



# Evaluation of spot patterns and carapace abnormalities of an Endangered freshwater turtle, *Clemmys guttata*, as a potential tool for population assignment

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**ABSTRACT:** Many of the world's contemporary species of turtle are extinct or threatened with extinction due to habitat loss, increases in anthropogenic sources of mortality, and poaching (illegal collection). The slow life-history strategy of most turtle species magnifies the effects of poaching because the loss of even a few mature individuals can impact population growth. Returning poached turtles to their population of origin, where possible, can mitigate these effects, but identifying the origin of these individuals can be challenging. We hypothesized that spot patterns might allow assignment of Endangered spotted turtles *Clemmys guttata* to their population of origin. We characterized and compared spot patterns from carapace photographs of 126 individuals from 10 sites. To explore other types of information these photographs might provide, we also documented carapacial scute abnormalities and quantified their association with genetic diversity and latitude. Spot pattern similarity was not higher within populations than among populations and did not accurately differentiate populations. Carapacial scute abnormalities occurred in 82% of turtles and were not correlated with estimates of neutral genetic diversity. Abnormalities were positively correlated with latitude, implicating thermal stress during the early stages of development in the generation of some scute deformities. However, this relationship became non-significant when line (scute seam) abnormalities were excluded from the data, suggesting a different primary cause for the more severe scute deformities. Further research should continue to investigate the drivers of these deformities, as monitoring shifts in the frequency of scute deformities may provide relevant information for conservation and recovery of endangered turtles.

**KEY WORDS:** Population assignment · Spotted turtle · *Clemmys guttata* · Digital photographs · Carapace abnormalities · Genetic diversity · Latitude

## 1. INTRODUCTION

Effective management and conservation of wildlife populations require a sound knowledge of population demographics, which involves unique marking or identification of individuals within a population. Photographs provide a non-invasive tool for individual identification of some species, and are especially useful for species that are large and difficult to catch, easily stressed by handling, or are difficult to capture

repeatedly. In some instances, photographic methods can reduce or eliminate the need for invasive marking methods that may cause discomfort, increase infection risk, alter animal behavior or performance, or become lost over time (Golay & Durrer 1994, Nichols et al. 1998, Dugger et al. 2006, Reisser et al. 2008, Saraux et al. 2011, Walker et al. 2012).

Naturally occurring spot patterns have been used to successfully identify individuals of many taxa, including leopards *Panthera pardus kotiya* (Miththa-

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pala et al. 1989, Kittle et al. 2017), sharks (Arzoumanian et al. 2005, Speed et al. 2007, Van Tienhoven et al. 2007, Holmberg et al. 2009), African penguins *Spheniscus demersus* (Sherley et al. 2010), and red-spotted newts *Notophthalmus viridescens* (Gill 1978). Facial or cranial scale patterns can be used to identify individual sea turtles (e.g. Reisser et al. 2008, Schofield et al. 2008, Rees et al. 2013, Dunbar et al. 2014) and European adders *Vipera berus* (Bauwens et al. 2018), and variations in whisker spots on the nose can be used to distinguish individual polar bears *Ursus maritimus* (Anderson et al. 2007) and Australian sea lions *Neophoca cinerea* (Osterrieder et al. 2015). Natural body markings can even be used to identify individual Archey's frogs *Leiopelma archeyi* (Bradfield 2004).

If markings are either heritable or are driven by environmental variation, then some may also be population- or site-specific. Heritability and environmental effects of natural markings vary among taxa. Some aspects of spot patterns in giraffes *Giraffa camelopardalis* are partially heritable (Lee et al. 2018), while natural markings of Andean bears *Tremarctos ornatus* are not related to kinship (i.e. not heritable; Van Horn et al. 2015). Five different coat patterns were described in Eurasian lynx *Lynx lynx*, and their frequency of occurrence varied among areas into which the animals were re-introduced (Thüler 2002), suggesting that environmental drivers of markings varied among areas. If markings are reliably population- or site-specific (suggesting either heritability or environmental drivers of natural markings), then they could be used to assign confiscated, illegally collected individuals to their populations of origin. Live wild animals are confiscated by local, regional, and national authorities for a variety of reasons (e.g. injury, habitat destruction, poaching for the illegal pet trade), and sometimes these animals can be returned to the wild, after considering associated costs and benefits (IUCN 2000). For species at risk, reinforcement of populations might improve the long-term conservation potential (IUCN 2000); thus, if it can be determined that the preservation and welfare of existing wild populations of the species will not be harmed, the addition/return of individuals to an existing population of the same taxon could be a powerful conservation tool for these species.

In addition to aiding in identification, photographs may also contain information about wildlife health. Wildlife diseases with external clinical signs, such as sarcoptic mange, can be monitored in large felids and canids using camera traps (Oleaga et al. 2011, Ryser-Degiorgis 2013). Similar techniques were used

to monitor dermal disease in wild bottlenose dolphins *Tursiops truncatus* (Thompson & Hammond 1992). Photographs have also been used to monitor the behavior of disease vector species such as the European badger *Meles meles*, a potential source of bovine tuberculosis (Chen et al. 2019), and to explore interactions of wildlife with human activities, predators, and conspecifics. Images of marine mammals can be used to quantify wounds and scars likely caused by entanglement with fishing gear (Kiszka et al. 2009), vessel collisions (Kraus 1990, Calambokidis 1995, COSEWIC 2002, Langtimm et al. 2004), and/or intra-/inter-specific social interactions (Heithaus 2001, Kiszka et al. 2009).

The Endangered spotted turtle *Clemmys guttata* is one of the smallest freshwater turtles in North America, and is distinguished by its smooth, black carapace covered with yellow-orange spots (Ernst & Lovich 2009). Spotted turtles are threatened by road mortality, illegal collection for the pet trade, and habitat loss and degradation (van Dijk 2011, COSEWIC 2014, Howell & Seigel 2019), threats that have placed approximately 61% of the 365 species of turtles recognized worldwide at risk of extinction over the past 500 yr (Lovich et al. 2018). Conservation of turtles is challenging because populations recover extremely slowly from perturbations or catastrophic events. This is largely due to their slow life-history strategy, which includes slow growth rate, late age of maturity, low fecundity, and low survival rate of eggs and juveniles (Enneson & Litzgus 2008, Keevil et al. 2018). Thus, the most effective strategy for conserving turtle populations may be the protection and maintenance of existing populations, rather than investing resources in recovery after declines have occurred (Congdon et al. 1994, Enneson & Litzgus 2008).

Adult survivorship has the greatest proportional effect on population growth rate in spotted turtles (Enneson & Litzgus 2008), so maximizing adult survival is essential to maintaining viable populations. Illegal collection of adult spotted turtles may not result in their death, but it mimics the effects of increased mortality by removing these individuals from a population. Returning confiscated spotted turtles to their population of origin can reverse the effects of their removal, but it is not always clear where these turtles should be released. Microsatellite markers can be used to assign confiscated turtles to known populations (Davy & Murphy 2009), but genetic testing can be expensive and difficult to access. If spot patterns on spotted turtle carapaces were more similar within populations than among

populations (i.e. among more closely related individuals than more distantly related individuals), perhaps these patterns could be used to assign confiscated individuals to their population of origin.

Photographs may also provide information useful for monitoring population viability or health. For example, scute abnormalities on turtle shells are easy to observe and quantify from photographs. Using photographs collected during routine field work, we were able to compare the occurrence of carapacial scute abnormalities among isolated populations of spotted turtles in Ontario, Canada. Turtles are excellent models for studying development instability because abnormalities can be easily seen in the form of shell malformations (Kazmaier & Robel 2001, Davy & Murphy 2009, Velo-Antón et al. 2011, Vas 2012, Farke & Distler 2015, Moustakas-Verho & Cherepanov 2015, Cherepanov 2016, Zimm et al. 2017). Scute abnormalities may result when developing eggs are subject to sub-optimal temperature, moisture, and/or salinity conditions (Lynn & Ullrich 1950, Kazmaier & Robel 2001, Telemeco et al. 2013); they have also been linked to loss of genetic diversity and latitude of the sampling site (Davy & Murphy 2009, Velo-Antón et al. 2011).

In this study, we explored the potential for digital photographs to enable population-level assignments or act as proxies for population health in the Endangered spotted turtle. We annotated spot patterns on mature spotted turtles from 10 sites and then compared patterns among sites to investigate whether they could be used to assign individuals to their population of origin. We also quantified the frequency of carapacial scute abnormalities at these sites. These abnormalities are partially determined by genetic factors but may also provide a proxy for physiological stress experienced during egg development, so we explored their association with genetic diversity and latitude.

## 2. MATERIALS AND METHODS

### 2.1. Sample collection

We photographed spotted turtles during wading surveys conducted between April and October (2008–2018) at 10 sites (fens, bogs, and coastal marshes) across southern Ontario in the course of long-term mark–recapture studies. The sites were located in the Mixedwood Plains Ecozone, ranging in latitude from 42.317 to 45.494° N. Pairwise distances between sites ranged from 3.5 to 638 km. With the

exception of BP1 and BP2, these sites represent isolated clusters of spotted turtles with little or no recent gene flow (Davy & Murphy 2014), and we treated each sampling site as an independent ‘population’ in our analyses. Turtles were captured by hand and marked by shell notching (Cagle 1939). We took standard measurements, including carapace and plastron length, documented any shell abnormalities (e.g. missing, extra, or odd-shaped scutes; wavy lines between scutes), and took photographs of each turtle’s carapace.

Hatchling spotted turtles emerge from the egg with only 1 spot per scute, and accumulate additional spots as they age (Ernst & Lovich 2009). As adult spot patterns are unique to individuals, we hypothesized that spot patterns might also be more similar within a population than among populations. Juvenile spot patterns are quite consistent among individuals and could confound this analysis. To ensure no sub-adult individuals were included in our analysis, we only used photographs of mature turtles (turtles with a straight carapace length [SCL] >97 mm). We excluded photographs that were very dark or blurry, and photographs of turtles that had moss or algae growing on the carapace that might have obscured spots.

### 2.2. Quantifying carapace spot patterns

We used pattern-based interactive individual identification software (I<sup>3</sup>S Spot) (Van Tienhoven et al. 2007, den Hartog & Reijns 2014) to annotate the spot patterns of 126 individual spotted turtles from the 10 sampling sites. I<sup>3</sup>S Spot is a free, computer-based photo-matching program that uses natural markings to identify individual study animals. We used the program to annotate spot pattern of each turtle and create a library of known individuals. Our hypothesis was that turtles could be assigned to population of origin based on their spot patterns, which predicts that spot patterns are more similar among individuals from the same population than among individuals from different populations. To test this prediction, we compared each individual’s spot pattern with that of every other turtle in the database. For each comparison, the program lists pairwise numeric similarity scores in order of decreasing similarity, with lower scores representing closer matches (details in Van Tienhoven et al. 2007).

I<sup>3</sup>S Spot requires the user to identify 3 fixed reference points on each photograph to correct for differences in viewing angle, rotation, and scaling among photographs. The location of these points should be

consistent and clearly distinguishable in all photos, and the resulting triangle should cover most of the region of interest for identification (den Hartog & Reijns 2014). Anterior and vertebral scutes were most consistently visible in our collection of photos, so we positioned reference points between the third and fourth marginal scutes posterior to the head, at the peripheral edge of the suture, and between the last 2 marginals at the posterior periphery of the carapace (Fig. 1). Within the annotation area, ellipses are fitted to each spot, and the spot pattern of each individual is established relative to these reference points in roughly the same 2D recognition space or coordinate system. Annotating too many spots may result in a computationally expensive comparison, while increasing the possibility of a false positive match between 2 fingerprints; annotating too few spots may not be distinctive enough (den Hartog & Reijns 2014). To determine the scute sampling strategy that would maximize our chance of correctly assigning spotted turtles to their population of origin, we chose 4 annotation areas (A1 to A4, Fig. 1) and compared pairwise similarity scores for a sub-sample of 29 turtles from 4 sites (EO2, EO3, GH1, and HC). Annotation area A2 resulted in the largest difference between both median and mean scores for within-site and among-site matches at each of the 4 sites (Fig. S1 & Table S1 in the Supplement at [www.int-res.com/articles/suppl/n045p159\\_supp.pdf](http://www.int-res.com/articles/suppl/n045p159_supp.pdf)); therefore, it was used to annotate spot patterns for all 126 turtles. To assess whether spot patterns could be used to identify the likely population of origin for spotted turtles, we calculated similarity scores for each pair of turtles and visualized these using a heat map.

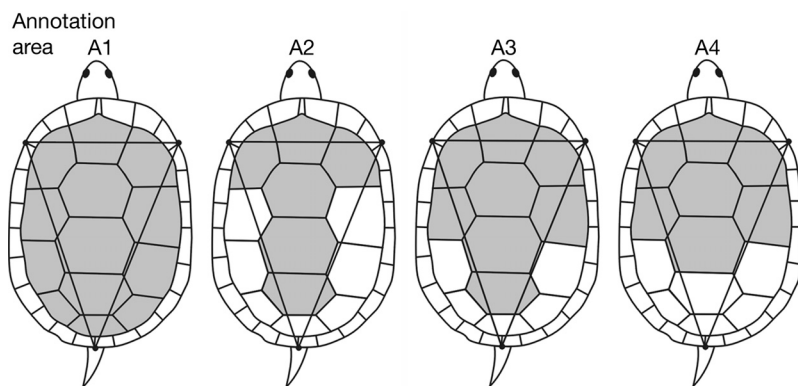


Fig. 1. We compared 4 annotation areas (A1–A4) to determine the scute sampling strategy that would maximize the chance of correctly assigning individual spotted turtles *Clemmys guttata* to their population of origin. All spots in shaded (gray) scutes were annotated using I<sup>3</sup>S Spot. The same 3 reference points (black dots) and reference triangle were used for each annotation area

### 2.3. Carapacial scute abnormalities

We also used photographs of spotted turtle shells to quantify carapacial scute abnormalities, and to explore associations between scute abnormalities and genetic diversity, and between scute abnormalities and latitude. We summarized carapace abnormalities from photographs of 206 spotted turtles; these included the 126 turtles described in Section 2.2 and additional individuals with SCL < 97 mm for which we had clear photographs. Each turtle received a binary score (yes/no) for the presence of carapacial scute abnormalities. These included extra or missing scutes, abnormally shaped scutes, split scutes, and any line (scute seam) abnormalities. Extra scutes were those that appeared to be embedded between 2 normally positioned scutes (Fig. 2A). Abnormally shaped scutes were positioned correctly, but appeared either very large, very small, asymmetric, or had a combination of unusual size and asymmetry (Fig. 2B). Split scutes were normally positioned scutes that were divided in 2, usually down the middle, either vertically or horizontally (Fig. 2D–F). Line abnormalities were cases where the separating line between scutes was wavy, zigzag, or otherwise uneven, even if the scutes themselves were of normal size and in the correct position (Fig. 2C,E).

We compared the frequency of scute abnormalities (proportion of sampled turtles that had some type of scute abnormality) at 9 study sites with previously published indices of genetic diversity (heterozygosity, allelic richness, and private allelic richness; Davy & Murphy 2014); we did not have genetic data for site EO3. The relationship between each index of genetic diversity and the frequency of scute abnormalities was tested by calculating standard Pearson's correlation coefficients. We also tested the relationship between latitude of study site and frequency of scute abnormalities for all 10 sites by calculating a standard Pearson's correlation coefficient.

Line abnormalities were the most subjective of the abnormalities we documented, so we also excluded line abnormalities from the dataset and reran the analyses considering only misshapen, split, extra, or missing scutes as abnormalities. We conducted all statistical analyses in R version 3.5.1 (R Core Team 2018).



Fig. 2. Examples of carapace abnormalities in spotted turtles *Clemmys guttata* caught during annual wading surveys in southern Ontario, 2008–2018. Common carapace abnormalities observed (indicated by arrows) included: (A) extra scutes, (B) abnormally shaped (asymmetrical) scutes (C,E) wavy or zigzag lines (seams) between scutes, and (D,E,F) split vertebral and costal scutes

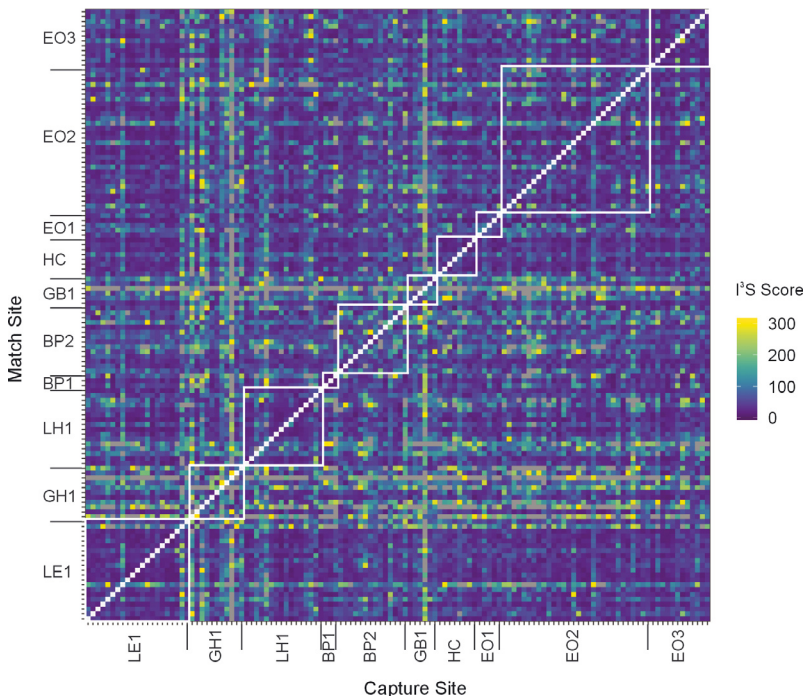


Fig. 3. Heat map of pairwise  $I^3S$  Spot similarity scores for spotted turtles *Clemmys guttata* ( $n = 126$ ) from 10 sampling sites in southern Ontario, Canada, ordered approximately from south-west to north-east, reflecting geographic proximity and genetic similarity among sites (Davy & Murphy 2014). In each comparison, the first match in the output file was always the subject turtle's own photo (white squares in the diagonal). Low  $I^3S$  scores (dark blue) indicate more similar spot patterns; high  $I^3S$  scores (yellow) indicate more dissimilar spot patterns. White boxes indicate within-population comparisons for each sampled site

### 3. RESULTS

#### 3.1. Quantifying carapace spot patterns

Pairwise  $I^3S$  Spot similarity scores based on spot patterns overlapped substantially between within-site and among-site comparisons (Fig. 3), indicating that spot pattern similarity assessed with the methods we applied cannot be used to reliably assign a spotted turtle to its site (or population) of origin.

#### 3.2. Carapacial scute abnormalities

Carapacial scute abnormalities were observed in 82% (170/206) of spotted turtles. Wavy or zigzag lines between scutes were the most common abnormality ( $n = 167$ ). These were most often located between vertebrals 3 and 4 ( $n = 114$ ), between the first vertebral scute and the first costals ( $n = 94$ ), and between vertebrals 2 and 3 ( $n = 67$ ). Fifty-one turtles had extra scutes, divided scutes, odd-shaped scutes, or some combination thereof, and 49 of those turtles also had uneven lines between scutes. Extra scutes were most common in this group ( $n = 33$ ) and occurred mainly along the marginal scutes, and between the first vertebral and the first costals, but they never occurred on the same turtle. Divided scutes ( $n = 14$ ) and odd-shaped

scutes ( $n = 13$ ) were less common; both occurred mainly along the vertebral scutes but rarely ( $n = 3$ ) on the same turtle.

None of the genetic diversity indices we tested were correlated with the frequency of carapacial scute abnormalities in our dataset, regardless of whether line abnormalities were included in the dataset (Table 1). The frequency of carapacial scute abnormalities was positively correlated with latitude ( $r^2 = 0.69$ ,  $p = 0.003$ ; Table 1). However, this correlation became non-significant when line abnormalities were excluded from the data (Table 1).

#### 4. DISCUSSION

In this study, we used digital photos to annotate and compare carapace spot patterns among populations of spotted turtles, document and quantify carapace abnormalities, and investigate possible relationships of the frequency of abnormalities with genetic diversity and latitude. Regrettably, comparison of carapace spot patterns did not allow assignment of individuals to their population of origin. Spotted turtles at our study sites exhibited carapace abnormalities similar to those seen in other temperate freshwater turtle species. Frequency of these carapace abnormalities would not be a reliable proxy for genetic diversity, but the association with latitude when line abnormalities are included suggests that minor carapacial malformations may be a proxy for thermal stress experienced during development, when scutation develops.

Given a certain amount of phenotypic variability in a population, theory predicts that individuals within groups should be less variable (i.e. more similar in heritable phenotype) than individuals from different groups (Krause et al. 1996). In Mexico, intraspecific analyses of the Cortes geoduck *Panopea globose* shell shape suggested an adaptive or phenotypic

response to environmental conditions at different sites (Leyva-Valencia et al. 2012). In California (USA), populations of topsmelt silverside *Atherinops affinis* from different regions were also morphologically distinct, suggesting the influence of environmental factors and local habitats (O'Reilly & Horn 2004). We investigated phenotypic differences in spot patterns among disjunct populations of spotted turtles in the hope that they could be used to determine site of origin for recovered and/or rehabilitated individuals. Despite distances of >10 km among our sites, spot patterning was not population- or site-specific. This result suggests that spot patterns are either not heritable or are not influenced by site-specific environmental factors, or that the analysis we performed failed to capture informative variation in these patterns. Although we have found that spot patterns and unique scute or scute seam abnormalities are useful for identifying individual spotted turtles, these were not informative for population-level assignment. For now, genetic testing remains the best option for assigning confiscated and/or rehabilitated Ontario spotted turtles to population of origin.

When developing embryos experience stressful conditions, such as heat or humidity near the boundaries of the tolerable range, mechanisms that regulate development can be challenged, resulting in increased phenotypic variance (Hoffmann & Hercus 2000, Badyaev et al. 2005). Observed morphological variability may provide a valuable early indicator of genetic and/or environmental stress (Palmer & Strobeck 1986, Parsons 1990), and detecting these can inform conservation of endangered populations (Leary & Allendorf 1989). Scute abnormalities do not appear to affect fitness in freshwater turtles (Bujes & Verrastro 2007, Davy & Murphy 2009, Farke & Distler 2015, Zimm et al. 2017). However, they may serve as indicators of developmental instability (Vas 2012), suboptimal thermal or hydric conditions during development (Zangerl & Johnson 1957, Moustakas-

Table 1. Associations (Pearson's correlation coefficient) between the frequency of carapacial scute abnormalities (where 'Line' refers to the scute seam) observed in spotted turtles *Clemmys guttata* at 10 sites in southern Ontario, Canada, and site latitude (as a proxy for thermal exposure while turtles are developing), as well as 3 measures of neutral genetic diversity, inferred previously with data from 11 microsatellite loci (Davy & Murphy 2014)

	Line abnormalities included				Line abnormalities excluded			
	<i>t</i>	df	$r^2$	<i>p</i>	<i>t</i>	df	$r^2$	<i>p</i>
Observed heterozygosity	-0.126	7	0.0023	0.9032	1.5485	7	0.2551	0.1654
Allelic richness	-1.302	7	0.195	0.2341	0.7906	7	0.082	0.4551
Private allelic richness	-0.093	7	0.0012	0.9284	0.2606	7	0.0096	0.8019
Latitude	4.2228	8	0.6903	0.0029	-0.225	8	0.0063	0.8276

Verho & Cherepanov 2015, Cherepanov 2016, Zimm et al. 2017), or potential exposure to environmental contaminants (Bishop et al. 1998, Bell et al. 2006). Thus, patterns in the occurrence of scute abnormalities may be worthy of further investigation, especially where endangered species are concerned.

We observed carapace abnormalities in 67–100% of spotted turtles from 10 sites, which is similar to the frequency of scute abnormalities (63–75%) reported in some studies of the European pond turtle *Emys orbicularis* (Fernández & Cordero-Rivera 2004, Velo-Antón et al. 2011). However, in most other species, the reported frequency of scute abnormalities (3–44%) is lower (MacCulloch 1981, Bishop et al. 1998, Kazmaier & Robel 2001, Bujes & Verrastro 2007, Davy & Murphy 2009, Vas 2012, Kosik et al. 2013, McKnight & Ligon 2014, Farke & Distler 2015, Loehr 2016, Saçdanaku & Haxhiu 2016, Zimm et al. 2017, Lada & Boldyreva 2018). Scute abnormalities in our turtles were biased towards the vertebral scutes, similar to findings in European pond turtles (Fernández & Cordero-Rivera 2004, Kosik et al. 2013, Saçdanaku & Haxhiu 2016) and sea turtles (Zimm et al. 2017). Extra scutes and divided scutes like those we observed were also reported in co-occurring populations of snapping turtles *Chelydra serpentina* (Bishop et al. 1998) and painted turtles *Chrysemys picta* (MacCulloch 1981, Davy & Murphy 2009).

Some studies have suggested an association between decreasing genetic diversity and the incidence of scute abnormalities (Velo-Antón et al. 2011, Kosik et al. 2013). However, genetic diversity estimated from microsatellite markers did not explain the variation in frequency of abnormalities seen in spotted turtles at our study sites. The only association we found between scute abnormalities and characteristics of the sampled population was between frequency of carapace abnormalities and latitude of the sampling sites, although this association became non-significant when line abnormalities were not included in the data. Potential links between latitude and carapace malformation are consistent with previous studies of painted turtles (Davy & Murphy 2009) and Iberian populations of European pond turtles (Cordero-Rivera et al. 2008, Velo-Antón et al. 2011).

Protection, maintenance, and enhancement of existing populations are essential for long-term conservation and recovery of endangered turtle species (Congdon et al. 1994, Enneson & Litzgus 2008). Unfortunately, spot pattern similarity did not prove useful for assigning confiscated individuals back to their populations of origin (at least in the case of Ontario spotted turtles), leaving genetic assignment tests as

the only available option, and even these are imperfect (Davy & Murphy 2014). Nevertheless, the association between carapace abnormalities and latitude implicates environmental stressors in the development of scute abnormalities, suggesting that long-term monitoring of carapace abnormalities could identify long-term shifts in stressors experienced by developing turtle embryos. Given the limited ability of turtle populations to recover after population declines (Brooks et al. 1991, Congdon et al. 1993, Heppell 1998, Enneson & Litzgus 2008, Keevil et al. 2018), monitoring the incidence of scute abnormalities among age-classes could provide a valuable, cost-effective 'early-warning system' for shifts in the severity of developmental stressors.

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