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THE DEVELOPMENT OF A COST EFFECTIVE METHOD FOR MEASURING THE VARIATION OF AREA IN CONTRASTING SCUTE PIGMENTATION IN CHELONIANS

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***Abstract.*—Metapopulations of chelonians often vary widely in the amount of contrasting carapace scute pigment. To date, measurement of such variation has been undertaken using a myriad of techniques from non-quantitative descriptive techniques to the use of expensive image capture techniques and processing software. This paper describes a rigorous, cost effective method using freely available software and a basic digital camera to quantify the comparative area of light and dark patterning for a species supporting high contrast carapace patterning. To validate the method, I used a sample population of Northern Spider Tortoises (*Pyxis arachnoides brygooi*) to test for age and sex dependent variation in scute color contrast. A reduction of dark pigmentation was correlated with increased age, and females were found to have a greater amount of dark pigmentation than males. This technique provides researchers with an alternative to expensive image capture software and processing techniques in cases where all that is required is a robust method for the quantification of contrasting dark and light area of patterning.**

Key Words.—Chelonians; image processing; Madagascar; *Pyxis arachnoides brygooi*; scute pigmentation; Spider Tortoise; Vidana 1.0.

INTRODUCTION

The study and measurement of morphology has historically played a major role in the taxonomic and ecological description of many species (Pritchard 1979; Ennen et al. 2010; Saey 2010; Weller et al. 2010). The assessment of color patterns, spectral radiance, and pigmentation are often critical for testing numerous ecological hypotheses (Saey 2010; Weller et al. 2010). Many species of reptiles often display marked variations in pigmentation (Crisp et al. 1979; Smith et al. 1991; Ennen et al. 2010) as a result of: (1) genetic variation within and among populations of the same species; (2) the influence of environmental variables, such as substrate color (Lewis 1949; Macedonia et al. 2003); (3) gender; and (4) ontogeny (Tucker et al. 1995). Such variation in pigmentation is particularly evident in the markings of the carapaces of some chelonian species (Dunn 1982; Bloxam et al. 1996; McGaugh 2008; Weller et al. 2010).

The study of carapace pigment variation within chelonians broadly can be split into two methodological categories (i.e., digital and non digital). Historically, studies prior to the advent of digital technology concentrated predominantly upon the dominant color or patterning of individuals or populations (Woolley 1957; Dunn 1982; Banks 1986). These studies were largely qualitative and subject to observer bias. The limited number of studies that sought to collect quantitative data were reliant upon the time consuming use of photographic film and subjective manual analysis techniques, such as tracing around different pigmentation in the photographs (Willemsen and Hailey 1999). More recently, the use of digital capture methods and image manipulation software has allowed for more quantitative and sophisticated analysis of the measurement of contrasting areas of pigmentation within individual animals (Cox et al. 2005; Whiting et al. 2006; Ennen et al. 2010; Weller et al. 2010). This paper describes a rapid, cost-effective, digital technique to quantitatively assess the relative coverage of light and dark pigment in chelonian carapace scutes using Vidana 1.0, an open source software package developed by the Exeter University Marine Spatial Ecology Laboratory. To demonstrate this method, I quantified the variation in the percentage coverage of dark pigmentation per tortoise for a population of Northern Spider Tortoises (*Pyxis arachnoides brygooi*) using digital photographs and Vidana 1.0.

MATERIALS AND METHODS

I collected photographic and morphometric data from a population of the Critically Endangered *P. a. brygooi* (IUCN. 2009. IUCN Red List of Threatened Species. Available from http://www.iucnredlist.org. [Accessed 10 April 2009]) across the species' small, fragmented range within the Mikea forests of southwest Madagascar

FIGURE 1. (right) *In situ* image capture of Northern Spider Tortoises (*Pyxis arachnoides brygooi*) using a tripod mounted digital camera, set to automatic settings. Image is in its JPEG form prior to cropping.

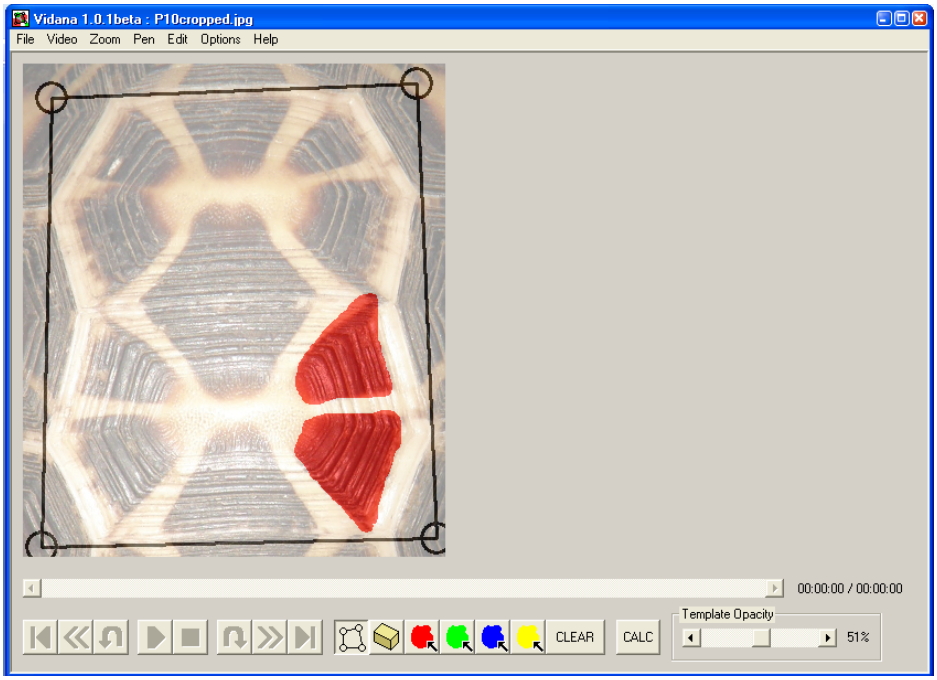

FIGURE 2. Cropped image of Northern Spider Tortoises (*Pyxis arachnoides brygooi*) carapace showing only the second and third vertebral scutes with partially completed manual digitizing of dark regions of scute pigmentation using Vidana 1.0.

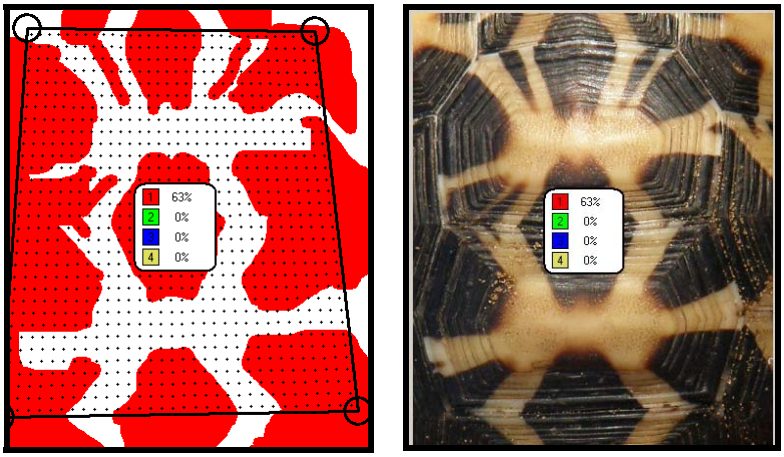

FIGURE 3. Left Manipulated cropped image using Vidana 1.0. to calculate variation in percentage coverage of dark scute pigmentation for the region within the second and third vertebral scutes with CALC (calculation) display box showing the percentage coverage of the manually digitized dark pigmentation. Right: Cropped image prior to manual digitizing.

(Walker 2009a, 2009b, 2010).

I collected data from 64 tortoises (males N = 25; Females N = 18; Juveniles N = 21) across 34 survey sites during February 2009. I determined the sex of each tortoise using the external morphology of the lower plastron and tail length as described by Jesu and Schimmenti (1995). Then, I assigned each tortoise an age class using the scute ring counting technique described by Germano (1998). I measured the curved carapace length (CCL) of each tortoise to the nearest mm. Each tortoise was then placed on a white board measuring 30 × 15 cm on flat ground and photographed *in situ* (Fig. 1). I used a tripod mounted Olympus SP560UZ (Olympus Imaging & Audio Ltd, Southend-on-Sea, Essex, United Kingdom) digital compact camera set 50 cm above the board and parallel to the ground. The camera was set to 8 megapixel resolution and automatic settings for focus, lighting, and shutter speed for each picture. Each image amounted to 2048 × 1536 pixel size. Finally, I stored the image as a 400–600 KB Joint Photographic Experts Group (JPEG) file. I maintained the consistency of light conditions by only using pictures for analysis that had been taken between 0730–0900 h during cloudless days (Oktas cloud cover scale = 0).

I cropped each image using the free software Photo Crop Editor 1.15 (iFoxSoft, Beijing, China; Available from http://download.cnet.com/Photo-Crop-Editor/3000-2192_4-10536710.html [Accessed 10 April 2009]) to reduce the image to include a quadrant containing the second and third vertebral scutes (Figs. 2–3). I used the second and third vertebral scutes to assess the extent of the area of dark pigmentation for each tortoise because this relatively small area of the carapace has only slight variation of carapace curvature among different individuals; therefore, only these two scutes can be consistently photographed from above and the researcher be confident of capturing the same area when collating photographic data from a large group of tortoises. This cropped image was loaded into the image processing software Vidana 1.0 (University of Exeter Marine Spatial Ecology Lab, Exeter, Devon, United Kingdom; Available from http://www.ex.ac.uk/msel [Accessed 10 April 2009]). I then laid the delineating quadrant function over the image. I maintained consistency in carapace area selection between tortoises through the consistent alignment of the top and bottom of the quadrant with the top and bottom edge of the second and third vertebral scutes respectively. The left and right boundaries of the quadrant were aligned with the left and right outer most portions of the second and third scutes (Fig. 2). Therefore the same respective area was always delineated by the quadrant for each image. Following this, I manually digitized around the black pigmentation contained within the quadrant (Figs. 2–3). Finally, by using the CALC button, the exact percentage cover of the dark scute pigmentation contained within the quadrant was calculated (Fig. 3). Both Vidana 1.0 and the cropping software were run on a laptop using Windows 2000 operating system, with a slow processor running at 266 MHz and 64 MB of RAM.

I assessed variation in scute pigmentation with age and sex using correlation and ANOVA ($\alpha = 0.05$). I used correlation analysis to determine if the percentage of dark scute pigmentation changed with age (the number of scute rings) of each of the 64 tortoises in the study. I used One-way ANOVA to compare variation in the percentage of dark scute pigmentation of 10 mature male and 10 mature female tortoises (age class of between 7–11 carapace scute rings). I only used 10 individuals of each sex to control for variation in age. I also compared curved carapace length (CCL) between males and females to test for size dependent variation in the measurement of pigmentation area using ANOVA. Minitab 14 (Minitab Inc. State College, Pennsylvania, USA) was used for all parametric statistical analysis. I adjusted all data to follow a normal distribution where adjustment was necessary.

RESULTS

The percentage area of dark pigment was significantly negatively correlated with the number of scute rings (age) for *P. a. brygooi* as the tortoise aged ($r = -0.51$, $P < 0.001$; Fig. 4). Although mature female tortoises had a higher mean percentage of dark pigmentation on their scutes (64.2 ± 12.1%) than males (57.9 ± 16.6%), the difference was not significant ($F_{1, 18} = 0.94$ $P = 0.346$; Fig. 5), and mean female CCL (148.8 ± 15.2 mm) was not significantly different than mean male CCL (138.3 ± 8.7 mm; F = 2.74, P = 0.124; Fig. 5).

DISCUSSION

The carapace of *P. a. brygooi* loses some of its dark

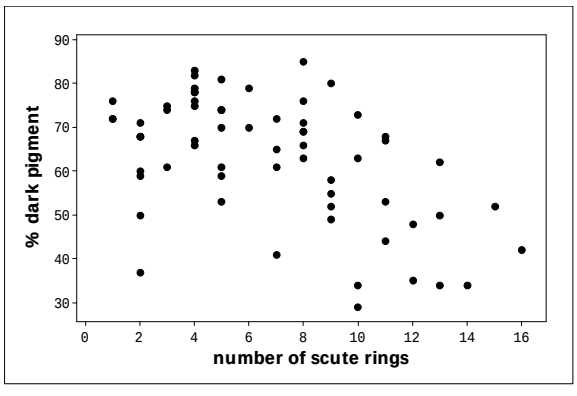

FIGURE 4. Scatter plot displaying the percentage of dark scute pigmentation of 64 Northern Spider Tortoises (*Pyxis arachnoides brygooi*) plotted against number of scute rings (age).

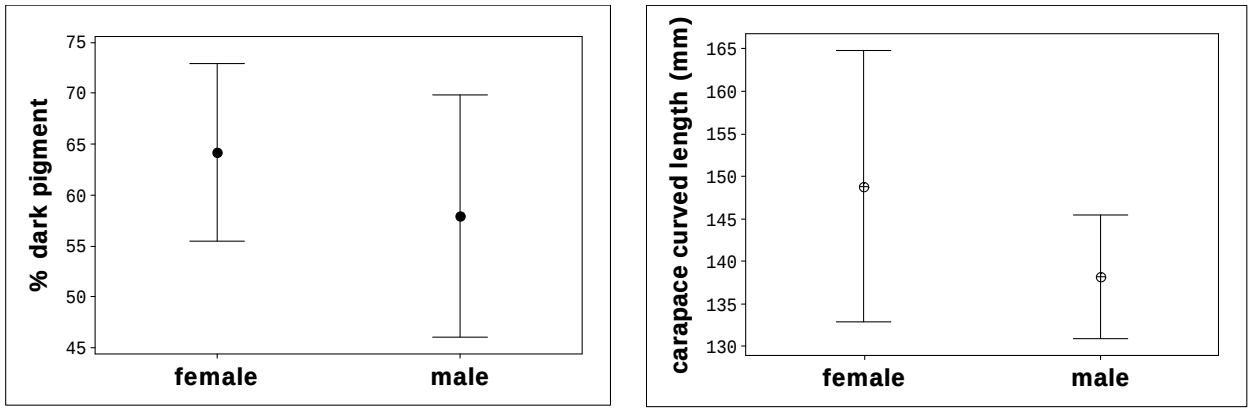

FIGURE 5. Left: Mean percentage of dark scute pigment for a cohort of 10 female and 10 male Northern Spider Tortoises (*Pyxis arachnoides brygooi*). Right: Mean curved carapace length (mm) for the males and females within the cohort.

pigmentation with age, in a manner similar to other chelonian species (Balazs 1986). Sexual dichromatism, whereby carapace size appears to be related to the amount of light and dark pigmentation, was evident for the tortoises within this study, as is the case with many chelonian species (Brophy and Ernst 2004; Brophy 2006). Females were larger and displayed darker carapace pigmentation than males.

Since the advent of digital technology, the use of image analysis software such as Fovea Pro (Reindeer Graphics, Asheville, North Carolina, USA; Davis and Grayson 2007; Davis and Maerz 2007) or SigmaScan Pro (Weller et al. 2010), costing up to about USD $1,000, has been common for measuring area of body parts, geometric shape of markings, and pigmentation in herpetofauna. However, a number of free image processing software packages , such as NIH Image (Cox et al. 2005; Whiting et al. 2006) and ImageJ (National Institutes of Health, Bethesda, Maryland, USA; Davis et al. 2008), are now available and have been used in a similar manner for digitally measuring area coloration differentiation on various species of herpetofauna external body parts. Vidana 1.0 is an easy to use addition to this suite of free software. This software was designed for performing marine substrate cover analysis from video footage or still images (Mumby et al. 2007) with the aim of making the processing of large amounts of data (i.e., multiple images) rapid and cost effective. While the technique employed in this study are not a replacement for more sensitive analysis of color (Endler 1991; Bortolotti et al. 2003; Eeva et al. 2008), this method works well for quantitatively assessing differences in the relative area of pigmentation (dark versus light) in chelonian species from digital images, especially when well differentiated patterns can be delineated. The technique can be applied rapidly to a large number of images in the study of pigmentation variability across large populations of chelonians, with a faster analysis time compared to some studies employing manual techniques such as tracing (e.g., Willemsen and Hailey 1999). This technique also provides a more rigorous alternative than subjectively analyzing 'high' or 'low' amounts of pigmentation on a carapace by eye (e.g., Kabigumila 2001). With the ever increasing accessibility of digital imagery capture hardware and image processing software, the use of measurement of pigmentation has recently become a popular method for establishing a greater knowledge of the ecology or taxonomy of species. Indeed, pigmentation measurement has recently played a major role in the description of a new species of chelonian; *Graptemys pearlensis* (Ennen et al. 2010) and the establishment of intergradation between two subspecies of a turtle; *Chrysemys picta bellii* and *C. p. marginata* (Weller et al. 2010). Saey (2010) describes how recent advances in image capture data are advancing our knowledge of the ecology and genetics of a wide variety of taxa including fish, invertebrates, and mammals.

The image analysis software used within this study could easily be applied to assessing the variation in pigmentation for a variety of taxa. However, as the technique relies to a degree on the assessment of areas to isolate in the images by the eye of the operator, it could lead to bias or subjectivity among species that do not have distinctive boarders in the variation of pigmentation. Developing scientifically rigorous, affordable, biological surveying and monitoring techniques, such as the methods described in this paper, is important when funds for conservation or biological research are limited (Hudson and Bird 2006). Cost effective techniques also make biological and ecological research more accessible to researchers and conservation biologists in developing countries. The advantage of Vidana 1.0 over some of the similar free software available is that is distributed as a single downloadable executable file, approximately 230 kb in size that will run on a PC or laptop with limited processor speed and memory and hard drive space. Thus, this makes the

software accessible to researchers in regions of the world where internet bandwidth and access to powerful computing systems is limited. Excluding the logistical and computing hardware costs, it is estimated that equipment costs for the 8 megapixel camera and tripod amounted to less than $200. I propose that this method could be used as a cost effective alternative to purely descriptive methods; as well as the use of expensive or bandwidth and memory intensive software.

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