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ASSESSING SOURCES OF VARIATION IN AMPHIBIAN SKIN THICKNESS: ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

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2017

A dissertation submitted for the degree of Doctor of Philosophy

ASSESSING SOURCES OF VARIATION IN AMPHIBIAN SKIN THICKNESS: ECOLOGICAL AND EVOLUTIONARY IMPLICATIONS

Collin S. VanBuren

Skin is the largest organ of the body and serves myriad functions. The skin of amphibians is semi-permeable and allows for water, gases, and other substances to be exchanged between the animal and its external environment. Although amphibian skin anatomy has been studied for over 100 years, sources of variation are rarely considered and have not been systematically assessed. This thesis quantitatively tests for the effects of sex, season, body region, and body size on skin thickness using histological preparation of the skin of amphibians from museum collections. The results collectively suggest that reported sexual dimorphism in skin thickness is explained by body size, which correlates with skin thickness within and across species. Seasonal differences in skin thickness are present in only a few species (e.g., *Lithobates catesbeianus*) and these analyses indicate that such differences can still be detected in museum specimens collected 70+ years ago. In regressions of skin data and body size for multiple species, winter specimens of *L. catesbeianus* were within the range of variation whereas summer specimens were outliers. Skin thickness (actual and size-corrected values) and body size were also regressed against environmental variables to test for a relationship between ecology and skin thickness. Surprisingly, relative skin thickness was not correlated with environmental variables, but body size and size-uncorrected skin thickness values were correlated with environmental variables, supporting a strong relationship between body size and ecology. However, the results of the interspecific regression and previously published studies suggest that skin thickness is ecologically significant at lower taxonomic levels (among populations or between closely related species). A potential explanation for this result is that an 'ideal' relative skin thickness exists for amphibians that is achieved through the evolution of integumentary structures that influence skin physiology (e.g., iridiophores, specialised glands). Before these adaptations evolve, or when habitat preference is variable, differences in skin thickness may exist to allow for this ecological plasticity. Future inter- and intraspecific studies are needed that test these hypotheses using a combination of field-based surveys, experimental manipulation studies, and macroevolutionary studies of amphibian skin anatomy.

DECLARATION

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text

The length of this dissertation does not exceed the maximum length specified by the Degree Committee of Earth Sciences.

Collin S. VanBuren

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ABSTRACT

The skin is the largest organ of the body and provides many functions, such as gas and liquid regulation and protection from the external environment. Among tetrapod vertebrates as a whole, amphibian skin is semi-permeable and responsible for a greater proportion of water absorption and gas exchange. Myriad factors, such as behaviour and morphology, affect the physiological performance of amphibian skin. Morphological traits linked to amphibian skin physiology or ecology have remained difficult to discern because of the paucity of quantitative comparative studies and sources of intraspecific variation that have been largely ignored in previous studies. This thesis aims to address the effects of these sources of variation by analysing a trait that is known to vary between sexes, among seasons, and between body regions and is assumed to be linked with physiology and/or ecology: skin thickness. The first source of variation addressed is sexual dimorphism. Specimens of the white-lipped treefrog (*Litoria infrafrenata*), which display sexual dimorphism in body size and skin thickness, were used to test if body size was the main determinant of sexually dimorphic skin thickness. Size corrected values did not differ significantly between males and females, although the sample size was small. Seasonal variation in skin thickness has also been documented in a few species. Specimens of the American bullfrog (*Lithobates catesbeianus*), the Northern leopard frog (*Lithobates pipiens*), and the spring peeper (*Pseudacris crucifer*) collected across multiple months of the year were sampled to determine if skin thickness changed during the autumn or winter months. Seasonal skin thickening was only detected in *L. catesbeianus*, and skin of specimens from autumn and winter months was significantly thicker than skin of specimens from earlier in the year. This pattern was also detectable in museum specimens collected up to 50 years before those that were used to detect the pattern initially. In the long-preserved specimens, the skin thickening signal was dampened, most probably due to an effect of preservation fluids on tissue structure. Using an interspecific dataset of 10 species, as well as data culled from the literature, a general pattern was uncovered whereby the dorsal skin is often the thickest region and the ventral thigh region is the thinnest. However, this pattern is not always true for every individual of every species (*L. pipiens* and *P. crucifer*) and in some species the dorsal skin is thinnest (*Bokermannohyla alvarengai* and *Litoria infrafrenata*). The same 10 species were used to ascertain whether skin thickness among species is significantly related to body size, as was found in the intraspecific study of *Litoria infrafrenata*.

Interestingly, summer specimens if *Lithobates catesbeianus* were outliers below the interspecific regression line and winter specimens fell within the range of variation of other species; this suggests that seasonal skin thickening might be more appropriately called 'seasonal skin thinning' in this species. Finally, a link between ecology and skin thickness was tested using the 10 species from previous analyses and data from the literature. At a phylogenetically broad scale, relationships between skin thickness and environmental variables from a species' range were weak, whereas body size explained a greater amount of the variation than environmental parameters. At lower taxonomic scales (between populations or congeners), skin thickness does appear more closely linked with ecology. It is concluded that amphibians follow a generally allometric trend for skin thickness and when faced with suboptimal conditions over long periods of time, integumentary structures, such as iridiophores, will evolve to compensate for any physiological disadvantage linked to the possession of a sub-optimal skin thickness. In the interim, however, skin thickness may change, thus sacrificing other attributes like mechanical support. These results represent one of the first attempts to understand the ecological and functional significance of amphibian skin thickness and focus primarily on anurans, which are the most taxonomically and ecologically diverse group of amphibians. Future studies are needed to build on this research to test the broad applicability of these conclusions in order to develop an understanding of the links between amphibian skin anatomy, physiology, and ecology.

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CHAPTER ONE: INTRODUCTION

In the end, we're all just semi-permeable membranes stretched across a surface area to volume relationship pre-determined by size and topography

Dr Haley D. O'Brien

The skin is the largest organ of the body and serves many functions in vertebrates. Primarily, these functions are protection (against disease, environmental abnormalities, and injuries), gas and fluid regulation, and mechanical support (Frolich, 1997). All vertebrates have skin that is comprised of a keratinised superficial epidermis and a thick dermis, which contains glands and blood vessels and lies deep to the epidermis. The dermis is separated into two layers: the superficial spongy dermis (papillary dermis or *stratum laxum*) and the deep compact dermis (reticular dermis or *stratum compactum*). Beneath the dermis lies the thin hypodermis that connects the skin to the underlying muscles.

The skin of amphibians is unique among vertebrates because it is relatively thin and semi-permeable (Duellman & Trueb, 1986). The exchange of water, gases, and salts is determined by hormones, concentrations of these substances in and outside of the body, and the activity of transport channels (Ussing & Zerahn, 1951; Hillman et al., 2009). Although water and gas exchange is ubiquitous across tetrapods, amphibians rely on these functions of the skin to a much greater degree than other clades (e.g. Frolich, 1997; Lillywhite, 2006). Amphibians utilise other organs to varying degrees to exchange these substances, such as the lungs, urinary bladder, and lymph sacs (e.g. Czopek, 1965; Hillman et al., 2009; Withers et al., 2014), but the skin has received far greater attention. The amphibian epidermis is only a few cell layers thick and is weakly keratinised with a few layers of alpha-keratin (Farquhar & Palade, 1965; Fox, 1986; Lillywhite, 2006). Amphibian skin also has a lower lipid concentration and is thinner overall than the skin of other tetrapods (e.g. Lillywhite, 2006). These anatomical features contribute to the semi-permeable nature of amphibian skin.

This 'leaky' skin is why amphibians are considered to be the terrestrial vertebrates that are most sensitive to environmental changes. Their skin function has even been used to explain, in part, why more species of amphibians are threatened with extinction than

mammals, birds, or reptiles (e.g. Wake & Vredenburg, 2008). However, global amphibian population declines are not affecting all species equally (Bielby, Cunningham & Purvis, 2006; Bielby et al., 2008; Cooper et al., 2008), so if skin function is linked to population declines in some species, then variation in skin anatomy or its physiological function could be used to identify species that are more at-risk than others. Determining the physiological basis for chytridiomycosis, the disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* that affects normal skin functioning, contributed to understanding the causal relationship between the disease and populations declines (Voyles et al., 2009). For others threats to amphibian species, such as that brought about by habitat loss, the role of skin function in population declines is less clear.

Despite the widespread perception of amphibian sensitivity, many morphological and behavioural strategies for avoiding desiccation and other unfavourable environmental conditions in amphibians have evolved in amphibians (e.g. Lillywhite, 1971; Duellman & Trueb, 1986; Toledo & Jared, 1993; Hillyard, 1999; Young et al., 2005; Hillman et al., 2009). The most effective strategies for reducing evaporative water loss are through the production of lipids by specialised granular glands (e.g. phyllomedusine treefrogs) or through burrowing underground and producing a cocoon made up of layers of dead skin (e.g. *Cyclorana* spp.) (Toledo & Jared, 1993; Barbeau & Lillywhite, 2005). Conversely, water absorption by the skin is facilitated by aquaporins, sculpted patterns on the skin, and the specialised skin regions that function to move water across the body and absorb it (Roth, 1973; Lopez & Brodie, 1977; Toledo & Jared, 1993; Suzuki et al., 2007). Some toads also display behavioural responses to substrate moisture (Hillyard, Hoff & Propper, 1998) and many treefrogs display a 'water conserving posture' to behaviourally control water loss (Barbeau & Lillywhite, 2005).

The morphological and behavioural traits related to gas exchange, protection against pollutants, or ecological niche partitioning have not been examined. Among the morphological traits, skin thickness is of particular interest because it is known to vary between sexes (Greven, Zanger & Schwinger, 1995; Wenying et al., 2011), seasons (Kun, 1959; Kobelt & Linsenmair, 1986), populations (Navas, Antoniazzi & Jared, 2004), body regions (Greven, Zanger & Schwinger, 1995; Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001; Centeno et al., 2015), and species (Czopek, 1965;

Schwinger, Zanger & Greven, 2001), yet the ecological and functional implications for these differences have not yet been explored as they have in other anatomical features related to terrestrialisation (e.g. Withers et al., 2014). Qualitative correlations between skin thickness and habitat type have been made. Czopek (1965) hypothesised that a link between skin thickness and ecology exists, and hypothesised skin thickness would be related specifically to gas exchanges; Le Quang Trong (1971, 1975) found relationships between skin thickness and ecology using qualitative comparisons among a handful of species of the West African genera *Ptychadena* and *Phrynobatrachus*. However, many factors were not considered in these studies, such as phylogenetic history, body size, or variation within a single individual between different body regions, all of which are known to affect other aspects of amphibian biology (e.g. Bentley & Main, 1972; Tracy, Christian & Tracy, 2010; Greenberg & Mooers, 2017). In principle, skin thickness should relate to the diffusion potential of a tissue because thicker tissues will have a greater resistance against the transmission of substances across it (Lillywhite, 2006). Tissue composition also contributes significantly to permeability, as does the location of the blood vessels for calculating the effective functional thickness of the skin (Lillywhite, 2006).

Although the basic anatomy of amphibian skin has been known for over 150 years (Ascherson, 1840), many questions remain regarding inter- and intraspecific sources of anatomical variation, how this variation might affect macroevolutionary studies and how such variation is related to ecology. This thesis aims to address these uncertainties using quantitative assessments of skin morphology. Focus has been placed on skin thickness because of the sources of variation outlined above and the currently unrecognised functional significance of this variation. The contents of each chapter are as follows:

Chapter Two: The first data chapter in this thesis tests for the presence of sexually dimorphic skin anatomy in the white-lipped treefrog (*Litoria infrafrenata*) using a dataset that controls for temporal and spatial sources of variation. Although the skin of males and females in this small dataset were significantly different in thickness, this difference was not recovered once size-corrected data were used. Difficulties in identifying polymorphic

skin glands in hylids and their potential function due to methodological differences among studies are also discussed.

Chapter Three: This chapter tests for the presence of seasonal skin thickening in three sympatric anurans from the Midwestern United States: the American bullfrog (*Lithobates catesbeianus*), the northern leopard frog (*L. pipiens*), and the spring peeper (*Pseudacris crucifer*). Seasonal skin thickening was only definitively detected in *L. catesbeianus*, despite it being sympatric with the other species and a congener of *L. pipiens*. In *L. catesbeianus*, skin thickness across the year is negatively correlated with the duration of daylight and positively correlated with precipitation. It is unclear exactly why skin thickening occurs but these results show that it cannot be assumed to be ubiquitous across all species that experience pronounced seasonality.

Chapter Four: To better understand the effects of preservation on the results obtained in this thesis, Chapter Four tests for seasonal skin thickening in a small sample of American bullfrogs collected in the 1930's and 1940's. Although the two datasets do seem to differ with the older dataset showing less pronounced seasonal skin thickening, the pattern was still detected qualitatively and quantitatively in some skin measurements. The differences between the datasets could relate to changes introduced by the preserving medium or due to larger scale global climate change. More studies are needed to determine which of these factors is more likely.

Chapter Five: Chapter five focuses on interspecific scaling relationships and regional variation in skin thickness across the body. In most (but not all) species, the dorsal skin is the thickest skin region, although multiple individuals of *Lithobates pipiens* and *Pseudacris crucifer* do not consistently show this pattern. In some hylids (*Bokermannohyla alvaregni* and *Litoria infrafrenata*), the dorsal skin is the thinnest region of skin. These species also have polymorphic skin glands that might compensate for water loss through their secretions, or it might be that vercuae on the ventral pectoral and ventral thigh regions cause the skin in these regions to appear thicker overall. Among species, skin thickness is tightly linked with body size with this relationship being

strongest for the compact dermis among tissue layers. Summer specimens of *Lithobates catesbeianus* fall as outliers below the interspecific regression line and create a weaker correlation between skin thickness and body size compared to winter specimens, suggesting that seasonal skin thickening might actually be more appropriately referred to as seasonal skin thinning in this species.

Chapter Six: The final chapter of this thesis tests for a relationship between skin thickness and ecology using the dataset from Chapter 5 and published datasets. Relationships between skin thickness and environmental data for phylogenetically disparate datasets were weak, and body size explained most of these relationships. However, at taxonomically restricted scales (conspecifics or congeners), skin thickness does not correlate with environmental parameters. The evolutionary and ecological significance of this result are discussed.

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CHAPTER TWO: EXAMINING THE RELATIONSHIP BETWEEN SEXUAL DIMORPHISM IN SKIN ANATOMY AND BODY SIZE IN THE WHITE-LIPPED TREE FROG *LITORIA INFRAFRENATA* (ANURA, HYLIDAE) WITH COMMENTS ON POLYMORPHIC GLANDS IN HYLIDAE

ABSTRACT

Amphibians transport water, oxygen, carbon dioxide, and various ions (e.g. sodium and potassium) across their skin. This cutaneous permeability is thought to affect their ability to respond to environmental change and play a role in global population declines. Skin anatomy sexual dimorphism has been documented in some amphibian species with conflicting results. In species that display body size sexual dimorphism, the skin of males is thinner than that of the larger females. It is unclear whether this difference in skin thickness manifests a functional difference or if it is related to body size alone. Skin anatomy attributes were examined in males and females of the white-lipped treefrog (*Litoria infrafrenata*): skin thickness, capillary depth, and gland density. Although the skin of males is absolutely thinner than that of females, this difference is explained by body size. Capillary depth and gland densities do not differ between the sexes. Regressions of skin thickness variables to body size found no statistically significant differences between the slopes of males and females, although sample sizes were low (n = 3 and 5, respectively). Overall, it was concluded that skin thickness in male and female *L. infrafrenata* correlates with body size dimorphism and suggest that future studies on amphibian skin anatomy include measures of body size, test the ecological significance of sexually dimorphic skin anatomy, and document the prevalence of sexually dimorphic skin anatomy more widely among amphibians.

INTRODUCTION

The skin of amphibians is semipermeable and allows gases, liquids, and ions (e.g., sodium, potassium) to be exchanged between the internal tissues and external environment (Duellman & Trueb, 1986). Because of its high permeability, amphibian skin is susceptible to evaporative water loss and desiccation and is often implicated in explanations addressing the observation that a high proportion of amphibian species are threatened with extinction compared with any other terrestrial vertebrate clade (Wake & Vredenburg, 2008). Studies on both interspecific (Le Quang Trong, 1971,

1975; Sever, 1976) and intraspecific (Kun, 1959; Kobelt & Linsenmair, 1986; Wenying et al., 2011) amphibian skin anatomy suggest that variation in the details of its structure can provide ecological insights. For example, the skin of the reed frog (*Hyperolius nitidulus*) is thicker in the dry season than it is in the wet season, which helps it to reduce evaporative water loss (Geise & Linsenmair, 1986; Kobelt & Linsenmair, 1986) and populations of the Cururu toad (*Rhinella schneideri*) from different habitat types differ in skin thickness (Navas, Antoniazzi & Jared, 2004). However, little is known about anatomical variation in the characteristics of skin across modern amphibians or its functional significance. As a consequence, skin anatomy and related physiological data have been excluded from quantitative studies on traits correlated with a higher extinction threat status across amphibian species (e.g., Bielby et al., 2008; Cooper et al., 2008) making their influence on population declines or more general aspects of amphibian ecology uncertain.

Research on one source of variation of amphibian skin (sexual dimorphism) has focused largely on specialised mucous and serous skin glands that are present in males and absent in females (e.g. Sever, 1976, 1989; Brunetti et al., 2015). In plethodontid salamanders, these glands are present under the chin (the mental region), as well as the tail and are thought to play a role in mating because they become enlarged during the breeding season (Weichert, 1945; Sever, 1976, 1989). In hylid frogs in the tribe Cophomantini, Brunetti et al. (2015) inferred that glands present in the mental and lateral regions of males are involved in the secretion of chemical signals during mating. While the specific function of these glands remains ambiguous, even fewer studies exist on other aspects of sexual dimorphism in amphibian skin, such as changes in dermal and epidermal thickness. Male African clawed frogs (*Xenopus laevis*) and some species in the genus *Ptychadena* have thinner skin than females (Le Quang Trong, 1975; Greven, Zanger & Schwinger, 1995); conversely, male Siberian wood frogs (*Rana amurensis*) have thicker skin in the breeding season than females (Wenying et al., 2011). In Dybowsky's frog (*Rana dybowskyii*), there is no consistent pattern across the body, where females have thicker dorsal skin and males have thicker ventral and lateral skin (Lili, Chuan & Shulan, 2013), and in the cane toad (*Bufo marinus*) and the green frog (*Pelophylax esculentus*), the sexes do not differ in skin thickness (Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001). These conflicting results could be related to ecology, behaviour, phylogenetic history, allometry, or a combination of factors, but which of these broad

factors explains the observed skin anatomy sexual dimorphism has not been established.

Measures of skin thickness and degree of vascularisation are traits that are linked to the ability of amphibians to transfer substances through the skin (McClanahan & Baldwin, 1969; Roth, 1973; Boutilier, Glass & Heisler, 1986; Katz, 1986; Toledo & Jared, 1993). Therefore, if these anatomical traits differ between males and females, then the two sexes might reasonably be expected to differ in their microhabitat preferences, particularly with regard to temperature and moisture requirements. Males and females of some species are known to differ in habitat preference outside of the breeding season, with males preferring to remain closer to water bodies (Regosin, Windmiller & Reed, 2003; Fellers & Kleeman, 2007). This difference could be strictly behavioural, for example being related to the location of territorial calling sites used by males rather than reflecting a physiological limitation; however, intrinsic factors, such as skin thickness or other anatomical features, is expected to exert some influence on habitat preference (Czopek, 1965; Roth, 1973; Wainwright & Reilly, 1994) and deserves to be examined. The role played by the skin is crucial to the refinement of our understanding because without precise documentation of skin microanatomy, potentially useful data may have been either overlooked or understudied.

Although amphibian skin anatomy has been studied for over 150 years (Ascherson, 1840), integrative studies seeking to answer broad evolutionary and ecological questions about this structure are lacking. Sexual dimorphism in body size is pervasive among amphibians (e.g. De Lisle & Rowe, 2015), yet previous studies of skin anatomy have not corrected for these, sometimes extreme, differences in body size. Larger frogs take longer to dehydrate to dangerous levels than smaller frogs but also take longer to rehydrate (Tracy, Christian & Tracy, 2010), suggesting a possible relationship between skin thickness and body size if this variation is not completely explained by differences in surface area to volume ratios. In the African grassland frogs (genus *Ptychadena*), savannah species seem to have relatively thicker skin than species inhabiting forest or mixed habitats (Le Quang Trong, 1975). Conversely, in the puddle frogs (genus *Phrynobatrachus*), body size and skin thickness seem to co-vary with habitat type (Le Quang Trong, 1971). The taxonomic breadth of each of the latter two studies is small, so drawing broad conclusions should be done with appropriate caution. Taken together, however, these data suggest that skin thickness

has ecological significance and reinforces the need for more rigorous studies on inter- and intraspecific variation in skin anatomy in order to clarify these relationships and interrelationships.

To investigate sexual dimorphism in the skin anatomy of amphibians, the skin was examined of the white-lipped treefrog (*Litoria infrafrenata*), which is native to the wet tropical forests of Southeast Asia and Australia. This species was chosen because it exhibits body size sexual dimorphism and is a close relative to the Australian green treefrog (*L. caerulea*), which is used commonly in laboratory-based studies of amphibians (e.g. Buttemer, 1990; Christian & Parry, 1997; Voyles et al., 2009). Moreover, the current study represents the first on skin anatomy sexual dimorphism of a terrestrial tropical rainforest amphibian species.

MATERIALS AND METHODS

Specimens and preparation

Eight formalin-fixed, alcohol preserved specimens of *Litoria infrafrenata* from the collections of the Museum für Naturkunde (MfN) in Berlin, Germany were sampled. All specimens were collected near Seru on the island of Yapen, Indonesia on 27 August 1995. Because the specimens were all collected on the same day and appear to represent full-grown adults, we are able to discount seasonal or ontogenetic effects, which are known to affect skin anatomy (Kun, 1959; Kobelt & Linsenmair, 1986; Rosenberg & Warburg, 1995). Body size was measured using snout-vent length (SVL). Three of the specimens were male (SVL 67–72 mm) and five were female (SVL 90–105mm).

Skin biopsies were taken from three regions: 0.5 mm^2 samples from the dorsal pectoral, ventral pectoral, and ventral thigh regions on the right-hand side of the body. The dorsal pectoral and ventral pectoral regions were chosen because they are commonly sampled in other studies on amphibian skin anatomy (e.g. Greven, Zanger & Schwinger, 1995; Zanger, Schwinger & Greven, 1995) and the ventral thigh region was selected because of the function of this area of skin for water absorption in at least some anurans (Roth, 1973). Dorsal pectoral and ventral pectoral samples were taken close to the pectoral girdle and adjacent to the midline; ventral thigh samples were taken from the ventral surface near the midshaft of the femur (Figure 2-1).

The methodology that was used to prepare the specimens for museum storage is unknown. In an attempt to create a more ‘life-like’ skin thickness and reduce the

effect of alcohol-induced shrinkage, the skin samples were first rehydrated by allowing them to sit in decreasing concentrations of alcohol (70%, 50%, 30%) and finally potassium base solution (PBS) for an hour each before being placed in 4% formalin overnight. The specimens were then placed in PBS for an hour before being progressively dehydrated and embedded in paraffin wax, which is a standard protocol for preparing even fresh tissues for histological preparation (Bancroft & Gamble, 2008). Although chemically mediated preservation protocols and histological preparation may be expected to shrink soft tissue, all eight specimens were stored in the same jar in the collections and were prepared using the same methodology. It is expected that any preservation or preparation biases will affect all specimens in a similar way, and therefore reduce their effects on the overall results. Sections were made at 5 μm thickness using a Leica SM2000 R Sliding microtome and then stained using azan staining modified after Geides (Geidies, 1954) and Masson Goldner's Trichome (Goldner, 1938) stains.

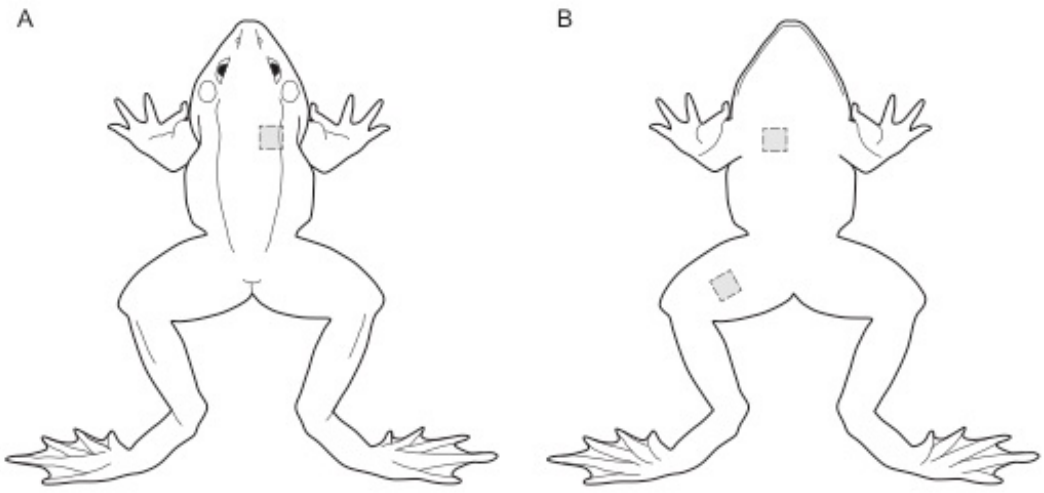

Figure 2-1. **Sampling locations across the body**. The locations where the dorsal (A; dorsal pectoral) and ventral (B; ventral pectoral and ventral thigh) skin was sampled. Sampling regions are indicated by the grey boxes.

Data collection

Photographs of the histological sections were taken with a Leica DFC490 camera mounted on an Axioskop light microscope and then measured in the program ImageJ

(Abràmoff, Magalhães & Ram, 2004). Linear measurements were recorded of the thickness of the epidermis, spongy dermis, and compact dermis, as well as capillary depth and gland density. For each variable, ten measurements were taken across the series of images for each specimen that were then averaged to produce a single value for each variable. The thickness of the epidermis was measured orthogonally from the basement membrane (stratum basale; Figure 2-2). The thickness of the spongy and compact dermis was measured using a line orthogonal to the orientation of the connective tissue layers in the compact dermis(Figure 2-2). Capillary depth was measured by taking the minimum distance between a capillary and the keratinised outer layer of the epidermis. Epidermis thickness was also measured by counting the number of cells between the basement membrane and the surface of the skin.

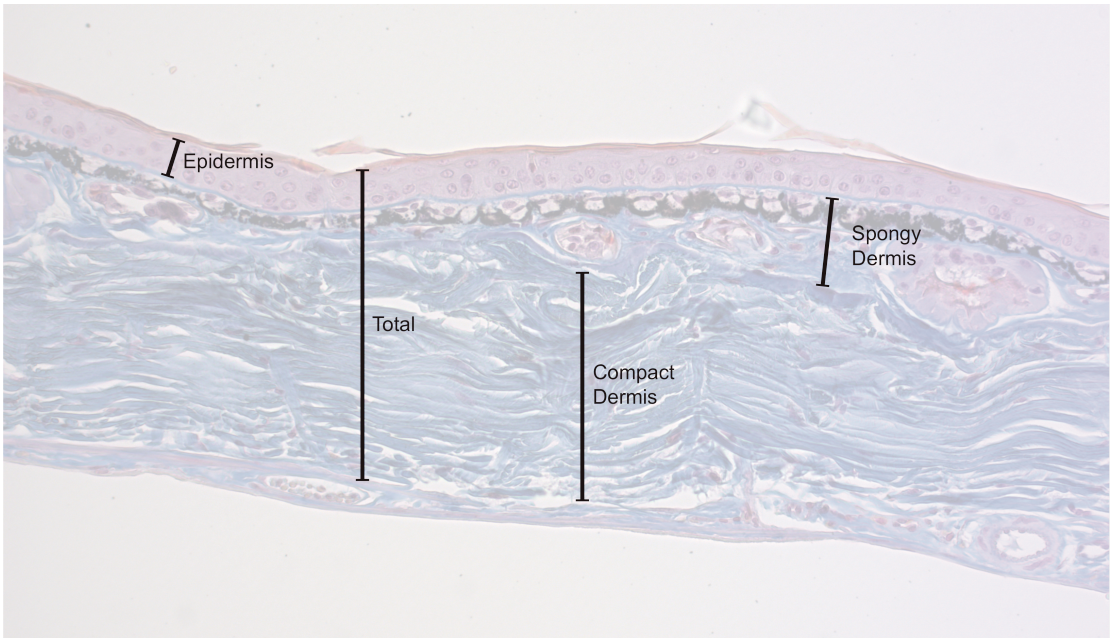

Figure 2-2. **Histology measurements**. Examples of how the various thickness measures were taking on images of stained tissue.

Gland density was calculated by measuring the length of the epidermis in the photograph and counting the number of glands present. In the dorsal sections, a straight line was used due to the uniformity of the skin in this section, with the exception of MfN 54644 which exhibited an abnormally crenulated epidermis. For the dorsal region of this specimen and for the ventral and thigh regions of all specimens, the length of the epidermis was measured along the stratum basale using a non-straight line as a better proxy for the amount of skin represented in the images. This measurement was taken on up to 10 images for each specimen, and then average values for the total number of all glands per mm and total number of each gland type per mm were calculated. There is no attempt to report on mucous and serous glands separately, as there is ambiguity concerning how many types of each gland are present or how such glands should be classified (see Discussion below). Nominally, gland classification was based on Delfino et al. (1998).

Analysis

Differences in the raw data were tested for using an analysis of variance (ANOVA) on each variable because the absolute difference in these variables is functionally and ecologically relevant. An ANOVA with two groups (males and females) was chosen instead of a standard t-test so that the results from the size corrected and size uncorrected analyses would be more directly comparable. To test for differences

independent of size, an analysis of covariance (ANCOVA) was performed for each variable with SVL as the coviariate. The use of simple ratios has been criticised for ignoring allometric affects in data and reduce degrees of freedom (Atchley, Gaskins & Anderson, 1976; Albrecht, Gelvin & Hartman, 1993), so were not used here. To examine possible allometric effects, ordinary least squares regressions of SVL versus the thicknesses of the skin layers were performed, firstly with all specimens to test if the slope significantly differed from zero and secondly to test for differences between regression lines for males and females. All data were log-transformed. All statistical analyses were performed in R v 2.1 (R Development Core Team, 2014).

RESULTS

Description of sampled skin regions and results from statistical tests of skin thickness

In both sexes, the skin is composed of the three standard cutis tissue layers: the epidermis, spongy dermis, and compact dermis (Figure 2-2). When the size-uncorrected values were analysed, males display significantly thinner compact dermis in the dorsal pectoral region; thinner epidermis and compact dermis in the ventral pectoral region; and thinner epidermis, spongy dermis, and compact dermis in the ventral thigh region, by comparison with females (Table 2-1). The total skin thickness in all three of the sampled body regions is less in males than in females before correcting for body size, and this difference is attributed to thinner cutis tissue layers in males (Table 2-1). However, once body size is taken into account, no significant differences were found by the ANCOVA.

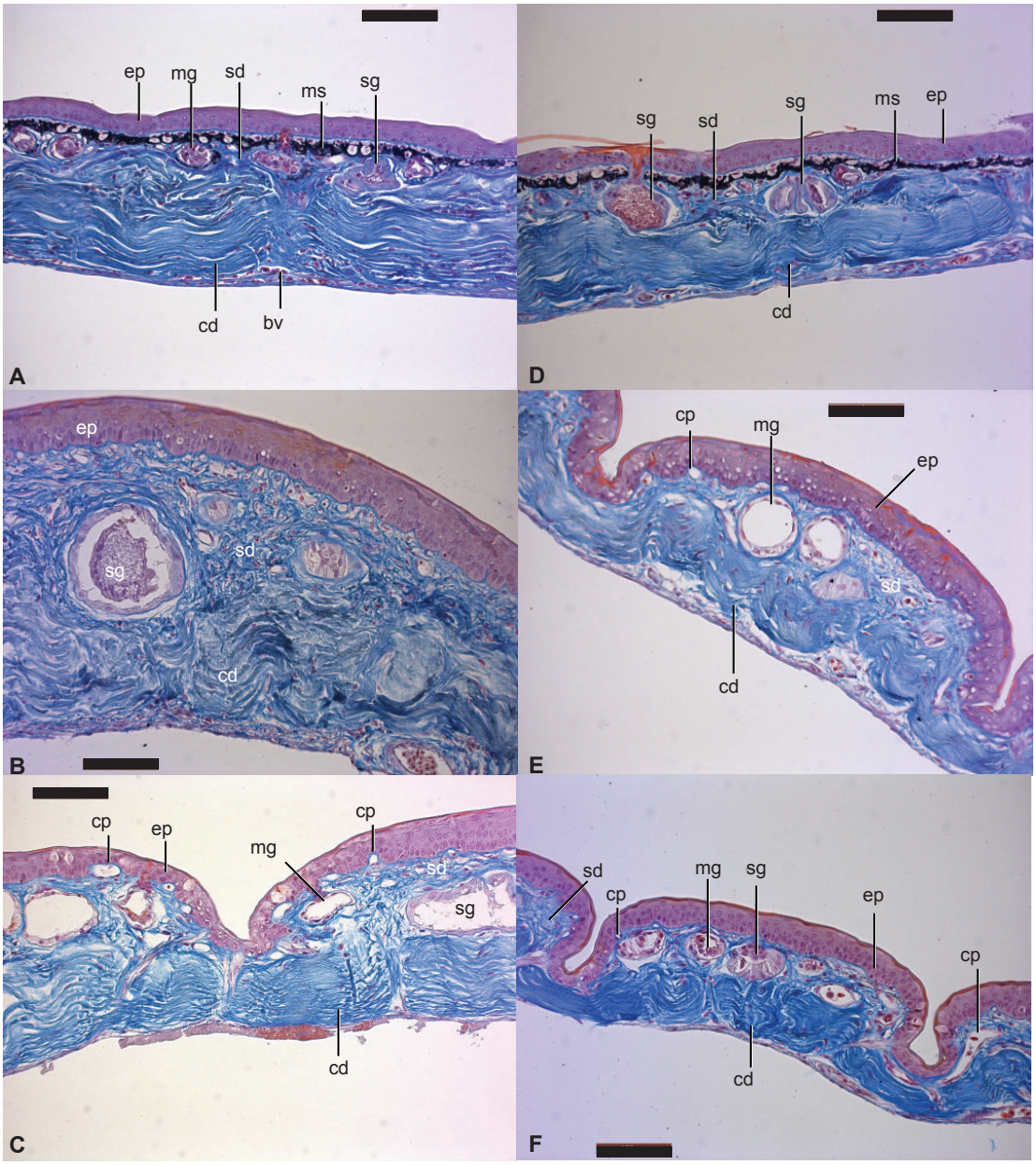

Figure 2-3. **Histological sections of the skin of male and female *Litoria infrafrenata*.** The skin of female (A–C) and male (D–F) *L. infrafrenata* is shown, sampled from the dorsal (A, MfN 54644; D, MfN 54644), ventral (B, MfN 54637; E, MfN 54642), and thigh (C, MfN 54647; F, MfN 54642) regions of the body; cd = compact dermis, cp = capillary, ep = epidermis, mg = mucous gland, ms = melanosomes, sd = spongy dermis, sg = serous gland; Scale bar = 100 µm.

Table 2-1. Average skin thickness of male and female *Litoria infrafrenata* reported ± standard deviation compared using uncorrected skin measurements alone (ANOVA) and with SVL as a covariate (ANCOVA).

				ANOVA Results			ANCOVA Results		
Region	Layer	Avg. (male)	Avg. (female)	Sum of squares	F (df = 6)	*p*-value	Sum of squares	F (df = 5)	*p*-value
Dorsal	Total	164 ± 45.4	249 ± 52.4	0.33	9.776	0.02	113.3	0.065	0.81
	Epidermis	24.9 ± 7.29	25.9 ± 3.25	0.01	0.182	0.68	17.44	0.656	0.46
	Spongy Dermis	50.6 ± 16.7	62.6 ± 17.5	0.10	1.086	0.34	1.04	0.003	0.96
	Compact Dermis	88.5 ± 13.9	160 ± 29.0	0.64	21.71	<0.01	29.68	0.051	0.83
Ventral	Total	188 ± 43.1	310 ± 72.2	0.48	20.57	<0.01	0	<0.001	0.99
	Epidermis	40.5 ± 6.46	53.5 ± 5.69	0.15	8.778	0.03	9.71	0.239	0.65
	Spongy Dermis	64.0 ± 23.1	103 ± 21.0	0.50	5.971	0.05	77.93	0.192	0.68
	Compact Dermis	83.5 ± 7.98	153 ± 24.0	0.68	24.68	<0.01	35.18	0.106	0.76
Thigh	Total	167 ± 50.4	295 ± 68.0	0.58	13.60	0.01	1328.3	0.309	0.60
	Epidermis	32.0 ± 3.92	39.8 ± 4.32	0.09	6.34	0.04	62.25	3.681	0.11
	Spongy Dermis	59.2 ± 8.49	120 ± 38.0	0.86	14.16	<0.01	183.5	0.161	0.70
	Compact Dermis	76.3 ± 21.5	136 ± 33.0	0.64	10.31	0.02	225.3	0.221	0.66

1. Dorsal skin sample: pectoral region. Based on average skin layer thicknesses (Table 2-1), the dorsal pectoral skin is the thinnest of the three regions sampled. The thickness of the tissue layers does not vary substantially, especially in comparison with the ventral pectoral and ventral thigh regions. The epidermis is 3–5 cells thick in females and 3–6 cells thick in males. The dorsal pectoral region is the only sampled region with clearly defined melanosomes and melanocytes (Figure 2-2A, D). The melanocytes are superficial to the melanin-filled melanosomes that they produce.

2. Ventral skin sample: pectoral region. Unlike the skin of the dorsal pectoral region, the ventral region is marked by vercuae, or regions of expanded spongy and compact dermis separated by troughs of thin dermis and slightly thinner epidermis (Figure 2-2B, E). The vercuae are larger in females than they are in males. Skin in the ventral region is the thickest on average; however it becomes much thinner in all three tissue layers in the troughs between the vercuae (Figure 2-2B, E). The epidermis is 3–7 cells thick in males and 3–8 cells thick in females, but it is 3–5 cells thick in the troughs between vercuae and 5–7/8 cells thick on the apex of the vercuae. Blood vessels within the spongy dermis push up into the region normally occupied by the epidermis at various points in the vercuae but never break through the basement membrane. The number of cells in the epidermis superficial to the blood vessels in these regions is lower than when blood vessels are not present.

3. Ventral thigh skin sample. Vercuae are present, much like those seen in the ventral region, and they are again smaller in males than they are in females (Figure 2-2C, F). The epidermis is 3–6 cells thick in males and 3–7 cells thick in females. Like in the ventral skin, the epidermis contains fewer cells in regions between vercuae, as well as above where blood vessels push up against the epidermis.

Description of gland types and gland density across sampled skin regions

Three types of glands can be distinguished in the sampled skin regions. Mucous and serous (granular) glands are conspicuous, and serous glands can be subdivided into two distinct types described by Delfino et al. (1998) (Figure 2-2). Mucous glands are typical of other amphibians and are characterised by possessing a thin epithelium and relatively small lumen, compared with the serous glands; this difference is more pronounced in females than in males (Figure 2-2C, F). The nuclei and cytoplasm of the mucous gland epithelial cells are more reactive to Azan staining, appearing darker in colour than the epithelial cells of either of the serous gland morphotypes (Figure 2-2). Mucous gland cells are also more ovoid and smaller, whereas the epithelial cells of both serous glands types have very elongate epithelial cells. The first type of serous gland (Type 1a, Delfino et al., 1998) is similar to that of all other amphibians (Figure 2-2D). Each has a thin epithelium and a relatively large lumen, usually filled with granules (Figure 2-2D). The second type of serous gland (Type 1b or II, Delfino et al., 1998) has relatively thick, bulbous epithelial cells that stain a lighter shade of pink

than the other two types of glands with the azan stain; they contain non-uniform granules that are roughly twice the size of the granules in the first serous gland. The anatomy of this second serous gland is similar to that of polymorphic serous glands reported in other hylids (Delfino et al., 1998). On this bases it is proposed that two serous gland types and one mucous gland type may be present in the skin of *L. infrafrenata*. There are no sexually dimorphic glands identified in the skin regions we sampled.

1. Dorsal skin sample: pectoral region. Glands are more densely distributed in the dorsal region than in the ventral or thigh regions. The first type of serous gland is much less common in females, as we only detected them in one female specimen (MfN 54646) but were found in all males. Gland density is significantly higher in males than in females before body size correction ($F = 8.771$, $p < 0.05$; Table 2-2). These values were not significantly different after correcting for body size.

2. Ventral skin sample: pectoral region. All three gland types are present in the ventral region. Gland density does not significantly differ between males and females in the ventral region (Table 2-2).

3. Ventral thigh skin sample. All three glands are present in the spongy dermis, suggesting that these glands are distributed across most of the ventral body. Gland density does not differ between males and females in the thigh region (Table 2-2).

Capillary depth

Capillaries have a very thin lumen and are usually identifiable because they still retain blood cells that stain bright red with Azan staining. They are usually present in the superficial spongy dermis, and push up into the epidermis in the ventral pectoral and ventral thigh regions. Blood vessels enter the spongy dermis through collagenous columns ascending from the hypodermis (Azevedo, de Jesus Santana & de Brito-Gitirana, 2006).

1. Dorsal skin sample: pectoral region. Capillary depth was not significantly different between males and females (Table 2-2).

2. Ventral skin sample: pectoral region. Many blood vessels lie just deep to the epidermis and they sometimes enter these spongy dermis outgrowths into the epidermis. Capillaries lie significantly deeper in the ventral skin of females than of males before body size correction ($F = 14.83$, $p < 0.01$, Table 2-2). These values were no longer significantly different after correcting for body size (Table 2-2).

3. Ventral thigh skin sample. Blood vessels push into the epidermis without breaking the basement membrane, although this feature is so extreme in one male (MfN 54641) that the basement membrane is difficult to identify, obscuring the separation between the epidermis and spongy dermis. In this specimen, the more highly vascularised regions of the epidermis are much thicker than the non-vascularised regions, and may be indicative of an unknown pathology. An attempt was made to exclude this specimen from statistical analyses of skin thickness measures to determine its effect on the results. When MfN 54641 was removed, the dorsal pectoral total skin thickness, ventral pectoral epidermis thickness, ventral thigh epidermis thickness, and ventral thigh compact dermis thickness were no longer significantly different between males and females before correcting for body size (results not shown). All other results were similar (results not shown), so we assume that the fewer significant differences are due to a smaller male sample size (two instead of three) rather than an effect of pathology. Capillary depth in the thigh region does not significantly differ between males and females (Table 2-2).

Table 2-2. Results from t-tests **co**mparing the capillary depth and gland density of male and female *Litoria infrafrenata* bot*h with and without s*ize correction.

				ANOVA Results			ANCOVA Results		
Layer	Variable	Avg. (male)	Avg. (female)	Sum of squares	F	*p*-value	Sum of squares	F	*p*-value
Dorsal	Capillary depth	44.1	45.6	0.005	0.226	0.65	1.899	0.040	0.8487
	Gland density	6.85	5.6	0.073	8.771	0.03	0.07992	0.202	0.6719
Ventral	Capillary depth	39.9	59.8	0.323	14.83	0.009	0.013	<0.001	0.9857
	Gland density	3.5	4.43	0.132	3.062	0.13	0.0889	0.161	0.7051
Thigh	Capillary depth	22.9	28.2	0.122	1.312	030	2.98	0.056	0.8225
	Gland density	3.54	4.1	0.042	1.055	0.35	0.6696	2.050	0.2255

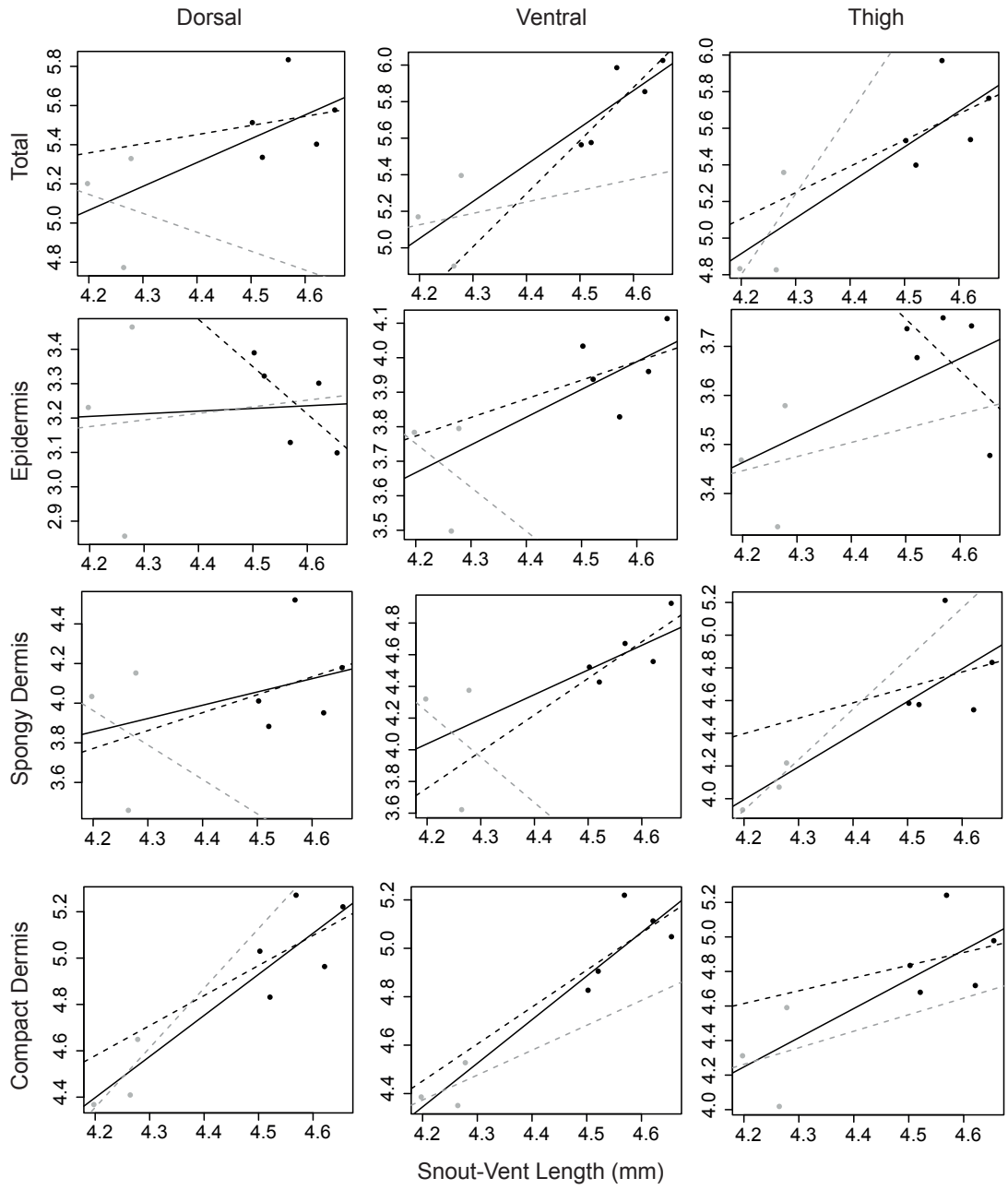

Figure 2-4. **Regressions of skin thickness measurements to body size (log-log scale).** Regression of the dorsal, ventral, and thigh (top) total, epidermis, spongy dermis, and compact dermis (left) thicknesses (µm) regressed against snout vent length (mm) on a log-log scale showing males (grey dashed lines), females (black dashed lines), and all specimens (solid black line).

Table 2-3. Results from regressions of skin thickness variables and body size and differences between males and females. (*$p < 0.05$; ** $p < 0.01$; ***$p < 0.001$)

Region	Layer	r^2	Slope	Intercept	M-F difference
Dorsal	Total	0.59	1.25*	-0.21	-
	Epidermis	-0.16	0.08	2.88	-
	Spongy Dermis	0.02	0.67	1.04	-
	Compact Dermis	0.81	1.77**	-3.05	-
Ventral	Total	0.79	1.52**	-1.21	-
	Epidermis	0.49	0.80*	0.29	-
	Spongy Dermis	0.46	1.55*	-2.49	-
	Compact Dermis	0.89	1.80***	-3.22*	-
Thigh	Total	0.64	1.62*	-1.76	-
	Epidermis	0.25	0.53	1.24	-
	Spongy Dermis	0.68	2.00**	-4.41	-
	Compact Dermis	0.56	1.69*	-2.85	-

Regression analyses

Regressions of skin thickness variables and body size did not identify any significant differences between males and females (Table 2-3, Figure 2-4). The slopes for the regressions of dorsal epidermis thickness, dorsal spongy dermis thickness, and thigh epidermis against body size did not have slopes significantly different from zero. The slope of the ventral epidermis was less than one. All other significant slopes were greater than one. The ventral compact dermis had the highest r-squared value (0.89) and the most significant slope ($p < 0.001$).

DISCUSSION

Skin anatomy sexual dimorphism in Litoria infrafrenata

Overall, the skin anatomy of *Litoria infrafrenata* is similar to that of other anurans (Fox, 1986a,b), with the exception of the polymorphic serous glands found in other hylids (Delfino et al., 1998). As in another species that demonstrate sexual dimorphism in body size, *Xenopus laevis* (Greven, Zanger & Schwinger, 1995), the skin is significantly thinner in the smaller males (Table 2-1). However, total average thickness of the skin layers and individual thicknesses of each layer in each region are not significantly different once body size is corrected for, suggesting that sexually dimorphic skin anatomy in *L. infrafrenata* is predominantly explained as a function of allometry. The absolute differences in epidermis thickness may be due to a slight difference in the number of cell layers between the sexes with males sometimes

having an epidermis that is one cell thinner than in females (except in the dorsal pectoral region).

It is worth noting that the small sample size inhibits the ability of statistical tests, particularly the ANCOVA, to recover significant differences. While the sample size is relatively large for a histological study (e.g., Bingol-Ozakpinar & Murathanoglu, 2011; Rigolo, Almeida, & Ananias, 2008), higher sample sizes may be required to capture adequate quantitative differences in tissue anatomy. Alternatively, there may be an allometric effect in the data that explains all significant difference in the uncorrected data. It is clear that further studies using quantitative measures of amphibian skin anatomy are required to determine which of these explanations is more likely.

Although significant differences were found, non-significant differences are also notable for their potential functional significance. For example, the dorsal epidermis thickness is nearly the same in males and females before body size correction. Treefrogs normally adopt a water-conserving posture that exposes only their dorsal surface to the external environment (Heatwole, 1963; Barbeau & Lillywhite, 2005). Since the epidermis provides a protective barrier between the internal and external environment, the dorsal epidermis of males might be thicker relative to body size to help prevent evaporative water loss (EWL) since rates of EWL are higher in smaller frogs (Tracy, Christian & Tracy, 2010). 'Typical' amphibians have rates of EWL that are equivalent to that of free standing water, but species of *Litoria*, along with other treefrog species, tend to have lower rates of EWL due to their glandular secretions (Shoemaker et al., 1972; Withers, Hillman & Drewes, 1984; Buttemer, 1990; Christian & Parry, 1997). *Litoria gracilenta* was found to have similar rates of EWL to *Phyllomedusa azure (Withers, Hillman & Drewes, 1984)*, suggesting that some species of *Litoria* might be just as efficient at reducing EWL as phyllomedusines, the latter being the group of anurans best able to resist EWL. Given the similarity in dorsal epidermis thickness between males and females, it is hypothesized that, along with lipid secretions and water-preserving behaviours, thickness of the dorsal epidermis assists in limiting EWL in *L. infrafrenata*.

The spongy dermis is thinner in males, but not significantly except in the ventral thigh region (Table 2-1). The glands lie in the spongy dermis and because of their location, their size limits the thinness of the spongy dermis where glands are present. The compact dermis, which provides structural support for the skin

(Duellman & Trueb, 1986), is absolutely thinner in males, suggesting their skin may be physically weaker than that of females (Greven, Zanger & Schwinger, 1995).

Centeno et al. (2015) described the presence of capillaries within the epidermis of the skin of the Santa Barbara treefrog (*Bokermannohyla alvarengai*) as a feature never before described in an anuran. Subepidermal capillaries are also present in both the ventral and thigh regions of *L. infrafrentata* (Figure 2-2), whereas Centeno et al. (2015) described only epidermal capillaries in the thigh region of *B. alverengai*. However, in *L. infrafrenata*, the capillaries never break the basement membrane whereas they might do so in *B. alverengai.* The skin of males appears to be more highly vascularised than that of females, although capillary density was not quantified here because these data cannot be collected using the methods employed here (e.g., Czopek, 1959, 1965). More highly vascularised regions of the skin are thought to be more important for regulating the animal's water budget and the location of these regions may vary with ecology (McClanahan & Baldwin, 1969; Roth, 1973), suggesting males may be able to uptake water more quickly than females.

Overall, it is concluded that, although some skin differences exist between adult males and females, these can be explained by body size alone. A paucity of research on the ecomorphology of amphibian skin inhibits the ability to fully understand the true differences between males and females of *L. infrafrenata* at this time, although it is hoped that this and similar studies documenting skin anatomy sexual dimorphism will inspire future studies to examine this relationship in more detail. For now, it is hypothesized that a relatively thicker dorsal epidermis and more highly vascularised ventral and thigh region are sexually dimorphic traits that allow males to better resist EWL and absorb water and other nutrients from the external environment, respectively.

Cutaneous gland types in hylid frogs

We identified polymorphic serous glands in *Litoria infrafrenata.* The anatomy of cutaneous glands of the many other hylid species has been described (Blaylock, Ruibal & Platt-Aloia, 1976; Delfino et al., 1998, 2002, 2006; Warburg et al., 2000; Nosi et al., 2002; Terreni et al., 2002; Barbeau & Lillywhite, 2005; Rigolo, Almeida & Ananias, 2008; Brunetti et al., 2015; Centeno et al., 2015). Most studies only report the presence of one type of mucous and one type of serous gland. However, sexually dimorphic mucous and serous glands have been reported in cophomantinis (Warburg

et al., 2000; Barbeau & Lillywhite, 2005; Brunetti et al., 2015). Four types of non-sexually dimorphic glands are present in *Phyllomedusa sauvegii* and *P. hypochondrialis*, and were classified by Delfino et al. (1998) as a single mucous gland along with Types Ia, Ib, and II serous glands. These classifications are based on the morphology of the gland and the granules of its secretion. Type Ia glands are the 'normal' serous glands found in all other amphibians for which the glands have been studied. Type Ib glands are grouped with type Ia glands because their secretory granules both have a spherical morphology. Type II glands are considered lipid-producing glands and are unique to phyllomedusine anurans (Blaylock, Ruibal & Platt-Aloia, 1976). In contrast, Barbeau & Lillywhite (2005) reported only one type of serous gland in *P. hypochondrialis*, although they note that some serous glands have "enlarged basal regions" (p. 2153) similar to the morphology described by Delfino et al. (1998) and reported here in *Litoria infrafrenata*. It is not clear, however, if the third gland in *L. infrafrenata* is a Type Ib or Type II serous gland (Delfino et al., 1998), as these two glands are in part defined by features only visible using electron microscopy methods, which is beyond the scope of this study.

Two types of mucous glands are present in *Bokermannohyla alvarengai*, and are differentiated by their affinities to different staining techniques (Centeno et al., 2015). Polymorphic mucous glands have not been reported in other hylids and it is unclear how these two mucous gland types differ in their secretions.

Methods for classifying polymorphic glands differ among researchers. One technique utilises morphological characters using light, transmission electron, and scanning electron microscopy techniques (Delfino et al., 1998, 2002, 2006), another uses histochemical attributes of the glands and their secretions (Barbeau & Lillywhite, 2005; Centeno et al., 2015), and some researchers prefer a combination of both (Blaylock, Ruibal & Platt-Aloia, 1976; Warburg et al., 2000). Although these methods are not tested or compared here, the presence of at least one polymorphic serous gland in *L. infrafrenata* and the reported lack of polymorphic glands in the closely related *L. caerulea* (Warburg et al., 2000) raise questions about the evolution and function of polymorphic glands in phyllomedusine and pelodryadine hylids that should be further investigated using these methods, considering the efficiency with which phyllomedusine hylids are able to avoid EWL (Shoemaker et al., 1972). Furthermore, given the morphological similarity between mucous glands in *B. alvarengai*, this suite of methods should also be applied outside Hylidae to confirm their absence in other

clades to better understand the evolution and function of these enigmatic structures in anurans.

The link between body size and skin anatomy sexual dimorphism in amphibians

Body size affects important physiological processes such as water loss and body temperature maintenance in amphibians (e.g. Tracy et al., 2010), so smaller males might be expected to have skin that is equally thick or thicker than that of the larger females. However, in *Litoria infrafrenata*, many layers of the skin are thinner in males than in females before body size is taken into account, suggesting that body size does not affect relative skin thickness with the notable exception of the dorsal epidermis and dorsal and ventral spongy dermis (Table 2-1). These results suggest that sexual skin anatomy dimorphism is largely explained by body size and this result might be applicable for other species in which body size is also sexually dimorphic, such as *Xenopus laevis* (Greven, Zanger & Schwinger, 1995). Males of *Rama amurensis* have thicker skin than females; however, this species was sampled in the breeding season and males were found to display sexually dimorphic skin glands (Wenying et al., 2011). Therefore, as with some tissue layers in *Litoria infrafrenata*, this difference in skin thickness may serve a yet unrecognised function. Studies with larger sample sizes are needed to test this hypothesis.

This study provides the first quantitative anatomical insights into the relationship between body size and skin anatomy sexual dimorphism in an amphibian and suggests that differences in skin anatomy between the sexes are broadly attributable to size alone. However, sexually dimorphic features were identified that are likely to be physiologically relevant (e.g., capillary density in the ventral pectoral region, before accounting for body size) and body size independent (e.g., relatively thick dorsal pectoral epidermis thickness) that should be investigated further. Future studies are needed to establish how prevalent sexual skin anatomy dimorphism is across amphibians, the processes that drive this dimorphism, and its ecological significance. It is critical that these studies also acknowledge other known sources of amphibian skin anatomy variation (e.g., seasonality) to increase the knowledge about this physiologically important organ.

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CHAPTER THREE: TESTING SEASONAL SKIN ANATOMY CHANGES IN THREE SYMPATRIC NORTH AMERICAN ANURAN SPECIES

ABSTRACT

Seasonal skin thickening is a strategy for resisting harsh seasonal environmental conditions in at least two anuran amphibian species. The function, pattern, and phylogenetic distribution of the seasonal skin thickening phenomenon are all poorly understood but could explain differences in the invasion potential, resistance to diseases like chytridiomycosis, or climatic niche breadth of different amphibians, because interspecific data suggest a link between skin thickness and ecology. The anuran species in which seasonal skin thickening has been documented live in regions with high winter-summer or dry-wet seasonality. To test the ubiquity of this strategy, skin samples were taken from three sympatric anurans (*Lithobates catesbeianus, L. pipiens,* and *Pseudacris crucifer*) across an annual cycle using recently collected museum specimens. These samples were qualitatively and quantitatively tested for changes in total skin thickness as well as that of the three primary skin layers (epidermis, spongy dermis, and compact dermis). Only *L. catesbeianus* showed seasonal skin thickening with thinner skin in the summer than in the winter in all body regions and skin layers, despite its ecological and physiological similarities to *L. pipiens* and an overlapping range with both other species. These data suggest this strategy is not utilised in all anurans and that more studies on seasonal skin thickening are required to better understand its evolutionary and physiological significance.

INTRODUCTION

Many plant and animal species exhibit seasonal variation in their life history characteristics. Understanding the timing and signals for these changes (e.g. intrinsic vs. extrinsic) and their ecological benefits is a necessary prelude to predict a species' local adaptations across its range (Wilczek et al., 2009), suitability for reintroductions to fringe areas of its historic range (Orizaola & Laurila, 2009), or its ability to establish itself in a new environment (Yeh & Price, 2004).

Amphibians are considered the most environmentally sensitive group of terrestrial vertebrates. They have evolved seasonal variation in a number of traits, including habitat preference (Cunjak, 1986; Sinsch, 1988), diet (Hodgkison et al., 2003; Kovács et al., 2007), physiology (Pasanen & Koskela, 1974), behaviour

(Runkle et al., 1994), ontogenetic pathways (Whiteman, 1994; Hector, Bishop & Nakagawa, 2012), and skin anatomy (Kun, 1959; Kobelt & Linsenmair, 1986). Variation in some traits is related to the breeding season. Males in many frog species develop 'nuptial pads' on the manus to help grip females during mating (amplexus) in the breeding season (e.g. Epstein & Blackburn, 1997), and male hairy frogs (*Trichobatrachus robustus*) develop filamentous outgrowths on their lateral body wall and dorsal thigh regions during the breeding season that are highly vascularised and serve a similar function to external gills (Noble, 1925). The males of some salamander species have different skin texture and increased body size in the breeding season and both sexes may modify their cloacal glands (Aoto, 1950; Sever, 1976). The function of these changes may relate to breeding behaviour as well as differences in habitat preference between the non-breeding and breeding seasons.

Beyond variation linked with the breeding season, there are various strategies that amphibians utilise in order to survive unfavourable seasonal conditions. For example, some Australian desert species form cocoons constructed from dead skin and mucus that significantly reduce evaporative water loss (Christian & Parry, 1997). Other species in both temperate and subtropical habitats are known to have thicker skin in the 'harsh' season (winter or dry season) compared to the more favourable season (summer or wet season) (Kun, 1959; Kobelt & Linsenmair, 1986). This strategy has only been documented in Chinese populations of the common toad (*Bufo bufo*), in a reed frog (*Hyperolius nitidulus*) native to East and Central Africa, and the smooth newt (*Triturus vulgaris*), which is native to Europe (Czopek, 1959; Kun, 1959; Kobelt & Linsenmair, 1986). In *Bufo bufo*, the epidermis is thickest while the animal is hibernating (winter), thinnest in the breeding season (summer), and is intermediate in thickness during the post-breeding season (Kun, 1959). In *Hyperolius nitidulus*, the skin of the dorsal and ventral regions increases in thickness by roughly 125 μm, and this increase is due to swelling of the iridiophores just below the epidermis to deflect UV radiation and reduce evaporative water loss (Kobelt & Linsenmair, 1986). In *Lissotriton vulgaris*, the skin is also thinnest in specimens in full breeding dress with an epidermis that is roughly 20 μm thick and a dermis that is roughly 69μm thick, compared to that of the 'normal' specimens, in which the epidermis averages 25.5μm in thickness and the dermis averages 83 μm in thickness (Czopek, 1959).

The link between anatomical changes and physiological function has only been investigated in *Hyperolius nitidulus* in which the rate of evaporative water loss in the dry season becomes so low that it is similar to that of desert reptiles (Geise & Linsenmair, 1986). However, differences in skin thickness have been shown to relate to habitat type in both inter- (Le Quang Trong, 1971, 1975) and intraspecific (Navas, Antoniazzi & Jared, 2004) studies, supporting a link between skin thickness and ecological requirements. In *L. vulgaris*, skin thinning has been documented only in males, so this example of skin thinning may be related to a breeding behaviour rather than a similar function to skin thickness changes in *B. bufo* or *H. nitidulus* (Czopek, 1959).

Seasonal skin anatomy changes have been documented in very few amphibian species, so little is known about this aspect of amphibian life history, including the timing of skin thickening, whether thickening is induced by intrinsic or extrinsic signals (or a mixture), or how widespread this strategy is among amphibians. Morphological traits limit an organism's behaviour and physiology, both of which are relatively more plastic; hence, constraints imposed by morphological features inhibit the ability of an organism to adapt quickly to unfavourable conditions in their environment (Wainwright & Reilly, 1994). Therefore, if amphibian skin changes in thickness due to environmental cues, it might provide a unique example of a morphological trait that can act to increase the size of the fundamental niche occupied by an organism in response to changes in environmental conditions.

To examine this phenomenon in amphibians, we examined three sympatric species of anurans that are native to the Midwestern and Eastern United states: the American bullfrog (*Lithobates catesbeiana*), the northern leopard frog (*L. pipiens*), and the spring peeper (*Pseudacris crucifer*). The American bullfrog is a widespread species native to the eastern United States that has been introduced outside its native range in North America, South America, Europe, and Asia (Govindarajulu, Price & Anholt, 2006; Adams & Pearl, 2007; Giovanelli, Haddad & Alexandrino, 2008; Barrasso et al., 2009). Its diet shifts post-metamorphosis from being composed largely of invertebrates just after metamorphosis to comprising both invertebrates and vertebrates once it attains full adult body size (Raney & Ingram, 1941; Govindarajulu, Price & Anholt, 2006). It increases the mass of fat deposits to prepare for winter and during torpor submerges itself in shallow water for hibernation (Byrne & White, 1975; Tattersall & Ultsch, 2008). The northern leopard frog is also a wide-ranging

species that is naturally found across the United States and Canada in a variety of habitats. Its diet consists primarily of fossorial or crawling invertebrates during both juvenile and adult life stages (Collier, Keiper & Orr, 1998). Like the American bullfrog, it also increases fat reserves in preparation for winter, but the Northern leopard frog tends to hibernate in deeper (~3 m) water than its congener (Mizell, 1965; Tattersall & Ultsch, 2008). Spring peepers are much smaller than the American bullfrog or northern leopard frog and are also naturally found across the US and Canada (Conant & Collins, 1991). As adults, they feed on small arthropods, (Smith, 1961; Bellocq, Kloosterman & Smith, 2000). These frogs hibernate terrestrially and are able to survive being frozen for short periods of time (Storey & Storey, 1986; Layne Jr & Kefauver, 1997).

Here, skin thickness of specimens collected in the majority of months of the year for these three species is documented for the first time to test if seasonal skin thickening is ubiquitous for all anurans native to habitats with high seasonality and if changes in skin thickening are synchronised among species. Environmental data are also used to test if changes in anatomy might be influenced by external signals (e.g. temperature and precipitation) or if they are more likely triggered by internal (e.g. genetic) factors.

MATERIALS AND METHODS

Sampling

Thirteen (13) *Lithobates catesbeianus*, seventeen (17) *L. pipiens*, and nineteen (19) *Pseudacris crucifer* specimens from the Midwestern United States (Illinois, Indiana, Michigan, and Wisconsin) were sampled from collections at the Field Museum of Natural History (FMNH; Chicago, IL, USA) and University of Michigan Museum of Zoology (UMMZ; Ann Arbor, MI, USA). Six specimens from Virginia (two *P. crucifer* and four *L. catesbeianus*) were also included from the Smithsonian Natural History Museum (USNM; Washington, DC, USA) to increase the sample size. Specimens from these regions were chosen because of the large temperature range between seasons present in this region relative to other areas of their range (e.g., in the southern US). All specimens were stored in alcohol and were not obviously dehydrated at the time of sampling; however, we have no information about the methodology used to preserve these specimens for museum storage. Only specimens collected more recently than 1985 were used to reduce the effect of preservation on

skin thickness. Both males and females were sampled so that any sex-related differences could also be assessed.

Of the specimens sampled, eight (8) specimens of *Pseudacris crucifer* (FMNH 257520, FMNH 263410, FMNH 275291, FMNH 276438, UMMZ 224980, USNM 467239, USNM 467243, and USNM 469723), five (5) specimens of *Lithobates pipiens* (FMNH 236044, FMNH 250083, FMNH 279726, UMMZ 218548, and UMMZ 243532), and two (2) specimens of *L. catesbeianus* (FMNH 262557 and USNM 514921) showed signs of skin abnormality or potential pathologies. These specimens were therefore excluded from analyses. Eleven (11) *P. crucifer*, 12 *L. pipiens,* and 11 *L. catesbeianus* were used to test for seasonal skin thickening.

Sampling protocols follow that of Chapter 2. Briefly, skin samples of between 0.1 and 0.5 cm^2 were taken from the dorsal pectoral, ventral pectoral, and ventral thigh regions. Dorsal and ventral pectoral samples were taken from just to the right of the midline in the pectoral region of the animal. Ventral thigh samples were taken below the approximate midshaft of the femur (Figure 2-1).

Samples were rehydrated and fixed overnight in formalin. They were then dehydrated and embedded in paraffin wax. Sections were cut at 5 µm thickness and stained with modified azan staining after Geides (Geidies, 1954) and Masson Goldner's Trichrome (Goldner, 1938) stains so that the different tissue layers could be identified and measured. Images of the histological sections to use for measurements were taken using a Leica DFC490 camera mounted on an Axioskop light microscope.

Measurements

Epidermis thickness was measured in two ways. Ten measurements (µm) were made using a line orthogonal to the stratum basale using ImageJ (Abràmoff, Magalhães & Ram, 2004) and then averaged. Epidermis thickness was also measured in number of cell layers between the external surface of the skin and the stratum basale. Spongy dermis thickness was measured as the minimum distance between the compact dermis and the stratum basale. Compact dermis thickness was measured using a line orthogonal to the direction of the connective tissue layers. Each of these measurements was also taken 10 times and then averaged.

Snout-vent length (SVL) was measured as a proxy for body size. Log-transformed skin thickness measurements were regressed against log-transformed SVL using an ordinary least-squares regression. Residuals from this regression were

then plotted against the month in which the specimen was collected. These patterns were then qualitatively compared among body regions and species to determine 1) if there are clear and identifiable differences throughout the year; 2) when increases and decreases in skin thickness begin (if any); and 3) which time of year does the species exhibit greatest skin thickness (if applicable).

Detecting seasonal skin thickening and preservation effects

Residuals were also used to quantitatively compare skin thickness by grouping months of the year into two groups. Specimens of *Lithobates catesbeianus* span the range from April until November, specimens of *L. pipiens* span the range from March to November, and specimens of *Pseudacris crucifer* span the range from March to October. Specimens collected before August were placed into one group and specimens collected during or after August were placed in the second. An analysis of variance (ANOVA) was performed on the twoe groups for each species and tested for differences between groups using a Tukey's Honestly Significant Difference test. An ordinary least squares regression of log-transformed epidermis thickness in μm and epidermis thickness in cell layers was also used to determine if the two variables are related. Finally, to test for any effects of preservation on tissue shrinkage that might affect our comparisons, a Spearman-ranked correlation test was used to compare year of collections and total skin thickness for all specimens and an ANOVA was used to compare specimens from different institutions to test for any institutional effects.

Ecological significance

All specimens had county locality data, as well as the date of collection. This information was used to extract daily minimum and maximum values at the county level for duration of daylight, precipitation, and minimum and maximum temperature for the 30 days preceding the specimen's date of collection using the function 'get_daymet' in the 'FedData' package (Bocinsky, Beaudette & Chamberlain, 2016). One specimen, FMNH 236044, only had a month and year of collection (April 1988), so the middle of the month was used as an estimate of the date of collection (15 April 1988). Mean, maximum, and minimum values of these data were used in subsequent analyses. Both maximum and minimum precipitation values were excluded in the analysis for minimum values of all variables because these values for all counties were zero.

Partial least squares regression (PLSR) was used to test for a relationship between skin thickness and the environmental data using the 'plsreg2' function in the package 'plsdepot' (Sanchez & Sanchez, 2012). This method is useful because it is able to handle multicollinearity better than traditional multiple regression techniques (Abdi, 2010), which is expected particularly in the skin dataset, and allows for multiple response and multiple predictor variables. The strength of the correlation was determined using the number of components that yielded Q^2 values above zero (Abdi, 2010) and the amount of variation summarised in those components. Variable of Importance for Projection (VIP) was used to determine which environmental variables were important for predicting skin thickness (Mehmood et al., 2012). Finally, regressions were run using both raw and size-corrected skin thickness values. All analyses were conducted using R (R Development Core Team, 2014).

RESULTS

Comparative skin anatomy of Lithobates catesbeianus, L. pipiens, *and* Pseudacris crucifer

All three species possess an epidermis, a spongy dermis, and compact dermis common to all amphibians thus far studied (Figures 3-1, 3-2, 3-3; Fox, 1986a,b). All three species have smooth dorsal pectoral skin (i.e., no vercuae). The glands are contained within the spongy dermis, which is separated from the compact dermis by a visible Eberth-Katshenko layer (Figure 3-1, 3-2). The dorsal pectoral skin of all three species contains melanosomes. Iridiophores are present in the dorsal pectoral skin of *Pseudacris crucifer* (Figure 3-3) but not in either of the *Lithobates* species. The compact dermis of the dorsal and ventral pectoral skin of *Lithobates catesbeianus* is thicker relative to the spongy dermis than in either of the other species (Figures 3-1, 3-2, 3-3). In the ventral pectoral and ventral thigh regions, vercuae are present in *P. crucifer* but not in either species of *Lithobates*.

The three species all possess at least two types of glands typical of amphibians: mucous and serous (granular) glands (Figure 3-1, 3-2, 3-2; Fox, 1986b). *Lithobates catesbeianus* seems to possess two different types of mucous glands based on the strength of the azan stain of the lumen. One stains dark magenta and the other is very light in colour. There do not appear to be any polymorphic glands in *L. pipiens* or *P. crucifer*.

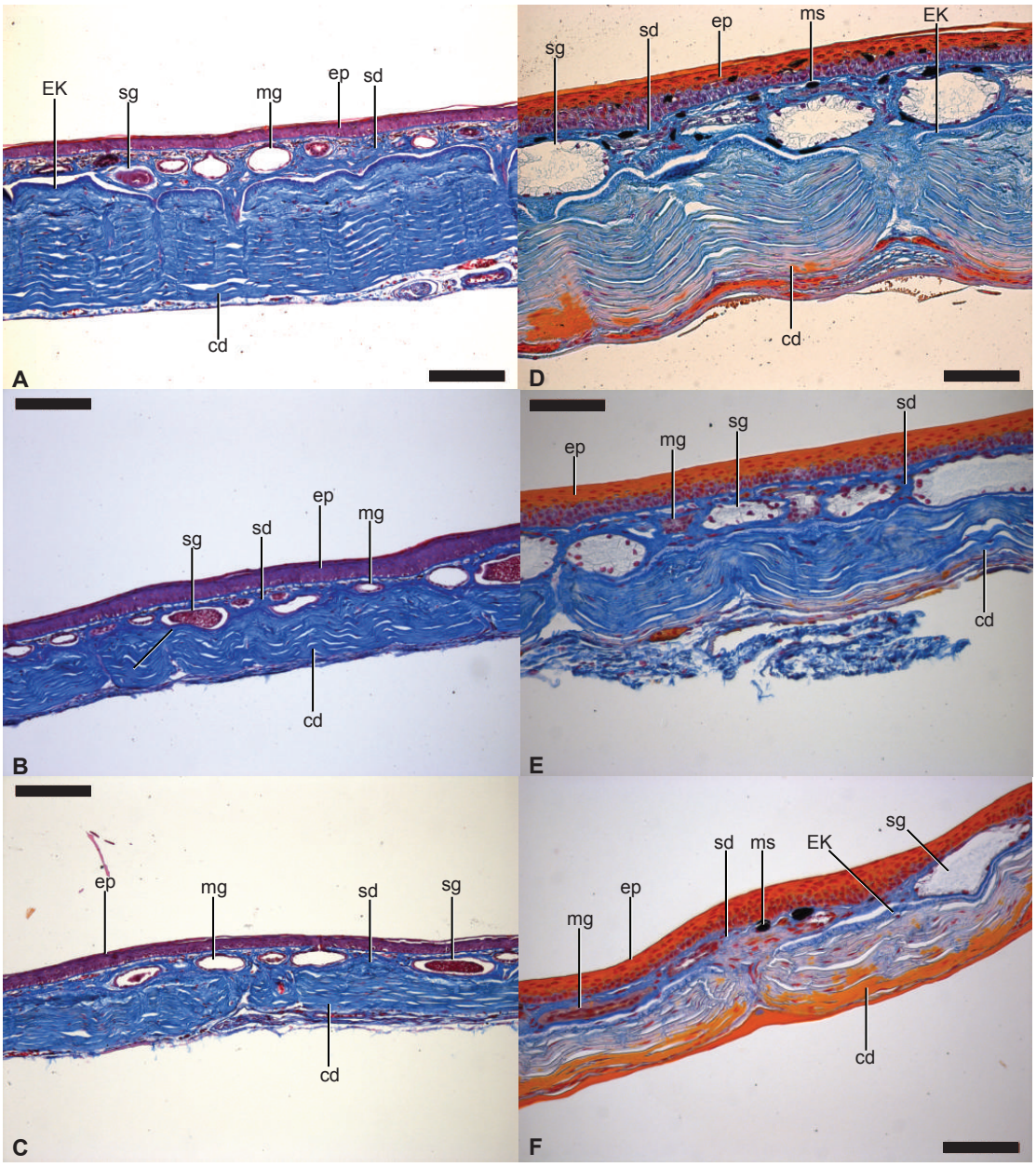


Figure 3-1. Histological sections of the skin of summer and autumn *Lithobates catesbeianus*. Histological sections of the dorsal pectoral (A, D), ventral pectoral (B, E), and ventral thigh (C, F) regions of *Lithobates catesbeianus* from July (A, B, C; USNM 347870), and October (D, E, F; FMNH 278931) stained with the azan stain modified after Geidies. **cd** = compact dermis; **EK** = EK-layer; **ep** = epidermis, **ir** = iridiophore; **mg** = mucous gland; **ms** = melanosome; **mus** = muscle; **sd** = spongy dermis; **sg** = serous gland. Scale bar = 100 µm.

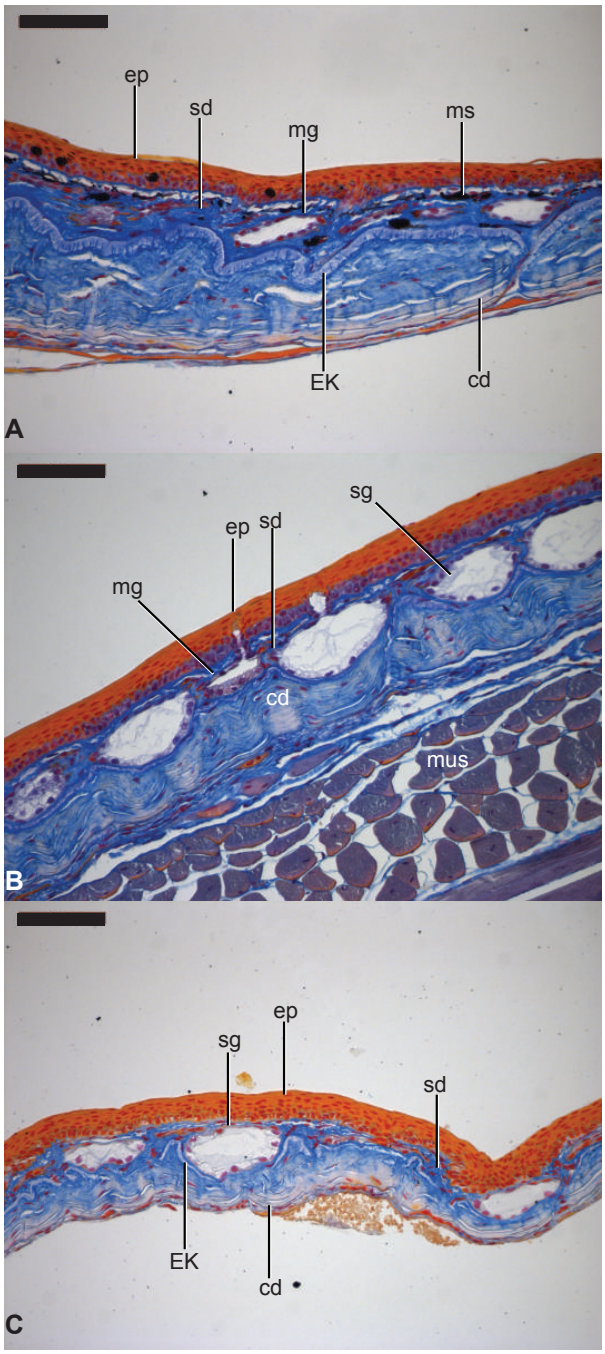


Figure 3-2 (left). Histological sections of the skin of *Lithobates pipiens*. Dorsal pectoral (A), ventral pectoral (B), and ventral thigh (C) skin sections of *Lithobates pipiens* (FMNH 279403) stained with azan stained modified after Geidies. Key follows that of Figure 3-1. Scale bar = 100 µm.

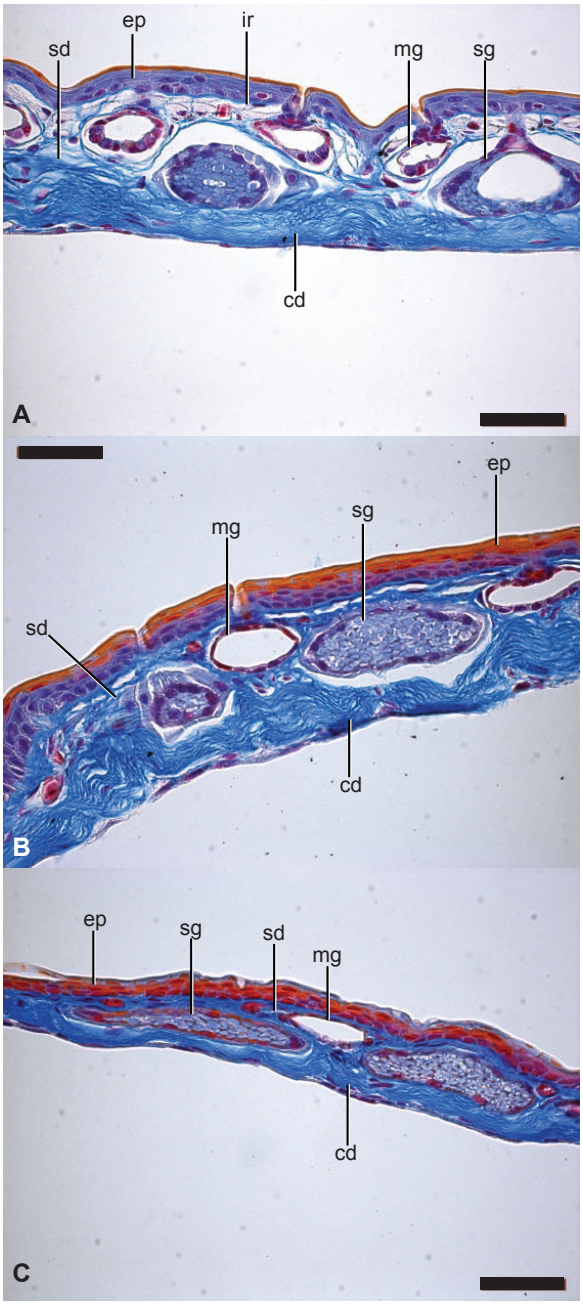


Figure 3-3 (right). Histological sections of the skin of *Pseudacris crucifer*. Dorsal pectoral (A), ventral pectoral (B), and ventral thigh (C) skin sections of *Pseudacris crucifer* (UMMZ 243630) stained with azan stained modified after Geidies. Key follows that of Figure 3-1. Scale bar = 50 µm.

Potential effects of preservation

ANOVAs and posthoc tests of specimens grouped by institution found that *Lithobates catesbeianus* specimens from the FMNH and USNM, *L. pipiens* specimens from the FMNH and UMMZ, and *Pseudacris crucifer* specimens from the FMNH, UMMZ, and USNM did not differ in total skin thickness in any of the three sampled skin regions (Table 3-1). The Spearman Rank Order Correlation tests found no significant relationships between year of collection and total skin thickness (Table 3-1).

Table 3-1. *p*-values for Spearman-ranked correlation test between skin thickness and year of collection and from the Tukey's posthoc test comparing specimens among institutions.

Species	Body Region	Collection year	FMNH-UMMZ	FMNH-USNM	UMMZ-USNM
Lithobates catesbeianus	Dorsal pectoral	0.92	-	0.14	-
	Ventral pectoral	0.84	-	0.11	-
	Ventral thigh	0.88	-	0.21	-
Lithobates pipiens	Dorsal pectoral	0.46	0.37	-	-
	Ventral pectoral	0.87	0.62	-	-
	Ventral thigh	0.87	0.94	-	-
Pseudacris crucifer	Dorsal pectoral	0.97	0.87	0.42	0.7
	Ventral pectoral	0.52	0.97	0.52	0.49
	Ventral thigh	0.99	0.59	0.32	0.82

Seasonal skin thickening in Lithobates catesbeianus

The 11 specimens of *L. catesbeianus* covered the months between April and November. Plots of residuals from the regressions of skin thickness to SVL and month of collection show a clear skin thickening pattern in *L. catesbeianus* (Figures 3-4, 3-5, 3-6). The skin is thickest in late Summer/early Autumn and appears to begin thinning again by November. This pattern appears in all three sampled skin regions and in all three cutis tissue layers (Figures 3-4, 3-5, 3-6). There is no discernable difference in skin thickening pattern between males, females, and juveniles using

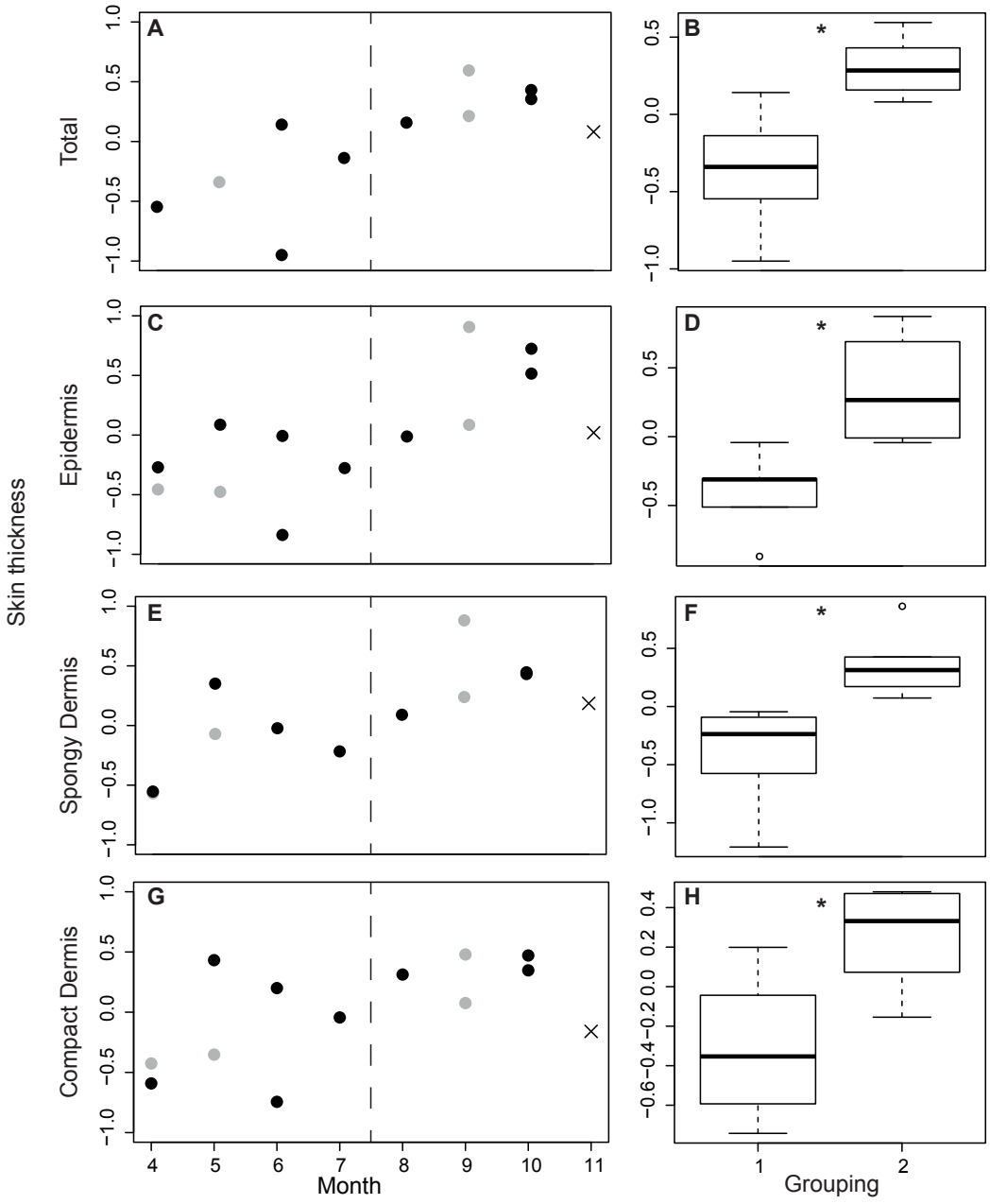


Figure 3-4. **Seasonal changes in dorsal pectoral skin thickness in *Lithobates catesbeianus*.** Plots of residual dorsal pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *L. catesbeianus*. Black dot represent female specimens, grey dots represent male specimens, and x's represent juvenile specimens. The dashed line represents the division for box plots. Significant relationships are denoted by an asterisk (*)

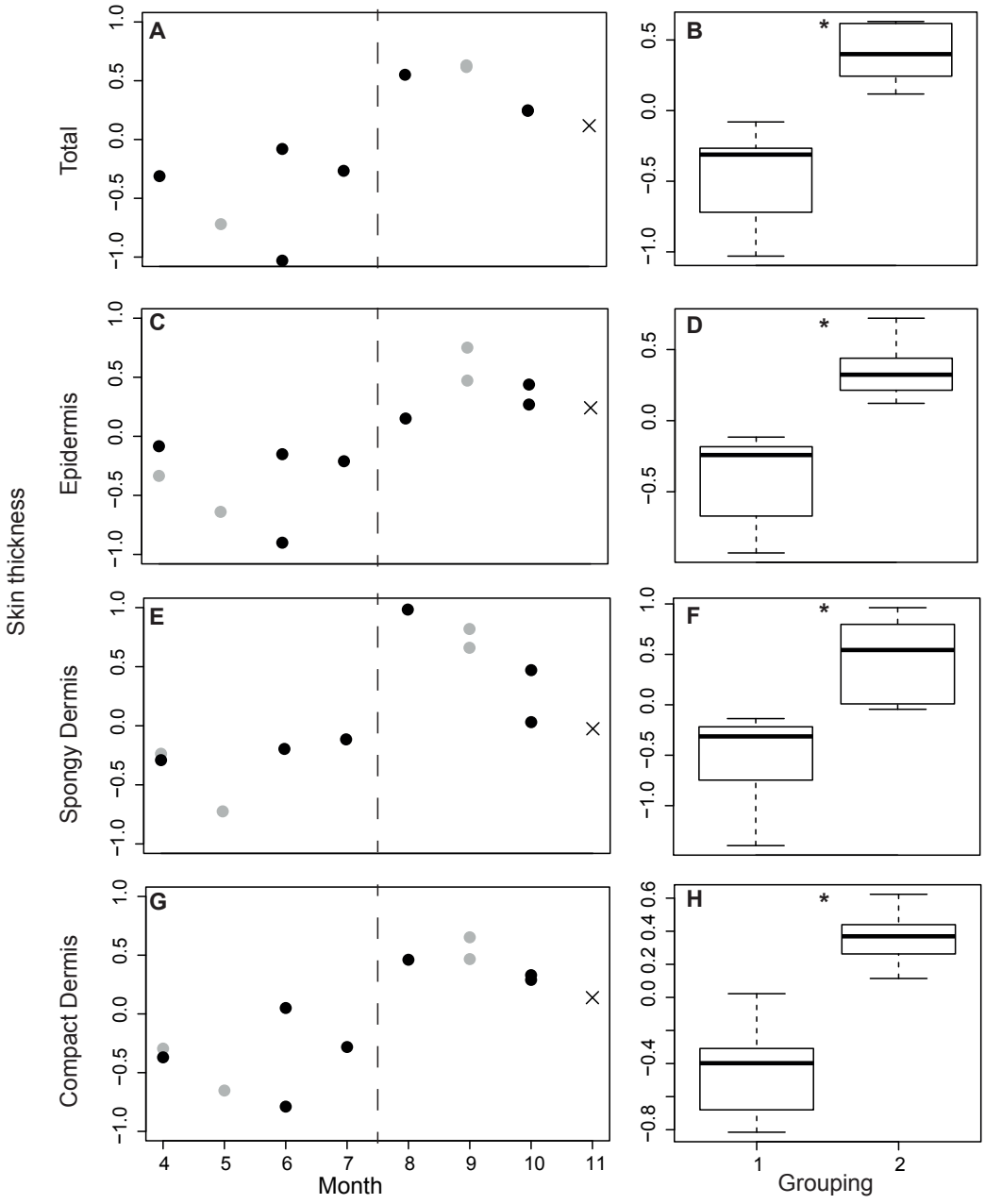


Figure 3-5. **Seasonal changes in ventral pectoral skin thickness in *Lithobates catesbeianus*.** Plots of residual ventral pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *L. catesbeianus*. Symbols follow Figure 3-4.

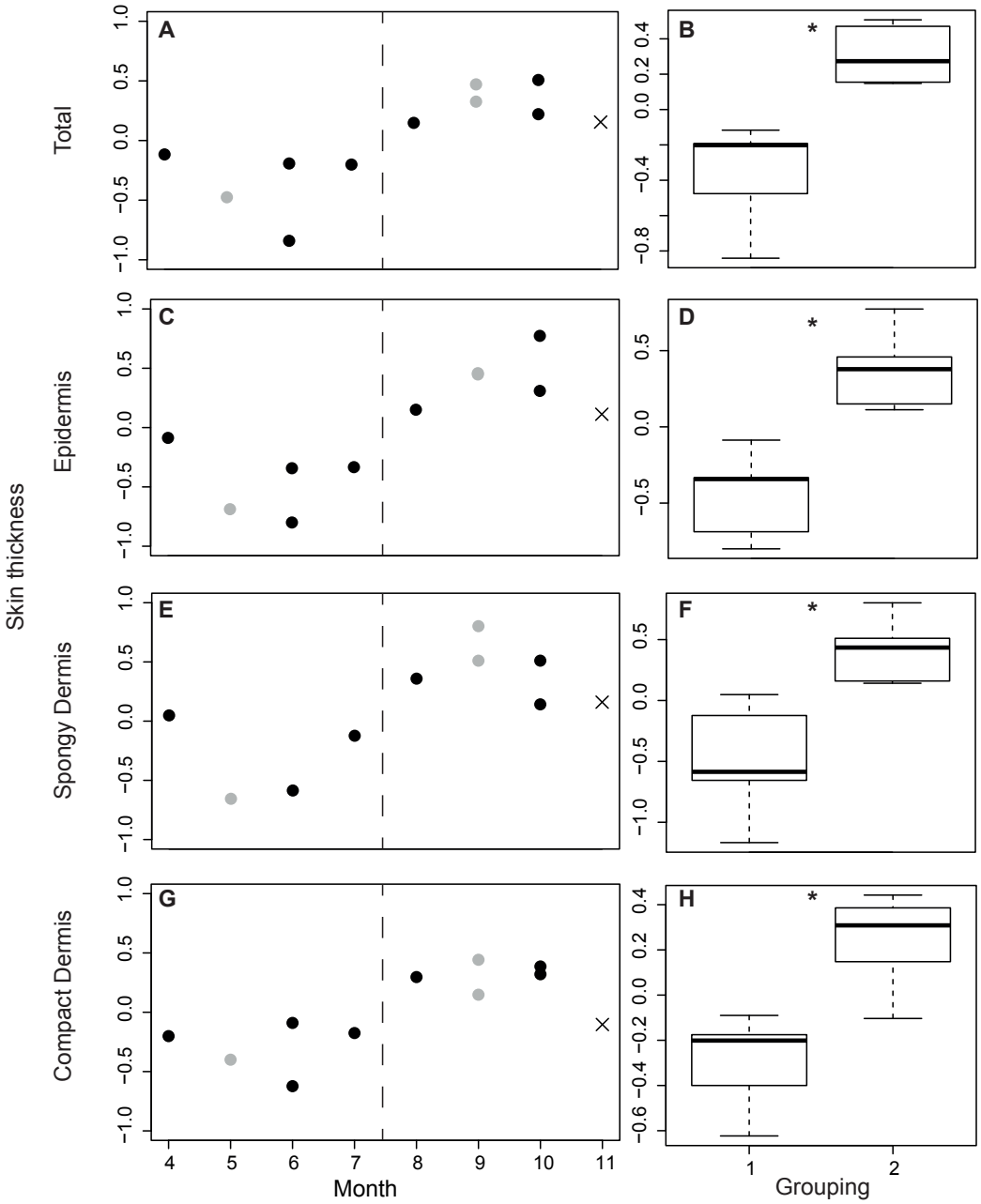


Figure 3-6. **Seasonal changes in ventral thigh skin thickness in *Lithobates catesbeianus*.** Plots of residual ventral thigh skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *L. catesbeianus*. Symbols follow Figure 3-4.

residuals. Thickness of the epidermis measured in μm and in number of cells is related significantly in all three sampled skin regions (Table 3-2). Grouping of the skin variables into three time bins found consistently significant differences between the two time bins for all variables (Table 3-3).

Seasonal skin thickening in Lithobates pipiens

The 12 specimens of *Lithobates pipiens* covered the months between March and November. Unlike in *L. catesbeianus*, the samples taken from specimens of *L. pipiens* did not show definitive skin thickening patterns across all skin layers and sampled skin regions (Figures 3-5, 3-8, 3-9). There does appear to be a common pattern of decreasing skin thickness from September to November, but the months before September vary across tissue layer and skin region (Figures 3-5, 3-8, 3-9). Males, females, and juveniles do not show different thickening patterns. The two measures of epidermis thickness were correlated in the dorsal pectoral and ventral pectoral regions, but not in the ventral thigh region (Table 3-2). Total thickness and compact dermis thickness in the ventral pectoral regions and all ventral thigh measurements except compact dermis thickness differed significantly between the two time bins (Table 3-3).

Table 3-2. Relationship between epidermis thickness and number of cells in epidermis.

Species	Region (epidermis)	r^2	Slope	Intercept	*p*-value
Lithobates catesbeianus	Dorsal pectoral	0.59	0.32	1.46	0.004
	Ventral pectoral	0.62	0.37	0.60	0.003
	Ventral thigh	0.41	0.36	0.96	0.02
Lithobates pipiens	Dorsal pectoral	0.84	0.49	0.60	<0.001
	Ventral pectoral	0.53	0.30	1.61	0.004
	Ventral thigh	0.13	0.18	2.33	0.14
Pseudacris crucifer	Dorsal pectoral	-0.12	0.03	2.35	0.81
	Ventral pectoral	-0.11	0.01	2.77	0.93
	Ventral thigh	0.01	0.16	1.99	0.32

Seasonal skin thickening in Pseudacris crucifer

The 11 specimens of *Pseudacris crucifer* span the months between March and October. There is no discernable pattern of skin thickening in the dorsal pectoral or

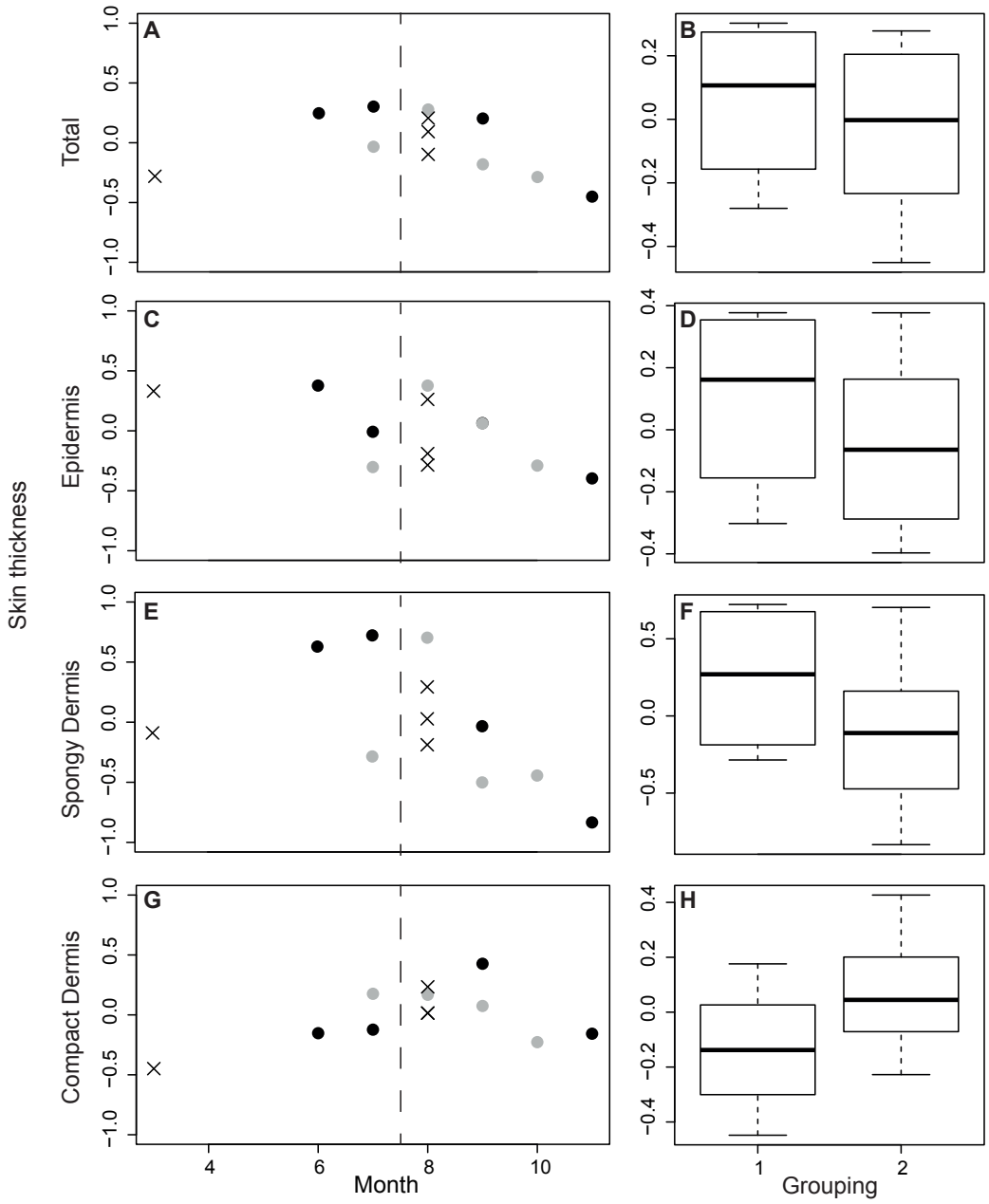


Figure 3-7. **Seasonal changes in dorsal pectoral skin thickness in *Lithobates pipiens.*** Plots of residual dorsal pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *L. pipiens*. Symbols follow Figure 3-4.

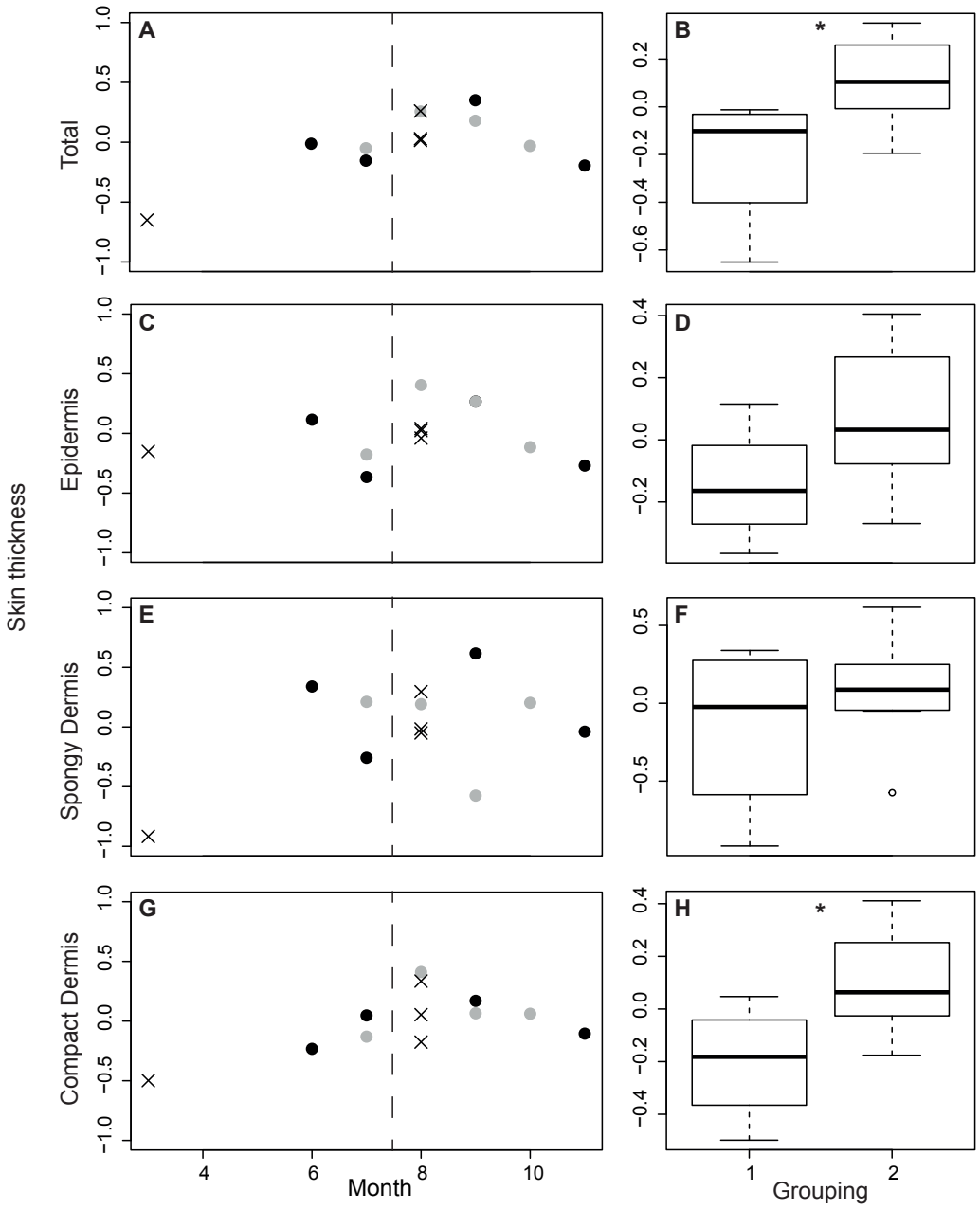


Figure 3-8. **Seasonal changes in ventral pectoral skin thickness in *Lithobates pipiens.*** Plots of residual ventral pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *L. pipiens*. Symbols follow Figure 3-4.

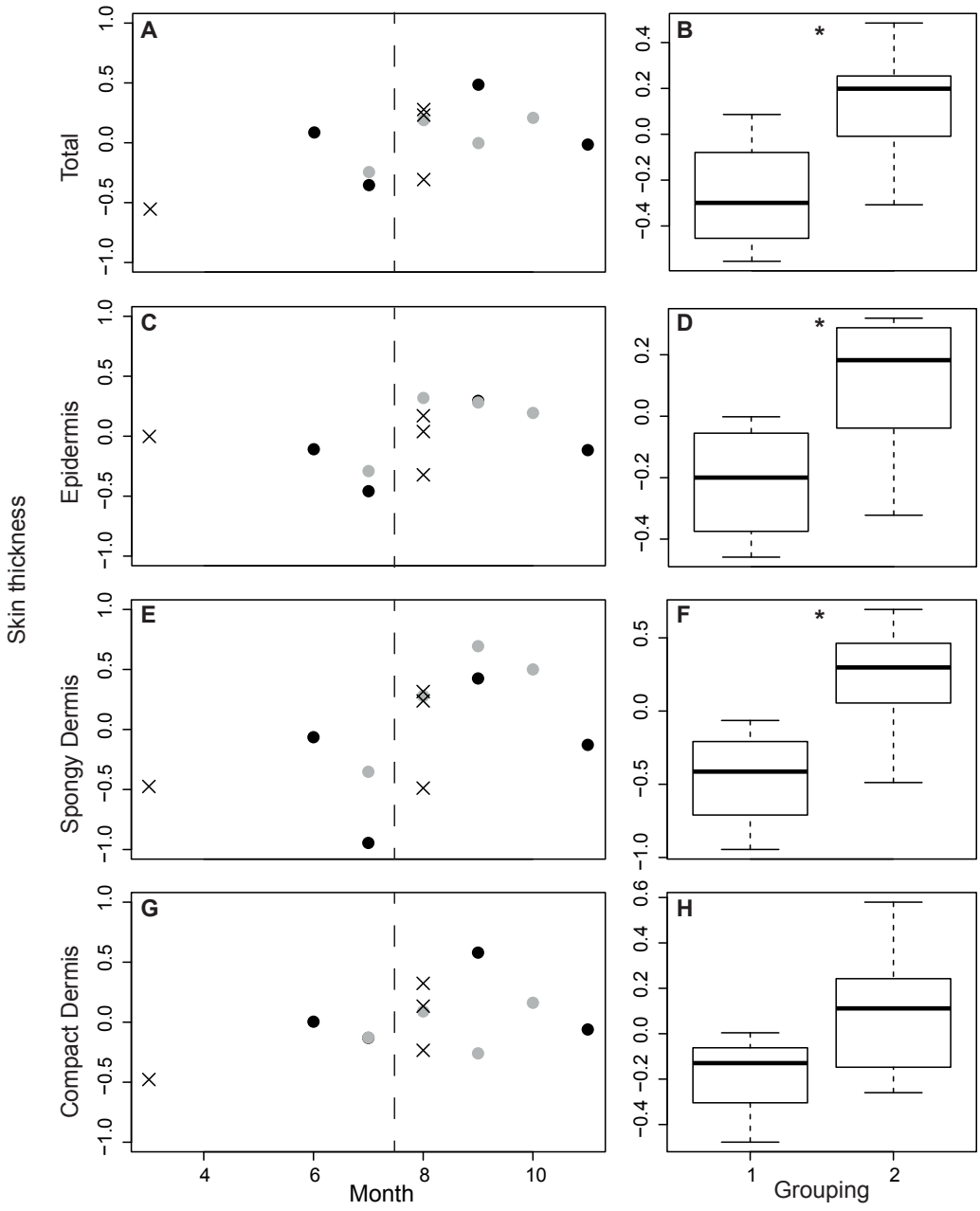


Figure 3-9. **Seasonal changes in ventral thigh skin thickness in *Lithobates pipiens.*** Plots of residual ventral thigh skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *L. pipiens*. Symbols follow Figure 3-4.

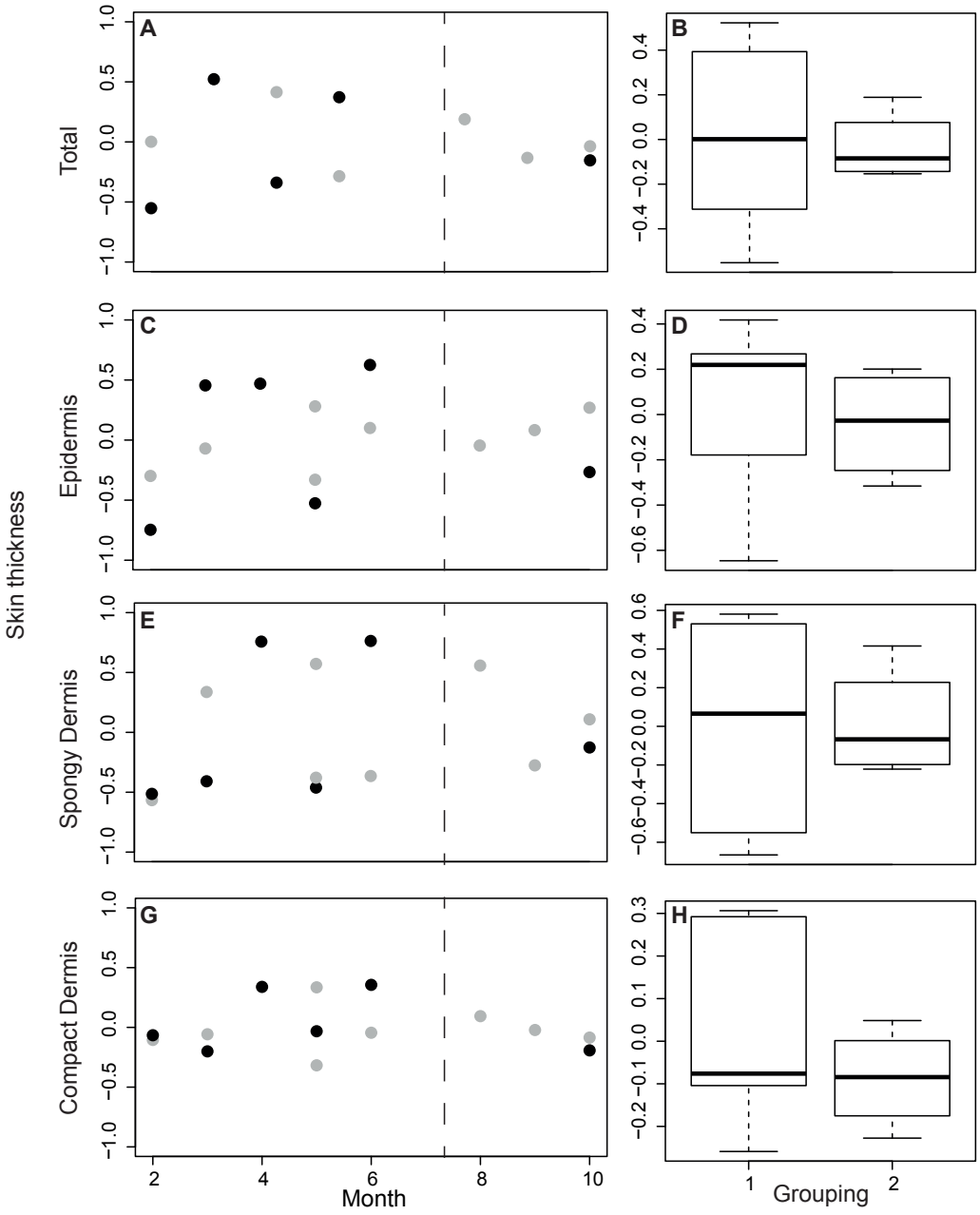


Figure 3-10. **Seasonal changes in dorsal pectoral skin thickness in *Pseudacris crucifer*.** Plots of residual dorsal pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *P. crucifer*. Symbols follow Figure 3-4.

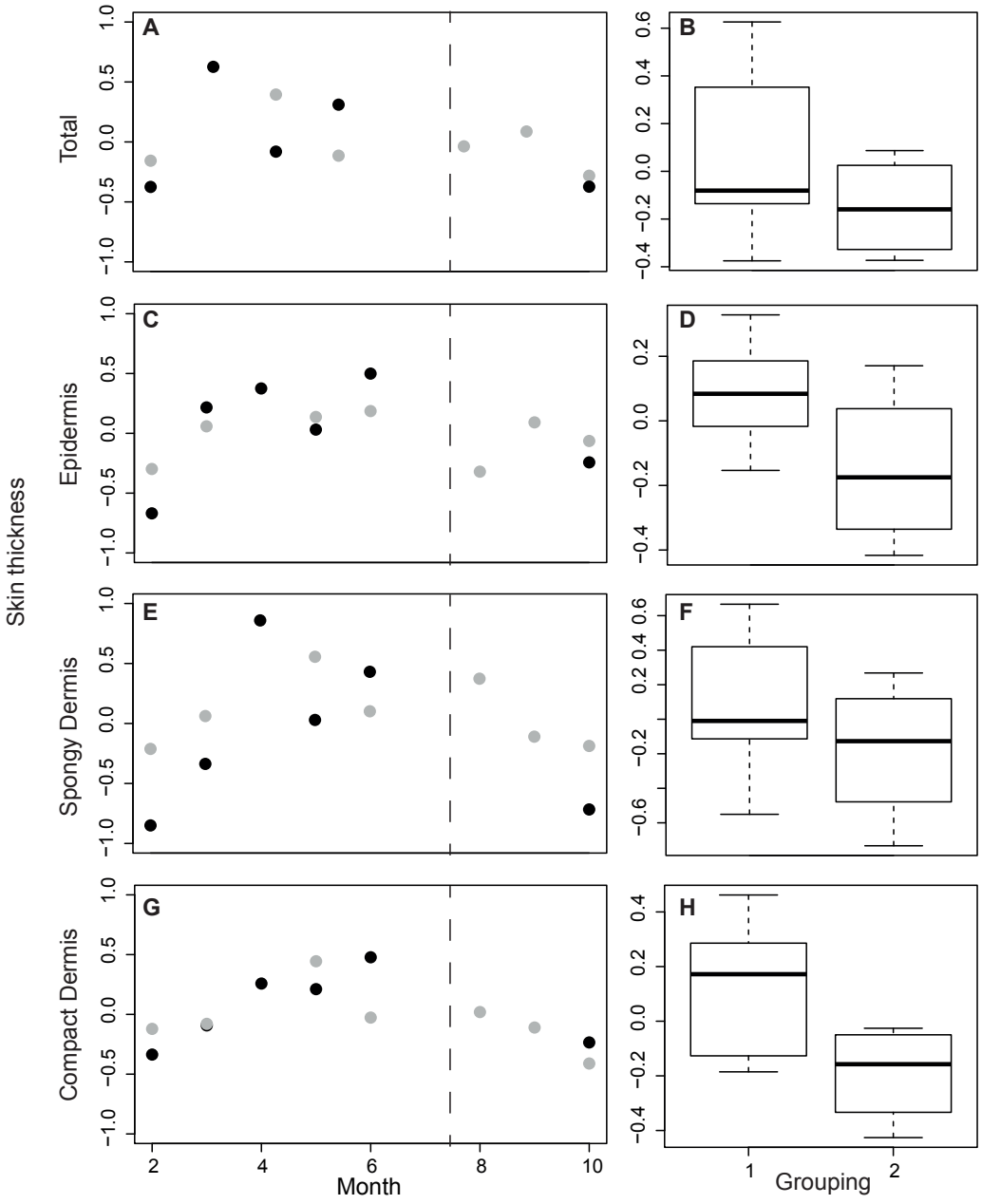


Figure 3-11. **Seasonal changes in ventral pectoral skin thickness in *Pseudacris crucifer.*** Plots of residual ventral pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *P. crucifer*. Symbols follow Figure 3-4.

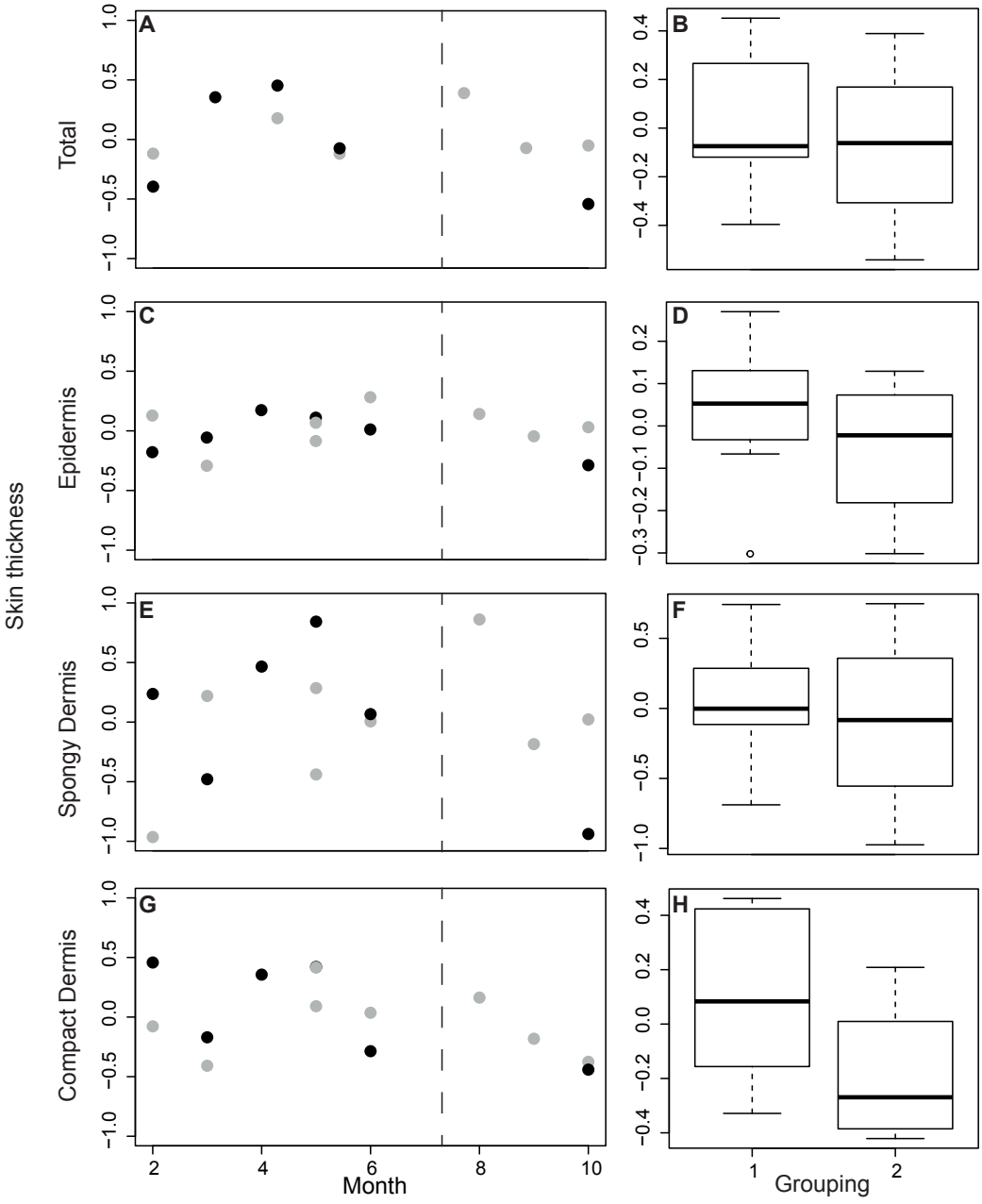


Figure 3-12. **Seasonal changes in ventral thigh skin thickness in *Pseudacris crucifer.*** Plots of residual ventral thigh skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for *P. crucifer*. Symbols follow Figure 3-4.

Table 3-3. Results (*p*-values) from ANOVAs comparing skin measurements from specimens collected before August and specimens collected after July.

Region	Layer	*Lithobates catesbeianus*	*Lithobates pipiens*	*Pseudacris crucifer*
Dorsal	Total	0.006	0.6	0.82
	Epidermis	0.007	0.43	0.76
	Spongy Dermis	0.008	0.25	0.94
	Compact Dermis	0.02	0.17	0.32
Ventral	Total	0.001	0.04	0.26
	Epidermis	0.001	0.13	0.09
	Spongy Dermis	0.005	0.39	0.32
	Compact Dermis	0.0006	0.04	0.08
Thigh	Total	0.001	0.02	0.61
	Epidermis	0.0006	0.04	0.48
	Spongy Dermis	0.003	0.01	0.66
	Compact Dermis	0.002	0.12	0.17

the ventral thigh regions (Figures 3-10, 3-11,3-10). There is no difference between males and females. The two measures of epidermis thickness were not correlated in any of the sampled skin regions (Table 3-2). Tukeys HSD posthoc tests revealed no significant differences between the two time bins for any skin layer of this species (Table 3-3).

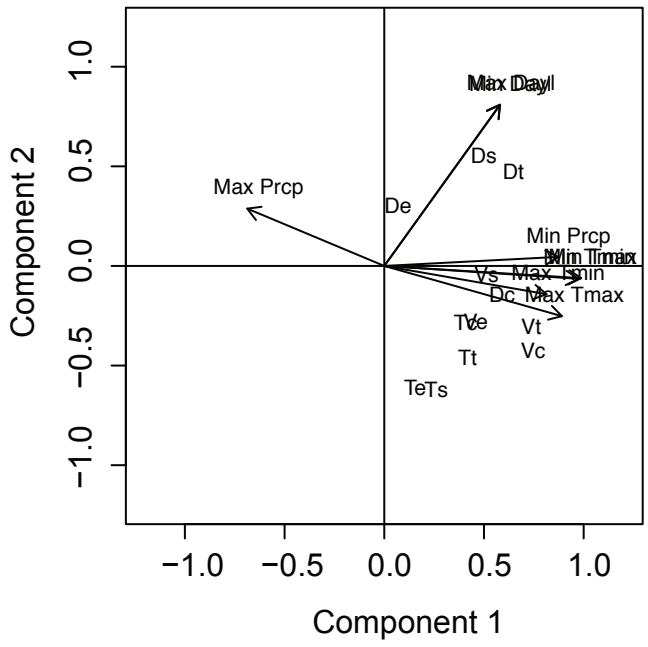
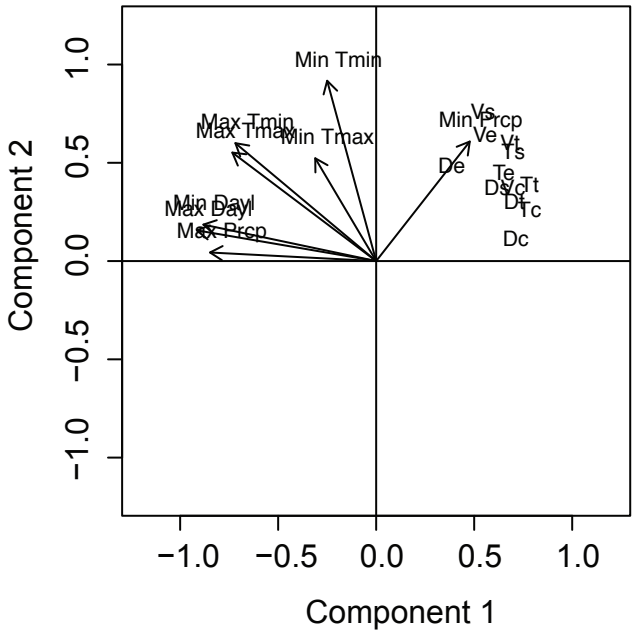


Figure 3-13. **Relationship between relative skin thickness and environmental variables in *Lithobates pipiens*.** Results from PLS regression using mean environmental values that recovered significant Q^2 values. D = Dorsal pectoral, V = Ventral pectoral, T = Ventral thigh, e = epidermis, s = spongy dermis, c = compact dermis, t = total



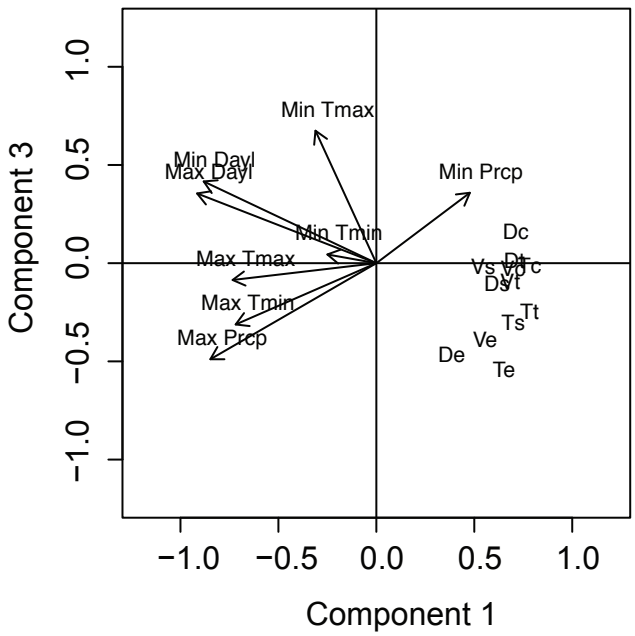


Figure 3-14. **Relationship between absolute skin thickness and environmental variables in *Lithobates catesbeianus*.** Results from PLS regression using maximum environmental values that recovered significant Q^2 values. Abbreviations follow figure 3-13.

Effects of environment

Using mean values of the environmental variables, only the relative skin thickness of *L. pipiens* recovered two important components with Q^2 values above zero (Table 3-4). This model summarised 85% of the variation in skin data between the two components. Variables that had VIP scores above one were maximum precipitation on the first component and maximum and minimum duration of daylight and maximum precipitation on the second (Table 3-4). Ventral pectoral total and compact dermis thickness loaded most strongly on the first component and ventral thigh total, epidermis, and spongy dermis loaded strongly on the second component along with dorsal pectoral total and spongy dermis thickness (Figure 3-12).

Using maximum values for the environmental variables produced more significant relationships (Table 3-5). For *L. catesbeianus*, both raw and size-corrected skin measurements yielded three important components (Table 3-5). Environmental variables summarised 90% of both sets of data (Table 3-5). VIP scores indicate that minimum and maximum duration of daylight and minimum and maximum precipitation are important variables for the raw values (Table 3-5), and skin variables load similarly on most axes (Figure 3-13). For the corrected skin values, minimum and maximum duration of daylight, maximum precipitation and maximum minimum temperature always had VIP values above one (Table 3-5). Again, skin variables loaded relatively similarly across the axes (Figure 3-14). For *L. pipiens*, the PLSR only recovered a significant component when raw skin values were used, and this component summarised 33% of the variation in skin data (Table 3-5). Minimum duration of daylight, minimum and maximum precipitation and minimum values for minimum and maximum precipitations had VIP values

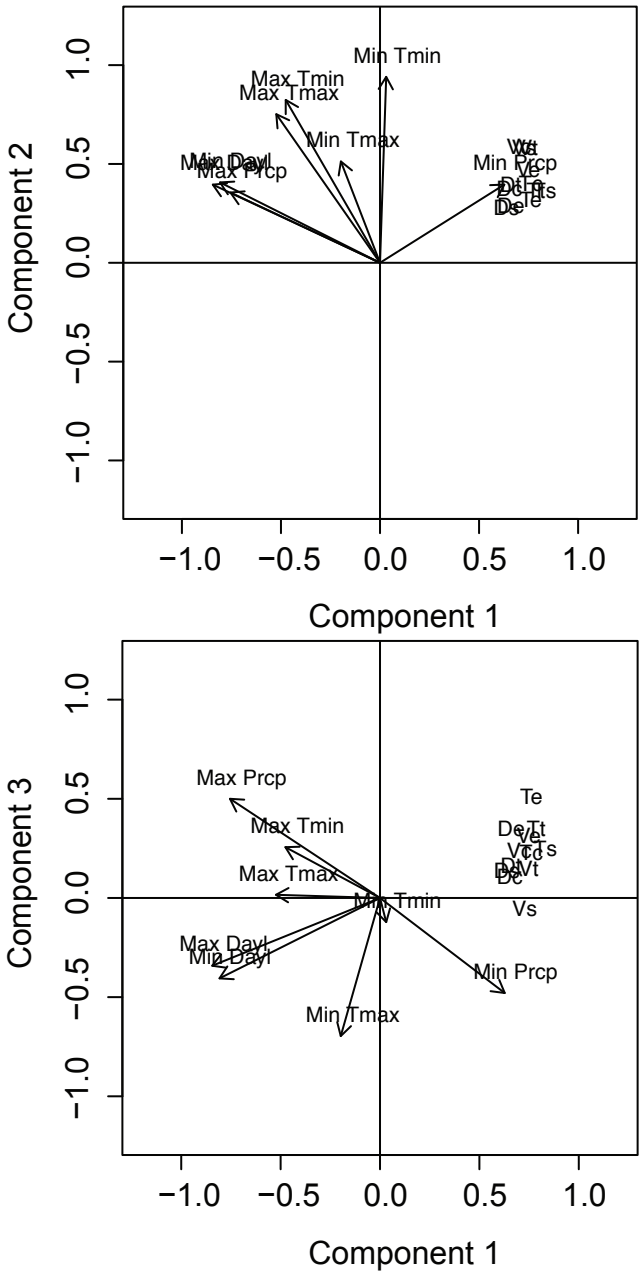


Figure 3-15. **Relationship between relative skin thickness and environmental variables in *Lithobates catesbeianus.*** Results from PLS regression using maximum environmental values that recovered significant Q^2 values. Abbreviations follow figure 3-13.

over one. Ventral pectoral and ventral thigh total thickness, spongy dermis thickness, and compact dermis along with dorsal pectoral compact dermis thickness loaded most strongly on this component. For *P. crucifer*, both raw and size-corrected skin values recovered one significant component that summarised 29% and 27% of the variation, respectively. Using the raw values, minimum and maximum duration of daylight and minimum values for minimum temperature had VIP values over one (Table 3-5). Ventral pectoral total thickness and spongy dermis thickness and compact dermis thickness for all three body regions loaded strongly on this component. Using the size-corrected values, the same environmental variables had VIP values above one (Table 3-5) and the same skin variables loaded most strongly on the component.

Table 3-4. Results from PLS regression using mean county-level environmental variables from the month before specimen collection showing the Variable of Importance (VIP) values and the variable loadings (Loadings) on each component (C) that had a Q^2 value above zero.

	L. pipiens (rel)		*P. crucifer* (raw)	*P. crucifer* (rel)
VIP	C1	C2	C1	C1
Min Daylight	0.47	1.29	1.29	1.47
Max Daylight	0.47	1.29	1.29	1.47
Min Prec	0.94	0.82	0.27	0.49
Max Prec	1.30	1.03	0.27	0.67
Min tmin	0.98	0.75	1.30	1.09
Max tmin	1.27	0.97	1.02	0.77
Min tmax	0.99	0.77	1.11	0.95
Max tmax	1.19	0.91	0.74	0.56
Loadings				
Dorsal Total	0.28	0.39	0.32	0.31
Dorsal Epidermis	0.02	0.25	0.19	0.27
Dorsal SD	0.21	0.46	0.26	0.24
Dorsal CD	0.25	-0.13	0.47	0.47
Ventral Total	0.32	-0.27	0.48	0.48
Ventral Epidermis	0.20	-0.24	0.17	0.43
Ventral SD	0.22	-0.04	0.52	0.52
Ventral CD	0.32	-0.37	0.46	0.46
Thigh Total	0.18	-0.40	0.32	0.34
Thigh Epidermis	0.06	-0.53	0.03	0.40
Thigh SD	0.11	-0.54	0.33	0.32
Thigh CD	0.17	-0.25	0.38	0.40
%Explained	0.66	0.19	0.39	0.35

Using minimum values for the environmental variables, only data for *P. crucifer* recovered important components (Table 3-6). Both the raw and size-corrected values recovered only one significant component that summarised 51% and 46% of the variation, respectively. Using both the raw and size-corrected values, minimum and maximum duration of daylight and minimum values for minimum temperature had VIP values above one (Table 3-6). Ventral pectoral total thickness and spongy dermis thickness and compact dermis thickness for all three body regions loaded strongly on the first component for both sets of data.

Table 3-5. Results from PLS regression using maximum county-level environmental variables from the month before specimen collection showing the Variable of Importance (VIP) values and the variable loadings (Loadings) on each component (C) that had a Q^2 value above zero.

	L.catesbeianus (raw)			*L. catesbeianus* (rel)			*L. pipiens* (raw)	*P. crucifer* (raw)	*P. crucifer* (rel)
VIP	C1	C2	C3	C1	C2	C3	C1	C1	C1
Min Dayl	1.50	1.22	1.19	1.46	1.27	1.24	1.02	1.48	1.63
Max Dayl	1.45	1.17	1.17	1.42	1.23	1.22	0.98	1.49	1.63
Min Prec	1.11	0.96	1.01	0.78	0.85	0.90	1.19	0.04	0.20
Max Prec	1.14	1.01	1.04	1.32	1.14	1.18	1.09	0.10	0.46
Min tmin	0.54	0.89	0.86	0.08	0.77	0.75	1.22	1.43	1.21
Max tmin	0.63	1.24	1.18	1.19	1.34	1.27	0.30	0.90	0.66
Min tmax	0.63	0.83	0.79	0.29	0.63	0.61	1.29	0.79	0.69
Max tmax	0.10	0.40	0.59	0.11	0.27	0.47	0.40	0.30	0.18
Loadings									
Dorsal Total	0.40	0.21	0.00	0.46	0.24	0.14	0.30	0.38	0.37
Dorsal Epidermis	0.39	0.42	-0.10	0.46	0.17	0.31	0.16	0.18	0.23
Dorsal SD	0.45	0.27	-0.23	0.44	0.16	0.11	0.18	0.32	0.31
Dorsal CD	0.21	0.34	-0.43	0.45	0.22	0.09	0.43	0.50	0.54
Ventral Total	0.39	0.42	-0.10	0.52	0.35	0.12	0.46	0.53	0.55
Ventral Epidermis	0.45	0.27	-0.23	0.52	0.28	0.27	0.31	0.21	0.39
Ventral SD	0.21	0.34	-0.43	0.51	0.35	-0.06	0.50	0.58	0.60
Ventral CD	0.31	0.45	-0.37	0.49	0.35	0.20	0.37	0.55	0.57
Thigh Total	0.45	0.27	-0.23	0.55	0.22	0.31	0.46	0.39	0.42
Thigh Epidermis	0.21	0.34	-0.43	0.53	0.19	0.45	0.21	0.11	0.42
Thigh SD	0.31	0.45	-0.37	0.58	0.21	0.21	0.37	0.39	0.41
Thigh CD	0.37	0.31	-0.50	0.53	0.24	0.20	0.43	0.47	0.48
%Explained	0.47	0.28	0.15	0.36	0.37	0.17	0.33	0.29	0.27

DISCUSSION

Seasonal skin thickening in three anurans studies

The three species we sampled overlap in large portions of their ranges and must survive very cold winters in at least a part of their range. All three species hibernate as a strategy to avoid the effects of harsh winters. Both species of *Lithobates* hibernate underwater after building up fat stores (Mizell, 1965; Byrne & White, 1975; Tattersall & Ultsch, 2008), whereas *Pseudacris crucifer* hibernates terrestrially and is able to survive being completely frozen for short periods of time (Layne Jr & Kefauver, 1997).

Table 3-6. Results from PLS regression using minimum county-level environmental variables from the month before specimen collection showing the Variable of Importance (VIP) values and the variable loadings (Loadings) on each component (C) that had a Q^2 value above zero.

	P. crucifer (raw)	*P. crucifer* (rel)
VIP	C1	C1
Min Daylight	1.13	1.33
Max Daylight	1.12	1.32
Min tmin	1.15	1.01
Max tmin	0.95	0.72
Min tmax	0.95	0.87
Max tmax	0.58	0.41
Loadings		
Dorsal Total	0.32	0.32
Dorsal Epidermis	0.14	0.23
Dorsal SD	0.23	0.23
Dorsal CD	0.48	0.47
Ventral Total	0.49	0.50
Ventral Epidermis	0.15	0.43
Ventral SD	0.49	0.49
Ventral CD	0.47	0.47
Thigh Total	0.28	0.30
Thigh Epidermis	-0.02	0.37
Thigh SD	0.28	0.25
Thigh CD	0.37	0.40
% Explained	0.51	0.46

Here, these species were examined to determine if they exhibit signs of seasonal skin thickening to help withstand unfavourable winter. *Lithobates catesbeianus* shows the strongest signs for this trait (Figures 3-4, 3-5, 3-6), and the

pattern of skin thickening closely mirrors that of the increase of fat body weight and liver weight as bullfrogs approach hibernation (Byrne & White, 1975). The presence of seasonal skin thickening in *L. pipiens* is slightly ambiguous compared to the results for *L. catesbeianus*, given that in the latter species, differences between the two times of year were found in all skin layers across the three body regions whereas they were only found in some measurements for *L. pipiens* (Table 3-2). In some skin regions (e.g. dorsal pectoral spongy dermis thickness), thickness decreases across the year, and although this difference isn't significant (Figures 3-7, 3-8, 3-9), it is the opposite pattern from what is observed in the skin layers that recovered significant differences between the earlier and later parts of the year (Table 3-2). The dataset for *L. pipiens* is heavily biased towards the later part of the year. Eight out of 12 specimens that were not found to show signs of disease or damage are from the month of August, which is the month at which the two groups were split. The unintentional unequal distribution of specimens in this dataset and the lack of consistent results across skin layers and body regions suggest that seasonal skin thickening in *L. pipiens* should be regarded as ambiguous at best.

The two species are sympatric and often utilise similar hibernation strategies (Tattersall & Ultsch, 2008). *L. pipiens* also increases the weight of its fat bodies in preparation for hibernation (Mizell, 1965). However, although their hibernation strategies are broadly similar, they do differ in their microhabitat use. Although both *Lithobates* species usually hibernate underwater, *L. catesbeianus* hibernates in shallow water near the shore where water has a higher oxygen concentration, whereas *L. pipiens* sometimes hibernates in water over 3 metres deep in more anoxic water (Tattersall & Ultsch, 2008). *Lithobates pipiens* is also known to infrequently hibernate terrestrially in caves (Tattersall & Ultsch, 2008). These differences make it difficult to predict if the same pattern of seasonal skin thickening should be expected in both species based on their ecology. No other species of *Lithobates* have been studied for seasonal skin thickening, so the evolution of this trait is also unknown. *Pseudacris crucifer*, however, overwinters terrestrially (Layne Jr & Kefauver, 1997), is much smaller in body size, and belongs to a different family than the other two species. Therefore, the lack of seasonal skin thickening in this species indicates that this trait should not be expected in all amphibian species that experience marked seasonality.

Environmental effects

To determine if any environmental patterns drive seasonal skin thickening, partial least squares regression was used to test for relationships between duration of daylight, precipitation, and minimum and maximum temperatures for the month before each specimen was collected. Mean values for environmental variables found strong relationships with relative skin thickness measures for *L. pipiens* and explained 85% of the variation (Table 3-4). Of the variables that were found to differ significantly between times of the year, ventral pectoral total and compact dermis thickness loaded highly on the axis that was determined by maximum values for precipitation, minimum temperature, and maximum temperature. The ventral thigh measurements (except compact dermis thickness) all loaded highly on the component that was determined by minimum and maximum values for duration of daylight and maximum precipitation (Figure 3-11). That the same environmental variables do not predict skin thickness in the ventral pectoral and ventral thigh regions further implies that seasonal skin thickening detected in those regions may be a sampling error instead of a true pattern. Otherwise, seasonal changes in skin thickness between these two regions are driven largely by different variables.

Maximum values explained 90% of the variation in the dataset for *L. catesbeianus* (Table 3-5). Minimum and maximum duration of daylight and maximum precipitation consistently determined the components. Maximum values for minimum temperature also determined the second and third component of the analysis using raw skin data and all components of the analysis using size-corrected skin data. Only on the third component are there obvious differences among the loadings of the skin variables, and these components only summarise 15% of the variation using raw skin data and 17% using size-corrected skin data. These results clearly indicate that seasonal skin thickening is correlated with decreases in the duration of daylight and minimum temperature and increases in maximum precipitation. However, these changes would be expected as seasons change, so the results here cannot test a causal relationship between skin thickening and changes in environmental variables. Furthermore, although PLS regressions are robust to datasets with a relatively small number of observations compared to variables, the limits of this property of this regression have not been assessed in biological systems and should be explored in future work with higher sample sizes and rarefaction analyses.

Potential effects of chytrid infection and specimen preservation on seasonal skin thickening

The fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) has been detected in museum specimens collected as far back as the late 1800's (Talley et al., 2015). It is therefore impossible to use our sample to determine which responses are typical and which might be induced by *Bd* infection. Although *Bd* is known to cause mortality in some North American amphibians, all three of the species sampled exhibit resistance to the pathogen (Gahl, Longcore & Houlahan, 2012). Specimens that showed obvious signs of disease were removed because of their unknown effects on seasonal skin thickening, but it is possible that some of the specimens were positive for *Bd* or other diseases that did not manifest obvious signs in the body regions sampled. Despite the potential effect diseases might have on the observed seasonal patterns, the clear and statistically significant pattern in *Lithobates catesbeianus* suggests that, unless *Bd* or other infections are shown to supress seasonal skin thickening in future studies of these or other species, this pattern can be identified using potentially infected animals. However, the number of *L.pipiens* specimens that needed to be removed and the potential effects that might have had on the results indicate that sampling efforts should be high for species that exist in areas where *Bd* has been detected.

The effects of specimen preservation on seasonal skin thickening were also examined (Table 3-1). There was no evidence that the length of time a specimen had been preserved or the conditions under which it had been preserved (using the institution as a proxy) influenced skin thickness. Only animals that were fixed in formalin before being stored in alcohol were selected, so it is possible that alcohol preservation might affect skin thickness measures. Furthermore, no fresh samples were used, so it is unclear how the results for *Lithobates catesbeianus* would compare to those collected from recently sacrificed individuals. However, given that there was no apparent effect of preservation, it is likely that the results reflect the true pattern of skin thickening in these species.

Seasonal skin thickening in Anura

Seasonal skin thickening has previously only been documented in two anuran species that differ geographically, ecologically, and phylogenetically, and one caudatan. The common toad (*Bufo bufo*), and the smooth newt (*Lissotriton vulgaris*), have thinner skin in the breeding season than in the non-breeding season (Czopek, 1959; Kun,

1959), and the reed frog (*Hyperolius nitidulus*), has thicker skin in the dry season than in the wet season (Kobelt & Linsenmair, 1986). In all of these studies, skin thickness was compared between two (Czopek, 1959; Kobelt & Linsenmair, 1986) or three (Kun, 1959) sampling periods taken over the year, so they do not document changes between sampling times. Here, seasonal skin thickening was detected in *Lithobates catesbeianus,* is ambiguous in *L. pipiens,* and not detected in *P. crucifer*. Therefore, among anurans, seasonal skin thickening has been confirmed in one bufonid, one hyperoliid, was not detected in one hylid, and remains ambiguous in ranids. This study is the first to test for seasonal skin thickening in an anuran and find negative results, demonstrating that this strategy is *not* ubiquitous for all anurans or all amphibians or even for all species that experience high seasonality within their native ranges.

The function of seasonal skin thickening has never been tested, and neither has the impact of skin thickness on skin physiological function. Drewes et al. (1977) examined the skin of over 50 species of anurans and found that, although there was some variation in skin anatomy among the species they examined, all were similar to that which had been previously described for the genus *Rana*. They did not disclose which species had been studied other than *Chiromantis petersii* so it is unclear how ecologically or phylogenetically broad their sample was.

However, there are other studies that suggest a functional link exists and it has been hypothesised by other authors (Czopek, 1965; Roth, 1973). The Cururu toad (*Rhinella schneideri*) inhabits both xeric and mesic habitats in its range. The skin of toads collected in xeric habitats is smoother (i.e., has fewer vercuae) and half as thick compared to that of toads collected in more mesic habitats (Navas, Antoniazzi & Jared, 2004). In the African genera *Phrynobatrachus* and *Ptychadena*, there appears to be a relationship between skin thickness and habitat types (Le Quang Trong, 1971, 1975). In *Phrynobatrachus*, skin thickness and body size seem to co-vary with habitat type (Le Quang Trong, 1971). In *Ptychadena*, simple ratios of skin thickness divided by body size suggest that species that live in drier habitats (e.g., savannah) have relatively thicker skin than species that live in forests (Le Quang Trong, 1975). This result contradicts the patterns observed in *Rhinella schneideri* in which the skin is thinnest in drier habitats (Navas, Antoniazzi & Jared, 2004), but these data were produced using ratios, which are known to be influenced by allometry (Albrecht, 1978). It should also be noted that many studies on amphibian have ignored other

factors known to affect skin anatomy, such as sex (Greven, Zanger & Schwinger, 1995; Wenying et al., 2011; Lili, Chuan & Shulan, 2013), seasonality (Kun, 1959; Kobelt & Linsenmair, 1986), body size (Chapter 2), and body region (Greven, Zanger & Schwinger, 1995) and should thus be viewed with caution.

Seasonal changes in skin thickness also suggest a function for differences in skin thickness. In *Hyperolius nitidulus*, the skin is thicker in the dry season, and skin thickening in this species occurs due to an increase in the size and number of iridiophores in the spongy dermis that efficiently limit evaporative water loss (Geise & Linsenmair, 1986; Kobelt & Linsenmair, 1986). However, the skin of *Lithobates catesbeianus* is thinner when the species is more terrestrial during the breeding season compared to when it hibernates underwater (Tattersall & Ultsch, 2008) and its skin thickens as the maximum precipitation increases. *Bufo bufo* is terrestrial throughout the year except when it breeds in the early spring and this species also has thinner skin in summer than when it hibernates either in burrows or under leaf litter (Sinsch, 1988). These latter two species are relatively large in body size, so skin thickening might be correlated with respiration, temperature regulation, or water balance when they are more active or exposed. However, so few species have been examined for seasonal changes in skin thickening that it is difficult to hypothesise the function of this trait and why this pattern is not observed in *Pseudacris crucifer* or if it should be expected in *L. pipiens*. More quantitative data on the relationship between skin thickness and ecology or physiology would be useful for determining these potential functions.

Seasonal skin thickening remains an enigmatic trait in amphibians. Here, seasonal skin thickening was found in *Lithobates catesbeianus*, as well as the absence of seasonal skin thickening in *Pseudacris crucifer*. That not all species sampled here display seasonal changes in skin thickness highlights an unrecognised complexity of the factors that drive these changes. The results show that there is no simple cause-and-effect scenario among amphibians and that different strategies may be employed by relatively closely related species inhabiting the same habitat. It furthermore suggests that there may be environmental cues that signal these changes in skin thickness, but these cues need to be verified by future experimental work. Although currently poorly understood, the importance of future work to determine the phylogenetic distribution and ecological significance of seasonal skin thickening to better predict its role in niche partitioning and environmental sensitivity among

amphibians is stressed; this work also highlights the broader impact that future work will have in relation to disentangling the ambiguous results from historic studies on the relationship between skin thickness and ecology to improve knowledge about the ecomorphology of this organ.

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CHAPTER FOUR: TESTING THE EFFECTS OF PROLONGED PRESERVATION ON DETECTING SEASONAL SKIN THICKENING PATTERNS IN THE AMERICAN BULLFROG (*LITHOBATES CATESBEIANUS*).

ABSTRACT

Natural history collections contain a wealth of information about biodiversity, but specimen preservation can damage tissues and limit the utility of museum specimens for certain analyses. Although the effects of preservation methods on some attributes, such as DNA, have been well studied, they have not been studied in detail for many soft tissue structures. Amphibian skin is of interest because of its physiological function and potential value in the assessment of global amphibian population declines. Some anurans thicken their skin seasonally as a likely adaptation to avoid harsher environmental conditions. Museum specimens of the American bullfrog (*Lithobates catesbeianus*) collected within the last 30 years have been used to detect seasonal skin thickening in this species. In this chapter, specimens of *L. catesbeianus* collected in the 1930's and 1940's were sampled to test whether seasonal patterns of skin thickening are still detectable. When using only the older specimens, fewer significant differences in relative skin thickness were obtained between times of the year than were obtained either in a previous study or a combined dataset of historically and recently collected specimens. Although length of preservation time did not have a statistically significant affect on skin thickness, the pattern of skin thickening was less apparent in the historic dataset using both quantitative and qualitative comparisons. The historic dataset contains a high proportion of juvenile samples, so it is unclear if the length of time the specimens have been preserved or their biological age at the point of fixation had a more significant impact on the results. However, given that measurable differences in skin thickness were recovered, these results suggest that historically collected specimens may contain data that are useful for detecting seasonal skin thickening in anuran amphibians.

INTRODUCTION

Natural history collections are an invaluable source of data about events that have impacted our planet and shaped past and current biodiversity that would have otherwise been lost. Specimens in such collections have been used to address many questions related to biodiversity dynamics, including on population genetics

(Wandeler, Hoeck & Keller, 2007), taxonomy (Helgen et al., 2013), historic population size baselines (Leonard, Vila & Wayne, 2005), disease epidemics (Talley et al., 2015), and effects of climate change (Tingley & Monahan, 2009). Although specimens in these collections are preserved in order to mitigate the processes of decay, they may degrade over time, thus limiting or progressively diminishing the amount and quality of data that can be extracted from them for scientific research (Wandeler et al., 2003). Determining the long-term effects of preservation on museum specimens is therefore key to their utilisation for assessing impacts of various processes on animal populations over time. Preservation effects on morphology have received much less attention than effects on DNA (Wandeler et al., 2003), yet morphological analyses of museum specimens has the potential to be used to elucidate historic patterns of organismal responses to environmental change that are not otherwise observable (Babin-Fenske, Anand & Alarie, 2008).

More species of amphibians are currently threatened with extinction than those of mammals, reptiles, or birds. Living amphibians are thought to be more vulnerable to environmental changes than other groups of terrestrial vertebrates (Wake & Vredenburg, 2008) and 2,023 of the over 7,000 species are currently threatened with extinction (http://www.amphibiaweb.org/). Museum specimens have been crucial for documenting the emergence and spread one of the greatest threats to amphibian species, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) (e.g. Berger et al., 1998; Ouellet et al., 2005; Lips et al., 2006). Amphibian population declines in Central and South America as well as Australia were enigmatic until the discovery of *Bd* and the documentation of its pathology (Berger et al., 1998). Specimens collected from affected regions (Lips et al., 2006) and natural history data documenting the geographic distribution of the wave of species disappearances or population declines were used to generate the first hypothesis for the origin of *Bd* in the Americas and its subsequent migration following its proposed introduction to California, USA, in the 1960's. Further findings of the presence of *Bd* before 1900 in Illinois, USA using specimens housed in natural history collections (Talley et al., 2015) complicate our prior understanding of both the geography and timing of the emergence of this pathogen; nevertheless, *Bd* provides a relevant example of the utility of museum specimens in the understanding the history of biological phenomena.

Seasonal skin thickening in amphibians is a poorly understood trait documented in only a few species (Kun, 1959; Kobelt & Linsenmair, 1986), but

museum specimens can be useful for better understanding its function and phylogenetic distribution (Chapter 3). However, it is unclear if unknown effects of prolonged museum preservation are likely to have an impact on the detection of seasonal variation in skin thickness. If this pattern can be detected reliably in historic collected museum specimens, then they can be used to study any changes in seasonal skin thickening in relation to climate change or chytrid outbreaks and determine the degree of connection between skin thickness and a range of potential ecological stressors.

Recently collected specimens (1985–2016) of the American bullfrog (*Lithobates catesbeianus*) were used to reconstruct seasonal patterns of skin thickening in the epidermis, spongy dermis, and compact dermis of three sampled skin regions (Chapter 3). No effect of preservation was found when comparing specimens collected throughout this range of dates (Chapter 3), but it is unclear if specimens collected more historically can still be used to recover patterns of seasonal changes in skin thickness. In this chapter, specimens from the 1930's and 1940's have been sampled and examined to test whether the pattern of seasonal skin thickening observed in recently collected specimens of *Lithobates catesbeianus* can be detected in historically collected museum specimens or if prolonged preservation leads to deterioration that obscures this pattern.

MATERIALS AND METHODS

Eight specimens of *Lithobates catesbeianus* collected in the 1930's and 1940's were sampled from collections at the Field Museum of Natural History (FMNH) in Chicago, IL, USA. Of these, one is male, two are female, and five are unsexed juveniles. Although the number of juveniles is high, Chapter 3 of this thesis found no obvious difference in skin thickness between adults and juveniles in more recently collected specimens of *L. pipiens*. All specimens were originally collected in the Midwestern United States (Arkansas, Illinois, Indiana, or Missouri) and were sampled in 2016. All of the specimens were fixed in formalin and preserved in ethanol, so the effects of variation in preservation technique unlikely to affect the results. Sampling methods, including histological preparation and morphometric data, are identical to those described in Chapter 3. Comparative skin thickness and snout-vent length data from 11 more recently collected (1985–2016) individuals of *L. catesbeianus* were used from Chapter 3 (recent dataset).

Ordinary least squares regressions were used to create residuals for 1) only the historically collected specimens; and 2) only the recently collected specimens (i.e. to recreate the dataset from Chapter 3); and 3) all specimens using log-transformed skin thickness measurements and snout-vent length. Residuals from the regressions of the historic and recent datasets separately and from the combined dataset were then plotted against month of collection to determine if differences exist between older and more recently collected specimens. The patterns of skin thickening were compared to those from the dataset of recently collected specimens from Chapter 3. Unfortunately, the skin data could not be compared to environmental data as in Chapter 3 because environmental data are not available for the time these specimens were collected.

Effects of preservation were examined by running a Spearman Rank Correlation test between skin thickness and year of collection. Snout-vent length and year of collection were significantly correlated ($p < 0.05$) due to the relatively high number of juvenile specimens from the older dataset, so residuals from an ordinary least squares regression of log-transformed skin thickness measurements and log-transformed snout vent length were used rather than raw data to remove the influence of body size on the correlation test. Finally, all specimens from both datasets were grouped into those collected from March–June (group 1), July–August (group 2) and September–November (group 3) and between-group differences were tested for using an ANOVA and a Tukey's HSD posthoc test. The same test was performed on only the older dataset, as well, but due to the small sample size of that dataset, the samples could only be divided into two time bins: March–July and August–October. All analyses were performed in R v. 2.1 (R Development Core Team, 2014).

RESULTS

Effects of preservation

Total skin thickness did not significantly correlate with year of collection in the dorsal pectoral ($p = 0.08$), ventral pectoral ($p = 0.31$) or ventral thigh ($p = 0.14$) body regions. T-tests comparing total skin thickness between the older specimens and those from Chapter 3 again found no significant differences between the dorsal pectoral ($p = 0.09$), ventral pectoral ($p = 0.39$), and ventral thigh ($p = 0.18$) body regions (Figure 4-7).

Seasonal skin thickening

In the older dataset, the skin of the specimens from August, September and October appears thicker than that of specimens collected earlier in the year. This pattern is most clear in the dorsal pectoral region, and this region shows the most significant differences between the two time bins, with significant differences recovered in the total thickness, spongy dermis, and compact dermis (Table 4-1). The pattern is less clear in the ventral pectoral and ventral thigh regions, as only the ventral pectoral compact dermis thickness and ventral thigh total thickness differed between the two time bins (Table 4-1). The ventral pectoral total skin thickness, ventral pectoral spongy dermis thickness, and ventral thigh epidermis thickness were significantly different at the $\alpha = 0.1$ level ($p = 0.06$ and 0.07, respectively; Table 4-1). There are many fewer significant differences recovered compared with Chapter 3.

Table 4-1. Seasonal differences in the historic dataset between the two time periods.

Region	Layer	*p*-value
Dorsal	Total	0.038
	Epidermis	0.11
	Spongy Dermis	0.015
	Compact Dermis	0.046
Ventral	Total	0.055
	Epidermis	0.11
	Spongy Dermis	0.5
	Compact Dermis	0.008
Thigh	Total	0.038
	Epidermis	0.07
	Spongy Dermis	0.37
	Compact Dermis	0.19

The regressions of skin thickness to body size differ among the two datasets and the combined dataset (Figures 4-1 to 4-6), which appears to affect the reconstructed pattern of skin thickening. When different regressions are used for the two datasets separately to produce residuals, the pattern of skin thickening shows a great deal of overlap. However, when the datasets are combined and a single regression line is used for all specimens, the older specimens collected in the autumn and winter months have relatively thinner skin than specimens collected in more recent years. This difference is less evident in specimens from the spring and summer months.

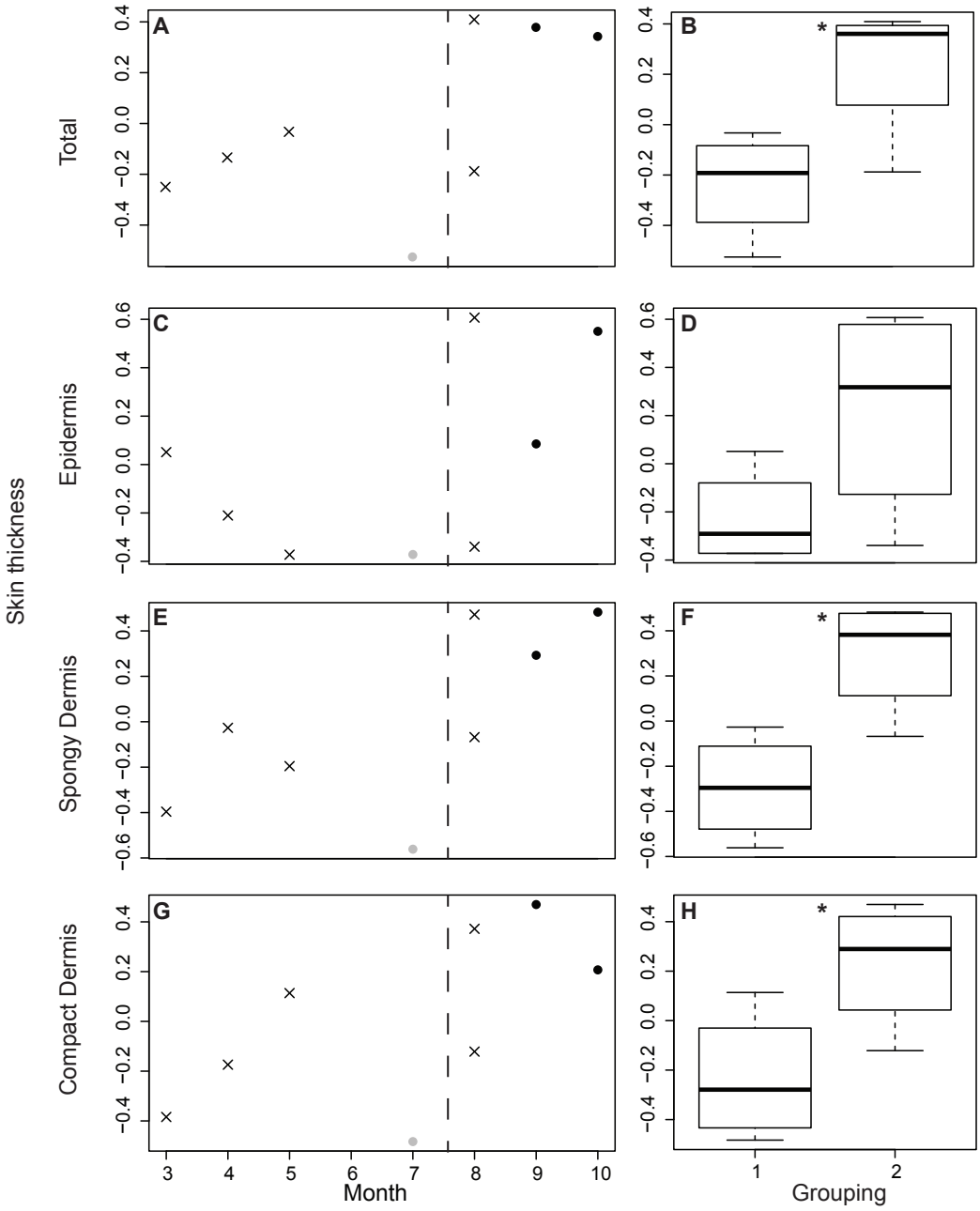

Figure 4-1. **Seasonal changes in dorsal pectoral skin thickness in historic specimens of *Lithobates catesbeianus.*** Plots of relative dorsal pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for historic specimens. Symbols follow Figure 3-4.

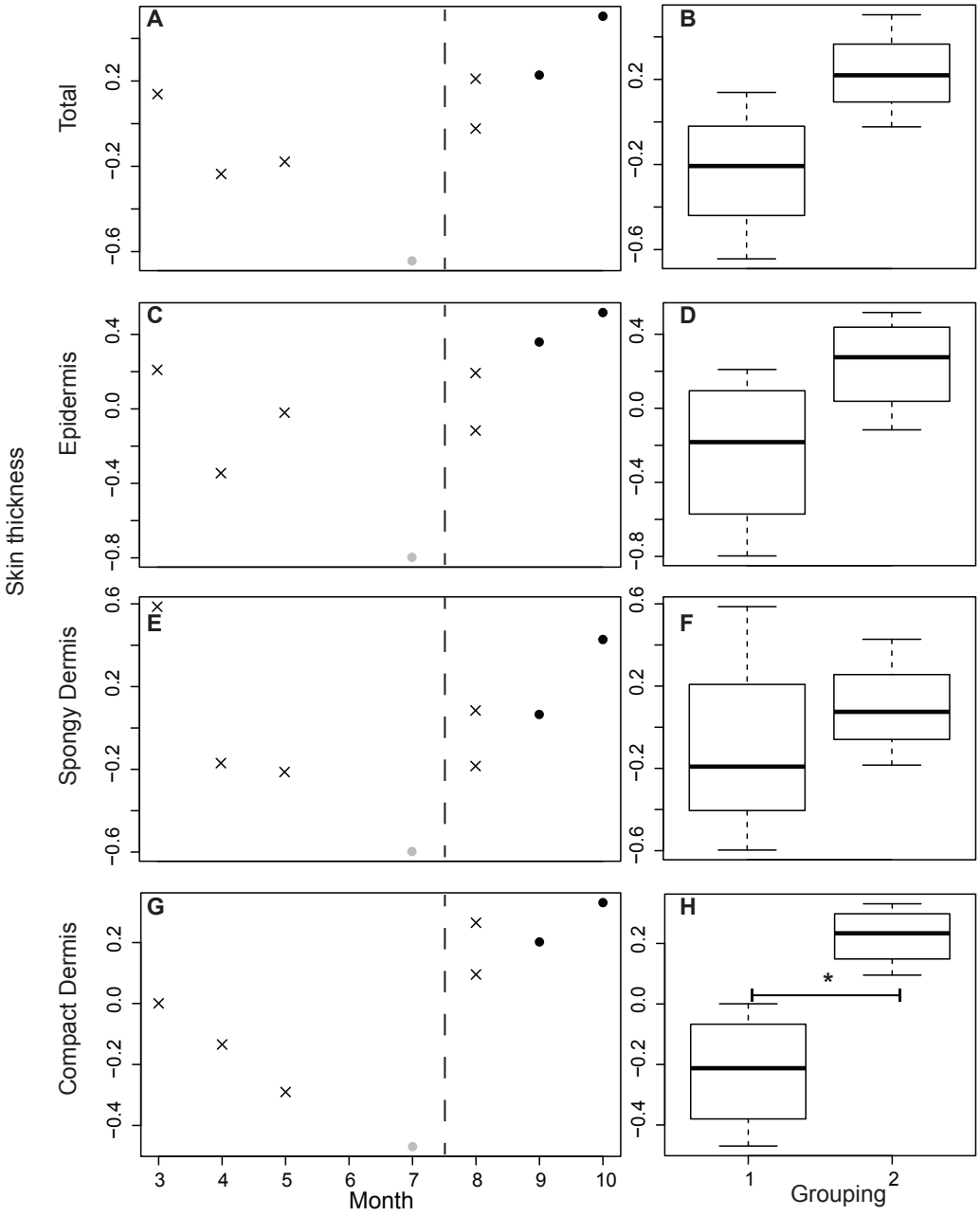

Figure 4-2. **Seasonal changes in ventral pectoral skin thickness in historic specimens of *Lithobates catesbeianus.*** Plots of relative ventral pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for historic specimens. Symbols follow Figure 3-4.

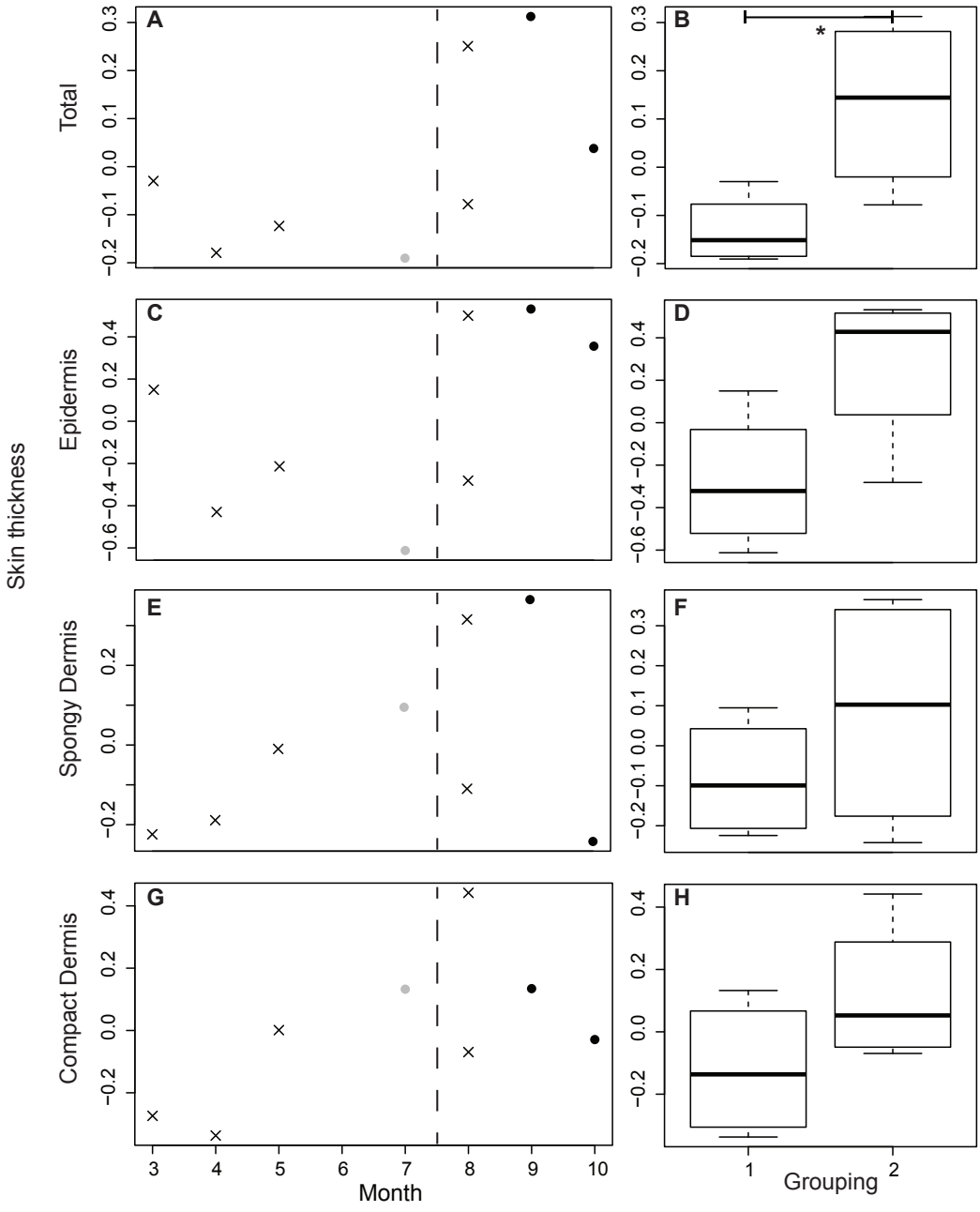

Figure 4-3. **Seasonal changes in ventral thigh skin thickness in historic specimens of *Lithobates catesbeianus.*** Plots of relative ventral thigh skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens from different halves of the year (B, D, F, H) for historic specimens. Symbols follow Figure 3-4.

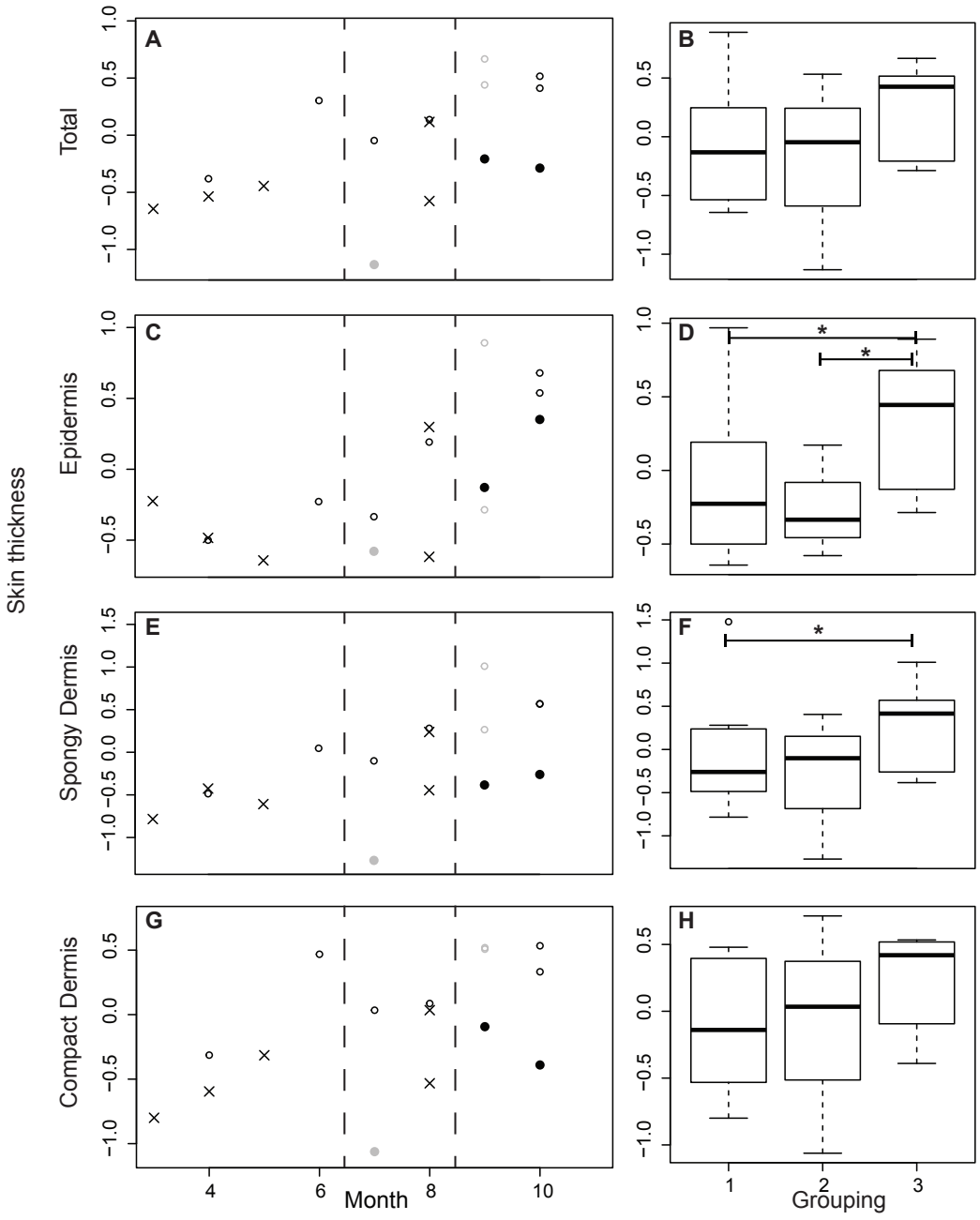

Figure 4-4. **Seasonal changes in dorsal pectoral skin thickness in the combined dataset of *Lithobates catesbeianus.*** Plots of relative dorsal pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens between three time slices (B, D, F, H) for the combined dataset. Symbols follow Figure 3-4.

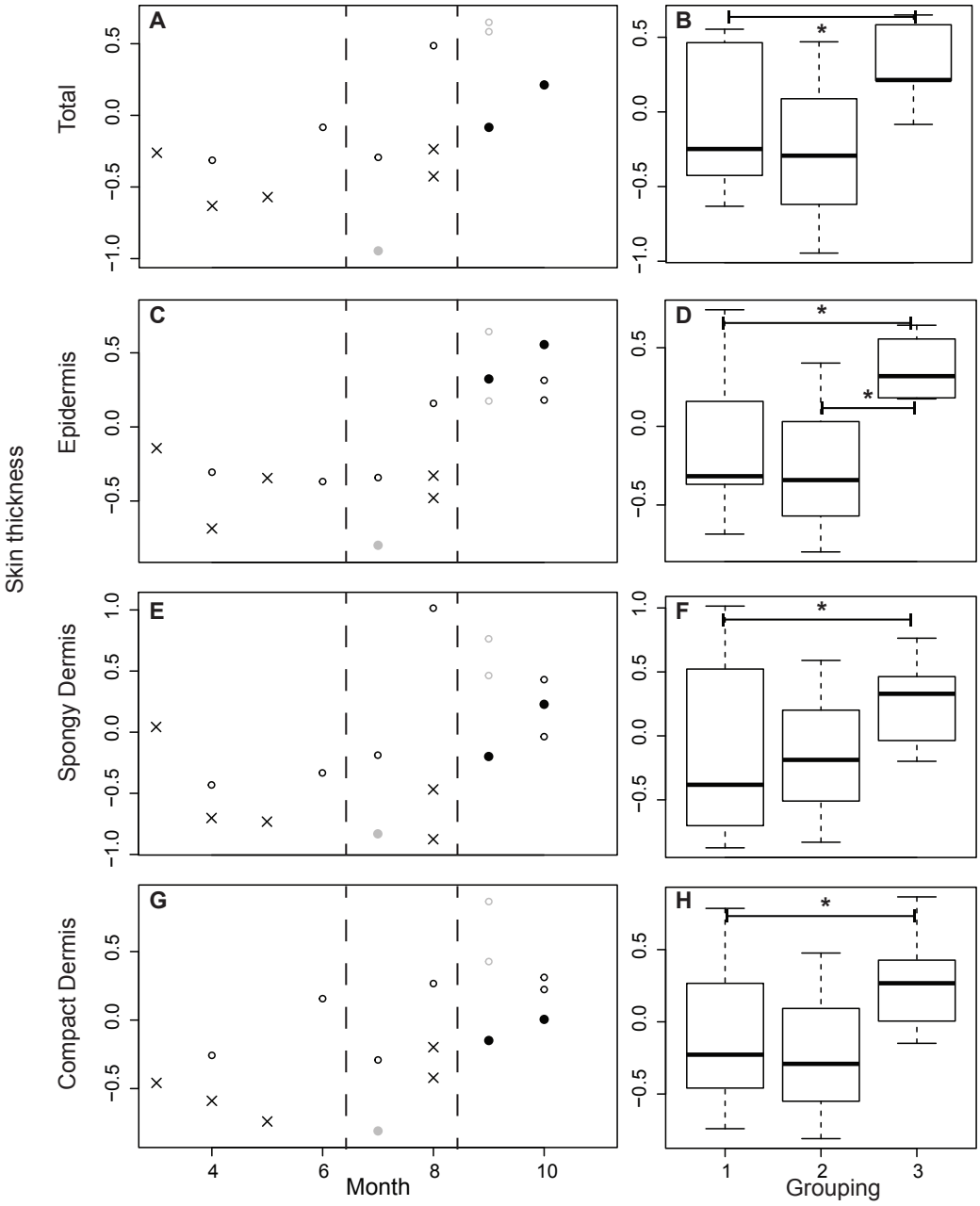

Figure 4-5. **Seasonal changes in ventral pectoral skin thickness in the combined dataset of *Lithobates catesbeianus.*** Plots of relative ventral pectoral skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens between three time slices (B, D, F, H) for the combined dataset. Symbols follow Figure 3-4.

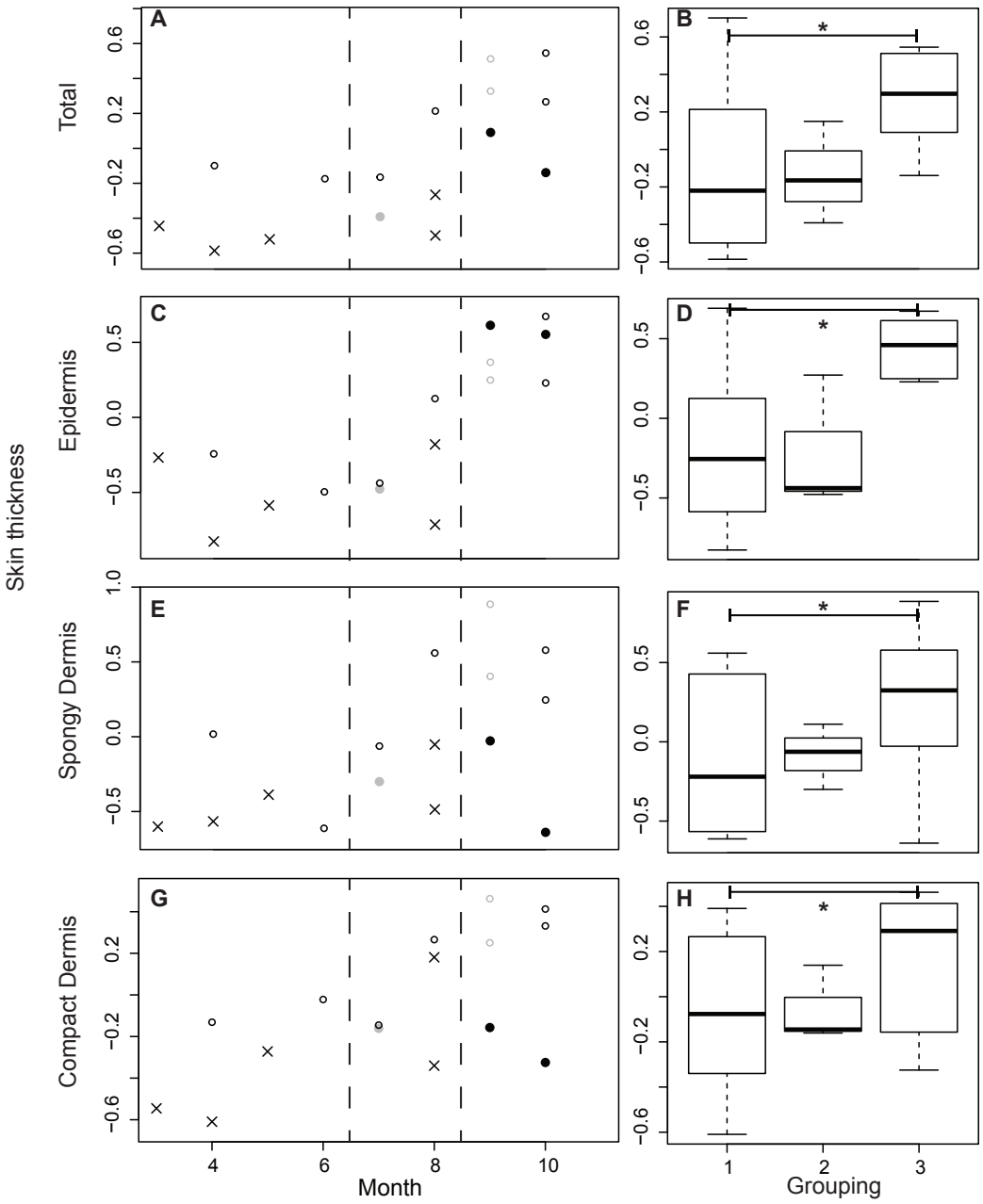

Figure 4-6. **Seasonal changes in ventral thigh skin thickness in the combined dataset of *Lithobates catesbeianus.*** Plots of relative ventral thigh skin thickness against month of the year (A, C, E, G) and box plots ranging from the first to third quartile and whiskers extending to a maximum of 1.5 times the interquartile range past the first and third quartile comparing specimens between three time slices (B, D, F, H) for the combined dataset. Symbols follow Figure 3-4.

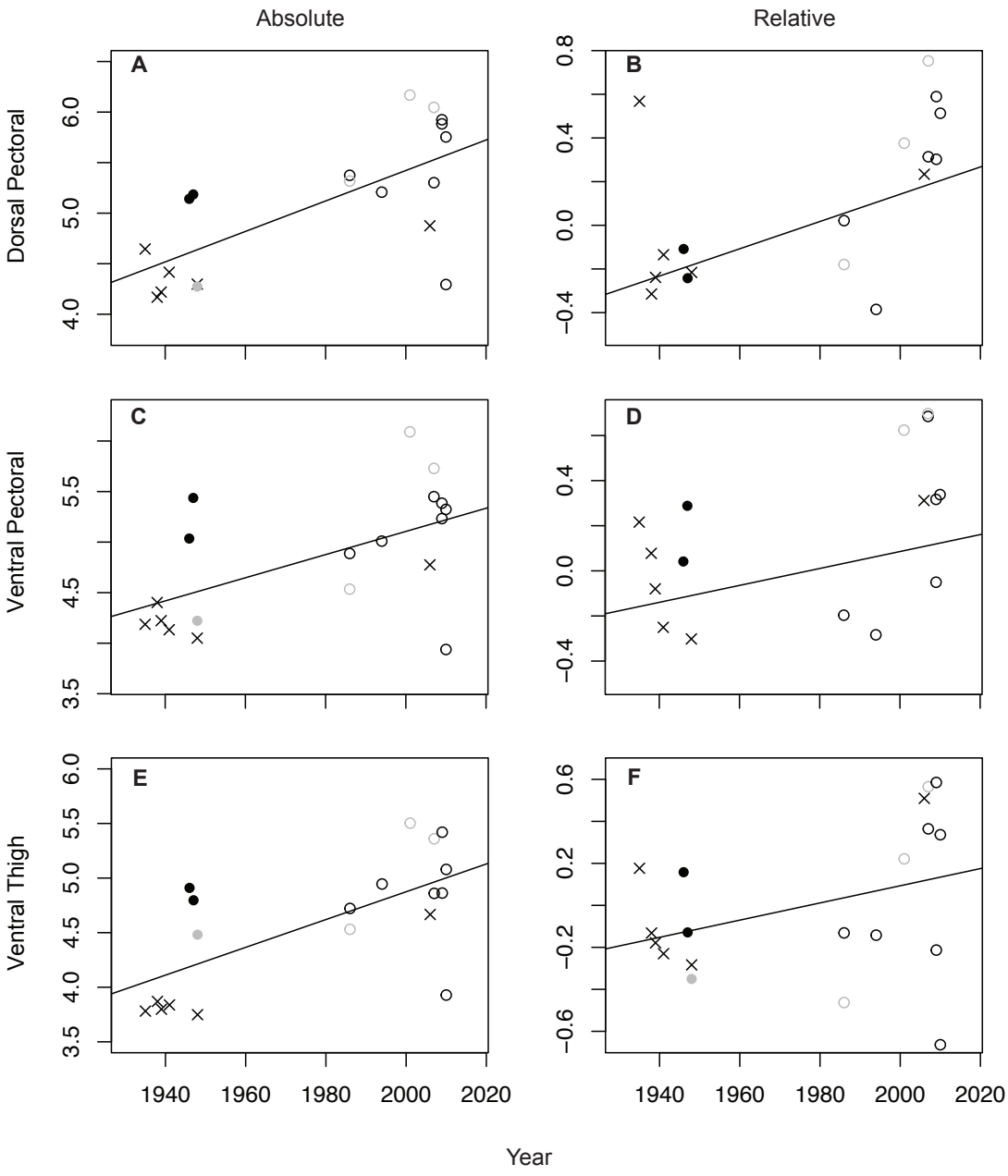

Figure 4-7. **Skin thickness against year of collection.** Plots of skin thickness against year of collection for the dorsal pectoral (A, B), ventral pectoral (C, D), and ventral thigh (E, F) body regions. Skin thickness is compared using log-transformed absolute thickness (A, C, E) and residuals from an ordinary least squares regression of log-transformed skin thickness against log-transformed SVL (B, D, F).

Using the combined dataset, there are significant differences in skin thickness between the first group (March–June) and the third group (September–November) for all variables except dorsal pectoral total thickness ($p = 0.08$) and dorsal pectoral compact dermis thickness ($p = 0.31$; Table 4-2). Significant differences were also found between the second (July and August) and third group in the dorsal pectoral epidermis thickness and ventral pectoral epidermis thickness (Table 4-2). All other comparisons were not significantly different.

DISCUSSION

Seasonal variation in skin thickness, in which the skin is thicker in the winter or dry season, has been documented in the American bullfrog (*Lithobates catesbeianus*) using recently collected specimens obtained from museum collections (Chapter 3). Historically collected specimens of this species were sampled to test if this pattern is detectable in specimens that had experienced prolonged preservation and also if preservation had a statistically significant effect on skin thickness.

Table 4-2. Results (*p*-values) of ANOVA comparing the three time periods in the year (1 = March–June, 2 = July–August, and 3 = September–November) using the combined dataset; significant results are shaded.

Region	Layer	1 vs 2	2 vs 3	1 vs 3
Dorsal	Total	0.79	0.32	0.08
	Epidermis	0.39	0.04	0.001
	Spongy Dermis	0.7	0.25	0.04
	Compact Dermis	0.8	0.74	0.31
Ventral	Total	0.39	0.17	0.007
	Epidermis	0.53	0.0002	0.005
	Spongy Dermis	0.37	0.45	0.03
	Compact Dermis	0.37	0.32	0.02
Thigh	Total	0.16	0.07	0.0006
	Epidermis	0.19	0.006	<<0.001
	Spongy Dermis	0.12	0.52	0.007
	Compact Dermis	0.08	0.93	0.02

Overall, it does not appear that the duration of preservation had a significant effect on the ability to detect seasonal skin thickening. The results show that the length of time a specimen was preserved did not have a significant effect on skin

thickness that might hinder the results. However, both the correlation test ($p = 0.08$) of relative dorsal pectoral skin thickness and year of collection and the t-test comparing historic and recent specimens for the same skin measurement ($p = 0.09$) had low p-values approaching the threshold for significance ($\alpha = 0.05$). Because these values are still not below the threshold, however, the results suggest that preservation does not have a significant effect on the thickness of amphibian skin. This result should be verified with future studies using larger datasets that cover a greater range of dates because, although the tests here did not detect a significant effect of preservation, the effect of preservation, if it exists, might act over longer periods of time than what was covered here or produce differences that were not detected statistically.

When seasonal skin thickening was compared quantitatively, fewer significant differences were recovered between the two time bins (March–July and August–October) using the older dataset than were recovered using the data Chapter 3 or the combined dataset. These differences may be because the sample size was too small ($n = 8$) to detect seasonal skin thickening statistically or that the specimens did not cover a wide enough range of months in the year, although the plots of relative skin thickness and month of collection do show an obvious pattern of seasonal skin thickening (Figures 4-1, 4-2, 4-3).

The skin in the dorsal pectoral region of specimens collected later in the year is relatively thinner in the older specimens, obscuring the clear pattern of skin thickening observed in the more recent specimens. This pattern is also observed in the ventral pectoral region and, to an even lesser extent, in the ventral thigh region (Figures 4-4, 4-5, 4-6). It is possible that seasonal skin thickening is not as pronounced in the dorsal skin as it is in other body regions, although this conclusion is contradicted by analyses using only the older specimens that found more significant differences in the dorsal pectoral region than either of the other two body regions.

The difference in skin thickness between the historically collected specimens and those collected more recently suggests that there may be an intrinsic difference between the two datasets. Older specimens from the spring and early summer months are not thinner than the more recent specimens when regressions are produced from the combined dataset, which would be expected if preservation had a general shrinking effect on skin thickness. However, the pattern of seasonal skin thickening

between the two datasets is much more similar when different regressions are used to produce residuals (Figures 3-4, 3-5, 3-6, 4-1, 4-2, 4-3).

There are several potential explanations for this pattern. The first is that the datasets are inherently different because of the relatively high number of juvenile specimens in the older dataset rather than due to preservation. Skin thickness variation through post-metamorphic ontogeny has never been studied in any amphibian species. Although residuals account for allometric effects in the data (Atchley & Anderson, 1978), the differences between the residuals produced by different regression lines suggest that the high number of juveniles in the historic dataset is having an unexpected effect on the data that will require future investigation. The second option is that skin thickening is predominantly caused by an increase in fat storage in the skin in preparation for overwintering and that preservation in alcohol strips fat from the tissues, thus masking the effects of seasonal skin thickening. Amphibians deposit fat in various parts of their body in preparation for overwintering (Byrne & White, 1975; Tattersall & Ultsch, 2008). They are not known to deposit fat within the epidermis, spongy dermis, or compact dermis, but they do sometimes deposit adipose tissue beneath the compact dermis (Wygoda, Garman & Howard, 1987). Therefore, it is unlikely that fat loss within the tissue as a result of prolonged preservation is the cause of the difference in thickness. A third possibility is that seasonal skin thickening is the result of developmental plasticity that reflects the impact of environmental change across the decades. This third 'hypothesis' is difficult to test using this dataset with the current, limited understanding of seasonal skin thickening and of the cumulative effects of chemical preservation on soft tissues. However, it is a provocative idea that should be examined in future work that integrates samples from museum and field-based experimental studies.

For the first time, the present study has shown that historically collected (more than 70 years) museum specimens can be used to detect the presence of seasonal skin thickening in anuran amphibians. However, the magnitude of change between the more recently collected (1986–2016) and historic specimens (60+ years old) differs, and it is unclear if this difference is due to chemical preservation-related degradation or currently unstudied ontogenetic effects. These results indicate that further studies are needed to better understand the effect of preservation on amphibian skin thickness, the postmetamorphic trajectory of skin thickness and seasonal skin

thickening, and the relationship between environmental disturbances and this enigmatic trait.

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CHAPTER FIVE: BODY REGION VARIATION AND ALLOMETRIC PATTERNS OF SKIN THICKNESS IN AMPHIBIANS

ABSTRACT

Various aspects of amphibian biology scale with body size and some traits linked to physiological processes may also vary among regions of the body. Amphibian skin is the organ through which amphibians exchange water, oxygen, carbon dioxide, and salts between the internal and external environment. Despite over a century of research on amphibian skin anatomy, the relationship between body size or body region and skin anatomy has never been tested systematically. In this chapter, the skin anatomy of 10 species of amphibians is histologically prepared and compared through qualitative anatomical description and regressions of quantitative measures of skin thickness against body size, here measured using snout-vent length. Skin anatomy is broadly similar across all taxa investigated with a few key differences such as epidermal spines in the spadefoot toad (*Scaphiopus couchii*) and a high degree of vascularisation in the wood frog (*Rana arvalis*) and clawed frog (*Xenopus laevis*). Skin in the dorsal pectoral region is the thickest across the body regions that were compared. Regressions of skin thickness measures against body size found significant relationships for all skin layers among all body regions. The relationship between body size and compact dermis thickness appears to be the strongest relationship because it was the most robust to bootstrapping and effects of species that exhibit seasonal skin thickening. Regressions also suggest that the American bullfrog (*Lithobates catesbeianus*), which demonstrates seasonal skin thickening, might actually thin its skin during the summer months rather than thicken it in the winter months. Taken together, these results suggest that skin thickness is tightly correlated with body size among species but that intraspecific variation due to ecological differences among populations or seasonal effects might provide better insights into the ecological significance of amphibian skin thickness.

INTRODUCTION

Amphibian skin is semi-permeable, allowing for water, gases, ions, and other substances to cross between the external and internal environment through a combination of active and passive mechanisms (Duellman & Trueb, 1986). This functionality of the skin ties most amphibian species to relatively warm, humid

habitats and is considered to make amphibians more sensitive to environmental disturbances than other terrestrial tetrapods (Duellman & Trueb, 1986; Wake & Vredenburg, 2008).

Despite over 150 years of research on the anatomy of amphibian skin (Ascherson, 1840) and centuries of research on its physiological function (Jørgensen, 1997), the links between the anatomy, physiology, and ecological significance of this structure are poorly understood. For example, anurans have evolved multiple strategies to limit rates of evaporative water loss (EWL) in extreme environments (Toledo & Jared, 1993). Some arboreal anurans have become 'waterproof' by evolving modified granular glands that produce a waxy substance that they then spread across their body (Blaylock, Ruibal & Platt-Aloia, 1976). Skin thickness is one variable that is thought to correlate with physiological function (Czopek, 1965; Roth, 1973) but its variation among amphibian ecomorphs is either completely unknown or, at best, poorly understood. The reed frog (*Hyperolius nitidulus*) thickens and changes the colour of its skin in the dry season to limit evaporative water loss, but these anatomical and aesthetic modifications are due to an increase specifically in the thickness of the layer of light-reflecting iridiophores in its skin (Geise & Linsenmair, 1986; Kobelt & Linsenmair, 1986). It is unknown if arboreal anurans that do not possess specialised granular glands or a high density of iridiophores have modified their skin thickness relative to non-arboreal species to limit evaporative water loss.

To better understand the relationship between skin anatomy and ecology in amphibians using large comparative datasets, sources of variation in skin anatomy need to be assessed to refine sampling protocols and determine how sources of variation may affect results. Skin thickness can vary based on sex (Greven, Zanger & Schwinger, 1995; Wenying et al., 2011), season (Kun, 1959; Kobelt & Linsenmair, 1986), body region (Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001; Navas, Antoniazzi & Jared, 2004), or age (Rosenberg & Warburg, 1995) or potentially due to a combination of these factors. Thickness, as well as other sources of anatomical variation such as gland density or capillary density, also varies interspecifically (Czopek, 1965), and neither inter- nor intra-specific variation has been systematically assessed against confounding covariates such as body size.

Two factors that are known to relate to measurements of skin-related physiological processes in amphibians are body region and body size (Whitford, 1973; Moalli et al., 1980; Pruett, Hoyt & Stiffier, 1991; Newman & Dunham, 1994).

Anuran amphibians absorb most water through the 'pelvic patch', an area of highly vascularised skin on the ventral surface of the thigh (Roth, 1973; Bentley & Yorio, 1979). Dorsal and ventral skin also exchange different amounts of oxygen and carbon dioxide (e.g. Talbot, 1992). The dorsal region of anurans is often thicker than the ventral region (Greven, Zanger & Schwinger, 1995; Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001), although differences in relative thickness among body regions have not been assessed systematically.

Body size also affects many aspects of amphibian biology. There is an allometric relationship between body size and osmotic exchange, urine flow, glomerular exchange, and sodium influx (Pruett, Hoyt & Stiffier, 1991). The correlation between body size and these physiological traits is higher when anurans are analysed alone than when all amphibians are grouped together or urodeles are analysed alone (Pruett, Hoyt & Stiffier, 1991), suggesting physiological traits are more linked to body size in anurans than in urodeles or gymnophionans. If skin anatomy and physiology are related, as has been suggested (Czopek, 1965; Roth, 1973), then anuran skin anatomy should also follow a strongly allometric trajectory. However, allometry of skin thickness has never been assessed. Species that show body size sexual dimorphism also often show skin anatomy sexual dimorphism (Greven, Zanger & Schwinger, 1995; Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001) but these differences disappear when body size is accounted for in statistical analyses (Chapter 2).

Although amphibian skin anatomy and physiology have been historically viewed as relatively uniform, it has become clear that both factors vary considerably across the body's surface and with body size. Recognition of these sources of variation has guided studies on amphibian skin physiology but considerably less attention has been paid to researching these sources of variation in skin anatomy research. This chapter will specifically test for a relationship between skin thickness and body size, which is expected given the strong link between physiological processes that involve the skin and body size. It will also compare regional differences in skin thickness across species with different ecologies. Taken together, these results will address how sources of variation, including body region and intraspecific variation, can be expected to affect future macroevolutionary analyses of amphibian skin anatomy.

MATERIALS AND METHODS

Adult specimens of nine amphibian species (eight anurans and one urodele) were obtained from the Field Museum of Natural History (FMNH; Chicago, IL), the Museum für Naturkunde (MfN, Berlin, Germany), and the Smithsonian Museum of Natural History (USNM; Washington, DC). These species are: *Acris crepitans, Anaxyrus cognatus*, *Lithobates catesbeianus, L. pipiens, Litoria infrefrenata Notopthalmus viridiscens, Pseudacris crucifer, Rana arvalis, Scaphiopus couchii,* and *Xenopus laevis.* These species were chosen because their skin anatomy has been studied previously (except *Litoria infrafrenata*) but quantitative data on their skin anatomy were reported only as mean values (Czopek, 1965). In this study, skin thickness data are reported for discrete body regions (dorsal pectoral, ventral pectoral, and ventral thigh regions) separately here. To control for seasonal effects on skin thickness, specimens that were collected in the summer months were used. The specimens of *Litoria infrafrenata* were collected in August (Chapter 2), and although this is a different time of year than the other specimens utilised, this species does not experience marked seasonality in the same form as species that are known to show changes in skin thickness between seasons (e.g., Chapter 3, Kobelt & Linsenmair, 1986). One male and one female specimen was sampled for each species except for *Rana arvalis* and *Scaphiopus couchii*. A female of *S. couchii* was sampled but the skin was too badly damaged to be measured. Data on multiple individuals of *Lithobates catesbeianus, L. pipiens, Litoria infrafrenata,* and *Pseudacris crucifer* from Chapters 2 and 3 were used to examine intraspecific differences.

Histological preparation of the specimens follows that outlined in Chapter 2. The only difference compared to the previous protocol is that only dorsal pectoral and ventral pectoral samples were taken for *Notophthalmus viridiscens* and not ventral thigh samples because urodeles do not have a 'pelvic patch' like that of anurans. Instead, the costal region seems to be the principal site of water absorption (Lopez & Brodie, 1977). The measurements made for quantitative comparison are the same as those outlined in Chapter 3.

Absolute skin thickness for each species was compared among the three body regions. These data from the species sampled here were compared with results from previous studies on relative skin thickness. Unfortunately, because body size is often not reported in studies of amphibian skin anatomy, these data could not be used in subsequent analyses.

Each of the twelve skin thickness measures was regressed against snout-vent length using ordinary least-squares regressions. The male and female specimen for each species was used except for in *Rana arvalis* and *Scaphiopus couchii* for reasons outlined above. For *Lithobates catesbeianus*, *L. pipiens, Litoria infrafrenata*, and *Pseudacris crucifer*, one male and one female specimen from the summer months were chosen at random to use as representative for the species. For *Lithobates catesbeianus*, regressions were performed using a male and a female specimen from just the summer months, just the winter months, and from both seasons to examine the potential effects of seasonal skin thickening on results. The slopes of the resulting regressions were then compared against isometry to test if they differed significantly using the R package 'smatr'. A regression containing all specimens of *Lithobates catesbeianus*, *L. pipiens, Litoria infrafrenata*, and *Pseudacris crucifer* and all other species was performed for comparison against the results of other regressions because it includes many more specimens. Although the sample size is relatively small and regressions using small datasets are prone to Type II error (Brown & Vavrek, 2015), the sample size is comparatively large for a histological dataset (e.g., de Brito-Gitirana & Azevedo, 2005; Bingol-Ozakpinar & Murathanoglu, 2011) and has the potential to serve as a basis for comparison against future work.

To test for the effects of individual variation on macroevolutionary studies, sensitivity analyses was performed. Measurements for *Lithobates catesbeianus, L. pipiens, Litoria infrafrenata*, and *Pseudacris crucifer* were replaced using bootstrap resampling of the complete datasets from Chapters 2 and 3 to determine the effects of individual variation and seasonality on the regression results. Bootstraps using 1,000 replicates were performed on each individual species and all species together. When resampling for *Lithobates pipiens, Litoria infrafrenata*, and *Pseudacris crucifer*, analyses were performed with the summer and winter specimens of *Lithobates catesbeianus* separately. Although some tissue layers showed potential signs for seasonal skin thickening in *Lithobates pipiens*, the presence of this trait was ambiguous so was not considered in these analyses.

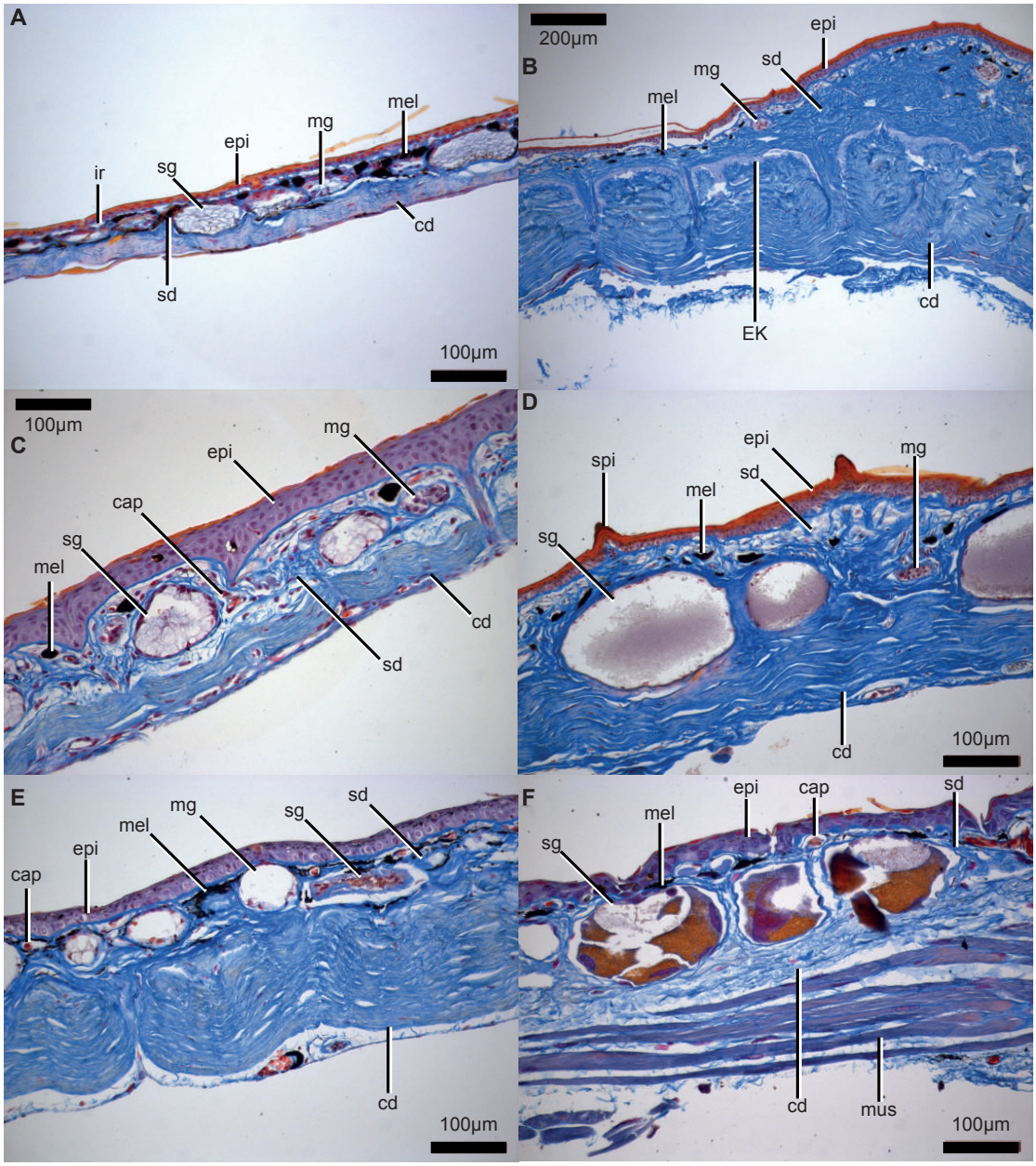

Figure 5-1. **Comparative histological sections of the dorsal pectoral skin of six amphibian species.** Histological sections of the dorsal pectoral skin of *Acris crepitans* (A; FMNH 284081), *Anaxyrus cognatus* (B, FMNH 259917), *Rana arvalis* (C; FMNH 234272), *Scaphiopus couchii* (D; FMNH 257215), *Xenopus laevis* (E; FMNH 251393), and *Notophthalmus viridiscens* (F; FMNH 275248) stained with azan stain modified after Geidies. **cap** = capillary; **cd** = compact dermis; **EK** = EK layer; **epi** = epidermis; **ir** = iridiophore; **mel** = melanosome; **mg** = mucous gland; **mus** = muscle; **sd** = spongy dermis; **sg** = serous gland; **spi** = spine.

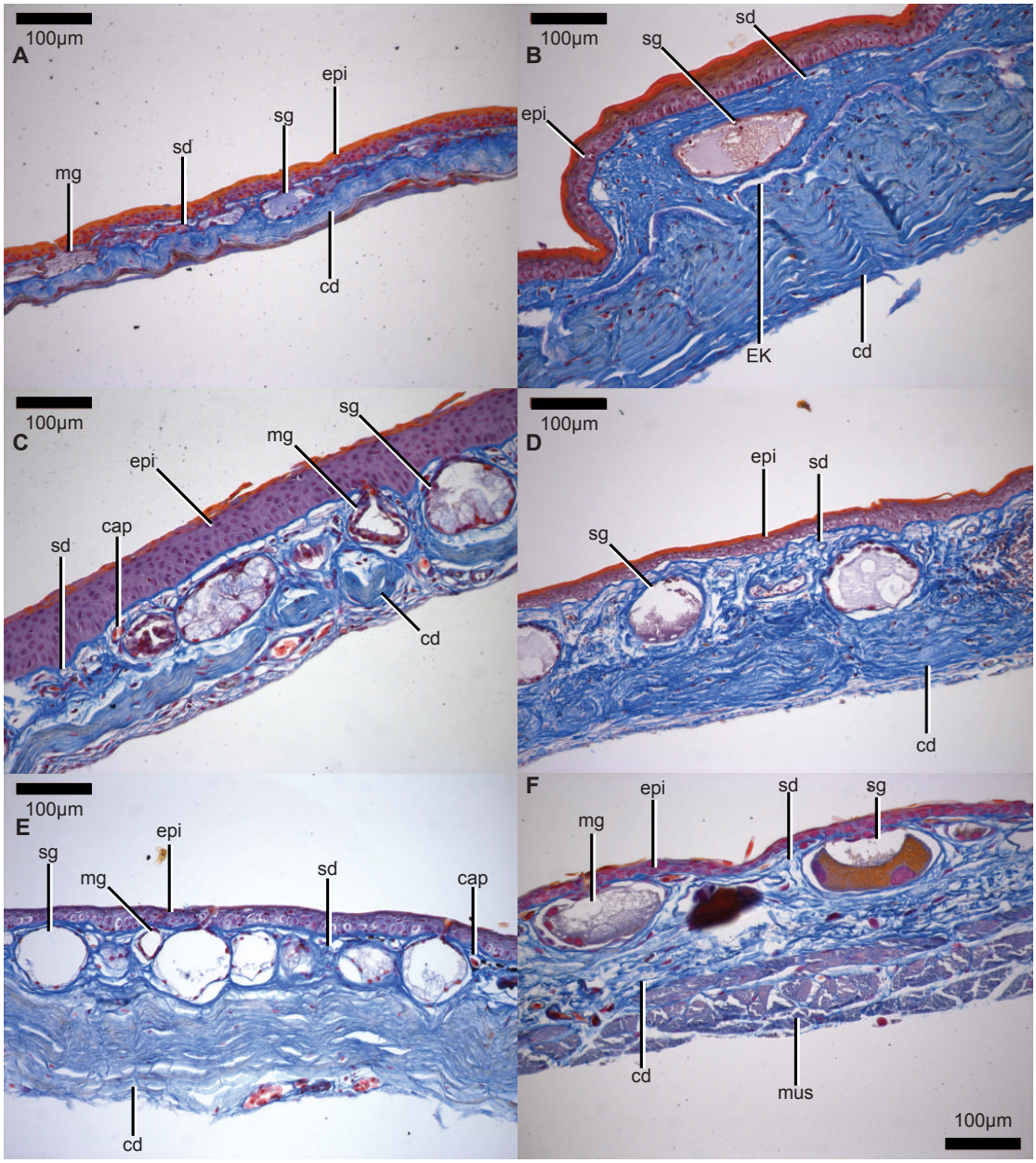

Figure 5-2. **Comparative histological sections of the ventral pectoral skin of six amphibian species.** Histological sections of the ventral pectoral skin of *Acris crepitans* (A; FMNH 284081), *Anaxyrus cognatus* (B, FMNH 259916), *Rana arvalis* (C; FMNH 234272), *Scaphiopus couchii* (D; FMNH 257215), *Xenopus laevis* (E; FMNH 251393), and *Notophthalmus viridiscens* (F; FMNH 275248) stained with azan stain modified after Geidies. Key follows Figure 5-1.

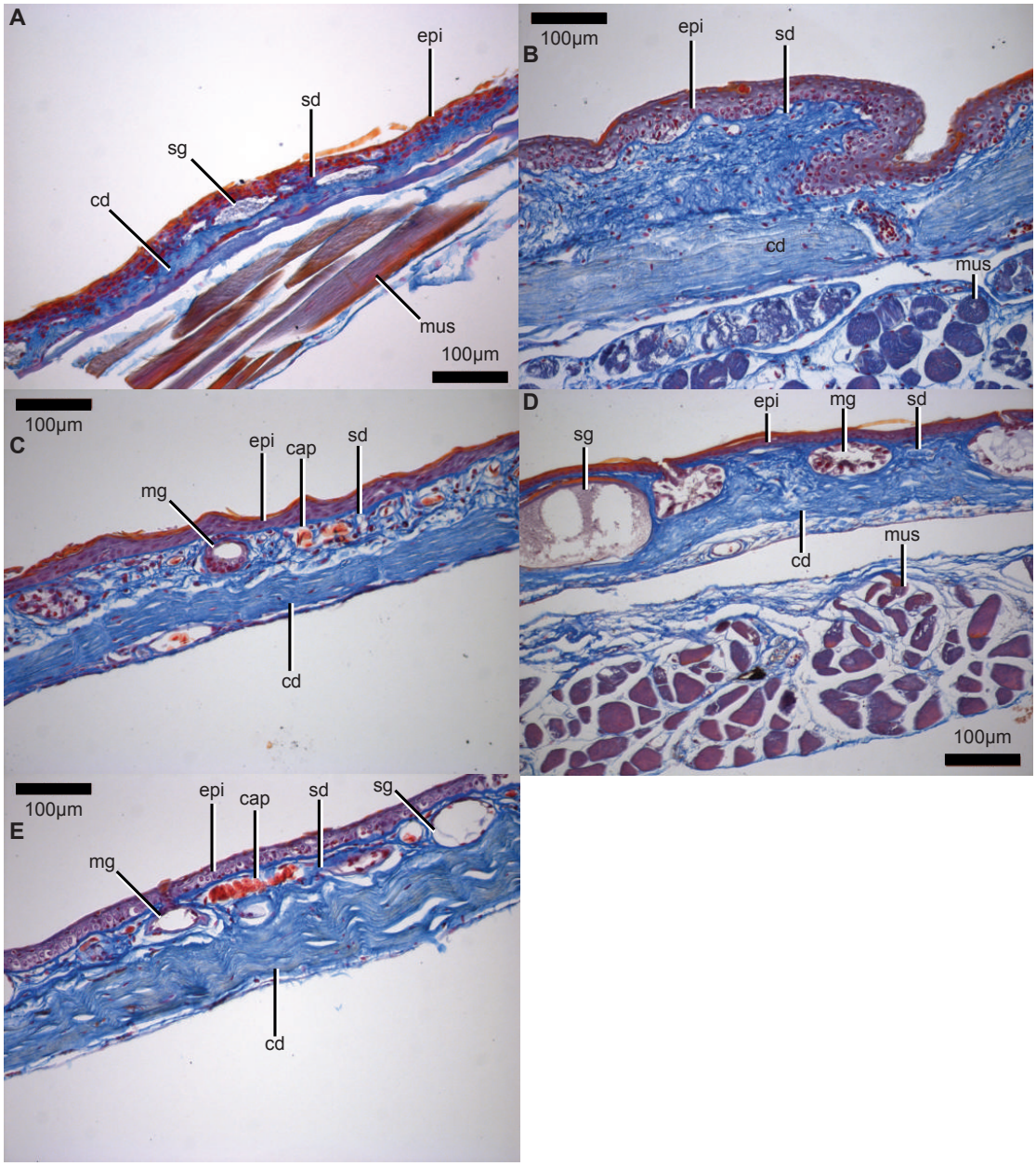

Figure 5-3. **Comparative histological sections of the ventral thigh skin of five anuran species.** Histological sections of the ventral pectoral skin of *Acris crepitans* (A; FMNH 284081), *Anaxyrus cognatus* (B, FMNH 259917), *Rana arvalis* (C; FMNH 234272), *Scaphiopus couchii* (D; FMNH 257215), and *Xenopus laevis* (E; FMNH 251393) stained with azan stain modified after Geidies. Key follows Figure 5-1.

COMPARATIVE DESCRIPTION OF SKIN ANATOMY

The skin of *Litoria infrafrenata* was described in Chapter 2, and the skin of *Lithobates catesbeianus, L. pipiens,* and *Pseudacris crucifer* was described in Chapter 3. The skin of the remaining six species will be compared to these previous descriptions.

All species show the three typical skin layers for amphibians: the epidermis, spongy dermis, and compact dermis (Fox, 1986a,b). In the dorsal pectoral skin, *Scaphiopus couchii* possesses cone-shaped 'spines' made of keratinised epidermal cells, similar to those found in *Rhinella ornata (Felsemburgh et al., 2009; Figure 4-1D)*. Below the epidermis, numerous capillaries are present in *Notophthalmus viridiscens*, *Rana arvalis*, and *Xenopus laevis* (Figure 4-1). Melanosomes are present in the spongy dermis in all species, and iridiophores are present in *Acris crepitans* (Figure 4-1). Glands, which also lie in the spongy dermis, are more numerous in *Notophthalmus viridiscens* and *Acris crepitans* than in other species. The glands of *Scaphiopus couchii* are larger in diameter than the glands of other species. Among the three primary tissue layers, *Anaxyrus cognatus* has a relatively much thicker compact dermis than any other species sampled. This layer is very thin in *Notophthalmus viridiscens,* which is aquatic in its adult form. There is also an obvious EK-layer in *A. cognatus* that lies just superficial to the compact dermis (Figure 4-1).

In the ventral pectoral skin, melanosomes are present in the spongy dermis of *Xenopus laevis* (Figure 4-2E). Subepidermal capillaries are present in *Rana arvalis* and *X. laevis* (Figure 4-2). Gland density is highest in *X. laevis* and *Notophthalmus viridiscens*. *Anaxyrus cognatus* again has a relatively thick compact dermis, and vercuae are also present.

The ventral thigh skin was the thinnest among the regions sampled for anurans. (Samples in this region were not taken for *Notophthalmus viridiscens*.) Again, subepidermal capillaries are present in *Rana arvalis* and *Xenopus laevis*, as well as in *Acris creptians* (Figure 4-3). There are very few glands in the spongy dermis of the ventral thigh region of *Anaxyrus cognatus*. As in the dorsal pectoral region, the glands of *Scaphiopus couchii* are large in diameter.

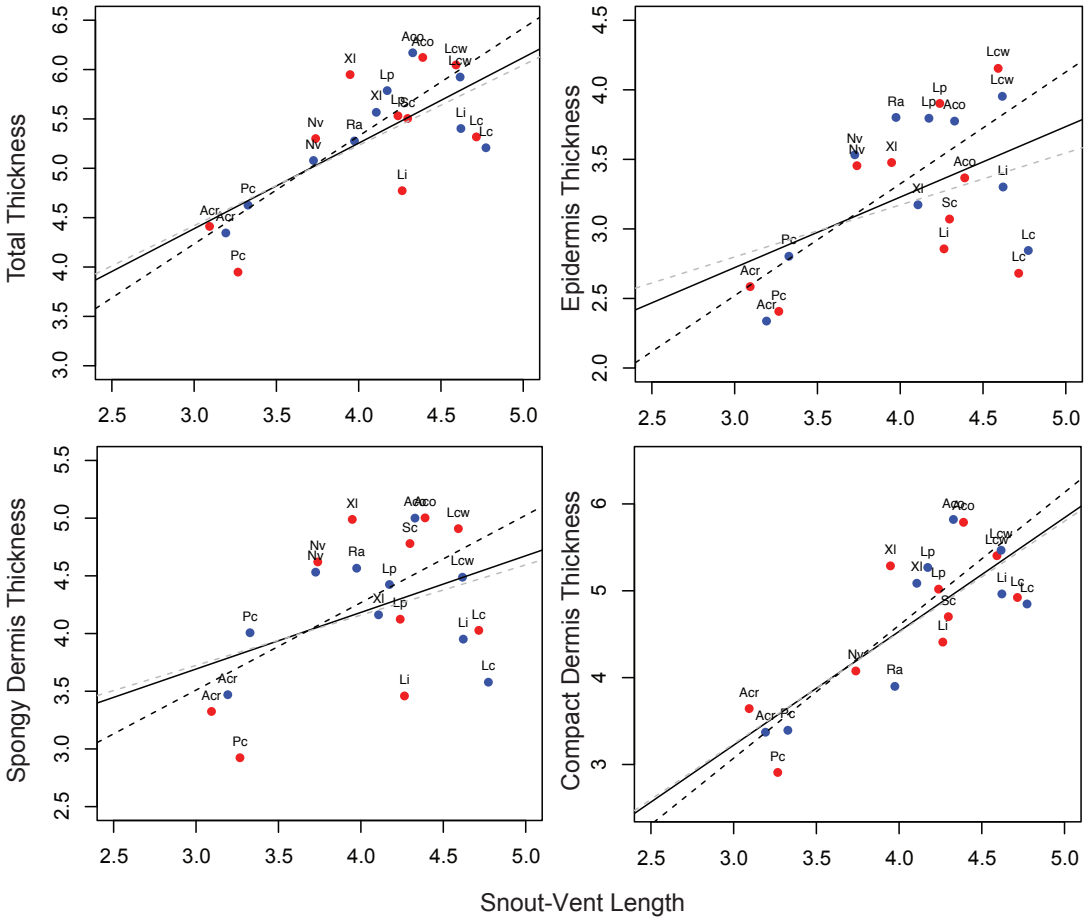

Figure 5-4. **Regressions of dorsal pectoral skin thickness against snout-vent length.** Measurements for total skin thickness, as well as epidermis, spongy dermis, and compact dermis thickness of the dorsal pectoral region for 10 amphibian species are regressed against body size. Red dots are female specimens and blue dots are males. Solid black lines represent regressions that include summer and winter specimens of *Lithobates catesbeianus*, broken black lines represent regressions that include only winter specimens of *L. catesbeianus*, and broken grey lines represent regressions that include only summer specimens of *L. catesbeianus*. Aco = *Anaxyrus cognatus*; Acr = *Acris crepitans*; Lc = *Lithobates catesbeianus* (summer); Lcw = *Lithobates catesbeianus* (winter); Li = *Litoria infrafrenata*; Lp = *Lithobates pipiens*; Nv = *Notophthalmus viridiscens*; Pc = *Pseudacris crucifer*; Ra = *Rana arvalis*; Sc = *Scaphiopus couchii*; Xl = *Xenopus laevis.*

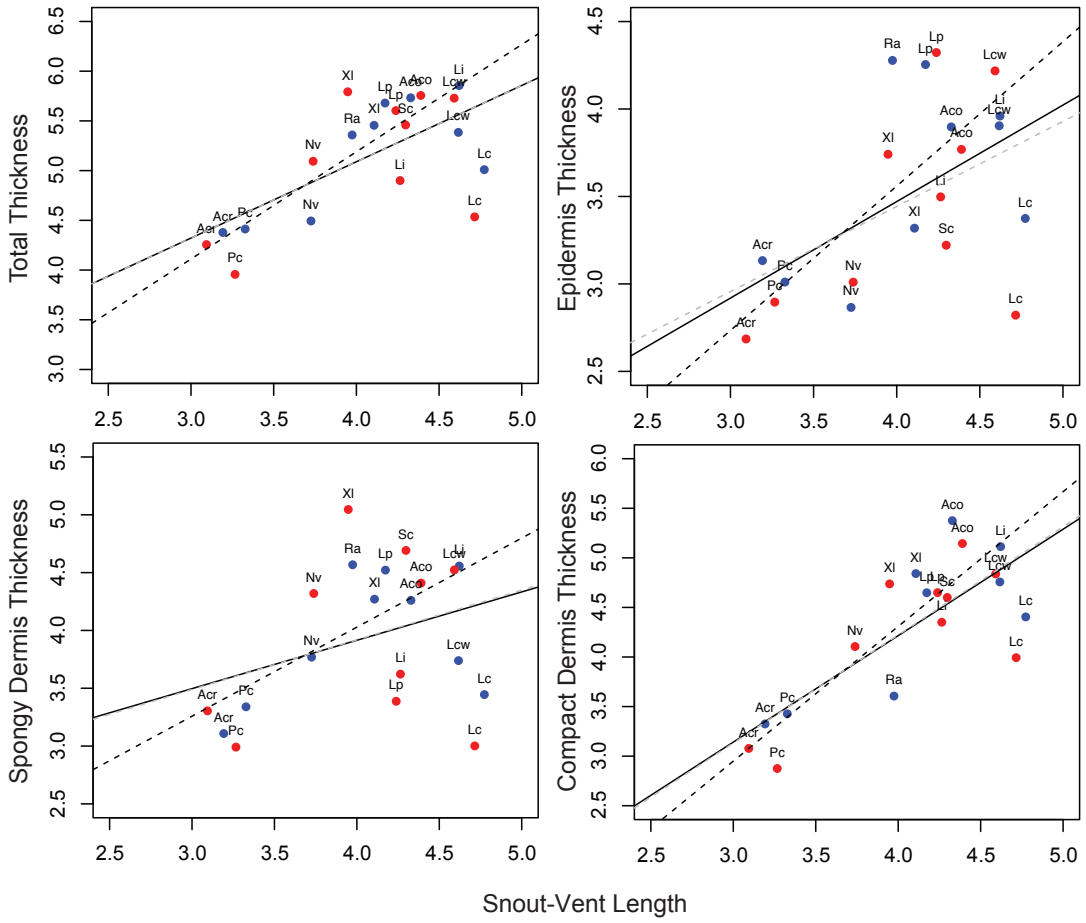

Figure 5-5. **Regressions of ventral pectoral skin thickness against snout-vent length.** Measurements for total skin thickness, as well as epidermis, spongy dermis, and compact dermis thickness of the ventral pectoral region for 10 amphibian species are regressed against body size. Key follows that of Figure 5-4.

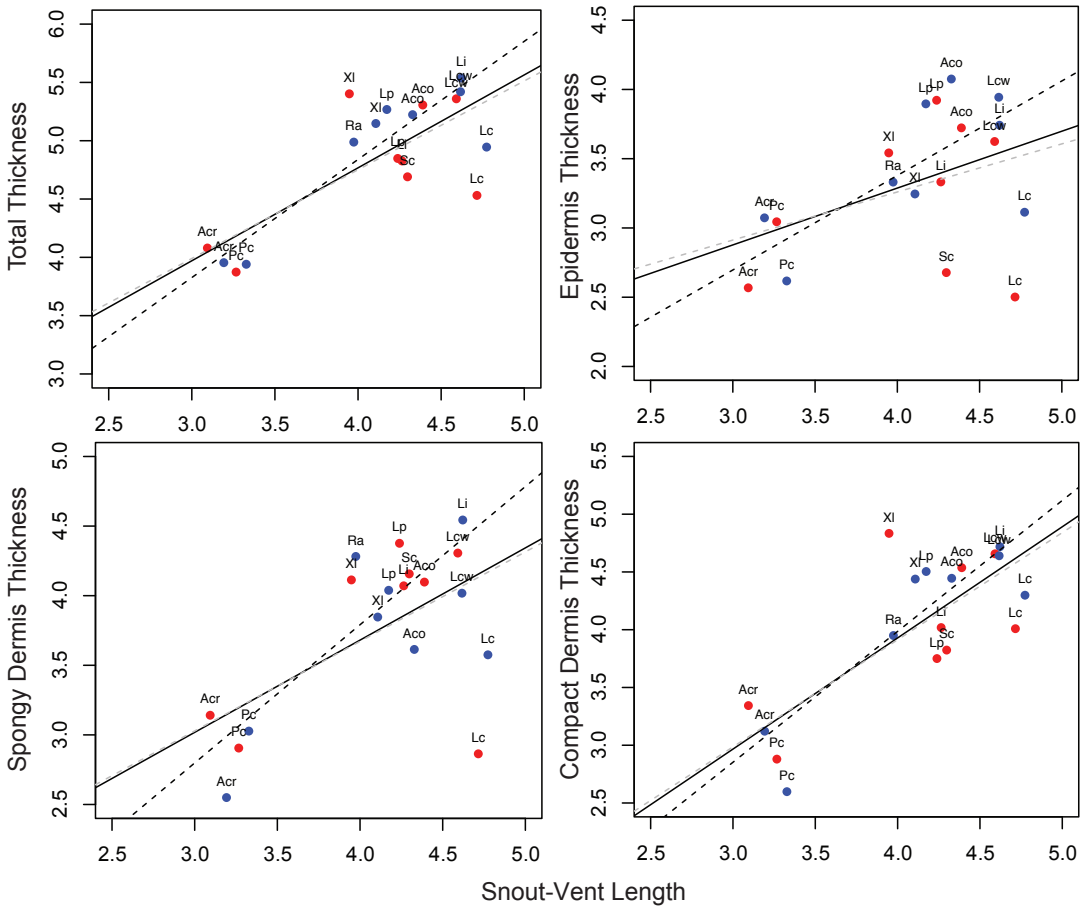

Figure 5-6. Regressions of ventral thigh skin thickness against snout-vent length. Measurements for total skin thickness, as well as epidermis, spongy dermis, and compact dermis thickness of the ventral thigh region for 10 amphibian species are regressed against body size. Key follows that of Figure 5-4.

RESULTS

Relative skin thickness among species

Comparing relative skin thickness among sampled body regions, dorsal skin is most commonly the thickest of the sampled skin regions. This result has also been found in previous studies that compared dorsal and ventral skin (Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001; Navas, Antoniazzi & Jared, 2004). The only species for which this pattern is not true are the white-lipped treefrog (*Litoria infrafrenata*) and the treefrog *Bokermannohyla alvarengai (Centeno et al., 2015),* in which the dorsal region is the thinnest skin region sampled in both total skin thickness and all three individual tissue layers in all but one specimen (MfN 54642). However, among species for which multiple specimens were available, this pattern was not always consistent. In *Lithobates catesbeianus*, the dorsal pectoral skin is almost always the thickest skin region, followed by the ventral pectoral region, and the ventral thigh region is the thinnest. In *L. pipiens* and *Pseudacris crucifer*, however, the ventral thigh region most often has the thinnest skin, but the thickest skin varies from being located in the dorsal pectoral or the ventral pectoral region, depending on the specimen considered.

Regression results

In the regressions using specimens of *Lithobates catesbeianus* collected in both summer and winter, ventral thigh epidermis thickness, dorsal pectoral spongy dermis thickness, and ventral pectoral spongy dermis thickness were not found to be significantly correlated with body size ($p = 0.07$, 0.07, and 0.14, respectively; Table 5-1) and had low r^2 values ($r^2 = 0.11$, 0.13, and 0.05, respectively; Table 5-1). Ventral thigh epidermis thickness also had the lowest slope (0.41). The highest slope was that of the dorsal pectoral compact dermis (1.31), which also had the highest r^2 value ($r^2 = 0.63$) and was the only regression to differ significantly from isometry ($p = 0.003$). Plots of the regressions show that the summer *L. catesbeianus* specimens fall noticeably below the regression line in all comparisons (Figures 5-4, 5-5, 5-6). The regression containing all specimens found that skin thickness measurements were significantly correlated with body size. The epidermis and spongy dermis measurements had the lowest r^2 values and, despite their low slopes, none of the epidermis measurements differed significantly from isometry.

Table 5-1. Regressions of skin tissue layers against body size and isometry using datasets of both winter and summer specimens *Lithobates catesbeianus*, only summer specimens of *L. catesbeianus*, and only winter specimens of *L. catesbeianus*.

Both summer and winter *Lithobates catesbeianus* specimens					
	Slope	Intercept	r^2	*p*-value	Isometry
Dorsal Total	0.87	1.79	0.5	<0.001	0.28
Thigh Total	0.8	1.58	0.57	<0.001	0.86
Ventral Total	0.77	2.02	0.41	0.001	0.43
Dorsal Epidermis	0.51	1.2	0.2	0.03	0.86
Thigh Epidermis	0.41	1.65	0.14	0.07	0.8
Ventral Epidermis	0.55	1.26	0.24	0.02	0.86
Dorsal Spongy Dermis	0.49	2.22	0.13	0.07	0.45
Thigh Spongy Dermis	0.66	1.03	0.32	0.009	0.61
Ventral Spongy Dermis	0.42	2.24	0.07	0.14	0.38
Dorsal Compact Dermis	1.31	-0.71	0.63	<0.001	0.003
Thigh Compact Dermis	0.96	0.08	0.59	<0.001	0.2
Ventral Compact Dermis	1.07	-0.08	0.59	<0.001	0.05
Summer *Lithobates catesbeianus* specimens					
	Slope	Intercept	r^2	*p*-value	Isometry
Dorsal Total	0.81	1.98	0.44	0.002	0.36
Thigh Total	0.76	1.71	0.52	<0.001	0.9
Ventral Total	0.77	2.02	0.38	0.004	0.37
Dorsal Epidermis	0.37	1.68	0.1	0.11	0.86
Thigh Epidermis	0.35	1.87	0.07	0.16	0.81
Ventral Epidermis	0.49	1.5	0.17	0.05	0.88
Dorsal Spongy Dermis	0.44	2.42	0.08	0.14	0.44
Thigh Spongy Dermis	0.64	1.1	0.27	0.02	0.57
Ventral Spongy Dermis	0.43	2.2	0.06	0.17	0.33
Dorsal Compact Dermis	1.28	-0.6	0.59	<0.001	0.006
Thigh Compact Dermis	0.93	0.2	0.54	<0.001	0.26
Ventral Compact Dermis	1.09	-0.14	0.56	<0.001	0.05
Winter *Lithobates catesbeianus* specimens					
	Slope	Intercept	r^2	*p*-value	Isometry
Dorsal Total	1.09	0.96	0.65	<0.001	0.06
Thigh Total	1.01	0.79	0.79	<0.001	0.32
Ventral Total	1.08	0.88	0.74	<0.001	0.1
Dorsal Epidermis	0.8	0.11	0.51	<0.001	0.6
Thigh Epidermis	0.68	0.65	0.49	0.002	0.77
Ventral Epidermis	0.83	0.26	0.54	<0.001	0.57
Dorsal Spongy Dermis	0.76	1.24	0.32	<0.001	0.25
Thigh Spongy Dermis	0.99	-0.18	0.74	<0.001	0.34
Ventral Spongy Dermis	0.77	0.95	0.35	0.006	0.28
Dorsal Compact Dermis	1.53	-1.52	0.71	<0.001	<0.001

Thigh Compact Dermis	1.13	-0.55	0.68	<0.001	0.05
Ventral Compact Dermis	1.36	-1.13	0.79	<0.001	0.002

When only the *L. catesbeianus* specimens from summer months were included, fewer variables were significantly correlated with body size, with almost half of them insignificantly related to body size with r^2 values below 0.2 (Table 5-1). Only the dorsal pectoral compact dermis differed significantly from isometry (p = 0.006). Intercepts were higher than that of the regression of the large dataset but similar to the subset dataset (Table 5-1). Conversely, when only the winter specimens were used, all variables were significantly correlated with body size and all r^2 values were above 0.5 except for the ventral thigh epidermis (r^2 = 0.49) and ventral pectoral spongy dermis (r^2 = 0.35; Table 5-1). The compact dermis thickness measurements were the only regressions to significantly differ from isometry, and intercept values were similar to those of the regressions from the large dataset (Table 5-1).

Skin thickness data in regressions including all specimens of all species were all significantly correlated with body size (Table 5-2). Regressions using epidermis thickness did not differ from isometry and ventral thigh total thickness was marginally significant (p = 0.05; Table 5-2). Regressions using the epidermis thickness values also had the lowest r^2 values (Table 5-2).

Table 5-2. Results from regressions of skin thickness against body size using a dataset containing all specimens of each species sampled.

	Slope	Intercept	r^2	p-value	Isometry
Dorsal Total	1.05	0.88	0.65	<0.001	0.002
Thigh Total	0.95	1.05	0.63	<0.001	0.05
Ventral Total	0.89	1.23	0.51	<0.001	0.04
Dorsal Epidermis	0.62	0.61	0.34	<0.001	0.67
Thigh Epidermis	0.5	1.4	0.29	<0.001	0.42
Ventral Epidermis	0.44	1.54	0.22	<0.001	0.44
Dorsal Spongy Dermis	0.69	1.16	0.28	<0.001	0.04
Thigh Spongy Dermis	0.82	0.38	0.41	<0.001	0.03
Ventral Spongy Dermis	0.71	0.78	0.25	<0.001	0.01
Dorsal Compact Dermis	1.51	-1.73	0.77	<0.001	<0.001
Thigh Compact Dermis	1.21	-0.84	0.73	<0.001	<0.001
Ventral Compact Dermis	1.27	-1.19	0.69	<0.001	<0.001

Resampling results

Species choice had noticeable effects on the results from bootstrap resampling. When all species were resampled, regressions of total thickness and compact dermis

thickness measurements against SVL most consistently recovered significant differences ($p < 0.05$; Table 5-3). The results were very similar when bootstrap was performed on only *Lithobates catesbeianus* with all other species remaining constant.

Resampling was performed for *Lithobates pipiens, Litoria infrafrenata*, and *Pseudacris crucifer* using two *Lithobates catesbeianus* specimens from either the winter or summer months (Table 5-3). When summer specimens of *L. catesbeianus* were used, significant differences in total thickness and compact dermis thickness were consistently found across all three species (Table 5-3). In bootstraps of *Litoria infrafrenata*, regressions of the ventral thigh spongy dermis also always recovered a significant relationship with body size (Table 5-3). Many of the other skin measurements either always recovered relationships that were not significant or did so in a majority of the time (Table 5-3).

When the winter specimens of *Lithobates catesbeianus* were used instead of the summer specimens, significant relationships between skin thickness and body size were almost always recovered for regressions of all skin measurements in all species (Table 5-3). For the bootstraps of *Litoria infrafrenata,* the dorsal pectoral spongy dermis thickness and ventral pectoral spongy dermis thickness infrequently recovered non-significant differences, whereas all bootstraps for all skin measurement in the other two species recovered significant differences (Table 5-3).

DISCUSSION

The goal of the present study was to test for a relationship between body size and skin thickness, as has been found for physiological processes, and for patterns of regional skin thickness variation among amphibian species. The skin anatomy of all species was similar to that of other amphibians for which skin anatomy has been described. Anatomical features that are noticeably different among the species included the epidermal spines in *Scaphiopus couchii*, the prominent EK-layer in *Anaxyrus cognatus*, and the highly vascularised skin of *Rana arvalis* and *Xenopus laevis*. All of these structures are thought to be involved in aspects of anuran water economy in some way. Spines in *Rhinella ornata* are thought to help with water uptake (Felsemburgh et al., 2009), and an EK-layer is hypothesised to limit water loss (Toledo & Jared, 1993; Azevedo et al., 2005). Subcutaneous capillaries are also used for water uptake, but they are often most prominent in the ventral regions. However, they are present across the body in both *Rana arvalis* and *Xenopus laevis*. *Xenopus*

laevis is a fully aquatic anuran that is not prone to evaporative water loss due to its ecology; however, *Rana arvalis* is semiaquatic and thus more prone to water loss. It is unclear why the latter species possesses such highly vascularised skin, especially when this condition is not present in any of the other ranids examined.

Table 5-3. Results from the bootstrap analysis showing the number of iterations (out of 1,000) that produced a non-significant relationship between skin thickness and body size. (*L. c. = Lithobates catesbeianus*; *L. p. = Lithobates pipiens*; *P. c*. = *Pseudacris crucifer*; *L. i. = Litoria infrafrenata*; summer or winter denote which *L. catesbeianus* specimens were used)

	All	*L. c.*	*P. c.* (winter)	*P. c.* (summer)	*L. p.* (winter)	*L. p.* (summer)	*L. i.* (winter)	*L. i.* (summer)
Dorsal Total	0	0	0	0	0	0	0	0
Thigh Total	4	0	0	0	0	0	0	0
Ventral Total	4	0	0	0	0	0	0	0
Dorsal Epidermis	148	282	0	813	0	1000	0	1000
Thigh Epidermis	236	443	0	1000	0	1000	0	1000
Ventral Epidermis	269	151	0	413	0	507	0	194
Dorsal Spongy Dermis	188	427	0	1000	0	1000	67	887
Thigh Spongy Dermis	46	97	0	263	0	228	0	0
Ventral Spongy Dermis	392	390	0	1000	0	1000	195	1000
Dorsal Compact Dermis	0	0	0	0	0	0	0	0
Thigh Compact Dermis	0	0	0	0	0	0	0	0
Ventral Compact Dermis	0	0	0	0	0	0	0	0

In nearly all species, the dorsal region of the body has the thickest skin. The dorsal skin is exposed more to the outside environment in terrestrial species, so thicker dorsal skin may limit EWL from this region of the body. Among the multiple

specimens of *Lithobates catesbeianus*, this pattern is consistent despite some individual variation. In *Lithobates pipiens* and *Pseudacris crucifer*, however, specimens showed inconsistent patterns in which the thickest skin region is either the dorsal pectoral or ventral pectoral region. In other species, the ventral pectoral region was the second thickest skin region. However, in *Litoria infrafrenata*, the ventral pectoral region is most often the thickest region of skin and the dorsal pectoral region is the thinnest. This pattern is also present in *Bokermannohyla alvarengai* (Centeno et al., 2015); however, it does not appear to be related to broadly defined ecological niches because *Pseudacris crucifer* is similar in relative skin thickness to the fully aquatic, semi-aquatic, and terrestrial species sampled.

Litoria infrafrenata is the only wet tropical species examined here and also the only wet tropical species for which skin anatomy has been assessed quantitatively. *Bokermannohyla alvarengai* is also a treefrog but inhabits drier regions. Therefore, it is possible that treefrogs exposed to warmer environments benefit from having a relatively thin dorsal skin. Unfortunately, because the link between skin anatomy and ecology has not been assessed and the current understanding of variation in skin anatomy among amphibians is so limited, it is difficult to draw specific conclusions at this time. Conversely, other species in the genus *Litoria* reduce evaporative water loss through secretions from modified granular glands (Toledo & Jared, 1993). *Litoria infrafrenata* possesses polymorphic skin glands (Chapter 2) that might produce similar substances to those found in congeners that would also help reduce EWL, but it is unclear what substances these glands produce in *L. infrafrenata*. Relative skin thickness across body regions in the so-called 'waterproof frogs' and further examination of polymorphic skin glands across may explain why this species is unique in relative skin thickness among the species examined here.

Body size and skin thickness

The results of the regression analyses (before resampling) suggest that there is a significant relationship between body size and skin thickness. In the analysis including every specimen of each species, a significant relationship was found between every skin measurement and body size. This relationship was so strong in some cases that it resulted in slopes that only deviated slightly from isometry to be recovered as significantly different (e.g., dorsal pectoral total thickness; Table 5-2). When a subset of the data was used so that each species was represented by only a

few specimens, similar results were recovered, although not every relationship was significant. In the dataset with both summer and winter *Lithobates catesbeianus* and the dataset with only the summer *L. catesbeianus* specimens, r^2 values were lower than those of the large dataset including all specimens and species. However, when only the winter specimens of *L. catesbeianus* were used, the r^2 values were similar or higher than those of the large dataset and all relationships were again significantly correlated with body size, although fewer relationships were significantly different from isometry due to variation (Table 5-1).

Bootstrap resampling also found differences based on the summer and winter specimens of *L. catesbeianus*. When the 'constant' component of the dataset contained summer specimens of *L. catesbeianus*, there was a much weaker relationship between many aspects of skin thickness and body size, whereas using a winter specimen of *L. catesbieanus* found consistently significant relationships between almost all skin thickness measurements and body size across the three other species on which subsampling was performed. Few differences were present among the three species between the summer and winter *L. catesbeianus* datasets.

Among the multiple analyses, both total skin thickness and compact dermis thickness were always significantly correlated with body size. The compact dermis contributes a significant portion of total skin thickness and is often the thickest tissue layer of the skin. Therefore, it would appear that a strong relationship between compact dermis thickness and body size drives the significant relationship between total skin thickness and body size, even when the relationship between body size and either epidermis thickness or spongy dermis thickness is weaker due to higher variation in the dataset.

When the individual datapoints are plotted, it becomes clear that the summer specimens of *L. catesbeianus* fall below the expected regression line produced by using only winter specimens and using specimens from both seasons (Figures 5-4, 5-5, 5-6). It is also clear that they influence the regression that includes only the summer specimens. This species is the only species within this dataset that is known to change the thickness of its skin in relation to season, in which it has thicker skin in the winter months and thinner skin in the summer months (Chapter 3, 4). Originally, it was hypothesised that this change in thickness was due to an above-average thickness of the skin to help protect against unfavourable conditions, as in the reed frog (*Hyperolius nitidulus*) (Geise & Linsenmair, 1986; Kobelt & Linsenmair, 1986) and

the common toad (*Bufo bufo*) (Kun, 1959). In *H. nitidulus*, skin becomes thicker due to an increase in the number of iridiophores, which are pigment-reflecting structures that lie below the epidermis of the skin of many frogs (Kobelt & Linsenmair, 1986). This anatomical change causes the skin to turn white in colour, which reflects sun and helps *H. nitidulus* reduce rates of evaporative water loss (Geise & Linsenmair, 1986). In *B. bufo*, the ecological significance of seasonal skin thickening is unclear, apart from the fact that it follows a similar pattern to that of *L. catesbeianus* (Kun, 1959; Chapter 3, 4). Both of the latter taxa are temperate neobatrachians, but sympatric species, such as *L. pipiens* and *Pseudacris crucifer* in the case of *L. catesbeianus*, do not show similar adaptations for overwintering, demonstrating the diverse strategies amphibians use to survive periods of unfavourable conditions.

Within the context of these regression analyses, it seems likely that the seasonal change in skin thickness in *L. catesbeianus* may be due to seasonal skin thinning in the summer months, rather than seasonal skin thickening in the winter months. This result is intriguing given that populations of the toad *Rhinella schneideri* from drier Caatinga habitats have thinner skin than populations from the Atlantic Forests (Navas, Antoniazzi & Jared, 2004). *Lithobates catesbeianus* overwinters underwater (Tattersall & Ultsch, 2008), so in both species, thicker skin is present in individuals that experience wetter habitats. Because amphibians are particularly sensitive to evaporative water loss, it would be expected that thicker skin should be present in species or individuals that experience higher rates of EWL. However, these results contradict that prediction. Unfortunately, *L. catesbeianus* is the only species in this dataset that is known to exhibit seasonal skin thickening, so this pattern cannot be tested using other species. Seasonal skin thickening (or thinning) has only been studied in a handful of species (Kun, 1959; Kobelt & Linsenmair, 1986; Chapter 3), and differences in skin thickness among populations from different habitats are even less common (Navas, Antoniazzi & Jared, 2004). Although there is a strong interspecific relationship between skin thickness and body size, intraspecific differences might be more related to specific environmental pressures and should thus be investigated in future work.

This is the first study to test for a relationship between skin thickness and body size using an intraspecific dataset. The dataset used here contains species adapted to arid (*Scaphiopus couchii*), fully aquatic (*Xenopus laevis*), arboreal (*Litoria infrafrenata* and *Pseudacris crucifer*), terrestrial (*Anaxyrus cognatus*), and semi-

aquatic (*Lithobates catesbeianus*) ecologies. It is possible that different clades or ecomorphs follow different allometric trajectories, but the current dataset does not allow for this hypothesis to be tested. One caveat is that the dataset only contained one urodele and no gymnophionans. Physiological attributes are more strongly linked to body size in anurans than other amphibians (Pruett, Hoyt & Stiffier, 1991), so datasets containing a higher proportion of non-anuran amphibians may recover different results. While future studies are needed to investigate this possibility, the results here support that amphibian skin thickness follows an allometric trajectory that may explain variation among species or individuals of different body sizes (e.g., body size sexually dimorphic species) as has been found for certain physiological processes involving the skin. The results also suggest that *Lithobates catesbeianus* thins its skin in the summer months as opposed to thickening it to overwinter, which provides a new perspective on seasonal changes in skin thickness among amphibians that should be explored further.

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CHAPTER SIX: EXPLORING THE RELATIONSHIP BETWEEN AMPHIBIAN ECOLOGY AND SKIN THICKNESS

ABSTRACT

Amphibian skin is an organ with which amphibians perform many physiological functions. Therefore, it is often assumed that a link exists between amphibian skin morphology and amphibian ecology. This hypothesis has received attention recently as potentially explaining the higher proportion of amphibian species threatened with extinction compared to mammals, reptiles, or birds. Despite over a century of research on amphibian skin anatomy, its ecomorphology has never been investigated. Here, published datasets are combined with environmental data to test for correlations between skin anatomy and ecological parameters. In phylogenetically restricted datasets using coarse habitat definitions, either body size or relative skin thickness negatively correlate with habitat aridity and only when fully aquatic species are removed from the analyses. In phylogenetically broad datasets, body size correlated with environmental variables better than did skin thickness measures or measures of gland density or degree of vasculaturisation; body size was positively correlated with moisture. These conflicting results make it difficult to determine the relationship between skin anatomy and ecology, but suggest that relative skin thickness may remain constant among species but differ at lower taxonomic levels to accommodate unfavourable environmental conditions. These results also highlight the need for more research on this topic before generalisation about amphibian skin ecomorphology can be made or skin physiology or function should be implicated in driving global amphibian population declines.

INTRODUCTION

Any species' range or habitat niche is controlled by myriad biotic and abiotic factors (e.g., Holt, 2009). Uncovering the traits that determine a species' niche is a primary goal of the fields of ecology and evolution to better understand the modern distribution of biodiversity and predict future distributions under projected scenarios, such as an increase in global mean temperatures due to climate change (Pearson & Dawson, 2003; Tingley & Monahan, 2009). These traits can be anatomical, physiological, behavioural, or developmental (e.g. Wainwright & Reilly, 1994; Sultan, 2007; Hillman et al., 2009).

Amphibians are a group of terrestrial vertebrates that are often restricted to warm and wet habitats because they require a moist environment for reproduction, are prone to losing water through evaporative water loss (EWL), and are ectothermic poikilotherms (Duellman & Trueb, 1986). Amphibian habitat preference is known to affect rates of EWL (Young et al., 2005). For example, arboreal anurans (frogs and toads) have evolved relatively high resistance to EWL because they are particularly prone to desiccation (Tracy, Christian & Tracy, 2010). Amphibians also use their skin to absorb water and salts (e.g., sodium, potassium) and to exchange oxygen and carbon dioxide using cutaneous respiration (Duellman & Trueb, 1986).

Despite a wealth of knowledge concerning the physiological function of the skin (Buttemer, 1990; Jørgensen, 1997), the relationship between amphibian skin anatomy and ecology is less well understood. A relationship between these attributes is expected given that skin anatomy is related to physiological function and physiology is linked with ecology (Canziani & Cannata, 1980; Tracy, Christian & Tracy, 2010; Hedrick et al., 2011). The latter link is assumed to be a driving force behind global amphibian population declines (Wake & Vredenburg, 2008). Determining the strength or presence of the relationship between skin anatomy and ecology is useful because morphological traits are generally considered to be less plastic within an individual than either physiological or behavioural traits; therefore morphologies that limit niche breadth are predicted to more strongly affect a species' ability to adapt to novel environmental conditions (Wainwright & Reilly, 1994).

Previous studies that quantitatively examined amphibian skin anatomy have produced hypotheses regarding the ecological significance of certain morphologies. Czopek (1965) provided the largest comparative dataset of morphological features associated with amphibian respiratory surfaces to date, including many measurements of the subcutaneous vascular network, gland density, and epidermis thickness. It was reported that lungless salamanders (plethodontids) have the thinnest skin and also the highest surface area of capillaries. Ranid anurans were found to have an epidermis that is roughly 40 μm thick, and toads (bufonids and *Bombina*) have skin that is 50–60 μm thick; the greater skin thickness was thought to limit oxygen exchange. Le Quang Trong (1971, 1975) measured skin thickness in the African anuran genera *Pychadena* and *Phyrnobatrachus*. In *Ptychadena*, relative skin thickness (calculated using simple ratios) was proposed to be correlated with habitat complexity and humidity whereas savannah species have thicker skin than forest species (Le Quang

Trong, 1975). In *Phrynobatrachus*, it is more difficult to draw definitive conclusions but skin thickness and body size seem to covary with habitat type, where larger species with thicker skin inhabit forests, but size-corrected skin measurements were not reported (Le Quang Trong, 1971). However, neither study performed statistical tests on the data. More recently, Navas et al. (2004) found that the skin of the toad *Rhinella schneideri* is thinner in the population that inhabits arid regions of the Caatinga than in Atlantic Forest populations, which is the opposite trend derived from interspecific studies (Le Quang Trong, 1971, 1975).

In these and other studies, the skin tends to be measured across regions of the body and averaged to report in order to generate a single measurement of thickness. However, it has long been known that amphibian skin is regionally specialised across the body and that these regions function differently (Bentley & Main, 1972). For example, the anuran pelvic patch is a region of the body that is well supplied with blood vessels and is the site where most water and salts are absorbed (Bentley & Main, 1972; Roth, 1973). This realization led to more precise studies on behavioural adaptations among species that have different rates of physiological processes such as water absorption (Hillyard, Hoff & Propper, 1998). Another concern with these studies is that body size is often not considered and in instances when it is, ratios are used instead of residuals (Czopek, 1965; Le Quang Trong, 1975). It has been shown that ratios ignore allometric trends in data and can produce misleading results (Atchley, 1978). Therefore, these studies contain methodological flaws that make their results difficult to apply to broader generalisations about patterns across amphibians.

It is likely that amphibian skin anatomy and ecology are linked in some way, but the previous studies that have been used to create an understanding of this relationship may be incorrect due to the issues highlighted above. Luckily, some of these studies report body size proxies so analyses can be re-run while accounting for body size, although raw values for skin thickness or other anatomical measurements for specific regions of the body were not reported (Czopek, 1965; Le Quang Trong, 1971, 1975). Using these data and data from a recent review of the effects of body region and body size variation on skin anatomy (Chapter 5), the goal of this study is to critically assess previous hypotheses concerning the ecomorphology of amphibian skin with an emphasis on skin thickness. Skin thickness is known to vary between seasons (Kun, 1959; Kobelt & Linsenmair, 1986) and among ecologically separated

species or populations (Le Quang Trong, 1971, 1975; Navas, Antoniazzi & Jared, 2004), yet such variation is poorly understood from both physiological and functional perspectives. Therefore, this measurement provides an opportunity to test how skin thickness relates to ecology as well as also how studies at different taxonomic scales compare in their results.

MATERIALS AND METHODS

Data from the literature

Skin thickness measurements from three sampled body regions (dorsal pectoral, ventral pectoral, and ventral thigh) and snout-vent length data for 10 species of amphibians were collected from Chapter 5. Ventral thigh data were not collected for the urodele *Notophthalmus viridiscens* in the Chapter 5 dataset, so two datasets were created, one with *N. viridiscens* excluded and a second with all species included but total ventral thigh skin thickness, ventral thigh epidermis thickness, ventral thigh spongy dermis thickness, and ventral thigh compact dermis thickness variables removed.

Data from Czopek (1965) includes seven of these species (excluding *Lithobates pipiens*, *Pseudacris crucifer,* and *Litoria infrafrenata*) along with 31 other species (the 'Czopek dataset'). Measurements from Czopek (1965) include: the thickness of the epidermis, number of glands in 1mm^2 of skin, number of meshes of capillary net per 1 mm^2 of skin, length of skin capillaries in meters per 1g body mass, surface area to volume, and the total length of capillaries of all respiratory organs in meters per 1g body mass. Body mass, as a proxy for body size, is also reported for each species.

The two datasets contain mostly measurements for different variables with the exception of epidermis thickness, and also different sampling methods (precise vs. whole-body averages). Epidermis thickness was therefore used to test if the measurements of the two datasets were similar enough to combine them for the seven species shared between them. Average epidermis thickness was calculated among the dorsal pectoral, ventral pectoral, and ventral thigh regions for the species in the Chapter 5 dataset. Both raw values and residual values from an ordinary least squares regressions of log-transformed body size measurements (SVL or weight) were compared using a correlation test to determine if the datasets could be combined for analyses. However, neither raw nor corrected values were correlated between the two

datasets, so they were kept separate. Although the methodological and variable choice differences between the datasets may limit the conclusions drawn from these comparisons, they allow for their effects to be observed.

Le Quang Trong (1971, 1975) reported skin thickness and body length values for five species of the genus *Ptychadena* (*Pt. maccarthyensis* = *Pt. bibroni*) and seven species of the genus *Phrynobatrachus* (*Ph. accraensis* = *Ph. latifrons*). [Taxonomic amendments follow that of Frost (2017).]

Environmental data

Data for the African genera *Ptychadena* and *Phrynobatrachus*, habitats were coded across a range based on the environmental classifications given in the original publication with the driest habitats (i.e. savannah) coded as '1' and the wettest (or most humid) habitats coded as '4' for *Ptychadena* and '5' for *Phrynobatrachus* (i.e. forest or humid forest). More detailed ecological data are available for the *Phrynobatrachus* species, including whether each species is terrestrial, semi-terrestrial, or semi-aquatic and the humidity of the habitat. Tests were also run on only the semi-terrestrial and terrestrial species because aquatic amphibians are not affected by factors such as EWL.

Environmental data for the species from the Chapter 5 and Czopek datasets were obtained using the Global Biodiversity Information Facility (GBIF) and the WorldClim database. GPS coordinates for each species were obtained using the 'rgbif' package (Chamberlain et al., 2017) and BioClim variables were extracted at a resolution of 2.5 arc seconds for each species using the 'raster' package (Hijmans & Etten, 2014). All environmental variables and further statistical analyses were performed using the statistical software R (R Development Core Team, 2014). One species in the Czopek dataset, *Pelophylax esculentus*, is not recognised in GBIF and was excluded. Each of the 19 BioClim variables were summarised into single values so that they could be used in multivariate analyses. For mean diurnal range, temperature seasonality, maximum temperature of the warmest month, mean temperature of the warmest quarter, precipitation seasonality, and precipitation of the wettest quarter, the average of the highest 100 values was used. For minimum temperature of the coldest month, mean temperature of the coldest quarter, precipitation of the driest month, and precipitation of the driest quarter, the mean of the lowest 100 values were used. Means of all values for the species were used for all

other variables. Because these variables have different units of measurement, they were standardised by converting them into Z-scores.

Statistical analyses

For the *Ptychadena* and *Phrynobatrachus* data, Pearson's Correlation was used to test for significant relationships between skin thickness measurements, body size, and environmental variables. Because ratios were used in the study on *Ptychadena*, both ratios and residuals were used to compare differences in results. For *Phrynobatrachus*, a range of values was reported, so minimum, maximum, and mean values were used for analyses.

Partial least squares (PLS) regressions were used to examine the relationship between skin thickness and environmental variables for the multivariate Czopek and Chapter 5 datasets. PLS summarises multiple variables, similar to traditional multiple regressions, but it has the advantage that it is able to cope with multicollinearity (Abdi, 2010), which is expected in these datasets given the similarity between certain environmental (e.g., precipitation in the wettest month and precipitation in the wettest quarter) and skin variables (e.g., dorsal pectoral total skin thickness and dorsal pectoral compact dermis thickness).

The 'plsreg1' and 'plsreg2' functions in the package 'plsdepot' (Sanchez & Sanchez, 2012) were used for regressions of environmental variables against body size and the multivariate skin datasets, respectively. Four species in the Czopek dataset, *Lithobates catesbeianus, L. sphenocephala*, *L. grylio*, and *Rhyacotriton olympicus* did not have data for all variables so were excluded from PLS regressions because they cannot handle missing data. Data from the Chapter 5 dataset was strongly correlated with body size (Chapter 5). Therefore, analyses were run with raw values, size-corrected values (residuals and simple ratios), and body size alone to determine the effects of body size on the results. Body size from the Czopek dataset was also regressed against the environmental variables.

The PLS regression was first run to identify the number of components that should be used. Only components with Q^2 values above zero were used because values that fall below zero indicate that the model has overfit the data (Abdi, 2010). These values are calculated by cross-validation using a 'leave one out' approach in the 'plsreg1' and 'plsreg2' functions. The strength of the model was assessed by the amount of variation explained by the useful components and, if two or more

components had Q^2 values above zero, the correlation between the actual skin thickness values and those predicted by the model. Unfortunately, the 'plsreg1' and 'plsreg2' functions do not allow for less than two components to be produced, so predicted skin values for models recovering less than two important components would be produced after overfitting of the data had occurred. To determine which anatomical variables related to which environmental variables, VIP (Variable of Importance for Projection) scores that were above one were considered significant contributors for that component (Mehmood et al., 2012) and were compared to skin measurements that loaded highly on the component. When body size was regressed against environmental variables, the strength of the correlation between body size and individual environmental variables on each important component was used instead of VIP scores.

RESULTS

Re-evaluation of Le Quang Trong (1971, 1975) datasets

Correlations between skin thickness and body size were not significant for the *Ptychadena* species, despite the use of ratios in the study by Le Quang Trong (1975). Ratio and residual values for relative skin thickness were both significantly correlated with habitat type. However, ratios recovered a significantly positive relationship in which forest species have thicker skin than savannah species, whereas residuals recovered the opposite result. Log-transformed skin thickness was not correlated with habitat type, suggesting skin thickness is not driving this correlation. Instead, body size was significantly negatively correlated with habitat type (i.e., forest species are smaller than savannah species).

Table 6-1. Correlations of environmental and skin data from Le Quang Trong (1975) for the genus *Ptychadena.*

	correlation	*p*-value
Body size vs. Skin thickness	0.49	0.33
Body size vs. Environment	-0.93	0.007
Skin thickness vs. Environment	-0.26	0.62
Ratios vs. Environment	0.96	0.003
Residuals vs.Environment	-0.92	0.009

Unlike in *Ptychadena*, minimum, maximum, and mean body size and skin measurement values for *Phrynobatrachus* were positively correlated (Table 6-2).

However, body size, skin thickness, and relative skin thickness (ratios or residuals) were not correlated with habitat type when all species were included. When semi-aquatic species were removed, correlations using ratios and residuals were significantly correlated with habitat types. Ratios were negatively correlated with habitat types (thinner skin in humid forest species) whereas residuals were positively correlated with habitat type (thinner skin in dry savannah species).

Table 6-2. Correlations using environmental and skin data from Le Quang Trong (1971) for the genus *Phrynobatrachus* using all species (all) and only non-aquatic species (terr).

	Mean		High values		Low values	
	Corr.	*p*-value	Corr.	*p*-value	Corr.	*p*-value
Body size vs. Skin thickness	0.9	0.006	0.84	0.02	0.93	0.002
Body size vs. Environment (all)	0.31	0.5	0.44	0.32	0.12	0.8
Skin thickness vs. Environment (all)	0.16	0.73	0.11	0.81	0.17	0.71
Ratios vs. Environment (all)	0.39	0.38	-0.63	0.13	0.03	0.94
Residuals vs. Environment (all)	0.39	0.39	0.65	0.11	-0.1	0.83
Body size vs. Environment (terr)	0.56	0.18	0.69	0.08	0.4	0.38
Skin thickness vs. Environment (terr)	0.35	0.44	0.31	0.5	0.36	0.43
Ratios vs. Environment (terr)	0.68	0.09	-0.85	0.02	-0.3	0.51
Residuals vs. Environment (terr)	0.6	0.15	0.8	0.03	0.17	0.72

Environmental Data

The PLS regression using the full Czopek dataset recovered zero important latent variables with all Q^2 values below zero. When body size (body weight in grams) was used alone, one latent variable had a positive Q^2 value and explained only 28% of the variation. Mean annual temperature, mean diurnal range, isothermality, maximum temperature of the warmest month, mean temperature of the driest quarter, and precipitation seasonality had the highest positive loading values on this axis (Table 6-3).

The Chapter 5 dataset with the urodele removed recovered only one important component that summarised 36% of the variation. On this axis, total skin thickness and compact dermis thickness measures had the highest loads and isothermality, mean temperature in the driest quarter, precipitation in the driest month, precipitation

seasonality, and precipitation of the driest quarter all had VIP values above one. Of these, precipitation of the driest month and precipitation of the driest quarter were loaded negatively on the first component, and the others were loaded positively. When the urodele was included but the number of variables was reduced, one component was recovered as important and explained 34% of the variation. The same variables had VIP scores above one in this analysis and again total skin thickness and compact dermis thickness measurements loaded strongly on the first component.

Table 6-3. Loadings for the PLS regressions of body size against environmental variables and the percentage of variance explained in each component (C).

	Czopek	Anurans and urodeles		Only anurans	
Variable	C1	C1	C2	C1	C2
Annual mean temp.	0.4	0.29	-0.25	0.28	-0.26
Mean diurnal range	0.36	0.07	0.37	0.06	0.36
Isothermality	0.33	0.31	-0.25	0.3	-0.26
Temp. seasonality	-0.17	-0.28	0.28	-0.27	0.29
Max temp. warmest month	0.33	0.14	0.3	0.15	0.28
Min temp. coldest month	0.23	0.28	-0.29	0.27	-0.29
Temp. annual range	-0.03	-0.25	0.29	-0.25	0.29
Mean temp. wettest ¼	0.25	0.14	-0.26	0.13	-0.26
Mean temp. driest ¼	0.33	0.32	-0.2	0.32	-0.21
Mean temp. warmest ¼	0.3	0.17	0.22	0.18	0.2
Mean temp. coldest ¼	0.27	0.28	-0.29	0.27	-0.29
Annual precipitation	-0.14	0.13	-0.3	0.13	-0.3
Precipitation of wettest month	0.09	0.27	-0.007	0.28	-0.02
Precipitation of coldest month	-0.18	-0.31	-0.19	-0.32	-0.2
Precipitation seasonality	0.34	0.35	0.15	0.36	0.14
Precipitation of wettest ¼	0.08	0.27	-0.03	0.27	-0.04
Precipitation of driest ¼	-0.18	-0.31	-0.19	-0.32	-0.19
Precipitation of warmest ¼	-0.07	0.13	-0.3	0.13	-0.3
Precipitation of coldest ¼	-0.12	0.11	-0.28	0.13	-0.28
Body Size	0.25	0.29	0.14	0.31	0.13
% Explained	0.28	0.61	0.13	0.59	0.1

When regressions or ratios are used, no latent variables had Q^2 values above zero. However, when body size (snout-vent length in mm) was used the dataset with the urodele included, two components had positive Q^2 values and summarised 61% of the variation. Precipitation seasonality loaded most highly on the first component; precipitation of the driest month and precipitation of the driest quarter had the lowest negative load on the first component (Table 6-4). The predicted values correlated significantly with the actual body size values ($r^2 = 0.81$; $p < 0.001$). When the urodele

was excluded, two components were again recovered as important and summarised 59% of the variation. The same variables loaded highest on the first component as in the analysis when urodeles were included. The predicted values are significantly correlated with the actual body size values ($r^2 = 0.83$; $p < 0.001$).

Table 6-4. VIP scores for environmental variables and loadings for skin variables for PLS regressions of skin thickness measures against environmental variables on each component (C). VIP scores above 1 are bolded.

	Only anurans	Anurans and urodeles
VIP	C1	C1
Annual mean temp.	0.77	0.74
Mean diurnal range	0.59	0.87
Isothermality	**1.09**	**1.03**
Temp. seasonality	0.62	0.52
Max temp. warmest month	0.59	0.87
Min temp. coldest month	0.58	0.48
Temp. annual range	0.76	0.61
Mean temp. wettest ¼	0.20	0.17
Mean temp. driest ¼	**1.12**	**1.11**
Mean temp. warmest ¼	0.50	0.74
Mean temp. coldest ¼	0.59	0.50
Annual precipitation	0.26	0.52
Precipitation of wettest month	0.87	0.80
Precipitation of coldest month	**1.94**	**1.74**
Precipitation seasonality	**2.04**	**2.10**
Precipitation of wettest ¼	0.84	0.76
Precipitation of driest ¼	**1.93**	**1.85**
Precipitation of warmest ¼	0.19	0.42
Precipitation of coldest ¼	0.34	0.62
Loadings		
Dorsal Total	0.28	0.27
Dorsal Epidermis	0.14	0.11
Dorsal Spongy Dermis	0.17	0.15
Dorsal Compact Dermis	0.34	0.35
Ventral Total	0.28	0.27
Ventral Epidermis	0.13	0.15
Ventral Spongy Dermis	0.20	0.16
Ventral Compact Dermis	0.35	0.34
Thigh Total	0.31	-
Thigh Epidermis	0.16	-
Thigh Spongy Dermis	0.24	-
Thigh Compact Dermis	0.34	-
% Explained	0.36	0.34

DISCUSSION

A link between amphibian skin anatomy and ecology has been suspected for decades (Czopek, 1965; Roth, 1973), yet a relationship has never been quantitatively assessed. Previous studies have suggested that species that inhabit drier or less complex habitats will have thicker skin than species that live in moister habitats (Le Quang Trong, 1975), whereas others have suggested that thinner skin and a lack of water-absorbing vercuae are traits in arid-adapted populations of the same species (Canziani & Cannata, 1980; Navas, Antoniazzi & Jared, 2004). The present study quantitatively examines this relationship using a combination of datasets from current and previously published work.

In the genus *Ptychadena*, it was suggested that relative skin thickness was related to habitat complexity and moisture (Le Quang Trong, 1975), but in the results found here, body size was negatively correlated with moisture in a habitat and skin thickness was not. Surprisingly, skin thickness was also not correlated with body size, a correlation that was found in a previous study using an interspecific dataset to detect allometric patterns in skin thickness (Chapter 5) and in *Phrynobatrachus*. It could be that small sample size (n = 5) and coarse data collection methods masked an allometric pattern in these data or correlations between skin anatomy and ecology. However, the limited dataset suggests that larger species of *Ptychadena* live in drier habitats and that these species do not have relatively thicker skin than the smaller species from forests.

Skin thickness was correlated with body size in *Phrynobatrachus*, but neither body size nor skin thickness was correlated with habitat type. It was only when size-corrected values were used for a dataset that contained only terrestrial and semi-aquatic species that significant relationships were recovered, but the two different size-correction methods (simple ratios and regression residuals) recovered opposing results. Given that residuals are widely accepted as a more appropriate method of correcting for size in morphometric data within the same group (Atchley, 1978), it is most probable that a positive relationship between relative skin thickness and habitat type (coded based on humidity from dry savannah to humid forest) also exists in *Phrynobatrachus*. Therefore, species that live in drier habitats have relatively thinner skin than species that live in more humid habitats, which is similar to the pattern

observed between populations of *Rhinella schneideri* (Navas, Antoniazzi & Jared, 2004).

In the multivariate datasets, results were less clear. The Czopek dataset, which contained primarily vasculature measurements, did not recover any important components (i.e., Q^2 for all components < 0). The Chapter 5 dataset only recovered important components when size uncorrected data were used, and the models only explained <50% of the variation in the skin dataset. Because the skin measurements used in this dataset were shown to correlate with body size (Chapter 5), residual and ratio values were regressed against environmental data. These regressions again recovered no important components. The PLS regressions that used body size alone, however, found correlations between the environmental dataset and body size for both the Czopek and Chapter 5 datasets, although the model using body size from the Czopek dataset explained <50% of the variation.

The environmental variables that correlated with either skin thickness or body size varied among the analyses. Body size from the Czopek dataset was most influenced by annual mean temperature. However, body size in the Chapter 5 dataset was most influenced by precipitation variables. The models (either without *Notophthalmus viridiscens* or without ventral thigh thickness measures) using size uncorrected skin data from Chapter 5 found correlations between the same skin layers and environmental variables, despite being different in species composition and number of skin variables. This similarity suggests that these relationships are robust to sampling. Many of the environmental variables that strongly related to body size or skin thickness were from the dry parts of the year (month or quarter) and were inversely related to body size or skin thickness (i.e., higher skin thickness correlated with less precipitation in the driest month or quarter). Therefore, like in *Ptychadena* and unlike in *Phrynobatrachus*, body size and skin thickness measures are smaller for species from wetter habitats.

Although the results from the *Phrynobatrachus* regressions seem counterintuitive and do contradict the results from the larger datasets, they are consistent with other studies on relative skin thickness in phylogenetically restricted contexts. Populations of the Cururu toad (*Rhinella schneideri*) that live in arid regions have thinner skin that has fewer vercuae than populations that live in forests (Navas, Antoniazzi & Jared, 2004). There is no known report of body size differences between the populations, so it is unclear how (or if) body size may affect this result.

However, a comparable difference between populations is seen in the Argentina horned frog (*Ceratophrys ornata*), in which populations from the arid region have smoother skin and have lower rates of both EWL and water uptake (Canziani & Cannata, 1980). Differences in skin thickness were not examined between *C. ornata* populations, but the parallel differences in skin sculpting suggests the population of *Rhinella schneideri* from the arid Caatinga may also have lower rates of EWL and water uptake (i.e., are less permeable) than the Atlantic Forest population. In the American bullfrog, *Lithobates catesbeianus*, specimens collected in summer months are outliers that fall below the regression line in an interspecific regression of skin thickness and body size, whereas specimens from winter months that have 'thickened' skin fall within the range of variation (Chapter 5). This species is more terrestrial in summer months compared to when it overwinters completely submerged underwater (Tattersall & Ultsch, 2008). Taken together, these results suggest that relative skin thickness is directly proportional to relative moisture in the preferred habitat either within a species or among closely related species.

The results presented here do not support a clear interspecific relationship between skin thickness and ecology among all species examined, which has been found in at least one previous (Drewes et al., 1977). In phylogenetically restricted datasets, relatively thinner skin seems to be present in populations that inhabit drier habitats. However, the interspecific data suggests that species are larger and therefore have thicker skin in drier habitats. Large body size decreases the surface area to volume ratio, so larger species can more easily avoid evaporative water loss (Tracy, Christian & Tracy, 2010) and might be expected particularly in arid-adapted species. However, species that are vastly different in body size are sympatric across much of their ranges (e.g., *Lithobates catesbeianus* and *Pseudacric crucifer*), and other studies on environmental parameters and body size in amphibians have not consistently recovered a relationship between these variables (Olalla-Tárraga & Rodríguez, 2007; Adams & Church, 2008). Microhabitat selection is important for many amphibian species, and data used in the analyses here were very coarse. These factors could explain why a strong relationship between skin thickness and environmental variables was not recovered. Alternatively, although skin thickness is predicted to relate to ecology (Czopek, 1965; Roth, 1973), other variables, such as the development of subcutaneous vasculature and fat content of the skin, may be more highly correlated

with ecological niche. Unfortunately, data on either of these variables are scarce for amphibians and are beyond the scope of this study.

Another explanation for the results lies in the phylogenetic history of the species examined. The large datasets utilised here cover a phylogenetically broad range of taxa that also span a wide range of ecologies, but most of them are native to temperate regions. Given the diverse strategies amphibians use to cope with environmental challenges (Toledo & Jared, 1993), detecting general ecomorphological patterns across higher clades may not yield meaningful results, as suggested by the contradicting pattern uncovered in the phylogenetically restricted datasets for *Ptychadena, Phrynobatrachus*, *Lithobates catesbeianus,* and *Rhinella schneideri* (Le Quang Trong, 1971, 1975; Navas, Antoniazzi & Jared, 2004). Skin thickness is one factor that affects physiological function of the skin by increasing resistance to liquid and gas exchange. However, tissue composition also plays a large part in determining the resistance of tissues. The difference between intra- and interspecific results may reflect differences between local adaptations that rely on relatively plastic skin thickness and species-specific adaptations that alter the environmental niche of the species through evolutionary changes in tissue composition or the evolution of apomorphic integumentary structures (e.g., iridiophores; Drewes et al., 1977; Kobelt & Linsenmair, 1986). Within a species or closely related species, altering skin thickness or skin sculpting among individuals or populations may be more evolutionarily advantageous than altering tissue composition, especially for species that have loose habitat requirements. Clearly, more studies are needed to better understand these relationships because currently very few quantitative inter- or intraspecific studies on amphibian skin exist. Documenting the prevalence of seasonal changes in skin thickness, population-level differences, and variation within genera or families are critical first steps to unravelling these seemingly contrasting results.

The results presented here demonstrate that the link between ecology and skin anatomy is not as clear as has been assumed in previous studies. Across distantly related species, body size is the best predictor of skin thickness (Chapter 5) and influenced many relationships between skin thickness and environmental parameters. Among closely related species or within a species, differences in skin thickness correspond with differences in habitat use or habitat preference in which thinner skin is present in individuals from drier habitats. However, datasets containing a

phylogenetically broad sample of taxa do not show obvious correlations with environmental variables and suggest that amphibian skin thickness does not correlate with interspecific differences in ecology at such coarse scales. Although these results are difficult to interpret given the lack of comparable studies, they highlight a great potential for future research on determining the ecological significance of amphibian skin.

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CHAPTER SEVEN: CONCLUSIONS

Amphibian skin anatomy has been studied for over 150 years (Ascherson, 1840). The work presented in this thesis has critically reviewed and analysed known sources of anatomical variation in the skin thickness of amphibians because of a widely held presumption of a causal link between skin thickness and physiology or ecology (Czopek, 1965; Roth, 1973). This presumed link has never been quantitatively tested, yet it has been implicated as a factor contributing to ongoing amphibian population declines on the basis that their skin makes amphibians more sensitive to environmental disturbances (Wake & Vredenburg, 2008). Despite the long history of research on amphibian skin anatomy (Ascherson, 1840), these sources of anatomical variation and their potential ubiquity were only recognised more recently (Kun, 1959; Kobelt & Linsenmair, 1986; Greven, Zanger & Schwinger, 1995; Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001; Wenying et al., 2011), along with the recognition that physiological processes differ across regions of the skin, in response to environmental parameters, as well as seasons of the year (Lillywhite, 1971; Roth, 1973; Pasanen & Koskela, 1974; Christensen, 1974; Byrne & White, 1975; Geise & Linsenmair, 1986; Hillyard, Hoff & Propper, 1998).

Among the sources of variation examined here, it has been shown that sexual dimorphism in skin anatomy is better explained as being controlled by body size because size-corrected values of skin thickness for males and females did not differ (Chapters 2, 3, 4). This result is consistent with studies comparing skin thickness for species that are not sexually dimorphic in body size (Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001). Conversely, seasonal differences in skin thickness are not only present in some species (but not all: Chapter 3), but were shown to have the most influential effect on interspecific analyses of skin thickness (Chapter 5). Previous studies only sampled two (Kobelt & Linsenmair, 1986) or three (Kun, 1959) times of the year to detect seasonal skin changes, and the results here support this methodology for detecting the presence of seasonal skin thickening in interspecific analyses.

Surprisingly, skin thickness was not correlated with ecology as defined by environmental parameters across species (Chapter 6). However, relationships were detected at low taxonomic levels (Chapter 6). It is therefore hypothesized that there is an ‘ideal’ relative skin thickness necessary to maintain mechanical support given that

the compact dermis, the tissue layer most responsible for the mechanical properties of the skin (Greven, Zanger & Schwinger, 1995; Zanger, Schwinger & Greven, 1995; Schwinger, Zanger & Greven, 2001) is most strongly correlated with body size, and that this thickness is maintained by the evolution of increased numbers of iridiophores, melanosomes, or other integumentary structures to compensate for any physiological disadvantage of this skin thickness. However, in instances where habitat occupation varies between populations of a species, or speciation has occurred relatively recently, modifying skin thickness to alter the resistance against the transportation of substances (e.g. water) across the skin may be a strategy to cope with frequent environmental fluctuations until selective pressure is strong enough, or has been present for long enough, to select for structural adaptations in skin morphology to environmental pressures.

Limitations

The results presented here have addressed sexual, seasonal, and body size sources of variation among amphibians for the first time. However, most of the data used here were from anurans and only one urodele was sampled for the interspecific regression studies. Anurans are taxonomically and ecologically more diverse than either urodeles or gymnophionans, so the results from this work will be applicable to a greater proportion of living amphibians. The sources of variation tested here have not been examined in urodeles [with the exception of skin thickness changes in male newts when they develop breeding ornamentation (Czopek, 1959)] or in gymnophionans, so it is unknown if any of these factors should be considered when sampling species in these clades. There are many differences among the three major groups of living amphibians (Duellman & Trueb, 1986), so it is likely that at least some of the results here may not apply across all groups. For example, although a strong relationship between skin thickness and body size was recovered, the dataset used was composed primarily of anurans, which also show a strong allometric relationship for rates of certain physiological processes that involve the skin compared to datasets containing members of all three groups or urodeles alone (Pruett, Hoyt & Stiffier, 1991). It should also be noted that, although this work contributes to filling a gap in our knowledge about the functional morphology and evolution of skin thickness in amphibians, the hypotheses proposed here are ultimately limited by a lack of

corroborating physiological and ecological data that are required to form a more holistic understanding of these patterns.

Future directions

This thesis addressed some of the sources of variation in skin anatomy; however, others should be considered in future studies. Obvious sources of potential variation are across latitudinal or altitudinal gradients for species that are wide-ranging. Although no study has ever explicitly tested for these factors as sources of variation in skin thickness, they may influence skin thickness given the differences documented between populations from different habitats (Navas, Antoniazzi & Jared, 2004). Latitudinal gradients may also affect seasonal changes in skin thickness for species whose range covers regions that experience different degrees of seasonality.

An additional source of anatomical variation that requires attention is polymorphic skin glands, as discussed in Chapter 2. Skin secretions are known to be effective in limiting evaporative water loss (Lillywhite, 1971; Toledo & Jared, 1993; Barbeau & Lillywhite, 2005) but the secretions of these polymorphic skin glands are not well understood and there is no consensus on the best practice for classifying these glands. Given the attention paid to understanding strategies for limiting evaporative water loss and the impact of polymorphic skin glands in some species, this area of research would be a useful next step in applying the results of studies focused mostly on phyllomedusine treefrogs more broadly.

Along with the addition of better ecological and physiological data, as mentioned above, experimental data would provide useful insights into the mechanisms behind some of these sources of variation, particularly population-level and seasonal differences. Tadpoles reared under different environmental conditions or frogs exposed to varying environmental conditions and sampled for differences in skin thickness would help to determine if intrinsic or extrinsic factors affect skin thickening in these contexts.

Ultimately, this body of work has addressed only some of the questions regarding variation in skin thickness in amphibians. It has also opened up new opportunities to expand our current understanding of this structure even further. Amphibians are often used as model organisms for understanding the transition of vertebrates onto land (e.g. Kawano & Blob, 2013) and studies like these can offer insights to the similarity and differences between modern amphibians and early

tetrapods to help refine hypotheses for this major transition in vertebrate history. Given the ongoing declines of amphibian species around the world (Stuart et al., 2004; Wake & Vredenburg, 2008), understanding how skin morphology and physiology are related to each other and to ecology is essential for predicting the response of species to environmental stressors or disturbances. The more information that is understood concerning the biology of amphibians, the easier it will be to predict their responses to environmental changes, the disproportionate extinction risk for the group, and develop strategic measures to ensure the future survival of this ancient clade of vertebrates.

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Appendix: Skin thickness measurements for specimens used in this thesis.

Species	Specimen Number	Sex	SVL	Dorsal Total	Thigh Total
Anaxyrus cognatus	FMNH 259917	M	80.59	456.01	201.64
Anaxyrus cognatus	FMNH 259916	F	75.81	478.09	185.57
Rana arvalis	FMNH 234272	F	53.22	195.74	146.64
Acris crepitans	FMNH 284081	F	24.35	77.09	52.17
Acris crepitans	FMNH 279689	M	22.06	82.39	59.13
Notopthalmus viridiscens	FMNH 275248	M	42.03	200.50	
Notopthalmus viridiscens	FMNH 275241	F	41.49	160.56	
Xenopus laevis	FMNH 251393	F	60.77	261.62	172.02
Xenopus laevis	FMNH 251398	M	51.82	383.28	221.88
Scaphiopus couchii	FMNH 257215	M	73.53	245.82	108.91
Pseudacris crucifer	FMNH 259639	F	25.77	48.87	53.61
Pseudacris crucifer	FMNH 267573	M	24.62	65.15	42.85
Pseudacris crucifer	FMNH 271427	F	24.2	57.61	38.78
Pseudacris crucifer	FMNH 272592	F	28.08	119.00	113.50
Pseudacris crucifer	FMNH 275291	M	25.39	44.00	
Pseudacris crucifer	FMNH 276430	M	26.08	83.18	56.36
Pseudacris crucifer	FMNH 276433	M	23.13	100.15	81.71
Pseudacris crucifer	UMMZ 243627	M	26.22	51.86	52.28
Pseudacris crucifer	UMMZ 243630	F	27.87	102.17	82.48
Pseudacris crucifer	UMMZ 243621	M	22.32	57.26	58.99
Pseudacris crucifer	USNM 535668	F	28.58	40.89	42.07
Pseudacris crucifer	USNM 535686	M	28.91	71.37	52.65
Lithobates pipiens	FMNH 252640	M	59.32	294.64	233.51
Lithobates pipiens	FMNH 269427	M	56.58	152.73	163.43
Lithobates pipiens	FMNH 275585	F	76.03	490.06	222.65
Lithobates pipiens	FMNH 279403	F	64.85	169.16	169.36
Lithobates pipiens	FMNH 279434	F	64.93	325.99	292.80
Lithobates pipiens	FMNH 279712	M	69.32	252.48	271.44
Lithobates pipiens	FMNH 259780	J	43.39	91.56	59.65
Lithobates pipiens	UMMZ 179173	M	39.98	124.33	136.03
Lithobates pipiens	UMMZ 218549	J	35.39	89.29	86.20
Lithobates pipiens	UMMZ 218553	J	32.79	63.63	78.17
Lithobates pipiens	UMMZ 218554	J	29.03	67.97	82.47
Lithobates pipiens	UMMZ 243074	M	55.89	192.10	157.42
Lithobates pipiens	UMMZ 243073	F	61.52	306.56	188.14
Lithobates catesbeianus	FMNH 278931	F	95.52	315.40	205.15
Lithobates catesbeianus	FMNH 275502	F	117.46	359.02	187.23
Lithobates catesbeianus	FMNH 271623	F	81.91	200.65	232.65
Lithobates catesbeianus	FMNH 271598	M	98.63	422.62	307.60
Lithobates catesbeianus	FMNH 267577	F	101.14	373.99	218.05
Lithobates catesbeianus	FMNH 259651	M	133.5	477.10	441.63
Lithobates catesbeianus	USNM 347870	F	102.28	215.82	132.60

Lithobates catesbeianus	USNM 514929	F	118.33	182.70	149.86
Lithobates catesbeianus	USNM 536865	M	111.65	203.95	93.19
Lithobates catesbeianus	FMNH 270102	J	66.38	131.01	118.49
Lithobates catesbeianus	FMNH 281068	F	86.99	73.25	51.30
Litoria infrafrenata	MFN 54637	F	105.09	264.42	318.58
Litoria infrafrenata	MFN 54638	F	101.61	222.02	254.18
Litoria infrafrenata	MFN 54641	M	71.12	118.26	124.80
Litoria infrafrenata	MFN 54642	M	66.52	181.56	125.59
Litoria infrafrenata	MFN 54643	F	91.92	207.59	221.13
Litoria infrafrenata	MFN 54644	M	72.1	206.29	212.45
Litoria infrafrenata	MFN 54646	F	96.43	341.71	391.40
Litoria infrafrenata	MFN 54647	F	90.23	247.89	252.79

Ventral Total	Dorsal Epidermis	Thigh Epidermis	Ventral Epidermis	Dorsal Spongy Dermis	Thigh Spongy Dermis
316.03	28.99	41.37	43.34	148.80	60.21
308.65	43.60	58.87	49.27	148.44	37.11
212.42	44.76	27.97	72.08	96.18	72.36
79.67	10.36	21.61	22.95	32.13	12.80
70.53	13.26	13.04	14.66	27.80	23.13
163.05	31.64		20.29	101.59	
89.44	34.23		17.56	92.93	
234.06	23.89	25.67	27.62	64.22	46.83
327.84	32.37	34.53	42.14	146.79	61.14
234.92	21.56	14.55	25.06	119.09	63.85
84.74	6.15	16.40	18.50	17.11	18.41
50.42	15.00	17.30	19.20	31.29	14.61
30.67	9.10	15.30	14.60	25.05	8.55
79.19	13.90	17.50	15.80	54.38	43.38
77.88	7.72		15.80	18.79	
79.87	9.70	11.10	18.50	46.99	26.06
62.01	17.30	25.90	23.40	52.02	30.10
48.12	11.10	18.10	21.00	18.61	19.90
51.45	16.50	20.30	13.70	54.98	28.20
47.69	15.30	27.80	22.90	22.89	15.29
37.62	13.20	14.10	12.00	16.72	13.19
49.83	7.62	11.60	9.20	34.96	19.73
131.38	52.30	73.40	47.70	138.46	55.54
127.46	24.80	41.50	40.90	38.97	53.90
98.77	53.90	44.20	25.50	266.10	44.04
117.49	28.00	41.10	32.60	37.38	47.69
193.98	44.50	70.40	49.20	83.50	91.98
127.47	49.50	75.40	50.50	61.82	29.59
45.15	29.60	30.20	28.60	28.19	13.95
110.76	26.70	40.70	37.50	44.11	38.36
80.11	12.50	29.00	26.30	18.83	27.78
43.19	10.00	25.10	17.50	12.49	26.86
68.32	14.10	23.90	26.60	14.80	33.00
80.00	24.00	38.50	25.00	44.26	53.77
123.07	55.60	57.10	31.80	141.12	66.51
160.63	44.10	42.00	32.10	84.80	64.65
129.35	22.50	27.30	17.40	60.62	34.43
129.00	29.10	37.60	26.60	55.22	105.09
212.62	63.70	67.90	37.50	135.49	92.12
225.90	52.10	49.60	51.60	88.94	42.06
245.42	22.50	50.60	39.30	84.72	82.88
112.35	19.00	25.90	17.10	46.13	36.43
140.53	17.20	29.20	22.50	35.82	31.35

92.86	14.60	16.80	12.20	56.13	20.11
106.33	35.00	41.60	24.60	53.85	36.94
50.82	12.20	13.10	10.40	15.89	10.04
413.79	22.17	32.38	61.15	65.31	125.60
348.89	27.15	42.17	52.46	52.00	94.03
134.31	17.40	28.00	33.04	31.79	58.60
175.88	25.30	32.08	43.98	56.47	50.97
264.01	27.72	39.53	51.29	48.56	97.07
220.47	31.97	35.84	44.47	63.58	67.93
397.69	22.85	42.87	46.00	91.92	183.60
260.83	29.66	41.93	56.46	55.20	97.87

Ventral Spongy Dermis	Dorsal Compact Dermis	Thigh Compact Dermis	Ventral Compact Dermis
82.25	326.56	93.38	171.49
70.84	337.36	85.21	215.98
96.35	49.37	51.92	36.82
22.38	29.12	22.68	27.79
27.23	38.23	28.31	21.71
75.14	58.95		60.68
43.34			
71.55	161.83	84.63	126.65
155.39	197.71	125.69	114.11
109.10	110.13	45.77	99.54
41.47	18.12	22.20	25.85
17.44	16.12	11.51	11.31
6.56	14.14	13.54	10.47
30.99	29.59	24.85	25.67
11.34	13.34		18.41
42.75	20.90	18.50	20.15
21.32	22.51	25.79	23.94
18.27	18.33	17.73	17.81
20.64	29.78	30.78	13.44
12.85	14.99	14.41	12.85
12.26	17.68	17.75	15.35
24.94	20.72	18.14	12.19
44.61	130.02	114.83	49.19
52.93	81.30	75.01	49.69
17.02	143.47	119.17	54.58
32.59	108.07	79.13	47.55
56.75	194.10	104.38	90.36
79.54	151.23	104.52	42.53
15.10	42.99	27.91	18.54
40.82	59.08	43.48	51.01
26.89	61.78	27.74	31.64
11.10	44.03	30.83	16.39
20.19	36.37	33.57	20.20
22.28	119.40	60.69	36.60
32.87	99.92	63.99	47.36
38.16	177.99	98.49	88.66
18.92	275.61	122.47	81.30
46.52	111.29	83.27	68.07
74.15	222.29	126.23	105.32
55.58	236.69	116.41	103.54
57.52	349.45	296.69	126.00
29.52	146.00	64.71	60.12
35.70	127.49	81.79	73.59

17.53	137.40	54.23	55.08
37.16	38.30	37.90	32.83
10.19	45.84	27.24	29.81
137.60	185.20	145.20	155.70
95.30	143.10	112.00	166.18
37.42	82.21	55.62	77.52
75.22	78.89	74.58	80.31
83.72	125.40	107.70	135.00
79.47	104.50	98.57	92.53
106.80	194.70	188.90	184.80
92.02	152.90	125.80	124.80