

Contributions to the Evolution and Development of Land Vertebrates

Schriftliche Habilitationsleistung

von

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Asterisk (*):	designates corresponding author(s)
Scientific idea:	definition of the research problem, experiment design, selection of the samples
Data generation:	sampling process, laboratory work, dataset management
Analysis and interpretation:	graph generation, selection of the experimental data, interpretation of the data
Paper writing:	organization of scientific ideas, figure preparation, text editing and review

All supplements to the papers are freely available online.

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SUMMARY

Introduction

When studying ontogeny and phylogeny of an organismic group such as land vertebrates, comprehensive methodological and philosophical concepts are required to traceably arrive at sound scientific conclusions. This morphological or “organism concept” requires a holistic approach to tackle the object of interest, including the observation of all relevant ontogenetic stages (from fertilization to dead), the use of different available techniques and methods, and the consequent application of the evolutionary theory (**PART A** of the habilitation).

The land vertebrates (Tetrapoda) evolved about 365 million years ago (Carroll 2009). During the transition from water to land, our fish-like ancestors experienced fundamental changes in their anatomy and life history (Laurin 2010). In an initial evolutionary step, tetrapods were still associated to aquatic life in the first phase of their development – eggs were laid in water and larvae were adopted to swimming and feeding in water. Later in ontogeny, early tetrapods lived on land, only returning to the water for reproduction. The descendants of these early tetrapod lineages, the Lissamphibia, often show highly derived modes of metamorphosis but also direct developers exist. The diversity of reproductive modes was already high in extinct early tetrapod lineages (Schoch 2014).

In a second evolutionary step, a specialized, cleidoic egg evolved within Reptiliomorpha, which characterizes the so-called ‘fully land-adapted vertebrates’, the amniotes (Sumida and Martin 1997). The cleidoic egg differs from the egg of non-amniotic vertebrates (fish, amphibians) in having a semipermeable shell, which prohibits evaporation of water out of the egg but permits gas exchange (Stewart 1997). In addition, the embryo is covered by extraembryonic membranes, which provide mechanical protection and embed the embryo in an enclosed liquid environment (amnion). With this key innovation (Maier and Werneburg 2014a), the amniotes were able to conquer habitats far

away from water, including deserts. Among amniotes different life history strategies evolved including the retention of the egg in the uterus of the mother – resulting in viviparity in therian mammals and in several squamate lineages. Also, the timing of hatching can be accelerated – resulting in altricial young, which need parental care (Starck 1996). All those strategies resulted in a great diversity of organismic life on land (Shedlock and Edwards 2009). The evolution of those life strategies in amniotes, in reptiles and mammals alike, and the evolution of their embryonic organs, their timing and their morphogenesis – all of which contribute to the diversification on land vertebrates – form one of my current research topics (**PART B** of the habilitation).

Morphological changes occurring with the conquest of the land include the evolution of limbs and the emergence of a neck in the postcranium (Laurin 2010). A neck separates the trunk from the head and permits a higher mobility, which is, for example, used to improve foraging strategies on land (Steiner 1977; Kuratani 2009). Fundamental changes occur in the cranium and involve eye, ear, tongue, and jaw anatomy. Life on land requires the use of food different to that of fish-like animals. For that, jaw muscle and the related skull architecture experienced comprehensive reorganizations. A key feature is the shape of the temporal skull region. Hypercarnivor animals among early amniotes, the Synapsida, evolved one opening in the temporal region, the strong edges of which support powerful jaw muscles (Barghusen 1972). Diapsid reptiles, on the other hand, developed two large temporal openings to assist highly differentiated jaw muscles, which initially evolved to handle arthropods as prey (Evans 2008). Among reptiles, turtles show the most peculiar morphotype of the skull (Romer 1956). Like early reptilian lineages, such as captorhinids or pareiasaurs, they have an anapsid morphotype of the skull with no temporal opening; however, molecular, neontological, and some paleontological studies of the last 20 years highlighted that turtles might have evolved from

diapsid ancestors (summarized by Rieppel 2008; Werneburg and Sánchez-Villagra 2009). One part of my research concentrates on the temporal skull region of turtles by focusing on the diversity of bone and muscle anatomy; and to tackle this complex issue properly (Scheyer et al. 2013), also the related neck anatomy needed a particular observation (**PART C** of the habilitation). Moreover, convergent evolutionary pathways need to be explored to get a better understanding of biomechanical, ontogenetic, and morphological relationships of the tetrapod head (**PART D** of the habilitation). Expanding the latter aspects will form the basis for my future research.

Methodology

The investigation of the morphological complexity of an organism and the study of key innovations in evolution need an integrated research program as is provided by the holistic “organism concept” (Maier 1999, **Figure 1**). In evolution, selection acts on an organism throughout ontogeny from fertilization to death and each developmental stage is adapted to the individual and environmental requirements at the time. Organs relevant and used for particular stages of development experience a change in function or are reduced later in ontogeny. Embryonic and adult organs (not stages!) that were in use in ancestors can be recapitulated through ontogeny of the descendent. Ontogeny triggers phylogenetic change (Olsson et al. in press). Detecting their identity can help understanding phylogenetic relationships and their functional origin. For that, a variety of methods should be used to identify all relevant morphological characteristics. Not only the organ itself but also the tissue in which the organ is embedded, its function, and its genetic and molecular background need to be analyzed (Maier and Werneburg 2014b: **Chapter 1**). However, the diverse subdisciplines in evolutionary and developmental research develop extremely fast and it is a challenge to integrate them in joint research programs or even to communicate the exchange among researchers but progresses are being made (Wilson and Werneburg

2014: **Chapter 2**).

One of the most important methods I elaborated myself during the last couple of years is the so-called continuous analysis using squared-changed parsimony to analyze heterochronic changes, which are changes in the developmental timing of characters (Gould 1977; Gould 1979; Arthur 2002; Arthur 2011), in a phylogenetic framework (Germain and Laurin 2009). For that method, developmental series of different species can be easily compared. Older methods trying to do so based their analyses mainly on the pairwise comparison of each single element (Eventpairing: Smith 1997, Parsimov: Jeffery et al. 2005). Those methods suffer from the non-independency of characters in the phylogenetic analysis (discussed by Werneburg and Sánchez-Villagra 2009; Werneburg and Sánchez-Villagra 2011).

Different animals have different time of development; for example, the mouse has a gestation of few weeks, whereas the elephant has a gestation of about two years. To make those ontogenies comparable, Germain and Laurin (2009) suggested to scale the whole developmental period from 0 to 1, where 0 could mean “fertilization” as the first and “1” could mean birth as the last developmental event to occur. It is important that these landmarks of development must be homologous in a structural sense (discussed by (Werneburg et al. 2016: **Chapter 6**). As a consequence of this scaling approach, each developmental event gains a timing number between 0 and 1 and those scores can be used for a continuous phylogenetic analysis (in contrast to a discrete character analysis) (Wiens 2001). As a result, ancestral developmental sequences can be reconstructed and can be compared to those of other nodes in a phylogeny. When changes are high and significant, macroevolutionary conclusions can be derived.

The continuous analysis was applied to organ development of external embryonic features (**Chapters 5-8**) and for pattern of bone ossification as well (**Chapters 9-13**). Both kinds of data were shown, although relevant for selected macroevolutionary aspects of morphological diversification (e.g., **Chapters 9-10**), to have some explanato-

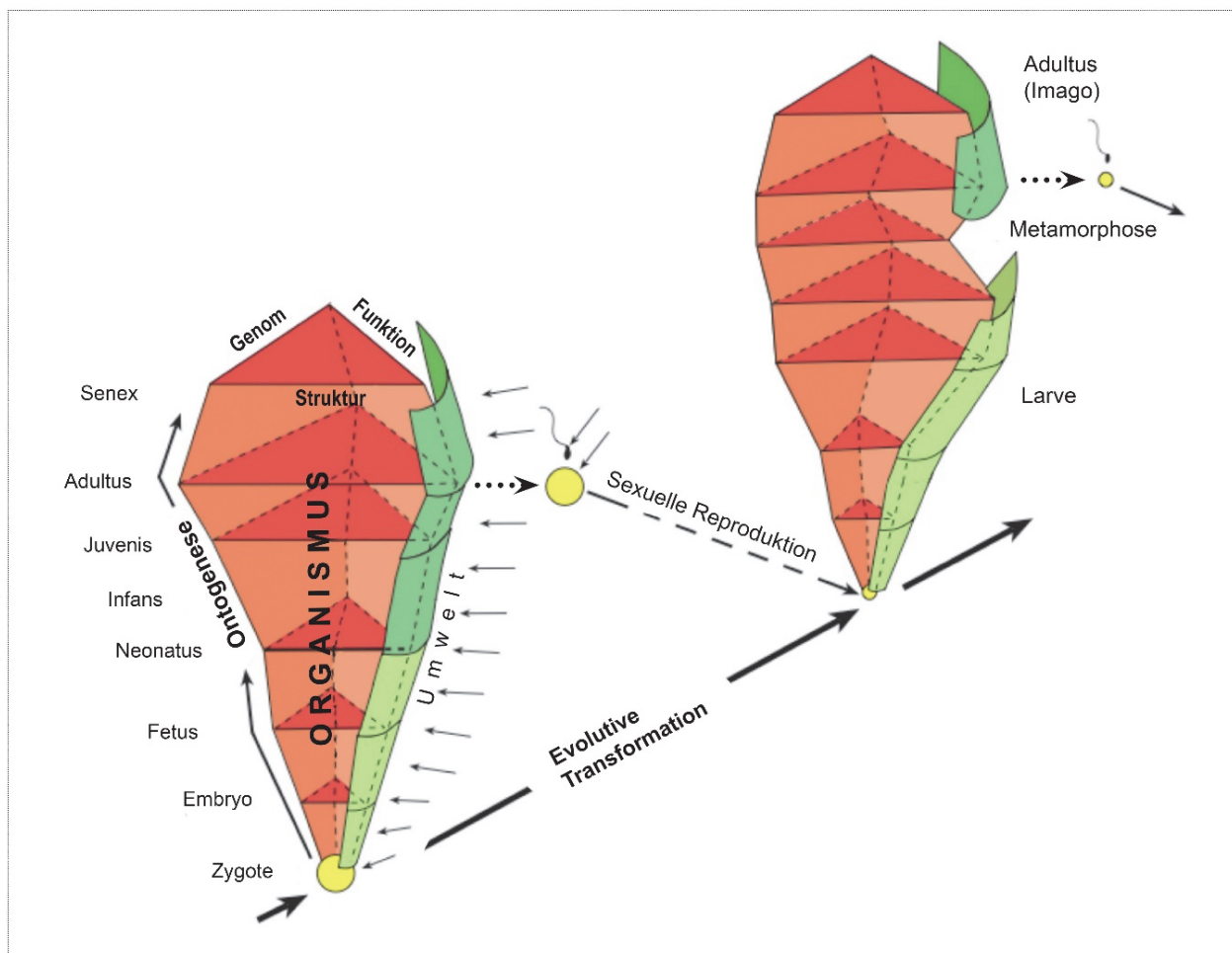


Figure 1. Schematic illustration of the organism concept, in which the whole ontogeny from fertilization to dead is considered. Left: A zygote develops into an organisms of different developmental stages through ontogeny. The organism is characterized by its genomic, its functional, and its structural components illustrating that different levels of organismic complexity need to be considered in synopsis to develop a holistic understanding of the organism. During ontogeny, different selection factors from the environment influence the shape of an organism (small arrows). Middle: In case these changes are implemented into the genome, they are transmitted via sexual reproduction to the next generation. Right: After the evolutionary transformation, ontogeny is structuralized into a larval and a postmetamorphic morphotype, both of which are adapted to the specific environment at the time. From Maier (2017).

ry limitations (**Chapters 12-13**). As such, I expanded my research from the quantitative patterns to a more qualitative, structure-based, and traditional approach when describing changes through skeletogenesis (**Chapter 11-15**). In order to study bone development, μ CT-methodology was used in a variety of land vertebrates taxa (**Chapters 9-15**).

We propagated the use of modern non-destructive techniques such as neutron tomography, synchrotron tomography, and micro-computed tomography (μ CT) as standard tools for the investigation and virtual reconstruction not only for embryos but also for adult anatomy and fossil material (**Chapters 13, 19, 20, 21, 24-25, 28-**

30). In order to highlight the significance of these techniques and to illustrate their application, we have recently chosen therapsids (Synapsida), a group of land vertebrates, which evolved on the stem line of mammals. Our investigations on *Diictodon* skulls (Therapsida, Anomodontia), partly stemming from Paläontologische Sammlung Tübingen, show that non-destructive methods are very useful to clearly distinguish fossil species. Two sexual morphs were described for *Diictodon feliceps* with the male having large tusks and the female having no tusks. In our case we have shown that inner ear labyrinth anatomy drastically differs between both morphotypes indicating that

both belong different species which inhabited completely different habitats (Laaß et al. revision submitted: **Chapter 3**).

Organogenesis in mammals

Mammals, which are extant synapsids, constitute a rich subject of study on evolution and development and provide model organisms for experimental investigations. They can serve to illustrate how ontogeny and phylogeny can be studied together and how the reconstruction of ancestors of our own evolutionary lineage can be approached. Likewise, mammals can be used to promote ‘tree thinking’ and can provide an organismal appreciation of evolutionary changes (Sánchez-Villagra and Werneburg 2016: **Chapter 4**).

Mammals are, in fact, of particular importance for evolutionary developmental studies as they show a great diversity of life history traits. While monotremes are ovipar, therian mammals – marsupials and placentals – give birth. Whereas monotremes and marsupials show highly altricial, less developed young at birth, most placentals have precocial newborns, which can walk independently within a few hours after confinement. The condition at birth is related to an extended retention of the fetus in the uterus of the mother which has important consequences for physiology, neonatal anatomy, and evolutionary fitness of a species (summarized and discussed by Werneburg and Spiekman in press: **Chapter 5**).

We wanted to understand the evolutionary origin of these diverse life history patterns, especially in maturity level at birth and compared different life history traits including gestation length, neonatal anatomy at birth, and other markers of life history. Based on the first synthesis of embryological data and the study of new ontogenetic series, we reconstructed estimates of the ancestral chronology of organogenesis and life-history modes in placental mammals (Werneburg et al. 2016: **Chapter 6**). We found that the ancestor of marsupial and placental mammals was placental-like at birth (see also fig. 10 in Werneburg and Spiekman in press: **Chapter 5**) but had a long,

marsupial-like infancy. We hypothesized that mammalian viviparity might have evolved in association with the extension of growth after birth, enabled through lactation, and that mammalian altriciality is inherited from the earliest amniotes (see also fig. 5d-e and the animation in Sánchez-Villagra and Werneburg 2016: **Chapter 4**). The precocial lifestyle of extant sauropsids and that of many placental mammals were acquired secondarily. We based our conclusions on the best estimates and provided a comprehensive discussion on the methods used and the limitations of our dataset. We provided the most comprehensive embryological dataset ever published, “rescued” old literature sources, and applied available methods and illustrate thus an approach on how to investigate comparatively organogenesis in macroevolution.

The characters used for this comprehensive investigation of embryonic anatomy are based on my proposal of the so-called *Standard Event System* (Werneburg 2009), a set of currently 166 homologous embryonic characters that everyone can easily recognize when observing any vertebrate species (see also: https://en.wikipedia.org/wiki/Standard_Event_System). This method was applied to a variety of land vertebrate species so far and the data base of characters and taxonomic sampling are constantly increasing (Werneburg and Sánchez-Villagra 2009, 2011, Werneburg et al. 2009, Werneburg et al. 2013b: **Chapter 7**, Roscito and Rodrigues 2012, Polachowski and Werneburg 2013: **Chapter 11**, Nunes Silva and Sobral Sampaio 2014, Werneburg and Spiekman in press: **Chapter 5**).

Studies of evolutionary developmental biology commonly use ‘model organisms’ such as fruit flies or mice, and questions are often functional or epigenetic (Jenner and Wills 2007; Tzika and Milinkovitch 2008). Phylogenetic investigations, in contrast, typically use species that are less common and mostly deal with broad scale analyses in the tree of life (Wilson and Werneburg 2014: **Chapter 2**). However, important evolutionary transformations have taken place at all taxonomic levels, resulting in such diverse forms as elephants and shrews among placental mammals. To understand the mechanisms underlying morphological diversi-

fication, broader sampling and comparative approaches are paramount. Using the *Standard Event System*, we described for the first time the development of soft tissues and, using μ CT-imaging, some parts of the skeleton of developmental series of *Echinops telfairi* and *Tenrec ecaudatus*, two tenrecid afrotherian mammals (Werneburg et al. 2013: **Chapter 7**). These mammals are of particular interest because the clade Afrotheria was only recognized recently as a monophyletic group by molecular analyses (Stanhope et al. 1998; Madsen 2009). The search for morphological apomorphies characterizing Afrotheria has inspired many research programs (Mess and Carter 2006; Sanchez-Villagra et al. 2007; Asher and Lehmann 2008; Tabuce et al. 2008; Agnolin and Chimento 2011) and we aimed at contributing to this research field using developmental timing characteristics. The developmental timing of soft tissue and skeletal characters described for the tenrecids was briefly compared with that of other mammals, including mouse, echidna, and the opossum. We found relatively few heterochronic differences in development in the armadillo vs. tenrec, consistent with a close relationship of Xenarthra and Afrotheria. Ossification in *T. ecaudatus* continues well into the second half of overall gestation, resembling the pattern seen in other small mammals and differing markedly from the advanced state of ossification evident early in the gestation of elephants, sheep, and humans. The interesting results of our case study highlighted the need for a better understanding of the meaning of heterochronies in vertebrate evolution (see also Ziermann et al. 2014).

The factors driving the evolution of developmental timing and hence the evolution of body forms is hardly understood. As major evidence for his evolutionary theory, Darwin (1859) pointed to the patterns of artificial selection, namely domestication, to understand the general mechanisms underlying selection (Darwin 1868; Diamond 2002), which is one of the major evolutionary factors (Mayr 2002). We have chosen one example of artificial selection to test the importance of developmental timing for evolutionary change and, again, the *Standard Event System* was used to define embry-

onic characters for phylogenetic comparisons. For postnatal growth, where discrete developmental features are hard to describe, morphometric measurements were taken into account to provide a comprehensive outline through a long part of ontogeny of our case study, in which we studied pre- and postnatal patterns of development and growth in the domesticated forms of three carnivoran species (Werneburg and Geiger in press: **Chapter 8**). Whereas hundreds of breeds of domestic dogs are known, only several dozen domestic cat breeds are currently recognized, and the ferret is not classified into specific breeds. For postnatal development, we presented analyses of new and literature measurements of cranial and limb proportions. We analyzed changes in the progress of growth among different domestic dog and domestic cat breeds. All three domesticated forms drastically differ in the relative timing of prenatal development. This is correlated with ontogenetic plasticity at birth, which enables artificial selection to act. For postnatal development, we detected a greater shape variance in domestic dog ontogeny when compared to that of the domestic cat. We concluded that ontogenetic preconditions as well as body size constrain the species' capability for artificial selection in domestic dogs and cats. However, we speculate that the human requirements for functional performance of their domesticates might render some developmental biases substantially. Although ferrets would be preferable for artificial selection given their plastic embryonic development, they have been of less interest for domestication due to their small body size - by which they were already well adapted for hunting in burrows - and due to the fact that other relevant tasks were already assumed by domestic cats and dogs since earlier phases of human cultural evolution (Werneburg 2014b).

Ossification.

Our studies on the timing of developmental characters and their meaning for evolutionary changes were largely expanded to land vertebrate skeletons (**Chapters 7, 9-15**). The multiple skeletal compo-

nents of the skull originate asynchronously and their developmental schedule varies across amniotes (Hanken and Hall 1994). Two kinds of ossifications can be distinguished: dermal ossification occurs in the skin and covers the skull externally (exocranium); enchondral ossification occurs as ossification within a primordial cartilage such as the embryonic neurocranium (chondrocranium) but can be also found in most postcranial bones (Cubo 2000; Hall 2005). We analyzed the embryonic cranial ossification sequence of 134 species, covering all major groups of mammals and their close relatives among sauropsids (Koyabu et al. 2014: **Chapter 9**). For that, we compared μ CT-scans and data on cleared and double stained embryos. This comprehensive dataset allowed the reconstruction of the heterochronic and modular evolution of the skull and the condition of the last common ancestor of mammals. We showed that the mode of ossification – either dermal or endochondral – unites bones into integrated evolutionary modules of heterochronic changes and imposes evolutionary constraints on cranial heterochrony. However, some skull-roof bones, such as the supraoccipital, exhibit evolutionary degrees of freedom in these constraints. Ossification timing of the neurocranium was considerably accelerated during the origin of mammals. Furthermore, association between developmental timing of the supraoccipital and brain size was identified among amniotes. We argued that cranial heterochrony in mammals has occurred in concert with encephalization but within a conserved modular organization.

As a second example of macroevolutionary changes in skeletal anatomy through heterochrony events, we analyzed the ossification pattern in snakes, which possess a derived anatomy, characterized by limb reduction and reorganization of the skull and internal organs (Burbrink and Crother 2011). To understand the origin of snakes from an ontogenetic point of view (Franklin 1945), we conducted comprehensive investigations on the timing of skeletal elements of the whole body, based on published and new data, and reconstructed the evolution of the ossification sequence among all

squamates (Werneburg and Sánchez-Villagra 2015: **Chapter 10**). There is comprehensive delay in the onset of ossification of most skeletal elements in snakes when compared to reference developmental events through evolution. We hypothesized, as a result, that progressing deceleration accompanied limb reduction and reorganization of the snake skull. We have shown that reconstruction of ancestral developmental sequences is a valuable tool to understand ontogenetic mechanisms associated with major evolutionary changes and to test homology hypotheses: The “supratemporal” of snakes could be, as we found, homologous to the squamosal of other squamates, which starts ossification early to become relatively large in snakes.

Challenging the ‘onset of ossification’ approach.

In recent years, developmental anatomy received increasing interest as a potential new source for phylogenetic research (Haas 2003; Germain and Laurin 2009; Laurin and Germain 2011). For skeletal development, studies mainly rely on the first appearance of ossification centers (e.g., **Chapters 9-10**). However, informative events occur during the whole course of skeletogenesis; interactions between external and internal development occur and morphometric changes take place (Clouse et al. 2010; Bhullar 2012) – all of which present potential sources for phylogenetic analyses. We applied micro-computed tomography (μ CT) (Ritman 2004), clearing and double-staining (Mulisch and Welsch 2010), and 2D and 3D morphometric methods (Mitteroecker and Gunz 2009; Catalano et al. 2010; Goloboff and Catalano 2010; Cardini and Loy 2013) to describe, illustrate, and analyze the development of the head of a snake (Polachowski and Werneburg 2013: **Chapter 11**) and of a varanid species (Werneburg et al. 2015b: **Chapter 12**) in great detail. In the snake, we found a 3D flattening of the skull during ontogeny, a pattern that is not reflected in external development. This may be explained by a different relationship of skeletogenesis and external characters to the developing jaw musculature or simply by the dif-

ferent type of data. Clearing and double-staining and CT-scanning revealed a broadly similar sequence in the onset of ossification. Minute differences may be due to the treatment of embryos. Bones of the dermatocranium are among the first to ossify and the development of the calcified endolymph may reflect its function as a calcium source during development. The value of phylogenetic observations using the sequence of first ossifications was critically discussed. The related heterochronic changes were interpreted to contribute at least to the very first phase of diverging skull formation among taxa.

We compared timing of ossification seen in snakes and varanids with the patterns seen in other squamates, using three major hypotheses of squamate interrelationship as phylogenetic templates (Gauthier et al. 2012; Wiens et al. 2012; Pyron et al. 2013), and were able to detect heterochronic patterns in ossification that are associated with adult anatomy in each phylogeny (Werneburg et al. 2015: **Chapter 12**). However, we refrained from preferring one topology given the current lack of congruence between molecular and morphological data sets. The rule of thumb that early appearance of developmental characters is correlated to larger prominence in adults (Mehnert 1897a, b; Sánchez-Villagra et al. 2008; Maxwell and Larsson 2009) was critically discussed and we concluded that such simple correlations are the exception rather than the rule (see also Spiekman and Werneburg 2017: **Chapter 13**). The entanglement of developmental processes detected highlights the non-independent formation of adult characters that are usually treated as independent in phylogenetic studies, which may bias the output of such studies (Kluge 1998a; Zherikhin 1998; Rieppel 1999; Wiens 2001; Wägele 2005). Our comprehensive descriptions of embryonic development of the snake and the varanid species (**Chapters 11-12**) may serve as a resource for future studies integrating the complex processes of embryogenesis into broad-scale phylogenetic analyses that are likely to show that change in embryonic timing is one of the major sources of morphological diversification.

We further tested the relevance of skeletal tim-

ing for biological conclusions and concentrated in another study on the development of marsupial mammals, which is specialized towards an extremely short gestation and highly altricial newborns. As a result, marsupial neonates display morphological adaptations at birth related to functional constraints (Werneburg and Spiekman in press: **Chapter 5**). However, little was known about the variability of marsupial skull development and its relation to morphological diversity. We studied bony skull development in five marsupial species (Spiekman and Werneburg 2017: **Chapter 13**). The relative timing of the onset of ossification was compared to literature data and the ossification sequence of the marsupial ancestor was reconstructed using squared-change parsimony (Felsenstein 1985). The high range of variation in the onset of ossification meant that no patterns could be observed that differentiate species. This finding challenges traditional studies concentrating on the onset of ossification as a marker for phylogeny or as a functional proxy (Abdala et al. 1997; Anderson Maisano 2001; Sánchez-Villagra 2002; Fröbisch 2008; Maxwell 2008; Maxwell and Harrison 2008; Sánchez-Villagra et al. 2008; Maxwell and Larsson 2009; Werneburg et al. 2009; Weisbecker and Mitgutsch 2010; Hautier et al. 2011; Mitgutsch et al. 2011; Hugi and Scheyer 2012). Our study also presented observations on the developmental timing of cranial bone-to-bone contacts and their evolutionary implications. Although certain bone contacts display high levels of variation, connections of early and late development are quite conserved and informative. Bones that surround the oral cavity are generally the first to connect and the bones of the occipital region are among the last. We concluded that bone contact is preferable over onset of ossification for studying cranial bone development.

Although having broad access to data, ossification analyses run into comprehensive statistical and methodological limitations, and the results are often difficult to interpret in a biological sense. At least they are valuable only for selected macroevolutionary aspects (**Chapters 9-10**) and apply only to specific taxonomic levels (e.g., all squamates, all

mammals). These general observations with modern quantitative ossification studies, however, highlight the great value of traditional morphological studies. In some initial case studies on skeletal formation (**Chapters 14, 15, 18**), I had to reduce the taxonomic sampling, due to the natural lack of material and time. However, a very close look into anatomy using histology and different staining methods still helps analyzing macroevolutionary changes at high taxonomic levels.

Modes of bone formation

As mentioned above, the phylogenetic position of turtles among amniotes is highly debated. The hooked element in the pes of turtles was historically identified by most palaeontologists and embryologists as a modified fifth metatarsal, and often used as evidence to unite turtles with other reptiles with a hooked element, namely lepidosaurs (see (Rieppel 2008). Some recent embryological studies, however, revealed that this element might represent an enlarged fifth distal tarsal (Sheil and Portik 2008; Fabrezi et al. 2009). Following the holistic ‘organism concept’ (Maier 1999), we provided not only data on skeletogenesis but also new myological observations (Walker 1973) on the hooked element of turtles, and re-evaluate its primary and secondary homology using all available lines of evidence (Joyce et al. 2013: **Chapter 14**). Timing of development, as expected, and digital count are uninformative. However, detailed skeletogenetic data on bone formation are consistent with the hypothesis that the hooked element of turtles represents a fusion of the fifth distal tarsal with the fifth metatarsal, with both bone parts forming a hook. The fifth distal tarsal dominates the hooked element in pleurodiran turtles, whereas the fifth metatarsal dominates the hooked element of cryptodiran turtles. The term ‘ansulate bone’ was proposed to refer to hooked elements that result from the fusion of these two bones. The hook in the true metatarsal in lepidosaurs is related to the hypertrophied insertion site of the gastrocnemius muscle to the diaphysis of this bone (Robinson 1975; Russel and Bauer 2008).

Earlier to enchondral ossification (Cubo 2000), cartilaginous and mesenchymal anlagen form and can be of phylogenetic significance (Yaryhin and Werneburg submitted: **Chapter 15**, Werneburg et al. 2013: **Chapter 18**). The neurocranium of vertebrates is mainly derived from early cartilaginous anlagen, the so-called chondrocranium, the base of the future skull (de Beer 1937; Rieppel 1993; Hanken and Hall 1994). Two initial bar-shaped and paired chondrifications flank the notochord, the rostral trabecles and the caudal parachordals. In most reptiles, there is an additional component, the transverse acrochordal, which is placed between trabecles and parachordals (Rieppel 1977). All these elements compose the base of the future chondrocranium. There are several drastically different hypotheses concerning the development and interrelationship of these elements. We reexamined the basicranial development in four squamates and found that all species show very similar conditions of early chondrocranial development (Yaryhin and Werneburg submitted, **Chapter 15**). The anterior part of the notochord is not embedded into the basal plate as it was previously reported. It remains free. The medial edges of the parachordals form the lateral walls of the basicranial fenestra. Only the posterior portions of the parachordals fuse and form the basal plate. The space in-between the parachordals is filled with a thin layer of cells, which never chondrify. The anterior tips of the parachordals later fuse with the posterior edge of the acrochordal, which ultimately delimitates, as crista sellaris, the basicranial fenestra anteriorly. We considered the observed processes a common development, at least in lizards, and review a variety of methodological approaches (Mulisch and Welsch 2010) and differences in data interpretation as reasons for the anatomical differences reported in the literature (De Beer 1930; EL-Toubi and Kamal 1959; Kamal and Abdeen 1972; Hernández-Jaimes et al. 2012). Moreover, based on our data we argue that the acrochordal is of mesodermal origin, which coincides with results of fate map experimental studies (Noden and Trainor 2005).

Genes, fossils and morphology

Even earlier than cartilaginous or mesenchymal condensation, developmental genetics are of great importance to understand pattern formation through ontogeny and subsequent differences in adult morphology. The development of distinct regions in the amniote vertebral column results from the timing and pattern of somite formation and *Hox* gene expression (Richardson et al. 1998; Morin-Kensicki et al. 2002; Piekarski and Olsson 2007; Wehner and Gehring 2007), with the adult morphology displaying remarkable variation among lineages (Gadow 1933; Lindell 1994; Christ et al. 2000; Pierce et al. 2013). Mammalian regionalization is reportedly very conservative or even constrained (Asher et al. 2011), but there has been no study investigating vertebral count variation across Amniota as a whole, undermining attempts to understand the phylogenetic, ecological, and developmental factors affecting vertebral column variation. We demonstrated that the mammalian (synapsid) and reptilian lineages show early in their evolutionary histories clear divergences in axial developmental plasticity, in terms of both regionalization and meristic change, with basal synapsids sharing the conserved axial configuration of crown mammals, and basal reptiles demonstrating the plasticity of extant taxa (Müller et al. 2010: **Chapter 16**). We conducted a comprehensive survey of presacral vertebral counts across 436 recent and extinct amniote taxa. Vertebral counts were mapped onto a generalized amniote phylogeny as well as individual ingroup trees, and ancestral states were reconstructed by using squared-change parsimony (Felsenstein 1985). We also calculated the relationship between presacral and cervical numbers to infer the relative influence of homeotic effects and meristic changes and found no correlation between somitogenesis and *Hox*-mediated regionalization. Although conservatism in presacral numbers characterized early synapsid lineages (Kammerer et al. 2014), in some cases reptiles and synapsids exhibit the same developmental innovations in response to similar selective pressures. Conversely, increases in body mass are not cou-

pled with meristic or homeotic changes, but mostly occur in concert with postembryonic somatic growth. Our study highlighted the importance of fossils in large-scale investigations of evolutionary developmental processes (Eernisse and Kluge 1993; Kluge 1998b; Lee et al. 2004; Rieppel 2009; Sterli 2010; Wiens et al. 2010; Scheyer et al. 2013).

The approach to integrate fossils, genes, and embryology was also applied to a case study on turtle necks. Despite the conservatism in vertebral count among turtles (eight cervicals), there is significant functional and morphological regionalization in the cervical vertebral column reflecting different modes of neck movement (see below). Since *Hox* genes play a fundamental role in determining the differentiation in function and form of vertebrae and based on our reconstruction of evolutionary genetics in deep time, we hypothesized genetic differences between the turtle groups and between turtles and other land vertebrates.

We correlated anterior *Hox* gene expression and the quantifiable shape of the cervical vertebrae to investigate the morphological modularity in the neck across living and extinct turtles (Böhmer and Werneburg submitted: **Chapter 17**). This permitted the reconstruction of the hypothetical ancestral Hox code pattern of the whole turtle clade. The scenario of the evolution of axial patterning in turtles indicates lineage-specific patterns. These include shifts in the expression of *HoxA-5* in relation to the reduction of cervical ribs in modern turtles and of *HoxB-5* linked with a lower morphological differentiation between the anterior cervical vertebrae observed in cryptodirans. Furthermore, by comparison with the mammalian pattern, we illustrate how the fixed count of eight cervical vertebrae in turtles is dependent on the emergence of the unique turtle shell.

The modular pattern in the neck of the analyzed turtles supports the observation that the *Hox* code reflects their unique body plan. Future genetic analyses will improve our knowledge of the Hox gene expression pattern of living turtles. Yet, the present work shows that vertebral morphological modularity offers the potential to study the evolutionary mechanisms responsible for the great mor-

phological adaptability of the cervical vertebral column.

The turtle neck.

The turtle neck continued to be in the focus of my research in the last couple of years. Turtles are one of the most enigmatic groups of vertebrates with their highly modified ‘body plan’ and, as such, they attracted the attention of researchers for a long time. Aside from the unique turtle shell, the head and neck in this group show also great differences in comparison to those of other amniotes. The neck has a mediating role in the morphology of the turtle ‘body plan’, namely between the head on one side and the shell on the other side.

A broad sample of extant turtles possesses a series of paired bones in the neck that are situated between the cervical vertebrae. These paired bones were originally proposed to be cervical rib remnants (Williams 1959), but have more recently been interpreted as vestiges of intercentra (Gaffney 1985). We documented, for the first time, the neck development of a pleurodire turtle, *Emydura subglobosa*, and identify blastematos structures, which partially recapitulate the ribs and intercentra of the plesiomorphic tetrapod condition (Werneburg et al. 2013a: **Chapter 18**). We identified blastematos “bridges” between intercentra and the corresponding ribs, which we homologized with the vestiges visible in extant turtles and with the remnant parapophyseal articulation processes of the intercentra of some stem taxa. Only the unpaired, median part of the intercentrum of the atlas is retained in adult turtles, but intercentra are recapitulated along the entire vertebral column during development; they are embedded in the cervical myosepta and serve as attachment sites for neck musculature. We also identified two rib rudiments in the occipital region, which may indicate that at least two vertebrae are integrated into the cranium of turtles in particular, and of amniotes in general.

The loss of ribs in the ontogeny of extant turtles is obviously related to the great mobility of their neck (Herrel et al. 2008). Turtles have the unparalleled ability to retract their heads and necks

within their shell but little is known about the evolution of this trait. Extensive analysis of neck mobility in turtles using radiographs, CT scans, and morphometry revealed that stem turtles possessed less mobility in the neck relative to their extant relatives, although the anatomical prerequisites for modern mobility were already established (Werneburg et al. 2015a: **Chapter 19**). Many extant turtles are able to achieve hypermobility by dislocating the central articulations, which raises cautions about reconstructing the mobility of fossil vertebrates in general. A 3D-model of the Late Triassic turtle *Proganochelys quenstedti* revealed that this early stem turtle was able to retract its head by tucking it sideways below the shell. The simple ventrolateral bend seen in this stem turtle, however, contrasts with the complex double-bend of extant turtles. The initial evolution of neck retraction therefore occurred in a near-synchrony with the origin of the turtle shell as a place to hide the unprotected neck. In this early, simplified retraction mode, the conical osteoderms on the neck provided further protection.

The unique ability of modern turtles to retract their head and neck into the shell through a side-necked (pleurodiran) or hidden-necked (cryptodiran) motion is thought to have evolved independently in crown turtles (Herrel et al. 2008). The anatomical changes that led to the vertebral shapes of modern turtles, however, are still poorly understood. We presented comprehensive geometric morphometric analyses that trace turtle vertebral evolution and reconstructed disparity across phylogeny (Werneburg et al. 2015c: **Chapter 20**). Disparity of vertebral shape was high at the dawn of turtle evolution and decreased after the modern groups evolved, reflecting a stabilization of morphotypes that correspond to the two retraction modes. Stem turtles, with their lateral head tuck, show increasing flexibility of the neck through evolution towards a pleurodiran-like morphotype. The latter was the precondition for evolving pleurodiran and cryptodiran vertebrae. There is no correlation between the construction of formed articulations in the cervical centra and neck mobility. An increasing mobility between vertebrae, associ-

ated with changes in vertebral shape, resulted in a more advanced ability to retract the neck. In this regard, we hypothesized that the lateral tucking retraction of stem turtles was not only the precondition for pleurodiran but also of cryptodiran retraction. For the former, a kink in the middle third of the neck needed to be acquired, whereas for the latter modification was necessary between the eighth cervical vertebra and first thoracic vertebra. Our study highlighted the utility of 3D shape data, analyzed in a phylogenetic framework, to examine the magnitude and mode of evolutionary modifications to vertebral morphology. By reconstructing and visualizing ancestral anatomical shapes, we provided insight into the anatomical features underlying neck retraction mode, which is a salient component of extant turtle classification.

Extant turtles are characterized by diverse marginal reductions in their temporal skull region. Among other minor factors, their modes of neck retraction were hypothesized to have a key influence for shaping that region through evolution. A small emargination in the “cheek” of *P. quenstedti* could be correlated to its neck tucking mode of retraction. By using a geometric morphometric approach, I correlated the curve shapes of retracted necks and other neck positions with the expansion of marginal reductions in turtle skulls (Werneburg 2015: **Chapter 21**). I hypothesized based on morphospace distributions that neck retraction evolved only once within turtle evolution and could prove that pleurodiran and cryptodiran turtle retraction are directly and independently derived from ancestral neck tucking. Pleurodires evolved a middle kink in their elongated neck for lateral retraction. At the dawn of turtle evolution, associated to the ancestrally retracted (laterally rotated) neck, the cervicals were less specialized than in extant taxa. For cryptodires, that condition may have permitted a transitional, intervertebral rotation towards the vertical neck orientation found in that group during retraction. It retained the ancestral characteristically oriented curvature of the cervical column. I found that the cryptodiran mode of retraction and the ventral neck flexion in all turtles are strongly correlated to the expansion of the oc-

ciput emargination. Pleurodiran retraction, however, does not influence skull shape to such a degree. The “cheek” emargination is correlated with the expansion of the “occiput” emargination and appears to occur in correlation to the fixation of the palatoquadrate to the braincase in crown turtles. Neck related forces acting on the skull and ventral neck flexion were also hypothesized to be crucial factors for the reduction of a potential temporal fenestration inherited from a potentially fenestrated turtle ancestor (see also **Chapter 23**).

The temporal skull region and head musculature

Although exhibiting various different marginal reductions (emarginations), turtles show a pure anapsid morphotype. As a result of their skull diversity, turtles can be taken as a model by which to understand the processes that may have resulted in the highly debated anatomy of the amniote temporal region in general. I reviewed almost forgotten literature and summarized, in addition to the mode of neck motion (see above), nine potential factors that may act on the skull to shape the temporal region of turtles (Werneburg 2012: **Chapter 22**). These are: (1) phylogenetic constraints, (2) skull weights, (3) type of food, (4) skull dimensions, (5) muscle bulging, (6) ear anatomy and jaw muscle bending mechanisms, (7) extent and nature of muscle attachment sites, (8) internal forces within the jaw adductor chamber, and (9) environmental pressure. In this review, particular focus was laid on the interrelationship of the jaw musculature and the dermatocranial armour, which were assumed to influence each other to a certain degree. In the literature, cranial dimensions were assumed to influence temporal bone formation within major tetrapod groups. Among these, turtles seem to represent a kind of intermixture, a phenomenon that may be reflected in their specific anatomy.

In addition to my research on the turtle neck (**Chapters 18-21**), I was always interested in turtle head muscle anatomy (Werneburg 2011, 2013a) and its influence in shaping the turtle skull anatomy. Recently, we have reviewed the current

knowledge of the turtle head, summarizing the general morphology of the skull as well as the different anatomical modifications characteristic of the main lineages of extant and extinct turtles (Ferreira and Werneburg submitted: **Chapter 23**). Using 3d models and ancestral state reconstructions, we inferred the arrangement and gross morphology of the jaw adductor musculature in the fully anapsid fossil *P. quenstedtii*, one of the earliest turtles with a complete shell, an important step towards the understanding of the evolution of those muscles in turtles.

For a better understanding of the original functional morphology of anapsid taxa, sea turtles (Chelonoidea) represent a valuable model as they exhibit almost fully closed temporal regions. Obviously, they are not directly comparable to stem turtles as they only evolved their anapsid skulls secondarily and as they feed in a completely different environment when compared to the semi- or fully terrestrial stem turtles (Gaffney 1990; Joyce and Gauthier 2004; Joyce 2015). However, the diversity and evolution of sea turtle feeding anatomy remained incompletely known. Using computed tomography and classical comparative anatomy, we described the cranial anatomy in two sea turtles, the loggerhead (*Caretta caretta*) and Kemp's ridley (*Lepidochelys kempii*), for a better understanding of sea turtle functional anatomy and morphological variation (Jones et al. 2012: **Chapter 24**). In both taxa, the jaw joint structure and muscle arrangement indicate that palinal jaw movement is possible. The tongue is relatively small, and the hyoid apparatus is not as conspicuous as in some freshwater aquatic turtles. We found several similarities between the muscles of *C. caretta* and *L. kempii*, but comparison with other turtles suggested only one of these characters may be derived: connection of the m. adductor mandibulae internus into the pars intramandibularis via the Zwischensehne. The large fleshy origin of the m. adductor mandibulae externus pars superficialis from the jugal seems to be a characteristic feature of sea turtles. In *C. caretta* and *L. kempii* the ability to suction feed does not seem to be as well developed as that found in some freshwater aquatic turtles. Instead, both have

skulls suited to forceful biting – maybe like stem turtles had. This, however, is consistent with the observation that both taxa tend to feed on relatively slow moving but sometimes armored prey. The broad fleshy origin of the m. adductor mandibulae externus pars superficialis may be linked to the cheek region being almost fully enclosed in bone but the relationship is complex, a feature also reconstructed for stem turtles (Werneburg 2013a; Ferreira and Werneburg submitted: **Chapter 23**).

The complexity of the jaw musculature in turtles and other amniotes is sometimes hard to compare resulting from the plasticity of muscles through development and the specific spatial and functional requirements among species (Edgeworth 1935; Rieppel 1987, 1988, 1990; Ericsson and Olsson 2004; Ziermann et al. 2014). To analyze homologies on higher taxonomic levels, another structure appears to be of greatest importance, namely, cranial neural crest cell (cNCC) derived soft tissue. cNCC develop early in ontogeny and form a variety of cranial tissues including facial bones (Donoghue et al. 2008; Hall 2009). In vertebrates, muscle tendons and ligaments between bones derive from cNCC and significantly influence the shape and positioning of head musculature (Olsson et al. 2001; Ericsson et al. 2004; Rinon et al. 2007; Schmidt et al. 2012). In this regard, homologization of these soft tissues gained a particular interest in the past (Iordansky 1994, 1996). For the discussion of turtle origin, one ligament played a particular role.

Lakjer (1926) hypothesized a replacement of the infratemporal bar in diapsid reptiles by a ligament spanning between quadrate and the upper jaw. As a similar ligament is also present in turtles, he argued for a diapsid origin of this group. Based on recent advances in the homologization of the tendinous framework in the reptile jaw adductor chamber (Iordansky 1994, 1996), one could argue for independent origins of the cheek ligaments in sauropsids. The quadratomaxillar ligament of turtles could, with reservation, be homologized with the quadrate aponeurosis of other sauropsids, as well as to the superficial tendon of m. masseter in mammals (Werneburg 2013b: **Chapter 25**). These

structures have a strong morphogenetic influence to cranial anatomy. Given such an identity, the hypothesis of a structural replacement of the lower temporal arcade in lizards would be refuted. Moreover, such a homology could be correlated to the evolution of the middle ear and to the origin of the chewing mechanism in mammals, which contributed to the evolutionary success of that group.

Feeding in vertebrates

Different modes of feeding largely influence the shape of the tetrapod skull in general. One of the key innovations in tetrapod evolution was the convergent emergence of herbivory in many tetrapod groups. Adaptations to plant feeding developed in the gut of ruminants with their large rumen or of rodents with their caecal digestion. For that, comprehensive symbioses evolved with gut bacteria, which are able to digest the cellulose of plants. In addition, other adaptations are characteristic for herbivores, including modifications of muscle, teeth, and skull anatomy. The combination of these features resulted in a great evolutionary success of these animals (Werneburg 2014a: **Chapter 26**).

Turtles are characterized by a fast evolutionary reduction of teeth. Whereas the half-shelled stem turtle *Odontobelys semitestacea* (Li et al. 2008) still had marginal teeth on the jaws, *P. quenstedti* (Gaffney 1990) and other fossil turtles only show palatal teeth (Joyce et al. 2016), and teeth are completely lost in extant turtles (Davit-Béal et al. 2009). Nowadays, ramphothecae, horny shields, are formed on the jaw margins, which serve as scissors to slice the food. Stem turtles most likely already had such ramphothecae and similar to the horny jaw shields in fossils, including Anomodontia (Synapsida) and Ceratopsia (Dinosauria), those have been herbivorous adaptations (King 1996; Hotton III et al. 1997; Weishampel 1997; Sues 2000). Understanding the transition from a plesiomorphically carnivorous to an herbivorous behavior is key to understand turtle cranial evolution. At the dawn of modern turtle evolution, a reversed transition from terrestrial (stem turtle) to aquatic, carnivorous

feeding behavior (crown turtles) took place (Werneburg 2013a). Only within modern cryptodires, in tortoises, a further transition from water to land and from carnivore to herbivore ecology evolved. Almost all extant tortoises (Testudinidae) are highly associated with terrestrial habitats and the few tortoises with high affinity to aquatic environments are found within the genus *Manouria*. *Manouria* belongs to a clade which forms a sister taxon to all remaining tortoises and is suitable as a model for studying evolutionary transitions within modern turtles. We analyzed the feeding behavior of *Manouria emys* and due to its phylogenetic position, we hypothesized that the species might have retained some ancestral features associated with an aquatic lifestyle (Natchev et al. 2015: **Chapter 27**). We tested whether *M. emys* is able to feed both in aquatic and terrestrial environments. In fact, *M. emys* repetitively tried to reach submerged food items in water, but always failed to grasp them - no suction feeding mechanism was applied. When feeding on land, *M. emys* showed another peculiar behavior; it grasped food items by its jaws - a behavior typical for aquatic or semiaquatic turtles - and not by the tongue as generally accepted as the typical feeding mode in all tortoises studied so far (Wochesländer et al. 1999). In *M. emys*, the hyolingual complex remained retracted during all food uptake sequences, but the food transport was entirely lingual based. We concluded that the feeding behavior of *M. emys* might reflect a remnant of the primordial condition expected in the aquatic ancestor of the tortoises. The kinematical profiles significantly differed from those described for other tortoises and from those proposed from the general models on the function of the feeding systems in “lower tetrapods”.

Among “lower tetrapods”, the temnospondyls form the stem group of the Lissamphibia and are of particular interest to understand the origin of cranial diversity in land vertebrates. Temnospondyls were the morphologically and taxonomically most diverse group of early tetrapods with a near global distribution during the Palaeozoic and Mesozoic. Members of this group occupied a range of different habitats (aquatic, amphibious, terrestrial),

reflected by large morphological disparity of the cranium throughout their evolutionary history. Like crocodiles and turtles, they have an akinetic skull with similar biomechanical requirements and can serve as model to understand these modern forms. A diagnostic feature of temnospondyls is the presence of an open palate with large interpterygoid vacuities, unlike the closed palate of most other early tetrapods, in which the vacuities are either slit-like or completely absent. Muscle attachment sites on neurocranium and palatal bones in temnospondyls allow the reconstruction of a powerful m. retractor bulbi and a large, sheet-like m. levator bulbi that formed the elastic floor of the orbit. This muscle arrangement indicates that temnospondyls were able to retract the eyeballs through the interpterygoid vacuities into the buccal cavity, like extant frogs and salamanders. In contrast, attachment sites on palate and neurocranium suggest a rather sauropsid-like arrangement of these muscles in stem-tetrapods and stem-amniotes. However, the anteriorly enlarged, huge interpterygoid vacuities of long-snouted stereospondyls suggest that eye retraction was not the only function of the vacuities here, since the eye-muscles filled only the posterior part of the vacuities. We propose an association of the vacuities in temnospondyls with a long, preorbital part of the m. adductor mandibulae internus (AMIa) (Witzmann and Werneburg in press: **Chapter 28**). The trochlea-like, anterior edge of the adductor chamber suggests that a tendon of the AMIa was redirected in an anteromedial direction in the preorbital skull and dorsal to the pterygoids. This tendon then unfolded into a wide aponeurosis bearing the flattened AMIa that filled almost the complete interpterygoid vacuities anterior to the orbits.

Our muscle reconstructions permitted comprehensive insights to the comparative soft tissue anatomy of early tetrapods and provided the basis for a biomechanic analysis of biting performances. Although the function of the interpterygoid vacuities has been discussed in the past, no quantitative studies have been performed to assess their biomechanical significance. Therefore, we applied finite element analysis, to test the possibility that the in-

terpterygoid vacuities served for stress distribution during contraction of the jaw closing musculature (Lautenschlager et al. 2016: **Chapter 29**). Different original and theoretical skull models, in which the vacuities differed in size or were completely absent, were compared for their mechanical performance. Our results demonstrated that palatal morphology played a considerable role in cranial biomechanics of temnospondyls. The presence of large cranial vacuities were found to offer the dual benefit of providing additional muscle attachment areas and allowing for more effective force transmission and thus an increase in bite force without compromising cranial stability.

Associated with cranial stability, a strongly ossified and rigid skull roof, which prevents parietal kinesis, has been reported for the adults of all amphibian clades (Carroll 2009; Schoch 2014). Our μ CT investigations revealed that the Buresch's newt (*Triturus ivanbureschi*) from Bulgaria possesses a peculiar cranial construction (Natchev et al. 2016: **Chapter 30**). In addition to the typical amphibian pleurokinetic articulation between skull roof and palatoquadrate associated structures, we found flexible connections between nasals and frontals (prokinesis), vomer and parasphenoid (palatokinesis), and between frontals and parietals (mesokinesis). This was the first description of mesokinesis in urodelans. The construction of the skull in the Buresch's newts also indicates the presence of an articulation between parietals and the exocipitals, discussed as a possible kind of metakinesis. The specific combination of pleuro-, pro-, meso-, palato-, and metakinetic skull articulations indicate to a new kind of kinetic systems unknown for urodelans to this date. We discussed the possible neotenic origin of the skull kinesis and pose the hypothesis that the kinesis in *T. ivanbureschi* increases the efficiency of fast jaw closure. For that, we compared the construction of the skull in *T. ivanbureschi* to the akinetic skull of the Common fire salamander *Salamandra salamandra*. We hypothesized that the design of the skull in the purely terrestrial living salamander shows a similar degree of intracranial mobility. However, this mobility is permitted by elasticity of some bones and not by

true articulation between them. We commented on the possible relation between the skull construction and the form of prey shaking mechanism that the species apply to immobilize their victims.

The variety of scientific topics united in this habilitation on “land vertebrate evolution and development” highlights the complex interdigitation of such diverse research fields like embryonic, adult skeletal, soft tissue, and functional anatomy. Land vertebrates provide an excellent group to study as all of them went through an adaptational bottleneck when leaving the shore. A variety of morphological, often convergent characteristics evolved to master the new challenges to survive. These include different life history traits and particular modifications of the craniocervical system exemplified for mammals, lizards, snakes, turtles, amphibians, and representatives of different stem groups. With my studies, I aim to reintegrate paleontological, embryological, and zoological approaches into a holistic organism-based morphological and evolutionary research program.

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