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SYMPOSIUM

Environmental Causation of Turtle Scute Anomalies *in ovo* and *in silico*

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Synopsis The turtle shell is often described as an evolutionary novelty that facilitated the radiation of the clade Testudines. The scutes, or keratinous plates, of the turtle shell are hypothesized to be patterned by reaction-diffusion dynamics, and this property of their development provides explanatory power to mechanisms of anomalous variation. A mathematical model of scute development predicts that anomalous variation in the phylogenetically stable pattern of scutes is achieved by environmental influence on the developmental program. We test this prediction with data on patterns of scute variation from natural nests and controlled incubation of sea turtle eggs in Florida and Western Australia. We find that high temperatures are sufficient to produce anomalous patterns in turtle scutes, and that this correlation is even stronger when conditions are dry. Furthermore, we find that the patterns of variation are not random; greater anomalous variation is found in the midline vertebral scutes and during a critical period of turtle development.

Introduction

The turtle shell is the most evident novelty of the clade Testudines and is believed to have triggered their diversification. Shells are composed of ribs, vertebrae, clavicles, interclavicles, and novel ossification covered by an array of modular epidermal structures called scutes. Together these components provide a mostly rigid axial body and efficient protection against predators. In some lineages, the ossified shell is reduced; this variation is thought to improve locomotor efficiency in aquatic habitats (Zangerl 1980; Wyneken 1997). The fossil record of turtle shells goes back ~220 million year ago (Li et al. 2008), and since then, a plethora of different shell shapes and architectures have evolved (Pritchard 1979; Cordero 2017; Moustakas-Verho et al. 2017). Much of the diversity of extant turtle shells lies in different scute shapes and color patterns with relatively few differences in scute arrangement. The overall high degree of conservation of turtle shell architecture in living turtles suggests a canalization from patterns in some of the earliest turtles that contained many more scutes in total (for example, as in *Proganochelys* (Gaffney 1990)). Most terrestrial turtles show the same basic carapacial (dorsal) scute pattern composed of a central column of five vertebral scutes, followed laterally by four costal scutes on each side, and rows of 12 smaller marginal scutes along the carapacial ridges, closing anteriorly with a single nuchal scute (Fig. 1A). The loss of scutes is seen in several freshwater turtles and the marine leatherback turtle, whereas in other marine turtles, different numbers of vertebral and costal scutes can be found and their precise numbers are diagnostic for the species (Wyneken 2001).

Turtle shell development begins with the formation of the carapacial ridge, which is a lateral protrusion of epithelium and dermatomal mesenchyme along the trunk of the turtle embryo (Burke 1989; Moustakas 2008). Scutes develop from epidermal placodes that are induced by signals coming from a row of 12 primary signaling centers along the carapacial ridge (Fig. 1B; Moustakas-Verho et al. 2014). The positions of those signaling centers correspond

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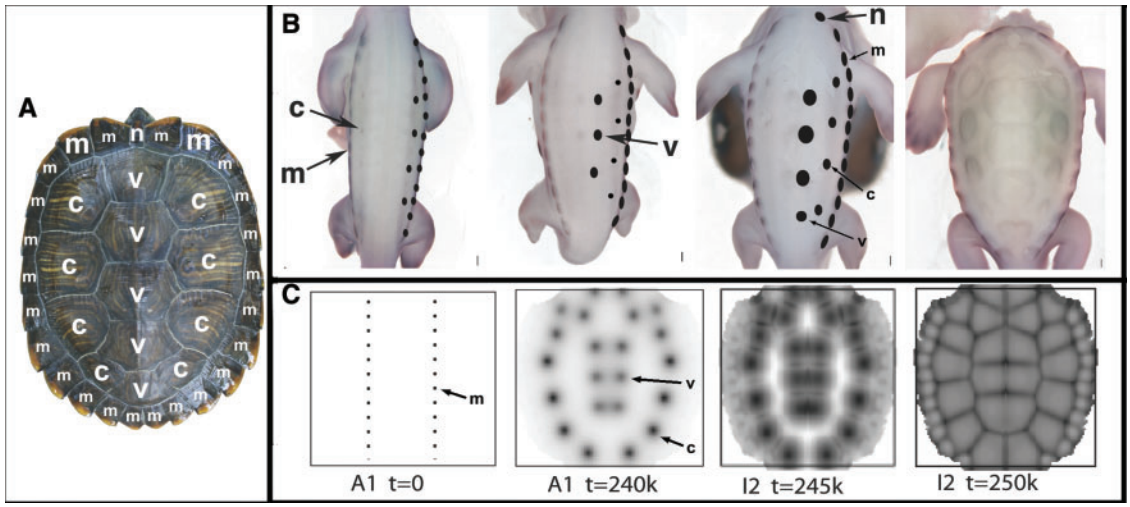

Fig. 1 Turtle scute pattern and development. (**A**) The adult carapace (here: *Trachemys scripta*; photo credit Bob Smither) consists of one nuchal scute (n), one column of five vertebral scutes (v), two columns of four costal scutes (c), and 24 marginal scutes (m). (**B**) Expression of *Bone Morphogenetic Protein 2* (*Bmp2*) visualized by in situ hybridization in developing scute primordia in *T. scripta* (modified from Moustakas-Verho et al. 2014). (**C**) Four different stages of *in silico* scute development derived from the mathematical model: initial conditions with two rows of 12 activation centers that initiate the first reaction-diffusion system, followed by the formation of presumptive costal, vertebral, and nuchal scute placodes that create the final scute pattern by the activation of a second reaction-diffusion system. Black foci in the model represent high concentration of Activator1 and Inhibitor2, respectively. For detailed explanation, see Moustakas-Verho et al. 2014 (model parameters for normal turtle shell development as in there, except: $m_3 = m_4 = 0.06$, $r_2 = 0.25$, $AZ = 100$)

with the underlying somitic mesoderm, suggesting an induction from the primary mesodermal segmentation. *In situ* hybridization data showed that costal scute placodes form before vertebral scute placodes. The nuchal and vertebral scutes develop from paired primordia that eventually fuse to form individual scutes. This same process has been studied histologically across several species of turtles (Cherepanov 2006), suggesting that scute development follows a common mechanism of pattern formation in turtles. In turtles that have lost their scutes, such as softshell turtles, epithelial placodes, and the placodal signaling centers do not form (Moustakas-Verho and Cherepanov 2015). The importance of these signaling centers for the development of the scutes was further emphasized in experiments where scute formation was suppressed *in vitro* by the addition of inhibitors of Hedgehog and Bone Morphogenetic Protein signaling (Moustakas-Verho et al. 2014). Although the molecular mechanisms underlying scute development are scarcely understood, an *in silico* model suggests that two coupled reaction-diffusion systems together with growth can explain, mechanistically, the development of the carapacial scute pattern (Fig. 1C).

A number of studies have approached the role of the environment in turtle development. For instance, sex differentiation is achieved by different incubation temperatures at crucial stages during development (Bull 1980). Different temperatures, humidity, and gas concentrations in nests have also been shown to have an effect on embryonic growth by changing the embryonic metabolism in the egg (Morris et al. 1983; Cordero et al. 2017). With respect to the shell, Coker (1910) proposed that pressures in the eggs' environment, e.g., the "intercrowding" between the eggs, may contribute to the internal pressure inside the egg and lead to deformation of the developing embryo. Other workers found correlations between scute abnormalities and dry nesting conditions and/or extreme temperatures (Lynn and Ullrich 1950; Telemeco et al. 2013). We, therefore, simulated environmental effects in the model of scute development and showed that interfering with growth, either mechanically or by modifying the growth rate, has a strong effect on the adult scute pattern (Moustakas-Verho et al. 2014).

Here we hypothesize that, apart from genetic interactions, environmental factors may play pivotal roles in the developmental stability of turtle scutes. We examine this proposal with qualitative and quantitative data of phenotypical scute pattern variation in order to connect real and model variation. We further test the hypothesis that environmental variation during the development of the turtle scutes can affect their patterning. We first consider scute

anomalies recorded from natural nests, and then compare with anomalies produced under controlled conditions in the laboratory. Our assessment of developmental stability in this system is a simple quantification of the normal number and pattern of scutes versus an abnormal phenotype that consists of fewer or additional carapacial scutes. We consider the environmental variables of temperature and humidity, and compare the effects of environmental variation on scute patterns seen in natural specimens with predictions from our mathematical model of scute development. We further quantify where along the carapace the anomalies occur *in vivo* and *in silico*.

The model of scute development

A mathematical model of development is a hypothesis about the mechanisms of morphogenesis. In our model of scute development (Fig. 1C; Moustakas-Verho et al. 2014), the developing carapace is represented as a 2D field of embryonic tissue in which morphogens can diffuse and interact locally. Four different morphogens were linked in a gene regulatory network and one of them was interpreted as a marker of scute differentiation. Based on localization of marker gene expression, the first morphogen is produced in regularly spaced spots along the margins of the developing carapace. By interacting as a reaction-diffusion system (Gierer and Meinhardt 1972), the first and second morphogens create a pattern of costal and vertebral scute primordia whose positions and sequence of appearance recapitulate experimental results. The second pair of morphogens, activated by the first pair, creates travelling waves from every scute primordium that collide and thereby mark the future seams between the scutes. Growth is implemented as an outgrowth of the 2D carapacial field from the midline, based on the cell cycle.

This model was created in order to test hypotheses of scute development quantitatively, but slight changes in the model parameters were able to reproduce the different scute architectures seen in the sea turtle species *Caretta caretta* (loggerhead), as well as *Lepidochelys olivacea* (olive ridley) and *L. kempi* (Kemp's ridley) (Moustakas-Verho et al. 2014). The ability of the model to recapitulate natural variation in the turtle shell suggests that scute pattern development follows a very similar mechanism in all turtle species and that the results gained from studies in the slider turtle *T. scripta* are very likely to apply to marine turtles, as well.

Developmental anomalies

Despite a high diversity of scute shapes and scute pigmentation, the basic scute composition of nearly all terrestrial turtles follows a very conserved pattern: 5 vertebral scutes, 4 costal scutes surrounded by an anterior nuchal scute, and 24 marginal scutes along the edge of the carapace. However, individuals with aberrant scute patterns are commonly described (Parker 1901; Newman 1906; Coker 1910; Lynn 1937; Zangerl and Johnson 1957; Zangerl 1969; Ernst 1971; MacCulloch 1981; Mast and Carr 1989; Hewavisenthi and Parmenter 2001; Türkozan et al. 2001; Fernandez and Rivera 2004; Özdemir and Türkozan 2006; Van Meter et al. 2006; Bujes and Verrastro 2007; Davy and Murphy 2009; Velo-Antón et al. 2011; Telemeco et al. 2013; Caracappa et al. 2016; Cherepanov 2014; Bárcenas-Ibarra et al. 2015; Moustakas-Verho and Cherepanov 2015; Loehr 2016; Saçdanaku and Haxhiu 2016; Mautner et al. 2017), though in different frequences amongst different species. Typical scute abnormalities are generally asymmetrical and comprise additional or fused scutes, as well as an offset of an entire carapacial hemisphere, causing a zig-zag structure of the vertebral scutes (Fig. 2A–C).

We used the model of scute formation to analyze the formation of such abnormalities. We found that temporary and permanent offsets to the growing carapacial field, as would be generated by mechanical stress or growth defects, were sufficient to reproduce the majority of abnormal scute patterns documented in nature (Fig. 2D). However, introducing mutations or noise into the genetic network underlying scute formation in the model could not reproduce them (Moustakas-Verho et al. 2014). This result emerges from the dynamics of reaction-diffusion systems. Hence, we proposed that anomalies primarily stem from mechanical effects the environment has on the developing embryo (epigenetic effects) rather than genetic mutations, as scute patterns are stable phylogenetically and anomalies are not known to be inherited across generations (although due to scarce cross-generation data this possibility cannot be completely ruled out). Though the mechanism whereby the scutes of the shell form was previously unknown, this same hypothesis of anomalies being created by perturbations during ontogenetic development has been proposed earlier (Zangerl 1969). Many turtles lay flexible-shelled eggs that permit mechanical effects onto the developing embryo. Such mechanical effects might result from extreme weather conditions, such as heat and drought, or any kind of accidental pressure. Another way that extreme weather conditions might affect the symmetrical development of the carapace is by affecting the speed of growth or by damaging tissue. Given that turtles lay their eggs in subterranean nests, temperature stress, and

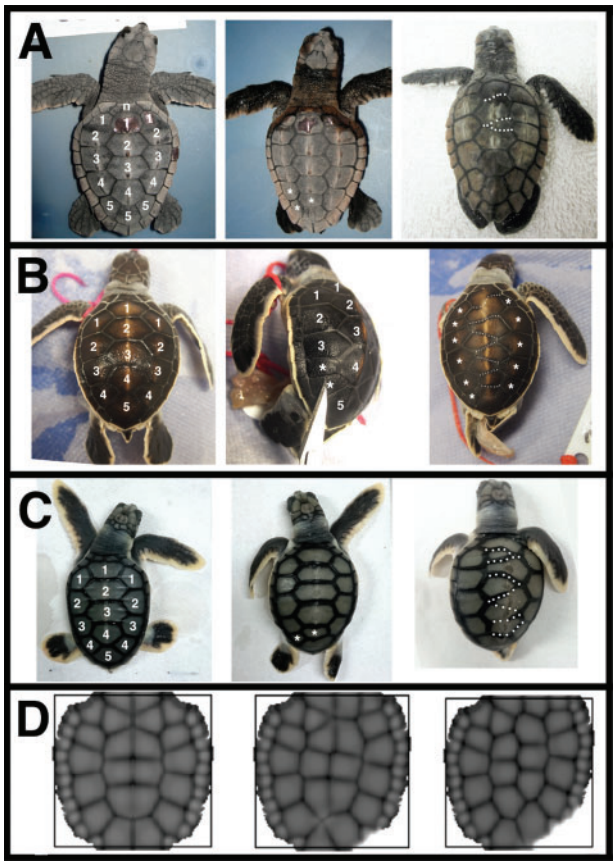

Fig. 2 Diversity of turtle scute patterns and anomalies. Dorsal views of normal and two different specimens with characteristic scute anomalies for (**A**) loggerhead (*C. caretta*), (**B**) green sea (*Chelonia mydas*), and (**C**) flatback (*Natator depressus*) sea turtles. For better visualization, the anomalies are outlined by a dotted line or emphasized by asterisks. (**D**) A normal pattern (left) versus two representative-*in silico* anomalies produced by lateral offsets in the growing carapace in the model.

gradients are likely to have a stronger effect on embryogenesis than in animals that tend their eggs or give birth to live young.

Independent of how exactly environmental stress translates into scute deformations, we examined the relationship between the frequency of scute anomalies with environmental conditions. We gathered quantitative data on anomalies in *C. mydas*, *N. depressus*, and *C. caretta*. We collected data on scute patterns in these species from (1) eggs that were monitored in their natural nests on beaches, (2) eggs incubated in controlled laboratory settings, and (3) individuals from rookeries that displayed scute anomalies to understand the prevalence of different types of abnormal patterns.

We observed scute patterns of hatchlings from natural nests of loggerhead (*C. caretta*) and green sea (*C. mydas*) turtles from Boca Raton, Florida, USA (located on the southeastern coast of Florida). These nests were each equipped with a calibrated temperature datalogger (Hobo model U22, Onset Computer Corp. Borne, MA, USA) positioned in the middle of each clutch (mean = 105 eggs). Dataloggers were placed within each nest during or just after oviposition and nests laid across the breeding season were sampled. Clutch temperatures were recorded every 10 or 15 min throughout incubation (45–59 days). Rainfall data collected from regional weather sites by the National Weather Service (www.srh.noaa.gov). Nests were sampled in 2014 (number of nests $n = 10$), 2015 ($n = 11$), and 2016 ($n = 10$, see Supplementary Tables 1–3). Because the turtles also served in another study that required rearing in the laboratory, no turtles were selected that had skin lesions or congenital nasophayngeal fissures (unilateral or bilateral), and dead individuals were not included. Hatchlings (10 individuals/nest) were photographed and carapacial scutes were examined and compared with the normal scute pattern for the species (Wyneken 2001). Nuchal, vertebral, and costal scutes were scored for each hatchling as normal or having an anomalous scute pattern. Scute anomalies were further characterized as being supernumerary, subnumerary, or deformed, with notes taken on the position and number of anomalous scutes.

Freshly oviposited green turtle (*C. mydas*, Supplementary Table 4) and flatback (*N. depressus*, Supplemenatry Table 5) eggs were collected from West Lacepede Island (16.853 S, 122.125 E), which lies ~120 km north-west of Broome, Western Australia, using methods described in Bentley et al. 2017. Under controlled laboratory settings, these eggs from *C. mydas* were incubated at temperatures ranging from 28°C to 32 °C with ~100% humidity. Hatchlings were collected as they emerged from their egg chamber and observations were taken on carapace scute patterns.

These datasets were supplemented with observations in flatback turtles from three populations (Eighty Mile Beach, Cape Domett, Thevenard Island) in Western Australia that showed scute anomalies. These data were used to understand the proportion of anomalies that affect the vertebral scutes, costal scutes, or both.

From these experiments and observations, we found an increase in the appearance of scute anomalies with increasing temperature (Fig. 3). According to climatological records, 2015 and 2016 were exceptionally warm and dry years, each being reported as the hottest years in the modern temperature record, with higher temperatures and less rainfall reported in Florida (Southern Climate Impacts Planning

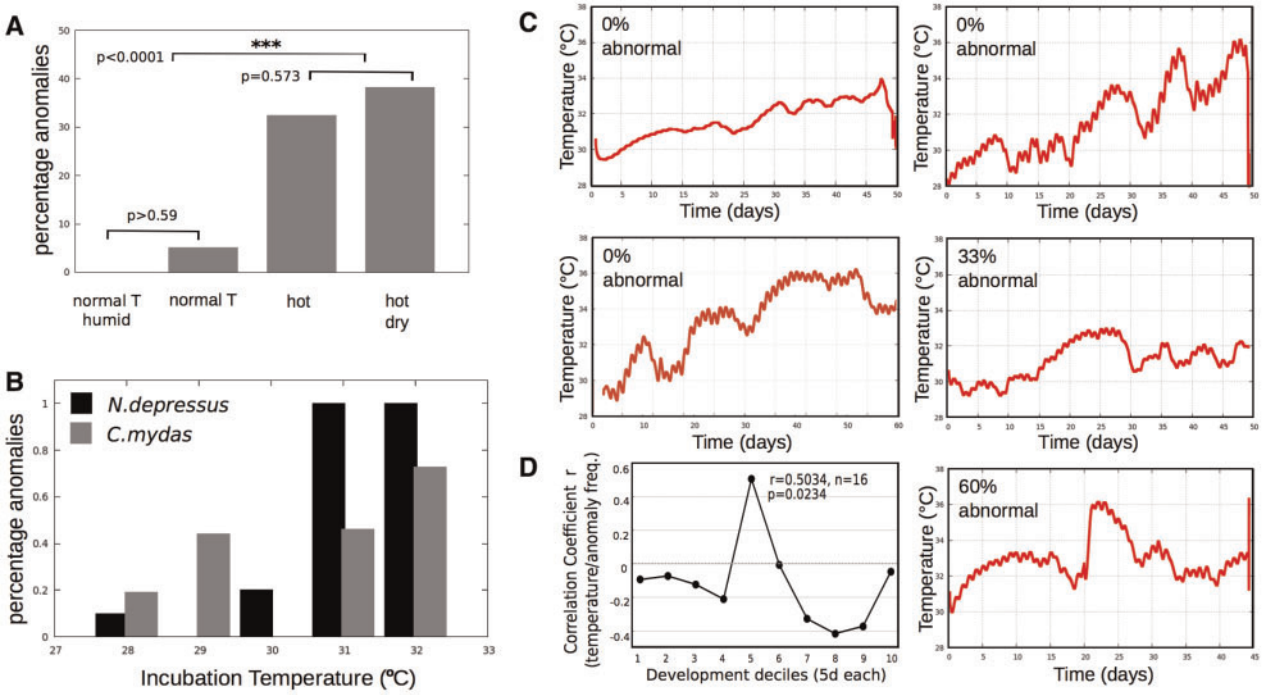

Fig. 3 Dependence of turtle anomaly occurrence on temperature and humidity. (**A**) Percentage of anomalies in turtle hatchlings (*C. caretta*) from natural nests with varying climatic and incubation conditions: normal temperature and higher humidity ($n = 10$), normal temperature and humidity ($n = 97$), hot with normal humidity ($n = 37$), and hot and dry ($n = 55$). Only temperature differences led to significantly different anomaly frequencies (X^2-test, $P < 0.0001$), whereas moisture differences did not ($P > 0.5$). (**B**) Percentage of *C. mydas* and *N. depressus* hatchlings with scute anomalies that were incubated at different constant temperatures in the laboratory. The higher incubation temperatures of 31 °C and 32 °C resulted in clutches where up to 100% of hatchlings showed carapacial scute anomalies. Positive correlations significant with $P < 0.0001$ (*C. mydas*) and $P < 0.0039$ (*N. depressus*). Note that no eggs from *N. depressus* were incubated at 30 °C. (**C**) Detailed temperatures from natural nests in Florida throughout the development of *C. mydas* and *C. caretta* for the 2016 nesting season. For each nest, $n = 10$ individuals were sampled, and the percentage of hatchlings with carapacial scute anomalies was calculated. (**D**) A substantial period of high temperatures during mid-development is seen to be associated with high anomaly counts. For each decile of the developmental period in ovo, correlation coefficients between decile mean temperature and anomaly frequencies in the hatchlings were plotted. In decile 5, the correlation coefficient $r > 0.566$ with $P = 0.065$ (t-test). We included only nests from which at least 80% of eggs hatched successfully. Variation in the marginal scutes was not considered.

Program (www.southernclimate.org/)). Consistent with our hypotheses, the percentage of turtles with anomalies collected in Florida during 2015 and 2016 was significantly higher than in the year 2014 (Fig. 3A and Supplementary Tables 1–3). Interestingly, we found that heat plus drought seems to be even more strongly associated with the occurrence of scute anomalies (38% versus 32% individuals with anomalies in humid versus dry hot weather. The differences between hot versus normal years are significant with $P < 0.0001$, X^2-test; Fig. 3A), suggesting that desiccation might be a key mechanism behind developmental deformations of the carapace. We performed an ANOVA test to infer statistically the contributions of the two variables heat and drought, and found that 73.4% of the variation is explained by heat, 7.26% by drought, and 19.3% by the interaction of both variables. Note that this still does not rule out that heat is actually inducing developmental stress by increased desiccation, as the two factors are not completely independent.

To test whether temperature itself sufficiently explains the increase in anomalies, we examined scute patterns from eggs of *C. mydas* and *N. depressus* at constant temperatures ranging from 28°C to 32 °C. We saw a clear increase of anomalies for incubation temperatures >30 °C (positive correlations significant with $P < 0.0001$ for *N. depressus* and $P > 0.004$ for *C. mydas*, t-test; Fig. 3B and Supplementary Tables 4–5). Overall, annual mean temperature and drought seem to be clearly correlated with occurrences of scute anomalies in turtles, independent of the species.

Critical developmental phases

We next sought to elucidate the developmental stages during which the developing carapace would be most susceptible to environmental stress.

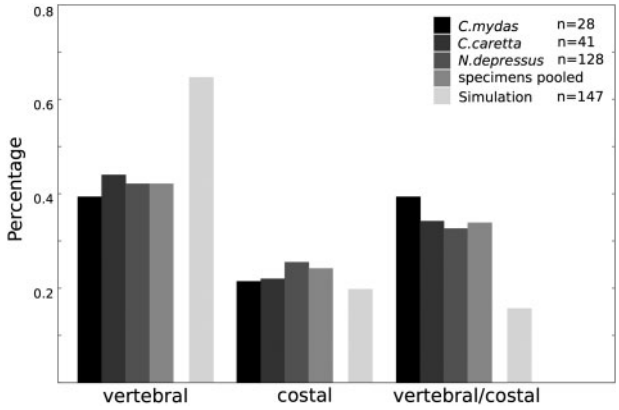

Fig. 4 Frequency of different scute anomalies is biased toward vertebral scutes. Relative frequency of vertebral and costal anomalies in the turtle shell ($n = 195$) in natural specimens with anomalies (gray to black) and in the model (light gray). Data from *N. depressus*, *C. mydas*, and *C. caretta* collected in different places and years were pooled. Anomalies that only affect marginal scutes are not included. In the model, anomalous scutes were counted as vertebral or costal based on their relative contact area with defined adjacent vertebral and costal scutes. Unclear cases and specimens in which both vertebral and costal scutes showed anomalies were counted as "vertebral + costal".

Although the exact timing of scute development in these sampled sea turtles is unknown, we infer that scute development in sea turtles is relatively similar to that of *T. scripta*, and that scutes are patterned during the second quartile of total development *in ovo* (Miller 1985; Billett et al. 1992; Kaska and Downie 1999). Thus, we assume a similar relative time-window for scute development in sea turtles. Using the detailed temperature data for the entire developmental period from different nesting sites in Florida, we identified that the strongest correlation between high temperature and scute anomalies is seen in mid-development ($P = 0.0234$, *t*-test; Fig. 3C). There was no significant correlation between average or maximal temperature during development and the frequency of anomalies.

The identification of mid-development as a critical phase for the appearance of scute anomalies makes intuitive sense as the developmental time period during which the positions of the scute primordia are being established by the first reaction-diffusion system. Furthermore, the model predicts that the occurrences of scute anomalies are biased toward the vertebral scutes. In the model, we systematically introduced temporal offsets of different strengths at different developmental timepoints, i.e., we shifted the cells in one of the carapacial hemispheres by different amounts and allowed them to relax thereafter (for more details see Moustakas-Verho et al. 2014). We characterized anomalous scutes as vertebral or costal, depending on their respective contact area to the surrounding vertebral and costal scutes. As a result, roughly $^2/_3$ of 147 severe in silico anomalies affect the vertebral scutes, whereas only 20% affect the costal scutes, and 15% affect both (Fig. 4). In 195 natural occurences of scute anomalies (in *N. depressus*, *C. caretta*, and *C. mydas*; Supplementary Table 6), 42% showed vertebral scute alterations, whereas 24% showed costal anomalies and 34% showed anomalies in both vertebral and costal scutes (vertebral-costal difference significant with $P < 0.0001$ in the turtle specimens and with $P = 0.0086$ in the simulated turtle shell patterns, binomial test; Fig. 4). Thus, we also find a strong bias toward vertebral abnormalities in nature, independent of the turtle species, although milder than in the simulation. The differences in severity of this bias between nature and computer model might be, amongst other reasons, attributed to the differences in tissue properties and growth between real and *in silico* animals. These findings suggest that developmental differences between the scute columns, such as the timing of their developmental onset, or geometrical constraints, may explain to a large extent the structure and frequency of specific scute anomalies.

Discussion

The low numerical variability of the scute pattern on the carapacial shell amongst most extant turtle species suggests that turtle shell development is highly conserved. Previous workers have noted that there is no phylogenetic significance to anomalous variations of the scutes (Coker 1910), though some species exhibit the same variations with a higher frequency (Zangerl 1969). Repeated variation would be expected given the hypothesis of patterning by reaction-diffusion dynamics. A consensus, however, has not been reached regarding the underlying causes for these variations more generally across turtles. Consistent with epigenetic mechanisms, scute anomalies have been attributed to combinations of genetic diversity, environmental stress, and DNA methylation (Hewavisenthi and Parmenter 2001; Bell et al. 2006; Van Meter et al. 2006; Velo-Antón et al. 2011; Telemeco et al. 2013; Caracappa et al. 2016). We combined data from nesting conditions of natural sea turtle populations with our hypotheses regarding the developmental dynamics of scute formation, and from this, we infer that the environment plays a central role in extant within-species variation. In three species of marine turtles, the frequency of scute anomalies clearly varies with temperature and

humidity, suggesting a mechanism by which the environment impinges on the phenotype generated through development. This is consistent with previous experiments that showed the production of scute anomalies under hot and dry incubation conditions (Lynn and Ulrich 1950; Telemeco et al. 2013).

Furthermore, we show evidence that the variation thus generated is not completely random; some anomalies appear to be clearly more frequent than others and the association of anomalies with mid-developmental stages might reflect the period of development during which the developing shell is particularly susceptible to environmental factors. In the case of the developing turtle shell, we find that vertebral scutes are more prone to developmental perturbations. Previous studies combining experimental and model approaches (Moustakas-Verho et al. 2014) suggest that this is due to the morphodynamic (Salazar-Ciudad and Jernvall 2004) way the vertebral scute placodes form: by fusion and outgrowth of primordia that were placed by a marginally induced reaction-diffusion system. These types of developmental processes are highly prone to noise amplification, as environmental noise, causing deformations, and changes in growth will easily prevent the primordia from fusing properly. Once this fusion has begun, developmental noise at later stages does not induce significant morphological anomalies. Consistently, we find that the correlation between high incubation temperatures and anomalies is strongest in mid-developmental stages.

More generally, it has been a long-standing debate in biology whether periods during development exist in which susceptibility to mechanisms generating variation is higher than in others, most famously in the debate about the "developmental hourglass". Many studies have argued either in favor (Irie and Kuratani 2014) or against (Bininda-Emonds Olaf et al. 2003) this hypothesis, whereas others have taken intermediate stances, emphasizing that the hourglass might only apply to some types of development (Salazar-Ciudad 2010). Although the concept of the hourglass model of development has been coined in the context of macroevolutionary patterns throughout the entire process of embryogenesis, we argue that an analogous framework can be usefully applied to the development of organs, as well. Since the development of discrete parts is usually better understood than the development of the entire body, we can understand in more detail why a certain pattern of time-dependent variation emerges. We wonder if the specific developmental reasons that lead to higher susceptibility of perturbations during mid-development in the turtle shell can be generalized or applied to other developing systems.

We note, however, that the actual mechanisms by which environmental stress affects developmental processes remain unresolved. Experiments that systematically perturb the developing scutes of embryos, in ways similar to those done *in silico*, are likely to shed light on this open question. The larger implication for the interaction between development and environment will then be addressed *in vivo*. Interestingly, since the variation observed is most likely not heritable, it merely emerges from the dynamics of development. Thus, it would be interesting to compare this pattern of carapacial scute variation to variation in organ systems that share developmental mechanisms.

The conclusion that the environment, and particularly climate, has an effect on variation has evolutionary implications. In particular, high temperatures may reveal cryptic variation present in populations. Might this increased phenotypic variability act as a buffering mechanism to changing environments? Many animals have been suggested to be suitable models to analyze phenotypic variation, sometimes termed plasticity, induced by environmental factors. Whereas in many organisms, such variation is part of an ecological strategy, it does not seem to be the case in turtles with respect to populational variation in the scutes. Consequently, the scute patterning of the turtle shell may well be an interesting model to quantify the morphospace of possible phenotypic variation. Because anomalous variation in the pattern of scutes on the turtle shell can be accompanied by other abnormalities, there is no clear pattern of how the presence of scute anomalies correlates with fitness. It is clear, however, that the anomalies form by deviation from normal development and the prediction from our analysis is that we will see an increase in the amount of scute anomalies on turtles if temperatures continue to rise. Thus, environmentally induced increases of phenotypic variation may help to monitor climatic changes in the past and the present.

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Supplementary data

Supplementary data available at *ICB* online.

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