum of larks.

desert birds have higher proportions of ceramides in their SC and lower proportions of free fatty acids because this combination allows the SC to exist in a more highly ordered crystalline phase (Bouwstra et al. 2003) and consequently creates a tighter barrier to water-vapor diffusion.

In birds, the current model posits that ceramides or their precursors can be found in membrane-bound multigranular bodies within the corneocytes, some held there as a reserve to be used when water conservation was necessary, whereas others are extruded into the intercellular matrix, the latter forming the permeability barrier (Menon et al. 1989). Our protocol for quantifying lipids of the SC would only recover intercellular

lipids and not those sequestered within corneocytes. Menon et al. (1989) further proposed that in zebra finches, intracellular lipids within multigranular bodies are normally degraded into free fatty acids and sphingosine bases as terminal differentiation of the SC proceeds, leading to a leakier barrier and permitting enhanced evaporative cooling when birds are fully hydrated. However, because larks do not use CWL as a thermoregulatory mechanism at high  $T_a$ 's but rely primarily on respiratory water loss under these circumstances (Tieleman and Williams 2002), it is unlikely that this explanation holds in these species. In water-deprived zebra finches, contents of multigranular bodies were universally extruded into the matrix of the SC, increasing the barrier to water-vapor diffusion.

The question remains, how does an alteration in the proportion of free fatty acids influence an increase in CWL? Application of unsaturated fatty acids such as oleic acid to the SC leads to disruption of barrier function, at least in mammals (Naik et al. 1995; Jiang and Zhou 2003). Two mechanisms have been proposed to explain how this might work: free fatty acids cause an increase in the fluidity of the ceramide bilayer, or alternatively, they cause lacunae to form in the ceramide bilayers, reducing the diffusional resistance to enhance water molecule transport through the SC. Because Tieleman and Williams (2002) have shown that differences in CWL between desert and mesic larks cannot be attributed to acclimatory processes, we think that larks in deserts have evolved a SC that contains fewer free fatty acids and more ceramides in lipid bilayers in the matrix.

In summary, larks from hot, dry environments have a lower CWL than birds from cool, moist environments. This reduction in CWL is associated with an increase in the proportion of polar ceramides in the intercellular matrix of the SC and a reduction in the proportion of free fatty acids. We are currently evaluating whether these changes in composition of the SC can be attributed to developmental phenotypic plasticity, acclimation, or natural selection.

#### Acknowledgments

We wish to express our appreciation to the National Commission for Wildlife Conservation and Development (NCWCD), Riyadh, Saudi Arabia, for supporting our research efforts. Wildlife research programs at the National Wildlife Research Center (NWRC) have been made possible through the generous support of His Royal Highness Prince Saud Al Faisal and under the guidance of Dr. Abdulaziz H. Abuzinada. Funding for this project was received from the NWRC, the National Science Foundation (IBN-0212092 to J.B.W.), the Ohio State University, the Schuurman Schimmel van Outeren Foundation, and the Schure Beijerinck Popping Foundation. Experiments were approved by NCWCD. For their support and assistance, we thank Naomi Chen, Dr. Gopinathan Menon, and Michael Buschur.

## Appendix A

Table A1: Mean amounts of free fatty acids (FFA), free sterols, and ceramides in the stratum corneum of larks

Species	N	FFA <sup>a</sup>	Free Sterols	Total Ceramides	Ceramide 1	Ceramide 2	Ceramide 3	Ceramides 4 and 5	Ceramide 6
Crested lark	7	36.43 <sup>AB</sup> (5.15)	5.78 <sup>AB</sup> (1.85)	39.83 <sup>D</sup> (6.70)	5.87 <sup>A</sup> (1.80)	1.79 <sup>A</sup> (.75)	6.57 <sup>ABC</sup> (1.14)	17.40 <sup>C</sup> (6.99)	4.98 <sup>ABC</sup> (1.60)
Black-crowned finchlark	5	46.21 <sup>AB</sup> (2.45)	7.92 <sup>AB</sup> (1.30)	34.31 <sup>BCD</sup> (8.17)	5.58 <sup>A</sup> (1.59)	1.58 <sup>A</sup> (.56)	9.15 <sup>BC</sup> (5.75)	10.69 <sup>ABC</sup> (4.14)	7.31 <sup>C</sup> (5.31)
Desert lark	7	39.57 <sup>AB</sup> (11.20)	10.56 <sup>B</sup> (2.56)	36.49 <sup>CD</sup> (6.00)	6.32 <sup>AB</sup> (2.28)	1.18 <sup>A</sup> (.40)	10.15 <sup>C</sup> (1.47)	14.36 <sup>BC</sup> (4.44)	4.46 <sup>ABC</sup> (1.46)
Calandra lark	2	17.41 <sup>A</sup> (1.15)	6.07 <sup>AB</sup> (3.30)	21.41 <sup>AB</sup> (4.79)	3.20 <sup>A</sup> (1.11)	2.46 <sup>A</sup> (6.08)	1.83 <sup>A</sup> (.007)	7.19 <sup>AB</sup> (.50)	6.74 <sup>BC</sup> (2.57)
Skylark	5	65.68 <sup>BC</sup> (27.88)	7.59 <sup>AB</sup> (3.38)	24.02 <sup>ABC</sup> (11.73)	6.53 <sup>AB</sup> (4.49)	2.23 <sup>A</sup> (1.40)	6.02 <sup>ABC</sup> (2.66)	5.66 <sup>A</sup> (4.64)	3.58 <sup>ABC</sup> (2.14)
Woodlark	6	112.40 <sup>C</sup> (60.57)	17.36 <sup>C</sup> (6.46)	30.64 <sup>ABCD</sup> (7.45)	10.89 <sup>B</sup> (4.63)	2.91 <sup>A</sup> (1.43)	9.43 <sup>BC</sup> (7.03)	8.16 <sup>AB</sup> (3.90)	3.08 <sup>AB</sup> (1.64)
Hoopoe lark	9	39.80 <sup>AB</sup> (13.99)	4.82 <sup>A</sup> (1.54)	26.10 <sup>ABC</sup> (5.14)	3.95 <sup>A</sup> (.92)	2.41 <sup>A</sup> (1.30)	6.07 <sup>ABC</sup> (1.85)	10.49 <sup>ABC</sup> (2.45)	3.19 <sup>AB</sup> (.58)
Dunn's lark	15	33.37 <sup>AB</sup> (6.21)	3.85 <sup>A</sup> (.49)	19.01 <sup>A</sup> (4.49)	3.01 <sup>A</sup> (1.80)	1.57 <sup>A</sup> (.61)	3.59 <sup>AB</sup> (1.67)	8.47 <sup>AB</sup> (3.46)	2.34 <sup>A</sup> (1.29)
ANOVA:									
<i>F</i>		8.76	16.15	9.83	7.03	2.47	4.90	5.37	4.35
<i>P</i>		<.001	<.001	<.001	<.001	.030	<.001	<.001	.001

Note: Mean numbers are followed by standard deviations in parentheses. The mean numbers represent homogenous subsets of means. Means with different superscripts are significantly different from other means in that category.

<sup>a</sup> Units for lipid means are mg lipid/g stratum corneum dry mass.

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