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The Spots of the Spotted Salamander Are Sexually Dimorphic

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Brilliant and conspicuous coloration in animals is often thought to signal quality to conspecifics (sexual selection) or to signal unpalatability to predators (aposematic selection). *Ambystoma maculatum* (spotted salamanders) have bilateral rows of conspicuous bright yellow dorsal spots against dark skin. Although this coloration has been long thought a classic example of warning coloration, to date, there are no quantitative measures of spot coloration. We captured adult male and female *A. maculatum* as they entered breeding ponds and measured body condition, spectral reflectance of the yellow spots, spot size, and coverage of the dorsal area in spots. We found evidence of sexual dichromatism; males had a larger area of their dorsum covered in spots, and tended to have more chromatic, but less bright yellow color. Moreover, our data suggest a tradeoff between coloration and body condition. Animals in better body condition expressed lower chroma and those with greater spot coverage expressed brighter spots and lower yellow chroma. Although these data are not entirely consistent with the predictions of sexual selection, they suggest a physiological tradeoff associated with coloration that has not been documented in salamanders.

EVOLUTIONARY explanations for the signaling function of conspicuous coloration of animals date back to theories by Darwin (1871), who argued that brightly colored males have better access to females, and by Wallace (1871), who argued that they reliably communicate their ability to defend themselves from predators. Indeed, a plethora of studies demonstrate that coloration can honestly signal resource-holding potential and mate quality to conspecifics (Andersson, 1994). The red coloration of house finches is a classic example of a sexually selected trait; males with more vibrant feathers honestly advertise their health and vigor to discriminating females (Hill, 2002). Sexual selection favors the evolution of costly traits whose expression reflects the survivorship and genetic quality of the individual or the 'truth in advertising' hypothesis (Kodric-Brown and Brown, 1984). Coloration can also honestly advertise inedible qualities to predators (Cott, 1957; Guilford, 1988). The bright coloration of poison dart frogs (Anura: Dendrobatidae) is a classic example (reviewed in Hagman and Forsman, 2003); more brightly colored *Dendrobates pumilio* are more toxic (Maan and Cummings, 2012).

Spotted salamanders (*Ambystoma maculatum*) have gray to black skin with bilateral rows of yellow spots that run down the dorsal part of the body. These irregularly shaped spots vary in number, size, arrangement, and color. The color of these spots is usually bright yellow, but some individuals have orange spots on their head (Pierce and Shayevitz, 1982), and rarely animals are found with gray spots or lack spotting completely (Petranka, 1998; M. Osbourn, unpubl. data). Although *A. maculatum* are nocturnal and inactive during the winter months (Homan et al., 2003), breeding occurs on a few nights during early spring when adults mass migrate to vernal pools and ponds and mate via scramble competition (Whitford and Vinegar, 1966; Shoop, 1968; Tennessen and Zamudio, 2003). *Ambystoma maculatum* use anti-predator postures and produce noxious secretions from parotid glands to deter predators (Brodie, 1977). Although not explicitly demonstrated, it seems logical to hypothesize that the yellow spots represent aposematic coloration to signal unpalatability.

The yellow, orange, and red coloration of amphibians can occur from carotenoid and/or pterin pigments (reviewed in Rudh and Qvarnstrom, 2013). The pterin pigment sepiapterin colors the yellow spots of spotted salamanders (Obika and Bagnara, 1964; J. Steffen, unpubl. data). Carotenoid-based colors are excellent models for studying the honesty-reinforcing mechanisms underlying sexually selected traits because integument pigments also have antioxidant and immunoregulatory properties that allow individuals to signal their superior health to prospective mates (reviewed in McGraw, 2006). It is unclear, however, whether pterin-based coloration signals similar physiological benefits (reviewed in McGraw, 2005). Moreover, although salamanders display a wide variety of integument pigmentation, little research has focused on sexual dichromatism and condition-dependent signaling in salamanders (reviewed in Rudh and Qvarnstrom, 2013; but see Todd and Davis, 2007; Davis and Grayson, 2008; Pokhrel et al., 2013).

The goal of this study was to determine whether the variation in spot characteristics (i.e., spot color, size of spots, and area of the dorsum covered in spots) in spotted salamanders is more consistent with the predictions of sexual selection or the aposematic coloration hypotheses. Because condition-dependent sexual traits signal information about aspects of the individual's phenotype and genotype, they are expected to show high variation with sex, age, and health (reviewed in Dale, 2006). Aposematic coloration, however, is expected to be less variable and not expected to vary with sex or body condition (reviewed in Dale, 2006). Thus, sexual dichromatism and more-elaborate coloration in individuals with better body condition would be consistent with sexual selection, but not aposematic coloration.

MATERIALS AND METHODS

On 29 February–1 March 2012 we hand-captured adult *A. maculatum* as they migrated to breeding ponds in Watauga County, NC, USA (36°15′50″N, 81°37′43″W). We measured snout–vent length (SVL; within 0.1 mm), total body length (within 0.1 mm), mass (within 0.1 g), spectral reflectance,

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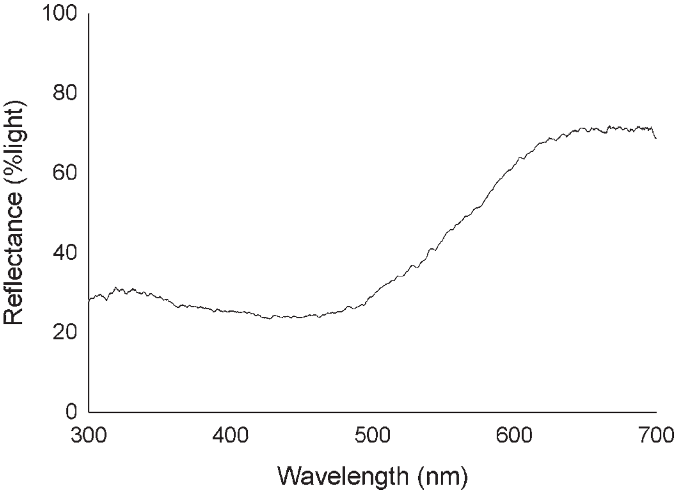

Fig. 1. Typical reflectance spectra of the yellow spots of spotted salamanders (*Ambystoma maculatum*).

and estimated sex using cloacal swelling (Peckham and Dineen, 1955; Hillis, 1977) while on site. We took photographs of each individual at a distance of 0.3 m and a 90° angle. Each photo had a size standard that allowed us to calculate the proportion of the dorsal area covered in spots and spot size (area mm^2; within 0.1 mm). We released all animals immediately afterward.

To measure spot coloration, we took spectral reflectance measurements with 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 anchoring the probe within a rubber stopper held flush with the salamander's skin surface. We illuminated a 2 mm measurement area with Xenon pulse light sources. We generated reflectance data by comparing spot color to a white standard (Labsphere, Inc.). We measured reflectance of two spots on each salamander: the most anterior spot on the right side of the head and the third spot of the right side of the body.

To quantify spot coloration we used the reflectance data to calculate pterin chroma and brightness. Pterin chroma is the measure of the difference of reflectance values from 450–700 nm divided by the maximum reflectance at 700 nm (Montgomerie, 2008) and multiplied by -1, so that an animal with a more saturated spot color should show a higher value. Brightness, or the total amount of light reflected by the spot, was calculated as the mean of the summed reflectance from 300–700 nm, and can be thought of as lighter (brighter) or darker coloration.

Using photographs, we measured the area (mm^2) of the third spot of the right side of the body using ImageJ 1.46 (U.S. National Institutes of Health). Procedures were modeled after the protocol of Miller (2011). We also used the photographs to measure total spot coverage of the dorsal area of the salamanders (excluding the legs). We used ERDAS IMAGINE to determine the total area of spot coverage and the total dorsal area and then calculated the percent of the dorsal area in spots, and refer to it as 'spot coverage.'

We used SPSS v. 20 to analyze data and set alpha $\leq$0.05. We tested for normality using Shapiro-Wilk tests and none of the data deviated significantly from normality. We used Student's t-test to explore whether sex influenced color, spot size, proportion of dorsal area in spots, mass, and SVL. Next, we used discriminate function analysis to assess our ability to correctly classify animals by sex category using spot characteristics or body size. We used separate Analyses of Covariance (ANCOVA) to explore whether 1) spot coloration, 2) spot size, or 3) spot coverage was influenced by sex or body condition, and whether the relationships between body condition and spot characteristics varied with sex (i.e., a statistical interaction).

RESULTS

We measured 30 male and 30 female salamanders; however, sample sizes are lower for some analyses because poor photograph quality limited quantification of spot characteristics. The reflectance spectra show very little reflectance in the short wavelength and greater reflectance in the longer wavelengths, typical of integument that looks yellow to the human observer (Fig. 1).

We found no significant relationships between spot coverage and spot size (Table 1). Although spot size did not correlate with spot color, animals with greater spot coverage displayed brighter and less chromatic yellow spots (Table 1).

Sexual dimorphism.—Females were significantly larger than males (Table 2), and our discriminate functions analysis model using body size categorized 83% of individuals correctly by sex (χ^2 = 30.48, df = 58, P < 0.001). Furthermore, we found that spot characteristics of *A. maculatum* were sexually dimorphic (Table 2; Fig. 2) and our discriminate functions analysis model categorized 82% of individuals correctly by sex (χ^2 = 13.24, df = 39, P = 0.02). Males had more chromatic yellow color, were less bright, and had smaller spots but greater spot coverage compared to females (Fig. 2).

Spot characteristics and body condition.—Because we found that female *A. maculatum* were heavier and longer than males, we divided the data by sex before creating proxies of body condition. In both males (R^2 = 0.53, $F_{1,29}$ = 31.30, $P < 0.001$) and females (R^2 = 0.46, $F_{1,29}$ = 23.81, $P < 0.001$), longer animals were heavier. We used the scaled mass index

Table 1. Relationships (Pearson correlations values [r]) between spot characteristics in spotted salamanders (male and female data combined), sample sizes in parentheses. *$P \leq 0.05$; ** $P \leq 0.01$.

	Brightness	Chroma	Spot coverage	Spot size (mm^2)
Body condition (z)	0.14 (n = 60)	−0.32* (n = 60)	−0.07 (n = 56)	0.37* (n = 47)
Brightness (%)		−0.78** (n = 60)	0.31* (n = 56)	0.02 (n = 47)
Chroma			−0.28* (n = 56)	−0.13 (n = 47)
Spot coverage (%)				0.19 (n = 47)

Table 2. T-tests comparing morphological characteristics, spot measurements, and spectral data of male and female *Ambystoma maculatum* (mean ± SD).

Variable	Male	Female		*n*	*P*
Mass (g)	23.59±4.01	33.23±7.43	6.25	30,30	<0.001
SVL (mm)	9.15±0.53	9.97±0.67	5.30	30,30	<0.001
Chroma	0.60±0.13	0.54±0.14	1.69	30,30	0.10
Brightness	0.37±0.13	0.43±0.13	1.75	30,30	0.09
Spot coverage	0.11±0.04	0.10±0.04	−1.97	28,28	0.05
Spot size (mm^2)	12.04±2.97	13.36±4.78	1.13	24,23	0.27

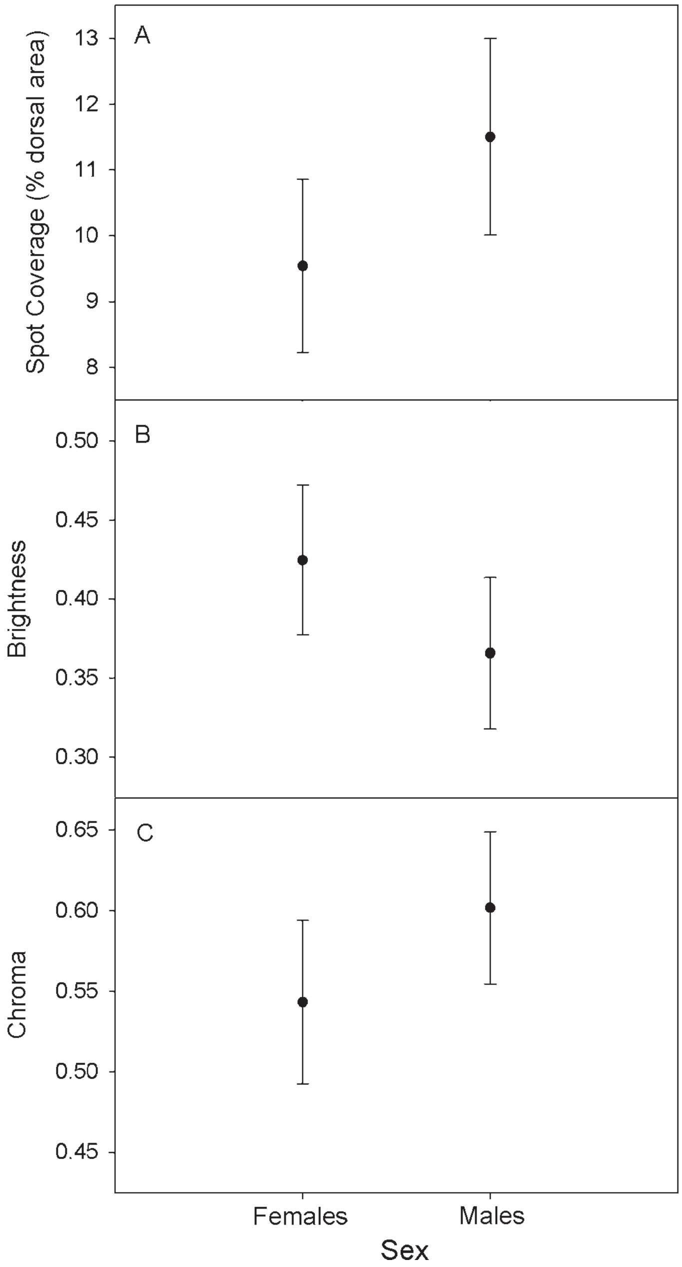

Fig. 2. Comparison of male and female spotted salamanders, *Ambystoma maculatum* in (A) spot coverage, (B) brightness of spots, and (C) chroma. Data represent means ±2 SE.

(SMI) as the proxy of body condition (Peig and Green, 2009) because an experiment demonstrated that SMI reliably reflects energy stores in amphibians (MacCracken and Stebbings, 2012).

We found a significant interaction between sex and body condition on chroma (ANCOVA: $F_{1,57} = 15.23$, $P < 0.001$). In both sexes, animals with better body condition displayed lower chroma; however, the relationship was stronger for males ($R^2 = 0.22$, $F_{1,28} = 7.50$, $P = 0.01$; Fig. 3A) than females ($R^2 = 0.19$, $F_{1,28} = 6.71$, $P = 0.01$; Fig. 3A). There was not a significant interaction between sex and body condition on brightness ($F_{1,57} = 3.39$, $P = 0.07$). In both sexes, there was no significant relationship between body condition and brightness (Males: $R^2 = 0.01$, $F_{1,28} = 0.27$, $P = 0.61$; Females: $R^2 = 0.09$, $F_{1,28} = 2.74$, $P = 0.11$).

There was a significant interaction between sex and body condition on spot size (ANCOVA: $F_{1,44} = 11.56$, $P = 0.001$). In both sexes, animals with better body condition had significantly larger spots; however, the relationship was stronger for males ($R^2 = 0.21$, $F_{1,21} = 5.60$, $P = 0.03$; Fig. 3B) than females ($R^2 = 0.19$, $F_{1,22} = 5.17$, $P = 0.03$; Fig. 3B). There was no significant interaction between sex and body condition on spot coverage (ANCOVA: $F_{1,53} = 0.20$, $P = 0.66$). In both sexes, there was no significant relationship between body condition and spot coverage (Males: $R^2 = 0.01$, $F_{1,26} = 0.15$, $P = 0.99$; Females: $R^2 = 0.00$, $F_{1,26} = 0.00$, $P = 0.99$).

DISCUSSION

Spot characteristics differ between the sexes, demonstrating that *A. maculatum* are sexually dimorphic. We found covariation between spot characteristics: brighter animals also had greater spot coverage and animals that were brighter were less chromatic. Finally, we found correlations between color and overall spot coverage and color varied with body condition. Associations between color, sex, and quality suggest that the yellow spots of *A. maculatum* could function as an honest signal of quality and therefore may be a product of sexual selection. However, because associations between spot characteristics were complex, we do not know whether elevated brightness or chroma represents the more-ornamented state. Without tests of how the spots function as signals, we can only speculate as to whether brighter or more chromatic animals are the most ornamented.

Using spot color, spot size, and spot coverage, we could correctly classify the sex of 82% of individuals. Compared to females, males tended to have more chromatic and less bright spots, and smaller spots but greater spot coverage. Female *A. maculatum* were heavier and longer than males, further corroborating the findings of previous researchers (Peckham and Dineen, 1955; Hillis, 1977; Flageole and

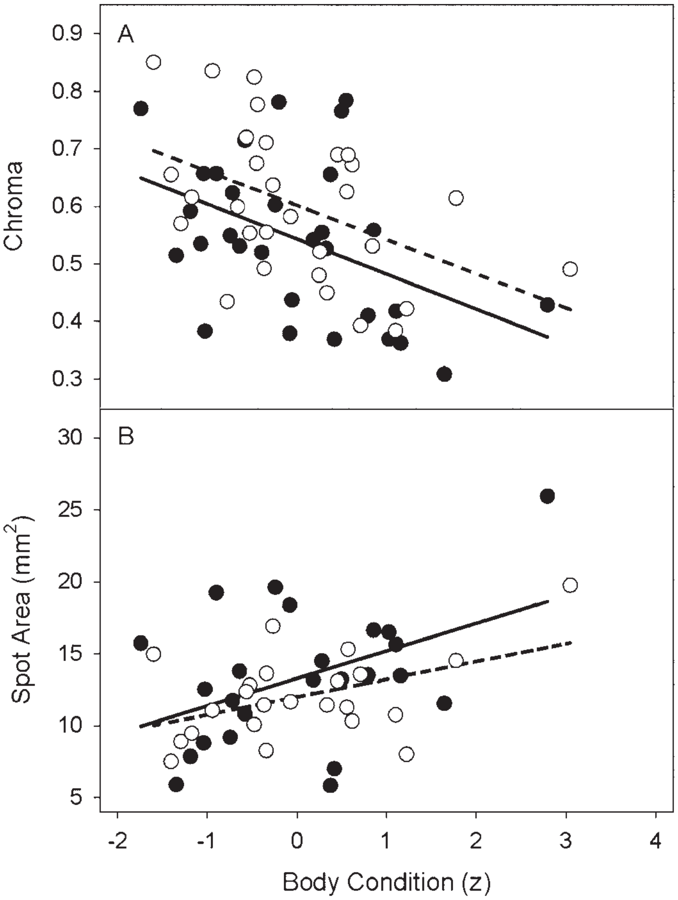

Fig. 3. Relationships between body condition (standardized for sex [z]) and (A) chroma and (B) spot size of spotted salamanders, *Ambystoma maculatum*. Females are open circles and dashed lines and males are solid circles and solid lines.

Leclair, 1992; Davis and Maerz, 2007). However, our ability to correctly classify salamander sex using body morphology (83%) was only slightly better than our ability to correctly classify salamander sex using spot characteristics (82%). The evidence of sexual dimorphism in ornamentation of *A. maculatum* is similar to the closely related marbled salamander (*Ambystoma opacum*), which displays silver-white saddles or crossbands against gray-black dorsal area (Petranka, 1998; Todd and Davis, 2007). Male *A. opacum* display brighter white dorsal saddles and have greater spot coverage compared to females (Todd and Davis, 2007; Pokhrel et al., 2013).

To test the expectations of the sexual selection hypothesis, we should have some expectation of the more ornamented state; yet, without knowing how the yellow traits function as a sexually selected signal, we can only speculate whether brighter or more chromatic animals are the most ornamented. We found that *A. maculatum* that were more chromatic were less bright, and animals with greater spot coverage displayed brighter and less chromatic spots. Animals in better body condition (animals with greater mass relative to their body length) displayed less chromatic spots. There may be tradeoffs between investing in energy reserves and depositing pterins into the integument. Some similarities between spotted and marbled salamanders occur; among *A. opacum*, the saddle coverage (but not the saddle brightness) is positively related to body condition in both males and females (Todd and Davis, 2007; Pokhrel et al., 2013). Our data show that animals in better body condition have larger body spots.

It is possible that sexual dichromatism is exaggerated in the salamanders just prior to breeding. In our study, the females that we measured were gravid and measured just prior to oviposition. The physiological strain could have limited their allocation of resources to pigmentation. Further, the females' spots could be temporarily stretched and, therefore, appear less chromatic and brighter. Because larger spot size, greater spot coverage, and greater individual body condition are associated with less chromatic and brighter (more pallid) yellow spots, it may be that larger individuals and females have greater energetic requirements and are therefore unable to allocate as many resources to pigmentation.

Because animals can either allocate carotenoid pigments toward important physiological functions or into integument, they are often honest indicators of individual quality and reliable sexual signals (reviewed in McGraw, 2006). The yellow coloration in *A. maculatum* is produced by pteridines and not carotenoids (Obika and Bagnara, 1964). Therefore, it is plausible that the pterin-based ornaments of *A. maculatum* serve as an honest indicator of quality. Unlike carotenoids, however, pteridines are produced endogenously and thus should be less sensitive to diet or individual health. Because pteridines, like carotenoids, have important immune and antioxidant functions (reviewed in McGraw, 2005), the production of pterin-based colors could also have physiological costs that ensure signal honesty. The antioxidant or immunopermissive action of colored pterins in animal tissues has not been studied in any taxa (reviewed in McGraw, 2005).

To date, researchers have not quantified how differences in pterin pigment types or concentrations of pterin pigments influence spectral reflectance (but see Steffen and McGraw, 2009 for a combination of carotenoid and pterin pigments). Although we are not sure whether a brighter or more chromatic yellow is the more ornamented state when signaling to conspecifics (i.e., sexual selection), a brighter spot should be a more conspicuous aposematic signal to potential predators (Prudic et al., 2007)—particularly nocturnal predators. *Ambystoma maculatum* exhibit gregariousness, antipredator postures, and noxious secretions (Brodie, 1977); these characteristics are believed to act in tandem with aposematic coloration to signal unpalatability and thus reduce the likelihood of predation. Although we may expect *A. maculatum* with brighter spots to be preyed upon less often than less bright males, without information about how potential predators behave in response to variation in spot characteristics, these arguments remain largely speculative. However, sexual and aposematic selection on coloration are not mutually exclusive hypotheses. It is possible that spot characteristics can function in both mate choice and as warning coloration to predators. In *Dendrobates pumilio*, more conspicuous males are more toxic (Maan and Cummings, 2012) and are favored by females (Maan and Cummings, 2008). At least in this species, the highly ornamented individuals benefit from both sexual and aposematic selections.

Little research has focused on how amphibian coloration functions in species with explosive breeding in large aggregations where scramble competition is thought to occur (reviewed in Rudh and Qvarnstrom, 2013). In *Bufo luetkenii*, dichromatism only occurs during the breeding aggregation (Doucet and Mennill, 2010), suggesting that coloration may act as a signal to potential mates or rivals. Sexually dichromatic males of *Rana arvalis* use color to discriminate between the sexes, suggesting that dichromatism allows males to quickly move between rivals while scrambling for females (Sztatecsny et al., 2012). Finally, female *Scaphiopus couchii* prefer brighter males (Vasquez and Pfenning, 2007) and sire coloration predicts offspring fitness in *Rana arvalis* (Sheldon et al., 2003). The function of dichromatism among aggregate breeding salamanders has not been tested.

Future studies should focus both the proximate influences and the evolutionary function of salamander coloration. First, researchers need to address the relationship between pigment concentration and integument coloration. Second, an investigation of the signaling function of the yellow spots is warranted. Specifically, how does color influence female choice of mates, male–male interactions, and predator deterrence?

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