

## Phenotypic flexibility in cutaneous water loss and lipids of the stratum corneum

Michael J. Haugen<sup>1</sup>, B. Irene Tieleman<sup>2,\*</sup> and Joseph B. Williams<sup>1,†</sup>

<sup>1</sup>Department of Evolution, Ecology and Organismal Biology, 318 W. 12th Avenue, Columbus, OH 43210, USA and

<sup>2</sup>Zoological Laboratory, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

\*Present address: Department of Biology, University of Missouri St Louis, St Louis, MO 63121-4499, USA

†Author for correspondence (e-mail: williams.1020@osu.edu)

Accepted 11 July 2003

### Summary

When vertebrates invaded land during the Carboniferous period, they were exposed not only to new ecological opportunities but also to a desiccating environment. To maintain cellular water homeostasis, natural selection modified the integument of pioneering terrestrial animals, enabling them to reduce water loss through the skin. In desert environments, where ambient temperatures ( $T_a$ ) can reach 50°C, relative humidities are low and drinking water is scarce, integumentary modifications that reduce cutaneous water loss (CWL) could be fundamental to survival. Previous research has shown that hoopoe larks (*Alaemon alaudipes*) from the Arabian desert reduced CWL when acclimated to 35°C compared with individuals at 15°C, but skylarks (*Alda arvensis*) and woodlarks (*Lullula arborea*), from The Netherlands, and Dunn's larks (*Eremalauda dunnii*), also from the Arabian desert, did not. Here, we test the idea

that hoopoe larks acclimated to 35°C would alter the lipid composition of their stratum corneum (SC), resulting in a decrease in CWL, but that skylarks, woodlarks and Dunn's larks would not. Specifically, we hypothesized that hoopoe larks, acclimated to 35°C, would increase the proportions of polar ceramide content and decrease the proportions of free fatty acids in their SC compared with individuals acclimated to 15°C. Results showed that hoopoe larks at 35°C had lower CWL and higher proportions of total ceramides but lower proportions of free fatty acids and sterols in their SC. We demonstrate that adjustments in ratios of lipid classes in the SC are associated with changes in CWL in hoopoe larks.

Key words: cutaneous water loss, stratum corneum, ceramide, free fatty acid, lipid, hoopoe lark, *Alaemon alaudipes*.

### Introduction

When vertebrates invaded land during the Carboniferous period, they were exposed not only to new ecological opportunities but also to a desiccating environment (Williams and Tieleman, 2002). To maintain cellular water homeostasis in such an environment, natural selection modified the integument of pioneering terrestrial animals, enabling them to reduce water loss through the skin. Nowhere is this modification of the integument more important than for animals living in hot, desert environments, where ambient temperatures ( $T_a$ ) can reach 50°C, relative humidity is low and drinking water is scarce (Noy-Meir, 1973; Williams and Tieleman, 2002). Given these harsh conditions, one might imagine that phenotypes with minimal evaporative water loss are favored in desert environments.

The sum of evaporative water loss from respiratory passages and from skin – total evaporative water loss (TEWL) – is the major avenue of water loss to the environment, especially for small species of birds in which TEWL is five times greater than urinary/fecal water loss (Bartholomew, 1972; Dawson, 1982; Williams and Tieleman, 2000). There exists substantial evidence that TEWL is reduced in species of birds that live in

deserts, with mesic species having rates of TEWL nearly double those of species from deserts (Williams, 1996; Williams and Tieleman, 2000; Tieleman et al., 2003).

Early investigators assumed that most evaporative water loss took place across respiratory passages and that cutaneous water loss (CWL) played a minor role in thermoregulation (Mount, 1979; Bartholomew and Cade, 1963). Subsequent work has shown that CWL can equal or exceed evaporation from respiratory passages at moderate  $T_a$ s (Dawson, 1982; Tracy and Walsberg, 2001; Webster and King, 1987; Wolf and Walsberg, 1996). In a study on larks, Tieleman and Williams (2002) found that CWL accounted for 50–70% of TEWL at 25°C and that surface-area-specific CWL was about 30% lower in arid species compared with ones living in mesic environments. They hypothesized that natural selection has equipped resident desert birds with a mechanism(s) that reduces water loss through the skin, at least at moderate  $T_a$ s, as a water conservation mechanism.

Natural selection could be responsible for reduced TEWL and CWL found in desert larks (Bennett, 1997; Feder et al., 2000; Williams and Tieleman, 2001; Tieleman et al., 2003).

We know that the magnitude of TEWL is a heritable trait, at least in mammals (Furuyama and Ohara, 1993), and, by inference, we assume that rates of CWL are influenced by heritable factors. Hence, it could be that phenotypes with a stronger barrier to water vapor diffusion have been selected in deserts. Alternatively, phenotypic plasticity, including reversible phenotypic flexibility (acclimation; Piersma and Lindstrom, 1997) and developmental plasticity (Pigliucci, 2001), can lead to covariation between physiological traits and environmental variables. Studies on the relative importance of phenotypic plasticity and genetic variation in the production of phenotypic traits among birds are few. Williams and Tieleman (2000) tested whether hoopoe larks (*Alaemon alaudipes*) acclimated to 15°C or 35°C for 3 weeks altered their TEWL; they found that those acclimated to 35°C had a TEWL 23% lower than the 15°C group. This acclimation experiment was repeated the following year and also included skylarks (*Alauda arvensis*) and woodlarks (*Lullula arborea*), from The Netherlands, and Dunn's larks (*Eremalauda dunnii*), from the Arabian desert. Results showed that skylarks, woodlarks and Dunn's larks did not change their TEWL significantly with acclimation temperature, but that hoopoe larks did, the latter finding being consistent with previous results; mass-specific TEWL for hoopoe larks was 22% lower in the 35°C group (Tieleman and Williams, 2002). In all experiments, TEWL was consistently lower in desert species, and acclimation could not account for the magnitude of differences (Tieleman and Williams, 2002).

Tieleman and Williams (2002) also showed that changes in TEWL were attributable to changes in CWL and not respiratory water loss (RWL). CWL in the hoopoe lark decreased by 22% in the 35°C-acclimated group but did not decrease for the other three species. These results suggest that hoopoe larks alter the permeability of their skin in response to acclimation to temperature but that the other three species do not.

The skin of birds is composed of a thicker (120 µm) vascularized dermis and a thin (13–22 µm) non-vascular epidermis that has two layers: (1) a viable layer composed of the stratum transitivum, stratum intermedium and the mitotically active stratum basale and (2) an outer layer of cornified non-living cells (corneocytes) embedded in a lipid matrix called the stratum corneum (SC; Lucas and Stettenheim, 1972). The lipid layers 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 mostly on mammals, but also a few domestic species of birds, evidence has accumulated that the SC forms the barrier to water vapor diffusion from the animal to the environment (Blank, 1953; Blank et al., 1984; Golden et al., 1987; Warner et al., 1988; Elias and Menon, 1991; Peltonen et al., 2000).

Haugen et al. (2003) have shown that free fatty acids,

cholesterol and ceramides are the major constituents of SC in wild larks (skylarks, woodlarks, hoopoe larks and Dunn's larks) from mesic, semi-arid and arid environments. They reported that CWL was reduced among larks inhabiting deserts, but their data did not support the hypothesis that birds from desert environments have larger quantities of lipids per unit dry mass of the SC compared with 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 CWL. This implies that subtle changes in the ratios of lipid classes alter the movement of water vapor through the skin. They proposed 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 (Bouwstra et al., 2003).

Given that hoopoe larks adjust their CWL when acclimated to 35°C compared with individuals acclimated to 15°C but that skylarks, woodlarks and Dunn's larks do not (Tieleman and Williams, 2002), we predicted that hoopoe larks acclimated to 35°C would alter the lipid composition of their SC, resulting in a decrease in CWL, but that skylarks, woodlarks and Dunn's larks would not. In particular, in light of the results of Haugen et al. (2003), we hypothesized that hoopoe larks acclimated to 35°C would increase the proportions of polar ceramide content and decrease the proportions of free fatty acids in their SC compared with individuals acclimated to 15°C but that skylarks, woodlarks (both mesic species) and Dunn's larks (another desert species) would not alter ratios of lipid classes in their SC with acclimation.

## Materials and methods

### Capture of birds

Using mist nets, we captured skylarks (*Alauda arvensis* L.) and woodlarks (*Lullula arborea* L.) in The Netherlands and Hoopoe larks (*Alaemon alaudipes* Desfontaines) and Dunn's larks (*Eremalauda dunnii* Shelly) in Saudi Arabia. Climate data and geographical locations 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 captured 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 maximum  $T_a$  in July is 40.2°C (National Wildlife Research Center, unpublished).

### Acclimation

In order to assess the flexibility in lipids of the SC as a result of acclimation, we randomly assigned individuals to one of two

groups ( $N=8$  in each group). In each group, half of the individuals from each species were housed for 3 weeks in a room with a constant  $T_a$  of  $15\pm 2^\circ\text{C}$  (12 h:12 h L:D), a temperature that is below the thermal neutral zone of all four species and close to the  $T_a$  experienced by skylarks and woodlarks in The Netherlands during the breeding season, and the other half were housed for 3 weeks in a room with a constant  $T_a$  of  $35\pm 2^\circ\text{C}$  (12 h:12 h L:D), which mimicked the  $T_a$  of the Arabian desert during spring. Each treatment group contained equal proportions of males and females and was similar in mean body mass. Humidity was not controlled in rooms where birds were housed but was recorded with a relative humidity probe (Vaisala, Woburn, MA, USA) or with a dew point hygrometer (M4; General Eastern, Woburn, MA, USA). The absolute humidity was  $5\text{--}7\text{ g m}^{-3}$  in the  $15^\circ\text{C}$  rooms and  $9\text{--}12\text{ g m}^{-3}$  in the  $35^\circ\text{C}$  rooms (Tieleman et al., 2003).

#### Cutaneous water loss

For measuring CWL [ $\text{mg H}_2\text{O cm}^{-2}\text{ day}^{-1}$ ] at  $25^\circ\text{C}$ , we followed the protocol of Tieleman and Williams (2002). In brief, we measured RWL and CWL simultaneously by placing a bird fitted with a plastic mask in a metabolism chamber into which dry,  $\text{CO}_2$ -free outside air was drawn. Steel metabolic chambers had an airtight Plexiglas lid and were water-jacketed to control  $T_a$  by a circulating water bath (RTE-140; Neslab, Portsmouth, NH, USA;  $+0.2^\circ\text{C}$ ). 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. We measured the water vapor density of air entering the chamber ( $\rho_{v\text{-in}}$ ) by routing air before and after experiments around the chamber and through the dewpoint hygrometer. Air pulled through the mask contained all respiratory gases and was routed through polytetrafluoroethylene (PTFE) tubing, a dew point hygrometer, columns of Drierite and ascarite (to remove  $\text{H}_2\text{O}$  and  $\text{CO}_2$ ), a previously calibrated Brooks mass flow controller (model 5850E; Levy, 1964) and a vacuum pump. Chamber volume and flow rates used are reported in Tieleman and Williams (2002). We calculated the flow rate through the dew point hygrometer ( $\dot{V}_{e1}$ ) by adjusting the value recorded at the mass flow controller for  $\text{H}_2\text{O}$  and  $\text{CO}_2$  added (Tieleman and Williams, 2002), the latter estimated assuming a respiratory quotient (RQ) of 0.71 (King and Farner, 1961). In practice, these adjustments were less than 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 flow rate of air leaving the chamber by summing the flow rates from the mask ( $\dot{V}_{e1}$ ) and from the chamber ( $\dot{V}_{e2}$ ) and determined CWL using the equation  $\text{CWL}=(\rho_{v\text{-chamber}} - \rho_{v\text{-in}})(\dot{V}_{e1} + \dot{V}_{e2})$ . 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 the  $T_a$  in the chamber using a data logger

model (21X or CR23X; Campbell Scientific, Logan, UT, USA). 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, together with water lost from the eyes. CWL measurements were taken for each bird before and after the 3-week acclimation period.

#### Separation of stratum corneum

After the final determination of CWL, we determined the mass of each bird, sacrificed it, plucked its feathers and removed its skin. We pinned the skin to a thin sheet of Teflon and dipped it in distilled water at  $65^\circ\text{C}$  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 ( $4^\circ\text{C}$ ) overnight. The following day, we rinsed the intact SC tissue with distilled  $\text{H}_2\text{O}$  to remove epithelial cells, re-immersed the remaining tissue in fresh 0.5% trypsin solution and allowed it to stand at  $38\pm 2^\circ\text{C}$  for 3 h. After a final rinse in distilled  $\text{H}_2\text{O}$ , 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 USA, we re-lyophilized 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 v/v for 2 h at each step (Law et al., 1995). Chemicals and tissues were placed in covered glass funnels equipped with Teflon stopcocks and pre-extracted 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  $200\text{ }\mu\text{l}$  chloroform:methanol 2:1 containing the antioxidant butylated hydroxytoluene ( $50\text{ mg l}^{-1}$ ).

We separated classes of lipids using analytical TLC on  $20\text{ cm}\times 20\text{ cm}$  glass plates coated with silicic acid (0.25 mm thick; Adsorbosil-Plus 1; Alltech Associates, Deerfield, IL, USA; Downing, 1968). Identification of lipids was achieved by comparison of 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, thereby removing any contaminants, activated them in an oven at  $110^\circ\text{C}$  and scored the adsorbent into 6 mm-wide lanes (Wertz et al., 1986). Using a Teflon-tipped Hamilton syringe (#80055; Hamilton, Reno, NV, USA), we pipetted  $5\text{ }\mu\text{l}$  of either lipid extract from the SC or a mixture of lipid standards onto a pre-absorbent 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 non-hydroxy 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, USA), and dissolved in chloroform:methanol (2:1) in concentrations from 0.625 mg ml<sup>-1</sup> to 10 mg ml<sup>-1</sup>.

Two solvent systems were employed to separate lipids of different polarities. For the more non-polar 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) and then placing the plate on a 20 cm×20 cm polished aluminum hotplate that was slowly heated to 220°C, 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 tnmage (<http://entropy.brni-jhu.org/tnimage.html>) to determine areas of charred regions. At the same time, we scanned lanes that contained a series of known concentrations of standards in order to establish standard curves (Vecchini et al., 1995; Johnson, 2000).

Although analytical TLC 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<sup>-1</sup> to 14.3 mg ml<sup>-1</sup>, onto plates, in triplicate, and followed our protocol for determining lipid concentrations (Haugen, 2003). We averaged values for the three lanes for each trial and compared these results with the known concentration. Calculated as (observed – actual/actual)×100, the mean error was ±0.82%, indicating that our method provides a reasonable estimate of quantities of lipids (Haugen et al., 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

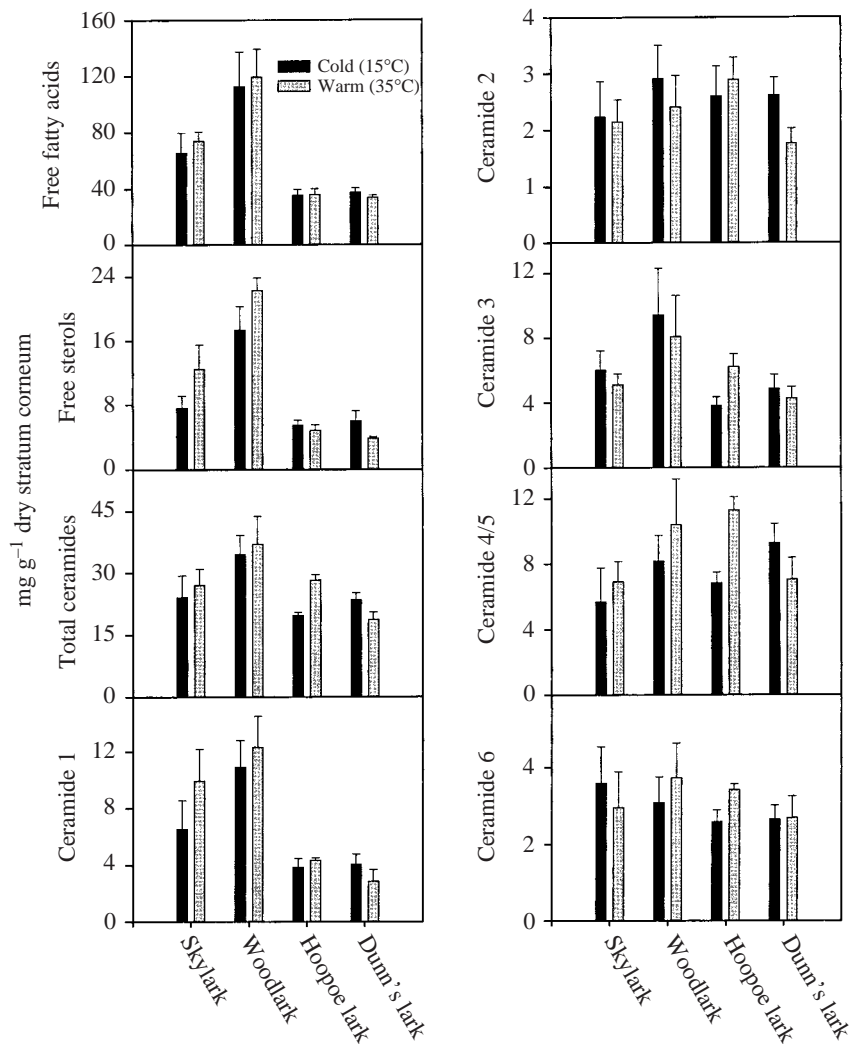


Fig. 1. The quantity of lipids (mg g<sup>-1</sup> dry stratum corneum) in the stratum corneum of skylarks, woodlarks, hoopoe larks and Dunn's larks. Black bars represent larks acclimated to 15°C for 3 weeks; gray bars represent larks acclimated to 35°C.

larks, we ran preparative TLC 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 tri-sodium salt (10 mg per 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×0.32 mm i.d. fused silica column and with a 0.25 µm 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  $P < 0.05$ . We used General Linear Model procedure for analysis of variance when comparing lipid quantities for each group of lipids from the SC of larks. Our model included lipid class as a dependent factor and both species and acclimation treatment as fixed factors. After testing for the significance of the two-way interaction term, we removed it when insignificant and tested for the significance of the fixed factors. For comparisons of lipid concentrations among species, we performed analysis of variance (ANOVA) followed by a Tukey's test to identify significant differences between species. To search for correlations, we calculated Spearman's rho. Means are in units of mg lipid  $g^{-1}$  dry mass of SC  $\pm 1$  S.E.M., unless otherwise specified. All proportions were arcsine transformed prior to statistical analyses.  $N=8$  for each species;  $N=8$  for each group. Experiments were carried out in The Netherlands under license #DEC2425, in Ohio under ILACUC permit #00A0161 and in Saudi Arabia with permission from the National Commission for Wildlife Research and Development.

## Results

### CWL and acclimation

Hoopoe larks acclimated to 35°C had a significantly lower CWL, by 22%, than individuals acclimated to 15°C when both groups were measured in a chamber at 25°C ( $F_{1,11}=4.92$ ,  $P < 0.05$ ; see also Tieleman and Williams, 2002). But CWL of Dunn's larks, skylarks and woodlarks, measured at 25°C, did not differ between acclimation treatments (Dunn's lark,  $F_{1,13}=0.05$ ,  $P=0.82$ ; skylark,  $F_{1,11}=2.94$ ,  $P=0.11$ ; woodlark,  $F_{1,11}=1.67$ ,  $P=0.22$ ).

### Among-species comparisons – lipid quantities

We investigated differences in quantities of lipid classes (mg  $g^{-1}$  dry mass SC) among species that were exposed to the different acclimation temperatures. There was no significant interaction between species and treatment for any of the lipid classes (all  $P > 0.05$ ), although the interaction term for percent total ceramides and for amount of cholesterol in the SC were nearly significant (Fig. 1;  $P=0.06$  for both). With the interaction term removed, we found significant effects for species for free fatty acids, free sterols, total ceramides, ceramide 1 and ceramide 3 ( $F=24.5$ ,  $P < 0.001$ ;  $F=32.4$ ,  $P < 0.001$ ;  $F=6.7$ ,  $P < 0.001$ ;  $F=15.2$ ,  $P < 0.001$ ;  $F=3.6$ ,  $P < 0.02$ ,

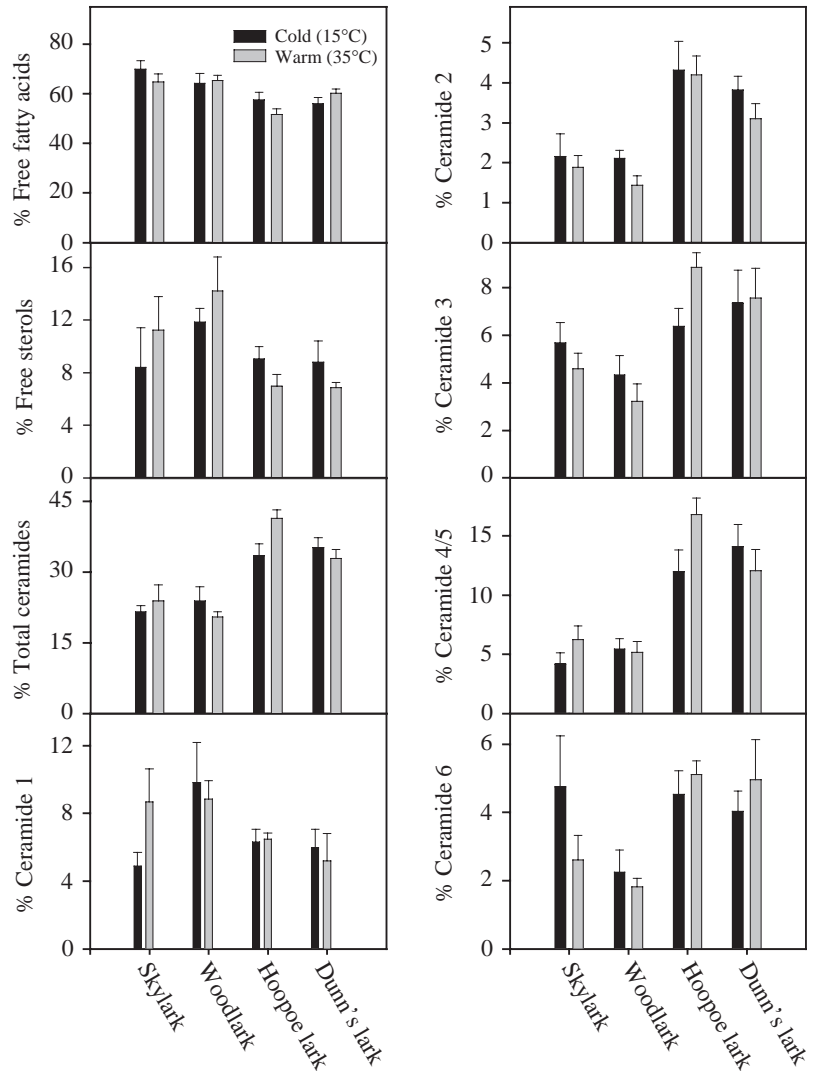


Fig. 2. Percentages of the major lipids in the stratum corneum of skylarks, woodlarks, hoopoe larks and Dunn's larks. Black bars represent larks acclimated to 15°C for 3 weeks; gray bars represent larks acclimated to 35°C.

respectively). A *post-hoc* test showed that hoopoe larks and Dunn's larks, both desert species, had significantly lower quantities of free fatty acids, cholesterol and ceramide 1 per unit dry mass of SC than did skylarks and woodlarks from The Netherlands. We did not find an effect of treatment in any species. However, for hoopoe larks, the 95% confidence intervals for total ceramides, ceramide 3 and ceramide 4/5 for the treatments did not overlap.

### Among-species comparisons – lipid proportions

Because proportions of lipid classes seem to be important in water barrier formation (Haugen et al., 2003), we also investigated the effect of acclimation on the proportion of each lipid class in the SC. With proportion of lipids as the dependent factor and species and acclimation treatment as fixed factors in our model, we found no significant interaction between species and treatment for any of the lipid classes (Fig. 2; all  $P > 0.05$ ).

When we removed the interaction term, we found significant differences between the species in proportions of free fatty acids, free sterols and total ceramides (Fig. 2;  $F=8.1$ ,  $P<0.001$ ;  $F=4.1$ ,  $P<0.02$ ;  $F=19.6$ ,  $P<0.001$ , respectively). Ceramides 2, 3, 4/5 and 6 were significantly different among species ( $F=13.6$ ,  $P<0.001$ ;  $F=6.2$ ,  $P<0.001$ ;  $F=16.4$ ,  $P<0.001$ ;  $F=4.2$ ,  $P<0.01$ ). *Post-hoc* tests revealed that the two desert species – Dunn's and hoopoe larks – formed homogeneous subsets for reduced proportions of free fatty acids and free sterols, while also forming a homogeneous subset for increased proportions of total ceramides when compared with the two mesic species – woodlarks and skylarks. Hoopoe larks and Dunn's larks had higher proportions of ceramides 2, 3, 4/5 and 6 in their SC than did woodlarks and skylarks. We found no significant effect of treatment in any of our comparisons.

#### CWL and lipids within hoopoe larks

Because we elucidated differences in CWL relative to acclimation  $T_a$  in hoopoe larks, but not other species, we explored changes among lipids in the SC of hoopoe larks

between treatment groups. Hoopoe larks acclimated to 35°C had significantly higher concentrations of total ceramide per unit dry mass of SC ( $F=30.6$ ,  $P<0.001$ ) and higher concentrations of polar ceramides: ceramides 3, 4/5 and 6 ( $F=6.4$ ,  $P<0.03$ ;  $F=17.7$ ,  $P<0.001$ ;  $F=5.8$ ,  $P<0.04$ , respectively). Hoopoe larks in the 35°C group also had significantly higher proportions of total ceramide in their SC ( $F=6.6$ ,  $P<0.03$ ) and higher proportions of ceramide 3 ( $F=6.5$ ,  $P<0.03$ ). Proportions of ceramide 4/5 were not significantly higher in the 35°C group ( $F=4.3$ ,  $P<0.06$ ). We note that the SC of hoopoe larks acclimated to 15°C contained ceramide that totaled 32.4% of the lipids that we measured, but the SC of birds acclimated to 35°C contained ceramide that totaled 40.8%, a significant increase for the latter of 26% ( $F=30.6$ ,  $P<0.001$ ). Hence, as CWL was reduced in the 35°C group, the proportion of total ceramide in the SC increased.

#### Correlations between CWL and lipids

We found a significant correlation with CWL and both % total ceramide (Fig. 3;  $r=-0.58$ ,  $P<0.03$ ,  $N=14$ ) and % ceramide 4 ( $r=-0.61$ ,  $P<0.03$ ,  $N=14$ ).

#### Correlations among lipids in the SC

Using all species, we found that both % free fatty acids and % free sterols were negatively correlated with % total ceramide (Fig. 4;  $r=-0.86$ ,  $P<0.001$ ,  $N=50$ ;  $r=-0.35$ ,  $P=0.013$ ,  $N=50$ , respectively). With values for individuals of each species averaged, % free fatty acids again correlated negatively with % total ceramides ( $r=-0.995$ ,  $P=0.005$ ,  $N=4$ ). However, there was no significant correlation between % free sterols and % total ceramides ( $r=-0.93$ ,  $P=0.07$ ,  $N=4$ ). The percentage of free fatty acids in the SC was also negatively correlated with the percentage of ceramides for fractions 2–6 ( $r=-0.52$ ,  $P<0.001$ ,  $N=50$ ;  $r=-0.34$ ,  $P=0.016$ ,  $N=50$ ;  $r=-0.78$ ,  $P<0.001$ ,  $N=50$ ;  $r=-0.49$ ,  $P<0.001$ ,  $N=50$ , respectively).

#### Discussion

Larks from the desert had higher proportions of ceramides, especially the more polar ceramides, and lower proportions of both free fatty acids and sterols (cholesterol) in their SCs compared with larks from mesic environments, a finding consistent with Haugen et al. (2003). Moreover, we found a negative correlation between the proportions of free fatty acids and total ceramides. We have argued that the combination of increased proportions of ceramides and decreases in proportions of free fatty acids results in a decrease in fluidity of the layers of the SC, which, in turn, causes a decrease in CWL (Wertz, 2000; Bouwstra et al., 2003).

Given that hoopoe larks adjust their CWL when acclimated to 35°C compared with individuals acclimated to 15°C, but skylarks, woodlarks and Dunn's larks do not (Tieleman and Williams, 2002), we predicted that hoopoe larks acclimated to 35°C would increase the proportions of polar ceramides and decrease the proportions of free fatty acids in their SC compared with individuals acclimated to 15°C, but that skylarks,

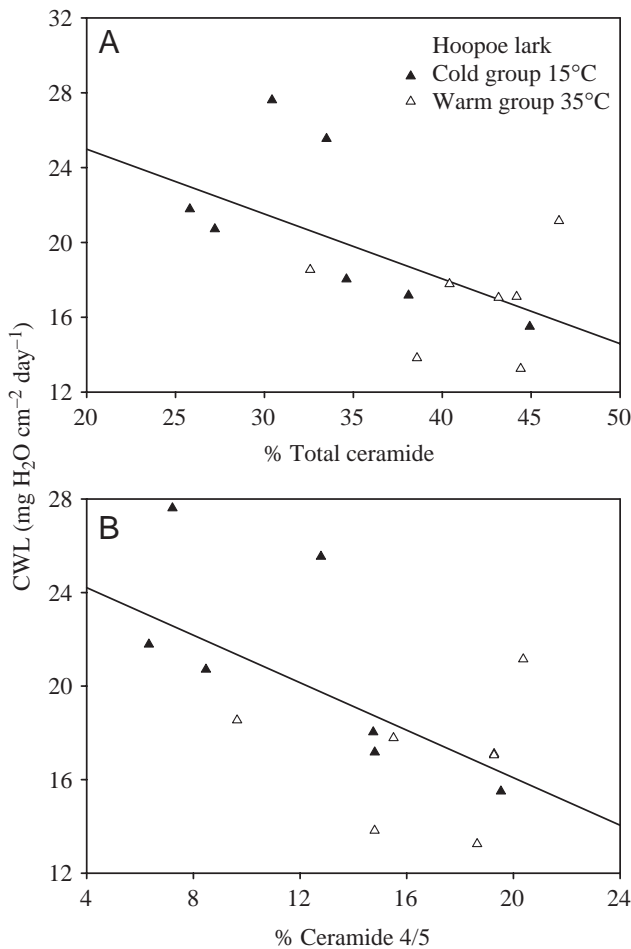


Fig. 3. Cutaneous water loss (CWL) in hoopoe larks as a function of (A) the percentage of total ceramides and (B) the percentage of ceramide 4/5 in cold-acclimated (15°C) and warm-acclimated (35°C) birds.

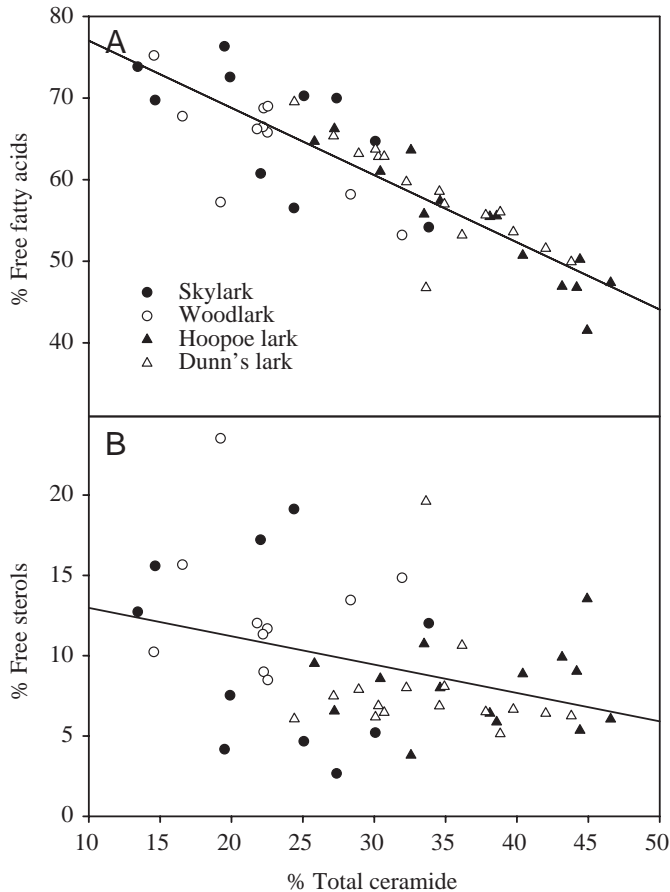


Fig. 4. The correlation between the proportions of free fatty acids and free sterols and the proportion of total ceramides from the stratum corneum among different species of larks.

woodlarks (two mesic species) and Dunn's larks (another desert species) would not. Hoopoe larks in the 35°C group had significantly higher proportions of total ceramide in their SC and higher proportions of ceramide 3. Hence, as CWL was reduced in the 35°C group, the proportion of total ceramide in the SC increased, in partial agreement with our hypothesis. We did not find a significant decrease in the proportion of free fatty acids, although the % of free fatty acids declined by 10.9%. Hence, we think that by adjusting lipid synthesis towards more polar ceramides and less free fatty acids, hoopoe larks can alter the permeability of their skin to water.

When comparing hoopoe larks acclimated to 15°C and 35°C, the 35°C group had a 19.2% decrease in total evaporative water loss and an 18.9% decrease in CWL, which corresponded with a 25.9% increase in the proportion of total ceramides in the SC. We did not see any significant changes in amounts or proportions of lipids in the SC among species that did not alter their CWL in response to acclimation. Why Dunn's larks do not adjust their CWL to acclimation temperature is not known, but it is noteworthy that hoopoe larks are permanent residents in the extreme arid regions in the Arabian Peninsula, including the Rub 'al Khali, whereas Dunn's larks emigrate from arid regions during severe drought.

In summary, the proportion of ceramides and free fatty acids in the stratum corneum in larks affects the rate of water loss through the skin. The lower CWL and TEWL rates found in species of larks (Williams and Tieleman, 2001; Tieleman and Williams, 2003; Haugen et al., 2003; Tieleman et al., 2003) can be partially explained by a difference in the proportion of total ceramides to free fatty acids in the SC, where an increased proportion of ceramides causes a decrease in the water flux across the skin. Hoopoe larks acclimated to 35°C had an 18.9% decrease in CWL compared with the group acclimated to 15°C, which was due to a change in the lipid composition in the SC. Specifically, it seems that an increase in the proportion of the more polar ceramides and a decrease in the proportion of free fatty acids caused a tighter barrier to water vapor in the skin of hoopoe larks.

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 HRH 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; J.B.W.) and The Ohio State University. Experiments were approved by NCWCD, Riyadh, Saudi Arabia. For their support and assistance, we thank P. Wertz, N. Chen, G. Menon and M. Buschur.

## References

- Bartholomew, G. A. (1972). The water economy of seed-eating birds that survive without drinking. In *Proceedings of the 15th International Ornithology Congress* (ed. K. H. Voous), pp. 1-16. Leiden, The Netherlands: E. J. Brill.
- Bartholomew, G. A. and Cade, T. J. (1963). The water economy of land birds. *Auk* **80**, 504-539.
- Bennett, A. F. (1997). Adaptation and the evolution of physiological characters. In *Handbook of Physiology* (ed. W. Dantzer), pp. 1-16. Oxford: Oxford University Press.
- Blank, I. H. (1953). Further observations on factors which influence the water content of the stratum corneum. *J. Invest. Dermatol.* **21**, 259-269.
- Blank, I. H., Moloney, J., Emslie, A. G. and Simon, I. (1984). The diffusion of water across the stratum-corneum as a function of its water-content. *J. Invest. Dermatol.* **82**, 188-192.
- Bleton, J., Gaudin, K., Chaminade, P., Goursaud, S., Baillet, A. and Tchaplal, A. (2001). Structural analysis of commercial ceramides by gas chromatography-mass spectrometry. *J. Chromatog. A* **917**, 251-260.
- Bouwstra J. A., Honeywell-Nguyen, P. L., Gooris, G. S. and Ponc, M. (2003). Structure of the skin barrier and its modulation by vesicle formations. *Prog. Lipid Res.* **42**, 1-36.
- Dawson, W. R. (1982). Evaporative losses of water by birds. *Comp. Biochem. Physiol. A* **71**, 495-509.
- Downing, D. T. (1968). Photodensitometry in the thin-layer chromatographic analysis of neutral lipids. *J. Chromatog.* **38**, 91-99.
- Elias, P. M. and Friend, D. S. (1975). The permeability barrier in mammalian epidermis. *J. Cell Biol.* **65**, 180-191.
- Elias, P. M. and Menon, G. K. (1991). Structural and lipid biochemical correlates of the epidermal permeability barrier. *Adv. Lipid Res.* **24**, 1-26.
- Feder, M. E., Bennett, A. F. and Huey, R. B. (2000). Evolutionary physiology. *Annu. Rev. Ecol. Syst.* **31**, 315-341.
- Furuyama, F. and Ohara, K. (1993). Genetic development of an inbred rat strain with increased Resistance adaptation to a hot environment. *Am. J. Physiol.* **265**, R957-R962.

- Golden, G. M., Guzek, D. B., Kennedy A. H., McKie, J. E. and Potts, R. O.** (1987). Stratum corneum lipid phase-transitions and water barrier properties. *Biochemistry* **26**, 2382-2388.
- Gray, G. M. and Yardley, H.** (1975). Lipid compositions of cells isolated from pig, human and rat epidermis. *J. Lipid Res.* **16**, 434-440.
- Haugen, M. J., Tieleman, L., Wertz, P. W. and Williams, J. B.** (2003). Lipids in the stratum corneum of the epidermis and cutaneous water loss among larks along a temperature-moisture gradient. *Physiol. Biochem. Zool.*, in press.
- Haugen, M. J.** (2003). Cutaneous water loss and lipids in the stratum corneum of larks. *PhD Thesis*, The Ohio State University.
- Johnson, M. E.** (2000). Simple quantitation in thin-layer chromatography using a flatbed scanner. *J. Chem. Edu.* **77**, 368-372.
- King, J. R. and Farner, D. S.** (1961). Energy metabolism, thermoregulation and body temperature. In *Biology and Comparative Physiology of Birds*, vol II (ed. A. J. Marshall), pp. 215-288. New York: Academic Press.
- Law, S., Wertz, P. W., Swartzendruber, D. C. and Squier, C. A.** (1995). Regional variation in content, composition and organization of porcine epithelial barrier lipids revealed by thin-layer chromatography and transmission electron microscopy. *Arch. Oral Biol.* **40**, 1085-1091.
- Levy, A.** (1964). The accuracy of the bubble meter for gas flow measurements. *J. Sci. Instr.* **41**, 449-453.
- Lucas, A. M. and Stettenheim, P. R.** (1972). Avian anatomy: integument. In *Agricultural Handbook 362*, pp. 673-696. Washington, DC: US Government Printing Office.
- Menon, G. K. and Menon, J.** (2000). Avian epidermal lipids: functional considerations and relationship to feathering. *Am. Zool.* **40**, 540-552.
- Mount, L. E.** (1979). *Adaptation to Thermal Environment*. London: Edward Arnold.
- Noy-Meir, I.** (1973). Desert ecosystems: environment and producers. *Annu. Rev. Ecol. Syst.* **4**, 25-41.
- Peltonen, L., Arieli, Y., Harjula, R., Pyörnilä, A. and Marder, J.** (2000). Local cutaneous water barrier in cold- and heat-acclimated pigeons (*Columba livia*) in relation to cutaneous water evaporation. *J. Morphol.* **246**, 118-130.
- Piersma, T. and Lindstrom, A.** (1997). Rapid reversible changes in organ size as a component of adaptive behaviour. *Trends. Ecol. Evol.* **12**, 134-138.
- Pigliucci, M.** (2001). *Phenotypic Plasticity: Beyond Nature and Nurture*. Baltimore: Johns Hopkins University Press.
- Raith, K. and Neubert, R. H.** (2000). Liquid chromatography-electrospray mass spectrometry and tandem mass spectrometry of ceramides. *Anal. Chim. Acta.* **403**, 295-303.
- Tieleman, B. I. and Williams, J. B.** (2002). Cutaneous and respiratory water loss in larks from arid and mesic environments. *Physiol. and Biochem. Zoo.* **75**, 590-599.
- Tieleman, B. I., Williams, J. B. and Bloomer, P.** (2003). Adaptation of metabolism and evaporative water loss along an aridity gradient. *Proc. R. Soc. Lond. B* **270**, 207-214.
- Tracy, R. L. and Walsberg, G. E.** (2001). Developmental and acclimatory contributions to water loss in a desert rodent: investigating the time course for adaptive change. *J. Comp. Physiol. B* **171**, 669-679.
- Vecchini A., Chiaradia E., Covalovo S. and Binaglia, L.** (1995). Quantitation of phospholipids on thin-layer chromatographic plates using a desk-top scanner. *Mol. Cell. Biochem.* **145**, 25-28.
- Warner, R. R., Myers, M. C. and Taylor, D. A.** (1988). Electron probe analysis of human skin: Determination of the water concentration profile. *J. Invest. Dermatol.* **96**, 495-499.
- Webster, M. D. and King, J. R.** (1987). Temperature and humidity dynamics of cutaneous and respiratory evaporation in pigeons, *Columba livia*. *J. Comp. Physiol. B* **157**, 253-260.
- Wertz, P. W., Stover, P. M. and Downing, D. T.** (1986). Lipids of the chicken epidermis. *J. Lipid Res.* **27**, 427-435.
- Wertz, P. W.** (2000). Lipids and barrier function of the skin. *Acta Derm-Venereol* **7-11**, Suppl. 208.
- Williams, J. B.** (1996). A phylogenetic perspective of evaporative water loss in birds. *Auk* **113**, 457-472.
- Williams, J. B. and Tieleman, B. I.** (2000). Flexibility in basal metabolism and evaporative water loss in hoopoe larks from the Arabian desert. *J. Exp. Biol.* **203**, 153-159.
- Williams, J. B., and Tieleman B. I.** (2001). Physiological Ecology and behavior of desert birds. *Curr. Ornithol.* **16**, 299-353.
- Williams, J. B. and Tieleman, B. I.** (2002). Ecological and evolutionary physiology of desert birds: A progress report. *Integ. Comp. Biol.* **42**, 68-75.
- Wolf, B. O. and Walsberg, G. E.** (1996). Respiratory and cutaneous evaporative water loss at high environmental temperatures in a small bird. *J. Exp. Biol.* **199**, 451-457.