Masterarbeit

Titel der Arbeit

Early development of jaws in the brown banded bamboo shark (*Chiloscyllium punctatum*, Müller and Henle 1838) in an evolutionary context

verfasst von

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1) Content

2) Abstract

Chiloscyllium punctatum, the brown banded bamboo shark, belongs to the Orectolobiformes, which is a basal order in the superorder of Galeomorphii. It is a small species of benthic, oviparous sharks, similar to *S. canicula* a well-studied carcharhiniform shark. They are bred in captivity all around the world and nowadays get more and more prominent in science for studying issues as jaw evolution and development. This inquiry aims at answering questions concerning the jaw and tooth development of this species in an evolutionary context.

Staging of the embryonic development was conducted after *S. canicula*, starting with a late pharyngula stage. Formation of the jaw starts at about stage 28 and the main developmental steps occur between stage 29 and stage 30. Tooth development starts a bit lagged at stage 30. Both teeth and jaw mineralize at stage 31 and mineralization of the jaw holds on during growth of the jaw apparatus until the adult organism. It was possible to show, that palatoquadratum and Meckel´s cartilage, as two counter parts, develop in a synchronous way and seem to be dependent on each other. The teeth grow in an alternating tooth patterning and equate each other in morphology throughout the whole jaw.

3) Zusammenfassung

Chiloscyllium punctatum, der braungestreifte Bambushai, ist eine Spezies der Ordnung Orectolobiformes, welche zu der Überordnung der Galeomorphii zählt. Ihre Stellung im Stammbaum ist dabei basaler anzusehen als jene von *S. canicula*, dem Katzenhai, der zu den Carcharhiniformes gezählt wird und heutzutage der Modellorganismus für die Untersuchung entwicklungsbiologischer und evolutionärer Fragestellungen in Hinblick auf die Kieferentwicklung ist.

Der Bambushai ist ein nachtaktiver, eierlegender Vertreter der benthischen Haie und ist in den letzten Jahren, aufgrund seiner leichten Haltung und Züchtung, immer beliebter in wissenschaftlichen Forschungseinrichtungen geworden. Wegen seiner eher basalen Stellung unter den modernen Knorpelfischen sollte er als besseres Modell der Kieferevolution dienen als der Katzenhai und ist deswegen auch Gegenstand dieser Studie. Anhand morphologischer Untersuchungen sollen Fragen die Kiefer- und Zahnentwicklung betreffen in einem evolutionären Kontext beantwortet werden.

Wegen der schon in anderen Studien gezeigten morphologischen Ähnlichkeiten zwischen *S. canicula* und *Chiloscyllium punctatum*, kann die Bestimmung der Stadien der Embryonen anhand von Studien am Katzenhai einfach durchgeführt werden. Das jüngste untersuchte Stadium ist eine späte Pharyngula und wird im Stadium 28 festgelegt. Ungefähr in diesem

beginnt die Kieferentwicklung, wobei die größten morphologischen Veränderungen in der Periode zwischen den Stadien 29 und 30 passieren. Ein wenig verzögert beginnt die Entwicklung der Zähne im Stadium 30, wobei sie nicht funktionsfähig, weil von einer Membran bedeckt, bleiben bis kurz vor oder kurz nach dem Schlüpfen. Sowohl Kiefer als auch Zähne mineralisieren ab dem Stadium 31. Dieser Prozess der Verstärkung der Kieferelemente ist nicht vollendet bis die Tiere völlig ausgewachsen sind. Mit geometrischer Morphometrie war es möglich zu zeigen, dass das Wachstum von Palatoquadratum und Meckels Knorpel recht synchron verlaufen und diese beiden Elemente, als morphologische Gegenstücke, sich offensichtlich nur abhängig voneinander entwickeln können. Die Zähne wachsen in einem alternierenden Muster, wobei es keinen Unterschied zwischen embryonalen und adulten Zähnen gibt. Außerdem ähneln sich die Zähne morphologisch in Ober- und Unterkiefer sehr.

4) Introduction

Jaws are the main novelty of gnathostomes, which led to their success in the animal kingdom (Compagno et al., 2005). The evolution and variety of this apparatus is one of the most prominent topics in science (Kuratani, 2005, 2012; Compagnucci et al., 2013; Medeiros and Crump, 2012; Depew and Compagnucci, 2008). Unfortunately it has proven to be one of the most difficult and controversial subjects nowadays too. Scientists do not even agree about which modern organism can be chosen as model organism for jaw evolution (Kuratani, 2005; Shigetani et al., 2002, Depew et al., 2002; Medeiros and Crump, 2012; Godard and Mazan, 2012). There are several proposals and all have their warranty so far.

Phylogeny

One suggested model group for jaw evolution research are the chondrichthyans, particularly the Neoselachii, which comprises all extant sharks and rays (Underwood, 2006; Marcelo R. de Carvalho, 2007; Hadfield et al, 2010). The sister group to chondrichthyans, which is summarized as the cartilaginous fishes, are the osteichthyans, the bony fishes, which constitute the evolutionary basis to all terrestrial vertebrates. The last common ancestor of these two taxa was dated back 525 million years (Gillis and Shubin, 2009). Most of modern osteichthyans have a very elaborate jaw apparatus, therefore, this thesis focuses on the "easier" and potentially more basal jaws of a modern Neoselachii, the brown banded bamboo shark (*Chiloscyllium punctatum*, Müller and Henle 1838). Always bearing in mind, that living neoselachians constitute a rather derived clade within the chondrichthyans (Underwood, 2006).

The monophyletic group of Neoselachii presumably evolved in the early Triassic and remained relatively insignificant until the Jurassic (Underwood, 2006). During the Jurassic and Cretaceous, this group underwent adaptive radiation, which led to a very high diversity in this era. Although the fossil record is relatively poor, because of the earliest Neoselachii being represented by isolated teeth only, it is clear that the two major clades within this ancestral taxon are the Galea and Squalea representing crown neoselachians (Klug, 2009).

The phylogeny within these taxa is not resolved up to now. The group of Squalea, which comprises all "skate-like" sharks is either monophyletic, Batoidea (skates) included, or paraphyletic, if the batoids form a sister clade (Marcelo R. de Carvalho, 2007). Depending on which data is used for the phylogenetic tree the outcome is different. Molecular data places the batoids outside the Squalea, whereas morphologists think they belong to this clade

(Underwood, 2006, Maisey et al. 2004). The second major clade, the Galea comprises all "real" sharks. This group is phylogenetically better resolved than the Squalea, still there are some controversies. The relations of the groups within the Galea of for example the Orectolobiformes, Carcharhiniformes and Lamniformes, are not absolutely clear yet (Figure 1). The oldest known fossil is dated back to the early Jurassic and intriguingly has a similar morphology to modern Hemiscylliidae (further information see Underwood, 2006), which is the group forming the focus of. *Chiloscyllium punctatum* is member of the Orectolobiformes, therefore pertains to a very basal position within Galea (Figure 1). Thus the bamboo shark presumably is a good model organism to study the evolution of jaws or other traits, respectively. Maybe it is even better than the lesser spotted dogfish (*Scyliorhinus canicula*), because the Scyliorhinidae are part of the Carcharhiniformes, which are more derived (Wilga, 2005; Maisey et al., 2004). For that reason, to study the evolutionary development of jaws in basal gnathostomes, the brown banded bamboo shark was chosen as model organism in this study.

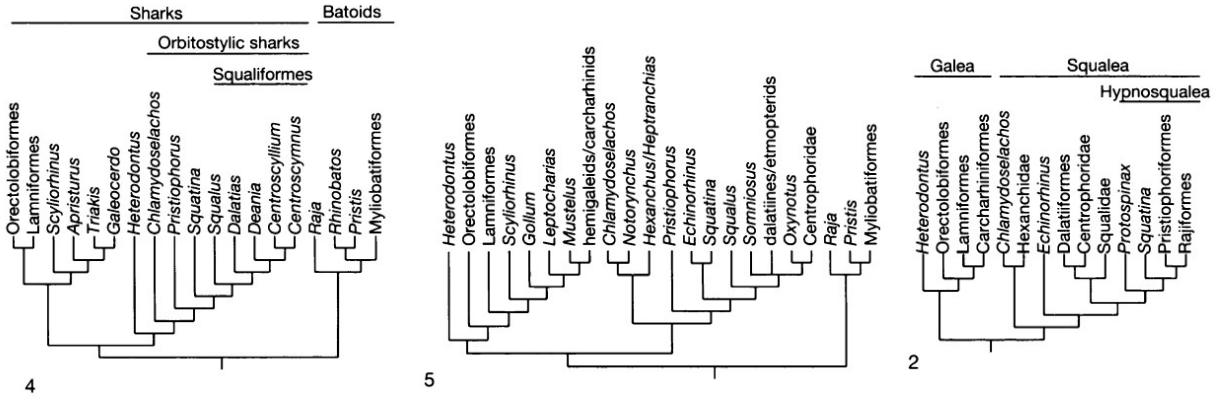


Figure 1: Summaries of the main neoselachian phylogenies showing differences in grouping of taxa. 4 and 5 are both based on molecular data and 2 on morphological characters. Note the different positions of the Orectolobiformes with regard to the Lamniformes and the Carcharhiniformes, respectively. Modified after Underwood (2006).

Anatomy

The skeleton of all chondrichthyans consists predominantly of cartilage, hence they are relatively light and extremely flexible, which makes them the perfect predator. Parts of the skeleton, which have to bear high pressures, like the jaws, vertebral column or fins have supportive calcium phosphate structures, reminiscent of bone (Compagno et al., 2005). These encompass the cartilage on its surface and form a layer of small apatite tiles, the so called tesserae, glued together by collagen fibres (Maisey, 2013). Jaws of species like *Carcharodon carcharias* (great white shark) or *Geleocerdo cuvieri* (tiger shark) would not sustain the

pressure during biting without these reinforcements (Dingerkus et al., 1990). Like all Galea, *Chiloscyllium punctatum* has a hyostylic jaw suspension, which means that the Palatoquadrate is free with only one joint to the neurocranium, the ethmopalatine articulation. The whole jaw apparatus is connected via the hyoid arch to the otic region of the neurocranium (Figure 2) (Wilga, 2005).

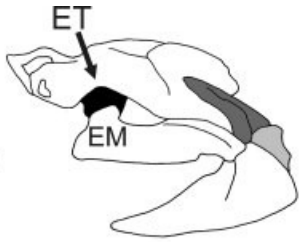


Figure 2: Schematic representation of a hyostylic jaw suspension. The ethmoid process connects the palatoquadrate through the ethmopalatine articulation with the neurocranium. Coloured in dark grey is the hyomandibula and in light grey the ceratohyal, both forming the second branchial arch (hyoid). EM, ethmoid process; ET, ethmopalatine articulation. Modified after Wilga (2005).

Orectolobiformes can be distinguished very easily by their mouth position with the opening being in front of the eye. They have five gill slits and a spiracle, an anal fin and two dorsal fins. Orectolobiformes is considered monophyletic and comprises seven families, among which the Hemiscylliidae with 16 recent species is the most diverse one (Compagno et al., 2005). The brown banded bamboo shark, as well as skates and other benthic sharks are bottom dwelling, searching the ground for prey. Therefore they cannot use their mouth, which is in close contact with the ground, to suck in water for breathing during prey capture. The spiracle is a pseudobranchie. Thus in the ancestral condition it was a branchia and important for breathing, but secondarily reduced it is used for water influx only (Romer and Parsons, 1983).

Distribution

Chiloscyllium punctatum is resident to an area from east India to Japan and North Australia (Harahush et al., 2007). It lives in coral reefs to maximum of 85 m depth and hides in caves or under corals. It is nocturnal and oviparous and feeds on bottom invertebrates or fish (Harahush et al., 2007). This shark inhabits a wide range of environmental conditions and is relatively easy bred in caption. Therefore nowadays it is very common in diverse scientific or educational facilities (Harahush et al., 2007).

Mating and embryology

Mating occurs after the change of temperature during seasonal changes in spring time only once a year. Sperms are then stored and eggs are laid from July to February (Harahush et al., 2007). Females stay constant to deposition locations and always lay the eggs in pairs. *Chiloscyllium punctatum* has a relatively long incubation time and juveniles hatch after 153 days post deposition (dpd) (Harahush et al., 2007). They leave the egg with 13-17 cm and mature up to 60-105 cm (Compagno et al., 2005). Before hatching they consume the external yolk completely and just retain an internal yolk sac, on which they feed the first 10 days after hatching. There is a natural variation between growth rates of the individuals, but the staging of Ballard et al. (1993) for the dogfish development corresponds well to *Chiloscyllium punctatum* according to Harahush et al. (2007).

Theories of jaw evolution

Kuratani (2005) as well as Minoux and Rijli (2010) mention the initial conserved patterns of differentiating cells in the developing facial region of vertebrates. Based on these and other observations in morphological and genetic studies among all gnathostome species, there occur three main evolutionary theories, which try to describe the way of jaw evolution from a jawless gnathostome ancestor to a jawed species of this group. In a review paper Kuratani (2012) describes these theories in detail. In this section only the main characters of each theory, respectively, will be described.

Each theory suggests a cyclostome-like common ancestor of all gnathostomes. The recent species of this group, for example amphioxus, have a differentiated mandibular arch, hence it is questioned if this relatively complicated structure can be compared to recent jaws of basal gnathostomes like sharks and fish, and if it is really less derived than these. Nevertheless, missing of a better starting point, cyclostomes are assumed a common ancestor of all gnathostomes (Kuratani, 2012).

Classical theory

Comparative morphologists in the 19th century and later on (Rathke, 1827; Romer, 1966; Mallat, 1996) elaborated a theory for jaw evolution, which is tightly linked to the traditional theory of head segmentation compiled in these days too. An idealized amphioxus-like ancestor is assumed, which has an undifferentiated, metamerical pharyngeal arch skeleton (Kuratani, 2012). One of the rostral arches is the mandibular arch, which enlarges and divides to become the jaw (Figure 3). This very simple description coincides well with the Hox-gene

expression program, but nowadays is more and more neglected, partly because of the assumption of premandibular arches (Kuratani, 2012). These structures neither have been found in any fossil record nor, does the theory work out the shift of the mouth posteriorly, if the premandibular arches really form the trabecula as suggested (Figure 3). Scientists generally agree that such a shift is not the most parsimonious way, which is thought to be sought for by evolution. Besides that not all versions of this theory assume premandibular arches. Nevertheless, there are many inconsistencies about the homologies between certain structures of the assumed ancestor and the recent organisms (Kuratani, 2012).

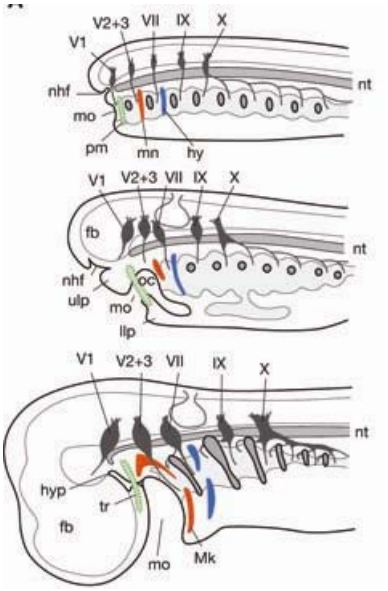


Figure 3: Schematic representation of the classical theory of jaw evolution. On top the idealized ancestor (amphioxus-like ancestral vertebrate) with three undifferentiated pharyngeal arches (coloured) is depicted. The mouth opening needs to take a posterior shift behind the premandibular arch (green) and first forms the upper and lower lip of the ammocoetes larva of lampreys (middle) and in vertebrates (pharyngula of shark at bottom) becomes the trabelucae (light green). The mandibular arch (red) stays relatively undifferentiated until it gives rise to the jaw apparatus, which then gets attached to the neurocranium by the hyoid arch (blue). V1, V2+3, VII, IX and X represent branchiomeric nerves, each innervating one pharyngeal arch. fb, forebrain; hy, hyoid arch; llp, lower lip; Mk, Meckel´s cartilage; mn, mandibular arch; mo, mouth opening; nhf, nasohypophyseal foramen; nt, notochord; oc, oral cavity; pm, premandibular arch; tr, trabeculae; ulp, upper lip. Modified after Kuratani (2012).

Neoclassical theory

The neoclassical theory is based on the consideration, that the ability of closing the mouth facilitates ventilation as well as prey caption and selection drove evolution in this direction (Kuratani, 2012). An ammocoetes larva-like ancestor with a premandibular oral cavity is assumed in this scenario. This cavity was fringed by the upper and lower lip and ended posteriorly at the mandibular arch (Kuratani, 2012). During evolution, the premandibular oral cavity got more and more reduced while the mandibular arch enlarged, divided dorso-ventrally and made up the new fringe of the mouth opening (Figure 4). The premandibular ectomesenchyme forms the trabeculae, which is the cranial base for the forebrain, as well as

the upper lip of the gnathostomes (Kuratani, 2012). The premandibular oral cavity exists in recent cyclostomes and the evolution of the trabeculae is taken into account as well, although recent studies have shown, that the cyclostome trabecula is not homologous with the gnathostome trabeculae (Kuratani, 2012). Among other points, like the differences in position of the nasal and adenohypophyseal placode (more detail below) between Cyclostomata and Gnathostomata, this lead to the refusal of this theory and a revision is needed.

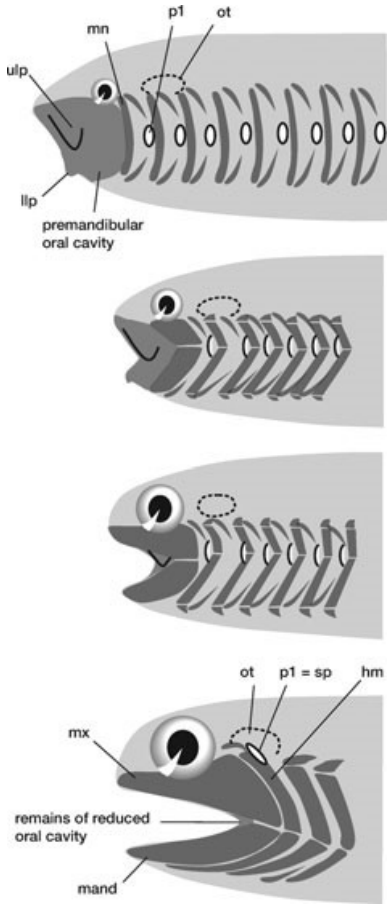


Figure 4: Schematic representation of the neoclassical theory of jaw evolution. From top to bottom are the evolutionary steps depicted. An ammocoetes larva of the lamprey is said to have a premandibular cavity, which gets reduced to make mouth closing possible. First fringed by the upper and lower lips, the new forming mouth is now supported by the maxillary and mandibula, which enlarged during evolution of the jaw. hm, hyomandibula; llp, lower lip; mand, mandibula; mn, mandibular arch; mx, maxillary; ot, otic capsule; p1=sp, spiraculum; ulp, upper lip. Modified after Kuratani (2012).

Heterotopy theory

According to Kuratani (2012) "jaw evolution has to be interpreted as changes in the neural crest derived ectomesenchyme". Hence to explain the heterotopy theory properly, first a revision of the neural crest cell patterning, which is essential for gnathostome rostral development, is necessary. In general patterns of neural crest cells between cyclostomes and gnathostomes are very similar and thought ancestral, but there is one important difference which has become the core feature of the heterotopy theory (Kuratani, 2012). The oral domain

in all chordates is *Dlx* positive. This gene marks all components in development, which form part of the mouth opening in the adult, but the neural crest contributions to this area are different between gnathostomes and cyclostomes. Thus between these two taxa a heterotopic (homologies between genes and anatomical elements have been uncoupled) shift has taken place (Kuratani, 2012). In gnathostomes there are two main streams out of the neural crest cells, which form the rostral region in development. The trigeminal crest cells differentiate into the mandibular arch only, whereas the premandibular crest cells partly migrate into two main regions, the preoptic (frontonasal ectomesenchyme) and the postoptic (trabeculae). Hence the jaw apparatus is exclusively of trigeminal crest cell origin. The ammocoetes larva upper lip is not a pure mandibular arch derivative, but is invaded by premandibular crest cells, which do not migrate to the pre- or postoptic region (Kuratani, 2012). Thus as mentioned above the *Dlx* expression pattern indicating the position of the oral region marks only the trigeminal crest cell population in gnathostomes and the trigeminal crest cells as well as the premandibular crest cells in cyclostomes (Figure 5). Additionally, Kuratani (2012) explains in detail that the separation of the nasal and oral cavities is conducted by a different set of crest cells in gnathostomes and cyclostomes, respectively, and that the anlagen of the olfactory and adenohypophyseal placodes are placed very differently in these two taxa.

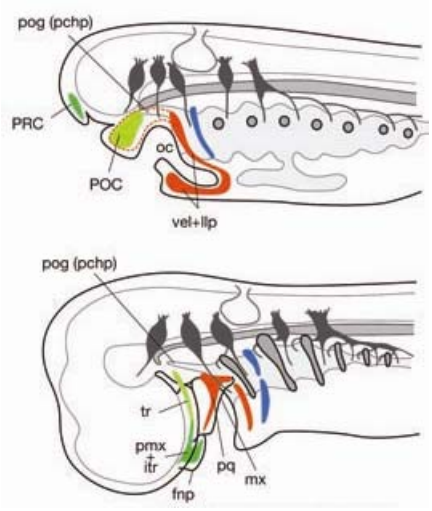


Figure 5: Schematic representation of the heterotopic theory of jaw evolution. The postoptic crest cells (light green) in the upper lip of the ammocoetes larva (top) are homologized with the trabeculae of the pharyngula of the shark (bottom). The fringe of mouth opening in ancestor is formed by two components, namely the post optic crest cells and the trigeminal crest cells (red), whereas in the gnathostomes only least form the jaw. Coloured in blue hyoid arch, coloured in dark green preoptic crest cells which form frontonasal process, premaxillary bone and inter trabecula in gnathostomes. llp, lower lip; fnp, frontonasal process; itr, intertrabecula; mx, maxillary process; oc, oral cavity; pmx, premaxillary bone; POC, post optic crest cells; pog (pchp), preoral gut (prechordal plate); pq, palatoquadrate; PRC, preoptic crest cells; tr, trabeculae; ulp, upper lip; vel,velum. Modified after Kuratani (2012).

There are three main commonalities of the neoclassical and the heterotopy theory, namely that both mention the premandibular origin of the cyclostome upper lip, suggest a posteriorly reduction of the oral domain, and assume the importance of premandibular structures (not arches!) in the evolution of jaws (Kuratani, 2012). Nevertheless, only the heterotopy theory depicts this process with least inconsistencies, but it has its gaps as well. For example, it equates the upper jaw of gnathostomes only with the palatoquadrate, although this morphology is only present in chondrichthyans. In higher gnathostomes additionally premaxillary elements form part of the jaw, whichsoever evolution cannot be described by this gene shift only (Kuratani, 2012).

Research question

This study aims at complementing the developmental staging of Harahush et al. (2007) with focus on the jaw development taking jaw evolution into consideration. Additionally questions concerning the tooth development and tooth patterning of *Chiloscyllium punctatum* just as possible mineralization patterns of the jaws should be answered. A central question here is: When exactly does jaw formation start and in which way does the mandibular arch differentiate into the palatoquadratum and Meckel´s cartilage?

Results gathered in this study should be compared to summarized, recent results of evolutionary studies in the fields of morphology as well as genetics to eventually find common patterns. As described above, there are three main theories of jaw evolution, which are in focus of many researches now, which try to identify the most likely one. This study intends to find hints which coincide with one of these theories and finally it should be possible to depict a model of evolutionary development of jaws in basal gnathostomes deduced from the findings of this inquiry.

5) Material and methods

Material

Altogether, nine embryos already fixed (either in a typical alcohol series 25%/ 50%/ 75%, Bouin or Formol) and separated from the eggshell (stage 28-32 of Ballard et al. (1993)), and two young adult sharks, all stored in 75 % alcohol, as well as three reconstructed jaws (table 3) were available for this study. All embryos were used for the staging and together with one of the young adults later on were cleared and stained. The other young adult was stained with

iodine and scanned with a microCT (SkyScan1173, Bruker microCT, Belgium). All cleared specimens and the three reconstructed jaws were analysed with geometric morphometrics.

Documentation

First all specimens were photographed (Panasonic Lumix DMC-FS7, lens Leica DC Vario-Elmarit 10MP), to document the characters used in the developmental staging. The dissected elements (procedure see below) were photographed with a microscope (VHX-1000 KEYENCE Digital Microscope, Double R lens). All pictures were then edited in Adobe Photoshop CS5 (Adobe Systems Incorporated, California USA). Addition of the scale bars, cropping of images and Auto-colouration and Auto-contrast, respectively, were performed. The determination of the tooth patterning and further labelling also were conducted in this program. Specimens with enough mineralization in the jaw elements were possible to be scanned with the microCT. For better contrast one young adult was stained with iodine (following adjusted protocol of Metscher 2009 I&II, for more detail see Supplement Table 6). E12 and E13 were scanned (Table 1) after the clearing and staining procedure (see below), without any further preparation, as was one adult jaw. These 3D scans were then cropped with Data Viewer (Bruker microCT Corporation, Belgium) to a handy data volume and only the jaw apparatus was reconstructed in Amira (FEI, Oregon USA).

Table 1: Listing of relevant data for CT scans. Specimens were all scanned with the same CT (SkyScan1173, Bruker microCT Corporation, Belgium).

Name animal	Image Pixel Size (um)	Source Voltage (kV)	Source Current (uA)
E11	9.98	50	160
E12	9.98	50	160
E13	12.11	50	160
Young adult	14.96	50	160
Adult 1	33.13	80	100

Clearing and staining

Whole specimen clearing and staining methods were employed to differentiate bone from cartilage. Therefore several protocols were combined and modified (Song and Parenti, 1995; Dingerkus et al. 1977; Nishikawa, 1987; Filipski and Wilson, 1884, 1986; Potthoff, 1984; Taylor, 1967; Wassersug, 1976).

In Table 2 all solutions are listed, which are needed for the following protocol. All specimens listed in Table 3 were used for clearing and staining. E10, E11, E12, E13 and one young adult got eviscerated. No skinning or removal of the eyes were necessary.

Table 2: Listing of all reagents used for the clearing and staining protocol. In brackets more information concerning the reagents.

Solution	Ingredients
Cartilage staining solution	10 mg Alcian Blue 8GS (C.I. 74240, Carl Roth GmbH Karlsruhe, dry matter >50%) 80 ml 96% EtOH 20 ml 100% Glacial acetic acid (Carl Roth GmbH Karlsruhe, density: 1.05)
Calcium staining solution	Stock solution: 0.1 g Alizarin Red S (C.I. 58005, Carl Roth GmbH Karlsruhe, M 360.40 g/mol) 100 ml dH_20 Working solution: 200 ml 0.5% potassium hydroxide solution (Carl Roth GmbH Karlsruhe, density: 1.5) 2 ml stock solution
Digestive solution	70 ml dH_2O 30 ml 6% di-sodium tetra borate decahydrate (Carl Roth GmbH Karlsruhe, density: 1.73) 1 g Trypsin from porcine pancreas (Sigma-Aldrich Chemie GmbH Steinheim, 101 U/mg)
Sodium borate (6%)	65 g borax 1000 ml dH_2O (60°C)
Nerve staining solution	Stock solution mix in warm water bath: 100 ml 70% EtOH 0.5 g Sudan Black B (Carl Roth GmbH Karlsruhe, M 456.55 g/mol) Working solution dilute stock solution with: 70% EtOH to a 5% nerve staining solution

The first step was the complete dehydration of the animals for approximately 24h in 96% Alcohol. It is important to work as water free as possible, because cartilage staining improves in a dehydrated environment (Potthoff et al. 1984). Small specimens to a maximum size of 3 cm lasted approximately 2h in the staining solution and bigger specimens maximum 24h. The entire specimen should be blue after this step. Differentiation and clearing were accomplished through hydration by descending alcohol concentrations. The specimens were put two times in 96%, 70% and 50% alcohol and dH_2O about two to three hours respectively (or until they sank). The next step required a higher temperature, so an incubator (Memmert, Germany) was used to accelerate the clearing step (Song and Parenti, 1995). At about 25 °C the animals were digested in the Trypsin-Borax solution. It is recommended to prepare the solution right before usage, hence the enzyme stays active (Taylor, 1967). Smaller specimens took about three days for this procedure, the larger ones about ten days. The digestive solution was changed every two days, or when it was stained blue. The specimen proceeded to the next step, when about three quarters of the body were cleared. All specimens were incubated for about one hour (or until they sank) in 0.5% KOH to prepare the bone staining procedure and then transferred into the bone staining solution for a maximum of three hours. KOH reacts very aggressively to the

tissue and dissolves the bodies very fast. Thus, when small specimens are stained it is recommended to use 75% Alcohol instead of 0.5% KOH (Taylor, 1967). For destaining and bleaching it is advised to leave the specimens in 0.5% KOH with some drops of H_2O_2 (Hydrogen peroxide 30%, Carl Roth GmbH Karlsruhe, density: 1.11) (Song and Parenti, 1995), but due to the aggressivity of KOH this step was skipped in most of the procedures and immediately the nerve staining was prepared. First three dehydration steps were conducted (30%, 50% and 70% alcohol), each 30 minutes lasting (or until it sank) and the following 24h the specimens remained in the nerve staining solution. The specimens needed to be destained again in 70% and/or 60% alcohol for 30´´ to two minutes, depending on how much colour had to be washed out. It can last now for about 1-2 days in 50% alcohol. When specimens were stained too dark or there still was too much tissue visible, if necessary a digestion step was conducted (Taylor, 1967). The 0.5% KOH/Glycerol series started after the specimens were cleared and stained well. Again, the first step in pure 0.5% KOH was skipped because of the strong chemic characteristics of KOH and they were transferred first into a 3:1 0.5% KOH/85%Glycerol (85% Glycerol, Otto Fischar GmbH Saarbrücken) mixture, then in a 1:1 and finally into a 3:1 0.5% KOH/85%Glycerol mixture, for about 24h (or until they sank). In the last step specimens were stored in pure 96% Glycerol (Carl Roth GmbH Karlsruhe, density 1.26) (for more details see Supplement Table 1-5).

Slight adjustments were taken in stages E2, 4 and 5. In these specimens no bone or nerve staining was conducted, thus directly after cartilage staining, the 0.5% KOH/Glycerol series was initiated (for more details see Supplement Table 1).

Dissection

Specimens E10-E13 and a young adult one, were big enough to make a dissection possible. The upper and lower jaw elements (left and right Palatoquadrate / Meckel´s cartilage) were extracted. The specimens E3 and E5 already dissolved during the clearing and staining procedure, so the palatoquadrate and Meckel´s cartilage had to be distinguished from the remaining elements. Only in E3 it was possible to detect an element defined as Meckel´s cartilage. Neither in E3 nor in E5, upper jaw elements could be found.

Dissection and preparation of the jaw elements were performed with two fine forceps and partially under a binocular (Leica wild M3C).

Geometric Morphometrics

The statistical analysis of the growth patterns of the palatoquadratum and Meckel´s cartilage respectively, was the major challenge of this study. The pictures of the dissected jaw elements were loaded into tpsUtil (by James Rohlf, life.bio.sunysb.edu) for constructing a tps file. Then tpsDig (by James Rohlf, life.bio.sunysb.edu) was used for setting landmarks. Further on, this data was saved as text file and opened in Past (by Øyvind Hammer, University of Oslo) for computing the Procrustres fit. The transformed data was now analysed with a principal component analysis and additionally shape changes were depicted in thin plate spline graphics (grids) if possible (Hammer et al. 2001).

Three main analysis were conducted: One of the palatoquadratum, one of the Meckel´s cartilage and one of both together, each computed with Procrustes fit. Additionally the last analysis was repeated without Procrustes fit, to include size change.

6) Results

After fixation, specimens were in a good condition. Only E6 was already beheaded as visible in Figure 6.

Staging

The staging procedure after Ballard et al. (1993) was conducted first on all specimens. In Table 3 the specimens with their individual designation, which does not coincide neither with their stage nor their age, are listed. The characteristics describe the main features which Ballard et al. (1993) used for their specimens and which were found in *Chiloscyllium punctatum* as well. Unfortunately not all animals´ birth and death dates were recorded, hence the evaluation of the exact age of all organisms was impossible (Table 3).

Specimens E2 (15d) and E4 (18d) were both categorized as stage 28. The main character of this stage is the hatching gland, which dissolves in this stage and is important for the extra embryonic environment inside the egg (Ballard et al. 1993). Stages 28 to 30 do not have any pigmentation, except for a circle of dark pigment around the eyes (Table 3). The main characters to distinguish stage 29 from stage 30 are the outer gill filaments, which are longest in stage 29 (E5, 21d) and already retracted in stage 30 (E6) (Table 3). Besides that, E6 already has the adult body shape and does not resemble the embryos from stage 28 to 29 anymore. The medial fin fold has split into the anal and caudal fins, respectively and the head to body axis already is fully developed (Figure 6).

Table 3: Designations and stages of individuals with corresponding characteristics. Staging after Ballard et al. (1993), using characteristics mentioned in the table. Names of specimens are not according to the stages nor their age. Not all animals' birth dates were known, thus age in days could not be evaluated.

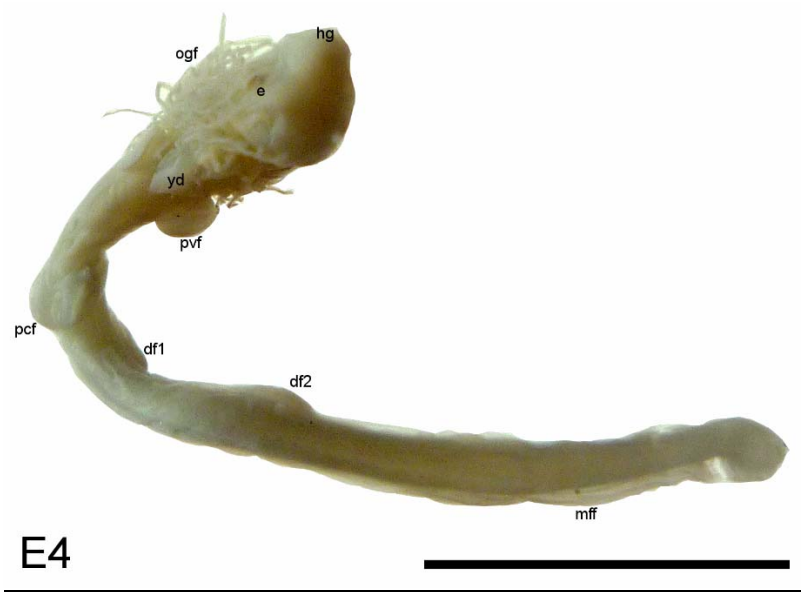
Name animal	Age/Stage of animal	Characteristics
E2	15d/ St.28	Fixed in Bouin, stored in 75% Alcohol Approx. 3 cm total length Yolk sack cropped, yolk duct visible No pigmentation, except for light pigmentation around eye Gill filaments outside body (approx. 3 mm length) Pectoral and pelvic fin fully developed, anal and dorsal fin still connate in medial fin fold Hatching gland visible Mouth opening oval
E3	-/ St.29	Fixation in alcohol series, stored in 75% Alcohol 3.5 cm total length Yolk sack cropped, yolk duct visible No pigmentation, except for light pigmentation around eye Gill filaments outside body (approx. 4-5 mm length) All fins fully developed except for anal fin, still connate in medial fin fold No hatching gland visible any more Rostrum differentiates and grows in 90° to body axis Mouth opening curved
E4	18d/ St.28	Fixed in Bouin, stored in 75% Alcohol 3 cm total length Yolk sack cropped, yolk duct visible No pigmentation, except for light pigmentation around eye Gill filaments outside body (approx. 1-2 mm length) Pectoral and pelvic fin fully developed, anal and dorsal fin Still connate in medial fin fold Hatching gland visible
E5	21d/ St.29	Fixed in Bouin, stored in 75% Alcohol 4 cm total length Yolk sack cropped, yolk duct visible No pigmentation, except for eye encircled in pigmented ring Gill filaments outside body (approx. 5 mm length) All fins fully developed except for anal fin, still connate in medial fin fold
E6	-/ St.30	Fixation in alcohol series, stored in 75% Alcohol 4.5 cm total length Yolk sack cropped, yolk duct visible No pigmentation, except for eye encircled in pigmented ring Gill filaments outside body (<5mm length) All fins fully developed Small barbels visible

E10	-/ St.31	Fixed in Formol, stored in 75% Alcohol 9 cm total length Yolk sack 2.5 cm diameter Light brown bands on light pink body colour dorsal and light brown ventral Residuals of outer gill filaments very short, swollen gills Fully developed barbels Placoid denticles on back and rostrum
E11	-/ St.31	Fixed in Formol, stored in 75% Alcohol 8.5 cm total length Yolk sack cropped, yolk duct visible Pigmentation darker than in E10 No outer gill filaments visible, swollen gills Fully developed barbels Placoid denticles on back and rostrum
E12	61d/ St.31	Fixed in Bouin, stored in 75% Alcohol Approx. 10 cm total length Yolk sack approx. 2 cm diameter Pigmentation darker than in E11 No outer gill filaments visible, swollen gills Fully developed barbels Placoid denticles on back and rostrum
E13	-/St. 32	Fixed in Formol, stored in 75% Alcohol Approx. 12 cm total length Yolk sack cropped, yolk duct visible Dark bands on light brown body colour dorsal and light brown ventral No swollen gills
Young Adult	>153d	Fixed in Formol, stored in 75% Alcohol Approx. 20 cm total length Brown bands on light brown body colour dorsal and light brown ventral
Adult 1 Adult 2 Adult 3	-	Only reconstructed jaws

Pigmentation of the whole body becomes visible in stage 31 (E11; E12, 61d). In this stage there can still be some residuals of the outer gill filaments apparent and the whole gill apparatus is still very swollen. On the dorsal side of the specimens, placoid denticles (Ballard et al., 1993) can be distinguished as little dots on the rostrum and back. These scales can be seen until stage 32 (Table 3). In this stage the gills are not swollen anymore and the specimens resemble the adult, only that the pigmentation is darker. The young adult has no yolk duct anymore and the placoid scales are fully developed (Figure 6).

Chiloscyllium punctatum embryos feed on yolk during embryogenesis, similar to the condition seen in *Scyliorhinus canicula* (Ballard et al. 1993). The yolk sac is, especially in the

early stages, relatively large and impractical during the fixation steps, so it was removed in all specimens, except for E10 and E12 (Figure 6). The so-called “red line” on the yolk sac, which is visible in ventral view of E10 and dorsal view of E12 (Figure 6), is mentioned by Ballard et al. (1993) too. It is the vein growing from the embryo into the yolk sac, supplying the animal with the essential nourishment (Ballard et al., 1993). As the young adult has claspers, which already is visible in E13, both can be categorized as males (Figure 6).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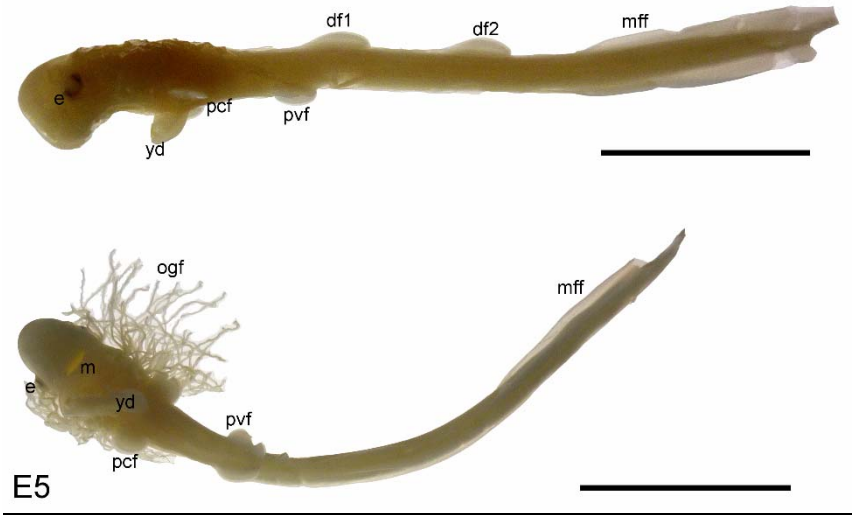
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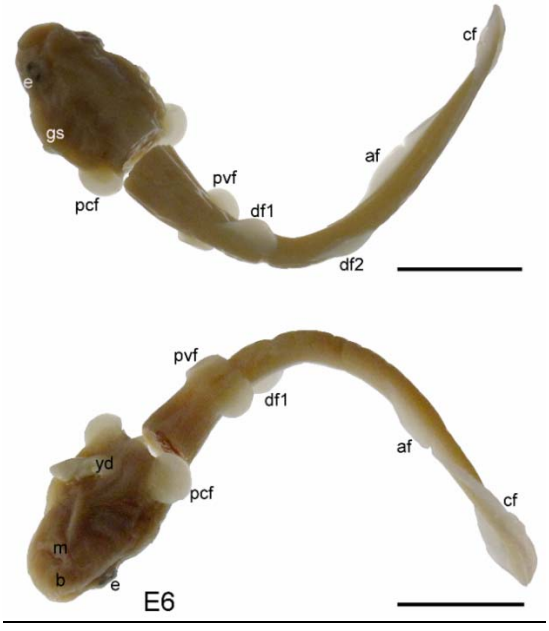
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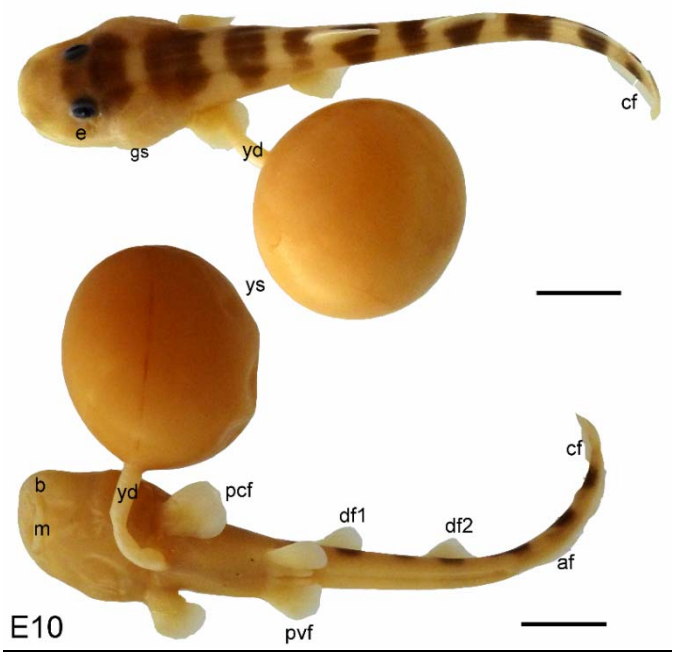
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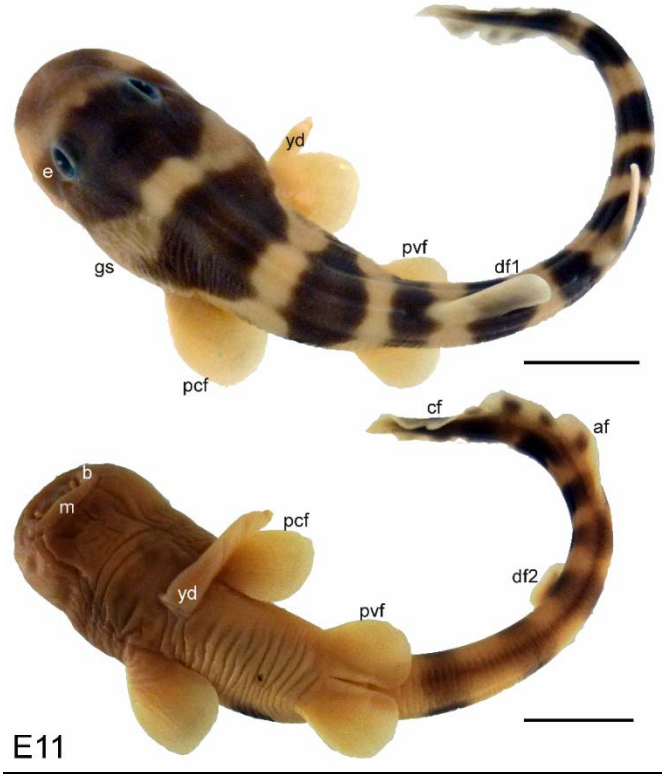
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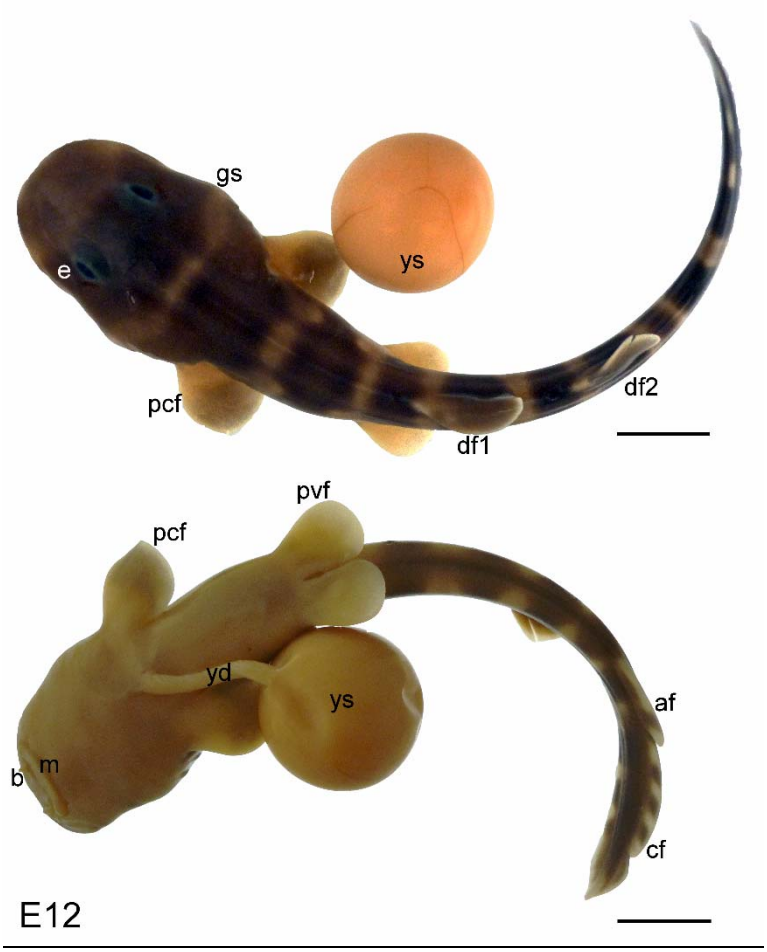
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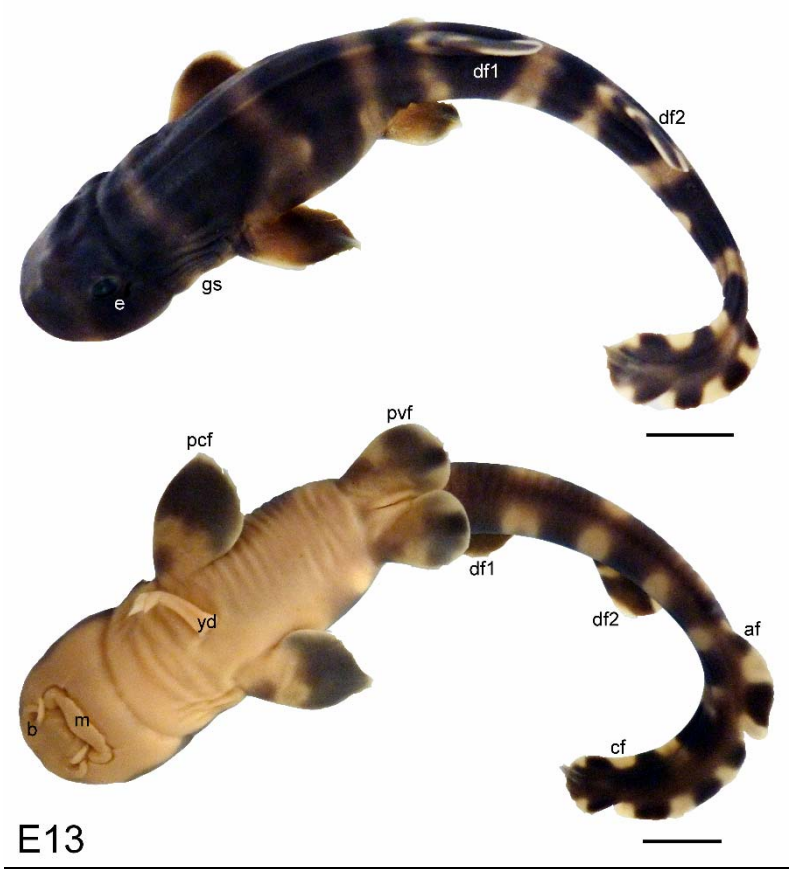
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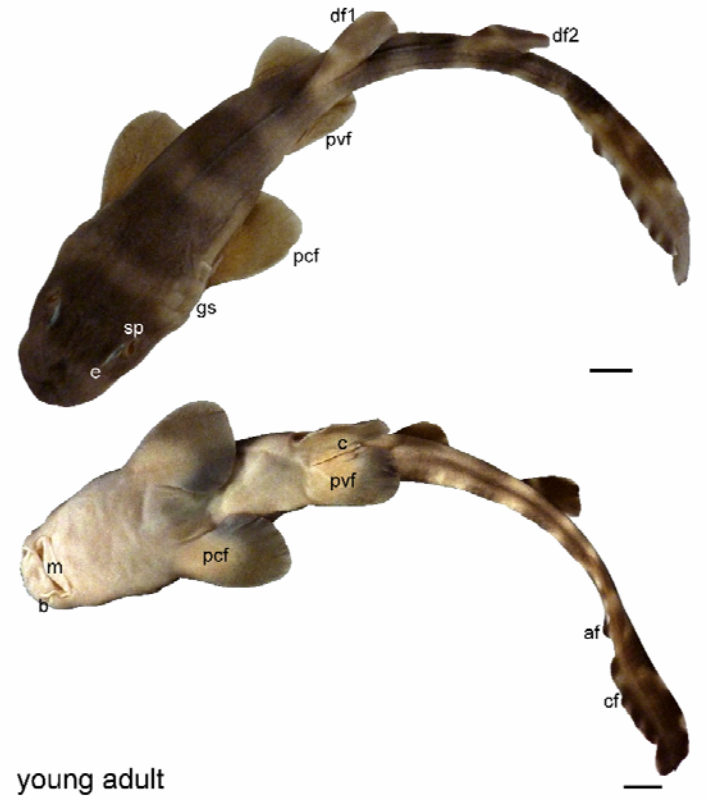


Figure 6: Description of the morphology of the specimens. Names of individuals according to table 3 on left bottom of every image, respectively. Scale bars 1cm. Anterior left, posterior right, dorsal top, ventral/lateral bottom. af, anal fin; b, barbel; c, clasper; cf, caudal fin; df1, first dorsal fin; df2, second dorsal fin; e, eye; gs, gill slits; hg, hatching gland; m, mouth; mmf, medial fin fold; ogf, outer gill filaments; pcf, pectoral fin; pvf, pelvic fin; sp, spiracle; yd, yolk duct; ys, yolk sack.

Clearing and staining

There are no pictures of the later stages (E6-ya), nor of E3 after the clearing and staining procedure, because it failed. Specimens mainly dissolved or were stained too dark by the nerve staining with no chance of clearing, hence documentation of these was not feasible. Nevertheless, this failure led to the following cleared and stained specimens of the youngest stages (E2, E4 and E5), which thanks to improvements of the treatment (for detail see Table 1 in Supplement) worked out well (Figure 7).

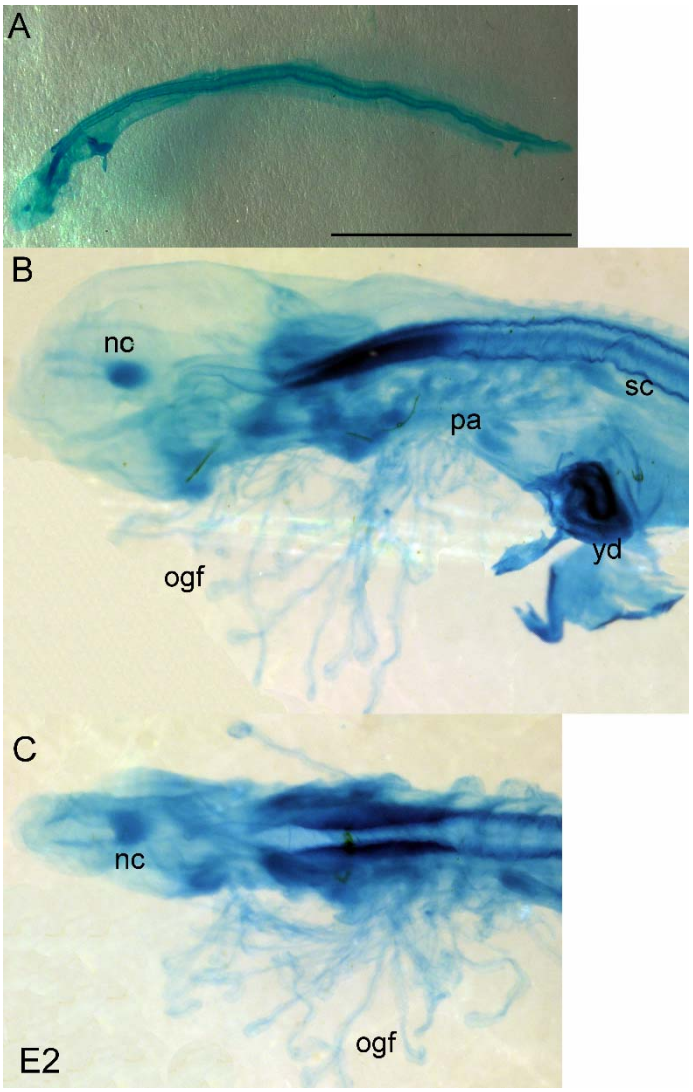
At the earliest stage investigated, there is still the hatching gland visible and beyond the presumptive nasal capsule is forming. This anterior part is very fragile, because there is no supporting skeleton in the head. The neurocranium just begins to grow. There are no fin rays visible, so the fins visible in Figure 7 are just dermal outgrowths with no backing. The pharyngeal arches just start to form, but are not clearly discernible yet, nor are the structures in the mouth region (Figure 7).

In E4 the pharyngeal arches are well differentiated and all six are discernible. The first, or mandibular arch is divided into two parts, which build up the presumptive jaw

anlagen. Posterior to the arches, the spiral valve is visible. It is already well developed. The hatching gland still forms a bladder on top of the nasal capsule anlagen and the neurocranium is still a dark stained extension of the spinal cord (Figure 7).

E5 is partly beheaded, the nasal capsule and tissue anterior to the developing neurocranium are missing. Nevertheless, the pharyngeal arches are well discernible and even more developed than in E4. The mandibular arch is not visible that well, but it grew bigger, hence the jaw anlagen are forming. The hyoid arch enlarged as well and clearly connects to the first arch. In this stage the fin rays are differentiated in the paired fins (pectoral and pelvic), but not yet in the medial fin fold (Figure 7).

The previous staining procedures showed no signs of mineralization in this early stages, hence no calcium staining was conducted. Mineralization arises relatively simultaneously with the growth of teeth at stage 31 (Figure 8 and Figure 9). At this stage the placoid denticles occurred for the first time too and stained light red. Concurrent with mineralization of the teeth and scales, tesserae (mentioned in introduction) are visible at the articulation site of the palatoquadratum and Meckel´s cartilage. Up to the adult stage, starting from the joint, mineralization mantels the whole upper and lower jaw respectively (Figure 8).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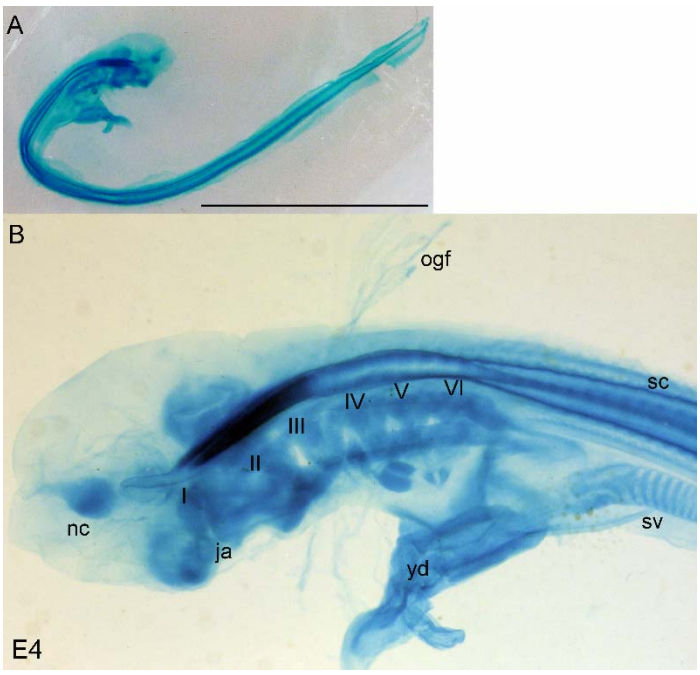
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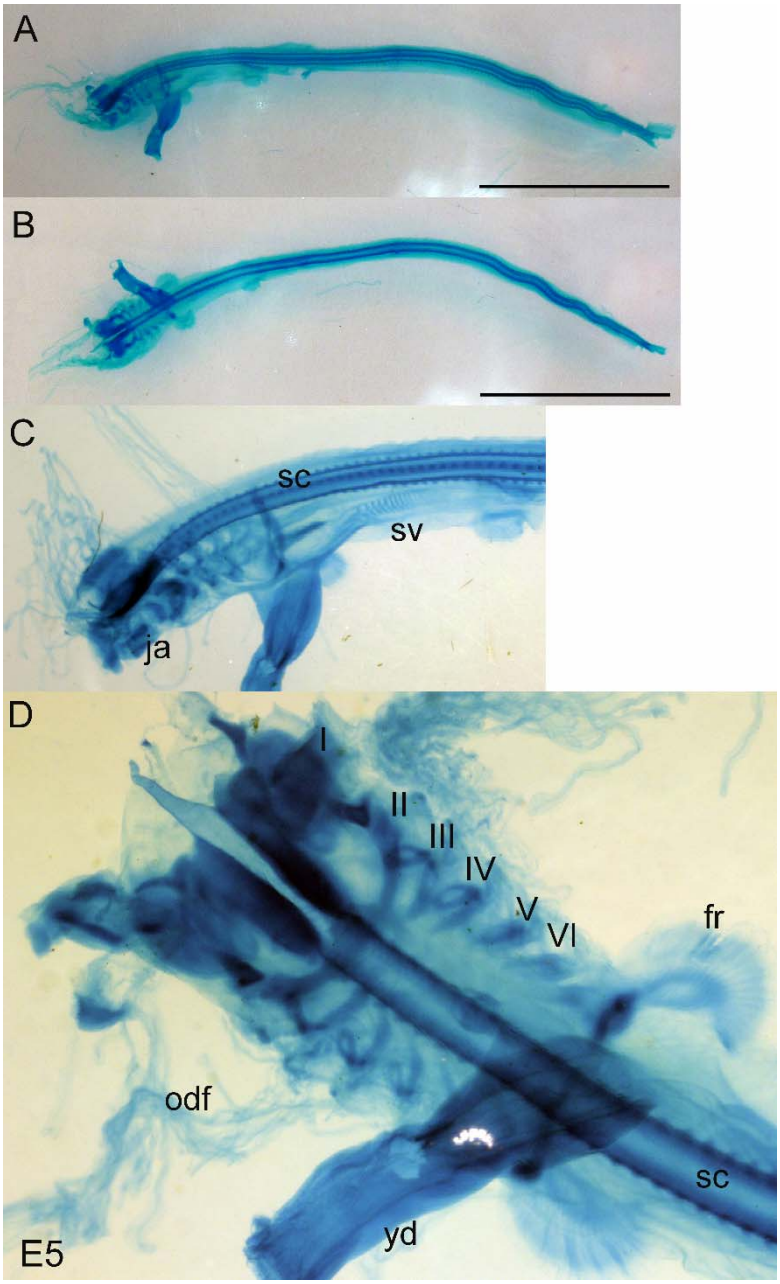


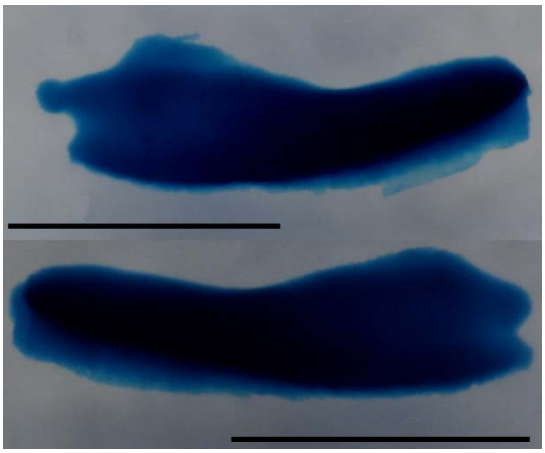
Figure 7: Clearing and staining results.E2, A, lateral view of whole animal. B, detail of lateral, anterior view, pharyngeal arches start developing but are not discernible exactly. C, detail of ventral, anterior view. **E4,** A, lateral view of whole animal. B, detail of lateral, anterior view, pharyngeal arches well discernible and spiral valve visible. **E5,** Partly beheaded animal, nasal capsule and tissue anterior to the developing neurocranium missing. A, lateral view of whole animal. B, ventral view of whole animal. C, detail of lateral, anterior view. D, detail of ventral, anterior view, arches well countable, but no jaw elements discernible, fin rays already developed. Anterior left, posterior right. Dark blue coloured anterior end of spinal cord is developing neurocranium. Note that spiral valve (E4 and E5) is developed before the jaw apparatus. Names of individuals according to table 3 on left bottom of every image, respectively. Scale bars 1cm. I, mandibular arch; II, hyoid arch; III-VI, pharyngeal arches; fr, fin rays; ja, jaw anlagen; nc, nasal capsule; pa, pharyngeal arches; ogf, outer gill filaments; sc, spinal cord; sv, spiral valve; yd, yolk duct.

As all specimens got cleared and stained in the first step. All subsequent procedures were conducted with this material. Hence all skeletal elements of the organisms are stained blue, except for the reconstructed adult jaws.

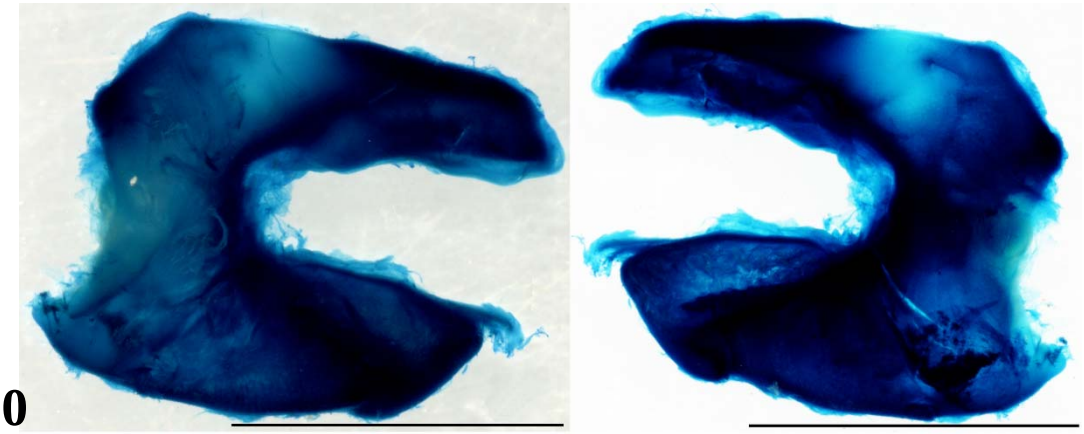
Dissection

After the failed clearing and staining try, the jaw elements were gathered from each individual and as much tissue was removed as possible.

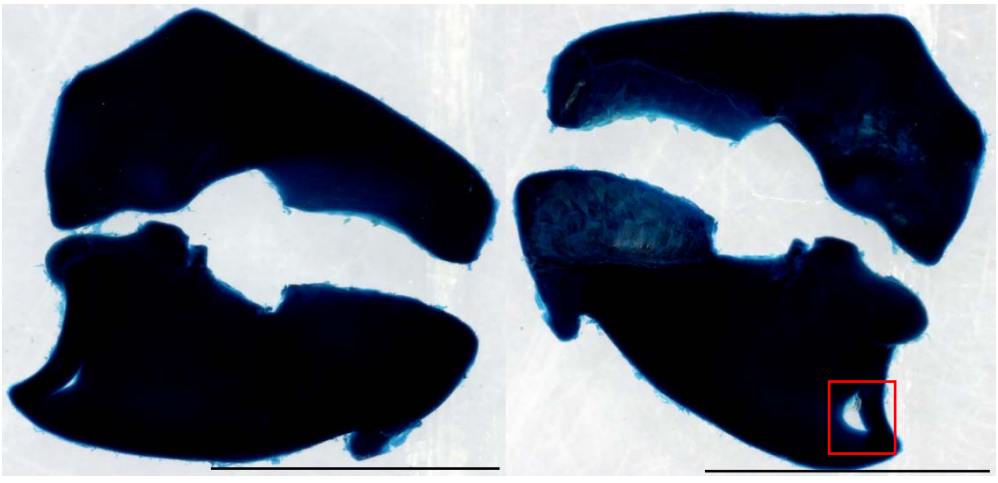
E3

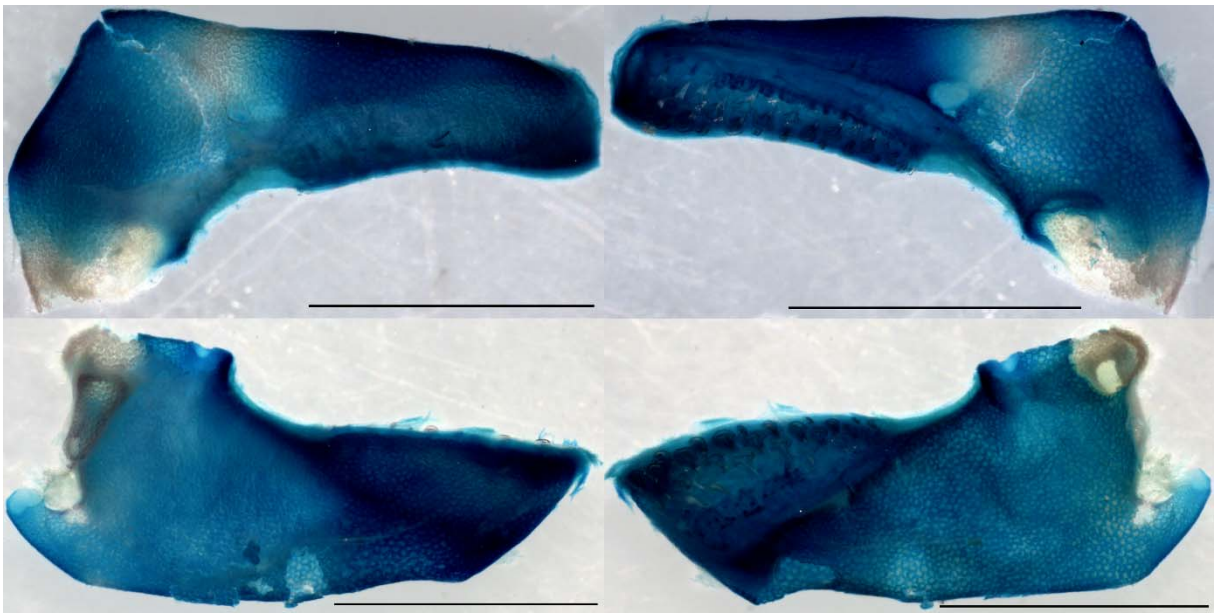


E10

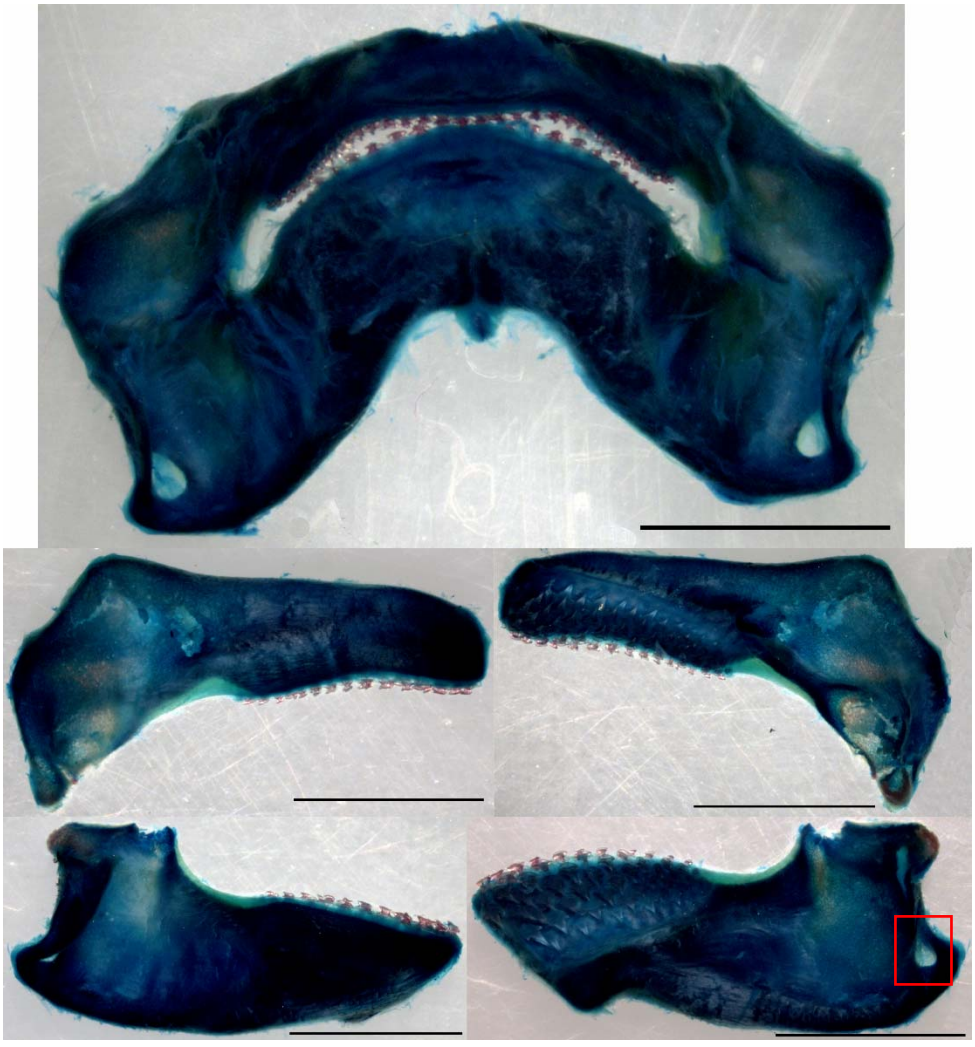


E11

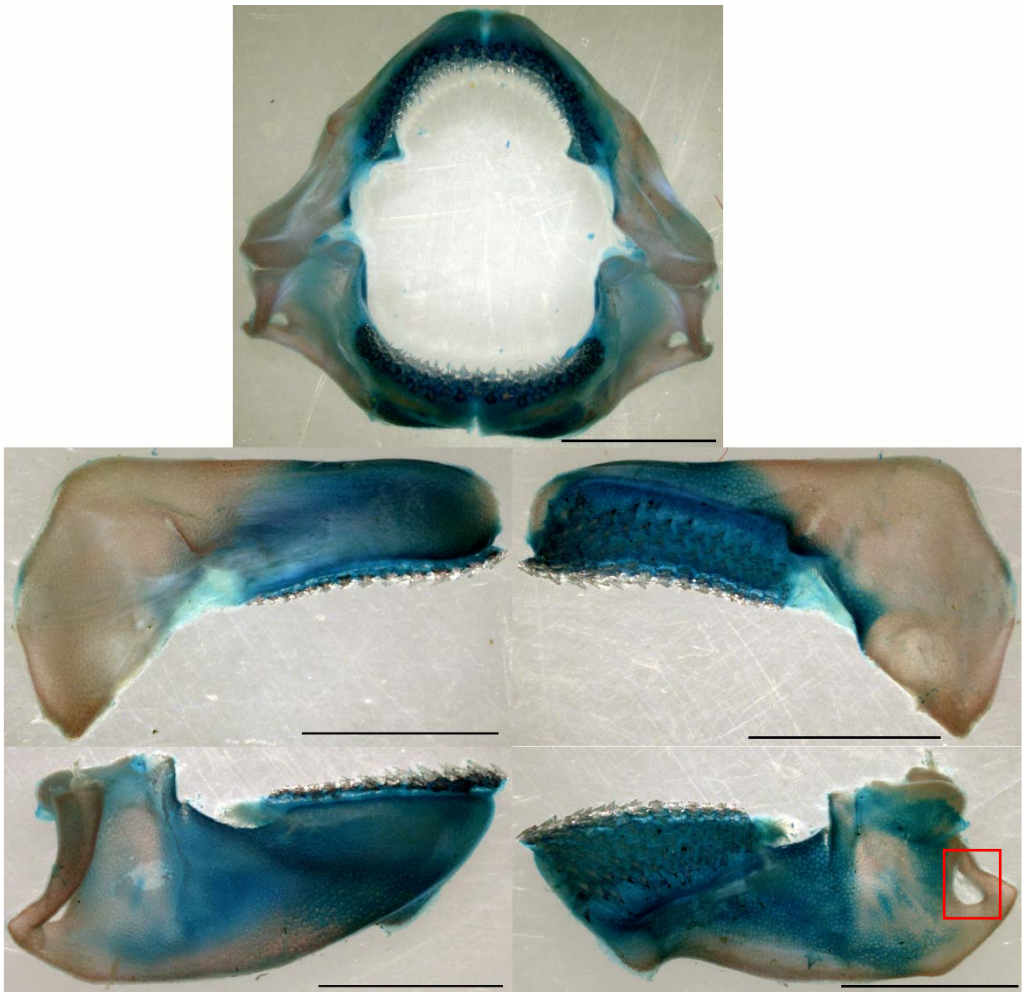




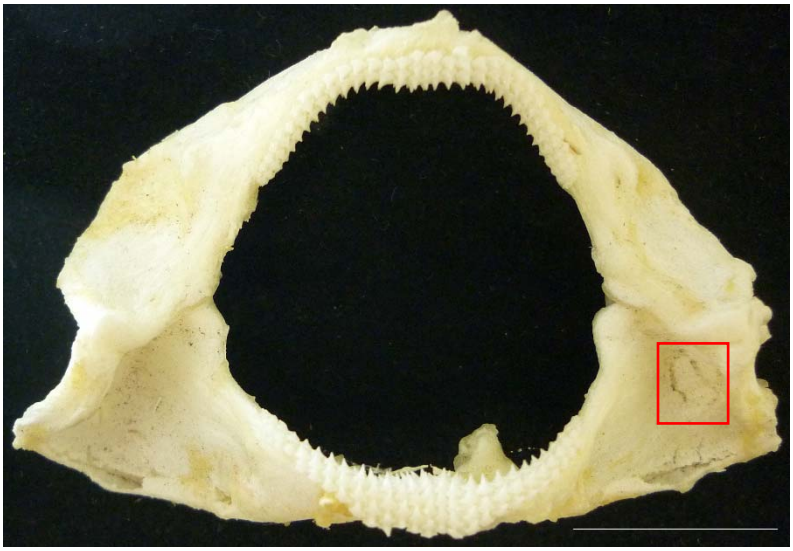
E12



E13



ya



a2

Figure 8: Dissected jaw elements. E3, only lower jaw left and right side. **E10-ya,** right side of jaw elements, on top, if possible whole jaw anterior view and below dissected elements, left labial view, right lingual view. First sign of teeth in E11, but still covered by membrane. First positive calcium staining (red) in E12. Note foramina visible in Meckel´s cartilage of E11, E13 and ya (red quadrate). Presumptive site of foramen in a2 (red quadrate). Scale bar 1cm each, except for E3 scale bar 1mm. a2, adult 2 ; ya, young adult.

The assumed lower jaw elements of E3 have a process (only element in image on top complete, Figure 8), which was defined as the posterior part of the Meckel´s cartilage. This will be shown in more detail in the geometric morphometrics part. E10 jaw elements already have a similar shape to the young adult. The dental region, where in E11 the first teeth arise, is in this earlier stage covered by a membrane, which disappears in E12, when teeth show first signs of mineralization. The skeleton of E10 is still very fragile and unfortunately got damaged during the dissection, hence the foramen visible in E11, cannot be depicted in E10, though it was already present (Figure 8).

Mineralization of the teeth and the skeletal elements is first visible in E12 and spreads further in later stages until it covers the entire jaw apparatus in the adult animal. E12 got damaged as well during the dissection, thus the foramen also is not visible . Nevertheless, it is present from stage 31 to the young adult and is not visible anymore in the adult jaws, although in these reconstructed jaws at this site the material is less stable and partly broken (left Meckel´s cartilage of adult 2 in Figure 8).

Tooth patterning

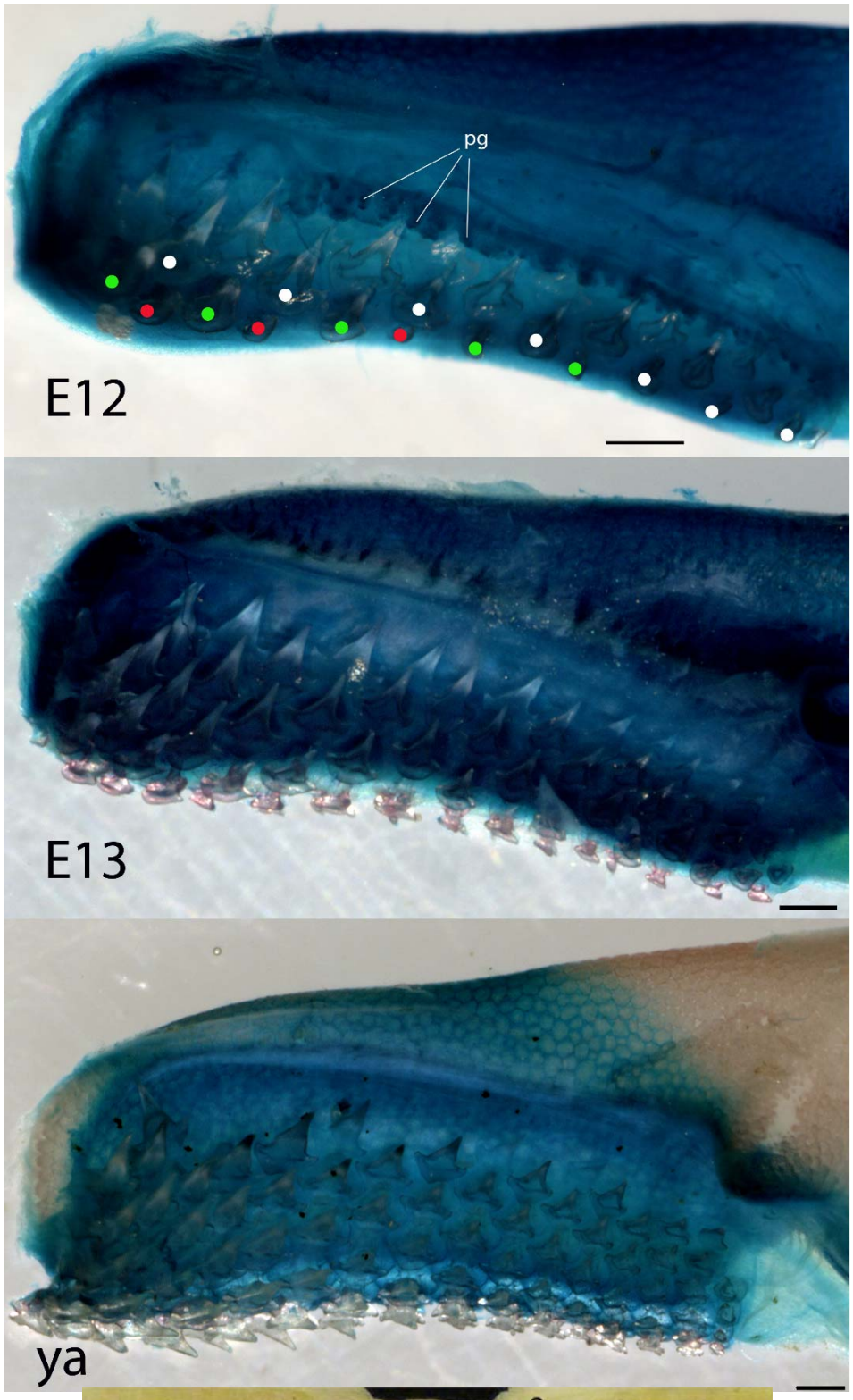
Analysis of the tooth patterning was conducted according to the paper of Smith et al. (2012). The earliest stage of *Chiloscyllium punctatum* with teeth in this study was stage 31. As already mentioned in the previous chapter, E11 was the first individual with teeth on the palatoquadratum and Meckel´s cartilage, respectively (Figure 9). E10, which was categorized stage 31 too, conversely lacks teeth at all (Figure 8).

It was very difficult to distinguish teeth in the jaw of E11, hence it was impossible to describe a certain patterning. It should be mentioned, that single teeth are present and the whole dental region is covered by a membrane (Figure 9). Attempts to remove this tissue failed, because, the teeth are caught in it and would have been removed too. As the size of the region where teeth grow enlarges, the number of teeth in a horizontal row increases as well and more and more teeth erupt at once (Figure 9 and Figure 1 of supplement). Teeth are bigger in the anterior part of the jaw and get smaller in the posterior part. In E12 the teeth were not covered by the membrane anymore and protogerms, the presumptive tooth anlagen (Smith et al., 2012), are visible in the upper and lower jaw lingually. The light red colour of the teeth is a sign of mineralization, visualized during the clearing and staining procedure. Hence in stage 31, the first teeth appear and after the membrane is reduced, teeth mineralize (Figure 9).

In E12 (St.31, 61d) upper and lower jaws, there are already tooth families (vertical rows) and the alternate tooth replacement patterning (named after Smith et al. 2013) of *Chiloscyllium punctatum* can be seen as well. From this stage on this patterning is visible in all individuals (Figure 9). This pattern can be described as following: Coloured teeth in Figure 9 have the same age, because they erupted at once and form one horizontal row. Teeth with the same colour do not neighbour each other, but are separated by an older and a younger tooth, respectively.

From stage 32 on (E13 to young adult and adult) the only difference between the jaws is the number in teeth and tooth families, which increases. Teeth grow from inside (lingual) out (labial), because protogerms form in a lingual position and teeth erupt in a labial position. In the young adult, as well as the adult jaws, the maximum number of tooth families in the upper jaw counted was 14 and in the lower jaw comprised 13 families, additionally to the symphysis tooth family, which is unpaired (Figure 1 of Supplement). The number of teeth in the sat unit enclosing the symphysis tooth family and the first tooth family does increase too. One sat unit combines the generations of teeth in two tooth families, hence the sat unit of the tooth families “one” and “S” accounts for all generations present in the jaw (Figure 9).

A



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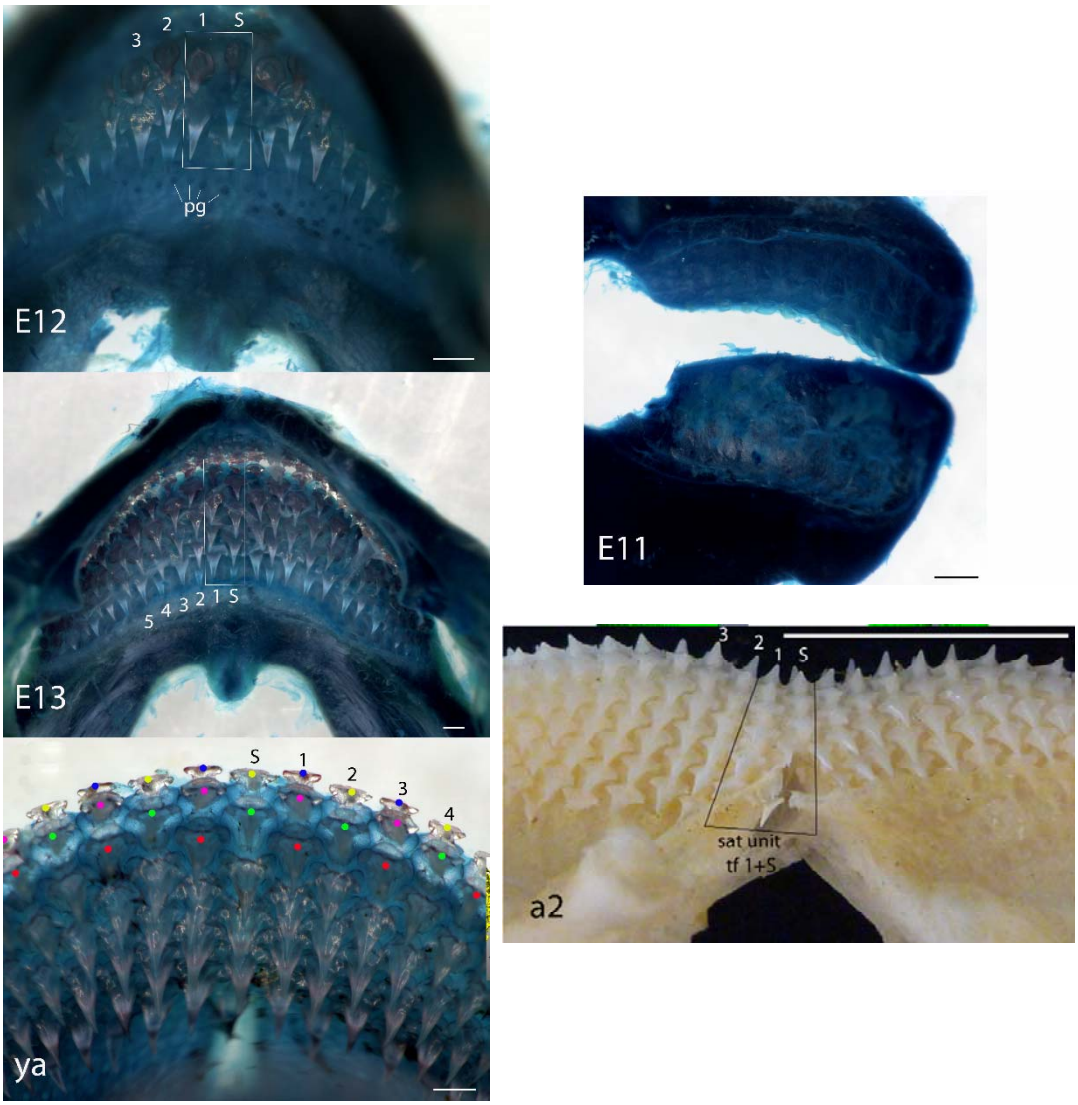
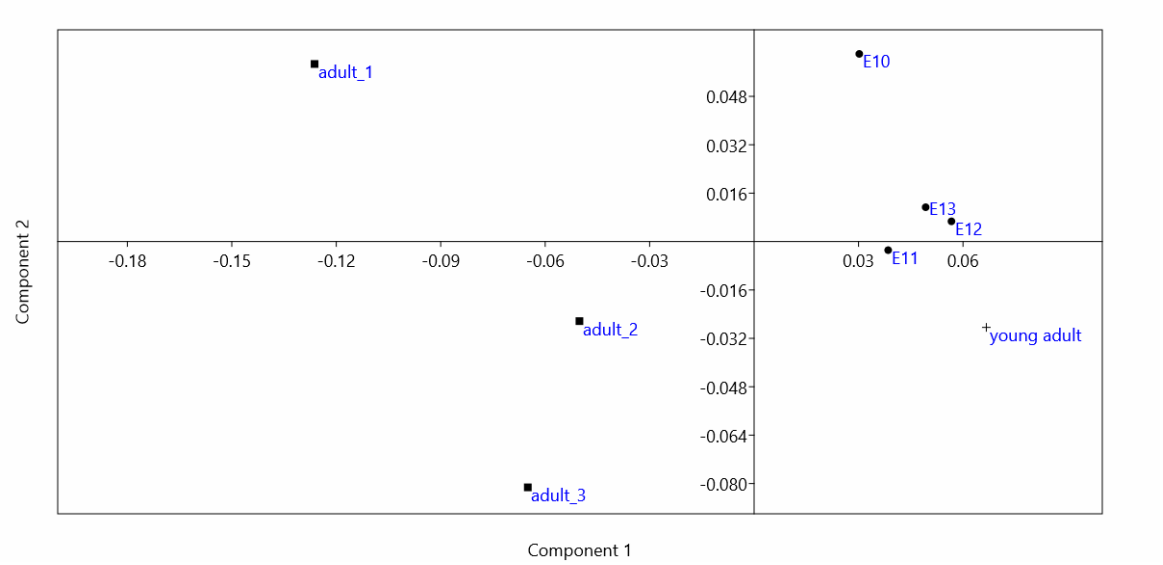


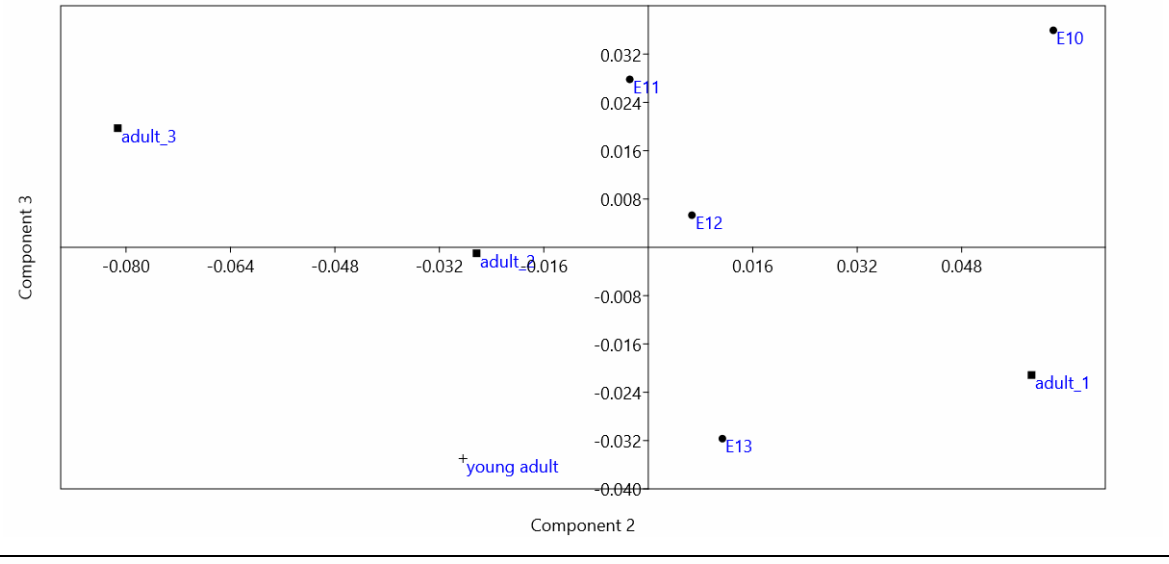
Figure 9: Tooth patterning of dissected jaw elements. A, upper jaw. **B,** lower jaw elements, except for **E11** upper and lower jaw shown. Names of individuals according to table 3 on left bottom of every image, respectively. Colouration shows teeth erupted at once. Numbers count vertical tooth rows, which are called tooth family. One sat unit (enclosed by rectangle) is made up by two neighbouring vertical rows. **E12**, protogerms, which are later forming teeth are visible at tooth forming sites lingual (Smith et al., 2012). Scale bar 1mm each, except for a2 scale bar 5 mm. a2, adult 2; pg, protogerm; S, teeth at symphysis; tf, tooth family; ya, young adult.

Geometric morphometrics

Geometric morphometrics analysis was conducted on the upper jaw and lower jaw elements, respectively, and on all combined as well. Graphs depicting the principal components of each result, and if possible the related grids are shown below.

The principal component analysis of the palatoquadratum was conducted with nine landmarks (Figure 11). As already described in Material and Methods, only shape data was computed. Principal component one describes 54%, principal component two 24% and principal component three 8% of all variance. The eigenvalues for each, respectively, are 0.0050, 0.0022 and 0.0007.





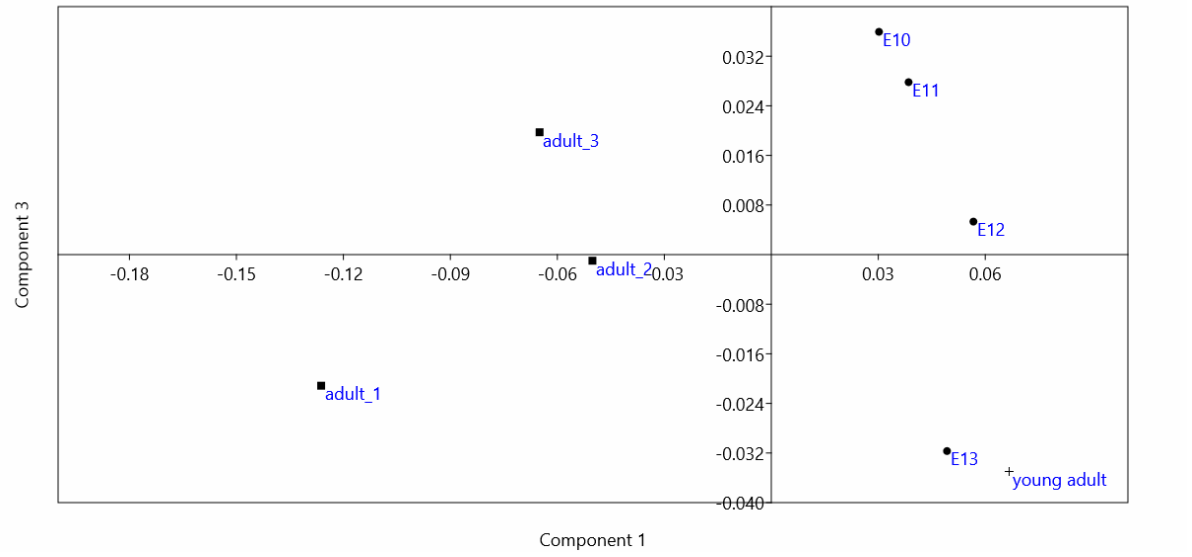


Figure 10: Principal component analysis graphs of palatoquadratum. Nine landmarks set on E10-E13, young adult and adult 1-3. Only shape data computed. Component one describes 54% (0.0050), the second 24% (0.0022) and the third 8% (0.0007) of variance. Eigenvalues in brackets.

Component one obviously separates the embryos and the young adult from the adult individuals. Whereas component two and three split the organisms into a developmental series, with the youngest embryo on top of the graph and the young adult at the bottom. The adult jaws do not fit into that pattern and cluster either outside the young individuals or, as illustrated in the graph showing component two and three, diagonal through this group (Figure 10).

The corresponding grids to this principal component analysis of the palatoquadratum are shown in Figure 11. The landmark set shown in red in the examples, is the same, but shown in black on the grid, which illustrates enlargement or compression between these landmarks through distortion of the grid (Figure 11 and 13).

The first developmental step from E10 to E11 is minimal and only a slight compression of the ventral side together with a slight enlargement of the dorsal part is visible. The next step is the opposite, because especially the dental region grows. From E12 to E13 the whole palatoquadratum increases in length and in the last step the angle, which is marked by landmarks 2-4 gets narrower again due to the growth of the articulation and dental site (Figure 11). The deformation grid depicting the developmental steps from the youngest embryo (E10) to the young adult illustrates that the main shape changes happen in the dorsal-ventral direction due to the narrowing of the angle between landmarks 2-4 (Figure 11).

The growth to a final adult condition is shown at the bottom of Figure 11. The three deformation grids all have a major commonality: Growth in length means that the angle between landmarks 2-4 widens. There are no main shape changes in the articulation area or where the teeth grow, but the central part of the palatoquadratum increases in size.

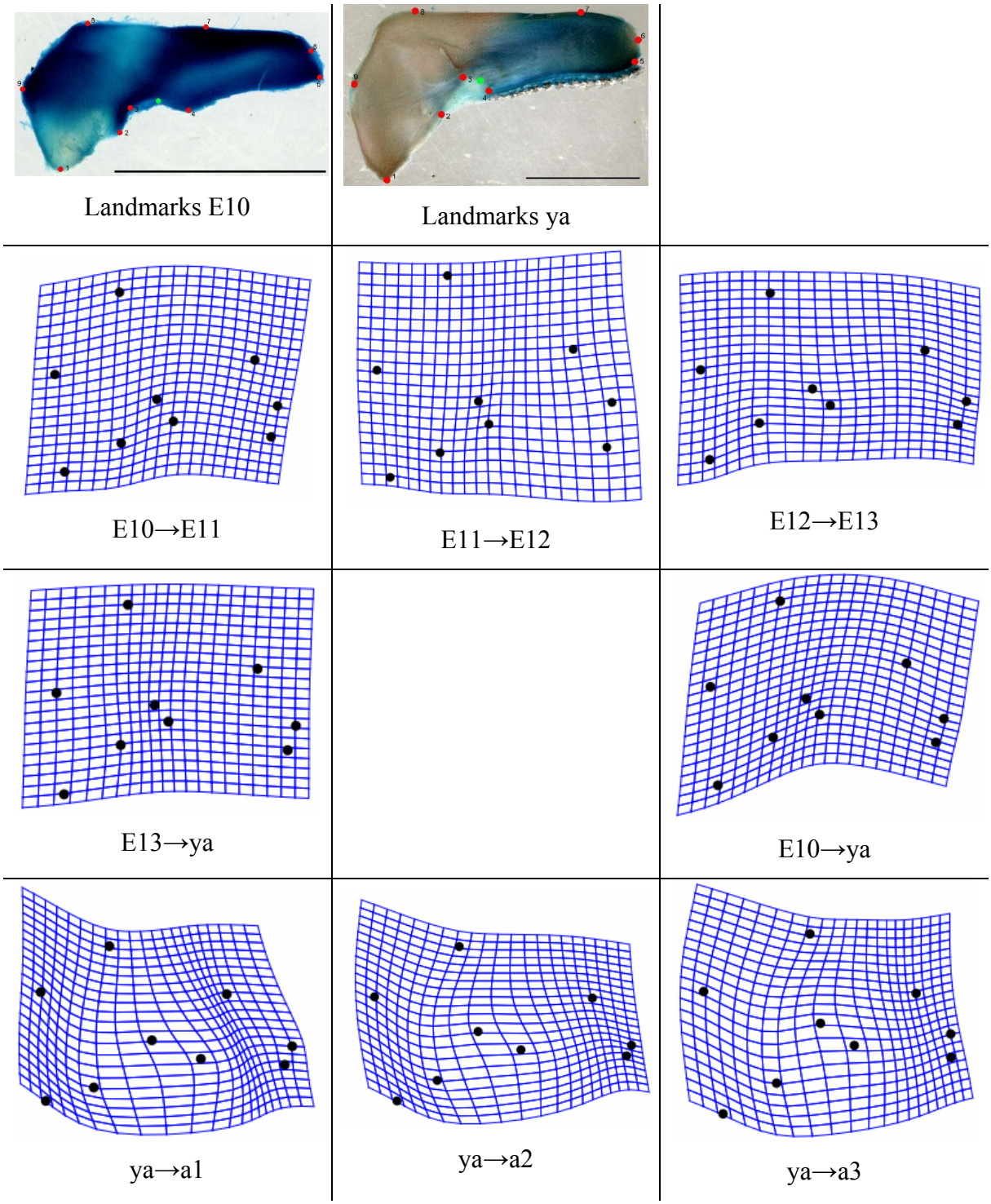


Figure 11: Principal components analysis deformation grids of palatoquadratum. On top examples of landmark set (red dots) (9). Green landmark used with others, except for landmark 6 in principal component analysis of all jaw elements together (Figure 14). Numbers define landmark order. Scale bars 1cm. Arrow between labels of specimens shows which grid is used as basis and to which individual the grid is computed. a1, adult 1; a2, adult 2; a3, adult 3; ya, young adult.

The Meckel´s cartilage was analysed with eleven landmarks (Figure 13) and resulting principal components describe following variance (and eigenvalues): first 57% (0.0170), second 26% (0.0076) and third 9% (0.0026).

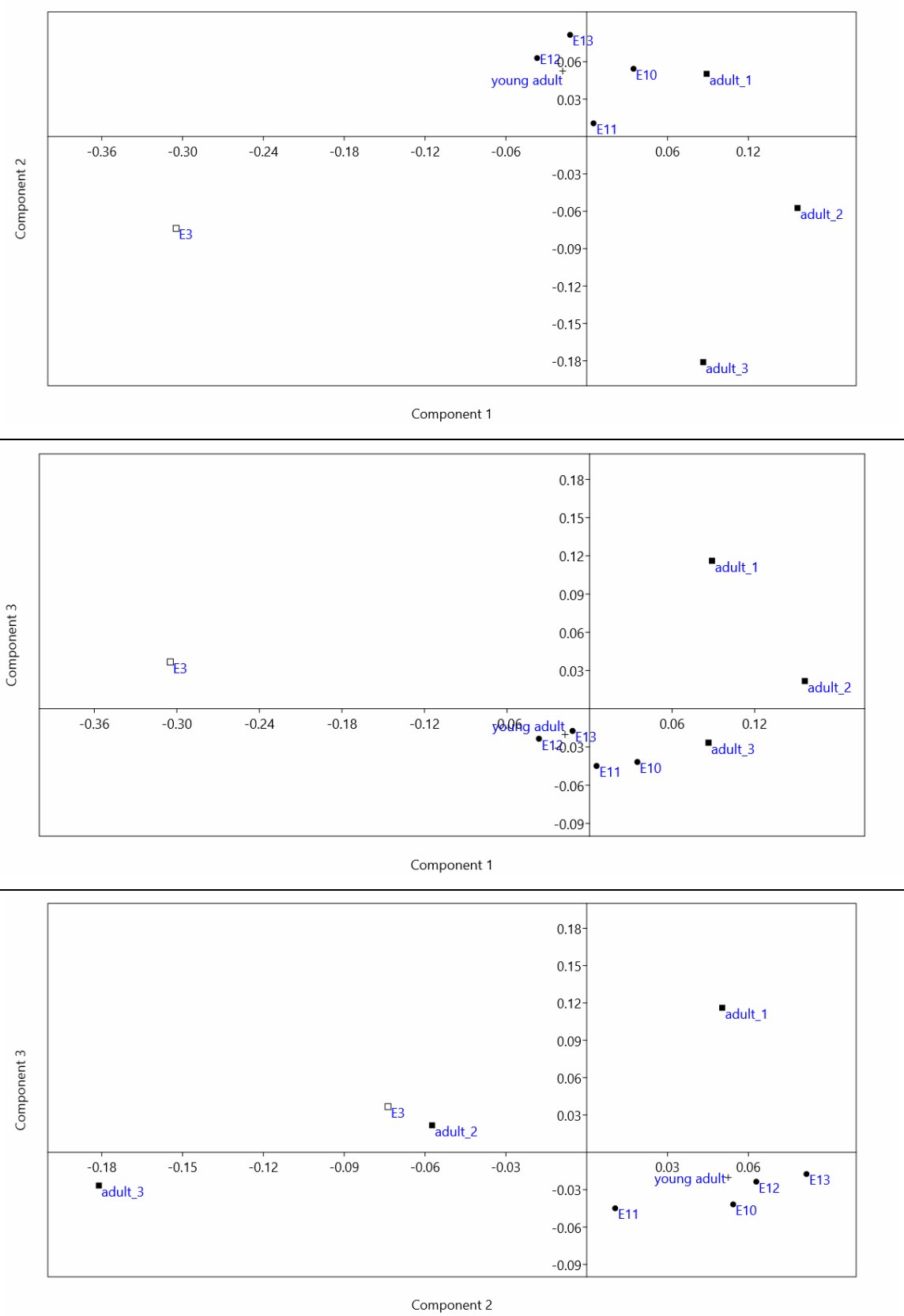


Figure 12: Principal component analysis graphs of Meckel´s cartilage. Eleven landmarks set on E3, E10-E13, young adult and adult 1-3. Only shape data computed. Component one describes 57% (0.0170), the second 26% (0.0076) and the third 9% (0.0026) of variance. Eigenvalues in brackets.

Components one and two similarly group E10-E13 and the young adult apart from E3 and the three adult individuals. Hence three main clusters can be differentiated, which are not present in component three. Principal component three groups all specimens used together (Figure 12).

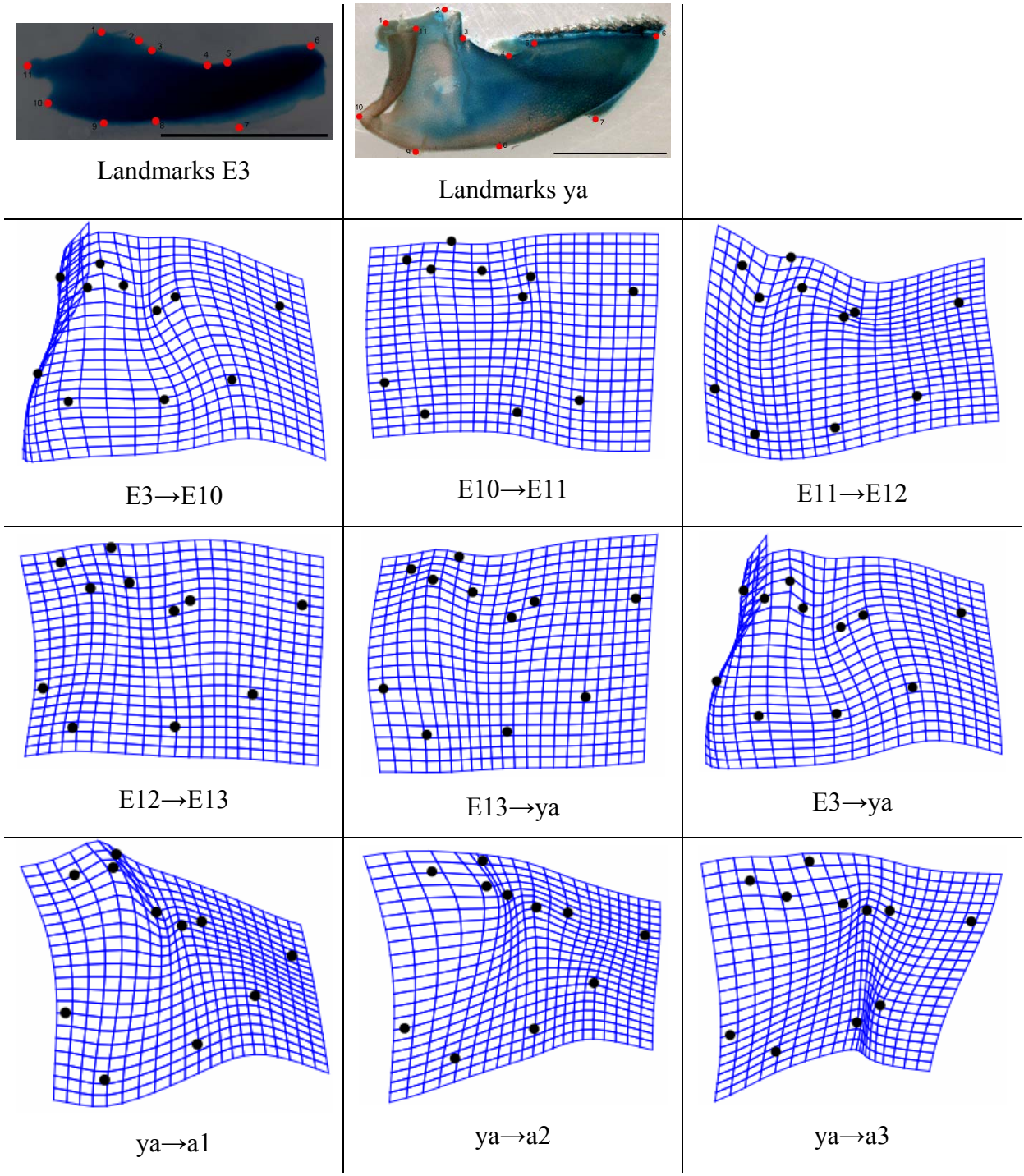


Figure 13: Principal components analysis deformation grids of Meckel´s cartilage. On top examples of landmark set (red dots) (11). Numbers define landmark order. Scale bar E3 1mm, scale bars young adult 1cm. Arrow between labels of specimens shows which grid is used as basis and to which individual the grid is computed. a1, adult 1; a2, adult 2; a3, adult 3; ya, young adult.

The main shape change of the Meckel´s cartilage is illustrated in the deformation grid E3→E10 (Figure 13). The eleventh landmark, which highlights part of the articulation site, is reeled into a more anterior and distal position, to form the posterior part of the Meckel´s cartilage where the foramen, described in Figure 8, is formed later on in development. As in the palatoquadratum, there is a constantly changing angle, here between landmarks 3-5 in the lower jaw as well. From stages E10 to E12 this angle gets narrower due to growth of the articulation and dental site and subsequently widens, when growth in length starts (Figure 13).

Growth to the adult condition mainly affects the more posterior region of Meckel´s cartilage. Over all, the articulation site seems to be strengthened (Figure 13).

The principal component analysis of both elements together was computed with nine landmarks. In the palatoquadratum all landmarks were used, except for landmark 6, which was changed for one in the angle position (marked green, Figure 11) and in Meckel´s cartilage landmarks 8 and 11 were skipped (Figure 13). Principal component one describes 44% (eigenvalue 0.0762), component two 32% (eigenvalue 0.0561) and component three 12% (eigenvalue 0.0202) of the variance. In Figure 14, the principal components of this analysis are depicted. The upper and lower jaws cannot be separated by component one or two. These two groups only can be conjectured in the third component. Nevertheless, these two elements cluster separately from each other in any principal component (Figure 14).

Additionally, a principal component analysis of combined upper and lower jaws computed without Procrustes fit was conducted to include size (see Figure 2 of Supplement). Principal component one accounts for most variety (99%), only the Meckel´s cartilage of adult two is separated from the group. Hence no clear separation of the palatoquadratum and Meckel´s cartilages, respectively, is visible. The adult elements cluster into the "embryonic group". Both principal components two and three intriguingly show a separation of each element of every individual, respectively. The Meckel´s cartilage of each individual is situated in the left upper region of the graph relative to the palatoquadratum of the same individual, which is situated below to the right. The slope of every pair is nearly the same (Figure 2 of Supplement).

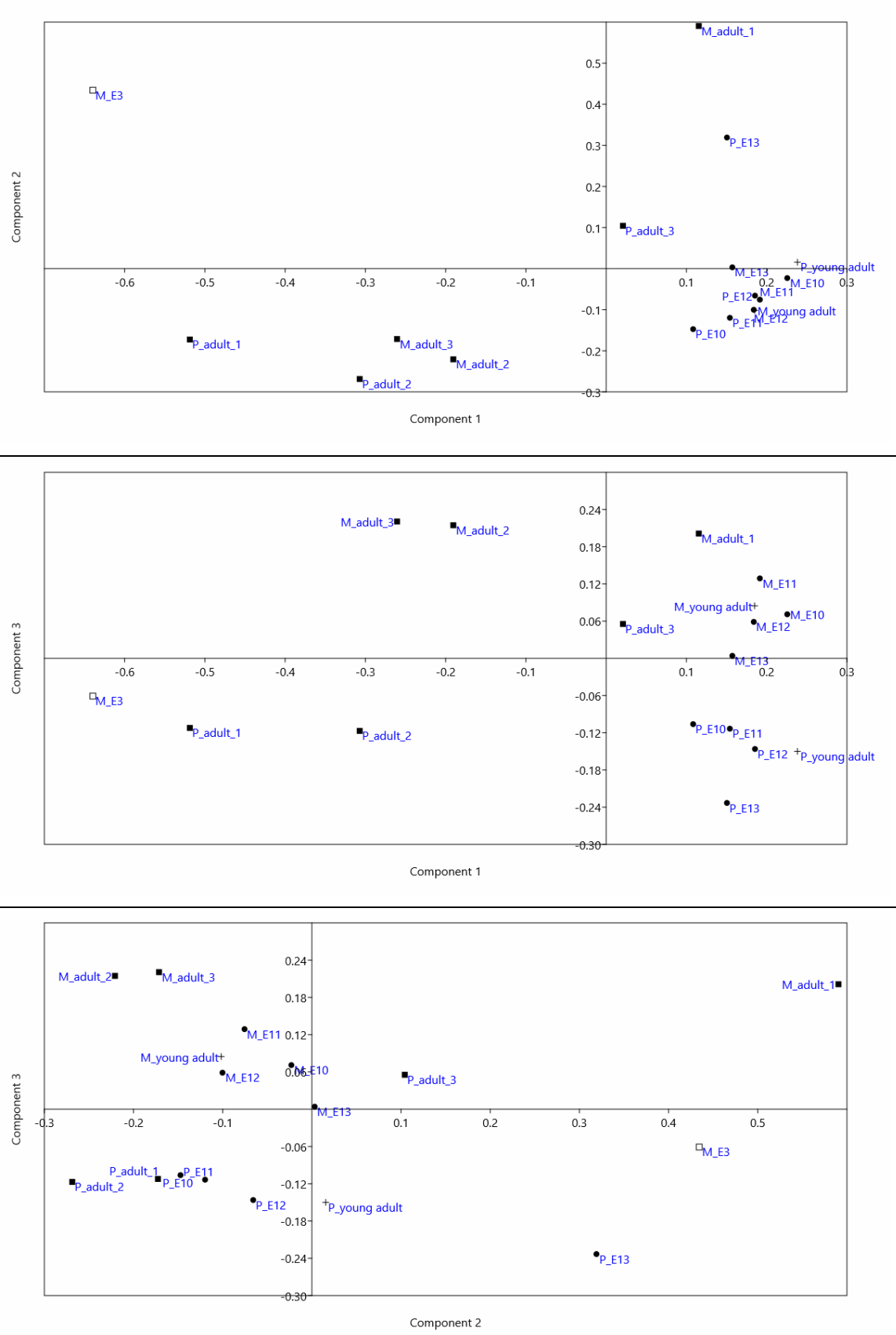


Figure 14: Principal component analysis graphs of palatoquadratum and Meckel´s cartilage together. Nine landmarks set on E3(only lower jaw), E10-E13, young adult and adult 1-3. Only shape data computed. Component one describes 44% (0.0762), the second 32% (0.0561) and the third 12% (0.0202) of variance. Eigenvalues in brackets.

7) Discussion

Staging

According to Harahush et al. (2007) the staging of Ballard et al. (1993) is well applicable in *Chiloscyllium punctatum*. Results of this study support this hypothesis, because the anatomical characters used by Ballard et al. (1993) are detectable in all bamboo sharks. The main problem of this study with staging the specimens was that Ballard et al. (1993) use the yolk sac and its volume, size or shape as a very important reference for defining a stage, which was impossible for most animals in this study. Nevertheless, determination of the stages was conducted successfully with other features.

Another point, which needs to be addressed is the duration of the stages. Unfortunately the specimens of this study could not be dated back exactly, therefore no time period for the stages was evaluated. However, considering the timetable elaborated by Ballard et al. (1993) the question is, whether this arrangement is really useful. Up to stage 24, the duration of one stage is three days maximum, but the duration might vary between three and 50 days in some stages. More specifically stage 25, for example, lasts seven days and stage 32 between 40 and 50 days. It is clear, that there should be some space for plasticity in duration of the developmental process, but 10 days of range in one stage and the high differences of duration between these late stages seems quite arbitrary. A revision of *Scyliorhinus canicula* development or a complete inquiry of the development of *Chiloscyllium punctatum* might shed more light on these later stages.

Clearing and staining

The successful clearing and staining protocols led to interesting results. On one hand it was very difficult to recognize the pharyngeal arches, especially the first and second ones. On the other hand was it possible to visualize the early formation of the fin rays and spiral valve in these specimens. These two features develop well before the neurocranium or the jaw is formed, even though the embryos do not need to swim or eat. It was not possible to clarify why fin rays do form that early, because the embryos can move in the egg long before that stage with the help of their posterior body (Harahush at al. 2007). Specimens E4 and E5 (Figure 7B-C) imply, that the spiral valve is connected through the yolk duct with the yolk sac and helps digesting. Ballard et al. (1993) documented embryos, that feed on the yolk through the “red line”, the main vein, formed in the early development to digest the yolk. Maybe the supply through this blood vessel is not sufficient in this developmental stage anymore and the

spiral valve accelerates nutrition. These presumptions should be considered in future functional morphology analyses.

Unfortunately, the clearing and staining procedure failed on most individuals of this study. One major disadvantage was the usage of the nerve staining, which should have stained only the brain and nerve system, but coloured the complete epidermis instead and it was impossible to destain and remove the dark blue colour. It is not clear why the staining failed, because it was conducted exactly after various descriptions, which were all tested successfully previously (see Material and Method). Possibly one of the ingredients was expired. Nevertheless, using KOH also turned out to be quite a disadvantage. For some reason it reacted very strong with the tissue and all animals used in the first try got destroyed. Due to several reviews of the concentration and the mixing process, it is certain, that it was no mistake of concentration. Maybe chondrichthyans do react more severely to this substance than osteichthyans or other bony animals, or it was a problem of the fixation. Summarizing, it can be said, that clearing and staining of chondrichthyans or at least small sharks, should be conducted with as much caution and as little use of KOH as possible.

Dissection

As dissection got necessary after the failed clearing and staining procedure, it was carried out with the most carefulness possible. Nevertheless, especially the young individuals were very fragile and unfortunately some got damaged, over all in the posterior region of the Meckel´s cartilage in the area of the foramen (described in Figure 8). This hole in the lower jaw surprisingly stays an enigma until now. Neither was a similar structure found in other species, nor could the function of this foramen be clarified. Another obstacle during the dissection was the numerous tendons and membranes in the jaw apparatus of *Chiloscyllium punctatum*. The jaw of elasmobranchs is a complex structure with numerous muscles and tendons attached, as already established (de Beer, 1931; Wilga, 2005). These posed the main problems in dissecting all elements.

It also should be mentioned, that teeth of specimens E10 and E11 were covered by a membrane, which already was mentioned by Shimada (2002) (Figure 8 and 9). For more details see next chapter.

Tooth patterning

Teeth evolved within the gnathostome stem lineage shortly after jaw evolution (Rücklin et al., 2012). Although in modern elasmobranchs the dentition is restricted to the mandibular arch,

in their ancestors it was augmented to the hyoid arch and neighbouring cartilages (Maisey et al. 2014).

Smith et al. (2012) tried to find a general arrangement to describe the dentition of modern sharks. According to this classification *Chiloscyllium punctatum* has an alternate tooth replacement patterning, because teeth forming at the same time do not neighbour each other directly but stand next to a tooth of an older and a younger generation, respectively (Figure 9). Teeth of the bamboo shark are all uniform, they do vary in size from the embryo to the adult individual only but not in shape. The sheath covering the dental region and first forming teeth was already mentioned by Shimada (2002). He claims that it is a sign for a non-functional embryonic dentition, if it is covered by a membrane. This makes sense in the context that *Chiloscyllium punctatum* embryos develop in an egg on their own and do not need their teeth until birth. Due to this fact it is possible that teeth from E12 to E13 were covered by this membrane as well and it got lost during the numerous manipulation steps.

A sat unit describes the present tooth generations of two tooth families in the jaw (Smith et al., 2012). It was very difficult, or even impossible to count correctly for the embryonic jaw elements, due to both the size of the teeth and the difficulties during the dissection, which could have led to the loss of teeth. Nevertheless, it can be said that the number increases during development to a maximum of 13 tooth families in the lower and 14 in the upper jaw of the adult individuals (Figure 2 of supplement). This number seems determined, because all three reconstructed jaws resembled the same phenotype. The number of the sat unit of tooth families one and the symphytic one was more than 13, but seems to be variable.

Summarizing, the number of tooth families seems to be determined and the number of erupted teeth in the jaw of *Chiloscyllium punctatum* is more variable, which appears to be very probable due to differing usage and nutrition habits of individuals of this species. An animal feeding mainly on bivalves could have a higher turnover of teeth than one rather feeding on fish.

Geometric morphometrics

It was the aim to describe presumptive modules of jaw development for finding any inclination to support one of the existing evolution theories. Klingenberg (2013a) describes modules as “relatively independent within the overall integration of the system they are integrated in”, thus “for testing jaw development as a module the whole head has to be studied.” Hence in this study there was no way to find real modules after Klingenberg

(2013a), but analysis of the jaw elements nevertheless, might provide insights into developmental patterns helping to understand jaw development and evolution.

The principal component analysis produces out of the multidimensional morphological data set a new coordinate system to reduce dimensions and shows the directions of most variance (Klingenberg, 2013b). First the palatoquadratum was analysed and the resulting principal components describe three main characters, which are most important for the development of the upper jaw. The first component describes the direction of shape variation, hence adult one and the young adult differ in shape, because they are distinctly separated in this direction. It can be seen too, that the embryos and the young adult are closer together than the adults. Thus this two groups form two different morphotypes (Figure 10). Principal component two describes the size of the angle between the articulation process and the dental region. This angle is very important in the development of the palatoquadratum, since it constantly changes and gives a hint, if shape changes (angle shrinks) or the element grows in length (angle widens) (Figure 11). Principal component three presumably describes the developmental steps themselves. In this direction the specimens are ordered by age, excluding the adult animals. The most important shape change happens between E12 and E13. This is the step between stage 31 and 32. In this phase of the embryonic development most changes in jaw morphology occur (tooth formation, calcification, Figure 8 and 9). The gap between E11 and E12 can be explained by the same procedures. Stage 31 and 32 are the two steps, which last the longest in embryonic development according to Ballard et al. (1993). Hence developmental shape change of the palatoquadratum is intriguingly peculiar and is illustrated in this analysis (Figure 10). It is evident that principal components one and three are most appropriate to describe the development of the palatoquadratum in *Chiloscyllium punctatum*, since they describe shape changes in combination with developmental steps (Figure 10).

The shape changes of the developmental stages of one individual to another are illustrated in the deformation grids as distortions of the part of interest of the first to the following stage (Mitteröcker and Gunz, 2009). The deformation grid of Figure 11, which shows the whole development of E10 to the young adult demonstrates the overall shape change. In this first phase the “I”- shape of the anlage in E10 changes to an “L”- shape in the young adult. This means, that the angle between landmarks 2-4 gets narrower. Later on, when the young adult grows in length only, the jaw also expands and the angle gets wider (Figure 11). This also coincides with the image that yields the principal component analysis. Here the angle is depicted by component three. E13 and the young adult are at the bottom of the graph, which have a narrow angle and at the top are E10, E11 and adult three, which have a wider

angle relatively to the others. The deformation grids show that when tooth development commences, the dental region increases in size (deformation grid E11→E12 and following, Figure 11). Only in the last step, E13→young adult, grows the articulation site, which could mean that relative to the dental area for instance, there is just little growth necessary where the joint is forming. The palatoquadratum is strengthened by mineralization starting from stage 31, especially in the posterior part. Hence there may be no need for further growth. The three adult jaws have a very similar shape, which can be seen in the deformation grids as well. The steps from the young adult to the adult jaws resemble each other. Hence growth after hatching seems to transpire in a uniform way. The young adult is a male, thus the three reconstructed jaws may be males too, or there is no dimorphism between male´s and female´s palatoquadratum.

The graphs of the principal component analysis of Meckel´s cartilage are more ambiguous and did not provide a clear result. Presumably the first component depicts shape changes of the articulation site, because all plot very close except for E3, which´s posterior part is very different and still has to differentiate. Component two seems to illustrate shape changes in regard of the angle, as it does in the palatoquadratum analysis, too. E3 and the adults two and three have a very wide angle, whereas adult one and the others cluster closer together in the top of the graph and have a narrower angle. Principal component three depicts the proportions between anterior and posterior regions. In the most positive area are the elements, which´s posterior part outweighs the anterior one and in the most negative vice versa (Figure 12). This indicates that E3 and adults one and two have a larger articulation area, relative to the dental region than, for example E11 or E10. This assumption is confirmed by the deformation grids. The joint area in E3 is relatively broad, because it is still undifferentiated. Grids ya→a1 and ya→a2 (Figure 13) support a major gain in size of the posterior part of Meckel´s cartilage.

Summarizing the graphs in Figure 12, it is obvious that there is no depiction of developmental steps as in the palatoquadratum. This could be due to the plasticity of the shape changes, which means that there is no linear development, but alternating steps of shape changes in different parts of the lower jaw. This assumption is supported by the deformation grids in Figure 13, too. In every step from one specimen to another the grid appears different and it seems that there are certain phases of growth, for example for the articulation site and another for the dental region, and so on. Most prominent are the steps from E3 to E10, when the posterior part of the joint curls up and resembles then the adult condition. This step is very peculiar and needs more specimens in between for confirmation. Adult growth (young adult

to adult1, 2 and 3, respectively) does not show a uniform direction, as shown for the palatoquadratum. All three growth steps (young adult to adult1, 2 and 3, respectively) coincide in the strengthening of the joint region, only adult three differs from this patterning in its growth of the dental region (Figure 13). It shortens and this could be due to sexual dimorphism, or it is normal plasticity. Harahush et al. (2007) already mentioned that during development individuals do differ in their growth rates. Maybe this variation has an impact on the adult phenotype, too.

Comparing the results of the palatoquadratum to those of the Meckel´s cartilage some parallels should be emphasized. During the onset of tooth formation and growth, the dental site of both upper and lower jaw enlarges. Growth of the articulation site from E12 to the young adult occurs in both elements at the same time and both angles already mentioned above do change in similar ways. This data suggests, as expected for counterparts, that the upper and lower jaw develop in similar ways.

This assumption also is confirmed in the principal component analysis of both elements in combination. The graphs in Figure 14 do not show any separation of the elements, which means that both shapes are very similar and the palatoquadratum seems to be an inverted Meckel´s cartilage. This emphasizes the parallels in shape change, but how about growth and resulting size? This question should have been answered by skipping the procrustes fit computation in the principal component analysis. Unfortunately the result was not that clear. The first component accounts for almost all variance but no certain pattern was found. It is very probable, that it shows the direction of size over all, but this information is relatively abundant, as it is clear, that during development these elements gain in size. However, as already mentioned in the results section principal component two and three in combination, which account for less than 1 % of the variance show an interesting pattern. Although distances are different between the pairs of Meckel´s cartilage and palatoquadratum, the slope and position do coincide intriguingly well (Figure 2 in Supplement). This could be another confirmation of the assumption that upper and lower jaw do differentiate and grow in a certain pattern parallel to each other.

Unfortunately it was difficult to acquire data of the early stages between E3 and E10, which makes this study still very incomplete. It even would not be sufficient to have one specimen of all stages, respectively, because of the great developmental steps between the older stages (stage 30 onward). It additionally was very difficult to find appropriate 2D landmarks on a 3D object, namely the reconstructed adult jaws, which has distorted the geometric morphometrics analyses. Since the focus of this study was on the embryonic

development this should influence the results marginally. Nevertheless, a new approach with a 3D method for all elements of the jaw, including even the head should give a complete image of jaw development and possible modularity.

8) Conclusion

This study can be considered a starting point for further evolutionary and developmental studies of the morphology of *Chiloscyllium punctatum*. This species is not only easy to keep and breed in captivity (Harahush et al., 2007), but also due to its basal position in phylogeny (Underwood, 2006; Marcelo R. de Carvalho, 2007; Hadfield et al, 2010) a representative of an ancestral state in elasmobranchs.

Due to the little number of specimens and not enough individuals of the early stages it was not possible to depict the whole development of the jaw and teeth unfortunately. Nevertheless, this study provides information of the early morphogenesis of this species. Formation of the jaw starts about stage 28, 15 days after the egg was laid and the main developmental steps occur between stage 29 and stage 30. Tooth development starts a bit lagged at stage 30. Both teeth and jaw mineralize at stage 31 and mineralization of the jaw continues during growth of the jaw apparatus until the adult shape is reached.

It was not possible to find modules because for such a study the whole head should have been investigated (Klingenberg, 2013b), but it was possible to show, that palatoquadratum and Meckel´s cartilage, as two counter parts, develop in a synchronous way and seem to be dependent on each other.

Teeth grow in an alternate replacement patterning (named after Smith et al. 2012) and equate each other in morphology throughout the whole jaw. It is probably that during embryogenesis they are covered by a sheath and become functional just before hatching, when they lose the protective membrane. The number of teeth increases naturally during development until a maximum of 14 tooth families in the upper and 13 in the lower jaw. The number of single teeth seems to be variable, maybe due to nutrition.

Unfortunately finally it has to be said that there was no way in finding clues for the previous mentioned theories of jaw evolution (Kuratani, 2012). Data, especially in the early embryonic development was too scarcely to make assumptions for this issue. This problem remains unsolved and should be the main topic of following inquiries of this and other species. Answering this question could not only help to understand the overall evolution and development of primary and secondary jaws, but also be of advantage in understanding other evolutionary processes.

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11) Supplement

Table 2: Clearing and staining protocol for specimen E2, E4 and E5.

1, Dehydration in 96% EtOH for 20h
2, Cartilage staining for 4h30´
3, Hydration two times in 96% EtOH (45´and 2h), 70% EtOH for 18h, 50% EtOH for 1h45´ and dH_2O 2h
4, Digest for 3d21h15´ at room temperature
5, 0.5%KOH/85%Glycerol series 1:1 for 22h30´
6, Stored in 96% Glycerol

Table 3: Clearing and staining protocol for specimen E3 and E6.

1, Dehydration in 96% EtOH for 24h 30´
2, Cartilage staining for 1h30´
3, Hydration two times in 96% EtOH (30´and 1h30´), two times in 70% EtOH (overnight and over weekend (2d), two times in 50% EtOH (1h and 30´) and once in dH_2O 45´
4, Digest for 23h at room temperature and 46h45´at 25°C in incubator
5, 0.5% KOH 1h
6, Calcium staining 22h15´
7, Destain und bleaching 15´
8, Dehydration 30% EtOH for 10´, 50% EtOH for 25´
9, Storage in 75% EtOH

Table 4: Clearing and staining protocol for specimen E10 and E11.

1, Dehydration in 96% EtOH for 24h 30´
2, Cartilage staining for 1h30´
3, Hydration two times in 96% EtOH (30´and 1h30´), two times in 70% EtOH (overnight and over weekend (2d), two times in 50% EtOH (1h and 30´) and once in dH_2O 45´
4, Digest for 23h at room temperature and 7d at 25°C in incubator (solution changed every

24h at first and every 48h in last 4d)
5, 0.5% KOH 50´
6, Calcium staining 3h 5´
7, Dehydration 30% EtOH for 15´, 50% EtOH overnight, 70% EtOH for 1h45´
8, Nerve staining for 24h
9, Differentiation in 70% EtOH for 15´
10, Digest for 4d at 25°C in incubator
11, 0.5%KOH/85%Glycerol series 3:1 for 4h 30´, 1:1 for 24h, 1:3 for 24h
12, Stored in 96% Glycerol

Table 5: Clearing and staining protocol for specimen E12 and E13.

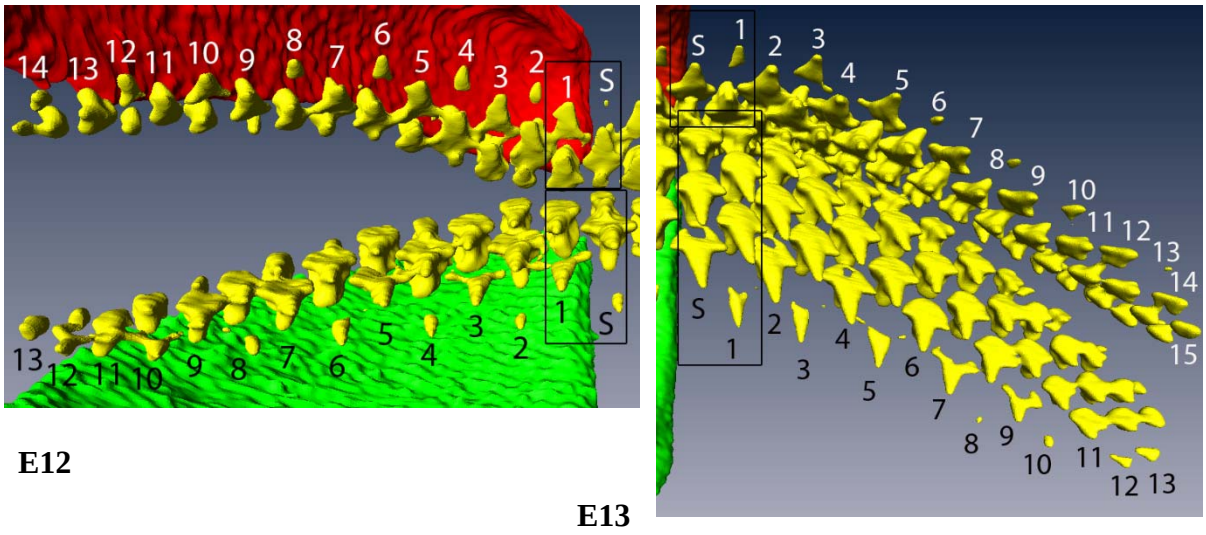
1, Dehydration in 96% EtOH for 24h 30´
2, Cartilage staining for 24h
3, Hydration in 96% EtOH for 5´, 70% EtOH over weekend (2d), two times in 50% EtOH (1h and 30´) and once in dH_2O 45´
4, Digest for 23h at room temperature and 8d at 25°C in incubator (solution changed every 24h at first and every 48h in last 4d)
5, 0.5% KOH 15´
6, Calcium staining 3h 15´ and after adding 1ml stock solution another 1h30´
7, Dehydration 30% EtOH for 15´, 50% EtOH overnight, 70% EtOH for 15´
8, Nerve staining for 24h
9, Differentiation in 70% EtOH for 15´
10, Digest for 5d at 25°C in incubator (solution changed once after 2d)
11, 0.5%KOH/85%Glycerol series 3:1 for 1h, 1:3 for 3d
12, Stored in 96% Glycerol

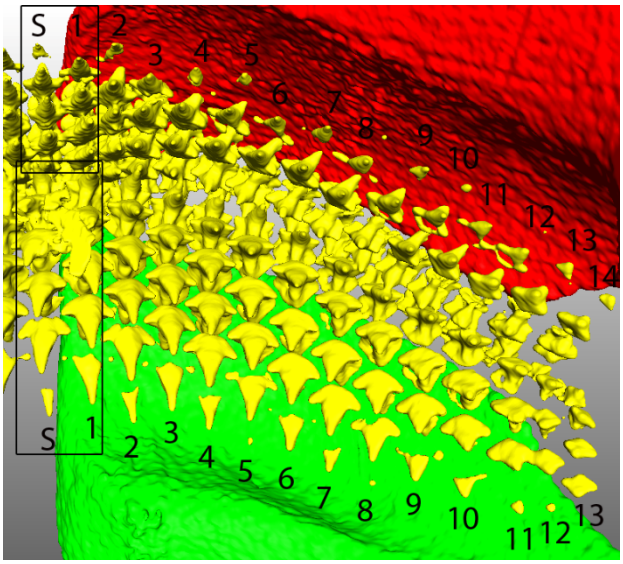
Table 6: Clearing and staining protocol for young adult

1, Dehydration in 96% EtOH for 24h 30´
2, Cartilage staining for 1h45´
3, Hydration two times in 96% EtOH (15´and 1h30´),two times in 70% EtOH (overnight and over weekend (2d)), two times in 50% EtOH (1h and 30´) and once in dH_2O 45´
4, Digest for 23h at room temperature and 8d at 25°C in incubator (solution changed every 24h at first and every 48h in last 4d)
5, 0.5% KOH 15´
6, Calcium staining 3h 15´ and after adding 1ml stock solution another 1h30´
7, Dehydration 30% EtOH for 15´, 50% EtOH over night
8, Storage in 75% EtOH

Table 7: Iodine staining of young adult

1, 95% EtOH 19h30´
2, Mixture of 20% Iodine and 80% dH_2O 24h
3, Mixture of 30% Iodine and 70% dH_2O 72h
4, Mixture of 40% Iodine and 60% dH_2O 72h
5, Mixture of 50% Iodine and 50% dH_2O 96h
6, Mixture of 60% Iodine and 40% dH_2O 72h
7, Mixture of 70% Iodine and 30% dH_2O 72h
8, Mixture of 80% Iodine and 20% dH_2O 192h
9, Mixture of 85% Iodine and 15% dH_2O 120h





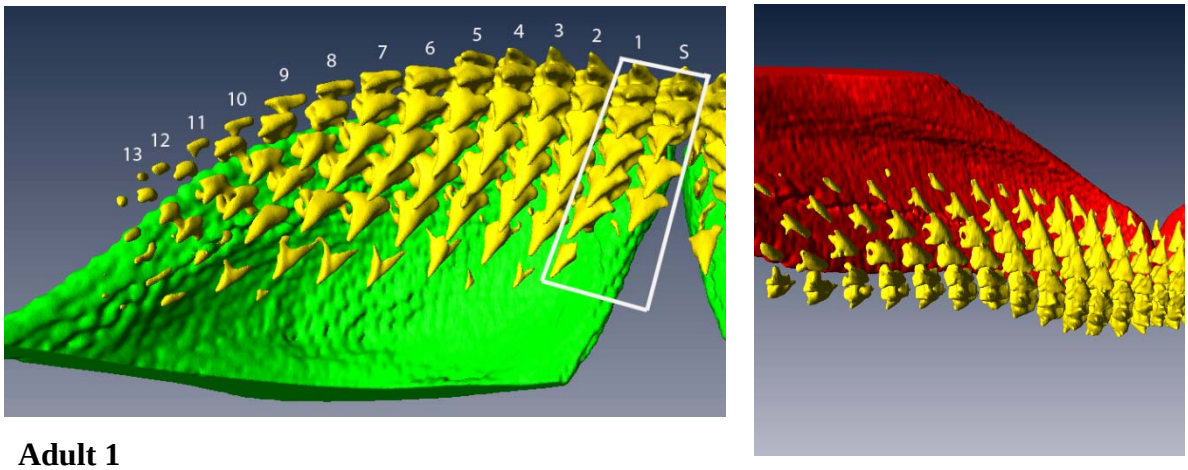


Figure 1: Micro-CT Scans of jaw elements depicting tooth morphology. Names of individuals on left bottom of every column respectively. Numbers count tooth family. Rectangles enclose sat unit of S+1 tooth family. S, teeth in symphysis tooth family. Annotations modified after Smith et al. (2012).

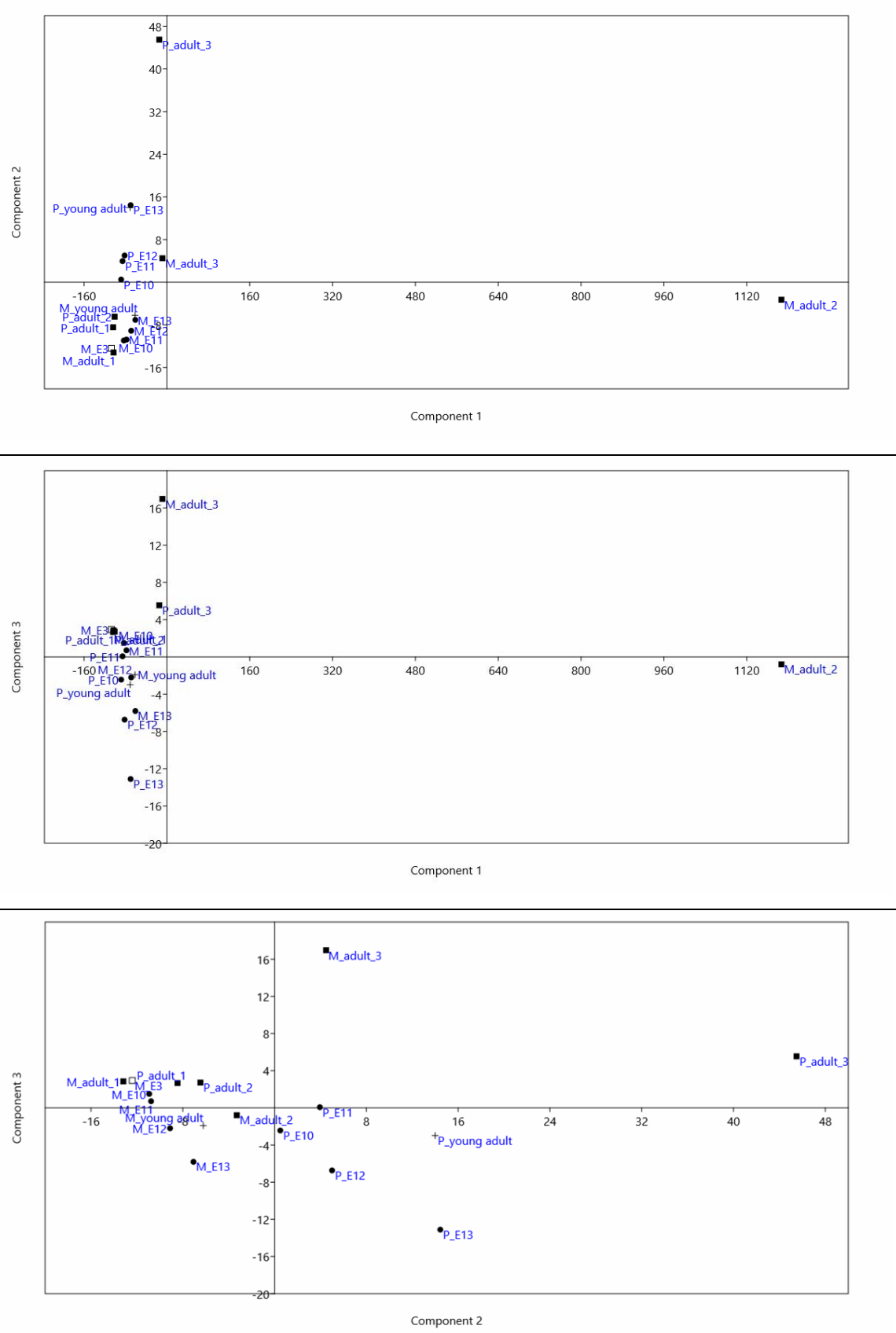


Figure 2: Principal component analysis graphs of upper and lower jaw together without Procrustes fit. Nine landmarks set on E3 (only lower jaw), E10-E13, young adult and adult 1-3. Shape and form data computed. Component one describes 99.68 %, the second 0.22 % and the third 0.04 % of variance.

12) Curriculum vitae

Personal Data

Name	Katharina Fritz
Place of birth	Vienna
Citizenship	Austria

Education

Since March 2013	University study at university of Vienna Field of study: Master zoology Master thesis: "Evolutionary development of jaws in gnathostomes"
October 2009 – March 2013	University study at university of Vienna Field of study: Bachelor biology (focus on zoology) Bachelor thesis: "The nervous system of *Aurelia aurita* planula larva"
01/06/08	School leaving examination
2004 - 2008	Secondary school (focus on sports) Maria Enzersdorf
1999 - 2004	Secondary school (focus on languages) Bruck an der Leitha
1995 - 1999	Elementary school Traiskirchen

Languages

English	First Cambridge Certificate
Spanish	Diploma de Español

Knowledge and abilities

29/07/09	PADI Divemaster, 86 dives
Excursions 2009 - 2013	March Auen (focus on phenology)
	Seewinkel (focus on birds)
	Riegersburg ("Knowledge of central-European living space") St. Pölten Land (focus on streams)
	Wien Land (focus on meadow)

WS 2012/13	Practical in histology, staining with fluorescent staining solutions, confocal microscopy and 3D reconstruction
SS 2013	Practical in neurobiology working with *Drosophila melanogaster*
WS 2013/14	Practical course in developmental biology: *in-situ* hybridization, Micro-transplantations
SS 2014	Practical in Barcoding
Clearing and staining methods	Very experienced
MS Office	Very experienced
Mathematica	Experienced
Photoshop	Very experienced
Literature inquest	Very experienced
Geometric Morphometrics (various programs)	Experienced
Amira	Experienced

Placements & jobs

2008 - 2009	Au pair (Tenerife)
August 2010	Monitoring of cetaceans in the street of Gibraltar
02.09. - 12.9.12	Course marine zoology in Elba
12.2012 - 4.2014	Tutoress for Spanish, mathematics, German, English and biology at Schülerhilfe
27.4. - 7.6.2014	Assistant at practical for marine biology in Mali Losinj/Croatia