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IMPROVING NATURAL HISTORY RESEARCH WITH IMAGE ANALYSIS: THE RELATIONSHIP BETWEEN SKIN COLOR, SEX, SIZE AND STAGE IN ADULT RED-SPOTTED NEWTS (*NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS*)

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Abstract.—**Natural history researchers are increasingly using digital cameras and computer software to measure their study animals. Adult Red-spotted Newts (*Notophthalmus viridescens viridescens*) are known to display a range of skin colors, from brown to green, but it has not been possible to quantify this variation until the advent of image analysis technology. We used an image analysis approach to compare skin color between sexes, across a range of sizes, and between aquatic and post-breeding (emigrating to the terrestrial habitat) stage adult newts. From 232 individuals (53% male, 47% female) we documented a wide but subtle range of skin colors, measured as the average hue value of all pixels in digital images of each newt body. We found that terrestrial post-breeding individuals were browner than aquatic individuals, consistent with the morphological adaptations of emigrating individuals for the terrestrial habitat. However, we also discovered that males were statistically greener than females but this effect depended on their stage. We suggest this difference may represent different degrees of adaptation for the terrestrial habitat between the sexes. Finally, we used image analysis to obtain measures of body length comparable to traditional snout-vent length and a measure of total body surface area, which provided a better correlation with newt mass than did body length and therefore could potentially serve as an alternative to mass or body condition. We suggest that image analysis methodology offers great promise for future questions relating to size and color in amphibians and provides researchers with an improved way to study aspects of the natural history of amphibians.**

Key Words.—breeding stage; body size; image analysis; *Notophthalmus viridescens viridescens;* Red-spotted Newt; sexual variation; skin color

INTRODUCTION

The recent widespread availability and lowering costs of digital cameras are changing the face of natural history research. The use of hand-held calipers to measure animals in the field is being replaced by photographs of the animal and computer software to measure organisms digitally. The advent of such computer programs have allowed researchers to measure features of animals in a broad range of research applications (e.g., Grether et al. 2001; Wright and Zamudio 2002; Davis et al. 2004). This method has proven especially useful to quantify insect coloration (Davis et al. 2004; Davis et al. 2005), to measure tail shapes of anuran larvae (Van Buskirk 2002; Relyea 2004), and to assess spot symmetry in salamanders (Wright and Zamudio 2002; Davis and Maerz In Press). Image analysis has the potential to elevate the quality and quantity of morphological data commonly gathered by field researchers, with the additional benefit of reducing the handling of animals. Amongst herpetologists for example, using calipers or rulers to measure the linear snout-vent length (SVL) may have high rates of error due either to elasticity of the organism, or movement of the animal in response to being handled (e.g., Luiselli 2005). The use of image analysis software allows researchers to obtain not only snout-vent lengths, but also other morphological measures from a single image of the animal.

Red-spotted Newts (*Notophthalmus viridescens viridescens*) are an excellent example of an organism where image analysis can not only improve the measurement of traditional morphological variables, but also provide additional biologically meaningful measurements such as body coloration variables. The natural history of Red-spotted Newts has attracted the attention of scientists for well over a century because of their unique and variable life history, with one of the most complex and flexible life cycles of any vertebrate. The aquatic larvae typically metamorphose into a terrestrial juvenile stage, commonly called a red eft, which may last for several years (Forester and Lykens 1991; Leclair et al. 2005). Efts are bright orange to red in color and highly toxic (Brodie 1968). However, in some populations larvae may skip the terrestrial eft phase altogether and can mature directly into an aquatic adult or reproduce as a gilled paedomorph (Noble 1926; Healy 1974; Harris 1987). In populations with the juvenile eft stage, individuals undergo a second transformation when they return to the pond to breed as adults, developing a dorsal tail fin and replacing bright red, granular skin with smooth, viridescent green skin (Gage 1891; Gill 1978). These adults may then remain permanently aquatic or emigrate to the terrestrial habitat during the non-breeding season. Adult newts that return to the terrestrial habitat in the non-breeding season undergo further morphological changes, including reduction of the tail fin and redevelopment of the granular skin that is browner in color (Brimley 1921; Walters and Greenwald 1977).

Image analysis offers the opportunity to provide quantitative information on skin color variation in Red-spotted Newts, and which may aid in our understanding of the transition between life phases. In this study, we used image analysis to measure features of Red-spotted Newts captured while conducting a long-term study of their population dynamics at Mountain Lake Biological Station (MLBS) in Giles County, Virginia. We addressed the following questions in the current paper: (1) does variation in skin color exist between the sexes; (2) is adult skin color in the aquatic stage quantitatively different from that of the terrestrial stage; and (3) is there a relationship between skin color and size? Because this is the first attempt at using these techniques on this species, a secondary goal of this study was to evaluate the utility of these methods for future research.

MATERIALS AND METHODS

Collection and Photography.—As part of a long-term study of the newt population at MLBS, drift fences were constructed in May 2005 of aluminum flashing completely encircling two man-made ponds. Numbered pairs of pitfall traps (19 l plastic buckets) were installed on each side of the drift fence every 6-8 m to capture animals encountering the fence during breeding migrations to and from the ponds. Aquatic adults were captured using a seine during initial sampling in June and July. Adults emigrating to the terrestrial habitat were captured in buckets on the inside of the fence, largely from the first week of July through the end of August. This difference in capture method effectively separated the newts in our data set into two stages – aquatic (those trapped by seining) and terrestrial (those captured leaving the ponds). Furthermore, we noted that emigrating individuals had fully developed the terrestrial morphology described by Walters and Greenwald (1977).

Captured newts were sexed using the yellow glandular spot on the posterior cloaca present in males. A random subset of captured newts (n = 68) were also weighed to the nearest 0.01 g. All newts were photographed on a constant white stage using a Nikon Coolpix E990 camera (Nikon, Inc., Mellville, New York, U.S.A.), with the camera at a fixed distance from the stage. Moreover, all pictures were taken indoors in the same room and with the same lighting conditions throughout. Further, all images were obtained with the same camera setup and distance from the stage as the ruler image. The initial purpose of photographing individuals was to use the dorsal pattern of red spots as an individual unique mark (Healy 1975; Gill 1978) so that a capture history of individuals could be obtained over time (Kristine Grayson, unpubl. data). Because the mucous texture and wetness of their skin resulted in glare when photographed in air, aquatic newts were photographed submerged in a small white container of clean water. Terrestrial newts captured at the drift fence were photographed dry in an identical white container; these individuals would not fully submerge in water and the granular texture of their skin trapped air bubbles, which tended to obscure parts of the newt. Several images were taken of the same individual and the photograph with the most complete dorsal view was selected for image analysis. A total of 232 newts were photographed from one of the two ponds (Horton Pond) and were used in the current paper.

Image Analysis Measurements.—All newt pictures were analyzed using Fovea Pro software (Reindeer Graphics, Inc., Asheville, North Carolina, U.S.A.). The image analyses methods we used generally followed Davis et al. (2004). Briefly, image analysis software calculates the dimensions of selected objects in digital images by calculating the number of on-screen pixels in the object,

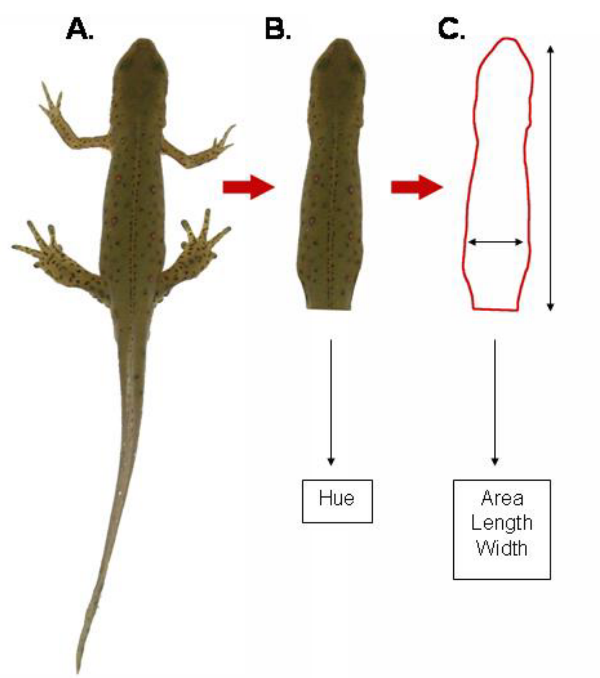

FIGURE 1. Image analysis methods used to measure newts. The newt was first separated from the background of the image (A) and the legs and tail were digitally removed (B). The image analysis software then measured the average hue of all pixels in the 'newt body'. The body area, maximum width and maximum length were measured based on a predetermined pixel-to-mm ratio (C).

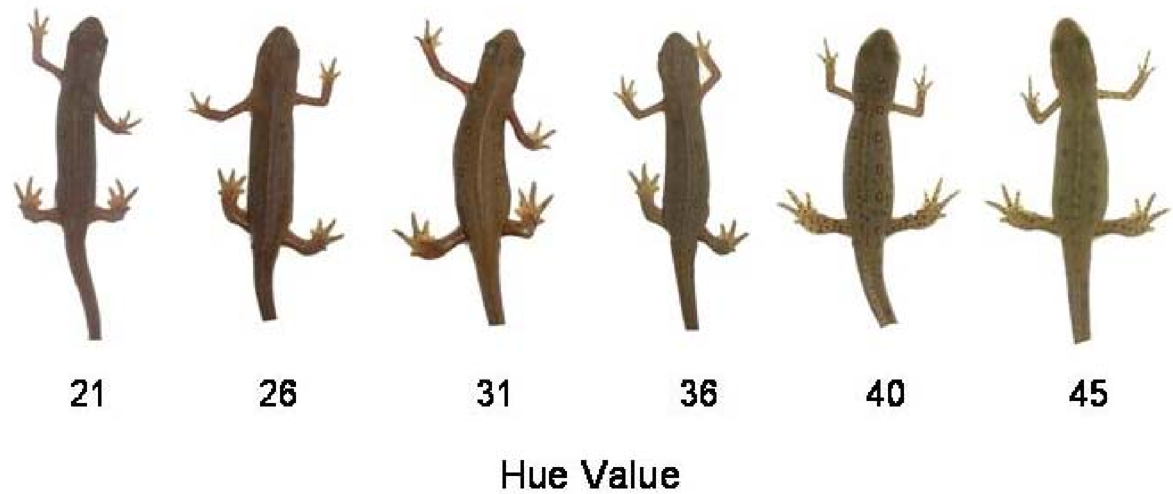

FIGURE 2. Composite of six Red-spotted Newts ranging in skin color from brownish-red (low hue values) to green (high values). Hue values assigned by the image analysis program are shown below each newt.

then outputting the actual dimensions of the object in millimeters based on a user-defined pixel-to-millimeter ratio. Before measuring the newts, the pixel-to-mm ratio for our newt images was obtained by importing and measuring a digital picture of a standard, metric ruler that was placed on the same stage as the newts, and taken with the same camera at the same distance from the stage.

On-screen measurement of the newts involved first digitally removing the background to leave only the newt on a white background (Fig. 1A), then digitally removing the limbs and tails from each image to leave only the torso (Fig. 1B). Next, this torso was selected and a FoveaPro color measure routine was applied to the selection. In this routine, the software returns the average of the hue, saturation and brightness values for all pixels in the selection (there are 136,781 pixels in the newt body in Fig. 1B). Based on visual inspection of the images, we determined that the average hue values alone could be used to indicate the relative skin color variations we observed, which ranged from dull brown (low hue values) to green (high values; Fig. 2). The area of dorsal spots accounted for a minor amount of the total area of the body (average of 1% of body area on a subset of 33 individuals), and therefore added little to the calculation of overall body hue (the average hue values of 33 individuals with and without spots selected were 33.99 and 33.94). Next, the area of the newt body (minus the limbs and tail) was measured (in mm^2), as well as the maximum width (hereafter body width) and the maximum length (hereafter body length), both in millimeters (Fig. 1C). The body length measurement was from the newt's snout tip to the point of tail attachment, a measure that corresponds to traditional snout-vent length.

Statistical Analyses.—To examine variation in skin color, we used two-way ANOVA ($\alpha = 0.05$), with skin hue as the dependent variable, sex and stage (aquatic or terrestrial) as dichotomous fixed factors, and body area as a continuous covariate. We used body area as our measure of size rather than body length because it was more closely related to body mass than length (see results). All two-way interaction terms were also included in this analysis. We then used model simplification procedures (Crawley 2002) by first removing each nonsignificant ($P > 0.05$) interaction term and comparing Akaike's information criteria (AIC) between the full model and the model with k-1 terms. Interaction terms were excluded from a simplified model if (1) their removal resulted in a lower value of AIC; or (2) their removal resulted in a small increase in AIC of less than 4.0, or if their associated p-value was greater than 0.2. We reported results from a final analysis that included main effects, covariates, and interaction terms for those parameters that explained substantial variation in each response variable as determined by model fitting. All tests were performed using SPSS software (SPSS 2005) and all tests were considered significant when $P < 0.05$.

TABLE 1. Mean size parameters obtained using image analysis for male and female Red-spotted Newts in this study (n = 232). Also shown are the average mass measurements obtained from a subset of individuals (n = 68). Values in parentheses are ± one standard deviation.

	Females (n=110)		Males (n =122)		Both Sexes	
Body Length (mm)	39.6	(2.5)	40.3	(2.1)	40.0	(2.3)
Body Width (mm)	9.5	(1.0)	10.4	(1.0)	10.0	(1.1)
Body Area (mm^2)	289.6	(36.5)	317.0	(33.4)	304.0	(37.4)
Mass (g)	2.4	(0.3)	2.6	(0.5)	2.4	(0.4)

TABLE 2. Summary of ANOVA examining the relationship between skin color (hue) and sex, size (body area) and stage (either aquatic or terrestrial) for all newts (n = 232). The interaction term of Stage*Body Area was not significant in the initial model; therefore, it was removed to improve the model fit based on model simplification procedures described in the methods.

Variable	*df*	Mean Square	F	*P*
Sex	1	18.9	1.65	0.201
Body Area	1	0.3	0.03	0.862
Stage	1	1809.2	157.36	<0.001
Sex * Stage	1	45.6	3.97	0.048
Sex * Body Area	1	36.9	3.21	0.074
Error	226	11.5		
Total	232			

RESULTS AND DISCUSSION

General Results.—Of the 232 newts we captured and photographed for this study, 122 (53%) were males while 110 (47%) were females. We captured 145 individuals by seining (female = 50, male = 95), and 87 individuals using drift fences (female = 60, male = 27). Basic size parameters that were obtained using image analysis (body length, body width, body area) are given in Table 1. Our use of image analysis allowed us to obtain estimates of individual body area for this species, which to our knowledge, has not been reported elsewhere. Interestingly, this variable appeared to be highly useful for indexing the size of newts. Using a subset of male and female newts that were weighed using a standard electronic balance, we found a significant correlation between body mass and body length obtained using image analysis software (Pearson Correlation, $r = 0.67$, $P < 0.001$). However, comparisons between mass and body area in these individuals yielded a stronger linear relationship ($r = 0.84$, $P < 0.001$; Fig. 3). This may be the result of the body area measure capturing more variation in structural size between individuals than body length (equivalent to the traditional SVL measurement).

Skin Color Results.—Our image analysis technique allowed us to quantify a wide range of skin color hue values for both males and female newts. The individuals ranged from a brownish-red color (lowest hue values) to brownish-green (middle-range values) to overall green (highest values; Fig. 2). There was no main effect of sex ($F_{1,232} = 1.6$, $P = 0.201$; Table 2). We did, however, find a significant interaction effect of sex by stage ($F_{1,232} = 4.0$, $P = 0.048$). The skin color difference between males and females was less pronounced during the aquatic stage and greatest during the terrestrial post-breeding stage (Fig. 4). Future research should investigate the relationship between skin color and the physiological adaptation of newts to their changing environments. We speculate that the significantly lower skin hue of emigrating females compared to males may indicate that females are further adapting for the terrestrial habitat (i.e., developing greater skin granulation, camouflage, and resistance to desiccation). Female newts have been shown to spend longer in the terrestrial habitat than males, often skipping breeding seasons (Gill 1985). Additionally, stage itself had a significant effect regardless of sex ($F_{1,232} = 157.4$, $P < 0.001$). Overall, newts were significantly greener during the aquatic stage and browner during the terrestrial stage (Fig. 4). Finally, body area alone did not significantly effect skin color hue, but our results suggest a weak interaction between sex and body area (Table 2).

Comments on image analysis.—Using image analysis methods on newt photographs, we were able to generate several variables relating to color and size, with more precise and detailed size variables than traditional methods. For example, we were able to obtain measures of body area for each individual, which appeared to be tightly correlated with body mass. This relationship suggests that body area calculated from photographs could potentially serve as an alternative to body mass or body condition. Furthermore, all of these measures were obtained from a

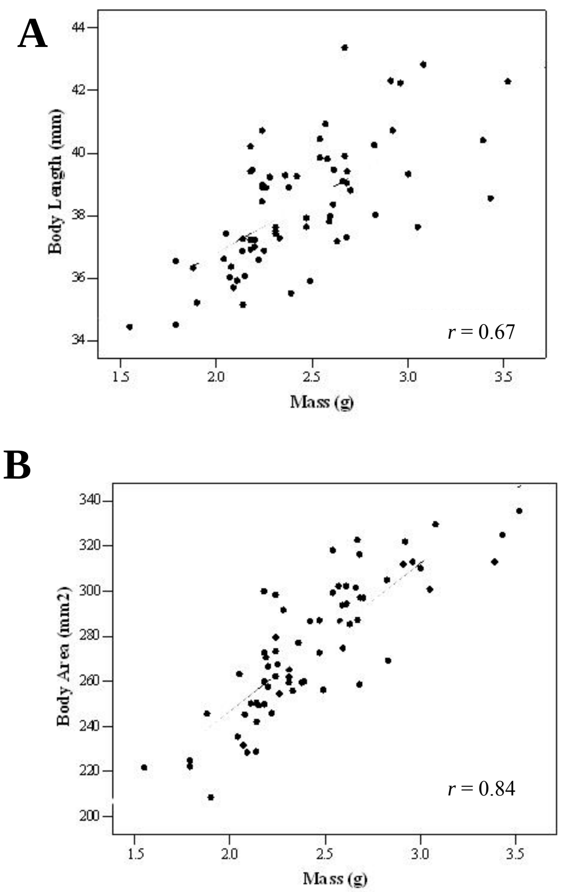

FIGURE 3. Comparison of mass and image analysis measures for a subset of newts from this study (n = 68).

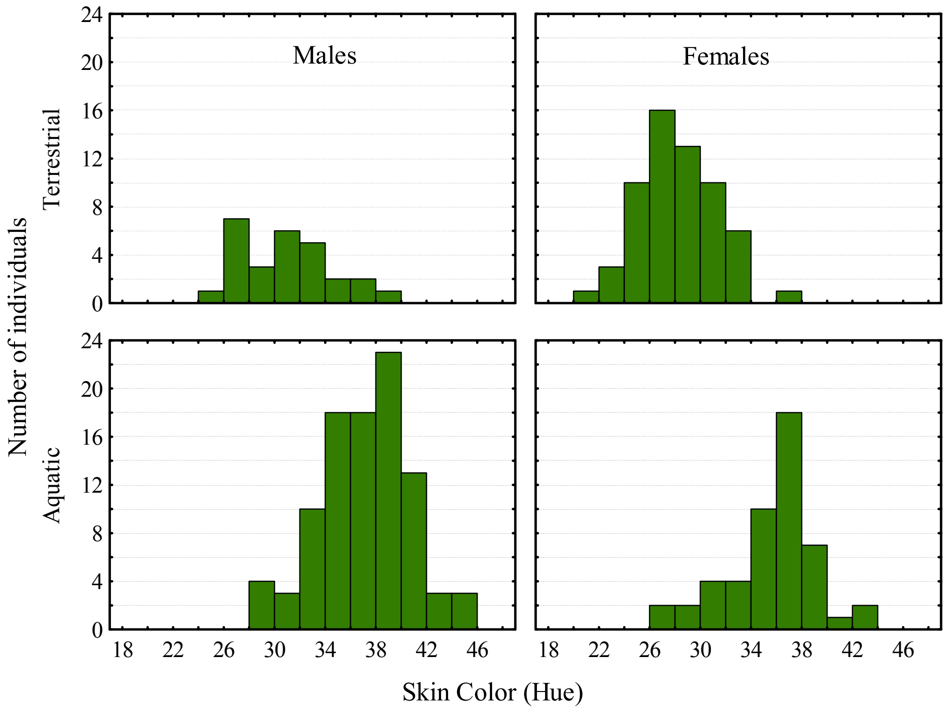

Figure 4. The distribution of skin color (hue) values for all newts in this study broken down by aquatic or terrestrial, males and females. Larger hue values represent greener individuals, while lower values indicate browner individuals.

single photograph, which reduced the handling time of newts considerably.

This paper demonstrates one of the greatest strengths of image analysis: the assessment of color in numerical terms, which then allows for statistical tests to be performed. Furthermore, with this method color is measured over the entire area of the newt body, in contrast to the use of portable spectrophotometers, which assess the color of a small, fixed, circular area (e.g., Hill 1998; Grill 1999). As in previous research using these techniques on insects, we observed a level of detail using the software beyond what could have been assessed visually (Davis et al. 2004; Davis et al. 2005). We found that females are generally browner than males, but are particularly browner when transitioning to the terrestrial habitat. We believe that studies of variation in coloration will be useful in studies of the ecology and natural history of several other taxa, such as insects and birds. We further point out that this result is just one example of the potential this technology now offers researchers studying the natural history of amphibians, and in particular we hope that our study provides a starting point for future research into the implications of variation in coloration.

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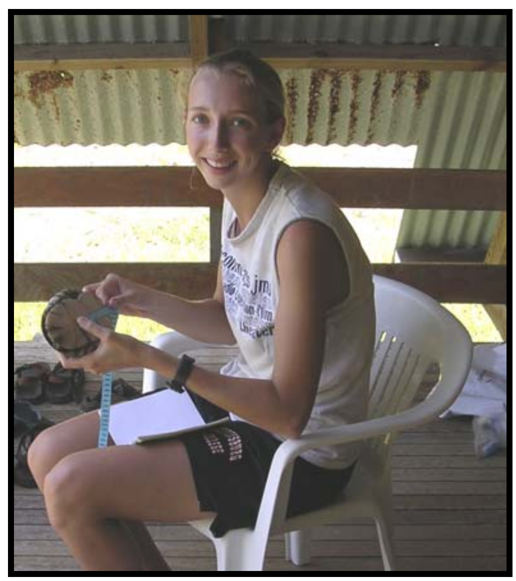

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