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Mapping the Dorsal Skin Pigmentation Patterns of Two Sympatric Populations of Ambystomatid Salamanders, *Ambystoma opacum* and *A. maculatum* from Northeast Tennessee.

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Mapping the Dorsal Skin Pigmentation Patterns of Two Sympatric Populations of Ambystomatid

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by

Lok Raj Pokhrel

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Key words: amphibian, pigmentation, *Ambystoma*, phenotypic variation, sexual dimorphism

ABSTRACT

Mapping the Dorsal Skin Pigmentation Patterns of Two Sympatric Populations of Ambystomatid

Salamanders, *Ambystoma opacum* and *A. maculatum* from Northeast Tennessee

by

Lok Raj Pokhrel

Because of growing concern of habitat fragmentation and its adverse effects on salamander

communities in Appalachian region, sympatric populations of ambystomatid salamanders *A.*

opacum and *A. maculatum* were studied in Northeast Tennessee to address a number of

questions: i) the extent of sexual size dimorphism (SSD) in both species, ii) what traits influence

the dorsal skin pigmentation and how, iii) whether gender differences in developmental stability

occur, and iv) the extent of phenotypic variation within each species. The findings of this study

revealed SSD in both species of salamanders. The most parsimonious statistical model was

developed that explained the influence of body mass, dorsal body area, and sex on development

of dorsal white pigmentation in marbled salamanders. Data on asymmetry indicate that females

are under more stress than males in marbled salamanders, while for spotted salamanders

nonsignificant asymmetry is indicative of similar level of stress in both sexes. Data on

coefficient of variation (CV) suggest stabilizing selection on optimal body size and mass in

female marbled salamanders compared to males; however, for spotted salamanders CV indicates

relatively similar selection pressure for body size and mass for both sexes.

2

DEDICATION

I dedicate this thesis to my wife Puja Karki (Pokhrel), my beloved daughter Angel Pokhrel, my father Sub. Narayan Prasad Pokhrel, and my mother Yashoda Devi Pokhrel. Without your love, patience, and encouragement I would not have made it far.

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I apologize for not mentioning those persons who have extended the help in one way or the other and deserved such thanks.

My vocabulary utterly fails in expressing my accolade to my revered parents who brought me to this stage. I would also like to thank my sisters Kamala and Sarita, brother Bishwa Raj, brother-in-law Bhawanath, and lovely nephew Anish whose encouragement strengthened me to stay away from home and complete my study.

Above all, I must thank my wife Puja for her constant support and encouragement, putting up with my late hours and my spoiled weekends. To Puja and my beloved daughter Angel, I owe an immeasurable debt and deep affection.

CONTENTS

LIST OF TABLES

9

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

Amphibians are declining at an unprecedented scale (Wyman 1990), and evidence suggests that they might be facing a large extinction crisis (Beebee and Griffiths 2005; Mendelson et al. 2006; McCallum 2007; Roelants et al. 2007). Global Amphibian Assessment (GAA conducted by IUCN 2005) revealed that between 33% and 50 % of the world's 6000 amphibian species are currently threatened with extinction and over 120 species have already disappeared since 1980 (Stuart et al. 2004; Moore and Church 2008). Researchers think the sudden decline in amphibian populations might reflect the environmental degradation, as amphibians may be considered as biomarkers of environmental health (Wyman 1990) because of their sensitivity to habitat perturbations and human-induced changes (Blaustein and Wake 1990). The global concern regarding amphibian decline originates from the important ecological roles that amphibians play in wetlands and the surrounding terrestrial habitats. In wetlands, amphibian larvae are found to be significant predators, prey (Duellman and Trueb 1994), and herbivores (Morin et al. 1990). In upland habitats, adult amphibians act as both predators and prey (Porter 1972). Amphibians account for much of the vertebrate biodiversity in uplands and wetlands (Duellman and Trueb 1994). Thus, loss of amphibians may have adverse effects on the wetland and its surrounding terrestrial communities (Beebee 1996).

With the growing human population, increase in anthropogenic activities is generally thought to decrease the viability of the local populations by degrading habitat quality (Francl and Gary 2002), limiting feeding and breeding opportunities, and subsequently increasing regional extinctions of wildlife species (Fernandez-Juricic et al. 2004). Anthropogenic changes in the environment are significant environmental stressors and may be linked to contemporary

evolution (Reznick and Ghalambor 2001). One of the major challenges in conservation and

evolutionary biology is to understand how natural populations respond to anthropogenically

induced environmental changes (Soderman 2006). With the ongoing changes of weather

patterns, depletion of atmospheric ozone, varied invasives, pollutants (Soderman 2006), and

emerging diseases like chytridiomycosis (Davidson et al. 2003) and iridoviruses (Jancovich et al.

1997), sensitive amphibians will be affected (see Davidson et al. 2003), and such effects may be

manifested during ontogeny as asymmetric phenotype (Wright and Zamudio 2002).

Developmental Stability and Bilateral Asymmetry

Developmental stability is the process by which an organism executes the genetically

programmed developmental pathways correctly, producing a phenotype without developmental

errors (Clarke 1995). Under normal conditions, development follows genetically determined

pathways and minor perturbations are controlled by developmental stability mechanisms.

However, under stress the efficacy of stability mechanisms may be reduced such that

development cannot be restored resulting in asymmetric phenotypes (Clarke 1995). As

developmental stability can be greatly affected by both genetic and environmental influences

during ontogeny (Palmer and Strobeck 1986; Leary and Allendorf 1989; Markow 1995), the

ability to buffer against stressors is viewed as a vital indirect fitness component (Clarke 1995).

Pigmentation Biology and Phenotypic Variation

The dorsal skin pigmentation system has long captured the interest of developmental

biologists, geneticists and ecologists (Hoekstra 2006). Color quality and/or pigmentation

patterns frequently exhibit variation both within and between species in quantifiable ways

(Endler 1990). Functions of pigmentation such as crypsis, thermoregulation, and sexual signaling

may impact the nature of variation in pigmentation and color patterns (Thayer 1909; Cott 1940).

In vertebrates, several mechanisms may contribute to regional variation in skin pigmentation. Though several genes have been identified that are linked to melanocyte development and dispersal (Baxter et al. 2004), very little is known about their spatial and temporal control. It has been hypothesized that developmental timing could play an important role in generating regular patterns. For instance, subtle differences in the timing of melanocyte differentiation could be responsible for phenotypic variation in the skin pigmentation patterns in zebra and other mammals (Bard 1977).

All pigment cells originate from a common neural crest precursor whose commitment towards a definite type of chromatophore is not established before its localization in a specific area of the body. Depending on localization, the pigment cells are differentiated from neural crest cells into melanophores, chromatophores, and leucophores/iridophores. Some pigment cells then migrate into epidermis earlier in life, while others remain in the dermis and migrate into the epidermis during metamorphosis (Bagnara 1987). Most neural crest cells are pluripotent, whereas a few are already committed toward a definite phenotype. Environmental effects are also thought to play important role in determining the final phenotype (Bagnara et al. 1979b). Ultrastructure of larval skin has revealed that pattern formation in spotted salamander is closely related to *Triturus alpestris*, where pattern formation is governed by environment. Phylogenetically, *A. maculatum* is closely related to *A. mexicanum* and *A. tigrinum* in which skin pattern formation is solely based on cell-cell interaction (see Epperlein et al. 1996).

Variation during ontogeny that is manifested in phenotype is typically induced by environmental heterogeneity or environmental stress (Berrigan and Scheiner 2004; Gabriel 2005). For instance, the larvae of Arizona tiger salamanders change their body color depending

on substrates in the ponds (Fernandez and Collins 1988). Temperature is also found to be crucial in the fate of development of the pigmentation patterns (Davison 1964).

In a small ecological niche where competition among the individuals of the same species may be intense, intra-population variation would be greater (Adolph 1931). Both inter- and intra-population variation are often related to phenotypic variation induced by local environmental differences (e.g., temperature, resources, competitors, predators) to which the individuals are exposed (Berven 1982; Reznick 1982; Berven and Gill 1983; Stearns 1983). Intra-population variation can be influenced by genotypic variation, non-genetic maternal effects, random effects among individuals (Travis 1980; Kaplan and Cooper 1984), and annual variation in environmental conditions (Collins 1979).

Amphibian metamorphs may differ both at age and size (Pfennig 1992; Collins et al. 1993). Differences are also seen in maturation patterns retaining larval phenotype and becoming sexually mature in the larval aquatic habitat (e.g., Sonora tiger salamander), or metamorphosing and becoming sexually mature in the typical upland terrestrial habitats (e.g., anurans and other salamanders) (Newman 1992; Collins et al. 1993). However, in rare occasion alternate morphs have also been encountered (Pfennig 1992).

To understand the evolution of morphological traits, it is vital to understand the ecological factors that may influence variation in the life history traits of the individuals in the local populations. Body size is possibly the most fundamental trait of an animal (Schmidt-Neilsen 1984). The ontogeny of body size and the overall shape of amphibians differ in predictable ways along environmental gradients, which is suggestive of adaptive response to food scarcity, predation and extreme environmental conditions (e.g., Lee 1993; Cummins and Swan 1995; Morrison et al. 2004; Phillips et al. 2006). Thus, body size has considerable

influence on organization of ecological communities and on the fitness and survival of the individuals (Lawton 1990).

As most amphibians are nocturnal and differentially colored, it has been regarded that the bright color patterns of nocturnal animals function to increase foraging success by providing attractive visual cues to nocturnal prey because bright parts of the animals stand out from the background (Heiling et al. 2003; Tso et al. 2004, 2006).

<div align="center">Sexual Size Dimorphism (SSD)</div>

In amphibians, sexual dimorphism in body size and morphology has drawn great attention (e.g., Shine 1979), but less is known about sexual dimorphism in regard to skin pigmentation patterns (Todd and Davis 2007). Intersexual niche divergence and/or dietary divergence can also amplify or restrain the degree of sexual dimorphism generated by aspects of reproductive biology (Shine 1988). Among amphibians, females are often larger than males because they are presumably selected for higher fecundity (Salthe and Duellman 1973). However, males are larger than females when male–male combat is present (Shine 1979; but see Halliday and Verrell 1986). Although sexual size dimorphism is typically associated with selection for reproductive roles (Shine 1988; Hedrick and Temeles 1989), ecological differences between the sexes in regard to habitat use, predation and diet have also been related to dimorphism (Shine 1989; Anderson and Vitt 1990; Reimchen and Nosil 2004).

Among salamanders, males are larger than females in most desmognathine species (Bruce 1993; Bakkegard and Guyer 2004). However, in the family Plethodontidae, dimorphism tends to be female-biased (Bruce 2000), except for *Hydromantes platycephalus* (Adams 1942).

This study investigated the influence of body size, mass, and sex on the extent of dorsal skin pigmentation in marbled salamander *Ambystoma opacum*. The most parsimonious

explanatory model using general linear model (GLM) was developed via the process of statistical eliminations to illustrate the relationships. This study also determined the extent of sexual size dimorphism in two species of ambystomatid salamanders as quantitative studies on SSD using several parameters of these species were lacking. Levels of developmental stability and phenotypic variation within the populations were also studied.

The following hypotheses were tested: (H1) There is sexual size dimorphism in marbled and spotted salamanders. The prediction was that sexual size dimorphism would be reflected in the extent of skin pigmentation, different body dimensions, and body mass. (H2) The dorsal body area, body mass, and sex of the individuals would influence the extent of pigmentation patterns on the dorsum of marbled salamanders. The prediction was that the body dimensions and their interactions with sex would show significant influence on the extent of white pigmentation in marbled salamanders because as dorsal white pigmentation develops soon after metamorphosis, pigmentation is thought to be somehow influenced by sex related body growth. (H3.1) Phenotypic variation is relatively greater in males than in females of marbled salamanders. Since female marbled salamanders spend more time in parental care during October-December for which there may be stabilizing selection on optimal body size and mass, and because such traits are correlated with increased clutch size, it was predicted that females would show less coefficient of variation than males of marbled salamanders. (H3.2) Phenotypic variation between sexes of spotted salamander is relatively similar. Because spotted salamanders have similar life history between sexes, it was predicted that both sexes would show relatively similar coefficients of variation. (H4) The degree of developmental stability will differ between sexes in both species. It is predicted that as the degree of stress differs between sexes, it would be manifested as subtle differences in bilateral morphological traits like skin pigmentation. So, it

was expected that the relative measure of asymmetry would be significantly different between sexes in both species of salamanders.

CHAPTER 2

MATERIALS AND METHODS

<u>Species Biology</u>

The study organisms are sympatric populations of the marbled salamander *Ambystoma opacum* and spotted salamander *A. maculatum* that inhabit disturbed and fragmented habitats near South Holston Dam in Northeast Tennessee, USA (Smith 2004). In Appalachian ecosystems, salamander communities are very important as they often exceed the combined biomass of other terrestrial vertebrates (Hairston 1987). Because habitat fragmentation and clear-cutting of the woodland have adverse impacts on salamander populations (Ash 1997, Harpole and Hass 1999), it is important to document phenotypic variation in salamander populations.

<u>Marbled Salamander</u>

Ambystoma opacum (Gravenhorst 1807) is widely distributed in eastern North America inhabiting mixed deciduous forests from eastern Texas and Oklahoma, northeast through Illinois and Indiana to southern New Hampshire and central Massachusetts, and south to north Florida (Scott 2005). Some disjunct populations can be found, such as along the southern edge of Lake Michigan (Anderson 1967) and at Osceola island, TN (Hamed et al. 2007) on which this study is based.

Ambystoma opacum is a stout, medium sized salamander that has a black ground color overlain by distinct cross-bands on the entire dorsum. Males have silvery white bands, whereas females have silvery gray bands (Petranka 1998), but there is no consistency in dorsal patterns within sexes (Nobel and Brady 1933).They may have a life span of 8-10 years (Graham 1971; Taylor and Scott 1997).

Males often arrive at potential breeding sites about a week before females (Petranka 1998). Breeding takes place from August to November when each female lays between 1 to 200 eggs on the soil under the cover of leaf litter in the dry pond. Eggs are often brooded by females for 1 to 2 months, and then leave the eggs before they are inundated (Green 1955; Worthington 1968; Petranka 1990). The marbled salamander is the only species of *Ambystoma* that shows parental care (Nussbaum 1985, 1987). The larvae are aggressive and are among the dominant predators in fishless ponds. They are voracious, and will eat both conspecific and heterospecific salamander larvae in ponds (Smith 1990) in addition to zooplankton, isopods, fairy shrimps, insects, snails, and even caterpillars (Petranka 1998). As aquatic larvae, animals are greenish-yellow to black and emerge from natal ponds as dark animals with minor white speckles, which become conspicuous and seen as white saddles or bands as they age (Conant and Collins 1998).
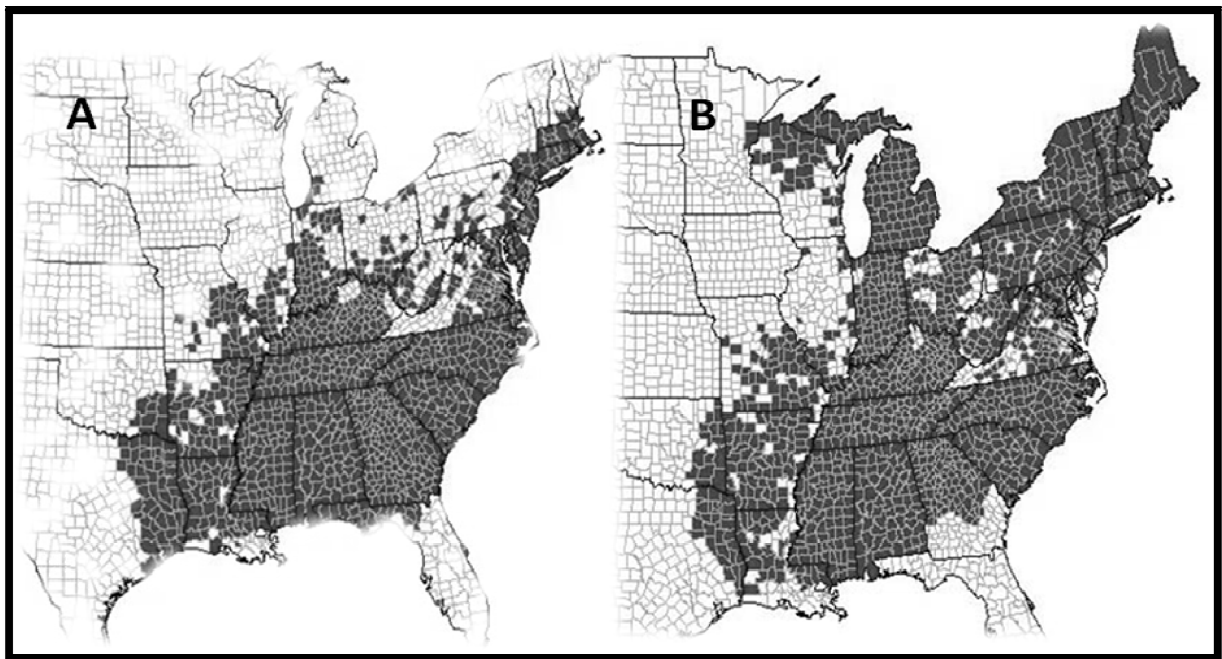
Figure 1 Distribution range (shaded dark) of marbled salamanders (**A**) and spotted salamanders (**B**) in USA (Modified from United States: ARMI National Atlas for Amphibians Distributions, 2004).

Spotted Salamander

The spotted salamander *Ambystoma maculatum* (Shaw 1802) is distributed from southern Canada to eastern and central United States (Petranka 1998). It is a slow moving animal with limited dispersal capabilities (Madison 1997) and inhabits deciduous hardwood and mixed bottomland forests along rivers, swamps, and fish-free vernal pools. It spends most of the time hiding in leaf litter, under fallen wood, or in tunnels below ground (Petranka1998).

The spotted salamander has brown to black skin with bilaterally oriented orange to yellow spots leading down its dorsum, which are acquired following metamorphosis (Petranka 1998). A few spots are also located on the head and legs. This species is an explosive breeder (breeding *en masse)*, and breeds between late winter to early spring when a female usually deposits 2 to 4 egg masses with 1 to 250 eggs in each clutch (Petranka 1998). Adults remain in the pond for only few nights every year (Tennessen and Zamudio 2003; Savage and Zamudio 2005). Larvae develop in ponds throughout the late spring and early summer (Zamudio and Wieczorek 2007). Adults migrate to breeding ponds during late winter to early spring, typically during rainy evenings. They exhibit strong homing behavior to their breeding sites (Whitford and Vinegar 1966), often even entering and exiting the pond repeatedly at similar locations. Homing behavior is sufficiently strong that when captured individuals were released into unfamiliar breeding habitats, they bypassed this habitat and returned to their natal breeding ponds (Shoop 1968; Stenhouse 1985; Sexton et al. 1986).

Study Site and Salamander Collections

The study site is located at Osceola Island Recreation Area in Sullivan County, Tennessee, USA. It is a vernal pool of about 900 feet in circumference (Smith 2004) and is situated one mile below the South Holston Dam on the Holston View Dam road (36.5239°N,

82.1100°W at altitude 1478 ft). This pond was inadvertently created when the Tennessee Valley

Authority (TVA) made a borrow pit to construct a parking lot at the recreational area. It fills with

water in the late winter but dries out by the end of the summer (Smith 2004). A mixed deciduous

forest primarily composed of Sycamore (*Plantus occidentalis*), Sweet Gum (*Liquidambar*

stryaciflua) and White Oak (*Quercus alba*), and non-native bamboo patch (*Phyllostachys*

aureosulcata; 0.5 ha) borders the south of the pond, while shrubby treeless habitat borders the

east, west and north of the pond (Figure 2). About 60 feet away from the pond toward the north

runs the Holston View Dam road, and across this road is a small isolated, fragmented, and

disturbed mixed deciduous forest (0.79 ha) mainly composed of Virginia Pine (*Pinus*

virginiana), Box Elder (*Acer negundo*), and Sycamore (*Plantus occidentalis*) (Hamed et al.

2008) .
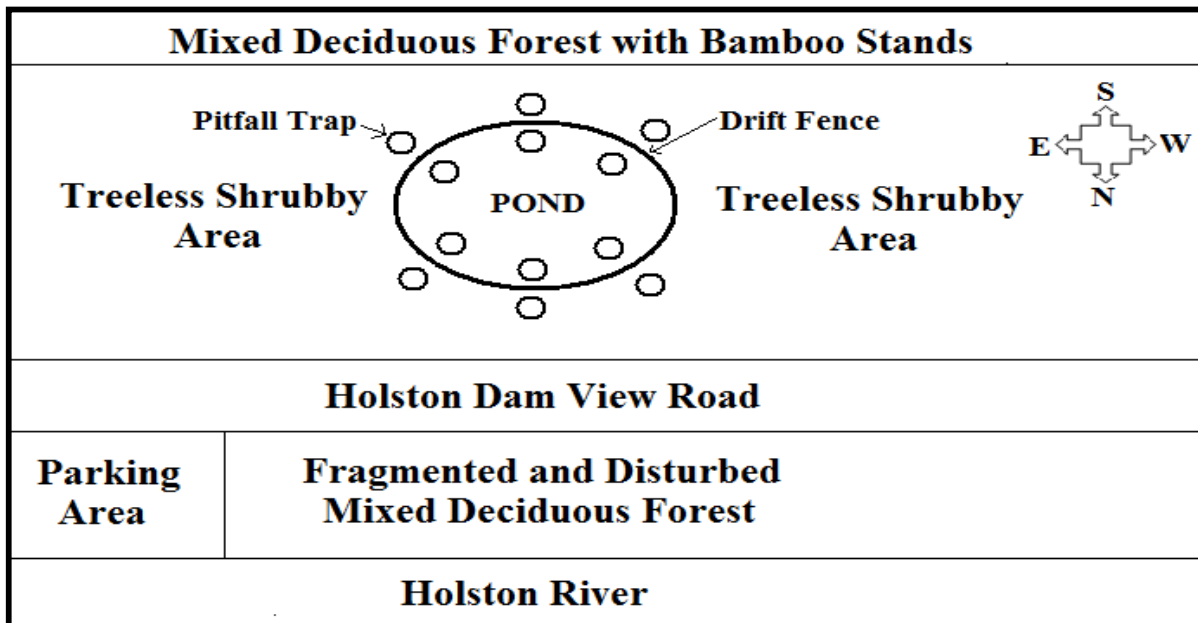
Figure 2 Schematic representation of the study area at Osceola Island, South Holston River, TN
(diagram not to scale)

All marbled salamanders were collected from the same study site during their post-

breeding migration during October and November 2008 by using pitfall traps and drift fence that

are installed around the pond. Spotted salamanders were also collected during post-breeding

migration during March 2008 using the same method mentioned earlier (for details see Table 1).

They were then transported into the laboratory in (2x1.5x1.5) cu. feet rectangular plastic

containers with moistened leaf litter, added to reduce dehydration of the animals, from the same

habitat. The procedures used for collections and handling of the salamanders for this study were

approved by the ETSU animal care and use committee (Protocol # P070902-AS).

Table 1 Collection of Salamanders from Osceola Island Recreational Area, TN

Specimen	Collection date	Male	Female	Total salamander collected (N)
Marbled salamander (*A. opacum*)	10/19/2008	100	0	275
	10/30/2008	6	36	
	11/28/2008	2	131	
	Total	108	167	
Spotted salamander (*A. maculatum*)	03/12/2008	8	14	131
	03/20/2008	56	53	
	Total	64	67	

Photography and Measurements

Salamanders were photographed (Fujifilm FinePix S5800 8MP digital camera) on the

same day of collection and were released to the collection site the next day. For photography, the

camera was mounted at a fixed distance from the stage and the specimen along with a standard

metric ruler was positioned directly under the camera lens so that both the left and right sides of

the specimen were present on each digital image of marbled salamanders. The purpose of using

the standard metric ruler was to provide a calibration scale for Image analysis. For spotted

salamanders, two images were taken for each specimen: one from the left side and the other from

the right side so that the spots could be precisely captured in the same plane. All images were

taken in the same room.

Image analysis software, ImageJ (NIH, Image Processing and Analysis in Java) was used to calculate the dimensions of selected objects in digital images by calculating the number of on-screen pixels in each selected object, then reporting the actual dimensions of the object in millimeters based on a user-defined pixel-to-millimeter ratio. The pixel-to-mm ratio for the images was obtained by measuring the digitized image of standard metric ruler. Depending upon the agility of the salamanders during photography, some were anesthetized using MS-222 (2%) for 15-30 minutes. The gender of animals was identified looking at the vent (for both species, males have parallel ridges inside the cloaca that run perpendicular to the cloacal slit).

For marbled salamanders, the following measurements were obtained (Figure 3): (1) eye-to-eye length (EEL) was measured from the distal part of left eye to the distal part of right eye; (2) neck length was measured at the constricted part of the neck; (3) width between fore limbs (WBFL) was measured from the anterior part of left fore-limb to the anterior part of right fore-limb; (4) dorsal body length was measured by drawing a line mid-dorsally that extended from anterior part of fore-limbs to anterior part of hind-limbs (DBL); (5) right body pigmented area (RBPA) was measured by tracing each band on the right side and taking their sum; (6) left body pigmented area (LBPA) was measured by tracing each band on the left side and taking their sum ; (7) dorsal body area (DBA) was measured by inverting the image so that the edge of the bands make the lateral boundaries and the fore limbs and the hind limbs make the anterior and posterior boundaries such that the area consists of both white bands and dark melanic areas; (8) width between hind limbs (WBHL) was measured from the anterior part of left hind limb to the anterior part of right hind limb; and (9) snout-vent-length (SVL) was measured from the tip of the snout to the distal margin of the vent in live animals. For spotted salamanders (shown right, Fig. 3), (1) total length (TL) was measured from the tip of the snout to the tip of the tail; (2)

snout-vent-length (SVL) was measured from the tip of the snout to the posterior part of vent in live animals; and (3) yellow spots were counted only from the main body.

All morphometric measurements obtained for both species were rounded to three decimal places. A line was drawn down the dorsum of marbled salamanders (see Figure 3) in order to separate bands on left and right sides so that a relative measure of asymmetry could be determined. The body mass was measured with an electronic balance to the nearest 0.001g.
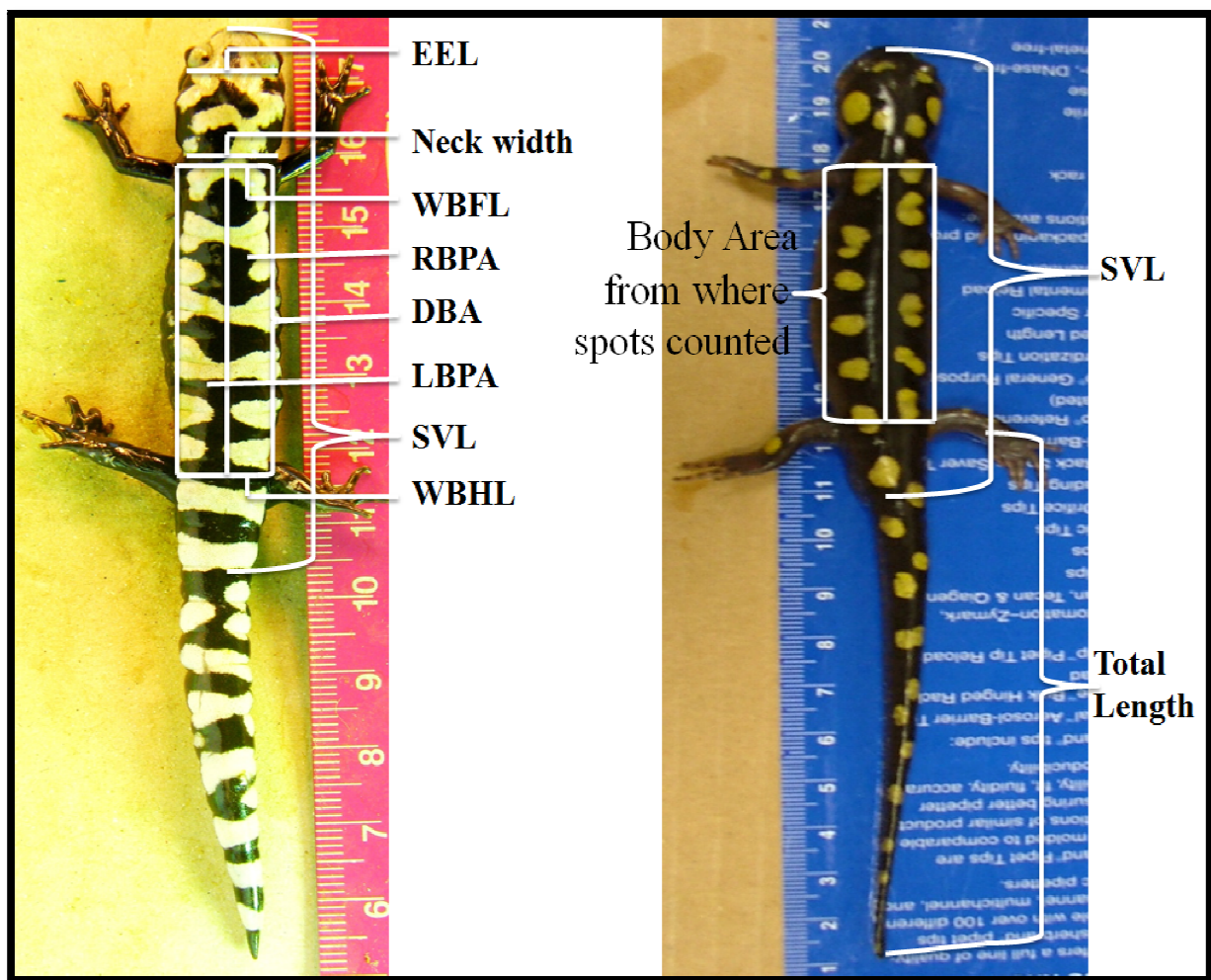
Figure 3 Image analysis procedure to generate morphological measurements for marbled salamander (left) and spotted salamander (right)

Statistical Analyses

All linear morphological traits measured were tested for normality using Kolmogorov-Smirnov test (Table 11) and the data distributions were visualized via frequency histograms. As all the traits showed normal distribution, transformations were not made unless otherwise stated.

Outliers were not removed from the analyses.

Sexual size dimorphism was explored using one way ANOVA for both species. Additionally Principal Component 1 (PC1-Bartlett's scores), which explained the body size, was used to investigate the extent of sexual size dimorphism in marbled salamander. The conditions for Principal Component Analysis (PCA) were met via Kaiser-Meyer-Olkin (KMO) test and Bartlett's test of sphericity. Data were rotated using Varimax rotation with Kaiser Normalization, and Principal Components were obtained by using correlation matrix. This was followed by Hierarchical cluster analysis by using the measure of Squared Euclidean distance and centroid method to generate a dendrogram in order to classify similar animals. Linear morphometric traits such as snout-vent-length, eye-to-eye length, distance between fore limbs, and distance between hind limbs were used to produce the dendrogram to classify the marbled salamanders into similar groups.

The most parsimonious explanatory model was developed to investigate the influence of different body dimensions, body mass, and sex on the extent of dorsal white pigmentation in marbled salamanders by using General Linear Model (GLM) via the process of statistical eliminations. Several candidate sets of plausible models were developed with and without interactions and observed for significance. The standardized residuals were plotted against the unstandardized residuals (fitted value) in order to test the validity of the fitted model. In several cases, frequency histograms of the standardized residuals were also plotted for visualizing

normality. When the model showed significance, the residual plot/histogram did not support linearity because of skewness. Transformations of the dependent variable improved the predicting power of the model. Then by removing unnecessary variables from the models, called model simplification, the most parsimonious explanatory model was designed that could predict the extent of dorsal skin pigmentation in marbled salamanders.

Coefficient of Variation (CV) is an index that may be used to describe the amount of phenotypic variation within and among populations.

Mathematically,

CV= (standard deviation/mean) x100 ………………………………… (I)

CV is particularly useful when comparing dispersion in datasets with different means or with different units of measurement. For different populations or sources, the mean and standard deviation of the traits often tend to change together so that the CV is relatively stable or constant (Snedecor and Cochran 1980). With the increase in normally distributed sample size, the sample CV provides the better estimate of population CV (Mahmoudvand et al. 2007).

Relative measure of asymmetry is a measure of developmental stability that is calculated as the ratio of absolute value of difference between trait on left and right sides to the trait size.

Mathematically,

Relative measure of asymmetry = |R-L| / (R+L) ……………………………… (IV)

Where

|R-L| = absolute value of the difference in means between right side trait and left side trait, and

(R+L) = summation of the mean values of the right side trait and left side trait.

CHAPTER III

RESULTS

Sexual Size Dimorphism (SSD)

1. Marbled Salamander

The correlation between white pigmentation and all morphological traits measured were found to be significant (P<0.001; see Table 2 for details). But the small correlation coefficient values indicate that total white pigmentation might also be correlated with third variable.

Table 2 Correlation Coefficients for different traits of marbled salamanders

Trait	Total White Pigmentation	P
	Correlation r	
Body Mass (g)	0.430	<0.001
Snout-Vent-Length (mm)	0.448	<0.001
Dorsal Body Length (mm)	0.411	<0.001
Dorsal Body Area (mm^2)	0.511	<0.001
Eye-to-Eye Length (mm)	0.367	<0.001
Neck Width (mm)	0.502	<0.001
Width b/w Fore Limbs (mm)	0.387	<0.001
Width b/w Hind Limbs (mm)	0.446	<0.001

Therefore, Principal Component Analysis (PCA) was carried out to investigate how the variables were interrelated (see Table 3 for details). Principal Component 1 (PC1) explained most of the variances for different linear body dimensions (variance=73.971% and eigenvalue=6.657), thus it was taken as body size; PC2 explained most of the variances for body mass (variance=8.942%), while PC3 explained most of the variances for total white pigmentation (variance=5.899%). However, PC1 was only used for further analyses of SSD as it showed greater eigenvalue (Eigen value=6.657). The eigenvalues for PC2 and PC3 were less than 1, and therefore were not used for further analyses.

Table 3 Rotated Component Matrix with Principal Components for marbled salamanders

Trait	Rotated Component Matrix		
	PC1	PC2	PC3
Body Mass (WT)	0.362	**0.870**	0.241
Snout-Vent-Length (SVL)	**0.845**	0.414	0.161
Dorsal Body Length (DBL)	**0.848**	0.383	0.127
Dorsal Body Area (DBA)	**0.862**	0.329	0.271
Eye-to-Eye Length (EEL)	**0.895**	0.292	0.098
Neck Width (NW)	**0.797**	0.059	0.432
Width b/w Fore Limbs (WBFL)	**0.898**	0.241	0.161
Width b/w Hind Limbs (WBHL)	**0.781**	0.217	0.332
Total White Pigmentation (TWP)	0.204	0.205	**0.932**

Results showed a significant difference between sexes in marbled salamanders in regard to body size obtained as PC1 (Welch-F=116.222, P<0.001; see Figure 4). This finding supported the hypothesis of SSD in several body dimensions of *A. opacum*.
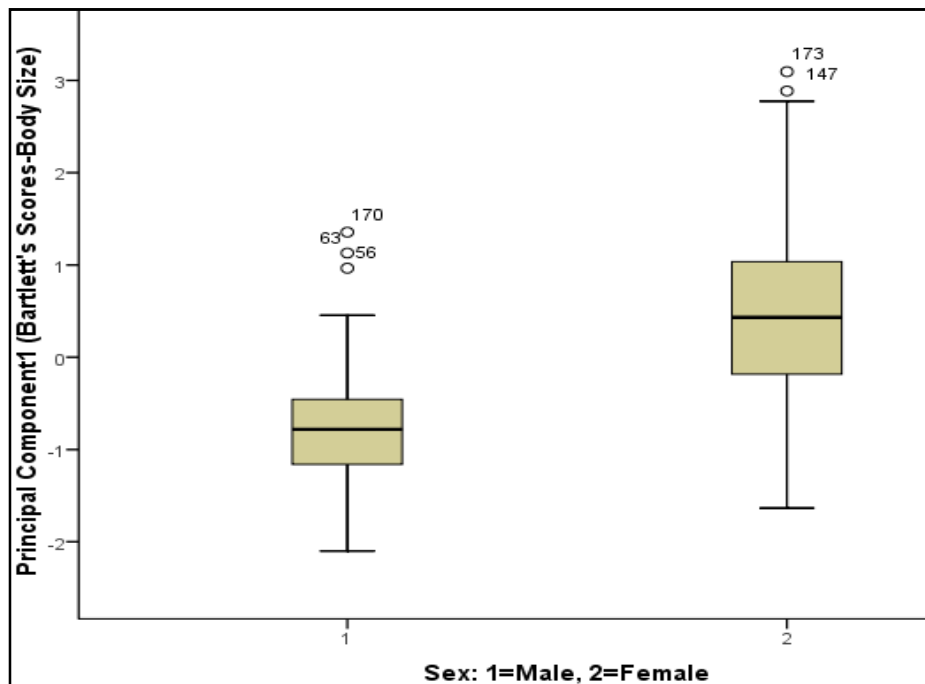
Figure 4 Marbled salamander: SSD in body size obtained as Principal Component 1(Bartlett's Score) using 8 morphological characters (as shown in Table 2)

Additionally one way ANOVA was used for each trait to test whether significant differences in body dimensions between sexes occur. A Mann-Whitney non-parametric test was used to test the significance for percentage of white pigmentation as it was scaled with dorsal body area. All nine morphological traits showed significant differences between sexes ($P<0.001$), except for total white pigmentation ($F=0.024$, $P>0.5$) and total number of white bands ($F=2.253$, $P>0.1$). Details on sexual size dimorphism are given in Table 4 (see Appendix for Figures 9-13 on SSD). This finding supported the hypothesis of sexual size dimorphism in different body dimensions of marbled salamanders.

Table 4   One way ANOVA test for sexual size dimorphism in marbled salamanders using several morphological traits

Trait	Mean±SD		F	P
	Male	Female		
Body Mass	6.685±1.886	7.817±1.355	33.443	<0.001
Snout-Vent-Length	60.426±6.424	70.567±6.314	166.314	<0.001
Eye-to-Eye Length	9.990±0.740	11.197±0.818	152.811	<0.001
Neck Width	9.399±1.042	10.105±1.075	28.843	<0.001
Width between Fore Limbs	10.322±1.144	12.079±1.104	160.614	<0.001
Width between Hind Limbs	9.799±1.079	10.832±1.151	55.283	<0.001
Dorsal Body Length	32.511±4.605	39.973±4.202	191.130	<0.001
Dorsal Body Area	325.160±78.092	444.996±89.009	130.119	<0.001
% White Pigmentation	54.866±9.535	41.134±9.563	2715.000	<0.001
Total White Pigmentation	179.473±55.377	180.445±47.987	0.024	>0.5
# White Bands	5.70±0.924	5.86±0.840	2.253	>0.1

Results showed that females are on average significantly larger (both in body length and body widths) and heavier (in body mass), but with significantly lesser coverage of percentage of dorsal white pigmentation than males. However, no significant difference was observed in total number of white bands and total white pigmentation between sexes. The mean number of bands was 5.70 and 5.86 for males and females respectively. The mean total white pigmentation was 179.473 mm^2 and 180.445 mm^2 for males and females respectively.

Hierarchical Cluster Analysis was performed using the measure of Squared Euclidean distance that classified the marbled salamanders into two large groups of males and females; but one small cluster of smaller males was also produced which was not expected. Linear morphometric traits such as snout-vent-length, eye-to-eye length, distance between fore limbs, and distance between hind limbs were used to produce the dendrogram (Figure 5) using centroid clustering method that showed the relative cohesiveness of the groups of marbled salamanders.
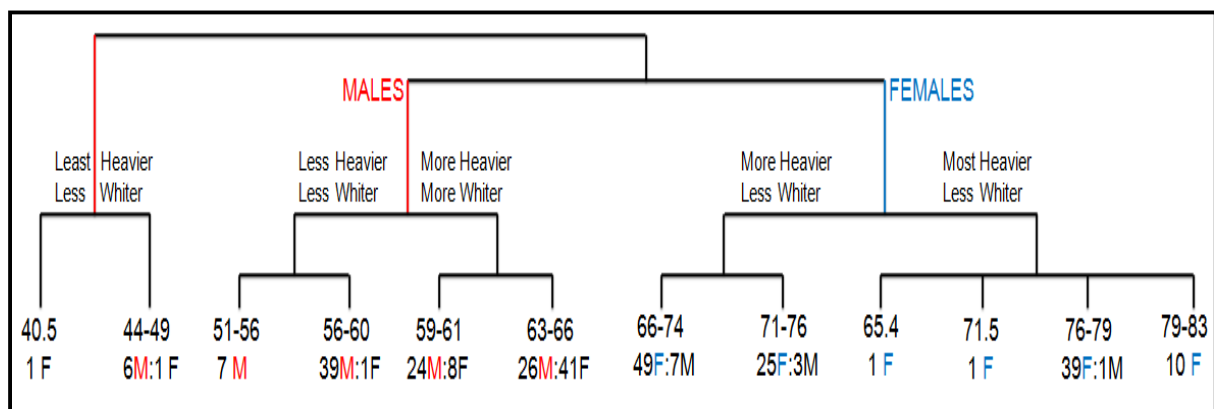
Figure 5 Dendrogram generated by Hierarchical Cluster Analysis of marbled salamanders using linear morphometric traits such as snout-vent-length, body length, eye-to-eye length, distance between fore limbs, and distance between hind limbs. Data just below the branch of dendrogram represent the range of SVL below which are the numerals representing the ratio of number of males (M) to females (F).

2. Spotted Salamander

There was a significant difference between sexes in spotted salamander in body mass, snout-vent-length and total length, but not for total spots number on the dorsum (Table 5).

Table 5 One way ANOVA test for sexual size dimorphism in spotted salamanders

Trait	Mean±S. D.		F	P
	Male	Female		
Weight (g)	16.403±3.240	18.327±3.781	9.740	<0.005
Snout-Vent-Length (mm)	91.406±6.529	97.074±7.406	21.528	<0.001
Total Length (mm)	177.937±13.614	187.194±16.721	12.007	<0.005
# Total Spot	10.670±1.985	10.810±1.964	0.151	>0.5

Data showed that females are significantly heavier (in body mass) and larger (in Snout-Vent-Length and Total length) than males in spotted salamanders; however, there is no intersexual difference in yellow skin pigmentation (measured as total number of spots counted on the dorsum). This finding supported the hypothesis of SSD in spotted salamanders.

<u>Designing Statistical Model for Dorsal White Pigmentation</u>

The most parsimonious statistical model that explained how the body mass, dorsal body area, and sex could influence the ontogeny of dorsal white skin pigmentation in the marbled salamander is developed via model simplification using a general linear model (GLM). The model showed main effects of body mass and dorsal body area, a significant interaction of sex with dorsal body area, and a significant interaction of sex with body mass and dorsal body area on the generation of dorsal white pigmentation in marbled salamander. This predictive model of white pigmentation for marbled salamander supported the hypothesis that skin pigmentation can be influenced by body mass, dorsal body area, and sex of the individual salamander.

The fitted model is as follows:

Ln (Total White Pigmentation) =3.398+ 0.139 Body Mass + 0.003 Dorsal Body Area + 0.002(Sex*Dorsal Body Area) - 0.0002 (Sex*Body Mass*Dorsal Body Area)

The statistical analyses and the parameters of final model (see Table 6) along with the residual plot (see Figure 6) are presented below.

Table 6   Most Parsimonious Statistical Model designed by using GLM via statistical eliminations that shows the influence of Body Mass, Dorsal Body Area (DBA), and Sex on logarithm of Total White Pigmentation

Dependant Variable: White Pigmentation Variable	Type III Sum of Squares	Mean Square	F	P
Intercept	13.238	13.870	316.794	<0.001
Body Mass	1.180	1.180	26.941	<0.001
DBA	2.801	2.801	63.979	<0.001

Table 6   Continued

Sex*DBA	0.820	0.820	18.725	<0.001
Sex*Body Mass*DBA	0.877	0.439	10.019	<0.001
Error	11.690	0.044		
Coefficients Table				
Predictor	B	Std. Error	t	P
Intercept	3.398	0.191	17.799	<0.001
Body Mass	0.139	0.027	5.190	<0.001
DBA	0.003	0.000	7.337	<0.001
Sex*DBA	0.002	0.000	4.327	<0.001
Sex*Body Mass*DBA	-0.0002	0.000	-3.998	<0.001
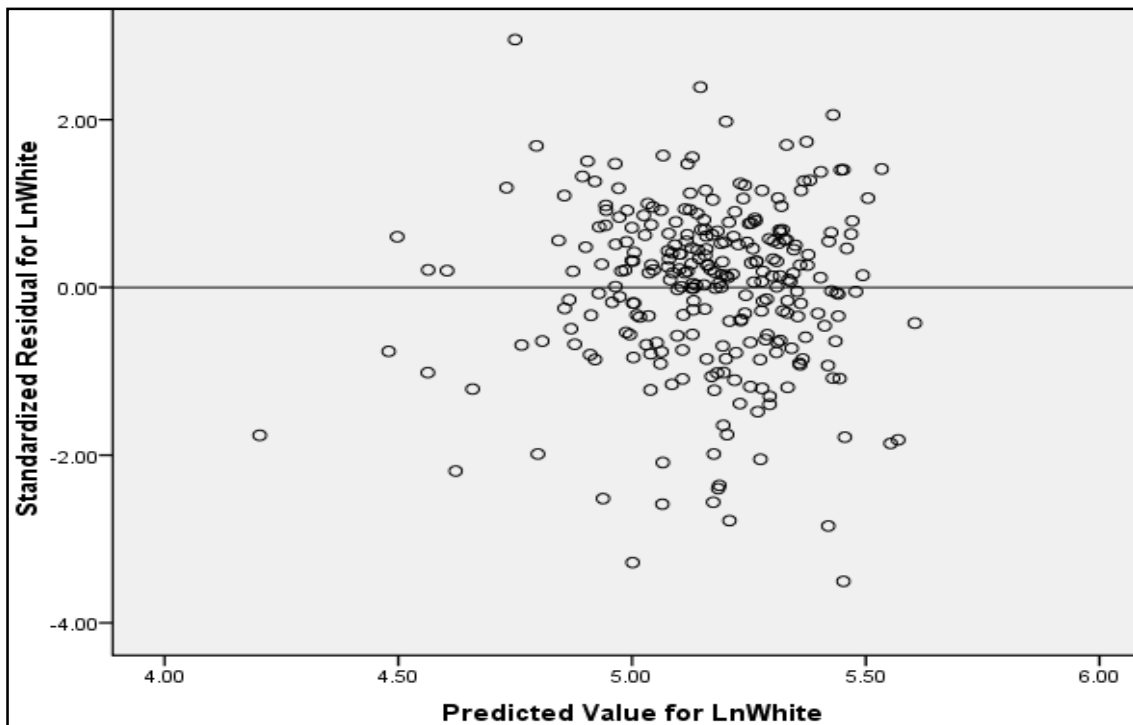
Figure 6 Residual Plot of the most parsimonious model: standardized residual of LnWhite vs. predicted residuals of LnWhite of marbled salamanders. As residuals did not show discernable pattern, the model is accepted.

Table 7 shows the initial General Linear Model that is followed by its residual plot shown in Figure 7.

Table 7   First candidate statistical model designed for White Pigmentation in marbled salamanders

Dependent Variable: White Pigmentation Variable	Type III Sum of Squares	Mean Squares	F	P
Intercept	4494.633	4494.633	3.285	>0.05
Sex	4507.920	4507.920	3.295	>0.05
Body Mass	1608.974	1608.974	1.176	>0.1
Snout-Vent-Length	9356.802	9356.802	6.839	<0.01
Body Length	15164.849	15164.849	11.084	<0.005
Body Area	27496.146	27496.146	20.096	<0.001
Eye-to-Eye Length	9768.323	9768.323	7.140	<0.01
Neck Width	9394.941	9394.941	6.867	<0.01
Width b/w Fore Limbs	1577.652	1577.652	1.153	>0.1
Width B/w Hind Limbs	2415.962	2415.962	1.766	>0.1
Sex*Body Mass	75.766	75.766	0.055	>0.5
Sex* Snout-Vent-Length	395.275	395.275	0.289	>0.5
Sex* Body Length	67.245	67.245	0.049	>0.5
Sex*Body Area	6779.593	6779.593	4.955	<0.05
Sex*Body Mass* Body Area	3907.883	1953.942	1.428	>0.1
Error	354365.769	1368.208		
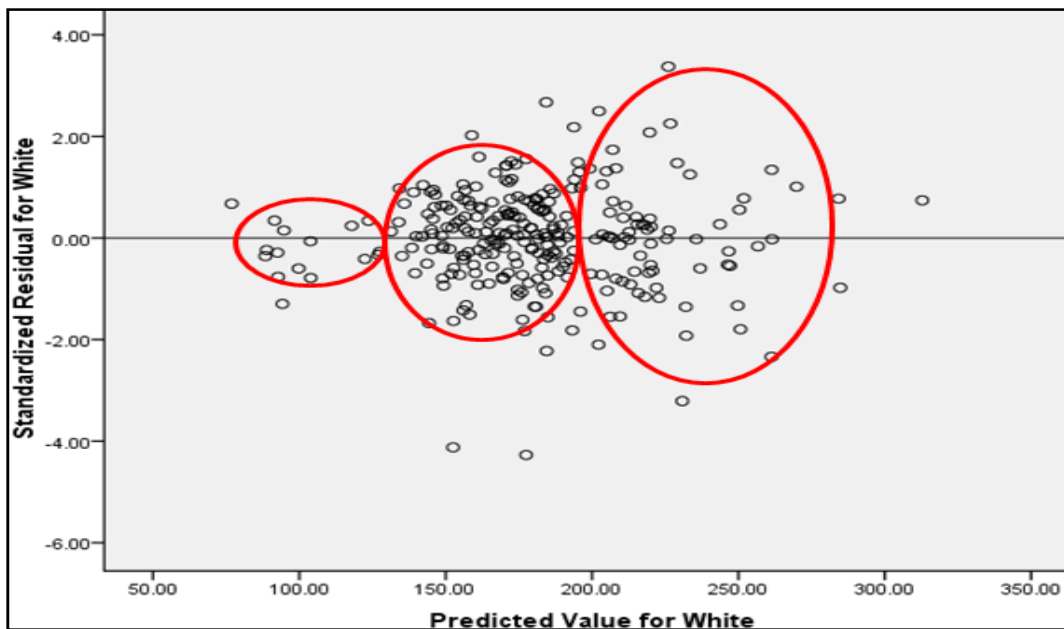
Figure 7 Residual Plot of first model: standardized residual of White Pigmentation vs. predicted residuals of White Pigmentation of marbled salamanders. As residual plot showed discernable pattern, the model was not accepted. So, new models were developed as shown in Tables 12, 13, 14, 15, and 16 (see Appendix).

<div align="center">Relative Measure of Asymmetry</div>

1. Marbled Salamander: Intersexual Difference

There was a significant difference in the relative measure of asymmetry between sexes in marbled salamanders in regard to dorsal white pigmentation. Females were more asymmetric in dorsal white pigmentation than males (Table 8) of marbled salamanders.

Table 8   One way ANOVA Test for inter-sexual difference in Relative Measure of Asymmetry of marbled salamanders. P<0.005 suggests significant difference in Relative Measure of Asymmetry between sexes of marbled salamander.

Sex	Marbled salamanders: Relative Measure of Asymmetry = $|R-L|/(R+L)$				
	N	Mean	Welch F-statistics	F-value	P
Male	107	0.052	12.360	9.421	<0.005
Female	168	0.077			

Asymmetry in white pigmentation was correlated with body length (Pearson r=0.119, P<0.05; see Figure 8 left) and dorsal body area (Pearson r=0.140, P<0.05; see Figure 8 right).
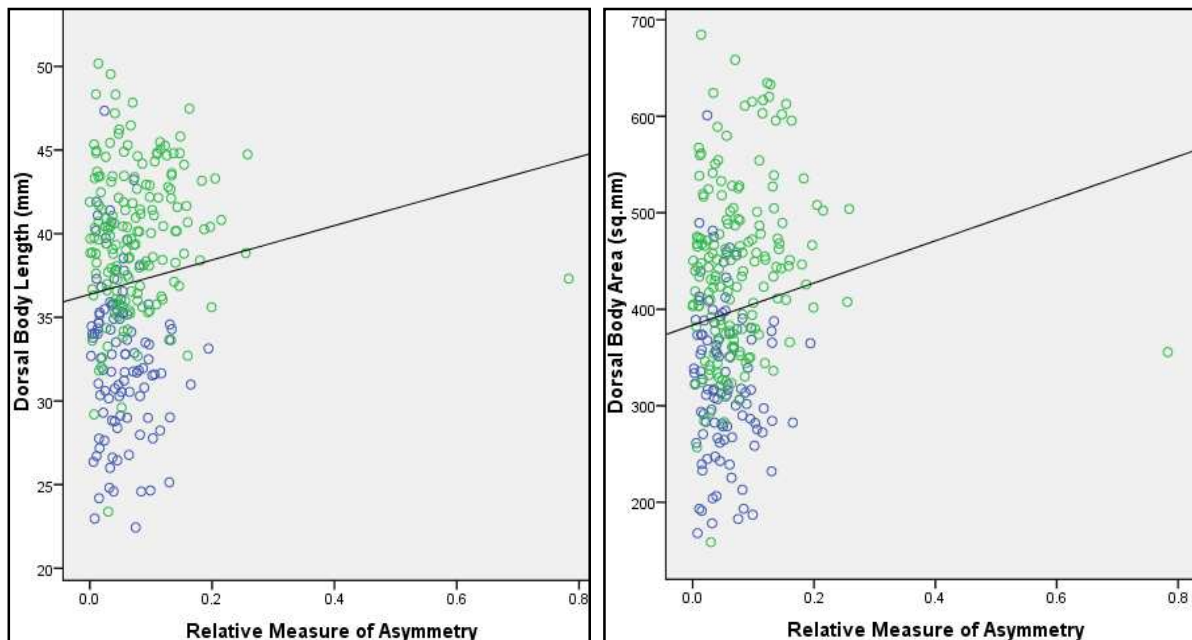
Figure 8 Scatter plots showing correlation of asymmetry in white pigmentation with dorsal body length (shown to left), and dorsal body area (shown to right) of marbled salamanders. Males are shown in blue and females are shown in green.

The findings supported the hypothesis that females are more asymmetric in white pigmentation than males of marbled salamanders, which suggests that females might be under greater stress than males.

2. Spotted Salamander: Intersexual Difference in Asymmetry

There was no significant difference in relative measure of asymmetry between sexes of spotted salamander in regard to mean yellow spot count (Table 9). This finding did not support the hypothesis of intersexual difference in spot count asymmetry. Spot asymmetry was neither correlated with body size (total length: Pearson r=0.037, P>0.5; snout-vent-length: Pearson r=0.155, P>0.05) nor with body mass (Pearson r=0.024, P>0.5).

Table 9 One way ANOVA test for inter-sexual difference in Relative Measure of Asymmetry of spotted salamanders. P>0.1suggests no significant difference in Relative Measure of Asymmetry in number of spots between sexes of spotted salamanders.

Sex	Spotted salamanders: Relative Measure of Asymmetry = |R-L|/(R+L)			
	N	Mean	Welch F-statistics	F (between groups)	P
Male	64	0.063	2.052	2.000	>0.1
Female	67	0.090			

Phenotypic Variation

The results showed that coefficient of variation (CV) of body mass/Snout-Vent-Length for males is relatively greater than that for females in marbled salamander, and within population variance was also found to be significantly different from mean variance for males (P<0.001), but not for females (P>0.1). This indicates the likelihood of strong selection pressure on body mass and body size for females compared to males. However, for spotted salamanders, males and females showed relatively similar CV, and within population variance was not significantly different from mean variance for both sexes (P>0.1; see Table 10 for details). This indicates the

possibility of similar selection pressure for both sexes in spotted salamanders supporting the

hypothesis that phenotypic variation between sexes of spotted salamander is relatively similar.

Table 10 Coefficient of Variation for Marbled and Spotted salamander populations obtained using Ratio statistics for Body Mass/Snout-Vent-Length. * indicates the significance within population variance at P<0.001 using Levene's test for equality of variance.

| Statistics | Ratio statistics for Body Mass/SVL | | | |
| | Marbled salamander | | Spotted salamander | |
	♂	♀	♂	♀
Std. Deviation	0.023	0.016	0.025	0.029
Mean	0.109	0.111	0.179	0.187
Coefficient of Variation (CV)	21.5%*	14.8%	13.9%	15.5%

Body size relationships of different morphological traits were also investigated which

showed isometric relationships that are presented in Appendix (see Tables 16 and 17).

CHAPTER 4

DISCUSSIONS

The results show that *A. opacum* differs significantly between sexes in overall body size including average body mass and percentage of dorsal white pigmentation. This study revealed significant sexual size dimorphism in nine morphological characters in marbled salamanders. In *A. opacum*, females are significantly heavier and larger than males, but males have significantly greater white pigmentation than females when scaled with body size. However, no statistically significant difference was found in total white pigmentation (not scaled with dorsal body area) or mean number of white bands. This result supports the findings of Todd and Davis (2007) for percentage of white pigmentation, but they did not measure other size related parameters. Likewise, for *A. maculatum*, females are, on average, heavier and larger than males but are not significantly different in yellow skin pigmentation (measured as number of spots on the dorsum per individual).

Dimorphic characters in both species of ambystomatid salamanders may reflect the adaptation of males and females to different social and/or reproductive needs. Previous studies in frogs indicate that larger body size in females is a character correlated with selection for increased clutch size when females are larger than males (Salthe and Duellman 1973; Crump 1974). Although color signaling is considered an important ecological character in diurnal systems (Bruce et al. 2003), its importance in nocturnal organisms is very poorly understood (Chuang et al. 2007). Greater white pigmentation might be of evolutionary significance because white coloration stands out against the dark background at night. Salamanders being nocturnal breeder, such coloration may possibly have a role in sexual signaling. However, these hypotheses remain to be tested in marbled salamanders.

The most parsimonious General Linear Model developed for dorsal white pigmentation of marbled salamander reflects the influence of body mass, dorsal body area, and gender in generation of skin pigmentation. Body mass and dorsal body area explained the main effects on white pigmentation, and sex was found to influence the pigmentation but only via interaction with body mass and dorsal body area. Though much is not known about how body mass, body size, and sex influence the generation of skin pigmentation in amphibians, it is understood that some pigmented cells migrate into epidermis earlier in life, and others remain in the dermis and migrate into the epidermis during metamorphosis (Bagnara 1987). Metamorphosis is followed by higher growth rate of body size, and with this growth follows the development of white and yellow pigmentation in marbled and spotted salamanders respectively (Petranka 1998). As very little is known whether the extent of skin pigmentation pattern is fixed in these two species of salamanders, Wright and Zamudio (2002) found that the spot pattern in *A. maculatum* had changed in the course of last 50 years in the areas adjacent to the golf course because of the high applications of herbicides and chemical fertilizers in the golf course. However, as meta-population dynamics exists in ambystomatid salamanders (Zamudio and Wieczorek 2007), the existing population can be replaced by a new population over time, thus rendering less validity of such studies. Moreover, skin pattern formation is hypothesized to be interplay between neural crest derived cells, environmental factors, most important of which are factors associated with growth and survival (Bagnara 1982; Tucker and Erickson 1986).

This study has established the base line data for developmental stability in marbled and spotted salamanders that may serve researchers in comparative studies to understand the effects of genetic/environmental stress. In addition, it has been accepted that lower asymmetry levels indicate higher developmental stability and higher fitness of the individuals (Moller and Swaddle

1997). Any subtle deviation from the perfect symmetry can indicate the body condition of the individuals and the environment they inhabit (Moller and Swaddle 1997). The significant difference in relative measure of asymmetry for average area of dorsal white pigmentation between sexes in marbled salamander indicates the possibility of different roles that the reproductive biology of each sex play in generating white pigmentation, and/or the possibility of different stress levels between sexes. It is known that female marbled salamanders breed on dry ponds during October-December, and also exhibit parental care via attending the nest with the clutch of eggs for 1-2 months during which it does not feed (Noble and Brady 1933). In contrast, parental care is lacking in males. So it seems conceivable that females are under more stress than males, which may be manifested as higher asymmetry in females than males in morphological traits like skin pigmentation. Likewise, it is also possible that the two sexes might have different buffering capacity that would result into significantly different asymmetry levels.

Relative measure of asymmetry in average spot count between sexes in spotted salamanders was not significantly different and suggests the possibility of both sexes being under strong genetic control and thus similar buffering capacity against stress. Likewise, they could also be under similar stress level, which is logical, as both sexes of the same species inhabit the same habitats.

Many ecomorphological studies suggest that if interspecific competition is driving the size spacing of species, the CV for the morphological traits should be severely constrained in each competing species to preserve the necessary spacing between them necessary for ecological coexistence (Grant 1968; Pulliam 1975; Ricklefs and Travis 1980). Relatively smaller CV for female marbled salamander suggests that they might be under higher selection pressure on body mass and body size as larger body size is thought to confer greater reproductive advantage

(Salthe and Duellman 1973; Crump 1974) that might have resulted into low variation compared to males. However, for spotted salamanders data showed relatively similar variation in body mass and snout-vent-length which might infer the possibility of similar selection pressure on both sexes. This is conceivable as both sexes have similar ecological roles in the ecosystem.

CHAPTER 5

CONCLUSIONS

The findings of this study supported the hypothesis (H1) that there is sexual size dimorphism in both species of ambystomatid salamaders: *A. opacum* and *A. maculatum*. In *A. opacum*, females are significantly heavier and larger than males, but males have significantly greater white pigmentation than females when scaled with body size. Likewise in *A. maculatum*, females are significantly heavier and larger than males, but with no significant intersexual difference in mean yellow spot number.

The most parsimonious general linear model (GLM) was developed which supported the hypothesis (H2) that the extent of dorsal white pigmentation could be influenced and predicted by body size, mass, and sex of marbled salamander. Though much is not known about how body size, body mass, and sex could influence the generation of skin pigmentation in amphibians, this necessitates further studies on ontogeny and biology of pigmentation.

As less is known about phenotypic variation in *A. opacum* and *A. maculatum*, this study showed that females may be under greater selection pressure on body size and mass of marbled salamanders, and thus showed less variation compared to males supporting the hypothesis (H3.1). For spotted salamanders, because phenotypic variation was found to be similar between sexes, which supported the hypothesis (H3.2), data on CV are indicative of similar selection pressure for body size and mass of each gender.

Data on relative measure of asymmetry indicated that females might be under more stress than males of marbled salamanders, and supported the hypothesis (H4), while for spotted salamanders asymmetry was not significantly different between sexes, which did not support the hypothesis (H4), indicating the possibility of similar levels of stress in both sexes.

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Tests for Normality of Data Distribution

Table 11 Tests for Normality of data distribution using Kolmogorov-Smirnov Z for several morphological traits. N is sample size.

Traits	Marbled salamanders (N=275)	
	Kolmogorov-Smirnov Z	P-value
Weight (WT)	0.697	>0.5
Snout-vent-length (SVL)	0.564	>0.5
Dorsal Body Length (DBL)	0.719	>0.5
Dorsal Body Area (DBA)	0.552	>0.5
Eye-to-Eye Length (EEL)	0.311	>0.5
Neck Width (NW)	0.884	>0.1
Width b/w Fore Limbs (WBFL)	0.580	>0.5
Width b/w Hind Limbs (WBHL)	0.677	>0.5
Total White Pigmentations (TWP)	0.871	>0.1
Traits	Spotted Salamanders (N=131)	
	Kolmogorov-Smirnov Z	P-value
Weight (WT)	0.808	>0.5
Snout-vent-length (SVL)	0.727	>0.5
Total Length (TL)	0.601	>0.5

Sexual Size Dimorphism in Marbled Salamanders
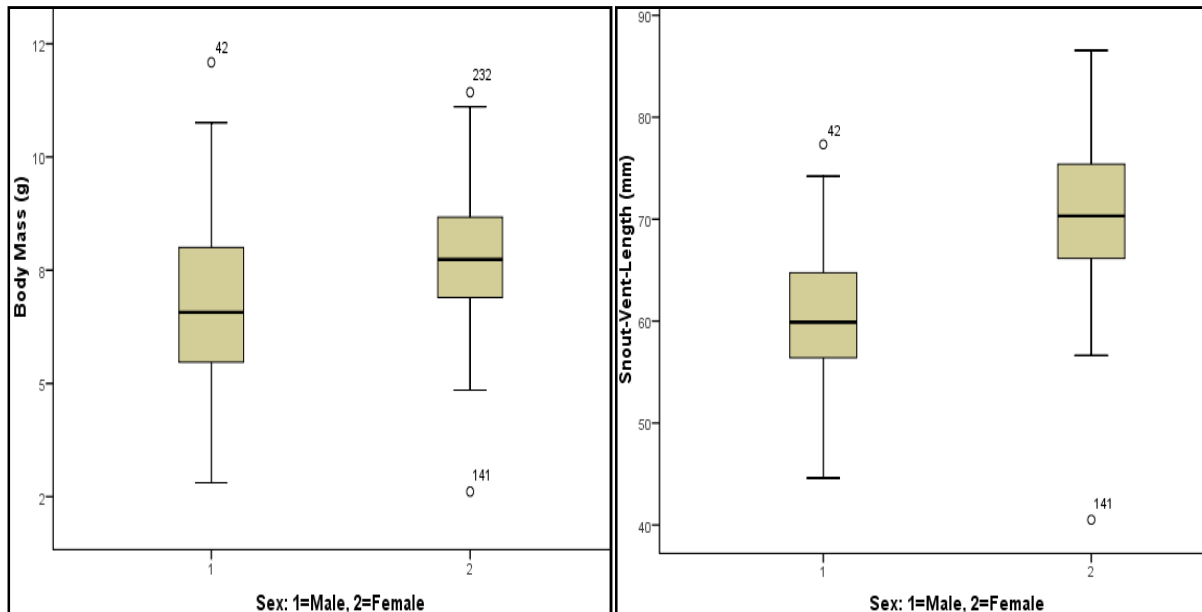
Figure 9 Marbled salamander: SSD in Body Mass (P<0.001; shown to left), and Snout-Vent-Length (P<0.001; shown to right)

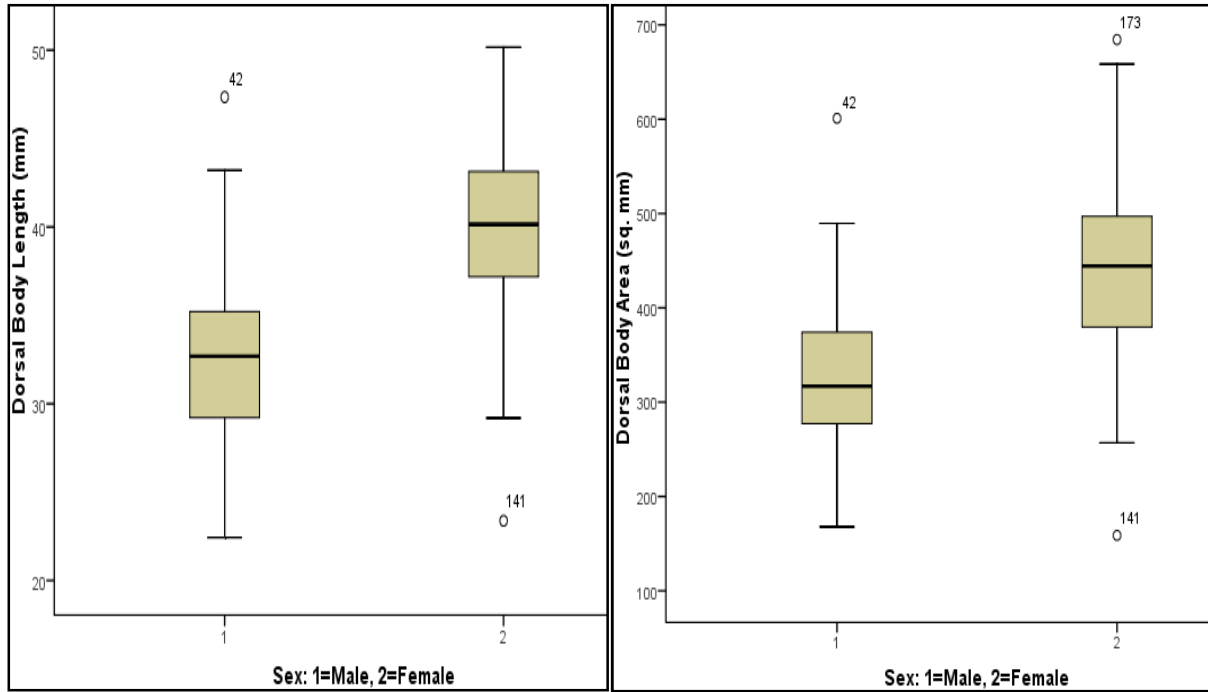Figure 10   Marbled salamander: SSD in Dorsal Body Length (P<0.001; shown to left), and
Dorsal Body Area (P<0.001; shown to right)
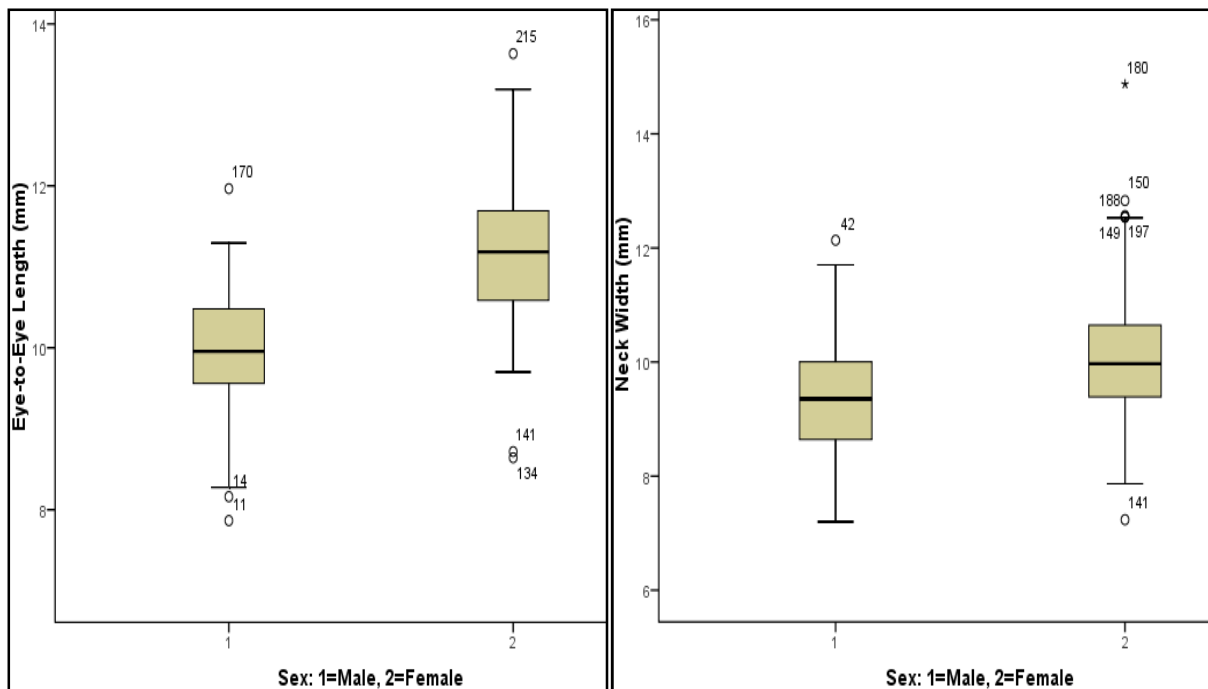
Figure 11 Marbled salamander: SSD in Eye-to-Eye Length (P<0.001; shown to left), and Neck
Width (P<0.001; shown to right)
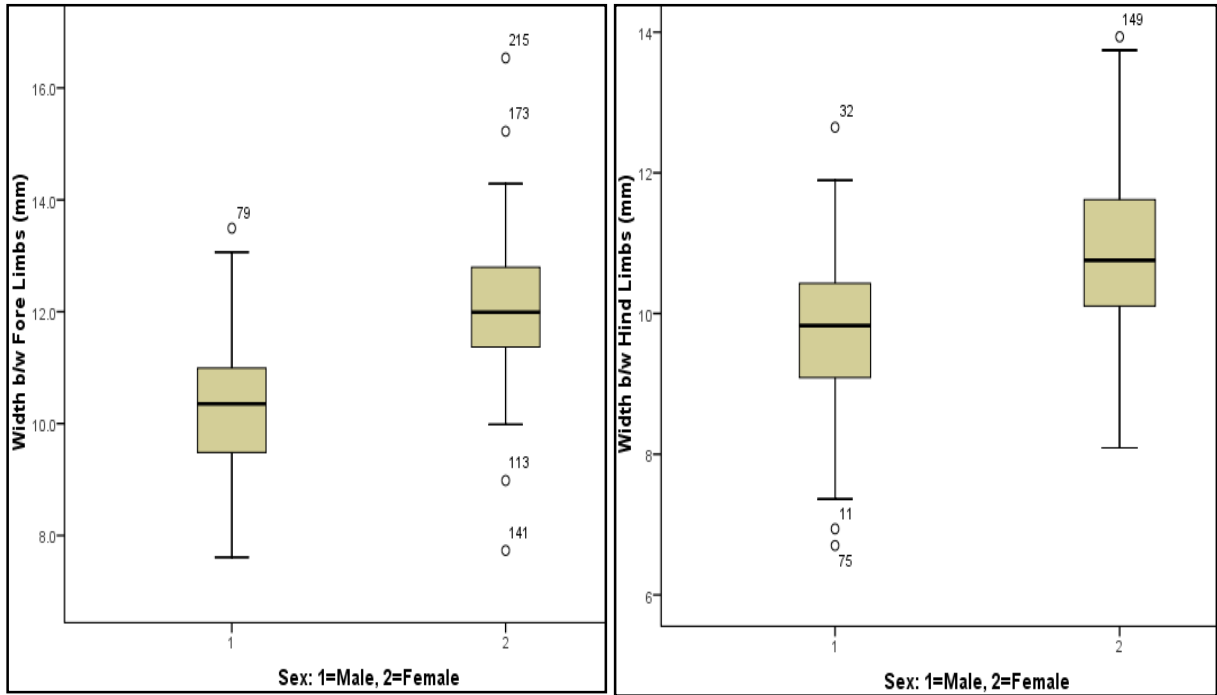
Figure 12   Marbled salamander: SSD in Width between Fore Limbs (P<0.001; shown to left),
and Width between Hind Limbs (P<0.001; shown to right)
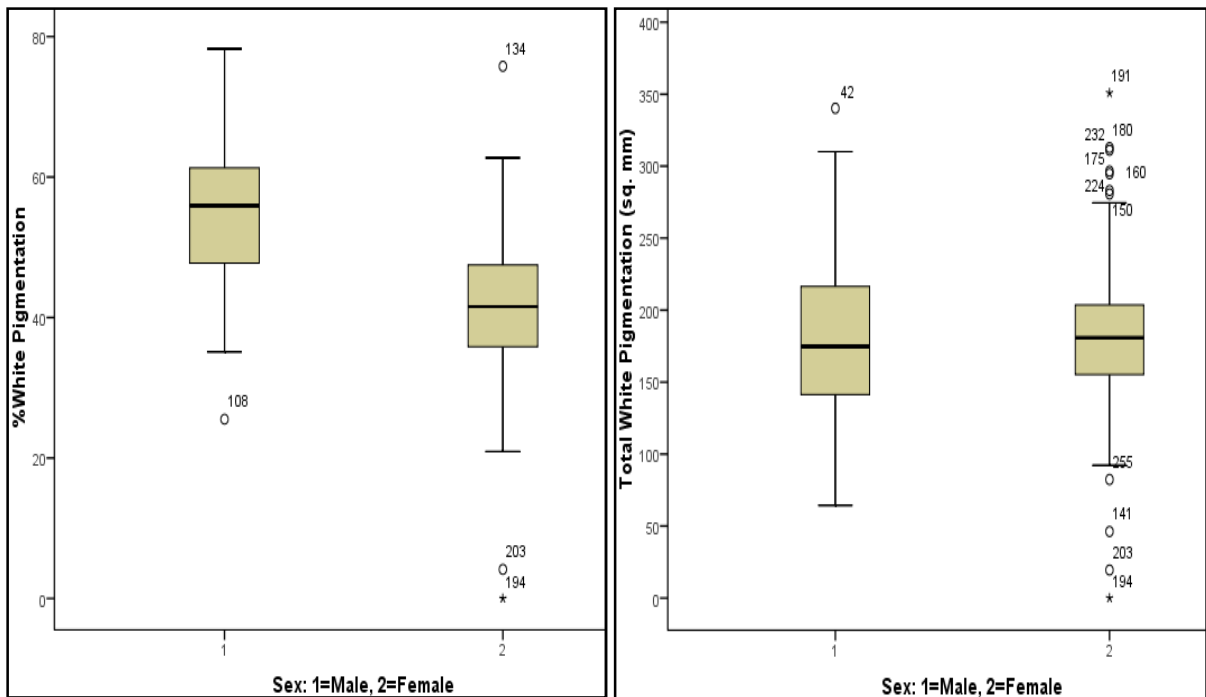
Figure 13 Marbled salamander: SSD in % of White Pigmentation (P<0.001; shown to left), and
Total White Pigmentation (P>0.5; shown to right)

Figure 14 Spotted salamander: SSD in Body Mass (P<0.001; shown to left), and Snout-Vent-
Length (P<0.001; shown to right)

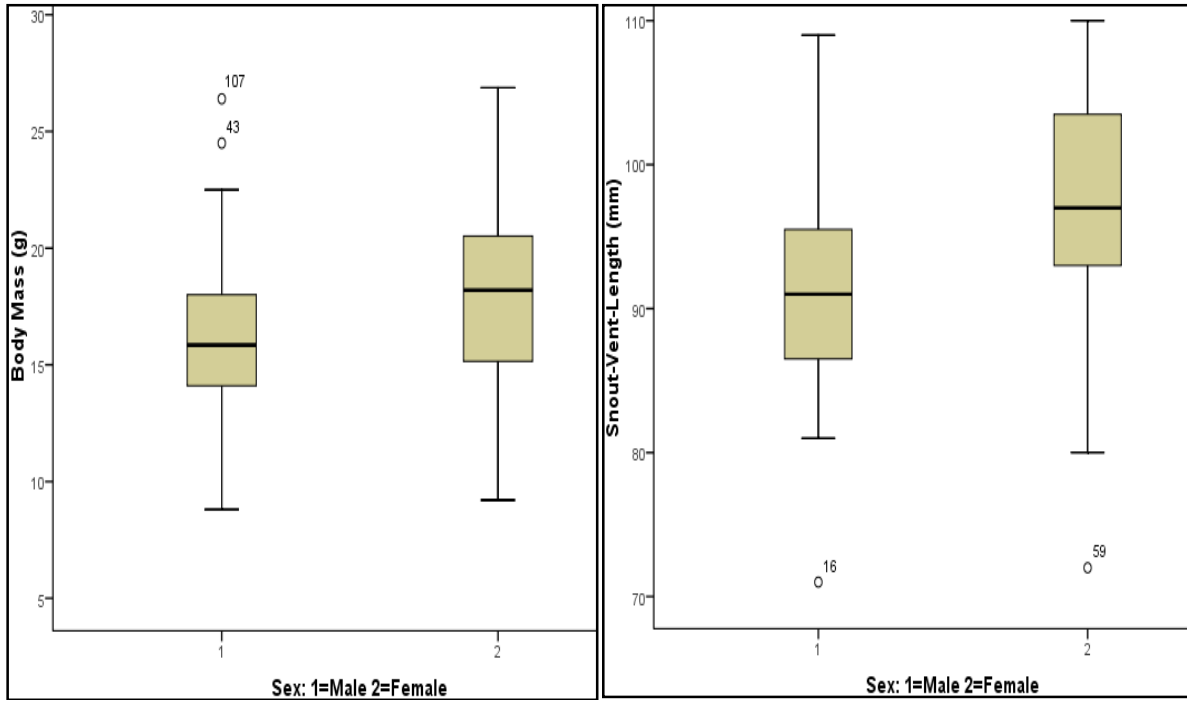Sexual Size Dimorphism in Spotted Salamanders
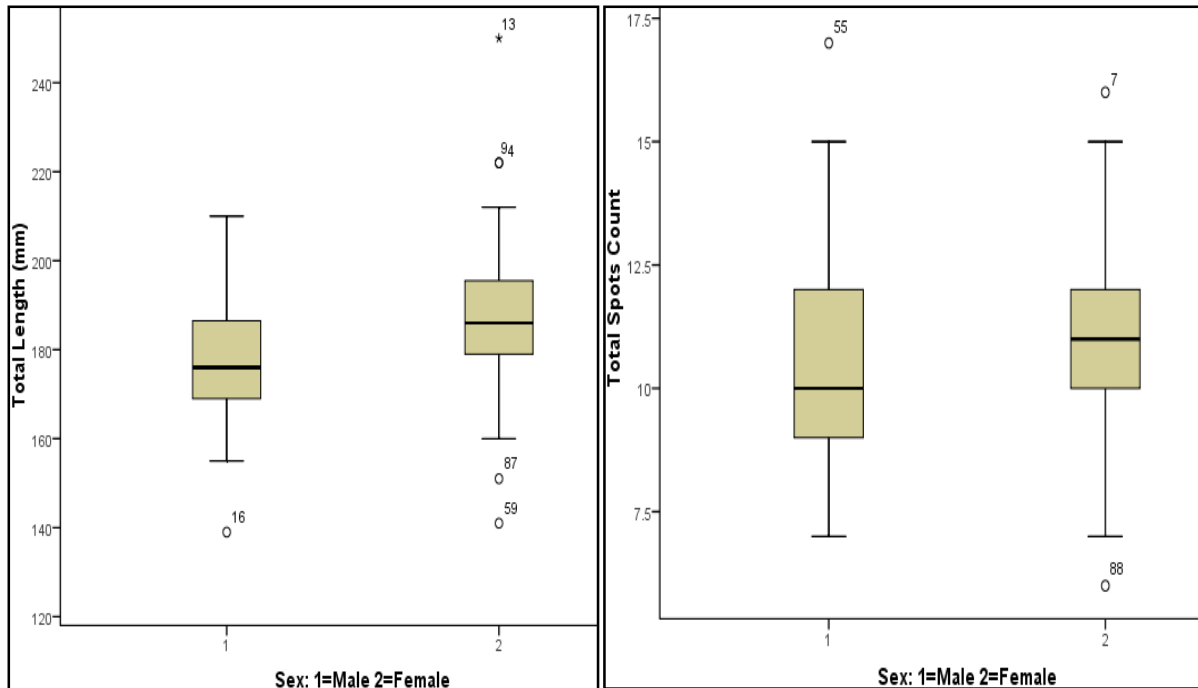

Figure 15 Spotted salamander: SSD in Total Length (P<0.005; shown to left), and mean Total
Spots Count (P>0.5; shown to right)

55

Sets of Candidate Models for White Pigmentation of Marbled Salamanders

Table 12 Second candidate statistical model designed for White Pigmentation of marbled salamanders by logarithmic transformation of dependant variable

Dependent Variable: Ln(White Pigmentation) Variable	Type III Sum of Squares	Mean Squares	F	P
Intercept	6.489	6.489	162.163	<0.001
Sex	0.084	0.084	2.107	>0.1
Body Mass	0.612	0.612	15.307	<0.001
Snout-Vent-Length	0.337	0.337	8.410	<0.005
Body Length	0.325	0.325	8.125	<0.005
Body Area	1.437	1.437	35.911	<0.001
Eye-to-Eye Length	0.189	0.189	4.729	<0.05
Neck Width	0.234	0.234	5.857	<0.05
Width b/w Fore Limbs	0.062	0.062	1.544	>0.1
Width B/w Hind Limbs	0.082	0.082	2.054	>0.1
Sex*Body Mass	0.012	0.012	0.299	>0.5
Sex* Snout-Vent-Length	0.030	0.030	0739	>0.1
Sex* Body Length	0.005	0.005	0.128	>0.5
Sex*Body Area	0.193	0.193	4.812	<0.05
Sex*Body Mass* Body Area	0.689	0.345	8.614	<0.001
Error	10.283	0.040		

Figure 16 Residual Plot of second model: standardized residual of LnWhite Pigmentation vs. predicted residuals of LnWhite Pigmentation in marbled salamanders. As residual plot showed no discernable pattern, the model was accepted.

Table 13 Third candidate statistical model designed for White Pigmentation of marbled salamanders

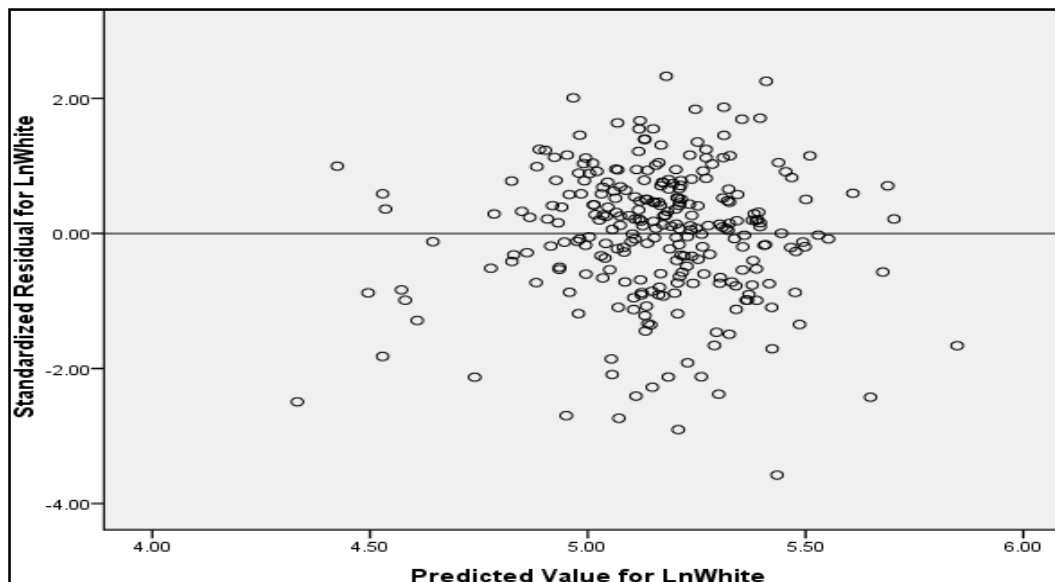| Dependent Variable: Ln(White Pigmentation) Variable	Type III Sum of Squares	Mean Squares	F	P
Intercept	7.310	7.310	171.083	<0.001
Body Mass	1.002	1.002	23.454	<0.001
Snout-Vent-Length	0.250	0.250	5.845	<0.05
Body Length	0.122	0.122	2.864	>0.05
Body Area	1.403	1.403	32.838	<0.001
Eye-to-Eye Length	0.287	0.287	6.711	<0.05
Neck Width	0.077	0.077	1.803	>0.1
Sex*Body Area	0.738	0.738	17.277	<0.001
Sex*Body Mass* Body Area	0.847	0.424	9.917	<0.001
Error	11.237	0.043		
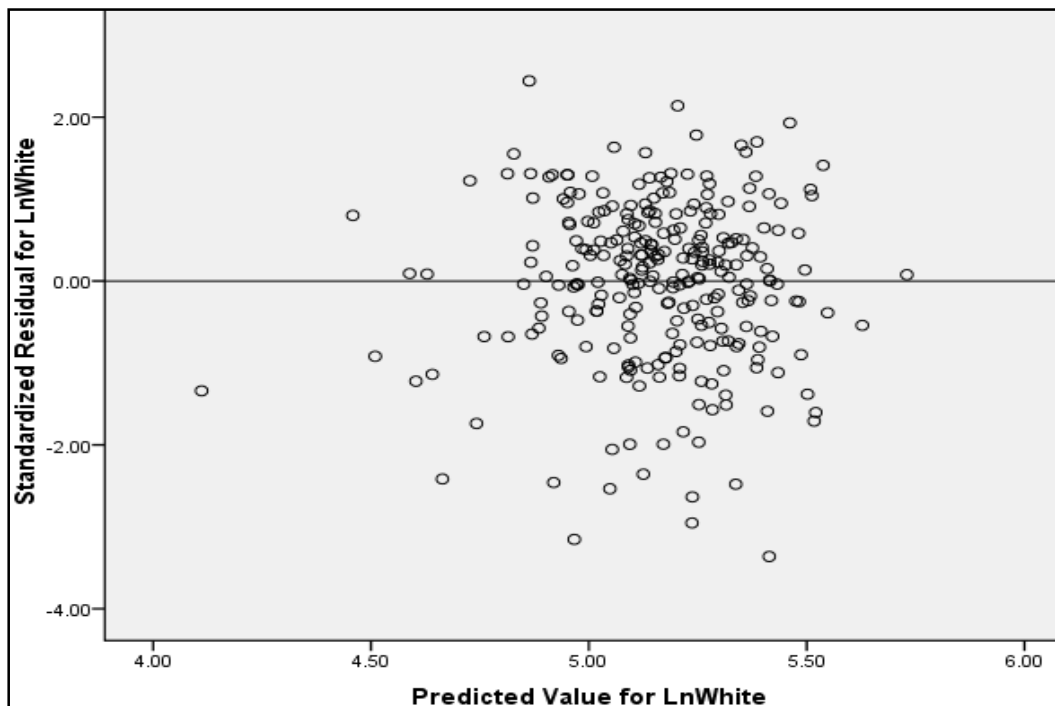
Figure 17 Residual Plot of third model: standardized residual of LnWhite Pigmentation vs. predicted residuals of LnWhite Pigmentation in marbled salamanders. As residual plot showed no discernable pattern, the model was accepted.

Table 14 Fourth candidate statistical model designed for White Pigmentation of marbled salamanders. As Snout-Vent-Length is nonsignificant in influencing white pigmentation in this model, it was eliminated in the next model.

| Dependent Variable: Ln(White Pigmentation) Variable	Type III Sum of Squares	Mean Squares	F	P
Intercept	7.982	7.982	184.740	<0.001
Body Mass	1.028	1.028	23.798	<0.001
Snout-Vent-Length	0.122	0.122	2.828	>0.05
Body Area	1.755	1.755	40.631	<0.001
Eye-to-Eye Length	0.214	0.214	4.944	<0.05
Sex*Body Area	0.885	0.885	20.507	<0.001
Sex*Mass* Body Area	0.860	0.430	9.949	<0.001
Error	11.450	0.043		
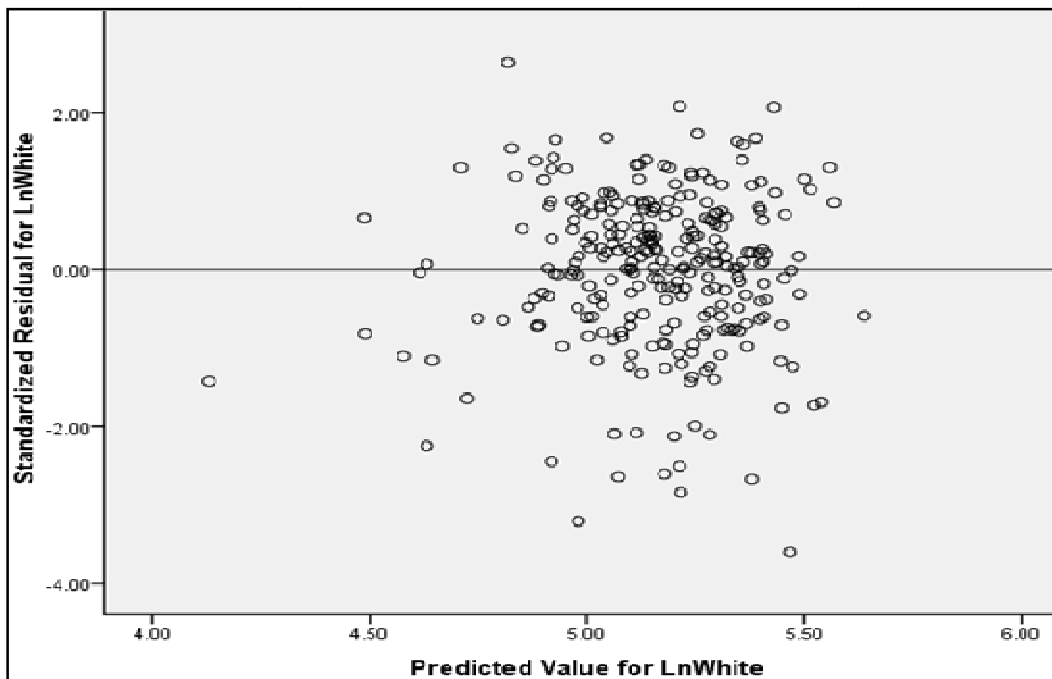
Figure 18 Residual Plot of fourth model: standardized residual of LnWhite Pigmentation vs. predicted residuals of LnWhite Pigmentation in marbled salamander. As residual plot showed no discernable pattern, the model was accepted.

Table 15 Fifth candidate statistical model designed for White Pigmentation of marbled salamanders. As Eye-to-Eye Length is non-significant in influencing white pigmentation in this model, it was eliminated in the next model.

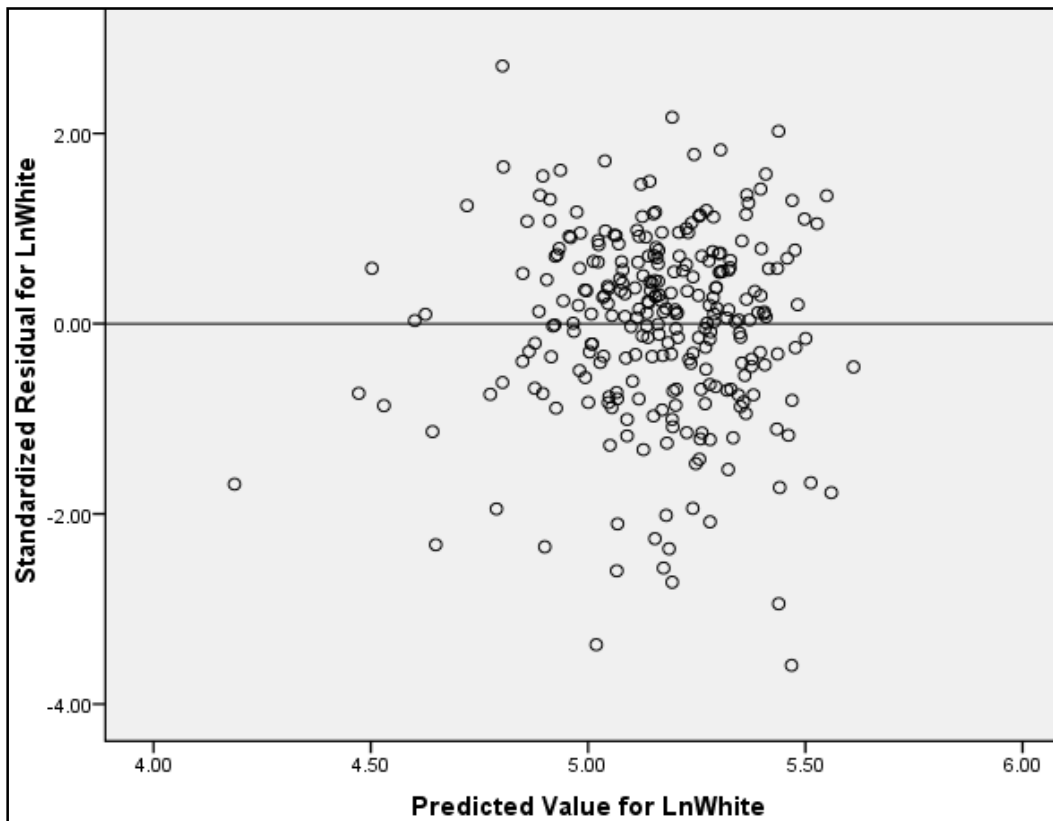| Dependent Variable: Ln(White Pigmentation) Variable	Type III Sum of Squares	Mean Squares	F	P
Intercept	8.754	8.754	201.233	<0.001
Body Mass	1.288	1.288	29.612	<0.001
Body Area	2.639	2.639	60.664	<0.001
Eye-to-Eye Length	0.188	0.188	2.702	>0.1
Sex*Body Area	0.824	0.824	18.938	<0.001
Sex*Mass* Body Area	0.970	0.485	11.145	<0.001
Error	11.572	0.044		

Figure 19 Residual Plot of fifth model: standardized residual of LnWhite Pigmentation vs. predicted residuals of LnWhite Pigmentation in marbled salamanders. As residual plot showed no discernable pattern, the model was accepted.

Body Size Relationships of Different Morphological Traits of Marbled and Spotted Salamanders

Table 16 Body size relationships of different morphological traits of marbled salamanders. Predictor variable (*x*) is snout-vent-length (SVL). Slope with * indicates that the slope is not significantly different from 1 (P>0.1) indicating isometric relationship. For acronyms, see Table 10.

Depend ent variable (*y*)	Male			Female		
	Intercept (*a*)	Slope (*b*)	Allometric equation $y = a\,x^{b}$	Intercept (*a*)	Slope (*b*)	Allometric equation $y = a\,x^{b}$
WT	-1.159^{-15}	0.722*	WT=-1.159^{-15}*SVL$^{0.722}$	-1.412	0.436*	WT=-1.412*SVL$^{0.436}$
EEL	-0.015	0.884*	EEL=-0.015*SVL$^{0.884}$	-8.856^{-15}	0.813*	EEL=-8.856^{-15}*SVL$^{0.813}$
NW	-0.025	0.748*	NW=-0.025*SVL$^{0.748}$	6.729^{-16}	0.601*	NW=6.729^{-16}*SVL$^{0.601}$
WBFL	-0.017	0.829*	WBFL=-0.017*SVL$^{0.829}$	-7.232^{-15}	0.695*	WBFL=-7.232^{-15}*SVL$^{0.695}$
WBHL	0.018	0.690*	WBHL=0.018*SVL$^{0.690}$	0.014	0.658*	WBHL=0.014*SVL$^{0.658}$
DBL	1.975^{-16}	0.882*	DBL=1.975^{-16}*SVL$^{0.882}$	-2.003^{-15}	0.877*	DBL=-2.003^{-15}*SVL$^{0.877}$
DBA	-3.687^{-16}	0.882*	DBA=-3.687^{-16}*SVL$^{0.882}$	-4.071^{-15}	0.852*	DBA=-4.071^{-15}*SVL$^{0.852}$
TWP	-2.810^{-16}	0.750*	TWP=-2.810^{-16}*SVL$^{0.750}$	0.003	0.370*	TWP=0.003*SVL$^{0.370}$

Table 17 Body size relationships between body mass (WT) and snout-vent-length (SVL) of spotted salamanders. Predictor variable (*x*) is snout-vent-length (SVL). Slope with * indicates that the slope is not significantly different from 1 (P>0.1) indicating isometric relationship.

Depend ent variable (*y*)	Male			Female		
	Inter-cept (*a*)	Slope (*b*)	Allometric equation: $y = a\,x^{b}$	Intercept (*a*)	Slope (*b*)	Allometric equation: $y = a\,x^{b}$
WT	-0.004	0.840*	WT=-0.004*SVL$^{0.840}$	-2.570^{-15}	0.759*	WT=-2.570^{-15}*SVL$^{0.759}$

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