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Effects of the egg incubation environment on turtle carapace development

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Abstract

Developing organisms are often exposed to fluctuating environments that destabilize tissue-scale processes and induce abnormal phenotypes. This might be common in species that lay eggs in the external environment and with little parental care, such as many reptiles. In turtles, morphological development has provided striking examples of abnormal phenotypic patterns, though the influence of the environment remains unclear. To this end, we compared fluctuating asymmetry, as a proxy for developmental instability, in turtle hatchlings incubated in controlled laboratory and unstable natural conditions. Wild and laboratory hatchlings featured similar proportions of supernumerary scales (scutes) on the dorsal shell (carapace). Such abnormal scutes likely elevated shape asymmetry, which was highest in natural nests. Moreover, we tested the hypothesis that hot and dry environments cause abnormal scute formation by subjecting eggs to a range of hydric and thermal laboratory incubation regimes. Shape asymmetry was similar in hatchlings incubated at five constant temperatures (26–30°C). A hot (30°C) and severely Dry substrate yielded smaller hatchlings but scutes were not overtly affected. Our study suggests that changing nest environments contribute to fluctuating asymmetry in egg-laying reptiles, while clarifying the conditions at which turtle shell development remains buffered from the external environment.

KEYWORDS
developmental instability, developmental noise, fluctuating asymmetry, turtle shell development

[Correction added on 24 March 2023, after first online publication: Author name Sara Ruane was corrected and the affiliations were updated.]

1 | INTRODUCTION

Organismal development is not fully insulated from the environment; many species feature internal systems that receive, interpret, and sometimes incorporate environmental cues during critical periods in early ontogeny (Gilbert, 2016; Pelster & Burggren, 2018; Waddington, 1957). An important property of such systems is the capacity to buffer the embryo from the potentially detrimental effects of environmental stochasticity (Hagolani et al., 2019; Irvine, 2020). Failure to do so may otherwise destabilize molecular signaling pathways and cellular dynamics that normally orchestrate the construction of tissues, organs, and ultimately the organism (Dongen, 2006; Hagolani et al., 2019; Waddington, 1957). Developmental instability might be particularly evident in species that undergo embryonic development in the external environment, as illustrated by Huxley's (1927) experimental induction of tissue asymmetry in chicken embryos that were exposed to thermal gradients in ovo. In bilaterian animals, asymmetry as a plastic response to the environment is a hallmark of developmental instability (Ludwig, 1932; Van Valen, 1962; Waddington, 1957).

Fluctuating (morphological) asymmetry is considered a proxy for environmentally induced developmental instability because it likely reflects random deviances in the rates at which cells undergo division, proliferation, and differentiation (Dongen, 2006; Hagolani et al., 2019; Waddington, 1957). Although experimental evidence is still lacking, developmental instability might be rather common in species that provide minimal parental care to their eggs. Many reptiles lay eggs in stable subterranean nests that are often carefully chosen by mothers to ensure suitable conditions for embryonic development (Refsnider & Janzen, 2010), yet nest environments are often disrupted by floods, droughts, and heat waves (Jergenson et al., 2014; Mainwaring et al., 2017; Telemeco et al., 2013). Such conditions may compromise pattern formation, growth, and survival (Sanger et al., 2021; Zimm et al., 2017). Overall, the capacity for reptile embryos to actively cope with extremes in temperature, moisture, and gas concentrations is limited while in the subterranean nest environment (Ackerman & Lott, 2004; Cordero et al., 2018; Telemeco et al., 2016). Even species whose embryos undergo development in utero are affected by unfavorable environmental conditions experienced by gravid mothers, for example, aberrant formation of epidermal scales (scutes) in snakes (Lowenborg & Hagman, 2017; Osgood, 1978).

In turtles, malformed scutes on the dorsal shell (carapace) are one of the most frequently observed indicators of developmental instability (Mast & Carr, 1989; Newman, 1906; Parker, 1901; Sim et al., 2014; Telemeco et al., 2013; Zimm et al., 2017). Carapacial scute patterns probably originate via two reaction-diffusion mechanisms that involve a regulatory loop comprising Shh, Bmp2, and Gremlin genes (Moustakas-Verho et al., 2014). The expression of these genes, and possibly others, maps directly onto the precursor placodes that eventually give rise to scutes (Moustakas-Verho & Cherepanov, 2015). In most living turtles, 44 placodes (12 paired + 32 unpaired) typically produce 38 carapacial scutes (Moustakas-Verho & Cherepanov, 2015; Moustakas-Verho et al., 2014) (Figure 1a,b). Mathematical simulations on scute pattern formation suggest that extreme temperatures probably exacerbate preexisting placode asymmetries that arise from inherent noise in the Shh-Bmp2-Gremlin signaling loop (Zimm et al., 2017). As such, high temperatures may contribute to the malformation of carapacial scutes.

The failure of paired placodes to properly align and fuse may underlie the development of supernumerary and morphologically asymmetric vertebral scutes (Cherepanov, 2014; Zimm et al., 2017). By contrast, supernumerary marginal and pleural scutes might mirror the evolutionary acquisition of additional placodes because the resulting phenotypes often mirror interspecific variation in some marine and fossil turtles (Cordero & Vlachos, 2021; Moustakas-Verho et al., 2014). In either case, field observations indicate that hot and dry nests are more likely to produce hatchlings with abnormalities (Telemeco et al., 2013; Zimm et al., 2017). However, because few studies have attempted to experimentally assess this hypothesis under controlled conditions (but see Hewavisenthi & Parmenter, 2001), the relative contributions of the environment versus genetic mutations remain challenging to tease apart (Velo-Antón et al., 2011).

We tested the hypothesis that carapacial scute formation is sensitive to temperature and moisture levels in the egg incubation environment of turtles. We first quantified morphological asymmetry and compared the frequency of supernumerary scutes in hatchlings of the Blanding's turtle (*Emys* [*Emydoidea*] *blandingii*, Emydidae) that were incubated in stable laboratory versus fluctuating natural conditions. We expected to observe a higher frequency of abnormalities and greater asymmetry in hatchlings from the wild (Figure 1c). In the laboratory, we exposed eggs to different (constant) thermal and hydric regimes to test whether hot and dry incubation environments explain morphological variance and abnormal scute phenotypes, while accounting for potential effects of body size and gonadal sex. Our study did not attempt to associate genetic alleles with observed carapace scute abnormalities.

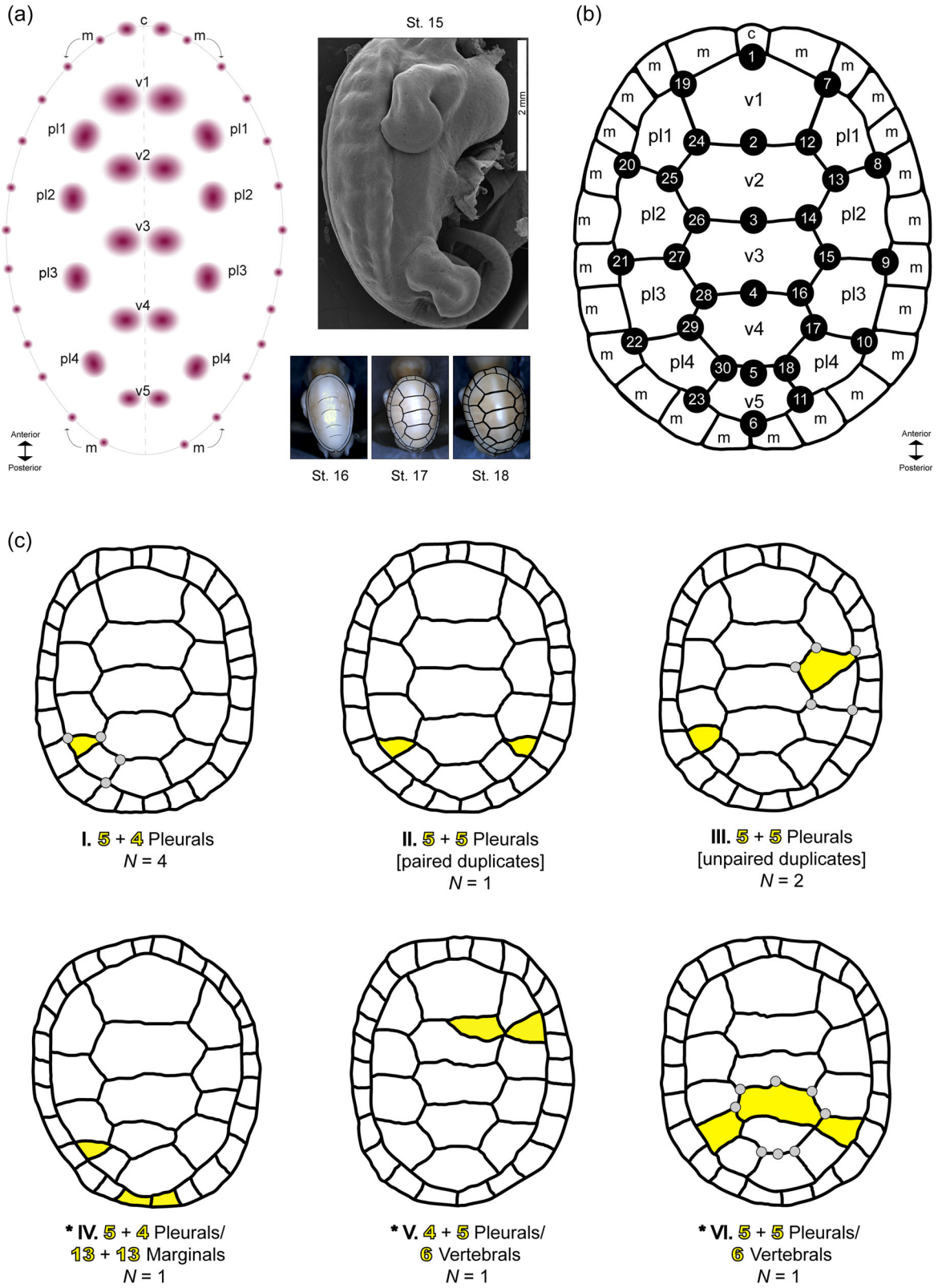

FIGURE 1 (a—left panel) Model of the epidermal placode arrangement associated with the carapace scutes of turtle embryos. Placodes for cervical (c) and vertebral (v) scutes are paired units that eventually fuse, whereas those for marginal (m) and pleural (pl) scutes constitute single units. (a—right panels) Marginal placodes are expected to arise on the myoseptal furrows along the carapacial ridge (shown at stage 15). Placodes give rise to scutes during stages 16–18, as shown in embryos (images not to scale) of *Chrysemys picta* from Cordero and Janzen (2014). (b) Terminal scute configuration with the landmark sampling scheme used to quantify shape in *Emys blandingii*. (c) Abnormal carapace scute phenotypes (types I–VI) observed in hatchling *E. blandingii*. Supernumerary scutes are shown in yellow; *Observed exclusively in hatchlings from natural nests. Also depicted are examples of how landmarks (grey circles) were placed in cases where supernumerary scutes were observed.

2 | MATERIALS AND METHODS

2.1 | Egg incubation and experimental design

Experimental procedures were approved by an Institutional Animal Care and Use Committee (University of Central Arkansas protocol 2004-004). Field sampling was undertaken with permission from the Nebraska Game and Parks Commission (permit # 758). As part of a series of experiments, eggs of *E. blandingii* were collected at Beem Lake and Doc Lake near Hyannis, Nebraska. Eggs ($N = 22$ clutches) were collected directly from females by using oxytocin injection. Eggs were initially maintained at room temperature after being randomly assigned to plastic boxes (with lids) with vermiculite set to a water potential of −150 kPa ($N = 5$ boxes) or −850 kPa ($N = 5$ boxes). Owing to logistical challenges, eggs were transported to Iowa State University 3 weeks after oviposition. The onset of carapace morphogenesis (stage 14) (Cordero & Janzen, 2014), as well as the temperature-sensitive period (stages 16–20) for gonadal differentiation in *Emys* (Pieau & Dorizzi, 1981), were expected to be included within the laboratory incubation period. Eggs were randomly distributed across five environmental chambers set to five constant temperatures ranging from 26°C to 30°C ($N = 2$–4 boxes/chamber). In *E. blandingii*, 26°C is expected to yield 100% males, whereas 30°C produces 100% females (Gutzke & Packard, 1987). The highest temperature (30°C) approached the thermal regime (31°C) at which hatching success is expected to diminish in *E. blandingii*, whereas development may fail altogether at <25°C (Gutzke & Packard, 1987). Egg boxes ($N = 16$) were rotated weekly because heat is sometimes not uniformly distributed within incubators. Nonetheless, infrared-based readings indicated that spatial thermal variance did not exceed ±1°C of set temperatures.

Eggs maintained in vermiculite with −150 kPa substrate moisture were treated as the control group. −150 kPa represents a wet, but not saturated, substrate condition (Packard et al., 1982), which typically serves as a wet or reference control (e.g., Bodensteiner et al., 2015). Eggs in the −850 kPa substrate were considered the dry experimental treatment. This water potential approximates commonly observed dry conditions in turtle nests near our field site (Packard et al., 1992). Up to two replicate egg boxes per substrate treatment were represented in each environmental chamber. Water potential was determined gravimetrically by adding different amounts of deionized water to 300 g of vermiculite, following water-to-vermiculite ratios previously validated by thermocouple hygrometry (Packard et al., 1987). The vermiculite water potential was checked weekly by weighing boxes and replenishing water if necessary. Eggs were not in contact with each other and were half-buried in vermiculite throughout the incubation period. Exposure of the upper egg hemisphere to air does not cause persisting thermal gradients within eggs (Telemeco et al., 2016). Viable eggs did not exhibit signs of dehydration during incubation. If necessary, nonviable eggs were removed.

Eggs that remained in natural nests ($N = 4$ clutches) were also sampled. Hatchlings from these eggs were assigned to a Natural reference group. Data loggers indicated that average temperatures (July to August) in three natural nests ranged from 24.5°C to 28.3°C, while nest soil moisture content rarely exceeded 20% water content. Time spent (up to 60 h) at extremely high incubation temperatures (34°C) was correlated with the frequency of carapace abnormalities in hatchling painted turtles, *Chrysemys picta* (Telemeco et al., 2013). On average, maximum daily air temperatures near our field site during the natural egg incubation period of *E. blandingii* in June, July, and August were 24.6°C [range: 13.3–32.8°C], 30.8°C [range: 15.5–39.4°C], and 28.1°C [range: 12.7–37.2°C], respectively (weather station data from the High Plains Regional Climate Center; https://hprcc.unl.edu). Total precipitation in June, July, and August was 106.4, 18.5, and 51.8 mm, respectively.

2.2 | Sample processing and data acquisition

A subset of 89 hatchlings representing all laboratory clutches, together with all hatchlings ($N = 44$) from natural nests, were selected for further processing. The hatchlings were euthanized (within a month after hatching) by severing the spinal cord. Gonadal sex in *E. blandingii* can be diagnosed by examining gross morphological features immediately after hatching (Gutzke & Packard, 1987). Following established protocols (Paukstis et al., 1984; Yntema, 1976), gonadal sex was assigned after dissection of the inguinal region and inspection of gonadal morphology, that is, the presence of complete Müllerian ducts in females. Care was taken to ensure that the carapace was not damaged during the gonadal sex diagnosis. Hatchling mass was also recorded.

Following preservation in 70% ethanol, the carapace was measured with digital calipers and photographed consistently in all specimens to ensure that images were taken in the same plane of orientation. Two-dimensional carapace shape was quantified, using TPSDIG (Rohlf, 2015), by digitizing 30 fixed homologous landmarks (symmetric = 24; midline = 6; Figure 1b). Landmark digitization was replicated three times per specimen. Following the protocol of Ceballos and Valenzuela (2011), landmarks were not placed on the outer margin of the carapace (on seams of marginal scutes) because this region is highly

pliable and susceptible to damage in preserved specimens. Also, marginal scutes are frequently missing. Using the GEOMORPH R package (Baken et al., 2021), the generalized Procrustes transformation was performed on digitized landmarks. This yielded a matrix of Procrustes-aligned coordinates that were used to represent shape in statistical analyses.

To avoid redundancy and inflation of degrees of freedom (Cardini, 2016), coordinates for symmetric landmarks (12 left + 12 right) were averaged together to test among-group differences in shape. Thin-plate spline wireframes were used to illustrate shape variation. Thin-plate splines interpolate shape across landmarks by computing vectors that represent the magnitude of shape change in a group relative to the mean shape of the entire sample. However, vectors describe shape changes at a single landmark without accounting for the surrounding space (Márquez et al., 2012). Thus, using the LORY program, local shape variation was modeled as infinitesimal differences mapped continuously over landmarks (Márquez et al., 2012).

The placement of corresponding landmarks was complicated by scutes that were in excess of the typical number found in *E. blandingii*, see supernumerary scutes in Figure 1. To account for this potential bias, landmarks were placed in an anterior-to-posterior sequential fashion (starting with the midline) and, if supernumerary scutes were present, landmarks were placed on the first scute seam that was encountered but not on the one that followed (see Figure 1b,c). This scheme may have underestimated shape variation in some individuals, though it reduced major shifts in landmark positions that may have otherwise amplified variance for the entire landmark configuration. In any case, statistical analyses on variances accounted for such abnormal supernumerary scutes and their impact on local shape variation was explored graphically.

2.3 | Statistical analyses

2.3.1 | Analyses on size, mass, and gonadal sex

To assess environmental effects on growth and physiology, generalized linear models (GLMs) were tested with hatchling mass or carapace length (CL) as a response and egg mass as a covariate. Temperature and moisture were categorical predictors and clutch of origin was a random effect. The replicate incubation box was also treated as a random effect. The GLMs included sex as a factor to address potential sexually dimorphic effects (Gutzke & Packard, 1987). Logistic regression was performed to validate the expected proportions of hatchling males, that is, the sex "ratio," in *E. blandingii*. The probability of observing supernumerary scutes was tested using a GLM with CL as a covariate and clutch as a random effect. GLMs were validated by examining plots of residual distributions and residual versus fitted values. Model fitting was performed in the *lme4* R package (Bates et al., 2015).

2.3.2 | Analyses on carapace shape

A principal component analysis (PCA) on Procrustes-aligned (averaged symmetric) coordinates was first performed to explore carapace shape variation. To test whether shape differed in Natural versus Laboratory hatchlings, a nonparametric multivariate analysis of variance (NP-MANOVA on 10,000 permutations) was performed using the *procD.lm* function of GEOMORPH. Shape (Procrustes-aligned coordinates) was the response and centroid size (CS) was the covariate, while clutch identity and the presence/absence of supernumerary scutes were random effects. CS was used as a proxy for body size because it was derived directly from the landmark set used to quantify shape. Using the *pairwise* function, between-group contrasts were performed and statistical significance was evaluated with the randomized residual permutation procedure.

Shape asymmetry was analyzed with the *bilat.symmetry* function of GEOMORPH. Fluctuating asymmetry (FA) was estimated as the deviation (from the mean) between the sides of a given specimen after adjusting for the mean deviation in all specimens. Between-group FA contrasts were performed using the *morphol.disparity* function, which randomizes (10,000 permutations) the vectors of residuals between groups. This analysis was based on a linear model wherein the FA variance component was the response and CS (including an interaction term for the presence/absence of supernumerary scutes) was the covariate, while substrate moisture type or temperature were categorical predictors. Among-individual variation was explored by plotting the unsigned asymmetry index, see Lazić et al. (2015).

An NP-MANOVA model tested environmental effects on shape in the laboratory. The model included shape as the response variable, with temperature and substrate moisture as predictors. Egg mass and CS were covariates that accounted for potential allometric effects. Clutch of origin, replicate egg box, and presence/absence of supernumerary scutes were random effects. Using the *trajectory.analysis* function, the interaction of temperature and substrate moisture was explored with a phenotypic trajectory analysis (Collyer & Adams, 2013). To secondarily assess sexually dimorphic carapace shape differences that might be detectable in hatchling turtles (Ceballos & Valenzuela, 2011), an

NP-MANOVA model with gonadal sex as a predictor and CS as a covariate was tested. To avoid colinearity, temperature was excluded from this model.

3 | RESULTS

3.1 | Prevalence of carapace scute abnormalities

Similar proportions of supernumerary carapace scutes were observed in hatchlings from Natural (9%) and Laboratory (7%) groups (LRT: $\chi^2 = 0.234$, $p = .629$; Table 1), regardless of body size (i.e., CL) variation ($p = .504$). Observed supernumerary scutes in Laboratory hatchlings were paired or unpaired pleural scutes (Figure 1c). Supernumerary vertebral scutes were not observed in the laboratory. Hatchlings from the Natural nests featured supernumerary vertebral scutes which sometimes co-occurred with supernumerary pleural scutes (Table 1; Figure 1c). Supernumerary marginal scutes were observed in one hatchling from the Natural group (Table 1; Figure 1c).

3.2 | Environmental effects on hatchling size, mass, and gonadal sex

Hatchlings from the Wet substrate were larger than those from the Dry substrate (Figure 2a; Table 1). Hatchling mass was not affected by gonadal sex (LRT: $\chi^2 = 1.47$, $p = .2245$), while the effect of CL on hatchling mass was weak (LRT: $\chi^2 = 3.67$, $p = .055$). Incubation temperature in the laboratory predicted hatchling sex (LRT: $\chi^2 = 77.45$, $p < .0001$). Proportions of males were 26°C: 100%; 27°C: 92%; 28°C: 63%; 29°C: 6%; 30°C: 0% (Figure 2a). The effect of temperature on sex was similar between Dry and Wet laboratory incubation groups (LRT: $\chi^2 = 0.036$, $p = .8486$). The Natural treatment was 14% males. Hatchlings from the Natural reference group were slightly larger than those reared in the laboratory (Table 1).

3.3 | Carapace shape asymmetry in natural versus laboratory conditions

Carapace scute shape (left–right averaged) in the Natural group was similar to that in the Laboratory group (pairwise difference: 0.0169; $p = .649$), though variance was higher in natural nests (see PCA in Figure 2b). When comparing shape asymmetry (i.e., FA), the Wet laboratory substrate treatment differed from the Natural treatment (pairwise difference: 0.0029, $p = .0127$; Figure 2c). Differences in FA were marginally supported in the Dry versus Natural groups (pairwise difference: 0.0026; $p = .0664$). Among-individual asymmetric shape variation was highest in the Natural group (Figure 3), owing to a few individuals with supernumerary and highly misshapen scutes (Figure 4). Differences in FA were not statistically significant when comparing constant temperature groups to the Natural group (p-values 0.14–0.26) (Figure 5a).

3.4 | Environmental effects on carapace shape in the laboratory

The effects of temperature and body size (i.e., CS) on scute shape were supported (Table 2). Egg mass and substrate level did not affect shape ($Z = 0.457$–0.572; $p > .32$), and there was no interaction of temperature and substrate ($Z = -0.817$; $p = .792$) nor of temperature and CS ($Z = 0.721$; $p = .233$). The effect of temperature remained significant in a model that excluded hatchlings with supernumerary scutes ($p < .0001$; $p = .0389$). In the NP-MANOVA model that tested sexual shape dimorphism, males and females differed in carapace shape ($Z = 2.14$; $p = .014$), while there was a marginally significant effect of CS ($Z = 1.57$; $p = .057$) and no interaction of CS and sex ($Z = -0.055$; $p = .519$). Phenotypic trajectories along different incubation temperatures were similar for Wet and Dry substrate treatments (Figure 5b). Between-group contrasts are summarized in Table 3 and shape visualizations at extreme temperatures (26°C and 30°C) are depicted in Figure 5c.

4 | DISCUSSION

Our study tested the effects of constant temperature and substrate moisture levels on the formation of carapace scutes in turtles, while comparing to natural conditions that were less stable. In wild hatchlings of *E. blandingii*, morphological asymmetry was higher than in laboratory hatchlings. The frequency of supernumerary scutes was similar between natural and laboratory groups, which suggests that scute pattern formation was robust in *E. blandingii*. Although the hot and dry laboratory egg incubation regime probably affected embryo growth and physiology, it did not substantially affect carapace scute formation.

4.1 | Evidence of developmental instability in natural nests

Naturalists have long hypothesized that perturbed nest environments contribute to shell abnormalities

TABLE 1 Raw means and sample sizes for phenotypic parameters measured in experimental groups of hatchling *Emys blandingii*

Substrate	Temperature (°C)	*N*	*N* [Abnormal]	Egg mass (g) [95% CI]	Hatchling mass (g) [95% CI]	Carapace length (mm) [95% CI]
Dry	26	9		12.9 [11.98–13.82]	8.65 [7.89–9.41]	32.2 [31.0–33.36]
Wet	26	9		12.2 [11.06–13.34]	8.75 [8.02–9.49]	32.9 [31.67–34.13]
Dry	27	5	1[a]	13 [11.90–14.10]	8.84 [7.69–9.99]	32.1 [30.61–33.59]
Wet	27	8	1[b]	12.8 [11.66–13.94]	8.95 [8.29–9.61]	32.5 [31.47–33.53]
Dry	28	9		12.6 [11.88–13.32]	8.24 [7.62–8.86]	32.3 [31.10–33.50]
Wet	28	10	1[b]	13 [12.35–13.65]	8.8 [8.34–9.26]	33.3 [32.72–33.88]
Dry	29	8		12 [10.89–13.11]	8 [7.20–8.80]	31.3 [30.01–32.59]
Wet	29	8	1[b]	12.7 [12.06–13.34]	8.65 [8.22–9.08]	32.7 [32.01–33.39]
Dry	30	9	1[a]	13.5 [12.75–14.25]	8.24 [7.50–8.98]	31.8 [30.77–32.83]
Wet	30	10	1[c]	13 [12.47–13.53]	8.7 [8.20–9.20]	32.9 [31.83–33.97]
Natural	Natural	42	4[b,d,e,f]	-	-	33.5 [32.98–34.02]

Note: Egg and hatchling mass could not be measured in the Natural group.

[a]5 + 5 pleurals [unpaired duplicates].

[b]5 + 4 pleurals.

[c]5 + 5 pleurals [paired duplicates].

[d]5 + 4 pleurals/13 + 13 marginals.

[e]4 + 5 vertebrals.

[f]5 + 5 pleurals/6 vertebrals.

(Cagle, 1950; Legler, 1954). Although subterranean nests dampen surface air temperatures, eggs are subject to cyclical temperature and moisture fluctuations (Packard et al., 1985). Floods, heat waves, and droughts further disturb nest microclimates (Jergenson et al., 2014; Mainwaring et al., 2017; Telemeco et al., 2013). Such heterogeneity and stochasticity within nests should, therefore, translate to increased phenotypic variance owing to developmental instability. Even so, the assumption that natural nests produce hatchlings with greater phenotypic variance, compared to in the laboratory, received inconclusive support when CL was examined (St Juliana & Janzen, 2007). Our assessment of carapace scute shape did corroborate an environmental effect on phenotypic variance. The asymmetric variance component of shape (i.e., FA) was augmented in wild *E. blandingii* hatchlings, which was driven by a few hatchlings with highly misshapen scutes. That elevated asymmetry was not uniformly expressed across hatchlings is expected owing to microclimatic gradients. For instance, turtle eggs situated at the top of nests are more likely to experience unusually high temperatures (Telemeco et al., 2016), or are more likely to suffer from periodic dehydration (Legler, 1954).

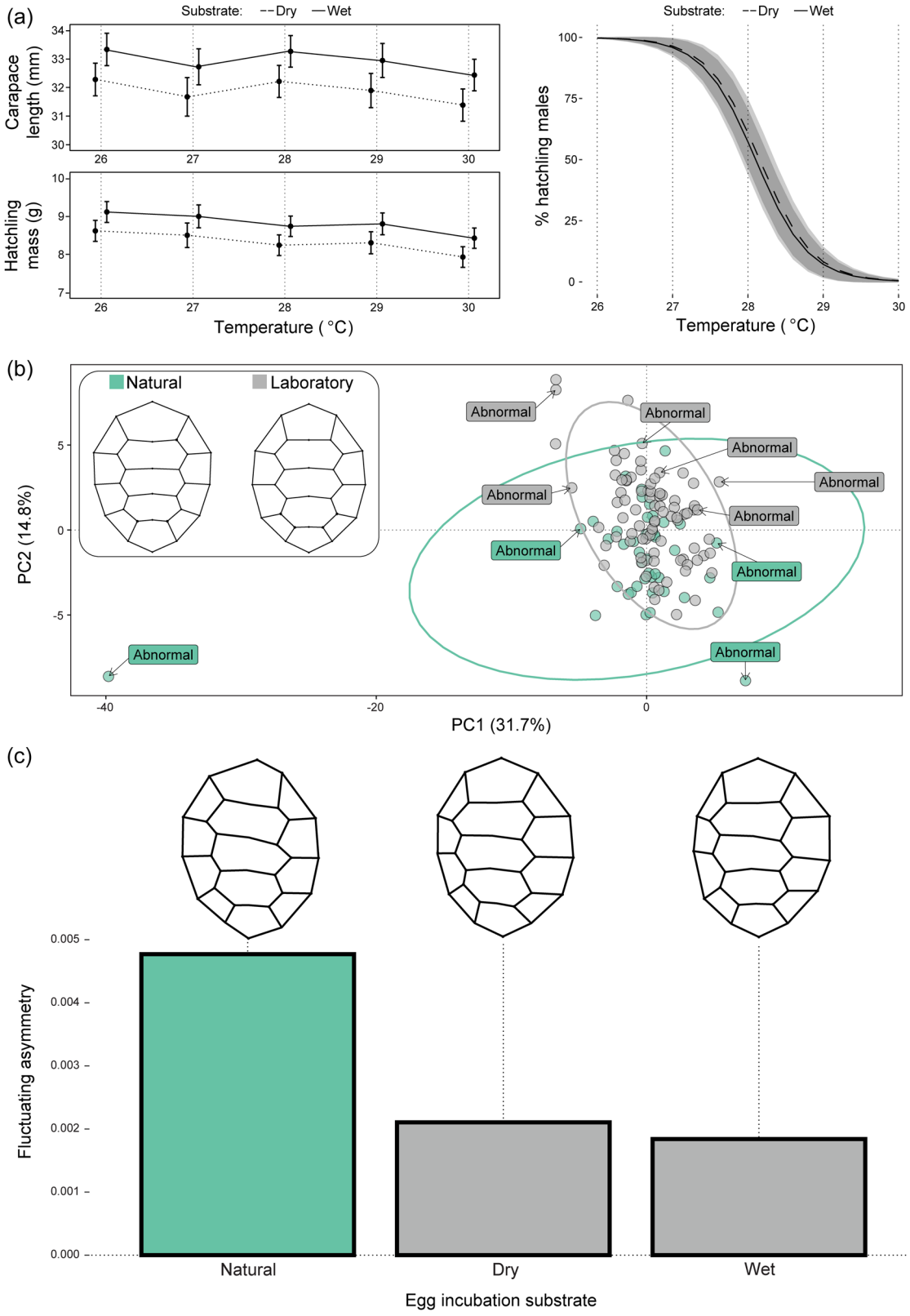

FIGURE 2 (See caption on next page)

Examining morphological asymmetry as a potential outcome of developmental instability may further elucidate the sensitivity of developing organisms to changing environments, especially in turtles and other reptiles that provide little, if any, parental care to their eggs. Measuring asymmetry after floods, droughts, and heat waves may further expose the phenotypic consequences of altered nest microenvironments. In particular, future experiments should aim to manipulate environmental parameters during critical windows in development. Carapace scute malformations are more likely to arise if environmental perturbation coincides with the pre-patterning of placodes during stages 14–16 (Zimm et al., 2017). Nesting in our *E. blandingii* population typically begins in June (Ruane et al., 2008). As such, the hottest and driest part of the incubation period (August; see Packard et al., 1985) typically does not overlap with the sensitive window during which scutes are patterned in embryos. Although the proportion of wild *E. blandingii* hatchlings with supernumerary scutes was rather low (9%), our analyses indicate that localized shape asymmetry was greater than in the laboratory. Crucially, supernumerary scutes on their own did not entirely inflate estimates of asymmetry because neighboring or even distantly situated scutes were also misshapen, particularly in hatchlings from natural nests. A similar observation was made in wild *E. blandingii* hatchlings from Nova Scotia, which otherwise featured low proportions (7%–16%) of supernumerary scutes (Standing et al., 2000).

Our findings agree with the expectation that carapace scute formation is environmentally sensitive. A key trend to evaluate is whether the incidence of abnormal carapace scute patterns will increase if heat waves occur earlier in the natural incubation period of *E. blandingii* and other turtles. The thermal sensitivity of carapace scute formation in natural nests has thus far been supported by the increased frequency of supernumerary scutes in hatchlings produced during hot summers in other freshwater turtles, that is, *C. picta* (Telemeco et al., 2013). Overall, the comparison of scute formation under natural versus controlled laboratory conditions is a promising model to address hypotheses concerning developmental instability in response to environmental disturbances.

4.2 | Carapace scute variation under controlled laboratory conditions

Carapace scute formation was not severely disrupted by laboratory manipulation of the egg incubation environment in *E. blandingii*, despite environmental effects on embryo growth and physiology (size, mass, and gonadal sex differentiation). Our study *E. blandingii* population might be locally adapted to dry conditions (Ruane et al., 2008). In particular, egg size, rather than clutch size, increases with maternal body size, potentially as a means to reduce the likelihood of egg desiccation (Ruane et al., 2008). In the laboratory, we showed that scute formation is robust to a constant −850 kPa substrate water potential. Even lower water potential of −3500 kPa did not induce carapace scute abnormalities in a marine turtle (Hewavisenthi & Parmenter, 2001). In *C. picta*, substrates below −600 kPa were presumably sufficient to cause abnormal shell development, though this species lays flexible-shelled eggs that are prone to dehydration (Tracy et al., 1978). Even though the semiflexible eggshell structure of *E. blandingii* confers a rather high water retention capacity, water intake is reduced if water potential is set to −700 kPa (Packard et al., 1982). Such a reduction in water intake is consistent with smaller *E. blandingii* hatchlings in our Dry treatment. This effect was likely amplified by high temperatures (28–30°C), as in previous laboratory studies (Gutzke & Packard, 1987; Packard et al., 1982). Consequently, carapace shape variation in our experiment might be partially explained by allometric or sexually dimorphic effects.

Although symmetric scute shape responded to constant temperature levels in the laboratory, FA was similar across hatchlings from different thermal incubation regimes. The persistence of supernumerary carapace scutes under such controlled laboratory conditions is intriguing because it points to a source of environmental perturbation that was unaccounted for in our experiment

FIGURE 2 (a—left panels) Incubation substrate had an effect on carapace length (LRT: $\chi^2 = 22.39$, $p < .0001$) and mass (LRT: $\chi^2 = 20.92$, $p < .0001$) in hatchling *Emys blandingii*, with Dry substrates and high temperatures yielding smaller hatchlings. Jittered points denote covariate-adjusted (marginal) means and bars represent 95% confidence intervals. (a—right panel) Low temperatures produced higher proportions of males, regardless of substrate treatment. (b) A principal component (PC) plot on left–right averaged shape (Procrustes-aligned coordinates) depicts greater dispersion around the mean (see 95% confidence ellipses) in the Natural reference group. (b—inset) Thin-plate spline wireframes represent the shape (relative to the mean for the entire data set) of Natural and Laboratory groups. Hatchlings with supernumerary scutes are labeled as "Abnormal." (c) Fluctuating asymmetry was greater in hatchlings from the Natural egg incubation substrate treatment. Thin-plate spline wireframes depict asymmetric shape (relative to the mean for the entire data set).

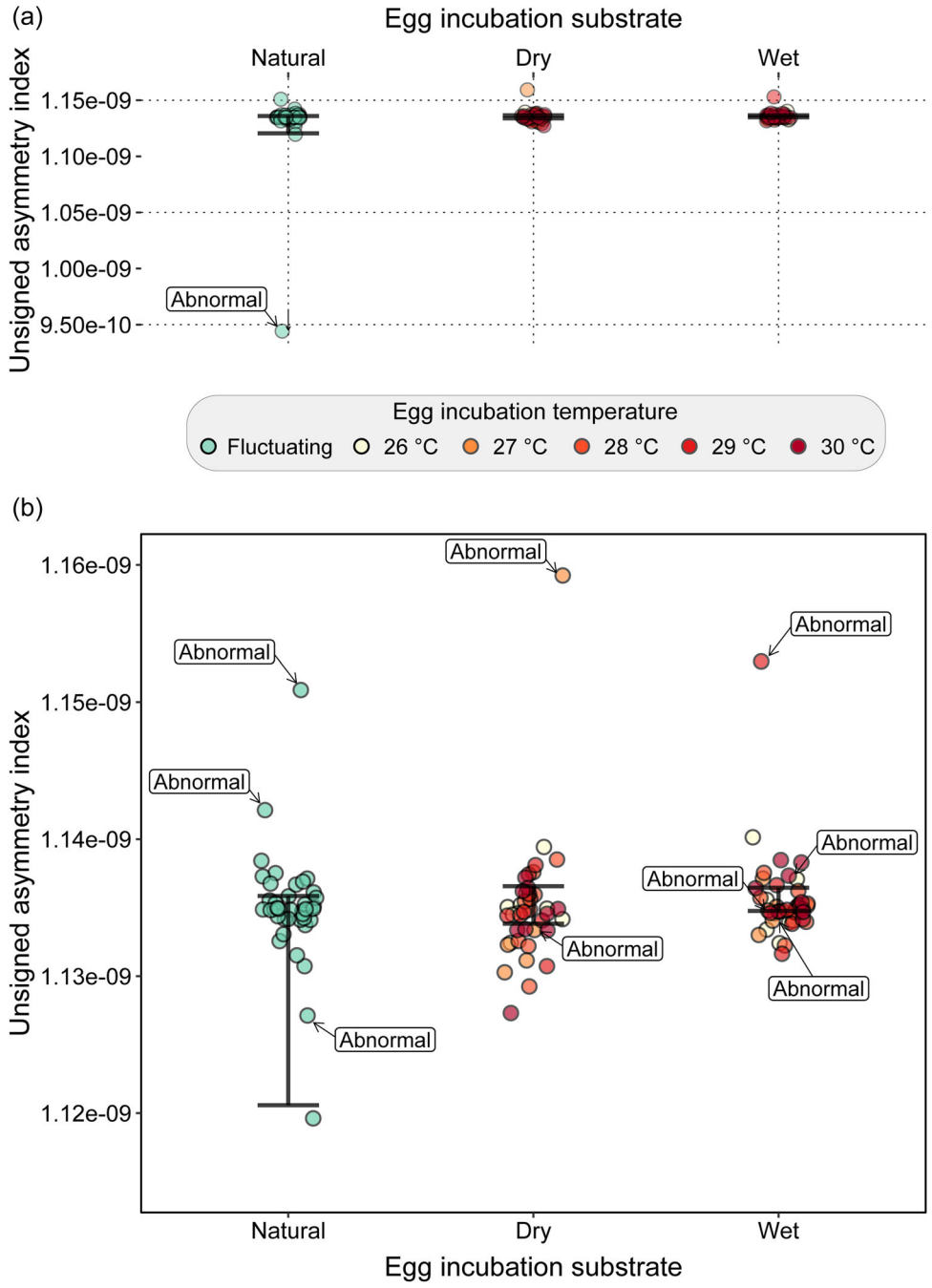

FIGURE 3 (a) Among-individual variation in carapace scute asymmetry in hatchling *Emys blandingii* from different thermal and hydric egg incubation regimes. Hatchlings with supernumerary scutes are labeled as "Abnormal." (b) A plot of unsigned asymmetry index values that excludes the extreme outlier shown in panel A and highlights additional individuals with Abnormal scute phenotypes. Colored points (jittered horizontally for clarity) represent hatchlings from different natural (fluctuating) and laboratory (constant) thermal regimes.

or perhaps a heritable genetic component. Along these lines, the random expression of supernumerary and irregularly shaped vertebral scutes in laboratory-reared turtles, that is, the "dovetail" syndrome, has not been linked to a particular environmental cue or genetic locus (Ewert, 1979). We could not address the environmental triggers of this abnormal phenotype because we only observed supernumerary vertebrals in three hatchlings, which were from natural nests.

Future laboratory experiments should impose moisture levels that are sufficient to cause mechanical stress owing to egg dehydration. When *C. picta* eggs were exposed to a semicontrolled laboratory drought event, the resulting hatchlings featured highly deformed and

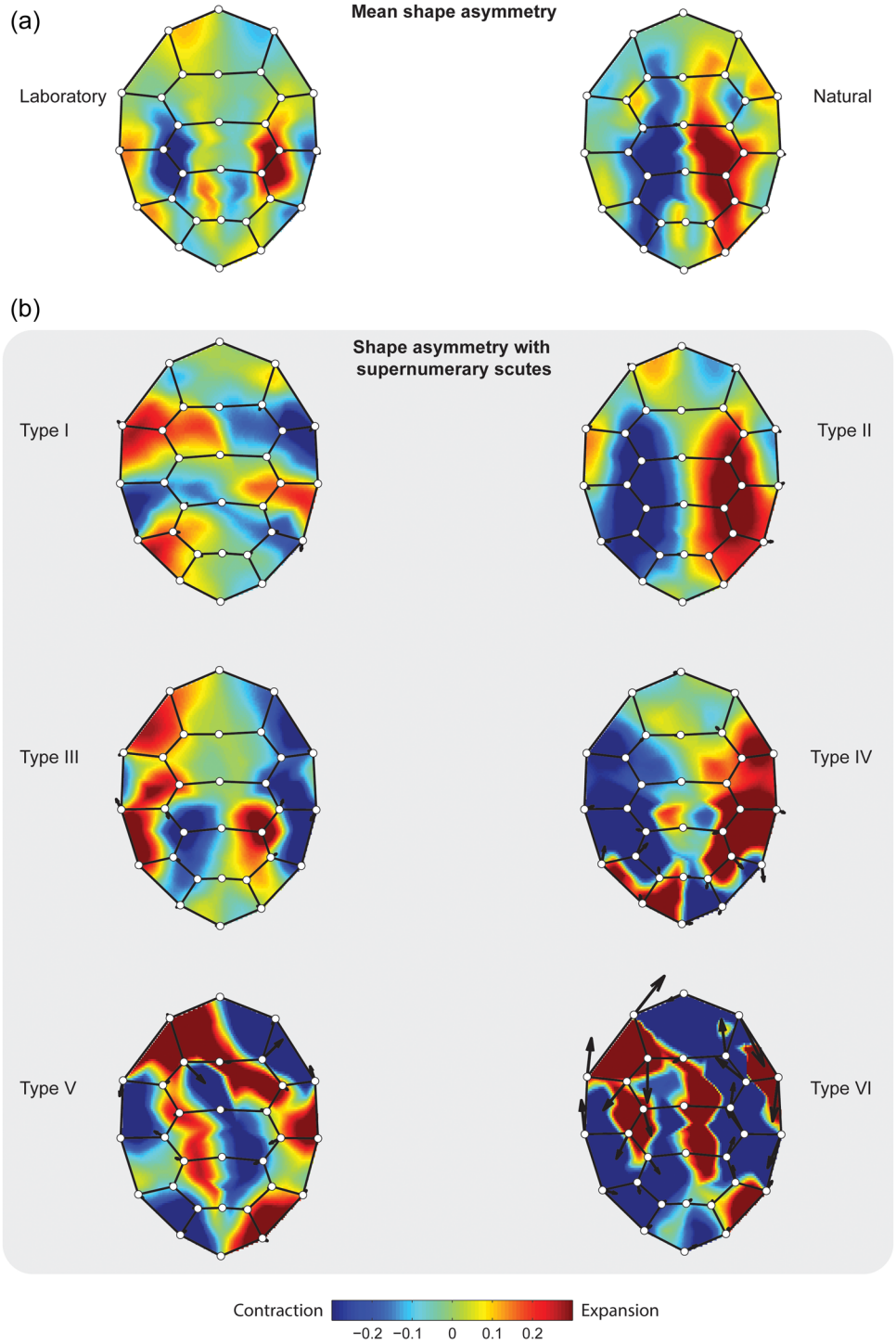

FIGURE 4 (a,b) Parrot plots of localized carapace scute shape asymmetry in *Emys blandingii*. The color gradient describes the base-2 logarithm of Jacobian determinants from thin-plate spline functions, wherein the intensity of red hues (see color gradient bar) corresponds to the magnitude of tissue expansion and blue hues indicate contraction. Green hues represent invariant regions. (a) Plots of mean shapes in hatchlings from laboratory versus natural egg incubation environments are shown. (b) Plots are also shown for hatchlings of representative abnormal carapace scute phenotypes, that is, supernumerary scutes. The vectors on landmarks represent the magnitude and direction of change relative to the mean for the entire data set. Type I = 5 + 4 pleurals; Type II = 5 + 5 pleurals (paired duplicates); Type III = 5 + 5 pleurals (unpaired duplicates); Type IV = 5 + 4 pleurals/13 + 13 marginals; Type V = 4 + 5 vertebrals; Type VI = 5 + 5 pleurals/6 vertebrals.

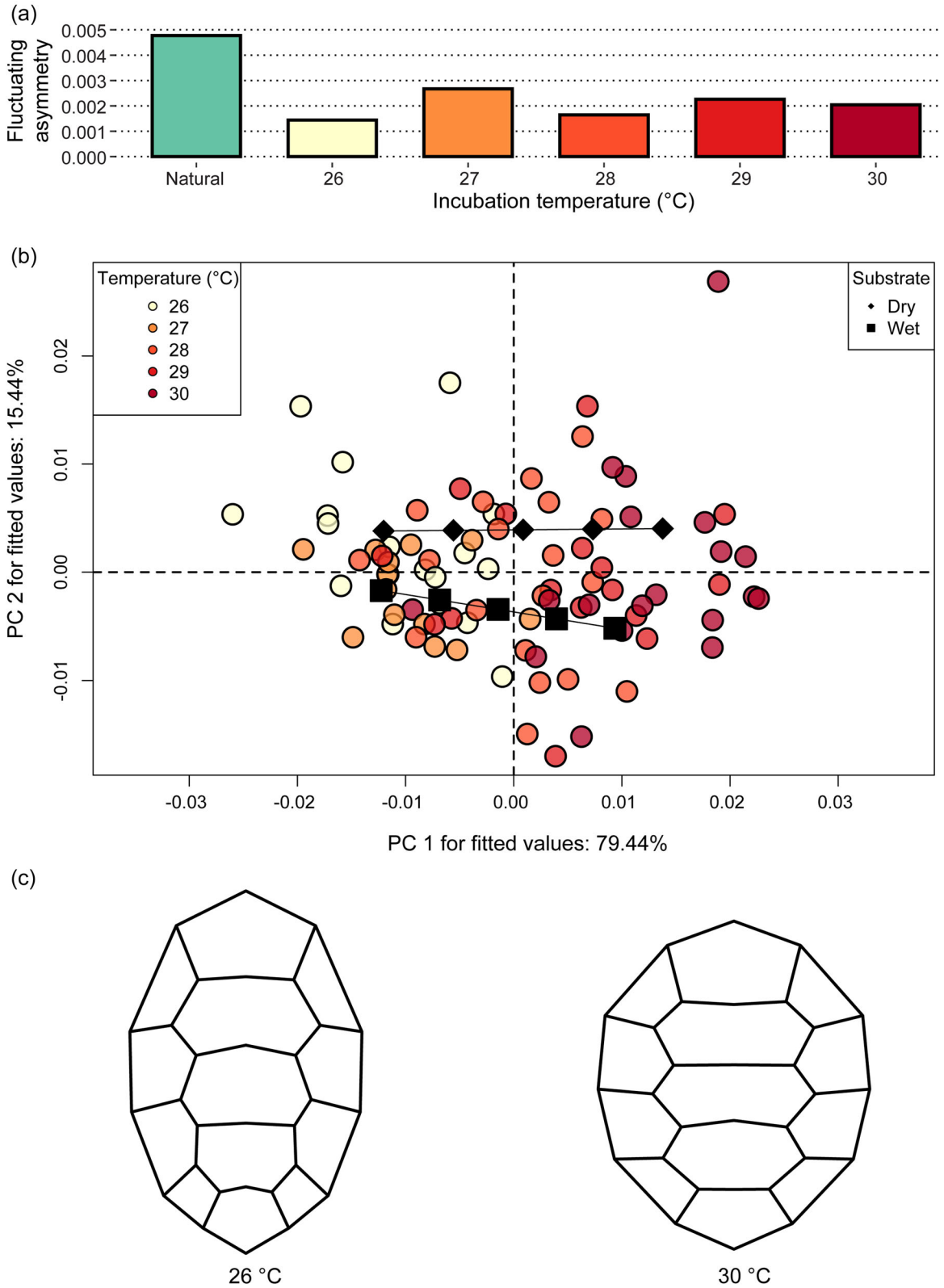

FIGURE 5 (a) Fluctuating asymmetry in hatchling *Emys blandingii* from natural versus laboratory egg incubation environments. (b) A plot of a principal component analysis (PCA on fitted values from the linear model used to test treatment differences) with phenotypic trajectories of carapace shape for hatchling *E. blandingii* incubated in Dry (mean shape = black diamonds) and Wet (mean shape = black squares) incubation substrates across different constant temperatures (see legend). (c) Thin-plate spline wireframes highlight differences in the mean shape, relative to the mean for the entire data set, of hatchlings incubated at 26°C versus at 30°C.

asymmetric shells owing to tissue shrinkage and contraction of eggshells (Lynn & Ullrich, 1950). A potential effect of mechanical stress on scute formation was also supported by a study that recorded a high incidence of abnormalities in hatchlings from eggs that were translocated (Mast & Carr, 1989). Periodically subjecting turtle embryos to environmental conditions beyond the limit that can sustain development in the laboratory may elevate asymmetry and the incidence of abnormal carapace scute phenotypes.

4.3 | Potential causes of aberrant scute formation

Random deviations in tissue growth should increase if developing organisms are exposed to stressful environments (Dongen, 2006; Hagolani et al., 2019; Waddington, 1957). Indeed, the inherent noise of molecular signaling pathways that govern carapace scute formation might be amplified by extremely hot temperatures (Zimm et al., 2017). The association of high nest temperatures and malformed scutes suggests that the dynamics of pattern formation mechanisms are destabilized at the thermal limits of development. Specifically, the Shh-Bmp2/4-Gremlin reaction-diffusion mechanism that first establishes the relative position of precursor scute placodes might be altered. Once placodes assume their normal spatial configuration, a second-reaction diffusion mechanism is hypothesized to induce cell behaviors necessary to construct scutes and determine their boundaries (Moustakas-Verho et al., 2014). In vitro and in silico disruption of these mechanisms, see Moustakas-Verho et al. (2014), produced similar scute abnormalities as we observed in wild and laboratory hatchlings of *E. blandingii*.

Paired placodes that give rise to vertebral scutes might be more susceptible to environmental perturbation because they must "fuse" during scute prepatterning. Consequently, supernumerary vertebral scutes tend to be more common than those related to pleural scutes. Another means by which supernumerary scutes may emerge concerns carapace growth. Moustakas-Verho et al. (2014) altered the spatial dimensions in which scute reaction-diffusion mechanisms operate and produced supernumerary scutes, some of which resembled species-specific configurations: A fifth or sixth pair of pleural scutes followed the fourth pair that normally lies at the posterior end of the carapace. As in other turtles (Cagle, 1950; Coker, 1910; Lynn, 1937), supernumerary or malformed scutes were mainly observed in the posterior region of the carapace in *E. blandingii* hatchlings. Even so, the low frequency of scute abnormalities precluded statistical tests on the relationship between carapace growth and scute malformation at different temperatures. This hypothesis awaits further exploration.

Mechanical perturbation of mesodermal somites or the turtle-specific carapacial ridge (CR) molecular signaling center that is critical to shell morphogenesis led to scute irregularities and asymmetry (Burke, 1991; Yntema, 1970). In ovo mechanical forces may later contribute to variation in scute morphology as the carapace grows. In late-term turtle

TABLE 2 Results of the nonparametric multivariate analysis of variance on carapace shape in hatchlings of *Emys blandingii* incubated at different temperatures in the laboratory.

Source	*df*	Type I sum of squares	Mean square	*F*	*Z*	*p*
Centroid size	1	0.002	0.002	2.668	2.218	.012
Temperature	1	0.006	0.006	8.322	4.318	<.0001
Residuals	45	0.035	0.001			
Total	84	0.092				

TABLE 3 Pairwise distances between carapace shape means of hatchling *Emys blandingii* incubated at different temperatures in the laboratory

Contrast	Distance	95% Upper confidence limit	*Z*
26°C versus 27°C	0.013	0.019	−0.678
26°C versus 28°C	0.015	0.018	−1.006
26°C versus 29°C	0.018	0.022	−0.834
26°C versus 30°C	0.024	0.028	−0.556
27°C versus 28°C	0.016	0.022	−0.502
27°C versus 29°C	0.018	0.024	−0.489
27°C versus 30°C	0.025	0.029	−0.388
28°Cversus 29°C	0.009	0.014	−1.735
28°C versus 30°C	0.015	0.020	−0.791
29°C versus 30°C	0.013	0.017	−0.984

embryos and hatchlings, the posterior carapace might ossify at a slower rate than the anterior (Cordero, 2021). This maturity gradient may explain why the posterior carapace is often malformed (Zangerl & Johnson, 1957). Alternatively, perturbation of the posterior-to-anterior emergence of the CR, see Cordero (2020), may yield misshapen scutes at the posterior end of the carapace if the propagation of pattern formation molecular signals is affected. In general, identifying the mechanisms that underlie scute malformation will require teasing apart molecular signaling dynamics from potential thermal, hydric, and mechanical stressors in the egg incubation environment. Our experiments demonstrated that carapace scute formation remained canalized within controlled laboratory conditions. To further elucidate the environmental causation of aberrant scute formation, additional in vivo experiments are needed to identify the precise conditions that are suboptimal to pattern formation mechanisms of the carapace.

4.4 | The evolutionary relevance of environmentally induced scute variation

Carapace scute number has the potential to undergo evolutionary change independently from carapace bones. As such, the progressive numerical reduction of scute elements in turtle macroevolution is not strictly correlated with a reduction in bone number (Cordero & Vlachos, 2021). In fact, scute number variation is considered a naturally occurring polymorphism in some species (Zangerl & Johnson, 1957). Moreover, most documented scute abnormalities in wild populations are considered benign because they seem to persist long after individuals reach reproductive maturity (Ewert, 1979). Out of 2220 (mainly adult) museum specimens, 43% featured abnormal scutellation (Zangerl & Johnson, 1957). However, there might be observational biases because the frequency of scute abnormalities might be higher in individuals that die prematurely. Thus far, selection against abnormal scutes has not been supported in marine turtles (Bentley et al., 2020), while abnormal scutes do not seem to interfere with hatchling performance (Sim et al., 2014).

Correlated bone-scute malformations are noteworthy because scute pattern formation is somewhat independent from skeletal development in turtles (Moustakas-Verho et al., 2014; Zimm et al., 2017). However, the congruence between scute and skeletal abnormalities is rarely described, but see Parker (1901), because it requires dissection, clearing, or x-ray visualization (Farke & Distler, 2015; Newman, 1906). By clearing specimens, Newman (1906) described a late-term embryo of *Graptemys* spp. with a supernumerary vertebral scute that was juxtaposed to a supernumerary and highly deformed thoracic rib. The literature supports that such structurally linked malformations might be more common in snakes because supernumerary scales often co-occur with neighboring supernumerary vertebral segments (see references in Lowenborg & Hagman, 2017). Similarly, carapace scute deformities were associated with the loss of neural or peripheral bones in captive tortoises (*Testudo* spp.) (Farke & Distler, 2015).

Whether environmentally induced carapace scute variation influences Darwinian fitness is an open question that necessitates research that integrates detailed anatomical analyses with egg incubation and offspring performance experiments. Toward this goal, our study of laboratory and field-incubated turtle hatchlings is one of a handful that has manipulated egg incubation parameters to experimentally validate that carapacial scute formation is environmentally sensitive. This work contributed toward clarifying the environmental conditions at which scute formation is seemingly canalized, while also supporting the assumption that fluctuating environments may destabilize embryonic development. Our study invites further research on how complex phenotypes, such as the turtle's shell, respond to changing environments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data will be made available upon request.

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