

Phenotypic innovation and repeatability in turtles

by

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I dedicate this work to the Cordero family for supporting me through this amazing journey: Gerardo, Sr., Carmen, Claudia, Maria Andreina, and Elías.

TABLE OF CONTENTS

	Page
DEDICATION.....	ii
ACKNOWLEDGMENTS	v
ABSTRACT.....	vii
CHAPTER 1. GENERAL INTRODUCTION.....	1
Introduction	1
Dissertation Organization	6
References	8
CHAPTER 2. AN ENHANCED DEVELOPMENTAL STAGING TABLE FOR THE PAINTED TURTLE, <i>Chrysemys picta</i> (TESTUDINES: EMYDIDAE).	13
Introduction	14
Materials and Methods.....	16
Results	18
Discussion	22
Acknowledgments	28
References	29
CHAPTER 3. SKELETAL REMODELING SUGGESTS THE TURTLE'S SHELL IS NOT AN EVOLUTIONARY STRAITJACKET	49
Introduction	50
Materials and Methods.....	51
Results	52
Discussion	53
Acknowledgments	55
References	56
Supplementary Material	62
CHAPTER 4. GENES, JOINTS, AND THE REPEATED EVOLUTION OF SKELETAL COMPLEXITY IN TURTLES.....	74
Introduction	75
Materials and Methods.....	79
Results	85
Discussion	88
Acknowledgments	93
References	94
Supplementary Material	108
CHAPTER 5. GENERAL CONCLUSIONS.....	111

APPENDIX A: REVIEW ON EVO-DEVO OF THE PAINTED	
TURTLE	116
References	120

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ABSTRACT

According to Darwin's principle of "descent with modification" closely related species are expected to share similar traits. Still, Darwin's critics pointed out that unrelated species often feature strikingly similar or even identical traits, i.e. phenotypic repeatability. To explain this trend, modern theory of evolutionary biology posits that natural selection favors phenotypic repeatability in unrelated species inhabiting similar environments.

Alternative perspectives from evolutionary developmental biology (evo-devo) propose that developmental constraints imposed by organismal design could promote phenotypic repeatability by limiting variation available to natural selection. Evo-devo questions other related issues in modern evolutionary biology, such as that selection operating gradually across evolutionary time is the sole process underlying the development of novel traits, i.e. phenotypic innovation. Examination of developmental processes could provide new insights on this and other conceptual issues concerning the rapid and repeated evolution of traits.

The overarching objective of this dissertation was to employ an evo-devo framework to uncover developmental underpinnings of phenotypic innovation and repeatability. This dissertation research aimed to address this fascinating topic in three data chapters. The focal study system of these projects is the enigmatic turtle body plan, which is a classic example of phenotypic innovation and developmental constraint.

In data chapter two, I seek to reevaluate classical descriptions of embryonic development in a "model" turtle species, the painted turtle (*Chrysemys picta*). This observational study served as the foundation to data chapter three, which entailed the most phylogenetically comprehensive comparison of turtle development. Data chapter three seeks to identify evolutionary divergence in processes governing development of phenotypic innovation in turtles. In data chapter four, I

attempt to link phylogenetic patterns of phenotypic repeatability with underlying genetic pathways. This culminating chapter examines the evolution of a repeatedly evolving trait, shell kinesis, to address one of the most intriguing questions in modern biology: Do unrelated species employ similar genetic solutions to similar ecological problems?

Altogether, these data chapters illuminate developmental underpinnings of striking patterns of trait evolution in turtles, and corroborate that both natural selection and developmental constraints influence the origins of phenotypic innovation and repeatability in nature.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

How do novel traits arise during the evolutionary history of organisms? Why do unrelated species share strikingly similar traits? Scientists have sought to answer these intriguing questions since the dawn of modern biology. Even so, how novel phenotypes (hereafter phenotypic innovation) and trait similarity arise in unrelated species (hereafter phenotypic repeatability) are conceptually problematic to modern theory of evolutionary biology (Wake et al. 2011; Hall 2012; Wagner 2014; Ochoa and Rasskin-Gutman 2015).

Related species are expected to share similarities following Darwin's iconic principle of "descent with modification" (Darwin 1859). Yet, it is not uncommon for species that did not descend from a most recent common ancestor to feature similar adaptive traits. This trend is concerning because it challenges a central tenet of evolutionary biology---homology, or phenotypic similarity due to shared ancestry of all life on Earth.

Definitions of phenotypic innovation and repeatability are subject to debate (Arendt and Reznick 2008; Ochoa and Rasskin-Gutman 2015). Herein, I define phenotypic innovation as a trait not present in a most recent common ancestor that enables a species to exploit a new ecological niche and undergo adaptive diversification (Wagner 2014). I define phenotypic repeatability, often referred to as convergent or parallel evolution, as the recurrence of an adaptive trait across separate branches of the tree of life of a particular group of species (McGhee 2011; Wake et al. 2011; Hall 2012). My discussion here is focused on morphological traits in animals (but see Donogue and Ree 2000), as phenotypic innovation and repeatability were initially examined from this perspective (reviewed in Bock 1963).

Phylogeneticists intensively debated patterns of phenotypic innovation and repeatability early in the 20th century, but could not reach an explanatory consensus (reviewed in Bock 1963). Instead, explanations provided by emerging theory based on Mendelian and population genetics were conventionally accepted (Dobzhansky 1959). This paradigm, known as the *modern synthesis* of evolutionary biology, provided a framework for empirical tests of Darwin's theory of evolution by natural selection.

Beginning in the 1930s, the assertion that slow and gradual change in gene frequencies translates into adaptive phenotypic variation within evolving populations, the *genotype-to-phenotype map*, became the central dogma of the *modern synthesis* (Wright 1968; Lewontin 1974). Still, empirical evidence addressing the rapid and repeated evolution of complex traits in nature remained unsatisfactory (Frazzetta 1976).

In the 1940s, a handful of evolutionary geneticists proposed unorthodox theory that questioned whether slow and gradual change in gene frequencies was the sole process underpinning phenotypic innovation (discussed in Alberch 1989; Gottlieb 1992). For example, Richard Goldschmidt introduced the concept of macromutation as a means to explain the rapid evolution of adaptive traits (Goldschmidt 1940). Evolutionary biologists were not receptive of his idea and, despite a lack of empirical evidence, continued to explain phenotypic innovation using population genetic theory (e.g. Bock 1959; Mayr 1959).

Although phenotypic repeatability was dismissed as a nuisance by phylogeneticists, architects of the *modern synthesis* felt compelled to address it (e.g. Dobzhansky 1959), because the phenomenon was used as evidence to criticize Darwin's principle of "descent with modification". Darwin explained phenotypic repeatability by suggesting that similar environments should select for similar traits (Darwin 1872). The *modern synthesis* enhanced

Darwin's explanation by proposing that similar environments select for genotypes that give rise to similar traits in evolving populations of unrelated species (Dobzhansky 1959). Eventually, this theoretical model was empirically validated, but only for structurally simple traits (Hoekstra and Coyne 2007; Arendt and Reznick 2008).

Understanding the potential for traits to evolve repeatedly and rapidly is critical to furthering our basic understanding of how organismal diversity arises in nature (Wake et al. 2011; Wagner 2014). However, promoting this research agenda under the *modern synthesis* paradigm remains challenging because: 1) phenotypic variation in evolving populations is not truly random, as it depends on the limits of inherited organismal design (e.g. developmental constraints; Brakefield 2006; Sears 2014); 2) phenotypic variation is influenced by historical contingency or phylogenetic constraints (McKittrick 1993; Donoghue and Ree 2000); and 3) the *genotype-to-phenotype map* is far from a simple one-to-one relationship (Davidson 2006; Salazar-Cuidad and Marin-Riera 2013; Sears 2014).

Determining the mechanistic basis of phenotypic innovation and repeatability is a major objective for evolutionary biology of the 21st century (Wake et al. 2011; Hall 2012; Wagner 2014). Interest in these concepts was renewed by the *developmental constraints* school of thought led by a small group of evolutionary and developmental biologists in the 1970s and 1980s (Gould 1977; Lewontin and Gould 1979; Alberch 1980; Oster and Alberch 1982). Their novel perspectives gave rise to the modern field of evolutionary developmental biology (evo-devo) by reintroducing classical embryology to modern evolutionary thought (Maynard-Smith et al. 1985).

In the 1980s, a series of revolutionary embryological experiments linked phylogenetic patterns of tetrapod digit reduction to underlying developmental processes (Alberch and Gale

1983; 1985). For the first time, embryological data complimented classical Mendelian experiments foundational to the *modern synthesis* (e.g. Wright 1934), which had been recently validated by population genetic models (Lande 1978). Eventually, developmental processes were even incorporated into the mathematical framework of quantitative genetics (Atchley 1987).

Perhaps the biggest breakthroughs towards improving our understanding of interesting patterns of trait evolution, such as phenotypic innovation and repeatability, came in the 1990s when developmental geneticists conclusively demonstrated that distantly-related animal lineages express the “same” or homologous genes during development of complex morphological traits (Wilkins, 2002; Carroll 2008; Shubin et al. 2009; Sears 2014). This discovery legitimized the foundational doctrine of an emerging evo-devo paradigm: phenotypic variation is the end product of intricate processes regulated by genes and influenced by the external and internal environment of developing organisms (Alberch 1980; Oster and Alberch 1983; Maynard-Smith et al. 1985; Arnold et al. 1989; Gottlieb 1992; Wilkins 2002; West-Eberhard 2003).

By the onset of the 21st century, it was generally accepted that developmental genes are highly evolutionarily conserved across the animal kingdom (Carroll 2008; Shubin et al. 2009). This result suggested that unrelated species might deploy similar genetic pathways during development of ecologically relevant traits, i.e. the *genetic tool kit* (Carroll 2008; Shubin et al. 2009). For example, genes encoding for bone morphogenetic proteins play a critical role in shaping beak morphology in Darwin’s Finches, defining variation in the feeding morphology of African cichlid fishes, and organizing shell morphogenesis in turtles (Young and Badyaev 2007). Evo-devo’s *genetic tool kit* hypothesis even expanded to address the origins of complex behavioral traits (Toth and Robinson 2007).

A key prediction of the *genetic tool kit* hypothesis is that morphological evolution ensues via adaptive change in a small number of developmental genes shared by most animal lineages. Then do unrelated species employ similar genetic solutions to similar ecological problems? Is selection for novel phenotypes associated with tool kit genes? Examining the expression of heritable (i.e. genes) and non-heritable (environment) factors that control morphological development is critical to answering these questions.

Characterizing differences in developmental processes is informative for several reasons. First, the formation of morphological traits is a cellular-scale process governed by molecular signals encoded by genomes during embryogenesis. Secondly, because genomic information is transmitted across generations, it is often assumed that phenotypic evolution is correlated with functional genetic variation (i.e. gene expression).

As acknowledged by some founders of the *modern synthesis*, phenotypic evolution is in many cases the result of selection acting across multiple hierarchical levels of biological organization: gene > protein > cell > tissue > organ > organism (Dobzhansky 1956; Wright 1968). Thus, a hierarchical approach integrating phylogeny, development, and genetics is foundational to examining phenotypic innovation and repeatability in nature (Serb and Oakley 2005).

First, a resolved phylogenetic tree is critical for such integrative studies. The character state of a given trait of interest must be scored, along with other correlated variables, and a hypothetical selective pressure must be identified. Also, genetic and phenotypic data are needed. These conditions are typically satisfied in laboratory study taxa, in which the influences of selection and developmental constraints on phenotypic evolution have been successfully demonstrated (Matos et al. 2002; Woods et al. 2006; Atallah et al. 2009). Still, distinguishing

between these two effects is challenging and little is known about how they drive adaptive morphological diversification in nature (Brakefield and Roskam 2006). Turtles are a promising natural study system that meets important methodological conditions for the study of phenotypic innovation and repeatability.

Turtle evolution is defined by phenotypic innovation, as exemplified by an unusually shelled body plan (Gilbert et al. 2008). Furthermore, turtle evolution is characterized by multiple examples of phenotypic repeatability involving behavioral, physiological, and morphological traits. For example, genotypic sex determination has arisen independently multiple times during the lengthy evolutionary history of turtles (Janzen and Phillips 2006).

Turtles have a relatively well-described fossil record (Lyson et al. 2013), and comprise the sister group to birds + crocodilians (Archosaurs) within class Reptilia (Crawford et al. 2015). Crucially, deep phylogenetic relationships (family or subfamily level) are well supported and resolved within turtle phylogeny (Crawford et al. 2015). This rich evolutionary history serves as an excellent phylogenetic framework to test hypotheses on phenotypic innovation and repeatability.

Dissertation Organization

Guided by an evo-devo framework, my dissertation research seeks to examine patterns of phenotypic innovation and repeatability in turtles. In Chapter 2, I begin by reevaluating embryonic development of a model turtle species in evo-devo, the painted turtle (*Chrysemys picta*; see Appendix A). I combine classical embryological techniques with modern imaging to enhance and update a previously published developmental staging table for *C. picta*. Furthermore, I endeavor to quantify duration of developmental stages, embryo growth rate, and

length of incubation period under controlled laboratory conditions. F.J.J. originally conceived the idea for Chapter 2, which was executed by G.A.C. Data collected in Chapter 2 provides an important foundation for the work conducted in Chapter 3 and Chapter 4, where I explicitly address developmental and genetic underpinnings of phenotypic innovation and repeatability in diverse turtle lineages.

Although distantly-related turtles deploy diverse processes during shell development, all extant species share in common a shoulder blade (scapula) that is encapsulated within the shell. Therefore, the evolution of novel shell morphologies, such as shell kinesis, likely depends on evolutionary change in the correlated development of the shell and scapula. In Chapter 3, I address this expectation by examining scapula growth and differentiation in embryos, hatchlings, and adults of 13 turtle species by combining embryological and histochemical assays with comparative phylogenetic statistical methods. This chapter entails the most phylogenetically comprehensive examination of turtle development to date, which included comparisons to chicken and alligator embryos and hatchling lizards. This research was primarily executed by G.A.C. with laboratory assistance provided by co-author K.Q. Chapter 3 provides important knowledge on developmental processes related to patterns of phenotypic innovation and repeatability examined in Chapter 4.

In Chapter 4, I conduct a large-scale comparison of developmental gene expression in two turtle lineages (Emydidae: *Emys* and *Terrapene*) that have independently evolved shell kinesis. This chapter implements a balanced-block experimental design for massively paralleled cDNA sequencing (i.e. RNA-Seq) of scapula tissue, which included sampling of *C. picta* as the reference ancestral condition. The field and laboratory components of this work were primarily executed by G.A.C. with assistance provided by co-authors K.Q. and R.W. Bioinformatic

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**CHAPTER 2. AN ENHANCED DEVELOPMENTAL STAGING TABLE FOR
THE PAINTED TURTLE, *Chrysemys picta* (TESTUDINES: EMYDIDAE).**

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Abstract

Normal developmental staging tables often undergo expansion and enhancement in response to advancing research paradigms and technologies. The Painted Turtle, *Chrysemys picta*, has long been a preferred reference taxon for comparative embryology and recently became the first turtle species to feature a sequenced genome. However, modern descriptive studies on embryogenesis are lacking and an earlier developmental staging table has been ignored. To address these problems, we reevaluated descriptions of developmental stages by studying embryos under standardized laboratory conditions. We created an enhanced normal developmental staging table that clarifies and validates previous descriptions of developmental processes in this species. Moreover, we emphasized description of turtle-specific developmental characters such as the carapacial ridge. We demonstrated that embryo growth rate, length of incubation period, and timing to developmental stages are predictable under controlled environmental conditions. The appearance of characters associated with eye, limb, and shell anatomy was congruent with observations made in other turtle species. To reduce experimental bias, we recommend the use of our enhanced staging table when describing embryogenesis in the Painted Turtle.

Introduction

One of the first steps in biological research requires the observation and accurate description of pattern or process. Developmental biologists and morphologists have thus created and enhanced normal developmental staging tables that describe embryogenesis in a handful of representative vertebrate animals. Moreover, the creation and revision of staging tables has been motivated by changes in technology, research paradigms, and preference of study species (Hopwood, 2007; 2011).

Non-avian reptiles represent a substantial portion of the amniote tree of life and are increasingly popular research subjects in developmental biology (Vickaryous and McLean, 2011; Nomura et al., 2013). Historically, these taxa have been overlooked because their physiology and life history renders their embryos difficult to manipulate experimentally (New, 1966; Billet et al., 1985). Even the application of modern developmental genetic assays continues to be challenging in taxa such as turtles (Gilbert, 2009). Consequently, research on the developmental origins of morphological novelties, such as the turtle's shell, has been hindered by lack of knowledge of underlying gene regulatory networks (Wagner and Lynch, 2010). The sequenced genome of the Painted Turtle (Emydidae: *Chrysemys picta*) (Shaffer et al., 2013) is likely to facilitate reconstruction of gene regulatory networks associated with shell development. Even so, modern descriptive studies on most aspects of embryonic development are lacking for this representative model taxon. Furthermore, many important questions concerning the evolution and developmental underpinnings of temporal bone arrangement of the skull (Werneburg, 2012) and sex-determining mechanisms of turtles (Janzen and Krenz, 2004; Janzen and Phillips, 2006) remain unanswered.

A lack of standardized laboratory conditions and descriptions of embryos became problematic for comparing and replicating studies of developmental processes in the Painted Turtle (discussed in Smith, 1914; Cagle, 1954; Ewert, 1985). Early attempts to standardize protocols for the study of embryos included descriptive criteria for five stages of pectoral girdle development (Walker, 1947). These stages cannot be identified reliably because detailed descriptions of whole embryos were not provided and a small sample size was used. The normal developmental staging table of Mahmoud et al. (1973) was created to address such issues, but has been underutilized because illustrations were not highly detailed and a fluctuating incubation temperature (21-23 °C) was used. As a result, investigators have traditionally referenced the staging table for the phylogenetically disparate Snapping Turtle (Chelydridae: *Chelydra serpentina*; Yntema, 1968) when describing embryogenesis in the Painted Turtle (discussed in Ewert, 1985, 2008).

Analyses of developmental data generated under the guidance of staging tables that were not modeled after the species of interest could be a potential source of bias in comparative studies (Werneburg, 2009). For example, temporal variation in developmental stages is considered a source of technical error in gene expression studies (Gallego-Romero et al., 2012). Lack of information on developmental stages could also bias studies of ecological and physiological embryology in reptiles (Andrews, 2004). Also, because reptilian embryos are highly sensitive to the environment, egg incubation conditions must be accounted for if staging criteria are to be compared across species (Miller, 1985). Given the imminent increase in research centered on the Painted Turtle as a representative model for the study of developmental processes among amniotes (Valenzuela, 2009), improving developmental staging criteria is timely and highly relevant.

The objective of this study was to enhance the developmental staging table for the Painted Turtle. Specifically, we: 1) Clarified and validated previous descriptions of normal developmental stages; and 2) Quantified embryonic growth rate, length of incubation period, and timing to respective developmental stages under highly controlled and replicable laboratory conditions.

Materials and Methods

During the nesting seasons of 2010-2013, 316 eggs of the Western Painted Turtle (*C. picta bellii*) were collected at the Thomson Causeway Recreation Area in Thomson, IL (lat. 41° 57' N; described in Schwanz et al., 2010) within the same day of oviposition. In addition, 35 eggs were collected at the Avocet and De Fair Lake Wildlife Management Areas (Hyannis, NE) for scanning electron microscopy (SEM; described below). Eggs began incubation under semi-natural conditions (1-3 days) before transport to Iowa State University where they were placed in environmental chambers at a constant 27 °C. Eggs were half-buried in moist (−150 kPa water potential) vermiculite. Weekly, the vermiculite was rehydrated and containers were rotated to control for potential thermal gradient effects. Oxygen levels in the chambers were typically at 20-21%.

Embryos ($N = 234$) were dissected from eggs and extraembryonic membranes using distilled water and phosphate-buffered saline before preservation. Embryos in the first week of development were stained (*in vivo*) with neutral red agar to facilitate dissection. Embryos were then fixed in 10% buffered formalin at a mean sampling interval of 1.14 d through day 40 of incubation. Some embryos of representative stages (15-21) were also fixed in Bouin's solution to facilitate imaging of the eye and skin. Most embryos were fixed overnight at room temperature

and then removed for washing and dehydration using an increasing ethanol series. Embryos from representative stages were chosen for imaging on compound (Nikon Eclipse 55i) and dissecting (Nikon SMZ 745T) microscopes with mounted digital camera (Nikon DS-Vi1). Some embryos were imaged while partially embedded in plates of 1.5 % agarose in Tris/Borate/EDTA. Images were edited and formatted using programs Preview, Paintbrush, and ImageJ. Fixed embryos were staged by direct examination on the microscope followed by review of photomicrographs. Some unfixed embryos were also examined. The Mahmoud et al. (1973) staging table (MHK table hereafter) was used to stage embryos. These descriptive criteria were reevaluated and enhanced following generally accepted guidelines for the staging of reptilian embryos (Miller, 1985).

Eggs showing early signs of pipping were sampled to estimate total length of the embryonic incubation period. Egg pipping is a generally accepted index for the end of embryogenesis (Gutzke et al., 1984). Museum (ISU research collection) specimens of hatchling turtles were studied to describe the developmental stage immediately after the end of embryogenesis. Dial digital calipers were used to record crown-rump length (CRL) and carapace length (CL) to assess the relationship between embryo size and developmental time (i.e. growth rate) using regression analysis for comparison to the MHK table. All embryo measurements were taken after fixation and dehydration and choice of fixative did not appear to have an effect on size. Statistical analyses were conducted in JMP 10.

Embryos (stages 13, 15-16) assigned to SEM imaging were submerged in half-strength Karnovsky's fixative (Karnovsky, 1965) and left to soak overnight at 4 °C. Embryos were then dehydrated in an ethanol series and submitted to the Iowa State University Bessey Microscopy and Nanoimaging Facility for the implementation of standard SEM methodology. Briefly,

embryos were subjected to critical-point drying and sputter coating procedures in preparation for imaging in a SEM. Images were edited and formatted as described above.

Results

Revisiting the MHK Table

Descriptions of the MHK embryonic developmental stages (3-22) for the Painted Turtle were validated and enhanced. The “hatching” stage (23) was also described. Descriptions were clarified and new diagnostic features were provided for some stages. Embryos progressed rapidly through stages 1-2; thus, observations were limited during that time. Images of lateral or ventral aspects of whole or parts of embryos are presented for stages 3-23 (Figs. 1-9). Early formation of the apical ectodermal ridge (AER) of the limb bud is shown in Fig. 4. Images of the carapacial ridge (CR), the turtle-specific structure involved in shell development (reviewed in Nagashima et al., 2013), are presented at stages 15-16 (Fig. 6).

Stages 1 (described in MHK): The blastopore is apparent on the dorsoposterior surface of the embryo. The chordamesodermal canal connects the blastopore to the ventral surface of the embryo. Mesoderm has begun to condense on the anterior and posterior periphery of the embryonic disc.

Stages 2 (described in MHK): The blastopore is U-shaped. The chordamesodermal canal expands (anteriorly) on the ventral surface of the embryo.

Stage 3 (Fig. 1a): Somites are not visible. The neural and head folds begin to form. The notochord is apparent (ventrally) and the head process begins to flex.

Stage 4 (Fig. 1b): Three paired somites are present. Formation of the neural folds extends along the entire dorsal length of the embryo.

Stage 5 (Fig. 1c): Five paired somites are present. The neural folds begin to fuse anteriorly. The anterior intestinal portal has formed.

Stage 6 (Fig. 2a-b): Seven paired somites are present. The prosencephalon is apparent and formation of the optic vesicle begins. The anterior intestinal portal is near the presumptive pericardial region. The neural folds have fused along most of the anteroposterior axis.

Stage 7 (Fig. 2c-d): Nine paired somites are present. The mandibular arch is apparent. The auditory pit appears. The mesencephalon is distinguishable. The pericardial protuberance is present and blood islands are visible on the area vacuosa. The anterior intestinal portal has shifted caudally.

Stage 8 (Fig. 2e-f): 14 paired somites are present. The stomodeum has formed and the pericardial protuberance features an S-shaped heart anlage. The lateral body folds have formed along the entire anteroposterior axis of the embryo. The embryo is slightly oriented to the left and has flexed dorsally. A slight tail process is present.

Stage 9 (Fig. 3a-b): 20 paired somites are present. The neuropores have closed. The first pharyngeal slit has opened and the second and third pharyngeal arches are visible. The maxilla and olfactory pit begin to form. The primordial heart has begun to pump blood. The optic fissure appears and formation of the lens vesicle has begun. The embryo has now shifted its orientation to the left side (anteriorly). Early limb buds appear as condensations on the Wolffian ridge.

Stage 10 (Fig. 3c-d): 25 paired somites are present. All four pharyngeal slits are visible and the first, second, and third ones are open. The posterior intestinal portal has formed and the tail process is longer and curved. Limb buds have begun to grow along the anteroposterior axes.

Stage 11 (Figs. 3e-f, 4): 30 paired somites are present. All four pharyngeal slits are now open and the fourth pharyngeal arch is visible. Pigmentation of the retina has begun. The tail process

is narrower distally. The allantois has begun to form. The limb buds are wider (anteroposteriorly) than they are long (proximodistally). The AER appears on the periphery of the limb buds.

Stage 12 (Fig. 5a-c): Somites are difficult to count. The fourth pharyngeal slit has closed and the fifth has become visible. The nasolacrimal groove and cervical sinus have formed. The urogenital papilla appears. The limb buds are longer than wide.

Stage 13 (Figs. 5d-f, 6a-b): External outgrowth of the pharyngeal arches is highly reduced. The mandible is posterior to the eye. The retina is pigmented uniformly. The anterior intestinal portal has shifted to a central position on the embryonic trunk. The limb buds assume a ventroposterior orientation and are “paddle” like in form.

Stage 14 (Fig. 5g-i): The pharyngeal arches and nasolacrimal groove have disappeared. The iris features light pigmentation. Early outgrowth of the CR is visible along the posterior flank of the embryo. The intestines have herniated into an external loop. The limbs feature a vaguely defined digital plate. The forelimb is slightly longer than the hindlimb.

Stage 15 (Figs. 6c-d, 7a-b): The cervical sinus has closed. The maxilla has fused with the frontonasal process and the external nares have formed. The iris is entirely pigmented and the optic fissure is no longer visible. The CR has extended along the entire flank of the embryo and carapacial ectoderm has begun to differentiate. The forelimb features an elbow.

Stage 16 (Figs. 6e-f, 7c-d): The caruncle has begun to form on the frontonasal process. The mandible is positioned at the axial level of the posterior margin of the eye. Scleral papillae have begun to form on the lower half of the retina. Concentric growth of the CR is complete and the primordial carapace now extends beyond the anterior and posterior limb buds. The plastron has begun to form peripherally and anterior to the pericardial protuberance. The digital rays are visible.

Stage 17 (Fig. 7e-f): The mandible is positioned at the axial level of the lens. The ectodermal plates (scutes) of the carapace are delineated and the plastron extends over and across a much reduced pericardial protuberance. The AER is reduced and serration of the digital plate has begun. Sparse pigmentation is visible at the base of the limbs.

Stage 18 (Fig. 8a-b): The mandible is positioned beyond the axial level of the anterior margin of the lens. Scleral papillae cover the entire retina and the lower eyelid is present. The plastron has completed its growth along the entire anteroposterior axis of the embryo. The carapacial scutes are clearly differentiated. Interdigital webs have begun to differentiate on the digital plate.

Stage 19 (Fig. 8c-d): The anterior margin of the mandible is positioned between the eye and frontonasal process. The lower eyelid has begun to grow over the sclera and the upper eyelid is present. Pigmentation of the carapacial scutes has begun. The digits extend beyond the intervening webbing. Pigmentation of the limbs has increased.

Stage 20 (Fig. 8e-f): The rhampothecae are differentiated. Scleral papillae are no longer visible. Cutaneous papillae have begun to grow on the neck. Plastral scutes are delineated and marginal carapacial scutes feature a pigmented line. The limbs feature ectodermal scales and presumptive claws are lightly pigmented. The cloaca is fully differentiated.

Stage 21 (Fig. 9a-b): The mandible is at its occlusion point with the maxilla. The upper eyelid has begun to grow over the sclera and the nictitating membrane has formed. Cervical constriction is reduced and a skin fold has formed on the dorsal neck. A lightly pigmented line demarcates the dorsal midline of the carapace. Both limbs feature lightly pigmented stripes. Claws feature skin folds proximally.

Stage 22 (Fig. 9c-d): Both eyelids extend their growth over the iris. The head features pigmented stripes. The carapacial keel has formed. The plastron is pigmented, particularly

around the umbilicus. The intestinal loop is withdrawn. The limbs and tail are thoroughly pigmented and uniformly covered by scales interspersed by skin folds.

Stage 23 (Fig. 9e-f): The pupil has darkened. Pigmentation (yellow and dark olive) of the carapace, plastron, head, tail, and limbs has increased. The number of scales on the head, limbs, and tail has increased. The turtle has hatched from its egg.

Embryo size, Growth Rate, and Incubation Period

Variation in embryo size was recorded beginning at stage 11 (CRL; Table 1). Beginning at stage 15, embryo size was estimated using CL (Fig. 10; Table 1). Variation in CL was strongly predicted by incubation period (Fig. 11; polynomial fit, $R^2 = 0.97$, $N = 131$, $F_{2, 128} = 2490$, $P < 0.0001$). Means for embryo size at 27 °C (this study; $R^2 = 0.99$, $N = 8$, $F_{2, 5} = 446$, $P < 0.0001$) and 21-23 °C (Mahmoud et al. 1973; $R^2 = 0.95$, $N = 8$, $F_{2, 5} = 51.3$, $P < 0.001$) were strongly predicted by incubation period in regression models fitted with polynomial curves (Fig. 12). Length of incubation period was 53.5 d at 27 °C.

Discussion

Enhancement and Validation of the MHK Table

The descriptive criteria of the MHK table, which were roughly modeled after Yntema's (1968) staging table for the Snapping Turtle, met the basic premise for the designation of developmental stages in vertebrate embryos (Hamburger, 1992): stages were clustered as closely possible and could be unequivocally identified by at least one external character. In addition, our enhanced criteria satisfy guidelines for the establishment of developmental stages in reptiles (Miller, 1985) because we carefully controlled for environmental variables (gas, moisture, substrate, and

temperature) during incubation. Although our observations were generally congruent with the MHK table, we did note some important differences. We observed the early formation of a tail process in stage 8 embryos rather than at stage 7. Also, the AER, which is first mentioned in stage 14 of the MHK table, became apparent on both limb buds at stage 11. Moreover, we observed earlier onset of digital plate serration (stage 17) than described in the MHK table (stage 18). The MHK table does not indicate the onset of claw differentiation, though completion of claw development is described in stage 22. We described the onset of claw differentiation and its increased pigmentation at stages 20 and 21, respectively. Because completion of mandibular development was not indicated in the MHK table (discussed in Greenbaum and Carr, 2002), we described the terminal differentiation of that structure at stages 20-21.

By carefully tracing the development of other structures, such as the eye, we noted stage-specific diagnostic features that were not reported in the original MHK table. For example, we described the early onset of scleral papillae and their growth around the retina (stages 16–20), whereas this character is first mentioned at stage 19 of the MHK table. We also made new observations on the appearance of a nictitating eye membrane during stage 21 and change in color of the pupil at stage 23. Overall, the original work of Mahmoud et al. (1973) and our enhanced description of their staging table should provide solid guidance for the study of embryogenesis in the Painted Turtle.

Accounting for Incubation Environment

Growth rates of vertebrate embryos can be highly variable within and among species and depend greatly on incubation temperature (Reiss, 1989). Investigators have thus relied on staging tables to reduce experimental error due to this variation (Hall and Miyake, 1995). We incubated

embryos of the Painted Turtle under controlled laboratory conditions, which included a constant temperature (27 °C) within the range of those used by most investigators (Fig. 13) and typical of nest temperatures in our Illinois population (Weisrock and Janzen, 1999). Temperature strongly influences embryonic growth rate and duration of incubation period in this ectothermic reptile (Fig. 14; Janzen and Morjan, 2002). Substrate moisture and oxygen availability can also affect growth rate and incubation period (reviewed in Packard and Packard, 1988). The methodology used to establish stages of the MHK table did not carefully control for these variables, as in many classical works on reptile embryology (Miller, 1985). Such neglect of the incubation environment has likely confounded attempts to compare developmental chronology in turtles (e.g. Renous et al., 1989; Tokita and Kuratani, 2001).

We found that incubation period, and thus duration and timing to developmental stages, might be replicable when controlling for hydric and thermal environment. Our estimate of incubation period was the same (53.5 d) as that of a study that sampled eggs from a similar latitude (42° 52' N) under similar laboratory conditions (53.5 d at constant 27 °C and -150 kPa; Gutzke et al., 1987). Developmental variation might not be explained solely by environment, as we observed embryo size variation within stages. Even so, we more effectively characterized embryonic growth rate and with larger sample sizes than Mahmoud et al. (1973). Future studies should carefully address incubation environment or adjust experimental designs accordingly. Also, we recommend increased awareness of embryogenesis in the Painted Turtle and other reptiles, as it could inform current knowledge on phenotypes that are strongly affected by the incubation environment (e.g. temperature-dependent sex determination; discussed in Andrews, 2004).

Comparisons to Other Turtle Species

Our observations of embryogenesis in the Painted Turtle not only enhance its developmental staging table, but also disprove assumptions made in previous comparative analyses. For example, following descriptions from the MHK table, Tokita and Kuratani (2001) speculated that embryogenesis in the Painted Turtle was “unique”, presumably due to delayed development of the carapace, eye, limb, and plastron. Although those authors had only observed embryogenesis in the Chinese Softshell Turtle (Trionychidae: *Pelodiscus sinensis*), they claimed that development in the Painted Turtle differed in comparison to that species, the Snapping Turtle, and marine turtles. We clarified that the timing of differentiation for the carapace, eye, limb, and plastron is not delayed in the Painted Turtle.

We confirmed that formation of the AER precedes digital plate differentiation, as demonstrated in most turtle species. This structure was already apparent in embryos of the Painted Turtle at the same developmental stage (12) as in the Chinese Softshell Turtle (Tokita and Kuratani, 2001) and Snapping Turtle (Yntema, 1968). Similarly, the appearance and maturation of the CR did not differ in the Painted Turtle. Our direct examination of embryos of the Snapping Turtle and Spiny Softshell Turtle (*Apalone spinifera*), a close relative of the Chinese Softshell Turtle, supports that pattern (Cordero, 2012; unpublished data). We observed some aspects of eye, limb, and shell differentiation earlier than in the MHK table, which was consistent with staging tables for the closely-related emydid Red-Eared Slider (Greenbaum, 2002), as well as the Chinese Pond Turtle (*Chinemys reevesii*, Tan et al., 2001) and the Japanese Pond Turtle (*Mauremys japonica*, Okada et al., 2011) of the sister family Geoemydidae. We demonstrated that important developmental processes are not delayed in the Painted Turtle relative to other studied turtles.

Overall, timing of differentiation of the basic turtle body plan and externally positioned sensory organs appear to be highly conserved across deeply divergent turtle lineages. However, we are cautious about these generalizations, as development has only been studied in a handful of taxa. Developmental staging tables or systems exist for representatives of 10 of 14 recognized turtle families (Danni et al., 1990; Okada et al., 2011; reviewed in Tokita and Kuratani, 2001; Greenbaum and Carr, 2002; Werneburg et al., 2009). Based on this work, we deduced that clade-specific phenotypic differences are generally established during the middle one-third of embryogenesis. We observed that embryos of the Painted Turtle could be distinguished, based on relative body size, craniofacial morphology, pigmentation, and tail length, from those of Chelydridae and Kinosternidae as early as stage 17. In contrast, they were indistinguishable from those of Map Turtles (Emydidae: *Graptemys*) until stage 21 (Cordero, 2011; unpublished data). Because differences in phylogenetically disparate species become apparent earlier in embryogenesis (e.g. Snapping Turtle vs. Painted Turtle), we recommend the use of staging tables designed specifically for the species of interest or a close relative.

Embryogenesis in the closely related Painted Turtle and Red-eared Slider is highly similar, except for differences in the latest stages (see Greenbaum, 2002). The Red-eared Slider has a longer incubation period and reaches larger hatchling and adult body sizes. Theory predicts that species with larger adult body size should have longer developmental incubation periods (Reiss, 1989), which could explain additional stages of development (24-26) described in the Red-eared Slider. These stages are characterized by minor species-specific differences in pigmentation patterns and ectodermal morphology. For example, a translucent sheath on the ventral claw of the Red-eared Slider was described, though we did not observe it in embryos of the Painted Turtle. This character was also observed in the late developmental stages (24-26) of the Japanese

Pond Turtle (Okada et al., 2011) and Snapping Turtle (Yntema, 1968), both of which are larger and have longer incubation periods than the Painted Turtle.

The staging table for the Red-eared Slider was designed to mirror the descriptive criteria of Yntema (1968). For example, both tables describe the appearance of scattered pigment cells on stage 15 limb buds. Yet this early onset of limb pigmentation in the Red-eared Slider would seem to be unusual, as it is not apparent until stage 17 in the Painted Turtle, though we have observed pigmentation as early as stage 16 in the Snapping Turtle. Efforts to adhere to the criteria of Yntema (1968) may have biased descriptions in the staging table of the Red-eared Slider. Our staging table is better suited to describe embryogenesis in emydid turtles before species-specific differences are established late in development. We acknowledge that some aspects of developmental staging tables are inherently subjective and encourage the combined use of alternative approaches, such as the Standard Event System for vertebrate embryology (Werneburg, 2009). In either case, diligent examination of embryonic anatomy is necessary to reduce bias in comparative studies. Furthermore, staging tables are based primarily on external characters; less is known about developmental differences that define diversity in the internal anatomy of turtles.

In summary, we recommend that investigators of the Painted Turtle preferentially use our enhanced developmental staging table over those designed for the Snapping Turtle (Yntema, 1968) and Red-eared Slider (Greenbaum, 2002) for the following reasons: 1) Estimates on the chronology of developmental stages could be affected by longer incubation periods in these species; 2) Species-specific developmental differences, both early (stage 17, Snapping Turtle) and late (stages 24-26, Red-eared Slider) could bias descriptions and, thus, staging of embryos.

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Table 1. Means for embryo size in the Painted Turtle (*Chrysemys picta*), timing, and defining characters of developmental stages.

Stage	CRL ^a (mm ± SE ^b)	CL ^c (mm ± SE ^b)	N	Day(s)	Week(s)	Defining character ^e
3			4	3	1	Notochord
4			3	4	1	Somites
5			6	5	1	Anterior intestinal portal
6			7	6	1	Optic vesicles
7			9	7	1	Mandibular arch
8			8	7-8	1-2	Stomodeum
9			6	7-9	2	First pharyngeal slit is open
10			5	10	2	Posterior intestinal portal
11	5.57 (0.30)		5	10-12	2	Limb buds with apical ectodermal ridge
12	6.49 (0.23)		7	10-15	2-3	Urogenital papilla
13	7.01 (0.14)		6	11-16	2-3	Retina is uniformly pigmented
14	7.30 (0.30)		6	14-17	3	Carapacial ridge
15		6.06 (0.42)	12	16-18	3	External nares
16		6.53 (0.13)	11	17-21	3-4	Caruncle
17		7.30 (0.12)	14	18-23	3-4	Serration of digital plate
18		8.67 (0.20)	18	22-27	4	Interdigital webs
19		10.2 (0.37)	17	24-31	4-5	Carapace pigmentation
20		12.2 (0.32)	14	26-33	4-5	Claws
21		15.6 (0.26)	20	27-36	4-6	Pigmented stripes on limbs
22 ^d		18.5 (0.18)	27	37-53	6-8	Plastron pigmentation

^a CRL = Crown-rump length (mm) measurements were used to estimate embryo size during stages 11-14.

^b ± SE = Standard error.

^c CL = Carapace length (mm) was used as an index of embryo size during stages 15-22.

^d = Size measurements were taken when embryos first reached stage 22 (~37-38 d). Embryos (*N* = 5) in pipped eggs (i.e. immediately before hatching; ~53.5 d) were > 22 mm in carapace length.

^e = A character that first appears or undergoes change during the listed stage of development.

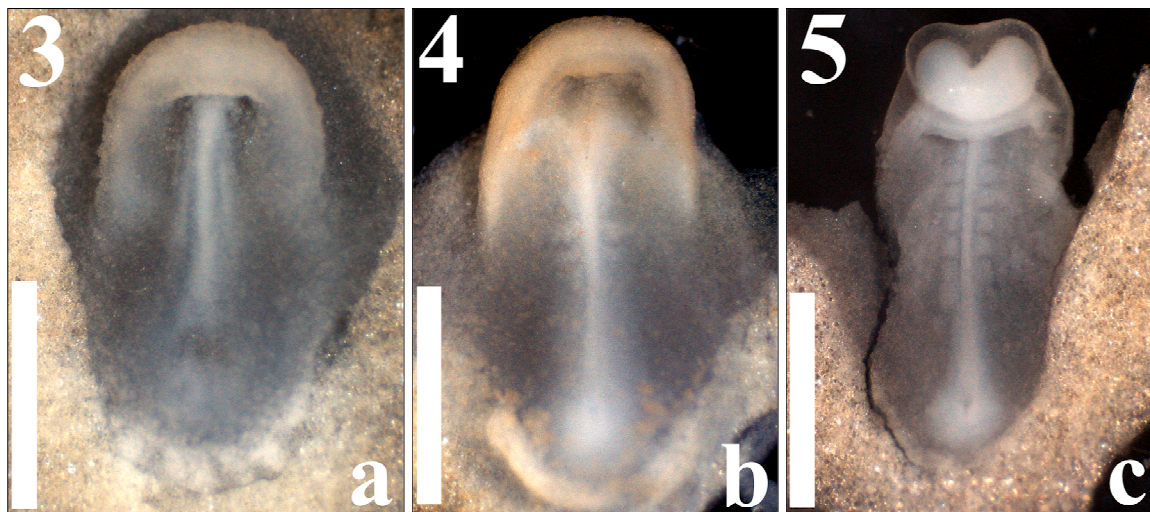


Fig. 1. Stages 3-5: Embryos in ventral view (a-c; scale bars = 1 mm).

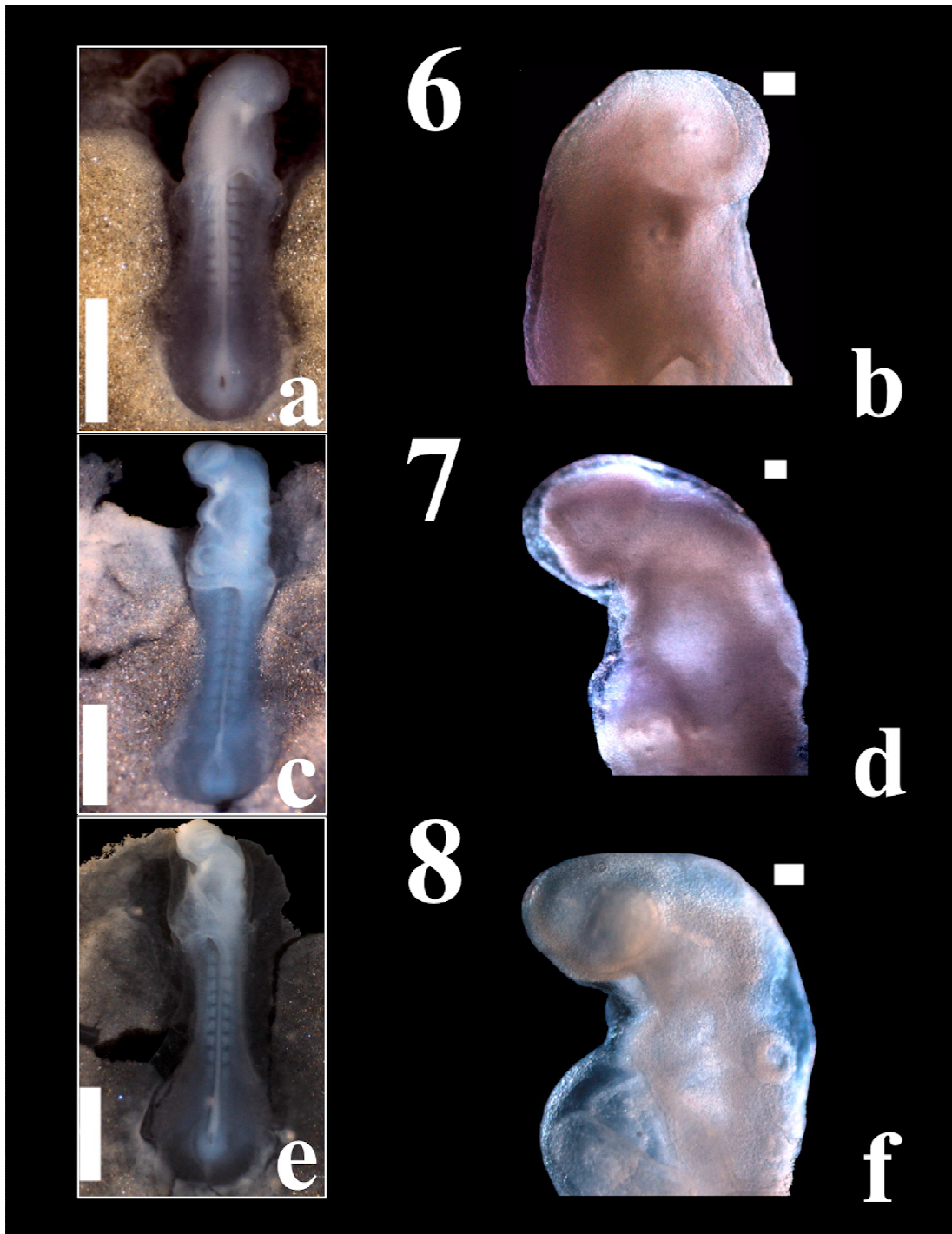


Fig. 2. Stages 6-8: Left column, embryos in ventral view (a, c, and e; scale bars = 1 mm). Right column, detail view of head and pharyngeal regions (b, d, and f; scale bars = 100 μm).

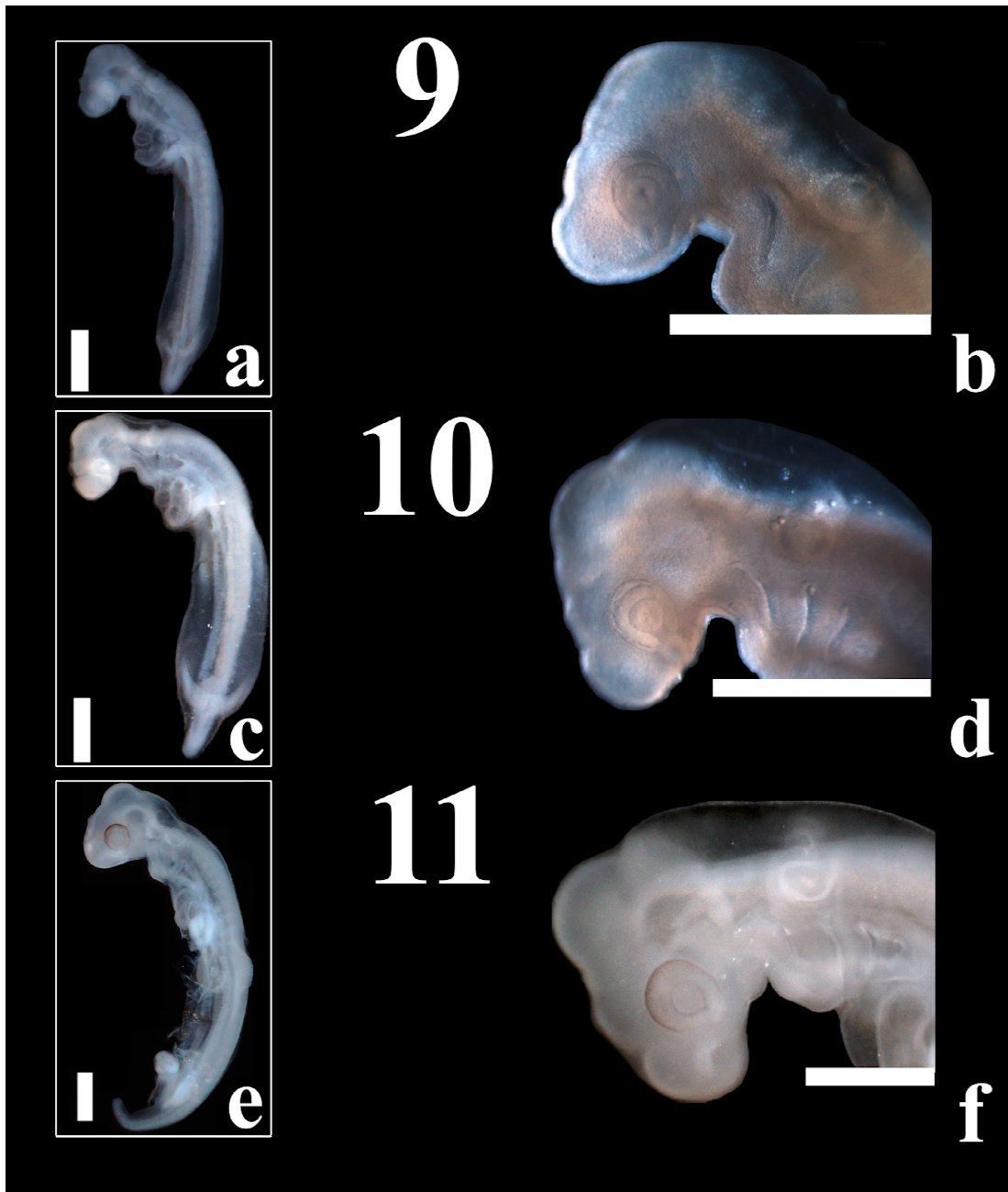


Fig. 3. Stages 9-11: Left column, embryos in lateral view (a, c, and e; scale bars = 1 mm). Right column, detail view of head and pharyngeal regions (b, d, and f; scale bars = 1 mm).

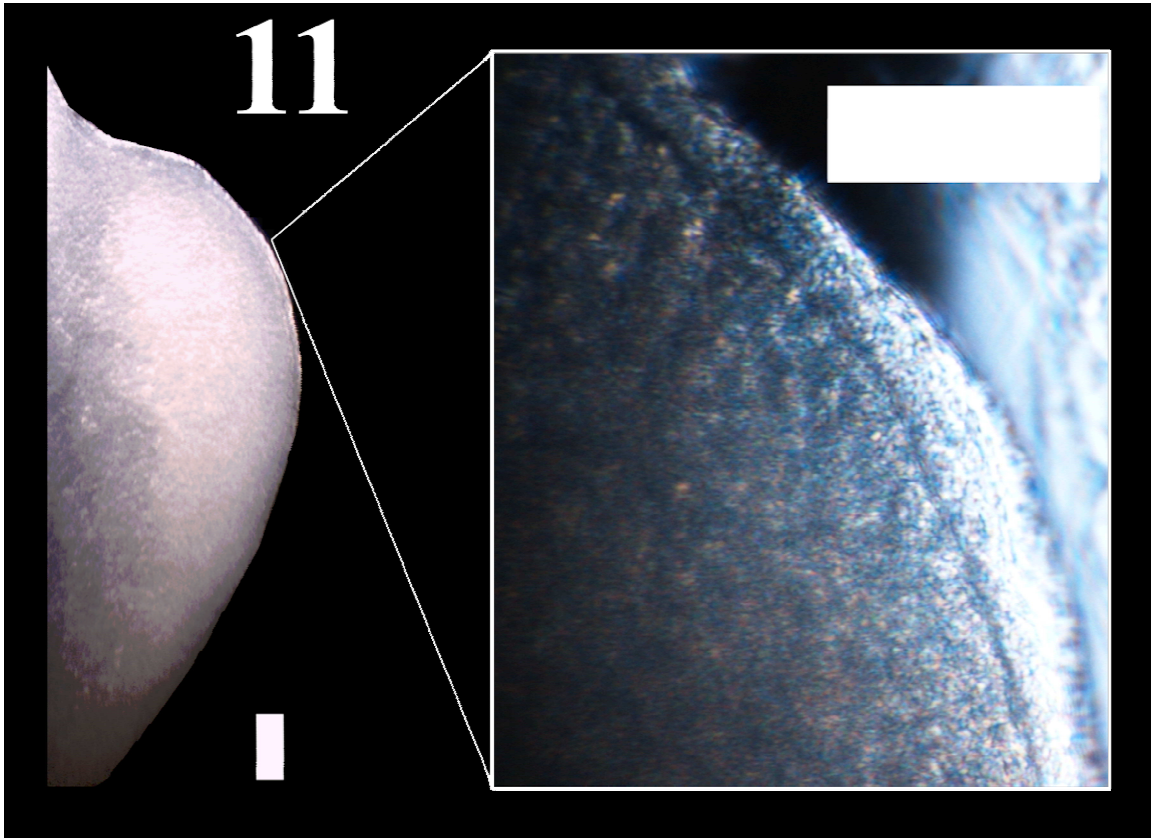


Fig. 4. Stage 11: Dorsal view of right forelimb bud (scale bar = 1 mm) and detail view of the apical ectodermal ridge (scale bar = 100 μ m).

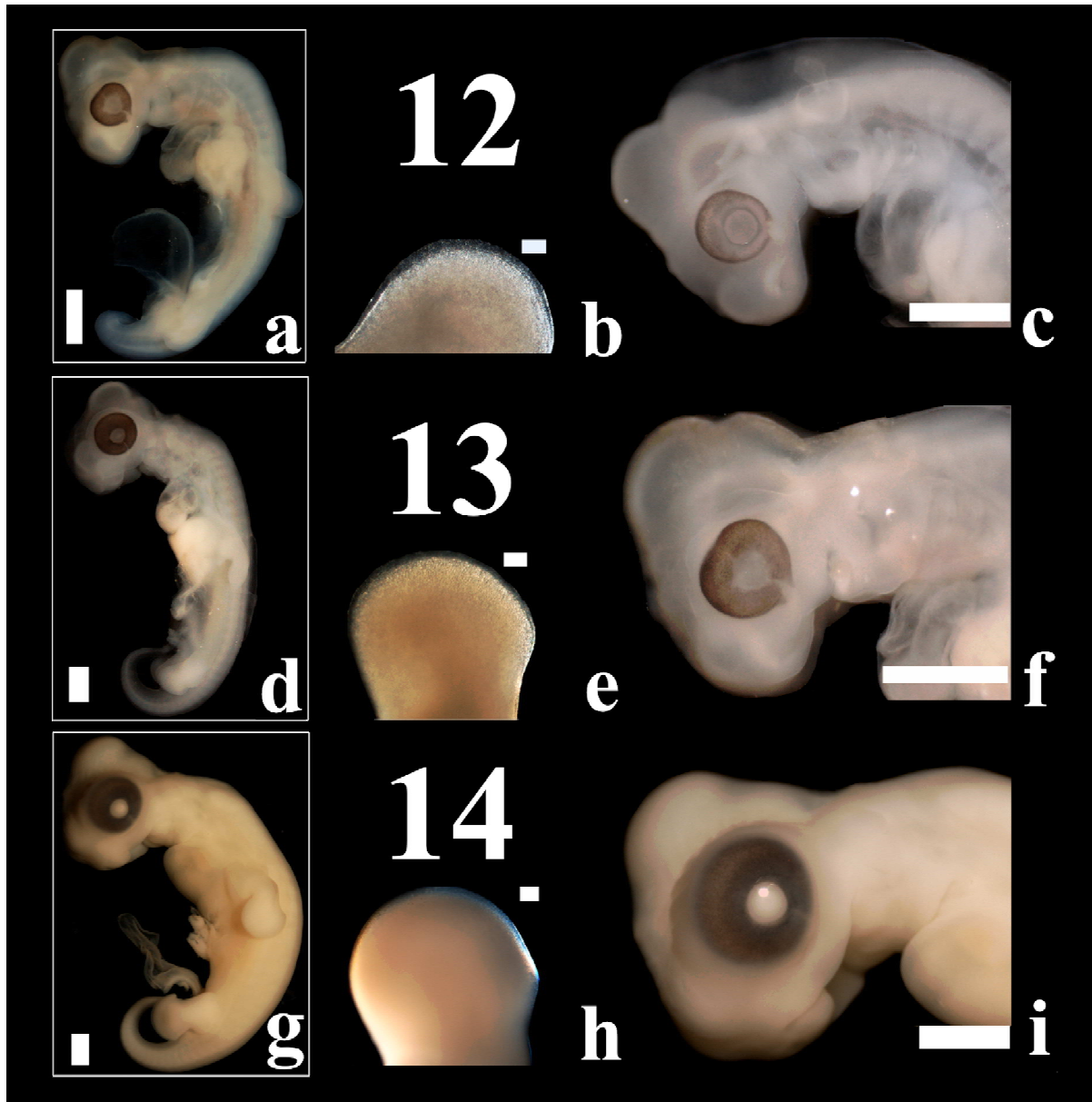


Fig. 5. Stages 12-14: Left column, embryos in lateral view (a, d, and g; scale bars = 1 mm).

Middle column, detail (dorsal) view of right forelimb buds (b, e, and h; scale bars = 1 mm).

Right column, detail view of head and pharyngeal region (c, f, and i; scale bars = 1 mm).

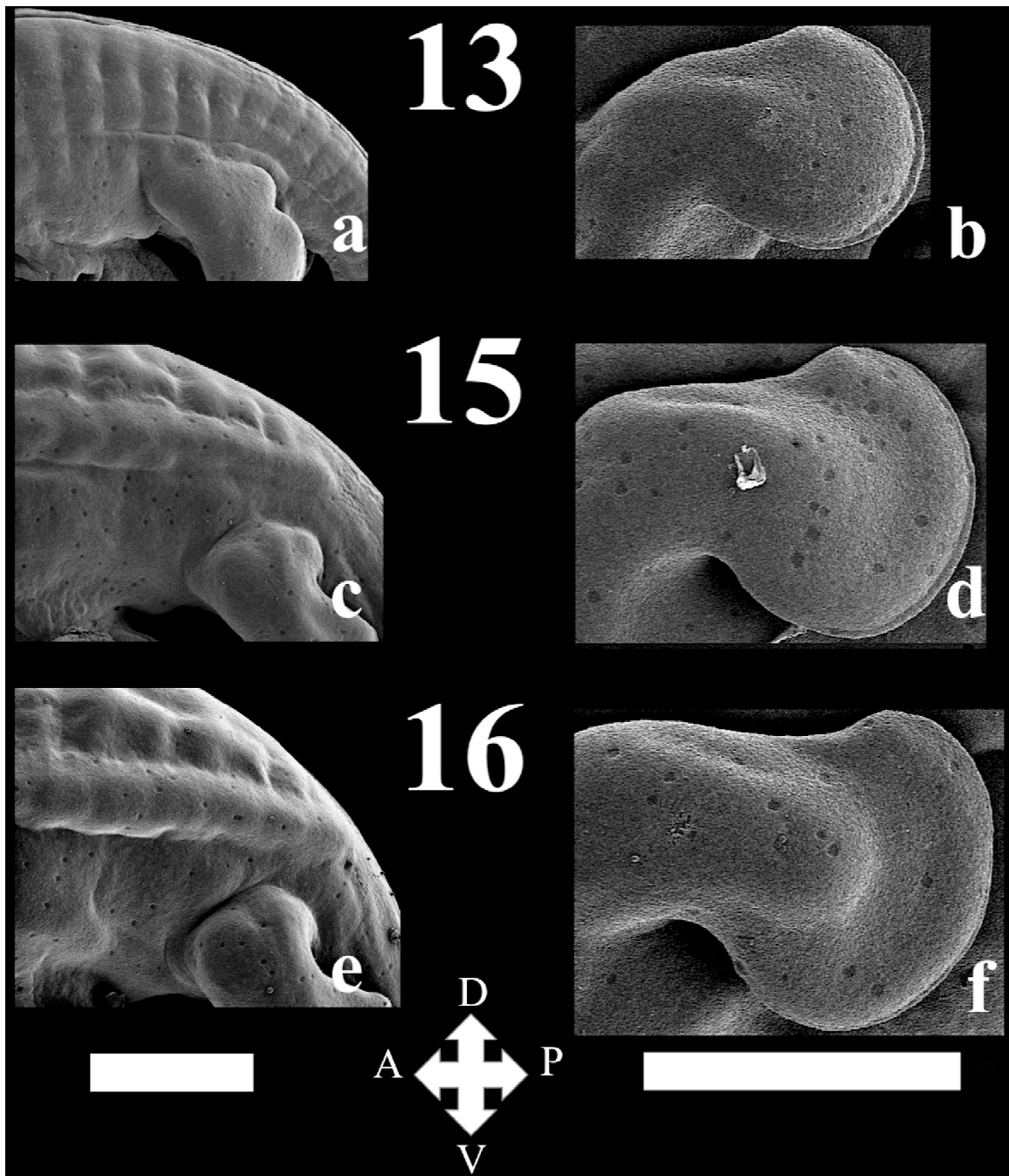


Fig. 6. Stages 13, 15-16: Left column, detail views of the posterolateral (left side) region of embryos where the carapacial ridge forms (a, c, and e; scale bar = 1 mm). Right column, dorsal view of right forelimb buds featuring the apical ectodermal ridge (b, d, and f; scale bar = 1 mm). Arrows (bottom, middle column) indicate planes of orientation (A = anterior, P = posterior, D = dorsal, V = ventral).

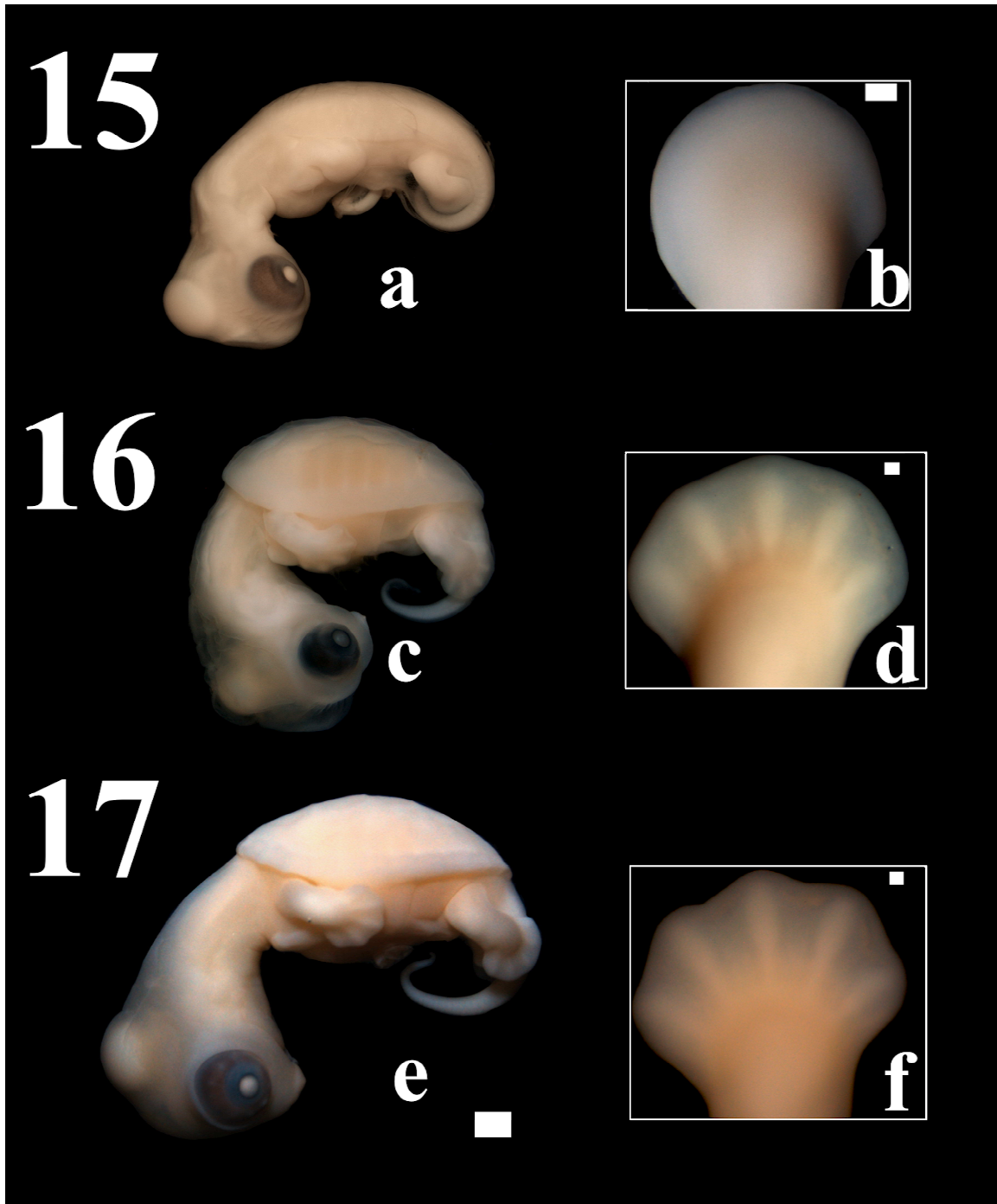


Fig. 7. Stages 15-17: Left column, embryos in lateral view (a, c, and e; scale bars = 1 mm). Right column, detail view (dorsal) of right forelimbs (b, d, and f; scale bars = 100 μ m).

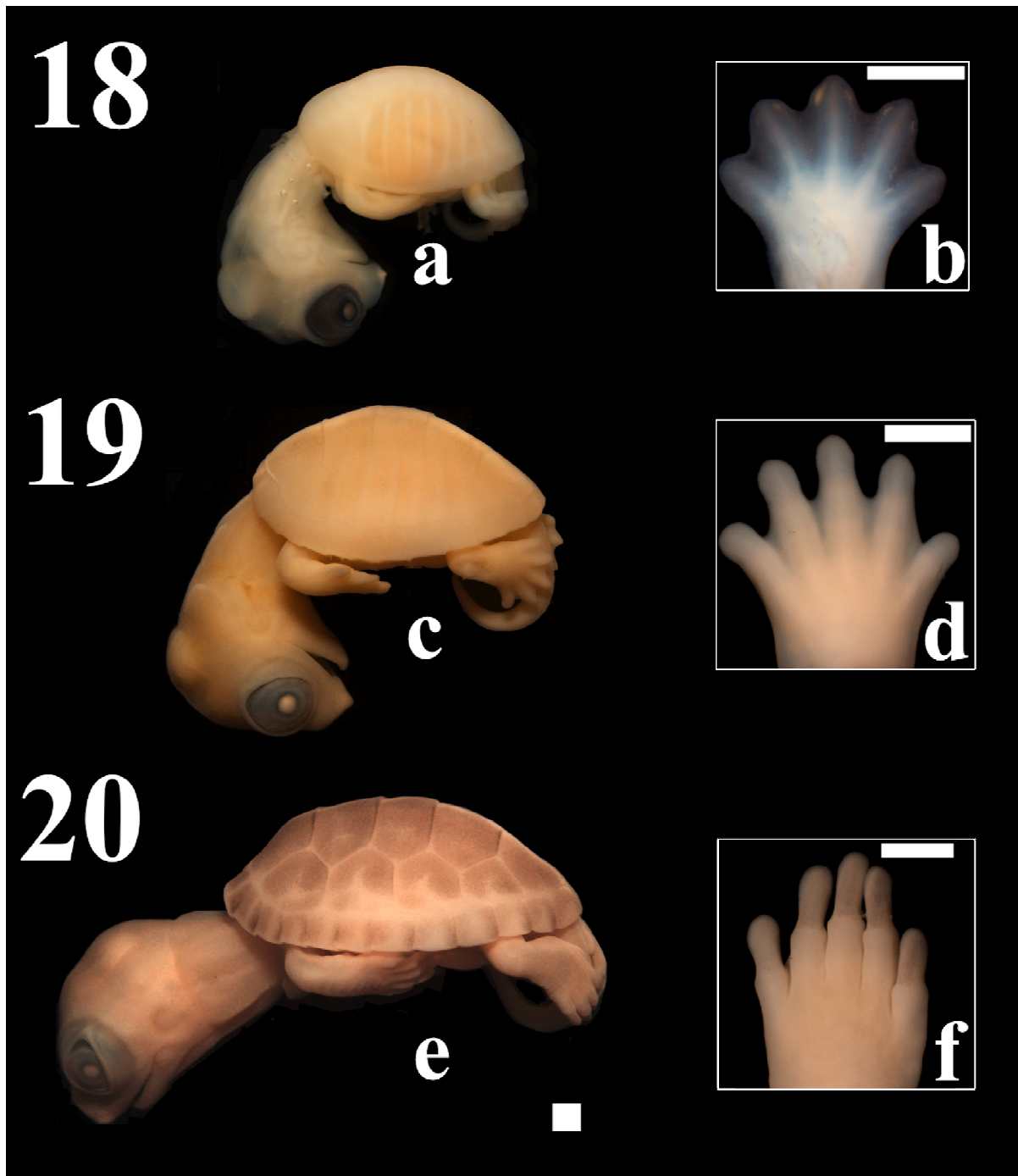


Fig. 8. Stages 18-20: Left column, embryos in lateral view (a, c, and e; scale bar = 1 mm). Right column, detail view (dorsal) of right forelimbs (b, d, and f; scale bars = 1 mm).

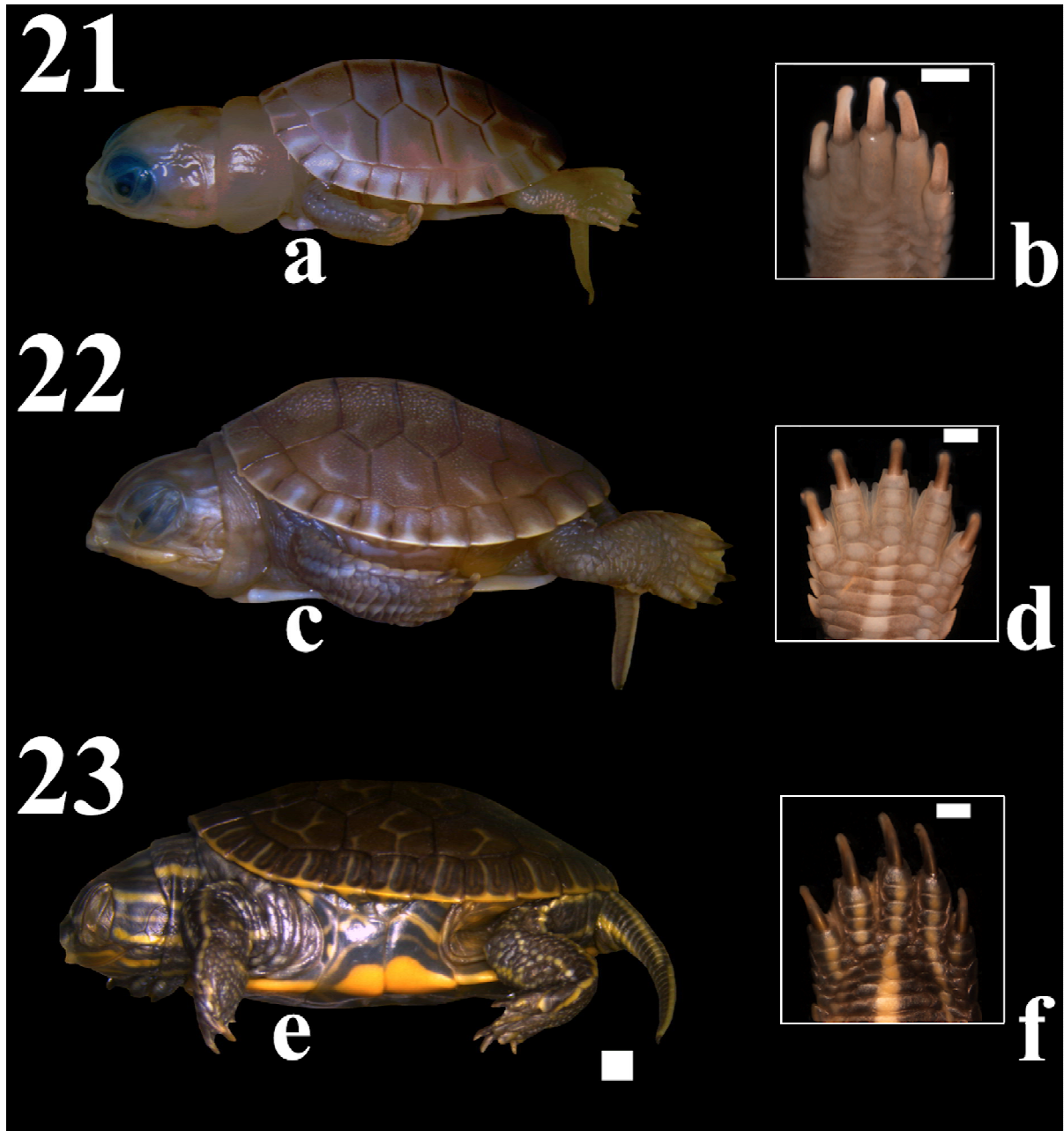


Fig. 9. Stages 21-23: Left column, embryos in lateral view (a, c, and e; scale bar = 2 mm). Right column, detail view (dorsal) of right forelimbs (b, d, and f; scale bars = 1 mm).

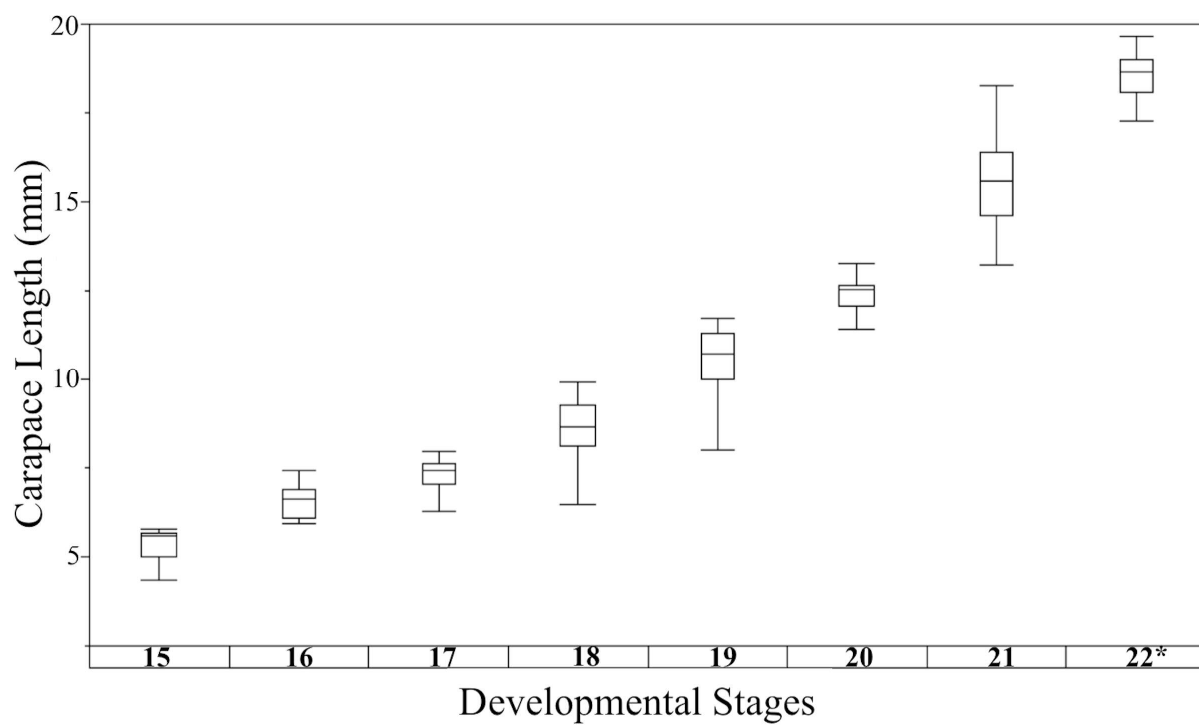


Fig. 10. Variation in size (estimated by carapace length) of embryos at stages 15-22. * = Measurements were taken when embryos first reached that stage (~37-38 d).

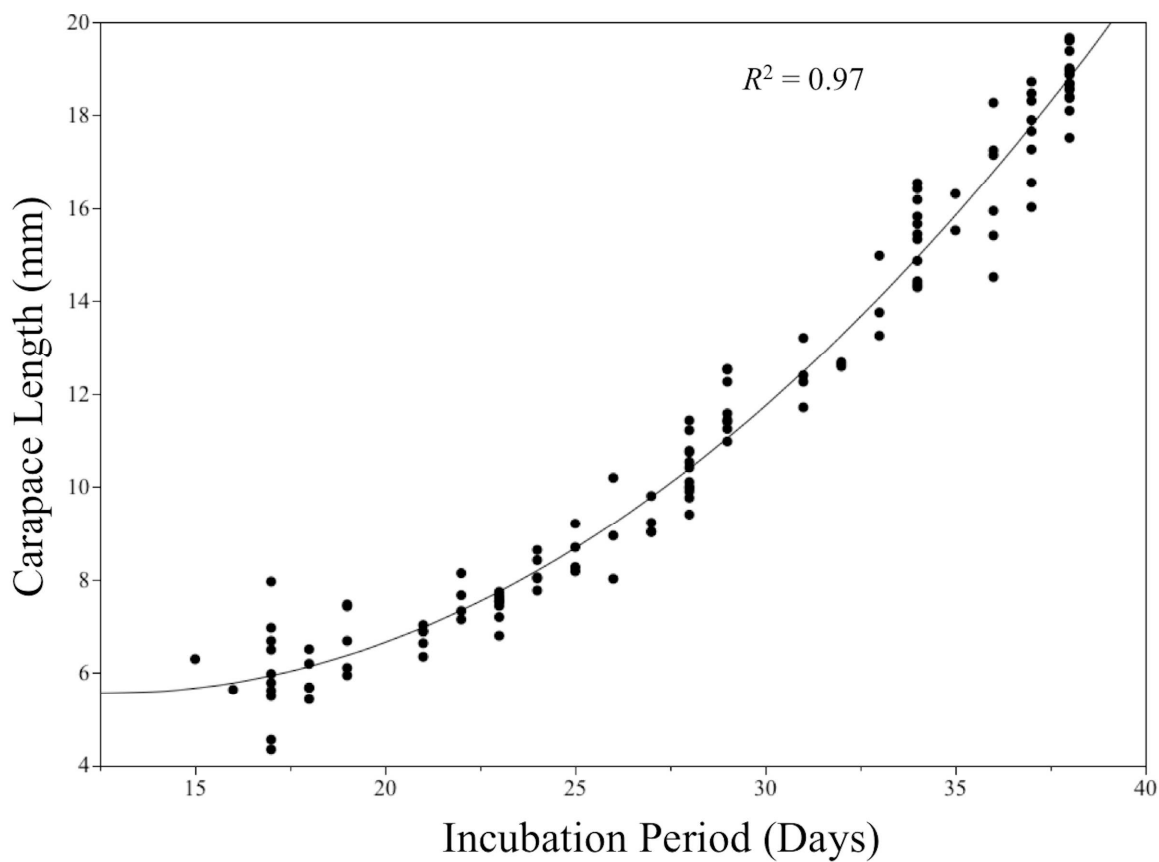


Fig. 11. Carapace length as a function of incubation period (at 27 °C).

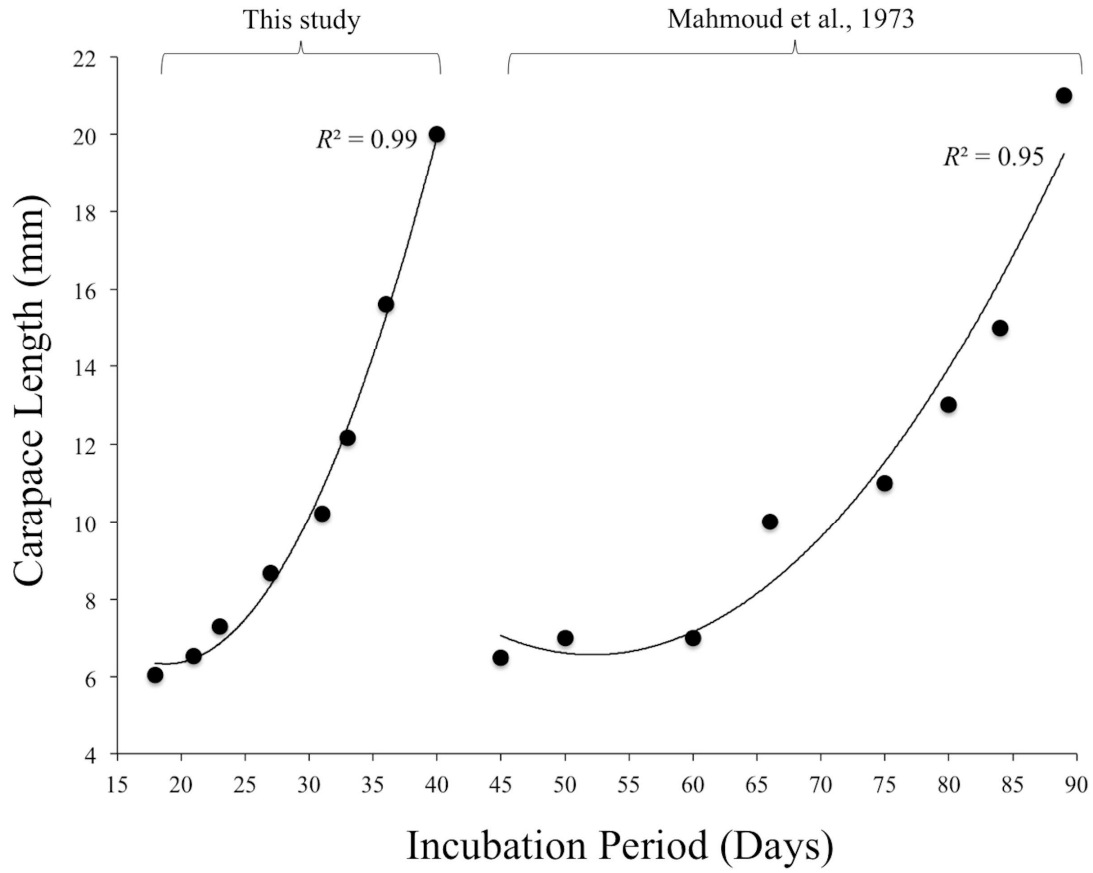


Fig. 12. Means (black circles) of carapace length as a function of incubation period at 27 °C (this study) versus at 21-23 °C (Mahmoud et al. 1973).

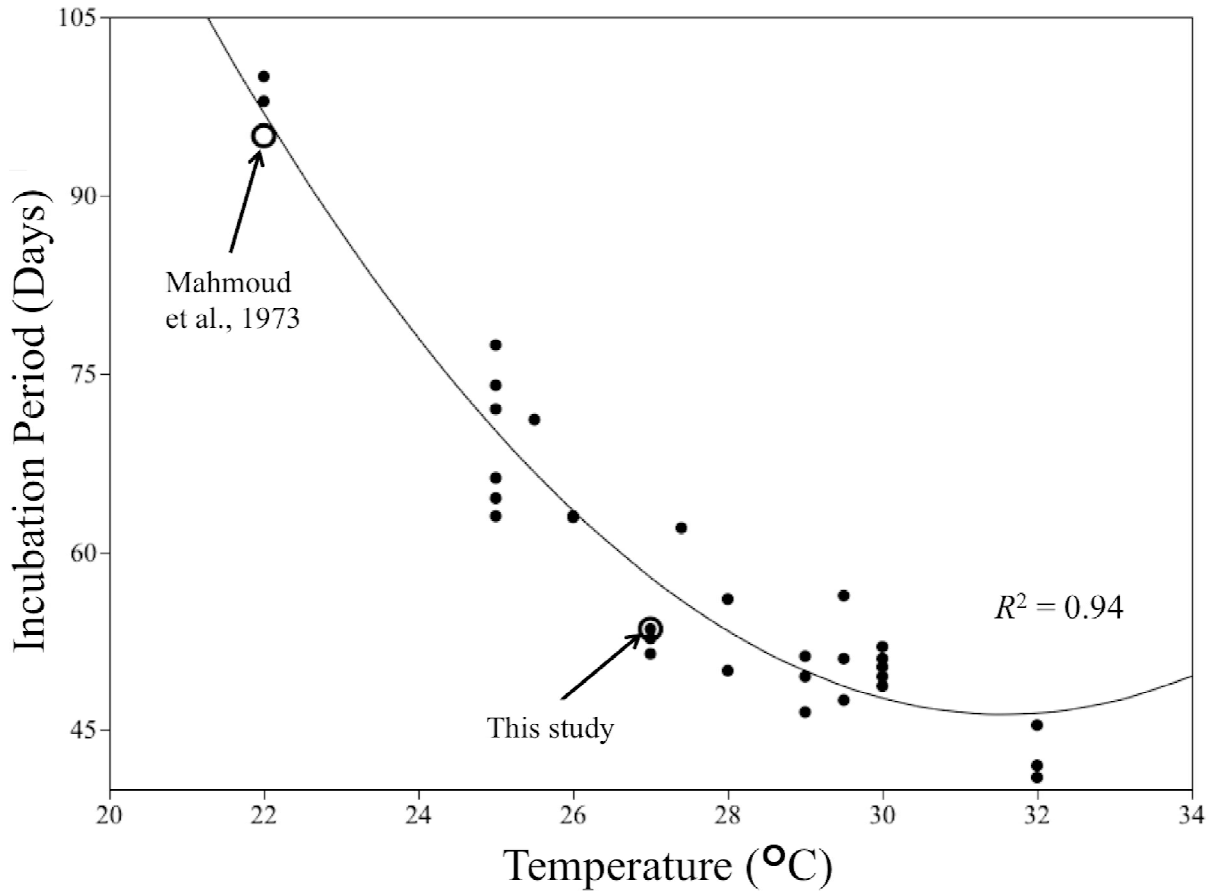


Fig. 13. Incubation period as a function of temperature. Black circles represent mean estimates of incubation period. Hollow circles represent means estimated by Mahmoud et al. (1973) and this study. Other data were referenced mostly from egg incubation studies of northern populations (Lynn and Von Brand, 1945; Ream, 1967; Ewert, 1979, 1985; Packard et al., 1981, 1983; Gutzke et al., 1987; Tucker, 2000; Peterson and Kruegl, 2005; Kitana and Callard, 2008; Warner and Janzen, unpublished data).

CHAPTER 3. SKELETAL REMODELING SUGGESTS THE TURTLE'S SHELL IS NOT AN EVOLUTIONARY STRAITJACKET

A paper published in Biology Letters

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Summary

Recent efforts to decipher the enigma of the turtle's shell revealed that distantly related turtle species deploy diverse processes during shell development. Even so, extant species share in common a shoulder blade (scapula) that is encapsulated within the shell. Thus, evolutionary change in the correlated development of the shell and scapula likely underpins the evolution of highly derived shell morphologies. To address this expectation, we conducted one of the most phylogenetically comprehensive surveys of turtle development, focusing on scapula growth and differentiation in embryos, hatchlings, and adults of 13 species. We report the first description of secondary differentiation due to skeletal remodeling of the tetrapod scapula in turtles with the most structurally derived shell phenotypes. Remodeling and secondary differentiation late in embryogenesis of box turtles (*Emys* and *Terrapene*) yielded a novel skeletal segment (i.e. the suprascapula) of high functional value to their complex shell-closing system. Remarkably, our analyses suggest that, in soft-shelled turtles (Trionychidae) with extremely flattened shells, a similar transformation is linked to truncated scapula growth. Skeletal remodeling, as a form of developmental plasticity, might enable the seemingly constrained turtle body plan to diversify, suggesting the shell is not an evolutionary straitjacket.

Introduction

The turtle's shell is a classic textbook example of evolutionary innovation [1-2]. Comprised of highly evolutionarily derived bones, it provides benefits related to predator defense, shelter from the environment, thermoregulation, and storage of fat, minerals and water [3-5]. Even so, it has been referred to as an evolutionary straitjacket due to presumed limitations on adaptive radiation and diversification [3]. Although the turtle's shell is unusual in the context of other vertebrates, its adaptive potential is not entirely constrained [3-4,6]. Over the last 210 million years, the slow pace of turtle morphological evolution has enabled successful colonization of freshwater, marine, and terrestrial ecosystems worldwide [6-7]. Exploring this ecological diversity may demonstrate how developmental change has contributed to subsequent evolution of the basic turtle body plan [4,8-9]. Recent studies focusing on two distantly related species revealed surprising variation in how the shell forms [8,10-12]. Still, as an outcome of shell development, all extant species share a similar anatomical configuration: the shoulder girdle is situated anteriorly to the rib cage and is encapsulated within the shell [13].

Key anatomical transformations associated with the evolution of modern turtles involved correlated changes of the shell and shoulder girdle [13-17]. In addition to deciphering the puzzle of shell morphogenesis, examination of shoulder girdle development is crucial to determining whether phenotypic evolution is constrained by the shell. Adaptive shell variation is closely linked to variation in shoulder blade (scapula) morphology and ecological niche of turtles [18]. In fact, the most ecologically diverse and anatomically derived species may deploy atypical modes of scapula development [19]. In terrestrial box turtles (*Terrapene*) with complex shell-closing systems, a moveable skeletal segment was hypothesized to develop via secondary subdivision of the scapula late in embryogenesis [19]. As a result, adult box turtles feature the

remarkable capacity to temporarily displace the shoulder girdle so that it may truly lie within the rib cage, unlike most turtles [13,19]. Embryonic growth of the turtle scapula is projected vertically until it eventually articulates with the dorsal shell (carapace) [13,15]. Thus, scapula development might also deviate from the norm in aquatic soft-shelled turtles (Trionychidae) because endoskeletal growth might be physically constrained by their extremely flattened shells [20].

Herein, we address the expectation that correlated change in shell and scapula development is associated with phenotypic diversification of turtles. We sampled diverse turtle lineages to determine whether variation in scapula development has arisen to accommodate adaptive modifications of the shell. We focused on the dorsal terminus of the scapula, the site of rapid tissue growth and articulation with the carapace.

Material and Methods

During 2010-2014, eggs of cryptodiran turtles were field-collected and incubated under controlled laboratory conditions to reduce environmental effects on growth (S-Table 1). Early shell morphogenesis and subsequent variation was examined to establish the temporal window for turtle developmental divergence. During this time frame, the shoulder girdle was excised and gross anatomical details of the developing scapula were examined across equivalent developmental stages of representative lineages; staging followed established criteria [21-23].

Dissected scapulae were preserved in 10 % buffered formalin and dehydrated in ethanol before staining with alcian blue and alizarin red to visualize cartilage and bone, respectively. This was followed by trypsin digestion and clearing with glycerol. Hematoxylin and eosin (H&E) tissue preparations and adult specimens were studied to further observe details of the scapula in some

species. Scapulae were also examined in hatchlings of representative lineages, including some museum specimens. Archosaurs, represented by *Gallus gallus* and *Alligator mississippiensis*, were an outgroup for comparisons because they exhibit the ancestral anatomy of the tetrapod dorsal scapula. Lepidosaurs were also included because some members (i.e. *Elgaria multicarinata*) resemble the condition of the dorsal scapula in box turtles.

To test the hypothesis that scapula growth is constrained by shell dimensions, we measured oven-dried mass of the forelimb system and carapace length and shell height in hatchling turtles. Dry scapula mass was normalized by dividing it by total forelimb mass. A Pearson's correlation test was used to assess the relationship of normalized scapula mass and shell height-to-length ratio, followed by correlation of phylogenetic independent contrasts (PICs) to correct for phylogeny (electronic supplementary material). Animals in this study were humanely euthanized with injection of sodium pentobarbital.

Results

Comparison of hatchlings indicated that change in dorsal scapula morphology is specified during embryonic development and revealed that soft-shelled and emydine box turtles diverged from the evolutionarily conserved pattern; these lineages feature a suprascapula (Figure 1A-C). Primary scapula differentiation, growth, and remodeling occurred during wk 3-6 of embryonic development, as exemplified in Emydidae (Figure 2A-B; S-Figure 1). During wk 6, the suprascapula is secondarily differentiated in emydine box turtles. Alligator (*A. mississippiensis*) and chicken (*G. gallus*) embryos did not exhibit secondary scapula differentiation (S-Figure 2). Remodeling (wk 5-6) in soft-shelled turtles (Trionychidae) resulted in differentiation of a “transient” suprascapula, which was absent in adults (Figure 2D, S-Figure 3). Compared to

lizards (*E. multicarinata*), suprascapula differentiation differs in turtles due to the presence of a synovial joint or dense fibrocartilage matrix (S-Figure 4; Figure 2D). Normalized scapula mass was positively correlated with shell height-to-length ratio in hatchlings (Figure 2E; $r = 0.95$, $P = 0.0003$; PICs: $r = 0.77$, $P = 0.0361$). These characters remained strongly correlated after exclusion of soft-shelled turtles from analyses ($r = 0.78$, $P = 0.066$; PICs: $r = 0.82$, $P = 0.091$).

Discussion

We identified variation in scapula development that has likely arisen to accommodate some of the most striking turtle shell phenotypes. Thus, our findings substantiate that change in the correlated development of the shell and scapula is related to major evolutionary transitions of the turtle body plan [13-17]. We present evidence suggesting that skeletal tissue remodeling underlies terminal additions to the developmental sequence of the shoulder girdle of box turtles. Furthermore, we discovered that soft-shelled turtles exhibit skeletal remodeling of the dorsal scapula, but possibly in response to constrained scapula growth. Notably, these transformations occurred remarkably late in embryogenesis. These results are illuminating because the search for evolutionary change in developmental mechanisms of adaptive traits has primarily focused on early stages of embryonic differentiation [25]. By examining skeletogenesis in late-term embryos, we were able to, for the first time, describe secondary differentiation of the scapula in a tetrapod animal. This process yielded an additional mesodermally derived skeletal element, the suprascapula, which eventually ossifies in some adult turtles and lizards [13,20].

The extraordinarily complex shell and shoulder girdle adaptations of emydine box turtles (*Emys* and *Terrapene*) enable muscle-induced movement of the ventral shell to fully conceal appendages during predator attacks [i.e. shell kinesis; 7,19]. The suprascapula is an innovation

that permits displacement of the shoulder girdle to the inside of the rib cage to create clearance for appendages [19]. Secondary scapula sub-division was hypothesized as the underlying process governing suprascapula differentiation late in embryogenesis of box turtles [19]. Here, we describe this turtle-specific process and propose that it is facilitated by changes in physical interactions of muscle and cartilage tissue of the dorsal scapula. In box turtles, scapula muscles are unusually large [19]. Future studies should aim to determine whether tensional forces exerted by muscles on developing cartilage underlies formation of novel skeletal segments or shell joints. Such a developmental process has been demonstrated late in embryogenesis of model laboratory vertebrates [2, 26].

Extreme shell height reduction in soft-shelled turtles (Trionychidae) has had profound developmental effects: decreased dorso-ventral growth of the skull and neck, reduction of the neck-shell joint, and relocation of the heart to accommodate neck retraction [19,27]. We demonstrated that, in late-term embryos, the growth zone of the dorsal scapula is disrupted by differentiation of a “transient” suprascapula. Our description of this unusual structure is congruent with previous work on hatchlings, e.g. *Pelodiscus* [28]. However, unlike in emydine box turtles, it does not persist in adults and has no clear adaptive value. Instead, its differentiation is likely a plastic side-effect of constrained scapula growth within an exceptionally flattened shell, as suggested by the positive correlation of scapula mass and shell height. These observations contribute to a growing consensus on fundamental developmental differences between hard- and soft-shelled turtles [3,8,10-12,20].

Skeletal morphology may vary due to epigenetic tissue remodeling induced by novel mechanical stimuli experienced in development [2,26]. Therefore, alteration to the mechanical environment of developing tissues could underlie the origins of skeletal innovation in animals

[2,26,29]. This form of developmental plasticity might have facilitated early steps in the mosaic evolution of the shell, congruent with gradual additive change early in turtle evolution [13-14,16]. Intriguingly, epigenetically induced plasticity could alter the developmental correlation of the shell and shoulder girdle, which may partially explain the notable diversity of extant turtle forms. Further research is needed to test such hypotheses and continue to refute the conjecture that the shell is an evolutionary straitjacket. Indeed, turtles have great potential to diversify, though by means that we have only begun to discover.

Acknowledgments. Sampling followed animal care protocol 2-11-7091-J. We thank Fredric Janzen for advice and John Iverson for help with egg collection. Funding by NSF (DEB-0640932, DDIG DEB-1310874).

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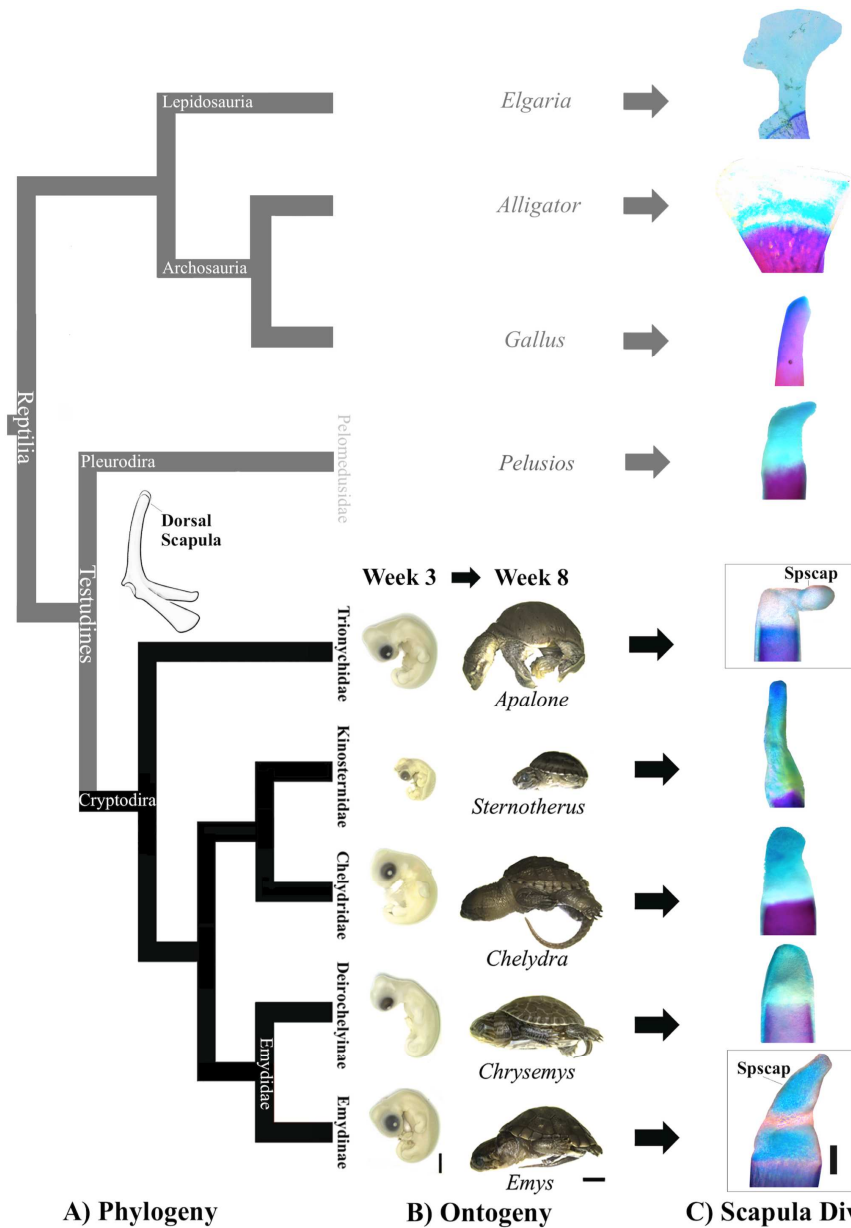


Figure 1. Phylogeny (**A**; after ref [24]) and ontogeny (**B**) of cryptodiran turtles suggests evolutionary change in dorsal scapula morphology (**C**) is specified during wk 3-8 of embryonic development, when lineages diverge phenotypically. Hatchlings of soft-shelled turtles (Trionychidae: *Apalone mutica*) and box turtles (Emydinae: *Emys blandingii*) diverged from the ancestral condition of the dorsal scapula (scale bars = 1 mm) by the presence of the suprascapula (SpScap) (**C**). Wk-3 scale bars = 2 mm; wk 8 = 5 mm. ISU collection specimens: *Apalone mutica* (FJ66831), *Elgaria multicarinata* (FJ63667), *Pelusios castaneus* (FJ66433).

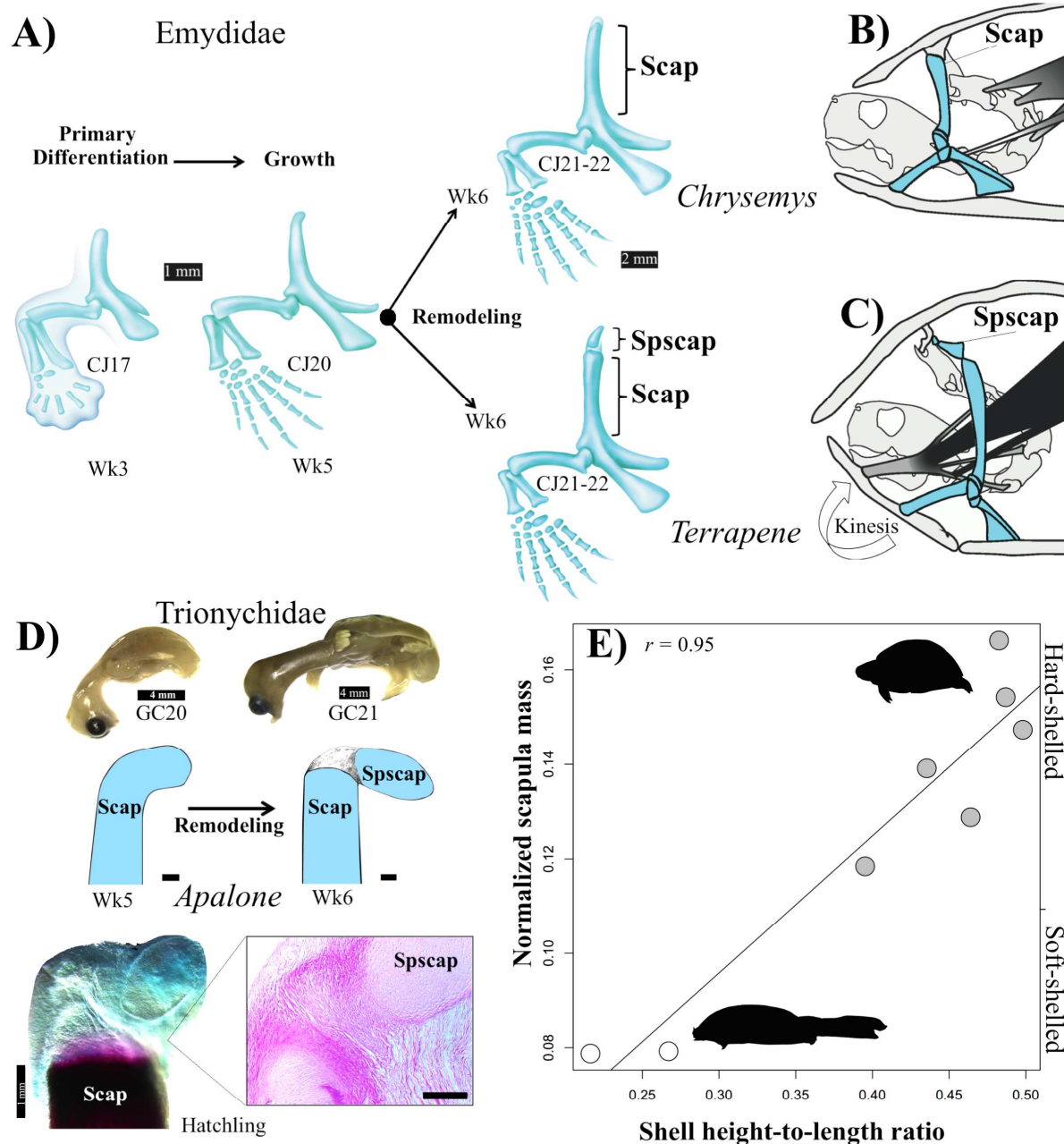


Figure 2. Primary differentiation (Wk3, Cordero & Janzen (CJ[23]) stage 17), growth (Wk 5, CJ20), and remodeling (Wk 6, CJ21-22) of the scapula (**Scap**; dorsolateral aspect) in Emydidae

(e.g. adult *Chrysemys*) (**B**). Remodeling follows secondary differentiation in box turtles (Emydinae), underlying formation of the suprascapula (**Spscap**; e.g. adult *Terrapene*) (**C**). In soft-shelled turtles (Trionychidae: *Apalone mutica*), differentiation of a “transient” suprascapula (scale bars = 100 μm) occurs via remodeling during Wk 5-6 at Greenbaum and Carr (GC stages 20-21 [20]) stages 20-21 (**D**); H&E sectioning (scale bar = 500 μm) confirmed separation of the suprascapula in hatchling *A. mutica* (FJ66436). Normalized scapula mass was positively correlated with shell height-to-length ratio in hard- versus soft-shelled turtles; each data point represents the mean for a species. Illustration (**A-C**) credit: Jessica Gassman.

Supplementary Material

Field sampling, Egg incubation, and Museum Specimen Data.

S-Table 1. Data on turtle egg collection and incubation; sampling was conducted with permits from: Arkansas Game & Fish Commission #020520132; Illinois Dept. of Natural Resources #NH13.0073; Iowa Dept. of Natural Resources #14; Nebraska Game & Parks Commission #310. The Iowa State University Institutional Animal Care and Use Committee (IACUC) approved this sampling methodology (IACUC protocol # 2-11-7091-J).

Family: subfamily	Species	Sample locality	No. of embryos	Stages ^c	Wk(s)	Incubation temperature ^d
Trionychidae	<i>Apalone mutica</i>	Muscatine, Iowa	23 ^b	GC14-15, 19-25	3,5-6,8	30 °C
Chelydridae	<i>Chelydra serpentina</i>	Hyannis, Nebraska; Thomson, Illinois	82	Y14-25	3-8	27 °C
Kinosternidae ^a	<i>Kinosternon subrubrum</i>	Lake Hamilton, Arkansas	14	Y14-17, 24-25	3, 7-8	30 °C
	<i>Sternotherus odoratus</i>	Lake Hamilton, Arkansas	6	Y15, 24-25	3, 7-8	30 °C
Emydidae: Deirochelyinae	<i>Chrysemys picta</i>	Thomson, Illinois	223	CJ14-22	3-8	27 °C

S-table 1 continued

Emydidae:						
Emydinae	<i>Emys blandingii</i> ^e	Hyannis, Nebraska	69	CJ14-15, 17-22	3-8	27 °C
	<i>Terrapene ornata</i> ^e	Oshkosh, Nebraska	52	CJ14-15, 19-22	3, 5-8	27 °C

a = staged using criteria of sister clade Chelydridae (see below).

b = wk-8 specimens (FJ57402, FJ57399) from the ISU collection.

c = stages 22-25 of Greenbaum and Carr (GC- [ref 20]) and Yntema (Y- [ref 21]) are equivalent to 21-22 in the emydid stages of Cordero and Janzen (CJ- [ref 22]).

d = constant temperature resulting in comparable egg incubation periods; egg substrate moisture was set to a constant -150 kPa.

e = see below for discussion of IUCN status of these species.

Note on IUCN Redlist status of *Emys blandingii* and *Terrapene ornata*:

Emys blandingii and *Terrapene ornata* are listed as “threatened” or “near threatened” by the IUCN Redlist because populations have declined across various localities within their geographic distributions in North America. Therefore, we took careful measures to only sample healthy and regionally non-threatened populations of these species in western Nebraska. Collection of eggs was conducted in accordance to regional and federal laws in the United States. Nonetheless, to minimize potential impacts, sampling of *E. blandingii* and *T. ornata* eggs spanned three and four nesting seasons, respectively. This yielded an average of 5.25 clutches of eggs per year collected for *T. ornata*, and 3.66 clutches of eggs per year collected for *E. blandingii*.

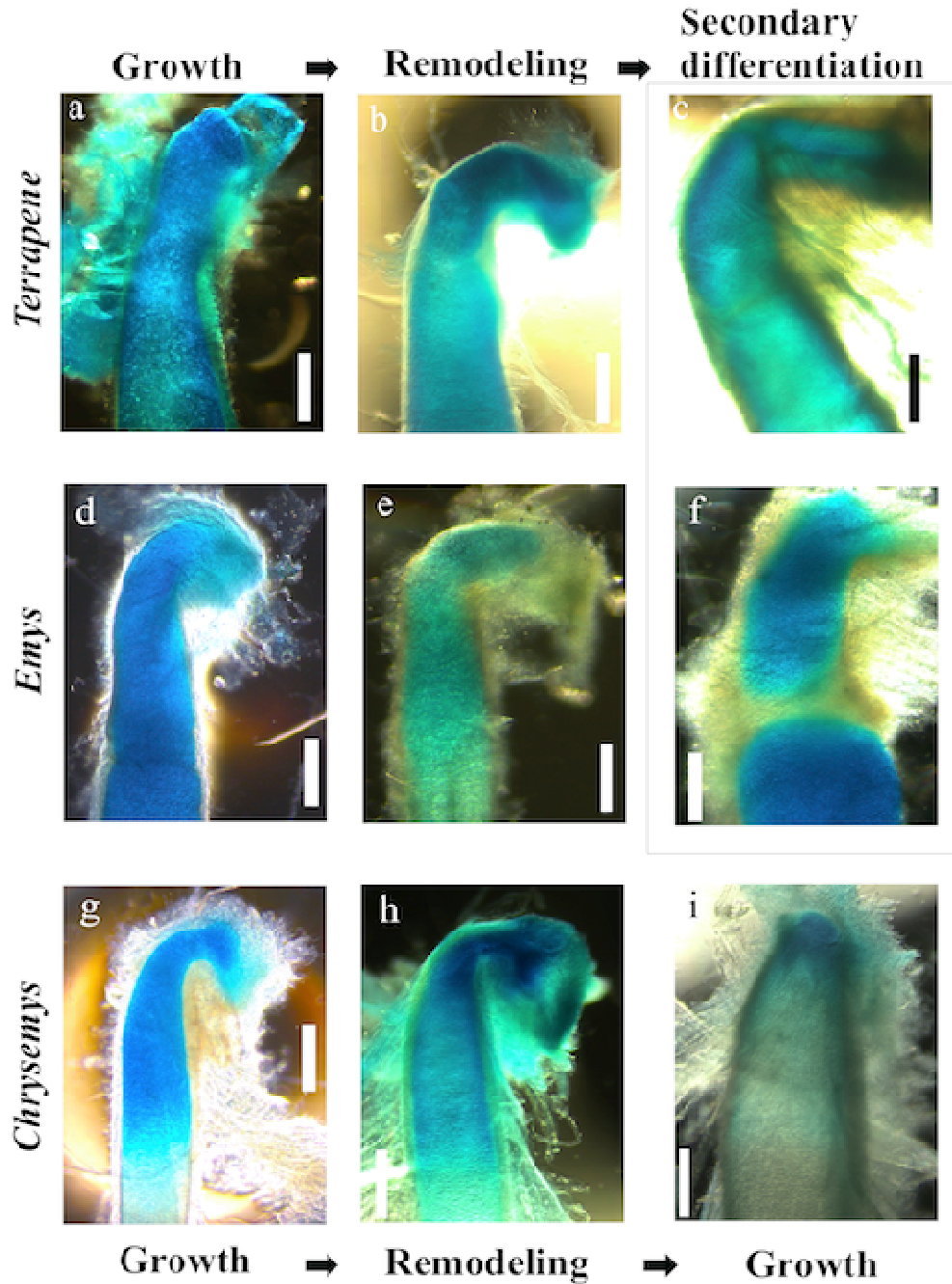
Our collection efforts were undertaken with advice from members of the IUCN Tortoise and Freshwater Turtle Specialist group: John Iverson and Fredric Janzen. The Nebraska Game & Parks Commission lawfully granted us the unique opportunity to sample healthy populations of *E. blandingii* and *T. ornata*. Therefore, harvested tissues were also made available to various other studies conducted by our colleagues, as well as in our laboratory. Our sampling was authorized by IACUC protocol # 2-11-7091-J.

S-Table 2. Hatchling specimen ID and data used in correlation analyses; FJ = Iowa State University herpetological collection, CM = Carnegie Museum of Natural History; ^ = dry whole forelimb mass includes the scapula. * = measurements were taken with digital calipers. Access to CM specimens was kindly facilitated by Steve Rogers.

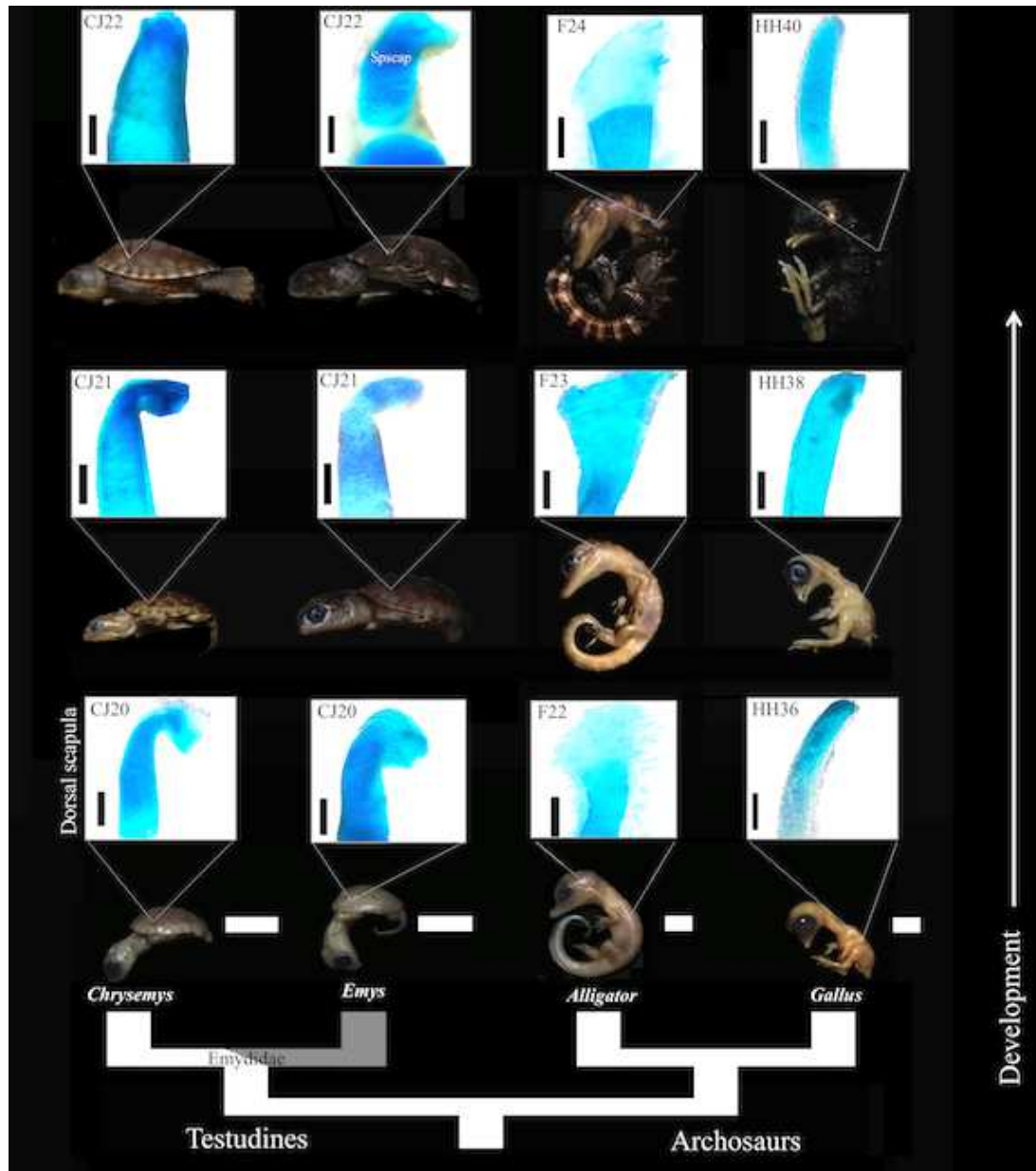
Specimen ID	Species	Family: Subfamily	Dry whole forelimb mass (g) ^	Dry scapula only mass (g)	Shell height* (mm)	Carapace length* (mm)
FJ60071	<i>Apalone mutica</i>	Trionychidae	0.057	0.003	8.01	36.13
FJ60441	<i>Apalone mutica</i>	Trionychidae	0.068	0.006	8.70	36.58
FJ60092	<i>Apalone mutica</i>	Trionychidae	0.049	0.005	7.44	37.98
FJ60090	<i>Apalone mutica</i>	Trionychidae	0.065	0.004	7.50	37.08
FJ60252	<i>Apalone mutica</i>	Trionychidae	0.056	0.005	7.25	32.48
FJ55844	<i>Apalone spinifera</i>	Trionychidae	0.079	0.007	9.59	36.38
FJ55878	<i>Apalone spinifera</i>	Trionychidae	0.090	0.013	10.12	37.76
FJ55847	<i>Apalone spinifera</i>	Trionychidae	0.111	0.005	10.10	36.58
FJ55879	<i>Apalone spinifera</i>	Trionychidae	0.124	0.008	11.57	41.51
FJ55874	<i>Apalone spinifera</i>	Trionychidae	0.112	0.006	9.50	38.10
FJ65384	<i>Chelydra serpentina</i>	Chelydridae	0.123	0.016	15.75	30.88
FJ65380	<i>Chelydra serpentina</i>	Chelydridae	0.133	0.018	13.10	31.76
FJ65370	<i>Chelydra serpentina</i>	Chelydridae	0.143	0.021	15.92	31.93
FJ65379	<i>Chelydra serpentina</i>	Chelydridae	0.145	0.019	15.65	32.06
FJ65369	<i>Chelydra serpentina</i>	Chelydridae	0.149	0.015	13.26	32.27
FJ65282	<i>Chrysemys picta</i>	Emydidae: Deirochelyinae	0.058	0.010	13.20	26.78
FJ65322	<i>Chrysemys picta</i>	Emydidae: Deirochelyinae	0.064	0.011	13.28	27.41
FJ65283	<i>Chrysemys picta</i>	Emydidae: Deirochelyinae	0.057	0.008	13.66	26.21
FJ65284	<i>Chrysemys picta</i>	Emydidae: Deirochelyinae	0.063	0.008	13.57	27.50
FJ65324	<i>Chrysemys picta</i>	Emydidae: Deirochelyinae	0.064	0.011	12.95	28.23

S-table 2 continued

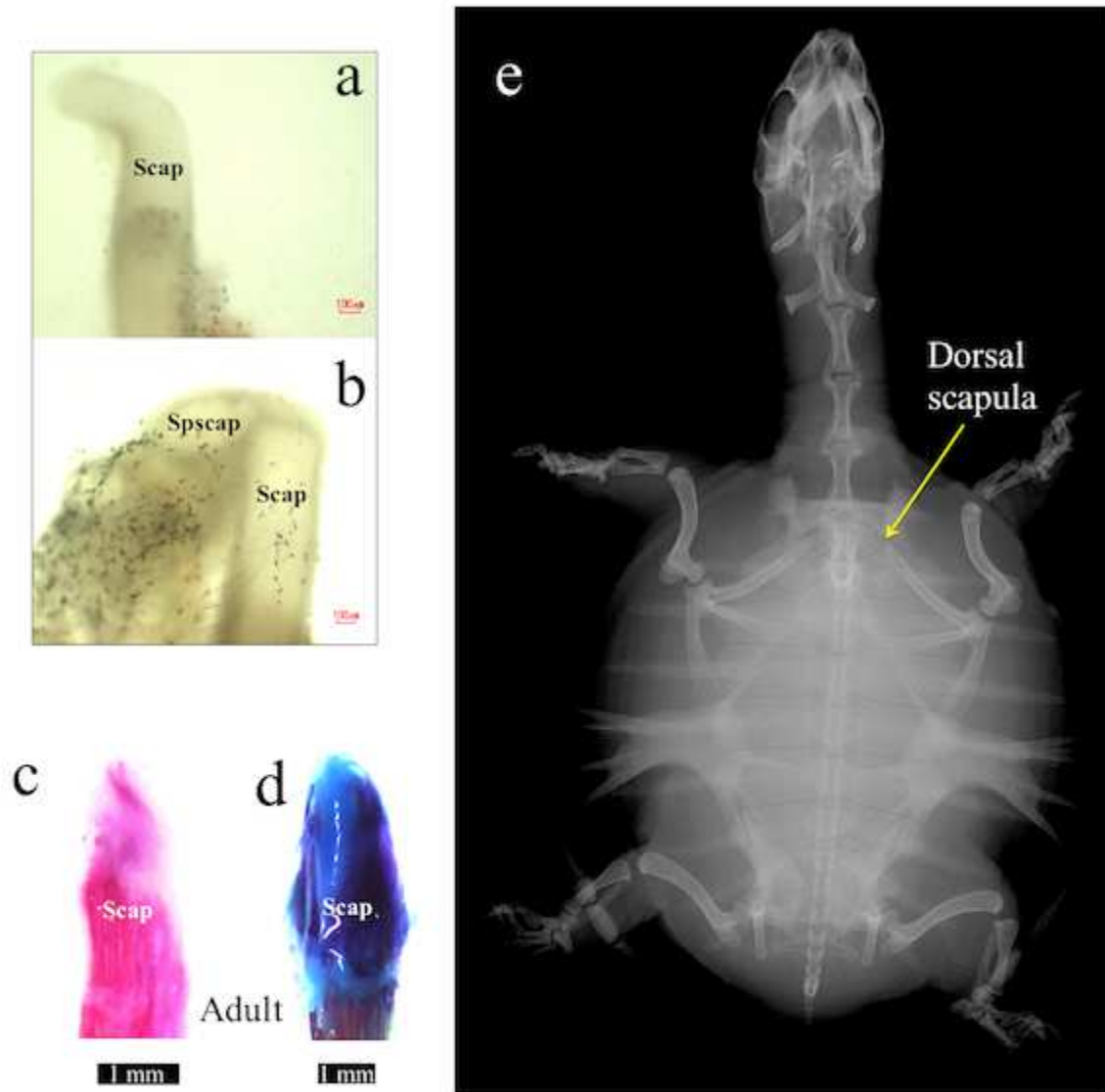
FJ52410	<i>Trachemys scripta</i>	Emydidae: Deirochelyinae	0.081	0.009	14.54	31.90
FJ52573	<i>Trachemys scripta</i>	Emydidae: Deirochelyinae	0.069	0.009	13.76	30.60
FJ52581	<i>Trachemys scripta</i>	Emydidae: Deirochelyinae	0.081	0.010	13.52	32.50
FJ52666	<i>Trachemys scripta</i>	Emydidae: Deirochelyinae	0.060	0.009	12.61	31.47
FJ52661	<i>Trachemys scripta</i>	Emydidae: Deirochelyinae	0.072	0.013	14.47	31.79
FJ59459	<i>Emydoidea blandingii</i>	Emydidae: Emydinae	0.082	0.011	14.34	35.27
FJ59403	<i>Emydoidea blandingii</i>	Emydidae: Emydinae	0.103	0.013	15.18	37.30
FJ59438	<i>Emydoidea blandingii</i>	Emydidae: Emydinae	0.094	0.012	14.44	37.38
FJ59458	<i>Emydoidea blandingii</i>	Emydidae: Emydinae	0.118	0.012	14.70	36.94
FJ59457	<i>Emydoidea blandingii</i>	Emydidae: Emydinae	0.078	0.008	13.41	35.43
CM156609	<i>Terrapene carolina</i>	Emydidae: Emydinae	0.086	0.015	18.75	37.67
CM156608	<i>Terrapene carolina</i>	Emydidae: Emydinae	0.066	0.010	15.79	34.89
CM156621	<i>Terrapene carolina</i>	Emydidae: Emydinae	0.071	0.013	17.35	36.61
CM156614	<i>Terrapene carolina</i>	Emydidae: Emydinae	0.076	0.012	17.99	34.53
CM156611	<i>Terrapene carolina</i>	Emydidae: Emydinae	0.061	0.010	15.72	33.61
FJ67639	<i>Terrapene ornata</i>	Emydidae: Emydinae	0.127	0.027	15.01	30.24
FJ67638	<i>Terrapene ornata</i>	Emydidae: Emydinae	0.104	0.020	13.08	27.60
FJ67636	<i>Terrapene ornata</i>	Emydidae: Emydinae	0.091	0.012	13.32	25.32
FJ67637	<i>Terrapene ornata</i>	Emydidae: Emydinae	0.126	0.010	13.61	28.37
FJ67640	<i>Terrapene ornata</i>	Emydidae: Emydinae	0.129	0.020	13.45	29.36



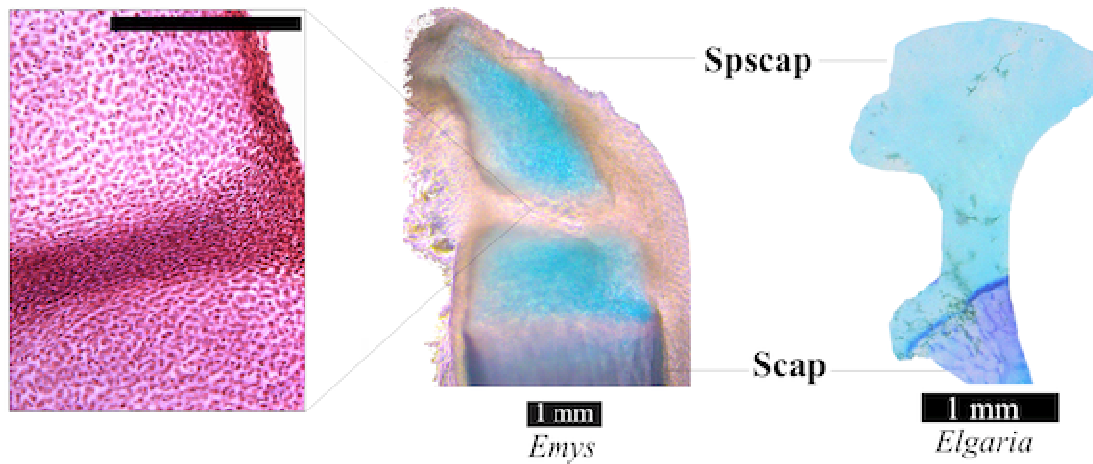
S-Figure 1. Lateral view of the dorsal scapula, stained in alcian blue, in embryos of *Terrapene ornata* (a-c), *Emys blandingii* (d-f), and *Chrysemys picta* (g-i) at Cordero and Janzen (CJ; ref 22) stages 20-22. Emydine box turtles (*Terrapene* and *Emys*) diverge from the general turtle condition (e.g. *Chrysemys*) in that tissue remodeling is followed by secondary differentiation of the scapula (c and f); this yielded the suprascapula. Imaging was performed with a Leica LDM2500 microscope. Scale bars = 500 μ m.



S-Figure 2. Lateral views of *Chrysemys picta*, *Emys blandingii*, *Alligator mississippiensis*, and *Gallus gallus* embryos (scaled to 5 mm) at approximately equivalent stages of development. Panels above embryos display the dorsal scapula stained in alcian blue (scale bars = 1mm). *Emys* has diverged from the conserved pattern of dorsal scapula development in Testudines and Archosaurs (phylogeny after ref 23) by differentiation of the suprascapula (Spscap) by CJ22. Staging of *Alligator* and *Gallus* followed the criteria of Ferguson (F, S-ref 1) and Hamburger and Hamilton (HH, S-ref 2), respectively. *Alligator* embryos were sampled by Ruth Elsey of the Rockefeller Wildlife Refuge, Louisiana. *Gallus* embryos were sampled from eggs purchased from the Murray McMurray Hatchery, Iowa.



S-Figure 3. Lateral view of unprepared dorsal scapulae in embryos of the soft-shelled turtle *Apalone mutica* at Greenbaum and Carr stages (GC; ref 20) 20-21 (a-b). The scapula (Scap) is a single continuous segment at GC20 (a) and is secondarily differentiated giving rise to a “transient” suprascapula by GC21 (Spscap, b). This transient “suprascapula” was absent in adult specimens of *A. ferox* (c, alizarin red) and *A. mutica* (d, alizarin+alcian blue) from the Iowa State University herpetological collection (FJ59594 and FJ59597). The suprascapula was not detected with x-ray imaging on a *Lyssemys scutata* specimen from the Smithsonian National Museum of Natural History (USNM520644). We thank USNM staff for assistance with x-ray imaging.



S-Figure 4. Comparison of the suprascapula (Spscap) in *Emys blandingii* and an Alligator Lizard (*Elgaria multicarinata*) from the ISU herpetological collection (FJ63667). The suprascapula in *Emys* features a synovial joint, evident by an interzone of condensed mesenchyme (H&E section, scale bar = 100 μ m) separating it from the scapula (Scap). Lizards, represented by *Elgaria*, do not feature such discontinuity. The *Elgaria* specimen was collected and kindly donated by Rory Telemeco.

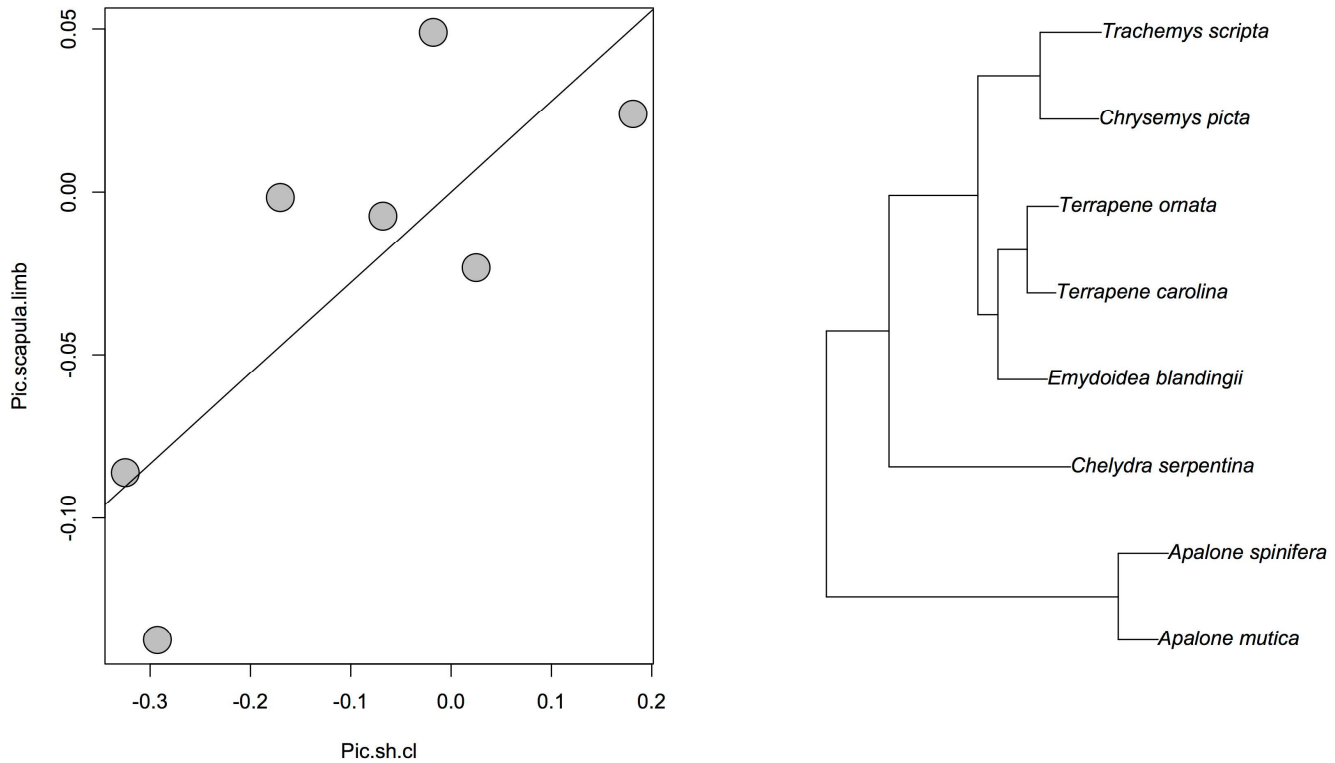
S-References

- S1. Ferguson M.W. 1985 Reproductive biology and embryology of the crocodilians. In *Biology of the Reptilia* (eds. F. Billet, Maderson P. F. A.), pp. 329-491. New York, John Wiley & Sons
- S2. Hamburger V., Hamilton, H. 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92.

Phylogenetic Analyses

S-Table 3 – Summary statistics of species data used in phylogenetic correlation analyses. Estimates are based on $N=5$ samples per species; means are followed by standard errors.

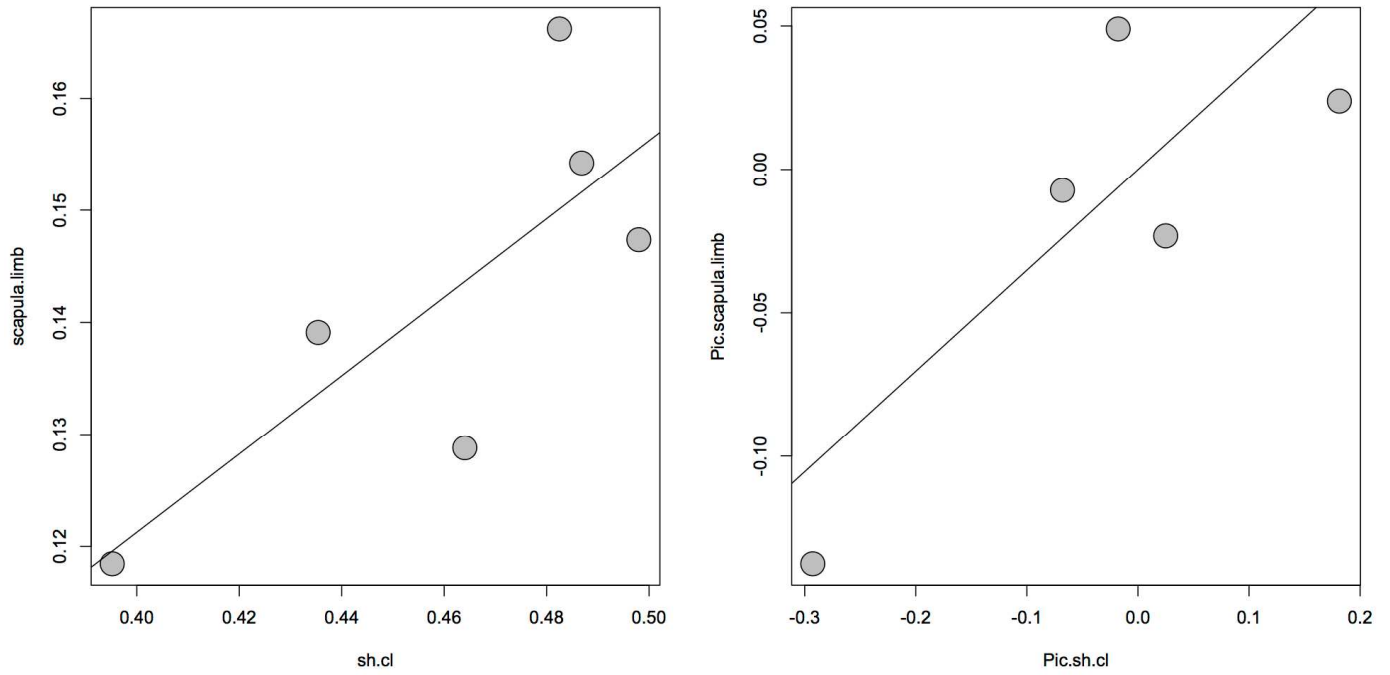
Species	Normalized scapula mass	Shell height-to-length ratio
<i>Apalone mutica</i>	0.0787 ± 0.0092	0.2162 ± 0.0075
<i>Apalone spinifera</i>	0.0792 ± 0.0178	0.2671 ± 0.0052
<i>Chelydra serpentina</i>	0.1287 ± 0.0076	0.4640 ± 0.0216
<i>Chrysemys picta</i>	0.1566 ± 0.0096	0.4980 ± 0.0100
<i>Trachemys scripta</i>	0.1391 ± 0.0121	0.4354 ± 0.0113
<i>Emys (Emydoidea) blandingii</i>	0.1184 ± 0.0067	0.3952 ± 0.0056
<i>Terrapene carolina</i>	0.1661 ± 0.0056	0.4825 ± 0.0120
<i>Terrapene ornata</i>	0.1542 ± 0.0234	0.4868 ± 0.0115



S-Figure 5 – Left: Correlation ($r = 0.77$, $P = 0.039$) of phylogenetic independent contrasts (Pic) on normalized scapula mass (Pic.scapula.limb) versus shell height-to-length ratio (Pic.sh.cl) generated using the Ape package of the R programming language. The analysis was conducted on a pruned topology (right) of Guillon et al. (see S-Ref[1]); the tree topology file (Tr63637), along with branch length data, is available from TreeBASE (<http://treebase.org/treebase-web/search/study/trees.html?id=14093>). Note: Our pruned topology is congruent with family-level relationships examined in Crawford et al. [ref 24]. However, our analyses were based on the data of Guillon et al. (see S-Ref[3]) because Crawford and colleagues examined only four out of the eight species in our study.

S-References

S3. Guillon J.-M., Guéry L., Hulin V., Girondot M. 2012 A Large phylogeny of turtles (Testudines) using molecular data. *Cont. Zool.* **81**, 147-158.



S-Figure 6 – Strong correlation of normalized scapula mass (scapula.limb) and shell height-to-length ratio (sh.cl) after exclusion of soft-shelled turtles (left; $r = 0.78$, $P = 0.066$). Phylogenetic independent contrasts (Pic) on normalized scapula mass (Pic.scapula.limb) versus shell height-to-length ratio (Pic.sh.cl) remained strongly correlated after exclusion of soft-shelled species (right; $r = 0.82$, $P = 0.091$).

CHAPTER 4. GENES, JOINTS, AND THE REPEATED EVOLUTION OF SKELETAL COMPLEXITY IN TURTLES

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Wimalanathan, Fredric Janzen

Abstract

Similarity in form and function in unrelated species, due to repeated evolution, is a common theme of life on Earth. Examining this trend provides an ideal framework to advance current understanding on processes that generate or limit organismal diversity. In recent years, comparative approaches revealed that morphological similarity in adults of unrelated species is the result of similar (parallel) or dissimilar (convergent) evolutionary change in developmental genes. Even so, direct examination of divergence in underlying developmental processes, presumably due to parallel or convergent genetic change, is rarely undertaken. Here, we compared gene expression associated with the embryonic development of a repeatedly evolving skeletal trait in turtles. Terrestrial and semi-aquatic emydid turtles with shell-closing systems independently evolved a complex synovial joint on the shoulder blade (scapula). Specification of synovial joint development in vertebrates is underpinned by epigenetic tissue remodeling triggered by muscle contraction late in embryogenesis. Thus, we tested the hypothesis that hypertrophied scapula muscles in embryos of species with shell-closing systems induces expression of genes related to cartilage remodeling during early development of synovial joints. Consistent with our prediction, transcriptomic and histochemical comparisons provided robust support for differential regulation of genes associated with muscle growth, decomposition of extracellular matrix of cartilage, and cell death. These data suggest that synovial joint

development in unrelated lineages with shell-closing systems is an epigenetic by-product of change in the mechanical environment governed by embryonic muscle contraction. For the first time, we provide evidence of repeated skeletal evolution via parallel epigenetic change in a vertebrate animal.

Introduction

Striking similarity in form and function in unrelated species suggests some degree of commonality in mechanisms that generate or limit diversity across the tree of life. Over the last decade, much progress has been made towards illuminating the genetic and molecular underpinnings of phenotypic similarity due to repeated trait evolution in laboratory and natural populations of a wide variety of taxa (Woods et al. 2006; Conte et al. 2012; Tenaillon et al. 2012; Blank et al. 2014; Gallant et al. 2014; Pankey et al. 2014; Berens et al. 2015; also see reviews by Arendt and Reznick 2008; Elmer & Meyer 2011; Losos 2011; McGhee 2011; Wake et al. 2011; Rosenblum et al. 2014; Ochoa and Rasskin-Gutman 2015). Whether unrelated species evolve similar traits due to similar (parallel) or dissimilar (convergent) changes in underlying genetic architecture depends on phylogenetic distance, trait complexity, and population demography (Conte et al. 2012; Wake et al. 2011; Rosenblum et al. 2014; Ochoa and Rasskin-Gutman 2015).

Many of these studies, however, assume that structurally similar traits in adults of unrelated species are homologous without examining potential divergence in underlying developmental processes (Hall 2012). With some exceptions (e.g. Sanger et al. 2012; 2013), research on repeated morphological evolution rarely accounts for variation in developmental processes. Functional gene variation (i.e. gene expression) associated with embryonic development of parallel or convergent morphological traits in tetrapods has not been examined within a

comparative phylogenetic framework. Even so, individual studies reveal that gene expression governs cellular-level processes underlying macroevolutionary patterns of skeletal variation in tetrapods (Wilkins 2002; Davidson 2006; Carroll 2008; Shubin et al. 2009; Peter and Davidson 2011; Wagner 2014). Intriguingly, these cellular-level processes respond and exhibit plasticity to mechanical force generated by embryonic motility and muscle contraction (Drachman and Sokoloff 1966; Hall 1986; Atchley and Hall 1991; Hogg and Hosseini 1992; Newman and Muller 2001; Muller 2003; Newman and Muller 2005; Young and Badyaev 2007; Kahn et al. 2009; Nowlan et al. 2010; Pollard et al. 2014). This form of epigenetic tissue remodeling has been linked experimentally to the origins of evolutionary novelty in the avian skeleton (Muller 1990; Newman and Muller 2001; Muller 2003; Newman and Muller 2005; Wagner 2014). In addition to parallel or convergent changes in *cis*-regulatory and protein-coding gene sequences (Rosenblum et al. 2014), epigenetic change driven by tissue-tissue interactions in development may underpin repeated evolution of skeletal traits in tetrapods (Maderson 1975). However, parallel or convergent genetic change typically is attributed instead to similarity in selective regimes of unrelated species or populations (Dobzhansky 1959; Bock 1963; Wake 1991, 1999; Powell 2007; Wake et al. 2011; Mahler et al. 2013; Rosenblum et al. 2014; Ochoa and Rasskin-Gutman 2015).

Examining epigenetic change driven by tissue-tissue interactions could provide novel insights into how development of highly derived skeletons, such as in turtles, influences repeated evolution of adaptive traits. The shelled body plan of modern turtles has changed little during 210 million years of evolution, even after multiple adaptive radiations across freshwater, marine, and terrestrial ecosystems worldwide (Zangerl 1969; Pritchard 2008; Lyson and Joyce 2012; Joyce et al. 2013; Lyson et al. 2013). Still, repeated morphological evolution is widespread in

turtles (Zangerl 1969; Bramble 1974; Bramble and Hutchison 1981, 1984; Feldman and Parham 2002; Claude 2006; Pritchard 2008; Angielczyk et al. 2010; Angielczyk and Feldman 2013), suggesting that development of their unusual body plan channels morphological evolution in a predictable manner. For instance, evolutionary transitions to terrestrial or semi-aquatic ecological niches are associated with at least seven independent origins of shell-closing systems in small-bodied turtles (Bramble 1974; Bramble and Hutchison 1981, 1984; Feldman and Parham 2002; Stephens and Wiens 2003, 2009; Pritchard 2008; Angielczyk et al. 2010). Shell-closing systems enable complete or nearly complete concealment of extremities, thereby increasing the likelihood of surviving predator attacks on land (Minckley 1968; Bramble 1974; Bramble and Hutchison 1981, 1984; Greene 1988). In addition, shell closure may decrease water loss while in terrestrial burrows (Wygoda and Chmura 1990).

The evolution of shell-closing systems follows a mosaic pattern of regionalized anatomical change in internal skeletal traits that substantially enhance the functional capacity of the shell (Bramble 1974; Bramble and Hutchison 1981, 1984; Cordero and Quinteros 2015). Within some clades, internal skeletal traits have evolved repeatedly as key biomechanical components of complex shell-closing systems (Figure 1A-C; Bramble 1974; Bramble and Hutchison 1981, 1984), particularly in North American box turtles (Emydidae: *Terrapene* spp.) exhibiting the most highly derived form of the shell-closing phenotype. Notably, the shell-closing system of box turtles features a specialized synovial joint with derived skeletal elements that enable displacement of the shoulder blade (scapula) to the inside of the rib cage, unlike most turtles (Figure 1B; Bramble 1974; Cordero and Quinteros 2015). The *de novo* formation of a synovial joint on the dorsal scapula of box turtles is highly unusual, as such a condition has not been described in adults of any other tetrapod lineage (Burke 1991; McGonnell 2001; Vickaryous and

Hall 2006; Piekarski and Olsson 2011; Lyson and Joyce 2012). Remarkably, the Blanding's turtle (Emydidae: *Emys blandingii*), a semi-aquatic relative of North American box turtles, has independently evolved this trait in conjunction with a less derived shell-closing system (Feldman and Parham 2002; Angielczyk et al. 2010; Cordero and Quinteros 2015).

Synovial joints are intricate skeletal articulations that feature lubrication and immune systems and are typically associated with the anatomy of knees and elbows (Firestein 2007; Hall 2015). In chicken and mouse, initiation of synovial joint development requires muscle contraction due to embryonic motility (Drachman and Sokoloff 1966; Hogg and Hosseini 1992; Kahn et al. 2009; Hall 2015). Chicken embryos exhibit a similar pattern of embryonic motility as box turtles (Tuge 1931). Embryonic motility reaches a peak about half way through development, coinciding with differentiation of synovial joints in distal limb elements in chicken (Drachman and Sokoloff 1966). In an earlier study, we showed initiation of synovial joint formation on the dorsal scapula through 60% of embryonic development in the ornate box turtle (*Terrapene ornata*) and *E. blandingii* (Cordero and Quinteros 2015). By ~75% of development, the primordial architecture of a synovial joint had formed following extensive cartilage remodeling and disproportionate growth of adjacent scapula muscles (Cordero and Quinteros 2015).

The unusually late transformation of the dorsal scapula in *T. ornata* and *E. blandingii* is likely induced, at least in part, by contraction of hypertrophied scapula muscles. These changes did not occur in an aquatic relative, the painted turtle (Emydidae: *Chrysemys picta*), exhibiting the ancestrally conserved condition of the dorsal scapula in emydid turtles (Figure 1C). Here, we test the hypothesis that the specialized synovial joint of emydid shell-closing systems arose independently via similar patterns of gene expression. We compared transcriptomes of developing scapulae in embryos of *C. picta*, *E. blandingii*, and *T. ornata*. We predicted that *E.*

blandingii and *T. ornata* express genetic pathways that govern cartilage remodeling and segmentation, including cell death and extracellular matrix decomposition.

Material and Methods

Comparative Phylogenetic Analyses

Ancestral state reconstruction: The independent origins of shell-closing systems were assessed using maximum likelihood ancestral reconstruction implemented in Mesquite (version 3.02, Maddison and Maddison 2015). Using the most recent molecular (nuDNA + mtDNA) phylogeny of extant Emydidae (Guillon et al. 2012), independent origins of shell-closing systems were confirmed by an Mk1 model of character state evolution (estimated rate 4.0996, -log-Likelihood: 11.7478). The proportional likelihood for the presence of a shell-closing system in the most common recent ancestor of *E. blandingii* and *T. ornata* was just 4% (Figure 1C).

Outgroup choice: Based on anatomical comparisons of adult emydid turtles (see Bramble 1974), we inferred that the most recent common ancestor of *E. blandingii* and *T. ornata* did not feature a synovial joint with derived scapular segments (Figure 1B-C). Sampling close relatives of *E. blandingii* and *T. ornata* was not feasible due to their threatened conservation status. Still, sister lineages of *Emys* and *Terrapene* display the ancestral ground state of the dorsal scapula in Emydidae, as exemplified by *C. picta*. Thus, *C. picta* was chosen as the comparative outgroup in subsequent analyses. Moreover, *C. picta* is the only emydid species with a sequenced nuclear genome and extensive data on developmental processes (Shaffer et al. 2013; Cordero 2014; Cordero and Janzen 2014).

Sample Processing

Egg collection and incubation: Eggs of *C. picta*, *E. blandingii*, and *T. ornata* were collected from non-threatened populations in Illinois and Nebraska following Cordero and Quinteros (2015). Eggs of *C. picta* were collected from a population in Thomson, Illinois. Eggs of *E. blandingii* and *T. ornata* were collected from Nebraska populations near Oshkosh and Hyannis, respectively. Based on the protocol of Cordero and Janzen (2014), temperature of egg incubation was set to a constant 27 °C, with eggs half-buried in moist vermiculite (−150 kPa water potential). Sampling followed an approved protocol (IACUC 2-11-7091-J) with scientific collection permits from the Illinois Dept. of Natural Resources (#NH13.0073) and Nebraska Game & Parks (#310).

Tissue microdissections: Microdissections of embryonic scapulae were conducted using autoclave-sterilized surgical instruments on a Nikon (SMZ1000) dissecting microscope. Briefly, embryos were removed from eggs and euthanized by decapitation, an incision was made on the anterior carapace, and the entire shoulder girdle was carefully excised and submerged in cold phosphate buffered saline (PBS) at pH 7.4 (Sigma P5368). This and other solutions were prepared with ultrapure dd H₂O. The dorsal scapulae were then dissected and quickly transferred to RNA-later (Ambion, AM7021). After overnight incubation at 4 °C, samples were stored at -80 °C before further processing. All instruments, glassware, and working surfaces were treated with RNase Zap (Ambion, AM 9798) to reduce the likelihood of RNase contamination. Plastic tubes in this experiment were manufacturer-certified to be RNase-free.

Sampling intervals: Embryonic scapulae of *E. blandingii*, *C. picta*, and *T. ornata* were dissected on days 34, 38, and 44 of development, which coincided with Cordero and Janzen (CJ; 2014) stages 20, 21, and 22, respectively. In addition, the distal tail in CJ21 *C. picta* was sampled

as a reference control tissue. Three pooled samples of four to six individuals were collected per stage and species; individuals were randomized by clutch identity. Sampling of embryonic scapulae began on day 34 of development, immediately before scapula segmentation in species with shell-closing systems (Cordero and Quinteros 2015). Day 38 of development was sampled because it corresponded with early specification of synovial joint development in embryos of *E. blandingii* and *T. ornata*. The time in between sampling intervals was increased, from four to eight, by sampling day 44. This arrangement was an attempt to observe and sample morphogenesis of the interskeletal cavity characteristic of adult synovial joints (Firestein 2007). However, cavitation in the scapula synovial joint of *E. blandingii* and *T. ornata* does not occur during embryonic development, as determined by subsequent serial histological sectioning in embryos and hatchlings.

Histochemical characterization: Following Cordero and Quinteros (2015), samples were stained in Alcian blue to confirm the presence of proteoglycan proteins characteristic of cartilage tissue matrix. Subsequent staining with Alizarin red did not indicate tissue mineralization, indicating that the cartilage of all dorsal scapula samples had not transitioned to bone. Hematoxylin and eosin (H&E) preparations of scapula samples were examined to confirm cartilage segmentation. Turnover of proteoglycan protein matrix during scapula segmentation was assessed by examining Alcian blue/nuclear fast red sections. Samples were imaged on Leica compound (LDM2500) and dissecting (S6D) microscopes.

RNA extraction: Total RNA was extracted following a modified cartilage-specific protocol that included additional purification procedures (Ruettinger et al. 2010). Before tissue homogenization and RNA extraction, RNA Later was removed from thawed samples by briefly washing and transferring to cold sterile PBS. This procedure minimized carry-over effects of RNA Later.

Samples were centrifuged to create a dense tissue pellet for homogenization in Tri Reagent (Sigma T924) using a PowerGen 125 rotor homogenizer (model FTH-115, Fisher Scientific). Thoroughly homogenized samples were treated with 1-bromo-3-chloropropane (Sigma B9673) before undergoing additional purification using the filter column procedures of the Qiagen RNeasy Mini Kit (N0. 74104). To remove any potential traces of DNA in our samples, total RNA was treated with DNase using the Qiagen RNase-Free DNase Set (No. 79254). RNA extractions were conducted in an AirClean 600 Workstation (AirClean Systems) using sterile pipettes wiped with RNase Zap and loaded with RNase-free barrier tips. We used molecular-biology grade 2-propanol (Sigma, I9516) and ethanol (Sigma, E7023) in RNA extraction procedures. Purified total RNA was re-suspended in ultrapure dd H₂O and stored in -20 °C.

RNA quality assessment: Total RNA quality and concentration were preliminarily assessed by spectrophotometry on a NanoDrop Spectrophotometer ND-1000, followed by isolation of mRNA transcripts for housekeeping genes GAPDH and β -actin using RT-PCR (see below). Once presence of RNA was confirmed in all samples, RNA integrity was estimated using an Agilent 2100 Bioanalyzer. Only samples with RNA integrity numbers (RIN; Schroeder et al. 2010) ≥ 8 (out of 10) were used in sequencing (see S-Table 1).

RNA-Seq

Sequencing: Samples that met our RNA quality criteria were submitted to the Iowa State University DNA facility for massively paralleled cDNA sequencing (RNA-Seq; Marguerat and Bahler 2010) on the Illumina sequencing platform (reviewed in Glenn 2011). Total RNA samples were subjected to indexed cDNA library construction using the TruSeq RNA Sample Preparation Kit (Illumina) and loaded onto the Illumina HiSeq 2500, following a balanced-block

experimental design to account for potential lane effects (Auer and Doerge 2010). Rather than separate each experimental condition according to sequencing lane, libraries were arranged such that each experimental condition (species by developmental stage) was represented once across three sequencing lanes, enabling technical replication (Auer and Doerge 2010). Sequencing yielded 30 cDNA libraries (10 per lane) of single-end 100-bp reads.

Read quality assessment: Read libraries were subjected to statistical assessment of quality using FastQC (version 0.11.2; Andrews 2010). The number of unprocessed reads per library and read count distributions per sample are provided in the supplementary information section (S-Figure 1; S-Table 1). Trimming of libraries was not required based on quality control statistics. Adapter sequences and potential sequencing artifacts were removed using “soft-trim” methods during read alignment procedures (see below).

Read alignment, mapping, and counts: Reads were aligned and mapped to the reference genome of *Chrysemys picta bellii* (GenBank assembly accession: GCA_000241765.2; Shaffer et al. 2013) using GSNAP (version 2013-04-30; Wu and Nacu, 2010). Pilot alignments were conducted to optimize the proportion of reads that could be mapped. This approach entailed minor modifications of algorithm parameters that account for indels, local or distant splicing, and intron-exon boundaries. The proportions of uniquely-mapping reads for species-specific transcriptomes were comparable: 83.9% in *C. picta*; 85.7% in *E. blandingii*; and 85.9% in *T. ornata*. High proportions of uniquely-mapping reads are expected if read alignments are performed against the reference genome of a relative that is <100 MY divergent (see Hornett and Wheat 2012). *Emys blandingii* and *T. ornata* last shared a common ancestor with *C. picta* at ~34 MYA (Spinks and Shaffer 2009). Read counts per gene were estimated using the featureCounts program (version 1.4.6, Liao et al. 2014) of the Subread R Bioconductor package (version 1.4.6;

Liao et al. 2013). The proportion of unambiguously assigned and counted genes ranged from 62.9 to 72.8% across all libraries.

Differential gene expression: Differential expression analysis was conducted using the edgeR package of the R programming language (version 3.6.8, Robinson et al. 2010). To account for among-sample variance in size of libraries, read data were normalized using the “TMM” method (Robinson and Oshlack 2010). A generalized linear model was then fitted to the data: read count \sim species + stage + species*stage. A preliminary model included parameters for lane, RIN, and sample concentration effects, but these were dropped because they were not statistically significant. Differential gene expression was assigned if false discovery rates (FDR) were less than 0.05 with absolute values of ≥ 1 for log₂-fold change. Principal component analysis (PCA) was used to explore differences among samples based on normalized count data for all differentially expressed genes (DEGs). Data were further explored using multi-dimensional scaling analysis based on Ward’s method for hierarchical clustering (S.-Figure 1). Pairwise comparisons of DEGs for subsequent analyses were chosen as follows: *C. picta* versus *E. blandingii* at CJ20-22; *C. picta* versus *T. ornata* at CJ20-22.

Gene Ontology and Pathway Analyses

Subsets of genes that were differentially regulated (i.e. DEGs) in *E. blandingii* and *T. ornata*, relative to *C. picta*, were queried for gene ontology (GO) and pathway enrichment using the Enrichr interactive suite (Chen et al. 2013). Gene subsets with FDRs < 0.05 were selected. Analyses focused on DEG lists shared in common by *E. blandingii* and *T. ornata* in stage-specific comparisons against *C. picta*. Gene and pathway enrichment analyses were performed against up-to-date databases, whereby DEG subsets are assigned as enriched for a particular

biological function based on Fisher's exact tests. Moreover, these procedures also scored enrichment for GO terms specifically related to molecular and cellular functions. These data were used to determine whether DEGs in *E. blandingii* and *T. ornata* were related to developmental processes associated with synovial joint development: cell death, muscle growth, and extracellular matrix decomposition.

Post-hoc Validation of Differentially Expressed Genes

Differential expression of a subset of genes, based on RNA-Seq analyses, was verified using real-time PCR. These assays followed standard experimental guidelines, including design of primers that reduce the likelihood of gDNA amplification (Bustin et al. 2010). Total RNA of similar concentrations across samples (~200 ng total) was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, No. 205311), pre-amplified using standard PCR, and purified using the Pure Link Quick PCR Purification Kit (Invitrogen, K310001). Purified PCR products were subjected to real-time PCR using the Quantifast SYBR Green Kit (Qiagen, No. 204054) on a Bio-Rad CFX384 thermocycler. Relative gene expression (ΔC_q) was estimated using the Bio-Rad CFX Manager 3.1 software. Reference genes GAPDH and β -actin were chosen because they are typically used in real-time PCR analyses of cartilage tissue (Quiroz et al. 2010).

Results

Similarity in development and transcriptome profiles

The dorsal scapula in emydid turtles with shell-closing systems undergoes segmentation, via tissue remodeling, late in embryonic development (Figure 2A). In *E. blandingii*, cartilage

segmentation yielded an additional skeletal derivative: a suprascapula. In *T. ornata*, cartilage segmentation yielded two additional skeletal derivatives: a suprascapula and an episcapula. The putative episcapula in *E. blandingii* was derived ectopically, as a sesamoid bone, from articular cartilage adjacent to the suprascapula. The structural complexity of the joint, in terms of skeletal segments and morphology, is correlated with shell-closing capacity (i.e. shell kinesis) in adults (Figure 2A).

Scapula transcriptomes before, during, and after cartilage remodeling in *E. blandingii* and *T. ornata* at Cordero and Janzen (2014; CJ) stages 20-22 clustered closely based on a PCA of gene expression data (Figure 2B). Overall, transcriptomes clustered according to species and developmental stage. The transcriptome of the reference control tissue (*C. picta* tail at CJ21) was an outlier in the PCA analysis (Figure 2B). Based on Fisher's exact tests, transcriptomes of *E. blandingii* and *T. ornata* shared a moderate proportion (50-60%; all P 's < 0.0001) of differentially expressed genes, relative to *C. picta*, across CJ 20-22 stages.

Similarity in cartilage remodeling pathways

Based on statistically significant Fisher's exact tests ($P < 0.05$), differentially regulated gene subsets shared by *E. blandingii* and *T. ornata* were overrepresented for GO terms of KEGG database pathways related to cartilage protein (glycan) breakdown at CJ20: Glycan structures degradation and n glycan degradation. At CJ21, shared upregulated genes of *E. blandingii* and *T. ornata* were enriched for GO terms related to oxidative phosphorylation, a metabolic function related to decreased glycan protein production ($P < 0.0001$). In both species, the extracellular matrix receptor interaction pathway was enriched at CJ21-22. The focal adhesion pathway was also enriched during those stages. At CJ22, pathways for the production of glycan protein were

differentially regulated: Glycan structures biosynthesis 1 and n glycan. Enriched pathways associated with cartilage remodeling in *E. blandingii* and *T. ornata* ranked in the top 15 out of 170 queried Kegg pathways. Moreover, both species differentially regulate genes of GO terms related to extracellular structure organization and extracellular matrix organization at CJ22 ($P < 0.0001$).

Similarity in genes governing muscle contraction and growth

Emys blandingii and *T. ornata* shared gene subsets enriched for muscle-specific GO terms, including specific cellular and molecular functions at stages CJ21-22. GO terms for muscle contraction, striated muscle contraction, and muscle system biological processes were overrepresented and highly ranked (CJ21: top 10 out of 4,704 terms; CJ22: top 10 out of 4,767 terms) according to significance of Fisher's exact tests ($P < 0.0001$; Figure 3A). The highest-ranking overrepresented GO terms of cellular and molecular functions were of contractile fiber part (CJ21: top one out of 588 terms; CJ22: top one out of 591 terms) and structural constituent of muscle (CJ21: top one out of 1,022 terms; CJ22: top one out of 1,028 terms), respectively (Figure 3A). The muscle hypertrophy GO term was uniquely enriched in the DEG list of *T. ornata* at CJ22 ($P = 0.0134$). This term was uniquely enriched in the DEG list of *E. blandingii* at CJ21 ($P = 0.0305$).

Dissimilarity in cell death processes

By CJ21, the primordial interzone of a synovial joint, not present in *C. picta*, had differentiated on the dorsal scapula of *E. blandingii* and *T. ornata* via tissue remodeling, as determined by alcian blue/nuclear fast red staining (Figure 3B-H). The process of tissue remodeling was

prominent at CJ21 in *T. ornata* in the region where hypertrophied anterior scapula muscle, the Latissimus dorsi, attaches to the dorsal scapula (Figure 3E). This process was spatially congruent with the site of synovial joint formation where cartilage tissue breakdown occurred, potentially via cell death. This apoptosis was indicated by the presence of large mononucleated and multinucleated chondroclast-like cells (Figure 3F-G). Histological evidence of cartilage remodeling via apoptosis was equivocal in *E. blandingii*.

Shared subsets of genes differentially regulated, relative to *C. picta*, in *E. blandingii* and *T. ornata* (Figure 2C) were not enriched for apoptosis-related biological processes. When gene identity lists were analyzed separately, differentially regulated genes in *T. ornata* only, relative to *C. picta*, were enriched for 15 GO terms related to apoptosis (Table 1). Total number and combinations of apoptosis-related GO terms varied according to developmental stage in *T. ornata*: CJ20 ($N = 7$), CJ21 ($N = 6$), CJ22 ($N = 6$). Differentially regulated genes in *E. blandingii* only, relative to *C. picta*, were enriched for GO terms of three apoptosis-related processes, particularly at CJ20 and CJ22; evidence for enrichment was marginally significant at CJ21 (Table 1). Terms for intrinsic and extrinsic apoptotic signaling pathways were overrepresented in *T. ornata*, but not in *E. blandingii* (Table 1).

Discussion

We provide evidence of parallel epigenetic change during the repeated evolution of key biomechanical components associated with shell-closing systems of emydid turtles. Complex synovial joints in unrelated emydid turtles with shell-closing systems develop as epigenetic by-products of change in the mechanical environment governed by embryonic muscle contraction. Hypertrophied scapula muscles in embryos of *E. blandingii* and *T. ornata* likely induced

expression of genes related to cartilage remodeling during early development of synovial joints. Consistent with our prediction, transcriptomic and histochemical comparisons provided robust support for upregulation of genes associated with muscle contraction and growth, decomposition of extracellular matrix of cartilage, and cell death in embryos of *E. blandingii* and *T. ornata*. Similar cellular-level processes are associated with initiation of synovial joint development via muscle contraction in chicken and mouse embryos (Drachman and Sokoloff 1966; Hogg and Hosseini 1992; Kahn et al. 2009; Hall 2015). Notably, cell death has only been described during synovial joint development in mouse digits (Hall 2015).

Because emydid embryos exhibit similar patterns of embryo motility and muscle contraction as chicken (e.g. *Terrapene carolina*, Tuge 1931), it is reasonable to assume that differentiation of synovial joints also requires muscle contraction in *T. ornata* and *E. blandingii*. Thus, the independent evolution of a complex synovial joint on the dorsal scapula of these species likely involved similar changes in epigenetic tissue remodeling. To our knowledge, *de novo* development of synovial joints has not been experimentally induced by altering gene expression of skeletal cells. Instead, morphological variation in developing skeletal tissue is intrinsically linked to muscle contraction and growth (Nowlan et al. 2010). Therefore, evolutionary change in genes controlling size of scapula muscles is likely required for synovial joint development to occur in *T. ornata* and *E. blandingii*.

Gene Expression is Congruent with Remodeling of Cartilage Matrix

Cells in the dorsal scapula region of embryonic *E. blandingii* and *T. ornata* expressed similar sets of genes across late stages of development. These genes were differentially expressed relative to embryos of *C. picta*, which feature the ancestral state condition of the dorsal scapula

in emydid turtles (see Walker 1947; Bramble 1974; Cordero and Quinteros 2015). Patterns of differential gene expression in *E. blandingii* and *T. ornata* corresponded with cartilage remodeling processes, including degradation of glycan proteins and regulation of glycan biosynthetic signaling. These results were congruent with the observation that cartilage segmentation occurred late in embryonic development of these species.

These processes were likely not intrinsically determined in cartilage tissue of the dorsal scapula, as some differentially expressed genes were associated with extracellular matrix interactions and focal cell adhesion signaling. This finding suggests that cartilage cells responded to mechanical input from the extracellular environment. Cartilage cells cannot exert mechanical force on muscle (Muller 1990; Atchley and Hall 1991; Newman and Muller 2005). Thus, upregulation of extracellular matrix signaling was likely in response to tensional forces exerted on cartilage by scapula muscles. However, gene expression patterns at the first stage sampled in our temporal transcriptomic series, CJ20, did not reflect processes related to muscle growth and contraction. Alternatively, early mechanical stress on the dorsal scapula may have been the result of disproportionate growth of the scapula. Such growth of the scapula induces tissue remodeling in turtle with extremely flattened shells (Cordero and Quinteros 2015).

Differential Regulation of Genes Related to Muscle Contraction and Growth

Genes associated with muscle contraction and growth were differentially regulated in *E. blandingii* and *T. ornata* at stages CJ21-22. This conclusion was supported by gene enrichment analyses, which unequivocally ranked cellular and molecular functions as highest, based on proportions, out of hundreds of differentially expressed gene subsets. These results support our expectation of cartilage tissue remodeling due to species-specific differences in muscle

contraction and growth. Larger muscle size in *E. blandingii* and *T. ornata* should translate into greater mechanical force exerted on cartilage via muscle contraction. Adults of these species feature substantially larger scapula muscles compared to *C. picta* (Bramble 1974). Size and morphology of scapula muscles in adult emydids is determined during stages of development sampled in this study (see Walker 1947). By hatching, scapula mass as a whole is substantially greater in *T. ornata* relative to *C. picta* (Cordero and Quinteros 2015). Differential regulation of muscle contraction and growth genes coincided with acceleration of tissue cartilage remodeling in *T. ornata* and *E. blandingii*. Furthermore, the main site of tissue remodeling in *T. ornata* was the attachment point for the primary musculature of the anterior scapula.

Evidence of Cell Death in Cartilage Remodeling

Remarkably, our analyses strongly suggest initiation of synovial joint development in conjunction with cell death in the dorsal scapula of *T. ornata*. Cell death plays a key role early in the differentiation of distal limb elements, such as interdigital webbing in chicken and synovial joints of digits in mouse (Hall 2015). Here, for the first time we demonstrate a potential role of cell death in differentiation of synovial joints of the proximal limb system of vertebrates. Our comparative transcriptomic analyses revealed differences in regulation of genes comprising various signaling pathways that regulate cell death. This finding is exceptional because cell death has not been detected during development of other synovial joints, not even in model laboratory systems.

Though it was not feasible to label characteristic enzymes associated with activity of cells involved in cell death, we did detect large mononucleated and multinucleated cells in areas undergoing turnover of cartilage matrix. The characteristics and location of these cells are typical

of cartilage-deconstructing cells, or chondroclasts (Hall 2015). Histological evidence of cell death in *E. blandingii* was equivocal and with fewer gene subsets related to apoptotic processes. This is likely because the dorsal scapula in this species only features one zone of cartilage segmentation giving rise to the suprascapula, and the rate of cartilage remodeling appeared slower. By comparison, the dorsal scapula of *T. ornata* features two zones of cartilage segmentation, giving rise to the suprascapula and episcapula.

Epigenetic “Side-effects” and Repeated Phenotypic Evolution

Many articulating skeletal surfaces have the potential to form synovial joints, but this would require the proper mechanical stimulus (e.g. the epigenetic side-effect hypothesis; Newman and Muller 2005). Thus, the capacity for synovial joints to form may not be inherited *per se* but rather acquired as a “side-effect” of evolutionary change in muscle connections that could provide the required mechanical stimulus. The roles of such complex tissue interactions in embryonic development have been recognized for decades (de Beer 1958; Maderson 1975; West-Eberhard 2003). Still, until recently, empirical data framing their relevance to developmental genetics of skeletal traits had not been presented (see Young and Badyaev 2007).

Phenotypic innovation often arises via change in developmental rates of pre-existing embryonic structures, presumably due to evolutionary change in underlying genetic architecture (de Beer 1958; Arnold et al. 1989; Muller 1990). However, origination of novel adaptive traits has proven difficult to quantify (Goldschmidt 1940; Frazzetta 1970, 1976; Muller 1990; Theissen 2009; Wagner 2014). Repeatedly-evolving structures may arise via evolutionary change in genes controlling developmental rates. In turn, those changes could trigger morphogenetic processes that alter spatial dimensions and physical interactions of anatomical modules in embryos (Muller

1990; Newman and Muller 2001; Muller 2003; Newman and Muller 2005; Wagner 2014). The phenotypic outcome of such complex tissue-tissue interactions could enhance functionality of adult phenotypes subjected to natural selection in evolving populations. Development of adaptive traits in this manner is considered a form of epigenetic tissue remodeling, because it does not involve evolutionary change in nucleotide sequences of genes governing development of the trait in question. This claim is supported by the observation that many structurally complex traits initiate differentiation late in embryogenesis, such as in emydid turtles with shell-closing systems. Gene mutations that could induce such transformation, without mechanotransduction of genetic pathways, would have high pleiotropic effects and would not persist in natural populations (Newman and Muller 2005; Young and Badyaev 2007).

In this study, we demonstrated that turtle species with independently-evolved complex shell-closing systems express similar gene regulatory networks during development of a highly derived synovial joint on the scapula. We conclude that repeated evolution of this trait is likely underpinned by parallel epigenetic change in development. Epigenetic tissue remodeling, as a form of developmental plasticity, may play a greater role in repeated phenotypic evolution than previously appreciated.

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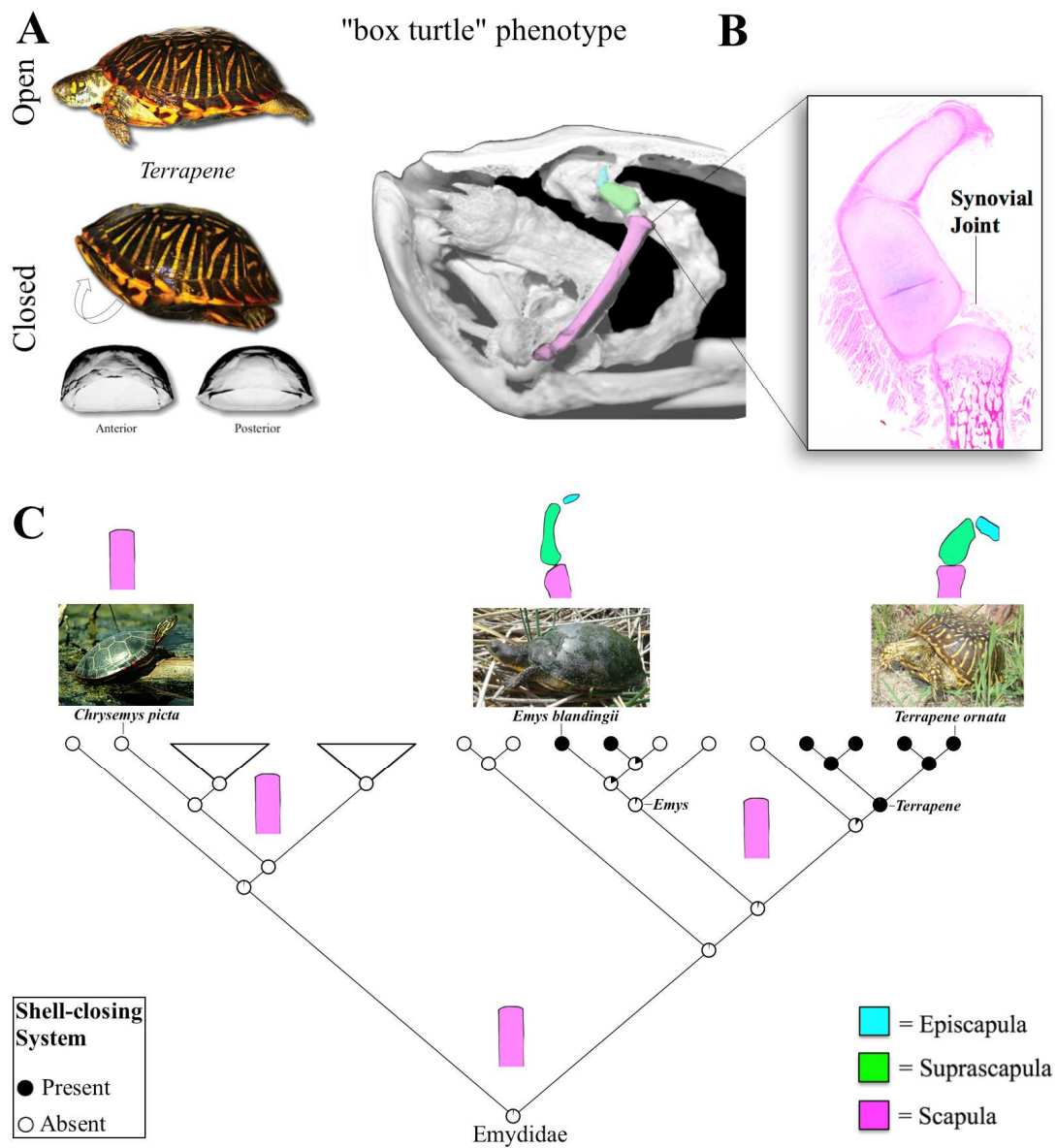


Figure 1. The shell-closing system of box turtles (*Terrapene*) (**A**) is characterized by a unique synovial joint articulation on the scapula (**B**). Maximum likelihood ancestral state reconstruction suggests this skeletal specialization has evolved twice in emydid turtles, *E. blandingii* and *T. ornata*, with shell-closing systems; phylogeny after Guillon et al. (2012) (**C**).

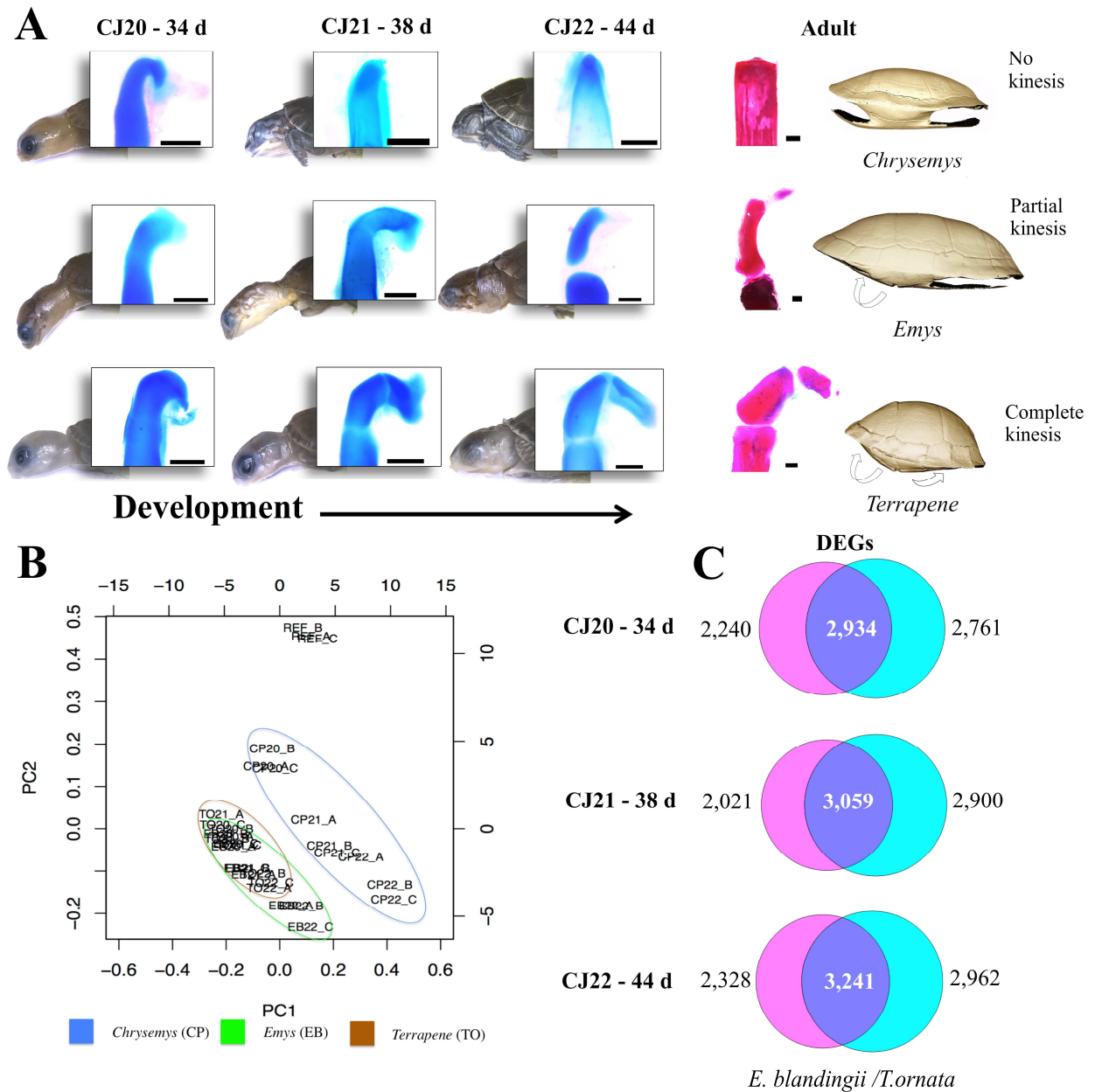


Figure 2. During early development of a synovial joint articulation, the dorsal scapula in emydid turtle embryos undergoes segmentation; embryos of *Chrysemys picta*, *Emys blandingii*, and *Terrapene ornata* at Cordero and Janzen (2014; CJ) stages 20-22 (A). The structural complexity of the joint is correlated with shell-closing capacity (i.e. shell kinesis) in adult emydid turtles. Based on a PCA on gene expression data, dorsal scapula tissues of emydid turtles, at CJ stages 20-22,

clustered according to species and developmental stage; each experimental condition was represented by three biological replicates (A-C), including a reference control tissue (REF) (**B**). Transcriptomes of *E. blandingii* and *T. ornata* shared a moderate proportion (50-60%) of differentially expressed genes (DEGs), relative to *C. picta*, across CJ 20-22 stages (all P 's < 0.0001; Fisher's exact tests) (**C**).

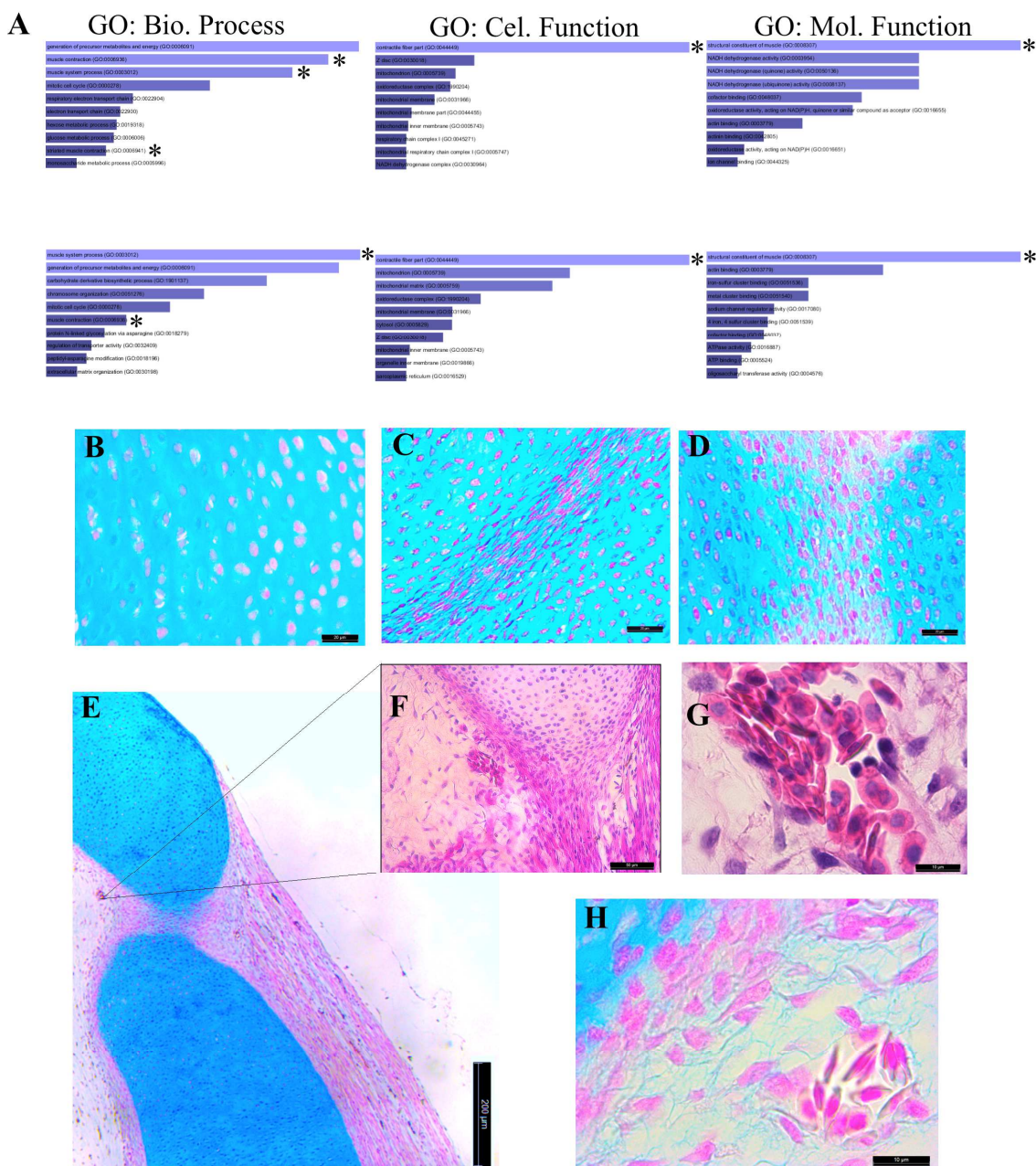


Figure 3. *Emys blandingii* and *T. ornata* shared gene subsets that were enriched for muscle-specific (*) gene ontology (GO) terms, including specific cellular and molecular functions at stages CJ21 (above) and CJ22(below) (**A**). GO terms for muscle contraction, striated muscle contraction, and muscle system biological processes were overrepresented. The highest-ranking

overrepresented GO terms were for contractile fiber part and structural constituent of muscle cellular and molecular functions, respectively; terms are ranked by statistical significance based on *P* values of Fisher's exact tests. By CJ21, the primordial interzone of a synovial joint, not present in *C. picta* (**B**), had differentiated on the dorsal scapula of *E. blandingii* (**C**) and *T. ornata* (**D**). The dorsal scapula of *T. ornata* underwent substantial remodeling, potentially via cell death (**E**). This was evident by the presence of chondroclast-like cells in the periphery of the synovial joint interzone (**F-H**).

Table 1. Differentially regulated genes in *E. blandingii*, relative to *C. picta*, were enriched for gene ontology (GO) terms of up to four apoptosis-related processes. By contrast, differentially regulated genes in *T. ornata*, relative to *C. picta*, were enriched for GO terms of up to 17 apoptosis-related processes. + = enriched pathway at given stage of development, based on Fisher's exact tests with $P < 0.05$; * = marginal statistical significance at $P = 0.05-0.07$.

Apoptotic Process	<i>E. blandingii</i>			<i>T. ornata</i>		
	CJ20	CJ21	CJ22	CJ20	CJ21	CJ22
Apoptotic signaling pathway (GO:0097190)				+	+	
Cellular component disassembly involved in execution phase of apoptosis (GO:0006921)					+	+
Intrinsic apoptotic signaling pathway (GO:0097193)					+	+
Intrinsic apoptotic signaling pathway by p53 class mediator (GO:0072332)				+		
Intrinsic apoptotic signaling pathway in response to oxidative stress (GO:0008631)			+			
Negative regulation of apoptotic signaling pathway (GO:2001234)	+			+		
Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043154)					+	
Negative regulation of extrinsic apoptotic signaling pathway via death domain receptors (GO:1902042)						+
Negative regulation of intrinsic apoptotic signaling pathway (GO:2001243)		*			*	
Negative regulation of neuron apoptotic process (GO:0043524)						+
Positive regulation of apoptotic signaling pathway (GO:2001235)					*	

Table 1 continued

Positive regulation of extrinsic apoptotic signaling pathway (GO:2001238)			+	
Positive regulation of neuron apoptotic process (GO:0043525)			+	
Regulation of apoptotic signaling pathway (GO:2001233)	+	*	+	+
Regulation of cysteine-type endopeptidase activity involved in apoptotic process (GO:0043281)	+		*	+
Regulation of extrinsic apoptotic signaling pathway (GO:2001236)			+	
Regulation of extrinsic apoptotic signaling pathway via death domain receptors (GO:1902041)				+
Regulation of neuron apoptotic process (GO:0043523)				*
				+

Supplementary Information

S-Table 1. Summary of RNA sample conditions and processing; CP = *Chysemys picta*, EB = *Emys blandingii*, TO = *T. ornata*, and REF = reference control; Biological replicates = A-B at stages 20-22.

Sample ID	Tissue dissection date	RNA extraction date	RIN	Raw sample concentration (ug/ul)	Adjusted concentration for sequencing (ug/ul)	Total no. of reads
CP20-A	12-Jul-12	5-Aug-14	10	0.301	0.05	17,715,742
CP20-B	17-Jul-12	5-Aug-14	10	0.1664	0.05	20,227,797
CP20-C	17-Jul-12	5-Aug-14	10	0.85	0.05	20,887,553
CP21-A	28-Jul-14	6-Aug-14	8	0.054	0.1	23,115,270
CP21-B	28-Jul-14	6-Aug-14	8	0.059	0.1	21,490,744
CP21-C	28-Jul-14	6-Aug-14	8	0.075	0.1	5,965,348
CP22-A	5-Aug-14	7-Aug-14	10	0.363	0.1	22,663,523
CP22-B	5-Aug-14	7-Aug-14	10	0.519	0.1	8,705,599
CP22-C	5-Aug-14	7-Aug-14	10	0.4407	0.1	6,804,917
CPREF-A	28-Jul-14	7-Aug-14	10	0.2267	0.1	18,668,006
CPREF-B	28-Jul-14	7-Aug-14	10	0.6113	0.1	28,371,148
CPREF-C	28-Jul-14	7-Aug-14	10	0.8686	0.1	24,722,970
EB20-A	13-Jul-12	11-Aug-14	9.1	0.437	0.1	14,610,120
EB20-B	13-Jul-12	11-Aug-14	9.1	0.2773	0.1	16,125,718
EB20-C	13-Jul-12	11-Aug-14	9.3	0.619	0.1	25,209,403
EB21-A	20-Jul-13	11-Aug-14	9.3	1.43	0.1	34,120,056
EB21-B	20-Jul-13	11-Aug-14	9.6	1.3	0.1	23,543,238
EB21-C	20-Jul-13	11-Aug-14	9.2	1.009	0.1	6,182,246
EB22-A	24-Jul-13	11-Aug-14	8.7	2.096	0.1	13,358,521
EB22-B	24-Jul-13	11-Aug-14	9.2	2.587	0.1	15,017,190
EB22-C	24-Jul-13	11-Aug-14	8.2	2.018	0.1	10,672,631.
TO20-A	16-Jul-13	12-Aug-14	10	0.487	0.1	20,000,920
TO20-B	16-Jul-13	12-Aug-14	9.2	0.497	0.1	19,923,985
TO20-C	16-Jul-13	12-Aug-14	9.5	0.409	0.1	26,693,902
TO21-A	20-Jul-13	12-Aug-14	10	0.367	0.1	23,543,790
TO21-B	20-Jul-13	12-Aug-14	9.8	0.343	0.1	17,809,194
TO21-C	20-Jul-13	12-Aug-14	9.6	0.3335	0.1	4,685,222
TO22-A	18-Jul-14	12-Aug-14	9.4	1.086	0.1	7,779,962

TO22-B	18-Jul-14	12-Aug-14	9.3	0.874	0.1	12,744,412
TO22-C	18-Jul-14	12-Aug-14	9.2	1.286	0.1	22,771,506

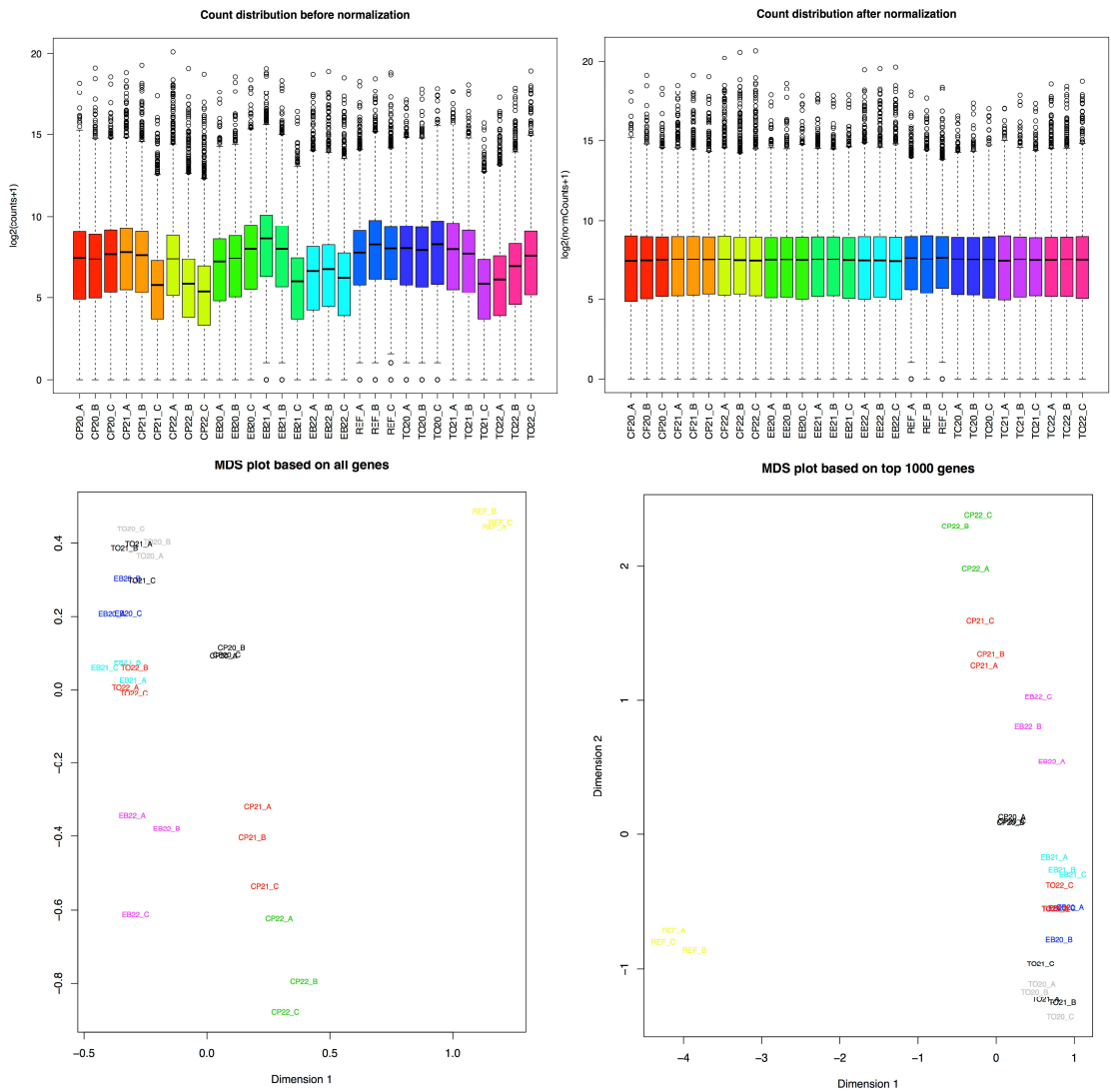
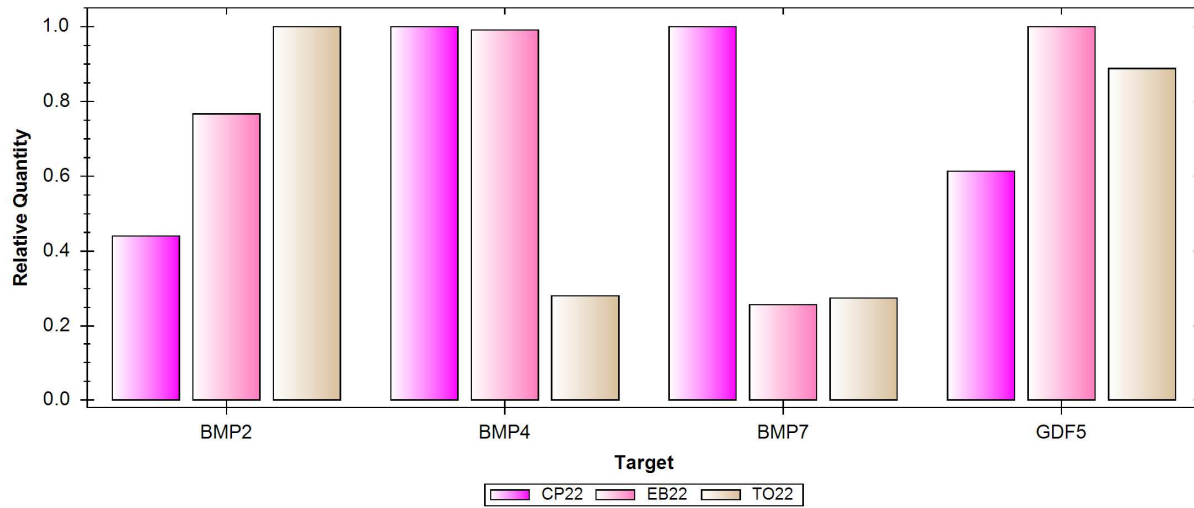


Figure 1. Above: Read count distributions of libraries before (left) and after (right) normalization. Below: multidimensional scaling of libraries on all reads (left) and top 1000 differentially expressed genes (right); Libraries generally clustered according to experimental condition and biological replicate.



S-Figure 2. Validation of differential gene expression, via real-time PCR, of key signaling molecules involved in cartilage growth and remodeling in *C. picta*, *E. blandingii* and *T. ornata* at stage 22. Analyses focused on members of the transforming growth factor beta gene family: BMP2, BMP4, BMP7, and GDF5. Although these were present in our differentially expressed gene lists, we only report marginal statistically significant mean differences for BMP4 and BMP7 using Wilcoxon/Kruskal-Wallis tests (P 's = 0.06).

CHAPTER 5. GENERAL CONCLUSIONS

Phenotypic innovation and repeatability have and will continue to be at the forefront of conceptual debates in evolutionary biology. My dissertation research is an important contribution towards understanding these key concepts. By combining classical embryology, comparative anatomy, phylogenetic comparative methods, and genomics, I illuminated developmental underpinnings of phenotypic innovation and repeatability in turtles. I conducted two core studies demonstrating the critical role of developmental plasticity in the evolution of novel and recurring skeletal traits of vertebrate animals.

In Chapter 2, I first reevaluated embryonic development in the painted turtle (*Chrysemys picta*), a reemerging model turtle species in evo-devo (see Appendix A). Embryonic development of this species had not been critically examined in more than three decades. Consequently, results of classical studies had been misinterpreted in the modern literature, particularly by authors who claimed that embryonic development in the painted turtle was not representative of the general turtle condition. I disproved this conjecture by meticulously illustrating nearly the entire developmental series of this reptile. Furthermore, my study was the first to employ scanning electron microscopy to examine a growth series of a turtle innovation, the carapacial ridge, which initiates shell development.

Crucially, unlike in classical embryological studies, my observations were recorded under highly controlled laboratory conditions, which rendered duration of developmental stages, embryo growth rate, and length of incubation period predictable. In addition to being a timely contribution to the field of evo-devo, Chapter 2 provided an essential foundation for the rest of

my dissertation. Important information on embryogenesis of the painted turtle enabled the successful implementation of laboratory studies in Chapter 3 and Chapter 4.

Over the last 210 million years, turtles successfully colonized and adapted to freshwater, marine, and terrestrial environments worldwide. This ecological diversification is reflected in the remarkable shell variation of living species, suggesting that processes governing the formation of the turtle body plan have the potential to evolve. In Chapter 3, I conducted the most phylogenetically comprehensive examination of turtle development of modern times. I established that evolutionary change in developmental processes related to morphological diversity in the Cryptodira sub-order occurs after ~60% of embryogenesis is completed. Notably, I demonstrated that the evolution of complex shell forms is underpinned by correlated changes late in development of the shoulder blade and shell.

In an unprecedented effort, I rigorously examined and compared embryonic development in the most structurally derived species: box turtles and softshell turtles. Box turtles of the Emydidae family are characterized by extraordinarily intricate shell-closing systems, which feature a disarticulating shoulder blade. This is an innovation with respect to the ancestral condition of tetrapod animals because it enhances withdrawal of extremities by allowing temporary displacement of the shoulder blade. I found that this unique function is enabled by the development of a complex skeletal element, the suprascapula. By making comparisons to the painted turtle, I showed that the suprascapula is a terminal addition to the developmental sequence of the box turtle shoulder girdle. Notably, the suprascapula was secondarily derived via remodeling, i.e. developmental plasticity, of skeletal tissue.

In Chapter 3, I also showed that a similar transformation is likely associated with truncated growth of the shoulder blade in softshell turtles with extremely flattened shells.

Findings of Chapter 3 advanced the frontiers of evo-devo by underscoring that: 1) developmental plasticity is crucial to the adaptive potential of developmentally constrained organisms; 2) developmental change giving rise to phenotypic innovation may occur exceptionally late in embryogenesis; and 3) the potential for turtles to undergo morphological diversification is greater than previously appreciated. Lastly, I described, for the first time, an unusual means by which the limb system of tetrapod animals can undergo adaptive diversification.

Chapter 4 was a follow-up of intriguing discoveries described in Chapter 3. Specifically, I addressed the repeated evolution of the box turtle suprascapula. In this study I focused on three emydid species: the painted turtle, ornate box turtle, and Blanding's turtle. I used ancestral state reconstruction methods to robustly support the hypothesis that Blanding's and ornate box turtles independently evolved shell-closing systems with moveable suprascapulae.

The repeated evolution of shell-closing systems is associated with terrestrial or semi-terrestrial ecological niches in turtles. The ornate box turtle is primarily terrestrial and is able to completely conceal extremities via activation of its shell-closing system. The Blanding's turtle is seasonally terrestrial and is considered an intermediate in terms of form and function of its less derived shell-closing system. The aquatic painted turtle resembles the general turtle condition with no shell-closing system. Thus, comparing development in these species provided an excellent framework to test one of the most fascinating questions in modern biology: Do unrelated species deploy similar genetic solutions to similar ecological problems?

I conducted an RNA-Seq experiment to answer this question. I employed a balanced-block experimental sequencing design to generate 30 cDNA libraries that represented gene expression profiles of scapula tissue across three stages of development in these three turtle species. This design also included tail tissue as a reference. Gene expression profiles of the

developing suprascapula in Blanding's and ornate box turtles were highly similar relative to the profile of the homologous tissue in painted turtles. Further exploration of differentially regulated genes still revealed high similarity in species with shell-closing systems that independently evolved a suprascapula. I used histological analyses to further support that similarity in gene expression profiles was not due to these species being closely related, relative to the painted turtle. I demonstrated evidence of extensive remodeling of the extracellular protein matrix of the developing scapula and suprascapula. Moreover, I provided evidence of specification and early morphogenesis of a complex synovial joint in Blanding's and ornate box turtles. This skeletal trait is not present in the painted turtle.

The evidence I presented in Chapter 4 suggests that the origins of phenotypic innovation are epigenetically induced. Specifically, tensional forces exerted by hypertrophied shoulder blade musculature in box turtles likely triggered skeletal remodeling, thereby initiating secondary differentiation of the suprascapula. I provided evidence of upregulation of genetic pathways that would be involved in such a process. These results are noteworthy because they support the *epigenetic side-effect hypothesis*, which posits that early steps in adaptive Darwinian evolution are likely facilitated by developmental plasticity of skeletal tissue. Thus, phenotypic innovation and repeatability may often arise as an epigenetic byproduct of complex tissue-tissue interactions. For the first time, I provided evidence to support this theory in a non-model laboratory organism.

The novel insights provided by my work warrant additional studies on: unraveling the potential for the enigmatic turtle body plan to undergo adaptive diversification, elucidating epigenetic control of skeletal differentiation of vertebrate animals, and clarifying the role of developmental plasticity in evo-devo.

Future studies should aim to explore molecular epigenetic mechanisms associated with developmental plasticity of skeletal tissues. At the same, examining *cis*-regulatory DNA sequences of genes controlling skeletal development is needed to address the alternative hypothesis that repeated phenotypic evolution in Blanding's and box turtles is due primarily to evolutionary change in underlying genetic architecture. Also, development in other close relatives of these species needs to be examined. Labeling the expression domains of key developmental genes, while experimentally assessing their function, could strengthen these research pursuits.

My dissertation research succeeded in achieving a highly ambitious objective - to link phylogenetic patterns of phenotypic innovation and repeatability with underlying developmental processes. In conclusion, phenotypic innovation and repeatability is often the end result of complex adaptive and developmentally plastic processes operating late in ontogeny.

APPENDIX A

REVIEW ON EVO-DEVO OF THE PAINTED TURTLE

From a paper published in Evolution & Development

Gerardo Antonio Cordero

Table 1. Literature on direct observations of developmental patterns and processes in the Painted Turtle (*Chrysemys picta*^a) reviewed, in part^b, by Ewert (1985).

Developmental Pattern/Process	Source
Cellular RNA distribution	Vervonek 1954
Cell division/proliferation (<i>in vitro</i> ; adult tissue)	Wolf et al. 1960 ^b Cunningham 1923; Pasteels 1937; Pasteels 1957a, 1957b
Cleavage	
Developmental gene expression	
Adrenal-kidney-gonad complex	Reviewed in Valenzuela et al. 2013 ^b Lynn and Von Brand 1945; Packard et al. 1983; Packard and Packard 1986 ^b ; Peterson and Kruegl 2005 ^b Agassiz 1857; Munson 1904; Thing 1918; Harris 1945 ^b ; Fisk and Tribe 1949
Embryonic metabolism	Brachet 1914; Cunningham 1923; Pasteels 1937; Nelsen 1953; Pasteels 1957a, 1957b
Extraembryonic tissues	
Gastrulation	

Neurulation
Organogenesis

	Crotty-Davenport 1896 ^b ; Brachet 1914; Ganfini 1914 ^{b, c} ; Nelsen 1953
Brain	Warren 1911; Bailey 1916; Johnston 1916 ^b ; Larsell 1919 ^b ; Holgrem 1925; Larsell 1932; Kallen 1951
Ear	Smith 1914; Fineman 1915; Keibel 1916; von Alten 1916; Terry 1919 ^b ; Gill 1930 ^b ; Toerien 1965 ^b
Eye	Bremer 1921 ^b
Nose	Seydel 1896; Brookover 1915 ^b ; Thater 1910; Parsons 1959
Endocrine glands	Zeleny 1901 ^b ; Baumgartner 1916 ^b ; Johnson 1922; Shaner 1926
Stomach	Sjogren 1945
Upper Intestines	Agassiz 1857; Brachet 1914
Lower Intestines	Moens 1911; Shaner 1925
Kidney	Allen 1906a, 1906b; Burlend 1912, 1913; DeRyke 1925; deWalsche 1929
Liver and pancreas	Shaner 1925; Siwe 1929
Heart and arteries	Shaner 1920 ^b , 1921; Johnson 1922; Kimball 1923; Bremer 1924 ^b ; DeRyke 1925; Shaner 1925; Rojas 1931; Burda 1965 ^b ; Hart 1968; Jamniczky and Russell 2008 ^b
Lung	Hesser 1905; Miller 1904; Jordan 1917; Broman 1940
Larynx	Edgeworth 1919 ^b
Gonads	Allen 1906a, 1906b, 1907, 1911; Dustin 1910; Schwarzkopf and Brooks 1987 ^{b, d} ; Ewert and Nelson 1991 ^b ; Etchberger et al. 1992 ^b ; Kitana and Callard 2008 ^b

Skeletal Development

Carapace	Vallen 1942; Yntema 1970 ^b
Plastron	Vallen 1942
Limbs	Holgrem 1933 ^b ; Vallen 1942; Walker 1947; Yntema and Borynsenko 1971; Fallon and Cameron 1977; Fallon and Crosby 1977; Burke and Alberch 1985 ^b ; Burke and Feduccia 1997 ^b
Skull	Edgeworth 1911 ^b ; Smith 1914; Shaner 1926; Brock 1929 ^b
Vertebrae	Mookerjee and Mukherjee 1934

^a *Chrysemys picta* is taxonomically synonymous with *Chrysemys cinerea*, *Chrysemys dorsalis*, and *Chrysemys marginata* species epithets used in the literature during 1857-1970.

^b Not reviewed in Ewert (1985), see references section in this paper for complete citation.

^c Discussed and cited in Yntema (1966).

^d Embryos of the Painted Turtle were examined and staged, using limb characters, to estimate the thermosensitive period of gonadal differentiation.

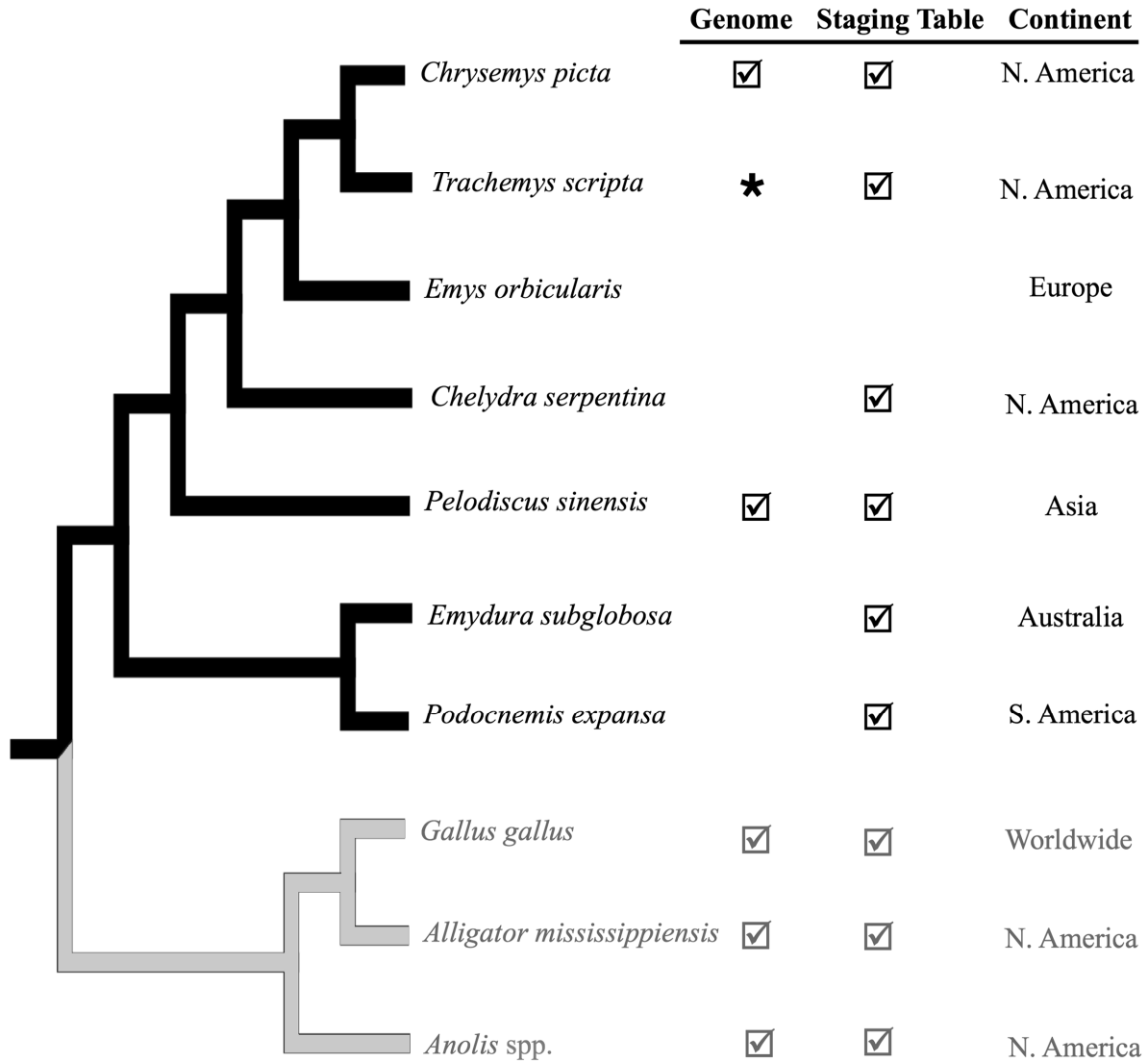


Fig. 1. Geographically-based reference species (labeled in black) for the study of evo-devo in turtles. Highly divergent species have both sequenced genomes ([*C. picta*, Shaffer et al. 2013] [*C. mydas* and *P. sinensis*, Wang et al. 2013]) and developmental staging tables ([*C. picta*, Mahmoud et al. 1973; Cordero and Janzen 2014] [*C. mydas*, Miller 1985; *P. sinensis*, Tokita and Kuratani 2001]). Other reference species feature developmental staging tables ([*T. scripta*, Greenbaum 2002] [*C. serpentina*, Yntema 1968] [*E. subglobosa*, Werneburg et al. 2009] [*P. expansa*, Danni et al. 1990]) and an embryonic transcriptome (**T. scripta*, Kaplinsky et al. 2013).

Outgroup amniote clades (labeled in grey) have been or are currently part of genome sequencing projects (reviewed in Rowan et al. 2011) and feature developmental staging tables ([*G. gallus*; Hamburger and Hamilton 1951] [*A. mississippiensis*; Ferguson 1985] [*Anolis*; Sanger et al. 2008]). Phylogenetic relationships were modeled after Chandler and Janzen (2009), Shen et al. (2011), and Crawford et al. (2012).

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