Nature or nurture: Can prey-based diets influence species-specific physiological performance traits of epidermal lipid content and cutaneous water loss?

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ABSTRACT

Epidermal lipids serve as the primary barrier to cutaneous water loss and play a significant role in water conservation and homeostasis. Previous studies have shown the correlation between increased aridity of habitats and the amount of epidermal lipids among species. Generally, increased amounts of epidermal lipids lower skin permeability. Species-specific differences in cutaneous water loss and prey preferences between two sympatric snake species, the Northern Cottonmouth (*Agkistrodon piscivorus*) and the Eastern Copperhead (*Agkistrodon contortrix*), motivated us to question if prey-base can result in these observed species-specific differences in cutaneous water loss. We experimentally controlled the diets for a captive colony of Northern Cottonmouths (*Agkistrodon piscivorus*) by feeding either fish (*Notemigonus crysoleucas*) or mice (*Mus musculus*) to investigate if diet can affect the quantity and quality of epidermal lipids and the rates of cutaneous water loss. Snakes fed mice gained consistently more mass, but diet treatments did not affect growth rate. We found no significant differences in quantitative lipid content nor rates of cutaneous water loss between diet treatments. An analysis for qualitative lipid content using IR spectrophotometry also showed no diet effect, thus suggesting that lipid content and cutaneous water loss are strong species-specific physiological performance traits not influenced by recent dietary history. While there is some evidence that epidermal permeability may be variable under certain environmental conditions (e.g., humidity), our findings show that diet has no effect and that a shift in prey preference may not influence or enhance physiological performance for decreasing cutaneous water loss.
1. Introduction

The physiological and behavioral conservation of water and protecting against dehydration is an important performance trait for many terrestrial species, especially those in arid climates. Species in arid habitats tend to have adaptations for lowering rates of evaporative water loss compared to those species from more mesic or aquatic habitats. Numerous studies have demonstrated the negative correlation between evaporative water loss and habitat aridity (e.g., Bentley and Schmidt-Nielsen 1966; Gans et al. 1968; Prange and Schmidt-Nielsen 1969; Elick and Sealander 1972; Cohen 1975; Baeyens and Rountree 1983; Roberts and Lillywhite 1983; Dmi’el 1998; Lillywhite 2006). Interestingly, evaporative water loss seems to correlate with habitat aridity regardless of taxonomic position (Dmi’el 1998) and is observed in other vertebrate taxa (e.g., Tieleman et al. 2003).

Although many of these studies have examined interspecific comparisons of evaporative water loss and habitat aridity among a broad range of ophidian taxa, fewer studies have compared congeneric (Dunson and Freda 1985; Dmi’el 1998; Moen et al. 2005) or conspecific (Agugliaro and Reinert 2005) taxa. Miller and Lutterschmidt (2014) compared copperheads and cottonmouths, two closely related congeneric sister species in the genus *Agkistrodon* (Parkinson et al. 2000), to investigate if species differences in mesic versus aquatic habitat preferences correlated with their rates of cutaneous water loss (CWL). The species-specific physiological ability for limiting CWL may reflect individual adaptations that serve an important role in differences in habitat preference and resource partitioning (Miller and Lutterschmidt 2014).

Because cutaneous (not respiratory) water loss is the primary source of evaporative water loss in squamates (Prange and Schmidt-Nielsen 1969; Cohen 1975; Dmi’el 1985; Dmi’el 2001), epidermal lipids in the integument serve a major role in regulating CWL in reptiles (Roberts and...
Lillywhite 1980). Potential species-specific differences in epidermal lipids may then serve as a potential mechanism allowing copperheads (*Agkistrodon contortrix*) to limit CWL and use and forage in more mesic and upland habitats, thus avoiding both direct and indirect competition with sympatric cottonmouths (*Agkistrodon piscivorus*). Copperheads consume mainly small mammals (Garton and Dimmick 1969; Brown 1979) which contain substantially more lipid than fish and amphibians consumed mainly by cottonmouths (Clark 1949; Kofron 1978). Thus, we raised the question: Is the lower CWL in copperheads (Moen et al. 2005; Miller and Lutterschmidt 2014) an adaptive species-specific physiological performance trait enhancing their ability to use more upland mesic habitats? Or, is the lower CWL in copperheads simply the product of a lipid-rich mammalian prey-preference more readily available in upland mesic habitats? Parkinson et al. (2000) investigated the phylogeography of the North American *Agkistrodon* species and found that *A. contortrix* exhibits the ancestral condition of terrestriality, with *A. piscivorus* exhibiting the only derived shift in aquatic habitat preference. Therefore, a more informed question may be: Is the higher CWL in cottonmouths a derived adaptive trait for lowering energetic cost in maintaining epidermal lipids less needed in an aquatic habitat? Or, is higher CWL in cottonmouths simply the product of lipid-poor prey (i.e., fish and amphibians) more readily available in aquatic habitats?

As epidermal lipids (Roberts and Lillywhite 1980) and diets deficient in essential fatty acids influence CWL (Menton 1970; Elias and Brown 1978; Williams and Elias 1987), we compared the rates of CWL and conducted both quantitative and qualitative analyses of epidermal lipids for Northern Cottonmouths (*Agkistrodon piscivorus*) fed either diets of fish (low-lipid) or mice (high-lipid). The cottonmouth served as an ideal model because this species is a diet generalist (Burkett 1966) and readily feeds on either fish or mice in the laboratory. This
unique opportunity to manipulate diet experimentally allowed us to investigate if skin permeability and increased CWL in the cottonmouth are influenced by prey-based lipid content. Negative results for the influence of diet would then suggest that CWL is a fixed species-specific trait reflective of selection pressures for increased physiological performance in preferred microhabitats.

2. Materials and methods

2.1. Experimental Subjects and Captive Care

Adult Northern Cottonmouths (*Agkistrodon piscivorus*) were collected in July 2016 from Harmon Creek located in Walker County, Texas (Texas Parks and Wildlife Scientific Research Permit SPR-0715-127 issued to WIL). Only female snakes (*n* = 24) were used in experiments to control for potential sex differences in skin lipids (Mason et al. 1987; Ball 2000). Beginning in August 2016, each snake was housed separately in plastic cages (38 x 26 x 22 cm) with aspen bedding (Harlan Teklad, Madison, Wisconsin) and water provided *ad libitum*. Snakes were kept in a laboratory and acclimatized to temperature (25 ± 2 °C), relative humidity (50 ± 3%), and photoperiod (12L:12D cycle) with the photophase centered on 1200 h. Snakes were randomly selected for one of two diet treatments; a low-lipid fish diet (*n* = 12) or high-lipid mice diet (*n* = 12). Generally, fish (i.e., golden shiners) have a mean body fat ≈9% of *M* _b_ (Lochmann and Phillips 2012) while mice have more than double the mean body fat ≈25% of *M* _b_ (Reed et al. 2007). Beginning September 2016, each of the two diet groups were fed weekly and offered either fish or mice equal to 20% of their *M* _b_ (Lutterschmidt and Rayburn 1993; Byars et al. 2010; Sparkman et al. 2010). We measured each snake’s initial snout-vent-length (SVL) to the nearest 0.1 cm (*mean* = 51.07, *SE* = 1.167, *n* = 24) and body mass (*M* _b_ ) to the nearest gram (*mean* =...
193.9, \( SE = 11.98, n = 24 \) prior to experimentation and \( M_b \) was measured monthly (September 2016 to July 2017) for growth. Food amounts were adjusted to ensure food per unit \( M_b \) remained constant. Fish (golden shiners, \textit{Notemigonus crysoleucas}) were purchased from Oakhurst Bait Co. (Oakhurst, Texas) and CD-1® IGS laboratory mice (\textit{Mus musculus}) were supplied by the Sam Houston State University Science Annex.

### 2.2 Shed Epidermis Collection

Snakes were maintained in captivity until all snakes produced a second shed epidermis for collection and study. The first shed was discarded and not used for experimentation as these shed integuments are affected by each snake’s natural diet regime and other differences such as surface abrasion from traversing habitat structures under field conditions. Thus, the second shed was used because all snakes experienced identical acclimatization and captive care regimes ensuring valid between-treatment comparisons of diet.

Cages were inspected daily for the presence of fresh sheds. Collected sheds were immediately dried, sealed in plastic bags, and frozen (-20°C) to preserve the integument (Burken et al. 1985a; Agugliaro and Reinert 2005;). All experiments on individual sheds were performed over the same time period (Summer 2017) and began once the second shed was collected from all 24 snakes. Shed epidermis samples were stored between 8 and 234 days prior to analysis. Dunson and Freda (1985) showed no changes in rates of water influx and efflux with snake skins stored for two years.

### 2.3 Measures of Cutaneous Water Loss

We used the \textit{in vitro} technique (Agugliaro and Reinert 2005; Miller and Lutterschmidt 2014) to measure cutaneous water loss (CWL) of intact epidermis (Dunson and Robinson 1976; Zucker and Maderson 1980; Stokes and Dunson 1982). Only the mid-dorsal region of the shed
epidermis was used to control for potential differences in CWL along the body’s dorsal surface (Miller and Lutterschmidt 2014). Three mid-dorsal samples (ca. 2 x 2 cm) were cut from each snake’s second shed and inspected microscopically for the presence of holes or tears in the integument. These shed samples \((n = 72)\), with the mucosal surface facing outward, were then stretched over the opening \((0.58 \text{ cm}^2)\) of a culture tube \((10 \times 75 \text{ mm})\) containing 1 mL of deionized water. We then secured the shed to the opening of the culture tube and created a tight seal using waxed tread and Parafilm® (Pechiney Plastic Packaging, Menasha, Wisconsin). The culture tube was then inverted and suspended inside a 30-mL specimen bottle containing 5 g of t.he.® desiccant (EMD Chemicals Inc., Gibbstown, New Jersey). Water is then drawn from the culture tube, through the shed epidermis under simulated natural physiological conditions (Burken et al. 1985a; Agugliaro and Reinert 2005). The initial and final mass of each culture tube was measured at 120 h. We calculated the rate of CWL for each sample from the difference of the initial and final mass of the culture tube, divided by the 120 h. The three shed samples from each snake were then averaged for a total of 24 independent mean values of CWL rate.

2.4 Epidermis Lipid Extraction and Quantitative Analyses

The quantitative lipid content of a shed \((\text{mg lipid / g of shed})\) was determined by the initial and final masses of a shed after lipid extraction. We separated both the dorsal and ventral surfaces of each shed allowing for comparison of lipid content between shed surfaces (dorsal vs. ventral) and the sum of these surfaces provided total lipid content for each shed. Standard techniques for lipid extraction from shed epidermis were used (Roberts and Lillywhite 1980; Stokes and Dunson 1982; Burken et al. 1985b; Agugliaro and Reinert 2005). The dorsal and ventral sheds were first placed in 240-mL specimen jars with 100 g of t.he.® desiccant for 24 h, removed and immediately massed to the nearest 0.0001 g using a Denver Instruments A-250
analytical balance (Denver Instruments Company, Bohemia, New York). We then placed each shed surface in a 400-mL jar for 24 hours containing a 120 mL, 2:1 chloroform to methanol solution (Folch et al. 1957) to extract total lipids. After lipid extraction, sheds were removed from the chloroform-methanol solution and washed once in fresh chloroform-methanol solution and rinsed three times in distilled water. We then placed the sheds in jars containing fresh desiccant for 24 h prior to measuring final dry mass. Quantitative lipid content (mg) was determined from the difference in initial and final dry masses (to the nearest 0.0001 mg) of each shed surface. The lipid content per shed epidermis mass (mg / g) was standardized for analysis by dividing lipid content by the initial mass of the dorsal and ventral surfaces.

2.5 Infrared (IR) Spectroscopy and Qualitative Lipid Analyses

Prior to total lipid extraction from sheds, a fourth mid-dorsal and a single mid-ventral shed sample (ca. 1 x 1 cm) was cut from each shed for qualitative lipid analyses. We used infrared (IR) spectrometry (Bruker Optics Alpha Fourier Transform IR) to examine and quantify the qualitative composition of lipids within the shed epidermis. Spectroscopy (Lin et al. 1994; Ismail et al. 1999; Zarini et al. 2019) is the study of how radiated energy and matter interact. Different chemical bond types respond to radiation differently allowing one to identify various functional groups and distinguish differences in chemical composition between samples. IR spectroscopy specifically uses infrared radiation to excite the molecules of a compound generating an infrared spectrum of energy absorbed by molecules as a function of frequency or wavelength. We used IR spectrometry, the application of spectroscopy, to examine the absorbance wavenumbers that correspond to molecular geometries of organic molecules within the shed epidermis. The qualitative composition of lipids was examined quantitatively by recording the absorbance values of threshold wavenumber peaks in each sample. Each shed
sample was dry, allowed to reach ambient temperature (23 °C), centered within the instrument, and measured once. We examined nine standardized wavenumber positions (corresponding to chemical bonds and molecular geometries, Table 1) to investigate qualitative differences in lipid content between sheds from the fish (low-lipid) and mice (high-lipid) diets. Additionally, we compared dorsal and ventral sheds. Molecular geometries of functional groups and the chemical composition within sheds were identified (Barry et al. 1993; Ripamonti et al. 2009).

2.6 Statistical Analyses

We used SigmaPlot® 11.0 and SPSS Statistics® 22.0 for all statistical analyses, for testing assumptions of normality (Shapiro-Wilks) and equal variance (Levene’s), and for graphing. Linear regression analysis (Zar 2010) was used to confirm that storage times between 8 and 234 days did not affect CWL or lipid content of shed epidermis samples. A Student’s t-test was used to test for differences in CWL between diets. To investigate differences in total shed lipid content, we used a multivariate analysis of variance (MANOVA) with diet (fish and mice) representing treatment groups and shed surface (dorsal and ventral) as dependent variables within treatments. This multivariate analysis appropriately tests the null hypothesis that snakes with different diets have the same dorsal and ventral lipid content because dorsal and ventral shed samples are obtained from the same shed of an individual and inter-correlated (Zar 2010). As shed surfaces were sampled from the same shed and individual, paired t-tests were used to investigate differences between shed surfaces from each within each diet treatment.

Principal components analysis (PCA) with the non-orthogonal oblique rotation method (Oblimin with Kaiser normalization), assuming non-independence among IR peaks, was used to investigate possible differences in molecular geometries and the variation in qualitative lipids between diets and shed surfaces. Group-mean PC scores of the first two principal components
are illustrated with 95% confidence intervals and were statistically analyzed for separation using one-way analysis of variance of component scores followed by Tukey’s a posteriori tests. Differences between treatments (diet) and variables (shed surface) were considered significant at $P < 0.05$.

3. Results

Linear regression analysis confirmed that storage time of shed epidermis did not affect CWL or quantitative lipid content in our samples. We found no time-effect on CWL for both the fish ($F = 3.16; df = 1, 10; P = 0.106$) and mouse ($F = 3.13; df = 1, 10; P = 0.107$) diets. Additionally, we found no effects of storage time on quantitative lipid content (fish diet, $F = 0.37; df = 1, 10; P = 0.554$ and mouse diet, $F = 0.01; df = 1, 10; P = 0.920$). These results confirm the stability of shed epidermal tissue for storage and the later testing of CWL and lipids (Dunson and Freda 1985; Miller and Lutterschmidt 2014).

We found no difference in the rates of CWL ($t = -0.456; df = 22; P = 0.653$) between the fish and mouse diet treatments (Fig. 1). Using an MANOVA, we also found no difference in quantitative lipid content between the fish and mouse diet treatments when considered jointly on the variables dorsal and ventral shed surfaces (Wilk’s $\Lambda = 0.983; F = 0.181; df = 2, 21; P = 0.835$; partial $\eta^2 = 0.017$). The MANOVA between-subject effects for each dependent variable indicated that there were no significant difference between fish and mouse diet treatments for dorsal sheds ($F = 0.180; df = 1, 22; P = 0.676$; partial $\eta^2 = 0.008$) or ventral sheds ($F = 0.380; df = 1, 22; P = 0.544$; partial $\eta^2 = 0.017$). Following the MANOVA, paired t-tests were used to investigate differences between shed surfaces within each diet treatment. We found that lipid content between dorsal and ventral shed samples differed significantly within both the fish ($t = 4.911; df = 11; p < 0.001$) and mouse ($t = 4.975; df = 11; p < 0.001$) diet treatments (Fig. 2).
Using IR spectroscopy, we found nine predominant peaks in both the dorsal and mid-ventral shed surfaces (Fig. 3), corresponding to nine molecular geometries (Table 1). Of the 48 shed samples, eight were not used due to poor condition. Thus, sample sizes were <12 for the mid-ventral sheds in both the fish ($n = 9$) and the mouse ($n = 7$) diet treatments (Fig. 4). The component correlation matrix supported the use of a non-orthogonal Oblimin rotation method as correlation coefficients between principle components PC1 and PC2 ($r > 0.427$) indicated nonindependence among IR peaks. The first two principal components explained 96.6% of the variation among these groups with respect to their molecular geometries (Fig. 4). The first principal component (x-axis) explained 92.8% of the variation (eigenvalue = 8.349) with peak numbers 4 and 7 correlating strongest with this axis ($r = 0.982$ and 0.981, $df = 38$). The second principal component (y-axis) explained 3.8% of the variation (eigenvalue = 0.340) with peaks 2 and 8 correlating strongest with this axis ($r = 0.350$ and 0.273, $df = 38$). The 95% confidence intervals of mean principal component scores for both PC1 and PC2 overlapped heavily between diet treatments (Fig. 5A) but showed significant separation between the dorsal and ventral shed surfaces for PC1 (Fig. 5B). One-way analyses of variance for both PC1 ($F = 0.639; df = 1, 38; P = 0.429$) and PC2 ($F = 0.629; df = 1, 38; P = 0.433$) scores indicated no significant differences between diet treatments (Fig. 5A). However, group mean differences between dorsal and ventral shed surfaces (Fig. 5B) differed significantly for PC1 ($F = 67.085; df = 1, 38; P < 0.001$; Tukey’s test, $q = 11.583; P < 0.001$) but not PC2 ($F = 1.105; df = 1, 38; P = 0.300$) scores.

As would be expected with captive feeding, there were significant relationships between cumulative $M_b$ gain and time in captivity for both the fish ($F = 127.87; df = 1, 10; P < 0.001; r^2 = 0.93$) and mouse ($F = 272.15; df = 1, 10; P < 0.001; r^2 = 0.96$) diets. Differences in lipid content between the prey-based diet treatments, where mice contain more than double the mean body fat
(Reed et al. 2007) of fish, also resulted in significant differences in growth (Fig. 6). Comparison
of regression coefficients and slopes (Zar 2010) indicated that the rate of growth did not differ
between fish and mouse diets ($t = -0.499; df = 18, P = 0.624$). However, snakes in the mouse-
diet treatment gained consistently more $M_b$ as indicated by the highly significant difference in
regression elevations ($t = -5.594, df = 19, P < 0.001$). The final accumulative gain in $M_b$ for
mouse-fed snakes ($mean = 234.4, SE = 35.13, n = 12$) averaged 37.0 g ($SE = 42.24, n = 12$)
greater than fish-fed snakes ($mean = 197.4, SE = 25.87, n = 12$) at the end of the 11-month
captivity period.

4. Discussion

Our results indicate that prey-based diets of either fish or mice do not affect CWL nor the
quantity or quality of epidermal lipids in the northern cottonmouth ($A. piscivorus$). Thus,
significantly greater rates of CWL in cottonmouths compared to copperheads (Miller and
Lutterschmidt, 2014; Moen et al, 2005) result most likely from differences in species-specific
physiological performance traits that are not influenced by diet. However, factors such as habitat
and microhabitat acclimatization (e.g., Kattan and Lillywhite 1989), phenotypic plasticity (e.g.,
Haugen et al. 2003; Lillywhite 2004), and ontogenetic changes (e.g., Agugliaro and Reinert
2005; Muñoz-Garcia and Williams 2008) may influence water barrier function that is typically
and relatively characteristic of a species (Lillywhite 2004). As copperheads are more terrestrial,
the derived shift from terrestrial to aquatic microhabitat preference by cottonmouths (Parkinson
et al. 2000) may reflect an adaptive trait for reducing epidermal lipids and the energetic costs
associated with maintaining these lipids (Miller and Lutterschmidt 2014). Epidermal
permeability does not seem to correlate with phylogeny (Dmi’el 1998; Teleman et al. 2003),
making the pronounced differences in CWL between these closely related sympatric species a
result of evolutionary adaptation, and not phenotypic plasticity. Miller and Lutterschmidt (2014) found species-specific differences in CWL and epidermal lipid content (under identical and controlled mouse diets for both species) and suggest that these performance traits may potentially serve as a physiological mechanism for the ecological partitioning of two closely related sympatric species. These authors add that such theoretical concepts in physiological ecology are well illustrated when traits for physiological performance and tolerance correlate with species natural history and ecology. Here, we questioned if cottonmouths (by consuming increased lipids in a prey-based diet) could decrease skin permeability and CWL thus creating potential overlap in niche space and competitive interactions with copperheads. While these two taxa do partition by diet and microhabitat, diet was not likely the driving force in the divergence of these two taxa. Our results suggest that CWL and epidermal lipids are not influenced by diet and may represent more relatively fixed species-specific traits (Lillywhite 2004) reflective of evolutionary selection for physiological performance.

Roth (2005) found the spatial distributions of cottonmouths to be mostly riparian with 83% of snake locations occurring within 10 m of water. This well-documented aquatic habitat preference by cottonmouths (Gloyd and Conant 1990; Dixon and Werler 2005) may have other associated adaptations for decreased epidermal lipids and increased CWL. Behavioral aggregation and increased social interaction in cottonmouths (Roth 2005; Roth and Lutterschmidt 2011) may be adaptive for limiting evaporative water loss by reducing the surface area of exposed integument (Graves et al. 1986; Tu et al. 2002; Agugliaro and Reinert 2005). Additionally, increased skin permeability may aid also in water absorption (Cohen 1975), evaporative cooling for thermoregulation, and cutaneous gas exchange (Standaert and Johansen 1974; Heatwole and Seymour 1978) for increased respiration in an aquatic environment (Winne
et al. 2001).

There is a well-established aridity gradient for skin permeability (Cohen 1975; Roberts and Lillywhite 1980; Stokes and Dunson 1982; Baeyens and Rountree 1983; Roberts and Lillywhite 1983; Dmi’el 1998; Lillywhite 2006) which correlates with the amount and quality of lipids in the epidermis (Lillywhite and Maderson 1982; Burken et al. 1985b; Lillywhite 2006; Miller and Lutterschmidt 2014). However, it seems that increased consumption of dietary lipids from prey does not necessarily result in increased epidermal lipids. Ingested lipids are not simply deposited into the epidermis (e.g., Sheridan 1994; Price 2017). Dietary triglycerides are hydrolyzed in the intestine by pancreatic lipase producing free fatty acids and monoacylglycerol (Patton 1975; Kammoun et al. 2008) where these components are then absorbed and re-esterified by enterocytes to produce triglycerides for transport throughout the body (Price 2017).

The anabolism of epidermal lipids results from sequestered acetate, the main carbon source for lipid synthesis (Wertz 1996). Multiple lipid bilayers obstructing the intercellular space of the outermost layer (i.e., stratum corneum) of the epidermis serve as the permeability barrier preventing transcutaneous water loss (Landmann 1988). Generally, the lipids in reptile epidermis consist of cholesterol, free fatty acids, and ceramides (Roberts and Lillywhite 1983; Burken et al. 1985b; Landmann 1988; Elias and Menon 1991; Ball 2004; Torri et al. 2014). A high concentration of ceramides may decrease permeability by allowing lipid lamellae to form tight, highly ordered crystalline phases (Velkova and Lafleur 2002; Bouwstra et al. 2003). A high concentration of cholesterol more tightly packs the lipid fatty acid chains together, creating a more impermeable barrier (Hadley 1989; Raffy and Teissié 1999). Thus, despite the majority of the epidermis being composed of keratin, lipids in the mesos layer provide the barrier to water loss (Roberts and Lillywhite 1980).
The closest parallels to our study are those studies that fed mice diets deficient in essential fatty acids, thus increasing rates of epidermal water loss (Menton 1970; Elias and Brown 1978; Williams and Elias 1987). The lamellar bodies of these mice were void of lipids (Elias and Brown 1978), suggesting that such diet deficiencies completely disrupt the lipid barriers to water loss. Although our study for increasing lipid consumption in a prey-based diet (mouse vs. fish) failed to show differences in epidermal lipids and skin permeability, we did observe significant growth differences between diet treatments (Fig. 6). This observed difference in growth is most likely due to the greater mean body fat in the mouse diet (≈25% of Mb) compared to the fish diet (≈9% of Mb). Similar differences in growth were observed in fish (e.g., Vergara et al. 1999) with diets of higher lipid content.

We found significant differences in quantitative lipid content between the dorsal and ventral shed surface of the northern cottonmouth for both the fish and mouse diet treatments (Fig. 2). Miller and Lutterschmidt (2014) also found similar results for both copperheads and cottonmouths. This greater amount of lipid in the dorsal integument most likely aids in reducing water loss from the dorsal surface. Here, we demonstrate that diet does not influence this difference in either the quantity (Fig. 2) or quality (Fig. 5a) of lipids in the dorsal or ventral shed surfaces. Additionally, the ventral integument is 50% thicker than the dorsal integument (Jayne 1988). The increased amount of keratin likely protects against ground abrasion during locomotion. The thinner dorsal surface likely has proportionally more lipids as scale thickness does not prevent water loss across the integument (Lillywhite and Maderson 1982).

The main motivating factor for this study was to further investigate the significant difference in dorsal CWL between copperheads and cottonmouths (Miller and Lutterschmidt 2014) and a potential correlate with lipid content in a prey-based diet. In addition to Miller and
Lutterschmidt (2014) finding no significant species difference in the mean amount of dorsal lipid, we found no significant difference in the mean amount of lipid in the dorsal and ventral shed surfaces between prey-based diets. This suggests that the observed differences in CWL between copperheads and cottonmouths is related to species-specific differences in the qualitative properties of dorsal lipids. Further investigation for qualitative differences in epidermal lipids between copperheads and cottonmouths would help inform the observed species-specific differences in CWL (Miller and Lutterschmidt 2014).

Reptiles have mainly cholesterol, free fatty acids, and ceramides in the mesos layer of the epidermis (Roberts and Lillywhite 1983; Burken et al. 1985b; Landmann 1988; Elias and Menon 1991; Lillywhite 2006; Torri et al. 2014). An increase in polar ceramides is associated with lower permeability in birds and bats (Haugen et al. 2003; Muñoz-Garcia and Williams 2007; Muñoz-Garcia et al. 2012) and a high amount of cholesterol in lipid bilayers also lowers permeability (Hadley 1989; Raffy and Teissié 1999). Our qualitative lipid analysis for the presence of particular molecular geometries indicated no effect of diet (Fig 5a). It is important to note that differences in molecular geometries may not be completely associated with lipid composition, as other molecular compounds are within epidermis. However, the dorsal and ventral surfaces were found to be significantly different (Fig. 5b) with the dorsal surface often lacking a discernible peak 6 (δCH₃). The significance for the absence of this molecular geometry in the dorsal integument is unknown.

Finally, we comment on the observed treatment effects of diet on the Mb of snakes. Although the growth rate between snakes in the fish and mouse diet treatments did not differ, snakes fed mice gained consistently more Mb each month (Fig. 6) and resulted in a significantly higher regression elevation than snakes fed fish. At the end of the 11-month captivity period,
mouse-fed snakes gained an average Mb 37.0 g greater than fish-fed snakes. Although there are numerous abiotic and biotic factors that influence growth in natural populations, it might be of interest to question if diet composition as a result of prey availability and prey preference for small mammals could influence growth and Mb within natural populations.

5. Conclusion

We found that diet and differences in the prey-based consumption of lipids has no influence on the quantitative or qualitative lipid content of the epidermal integument, and thus no influence on CWL. The observed species-specific difference in CWL between copperheads and cottonmouths (Moen et al. 2005; Miller and Lutterschmidt 2014) suggest a species-specific physiological performance trait not influenced by diet. Because epidermal permeability does not seem to be correlated with phylogeny (Dmi’el 1998; Teleman et al. 2003), these pronounced differences are most likely the result of evolutionary adaptation, and not phenotypic plasticity. While these two sympatric and closely related sister taxa are partitioned by diet and microhabitat, diet was likely not the driving force for species divergence and the derived shift in aquatic habitat preference by cottonmouths.

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Table 1. Molecular geometries of each peak and wavenumber (cm$^{-1}$) identified by IR spectrometry.

| Peak Number | Wavenumber (cm$^{-1}$) | Assigned Molecular Geometry |
|-------------|------------------------|----------------------------|
| 1           | 3268                   | νOH                        |
| 2           | 2927                   | νCH$_2$                    |
| 3           | 1630                   | νCO, amide I               |
| 4           | 1526                   | νCO and δNH, amide II      |
| 5           | 1450                   | δCH$_2$                    |
| 6           | 1397                   | δCH$_3$                    |
| 7           | 1236                   | νCN, amide III             |
| 8           | 1066                   | νCC                        |
| 9           | 486                    | νSS                        |
Fig. 1. Mean (± 95% CI) rates of cutaneous water loss (CWL) for the fish (0.649, $SE = 0.0798$, $n = 12$) and mouse (0.702, $SE = 0.0828$, $n = 12$) diet treatments are show in black. Gray box plots show the median (central gray line within box), the 25th and 75th percentiles (bottom and top lines of box), and the 10th and 90th percentiles (gray error bars).
Fig. 2. Quantitative lipid content of shed epidermis for both dorsal (84.15, \(SE = 5.515, n = 12\)) and ventral (59.91, \(SE = 6.445, n = 12\)) shed surfaces in the fish diet treatment are shown in the first pair of box plots. The second pair of box plots show both mean (± 95% CI) for dorsal (80.06, \(SE = 7.899, n = 12\)) and ventral (54.12, \(SE = 6.840, n = 12\)) shed surfaces in the mouse diet treatment. Group means (black circles) and ± 95% CI (black error bars) are shown with gray box plots showing the median (central gray line within box), the 25\(^{th}\) and 75\(^{th}\) percentiles (bottom and top lines of box), and the 10\(^{th}\) and 90\(^{th}\) percentiles (gray error bars). Paired \(t\)-tests indicated statistically significant differences between the dorsal and ventral shed surfaces within both diets.
Fig. 3. An example output of the infrared (IR) spectroscopy showing the nine predominant absorbance peaks of a shed sample. The x-axis (wavenumber) represents the vibration frequency of molecular bonds within the sample. Stronger bonds and lighter atoms vibrate at higher frequencies, so their location along the x-axis corresponds to different functional groups. Peaks indicate the IR absorbance units (y-axis) specific to those wave numbers or frequencies characteristic of molecular geometries.
Fig. 4. Principal components analysis (PCA) of IR absorbance units for the nine predominant peaks and molecular geometries within lipids of both the dorsal and ventral shed surfaces and within each diet treatment. This illustrates that the primary source of variation is associated with differences between dorsal and ventral epidermal samples and not diet.
Fig. 5. Principal components analysis (PCA) of IR absorbance units for the nine predominant peaks and molecular geometries within lipids with 95% confidence intervals of mean component scores for each group illustrated on both axes. Extensive overlap of confidence intervals of the combined dorsal and ventral shed surfaces within the fish and mouse diet treatments (A) indicate that diet had no effect on qualitative lipid content. Separation of the 95% confidence intervals for both principal components of the combined diet treatments within each shed surface (B) indicate that the dorsal and ventral shed surfaces differed in qualitative lipid content.
Fig. 6. Mean (± SE) cumulative gain of body mass ($M_b$) for snakes in both the fish ($n = 12$) and mouse ($n = 12$) diet treatments. The grey square represents the initial gain of $M_b$ equal to zero and resulting regression lines for both diets include zero.