CONSPICUOUS COLORATION MAY FUNCTION TO DETER AVIAN PREDATORS IN APPALACHIAN SALAMANDERS

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by
MONICA MARIE WINEBARGER

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APPROVED BY:

Lynn M. Siefferman, Ph.D.
Chairperson, Thesis Committee

Michael S. Osbourn, Ph.D.
Member, Thesis Committee

Carol M. Babyak, Ph.D.
Member, Thesis Committee

Zack E. Murrell, Ph.D.
Chairperson, Department of Biology

Max C. Poole, Ph.D.
Dean, Cratis D. Williams School of Graduate Studies

Abstract

CONSPICUOUS COLORATION MAY FUNCTION TO DETER AVIAN PREDATORS IN APPALACHIAN SALAMANDERS

Monica Marie Winebarger
B.S. Appalachian State University
M.S. Appalachian State University

Chairperson: Lynn Siefferman

Amphibians are renowned for the variation in the color and patterns of their skin, both within and between species. In the southern Appalachians, three closely related salamander species (*Plethodon* spp.) display vastly different coloration; yet, the signaling function of integument coloration is not well studied. *Plethodon yonahlossee* have a large red dorsal patch that covers ~40% of their dorsal region, while *P. cylindraceus* are black with white spots, and *P. montanus* are uniformly gray. *Ambystoma maculatum* salamanders also occur in sympatry with these species and display conspicuous yellow spots on dark bodies. Variation in integument coloration within and among species offers opportunities to explore hypotheses of adaptive signaling. Conspicuous coloration may serve as an aposematic signal in which the conspicuous coloration of prey is used to signal unpalatability to potential predators. It is hypothesized that larger body size, larger integument patterns, and larger group size increase the efficacy of aposematic signals. There is evidence that the integument secretions of species in both *Plethodon* and *Ambystoma* are unpalatable to avian predators. Thus, I hypothesize that the integument coloration of *P. yonahlossee* and *A. maculatum* is an aposematic signal to passerine avian predators. Here, I use three complementary approaches to investigate the potential for aposematic signaling in conspicuous salamanders. First, I used avian vision models to quantify the conspicuousness of *P. yonahlossee* and *A. maculatum* to avian predators. I found that both species are distinguishable from typical forest backgrounds and are chromatically distinct from two duller sympatric heterospecifics (*P. montanus* and *P. cylindraceus*). Second, I use plasticine models of *P. yonahlossee* and *P. montanus* to experimentally test whether predators depredate conspicuously colored models less frequently than dull models. Predation rates on grey models were significantly higher compared to that of red models, suggesting that the red dorsal coloration of *P. yonahlossee* is interpreted as a warning signal that deters predation. Third, I use a comparative approach to investigate associations between body size and conspicuous coloration in the genera *Ambystoma* and *Plethodon*. I found that increased conspicuous coloration co-evolved with increased body size in *Ambystoma*, but that evolution in *Plethodon* salamanders has favored a negative relationship between these two traits. These results suggest that both *P. yonahlossee* and *A. maculatum* possess traits consistent with aposematism, but more information on unpalatability is needed for each species to further explore this hypothesis.

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FOREWORD

This manuscript has been formatted according to the style guide for *Copeia*, a peer-reviewed journal focused on ichthyology and herpetology.

CHAPTER 1

General Introduction

Biological communication is one of the most well studied aspects of animal behavior (Wallace, 1867; Dawkins and Guilford, 1991; Stevens, 2015). Communication involves using either morphology or behavior of an organism to signal information (i.e. the signaler) to another organism (i.e. the receiver) that alters the receivers' behavior in some way (reviewed in Scott-Phillips, 2008). Animals use a diversity of signals to convey a variety of information, from individual quality to unpalatability (Dale, 2006). Signals become established in a population when they increase an individual's own fitness, the fitness of the individual's kin, or the fitness of descendants in later generations (Owren et al., 2010).

Integument coloration is widely used as a signal by both plants and animals. For example, conspicuously colored flowers attract pollinators, conspicuously colored avian plumage attracts mates, and some animal coloration deters predators, either through concealment or advertisement of toxicity (Vorobyev et al., 1999). The conspicuousness of a color signal is dependent on the visual system of the receiver, the reflectance or patterning of the integument, the light environment, and the background; the effect is that more conspicuous signals are those in which the integument is more easily distinguished from the background noise (Siddiqi et al., 2004; Uy and Endler, 2004; Endler and Mielke, 2005).

When conspicuous coloration in potential prey items is coupled with a secondary defense, such as venom or poison, color is thought to alert predators to the unprofitability of the prey in a phenomenon known as aposematic signaling (Summers et al., 2015). Cott (1940) suggested that the colors red, yellow, and white, often in combination with black, are used in aposematic signaling because they are easily detected and recalled; thus, they increase predator recognition of signals of unpalatability. Predators either innately avoid certain conspicuous colors (Smith, 1975) or learn over time to associate the color with the defense, and thus alter their behavior to attack more profitable prey (Mappes et al., 2005). Among organisms that have the potential to exhibit warning color (i.e. are unpalatable), these signals are expected to be more conspicuous on larger organisms (Gamberale and Tullberg, 1996) and are expected to be more effective among gregarious species which tend to occur in high densities (Fisher, 1930; Cott, 1940; Edmunds 1974). Both body size and gregariousness can amplify the conspicuousness of the color signal, increase predator recognition, and increase initial aversion in naïve predators (Gamberale and Tullberg, 1998).

Amphibians possess great variation in the color and patterns of their integument, both within and between species (reviewed by Rudh and Qvarnstrom, 2013). Poison dart frogs (family Dendrobatidae) are a well-known example of animals that use conspicuous coloration to signal their unpalatability to predators. For example, the strawberry poison frog (*Dendrobates pumilio*) displays bright red, yellow, orange, metallic green, or blue integument coloration and secretes toxins that are lethal to predators (Siddiqi et al., 2004). Color is an honest signal of unpalatability within *D. pumilio*; the most conspicuous individuals are also the most unpalatable (Maan and Cummings, 2012). Additionally, comparative analyses of species within the poison dart frog family Dendrobatidae have demonstrated evidence of coevolution of conspicuousness and body size; larger bodied species are often the most colorful (Hagman and Forsman, 2003; Santos and Cannatella, 2011).

In addition to aposematic signaling, color can allow organisms to avoid predation by mimicking an aposematic species. Batesian mimicry occurs when a relatively scarce, palatable species resembles an abundant unpalatable species; while Mullerian mimicry occurs multiple aposematic species resemble one another, thus amplifying the signal of unpalatability (Cott, 1940). There are several proposed examples of Batesian mimicry in salamanders (Howard and Brodie, 1971; Brodie and Howard, 1973; Kutcha, 2005), and snakes (Brodie III and Janzen, 1995). Several species of butterfly have recently been discovered to be part of a Mullerian mimicry system (see Kapan, 2001). However, it is difficult to determine whether one species is a mimic of another without an exhaustive study of antipredator mechanisms and ecological characteristics of all species involved, including unpalatability (Wollenberg and Measey, 2009). Thus far, mimicry in salamanders has been experimentally tested using plasticine models (Kuchta, 2005), and with feeding experiments using avian predators (e.g. Howard and Brodie, 1973; Brandon et al., 1979; Brodie and Brodie III, 1980).

Amphibian unpalatability is often due to the chemical compounds present in integument secretions, which can be a rich source of biologically active compounds, including biogenic amines, peptides, bufadienolides, tetrodotoxins, and lipophilic alkaloids (Clarke, 1997; Daly et al., 2005; Saporito et al., 2012). Integument secretions serve multiple physiological purposes, and if poisons are present they are usually stored in granular glands in the integument, which are controlled by the sympathetic nervous system and secrete their contents onto the dorsal surface of the animal in response to various stimuli (Barra and Simmaco, 1995).

Behavioral studies of *Ambystoma* species conducted by Brodie and Gibson (1969) demonstrate that salamanders display defensive posture when confronted by predators (mammalian, avian, and reptile predators). This defensive posture involved orienting the body such that the predator is exposed to the granular glands in the parotid region and tail ridge, increasing the probability that an attacking predator will be contacted. Researchers described the granular gland secretions as being insoluble in water, ethyl alcohol, and acetone, and causing mortality when injected into mice, voles, and rats (Brodie and Gibson, 1969). Predation studies involving *Plethodon* species have also shown evidence of unpalatability in these salamanders. Avian species exhibit signs of distress upon eating (or attempting to eat) *P. jordani* salamanders and avoid both *P. jordani* and similarly colored *Desmognathus* species in subsequent trials (Hensel and Brodie, 1976). In addition, the integument secretions of *Plethodon shermani* have been described as sticky and noxious and are hypothesized to play a role in predator avoidance (Largen and Woodley, 2008). Several other species of *Plethodon* have also historically been described as noxious (Brodie, 1977), and some species, particularly *Plethodon jordani*, have been hypothesized to display aposematic coloration because there is experimental evidence that they are avoided by avian predators (Hensel and Brodie, 1976). However, there are no published studies describing the chemical composition of integument secretions in any amphibian family except Salamandridae, and very little research has focused on potential aposematic coloration in salamanders.

In the Southern Appalachian Mountains, several species of salamander display conspicuous coloring and patterning that may have evolved via aposematic signaling. *Ambystoma maculatum* are large black-bodied salamanders with large (~2.0 mm in diameter) bright yellow to orange spots on their dorsal side. *Plethodon yonahlossee* have a red dorsal patch that covers ~40% of their dorsal region. Two other species appear more cryptic to the human eye; *P. cylindraceus* are black with small (~0.5 mm diameter) white flecks, and *P. montanus* are uniformly gray. All four species occur sympatrically in the montaine forests of Watauga County, North Carolina (Petranka, 1998). Here, I explore the function of integument coloration of *Plethodon yonahlossee* and *Ambystoma maculatum*. In Chapter 2, I quantify conspicuousness of each species as viewed by an avian predator against typical forest backgrounds as well as quantify whether avian predators could distinguish these conspicuous species from duller sympatric heterospecifics. In Chapter 3, I conduct a clay model experiment to assess the effect of the red dorsal patch of *P. yonahlossee* on avian predation rates. In Chapter 4, using a comparative approach to determine whether conspicuous coloration co-evolved with body size in salamander genera: *Plethodon* and *Ambystoma*.

In Chapter 2, modeling of avian predator vision to demonstrates that *P. yonahlossee* and *A. maculatum* are easily distinguishable from typical forest backgrounds and thus should be highly conspicuous to avian predators. Moreover, avian vision models suggest that *P. yonahlossee* and *A. maculatum* are chromatically distinct from two duller sympatric heterospecifics (*P. montanus* and *P. cylindraceus*), suggesting that birds could easily distinguish the two putatively aposematic species from duller heterospecifics. In Chapter 3, I demonstrate that the plasticine models of the conspicuous *P. yonahlossee* are depredated by avian predators significantly less often than the duller *P. montanus.* This experiment suggests that the red dorsal coloration of *P. yonahlossee* is interpreted by avian receivers as a warning signal that deters predation. In Chapter 4, the comparative approach suggests that increased conspicuous coloration co-evolved with increased body size in *Ambystoma*, but that evolution in *Plethodon* salamanders has favored a negative relationship between these two traits. It may be that *Ambystoma* are more likely to benefit from an aposematic signal than *Plethodon*. *Ambystoma* are generally larger than *Plethodon* (Mitchell and Gibbons, 2010), exhibit gregarious behavior while *Plethodon* do not (Petranka, 1998), and there is better evidence of toxicity in *Ambystoma* compared to *Plethodon* (Brodie, 1977). Proposed explanations for the loss of conspicuous coloration with increased body size in *Plethodon* are alternative uses of color, such as mimicry or thermoregulation. Overall results from my thesis suggest that both *P. yonahlossee* and *A. maculatum* possess traits consistent with aposematism, and that avian predators could discriminate these signals and do actively avoid depredating conspicuous salamander models. However, to further explore the hypothesis that local salamanders use aposematic signaling, research should focus on identifying unpalatable chemicals and testing behavioral responses of live predators towards salamanders that vary in conspicuousness.

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CHAPTER 2

Two Southern Appalachian salamanders may use color as an aposematic signal to avian predators

ABSTRACT

Color may serve as an aposematic signal in which the conspicuous coloration of prey is used to signal unpalatability to potential predators. For aposematic signals to be effective, they must be conspicuous to predators. Conspicuousness is dependent upon several factors including: the receiver's visual capabilities, the ambient light environment, and the coloration of both the signaler and the background. Here, I quantify the conspicuousness of two salamanders, *Plethodon yonahlossee* and *Ambystoma maculatum*, from an avian perspective to investigate whether their integument coloration could be an aposematic signal to avian predators. *Ambystoma maculatum* are large black-bodied salamanders with bright yellow to orange spots on their dorsal side. *Plethodon yonahlossee* have a red dorsal patch that covers ~40% of their dorsal region. Using an avian vision model approach, I found that both species are distinguishable from typical forest backgrounds and are chromatically distinct from two duller sympatric heterospecifics. I also found that larger *Plethodon yonahlossee* individuals have more spectrally pure red coloration (greater red chroma) of their dorsal patches. Though these results are consistent with aposematic theory, future investigation should focus on quantifying unpalatability of both species to further explore whether the signals are reliable and whether predators avoid the prey.

INTRODUCTION

Integument color is widely used in animal communication to signal information about one organism (i.e. the signaler) to another organism (i.e. the receiver) that may alter the receivers' behavior in some way (discussed by Owren et al., 2010; reviewed in Scott-Phillips, 2008). Conspicuous color may convey information about individual quality or profitability as a prey item, while cryptic coloration enables organisms to avoid detection (Dale, 2006). When conspicuous coloration is combined with some form of secondary defense, such as venom or poison, color is thought to serve as an aposematic signal in which the signaler alerts potential predators to their unprofitability (Wallace, 1867; Cott, 1940).

Certain color combinations are commonly used in aposematic signaling, such as red or yellow pigmentation against a black integument background (Wallace, 1867; Cott, 1940). These colors are either innately avoided by some predators (Smith, 1975), or are easily recalled and associated with the defense over time (Mappes et al., 2005). Among organisms that have the potential to exhibit warning color (i.e. are unpalatable), warning colors are expected to be more conspicuous when occurring in larger organisms (Gamberale and Tullberg, 1996a) and are expected to be more effective among gregarious species which tend to occur in high densities (Fisher, 1930; Cott, 1940; Edmunds 1974; Gamberale and Tullberg, 1996b). Body size, the size of the conspicuous color patch, and gregariousness can amplify the conspicuousness of the color signal, increase predator recognition, and increase initial aversion in naïve predators (Gamberale and Tullberg, 1998). It is also expected that aposematic species will exhibit little intraspecific variation in color, as natural selection should favor a convergence of color and pattern, likely to increase predator recognition and reinforce association with the defense (Summers et al., 1997; Dale, 2006). However, if there is intraspecific variation in aposematic species, it could be attributable to variation in toxicity (some studies suggest an increase in toxicity should coincide with an increase in signal strength (i.e. Maan and Cummings, 2012)), or color serving as both an aposematic signal and sexual signal (Siddiqi et al., 2004).

Amphibians display a diversity of integument coloration and patterns, and nearly every color combination is present in the three amphibian orders: Caudata, Anura, and Gymnophiona (Rudh and Qvarnstrom, 2013). Amphibians typically possess two cell types that control integument color and pattern: chromatophores, which contain pigments such as melanin and pterins, and iridiphores, which contain reflective structural elements, such as guanine (Summers et al., 2003). The pigments and/or structures absorb and reflect specific wavelengths of light that are perceived as color by viewers (Bagnara and Hadley, 1973).

Avoiding predation is essential for survival and reproduction of all animals, including amphibians, resulting in the evolution of diverse predator avoidance tactics, many of which involve integument color (Darst et al., 2006). Because amphibians typically have small, soft bodies that lack protective structures, many have taken advantage of conspicuous coloration as a first line of defense against predation (Rudh and Qvarnstrom, 2013). Previous research has also shown that many amphibians produce noxious integument secretions when threatened by a potential predator, which decrease their profitability as prey (reviewed in Clarke, 1997). There are several examples of amphibian species that use aposematic coloration. Strawberry poison dart frogs (*Dendrobates pumilio*) which occur in the neotropics, are brightly colored and secrete some of the most potent toxins known to exist in nature (Siddiqi et al., 2004). Eastern newts (*Notophthalmus viridescens*), which occur in eastern North America, secrete the neurotoxin tetrodotoxin (Mosher et al., 1964) and undergoes a terrestrial juvenile stage characterized by brilliant orange integument (Petranka, 1998; Mitchell and Gibbons, 2010).

For an aposematic signal to be effective, it must be conspicuous to the intended receiver. The conspicuousness of integument color is dependent upon multiple factors including the visual system of the receiver, the reflectance or patterning of the integument, the light environment, the background upon which the signaler is located, as well as the medium between the signaler and the receiver (Siddiqi et al., 2004; Uy and Endler, 2004; Endler and Mielke, 2005). Though natural predators of amphibians are often poorly documented, avian predators have been most commonly used in predation simulations using clay model replicas of amphibians (i.e. Saporito et al., 2007; Hegna et al., 2011; McElroy, 2015), and are speculated to be common predators of amphibians in North America (Petranka, 1998 and the references therein). It is therefore typical to examine color from the point of view of an avian predator when quantifying conspicuousness coloration in amphibians (e.g. Siddiqi et al., 2004; Maan and Cummings, 2012).

Differences in visual sensitivity cause humans and birds to perceive color differently (reviewed in Cuthill, 2006), and recent advances in the understanding of avian vision have made it possible to model color from an avian perspective. Humans have trichromatic color vision (three photoreceptors that process light between 400 nm and 700 nm on the electromagnetic spectrum); every visible color is simulated by specific combinations of red, green, and blue wavelengths (Cuthill, 2006). Most birds, however, have tetrachromatic color vision (four photoreceptors that process the same wavelengths as humans, plus wavelengths from the ultraviolet part of the spectrum, 300 nm to 400 nm); every visible color is simulated by specific combinations of red, green, and blue and UV (or near UV) wavelengths (Fig. 1;

reviewed in Osorio and Vorobyev, 2008). Also, birds also have doubles cones which allow to have better ability to discriminate achromatic differences (i.e. brightness) between two objects (Vorobyev et al., 1998) compared to humans. Using vision models, we can apply our understanding of avian vision to studies of color signaling and theoretically "see" signals as birds do (Maia et al., 2013) from both the chromatic (spectral purity or hue) and achromatic (brightness) perspectives.

Here, I investigate potential aposematic signaling in two species of salamanders that occur in eastern North America. In the southern Appalachians, salamanders across genera display markedly distinct coloration; yet, the signaling function of integument coloration in salamanders is not well studied. Two locally abundant salamanders that, to the human eye, appear to display conspicuous coloration which could have evolved via aposematic signaling include: *Ambystoma maculatum* which are large black bodied salamanders with large (~2-3mm diameter) bright yellow to orange spots on their dorsal side (Fig. 2b) and *Plethodon yonahlossee*, which have a red dorsal patch that covers ~40% of the dorsal region (Fig. 2a). Two other species appear more cryptic to the human eye; *P. cylindraceus* are black with small (~0.5 mm diameter) white flecks (Fig. 2d), and *P. montanus* are uniformly gray (Fig. 2c; Petranka, 1998). All four species occur sympatrically in the montaine forests of Watauga County, North Carolina. The Plethodon species are terrestrial while *Ambystoma maculatum* salamanders are seasonal pool breeders that migrate from underground burrows to shallow, stagnant bodies of water at night during late winter/early spring rains (Petranka, 1998). Previous research exploring unpalatability in these genera has shown that species in both Plethodon and Ambystoma are distasteful to some predators (Hensel and Brodie, 1976; Brodie, 1977). Further, the integument secretions of some Ambystoma are potentially lethal to mammalian predators including rats and voles (Brodie and Gibson, 1969), indicating that color in both genera may signal unpalatability to potential predators. If their coloration is aposematic, it is expected that these species are distinguishable from an avian perspective against typical background substrate under various lighting conditions, and that they are distinguishable from heterospecifics that do not have red or yellow pigmentation in their integument.

I test the hypothesis that *P. yonahlossee* and *A. maculatum* are conspicuous to avian predators, which would be necessary if such colors have evolved via aposematic signaling. I predict greater conspicuousness of the integument coloration of the brightly colored *P. yonahlossee* and *A. maculatum* compared to the duller *P. cylindraceus* and *P. montanus* species. First, I measure the spectral reflectance of the dorsal body region of salamanders and use models of the 'forest shade' light environment to quantify whether each species is visibly discriminable (both achromatically and chromatically) to avian predators against typical forest background coloration (moss, leaf litter, etc.). Next, because *P. yonahlossee* and *A. maculatum* display putative aposematic coloration (red and yellow coloration, respectively), I model whether each of species is visibly discriminable from duller heterospecifics (*P. montanus* and *P. cylindraceus*). I predict that *P. yonahlossee* and *A. maculatum* should display greater chromatic variation (i.e., shape of the spectral curve) visible to potential avian predators. Further, because 1) aposematic theory predicts positive associations between body size, signal size, and signal effectiveness and 2) comparative analyses show that larger Ambystoma species have more conspicuous coloration (Chapter 4, p. 86), I investigate intraspecific correlations between body size and spectral reflectance in both *P. yonahlossee* and *A. maculatum.* In these two species, I also investigate potential correlations between the size of the colorful body region and the spectral reflectance to determine whether animals with larger (more conspicuous patches) also display more conspicuous spectral reflectance. Finally, I model whether the extremes of intraspecific variation in both *P. yonahlossee* and *A. maculatum* would be visibly discriminable to avian predators.

MATERIALS AND METHODS

Data collection.**---**I opportunistically captured Plethodon salamanders during night surveys in forest habitat of Watauga and Avery counties, North Carolina from July 2015 to November 2017. I marked the location of each *Plethodon* captured using survey flags. During the migration/breeding period of 2016 (late February/early March), I captured adult *Ambystoma* salamanders as they migrated between their terrestrial home ranges and breeding pools at one site in Watauga County, North Carolina. For each species, I collected small samples of substrate upon which salamanders were found, including twigs, leaves, moss, and soil. I transported all individuals to Appalachian State University, for measurement. In the laboratory, I measured body size (total length), determined sex of *Ambystoma* salamanders (I excluded *Plethodon* because determining sex in individuals outside of breeding condition is difficult (Petranka, 1998)), and measured spectral reflectance (methods below) of the dorsal region. I photographed the dorsal region of *P. yonahlossee* and *A. maculatum* to quantify the size of the colorful body patches (methods below). Upon completion of data collection, I returned all salamanders to the exact site of capture.

Spectral measurements.---To measure the spectral reflectance of the dorsal region, I used an Ocean Optics reflectance spectrometer (S2000: Range 250-880 nm: Dunedin, FL, USA) equipped with both a deuterium bulb (UV light source) and a tungsten-halogen light source (visible light source). I generated reflectance measurements relative to a white standard (100% reflectance from 300-700 nm; Labsphere, Inc.). I then used two standard descriptors of color for each species: a chromatic and an achromatic descriptor. Following protocol of Jones and Siefferman (2014), I measured reflectance on five dorsal body regions of each *Plethodon* species, beginning at the cervical vertebrae and moving along the spine to end at the start of the caudal vertebrae (sacral plate) and averaged the spectral data. I quantified the red chroma of the dorsal patch using the S1R chroma descriptor of reflectance spectra: S1R = $((R_{\lambda 605} - R_{\lambda 700}) / R_{\lambda 700}$, where $R_{\lambda i}$ is the percent reflectance at the i^{th} wavelength (λ_i) (Montgomerie, 2006). Additionally, to attain spectral reflectance data of the yellow spots of *A. maculatum*, I followed the protocol of Morgan et al. (2014) and took two measurements each of the first and third dorsal spot from the anterior dorsal region and averaged the spectral data. I then quantified the yellow chroma of the averaged spots using the S1Y chroma descriptor of reflectance spectra: S1Y = $((R_{\lambda 550} - R_{\lambda 625}) / R_{\lambda 700}$, where $R_{\lambda i}$ is the percent reflectance at the i^{th} wavelength (λ_i) (Montgomerie, 2006). I also took five reflectance measurements of each substrate type (twigs, leaves, moss, and soil) collected from capture sites and averaged the measurements from the five replicates of each substrate to create each background (moss, soil, leaf litter, twig).

Vision model measurements.---To model whether salamanders are discriminable from one another within a species, and whether salamanders are discriminable from backgrounds upon which they are found, I ran the full-spectrum (300–700 nm) reflectance data through models of avian vision: Perceptual, Analysis, Visualization, and Organization of Spectral Color Package (pavo) in the R v.3.3.3 statistical program (Maia et al., 2013, R Core Team 2013). I ran the model from the perspective of the European starling (*Sturnus vulgaris*), the blue tit (*Parus caeruleus*), and the default average UV visual system function (avg.uv) in pavo, which is based on the average peak sensitivity found in birds that have the UV type of visual system (Endler and Mielke 2005). As results were almost identical between the three perspectives, I only present the results from the avg.uv.

I used the color distance function, coldist, which calculates color distances based on the receptor-noise model of Vorobyev et al. (1998), to estimate interspecific discriminability, intraspecific discriminability, and discriminability between salamanders and backgrounds. The coldist function calculates both chromatic differences (i.e., shape of the spectral curve) and achromatic differences (overall % reflectance) between two objects; units of the output of this function are just-noticeable-differences (JND) (see Vorobyev et al., 1998). Just-noticeable-difference values > 1.0 are considered to be discriminable, with discriminability increasing as JND values increase (Vorobyev and Osorio, 1998). Following the method of Siddiqi et al., (2004), I considered JND values < 4 to be relatively poorly discriminable. Achromatic differences are calculated based on the double cones responsible for chromatic processing (Siddiqi et al., 2004); I used the double cone abundance for European Starlings in this study (Hart et al., 1998) as I assume most crepuscular and diurnal avian predators are passerines.

This model also incorporates ambient light environment; options are “ideal”, “blue-sky”, “forest shade”, and standard (“D65”). Because salamanders would be viewed by most crepuscular and diurnal avian predators in the early morning and evening in the forest environment, I used “forest shade” and “D65”. As results were almost identical between the “forest shade” and “D65” light environments, I only present the results from “forest shade” here.

Measurement of aposematic spot characteristics.---I photographed each salamander using an 8-megapixel camera from a distance of 0.3 m (at a 90^o angle next to a cardstock rectangle with a 20 mm by 20 mm square for a size standard). I used Adobe Photoshop to quantify the patch surface area (mm^2) and percent patch cover of each *P. yonahlossee* and spot surface area (mm^2) spot cover of *A. maculatum*. To do so, I first calculated the total surface area in millimeters of the salamander in each standardized photograph, then calculated the total surface area of the patch (*P. yonahlossee*) or spots (*A. maculatum*), then divided patch surface area by total surface area to determine the percentage of the body covered by the patch or the spots.

Statistical methods.---Statistical analyses were performed in SPSS v. 23. For *P. yonahlossee* and *A. maculatum*, I used Pearson correlations to investigate relationships between spectral variables (brightness and chroma), body size and patch characteristics. I also used Student’s t-tests to identify sex differences in body size, spot area and color measurements of *A. maculatum*.

RESULTS

Visual discriminability of salamanders against backgrounds

I contrasted each species separately against 4 backgrounds as viewed by a typical passerine bird under the "forest shade" light environment. Results are similar between backgrounds so I present only the results for "leaf litter" background; results for other backgrounds can be found in the Appendix.

***Plethodon yonahlossee*.---**Spectra of 61 *P. yonahlossee* were compared to a "leaf litter" background for both chromatic and achromatic differences. For chromatic discriminability, 100% of *P. yonahlossee* were discriminable, 4.9% were relatively poorly discriminable, and the highest JND value was 8.15 (Fig. 3a). For achromatic discriminability, 100% were discriminable, and JND values ranged from 7.6 to 34.9 (Fig. 4a).

***Ambystoma maculatum*.---**Spectra of 73 *A. maculatum* were compared to a "leaf litter" background for both chromatic and achromatic differences. For chromatic discriminability, 100% of *A. maculatum* were discriminable, but 80.8% were relatively poorly discriminable (JND values ranged from 1.8 to 8.0 (Fig. 3b). For achromatic differences, 11.0% were not at all discriminable, 39.7% were relatively poorly discriminable, and JND values ranged from 0.005 to 19.7 (Fig. 4b).

***Plethodon montanus*.---**Spectra of 74 *P. montanus* were compared to a "leaf litter" background for both chromatic and achromatic differences. For chromatic discriminability, 100% of *P. montanus* were discriminable, and JND values ranged from 5.5 to 11.9 (Fig. 3c).

For achromatic discriminability, 100% were discriminable and JND values ranged from 15.9 to 38.6 (Fig. 4c).

***Plethodon cylindraceus*.---** Spectra of 48 *P. cylindraceus* were compared to a “leaf litter” background for both chromatic and achromatic differences. For chromatic discriminability, 100% were discriminable and JND values ranged from 6.1 to 13.6 (Fig. 3d). For achromatic discriminability, 100% were discriminable and JND values ranged from 8.8 to 37.6 (Fig. 4d).

Interspecific comparisons

I contrasted *P. yonahlossee* and *A. maculatum* separately against both *P. cylindraceus* and *P. montanus* to determine whether species could be distinguished using only chromatic contrasts as viewed by a typical passerine bird under the “forest shade” light environment.

***Plethodon yonahlossee* vs *Plethodon cylindraceus*.---**I compared 61 spectra of *P. yonahlossee* to 48 spectra of *P. cylindraceus*, resulting in a total of 2,928 comparisons. One hundred percent of *P. yonahlossee* are discriminable against *P. cylindraceus*, but of those, 10.4% are relatively poorly discriminable (Fig. 5a).

***Plethodon yonahlossee* vs *Plethodon montanus*.---**I compared 61 spectra of *P. yonahlossee* to 74 spectra of *P. montanus*, resulting in a total of 4,514 comparisons. One hundred percent of *P. yonahlossee* are discriminable against *P. cylindraceus* and, of those, 11.9% are relatively poorly discriminable (Fig. 5b).

***Ambystoma maculatum* vs *Plethodon cylindraceus*.---**I compared 73 spectra of *A. maculatum* to 48 spectra of *P. cylindraceus*, resulting in a total of 3,504 comparisons. One hundred percent of *A. maculatum* are discriminable against *P. cylindraceus* and, of those, 4.9% are relatively poorly discriminable (Fig. 5c).

***Ambystoma maculatum* vs *Plethodon montanus*.---**I compared 73 spectra of *A. maculatum* to 74 spectra of *P. montanus*, resulting in 5,402 comparisons. One hundred percent of *A. maculatum* are discriminable against *P. montanus* and, of those, 2.1% were relatively poorly discriminable (Fig. 5d).

Intraspecific morphology-color associations

Coefficients of variation (COV) suggest that within species variation in color measures are greater for *P. yonahlossee* than *A. maculatum* (*P. yonahlossee*: n = 61, mean = 0.35, SD = 0.06, COV = 0.18; for *A. maculatum*: n = 73, mean = 0.28, SD = 0.03, COV = 0.10).

***Plethodon yonahlossee*.---**I measured spectral reflectance and body size for 30 *P. yonahlossee* individuals. Red chroma was significantly positively correlated with total length; larger individuals had more spectrally pure red coloration, while brightness was not significantly related to body size (Table 1). Red chroma was not significantly correlated with the surface area of the red patch, nor the percentage of the body covered by the patch (Table 1). Modeling of chromatic discriminability suggests that the *P. yonahlossee* individual with the greatest red chroma should be clearly distinguishable from the individual with the lowest red chroma (chromatic JND = 9.8).

Ambystoma maculatum.**---**I measured spectral reflectance and body size for 75 *A. maculatum.* Because both body size and spectral reflectance differ significantly with sex (Table 2), I investigated correlations between body size, spectral reflectance, and % spot coverage separately for females and males. There were no significant associations between body size and yellow chroma or body size and brightness in either sex (Table 3). Modeling of chromatic discriminability suggests that the *A. maculatum* individual with the highest yellow chroma is only relatively poorly discriminable from the individual with the lowest yellow chroma (JND = 2.3).

DISCUSSION

To elucidate the effectiveness of potential aposematic signaling in two salamander species, I modeled how well potential predators can discriminate spot and patch coloration against natural backgrounds and against less conspicuous sympatric heterospecifics. My results indicate that *Plethodon yonahlossee* is both chromatically and achromatically discriminable against a leaf litter background from an avian perspective. Further, although *Ambystoma maculatum* is also both chromatically and achromatically discriminable against a leaf litter background, most individuals are relatively poorly discriminable chromatically. However, both *P. yonahlossee* and *A. maculatum* are chromatically discriminable from the duller *P. montanus* and *P. cylindraceus*. Previous research has shown that species in both *Plethodon* and *Ambystoma* are distasteful to some predators (Hensel and Brodie, 1976; Brodie, 1977), and that integument secretions of some *Ambystoma* are potentially lethal to mammalian predators (Brodie and Gibson, 1969), indicating that color in both genera may signal unpalatability to potential predators. My results support the hypothesis that integument color in *P. yonahlossee* and *A. maculatum* is conspicuous to passerine predators and these findings support previous research suggesting that these two species could be using aposematic signaling.

Among *P. yonahlossee,* larger individuals had greater red chroma in their red dorsal patches, but there were no significant associations between relative patch size and chroma. Moreover, from an avian perspective, the *P. yonahlossee* individual with the highest red chroma is distinguishable from the individual with the lowest red chroma. From an interspecific comparison perspective, positive associations between body size and conspicuousness are expected because larger organisms are easily detected and therefore more likely to benefit from conspicuous coloration (Hossie et al., 2015). That larger *P. yonahlossee* individuals had greater red chroma could also be interpreted as being consistent with aposematic theory. *Plethodon yonahlossee* also exhibited greater chromatic variation than predicted, as species that use aposematic signaling generally show little intraspecific variation in color (Dale, 2006). However, some aposematic theory suggests that an increase in signal strength (like more intense pigmentation) should coincide with an increase in unpalatability (Speed and Ruxton, 2007). Indeed, in poison dart frogs, the more conspicuous morphs have greater concentrations of toxins in their integument (Maan and Cummings, 2012).

Among *A. maculatum*, body size and yellow chroma were not correlated. However, from an avian perspective, the *A. maculatum* individual with the greatest yellow chroma would not distinguishable from the individual with the lowest yellow chroma. Therefore, the intraspecific variation in color of *P. yonahlossee,* but not *A. maculatum,* should be visible to predators. This could be due to greater spectral sensitivity in the red portion of the visible spectrum than in the yellow portion, but there is also more variation in red chroma in *P. yonahlossee* than in yellow chroma in *A. maculatum* (greater coefficient of variation for chroma). However, predators may be better able to detect *A. maculatum* with greater spot coverage.

Ambient light also influences the conspicuousness of a color signal, and this study only examined perception under forest shade light conditions. *Plethodon* species are most active at night and the *Ambystoma* species is largely both nocturnal and fossorial, except during the explosive annual breeding migration during which they are active above ground for several weeks (Petranka, 1998). However, there is evidence that all species are, on occasion, active during daylight hours, and the *Plethodon* species are active in the leaf litter and under cover objects in the day, as well (Brandon and Huheey, 1975). Any opportunity of being viewed during the day, however slight, may be enough to drive selection of integument coloration that is conspicuous to diurnal predators (e.g. caecilians; Wollenberg and Measey, 2009). In addition, many nocturnal predators have evolved physiological adaptations to a dim light environment, including increased perception of achromatic signals (Osorio and Vorobyev, 2005). Thus, the high achromatic discriminability of both *P. yonahlossee* and *A. maculatum* against forest backgrounds could be useful in signaling to nocturnal predators, in addition to the chromatic signals that may be more accurately perceived by diurnal predators.

In addition to chromatic and achromatic discriminability, integument patterning of both *P. yonahlossee* and *A. maculatum* likely add to their conspicuousness. In other fossorial amphibians (i.e. caecilians), both contrasting integument patterns and yellow pigmentation evolved in tandem with an increase in surface activity (Wollenberg and Measey, 2009).

Contrasting patterns elements (such as red and black or yellow and black spots or stripes) are thought to increase conspicuousness and thus increase predator learning, recognition, and avoidance of aposematic species, while an increase in the size of pattern elements (larger spots, for example) has been shown to further increase signal efficacy (Gamberale and Tullberg, 1996a, 1998; Lindstrom et al., 1999). The greater yellow chroma in *A. maculatum* individuals with a greater percentage of the body covered in spots is consistent with aposematic theory. Therefore, though many *A. maculatum* individuals were only relatively poorly discriminable from the leaf litter background, the integument pattern itself may amplify the signal and increase predator avoidance.

Though these results indicate that *P. yonahlossee* and *A. maculatum* are conspicuous to potential avian predators, color signals can be viewed by any visually-oriented organism. Because conspicuousness is dependent upon multiple factors, *P. yonahlossee* and *A. maculatum* could be conspicuous to some organisms but not to others. Color can therefore serve multiple functions simultaneously. For example, swallowtail butterfly larva (*Papilio machaon*) are inconspicuous to humans at a distance but conspicuous up close, indicating coloration is used in both crypsis and aposematism (Tullberg et al., 2005). Color in the polymorphic *Dendrobates pumilio* is discriminable to both avian predators and conspecifics and is thought to act simultaneously as an aposematic and sexual signal (Siddiqi et al., 2004). Thus, it is possible that some variation in coloration can be attributed to sex and that sexual selection could lead to greater conspicuousness of *P. yonahlossee.* I did not include sex as a factor in analyses of *P. yonahlossee* coloration because determining sex using external characteristics is difficult and likely would be inaccurate, and thus I cannot exclude the possibility that color is related to sexual differences within the species. *Ambystoma* *maculatum* coloration is sexually dimorphic; females have brighter (lighter yellow) and larger spots (my results; Morgan et al., 2014) and are also heavier than males (Morgan et al., 2014). Therefore, it is possible that *A. maculatum* coloration is simultaneously acting as an aposematic and sexual signal. Additional exploration of perception of *A. maculatum* and other potential signal receivers is needed.

This study suggests that both *P. yonahlossee* and *A. maculatum* are distinguishable to passerine avian predators against typical forest backgrounds in forest shade ambient light conditions. Further, both are distinguishable from duller heterospecific species that occur in the same habitat. These results are consistent with aposematic signaling, as are the morphological associations with color in both species. Although the results support the hypothesis of aposematic coloration in the two species, much more work is needed to fully address the possibility. More information on the chemical composition of integument secretions in both species is crucial to understanding their defensive strategies and the function of their bright integument coloration.

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TABLES

Table 1. Pearson's correlations between color variables and morphology of *Plethodon yonahlossee*, n = 30 for total length and n = 27 for patch surface area and % patch cover.

Variable	**Brightness**		**Red Chroma**	
	r	p	r	p
Total Length (mm)	0.093	0.623	0.437	0.016
Patch Surface Area	0.194	0.333	0.312	0.113
% Patch Cover	0.148	0.460	-0.136	0.499

Table 2. Sex differences (Student's T-tests) in morphology, coloration, and spot characteristics of *Ambystoma maculatum.* For females, n = 25 for total length and % spot cover and n = 24 for chroma and brightness, n = 50 for males.

Variable	**Female mean +/- SD**	**Male mean +/- SD**	**T**	**p**
Total Length (mm)	204.6 +/- 8.8	191.2+/-12.0	5.486	<0.001
Yellow Chroma	0.2652+/-0.0616	0.2804 +/-0.0297	-1.151	0.259
Brightness	0.1769+/-0.0970	0.1131+/-0.0483	3.044	<0.001
% Spot over	10.9+/-4.3	12.7+/-2.9	-1.826	0.076

Table 3. Pearson's correlations between color variables and morphology of female (n = 24) and male (n = 50) *Ambystoma maculatum.*

Sex	**Variable**	**Brightness**		**Yellow chroma**	
		r	p	r	p
Female	Total Length (mm)	0.184	0.390	-0.222	0.296
Female	Spot Surface Area (mm^2)	0.310	0.141	0.352	0.092
Female	% Spot Cover	0.294	0.163	-0.133	0.544
Male	Total Length (mm)	0.061	0.674	-0.155	0.282
Male	Spot Surface Area (mm^2)	0.041	0.775	-0.174	0.226
Male	% Spot Cover	0.149	0.301	-0.177	0.220

FIGURES

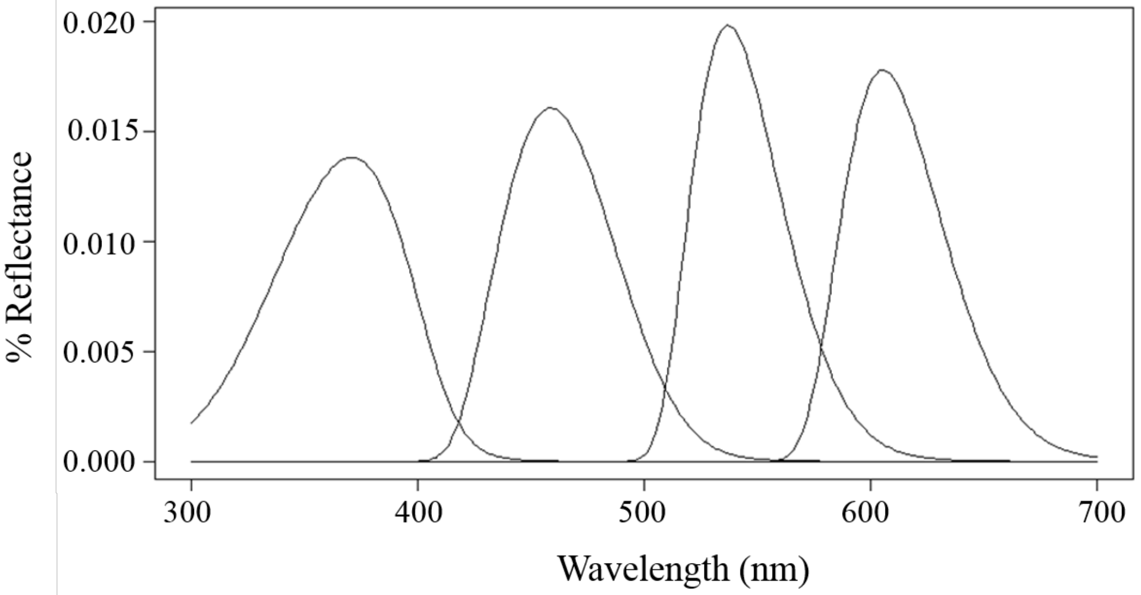

Figure 1. Receptor spectral sensitivity of the Blue tit (*Cyanistes caeruleus*); adapted from Hart et al., 2000).

Figure 2. Photographs of A) *Plethodon yonahlossee*, B) *Ambystoma maculatum*, C) *Plethodon cylindraceus,* D) *Plethodon montanus.* Photographs courtesy of M. Worth Pugh.

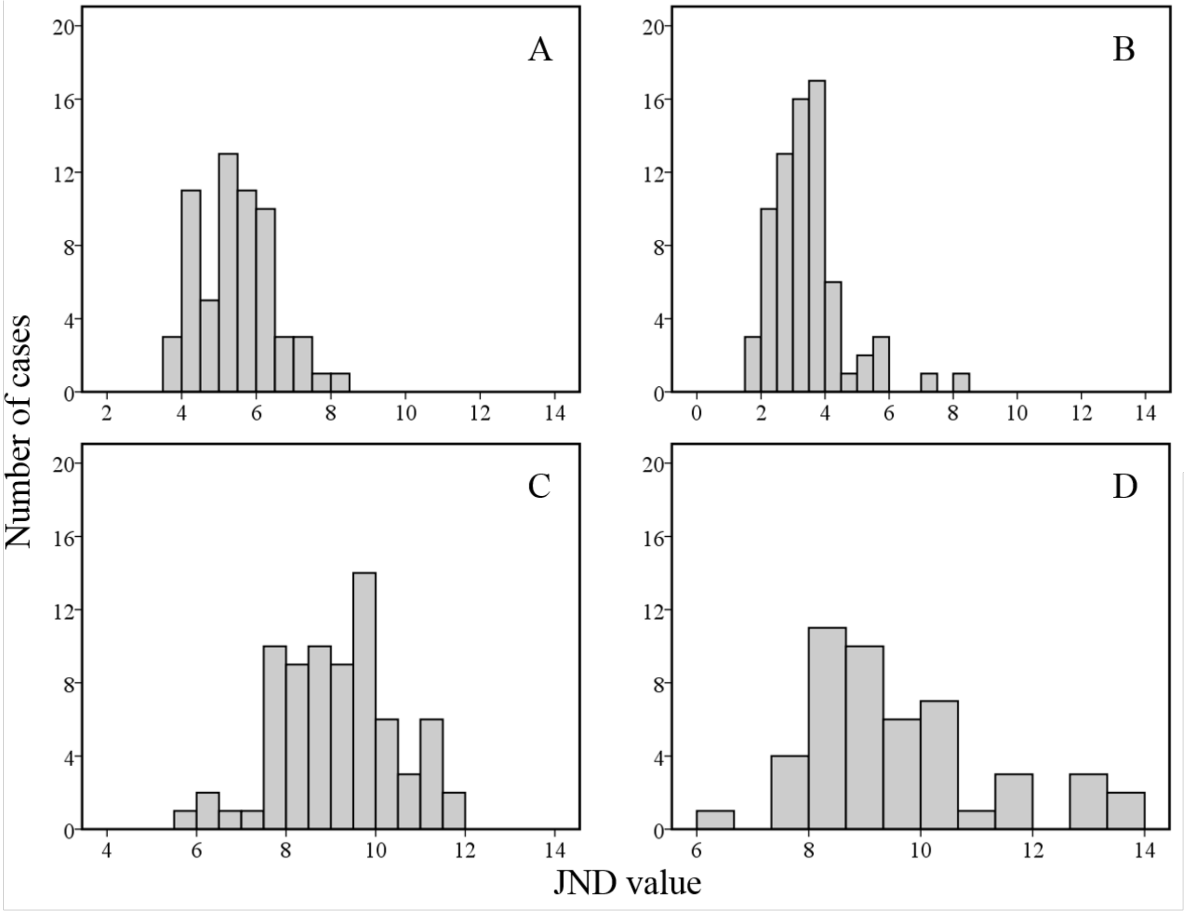

Figure 3. Histogram of chromatic Just Noticeable Differences for A) *Plethodon yonahlossee,* B) *Ambystoma maculatum*, C) *P. montanus*, D) *P. cylindraceus* on a leaf litter background.

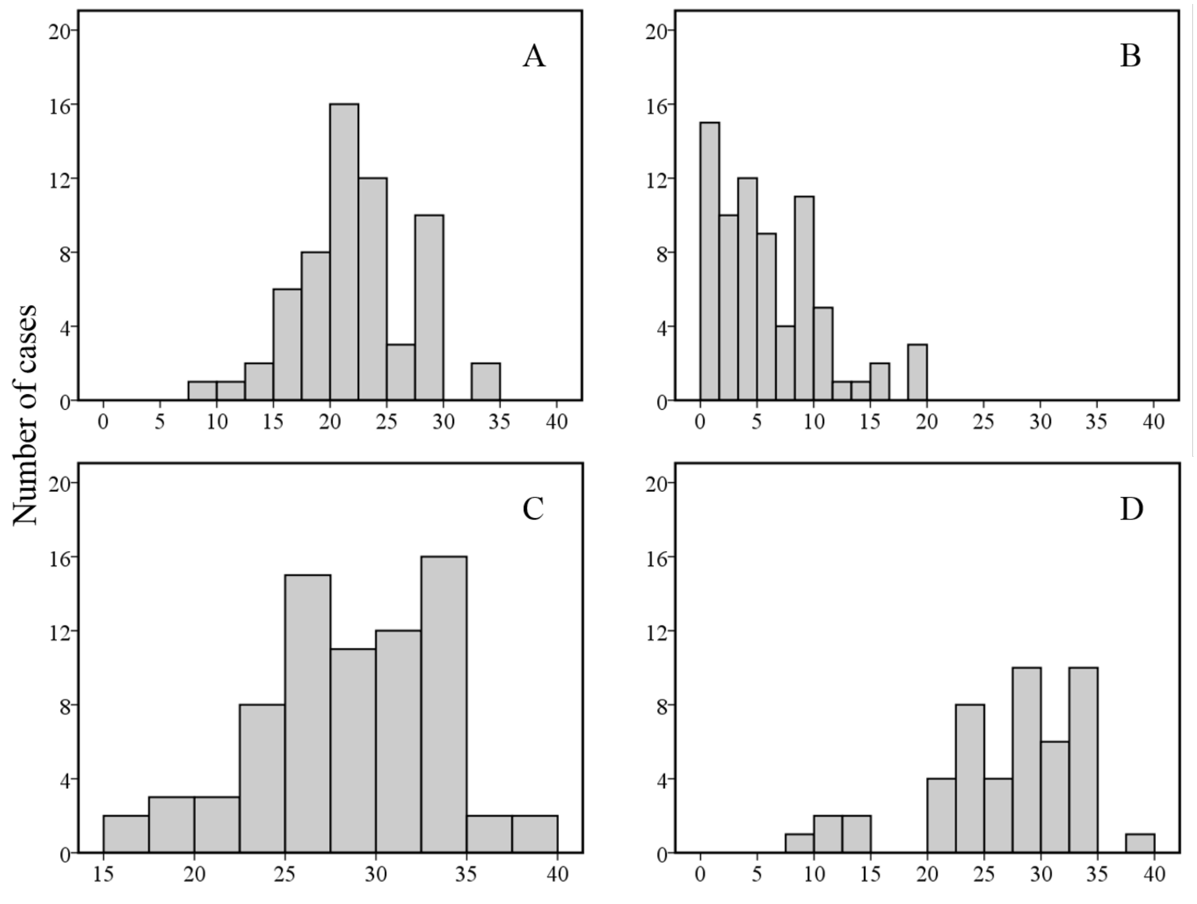

Figure 4. Histogram of achromatic Just Noticeable Differences for A) *Plethodon yonahlossee,* B) *Ambystoma maculatum,* C) *P. montanus,* D) *P. cylindraceus* on a leaf litter background.

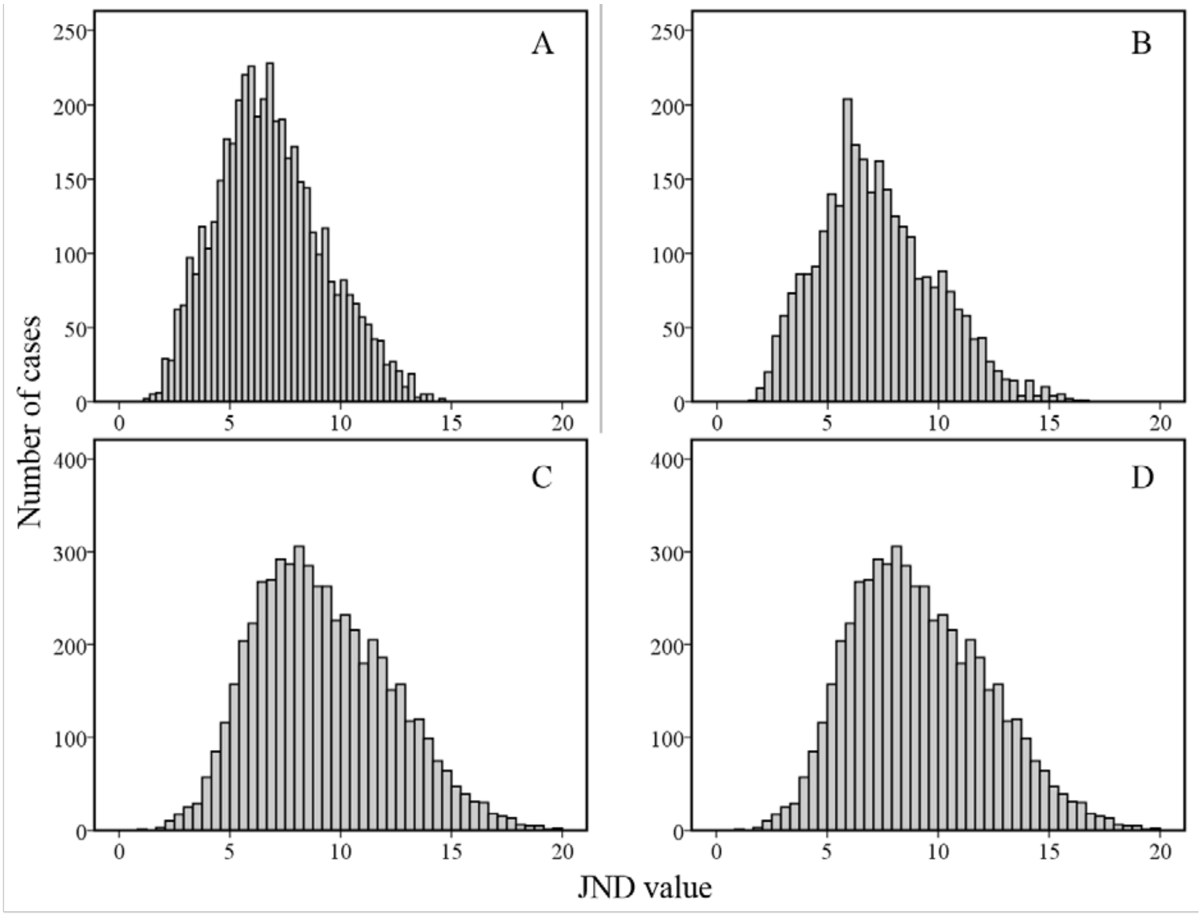

Figure 5. Histogram of chromatic Just Noticeable Differences for interspecific comparisons. A) *P. yonahlossee* vs *P. montanus,* B) *P. yonahlossee* vs *P. cylindraceus*, c) *A. maculatum* vs *P. montanus,* d) *A. maculatum* vs *P. cylindraceus*.

APPENDIX

Supplemental Figures

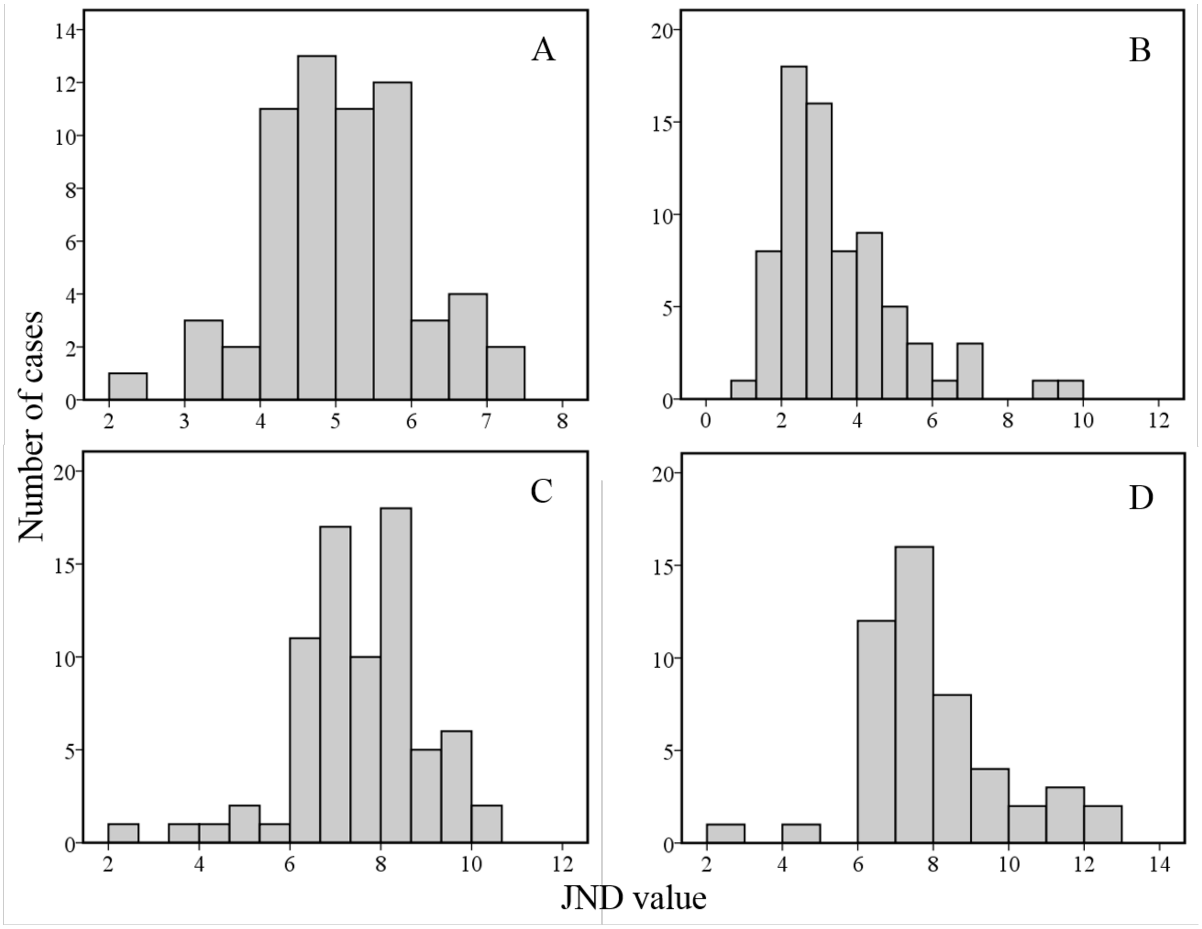

Figure S1. Histogram of chromatic Just Noticeable Differences for A) *Plethodon yonahlossee,* B) *Ambystoma maculatum,* C) *P. montanus,* D) *P. cylindraceus* on a moss background.

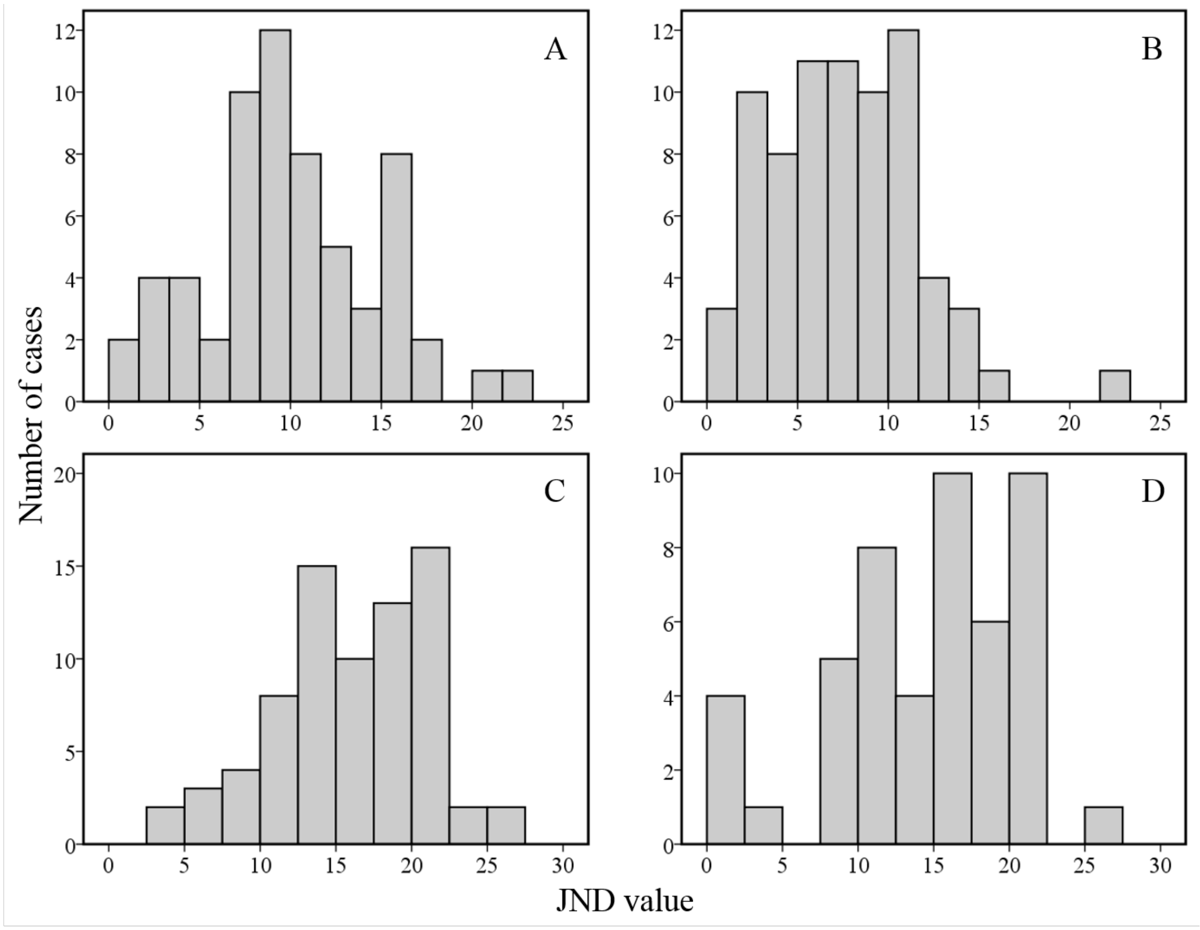

Figure S2. Histogram of achromatic Just Noticeable Differences for A) *Plethodon yonahlossee,* B) *Ambystoma maculatum,* C) *P. montanus,* D) *P. cylindraceus* on a moss background.

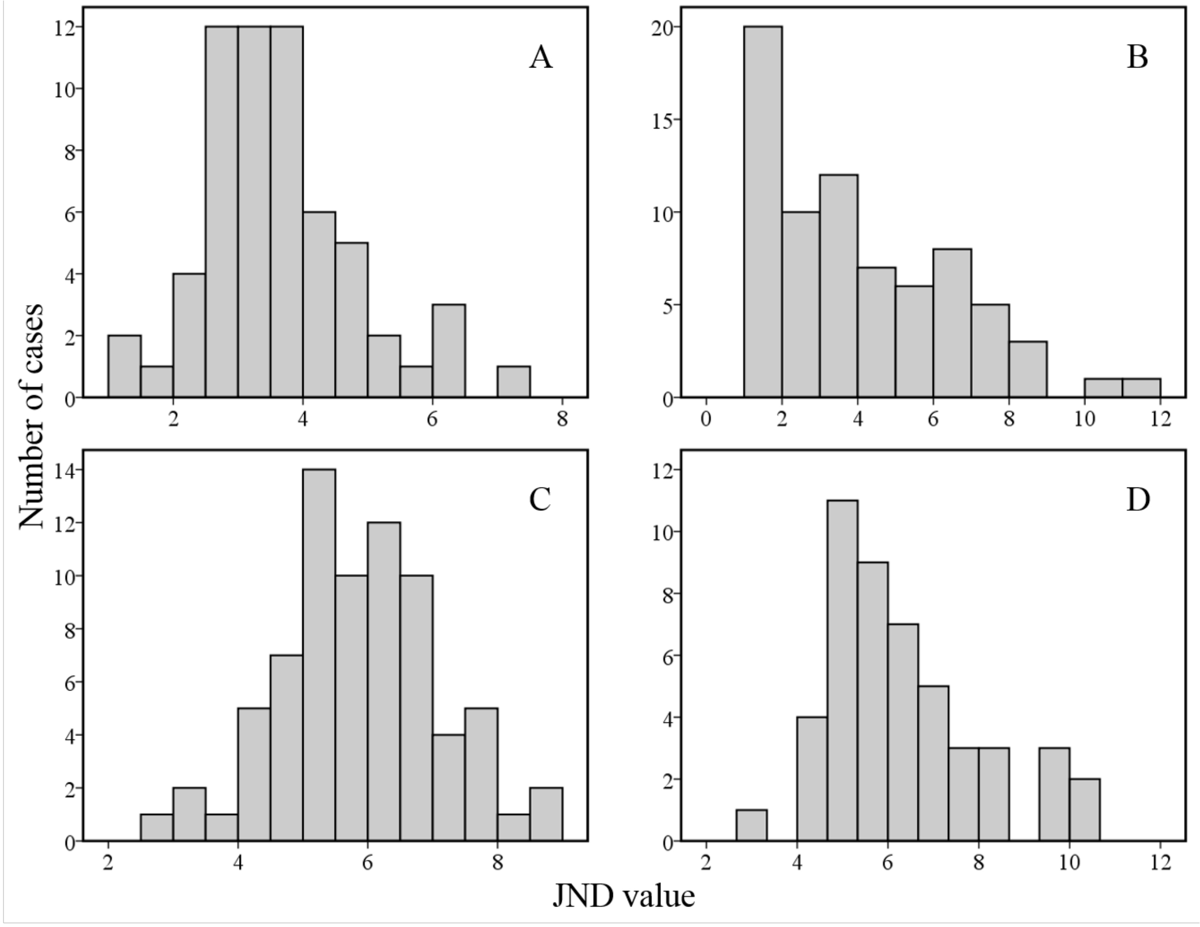

Figure S3. Histogram of chromatic Just Noticeable Differences for A) *Plethodon yonahlossee,* B) *Ambystoma maculatum,* C) *P. montanus,* D) *P. cylindraceus* on a soil background.

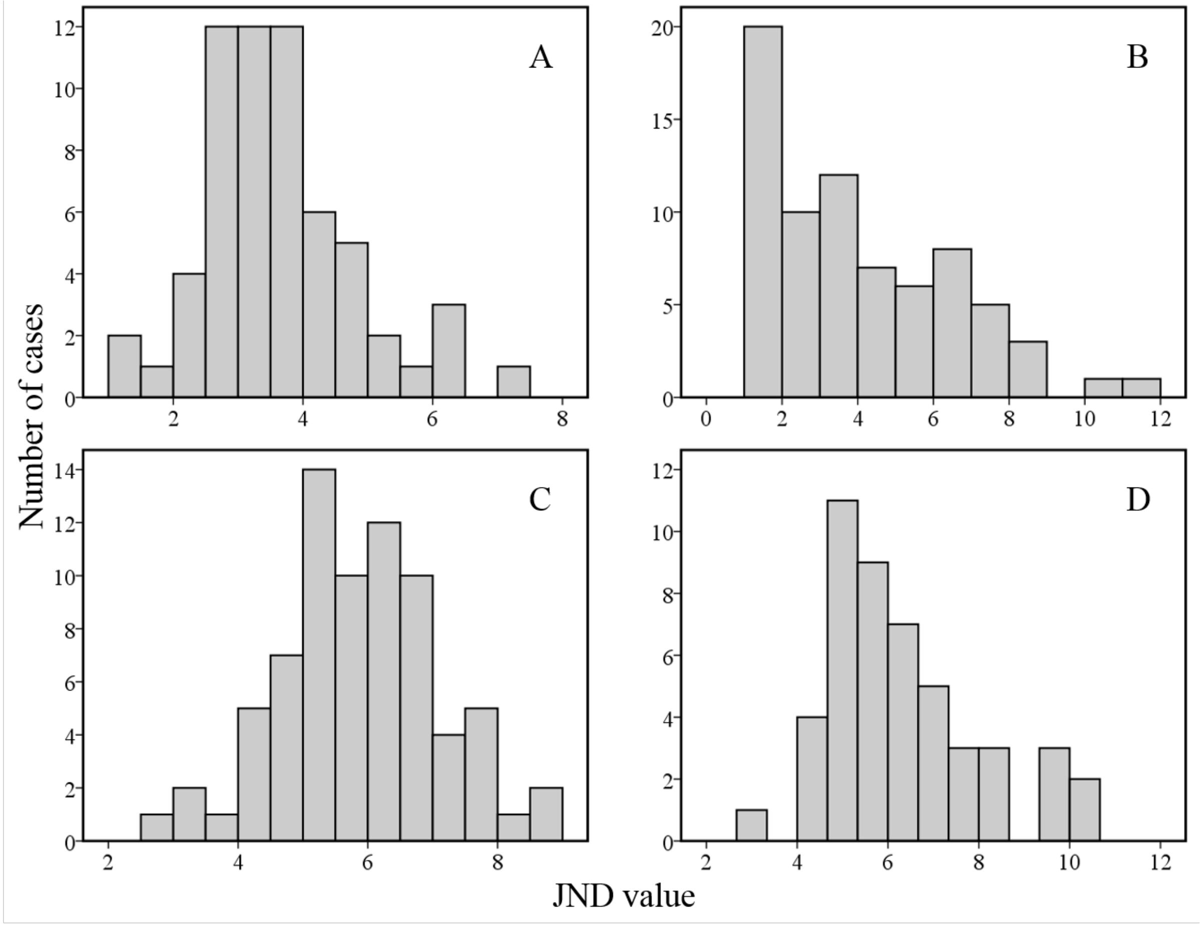

Figure S4. Histogram of achromatic Just Noticeable Differences for A) *Plethodon yonahlossee,* B) *Ambystoma maculatum,* C) *P. montanus,* D) *P. cylindraceus* on a soil background.

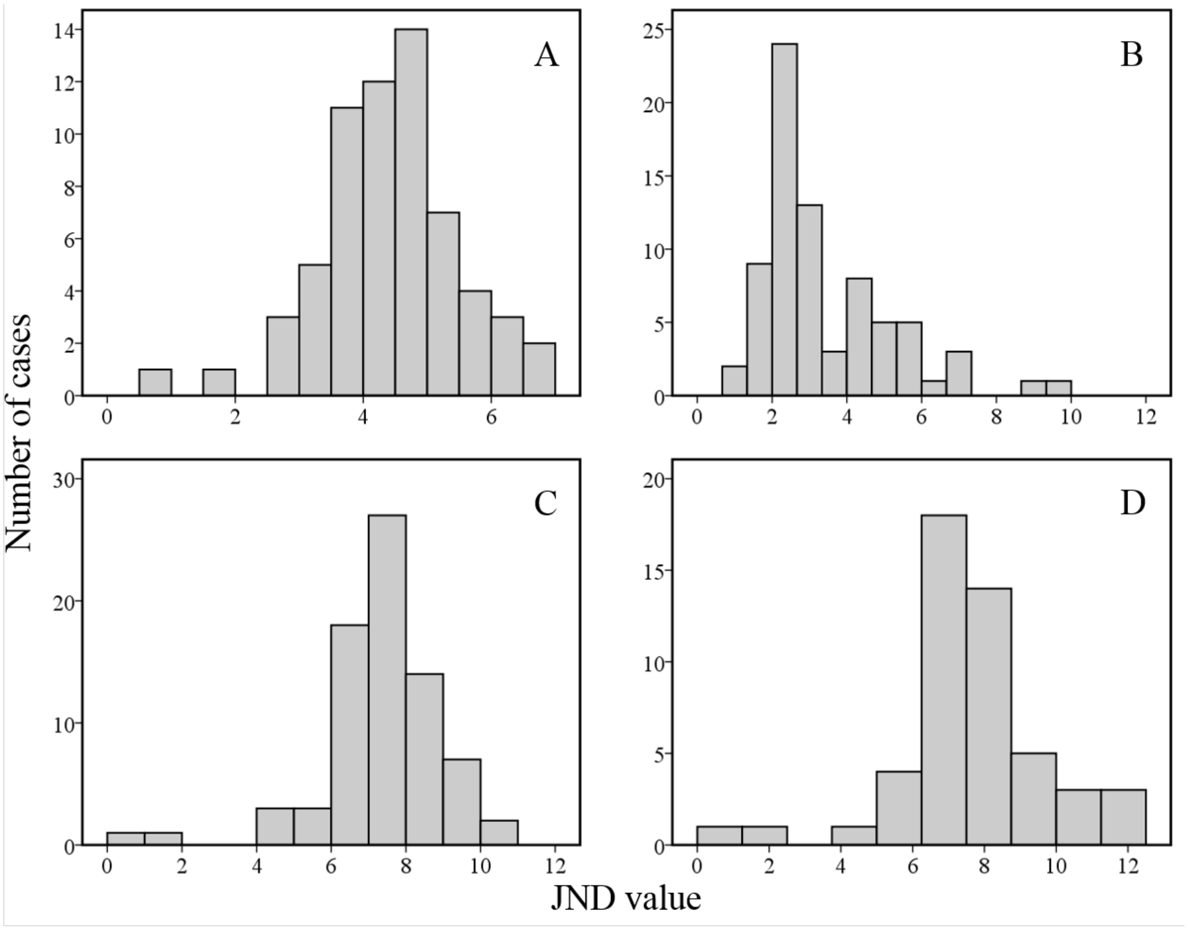

Figure S5. Histogram of chromatic Just Noticeable Differences for A) *Plethodon yonahlossee*, B) *Ambystoma maculatum,* C) *P. montanus,* D) *P. cylindraceus* on a twig background.

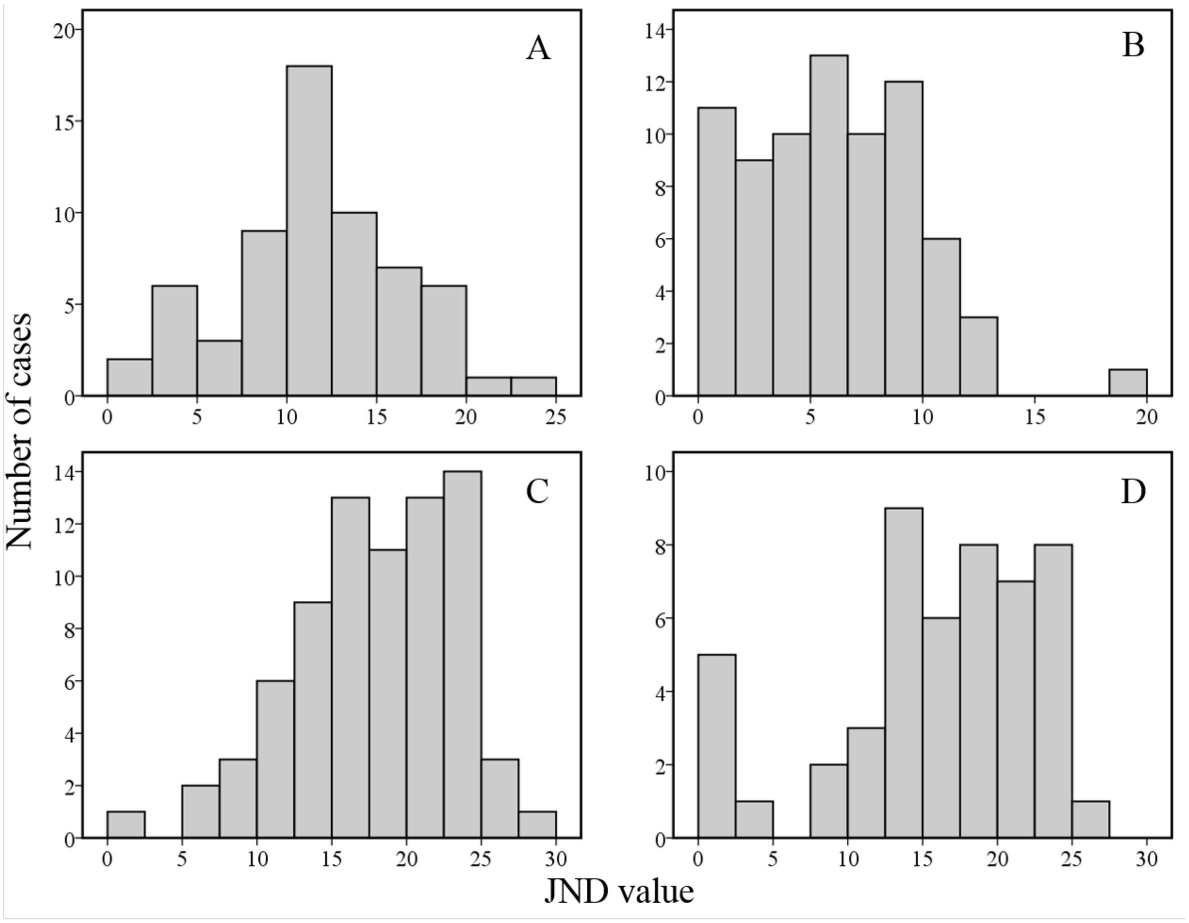

Figure S6. Histogram of achromatic Just Noticeable Differences for A) *Plethodon yonahlossee*, B) *Ambystoma maculatum*, C) *P. montanus*, D) *P. cylindraceus* on a twig background.

CHAPTER 3

Experimental evidence for conspicuous coloration as a predator deterrent in Yonahlossee salamanders

ABSTRACT

Amphibians are renowned for the variation in the color and patterns of their integument, both within and between species. Some amphibian taxa, particularly the poison dart frogs, are well known for using conspicuous coloration to signal their unpalatability to potential predators. Integument secretions contain biologically active compounds that are the source of unpalatability. Although relatively poorly studied, salamanders are often brightly colored and secrete mucous-like substances that may serve physiological and defensive functions. In the southern Appalachians, two closely related salamander species, *Plethodon yonahlossee* and *P. montanus* display vastly different coloration; yet, the signaling function of integument coloration is not well studied. *Plethodon yonahlossee* has a large red dorsal patch, while *P. montanus* is uniformly grey. I hypothesize that the red coloration of *P. yonahlossee* is an aposematic signal that communicates unpalatability to potential predators and predict that avian predators will avoid depredating this species. Here, I used plasticine models of both species to experimentally test whether predators depredate conspicuously colored models less frequently than dull models. Predation rates on grey models were significantly higher compared to that of red models, suggesting that the red dorsal coloration of *P. yonahlossee* is interpreted as a warning signal that deters predation. Future research should address whether *P. yonahlossee* is unpalatable or is effectively mimicking a sympatric aposematic species.

INTRODUCTION

Animals use a variety of signals to communicate with one another, including conspicuous coloration. When conspicuous coloration of potential prey is coupled with a secondary defense, such as venom or poison, color is thought to alert predators to the unprofitability of the prey in a phenomenon known as aposematic signaling (reviewed by Summers et al., 2015). Signals are considered honest when they accurately relay information, dishonest when they do not (Dawkins and Guilford, 1991), and become established in a population when they increase individual fitness (Owren et al., 2010). Cott (1940) suggested that some colors and color combinations (red, yellow, and white, often in combination with black) are broadly used in aposematic signaling because they increase predator recognition of signals of unpalatability. Predators either innately avoid certain conspicuous colors (Smith, 1975) or learn over time to associate the color with the defense, and thus alter their behavior to attack more profitable prey (Mappes et al., 2005).

Poison dart frogs are a particularly well known example of animals that use conspicuous coloration to signal their unpalatability to predators. Several species of the frog family Dendrobatidae, such as the strawberry poison frog (*Dendrobates pumilio*), display bright red, yellow, orange, metallic green, or blue integument coloration (Siddiqi et al., 2004), and recent field experiments using plasticine models demonstrate that predators avoid poison dart frogs with conspicuous coloration (Saportio et al., 2007). Integument secretions of some Dendrobatidae species contain a variety of biologically active compounds that are distasteful or harmful to most predators (Daly et al., 2005).

Other amphibians, including salamanders, also secrete mucous-like substances that may serve physiological and defensive functions (Toledo and Jared, 1995). In the salamander family Salamandridae, representatives of the genera *Taricha, Notophthalmus, Cynops*, and *Titurus* secrete varying amounts of the neurotoxin tetrodotoxin (Wakely et al., 1966), and *Salamandra salamandra terrestris* have been found to secrete the steroidal alkaloids samandarine and samandarone which are also thought to be used in chemical defense (Mebs and Pogoda, 2005). However, salamanders outside of the family Salamandridae are underrepresented in published literature involving chemical analysis of integument secretions, but several studies have used behavioral trials and toxicity assays to determine level of unpalatability in the families Plethodontidae and Ambystomatidae. Representatives of both families secrete mucous-like substances when threatened, some of which seem to deter predation by vertebrates and sicken or kill them when injected into the skin (Brodie and Gibson, 1969; Dodd et al., 1974; Hensel and Brodie, 1976; Brandon and Huheey, 1981).

In the Southern Appalachian Mountains, several species of salamanders display conspicuous integument patterning and coloration, but the signal function of integument color and chemical makeup of integument secretions are largely unexplored in the literature (aside from *Notophthalmus viridescens*, which occurs throughout much of eastern North America and has been extensively studied (Petranka, 1998)). To experimentally test whether one species of conspicuously colored salamander is using integument coloration to avoid predation, potentially as an aposematic signal, I used plasticine models of two local, sympatric salamander species, one with conspicuous coloration (*Plethodon yonahlossee*) and one without (*P. montanus*), to investigate predator response to differences in their coloration. I expected that predators would avoid the more conspicuous models, as they would associate the coloration with unpalatable integument secretions.

MATERIALS AND METHODS

Study species.--- Both *P. yonahlossee* and *P. montanus* occur sympatrically in the Blue Ridge Mountains of North Carolina, northeastern Tennessee, and southwestern Virginia (Petranka, 1998) and occur in the same habitat. *Plethodon yonahlossee* has a dark dorsum with lateral white flecking (giving it a frosty appearance) and a large red to copper dorsal patch which extends from the base of the head to the base of the tail; *Plethodon montanus* is uniformly grey. The close phylogenetic relationships coupled with differences in integument coloration make these model species to explore the signaling function of salamander coloration.

Clay model replicas.---I used pre-colored, non-hardening, non-toxic modeling clay (Sculpey III) to make model replicas of both *P. yonahlossee* and *P. montanus.* Plasticine models are useful for field experiments as they retain impressions from predation attempts and have been used successfully in previous studies of aposematic coloration and mimicry in insects, amphibians, and reptiles (Brodie III, 1993; Brodie III and Moore, 1995; Kuchta, 2005; Saporito et al., 2007). To make the models, I used a hardened clay mold of a *P. yonahlossee* specimen that measured 15 cm total length which falls within the natural size range of both *P. yonahlossee* (11-22 cm) and *P. montanus* (9-18.4 cm). *Plethodon yonahlossee* models were uniformly dark grey with a large red dorsal patch extending from the base of the head to the base of the tail while *P. montanus* models were uniformly medium grey (Fig. 1).

To select clay colors that closely matched reflectance spectra of live salamanders (Fig. 2), I measured the spectral reflectance of 60 *P. yonahlossee* and 76 *P. montanus* as well as each color of clay using an Ocean Optics S2000 spectrophotometer (range 250–880 nm:

Dunedin, FL, USA) using a bifurcated micron fiber optic probe (see Steffen and McGraw, 2007). The probe was maintained at a fixed distance (1 mm) and angle (90) from the skin surface by placing the probe within a rubber stopper held flush with the salamander's skin surface. I illuminated a 2 mm measurement area with a tungsten-halogen bulb. I generated reflectance data by comparing integument reflectance to a white standard (Labsphere, Inc.). To quantify dorsal coloration, I used the reflectance data to calculate red chroma and brightness. Red chroma is the measure of the proportion of light reflected in the red region, calculated as reflectance from 605 – 700 nm divided by the total reflectance (300-700 nm; Montgomerie, 2008), so that an animal with greater red color will have a higher value of chromatic variation in spectral reflectance. Brightness, or the total amount of light reflected by the skin, was calculated as the mean of the summed reflectance from 300 –700 nm, and can be thought of as lighter (brighter) or darker coloration (achromatic variation in spectral reflectance).

Experimental design.---To assess predation under natural conditions, I surveyed Appalachian State University's Gilley Field Station (Watauga County, NC) in May 2016, to ensure both study species occurred in the area. I then conducted three separate trials, each with 800 models: 400 *P. yonahlossee* and 400 *P. montanus*. I arranged models in a 10 x 10 model grid in 8 30 m^2 plots, located throughout the study area. Each plot consisted of 50 models of each species (100 total), spaced at least 0.6 m between each model on all sides. Plots were separated by at least 100 m. To avoid spurious loss of models, I used black biodegradable sushi trays filled with leaf litter as foraging units. To avoid bias in model placement, I flipped a coin or rolled a die to determine which species model to place in each tray. I conducted the first trial June 16, 2016, through June 23, 2016 (8 days), the second trial July 8, 2016, through July 1l, 2016 (4 days), and the third trial September 13, 2016, through September 20, 2016 (8 days), and used new models for each trial.

Quantifying predation.---I assessed each model for presence/absence of attacks and assigned each attack mark to a predator type, including only birds and mammals and disregarding those marked by invertebrates (many marks resulted from snails). Following the method of Saporito et al. (2007), I considered multiple marks on a single model as a single predation attempt. For statistical analysis, I only included avian predation attempts because many of the models appeared to have been attacked by shrews (*Blarina* and *Sorex* sp), and shrews have poor color vision in longer wavelengths (605-700 nm) (Jacobs and Neitz, 1986), making it unlikely that they were able to discriminate between the colors of the models.

Over the course of the 3 trials, 152 models were missing (6.8 %). I did not include missing models in the analysis as there was a storm during the second trial and a tree fell on one of the plots, making it impossible to recover all models from trials.

Statistical design.---All statistical analyses were performed using SPSS v. 23. To determine whether salamander model color was a significant predictor of predation, I used a generalized linear mixed model with a binomial error distribution and binary probit link term. I used model type as a fixed effect and, to account for the possibility that trial influenced predation (all trials were not of equal length), I also used trial as a fixed effect. To account for possible non-independence of samples within the plots, I used plot ID as a random effect. To qualitatively assess similarity of clay models to live animal coloration, I compared the range of red chroma and brightness measures of all the live animals with the values generated from the clay models.

RESULTS

Over the course of the 3 trials (2,248 models), 179 were attacked by avian predators (8.0%) and, of the models attacked by avian predators, 107 (59.8 %) were *P. montanus* and 72 (40.2%) were *P. yonahlossee* (Fig. 3). Salamander model color was a significant predictor of avian predation; grey models were depredated more often compared to red models ($F = 7.770$, $p = 0.005$, df = 1, 2244; Fig. 3). Trial was also a significant predictor of avian predation, predation was lower during the 2nd (and shortest) trial ($F = 28.478$, $p < 0.0001$, df = 2, 2244), however, plot ID was not a significant predictor of avian predation ($Z = 1.457$, $p = 0.145$).

The red chroma of the clay models fell within the range of the red chroma measured from the live animals for both *P. yonahlossee* (live model range: 0.23-0.50, clay model: 0.37) and *P. montanus* (live model range: 0.09-0.29, clay model: 0.27). However, the clay models were brighter than the live models (*P. yonahlossee* (live model range: 0.01-0.11, clay model: 0.20) and *P. montanus* (live model range: 0.004-0.15, clay model: 0.21).

DISCUSSION

I found that avian predators are more likely to attack uniformly grey models than models with a large red dorsal patch. These results are consistent with the hypothesis that the red dorsal patch of *P. yonahlossee* acts as an aposematic signal to potential predators. The plots were designed to be analogous to a choice test such that predators would be able to view the two different models simultaneously and choose which to attack. Although the data support the hypothesis that the red patch is an aposematic signal, because of the lack of available data on unpalatability in salamanders, it is difficult to determine whether predators avoided models with the red patch because *P. yonahlossee* is unpalatable, because predators have an innate wariness of certain colors, or because *P. yonahlossee* may be similar in coloration to an aposematic species (mimicry). Nonetheless, the red coloration is likely interpreted by predators as a warning signal that deters predation.

It is possible, but untested, that the *P. yonahlossee* is a mimic of *N. viridescens*. *Notophthalmus viridescens* secretes the neurotoxin tetrodotoxin and occurs sympatrically with *P. yonahlossee* throughout *P. yonahlossee*'s range (Petranka, 1998). During its intermediate terrestrial stage of development (red eft stage), which can last up to 7 years, *N. viridescens* displays a brilliant orange-red color (Mitchell and Gibbons, 2010). Many other salamanders with red coloration occur sympatrically with *N. viridescens*, and these geographical associations have been used as an argument in support of Batesian mimicry of the toxic species (Brodie, 1977). However, the *P. yonahlossee* may be unpalable or even toxic. Some predominantly red species of Plethodontid salamanders, such as *Pseudotriton ruber* and *Pseudotriton montanus*, secrete toxic compounds that can induce death of chickens and mice (Brandon and Huheey, 1981). Further, *Plethodon jordani*, which typically has red patches on its cheeks, has also been found to be unpalatable to some predators in behavioral trials (Brodie and Howard, 1973; Hensel and Brodie, 1976). Although the toxicity of *P. yonahlossee* has yet to be tested, the unpalatability of other plethodontids suggest evidence in support of signaling warning coloration or Mullerian mimicry.

Most plethodontid salamanders, including both *P. yonahlossee* and *P. montanus*, are nocturnal species and are primarily active on rainy nights (Petranka, 1998), thus evolving conspicuous signals to potential predators may seem counterintuitive. However, *Plethodon* species are often active in the leaf litter on overcast days and can be found under cover objects on most days (Brandon and Huheey, 1975). While *P. yonahlossee* is not often seen during the day, some potential predators (such as grouse and turkeys) scratch in the leaf litter and can uncover individuals (author, pers. obs.). Moreover, despite nocturnal activity patterns, *P. yonahlossee* could have evolved warning coloration through selection pressure caused by being uncovered during the day or from nocturnal predators like owls. Indeed, within the caecilian clade (Amphibia: Gymnophiona), in which nearly all activity occurs underground, species slightly more prone to surface activity have also evolved conspicuous contrasting patterns and yellow integument pigmentation (Wollenberg and Measey, 2009).

Although color was a significant predictor of predation, trial also had a significant effect, which could be due to a number of factors. First, trial 2 was shorter than the other trials by 4 days and 91.1% of attacks occurred during the longer trials, likely because the predators simply had extended opportunity to attack. I conducted trial 2 for only 4 days rather than 8 days to follow the methods of Saporito et al. (2007) and Hegna et al. (2011); both studies using plasticine models conducted research in the tropics. However, I returned to the 8 day protocol after finding few attacks on the models. Predator densities may be much higher in the tropics compared to the temperate climate of Boone, North Carolina. In a similar study conducted in California on *Ensatina eschscholtzii xanthoptica*, the models were presented for 24-25 days (Kuchta, 2005). In addition to trial length, seasonality could have influenced predator attacks. Trials 1 and 2 occurred in early-mid-summer while trial 3 occurred at the very end of summer, and predation rates were higher in trial 3 than trial 1 (95 vs 68 of 163 total). Predation rates may have been higher in late summer because most bird species change from territorial to non-territorial behavior and thus forage over larger ranges.

The red-orange and grey clay models were good chromatic matches as the red chroma fell well within the natural range of red chroma of each respective live species. However, for both species, the clay models were brighter (expressed greater achromatic coloration) compared to the live animals. My goal was to match the chromatic variation as the color of two species differ mainly in chroma (spectral shape) rather than brightness (achromatic color aspects measured as overall area under the curve). Moreover, because the clay models of both species were brighter (~15% brighter) than their live counterparts, and because the experimental design was set up as a choice test, I think it unlikely that greater brightness of the clay models influenced predator choice. To my knowledge, this is one of the first studies to compare reflectance spectra of models and live animals across all wavelengths visible to birds (300 to 700nm).

These data suggest that avian predators avoid the conspicuously colored model salamanders and, while the results of the experiment do not rule out the possibility of mimicry, no studies have rigorously tested whether *P. yonahlossee* is a mimic of *Notophthalmus viridescens*. Further, *Plethodon* species have large granular glands in their integument and future research should focus on identification and quantification of potentially noxious compounds derived from *Plethodon* integument.

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FIGURES

Figure 1. Images of salamander models. Top: *Plethodon montanus* Bottom: *Plethodon yonahlossee*.

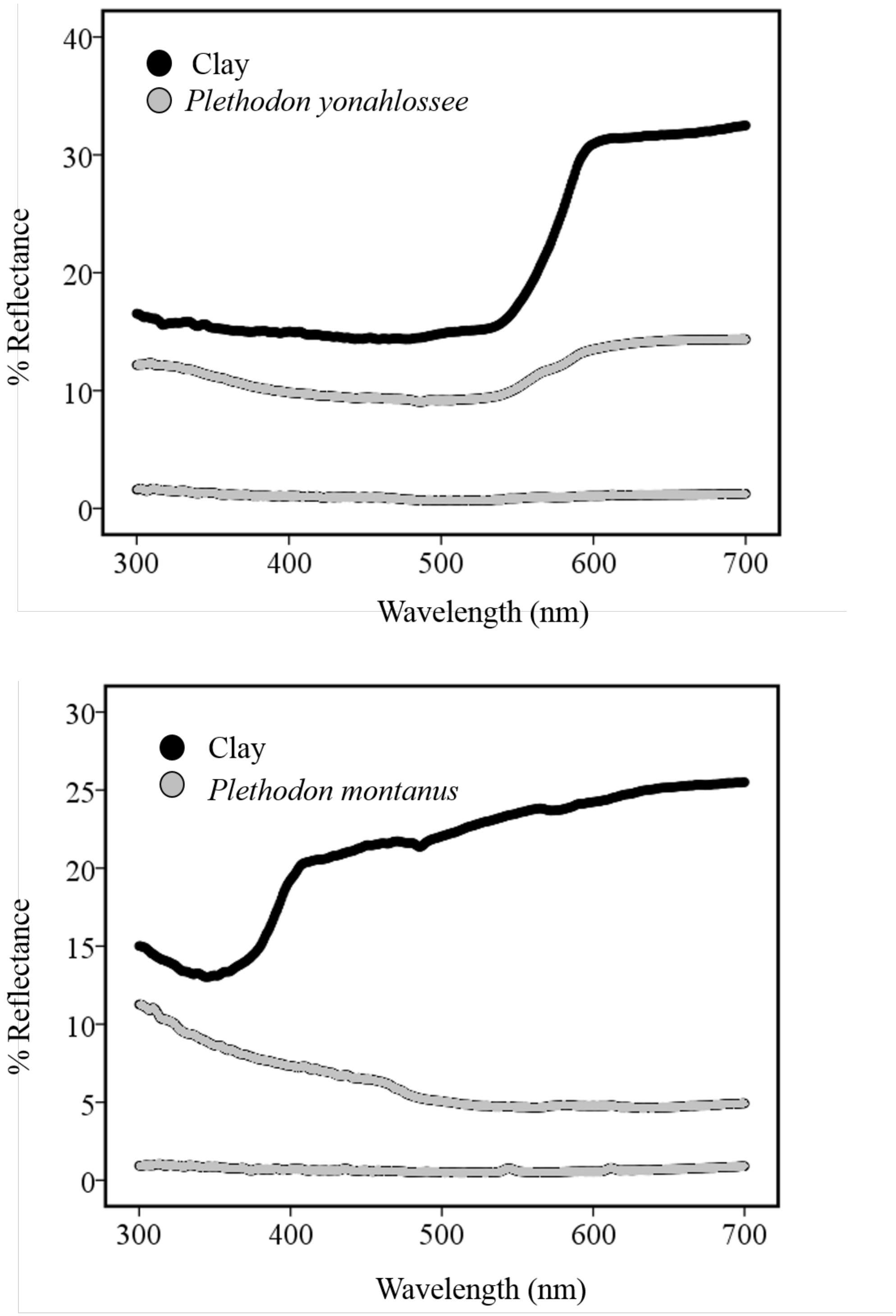

Figure 2. Reflectance spectra of clay color vs two live salamanders of each species (the most and least bright individuals).

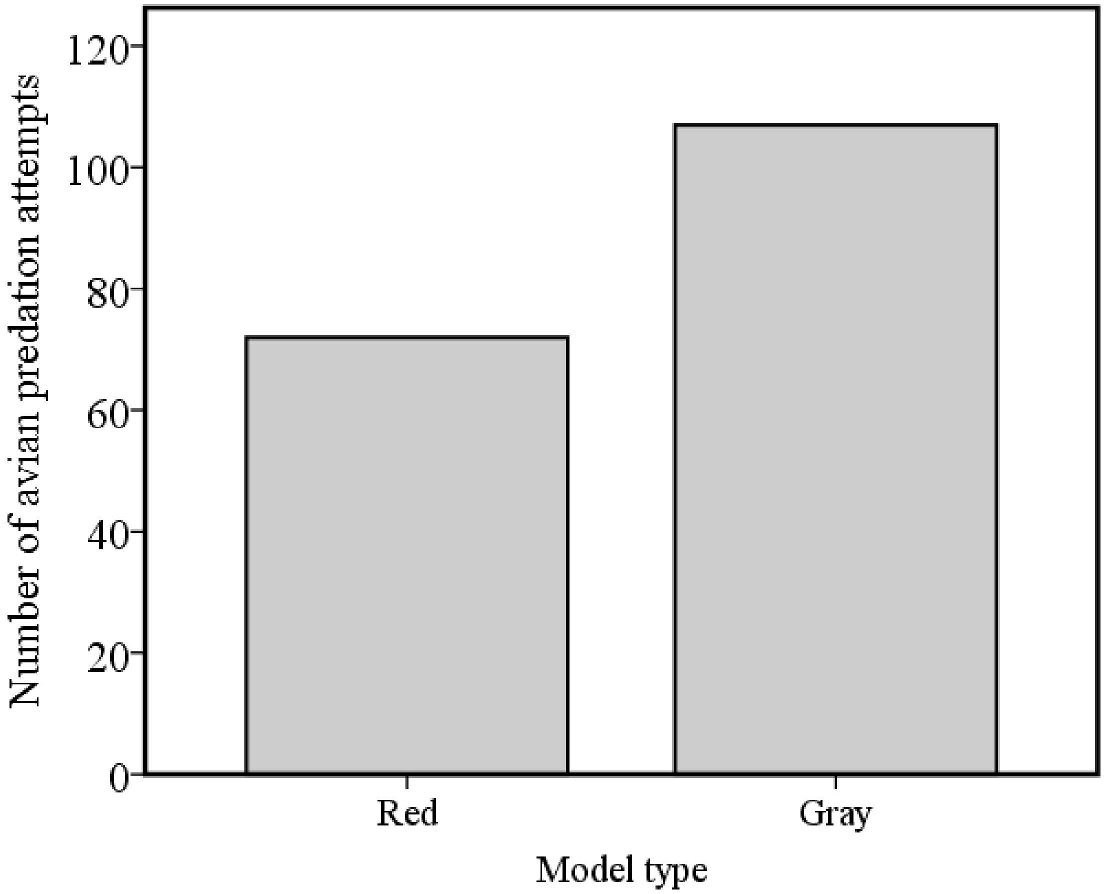

Figure 3. Total number of avian predation attempts on gray and red salamander models.

CHAPTER 4

An investigation of correlated evolution of conspicuous coloration and body size in two salamander genera: *Ambystoma* and *Plethodon*

ABSTRACT

Conspicuous coloration coupled with secondary defensive mechanisms is known as aposematic coloration and is used in predator avoidance and defense. Aposematic signals tend to be more effective in larger organisms as they are intrinsically more easily detected by predators and are also more profitable prey items. Therefore, it is hypothesized that conspicuous coloration evolves in tandem with increased body size in aposematic prey. This study uses a comparative phylogenetic approach to investigate associations between body size and conspicuous coloration in two North American salamander genera: *Ambystoma* and *Plethodon*. Results of the analysis show that increased conspicuous coloration co-evolved with increased body size in *Ambystoma*, but that evolution in *Plethodon* salamanders has favored a negative relationship between these two traits. This is likely because *Ambystoma* are more likely to benefit from an aposematic signal; *Ambystoma* are generally larger than *Plethodon*, exhibit gregarious behavior, and are thought to be toxic to some predators; *Plethodon* are smaller, exhibit no gregarious behavior and are only thought to be noxious to some predators. Proposed explanations for the loss of conspicuous coloration with increased body size in *Plethodon* are alternative uses of color, such as mimicry or thermoregulation.

INTRODUCTION

Animals use a variety of visual signals to convey information to one another, including conspicuous coloration (Wallace, 1867). When conspicuous coloration is coupled with a secondary defense, such as venom or poison, coloration is thought to serve as a signal of unpalatability to potential predators via aposematic signaling (Bates, 1862). Cott (1940) suggested that most often aposematic signals involve red, yellow, or white patterns on a black background, likely because they are easily detected, recalled, and associated with the defense by predators (Gittleman and Harvey, 1980). It is also hypothesized that larger animals are more likely to evolve conspicuous coloration compared to smaller animals for several reasons. First, larger animals are intrinsically more obvious to predators than smaller animals and are therefore more likely to evolve secondary defensive mechanisms (Hossie et al., 2015), and are less likely to benefit from cryptic coloration (Hagman and Forsman, 2003). Second, because smaller animals are less easily detected, they are less likely to benefit from conspicuous coloration (Hossie et al., 2015). Third, larger prey items are expected to be more profitable than smaller prey items, increasing selection pressure for defensive mechanisms (Penney et al., 2012). Indeed, experimental evidence from a study examining the responses of naïve domestic chicks (*Gallus gallus domesticus*) to varying sizes of conspicuously colored prey items (*Tropidothorax leucopterus*) suggests that the efficacy of an aposematic signal increases with increasing body size (Gamberale and Tullberg, 1996a).

There is conflicting evidence in the literature, however, as to whether conspicuous coloration coevolves with increased body size across taxa that employ antipredator coloration. In the caterpillar subfamily Macroglossinae, a phylogenetically controlled analysis found that conspicuous eye-like markings (eyespots), which are thought to deter predation, are associated with larger body size (Hossie et al., 2015). Using a comparative approach, Hagman and Forsman (2003) found that increased conspicuousness also evolved in tandem with increased body size in poison dart frogs (Dendrobatidae); these findings are supported in an alternative study focusing on one species of poison frog, *Oophaga pumilio*, or the Strawberry Poison frog (Rudh, 2013). This species shows remarkable phenotypic diversity throughout its range, and more conspicuous populations also have a larger mean body size, and the phylogenetic relationships suggest that a loss of conspicuous coloration co-evolved with a decrease in body size in several populations (Rudh, 2013). Similarly, a phylogenetic investigation of camouflage morphology in the crab superfamily Majoidea demonstrates that decreased camouflage decoration behavior is associated with an increase in body size as well as an increase in alternative defensive strategies such as color change (Hultgren and Stachowicz, 2009). All the aforementioned studies support the hypothesis that conspicuous coloration is positively associated with body size. However, the opposite pattern occurs in nudibranchs, with conspicuous coloration decreasing with increasing body size (Cheney et al., 2014), despite the presence of secondary chemical defenses (indicating their color is used as an aposematic signal). Finally, in lepidopteran larvae there is no apparent relationship between body size and conspicuousness (Nilsson and Forsman, 2003).

In addition to body size, experimental evidence also suggests that the efficacy of an aposematic signal increases with the size of the conspicuous body pattern (see Gamberale and Tullberg, 1996b), and that avian predators (*Parus major*) are better able to discriminate between palatable and unpalatable prey when the pattern elements are larger (Lindstrom et al., 1999). Behavioral traits are also expected to influence the efficacy of aposematic signals;

efficacy of aposematic signals should be positively associated with group size because there is greater initial unconditioned aversion by predators (Gamberale and Tullberg 1996b; 1998) and because predators will learn more quickly to avoid unpalatable and conspicuous prey (Gagliardo and Guilford, 1993). Finally, conspicuous behavior may both increase the likelihood that the signal is detected and potentially help protect the prey by increasing the likelihood of engaging the predator with any defensive compounds (Brodie, 1977; Toledo and Jared, 1995). For example, the tail is where many of the granular (defensive secretion) glands are located in salamanders (Toledo and Jared, 1995), and when threatened, most salamanders will position their bodies so that the tail is closest to the threatening stimuli (e.g. Brodie and Gibson, 1969).

Here, I use a comparative approach to investigate relationships between conspicuous coloration and body size in two genera of North American salamanders: *Plethodon* (family Plethodontidae) and *Ambystoma* (family Ambystomatidae). *Ambystoma* and *Plethodon* present an interesting contrast for this type of comparative study: *Ambystoma* salamanders are generally larger than *Plethodon* salamanders and nearly all *Ambystoma* species undertake annual breeding migrations, whereas no *Plethodons* are known to exhibit any type of gregarious behavior (Petranka, 1998). Species within both genera display a diversity of color patterns and body sizes (Petranka, 1998) and their integument secretions are unpalatable to some predators (Brodie, 1977).

Behavioral studies of *Ambystoma* species demonstrate that most species display defensive posture when confronted by predators (mammalian, avian, and reptile predators) (Brodie and Gibson, 1969; Brodie, 1977; Williams and Larsen, 1986). This defensive posture involves orienting the body such that the predator is exposed to the granular glands in the parotid region and tail ridge. Researchers described the secretions from the granular glands as being insoluble in water, ethyl alcohol, and acetone, and fatal when injected into mice, voles, and rats (Brodie and Gibson, 1969). *Plethodon* species also exhibit defensive postures, and the genus is generally described as "noxious" (Brodie, 1977). Some species, such as *Plethodon jordani*, have been hypothesized to display aposematic coloration based on feeding trials with avian predators (Hensel and Brodie, 1976), although most species with red coloration are thought to mimic *Notophthalmus viridescens* (Brodie and Brodie, 1980). However, to date, there are no published chemical analyses exploring the chemical composition of integument secretions in any amphibian family except Salamandridae, and very little research has focused on potential aposematic coloration in salamanders.

In addition to interesting similarities and differences between the *Ambystoma* and *Plethodon* genera, there are also recent phylogenies available for each genus which allow for the distinction of associations among traits that are caused by selection versus phylogenetic inertia, a necessity for analysis of evolutionary change (Felsenstein, 1985). The aim of this study is to use hypothesized evolutionary relationships in a comparative analysis of independent contrasts to investigate associations between the evolution of body size and conspicuousness in the *Ambystoma* and *Plethodon* genera. I predict that larger-bodied species will have more conspicuous coloration. I expect the positive trends between body size and conspicuousness to be more pronounced in the *Ambystoma* genus for three reasons: 1) *Ambystoma* are larger than *Plethodon*, 2) there is more empirical support for toxicity in *Ambystoma* than *Plethodon,* and 3) because *Ambystoma* aggregate during breeding while *Plethodon* are solitary year round.

MATERIALS AND METHODS

Data collection.--- My dataset included 50 *Plethodon* species from Wiens et al. (2006) and 17 *Ambystoma* species taken from Williams et al. (2013). To obtain information on conspicuousness for each species, I followed the method of Summers and Clough (2001) and surveyed 43 and 46 undergraduate students for *Plethodon* and *Ambystoma*, respectively. I removed the backgrounds from images of one individual of each species and asked students to rate level of conspicuousness on a scale of 1 to 10 (1 being least conspicuous and 10 being most conspicuous) and then averaged the responses (Summers and Clough, 2001). I used this method to prevent bias in conspicuousness ratings due to background color, while acknowledging that attempts to classify coloration as cryptic or conspicuous without accounting for background can be prone to error (Endler, 1978). Although this method uses photographs and human observations to determine conspicuousness rather than the reflectance spectra of animals and visual systems of natural predators and conspecifics, I considered it sufficient for applying the aposematism theory to these taxa.

I also compiled a database of maximum and minimum total lengths for adults from each species from published literature (Beane et al., 2010; Mitchell and Gibbons, 2010; Petranka, 1998). I used total lengths rather than snout-vent lengths because tail length is variable between species and tail integument is relevant in coloration studies. I found that in both genera maximum total length and minimum total length were significantly correlated (*Ambystoma*: $r = 0.96$, $n = 17$, $p < 0.001$; *Plethodon:* $r = 0.82$, $n = 50$, $p < 0.001$), and thus used average total length in analyses. I obtained phylogenetic information for each species from the published literature (Wiens et al., 2006; Williams et al., 2013). Appropriate phylogenetic trees were available for each genus (*Ambystoma*: Williams et al., 2013; *Plethodon*: Wiens et al., 2006).

Statistical analysis.---To investigate general relationships between body size and conspicuousness, I compared the average total length of each species with the average observer response. First, I treated each species as an independent data point and used Pearson and Spearman correlations (SigmaPlot 12.5). Then, because closely related taxa cannot be considered independent because of shared ancestry, I calculated independent contrasts (Felsenstein, 1985) using the computer software Mesquite (Maddison and Maddison, 2011), with the PDAP module (Phenotypic Diversity Analysis Programs, Garland et al., 1992). For *Ambystoma* species, I used a gradual model of character evolution and obtained branch length information Williams et al. (2013), which was based on the number of substitutions per site in mitochondrial DNA sequences. For *Plethodon* species, I also used a gradual model of character evolution and obtained branch length information from Wiens et al. (2006), which was based on the number of substitutions per site in both nuclear and mitochondrial DNA sequences. To ensure that the data met the statistical assumptions that body size and coloration evolution are related to phylogenetic data, I then regressed contrasts for conspicuousness on contrasts for body size through the origin for both species and found that the slope did not differ significantly from zero (Harvey and Pagel 1991; Garland et al., 1992; Pagel 1993).

RESULTS

A Mann-Whitney Rank Sum Test shows that *Ambystoma* salamanders are larger than *Plethodon* salamanders (*Mann-Whitney U statistic* = 315, $T = 774$, $n(Ambystoma) = 17$, $n(Plethodon) = 50$, $p = 0.049$; Table 1).

***Ambystoma.*---**Pearson correlations revealed no significant relationship between average total length and conspicuousness ($r = 0.36$, $n = 17$, $p = 0.16$). However, regressions of independent contrasts showed that conspicuousness was positively and significantly correlated to average total length ($R^2 = 0.75$, $n = 17$, $p < 0.001$) (Fig. 1). As this analysis used independent contrasts, it is likely that these correlations are not simply the result of phylogenetic inertia. A side-by-side comparison of phylogenies colored to indicate character differences between species and ancestral states provides a visual representation of the relationships parsed by independent contrast analysis (Fig. 2).

***Plethodon.*---**Spearman correlations show that average total length and conspicuousness are negatively correlated ($r = -0.36$, $n = 50$, $p = 0.01$). When I regressed the independent contrasts, conspicuousness was still negatively correlated with average total length ($R^2 = 0.14$, $n = 50$, $p = 0.01$) (Fig. 3). Because the significant relationship between average total length and conspicuousness is still present when independent contrasts are used, it is likely the association is not only influenced by common ancestry. A side-by-side comparison of phylogenies colored to indicate character differences between species and ancestral states provides a visual representation of the relationships parsed by independent contrast analysis (Fig. 4).

DISCUSSION

The results for *Ambystoma* salamanders are consistent with the hypothesis that conspicuousness is associated with body size and the prediction that larger species are more conspicuous than smaller species within a genus. Moreover, in this group, the phylogeny suggests relatively recent evolution of conspicuousness in some species (Fig. 2), suggesting that ecological selection pressures have favored the co-evolution of larger body size and more conspicuous coloration. The initial non-significant associations between body size and color found in *Ambystoma* using the Pearson correlations are likely the result of a small sample size and weaker relationships, which became stronger when independent contrasts were used in lieu of raw data. The results for *Plethodon* salamanders, however, are not consistent with the prediction; smaller animals tend to be more conspicuous. The phylogeny suggests that the ancestral *Plethodon* state is more conspicuous and smaller in body size than current taxa (Fig. 4), suggesting that evolution has favored increasing body size but not conspicuousness. Although initially these results suggest equivocal support for the hypothesis of coevolution between body size and conspicuousness in salamanders, differences between the genera may explain these discrepancies.

The evolution of conspicuous coloration can be influenced by several factors including unprofitability, kin selection, sexual selection, and mimicry of unprofitable organisms (Rudh and Qvarnstrom, 2013). For coloration to be considered aposematic it must convey information of unpalatability to potential predators, and it is expected that conspicuousness should be positively correlated with unprofitability (Summers and Clough, 2001). There is evidence to suggest that both *Ambystoma* and *Plethodon* are unpalatable to certain predators, as well as exhibit behavior consistent with possession of noxious integument secretions (Brodie, 1977). The available evidence in the literature, however, suggests that *Ambystoma* may be less profitable prey than *Plethodon*. *Ambystoma* are known to produce integument secretions which have strong adhesive properties (Evans and Brodie, 1994), neurotoxic components (Hamning et al., 2000), and are lethal to animals such as mice and voles (Brodie and Gibson, 1969). *Plethodon* species are typically avoided in feeding trials (Hensel and Brodie, 1976; Brodie et al., 1979), but their integument secretions have not been shown to be lethal to any potential predators. Therefore, *Ambystoma* may be unpalatable enough for evolution to have favored an increase in conspicuousness even as conspicuousness was lost in *Plethodon.* More comprehensive information on unpalatability would be needed to further address this possibility.

Within the genus *Plethodon*, the negative trend between conspicuousness and body size appears to be driven by a group of morphologically similar and closely related species that consistently received low conspicuousness ratings and are among the largest species in the genus. Phylogenetic studies of *Plethodon* have found that the genus underwent a rapid diversification event roughly 5 million years ago (Highton, 1995; Wiens et al., 2006); several lineages are currently incompletely isolated and are introgressively hybridizing, most notably the *glutinosus* group. The *glutinosus* group encompasses over half of the currently recognized species (Wiens et al., 2006), are among the largest *Plethodons*, received some of the lowest conspicuousness ratings (Table 1), and until recently were considered geographic variants of only two species (*P. glutinosus* and *P. jordani*) based on morphology (Petranka, 1998). Interestingly, *P. glutinosus* is considered one of the more unpalatable species in *Plethodon* based on behavioral trials with shrews (Brodie et al., 1979), and yet is relatively inconspicuous compared to other species in the genus. Perhaps unpalatability in this group is not sufficient to warrant conspicuous coloration as a signal and therefore conspicuousness has decreased as other selection pressures have favored an increase in body size. More data on the ancestral state of toxicity is needed for further investigation of this hypothesis, but this information is currently unavailable in existing literature.

Ambystoma and *Plethodon* salamanders have important lifestyle differences; *Ambystoma* species are largely fossorial with aquatic larvae and a few (*A. mexicanum, A. ordinarium*, and *A. dumerilii* (a data deficient species excluded from analyses)) are fully aquatic (Petranka, 1998). Terrestrial species of *Ambystoma* are largely fossorial, but explosive annual breeding migrations occur in almost all terrestrial species such that large numbers of individuals aggregate out above ground at least once per year (Petranka, 1998). One exception is the inconspicuously colored *A. barbouri*, a streamside species that does not travel long distances to breed. Gregarious behavior is thought to amplify aposematic signals, as large numbers of organisms gathered in one place increases the likelihood of being seen and encountered by predators (Poulton, 1890). Models of the evolution of aposematic coloration and gregariousness suggest that they should evolve in tandem in insects (Sillen-Tullberg, 1988), and indeed gregarious behavior evolved with both chemical defenses and warning coloration in Macrolepidopteran larvae (Tullberg and Hunter, 1996), but comparative studies of gregariousness and conspicuousness are thus far limited to insects. *Plethodon* species are solitary and terrestrial throughout their life cycle and tend to occur in high densities. Most are active on warm rainy nights, as well as under leaf litter and cover objects during the day (Brandon and Huheey, 1975; Petranka, 1998). Though they are rarely seen on the surface during the day, some potential predators (such as grouse and turkeys) scratch in the leaf litter and can uncover individuals (author, pers. obs.). Moreover, despite nocturnal activity patterns, species could have evolved warning coloration through selection pressure from being uncovered during the day or from nocturnal predators like owls. In other amphibian groups, such as the largely fossorial caecilians (Order: Gymnophiona), a slight propensity to surface activity coincided with the evolution of bright yellow integument patterns in 3 separate evolutionary events (Wollenberg and Measey, 2009).

The difference in body size between *Ambystoma* and *Plethodon* may also be relevant, as previous research has shown that the efficacy of warning signals increases with increasing body size (Gamberale and Tullberg, 1996a), and larger animals are thought to be more likely to evolve conspicuous coloration than smaller animals (Hagman and Forsman, 2003; Hossie et al., 2015; Penney et al., 2012). *Ambystoma* are generally larger than *Plethodon* (average *Ambystoma* length = 15.9 cm, average *Plethodon* length = 12.7 cm); *Ambystoma* typically have stout bodies with broad heads and tails, while *Plethodon* tend to be slender with narrow heads and tails (Petranka, 1998). Smaller body size in *Plethodon* salamanders may reduce any potential benefit they would glean from conspicuous coloration, as they are more difficult to distinguish than larger *Ambystoma* and are also less profitable prey. Conversely, larger *Ambystoma* would benefit more from conspicuous coloration and secondary defense mechanisms as they are more distinguishable and more profitable prey. This idea is supported by the exception to the negative relationship in *Plethodon* between body size and conspicuousness: *Plethodon yonahlossee*.

Plethodon yonahlossee is the largest species in the genus, as well as the most conspicuous, and, when removed from the analysis, changes the results rather dramatically; the relationship between average total length and conspicuousness changes from $r = -0.36$ ($n = 50$), to $r = -0.45$ ($n = 49$) (Spearman Correlation) and $r = -0.55$, to $r = -0.29$ ($n = 49$)

(Independent contrasts). Furthermore, recent experimental evidence supports the hypothesis that predators avoid the red coloration of *P. yonahlossee*. Clay models of *P. yonahlossee* are depredated by avian predators at a lower rate than *P. montanus* salamanders and *P. montanus* are much duller than the conspicuous *P. yonahlossee* (Chapter 3, p. 55). Within a *P. yonahlossee* population, larger individuals also display greater red coloration (Chapter 2, p. 22), suggesting some potential benefit of conspicuousness in larger individuals. Such positive associations between conspicuousness (yellow pigmentation) and body size are also present in populations of the poison frog species *Phyllobates terribilis* and *P. bicolor* (Zimmerman, 1986; Walls, 1994). These within-population relationships suggest ontogenetic shifts toward increased conspicuousness and body size, which corroborates the hypothesis that larger animals may benefit more from being highly conspicuous (Hagman and Forsman, 2003). Though the data show that selection in *Plethodon* currently favors large body size and inconspicuous coloration, *P. yonahlossee* has either not undergone the same selection pressures as other *Plethodon* species, is large enough to benefit from conspicuous coloration, or has acquired/maintained a defense mechanism that warrants conspicuous coloration. Because *P. yonahlossee* occurs sympatrically with several other inconspicuous species in the *glutinous* group (and shares many of the same habitat preferences and potential predators), it seems unlikely that differences in ecological selection pressures are driving its coloration, indicating that some combination of the latter two hypotheses is a more likely scenario.

There are possible alternative functions of aposematism to explain coloration in *Plethodon*; the most often explored hypothesis is mimicry. *Plethodon* salamanders occur exclusively in North America and a large portion of their range overlaps with that of the eastern newt, *Notophthalmus viridescens,* which occurs from southern Canada to the tip of Florida and as far west as central Texas (Petranka, 1998). Juvenile *N. viridescens* and *Plethodon* also overlap in size (*Plethodon* total lengths range from 5 to 22 cm (Table 1); *N. viridescens* red eft total lengths range from 3.5 to 8.6 cm (Beane et al., 2010). *Notophthalmus viridescens* secretes the neurotoxin tetrodotoxin (Mosher et al., 1964) and undergoes an intermediate terrestrial stage of development (red eft stage). The red eft stage can last up to 7 years, during which the entire integument is a brilliant orange-red (Mitchell and Gibbons, 2010). Species of *Plethodon* with red coloration on their integument are sometimes considered to be mimics of *N. viridescens*, especially the polymorphic *P. cinereus*, which has an all-red (erythristic) morph (Tilley et al., 1982). Red coloration is common in *Plethodon* species that received high conspicuousness ratings, and though most species with red coloration are not entirely red (*N. viridescens* is entirely red) but a red patch could be sufficient to induce avoidance in predators that have an established innate or learned avoidance response of red eft *N. viridescens*. There are several examples of imperfect mimics (prey items that inaccurately or superficially mimic model species) in the literature, and experimental evidence suggests this may be because predators impose less selection for mimetic fidelity in smaller species because they are less profitable prey (Penney et al., 2012). Sexual signaling is another possible use of conspicuous coloration in *Plethodon,* although this seems unlikely to adequately explain conspicuous coloration in this group. First, there is little evidence of sexual dichromatism in this group (Petranka, 1998) and such sexual dimorphism is a general indicator of sexual selection (reviewed in Andersson, 1994). Second, many *Plethodon* species have evolved an “olfactory” approach to sexual communication, with males using a proteinaceous pheromone stored and dispensed from an enlarged mental gland to increase female receptivity during courtship (Palmer et al., 2005). Further, there may be physiological selection pressure for darker (less conspicuous) coloration in amphibians. Melanin pigmentation gives the body a darker color and aids in thermoregulation and UV protection (Rudh and Qvarnstrom, 2013). Perhaps evolution favored the loss of conspicuousness in larger bodied *Plethodon* species via physiological pressures to increase melanin content in the integument.

The data in this comparative study show that increased body size and increased conspicuous coloration have evolved in tandem in *Ambystoma* salamanders, while increased body size has co-evolved with a loss of conspicuous coloration in *Plethodon* species. Differences between the genera help explain these trends; *Ambystoma* are larger, presumably more noxious, and exhibit gregarious behavior during annual breeding migrations. *Plethodon* are smaller, with evidence indicating they are less noxious than *Ambystoma*, are not known to exhibit gregarious behavior, and several species could potential be imperfect mimics of the toxic *N. viridescens*. More information on toxicity in each genera will increase understanding of antipredator defense mechanisms as well as the signal function of conspicuous coloration.

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TABLES

Table 1. Average conspicuousness rating, maximum total length, and average total length ± 1 standard deviation for each species of *Ambystoma* and *Plethodon.*

Species	**Average Conspicuousness Rating**	**Maximum Total Length (cm)**	**Average Total Length (cm)**
A. annulatum	5.80	23.5	18.75 ± 4.75
A. barbouri	2.50	17.0	14.00 ± 3.00
A. bishopi	2.63	10.5	9.00 ± 1.50
A. californiense	5.67	22.0	18.50 ± 3.50
A. cingulatum	4.59	13.5	11.25 ± 2.25
A. gracile	2.83	22.0	18.00 ± 4.00
A. jeffersonianum	2.72	18.0	14.50 ± 3.50
A. laterale	4.80	14.0	11.00 ± 3.00
A. mabeei	4.74	12.0	10.00 ± 2.00
A. macrodactylum	8.50	17.0	13.50 ± 3.50
A. maculatum	7.15	25.0	22.50 ± 2.50
A. mexicanum	3.13	30.0	25.00 ± 5.00
A. opacum	6.78	10.7	9.85 ± 0.85
A. ordinarium	3.01	16.5	15.25 ± 1.25
A. talpoideum	3.17	12.0	10.00 ± 2.00
A. texanum	3.11	19.0	15.00 ± 4.00
A. tiginum	9.07	35.0	34.00 ± 1.00
P. albagula	3.10	20.0	15.75 ± 4.25
P. amplus	2.60	18.4	13.70 ± 4.70
P. angusticlavius	6.70	12.5	9.53 ± 3.00
P. aureolus	3.70	15.1	12.55 ± 2.55
P. caddoensis	5.20	11.0	10.00 ± 1.00
P. chattahoochee	3.10	20.0	15.75 ± 4.25
P. cheoah	5.20	18.4	13.70 ± 4.70
P. chlorobyronis	3.10	20.0	15.75 ± 4.23
P. cinereus	6.70	12.5	9.50 ± 3.00
P. cylindraceus	3.10	20.0	15.75 ± 4.25
P. dorsalis	6.70	11.0	8.75 ± 2.25
P. electromorphus	2.50	14.5	11.00 ± 3.50
P. elongatus	3.30	15.0	13.00 ± 2.00
P. fourchensis	3.10	17.8	14.65 ± 3.15
P. glutinosus	3.10	20.0	15.75 ± 4.25
P. grobmani	3.10	20.0	15.75 ± 4.25
P. hoffmani	2.50	13.0	10.40 ± 2.60
P. hubrichti	3.00	13.0	10.50 ± 2.50
P. idahoensis	5.20	13.0	9.00 ± 4.00

Species	Average Conspicuousness Rating	Maximum Total Length (cm)	Average Total Length (cm)
P. jordani	6.30	18.5	13.50 ± 5.00
P. kentucki	3.10	17.0	13.25 ± 3.75
P. kiamichi	3.10	20.0	15.75 ± 4.25
P. kisatchie	3.10	20.0	15.75 ± 4.25
P. larselli	5.10	10.5	9.00 ± 1.50
P. meridianus	2.60	18.4	13.70 ± 4.70
P. metcalfi	2.60	18.4	13.70 ± 4.70
P. mississppi	3.10	20.0	15.75 ± 4.25
P. montanus	2.60	18.4	13.70 ± 4.70
P. neomexicanus	2.30	14.3	11.90 ± 2.90
P. nettingi	2.50	11.0	9.50 ±1.50
P. ocmulgee	3.10	20.0	15.75 ± 4.25
P. ouachitae	4.60	17.0	13.50 ± 3.50
P. petraeus	6.00	18.0	14.75 ± 3.25
P. punctatus	3.10	17.0	13.50 ± 3.50
P. richmondi	2.50	14.5	11.00 ± 3.50
P. savannah	3.10	20.0	15.75 ± 4.25
P. sequoyah	3.1	20.0	15.75 ± 4.25
P. serratus	6.7	10.5	8.50 ± 2.00
P. shenandoah	3.9	10.0	8.50 ± 1.50
P. shermani	5.2	18.4	13.70 ± 4.70
P. teyahalee	3.1	20.7	16.40 ± 4.30
P. vandykei	5.9	10.5	10.00 ± 0.50
P. variolatus	3.1	20.0	15.75 ± 4.25
P. vehiculum	5.5	11.0	9.25 ± 1.75
P. ventralis	6.7	11.0	8.75 ± 2.25
P. virginia	3.3	11.2	10.40 ± 0.80
P. websteri	5.9	8.2	7.55 ± 0.65
P. wehrlei	3.9	17.0	13.50 ± 3.50
P. welleri	3.8	9.2	7.80 ± 1.40
P. yonahlossee	8.2	22.0	16.50 ± 5.50

FIGURES

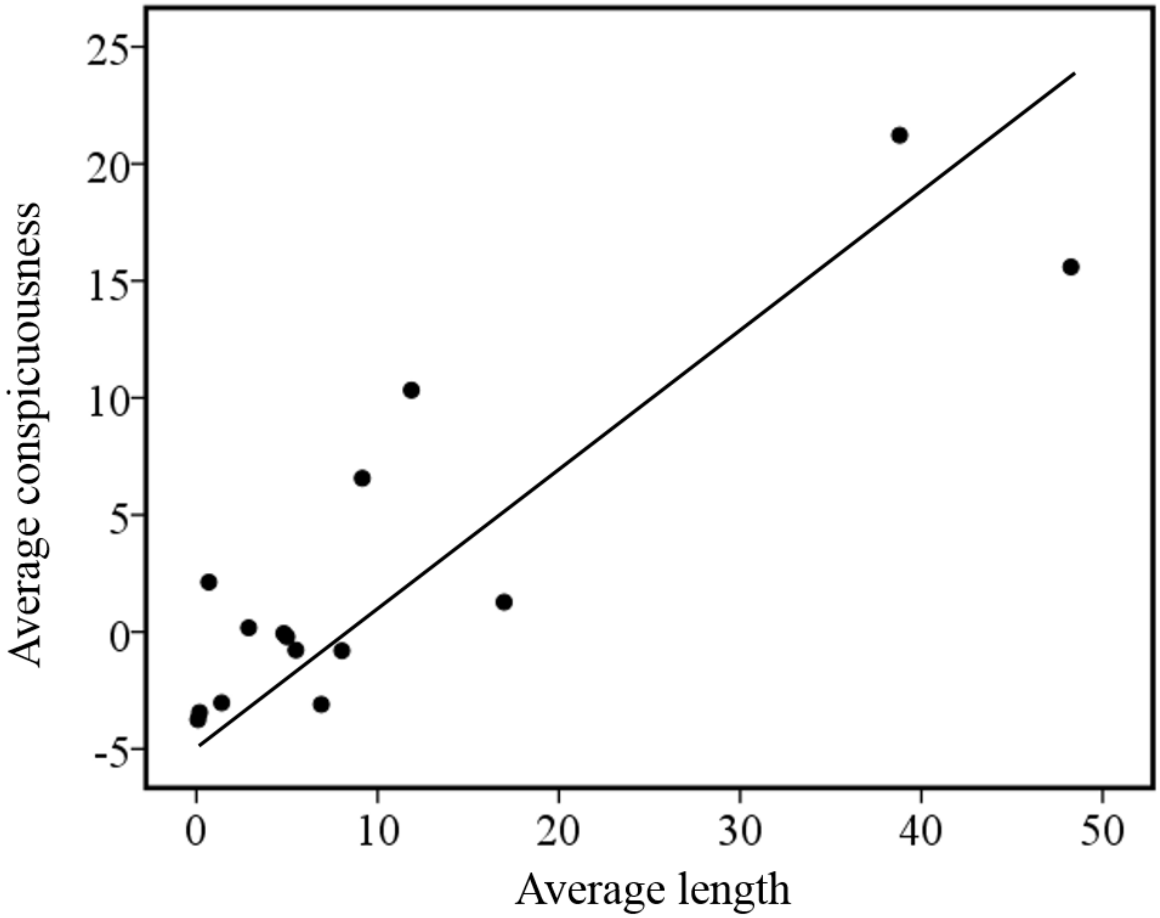
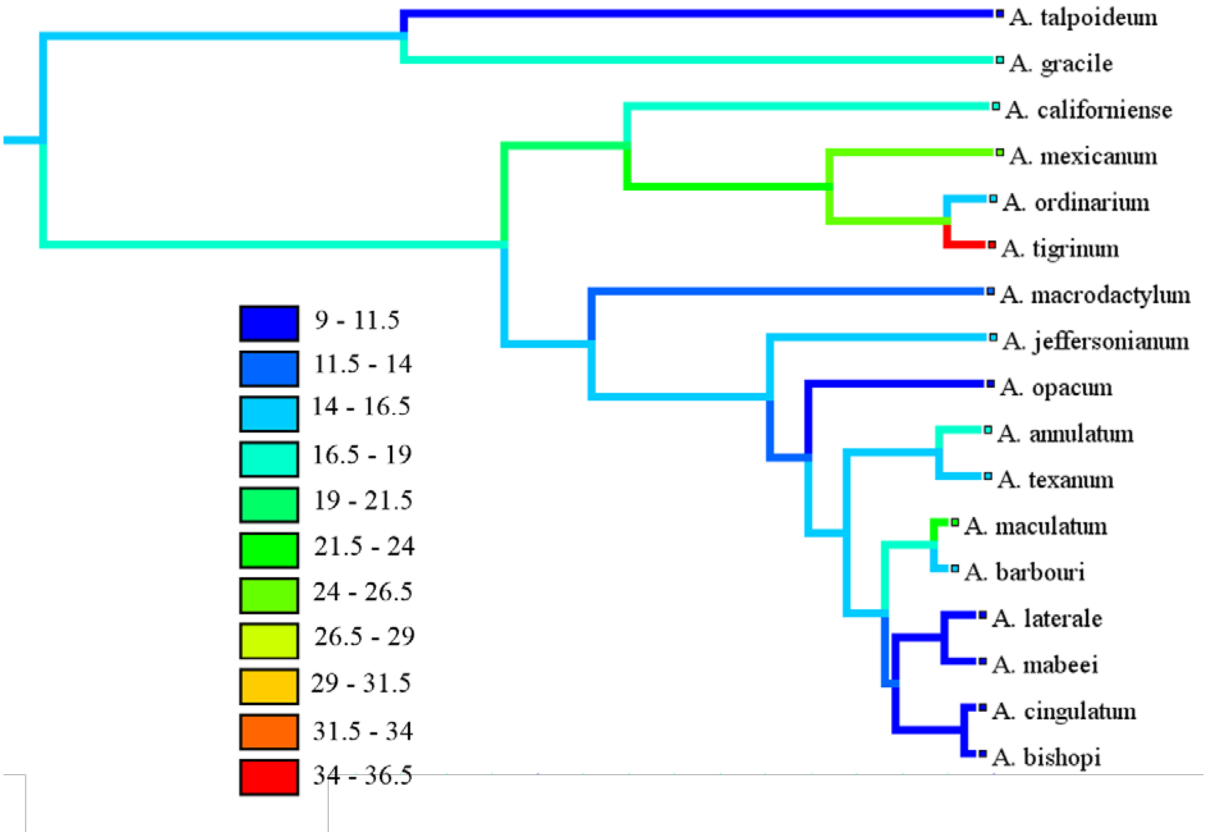


Figure 1. Relationship between coloration and body size in *Ambystoma* salamanders estimated by phylogenetically independent contrasts using a gradual model of evolutionary change. The figure shows standardized contrasts for average total length against standardized contrasts for average ratings of conspicuousness.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A. gracile
A. californiense
A. mexicanum
A. ordinarium
A. tigrinum
A. macrodactylum
A. jeffersonianum
A. opacum
A. annulatum
A. texanum
A. maculatum
A. barbouri
A. laterale
A. mabeei
A. cingulatum
A. bishopi
9 - 11.5
11.5 - 14
14 - 16.5
16.5 - 19
19 - 21.5
21.5 - 24
24 - 26.5
26.5 - 29
29 - 31.5
31.5 - 34
34 - 36.5

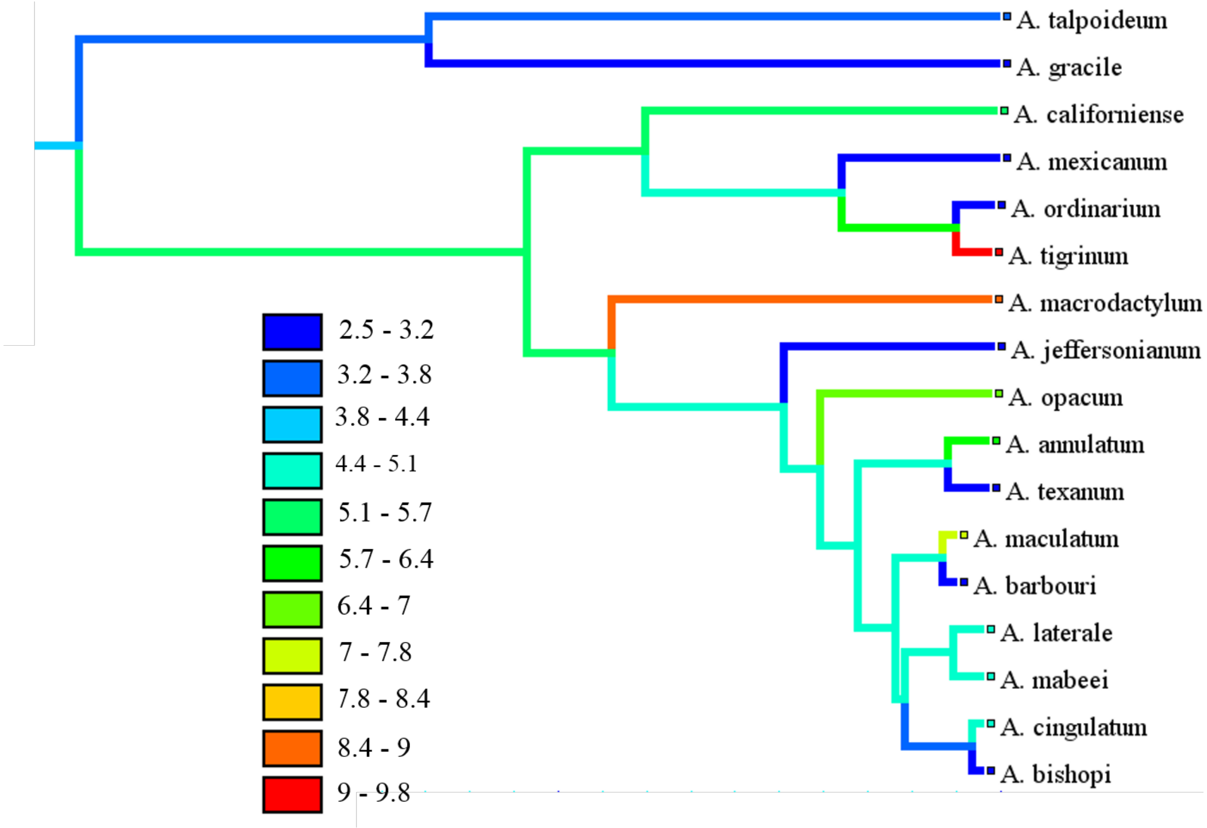

A. talpoideum
A. gracile
A. californiense
A. mexicanum
A. ordinarium
A. tigrinum
A. macrodactylum
A. jeffersonianum
A. opacum
A. annulatum
A. texanum
A. maculatum
A. barbouri
A. laterale
A. mabeei
A. cingulatum
A. bishopi
2.5 - 3.2
3.2 - 3.8
3.8 - 4.4
4.4 - 5.1
5.1 - 5.7
5.7 - 6.4
6.4 - 7
7 - 7.8
7.8 - 8.4
8.4 - 9
9 - 9.8

Figure 2. Evolution of average total length (top) and conspicuous coloration (bottom) in *Ambystoma* salamanders using the squared-change parsimony option in computer software Mesquite version 2.75. Orange and red shades represent a larger body size (top) and more conspicuous coloration (bottom).

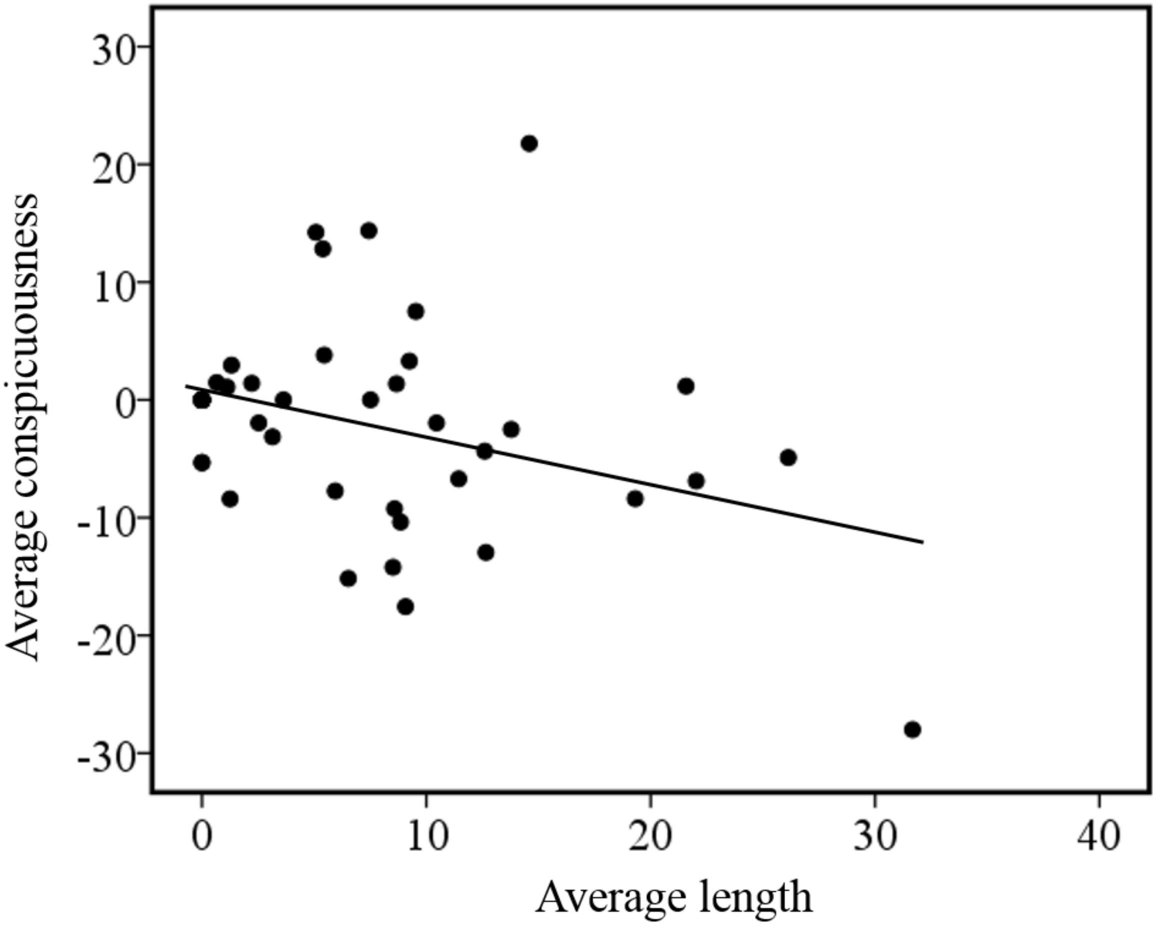
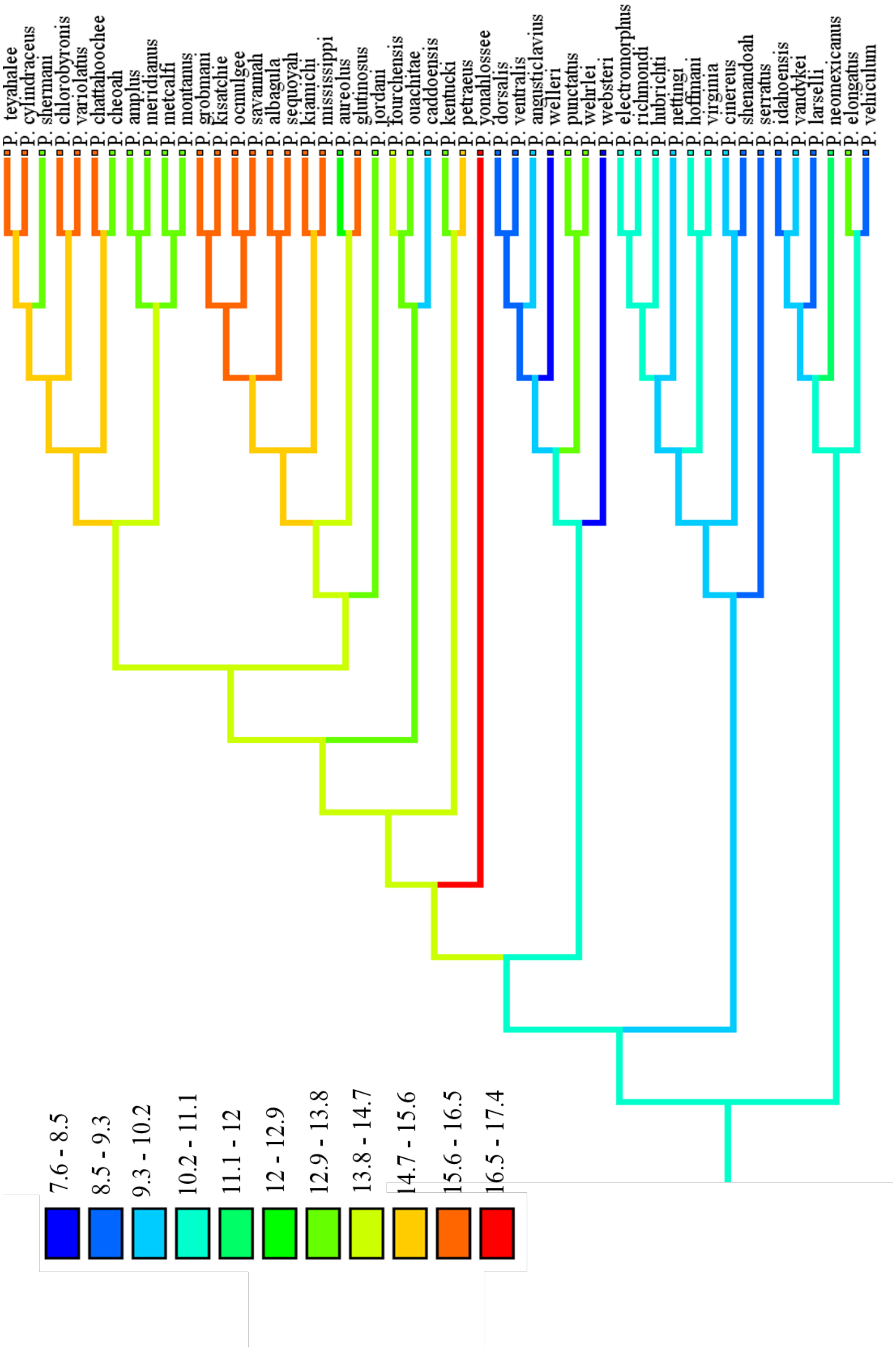


Figure 3. Relationship between coloration and body size in *Plethodon* salamanders estimated by phylogenetically independent contrasts using a gradual model of evolutionary change. The figure shows standardized contrasts for average total length against standardized contrasts for average ratings of conspicuousness.


P. teyahalee
P. cylindraceus
P. shermani
P. chlorobyronis
P. variolatus
P. chattahoochee
P. cheoah
P. amplus
P. meridianus
P. metcalfi
P. montanus
P. grobmani
P. kisatchie
P. ocmulgee
P. savannah
P. albagula
P. sequoyah
P. kiamichi
P. mississippi
P. aureolus
P. glutinosus
P. jordani
P. fourchensis
P. ouachitae
P. caddoensis
P. kentucki
P. petraeus
P. yonahlossee
P. dorsalis
P. ventralis
P. angusticlavius
P. welleri
P. punctatus
P. wehrlei
P. websteri
P. electromorphus
P. richmondi
P. hubrichti
P. nettingi
P. hoffmani
P. virginia
P. cinereus
P. shenandoah
P. serratus
P. idahoensis
P. vandykei
P. larselli
P. neomexicanus
P. elongatus
P. vehiculum
7.6 - 8.5
8.5 - 9.3
9.3 - 10.2
10.2 - 11.1
11.1 - 12
12 - 12.9
12.9 - 13.8
13.8 - 14.7
14.7 - 15.6
15.6 - 16.5
16.5 - 17.4

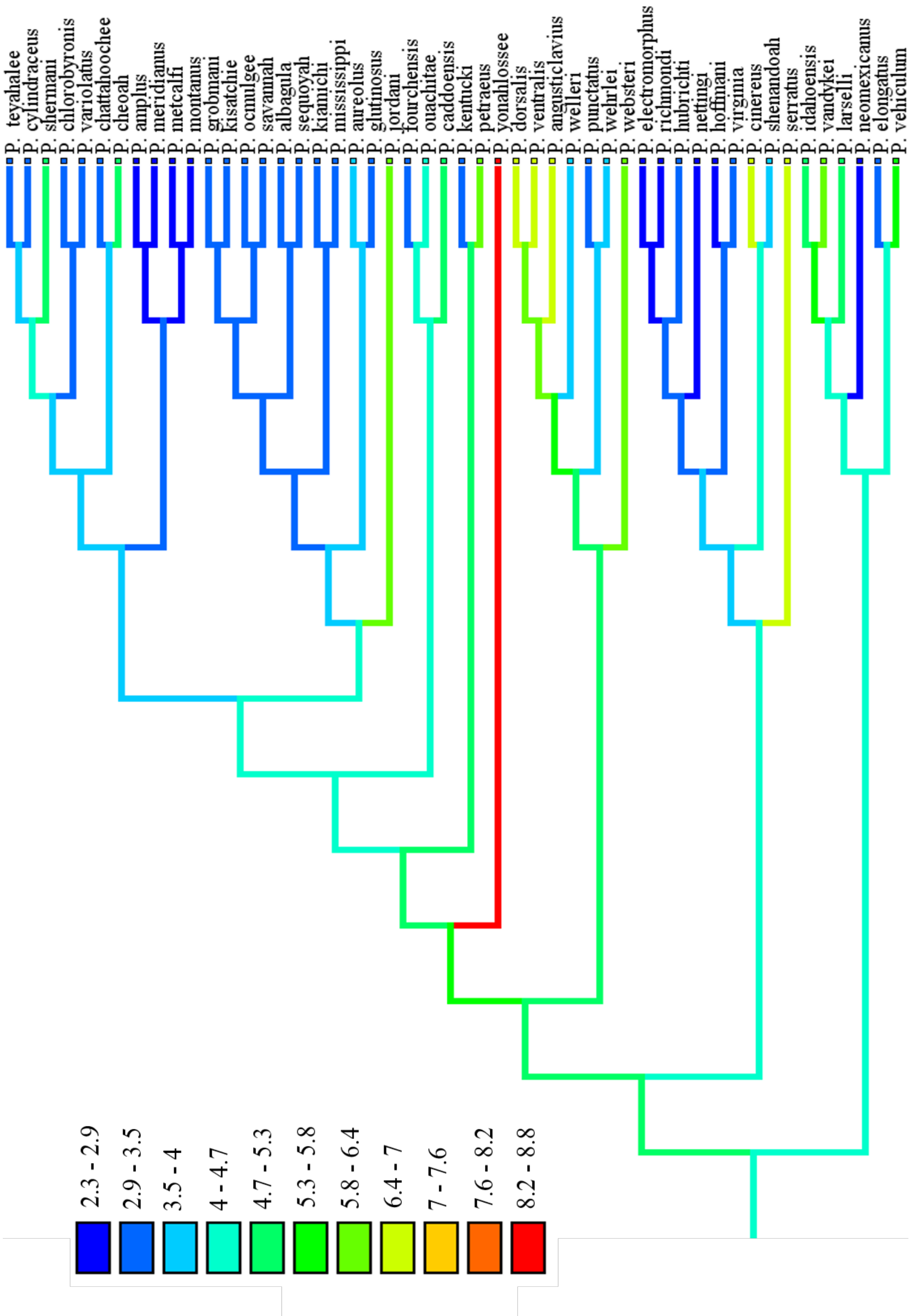

P. teyahalee
P. cylindraceus
P. shermani
P. chlorobyronis
P. variolatus
P. chattahoochee
P. cheoah
P. amplus
P. meridianus
P. metcalfi
P. montanus
P. grobmani
P. kisatchie
P. ocmulgee
P. savannah
P. albagula
P. sequoyah
P. kiamichi
P. mississippi
P. aureolus
P. glutinosus
P. jordani
P. fourchensis
P. ouachitae
P. caddoensis
P. kentucki
P. petraeus
P. yonahlossee
P. dorsalis
P. ventralis
P. angusticlavius
P. welleri
P. punctatus
P. wehrlei
P. websteri
P. electromorphus
P. richmondi
P. hubrichti
P. nettingi
P. hoffmani
P. virginia
P. cinereus
P. shenandoah
P. serratus
P. idahoensis
P. vandykei
P. larselli
P. neomexicanus
P. elongatus
P. vehiculum
2.3 - 2.9
2.9 - 3.5
3.5 - 4
4 - 4.7
4.7 - 5.3
5.3 - 5.8
5.8 - 6.4
6.4 - 7
7 - 7.6
7.6 - 8.2
8.2 - 8.8

Figure 4. Evolution of average total length (top) and conspicuous coloration (bottom) in *Plethodon* salamanders using the squared-change parsimony option in computer software Mesquite version 2.75. Orange and red shades represent a larger body size (top) and more conspicuous coloration (bottom).

VITA

Monica M. Winebarger was born in Boone, North Carolina, in 1990. She attended elementary and high school in Watauga County, NC, and graduated from Watauga High School in 2009. She was accepted into Appalachian State University for Fall 2009, and was awarded a Bachelor of Science degree in Biology, Secondary Education in May 2014. In August 2015, she began her Master's program at Appalachian State University. She was awarded her Master of Science degree in General Biology in May 2017. Monica has been accepted into the Biological Sciences Ph.d. program at the University of Alabama, where she will study freshwater ecology with a focus on freshwater mussels.