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Autopodial Development in the Sea Turtles *Chelonia mydas* and *Caretta caretta*

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The manus and pes were studied using whole-mount and histological preparations of ontogenetic series of *Chelonia mydas* and *Caretta caretta*. Patterns of connectivity and sequences of chondrification events are similar to those reported for other turtle species, with respect to both the primary axis and the digital arch. There is no evidence of anterior condensations in the region distal to the radius and the tibia, supporting the hypothesis that the radiale and tibiale are absent in turtles. The three middle metacarpals are the first elements to start ossification in the manus of *C. mydas*, while ossification has not started in the pes. In the hatchling of *C. mydas*, most carpals have started ossification, whereas tarsals are mostly still cartilaginous. In *C. caretta*, the first carpals to ossify are the ulnare and intermedium, followed by the pisiform. Among metatarsals, the fifth hooked metatarsal is the last one to start ossification. The fibulare and intermedium fuse early in chondrogenesis, later becoming the astragalocalcaneum. Ossification in the carpals of *C. caretta* starts while tarsals are still cartilaginous. The derived autopodial proportions in each autopodium of adults are laid out at the condensation stage, and features that were present in basal turtles are absent at all stages examined (developmental penetrance). In contrast to this, conservatism is expressed in the presence of similar patterns of connectivity during early chondrogenesis, and in the development of overall proportions of the manus versus pes. As in adult anatomy, the development of the autopodium of marine turtles is a mosaic of derived and plesiomorphic features.

Key words: limb, ontogeny, Testudines, Cheloniidae, cartilage, bone

INTRODUCTION

Turtles have diversified from a likely terrestrial origin (Joyce and Gauthier, 2003) and occupy a variety of freshwater and marine environments. Sea turtles exhibit some of the most derived morphological and physiological features in the group. Correlated with a mode of locomotion involving upward and downward strokes, the limbs are specialized (Walker, 1973; Raynaud, 1985). The anterior feet are paddles or flippers, with little individual finger movement, since digits are joined by a web (retention of embryonic interdigital membrane), and the skeleton of the forearm is shortened. The digits, especially the middle three, are greatly elongated. This is accomplished not by hyperphalangy, but instead via an elongation of the metacarpals and phalanges (Richardson and Chipman, 2003). An enlarged pisiform helps to support the ulnar border of the blade which, to a large extent, moves as a unit. The articular surfaces of the phalanges are flat and without round condyles, and the

radius is closely bound to the palmar side of the ulna. Walker (1973) discussed some of the muscular adaptations in the limbs of sea turtles.

The Green Sea Turtle, *Chelonia mydas*, and the Loggerhead Sea Turtle, *Caretta caretta*, are two of the few living species of sea turtles and belong to two different clades within the Cheloniidae (Dutton *et al.*, 1996). Both species exhibit the limb specializations described above (Fig. 1). Their phalangeal formula, for both manus and pes, is 2:3:3:3:2, as in other sea turtles (Walker, 1973). This phalangeal formula is most likely close to the primitive turtle condition, as the 2.2.2.2.2 formula of the basal turtle *Proganochelys* is hypothesized to be secondarily reduced and the 2.3.3.3.3 formula to be plesiomorphic for Pleurodira (Rabl, 1910; Sánchez-Villagra *et al.*, unpublished).

This paper addresses the ontogenetic origin of the skeletal anatomy of the manus and pes in two sea turtles. Examination of *C. mydas* and *C. caretta* is of interest because it widens the phylogenetic coverage of what we know about autopodial development in turtles. This can provide a test of current ideas about homology and patterns of connectivity of autopodial elements, and clarify whether the modifications involved in having a flipper have altered the early ontogeny

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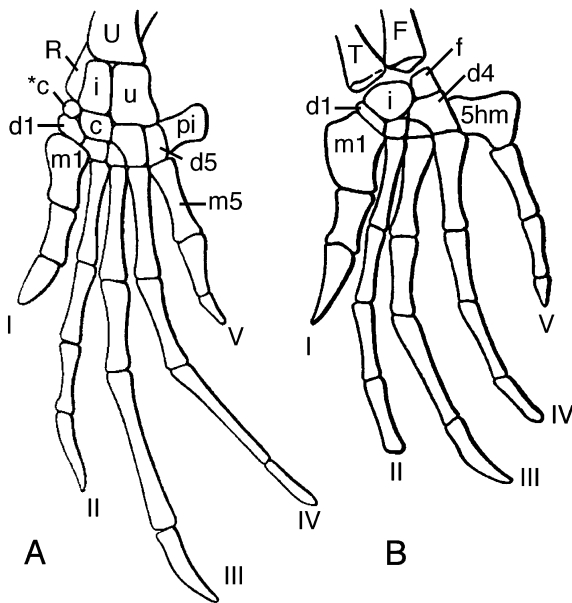


Fig. 1. Adult skeleton of the (A) dorsal left manus and (B) left pes in *Caretta caretta* (modified from Romer, 1956 and Walker, 1973). Not to scale. The autopodials of *Chelonia mydas* (Rabl, 1910; pers. obs.) are similar in proportions to those of *C. caretta*, having as well the same number and arrangement of carpals and tarsals. In *C. caretta*, the pisiform articulates with the ulnare and distal carpal 5 in some specimens, whereas in *C. mydas*, it does so just with the latter. Another noteworthy difference is that in *C. caretta*, the centrale just distal to the radius (*) is cartilaginous, whereas it is ossified in *C. mydas*. In *C. mydas* the astragalus and the calcaneum of the proximal tarsal row are usually fused together, a feature present in several other cryptodire turtles and which can vary intraspecifically (Zug, 1971). Abbreviations: c, centrale; d1–5, distal carpals 1–5; d1–4, distal tarsals 1–4; f, fibulare (calcaneum); i, intermedium (astragalum in the tarsus); m1–m5, metacarpals 1–5; m1, metatarsal; pi, pisiform; R, radius; T, tibia; u, ulnare; U, ulna; 5hm, fifth hooked metatarsal.

of the manus and pes. In this way, the developmental penetrance (Richardson, 1999) of the derived adult autopodial skeletal anatomy in these species can be examined. By developmental penetrance, we mean the situation in which derived features of the adult are already expressed earlier in development (Richardson, 1999), the alternative being embryonic conservatism. Furthermore, a consideration of ontogenetic processes can help to understand the generation in evolution of adult diversity in autopodial structures, as exemplified by a recent study on tortoises (Crumly and Sánchez-Villagra, 2004).

Recognition of the processes involved in the formation of cartilaginous limb elements and the digital arch and primary axis (Shubin and Alberch, 1986) have been a matter of research on the development of limbs of turtles (Burke and Alberch, 1985; Crumly and Sánchez-Villagra, 2004) and of tetrapods in general. Shubin and Alberch (1986) identified three patterns of connectivity in the formation of cartilaginous limb elements in tetrapods. A mesenchymal chondrogenetic element may form without connectivity, usually near the beginning of the process of limb formation, as in the origination of the humerus or femur. A second alternative is that

a single element branches, resulting in two elements distal to the division. This process is called bifurcation. Last, a single mesenchymal condensation may either bud off a new condensation distally or become subdivided into two separate elements. This last process is called segmentation. There is a conserved sequence in which these processes occur in the development of the turtle autopodials studied so far (primary axis, digital arch), so that the appearance of some elements is a prerequisite to the development of other, subsequent elements, and so forth. The linear array of cartilaginous primordia called the primary axis consists of the ulnare (fibulare), distal carpal (tarsal) 4, and metacarpal (metatarsal) 4. This hierarchy of events has been documented for only a handful of turtle species.

The sequence of autopodial chondrification and/or ossification was described and discussed by Burke and Alberch (1985, *Chrysemys picta* and *Chelydra serpentina*), Rieppel (1993a, *Chelydra serpentina*), Sheil (2003a, *Apalone spinifer*; 2005, *Macrochelys temminckii*), and by Sheil and Greenbaum (2005, *Chelydra serpentina*). Only few stages of some sea turtle species have been discussed in this regard (Rabl, 1910; Miller, 1985; Rieppel, 1993a; Sheil 2003b).

Earlier studies on development in sea turtles have been comprehensively cited and summarized by Ewert (1985) and Miller (1985). More recent contributions have been published (Renous *et al.*, 1989), but most have concentrated primarily on skeletochronology in post-hatchling stages (e.g., Zug *et al.*, 2002). There have been some studies considering limb development in sea turtles. The relative growth of the forelimb versus hindlimb in *Eretmochelys*, *Caretta* and *Chelonia* was documented in a series of photographs by Miller (1985). The relative timing of overall forelimb and hindlimb development across turtles, including some marine species, was compared by Tokita and Kuratani (2001). Renous *et al.* (1989) measured the total length of the two members across growth in *Dermochelys coriacea*. In *C. mydas* (Fig. 2) and *C. caretta*, the overall limb proportions characteristic of the adult are already present at hatching.

MATERIALS AND METHODS

Embryological specimens of *Chelonia mydas* were obtained from the Hubrecht collection (at the time of the loan in Utrecht, now based in Berlin). The series consists of eight stages labelled Cm 5-I, 9-V, 10-VI, 11-VII, 13-IX, 14-X, 15-XI, and 16-XII, most stages containing a single animal, some two. They comprise approximately Yntema (1968) Stages 17–22. The age of the specimens is unrecorded. They were in a flask containing a label with the following markings: "Collectie v.d. Meer-Mohr, Poeloe Berhalla, Straat Malakka, 1926–1927", presumably indicating the collectors and the location. A hatchling of *C. mydas* from the collections of the Natural History Museum, London (BMNH 1970.1287) was also studied (Fig. 2).

The series of *Caretta caretta* consists of 10 specimens and is part of the same series studied in investigations of cranial development by Kuratani (1999 and references therein), who provided details of its provenance and preparation. We did not capture or sacrifice any animals for this study, but instead used specimens in existing collections; therefore, animal care procedures and collecting permits were not required for these study animals.

Several stages were cleared and stained as whole mounts using a standard technique that permits one to visualize chondrogenesis and ossification (Dingerkus and Uhler, 1977). In addition to

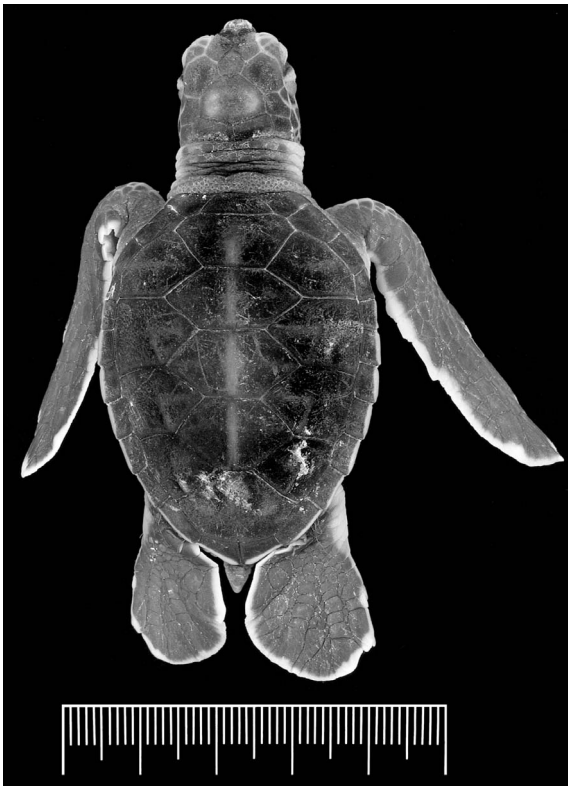


Fig. 2. Hatchling of *Chelonia mydas* (BMNH 1970.1287), dorsal view. Locality: Heron Island, Capricorn Group, Great barrier reef, Australia. Scale=50 mm.

this, histological sections of the autopodials of seven *C. mydas* and four *C. caretta* specimens were examined.

Three-dimensional reconstructions of serial histological sections were made of the manus and pes of *Chelonia mydas* specimens Cm X and Cm XI, of crown-rump length (CRL) 13.5 mm and 19.5 mm, respectively. Histological sections (15 μ m thick) of these two specimens were embedded in paraplast and stained with Azan/Dogmak. The method of 3-D computer reconstruction was described by Sánchez-Villagra *et al.* (2002).

For comparisons with adults, we examined articulated dry skeletons, including those of *C. mydas* (BMNH 7.2.3.7.2, 14a) and of *C. caretta* (BMNH unnumbered, 122). We also made comparisons with the drawings and descriptions of adult skeletons in the literature (e.g., Rabl, 1910; Romer, 1956; Walker, 1973).

RESULTS

Chondrification patterns in *Chelonia mydas*

The major features of the chondrification sequence in *Chelonia mydas* are described as follows.

Manus. In the youngest stages examined (Cm-12, Fig. 3A), a primary axis is clearly visible, with a main axis of condensation in the manus extending from the ulna to the ulnare to distal carpal 4 to the fourth digit, and a decreasing gradient of condensation in the same proximodistal sequence. The anlage of the intermedium is visible next to the ulnare, with mesenchymal connectivity to the ulna. There is no sign of any condensation distal to the radius. There is a decreasing gradient of condensations from the fourth to distal carpal (dc) 1. A faint condensation distal to the intermedium and ulnare is present, as well as clearer

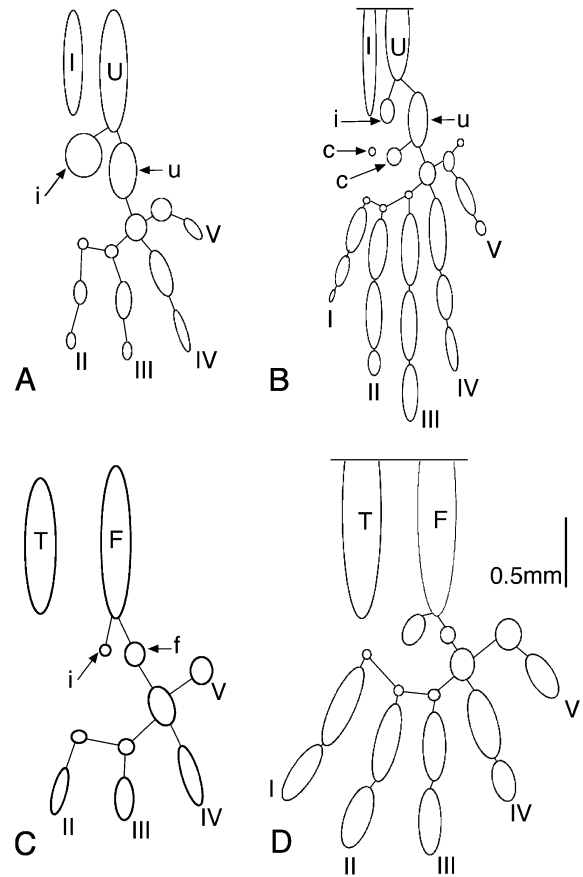


Fig. 3. Schematic representation of the early development of the left manus and pes in *Chelonia mydas* in dorsal view. Proportions and shapes of elements are only approximate. Lines signify connectivity between elements where visible. (A, B) Manus. (C, D) Pes. A, C represent the same individual (Cm-12), as do B, D (Cm-14). A faint condensation distal to the intermedium and ulnare is present in the manus of the smaller specimen (A), but it is not illustrated at this stage because it is too faint to identify with any certainty. The solid black line intersecting the radius/ulna and tibia/fibula in 3B and D, respectively, signify that these elements are relatively longer than depicted in here. Abbreviations refer to anlage of several elements and include: c, centrale; f, fibulare; F, fibula; i, intermedium; R, radius; T, tibia; u, ulnare; U, ulna. Scale=0.5 mm.

signs of dc5 (darker than the dc3) and digit V. At this stage, the phalangeal formula is 0:1:1:1:0, with no clear boundaries between the metacarpals and phalanges of each of "digits" 2, 3, and 4. The following stage (Fig. 3B) shows similar relations to those just described, plus additional phalangeal condensations, so the formula has become 2:2:2:2:1. There are two condensations situated distal to the intermedium which we homologized with two centrale, based on their topographical relations. The smaller one is particularly faint in comparison to the other carpal condensations. The homology of these two centrale with those of other turtles and saurosideps (Gaffney, 1990) is unclear to us, so we refrain from assigning them specific identities. In addition to the centrale, there is a relatively small condensation (as faint as those of the centrale) in the position of the adult pisiform. In this stage, the second phalanx of the third digit is already much longer than the other second phalanges present, laying out

at this early stage of differentiation what will become the typical proportions of the adult (Fig. 1). The relations described in this stage become more clear in the next one examined (Cm15-XIb). The following stage (Cm15-XI) exhibits, at a more advanced stage, the adult proportions of the second phalanges among hand digits.

Pes. In the earliest stage examined (Cm 12, Fig. 3C) the primary axis is clearly visible, with a proximodistal gradient of condensations of the fibula, fibulare, and distal tarsal 4. A small condensation next to the fibulare is also seen (more clearly in the next stage, Fig. 3D), interpreted as the anlage of the intermedium. The tibia is as visible as the fibula, but there is no sign of any element distal to it. Condensations corresponding to distal tarsals 3, 2, and 5 also are visible, as well as the those of metatarsals 4, 3, and 2. In the following stage (Fig. 3D), other elements of the digital arch have formed, and the phalangeal formula reaches 1:1:1:1:0. In the next stage available (Cm15-XIb), the anlage of the fifth hooked metatarsal is slightly larger than that of distal tarsal 4. The anlage of the intermedium is also clearly visible. It is not possible to discern clearly distal tarsals from metacarpals because their boundaries are diffuse, and perhaps they are not separated yet. The phalangeal formula is 1:2:2:1:1. In the next stage (Cm15-XI), the anlage of the fibulare is very small and next to a much larger intermedium. The other condensations seen, in decreasing order of size, are: fifth hooked metatarsal, distal tarsals 4, 3, 2, and 1. The phalangeal formula at this stage is 2:3:3:2:1. Later stages examined histologically show that after the initial formation of two elements distal to the fibula (fibulare and intermedium), these fuse to become a single cartilaginous element, the astragalocalcaneum of the adult.

The number, arrangement, and proportions of carpals and tarsals in stage Cm IX (carapace length=13.5 mm) are those of the adult, as shown by 3D reconstructions of manus and pes skeletons (Fig. 4).

Ossification patterns

Only one prenatal stage available to us exhibits bone formation in *Chelonia mydas*, showing that the three middle metacarpals are the first elements to start ossification in the manus. At this stage, the pisiform is still cartilaginous but already conspicuous. In the same stage, ossification has not started in the pes. A hatchling specimen (BMNH 1970.1287) has all the metapodials and phalanges of both manus and pes mostly ossified. Among carpals, the intermedium, ulnare, and pisiform are almost all ossified; distal carpals (dc) 5 and dc4 are greatly ossified, whereas dc1 and dc2 and centrale A show small centers of ossification, absent in dc3 and in centrale B distal to the radius. Tarsals are cartilaginous in this specimen, except for one ossification center in distal tarsals (dt) 1–3. The fifth hooked metatarsal is almost all ossified.

Fig. 5 illustrates the ossification sequence in the autopodials of *Caretta caretta* available in this study. In the manus, the last phalanges of digits II–V are still not ossified. The first carpals to ossify are the ulnare and intermedium, which are then followed by the pisiform. In the pes, most phalanges have already started ossification in the earliest *Caretta* stage available to us showing bone formation. Based on the relative degree of ossification of the different elements, we

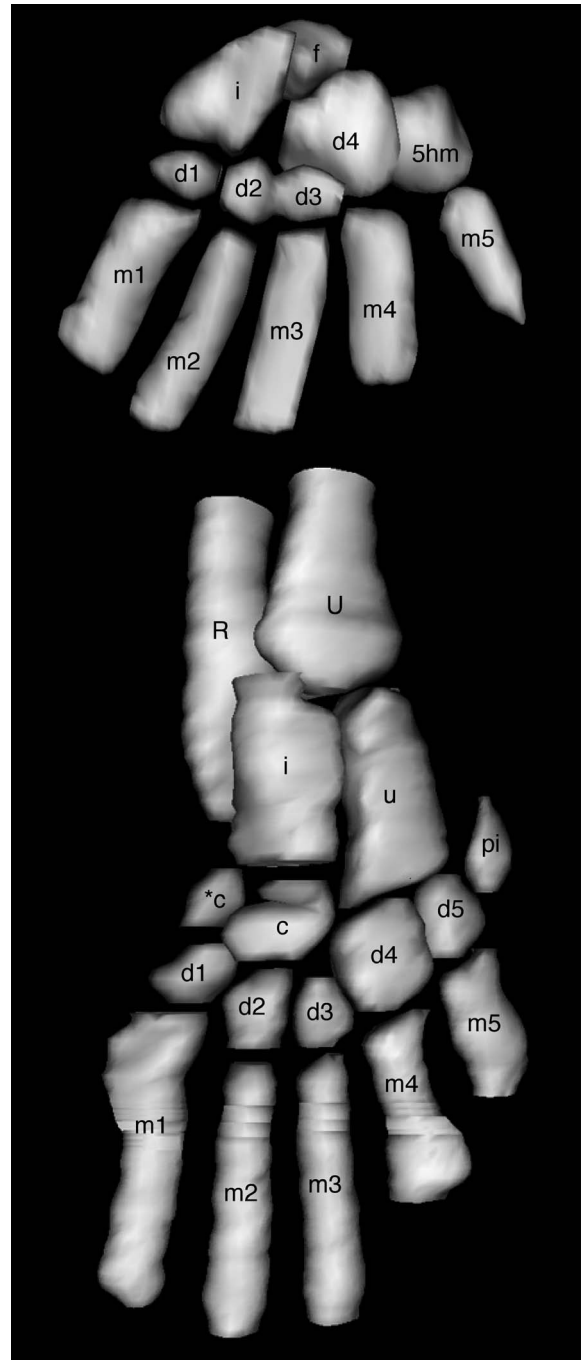


Fig. 4. Three-dimensional reconstructions of the partial left foot (top) and hand (bottom) skeleton anlage in *Chelonia mydas* (Hubrecht collection, specimen Cm-X, crown-rump length=13.5 mm), dorsal view. Abbreviations: c, centrale; d1–5, distal carpalia 1–5; d1–4, distal tarsalia 1–4; f, fibulare (calcaneum); i, intermedium (astragalum in the tarsus); m1–m5, metacarpals 1–5; m1–m5, metatarsals 1–5; pi, pisiform; R, radius; T, tibia; u, ulnare; U, ulna; 5hm, fifth hooked metatarsal. Not to scale.

observe that metatarsal IV is the first metatarsal to ossify. Also among metatarsals, the fifth hooked metatarsal is the last to start ossification. Tarsals are the last elements to start ossification in the pes, and the first one is distal tarsal 4.

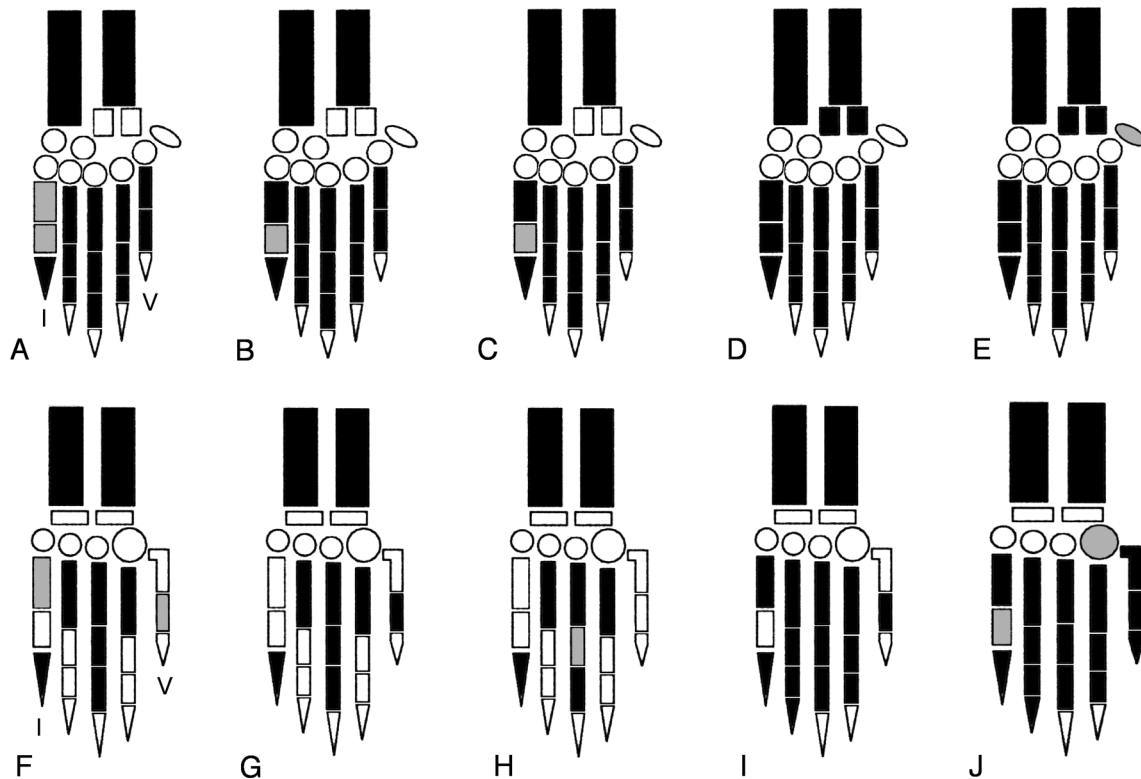


Fig. 5. Ossification sequence in the left manus (A–E) and pes (F–J) in *Caretta caretta*, dorsal view. In the same column (A–F, B–G, C–H, D–I, E–J) are autopodials of the same individual, from left to right: Kuratani-200036, 1600, 2200, 1600b, and 2100. Black signifies a clear intake of alizarin red in at least the entire diameter of a visible portion of the element in question, whereas gray refers to elements which show only a very small intake of alizarin and only on one side. Roman numerals refer to digit numbers.

DISCUSSION

Chondrification patterns and homologies of carpals and tarsals

Examination of early autopodial skeletal development in *Chelonia mydas* and *Caretta caretta* reveals more similar basic patterns of connectivity and sequence of events than has been reported for other turtle species. As for most other turtles and tetrapods for which the chondrogenetic pattern is described, we identified both the primary axis and the digital arch.

Not all details of the sequence of chondrification could be recorded because of lack of enough stages of development, but some patterns are clearly apparent. As in *Chrysemys picta* and *Chelydra serpentina* and in contrast to frogs (Burke and Alberch, 1985), digit IV develops well before the third, rather than at the same time as the third.

The ontogenetic data presented here serve to address the issue of homology of the preaxial autopodial elements. Whereas some authors have argued that turtles have a radiale and a tibiale in the manus and pes respectively, others have suggested they are absent. Burke and Alberch (1985) reviewed this issue, that goes back to older literature (e.g., Rosenberg, 1892; Rabl, 1910; Romer, 1956; Walker, 1973). Sheil (2003a) labeled the same element radiale and centrale 3 in two different parts of his comprehensive paper describing skeletogenesis in *Apalone spinifer*; Wyneken (2001) identified a radiale in her anatomical treatment of sea turtles.

If we are to base homologies on embryonic origins and connectivity patterns, as opposed to anatomical position in the adult, then we can hypothesize with certainty that the radiale and tibiale are missing in sea turtles, and most likely, in turtles in general. We support then the view of Burke and Alberch (1985).

At the chondrification level for *Chrysemys* and *Chelydra* according to Burke and Alberch (1985), the astragalus is formed by fusion of the intermedium with a centrale. This is not what we saw in *Chelonia mydas*. After the initial formation of two elements distal to the fibula (fibulare and intermedium), these fused to become a single element. This is the element which becomes the astragalocalcaneum in adults. Based on the material available to us, we cannot confirm the participation of an additional element (a condensation corresponding to a centrale anlage) in the astragalus.

There are two centers of hyperchondric cells in the large element distal to the fibula of *C. caretta* (Cm 39d, slide 65). Based on the available evidence, it appears that the two elements that fuse early in chondrogenesis, the fibulare and intermedium, become the astragalocalcaneum, a single element at the cartilaginous stage, with two later centers of ossification. Concerning ossification patterns, in *Caretta* two centers of ossification can be seen in the proximal tarsal element, corresponding to the calcaneum (fibulare) and astragalum (intermedium).

As discussed above, the clearest signal that the available material provides at the chondrogenetic level is the

absence of a condensation connected to the distal part of the radius and the tibia. Concerning the identity of elements in the middle carpal and tarsal rows, our material did not offer much resolution. In many cases it was not possible to establish if there was connectivity between elements or whether an element had arisen independently, because the number of samples available for study was low. Early in development, we observed diffuse centers continuous with each other, rather than clearly defined and discrete condensations.

Ossification sequence

In this study, as in previous ones concerning several reptilian groups (e.g., Rieppel, 1993a; Maisano, 2002; Sheil and Greenbaum, 2005) it is shown that the sequence of events in chondrogenesis and osteogenesis are disparate. There are some commonalities in the limb ossification sequence between *C. mydas* and *C. caretta*, and differences with the leatherback turtle, *Dermochelys coriacea*.

The three middle metacarpals are the first hand elements to start ossification in *Chelonia mydas*, as in the trionychid *Apalone spinifera* (Sheil, 2003a), and contrary to the condition of *Chelydra serpentina* studied by Rieppel (1993a), in which distal phalanges start ossification before the metacarpals. In *C. serpentina* (Sheil and Greenbaum, 2005), metacarpalia III–IV are ossified, as are the distal phalanges. In the trionychid *Macrochelys*, lack of resolution in the available data does not permit one to distinguish whether the metacarpals or distal phalanges start ossification first (Sheil, 2005). Based on information gathered from these studies and our own examination of a fine ontogenetic series of the trionychid *Pelodiscus sinensis* (Sánchez-Villagra *et al.*, unpublished), we consider that the onset of ossification in the metapodials and phalanges occurs during a very short period of time in turtles.

As in other sea turtles (Raynaud, 1985), the pisiform is enlarged (Fig. 1) in comparison to other turtles. Rieppel (1993a, p. 508) mentioned that the early ossification (first among carpals) of the pisiform in *Dermochelys* “is the most distinctive example” of a “modification of the sequence of ossification which may be related to adaptive modification of the limb skeleton.” The adult limb proportions of *Dermochelys* are even more derived than those of *C. mydas* and *C. caretta* (Rabl, 1910). In *Chelonia mydas*, the pisiform appears as a condensation later than most other carpals. In the hatchling of *C. mydas* available to us, the pisiform is greatly ossified, as are the ulnare and intermedium, more so than the distal carpals. In *C. caretta*, the ulnare and intermedium start ossification before the pisiform.

In *C. caretta* the ulnare and radiale are the first carpals to ossify, as is probably also the case in *C. mydas*, based on our observation of different degrees of ossification among carpals. This is different from the condition in the American mud turtle *Kinosternon* (Rieppel, 1993a), in which distal carpal (dc) 4 is the first carpal to start ossification, from *Apalone spinifera* (Sheil, 2003a), in which the first four distal carpals are the first carpals to start ossification, and from *Chelydra serpentina* (Sheil and Greenbaum, 2005), in which dc1-2 are the first carpals to ossify.

In *C. caretta*, distal tarsal (dt) 4 is the first tarsal to start ossification, confirming what was reported for sea turtles

with the exception of *Dermochelys*, in which the astragalus was reported by Rieppel (1993a) to ossify first. In the hatchling of *C. mydas* available to us, ossification has started in dt1–3 but not in dt4. In *Chelydra serpentina* (Rieppel, 1993a; Sheil and Greenbaum, 2005), the astragalus is the first tarsal element to start ossification, whereas in the trionychid *Pelodiscus sinensis* (Sánchez-Villagra *et al.*, unpublished), dt4 is the first tarsal to start ossification, a condition commonly found in other reptiles as well (Rieppel, 1993b).

The elements of the manus ossify earlier than those of their serial homologs in the pes in the ossification series of *Caretta caretta* available to us (Fig. 5). Whereas most phalanges have started ossification in the manus of the smallest specimen examined, the same is not true for the pes. The largest specimen has three elements of the carpus already partially ossified, whereas in the pes only the fourth distal tarsal has started ossification.

Miller (1985, p. 313) stated that in *Chelonia mydas* and *Caretta caretta* “the carpals ossify faster” than the tarsals, so at some stage there are more ossified carpals than tarsals, something we confirm here for both species. We recorded in a specimen of *C. caretta* that ossification in the carpals (ulnare and intermedium) started while the tarsals were still all cartilagenous.

The state of ossification in the hatchling of *Chelonia mydas* is much more advanced than that of *Dermochelys* (Rieppel, 1993a, p. 504). Variation in ossification state in hatchlings of this group of marine turtles also characterizes squamates (Maisano, 2001).

Mosaic of derived and plesiomorphic developmental features

Some derived autopodial proportions in adult skeletons are laid out at the condensation stage, and features that were present at some point in phylogeny in turtle ancestors (e.g., a radiale as defined above) are absent at all stages examined. This is an example of developmental penetrance, *i.e.*, the expression of derived features of the adult early in development (Richardson, 1999). On the other hand, conservatism is expressed in the presence of similar patterns of connectivity during early chondrogenesis, and in the overall proportions between forelimb and hindlimb. For example, the manus and pes are similar in size in the earliest stages examined, whereas in only a few later stages, well before the onset of ossification, are the adult proportions between these two elements laid out.

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