Lipid composition of the stratum corneum and cutaneous water loss in birds along an aridity gradient

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Abbreviations: SC = stratum corneum, CWL = cutaneous water loss, CBL = covalently bound lipids, BHT = butylated hydroxytoluene, TLC = thin layer chromatography, PIC = phylogenetic independent contrast, PCA = principal component analysis

Abstract

Intercellular and covalently bound lipids within the stratum corneum (SC), the outermost layer of the epidermis, are the primary barrier to cutaneous water loss (CWL) in birds. We compared CWL and intercellular SC lipid composition in 20 species of birds from desert and mesic environments. Furthermore, we compared covalently bound lipids with CWL and intercellular lipids in the lark family (Alaudidae). We found that CWL increases in birds from more mesic environments, and this increase was related to changes in intercellular SC lipid composition. The most consistent pattern that emerged was a decrease in the relative amount of cerebrosides as CWL increased, a pattern that is counterintuitive based on studies of mammals with Gaucher disease. Although covalently bound lipids in larks did not correlate with CWL, we found that covalently bound cerebrosides correlated positively with intercellular cerebrosides and intercellular cholesterol ester, and intercellular cerebrosides correlated positively with covalently bound free fatty acids. Our results led us to propose a new model for the organization of lipids in the avian SC, in which the sugar moieties of cerebrosides lie outside of intercellular lipid layers, where they may interdigitate with adjacent intercellular cerebrosides or with covalently bound cerebrosides.

Introduction

Among endotherms, birds have the highest mass-specific rates of total evaporative water loss (Williams and Tieleman, 2005), accounting for five times the water lost through urine and feces in many small bird species (Bartholomew, 1972; Dawson, 1982; Williams and Tieleman, 2000). Because cutaneous water loss (CWL) accounts for about 65% of total evaporative water loss (Tieleman and Williams, 2002; Ro and Williams, 2010), one can imagine that selection has modified the avian integument to minimize CWL, especially for species living in dry environments.

The outermost layer of the avian integument, the stratum corneum (SC), is composed of flat, dead cells called corneocytes embedded within a lipid matrix (Bouwstra, 1997). This lipid matrix is the primary barrier to water vapor diffusion from the animal to the environment (Menon et al., 1992; Simonetti et al., 1995; Meuwissen et al., 1998). The composition and arrangement of the lipids within this matrix appears to be critical in determining the rate of CWL.

The lipids of the avian SC consist primarily of cerebrosides, ceramides, cholesterol, free fatty acids, triacylglycerides, fatty acid methyl esters, and cholesterol esters (Menon et al, 1986, Haugen 2003a, Muñoz-Garcia et al., 2006; Ro and Williams, 2010). These lipids may be divided into two categories based on their arrangement within the SC, covalently bound lipids (CBL) and intercellular lipids. The CBL covalently bind to glutamate residues of the protein involucrin on the corneocyte surface at the ω end of each fatty acid chain (Swartzendruber et al. 1987, Wertz and Downing 1987, Wertz et al. 1989, Downing 1992, Stewart and Downing 2001). Although the exact mechanism is unknown, CBL are thought to orchestrate the structure of the intercellular lipids, which are organized into layers called lamellae (Wertz 2005; Koch et al. 2005; Gu et al., 2008). Current models of the SC, mostly constructed for the skin of mammals, envision that lipid molecules within lamellae of the SC interact to form bilayers (Wertz and Downing, 1982) or arrange in trilayers to form sandwich-like structures (Bouwstra, 2000).

A striking difference between mammalian and avian SC is the presence of cerebrosides in the latter (Muñoz-Garcia and Williams, 2005; Gu et al., 2008).

Cerebrosides are ceramides with a hexose moiety attached to the headgroup. In mammalian SC, the enzyme β-glucocerebrosidase cleaves the sugar group from cerebrosides before they reach the SC to convert them to ceramides. In mammals, a defect in this enzyme causes a pathological condition called Gaucher disease that results in scaly skin and high rates of CWL (Holleran et al., 1994). CWL may increase in patients with Gaucher disease because the bulky sugar moieties of the cerebrosides disrupt lipid packing and the hydroxyl groups of these sugars bind with water to increase SC hydration, and therefore permeability (Rawlings, 2005). In birds, β-glucocerebrosidase converts some, but not all, cerebrosides to ceramides (Cox et al., 2008), resulting in cerebrosides making up a substantial portion of the lipids in the SC (Muñoz-Garcia and Williams, 2005, Gu et al., 2008). However, despite the fact that birds have higher rates of CWL than do mammals, this does not seem to be a result of cerebroside content. In several studies, increases in cerebroside content in the SC were associated with decreases in rates of CWL. House sparrows (*Passer domesticus*) from the desert of Saudi Arabia exhibit rates of CWL 25% lower than conspecifics from mesic Ohio, and these desert house sparrows have higher percentages of both intercellular and covalently bound cerebrosides than house sparrows from Ohio (Gu et al., 2008; Muñoz-Garcia and Williams, 2005). Desert birds also tend to have a greater proportion of more polar ceramides with longer fatty acid tails, in agreement with mammalian models (Shaefer and Redelmaier, 1996; Haugen et al., 2003, Lillywhite, 2006; Muñoz-Garcia et al, 2008).

The correlation between low rates of CWL and higher amounts of cerebrosides in birds suggests a fundamental difference in the organization of intercellular and covalently bound lipids between mammals and birds. Furthermore, this correlation suggests that the arrangement of lipid classes, and the manner in which they interact, is more important than the composition of lipid classes per se. Models of the arrangement of lipids in the SC are valuable tools in predicting the roles of certain lipid classes in regulating CWL. In current models, intercellular lipids in avian SC are organized in trilayers (Muñoz-Garcia et al., 2005), and are separated from covalently bound lipids by a layer of water (Gu et al., 2008; Clement et al, 2012). However, these models are based only on data from house sparrows. Studies across a number of bird species from multiple

environments may help us understand how SC lipids are organized in birds relative to CWL. Haugen et al (2003) compared intercellular lipid composition in eight species of larks (Alaudidae) across an aridity gradient, but did not test for cerebrosides in their study, and Ro et al (2010) compared intercellular lipid composition in 12 species of birds from a mesic environment. Neither of these studies included covalently bound lipids in their analysis.

In this paper, we correlate CWL with environment and intercellular lipid composition in 20 species of birds from both mesic and arid environments. Furthermore, we correlate covalently bound lipids with environment and CWL for seven species of larks (Alaudidae) across disparate environments. Finally, we make comparisons between intercellular and covalently bound lipids in larks to make inferences about the potential interactions between intercellular and covalently bound lipids. Our results offer compelling evidence that an increase in cerebroside content in the avian SC is associated with a decrease in CWL, and that intercellular and covalently bound lipids interact in concert to form a barrier to water loss. Our findings prompted us to proffer a new model for lipid organization in the avian SC.

Methods

Capture of Birds

We captured larks with mist nets at several locations along an aridity gradient. Hoopoe larks (*Alaemon alaudipes*, Desfontaines), Dunn's larks (*Eremalauda dunni*, Shelley), desert larks (*Ammomanes deserti*, Lichtenstein), black-crowned finchlarks (*Eremopterix nigriceps*, Gould), and crested larks (*Galerida cristata*, Linnaeus) were captured in various sites in Saudi Arabia, as described by Tieleman and Williams (2002) and Tieleman et al. (2003). We captured skylarks (*Alauda arvensis*, Linnaeus) in various locations in the Netherlands and western Germany, and horned larks (*Eremophila alpestris*, Linnaeus) near Columbus, OH, United States. Birds were either processed for lipid analysis immediately or frozen in an atmosphere of nitrogen gas for later processing. In addition to the larks that we directly captured, we also used data from Ro (2009) and

Ro and Williams (2010) to add 13 non-lark species for our analysis of CWL and intercellular SC lipids.

Environments of bird sampling locations

To quantify environmental conditions for each species in terms of temperature and moisture availability, we calculated an aridity index for each species' environment as $Q = P/((T_{max} + T_{min})(T_{max} - T_{min}))$ x 1000, where P is the average annual precipitation (mm), T_{max} is the mean maximum temperature of the hottest month (°C) and T_{min} is the mean minimum temperature of the coldest month (°C) (Emberger, 1955). Q is low in hot, dry deserts and high in cool, wet areas. We collected climatic data from the literature (Tieleman et al, 2003, Walter & Lieth, 1967; Williams, 2001), and from http://www.worldclimate.com/ and http://www.onlineweather.com/. Because Q increases rapidly when environments become more mesic, we avoided unequal weighting of data for mesic species by using log Q in our analyses (Tieleman et al., 2003).

Cutaneous Water Loss

For every lark species except the horned lark, we did not measure cutaneous water loss for the same individuals as used for lipid analysis. Therefore, we used mean values of CWL measured at 25°C for skylarks, hoopoe larks, Dunn's larks, and desert larks from data in Tieleman and Williams (2002) and Haugen et al., (2003). We measured CWL of horned larks with a standard flow-through respirometry method identical to the methods used to measure the larks above, except that we measured Horned Larks at 30°C. Measurements of desert and mesic lark species have shown no differences in CWL between 25 and 30°C (Tieleman and Williams, 2002). For the 13 non-lark species, we used mean values of CWL measured at 30°C. For all non-lark species, the individuals were the same for CWL and lipid analysis (Ro and Williams, 2010, Ro, 2009). In addition to the direct measurements of CWL for the species listed above, we used estimates of CWL values for black-crowned finchlarks and crested larks from Haugen et al. (2003).

Extraction of intercellular lipids from the SC of larks

To extract lipids from the SC of larks, we plucked feathers, peeled the skin away from the body, and pinned the skin on a Teflon sheet covered with filter paper. We saturated the filter paper with a 0.5% trypsin (FisherChemical laboratory grade) solution in phosphate buffered saline (PBS), and incubated the skin at 4°C for 24-48 h to separate the epidermis from the dermis. After this period, the epidermis was immersed in a fresh 0.5% trypsin solution in PBS and incubated for 3 hours at 37°C to isolate the SC from the rest of the epidermis. The trypsin solution was rinsed with distilled water, and the SC was placed in a 10 mL vial and freeze-dried overnight to extract water. Thereafter, we determined the dry mass of the SC, and extracted the intercellular lipids by placing the SC in successive baths of chloroform:methanol 2:1, 1:1, and 1:2 v/v for 2 h each. Each bath also contained 50 mg/L of the antioxidant butylated hydroxytoluene (BHT). We combined the extracts and evaporated the solvent with a stream of nitrogen in a nitrogen manifold (N-EVAP, model 11155-O, Organomation Associates, Inc., Berlin, MA, USA). We then re-constituted the lipids in enough 2:1 chloroform:methanol with 50 mg/L BHT to fully dissolve the sample (~100 – 300 μL).

Extraction of covalently bound lipids from the SC of larks

To confirm that all intercellular lipids of the SC were extracted, we re-soaked the SC for each bird for 2 h in chloroform:methanol 1:2 (v/v), and examined extracts for lipids with thin layer chromatography (TLC). No lipid bands were detected in our plates, indicating that all the intercellular lipids had been removed. We then extracted covalently bound lipids (CBL) by immersing the SC in 2 ml of 1 mol l^{-1} NaOH in 90% methanol at 60°C for 2 h (Wertz and Downing, 1987). This mild alkaline hydrolysis breaks the ester bonds between lipids and proteins (Wertz and Downing, 1987). We then adjusted the pH to 6 by adding 3 mol l^{-1} HCl, and added 2.5 ml of chloroform. The solution was then passed through a sintered glass filter, and centrifuged at 3000 ***g*** for 15 min. After a few minutes, the solution separated into two layers, an aqueous layer and an organic layer that

contained lipids. The organic phase was washed twice with distilled water to remove contaminants. The aqueous phase was mixed with 1 ml of chloroform to extract any lipids that might be in this phase, and centrifuged again at 3000 ***g*** for 10 min. We combined the organic fractions, and removed any remaining small particles by passing the solution through a PTFE filter, 0.45 μm pore size (Millex, Millipore Corp., Bedford, MA, USA). This method has been verified through TLC of the aqueous fraction to extract all lipids to the organic fraction (Calhoon and Williams, unpublished). We dried the filtrate with a stream of nitrogen and re-constituted the sample in enough chloroform:methanol (2:1, v/v) with 50 mg/L of BHT to fully dissolve the sample (30 – 170 μL).

Quantification of intercellular and covalently bound lipids

We used thin layer chromatography (TLC) to analyze the amounts of lipid classes in the SC of larks. For our analysis, we used 20 cm X 20 cm glass plates coated with silicic acid 0.25 mm thick (Adsorbosil-Plus 1, Altech, Deerfield, IL, USA). A 2:1 $CHCl_3$:MeOH solution run to the top of the plates removed contaminants prior to sample loading. Following this washing, we activated the plates by heating them in an oven for 30 minutes at 110^{o}C, and then scored the silicic acid to create 13 separate lanes on each plate. For each sample, we ran one plate to detect relatively polar lipids, such as cerebrosides, ceramides, and cholesterol, and a separate plate to detect non-polar lipids, such as free fatty acids, triacylglycerides, methyl esters, and cholesterol esters. On the polar plate, we classified ceramides as ceramide I, the least polar, through ceramide III, the most polar, because polarity of ceramides has been shown to be important in CWL (Muñoz-Garcia et al, 2008). We used a Hamilton syringe with a Teflon-coated tip to load a set of five standards comprised of each lipid class dissolved in 2:1 chloroform:methanol at known concentrations on each plate. Our standard was serially diluted relative to the most concentrated standard to produce a range of lipid concentrations with which we compared the samples. After loading the standards, we loaded each sample into two lanes, and then developed the plates. To separate polar lipids, we developed the plate with chloroform:methanol:water 60:40:5 run 10 cm from the bottom, followed by

chloroform:methanol:acetic acid 190:9:1 run 15 cm from the bottom, and finally hexane:ethyl ether:acetic acid 70:30:1 run to the top. We developed nonpolar plates with hexane:ethyl ether:acetic acid 80:20:2 run to the top. After developing the plates, we allowed them to dry, sprayed them with a solution of 3% cupric acetate in 8% phosphoric acid, and heated them in an oven for 30 minutes at 180^{o}C. This procedure chars the lipids to allow visualization. We scanned the plates on a Hewlett-Packard (Scanjet 5590) scanner and quantified each lipid class with TN image (Nelson, 2003). For the analysis of intercellular lipids in non-larks, we used data from Ro (2009) and Ro and Williams (2010), in which the procedure for lipid extraction and TLC was identical to ours.

Statistics

We performed all statistical tests with SPSS 19.0 (Chicago, IL, USA), with statistical significance set at $P<0.05$. We tested for correlations between mean lipid class amounts or percentages and CWL or log Q for each species with stepwise multiple linear regression. Percentages were logit transformed ln(Y/1-Y) to normalize data.

Our data consisted of 15 mesic species and five desert species, the latter of which were all larks. Because the only desert species were larks, we calculated phylogenetic independent contrasts (PIC) of our physiological data to account for relatedness among species. We constructed our phylogenetic tree based on Sibley and Alquist (1990), modified by Boyd (2011) (Figure 1). Branch lengths were based on Sibley and Alquist (1990). From this tree, we calculated PIC on CWL, and amounts, percentages, and ratios of lipid classes, with the Phenotypic Diversity Analysis Program (PDAP) module of PDTREE (Garland et al., 1999; Garland and Ives, 2000).

To explore the interactions between lipid classes and their effects on CWL, we used principal component analysis (PCA) on the percent (logit transformed) of each class of lipid (Shaw, 2003). This analysis yielded uncorrelated composite variables, the principal components. We used the program 'Factor analysis' in SPSS without rotation to extract components with eigenvalues greater than one as our selection criterion. We then used linear regression to determine associations between each principal component and mean CWL for each species. We also attempted to use PCA on the amounts of each lipid

class, but we did not find enough correlation between variables to justify the use of PCA (Kaiser-Meyer-Olkin measure of sampling adequacy = 0.577).

Within larks, we used the same tests as described above to test for the effects of covalently bound lipids on CWL. Because all species were members of the same family, we did not calculate PIC, as previous studies on CWL in larks have shown no differences between phylogenetically informed and standard linear regression methods (Tieleman et al, 2003). We also attempted to perform PCA for covalently bound lipids in larks, but there was not enough covariance between variables to adequately reduce the number of variables. Finally, to test for similarities between the intercellular and covalently bound lipids, we tested for correlations between covalently bound and intercellular lipid classes among all individual larks by calculating Pearson's correlation coefficients. Because we correlated multiple lipid classes, we applied a Bonferroni correction to account for multiple comparisons (Zar, 1996).

Results

Cutaneous Water Loss and Environment

For all 20 species of birds for which we had physiological data, we found a significant positive association between log Q and CWL (Figure 2; $R^2 = 0.42$, $p = 0.003$), demonstrating that rates of CWL in birds are higher in more mesic regions than more arid regions.

Intercellular Lipids and CWL

Among all 20 species, we found that the main lipid classes in the intercellular spaces of the SC of birds were fatty acid methyl esters, triacylglycerides, ceramide III, and cerebroside, which made up over 70 percent of all lipids (Table 1). The amount of triacylglycerides and fatty acid methyl esters correlated positively with CWL, whereas the amount of ceramide I correlated negatively with CWL (Figure 3; $p < 0.001$, 0.007 and 0.030, respectively). The percentage of cerebrosides was negatively correlated with CWL (Figure 4; $R^2 = 0.61$, $p < 0.001$).

When we correlated ratios of lipid classes with CWL, the ratio of ceramides to cerebrosides correlated positively with CWL (Figure 4; $R^2 = 0.47$, $p = 0.001$), providing evidence that cerebrosides may decrease CWL.

In a PCA analysis of the percentage of each lipid class relative to total lipids, two axes accounted for 71.3% of the variance. A plot of scores for each species along these axes revealed that principal component 1 (PC1) separated desert species from mesic species (Figure 5a). Stepwise regression analysis of both principal components against CWL demonstrated that PC1 was negatively correlated with CWL ($R^2 = 0.57$, $p < 0.001$). Furthermore, a plot of the eigenvector loadings of each lipid class (Fig 5b) showed a separation of lipid classes into three distinct groups. PC 1 separated free fatty acids, cholesterol, ceramide I, and cerebroside from fatty acid methyl esters, ceramide II, and ceramide III. Principal component 2 (PC2) separated these lipid classes from triacylglycerides. Combining the species plot with the lipid plot suggests that a combination of free fatty acids, cholesterol, ceramide I, and cerebroside were associated with desert species, whereas a combination of fatty acid methyl esters, ceramide II, and ceramide III were more associated with mesic species. Because the scores for most desert birds and the score for triacylglycerides were negative along PC2, it is also possible that the modification of triacylglycerides is important for desert birds.

Although we could find no clear pattern in polarity or molecular structure between the groups of lipid classes separated by PC1 and PC2, the interactions between these lipid classes may provide insight into regulation of CWL by the stratum corneum. Therefore, we tested for an association between each principal component and CWL. In a stepwise regression analysis, we found that PC1 was negatively associated with CWL ($R^2 = 0.64$, $p < 0.001$).

Covalently Bound Lipids of larks

Within larks, we found that the main classes of CBL in the SC were cerebrosides, three classes of ceramides, cholesterol, free fatty acids, triacylglycerides, fatty acid methyl esters, and cholesterol esters (Table 2). Together, free fatty acids, cerebrosides, and ceramides accounted for over 80 percent of all covalently bound lipids. We found a positive correlation between the amount and percentage of triacylglycerides and CWL

(R2 = 0.76, p = 0.047 and R2 = 0.76, p = 0.048, respectively), despite the fact that triacylglycerides made up less than two percent of all CBL. When we performed multiple regression analysis on how amounts of covalently bound lipids in larks correlated with Log Q, we found that ceramide III correlated negatively with Log Q, whereas cholesterol correlated positively with Log Q ($R^2 = 0.327$, $p < 0.001$, $p = 0.004$, respectively.

Correlations between intercellular and covalently bound lipids of the SC of larks

Within larks, we found significant positive correlations between the amount of intercellular and covalently bound cerebrosides ($R^2 = 0.21$, $p = 0.001$), intercellular cholesterol ester and covalently bound cerebrosides ($R^2 = 0.23$, $p < 0.001$), and intercellular cerebrosides and covalently bound free fatty acids (Figure 6; $R^2 = 0.22$, $p < 0.001$).

Discussion

We found that birds from arid environments had lower rates of cutaneous water loss (CWL) than birds from more mesic environments, and a PCA analysis revealed that these lower rates of water loss may be associated with interactions between intercellular free fatty acid, cholesterol, ceramide I, and cerebroside. This potential interaction is difficult to interpret, because there were no clear patterns of polarity or molecular structure within this group compared with lipids associated with higher rates of CWL.

One pattern that emerged in both PCA and linear regression analyses was a consistent negative association between cerebrosides and CWL. This association is counterintuitive, as the sugar moieties of the cerebrosides are bulky and potentially bind with water to disrupt lipid packing and increase CWL (Rawlings, 2005). Furthermore, mice with Gaucher disease, with approximately 7 μg of intercellular cerebrosides per cm^2 of SC, have an average CWL rate of 9.8 $mg/cm^2/day$, whereas controls average only 0.24 $mg/cm^2/day$ (Holleran et al, 1994). Using Meeh's equation (Meeh, 1879), we calculated skin surface for all species in this study based on body mass, and thus were able to calculate intercellular cerebrosides in units of $\mu g/cm^2$ SC. We found that birds averaged

approximately 14 μg cerebrosides/cm^2 SC, approximately twice as much as mice with Gaucher disease. Despite this large discrepancy, this study and others have found that an increase in cerebrosides is associated with lower CWL in birds (Munoz-Garcia and Williams 2005, Gu et al. 2008). These studies taken together suggest that in avian SC, cerebrosides are arranged in a way that prevents, rather than facilitates water loss, a pattern opposite to that found in mammals.

The current model for intercellular organization in avian SC is a modification of the sandwich model proposed by Bouwstra (2000) for mammalian systems (Muñoz-Garcia et al, 2008). In this model, cerebrosides are arranged in the center of two outer layers of ceramides to form a trilayer of lipids. The hexose moieties of the cerebrosides therefore interdigitate with the lipid tail groups of ceramides and free fatty acids (Figure 7a). However, under this model, the hexose moieties would potentially attract water molecules inside the lipid layers, where they may disrupt lipid packing, and increase CWL (Golden, 1986). Because our data suggest that an increase in intercellular cerebrosides decreases water loss through the SC, we suggest that lipids are arranged in the avian SC in a bilayer, rather than in trilayers as previously envisioned. We hypothesize that lipid molecules are arranged within each layer with hydrophilic headgroups facing outward and hydrophobic tails facing inward. We argue that the sugar moieties of the cerebrosides are located on the outside of the lipid layers, where they could interact with water molecules without disrupting lipid packing (Figure 7b). Each cerebroside molecule interacts with four to nine water molecules (Bach et al, 1982), and these water molecules can influence the hydrogen bonding of surrounding water molecules to form solvation shells. In this way, the cerebrosides could potentially sequester water molecules within the SC by ordering them around the hydroxyl groups of the hexose moieties. This ordered water would potentially form aggregates in which water molecules exhibit strong hydrogen bonding. These hydrogen bonds would raise the energy required for each water molecule to percolate through the SC, thus lowering CWL (Clement et al, 2012). Hydrogen bond strength of water and lipid packing can be evaluated by infrared spectroscopy (Golden et al, 1986, Du et al, 1993). Thus, if the cerebrosides lie outside lipid bilayers, the strength of hydrogen binding in water molecules should increase as cerebroside content increases, and lipid packing will be

unaffected by the addition of water into the SC. However, if cerebrosides lie inside lipid trilayers, hydrogen binding strength will remain high, but the addition of water to the SC will disrupt lipid packing as the water molecules associate with cerebrosides and consequently interact with lipid tail groups (Williams et al, 2012).

Covalently bound lipids add a layer of complexity to current models of lipid organization in the avian SC (Clement et al, 2012). In current thinking, covalently bound ceramides and cerebrosides align with their polar headgroups facing away from the corneocytes, where the cerebrosides may interact with water molecules. Shorter-chained, less polar lipids such as free fatty acids and cholesterol esters occupy the remaining binding sites on the corneocytes to create a covalently bound lipid layer that inhibits water permeation through the corneocytes, and potentially interacts with the nearest intercellular lipid bilayer to create a water shell between the covalently bound and intercellular lipid layers.

Models of mammalian SC have suggested that the covalently bound and intercellular lipids interdigitate at their respective head groups to create links between adjacent corneocytes (Wertz, 2000). However, if cerebrosides are packed too tightly together, lamellar structure breaks down (Maggio et al, 2006). Therefore, if intercellular and covalently bound lipids interdigitate in avian SC, the cerebrosides must be spaced far enough apart to prevent disruptions of the lamellar structure. Although we found a positive correlation between intercellular and covalently bound cerebrosides, we also found that as the amount of intercellular cerebroside increased, covalently bound free fatty acids also increased, and as covalently bound cerebrosides increased, intercellular cholesterol ester increased. These correlations suggest that as the amount of cerebroside increases in either the covalently bound or intercellular lipid layer, the opposing layer incorporates more short-chained, less polar lipids to increase spacing between cerebroside molecules. This increase in spacing may allow the lipid layers to interdigitate even when high amounts of cerebrosides are present (Figure 8).

In conclusion, we found that birds in arid environments have lower rates of CWL than birds from mesic environments. These differences in water loss may be explained by differences in the interactions between and among intercellular and covalently bound lipids. Although these interactions are unclear, a consistent pattern that has emerged is

the negative correlation between cerebrosides and CWL. This finding represents an enigma given how cerebrosides affect CWL in mammals (Holleran, 1994, Rawlings, 2005), which has prompted us to evaluate a new model for the organization of lipids in the avian SC. In this model, cerebrosides are arranged in a way that potentially prevents, rather than facilitates water loss. While this model is speculative, tests on this model using infrared spectroscopy (Williams et al, 2012) may allow us to gain a better understanding of how lipids interact within the SC. Such an understanding will allow us to make better predictions of how birds change their physiology in response to environmental change. As the climate becomes hotter and drier in many regions (IPCC), birds may increase cerebroside content in their SC, either through natural selection, or phenotypic plasticity.

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Figure and Table Legends

Figure 1: Phylogenetic tree of all species in this study.

Figure 2: Phylogenetically independent contrast scores for average cutaneous water loss (CWL) for 20 species of birds (19 contrasts) vs. Aridity index (log Q). Values are positivized on the x-axis.

Figure 3: Phylogenetically independent contrast scores for average A) Triacylglycerides (TAG), B) Fatty acid methyl esters (FAME), and C) Ceramide I vs Average cutaneous water loss (CWL) in 20 species of birds. Values are positivized on the x-axis.

Figure 4: Phylogenetically corrected A) Percent cerebrosides and B) Ratio of ceramides to cerebrosides vs. cutaneous water loss (CWL). Values are positivized on the x-axis.

Figure 5: A) Principal component scores for five desert birds (●), and 15 mesic birds (○). B) Eigenvector loadings for cerebroside (♦), ceramide III (▼), ceramide II (◊), ceramide I (●), cholesterol (Δ), free fatty acids (○), triacylglycerides (□), and fatty acid methyl esters (■).

Figure 6: Correlations of covalently bound lipids with intercellular lipids for individual larks. A) Covalently bound cerebrocides vs intercellular (IC) cerebrosides, B) covalently bound cerebrosides vs IC cholesterol esters, and C) covalently bound free fatty acids (FFA) vs IC cerebrosides.

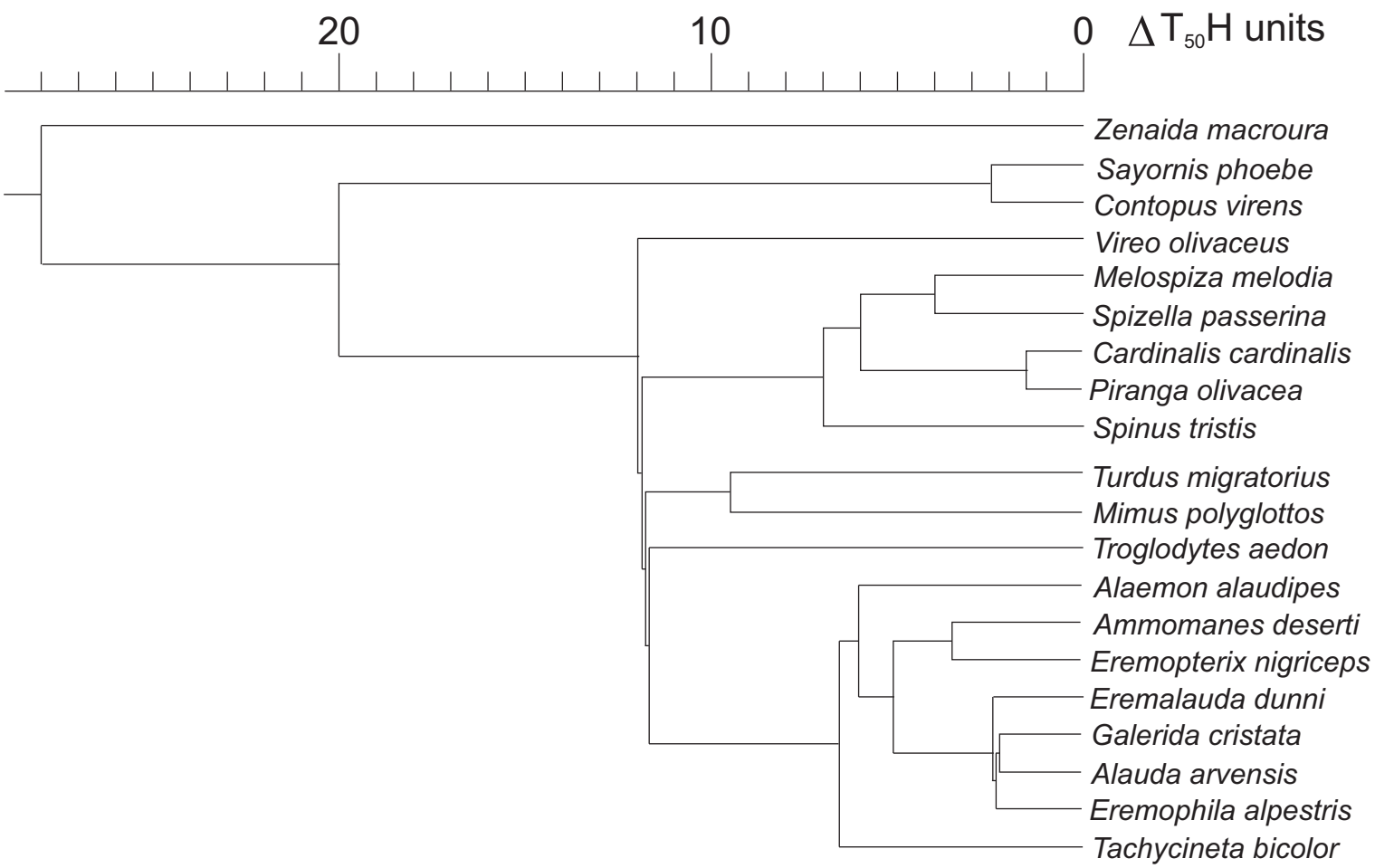
Figure 7a: The sandwich model for intercellular lipids in the avian stratum corneum. Adapted from Muñoz-Garcia et al. (2005).

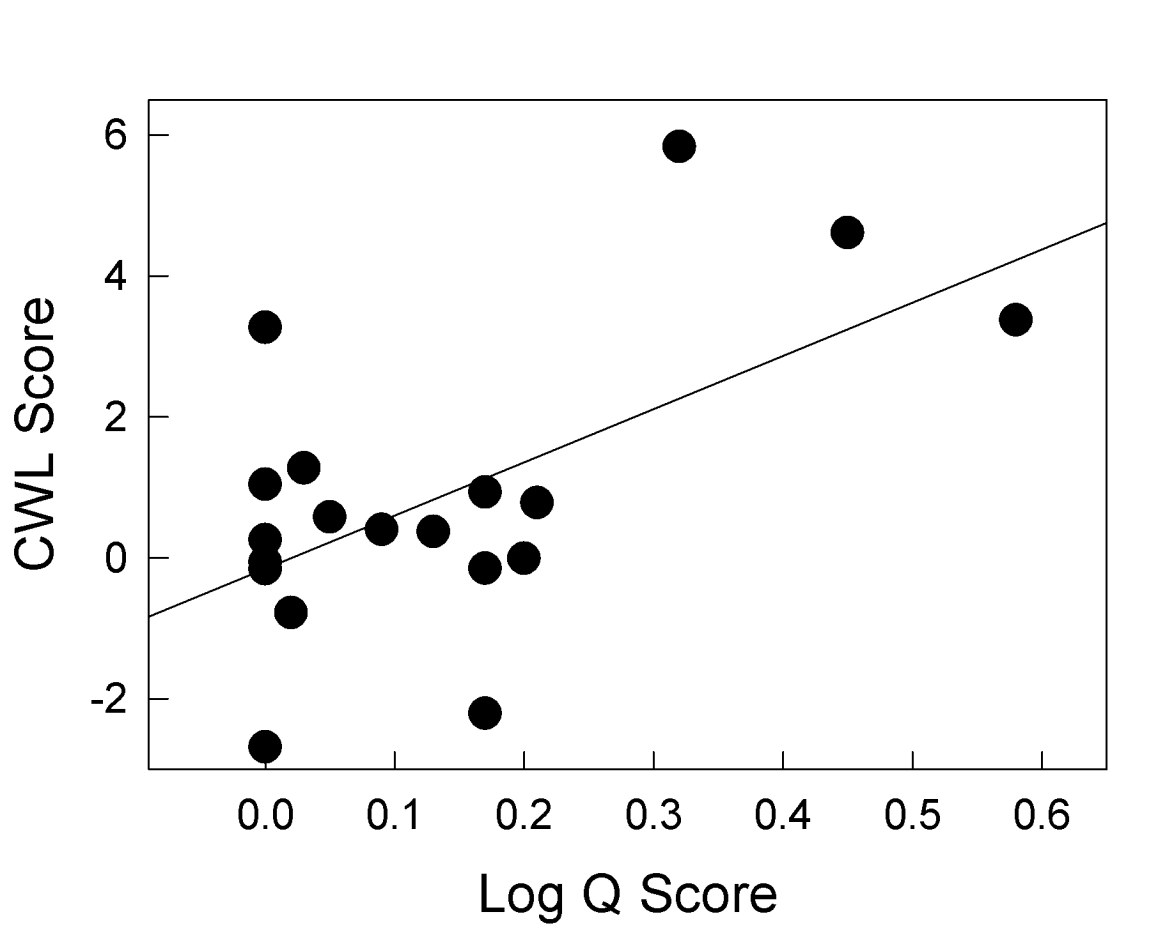
Figure 7b: The bilayer model for intercellular lipids in the avian stratum corneum.

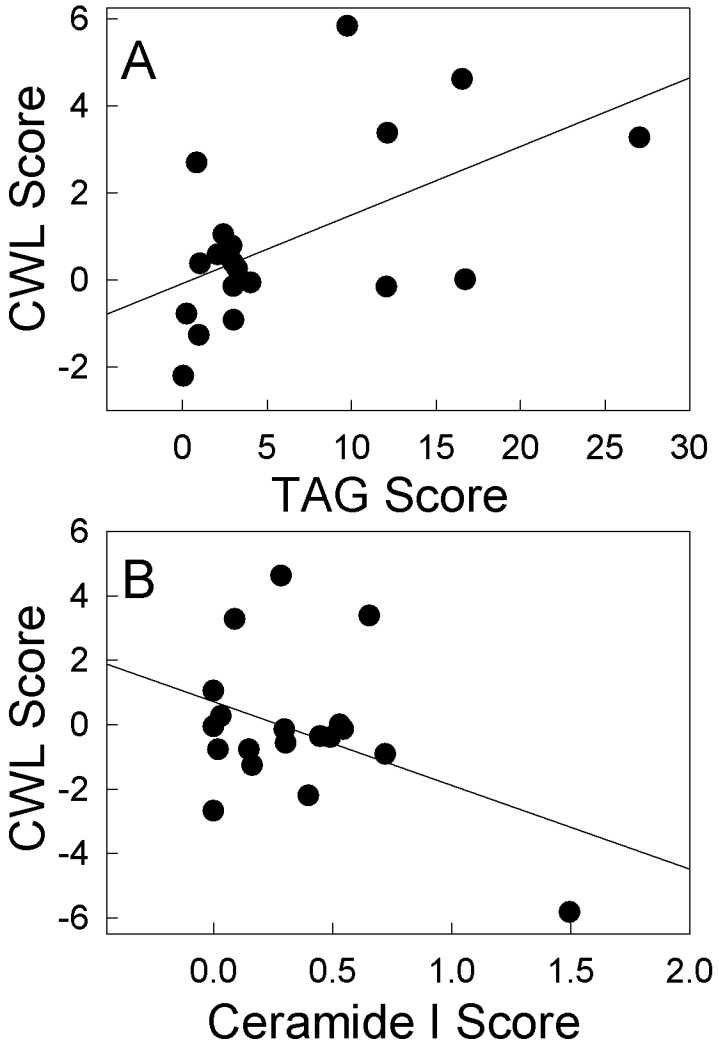
Figure 8: Model for the interface of covalently bound and intercellular lipids within the avian stratum corneum, showing interdigitation between intercellular and covalently bound cerebrosides. Covalently bound lipids are colored black, and intercellular lipids are colored blue.

Table 1: Cutaneous water loss ($mg/cm^2/day$) and intercellular lipid values for all species of birds (mg/g SC). Data for CWL for larks, except horned lark, are taken from Tieleman and Williams (2002) and Haugen (2003), and all data for nonlarks are taken from Ro and Williams (2010) and Ro (2009). All values are means ± SE, where information on SE was available.

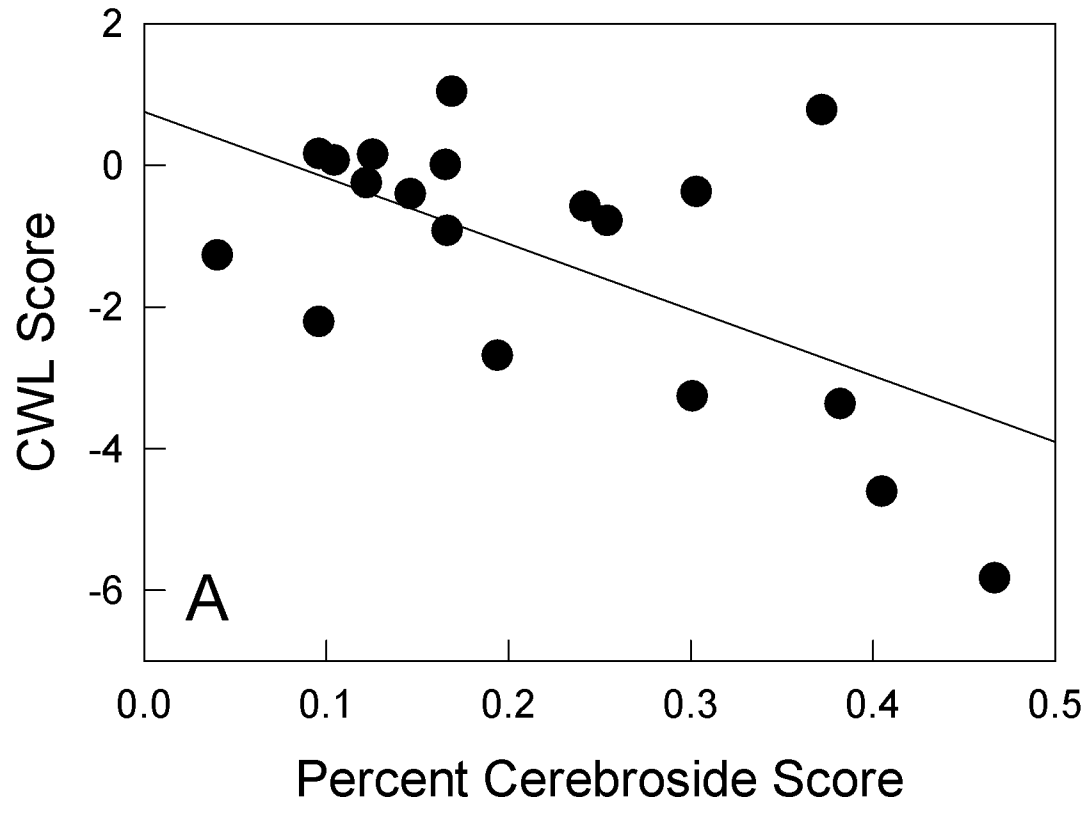
Table 2: Covalently bound lipid amounts for all lark species (mg/g SC). All values are means ± SE.

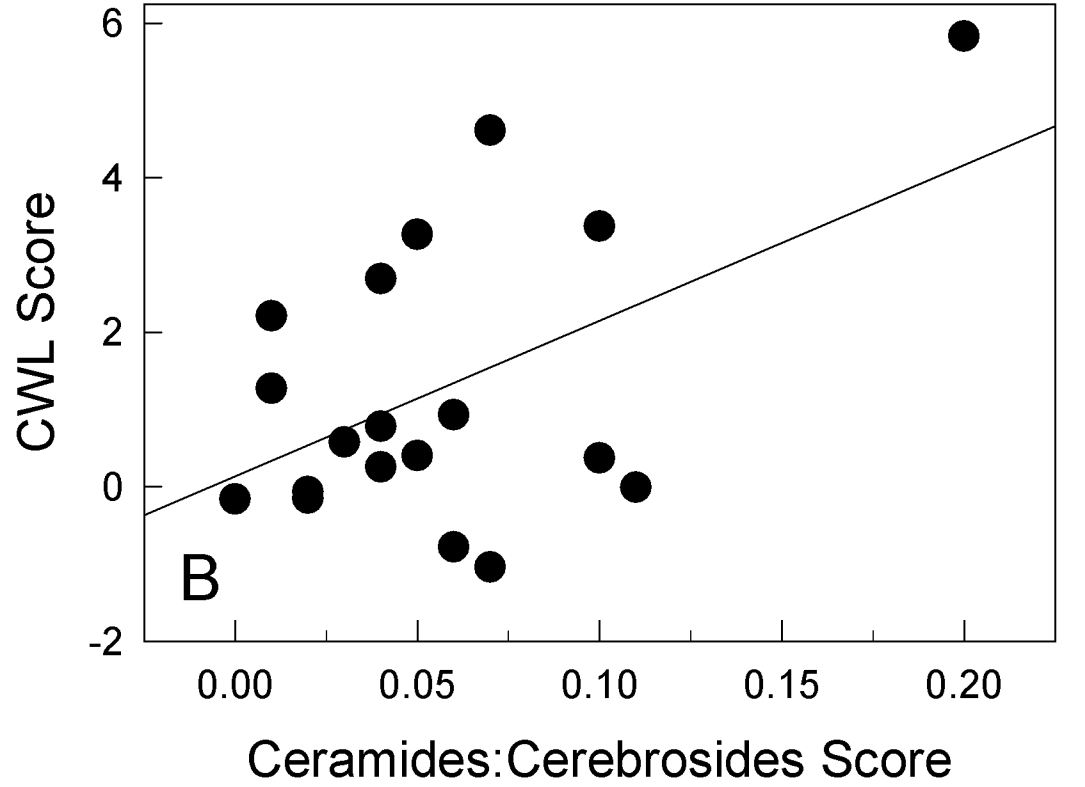


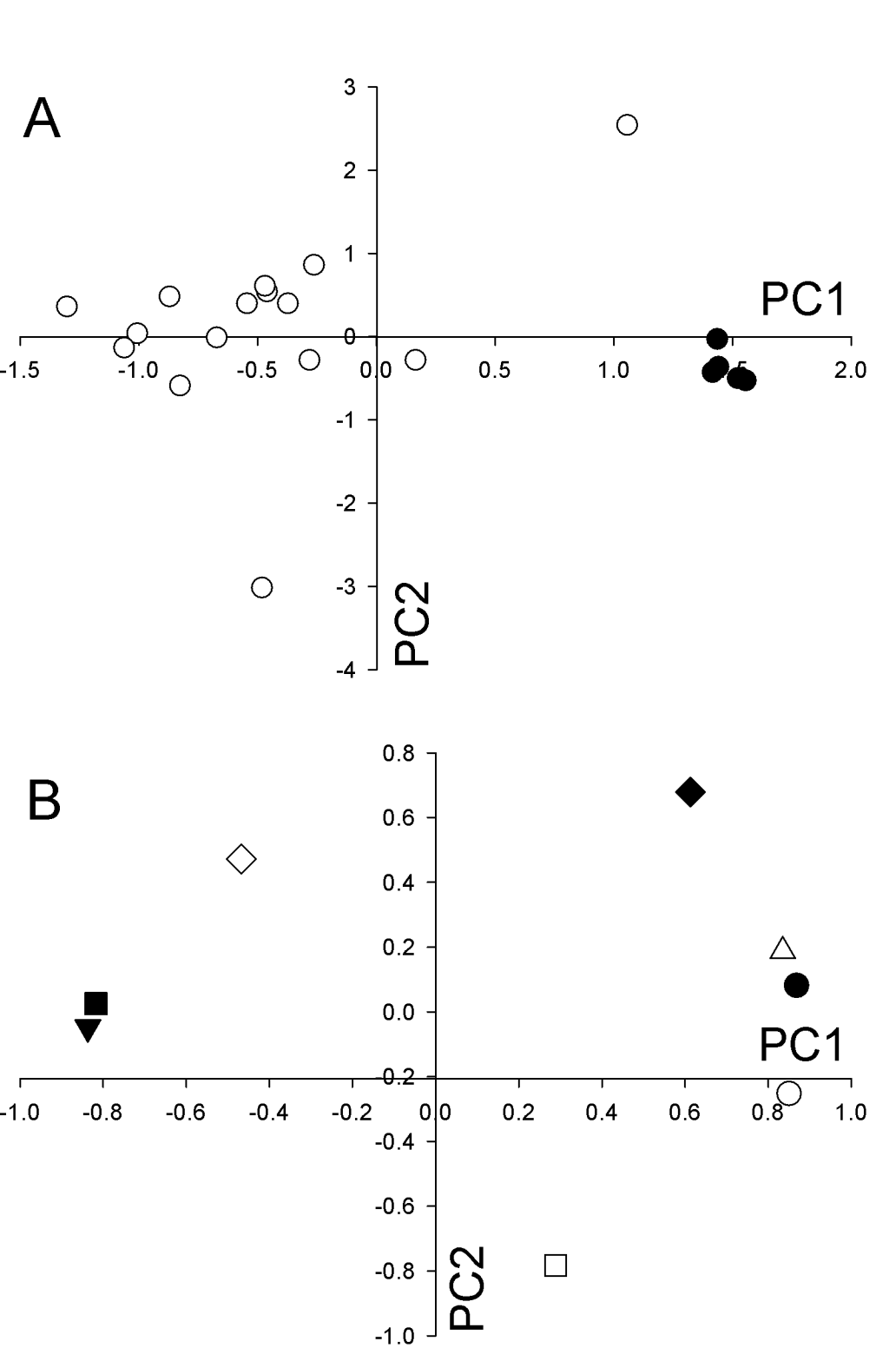

CWL Score
6
4
2
0
-2
0.0
0.1
0.2
0.3
0.4
0.5
0.6
Log Q Score

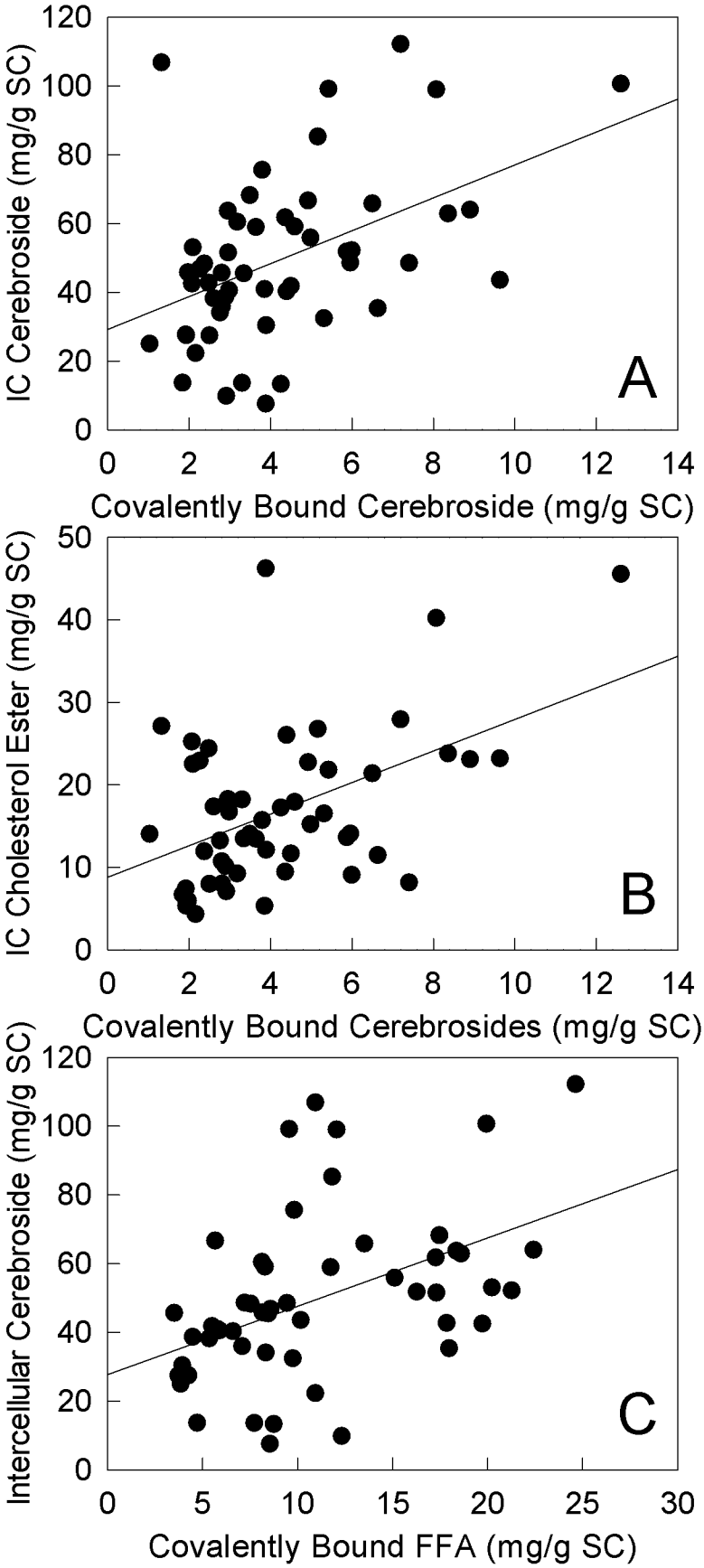

A
CWL Score
TAG Score
6
4
2
0
-2
0
5
10
15
20
25
30

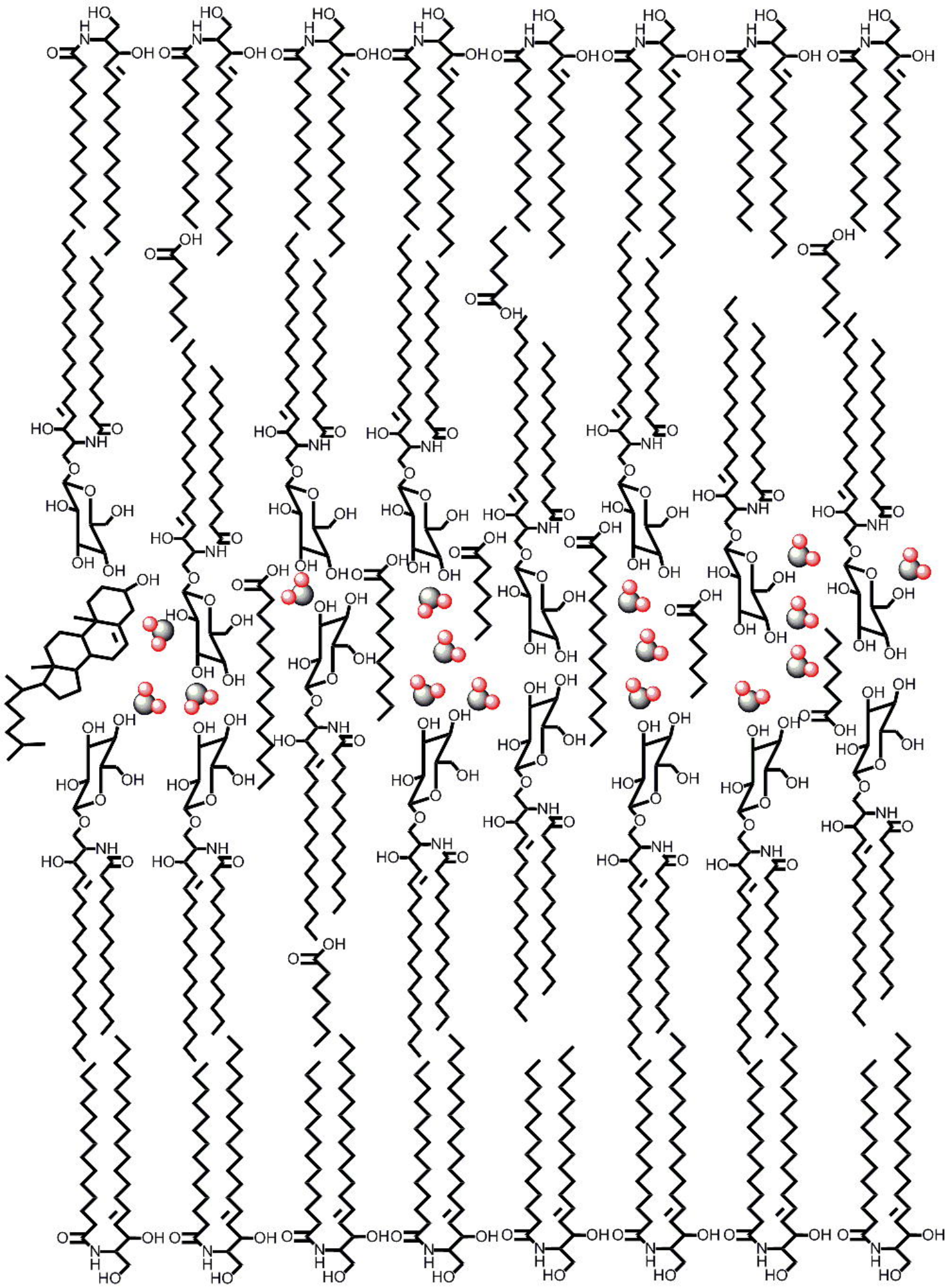
B
CWL Score
Ceramide I Score
6
4
2
0
-2
-4
-6
0.0
0.5
1.0
1.5
2.0

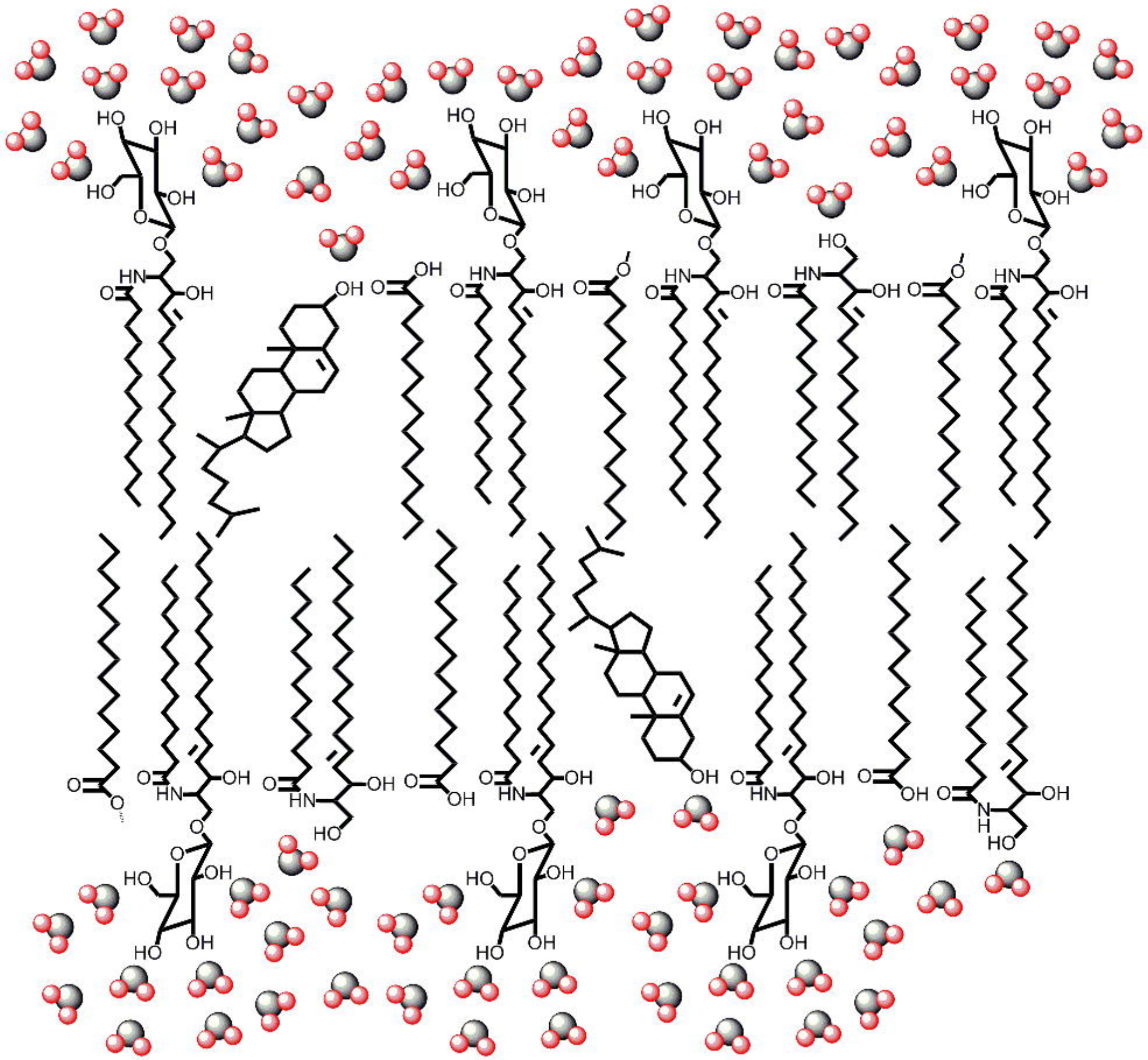

2
0
-2
-4
-6
CWL Score
A
0.0
0.1
0.2
0.3
0.4
0.5
Percent Cerebroside Score

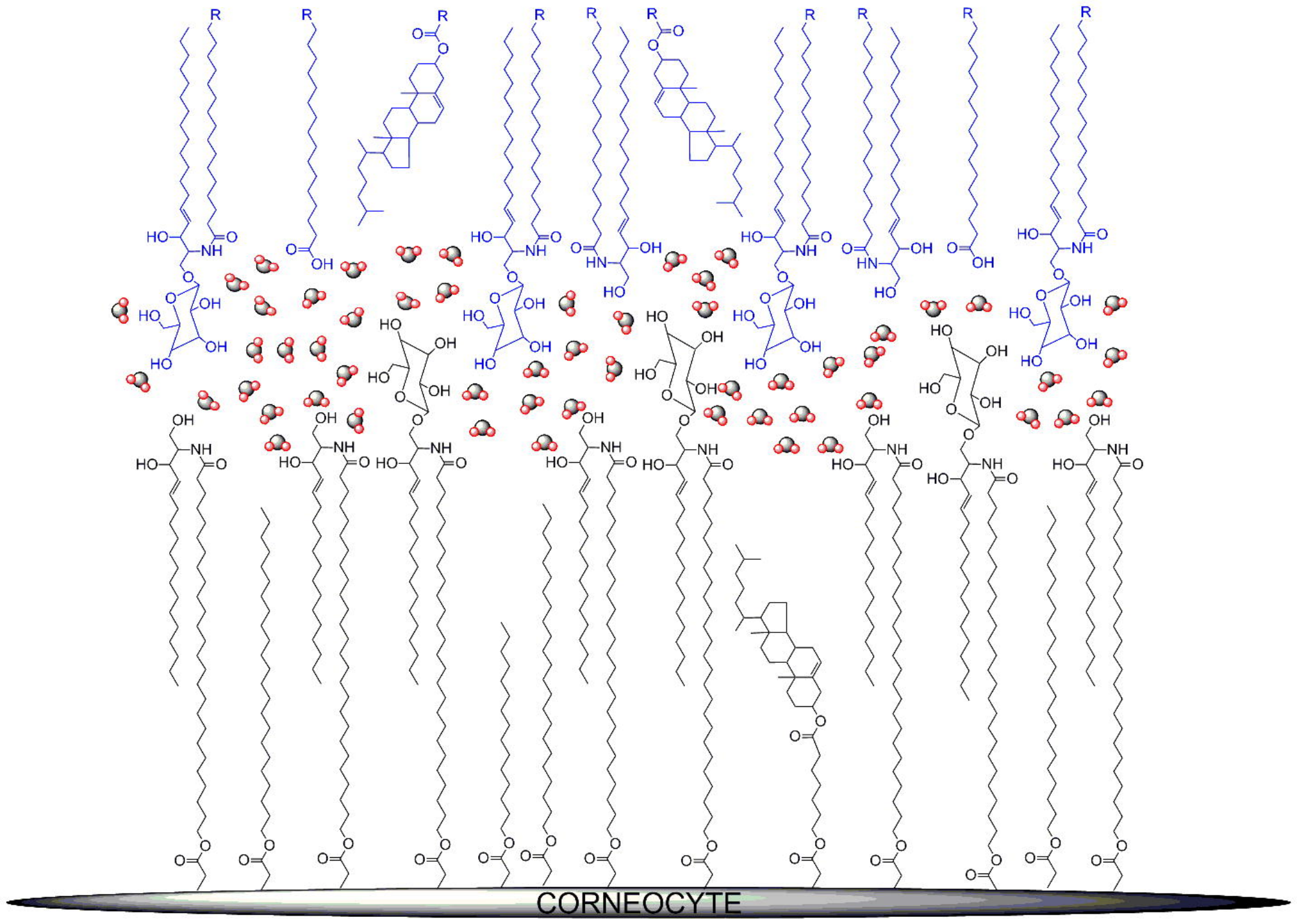

6
4
2
0
-2
CWL Score
B
0.00
0.05
0.10
0.15
0.20
Ceramides:Cerebrosides Score


A
PC1
PC2
3
2
1
0
-1
-2
-3
-4
-1.5
-1.0
-0.5
0.0
0.5
1.0
2.0
B
PC1
PC2
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.0
-0.8
-0.6
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0


IC Cerebroside (mg/g SC)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
14
Covalently Bound Cerebroside (mg/g SC)
A
IC Cholesterol Ester (mg/g SC)
50
40
30
20
10
0
0
2
4
6
8
10
12
14
Covalently Bound Cerebrosides (mg/g SC)
B
Intercellular Cerebroside (mg/g SC)
120
100
80
60
40
20
0
0
5
10
15
20
25
30
Covalently Bound FFA (mg/g SC)
C






CORNEOCYTE

Common Name (n)	Latin Name	CWL (mg/cm^2/day)	Cholesterol Ester	FAME	TAG	FFA	Cholesterol	Ceramide 1	Ceramide 2	Ceramide 3	Cerebroside
Black-crowned Finchlark (8)	*Eremopterix nigriceps*	14.0	25.1 ± 3.5	8.7 ± 1.0	26.5 ± 3.4	36.8 ± 4.9	8.8 ± 0.8	5.7 ± 0.7	8.5 ± 1.1	32.6 ± 3.2	78.4 ± 11.1
Dunn's Lark (7)	*Eremalauda dunni*	14.5	20.0 ± 5.0	7.3 ± 1.2	17.6 ± 2.5	26.2 ± 3.2	6.3 ± 1.0	3.9 ± 0.5	7.5 ± 0.7	19.7 ± 3.8	52.9 ± 10.8
Crested Lark (9)	*Galerida cristata*	15.8	19.0 ± 2.3	10.1 ± 1.3	33.2 ± 4.2	47.1 ± 8.6	7.1 ± 0.5	4.4 ± 0.4	8.2 ± 0.6	22.4 ± 2.7	55.7 ± 5.7
Hoopoe Lark (9)	*Alaemon alaudipes*	18.5	18.4 ± 3.6	8.0 ± 1.2	21.3 ± 2.3	23.0 ± 4.2	5.9 ± 0.6	3.3 ± 0.5	7.4 ± 1.3	20.6 ± 3.3	49.5 ± 8.4
Desert Lark (6)	*Ammomanes deserti*	20.0	19.1 ± 1.9	16.0 ± 2.3	26.3 ± 3.9	36.6 ± 9.1	10.6 ± 2.2	4.6 ± 0.5	8.6 ± 1.3	25.1 ± 2.3	60.5 ± 3.8
Horned Lark (11)	*Eremophila alpestris*	20.8 ± 1.4	8.8 ± 1.0	168.7 ± 31.7	19.0 ± 5.0	13.8 ± 1.7	8.4 ± 0.5	5.7 ± 0.4	2.7 ± 0.7	44.3 ± 5.7	42.7 ± 5.3
Chipping Sparrow (5)	*Spizella passerina*	21.8 ± 2.3	34.2 ± 7.2	44.9 ± 29.9	41.9 ± 11.3	8.2 ± 3.6	3.0 ± 1.3	0	7.6 ± 1.1	58.9 ± 5.3	32.8 ± 4.8
Mourning Dove (5)	*Zenaida macroura*	22.6 ± 3.5	4.4 ± 1.7	46.2 ± 17.7	35.4 ± 10.7	38.2 ± 26.9	2.1 ± 0.6	0.8 ± 0.8	25.0 ± 8.6	25.5 ± 5.4	11.6 ± 2.5
Northern Cardinal (6)	*Cardinalis cardinalis*	24.9 ± 3.7	29.8 ± 7.2	51.1 ± 13.4	28.7 ± 3.7	4.6 ± 2.1	0.8 ± 0.2	0	10.8 ± 1.4	45.0 ± 3.8	24.3 ± 3.4
Northern Mockingbird (5)	*Mimus polyglottos*	25.1 ± 3.1	15.9 ± 4.1	33.1 ± 17.6	60.2 ± 15.4	8.2 ± 6.2	1.5 ± 0.5	1.3 ± 0.7	11.4 ± 4.3	37.6 ± 3.9	18.4 ± 1.7
Skylark (10)	*Alauda arvensis*	25.7	13.2 ± 2.1	10.2 ± 1.6	68.7 ± 13.0	67.0 ± 7.2	9.1 ± 0.6	5.0 ± 0.6	0.0 ± 0.0	19.8 ± 2.2	27.9 ± 4.8
American Robin (5)	*Turdus migratorius*	25.8 ± 3.4	22.8 ± 3.7	10.1 ± 3.3	75.6 ± 18.1	6.9 ± 4.2	6.0 ± 3.5	0	16.5 ± 3.6	52.1 ± 6.8	26.0 ± 6.9
American Goldfinch (6)	*Spinus tristis*	26.5 ± 3.2	27.3 ± 6.0	41.3 ± 16.9	48.2 ± 15.8	26.2 ± 12.8	4.0 ± 3.4	0.1 ± 0.1	8.6 ± 0.8	63.8 ± 3.8	24.4 ± 2.8
House Wren (6)	*Troglodytes aedon*	26.7 ± 2.1	46.4 ± 6.4	42.0 ± 16.9	22.1 ± 3.3	13.9 ± 7.3	1.9 ± 0.6	0.2 ± 0.2	9.1 ± 1.1	58.0 ± 9.1	37.4 ± 4.8
Scarlet Tanager (4)	*Piranga olivacea*	26.7 ± 2.3	10.9 ± 2.2	69.8 ± 23.9	32.9 ± 8.0	2.9 ± 2.2	1.8 ± 0.3	0	13.5 ± 2.0	51.7 ± 8.1	37.4 ± 4.9
Red-eyed Vireo (6)	*Vireo olivaceus*	27.2 ± 2.6	52.1 ± 13.2	123.7 ± 58.1	42.2 ± 12.2	21.2 ± 15.1	0.4 ± 0.1	0	13.4 ± 2.5	59.5 ± 11.8	30.5 ± 4.8
Eastern Wood-pewee (1)	*Contopus virens*	28.5	6.6	52.2	0.1	0.7	0.8	0	11.2	32.6	19.6
Song Sparrow (6)	*Melospiza melodia*	29.4 ± 2.3	44.2 ± 11.7	53.3 ± 14.8	44.3 ± 17.8	18.9 ± 9.8	1.5 ± 1.2	0	9.0 ± 1.9	52.0 ± 7.4	23.6 ± 4.2
Tree Swallow (5)	*Tacycineta bicolor*	35.6 ± 4.9	13.4 ± 3.4	64.9 ± 29.8	56.7 ± 25.8	4.5 ± 2.8	2.4 ± 0.6	0	14.8 ± 3.4	59.5 ± 12.5	27.6 ± 2.2
Eastern Phoebe (5)	*Sayornis phoebe*	35.8 ± 2.1	40.7 ± 16.6	40.8 ± 23.7	60.6 ± 18.6	4.2 ± 3.1	3.5 ± 0.4	0.2 ± 0.2	5.9 ± 0.1	48.7 ± 5.1	22.8 ± 3.6

Table 1

Species (n)	ChEster	FAME	TAG	FFA	Cholesterol	Ceramide 1	Ceramide 2	Ceramide 3	Cerebroside
Black-crowned Finchlark (7)	0.4 ± 0.4	2.1 ± 0.4	0	15.6 ± 2.3	0.5 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	3.4 ± 0.9	5.9 ± 1.6
Dunn's Lark (7)	0.3 ± 0.3	2.0 ± 0.4	0	11.4 ± 1.8	0.1 ± 0.1	0.9 ± 0.2	0	3.0 ± 0.7	3.6 ± 0.6
Crested Lark (7)	0.9 ± 0.4	3.3 ± 0.8	0.1 ± 0.1	10.1 ± 1.6	0.4 ± 0.1	0.3 ± 0.2	0	3.6 ± 1.6	4.6 ± 0.8
Hoopoe Lark (8)	0.3 ± 0.1	4.8 ± 1.5	0.1 ± 0.1	7.9 ± 1.7	0.1 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	7.4 ± 2.6	4.4 ± 0.6
Desert Lark (6)	3.6 ± 1.7	2.9 ± 0.3	0.2 ± 0.2	16.9 ± 1.5	0.7 ± 0.3	0.4 ± 0.2	0	8.5 ± 1.8	4.7 ± 1.0
Horned Lark (9)	0.1 ± 0.1	1.1 ± 0.2	0.3 ± 0.1	8.8 ± 2.1	0.6 ± 0.2	0.7 ± 0.1	0	1.3 ± 0.1	3.8 ± 0.6
Skylark (9)	0.1 ± 0.1	1.3 ± 0.2	0.2 ± 0.1	8.7 ± 1.5	0.5 ± 0.1	0.6 ± 0.1	0.1 ± 0.1	1.5 ± 0.2	3.3 ± 0.6

Table 2