AN EXPERIMENTAL MANIPULATION OF DIET AND ITS INFLUENCE ON GROWTH AND EPIDERMAL LIPIDS IN THE NORTHERN COTTONMOUTH (*AGKISTRODON PISCIVORUS*)

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by

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ABSTRACT

Weidler, John M., An experimental manipulation of diet and its influence on growth and epidermal lipids in the northern cottonmouth (A*gkistrodon piscivorus*). Master of Science (Biology), May, 2018, Sam Houston State University, Huntsville, Texas.

I investigated the effect of diet on growth, skin permeability, and lipid content of snake skin. Lipids are a vital source of energy for life and provide the barrier to water loss in snake epidermis. I conducted a study on a captive colony of snakes (*Agkistrodon piscivorus*), controlling for either a fish (*Notemigonus crysoleucas*) or a mouse (*Mus musculus*) diet. Snakes fed a diet of mice gained significantly more mass than snakes on a diet of fish, indicating that increased lipid content in diet has a significant effect on growth. However, I found no significant difference in cutaneous water loss or lipid content between the two diet groups, indicating that lipid content and cutaneous water loss are strong species-specific physiological performance traits not influenced by recent dietary history. Using IR spectroscopy, I found qualitative differences in absorbance and molecular geometry between the dorsal and ventral surfaces of snake skin. The physiological ability to limit water loss likely plays an important role in microhabitat partitioning between copperheads and cottonmouths but is not influenced by the different diets of these sympatric (but not syntopic) species.

ACKNOWLEDGMENTS

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This research was funded in part by a student research award from the Texas Academy of Science for the purchase and supply of golden shiner minnows, and I thank the Oakhurst Bait Shop for working with me as a private buyer and within my research budget.

I would also like to thank the faculty and staff at the Texas Research Institute for Environmental Studies (TRIES). Dr. Rachelle Smith assisted with the IR spectroscopy and compact mass spectrometry for lipid analysis and Ms. Kathy Allen was always helpful with logistics and contacts of TRIES personnel.

I would like to thank Edward J. Tivador III for supplying mice from the Science Annex, and always being willing to take time out of his day to come over to help me with captive care of my snakes. Most of all, I thank "Ed" for all the fun conversations about herps, NBA, and everything in between.

Tara Martin was helpful in just about everything, even when I had random questions to ask (which was frequent).

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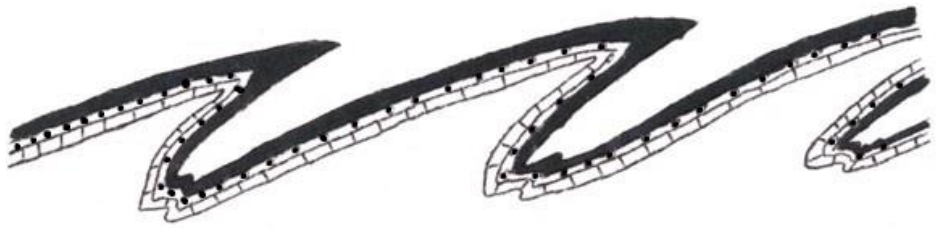
CHAPTER I

INTRODUCTION

The tetrapod integument has evolved to serve a variety of purposes for a myriad group of organisms all over the world. From the iridescent feathers of birds of paradise, the shells of turtles, to the armor of pangolins, the variety of integuments and outer appendages is truly breathtaking. In "higher" vertebrates, two important functions of the integument are to provide mechanical protection and a barrier to water loss.

The outer layer of integument, the epidermis, is primarily made of keratin. Keratins are a family of proteins that provide rigidity, support, and protection. Keratins are the main component in feathers, scales, hair, horns, and a variety of outer appendages. Figure 1.1 illustrates diagrams of the epidermis (scales and skin) of different vertebrate taxa. The two main types of epidermal keratin in reptiles are the hair-like alpha (α) keratin and feather-like beta (β) keratin (Baden et al. 1966, Lillywhite and Maderson 1982, Rudall 1947). The α layer has helically arranged α proteins, while the β layer has β proteins, which are mainly deposited as horizontal sheets. As Figure 1.1 illustrates, there are general differences between taxa. Crocodilians and birds have horizontal alternation of α and β keratin. Testudines can have α keratin, or horizontal alternation of α and β keratin as seen in birds and crocodilians. Mammals possess only α keratin. Lepidosaurs are unique in having vertically alternating layers of both α and β keratin. These different epidermal structures are notable, but their significance is not entirely clear (Lillywhite and Maderson 1982).

A.



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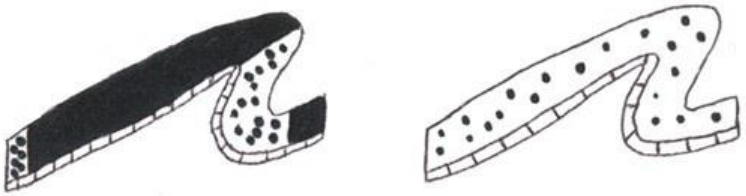

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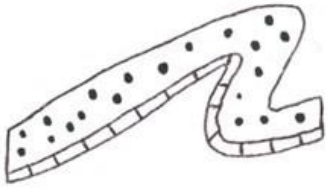

Figure 1.1 – Epidermal structure of higher vertebrates, including (a) Lepidosaur (b) Crocodilian/Avian (c) Testudine (d) Mammal. Shaded areas indicate β keratin, spotted areas indicate α keratin, and blocks are viable layers.

The other important role of the outer integument in tetrapods is to provide a barrier to water loss. Desiccation is typically not a concern for a fish surrounded by water, so fish generally do not have effective barriers to water loss. With the transition to a completely terrestrial lifestyle, a way for terrestrial vertebrates to keep water in proved to be essential. The first tetrapods (basal amphibians) remained close to water bodies, and presumably had very permeable skin. Extant amphibians also generally have highly permeable skin, with many amphibians having rates of water loss equal to, or even exceeding, evaporation from a free surface (Amey and Grigg 1995). However, there are a few notable exceptions. Certain species of desert frogs secrete a cocoon to survive dry seasons (Lee and Mercer 1967).

The reptilian integument likely evolved over 300 million years ago (Matoltsy and Bereiter-Hahn 1986). This remarkable skin "has enabled reptiles to colonize the hottest and driest places on earth" (Life on Earth 1979). A more effective barrier to water loss is one of the things that separates the epidermis of higher vertebrates from fish and amphibians.

Lepidosaurs are characterized by an epidermis made up of outer dead layers and underlying viable layers (Figure 1.2). There are four outer epidermal layers (Allam et al. 2016). From the outermost inward, there is the Oberhäutchen, the β layer, the mesos layer, and then the α layer. The Oberhäutchen is a specialized type of β keratin that has a role in shedding (Zucker 1980) and micro-ornamentation (Alibardi and Toni 2006). The mesos layer has the "bricks and mortar" composition of alternating lipids and protein (Elias 1983, Elias and Menon 1991, Williams and Elias 1987).

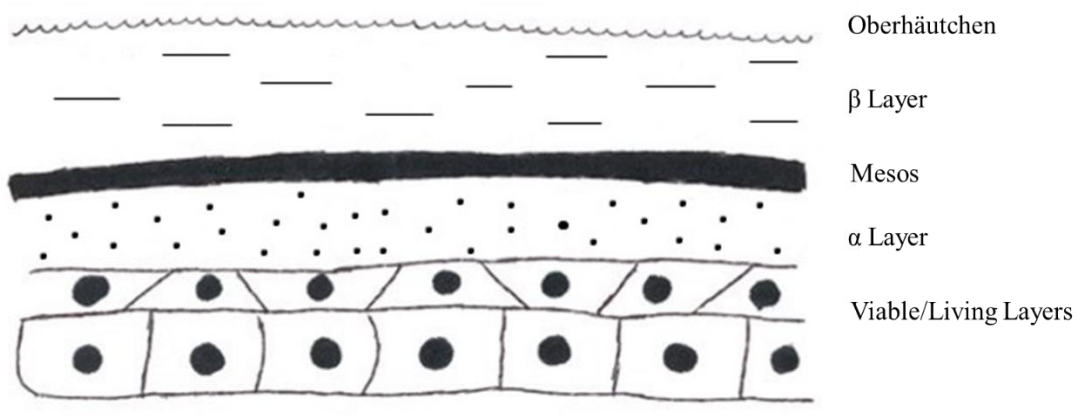

Figure 1.2 – Lepidosaur Epidermal layers

It was once widely believed that a reptile's skin was almost completely waterproof (e.g., Chew 1961, Chew and Damann 1961). However, a reptile's skin is not completely impermeable as it was once thought to be (Bentley and Schmidt-Nielsen 1966, Tercafs and Schoffeniels 1965). Neither are scales the barrier to water loss. For instance, scaleless snakes have rates of water loss equal to normal scaled snakes (Bennett and Licht 1975, Licht and Bennett 1972). Scale coloration can affect thermal ecology (Grover 1996) but the thickness of scales per se is not what determines rates of water loss. First, keratins are hygroscopic and absorb water (Roberts 1986), so they are ineffective at blocking water loss. Second, if keratins were the barrier to water loss then skin permeability would adhere to Fick's first law of diffusion, but demonstrating this experimentally has proven problematic (Elias et al. 1981, Kramer and Myers 2012, Mautz 1982).

Thus, despite the majority of the epidermis being composed of keratin, it is the lipids in the mesos layer that provide the barrier to water loss (Roberts and Lillywhite 1980). Lipids are primarily nonpolar and hydrophobic, making them ideal to prevent

water loss. As noted above, the epidermal keratins seem to be more for support and mechanical protection.

There is a well-established aridity gradient for skin permeability (Baeyens and Rountree 1983, Cohen 1975, Dmi'el 1998, Lillywhite 2006, Roberts and Lillywhite 1983, Stokes and Dunson 1982) which seems to be due to the amount and quality of lipids in the epidermis (Baeyens and Rountree 1983, Lillywhite 2006, Lillywhite and Maderson 1982, Roberts and Lillywhite 1980). Species dwelling in arid environments have more watertight skin than species inhabiting more humid or aquatic habitats (such as a rainforest). As species in drier environments are in much greater danger of desiccation, it makes sense that species in these types of habitats would evolve a more water-resistant skin.

The lipid makeup in reptile epidermis is generally cholesterol, free fatty acids, and ceramides (Ball 2004, Burken et al. 1985a, Elias and Menon 1991, Landmann 1988, Roberts and Lillywhite 1983, Torri et al. 2014). A high concentration of ceramides may decrease permeability by allowing lipid lamellae to form tight, highly ordered crystalline phases (Bouwstra et al. 2003, Velkova and Lafleur 2002). A high concentration of cholesterol packs the lipid fatty acid chains more tightly together, creating a more impermeable barrier (Hadley 1989, Raffy and Teissié 1999).

This research involved two main lines of inquiry with regards to lipids. My first research inquiry focused on how consuming food sources with different amounts of lipids might affect growth. Does diet (with differeing amounts of lipid quantity and quality) affect growth? Lipids are a greater source of energy than carbohydrates and protein

(Vander et al. 1998). Therefore, would a doubling (9% vs. 25%) in lipids significantly increase growth?

Second, I investigated how ingested lipids affect the epidermal permeability barrier. Could a difference in diet affect the permeability barrier? For instance, would consuming more lipid rich food result in more lipid deposition in the epidermis, resulting in a greater barrier to water loss?

CHAPTER II

MICE OR FISH? THE INFLUENCE OF DIET ON GROWTH AND GROWTH RATE IN THE NORTHERN COTTONMOUTH, *AGKISTRODON PISCIVORUS*[1]

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[1] J.M. Weidler and William I. Lutterschmidt, formatted for submission to *Herpetological Review*.

Introduction

Diet plays a significant role in the life history of reptiles. Reptiles are ectotherms, and ectotherms are often very efficient at converting ingested energy into growth and reproduction. For instance, snakes can convert over 40% of the energy from food into growth and reproduction (Dutton et al. 1975; Gans and Pough 1982).

Prey availability and abundance also plays a significant role in reproduction and growth. The number of offspring and the frequency of reproduction were significantly reduced in the lizard *Urosaurus ornatus* when there was low prey availability (Ballinger 1977). Diet can lead to earlier dates of sexual maturity and reproduction in brown house snakes (Byars et al. 2010). Clutch size is significantly greater in higher food source diets (Andrén and Nilson 1983; Ford and Seigel 1989; Seigel and Fitch 1985). A higher mass diet leads to significantly greater amounts of mass gain (Byars et al. 2010; Ford and Seigel 1989).

Diet is also important for conservation biology (Oftedal and Allen 1996). Zoos in the past often assumed that diet quality was not a concern (Oftedal and Allen 1996). However, providing a nutrient rich diet led to more rapid growth and decreased juvenile Galapagos land iguana mortality from 25% to 3% a year (Oftedal and Allen 1996).

In this study I fed cottonmouths one of two diets, mice (*mus musculus*) or golden shiners (*Notemigonus crysoleucas*). Laboratory mice have a mean body fat of roughly 25% of body mass (Reed et al. 2007) while fish (i.e., golden shiners) have a mean body fat of roughly 9% of body mass (Lochmann and Phillips 2012). Snakes on a diet of mice are thus ingesting more lipids than snakes on a diet of golden shiners. Does diet (with differeing amounts of lipid quantity and quality) affect growth? Since lipids are a richer

source of energy than proteins and carbohydrates (Vander et al. 1998), I sought to find out if snakes that ate a diet of mice experienced more growth (in terms of mass) than snakes that ate a diet of fish.

Materials and Methods

Adult snakes (*Agkistrodon piscivorus*) were collected (IACUC # 8403310 and # 16-10-27-1003-3-01) in July 2016 from the Sam Houston State University biological field station, the Center for Biological Field Studies (CBFS) located in Walker County, Texas. Only female snakes (N = 24) were used in experiments to avoid any potential intraspecific sex differences (Ball 2000; Mason et al. 1987). I measured body mass (M_b) for all snakes prior to experimentation and snakes were massed each month thereafter. Beginning in August 2016, each snake was housed separately in a plastic cage (38 x 26 x 22 cm) with aspen bedding (Harlan Teklad, Madison, Wisconsin, USA) and water provided *ad libitum*. Snakes were kept in a laboratory at 23 ± 2°C with a constant photoperiod (12L:12D) with the photophase centered at 1200 h.

Snakes were randomly selected for one of two diet treatments; mice (N = 12) or fish (N = 12). Snakes were fed weekly and offered mice or fish equal to 20% of their M_b (Byars et al. 2010; Lutterschmidt and Rayburn 1993; Sparkman et al. 2010). Thus, as a snake's mass increased, the amount of food offered also increased. Mice (*Mus musculus*) were supplied by the Sam Houston State University Science Annex and golden shiners (*Notemigonus crysoleucas*) were purchased from Oakhurst Bait Co. (Oakhurst, Texas, USA). I recorded whether or not snakes ate every week, but not the exact amount consumed. Snakes usually consumed all the food offered; only on a few occasions one individual had to be force fed.

Results

Using a repeated measures analysis of variance with the same group of snakes (mouse and fish diet) serving as the repeated measure among months, I found significant differences in mass gain ($F_{1,9} = 384.602$, $P < 0.001$) between my two diet groups (Fig. 2.1). Snakes in the mouse diet group gained significantly more mass than snakes in the fish diet group throughout nearly the entire study period (Fig. 2.1). The mouse and fish diet groups diverged immediately in the amount of mass gained. However, by the end of the study period there was no significant difference in mass gained as indicated by the overlap in standard error for the months of May, June, and July (Fig. 2.1).

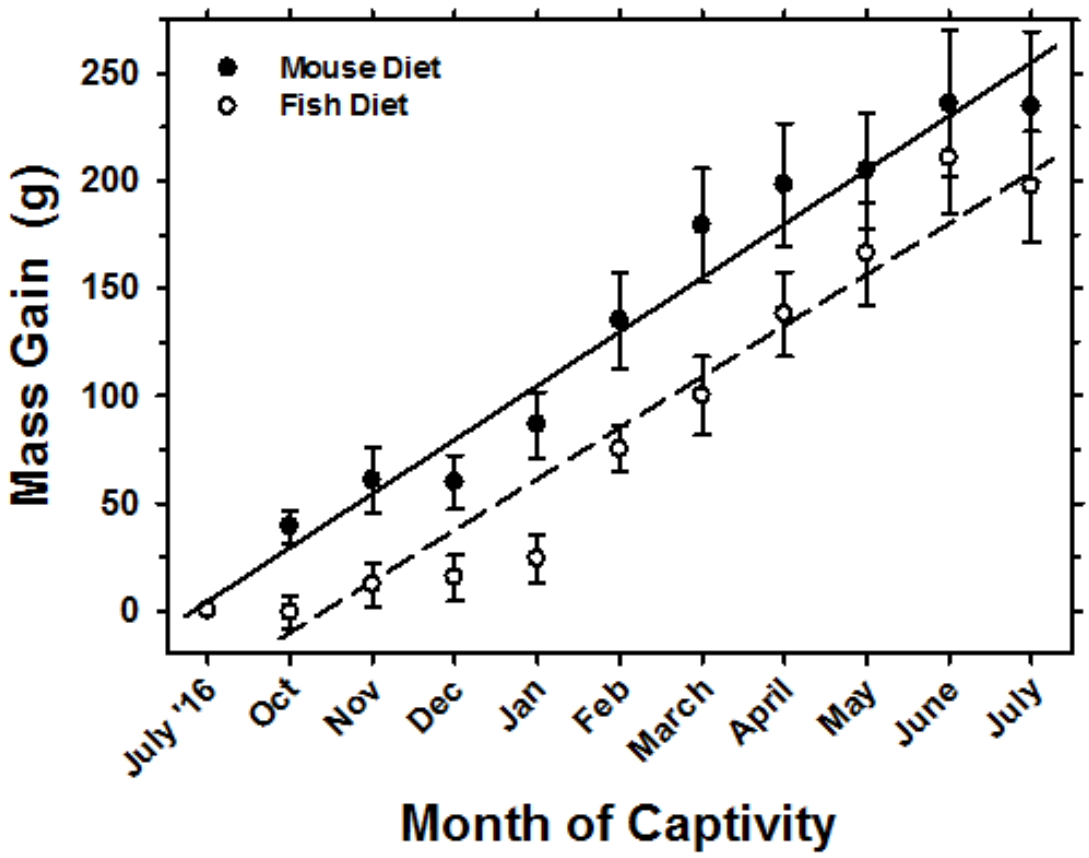

Fig. 2.1 – Average mass gain of snakes in both the mouse and fish diet treatments is shown for each month from October 2016 to July 2017. Error bars represent standard error of average mass calculated for the snakes in each diet treatment (N =12).

Discussion

My results for the effects of diet on growth are similar to those found for gilthead seabream (Vergara et al. 1999). Gilthead seabream fed diets equal in amount but differing in lipid content experienced different amounts of growth. The fish that ate the more lipid rich diets experienced more mass gain (Vergara et al. 1999). In this study, snakes that ate mice experienced more mass gain than snakes that ate fish.

My results are tempered by the fact that the snakes in the two diet treatments had significantly different average masses to start ($t = -2.198$; $df = 22$; $P = 0.039$). The snakes in the fish diet treatment massed 169.6 g ± 16.23 g SEM on average, and the snakes in the mouse diet treatment massed 218.3 g ± 15.12 g SEM on average. However, by the end of the study period there was no significant difference in average mass between the two diet treatments ($t = -1.699$; $df = 22$; $P = 0.103$).

All snakes were kept in identical plastic tubs, and there were no noticeable differences in activity between snakes in the two diet treatments. Snakes were typically coiled in their cages and displayed little activity except when food was offered.

Interestingly, snakes in the two diet treatments showed differences in how often they ate. Snakes in the fish diet treatment ate 72% of the time, but snakes in the mouse diet treatment ate only 52% of the time. One possible explanation is the morphology and size of the prey involved. Snakes on the fish diet were consuming multiple small fish prey items, while snakes on the mouse diet were consuming fewer large mouse prey items. Larger prey consumed by snakes can lead to more distension and slower crawling speed times (Willson and Hopkins 2011). For instance, *Nerodia fasciata* that consumed sunfish (*Lepomis marginatus*) took longer to ingest their prey, were more distended, and

had slower crawling speeds than *Nerodia fasciata* that consumed salamanders (*Ambystoma talpoideum*) (Willson and Hopkins 2011). The authors found that snakes would readily consume small and large salamanders. However, the snakes would refuse sunfish over 65% of body mass, while eating salamanders up to 105% of body mass (Willson and Hopkins 2011). This was the case even though both prey items had similar nutritional composition. (Willson and Hopkins 2011).

Similar to the study above, snakes ate mice (a larger prey item) less often than golden shiners (a smaller prey item). Perhaps snakes were less inclined to consume mice due to the greater distension that may have followed. Alternatively, perhaps snakes eating mice were gaining more energy content from a mouse than a similarly sized diet of golden shiners, and so it may not have been necessary to eat as often.

Acknowledgments

I thank Oakhurst Bait Shop for supplying golden shiners. This research was funded in part by a student research award from the Texas Academy of Science. Edward J. Tivador III was helpful with captive care of my snake colony.

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CHAPTER III

THE POTENTIAL INFLUENCE OF DIET ON QUANTITATIVE AND QUALITATIVE EPIDERMAL LIPIDS AND CUTANEOUS WATER LOSS RATES OF SNAKE SKIN[2]

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[2]J.M. Weidler and William I. Lutterschmidt, formatted for submission to *Copeia*.

ABSTRACT

As the primary barrier to cutaneous water loss in tetrapods, epidermal lipids play a vital role in water conservation and homeostasis. Previous studies have shown the correlation between the aridity of a species' habitat and the quantity of lipids in the epidermis. In general, the more arid the environment, the greater the amount of epidermal lipids, and the lower the skin permeability. I conducted a study on a captive colony of snakes (*Agkistrodon piscivorus*), controlling for either a fish (*Notemigonus crysoleucas*) or mouse (*Mus musculus*) diet. I found no difference in cutaneous water loss or lipid content between snakes in either diet group, indicating 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), my 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.

The importance of water relations and water balance in reptiles cannot be overstated. Conserving water and protecting against water loss is vitally important for terrestrial species, especially those in drier climates where desiccation can occur quickly. Evaporative water loss (EWL) can be broadly divided into two categories, cutaneous water loss (CWL) and respiratory water loss (RWL). Most EWL is CWL (Dmi'el, 1985; Dmi'el, 2001). Lipids are the main barrier to water loss in the epidermis of reptiles (Roberts and Lillywhite, 1980) and rates of EWL are generally correlated with aridity. Species from arid habitats tend to have lower rates of EWL than species from more aquatic habitats. Roberts and Lillywhite (1983) determined the EWL of 11 snake species; species from xeric habitats (e.g., *Crotalus vegrandis*) had lower rates of EWL than species from aquatic habitats (e.g., *Herpeton tentaculatum*). In a study of 15 snake species, average rates of EWL were lowest for species in xeric habitats, followed by mesic, and then aquatic habitats (Cohen, 1975). EWL seems to be correlated with aridity regardless of taxonomic position (Dmi'el, 1998). This correlation with aridity is true not only of reptiles, but also other vertebrates, such as birds. In a study of 12 species of larks, EWL decreased as aridity increased (Tieleman et al., 2003).

While EWL across species is generally correlated with the aridity of a species' habitat, other factors can influence rates of EWL as well. For instance, anoles conditioned to humid air have higher rates of EWL than anoles conditioned to dry air (Kobayashi et al., 1983; Kattan and Lillywhite, 1989). This is also true of mice (Denda et al., 1998) and other small vertebrates (Chew and Dammann, 1961). An increase in temperature results in an increase in permeability in human (Grice and Bettley, 1967; Grice et al., 1971) and porcine skin (Potts and Francoeur, 1990). CWL in tokay geckos is

highest during shedding (Zucker and Maderson, 1980). Snakes subjected to ultraviolet B radiation experience higher lipid peroxidation (Chang and Zheng, 2003), which may increase skin permeability. Cellophane stripping also increases CWL (Maderson et al., 1978). Even altering skin surface pH can change skin permeability in mice (Hachem et al., 2003). All of these examples demonstrate that while species-specific EWL for a species is often correlated with the aridity of a species' habitat, there is a certain plasticity when it comes to an individual's rate of EWL.

The physiological ability for limiting CWL may serve an important role in habitat selection (Miller and Lutterschmidt, 2014). In addition, species-specific differences in cutaneous water loss have been documented between two sympatric (but not syntopic) snake species, which suggests that these differences may reflect their individual adaptations to differences in microhabitat preference (Miller and Lutterschmidt, 2014). Such species-specific differences in lipids and CWL may serve as a potential mechanism allowing sympatric eastern copperheads (*Agkistrodon contortrix*) to use more upland habitats and avoid direct competition with northern cottonmouths (*Agkistrodon piscivorus*). Alternatively, copperheads' prey preference and diet on the increased availability of small mammals in upland habitats may be the source for increased epidermal skin lipids and reduced CWL. With regards to diet there is a distinct difference in diet between copperheads and cottonmouths. Copperheads mainly consume small mammals, especially rodents (Garton and Dimmick, 1969; Brown, 1979), while cottonmouths mainly consume amphibians and fish (Clark, 1949; Kofron, 1978).

In the *Agkistrodon* genus, copperheads exhibit the ancestral terrestrial state, while cottonmouths exhibit a derived semi-aquatic state (Parkinson et al., 2000). An interesting

question is whether higher skin permeability preceded cottonmouths' shift to being semi-aquatic. Perhaps cottonmouths first exploited novel aquatic habitats, with a subsequent increase in skin permeability. If diet (e.g. fish) increases skin permeability, it is conceivable that cottonmouths first exploited a novel food source, leading to higher skin permeability. A snake with higher skin permeability would be more susceptible to desiccation (Cohen, 1975), so spending more time in and around water would circumvent potential risk of desiccation (but see Aubret et al., 2015). Conversely, higher skin permeability could reflect relaxed selective pressure for energetic expenditure of lipid production (Miller and Lutterschmidt, 2014) and have nothing to do with diet. Is it possible that diet was the primer for cottonmouths' shift to being aquatic, perhaps to avoid competition with copperheads and vice versa?

Snakes represent a unique model organism because they shed their skin all at once, unlike other tetrapods which shed their skin piecemeal. Northern cottonmouths are also generalists in diet (Burkett, 1966) and will readily eat fish or mice in the laboratory. This presents a unique opportunity to test if diet differences can influence both lipid quantity and CWL of the skin. Laboratory mice have a mean body fat of roughly 25% of body mass (Reed et al., 2007) while fish (i.e., golden shiners) have a mean body fat of roughly 9% of body mass (Lochmann and Phillips, 2012). Snakes on a diet of mice are thus ingesting more lipids than snakes on a diet of golden shiners. Specifically, I was interested in whether a diet of mice would lead to a greater deposition of lipids in the epidermis than a diet of fish, and whether this would lead to lower rates of CWL.

MATERIALS AND METHODS

Experimental Subjects – Adult snakes (*Agkistrodon piscivorus*) were collected (IACUC # 8403310 and # 16-10-27-1003-3-01) in July 2016 from the Sam Houston State University biological field station, the Center for Biological Field Studies (CBFS) located in Walker County, Texas. Only female snakes (n =24) were used in experiments to avoid potential sex differences in skin lipids (Mason et al., 1987; Ball, 2000). I measured both snout-vent-length (SVL) and body mass (M_b) for all snakes prior to experimentation and snakes were weighed each month thereafter. Beginning in August 2016, each snake was housed separately in a plastic cage (38 x 26 x 22 cm) with aspen bedding (Harlan Teklad, Madison, Wisconsin) and water provided *ad libitum*. Snakes were kept in a laboratory at 23 ± 2 °C with a constant photoperiod (12L:12D) with the photophase centered at 1200 h. Snakes were randomly selected for one of two diet treatments; mice (n = 12) or fish (n = 12). Snakes were fed weekly and offered mice or fish equal to 20% of their M_b (Lutterschmidt and Rayburn, 1993; Byars et al., 2010; Sparkman et al., 2010). Thus, as a snake's mass increased, the amount of food offered also increased. Mice (*Mus musculus*) were supplied by the Sam Houston State University Science Annex and golden shiners (*Notemigonus crysoleucas*) were purchased from Oakhurst Bait Co. (Oakhurst, Texas).

Shed skins – Shed skins were collected from each snake. The first shed was not used for experimentation as these sheds and their lipid content have uncontrolled and possible environmental influences from the field. These influences may include a diverse diet and differences in surface abrasion from traversing over differing habitat structure. The second shed of each snake resulted from growth under controlled diet, captive care, and laboratory regimes. The second shed of each snake was thus used for analysis. All sheds

were air dried, sealed in airtight Ziploc® bags, and then frozen within 24 hours of shedding.

Cutaneous Water Loss (CWL) – An in vitro technique to determine CWL was performed, similar to previous studies (Agugliaro and Reinert, 2005; Miller and Lutterschmidt, 2014). CWL was only determined for the dorsal surface of each snake shed. Three dorsal samples were cut out from each dorsal half and examined for tears. The samples (with the outer mucosal surface facing outward) were then stretched over the opening (0.58 cm^2) of a culture tube (10mm X 7.5 cm) containing 1 mL of deionized water. After the sides of the skin were securely wrapped around the outside of the culture tube, the skin was attached and sealed with nylon string and Parafilm® (Pechiney Plastic Packaging, Menasha, Wisconsin). The culture tube was then inverted and suspended inside a 120 mL specimen bottle containing 5 g of t.h.e.® desiccant (EMD Chemicals Inc., Gibbstown, New Jersey). The mass of the culture tube was measured at the start and after five days to determine the rate of CWL for each sample. The amount of water lost for all three samples was averaged for each snake to get a single value. All dorsal samples were cut from approximately the same location from each snake shed. Desiccant was replaced as needed, and any samples that experienced tears were discarded. The dorsal samples used for CWL were not used for lipid extraction.

Lipid Extraction –The quantitative lipid content of a shed (mg lipid / g of shed) was determined by the mass difference of a shed before and after lipid extraction. The dorsal and ventral surfaces of each shed were tested separately to determine potential differences in lipid content between these surfaces. Samples were placed in 240 mL jars with 100 g of t.h.e.® desiccant for 24 h both before and after lipid extraction to determine

dry mass. Total lipids were extracted from each sample by being placed in a 400 mL jar for 24 hours containing a 120 mL, 2:1 chloroform/methanol solution (Folch et al., 1957). After lipid extraction, samples were rinsed first with 2:1 chloroform/methanol solution, then three times with distilled water. After rinsing, sheds were placed in jars with desiccant as described above. Sheds were massed 24 h later to obtain final mass, which was then subtracted from initial mass to determine lipid content.

Infrared (IR) Spectroscopy – Additional dorsal and ventral shed samples were cut from the sheds of 21 snakes and examined using IR spectroscopy. The samples were cut from approximately the same locations dorsally and ventrally. The IR analyses provided absorbance values for wavenumbers that correspond to molecular geometries. The wavenumber and absorbance values of threshold peaks were recorded for each sample. As samples had the same peaks but slightly different wavenumbers for those peaks, I averaged the wavenumber values across all samples to get an average wavenumber for each peak. Then, I found the absorbance values at these average peak wavenumber values for all shed samples. Molecular geometries were assigned according to Ripamonti et al., 2009 and Barry et al., 1993.

Statistical Analyses –The statistical software SigmaPlot 11.0® was used for analysis and graphing. A Student's T-test was done to test for differences in CWL between the fish and mouse diet groups. For lipid content, a 2 X 2 two-way analysis of variance was used to investigate differences, with shed surface and diet as factors. Linear regressions and scatter plots were made to test the number of days to second shed. Peaks from the IR data were analyzed using a multivariate discriminate function analysis. All data passed statistical tests for normality and equal variance.

RESULTS

I found no significant difference in CWL (t = -0.549; df = 20; P = 0.589) between the mouse and fish diet groups (Figure 3.1). Neither did I find any significant difference in lipid quantity between my two diet groups (Table 3.1, Figure 3.2). However, I did find significant differences in lipid content between the dorsal and ventral surfaces for each diet treatment, but no diet-specific differences (Table 3.1, Figure 3.2).

Shed snake skins kept for two years in dry plastic bags have shown no change in rates of water influx and efflux (Dunson and Freda, 1985). To test whether the number of days to second shed had any significant effect on lipid content, I ran linear regressions on the number of days to second shed and mean lipid content. This was done for both the dorsal and ventral surfaces (Figures 3.3 and 3.4). There was no significant correlation found between the number of days to second shed and mean lipid content for either the dorsal or ventral surfaces (Figures 3.3 and 3.4).

Using IR spectroscopy, I found nine prominent peaks in the dorsal and ventral surfaces of snake sheds (Figure 3.5), corresponding to nine molecular geometries (Table 3.2). I used a discriminant function analysis to discriminate and identify differences between my known groups of diet and shed surface (dorsal and ventral). The first two factors explained 97.5% of the variation among these groups with respect to their molecular geometry (Figure 3.6). The first factor (x-axis) explained 71.1% of the variation with peaks 4 and 8 loading heaviest on this axis. The second factor (y-axis) explained 26.4% of the variation with Peaks 2 and 7 loading heaviest on this axis. There was complete overlap between diet treatments, but separation between shed surface (Figure 3.7).

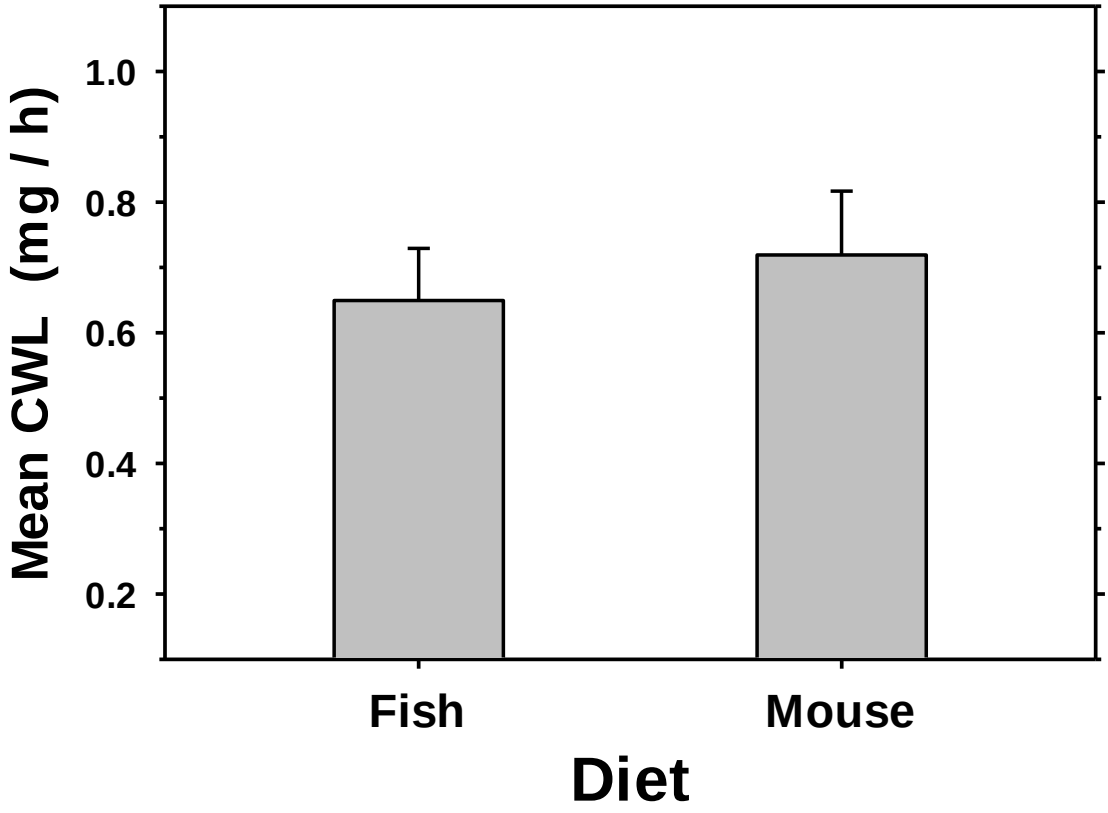

Figure 3.1 – Mean CWL (milligrams per hour) for the fish diet group (n = 12) and the mouse diet group (n = 10). Error bars above each bar indicate SEMs.

Table 3.1 – Results of the 2 X 2 ANOVA showing the source of variation in mean lipid content among different shed surfaces (i.e., dorsal and ventral) and diet (i.e., mouse and fish).

Source of Variation	DF	SS	MS	*F*	*P*
Diet	1	16.22	16.22	0.0342	0.854
Shed Surface	2	8259.681	4129.841	8.699	<0.001**
Diet x Shed Surface	2	92.052	46.026	0.097	0.908
Residual	38	18039.75	474.73		
Total	43	26325.84	612.229		

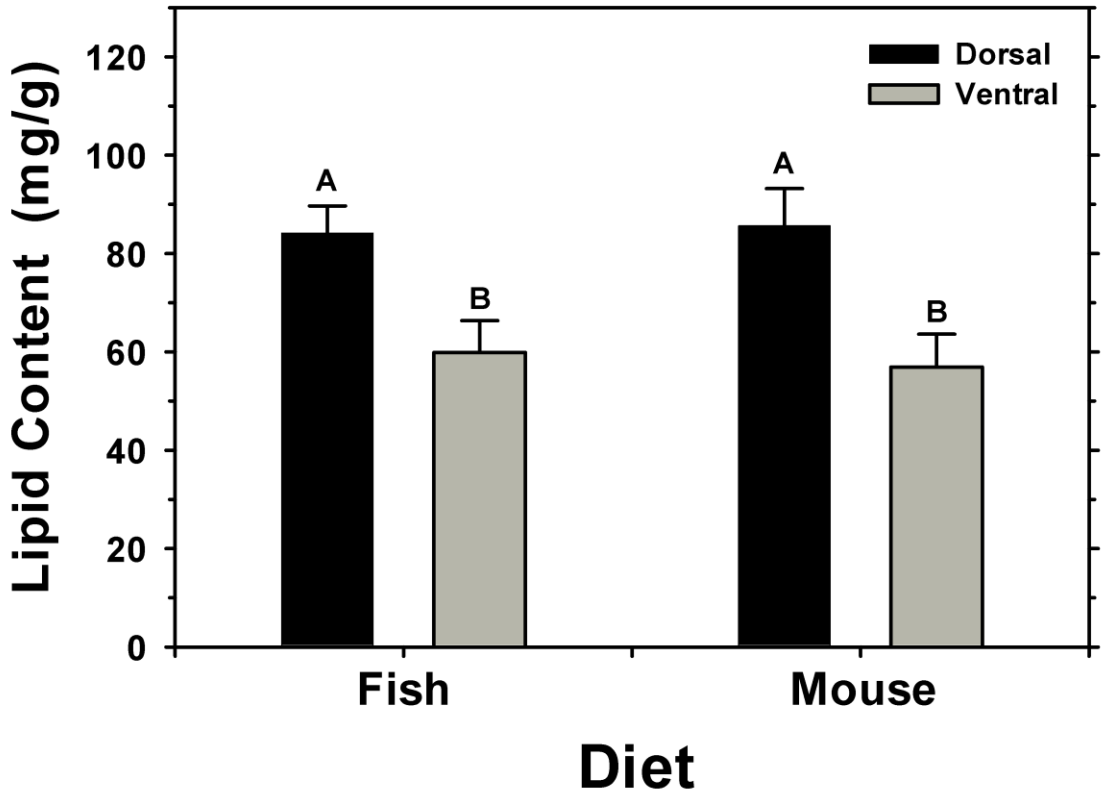

Figure 3.2 – Mean lipid content (milligrams per gram of tissue) for fish (*n* = 12) and mouse (*n* = 10) diet groups showing no diet-specific differences in dorsal or ventral samples. Mean lipid content between dorsal and ventral samples differed significantly for both diet treatments. Error bars above each bar indicate SEMs.

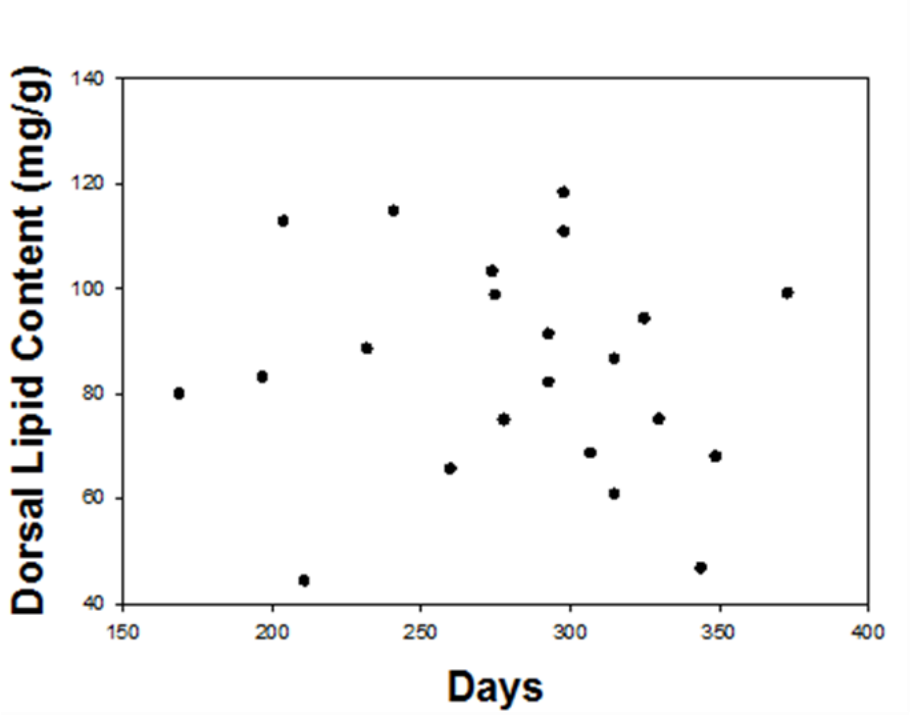

Figure 3.3 – Scatter plot of dorsal lipid content and the number of days to second shed. A linear regression showed no significant correlation (F = 0.175; df = 1, 20; P = 0.680).

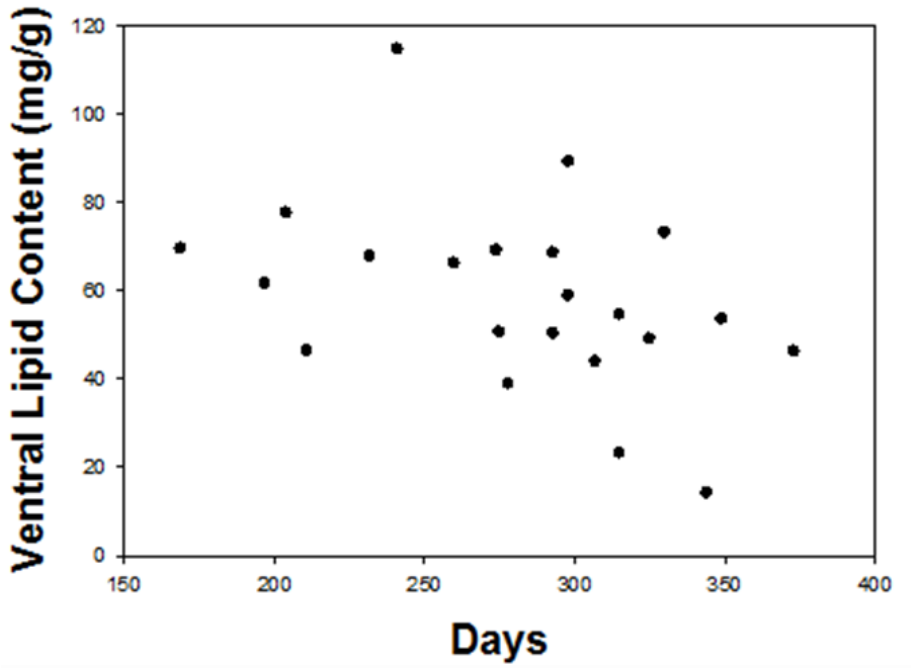

Figure 3.4 – Scatter plot of ventral lipid content and the number of days to second shed. A linear regression showed no significant correlation (F = 4.284; df = 1, 20; P = 0.052).

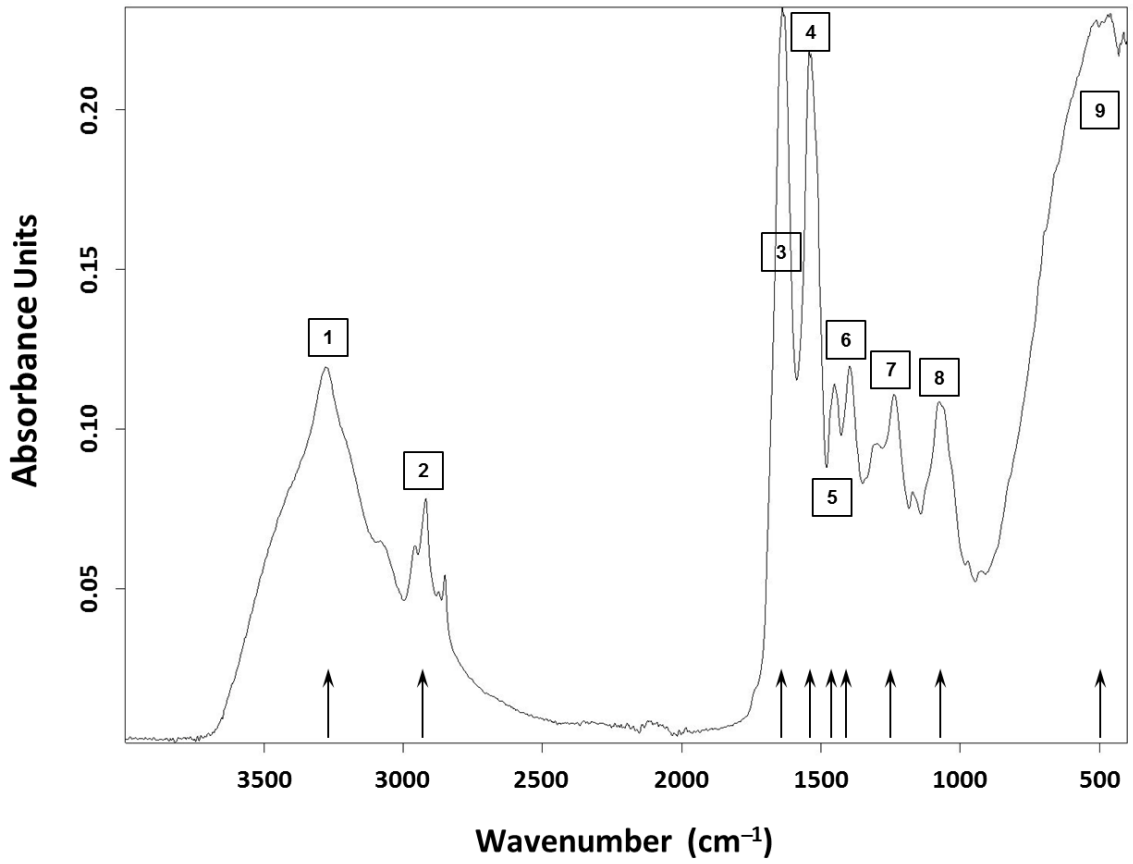

Figure 3.5 – Sample read out of IR spectroscopy with nine absorbance peaks.

Table 3.2 – IR spectroscopy peaks and molecular geometry identifications.

Peak Number	**Wavenumber (cm^{-1})**	**Assignment**
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

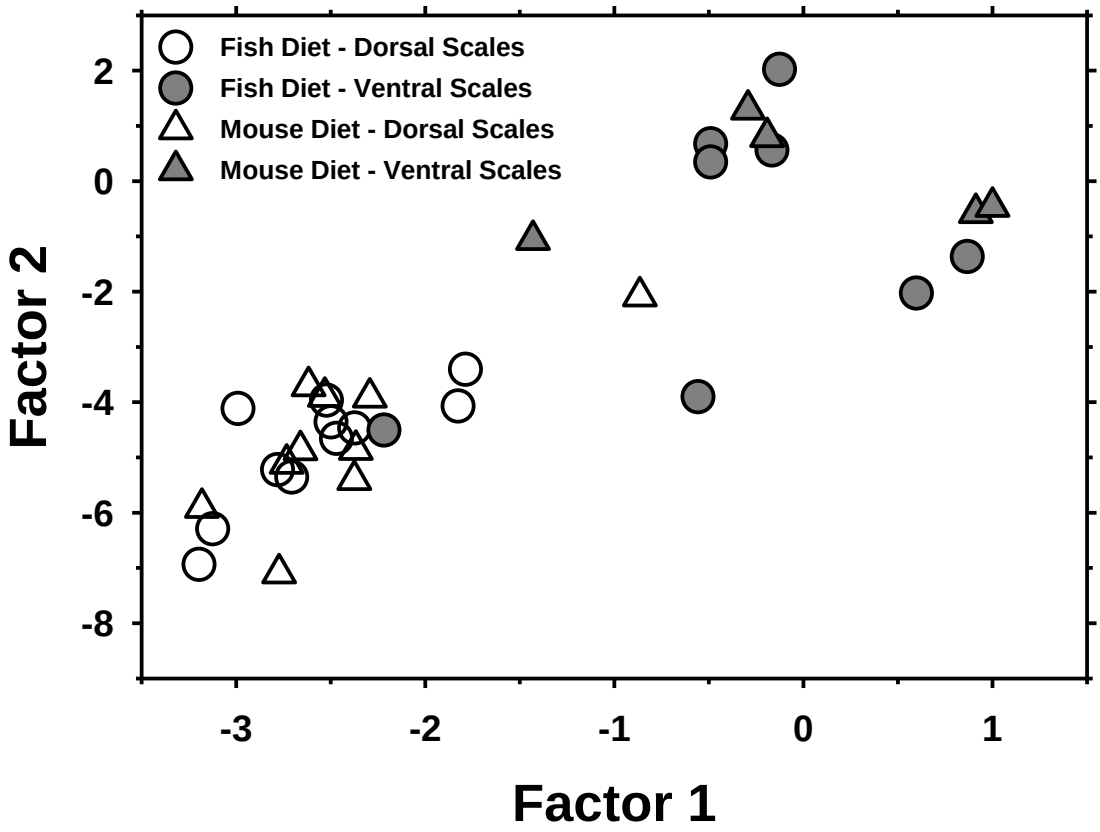

Figure 3.6 – Multivariate discriminate function analysis of IR absorbances at wavenumber peaks.

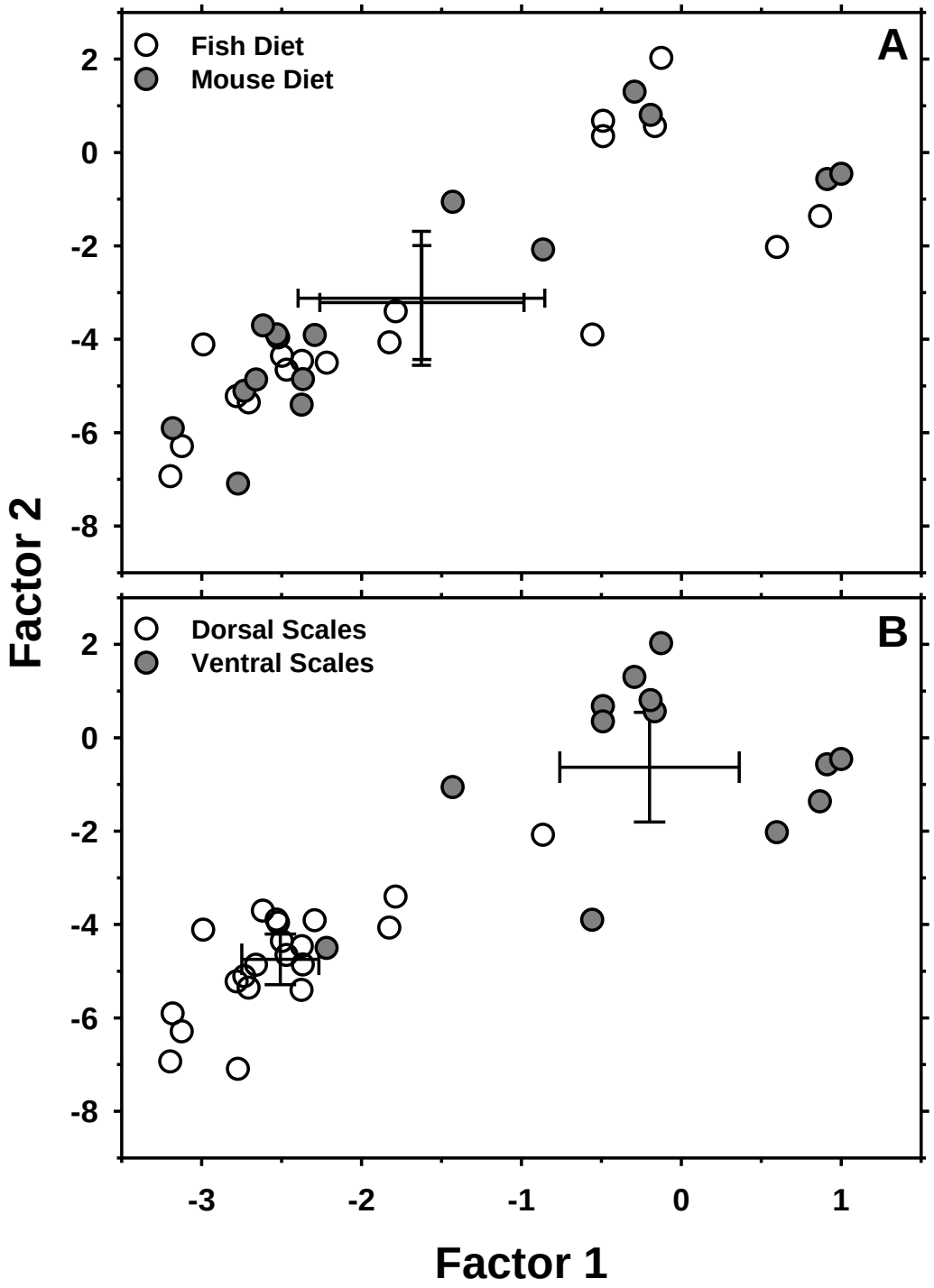

Figure 3.7 – Multivariate discriminate function analysis of IR absorbances at wavenumber peaks. (a) Overlap of mouse and fish diet treatments. (b) Separation between dorsal and ventral surfaces of sheds. Error bars indicate 95% confidence intervals.

DISCUSSION

Adaptations for limiting water loss are widespread in the natural world, and are not limited to epidermal lipids. Many desert taxa exhibit behavioral adaptations, such as being nocturnal. Reptiles and birds are uricotelic, which results in reduced water loss compared to ureotelic mammals and amphibians (but see Loveridge, 1970 and Shoemaker et al., 1972). In addition, many snakes do not drink and satisfy all of their water needs from prey.

My results indicate that diet does not significantly affect CWL or mean lipid content. Diet likely does not significantly affect epidermal lipid content due to how lipids are digested and processed. Lipids from prey items are not simply deposited into the epidermis. Lipids from the diet are first broken down into fatty acids and monoglycerides in the process of emulsification. After diffusing across epithelial cells of the microvilli, they are then reconstituted into triglycerides and are then transported throughout the body.

In addition, epidermal lipids appear to be primarily manufactured in the epidermis using acetate from circulation (Wertz, 1996). Epidermal lipids (such as cholesterol) are manufactured by the endoplasmic reticulum (Williams and Elias, 1987) and processed by Golgi bodies (Landmann, 1988). These stacks of lipid bilayers are packaged together into lamellar bodies and extruded into the epidermis (Lillywhite, 2006).

The closest parallels to the current study are studies of mice fed essential fatty acid deficient diets. Mice that are fed a diet lacking in essential fatty acids have greatly increased rates of epidermal water loss with their fur matted and wet as a result (Menton, 1970; Elias and Brown, 1978; Williams and Elias, 1987). The lamellar bodies of these

mice are empty (Elias and Brown, 1978), suggesting that the lack of essential fatty acids leads to a completely defective, if not absent, barrier to water loss. While these studies are intriguing, my study aimed to determine whether different diets (both complete in essential fatty acids) would lead to different rates of epidermal permeability. This turned out not to be the case.

I found significant differences in lipid content between the dorsal and ventral surfaces of sheds (Figure 3.2). My results agree with Miller and Lutterschmidt (2014) that there is a significantly higher mean lipid content in the dorsal surface of cottonmouths compared with the ventral surface. There are likely proportionally more lipids in the dorsal surface of snakes because the ventral surface is approximately 50% thicker than the dorsal surface (Jayne, 1988). If the majority of CWL occurs on the dorsal surface of snakes, there may be proportionally more lipids in the dorsum to better protect against water loss. Alternatively, the ventral surface of snakes may have greater amounts of keratin to protect against abrasion as snakes engage in locomotion across their environments.

Interestingly, Miller and Lutterschmidt (2014) found a significant difference in dorsal CWL between copperheads and cottonmouths, but not in dorsal mean lipid content. This implies that the difference in dorsal CWL between copperheads and cottonmouths is due to lipid quality, not quantity. Reptiles have mainly cholesterol, free fatty acids, and ceramides in the mesos layer of the epidermis (Roberts and Lillywhite, 1983; Burken et al., 1985a; 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). The exact differences (lipid type and quantity) in the epidermal lipid classes of copperheads and cottonmouths would be most informative as relates to their different rates of CWL.

When comparing the dorsum to venter, Miller and Lutterschmidt (2014) found a significant difference in CWL for cottonmouths, but not copperheads. Other studies have also not found significant differences in permeability between the dorsal and ventral surfaces of snakes (Stokes and Dunson, 1982; Roberts and Lillywhite, 1983; Burken et al., 1985b). Whether cottonmouths are unique among snakes in having different rates of CWL between the dorsal and ventral surfaces is unknown. Other aquatic and semi-aquatic snakes have highly permeable skin, which is useful for cutaneous oxygen uptake. Snakes such as sea snakes (Graham, 1974; Standaert and Johansen, 1974; Heatwole and Seymour, 1978), garter snakes (Costanzo, 1989) and diamondback water snakes (Gratz, 1978) are all capable of cutaneous respiration. Perhaps the increased dorsal permeability of cottonmouths is for facilitating cutaneous respiration. It is unknown if cottonmouths are capable of cutaneous oxygen uptake, but it is a distinct possibility.

My IR spectroscopy multivariate analysis underscored the differences seen between the dorsal and ventral surfaces of cottonmouth skin. There was complete overlap between the fish and mouse diet groups, indicating diet had no significant effect. However, the dorsal and ventral surfaces of cottonmouth skin separated out with 95% confidence intervals (Figure 3.6). This indicates that the absorbance values and mixture of molecular geometries differed between the dorsal and ventral surfaces. In addition,

there was no discernible peak 6 (δCH_3) on the dorsal surface of cottonmouth skin. The significance of this is unknown.

In comparing cottonmouths to copperheads, my results suggest that the difference in water loss between these two sister taxa (Moen et al., 2005; Miller and Lutterschmidt, 2014) is a truly species-specific trait that is not influenced by diet. Epidermal permeability does not seem to be correlated with phylogeny (Dmi'el, 1998; Teleman et al., 2003), making the pronounced differences between these two species a result of evolutionary adaptation, and not phenotypic plasticity. While these two taxa do partition by diet and microhabitat, diet was likely not the driving force in the divergence of these two taxa.

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CHAPTER IV

CONCLUSION

"Higher" vertebrates are distinct from amphibians and fish in that their epidermis provides a barrier to water loss. The evolution of the epidermal permeability barrier was a defining moment in higher vertebrate evolution, allowing many vertebrates to become completely terrestrial. For reptiles specifically, the lipids in the mesos layer provide the barrier to water loss (Roberts and Lillywhite 1980). Lipids are likely manufactured in the endoplasmic reticulum and processed by the Golgi apparatus (Landmann 1988, Williams and Elias 1987). Epidermal lipids have a bilayer configuration (Figure 4.1) with the polar heads facing outward, and the nonpolar fatty acid chains pointing inward. The configuration of epidermal lipid bilayers is strikingly similar to the lipid bilayers of cell plasma membranes. Epidermal lipid bilayers are stacked on top of each other and packaged in lamellar bodies. These lamellar bodies are then extruded into the intercellular spaces of the epidermis (Figure 4.2).

The use of lipids to prevent water loss is surprisingly ubiquitous in the natural world. For instance, plants and arthropods have waxy cuticles made of lipids (Hadley 1989). Although amphibians usually do not have substantial barriers to water loss, some species of tree frogs excrete lipids and engage in wiping motions to spread them over their bodies (McClanahan et al. 1978, Shoemaker et al. 1972).

Figure 4.1 – Illustration of lipid bilayers

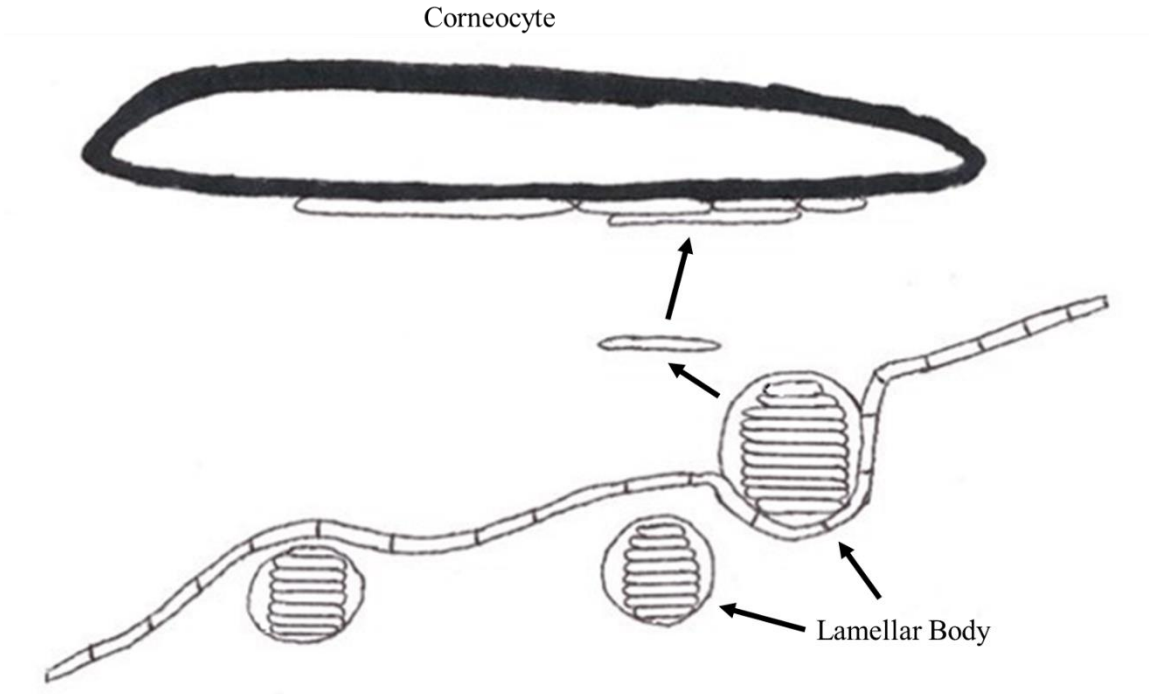

Figure 4.2 – How lipids are extruded into the epidermis.

Lipids are crucial to life on earth as they serve as the main barrier to water loss in countless organisms. Lipids are also a vitally important source of energy and important for growth rates, reproduction, and other life history traits. In this study, I found significant differences between the dorsal and ventral surfaces of snake skin in both lipid content and molecular geometry. I found that diet did not significantly affect cutaneous water loss. Cottonmouths and copperheads are two sister taxa with differing properties of epidermal permeability, which I have demonstrated is a truly species-specific trait that is not influenced by diet, but instead is likely a result of evolutionary adaptation.

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CURRICULUM VITAE

J.M. Weidler

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EDUCATION

Sam Houston State University, Huntsville, Texas
M.S., May 2018 (Biological Sciences with a Concentration in GIS)
Advisor: William I. Lutterschmidt
Thesis: An experimental manipulation of diet and its influence on growth and epidermal lipids in the northern cottonmouth (*Agkistrodon piscivorus*)

Texas A&M University, College Station, Texas
B.S., December 2013 (Wildlife & Fisheries Sciences)

PROFESSIONAL EXPERIENCE

Teaching Assistant
Sam Houston State University, August 2016 – May 2018
Taught multiple laboratory sections (Zoology, Foundations of Science, and Contemporary Biology)
Led class discussions, group experiments, and dissections
Assisted students inside and outside of the classroom

Fish Farm Technician
Herrmann's Fish Farm, September 2015 – July 2016
Cared for and raised fish for private stocking (channel catfish, blue catfish, largemouth bass, fathead minnows, etc.)
Frequently seined for fish. Participated in electroshocking surveys to gauge relative fish species diversity and abundance
Delivered and installed fish feeders, water fountains, and aerators. Applied chemical treatment for weeds, especially cattails and algae

Gypsy Moth Trapper
Minnesota Department of Agriculture, May – September 2015
Worked as part of a survey program to assess range and spread of gypsy moths in Minnesota

Worked independently to build, set, and check traps throughout the summer
Frequently interacted with landowners to gain permission to set up traps on their property

Fish Hatchery Technician
Colorado Parks and Wildlife, October 2014 – April 2015
Stocked fish in public lakes and ponds
Fed fish, maintained raceways, and cleaned fish tanks
Performed lab work measuring suspended and dissolved solids of Colorado hatcheries
General maintenance with power tools (including welding)

Land Management Internship
The Crane Trust, April – October 2014
Installed and upgraded fence for a bison herd. Took out old T-posts, dug post holes, welded H-braces, strung barbed wire, etc.
Participated in controlled burns to facilitate grazing
Applied herbicide to combat invasive species, especially musk thistle and poison hemlock
Participated in surveys of native flora and fauna (western prairie fringed orchid, Henslow's sparrow, and a vegetation survey)

State Park Internship
Colorado Bend State Park, January – April 2014
Led wild cave tours for park visitors
Cleared brush from trails, cleaned campgrounds, and put up trail signs and markers
Created a site book for the park campsites
Participated in a bat survey of cave myotis and tricolored bats

Field Technician
Texas A&M AgriLife Research Project, May – August 2013
Ran a system of pitfall traps to mark and recapture the dunes sagebrush lizard and other reptiles and amphibians
Conducted telemetry work on the dunes sagebrush lizard by attaching radio transmitters

Veterinary and Kennel Technician
Highland Park Animal Hospital and the Canine Country Club, May 2012 – January 2013
Assisted veterinarians in surgeries and administered vaccinations and medications
Monitored dogs in dog daycare
Cleaned cages. Provided food, water, and walks on a daily basis

Independent Contractor
Southwestern Company, summers 2010 and 2011
Sold educational books door to door, 70 – 80 hours per week
Gave over 3,500 presentations
Helped organize, manage, and lead a sales team
Sold over $15,000 of merchandise

CERTIFICATIONS

GIS Certificate (Sam Houston State University)
Firefighter Type II
Advanced Open Water Diver
S-212 Wildland Fire Chain Saws
Fish & Wildlife ATV-UTV Safety Training

SKILLS

Python programming
Welding (stick and wire)
Plasma cutter and acetylene torch
Four-wheel drive and manual transmission
Chainsaws
ATV and UTV

PUBLICATIONS

Weidler et al. 2017. Unusual Field Injury. Herpetological Review 48(3):664-665

GRANTS AND HONORS

Texas Academy of Science Student Research Award – 2017
College of Agriculture and Life Sciences Scholarship – 2013
Dean's List – Spring 2012, Fall 2013