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Ontogeny and abnormalities of the tortoise carapace: a computer tomography and dissection study

Christina M. Farke[1] & Claudia Distler

Allgemeine Zoologie & Neurobiologie, Ruhr-Universität Bochum, Universitätsstr. 150, ND 7/31, 44780 Bochum, Germany
[1)] present address: Schildkrötenhilfe TestuDO, Rübenkamp 91, 44319 Dortmund

Corresponding author: Claudia Distler, e-mail: distler@neurobiologie.rub.de

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Abstract. Abnormalities and the postnatal development of the carapace were investigated in 106 captive tortoises (Testudinidae, *Testudo hermanni boettgeri, Testudo graeca, Testudo marginata, Testudo horsfieldii*) using computer tomography (CT) in live animals and/or dissection of preserved specimens. The carapace was reconstructed two-dimensionally through combining sectional images. The postnatal ossification was demonstrated in 3D CT images combined with dissection results. We found that abnormal numbers and arrangements of horny scutes and bony plates may occur independently at different locations and different ontogenetic stages. Abnormalities of the horny scutes are present at hatching when the bony plates are not yet fully formed. The temporal course of carapace ossification appears to be species-specific. We demonstrate that computer tomography is a non-invasive and convenient method suitable for studying abnormalities and the postnatal ossification process of the bony carapace, as well as for diagnostics in live chelonians. However, the resolution limit of the method will be reached in the case of very young or metabolically challenged subjects.

Key words. Computer tomography, tortoise, carapace, development, abnormality, raising conditions, *Testudo*.

Introduction

The chelonian shell is composed of a dorsal carapace and a ventral plastron connected by a lateral bridge (Bojanus 1819). Thoracic vertebrae, ribs, specialized dermal bones, the cleithrum and/or neural crest cells together form the carapace, whereas the plastron includes the clavicles, the interclavicle, and possibly derivates of the gastralia and neural crest cells (e.g., Gegenbaur 1898, Procter 1922, Goodrich 1930, Romer 1958, Clark et al. 2001, Lyson et al. 2013). The standard tortoise carapace consists of 49 bony plates, however, many species- and family-specific variations exist (for a comprehensive overview see Pritchard 2008). These bony components are covered by horny scutes: the nuchal scute (anteriorly), 5 vertebral and the suprapygal scutes (posteriorly), 4 pleural scutes, which border the vertebral scutes, and 11 marginal scutes (Procter 1922, Thomson 1932, Zangerl 1939, 1969, Loveridge & Williams 1957, Pritchard 2008).

The development of the turtle shell has been debated controversially for years (e.g., Gilbert et al. 2001, 2008, Cebra-Thomas et al. 2005, 2007, Moustakas 2008, Scheyer et al. 2008, Delfino et al. 2010, Hirasawa et al. 2013, Nagashima et al. 2012, 2013, Lyson et al. 2013). In the "emergentist" view, ontogenetic deviations, namely entering of rib precursors into the dermis and the formation of the carapacial ridge (CR) led to the lateral rather than ventral growth of the ribs, and by that to the inward displacement of the pectoral girdle relative to the ribs. This implies a sudden de novo evolution of the turtle bauplan without apparent intermediate states (for review Gilbert et al. 2008). By contrast, in the "transformationist" view, the turtle bauplan evolved gradually and is based on the fact that turtle ribs lack the ventral component and do not enter the lateral body wall. In the context of this hypothesis, marginal growth of the lateral domain leads to the formation of the CR, which in turn causes the body wall to fold inward. As a consequence, the shoulder girdle lies beneath the ribs (for reviews, see Nagashima et al. 2013, Rieppel 2013). This view is also supported by fossil evidence (e.g., Joyce et al. 2009, Lyson & Joyce 2012). It was suggested that the exact mechanisms of shell formation might differ between hard- and soft-shelled turtles. In a recent report (Nagashima et al. 2014), however, such species-specific differences were disclaimed, and the paracrine hypothesis of shell formation (e.g., Gilbert et al. 2001, 2008, Cebra-Thomas et al. 2005, 2007) was dismissed in favour of the folding theory (e.g., Nagashima et al. 2012, 2013, Hirasawa et al. 2013) thereby also implying a gradual as opposed to a saltatory evolution of chelonians.

The bony shell is incomplete in hatchlings. Ossification of the carapace starts at the neurals and will proceed

mediolaterally. The ribs grow by apical apposition, and the periosteal collar of the ribs acts as initiation centre for the ossification of the costal bones. During postnatal development, the non-ossified fontanels close and the peripheral plates connect to the nuchal and pygal plates. In the plastron, both the epi- and hyoplastra (anteriorly) and the hypo- and xiphiplastra (posteriorly) grow from lateral to medial. Complete ossification of the shell is reached at more than 1 year of age (Cheylan 1981, Cebra-Thomas et al. 2005, 2007).

Abnormalities of the horny scutes and bony plates have been described both from wild populations and captive breeding colonies. A comprehensive review of the literature concerning these deformities is provided by Rothschild et al. (2013). Suboptimal incubation conditions, partial drying, and temperature variations during the early stages of gestation have been proposed as being the primary causes of these abnormalities, but detrimental environmental influences, nutrition, humidity, disease, infections, and parasitic load have also been thought to cause shell pathologies (Frye 1991, Gabrisch & Zwart 2001, Wiesner & Iben 2003, Liesegang et al. 2007, Pritchard 2008, Rothschild et al. 2013).

Computer tomography (CT) has been applied in turtles to investigate lesions and trauma to the skeleton and for functional anatomical studies (e.g., McKlveen et al. 2000, Abou-Madi et al. 2001, 2004, Arencibia et al. 2006, Valente et al. 2007, Werneburg et al. 2014). In these studies, slice thickness varied from 0.6–5 mm, and specimens were scanned either in frontal or sagittal planes. In order to avoid detrimental movements of the animals during the examination, some authors strongly recommend that live reptiles be sedated for CT scans (Schildger et al. 1992, Stetter 2000, Werneburg et al. 2014), whereas other authors fix the limbs in the shell or close the shell with tape (Gumpenberger 1996, Gumpenberger & Hittmair 1997, Stetter 2000, Straub & Jurina 2001).

We investigated a large cohort of captive-bred live tortoises using computer tomography and supplemented this survey with dissections of preserved material to 1) detect abnormalities of bony plates of the carapace; 2) investigate the postnatal development of the bony carapace; and 3) evaluate the fitness of the CT technique for diagnosis and analysis of the influence of breeding conditions on ossification and formation of abnormalities in captive animals.

Material and methods

Animals

Altogether 106 tortoises (91 *Testudo hermanni boettgeri*; 8 *Testudo graeca marokkensis*; 5 *Testudo marginata*; 2 *Testudo horsfieldii*) (Fritz & Havaš 2007, van Dijk et al. 2012) originating from 3 private breeding colonies (A, H, and F) were investigated with authorization and monitoring by the local authorities. Accordingly, animals belonging to the different breeding groups are identified with the appropriate prefix and a number assigned to them in the course of an independent breeding scheme investigating the effects of different incubation parameters on the formation of abnormalities of the carapace. The project was approved by the local authorities (Umweltamt Stadt Dortmund), and carried out in accordance with the German Animal Welfare Act. In addition to the 77 tortoises investigated with CT, 31 tortoises were dissected post-mortem, two of which were subjected to both procedures. Most of the dissected specimens remain in the care of the authors. The species, age, abnormalities, manner of investigation, and parents (if known) of the animals included in this study are summarized in Table 1.

Husbandry conditions

Animals in colony A were housed in a 20 m^2 outdoor enclosure on lava rocks with grass and bushes and a 0.6 m^2 greenhouse with a basking lamp, juveniles were raised in a 1 m^2 outdoor enclosure attached to an unheated 1 m^2 greenhouse. The animals were fed twice a week with dried and fresh herbs, water was available ad libitum. Tortoises in colonies H and F were housed in outdoor enclosures (H: 45 m^2, F: 100 m^2) with natural soil, grass, and sand with free access to water and food consisting of natural dried and fresh herbs and, in the case of colony H, fruit and vegetable. In colony H, shelter was provided by unheated wooden boxes whereas tortoises in colony F had free access to a 7.2 m^2 indoor enclosure with basking lamps.

The parental animals in colony A had all been bred in captivity and co-housed for 9 years. Animals H01–H10 had been taken over from previous owners where they had lived for > 25 years. Their pedigree therefore is unknown. Tortoises F01–F04 had been living in colony F for 20–46 years; their origin is unknown. Animals F05 and F08 were taken over from previous owners, and their origin is also unknown. Male F07 was bred in captivity. In colony F, males were co-housed with the females only in spring whereas in colony A and H, males and females lived together constantly. All animals younger than 6 years were bred in our colonies (Table 1).

Computer tomography

The CT analysis was performed with a Philips Mx-8000IDT 8 slice at the Centre for Veterinary Radiological Diagnostics (VMD-Zentrum) in Holzwickede, Germany. For scans that lasted about 1–2 minutes, alert animals were taped to a custom-made arresting device, and orientated in a vertical position that allowed free movement of the head and legs without contact to any substrate (Fig. 1). In this posture, animals calmly extended head and legs without struggling so that no superposition of legs and carapace occurred. All scans were made with the orthopedics software for small joints using a slice thickness of 1.3–2.0 mm

at ultra high resolution (Dicom 3.0). To visualize the entire carapace, single images were combined two-dimensionally by superposition of the maximal values of each image. Contrast was adjusted appropriately for bone. Some data sets, especially of very young animals, were additionally reconstructed three-dimensionally.

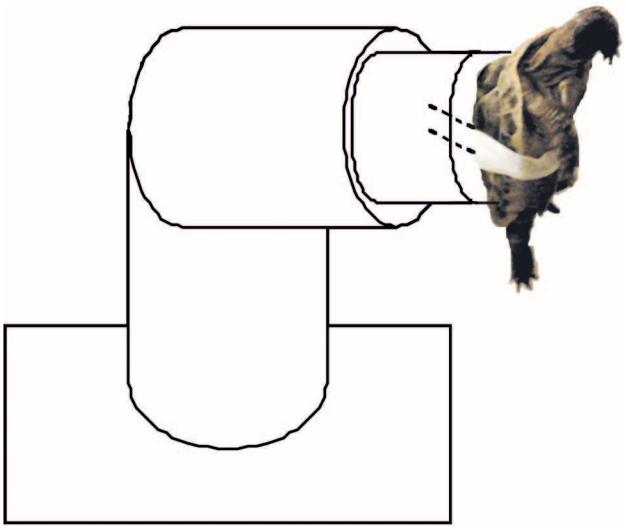

Figure 1. Arresting device and the position of the animal during scanning.

Results

Altogether 77 live tortoises from three captive colonies were analysed with CT. Figure 2A demonstrates the CT image of an adult tortoise (F01) with a normal carapace. On this image, the borders of the bony plates appear dark grey, whereas the borders of the horny scutes appear whitish. For the sake of clarity, in Figure 2B, based on the CT image, the horny scutes have been drawn schematically on the left side (shaded areas), the bony plates on the right side. The medial row of bony plates consists of the nuchal plate anteriorly, followed by 8 neural plates, a suprapygal, and a pygal plate. Laterally, 8 costal plates are surrounded by 11 peripheral plates (Amiranashvili 2000).

Abnormalities of the horny scutes and bony plates

Of 43 semi-adult and adult captive tortoises aged 5–83 years, eight (A03, H12, H14, F04, F11, F21, F23, F42) displayed abnormalities of the horny scutes, seven (H07, H08, H09, H10, F02, F11, F13) abnormalities of the bony plates, and two additional tortoises (H05, F14) abnormalities of both the horny scutes and the bony plates (Table 1). These abnormalities ranged from abnormally subdivided or fused horny scutes and abnormal fusions to the lack and/or presence of supernumerary bony elements, as judged by counting vertebrae and ribs in the individual frontal CT images (Figs. 3–5). They are listed in detail for all animals of this study in Table 1.

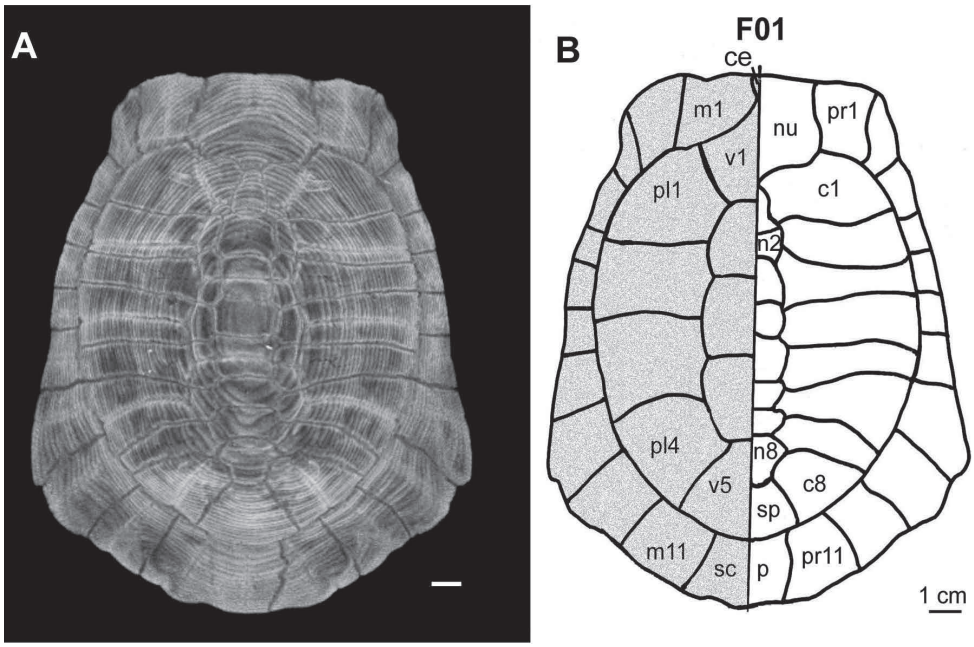

Figure 2. CT image of adult *T. hermanni boettgeri* male F01 (A) and drawing (B) of the horny scutes (left-hand side of the diagram, shaded) and bony plates (right-hand side of the diagram) displaying the standard arrangement of scutes and plates for tortoises. c – costal plate; ca – caudal scute; ce – cervical scute; m – marginal scute; n – neural plate; nu – nuchal plate; p – pygal plate; pl – pleural scute; pr – peripheral plate; sp – suprapygal plate; v – vertebral scute. Scale bars = 1 cm.

Table 1. Identification number (ID), species, age at the time of investigation (years), sex, weight (g), origin, and method of investigation of the animals used in this study. Scute – abnormality of scutes; bone – bone abnormality; CT – computer tomography; diss – dissection; ♂♂ – father; ♀♀ – mother; ? – unknown; √ – missing. Parents with abnormalities are marked with an asterisk and s (scute) if the abnormality involved the scutes. T.g.m. – *Testudo graeca marokkensis*; T.h.b. – *T. hermanni boettgeri*; T.hors. – *T. horsfieldii*; T.m.m. – *T. marginata marginata*. For further abbreviations see Figure 2.

ID	species	age	sex	weight	scute	bone	CT	diss	♂♂	♀♀
A01	T.h.b.	21	♂	985			X			
A02	T.h.b.	14	♀	1831			X			
A03	T.h.b.	13	♀	1644	divided v4 and v5		X			
A04	T.h.b.	12	♀	1824			X			
A05	T.h.b.	3	♀	127			X		A01	A02
A06	T.h.b.	3	♀	158	p* anterior to pl1 left		X		A01	A02
A07	T.h.b.	3	♀	192			X		A01	A03*s
A08	T.h.b.	3	♀	151			X		A01	A03*s
A09	T.h.b.	3	♀	151			X		A01	A03*s
A10	T.h.b.	3	♀	142	reduced v3, pl* left, 2pl* right		X		A01	A03*s
A11	T.h.b.	3	♀	152			X		A01	A03*s
A12	T.h.b.	3	♀	164			X		A01	A04
A13	T.h.b.	3	♀	146			X		A01	A02
A14	T.h.b.	3	♀	103	2 v* between v3 and v5, c* left		X		A01	A02
A15	T.h.b.	3	♀	115			X		A01	A02
A16	T.h.b.	2	♀	40	divided v5	skull	X		A01	A03*s
H01	T.h.b.	~50	♀	2682			X			
H02	T.h.b.	~50	♀	1763			X			
H03	T.h.b.	~50	♀	2279			X			
H04	T.h.b.	~50	♀	1873			X			
H05	T.h.b.	~20	♀	1365	joined pl3 / pl4, joined pl / v5	√ pr2 right, asym. c8	X			
H07	T.h.b.	~50	♂	1452		n9*	X			
H08	T.h.b.	~50	♂	1416		laterally shifted nu	X			
H09	T.h.b.	~50	♂	1266		√ n7, n6 and n8 joined right	X			
H10	T.h.b.	~50	♂	1504		√ n8, joined c8, c9* bilateral	X			
H12	T.h.b.	5	♀	128	√ v3		X		?	H03
H13	T.h.b.	5	♀	105			X		?	H03
H14	T.h.b.	5	♀	68	2 v* between v4 and v5		X		?	H03
H15	T.h.b.	3	♀	79	pl5* left		X		?	H04
H16	T.h.b.	1	?	60			X		?	H01
	T.h.b.	1	?	46				X	?	H01
H17	T.h.b.	1	?	38	joined v2 / v3		X		?	H01
H18	T.h.b.	1	?	27	v* between v3 and v4, pl* left		X		?	H01
H19	T.h.b.	1	?	32	joined v2 / v3, left pl* between pl2 and pl3			X	?	H03
H20	T.h.b.	0	?	4	pl5* right, pl5* and pl6* left, joined v2 / v3			X	?	H03
F01	T.h.b.	83	♂	1258			X			
F02	T.h.b.	79	♂	1380		c* bilateral, √ pr11 left, pr10 joined to p	X			
F03	T.h.b.	61	♀	1699			X			
F04	T.h.b.	61	♂	1646	bilateral pl* joined to v5		X			
F05	T.h.b.	71	♀	2184			X			
F07	T.h.b.	51	♂	1270			X			
F08	T.h.b.	45	♀	1088			X			
F11	T.h.b.	9	♂	645	pl5* left	√ n1	X		F04*s	F03
F12	T.h.b.	9	♂	617			X		?	?
F13	T.m.m.	9	♂	920		c* left	X		?	?
F14	T.m.m.	9	♂	721	√ v4, pl4 reduced bilaterally	√ n7, joined c7, scoliosis	X		?	?
F20	T.h.b.	8	♂	399			X		?	F03
F21	T.h.b.	8	♂	355	divided v5		X		?	F03
F22	T.h.b.	8	♂	260			X		?	F03
F23	T.h.b.	8	♂	274	reduced m left		X		?	F03

ID	species	age	sex	weight	scute	bone	CT	diss	♂♂	♀♀
F31	T.h.b.	12	♀	834			X		?	?
F42	T.h.b.	6	♀	266	divided v5		X		?	F03
F57	T.m.m.	6	♂	188				X	?	?
F61	T.m.m.	5	?	76				X	?	?
F62	T.m.m.	5	♀	379			X		?	?
F70	T.hors.	9	♀	729			X		?	?
F72	T.h.b.	64	♀	1723			X		?	?
F73	T.h.b.	44	♀	2268			X		?	?
F74	T.g.m.	49	♀	1620			X	X	?	?
F82	T.h.b.	3	♀	123			X		?	F03
F87	T.h.b.	2	♀	170	v* between v2 and v3, pl5* bilat.		X		?	F08
F89	T.h.b.	2	?	21	√ m1-3 right, divided v3	√ 8th rib, skull		X	?	F08
F90	T.h.b.		♂	79	doubled v3, reduced v4		X		?	F03
F91	T.h.b.	2	?	78				X	?	F03
F95	T.h.b.	2	?	92				X	?	F03
F96	T.h.b.	2	♂	339			X		?	F03
F97	T.h.b.	2	♂	85			X		?	F03
F98	T.h.b.	2	♂	73			X		?	F03
F99	T.h.b.	2	♂	74			X		?	F03
F100	T.h.b.	2	♂	84			X		?	F03
F101	T.h.b.	2	♂	965			X		?	?
F102	T.h.b.	2	♂	79	pl5* left		X		F04*s	F72
F103	T.h.b.	2	♂	245	pl5* left,,v* between v3 and v4		X		F04*s	F72
F104	T.h.b.	2	♂	86			X		F04*s	F72
F106	T.h.b.	2	♂	212			X		?	F08
F108	T.h.b.	2	?	76				X	F04*s	F72
F109	T.h.b.	2	?	79				X	F04*s	F72
F110	T.h.b.	2	?	31	√ v3, pl* between pl3 and pl4 right			X	?	F08
F115	T.h.b.	≤1	?	63	divided v5			X	?	F03
F116	T.h.b.	≤1	♀	65			X		?	F08
F117	T.h.b.	≤1	?	66	joined v2 / v3, partly divided v5			X	F01	F03
F118	T.h.b.	≤1	?	63				X	F01	F03
F119	T.h.b.	≤1	♀	62			X		F01	F03
F120	T.h.b.	≤1	♀	64	divided v5		X		F01	F03
F121	T.h.b.	≤1	?	15	√ v4, pl* left	skull		X	F04*s	F22
F122	T.g.m.	≤1	?	69				X	F112	F74
F123	T.h.b.	≤1	?	64	pl5* left			X	F01	F03
F124	T.g.m.	≤1	?	28			X	X	F112	F74
F125	T.h.b.	≤1	♀	60	pl5* left		X		F01	F03
F126	T.h.b.	≤1	?	55	pl1*, pl6* left			X	F01	F03
F127	T.g.m.	≤1	?	67	joined pl4 / v5 left			X	F112	F74
F128	T.h.b.	≤1	?	56	reduced v4, partially divided v5			X	F01	F03
F129	T.h.b.	≤1	?	61				X	F01	F03
F130	T.g.m.	≤1	?	68				X	F112	F74
F131	T.h.b.	≤1	?	64				X	F01	F03
F132	T.h.b.	≤1	?	63	reduced v4, partially divided v5			X	F01	F03
F133	T.g.m.	≤1	?	54				X	F112	F74
F134	T.h.b.	≤1	♀	59			X		?	F08
F136	T.g.m.	≤1	?	53				X	F112	F74
F137	T.g.m.	≤1	?	51				X	F112	F74
F138	T.h.b.	<1	?	16	v* between v4 and v5	bicephalic		X	H09	H04
F139	T.h.b.	<1	?	15	pl* right, v* between v4 and v5	skull		X	A01	A03
F140	T.h.b.	29	♀	2965			X		?	?
F142	T.h.b.	47	♀	2247			X		?	?
F143	T.h.b.	36	♂	1019			X		?	?
F147	T. hors.	<1	♀	20	asym. pl1, plastron		X		F30	F10

Animal F04 (Fig. 3A) exhibits bilateral additional pleural scutes (pl*) that fuse with the 5th vertebral scute; numbers and positions of the bony plates are normal (Fig. 3B). Animal F02 (Fig. 3C) has a standard set of scutes, but possesses bilaterally additional costal plates (c*) that merge sagitally between the nuchal and the first neural plate; additionally, the left 11th peripheral is absent and the 10th peripheral melts into the pygal plate. Animal H07 possesses an additional neural plate (Fig. 3D, n*), and in animal H08, the nuchal plate is shifted towards the right-hand side (Fig. 3E). In animal H10, the 8th neural plate is absent, the 8th costal plates of both sides merge medially, and supernumerary 9th costal plates are present bilaterally (Fig. 3F). In animal H09, the 7th neural plate is absent, the 6th and 8th neural plates are partly fused on the right-hand side, whereas on the left side, the 6th costal plate extends into the gap where the 7th neural plate should be. Animal H05 is characterized by fusion of the left 3rd and 4th pleural scutes (pl3/4), fusion of an additional pleural scute (pl*) with the 5th vertebral scute, and the presence of an additional vertebral scute (v*) between the 4th and 5th vertebrals (Fig. 4A). These abnormalities are accompanied by the loss of a right-hand side cranial peripheral, and an asymmetric shift of the 8th costal and a small 8th neural plate towards the left (Figs 4B, C). In tortoise F11, an extra pleural scute is present. In the bony carapace, the 1st neural plate is absent, and the first costal plates are fused medially. The numbers of vertebrae and ribs are normal, indicating that only the plate formation was irregular. In tortoise F14, the 4th pleural scutes are reduced bilaterally, and the 4th vertebral scute is absent (Fig. 5A). This animal misses the 7th neural plate despite having typical numbers of vertebrae and ribs, and the 7th costal plates are fused medially (Fig. 5B). Cranial to these abnormalities, this specimen exhibits signs of scoliosis in the region of the transition between the 2nd and 3rd vertebral scutes (Fig. 5C). These data indicate that abnormalities of the horny scutes and bony plates are in most cases not spatially correlated, but occur independently at different locations of the carapace of captive tortoises.

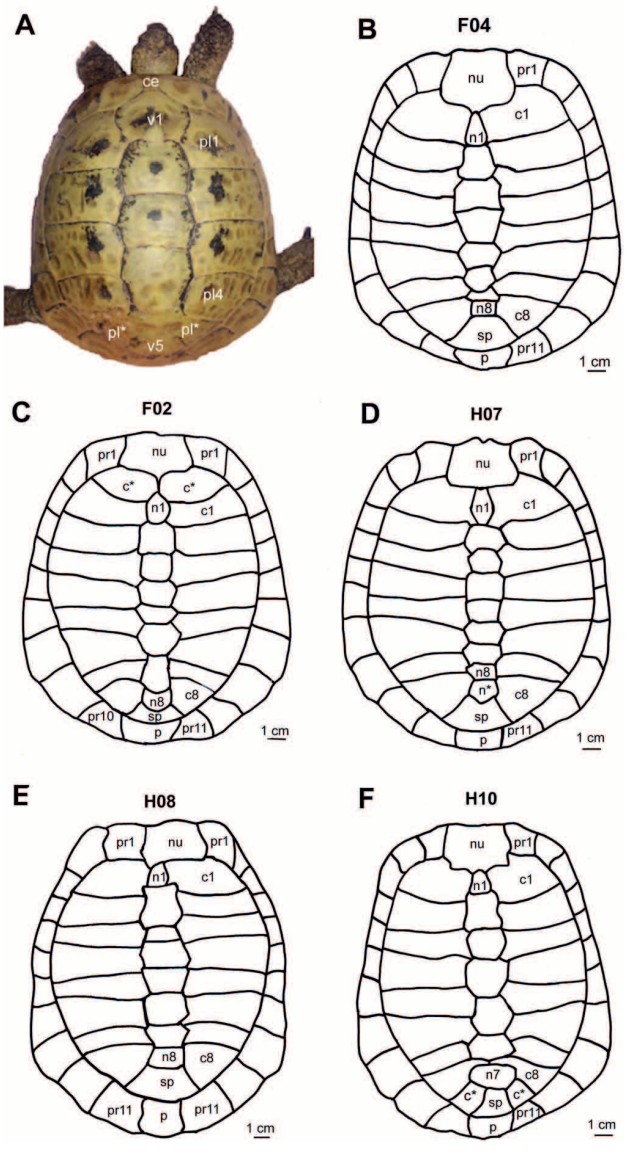

Figure 3. Examples of adult *T. hermanni boettgeri* displaying abnormalities of the horny scutes (A) or bony plates of the carapace (C–F). (A, B) male F04 with supernumerary pleural scutes bilaterally but normal bony plates; (C) male F02 with supernumerary costal plates bilaterally and lacking one peripheral on the left-hand side, the 10th peripheral is joined to the pygal plate; (D male H07 with a supernumerary neural plate; (E) male H08; the nuchal plate is shifted to the right; (F) male H10 misses the 8th neural plate, the 8th costal plates are joined at the midline, and supernumerary costal plates are present bilaterally. Supernumerary elements are marked with an asterisk. For abbreviations, see Figure 2. Scale bars = 1 cm.

Of altogether 63 juvenile captive tortoises ranging from 1–3 years of age, 28 presented abnormalities of the horny scutes. Of 13 juveniles from breeding colony A, five animals (A06, A10, A14, A16, A17) had abnormalities of the horny scutes, and two also had a cleft palate accompanied by a shortened upper jaw and a lack of nostrils (A16, A17). Six out of eight animals of breeding colony H (H15, H17, H18, H19, H20, H21), and 17 out of 42 tortoises from colony F (F87, F89, F90, F102, F103, F110, F115, F117, F120, F121, F123, F125, F126, F127, F128, F132, F147) had supernumerary, fused, or reduced horny scutes. Two tortoises from colony F additionally had abnormalities of the skull (F89, F121), while one animal from colony H was bicephalic (H21, Table 1).

Postnatal development of the bony carapace

Because of their small size, bony structures are difficult to visualize with CT in animals smaller than about 100 g. Therefore, hatchlings of 15–20 g were usually excluded from this study. Nevertheless, 34 juvenile tortoises ranging from 0–3 years of age and weighing 4–192 g were investigated with CT. Mainly the neural plates and ribs of these specimens were visible in the CT images.

Figure 6 depicts CT images of tortoises of different ages and weights to demonstrate the course of postnatal ossification of the carapace in our captive tortoises. In tortoises ranging in age from one year (F125, 60 g, Fig. 6A) to eight years (F20, 399 g, Fig. 6E), the CT images reveal a continuously increased ossification. Whereas mostly the ribs and the vertebrae could be discerned in the 1–2 year-old animals (F125, F97, Fig. 6A, B), ossified costal and neural plates could first be clearly detected with CT at an age of three years and a weight of 123 g (F82, Fig. 6C). At later stages of development, ossification increased and the fontanels decreased in the CT images until they vanished around nine years of age.

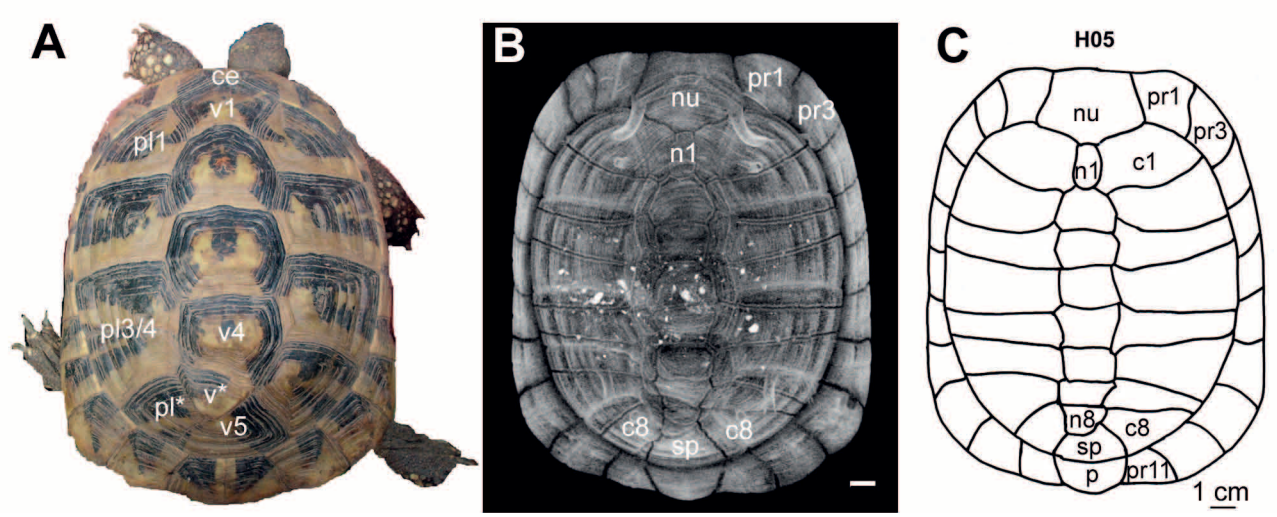

Figure 4. Adult female *T. hermanni boettgeri* (H05) displaying fusion of the left 3rd and 4th pleural scutes, fusion of a supernumerary pleural scute with the 5th vertebral scute, and a supernumerary vertebral scute between 4th and 5th vertebral scutes (A), and a missing right 2nd peripheral and asymmetric 8th costal plates (B, C). The white dots represent ingested sand particles. For abbreviations, see Figure 2. Scale bars = 1 cm.

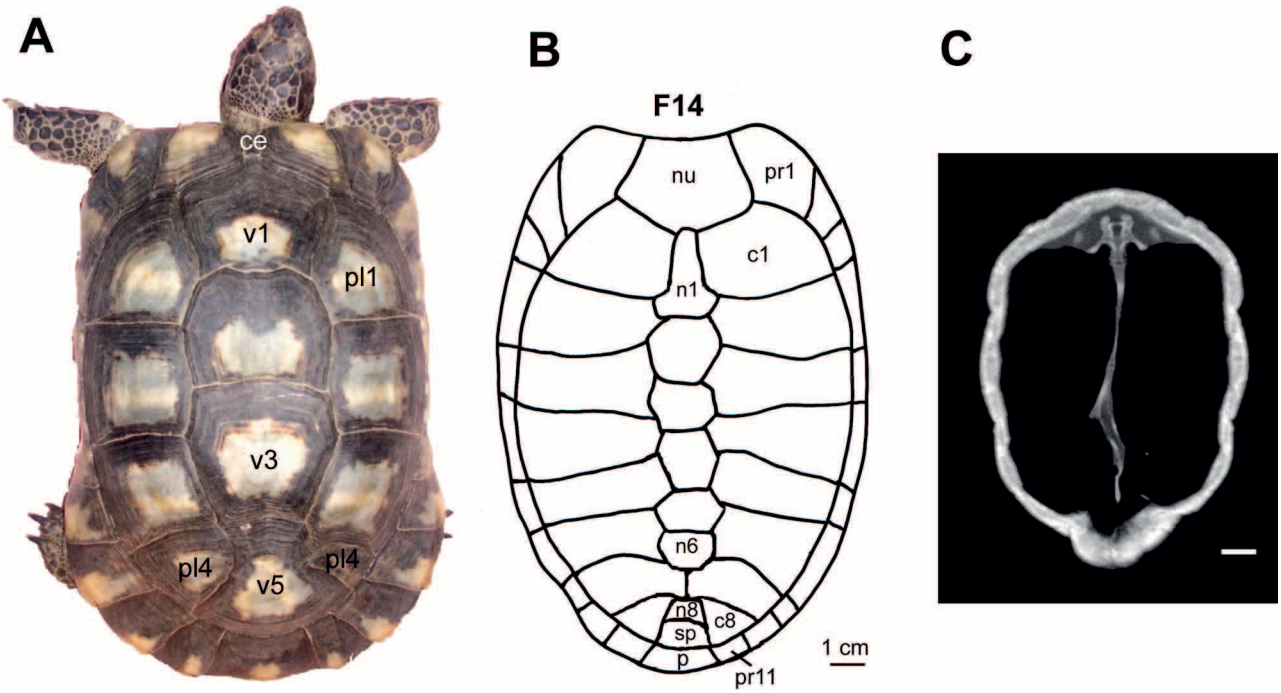

Figure 5. Adult male *T. marginata* (F14), exhibiting a missing 4th vertebral scute and reduced 4th pleural scutes bilaterally (A). In the bony carapace (B), the 7th neural plate is missing, and the 7th costal plates are joined at the midline. In addition, the animal displays signs of scoliosis anterior to the deformed bony plates (C). For abbreviations, see Figure 2. Scale bars = 1 cm.

The tortoise shown in Figure 7 was one year old but weighed only 28 g because of malformations afflicting the skull. After 2-D reconstruction, only the ribs and the vertebral column were visible in the CT image (Fig. 7A). However, the 3D reconstruction revealed considerable ossification of the carapace (Fig. 7B) that matched the ossification detected after post-mortem dissection (Fig. 7C). Similar results were obtained for tortoise F125 (Fig. 6A) whose sibling F126 was dissected post-mortem at 11 months of age and also showed extensive ossification of the neural and costal plates (Fig. 8B). Obviously, even though considerable ossification was present at this age, the bones were too thin to be detected by the CT scanner.

These data show that CT is a valuable tool for analysing abnormalities of the bony carapace and some aspects of ossification throughout development in live tortoises. However, these data also disclose the limits of the method in detecting anatomical details in very young or metabolically challenged animals. Therefore, we complemented our study with the dissection of 31 deceased tortoises, five of which exhibited severe malformations of the head (cleft palate, shortened jaw, lacking nostrils) and/or horny scutes (Table 1). Only one of these animals also had a skeletal abnormality. Figure 8 illustrates examples of dissected animals of different age. The hatchling in Figure 8A was born with a cleft palate, a shortened upper jaw, and lacked nostrils. It died shortly after hatching. Even though its skeleton is extremely fragile, formation of the costal plates had progressed over one third to half of the mediolateral distance. Also, more than half of the plastron area was ossified; however, the bone matter is very thin. Animal F126 (Fig. 8B, sibling of F125 figured in Fig. 6A) died at 11 months post-hatching. Ossification of its neural and costal plates was almost complete, but open fontanels remained between the costal and the peripheral plates. At two years of age, the fontanels were almost completely closed (F95, Fig. 8C).

Environmental influences on shell ossification

Environmental influences appear to affect ossification of the carapace of captive tortoises. The animal depicted in Figure 9A (H18) was raised with little UV-B radiation and suffered from high parasitic load. At one year of age,

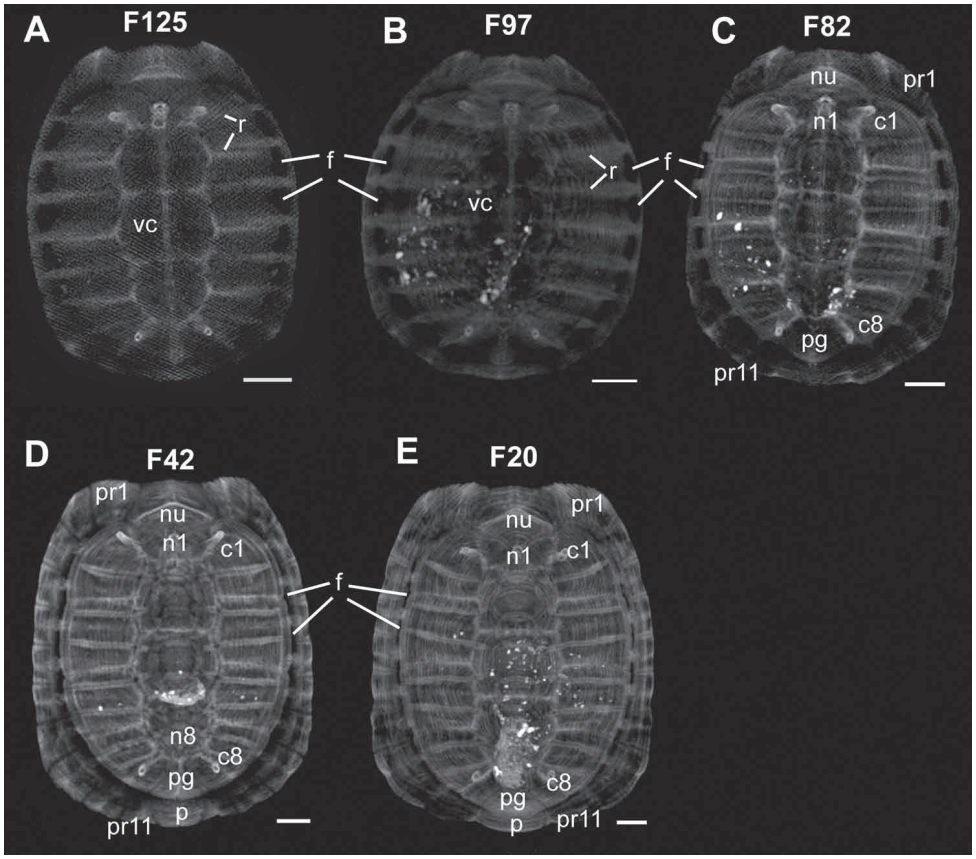

Figure 6. CT images of various developmental stages of *T. hermanni boettgeri*. A) F125, 1 year old, 60 g; B) F97, 2 years old, 85 g; C) F82, 3 years old, 123 g; D) F42, 6 years old, 266 g; E) F20, 8 years old, 399 g; the white dots represent ingested sand particles. f – fontanels; r – ribs; vc – vertebral column. In the 1 and 2 year-old animals, only the ribs and vertebral column are visible; the entire bony carapace is first recognizable in the 3 year-old animal. At 3 years of age, the fontanels between the costal and peripheral plates are clearly discernible and will decrease during further development. For further abbreviations, see Figure 2. Scale bars = 1 cm.

it weighed 27 g, and only the ribs and the vertebrae could be detected in the CT images. On the other hand, animals F103 (Fig. 9B) and F96 (Fig. 9C) were raised by the present owner in a small terrarium with high UV-B radiation and without hiding places. Both animals were completely ossified at two years of age, and the carapace exhibited pyramidal growth; they weighed 245 and 339 g, respectively. Thus, the rate of ossification of captive tortoises is influenced by exposure to UV-B basking lamps, food, the size of the enclosure, and general health.

The rate of ossification also appears to depend on the species. In the one year-old *Testudo marginata* F61 figured in Figure 10A, large fontanels remained between individual

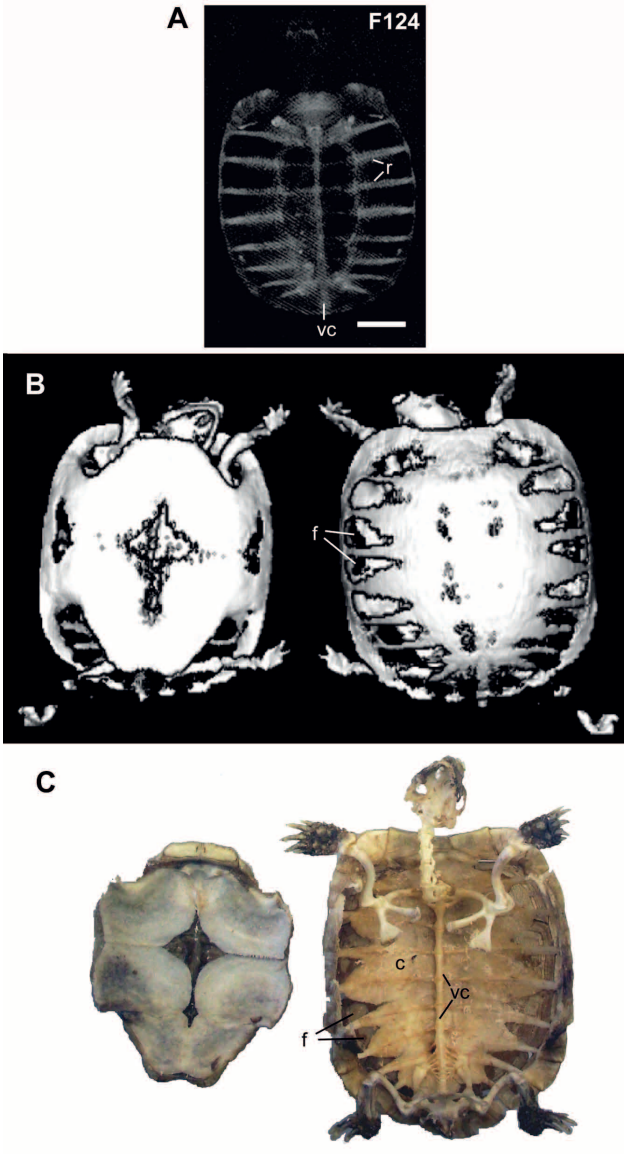

Figure 7. Comparison of a two-dimensional reconstruction (A) of the carapace of animal F124 (*T. graeca marokkensis*, 1 year, 28 g) in which only the ribs and vertebral column are visible with the three-dimensional reconstruction (B) of the ventral (left) and the dorsal (right) views of F124. C) Ossification of the plastron and carapace of F124 as presenting itself after dissection. Both the 3D reconstruction and the internal view show the extensive ossification of the neural and costal bones that is not evident in the 2D reconstruction.

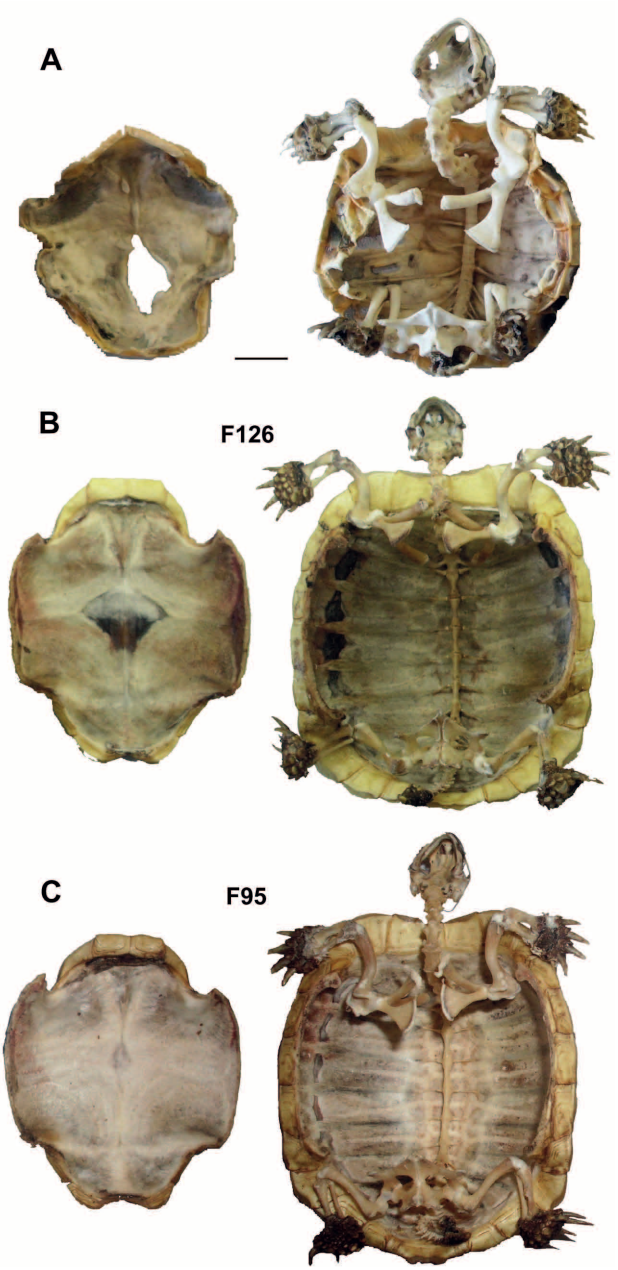

Figure 8. Development of ossification in *T. hermanni boettgeri* as demonstrated by dissection. A) Hatchling with a very thin carapace. The fontanels cover up to half the distance between vertebral column and edge of carapace. B) Animal F126, 11 months old, 55 g; The fontanels are still clearly visible and cover up to one fifth of the carapace. In the plastron, the bones are not yet fused at the midline. C) Animal F95, 2 years old, 92 g. At this age, the fontanels are reduced to narrow slits, and the plastron is completely ossified. Scale bars = 5 mm (A) and 1 cm (B, C), respectively.

costal plates and between costal and peripheral plates. In its plastron, the hyo- and hypoplastra were not yet fused anterioposteriorly or at the midline. In the 11 month-old *T. hermanni boettgeri* F126 figured in Figure 8B, fontanels between the costal and peripheral plates were smaller, and the hyo- and hypoplastra extended farther than in F61. By contrast, in the 11 month-old *T. graeca marokkensis* F130 shown in Figure 10B, the fontanels were largely closed, and the plastron was almost completely ossified. Environmental influences can be excluded here, as all three animals were raised together under identical husbandry conditions.

Discussion

In the present study, we investigated carapace abnormalities and the ontogenetic time course of shell formation in captive tortoises using CT scanning and dissection. We developed an easy, quick, and minimally invasive procedure that produced reliable data in live, alert animals older than one year and weighing about 150 g. With our arresting device, we avoided potentially harmful sedation. Because contact to substrate was prevented with this fixation method, the animals extended their legs and head, thereby avoiding superposition of the appendicular skeleton or the head with the bony elements of the carapace. This method is also suitable for diagnostic CT in sick animals.

Comparison of our 2D reconstructions of CT images with dissected individuals reveals that the limits of resolution of the CT are reached in animals younger than one year, weighing less than about 100 g, or in metabolically challenged individuals, i.e., very thin bones can not be detected with this type of scanner. However, performing 3D reconstructions of the whole animals can circumvent this limitation to a certain degree.

In our study group of 106 captive tortoises, about 36% of the animals displayed abnormalities of the horny scutes. Among the 43 animals older than three years, in which the bony elements can be discerned with the CT method, abnormalities of the bony plates of the carapace were found in 21% of the samples. These rates are well within the range of natural populations (2–69%, Zangerl 1969, McEwan 1982, Meek 1985, Cherepanov 1994, Cheylan 2012, Velo-Antón et al. 2011, Rothschild et al. 2013, McKnight & Ligon 2014). Shell abnormalities have also been reported for captive populations in a number of earlier studies (e.g., Wermuth & Mertens 1961, Calmonte 1968, Kirsche 1972, 1983, Highfield 1990, Rothschild et al. 2013). Most authors suggest environmental influences such as temperature, humidity, nutrition as well as toxic substances, e.g., pesticides, as causative agents (Gadow 1899, Parker 1901, Newman 1906, Coker 1910, Vogel 1912, Hildebrand 1930, Cagle 1950, Lynn & Ullrich 1950, Mlynarski 1956, Zangerl & Johnson 1957, Frye 1991, Bishop et al. 1998, Gabrisch & Zwart 2001, Kazmaier & Robel 2001, Wiesner & Iben 2003, Fernandes & Rivera 2004, Bujes & Verrastro 2007), but low genetic diversity and inbreeding could also play a significant role (Fernandes & Rivera 2004, Velo-Antón et al. 2011, McKnight & Ligon 2014). To our knowledge, the genetic basis of shell formation is unknown at this time. Nevertheless, we compared the location of abnormalities between parents and offspring of our captive animals (Table 1). Mother A03 had divided 4th and 5th vertebral scutes. She produced two normal and two offspring with abnormalities, with one exhibiting a divided 5th vertebral scute. Father F04 had supernumerary pleural

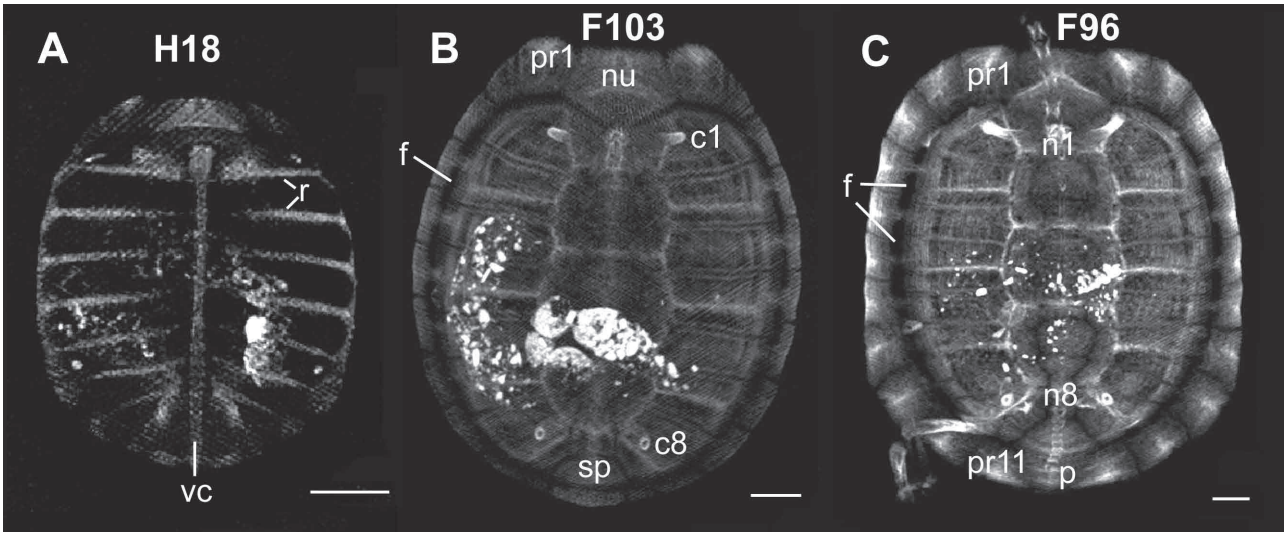

Figure 9. CT images of *T. hermanni boettgeri* demonstrating the effects of different raising conditions in captivity. A) H18 from breeding colony H, 1 year old, 27 g, compromised by parasites, UV-B underexposure and malnutrition; B) F103, 2 years old, 245 g; C) F96, 2 years old, 339 g, both raised under UV-B overexposure and excessive feeding in a small enclosure. The white dots represent ingested sand particles. For abbreviations, see Figure 2. Scale bars = 1 cm.

scutes bilaterally; he produced three normal offspring and four animals that all had a supernumerary pleural scute on the left-hand side. Thus, parents with abnormalities apparently can produce both normal and malformed offspring. However, the same is true for normal parents (H01: 2 normal, 2 abnormal, H03: 1 normal, 4 abnormal, F01: 4 normal, 7 abnormal, F03: 14 normal, 13 abnormal, F08: 3 normal, 3 abnormal, F74: 6 normal, 1 abnormal, F112: 3 normal, 1 abnormal; note, however, that both parents are known only for 40 of the 106 animals in our cohort). Thus, based on our data from three captive colonies it cannot be assessed whether environmental parameters or heredity are the major drivers for causing carapace abnormalities. Only four of our animals exhibited abnormalities both in the horny and the bony carapace. Generally, with the possible exception of H05 where the malformations occurred at similar locations, the abnormalities were not co-localised (Table 1), indicating that abnormalities of the scutes and bony plates occur independently and might not be correlated at least in captive tortoises. By contrast, Parker (1901) suggested that these abnormalities are correlated even though two of the three abnormalities described do not support his notion. Our view is supported by the fact that scute abnormalities are present at hatching at a time when the bony plates of the carapace are yet to form. Nevertheless, if the cause of abnormalities were genetic rather than epigenetic, this disposition would of course be present already during the embryonic stages.

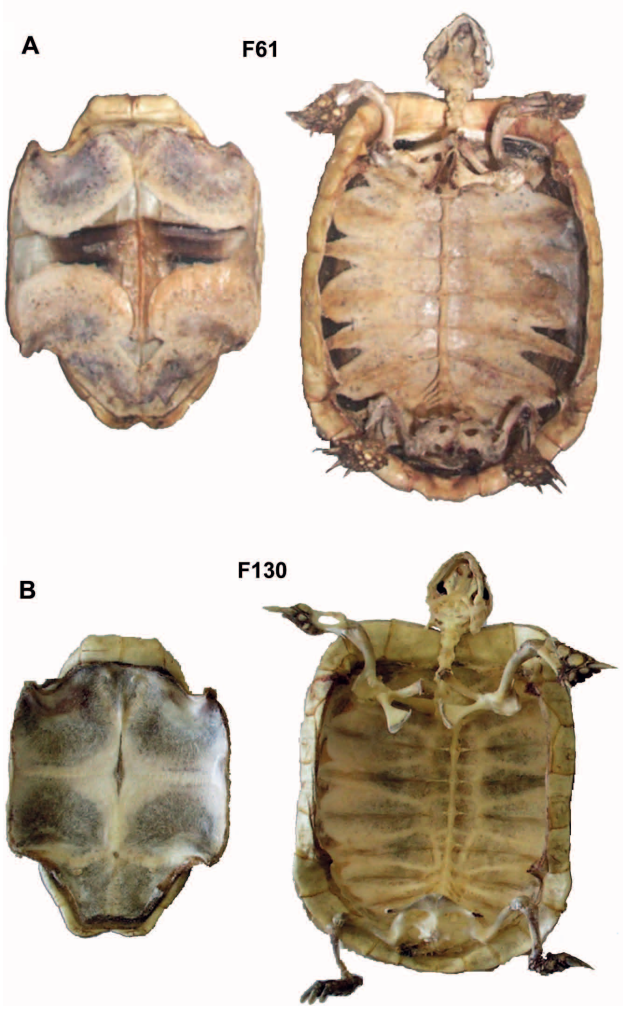

Figure 10. Species-specific ossification in 11–12 month-old animals. A) F61 (*T. marginata),* 1 year old, 76 g; B) F130 (*T. graeca marokkensis)*, 11 months old, 68 g. For comparison, see also F126 (*T. hermanni boettgeri*) in Figure 8B. All animals were raised together thereby excluding variability of environmental influences on ossification. Ossification proceeds slowest in *T. marginata* and fastest in *T. graeca marokkensis*.

The bony carapace is incomplete at hatching. Interestingly, a similar ontogenetic development of the shell could recently be demonstrated in a fossil turtle from the Early Cretaceous (*Changmachelys bohlini*; Brinkman et al. 2013). At birth, the bony plates begin to form, but are still too thin to be detected with CT. Within the first year of life, the neural and peripheral plates ossify completely, and the costal plates are not yet connected to the peripherals thereby leaving distinct fontanels. These fontanels close during later development until the shell will be complete at 6–9 years of age. Even though we did not test this experimentally, circumstantial evidence from our breeding colonies suggests that developmental time course is influenced by the state of nutrition and health of the animal and the raising conditions it is exposed to. We could demonstrate in case studies of malnourished and parasitised animals that ossification lags behind healthy age-mates and is hardly visible in 2D CT reconstructions. On the other hand, overexposure to UV-B radiation combined with overfeeding and limited opportunity for locomotion leads to premature ossification and pyramidal growth of the carapace of captive tortoises. Other parameters were not analysed.

The time course of shell formation is species-specific. Even though most of our data come from *T. hermanni boettgeri,* we were able to compare age-mates of this species with individuals of *T. marginata* and *T. graeca marokkensis* that were raised together, thereby excluding influences of husbandry conditions. At about one year of age, ossification had progressed farthest in *T. graeca marokkensis*, and least in *T. marginata*. No data are available for wild specimens of these species. Comparison with a study of *T. hermanni hermanni* from a natural habitat shows that ossification had progressed farther in our animals (Cheylan 1981). These differences could be due to variations between the two subspecies. Alternatively, even the animals from breeding colony F were raised with supernatural UV-B exposure and/or nutrition even though they were fed only dried and fresh herbs.

Further studies should include specimens from natural habitats to investigate the natural maturation process in comparison with captive-bred individuals. Additionally, more data on nutrition in natural habitats are needed. Such studies in combination with targeted experiments varying the degree of UV-B exposure, nutrition intake and quality, temperature, humidity, and other habitat parameters are

necessary to determine species-specific optimal breeding and raising conditions to improve animal welfare in breeding programs for conservation and the pet trade.

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