The Anatomical Record **WILEY**

Skeletal repatterning enhances the protective capacity of the shell in African hinge-back tortoises (*Kinixys*)

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Abstract

Changes in the structural association of skeletal traits are crucial to the evolution of novel forms and functions. In vertebrates, such rearrangements often occur gradually and may precede or coincide with the functional activation of skeletal traits. To illustrate this process, we examined the ontogeny of African hinge-back tortoises (*Kinixys* spp.). *Kinixys* species feature a moveable "hinge" on the dorsal shell (carapace) that enables shell closure (kinesis) when the hind limbs are withdrawn. This hinge, however, is absent in juveniles. Herein, we describe how this unusual phenotype arises via alterations in the tissue configuration and shape of the carapace. The ontogenetic repatterning of osseous and keratinous tissue coincided with shifts in morphological integration and the establishment of anterior (static) and posterior (kinetic) carapacial modules. Based on *ex vivo* skeletal movement and raw anatomy, we propose that *Kinixys* employs a "sliding hinge" shell-closing system that overcomes thoracic rigidity and enhances the protective capacity of the carapace. Universal properties of the vertebrate skeleton, such as plasticity, modularity, and secondary maturation processes, contributed to adaptive evolutionary change in *Kinixys*. We discuss a hypothetical model to explain the delayed emergence of skeletal traits and its relevance to the origins of novel form-to-function relationships.

KEYWORDS

carapacial hinge, delayed development, tissue remodeling, turtle shell evolution

1 | INTRODUCTION

Phenotypic evolution often depends on the propensity for developing traits to undergo coordinated structural rearrangements (Gawne et al., 2020; Riedl, 1975; Zelditch et al., 2001). During development, the redistribution of cells and energy for tissue accretion and expansion (i.e., growth) may produce regionalized changes in the morphology of some traits while not affecting others (Gawne et al., 2020;

Zelditch et al., 2001). For instance, the craniofacial region of vertebrates may grow somewhat independently from the rest of the cranium (Morris et al., 2019; Thompson, 1945). As such, bones that comprise the snout are considered a morphological module that is subject to evolutionary change in response to functional demands, for example, elongate snouts in fishes and reptiles (Morris et al., 2019; Thompson, 1945).

Functionally relevant modules may also originate via the *de novo* modification (heterotopy) or acquisition of a

discrete trait. In some tetrapods, modified skeletal articulations enhance function while unequivocally establishing spatial boundaries among bones that would otherwise remain contiguous in the ancestral skeletal configuration (Frazzetta, 1970, 1976). This is perhaps best exemplified by moveable joint articulations that structurally divide the cranium in a condition referred to as cranial kinesis (Bailleul & Holliday, 2017; Frazzetta, 1976; Holliday & Witmer, 2008). One of the most derived forms of cranial kinesis concerns a "hinge" joint that presumably bisects the maxillary bone of the upper jaw in bolyeriine snakes and is thus viewed as an innovation among tetrapods (Frazzetta, 1970, 1976). This maxillary hinge widens the gape and is hypothesized to be an adaptation for better grasping and processing of large prey items. Similarly, in parrots, cranial kinesis via modified sutural contacts of the frontal and nasal bones is related to an ontogenetic transition in the feeding biomechanics of the skull (Tokita, 2003).

Beyond histological descriptions in parrots (Tokita, 2003), few studies have managed to capture the tissue alterations associated with the construction and functional activation of skeletal kinesis. Nonetheless, similar histological changes (suture remodeling) were linked to the convergent evolution of shell kinesis in turtles (Cordero et al., 2018; Cordero et al., 2019). Dermally derived bones of the ventral shell (plastron) initially exhibit normal growth and suture formation in most kinetic-shelled turtles studied thus far, that is, *Kinosternon*, *Sternotherus*, *Pelusios*, and Emydinae (Cordero, 2021; Cordero et al., 2018). Subsequently, precursor sutures gradually undergo remodeling and acquire thick connective tissue, thereby giving rise to a functional hinge joint that permits elevation of the plastron to better conceal soft body parts when the adult life stage is reached (Bramble, 1974; Legler, 1960). The delayed emergence of this kinetic hinge underscores the evolutionary and functional significance of secondary skeletal transformations, many of which unfold across post-embryonic life stages (Otero et al., 2019; Salmon et al., 2018; Standen et al., 2014). Indeed, organismal growth is accompanied by plastic changes in the architecture and biomechanics of bone (Gawne et al., 2020; Zelditch et al., 2001). As such, the slow growth and maturation of the turtle's shell is an interesting model to investigate how skeletal plasticity contributes to the evolution of novel form-to-function relationships (Cordero, 2017, 2020).

A remarkable example of skeletal plasticity concerns the atypical hinge on the dorsal shell (carapace) of African hinge-back tortoises (Testudinidae: *Kinixys*; Broadley, 1997; Siebenrock, 1916). *Kinixys* is the only member of Testudines that features a carapacial hinge (Shah, 1960), which is well differentiated and functional in adults but is absent in juveniles. This hinge is situated

between the fourth and fifth costal and the seventh and eighth peripheral bones. It permits lowering (i.e., closure) of the posterior portion of the carapace in response to, for instance, potential predatory threats (Shah, 1960). This condition suggests that *Kinixys* has regained some degree of thoracic flexibility by altering the structural co-dependence of the ribs, vertebrae, and other bones that are integrated within the carapace (Broadley, 1997; Siebenrock, 1916). In fact, some thoracic vertebrate may even exhibit dorsoventral flexion in *Kinixys* (Siebenrock, 1916). Here, we describe how skeletal structures are altered during the delayed development and functional activation of the carapacial hinge joint. Joint development requires the translation of muscle-derived strain to molecular cues used by cells to remodel skeletal tissue (Carter & Beaupre, 2007). Changes in such internal forces often mirror changes in the shape of developing bones (Currey, 2002). Thus, we compared carapace shape across the ontogeny of *Kinixys erosa*. We also explored the size dependence and consistency of plastic developmental changes during hinge differentiation in other *Kinixys* species. Lastly, we examined the *ex vivo* movement and external anatomy of the hinge to validate previous descriptions and further clarify the developmental origins of this complex phenotype.

2 | MATERIALS AND METHODS

2.1 | Anatomical and functional comparisons

Anatomical illustrations of the carapacial hinge and associated bones and sutures were based on observations of prepared skeletons and x-rayed specimens of representative *Kinixys* species. *Kinixys erosa* was selected as a focal species because it was one of the most abundant *Kinixys* representatives that was accessible in museum collections (Table 1), but also because the most hatchlings ($N = 7$) and juveniles ($N = 13$) could be examined. Also, *K. erosa* is the largest member of the genus and exhibits the most developed carapacial hinge (Broadley, 1997). The gross anatomy of the carapace was examined in dry skeletal preparations, including specimens housed at the Museum of Natural History in Vienna (NMW) which Siebenrock (1916) used in the first detailed anatomical depiction of *K. erosa*. Skeletal preparations of *K. erosa* from the herpetological collection of the Smithsonian National Museum of Natural History (USNM) were also externally inspected and an ethanol-preserved juvenile specimen was x-rayed by the curatorial staff. Further observations were made on dry skeletal preparations from other museums (see Section 2.2 below). The *ex vivo* movements

TABLE 1 Summary of *Kinixys* spp. specimens examined in this study

Species	Ecomorph	Hatchlings		Juveniles		Adults	
		N	SCL range (mm)	N	SCL range (mm)	N	SCL range (mm)
Kinixys erosa	Forest	7	44.0–55.0	13	67.0–90.0	11	106.0–280.0
Kinixys homeana	Forest	2	48.0–53.0	6	56.0–92.0	16	101.0–200.0
Kinixys belliana	Savannah	1	55.0	2	88.0–98.0	42	110.0–219.0
Kinixys lobatsiana	Savannah			4	70.8–100.0	59	105.0–201.7
Kinixys natalensis	Savannah			4	71.2–98	5	104.2–136.6
Kinixys nogueyi	Savannah	1	49.0	3	65.0–100.0	15	104.0–186.0
Kinixys spekii	Savannah			4	83.8–95.3	38	101.0–178.2
Kinixys zombensis	Savannah			6	66.7–96.6	34	102.0–224.0

Note: Sample sizes per age class and ranges for the straight-line carapace length (SCL) of specimens are shown.

of the carapacial hinge and pelvic girdle, see Shah (1960), were evaluated in a partially decomposed specimen from the Iowa State University herpetological collection that was labeled as *K. belliana*.

2.2 | Quantification of shell size and shape

Landmark-based geometric morphometrics was employed to quantify the two-dimensional shape of the lateral carapace in 273 individuals representing all presently recognized *Kinixys* species (Rhodin et al., 2021). The carapace was photographed in preserved specimens ($N = 58$ dry shells; $N = 135$ ethanol-preserved specimens) from the following collections: Zoologisches Forschungsmuseum Alexander Koenig (ZFMK), Bonn; Center of Natural History (CeNak), Hamburg; Bavarian State Collection of Zoology (ZSM), Munich; Natural History Museum of Berlin (ZMB), Berlin; Natural History Museum (NHMUK [previously BMNH]), London; Museum of Zoology, Senckenberg Dresden (MTD); Senckenberg Museum Frankfurt (SMF); Ditsong National Museum of Natural History (DNMNH), Pretoria; and KwaZulu-Natal Museum (NMSA), Pietermaritzburg. In addition, live individuals ($N = 80$) were photographed in the field. Individuals with malformations and specimens that were damaged were excluded from the analyses.

Fieldwork in South Africa was performed under the following national and provincial permits: Limpopo: ZA/LP/80202, ZA/LP/91608, ZA/LP/99439; Northwest Province: NW6124/10/2018; Gauteng: CPF6-0210; KwaZulu Natal: OP 139/2017, OP 3529/2019; and Mpumalanga: MBP5660. Using digital calipers, the maximum straight-line carapace length (SCL) was measured to the nearest 1 mm in live and museum-preserved specimens. Selecting a size threshold to categorize juvenile and adult age classes is challenging owing to interspecific differences in the time to, or size at, reproductive maturity. For the purposes of this study, individuals that displayed umbilical scarring (≤ 55 mm SCL) were considered hatchlings, while those at 55–100 mm SCL were treated as juveniles. Tortoises exceeding an SCL of 100 mm were mostly classified as adults because, at this size, secondary sexual characteristics are generally recognizable across *Kinixys* species (Broadley, 1993; Lawson, 2001). Both sexes develop a carapacial hinge and sexually dimorphic differences might be mainly confined to the morphology of the plastron and tail (Broadley, 1993; Lawson, 2001). Although adult females tend to feature a more rounded carapace (dorsally) relative to males, this difference was likely negligible in analyses on the lateral carapace. Also, many juveniles and dry adult shells could not be confidently sexed, thus female-specific differences related to hinge movement during oviposition could not be investigated.

Using TPSDIG (Rohlf, 2015), 28 landmarks were digitized onto photographs (Figure 1a). All photographs contained a scale for reference and specimens were maintained in the same plane of orientation to reduce distortion. The landmarks were generally placed on the sulci of the pleural, marginal, and vertebral scutes of the carapace (Figure 1a). In younger individuals, landmarks were placed on the sulci of marginals 7–8 and pleurals 2–3, such that the expected ontogenetic transformation of this region was adequately sampled. These adjacent landmarks were not in contact with each other and were expected to shift their positions relative to one another during hinge differentiation. This ontogenetic shift was expected to be less prominent in small-bodied species in which the carapacial hinge is often confined to the peripheral bones and marginal scutes (Boycott & Jacobsen, 1988; Broadley, 1997). The landmark configuration was also expected to capture interspecific shape
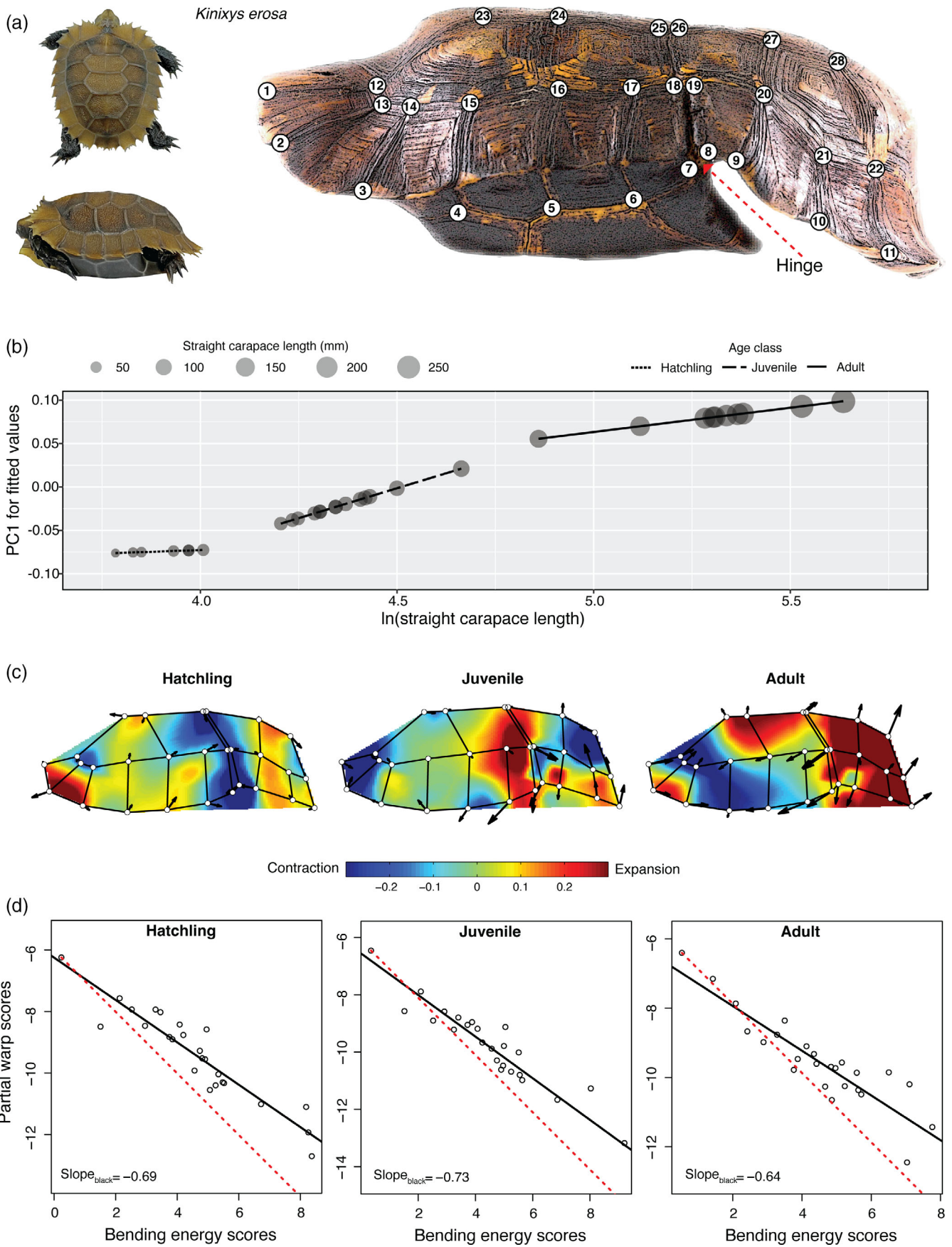
FIGURE 1 Legend on next page.

differences associated with the flaring, downward curvature, and serration of the marginal scutes, as well as the protuberance of vertebral scutes 4–5 in the forest-dwelling species *K. erosa* and *K. homeana* (Broadley, 1993; Ernst & Barbour, 1989).

The generalized Procrustes procedure was performed to remove non-shape variation (Gower, 1975). Using the GEOMORPH R package (Baken et al., 2021), landmarks were superimposed and translated to a shared origin. Landmarks were rotated to minimize the sums-of-squares differences among landmarks and rescaled to units of centroid size (CS). We chose SCL, rather than CS, to represent size because it is commonly used to categorize turtles by age class or sex, thus rendering our analyses comparable to previous and future studies on *Kinixys*. Nonetheless, SCL and CS were highly correlated ($r = .96$). Aligned Procrustes coordinates represented shape variation after orthogonal projection into a linear tangent space. The Procrustes-aligned coordinates were used as the response variable in statistical analyses on shape. Prior to analyses, the Procrustes distance of each specimen from the mean shape was graphically inspected and outliers were removed if they were 1.5 times greater than the upper quartile.

2.3 | Local shell shape variation in *K. erosa*

Kinixys erosa was used as a model to examine localized shape changes across ontogeny in hatchlings (SCL: 44–55 mm), juveniles (SCL: 67–90 mm), and adults (SCL: 129–280 mm) (Table 1). Five of the ten adult *K. erosa* were dry shell preparations, whereas the remaining specimens were ethanol-preserved. Morphological integration was expected to be high in hatchlings, as they lack the hinge that otherwise structurally divides the carapace in juveniles and adults (Broadley, 1997). To test this prediction, global integration tests were conducted by computing the slope of the log-variance of partial warp scores and their bending energy (Bookstein, 2015). This was performed via the globalIntegration function of GEOMORPH. Slopes between 0 and -1.0 indicate weak integration, whereas slopes equal to -1.0 indicate self-similarity

across the landmarks (Cardini, 2019). Modularity tests were not performed because the number of landmarks ($N = 28$) far exceeded specimen sample sizes ($N = 7–13$) per age class. Under such a scenario, even modularity tests with a low type-I error, such as the covariance ratio of Adams and Collyer (2016), are somewhat underpowered (Adams & Collyer, 2019). Otherwise, the global integration test is robust to variation in sample sizes while accounting for different spatial scales of landmark configurations (Bookstein, 2015; Cardini, 2019).

Because shape variation was expected to be highly localized to the area of the carapacial hinge, it was important to consider potential interpretation biases based on the displacement of a subset of landmarks after Procrustes superimposition, that is, the "Pinocchio effect" (discussed in Klingenberg, 2013). To account for this, differences among age classes were graphically inspected by modeling local shape variation in the LORY program (Márquez et al., 2012). LORY estimates local shape differences relative to the Procrustes mean shape of the data set, while defining local shape as infinitesimal differences mapped continuously over the landmarks (Márquez et al., 2012). Thin-plate splines visualizations are typically used to interpolate shape across the sampled landmarks by computing vectors that represent the magnitude of shape change relative to the overall Procrustes mean (Klingenberg, 2013). A potential limitation of this approach is that landmark vectors model shape changes at a single point without accounting for the surrounding space (Márquez et al., 2012). To address this, LORY computes the Jacobian matrix of the interpolation function used in the computation of the thin-plate spline.

Jacobian matrices provide information on the local shape change of the sampled specimen (Márquez et al., 2012). The determinants of the Jacobian matrices are used because they convey information on the size of the area (fields) that has undergone contraction or expansion. These fields were depicted as heat maps (i.e., parrot plots) wherein the intensity of red hues corresponded to the magnitude of tissue expansion. The intensity of blue hues corresponded to the magnitude of contraction, while green hues indicate invariant regions (Márquez et al., 2012). Parrot plots and their corresponding thin-

FIGURE 1 (a) Lateral and dorsal views (left panels) of a hatchling forest hinge-back tortoise, *Kinixys erosa*, which lacks the characteristic carapacial hinge that later defines the shell morphology of adults (right panel). Landmarks used in geometric morphometric analyses are shown (lateral view). (b) Differences in shell shape allometric trajectories of hatchlings, juveniles, and adults of *K. erosa*. Shown is the first axis of a principal component (PC1) of fitted values from a linear model of shape against straight-line carapace length. (c) Parrot plots of localized shell shape change (relative to the overall sample mean) for each age class. The color gradient represents the base-2 logarithm of Jacobian determinants from thin-plate spline functions. Note that juvenile and adult specimens exhibit localized expansion near the hinge region. (d) Global integration tests on landmark configurations for each age class. Black solid lines represent the observed slopes, whereas red dashed lines with slopes of -1 represent the hypothesis of self-similarity of the landmark configuration

plate splines were inspected to describe the location and magnitude of ontogenetic shape changes associated with the development of the carapacial hinge in *K. erosa*.

To test the effect of size (44–280 mm SCL) on shape, a nonparametric multivariate analysis of variance (NP-MANOVA on 1,000 permutations) was evaluated using the procD.lm function of GEOMORPH. Age class was the categorical predictor and ln-transformed SCL was the covariate in the model. Significance was assessed using the randomized residual permutation procedure (Collyer et al., 2015). Pairwise comparisons of slope vector lengths for age class were conducted and effect sizes (Z scores) were compared using the pairwise function of GEOMORPH.

2.4 | Interspecific shell shape differences across *Kinixys*

Interspecific shell shape differences across the ontogeny (all age classes: 44–280 mm SCL; Table 1) of all *Kinixys* species, including *K. erosa*, was also tested with the procD.lm function. Shape was the response, ln-transformed SCL was the covariate, and species was the categorical predictor. Whether individuals were preserved museum specimens (dry or ethanol-preserved) or live individuals was entered as a random factor. Slope vector lengths for species were compared. Ontogenetic shell shape trajectories were visualized by plotting size (SCL) against the first axis of a principal component analysis (PCA) on the fitted values of the aforementioned regression models (Adams & Nistri, 2010). To further explore interspecific differences in allometry, regression scores were also plotted against SCL (Drake & Klingenberg, 2008). These visualization methods were preferred because they describe allometry based on the same linear models that were used to test age class or interspecific differences (Adams et al., 2013).

Interspecific mean shape differences in adults (>100 mm SCL; Table 1) were tested with NP-MANOVA, as described above, and shape was graphically compared with thin-plate splines. Adult shape variation was initially explored via a phylogenetic PCA on a pruned topology from Kindler et al. (2012) with branch lengths adjusted according to the age of the oldest known *Kinixys* fossil (ca., 19–20 Ma; Lapparent de Broin, 2000; Meylan & Auffenberg, 1986). Using the physignal function, the phylogenetic signal was determined to be weak (Blomberg's $K = 0.059$; $p = .849$). Thus, a nonphylogenetic PCA was used to examine interspecific shape differences in adults. To account for size differences that may remain after Procrustes superimposition, this PCA was performed on the residuals of a linear model wherein SCL was the covariate (Klingenberg, 2016).

3 | RESULTS

## 3.1 | Localized shell shape changes in the ontogeny of *K. erosa*

Shell shape covaried with size (i.e., SCL; MANOVA: $F_{1,30} = 24.89$; $Z = 5.16$; $p <.0001$), with unique allometric trajectories for age classes of *K. erosa* (SCL by age class interaction: $F_{1,30} = 1.36$; $Z = 1.03$; $p = .02$; Figure 1b). The mean shape in hatchlings differed from juveniles ($Z = 2.20$, $p = .02$) and adults ($Z = 2.63$, $p = .01$). The region where the hinge is expected to eventually develop was contracted in hatchlings (Figure 1c). This corresponding region in juveniles and adults exhibited expansion (Figure 1c). The posterior-most carapace displayed expansion in adults (Figure 1c), while the anterior-most carapace exhibited expansion in hatchlings (Figure 1c). Global shape integration was highest in juveniles and lowest in adults (Figure 1d).

3.2 | Ontogenetic shape change across *Kinixys*

Shell shape covaried with size across the ontogeny of *Kinixys* species (MANOVA: $F_{1,272} = 81.99$; $Z = 9.13$; $p <.0001$) (Figure 2a). A comparison to a (full) model with a SCL by species interaction term indicated that species did not share a common allometric trajectory ($F_{1,7} = 2.28$; $Z = 4.91$; $p <.0001$). Although there was an effect of species on shell shape variation across ontogeny (MANOVA: $F_{7,272} = 2.28$; $Z = 4.91$; $p <.0001$), pairwise differences of species slope vector lengths were minimal ($Z = -1.128$–0.304, $p >.31$; Figure 2a). All species exhibited expansion of the carapace along with the region where the carapacial hinge was expected to develop in adults (Figure 2b,c).

3.3 | Interspecific differences in adult shape

The Procrustes mean of carapace shape differed across species of adult *Kinixys* (MANOVA: $F_{7,219} = 11.7$; $Z = 13.3$; $p <.0001$). The forest species, *K. erosa* and *K. homeana*, displayed the greatest mean shape differences when compared to the remaining species (Table 2; Figure 3a). Interspecific variation was related to the flaring of the anterior marginal scutes and curvature of the posterior marginals (Figure 3a). With some exceptions, savannah species generally resembled each other (Table 2, Figure 3a). Unlike in savannah species, the shape of the carapace in forest
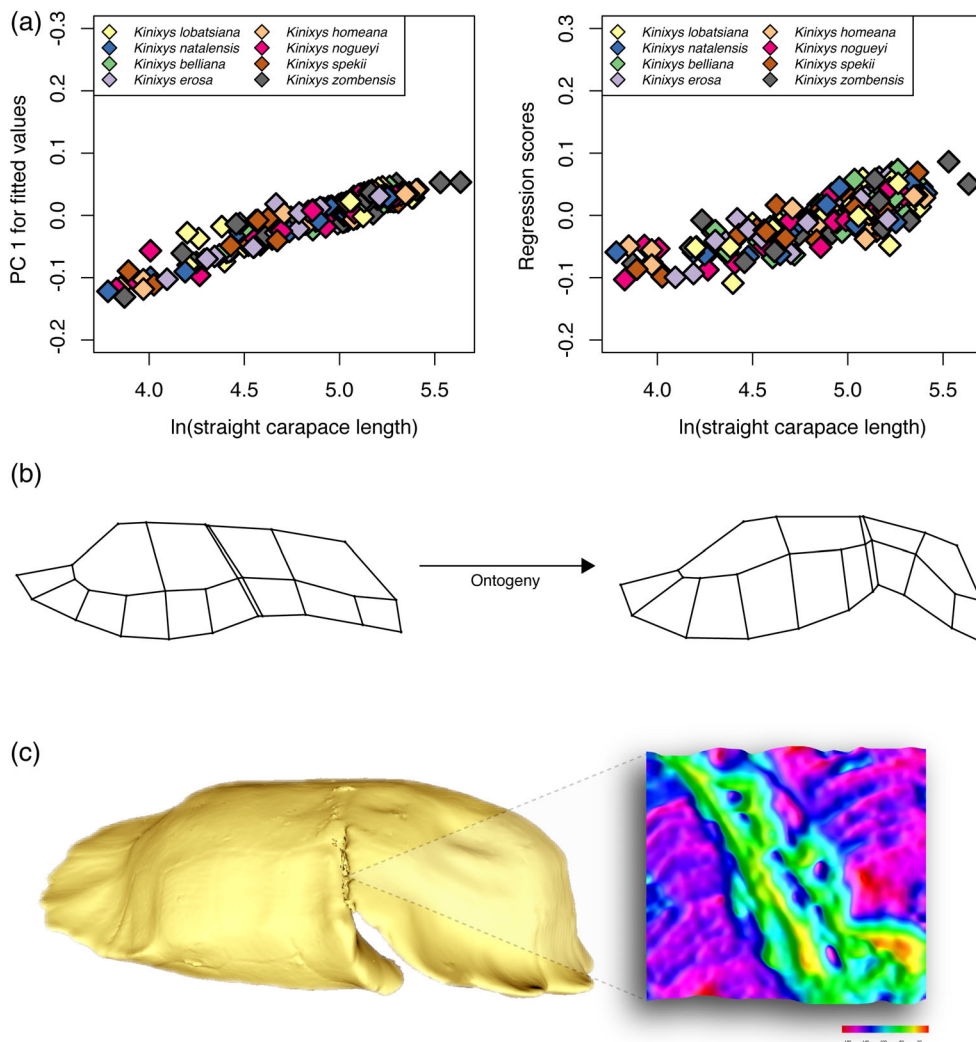
FIGURE 2 (a) Shell shape allometric trajectories of *Kinixys* species based on the first axis of a PCA (PC1 shown) of fitted values (left) and regression scores (right) of a linear model wherein straight-line carapace length (SCL) was the covariate. (b) Wireframes depict shell shape changes (relative to the overall mean) in the smallest hatchling specimen (left; 44 mm SCL) versus the largest adult specimen (right; 280 mm SCL) in the data set. The most striking shape change observed consistently across species coincided with the hinge region. (c) A surface plot describes the roughness and topology after carapace tissue underwent remodeling during differentiation of the hinge. The color gradient bar indicates distance from the surface

TABLE 2 Statistically significant ($p < .05$) pairwise tests on Procrustes mean differences in the carapace shape of adult *Kinixys*

Species contrast	Pairwise absolute difference	UCL (95%)	*Z*
Kinixys homeana—Kinixys lobatsiana	0.0006	0.0005	3.1033
Kinixys homeana—Kinixys zombensis	0.0007	0.0005	3.0468
Kinixys lobatsiana—Kinixys nogueyi	0.0008	0.0006	3.0265
Kinixys nogueyi—Kinixys zombensis	0.0008	0.0006	2.9806
Kinixys erosa—Kinixys nogueyi	0.0008	0.0007	2.5036
Kinixys erosa—Kinixys homeana	0.0006	0.0006	2.4590
Kinixys belliana—Kinixys nogueyi	0.0007	0.0006	2.1115
Kinixys spekii—Kinixys zombensis	0.0005	0.0005	2.0289
Kinixys lobatsiana—Kinixys spekii	0.0004	0.0004	2.0107
Kinixys belliana—Kinixys homeana	0.0005	0.0005	1.9492

Abbreviation: UCL, upper confidence level.

species was elongated and defined by a sharp protuberance (dorsally) on the posterior carapace, particularly in *K. homeana* (Figure 3a). The expansion of the hinge region was most prominent in the forest species (Figure 3a). This pronounced expansion, along with the sharp downward curvature of the posterior marginals, was also predicted by an analysis on size-corrected shape (Figure 3b).

FIGURE 3 (a) Wireframes describe the mean shape in adults (>100 mm straight-line carapace length) relative to the sample mean; * = species with relatively underdeveloped carapacial hinges.
(b) Interspecific carapace shape differences (adjusted for size) in adults are represented by a principal component analysis (PCA) on the residuals of a linear model wherein straight-line carapace length was the covariate. Wireframes describe the predicted size-adjusted shape, relative to the sample mean, at extremes of the PC axes
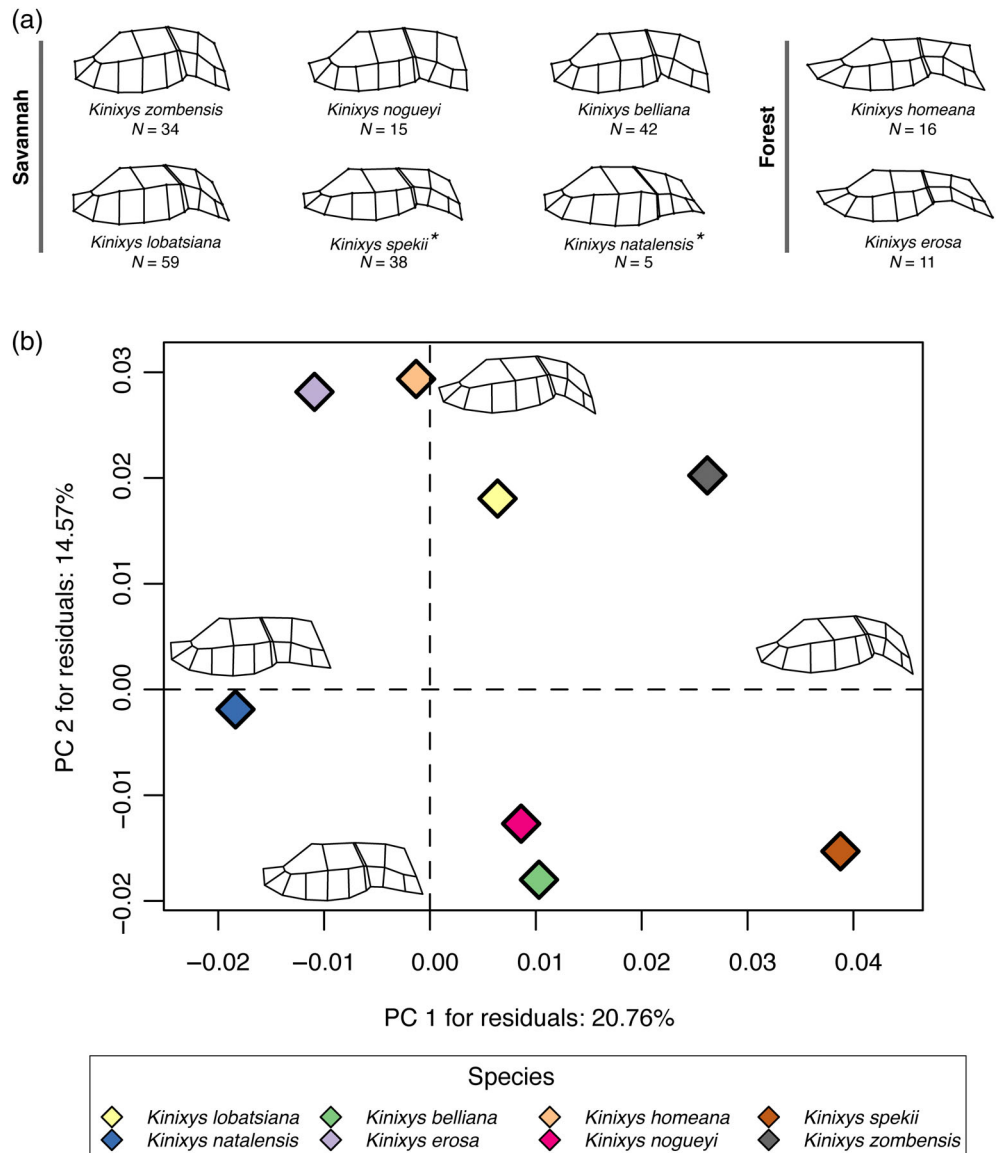
3.4 | Anatomical transformations and functional correlates

In juveniles, the sulci of marginal and pleural scutes remain intact and underlying peripheral bones 7–8, costal bones 4–5, and thoracic vertebrae exhibit a normal skeletal ossification pattern (Figure 4a,b). In individuals at the juvenile-adult transition (SCL 95–105), early signs of skeletal repatterning at the presumptive hinge region (peripherals 7–8) were evident while scutes remained externally intact (Figure 4c). In mature adults, the corresponding scute sulci and bone sutures are transformed and are lined by thick connective tissue that facilitates movement of the posterior-most shell elements (Figure 5a,b). Marginal scutes 7–8 become smaller, relative to other marginals, and their sulci are spatially aligned with the sutures of peripheral bones 7–8. Pleural

scutes 2–3 achieve partial alignment with the sutures of costals 4–5 (Figure 5b). Vertebral scutes do not undergo remodeling but remain somewhat pliable as costal bones 4–5 undergo dorsoventral flexion and peripheral bone 8 is displaced over peripheral bone 7 during shell closure (Figure 5b,c). Moreover, the pelvis rotates along its anterior–posterior axis as hind limbs are retracted and the shell is closed (Figure 6a). A summary of transformations associated with peripheral bones 7 and 8 and how they relate to the differentiation and function of carapacial kinesis is depicted in Figure 6b,c.

4 | DISCUSSION

Our study describes how the unique carapacial hinge of African hinge-back tortoises (Kinixys) emerges via
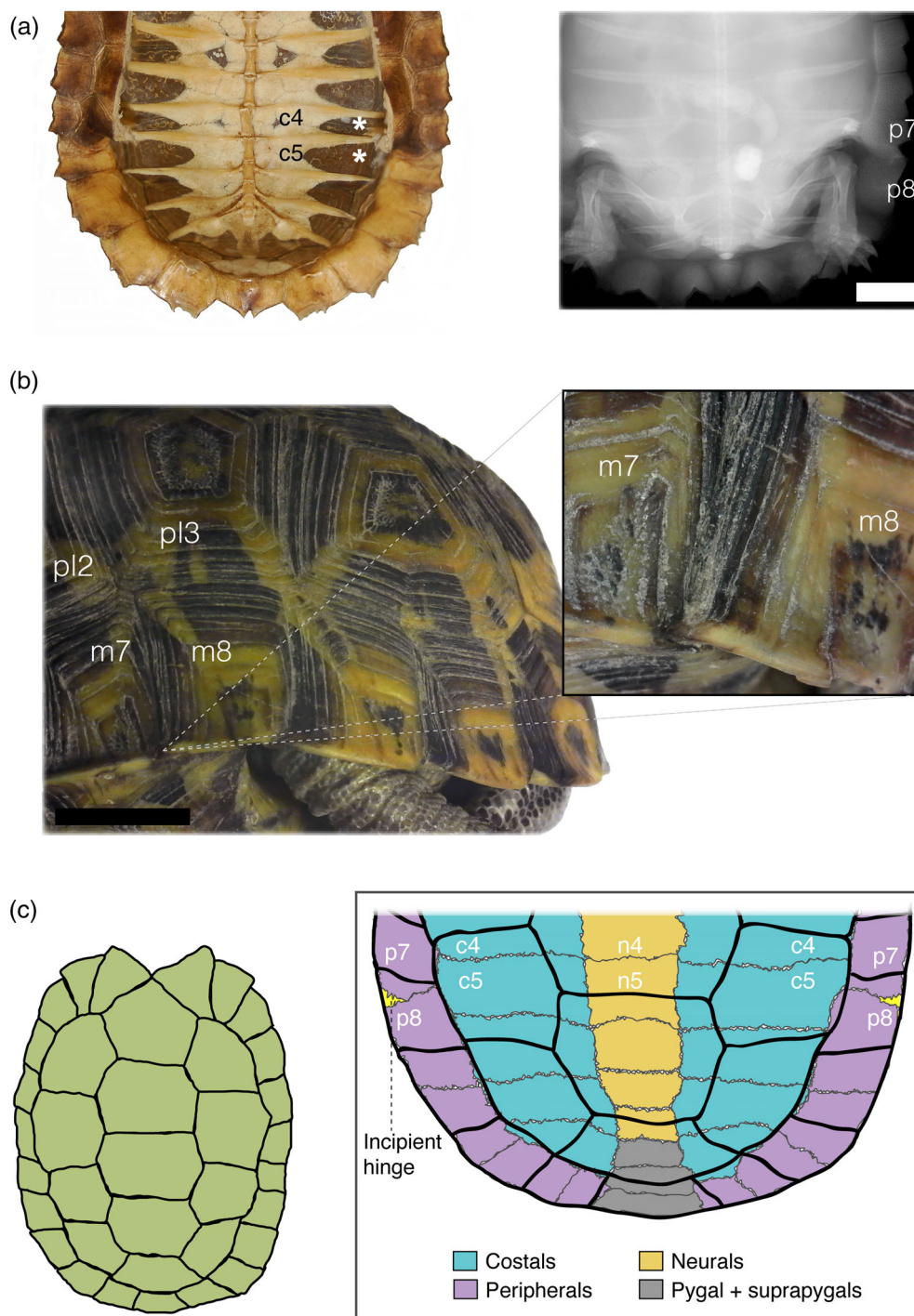
FIGURE 4 (a) In smaller juveniles (56–85 straight-line carapace length), the region of the incipient hinge exhibits the typical condition for tortoises: Carapacial fontanelles can be observed (see asterisks on a *Kinixys belliana* specimen) and peripheral (p) bones 7–8 exhibit normal ossification and suturing (*K. erosa* in right panel); scale bars = 10 mm. (b) Moreover, the sulci of pleural (pl) 2–3 and marginal (m) 7–8 scutes remain intact. (c) By the time 105 mm straight-line carapace length is reached, all carapace scutes remain externally intact (left), while the onset of skeletal repatterning at the p7–p8 suture, that is, the incipient carapacial hinge, can be observed (right panel). At this ontogenetic interval, the scute sulci (black lines) are not aligned with p7–p8 or costal (c4–5) sutures that are eventually remodeled in larger adults

progressive and localized shell shape changes. The delayed differentiation of the carapacial hinge, as well as ontogenetic shifts in morphological integration, were congruent with distinct anterior and posterior functional modules of the carapace. Based on *ex vivo* movement and raw anatomy, we propose that *Kinixys* employs a "sliding hinge" shell-closing system that overcomes thoracic rigidity and thus optimizes the protective capacity of the carapace. These findings exemplify how inherent properties of the skeletal system (plasticity, modularity, and secondary maturation processes) contribute to the origins of novel form-to-function relationships, even in clades with low rates of morphological evolution, such as Testudines.

4.1 | Localized and delayed shell shape changes in the ontogeny of *K. erosa*

D'Arcy Thompson (1945, pp. 517–519) proposed that aberrant carapace phenotypes may arise if the

FIGURE 5 (a) The sulci of pleurals (pl) 2–3 and marginals (m) 7–8 are obliterated and separated by dense connective tissue once the carapacial hinge is differentiated in adults (*K. homeana* shown; scale bar = 10 mm). During retraction of head and limbs (top right panel: *K. lobatsiana*), dorsoventral flexion of the posterior carapace can be achieved (bottom right panel: *K. belliana*). (b) Sutures of neural (n) 4–5 and costal (c) 4–5 bones are often reduced, whereas those of p7–p8 tend to be entirely remodeled (black lines denote scute outlines). Scute sulci are not spatially aligned with sutures of n4–5, but may achieve partial alignment with those of c4–5 bones. Right panel: The p8 bone "slides" over the p7 bone (scutes m7–8 were removed for clarity). The p7 bone serves as a pivot point while structurally bridging the plastron (inguinal [ig] and femoral [fe] scutes shown). Vertebrals (v) 3–4 do not undergo remodeling but exhibit some structural flexibility during dorsoventral flexion of the costals (c4–5; left panel in c). (c—right panel) Dorsoventral flexion of the posterior carapacial lobe is facilitated by the displacement of p8 over p7 once the carapacial hinge is fully developed and functionally activated in adults
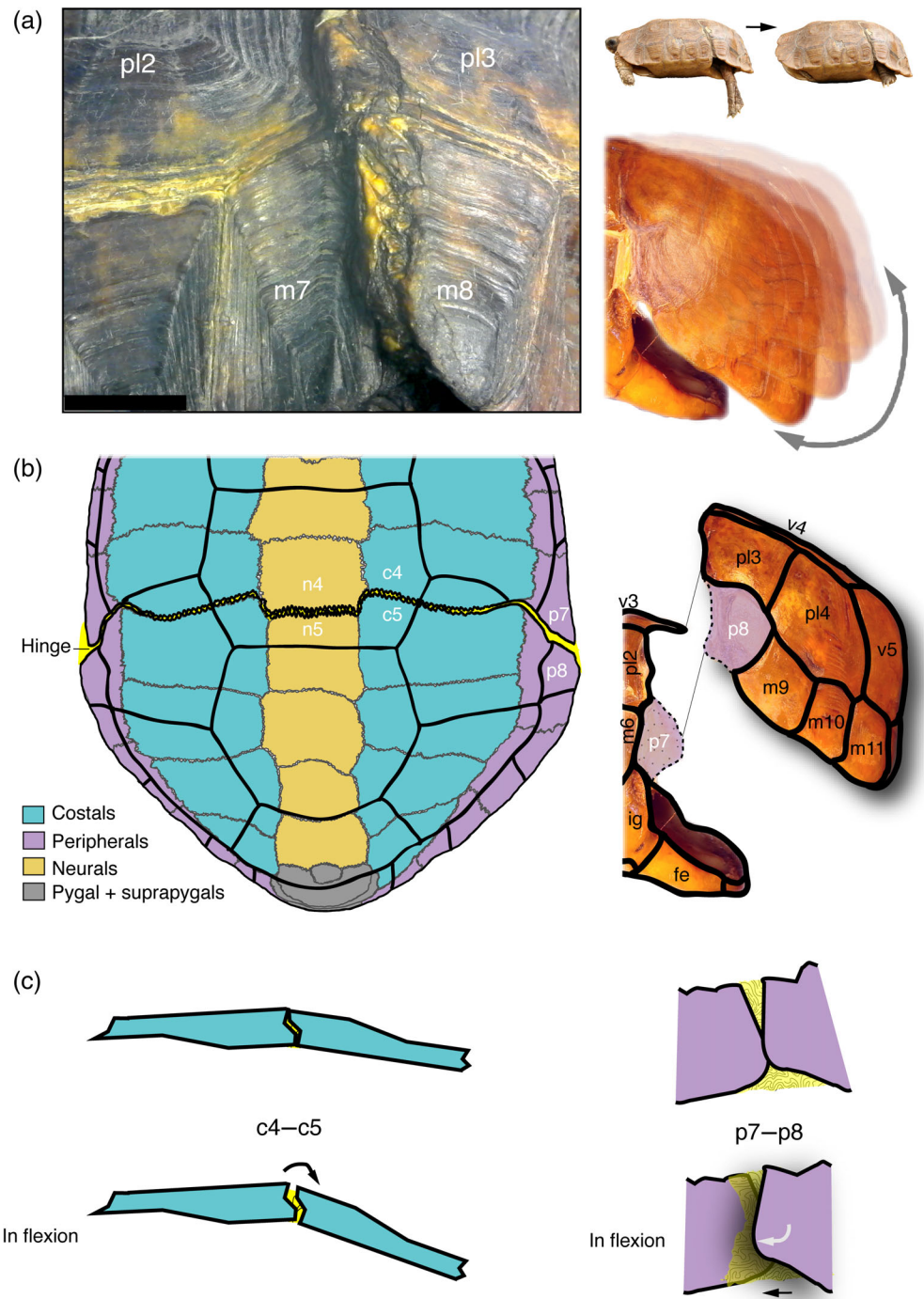
coordinated expansion of serially arranged developmental units is modified: "If the horny plates grow ever so little faster than the bones below, they will fail to fit, will overcrowd one another, and will be forced to bulge or wrinkle." The resulting phenotypes may be subject to natural selection, as supported by experimental and mathematical models on the genetic pathways that control the spatial configuration of carapace scutes in turtle embryos (Moustakas-Verho et al., 2014). The phenotypic effects of such heritable genetic changes may emerge much later in life, as exemplified by *Kinixys* and emydine

box turtles (Cordero et al., 2018; Cordero et al., 2019). The observed ossification pattern in juvenile *Kinixys* suggests that this clade retains the ground state condition (i.e., an incompletely ossified carapace) for Testudinidae at the onset of post-hatching development (see *Testudo*; Cherepanov, 2017). In general, ossification of the carapace is expected to be incomplete in hatchlings of most turtles (Cordero, 2021). Nonetheless, putative alterations in skeletal development that lead to hinge differentiation (see Figure 6) likely originate in embryos, but their phenotypic effects remain latent until reaching the body size
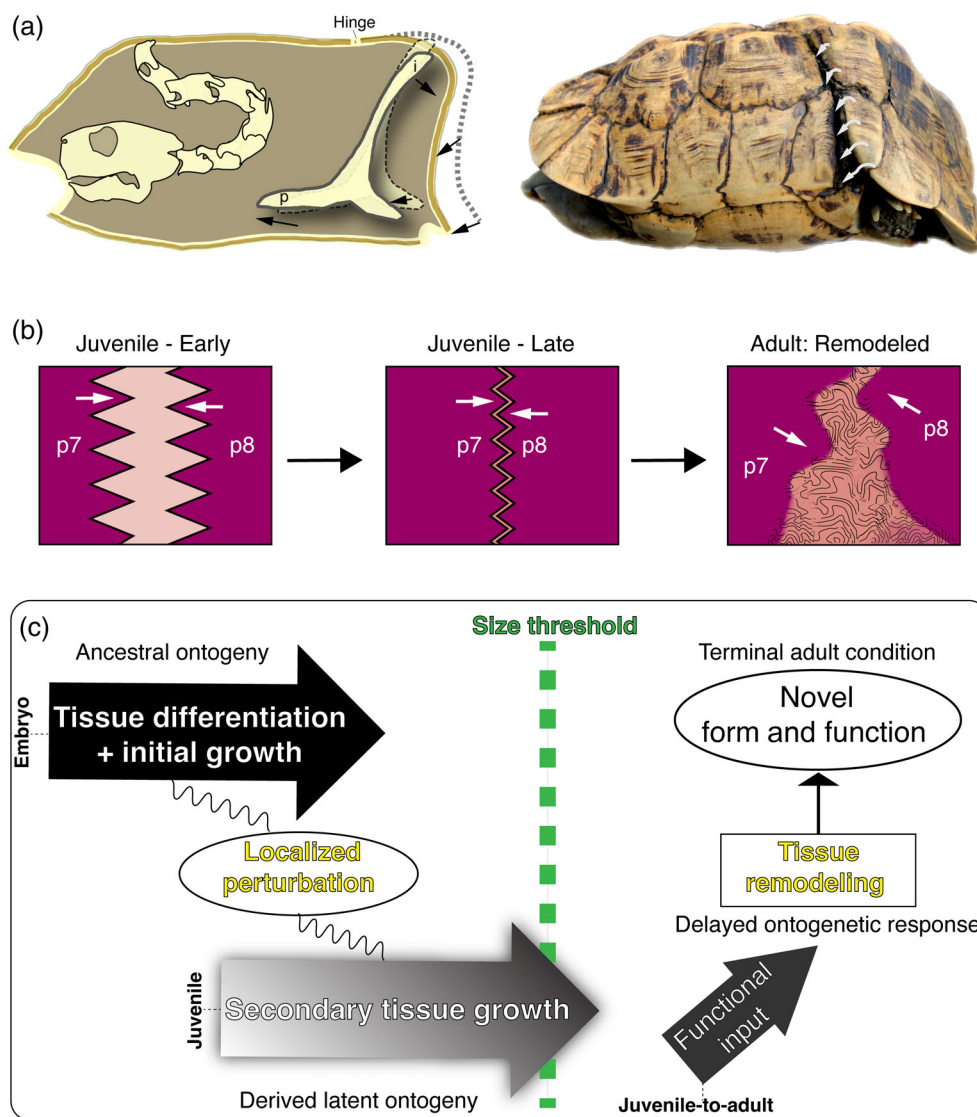
FIGURE 6 (a—left panel) Simplified diagram (in longitudinal view) of *Kinixys* during retraction (limbs not shown). Arrows point to the expected direction of movement of the pelvic girdle (see pubis [p] and ischium [i] bones), as well as the moveable component of the posterior carapace (see dashed lines). Externally (right panel), the hinge facilitates movement of posterior carapacial bones (plus scutes) (*K. zombensis* shown). (b) Summary of progressive transformations at the suture of peripheral (p) bones 7–8. In juveniles (left and central panels), p7–p8 bones grow toward each other (white arrows) and eventually interdigitate to form a typical suture. Following a change in the coordination of bone growth, this suture is secondarily remodeled to give rise to the carapacial hinge (right panel). (c) Hypothetical model of how heritable changes in early ontogeny, such as a localized perturbation, lead to delayed tissue-level responses that generate novel morphologies and functions once size constraints are overcome in ontogeny. Tissue alterations remain latent in ontogeny but gradually manifest if the proper functional input (e.g., behavior or muscle contraction) is applied, thereby facilitating secondary tissue remodeling (e.g., skeletal repatterning)

threshold at which internally derived mechanical stimuli may trigger skeletal repatterning, that is, bone resorption and connective tissue formation. Such tissue changes were supported by our analyses.

By focusing on *K. erosa*, we elucidated tissue expansion at or in the vicinity of the presumptive hinge. The highest slope of any global integration test suggested that tissue-tissue structural interactions are probably accelerated in juveniles. This is sensible because we observed

the first external signs of hinge differentiation at this life stage. In agreement, the rate of shell shape change (relative to body size) was greatest in juveniles. In captive *K. nogueyi*, external tissue changes related to hinge differentiation were observed 3 years after hatching (van Pelt & van Putten, 2006). Prior to the overgrowth of connective tissue, marginal scutes 7–8 undergo reduction and assume a more triangular shape, as supported by local deformation analyses on *K. erosa* and allometric shell

shape trajectories in other *Kinixys* species. The homologous region in Galapagos tortoises (Chiari & Claude, 2011), as well as in other tortoises of diverse body sizes (Dosik & Stayton, 2016), did not exhibit the same shape changes as *Kinixys*. Shape variation linked to skeletal repatterning in emydine turtles with plastral kinesis (see Cordero et al., 2019) was not as extensive as in *Kinixys*. Also, the only other tortoise species with a flexible carapace, *Malacochersus tornieri*, employs a different ontogenetic strategy. Specifically, *M. tornieri* exhibits a major slowdown in shell ossification, such that it develops a well fenestrated shell that is flexible but without a carapacial hinge (Mautner et al., 2017).

Localized shape variation in *K. erosa* could perhaps be explained by normal growth along the anterior–posterior axis (body elongation) (Zelditch et al., 2001). However, the expansion of the anterior carapace was restricted to a small area on marginal scutes 1–2, while other patterns of expansion beyond the hinge region were primarily confined to the posterior carapace: Marginal scutes 10–11, vertebrals 4–5, and pleurals 2–4. We interpret this as being related to the emergence of a functional (kinetic) module that is demarcated by the well-developed hinge in adults of *K. erosa*. Accordingly, adults featured the lowest slope for global integration tests. This modularity hypothesis will need to be revisited via histological assays and comparisons to outgroup taxa (e.g., *Centrochelys*) that share a recent common ancestor with *Kinixys*. Also, shape ontogenetic change in *Kinixys* could be further evaluated with different landmark configurations and larger sample sizes that are balanced across ontogeny and sexes, such that within-module integration and potential measurement error related to the position of the hinge is better understood. Potential sexually-dimorphic differences related to oviposition could also be explored, as the dorsal carapace appears to be broader in females and the passing of large eggs might be facilitated by carapacial kinesis. Overall, our qualitative anatomical comparisons corroborated morphometric models that predicted the expansion and distortion of the incipient hinge as a consequence of major tissue transformations, including suture remodeling, as kinesis is gradually acquired in ontogeny.

4.2 | Body size, repatterning, and functional correlates of carapacial kinesis

As in emydine box turtles (Bramble, 1974; Cordero et al., 2019), hinge differentiation depends on body size in *Kinixys*. Adults of *K. erosa* are the largest in the genus and display a highly developed hinge with extensive fibrous connective tissue that intersects the sutures of peripherals 7–8, costals 4–5, and even neural bones 4–5 in the largest individuals (Broadley, 1993, 1997). By contrast, the carapacial hinge in *K. natalensis*, the smallest species in the genus, is confined to peripherals 7–8 (Boycott & Jacobsen, 1988; Broadley, 1997), which is similar to smaller adults of the larger *Kinixys* species. This size dependence may be explained as follows: (a) The mechanical strain exerted on the carapace by hindlimb retraction scales positively with body size in turtles (Aiello et al., 2013; Fish & Stayton, 2014), (b) As shell size increases, internal space to accommodate the limbs and head might also increase during retraction (Cordero et al., 2019), thereby augmenting the mechanical strain transferred to the carapacial buttresses (Polly et al., 2016); and (c) Disproportionate and redirected growth of peripheral 7 may further stimulate suture degeneration as body size increases.

The unusual size of peripheral bone 7, as first depicted by Siebenrock (1916), might be critical to the development and function of the carapacial hinge in *Kinixys*. Peripheral 7 comprises the posterior carapacial buttress and appears to act as pivot point over which peripheral 8 slides over during hinge rotation. Growth of peripheral 7 appears to be deflected similar to a plate-buckling process (Cowin, 2004). Indeed, mechanical instability (i.e., buckling) or stress can directly generate novel structures during skeletal pattern formation (Cowin, 2004; Love & Wagner, 2022; Newman & Müller, 2001). This likely triggers a skeletal tissue repair response (Nomura & Takano-Yamamoto, 2000), thus explaining the proliferation of fibrous connective tissue within the hinge joint that discretely defines anterior (static) and posterior (kinetic) morphofunctional modules in *Kinixys*. Because joint development typically involves the transduction of mechanical stress to molecular signals used by cells to repattern skeletal tissue (Carter & Beaupre, 2007), we hypothesize that hinge maturation in *Kinixys* is a response to mechanical instability during post-hatching development.

Defensive behaviors may also reinforce hinge differentiation as interactions with predators and conspecifics increase as kinetic-shelled turtles mature, for example, *Kinosternon* (Peno et al., 2016). In box turtles (*Terrapene*), hindlimb retraction during shell closure generates a "pinch" force that increases from 2 kg in juveniles to 4 kg in adults (Preston et al., 2020). Crucially, a similar force may act as the intermittent mechanical stimulus required to sustain hinge joint formation as carapace bone sutures are remodeled in *Kinixys*. Changes in static forces associated with the altered growth trajectory of individual bones, as well as muscle-derived strain during shell closure, may collectively promote the emergence of the unique "sliding hinge" shell-closing system in *Kinixys*.

The carapacial hinge in *Kinixys* involves the partial displacement, via fibrous joint tissue, of peripheral bone 8 over the exterior surface of peripheral bone 7, while costal bone 5 rotates about its articulation with costal bone 4. In all other kinetic shelled species, adjacent plastral bones are not displaced over one another when the hinge joint undergoes flexion, for example, *Cuora*, *Emys*, *Terrapene*, *Kinosternon*, *Sternotherus*, and *Pelusios* (Bramble, 1974; Bramble et al., 1984; Bramble & Hutchison, 1981; Cordero et al., 2018; Cordero et al., 2019; Shah, 1960). By describing morphological transformations associated with carapacial hinge differentiation in *Kinixys*, we provide a foundation for future work on this unusual phenotype. Already, the capacity for shell bone microstructures to respond to mechanical stressors has been demonstrated in *K. erosa* (Ampaw et al., 2019), though the gradual histological changes that give rise to the carapacial hinge await description.

The role of pelvic and hindlimb movement and related muscle contractile forces warrants further examination in *Kinixys*. Our *ex vivo* analyses did substantiate that hinge rotation occurs in concert with movement of the pelvic girdle as hind limbs are retracted (Shah, 1960). However, the potential role of the neck-retracting muscles, see Shah (1960), and the connective tissue components that transfer muscle strain to the presumptive hinge region remain obscure. Describing how thoracic vertebrae adjacent to neural bones 4–5 rotate together with the carapacial hinge is also of interest because it would clarify how structural limitations imposed by a highly rigid (shelled) body plan are moderated in turtle evolution (Cordero & Quinteros, 2015; Cordero & Vlachos, 2021). Our study corroborated that carapacial bones may acquire some movement capacity even if their sutures are not entirely aligned with boundaries of the overlying scutes, which is a key difference between carapacial kinesis and plastral kinesis. Furthermore, we demonstrated that the process of skeletal repatterning begins before the marginal scutes undergo size reduction and their sulci achieve alignment with the sutures of underlying bones.

4.3 | Broader evolutionary implications of skeletal repatterning in development

The turtle's shell is far from an inert tissue composite that solely serves as a shield. Shell tissue is highly dynamic and responds physiologically, neurologically, and mechanically to internal and external stimuli (Fish & Stayton, 2014; Polly et al., 2016; Rosenberg, 1980, 1986; Sarnat & McNabb, 1981). In *Kinixys*, an interesting hypothesis to test is whether hinge development involves the deployment of the canonical skeletal tissue repair program of vertebrate animals. Turtles do exhibit impressive regenerative properties via skeletal repatterning (reviewed in Kuchling, 1999). Additional research on the process of skeletal tissue degeneration and reconstruction is necessary in turtles and reptiles in general. The capacity for skeletal tissue to respond to changes in the internal and external environment is an inherent property of the skeletal system of vertebrates (Hall, 2015). For instance, muscle contraction is required for proper jaw development in some fishes (Hu & Albertson, 2017), which may be considered a form of epigenetic tissue remodeling (Young & Badyaev, 2007). The proper orientation of toes is also determined by muscle contraction during the embryonic development of birds (Botelho et al., 2015). Moreover, pectoral girdle bones in mudskippers exhibit shape plasticity according to the locomotor style (aquatic versus terrestrial) employed by growing individuals (Standen et al., 2014).

Function-induced (plastic) skeletal transformations are considered a type of ancestral reaction norm that is widely employed as part of secondary pattern formation processes (Newman & Müller, 2001; Wagner, 2014). This may be adaptive if the functional trigger (see Figure 6c) is consistently applied to individuals and the phenotypic consequence (e.g., skeletal repatterning) is transmitted across generations (Wagner, 2014). The challenge then is to identify the heritable causal factors that elicit the delayed differentiation of skeletal traits. In addition, whether plastic skeletal responses can be genetically accommodated has long been debated (Waddington, 1957). Although much experimental work is needed in this area of animal developmental biology (Newman & Müller, 2001), describing skeletal repatterning as a reaction norm in the development of diverse species, such as *Kinixys*, is informative to future mechanistic studies. Altogether, we have shown that skeletal repatterning is integral to the origins of novel form-to-function relationships.

AUTHOR CONTRIBUTIONS
Gerardo A. Cordero: Conceptualization (lead); formal analysis (lead); investigation (lead); methodology (lead); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Melita Vamberger:** Data curation (supporting). **Uwe Fritz:** Conceptualization (supporting); writing – review and editing (supporting). **Flora Ihlow:** Conceptualization (equal); data curation (lead); methodology (equal); writing – original draft (equal); writing – review and editing (equal).

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