Embryology of the Gekkota: Insights into the Evolution and Development of Morphological Diversity

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EMBRYOLOGY OF THE GEKKOTA: INSIGHTS INTO THE EVOLUTION AND DEVELOPMENT OF MORPHOLOGICAL DIVERSITY

by

Aaron Harp Griffing, B.A., M.S.

A Dissertation submitted to the Faculty of the Graduate School, Marquette University, in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

Milwaukee, Wisconsin

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Understanding the patterns and processes which result in morphological diversity is a central goal of evolutionary developmental biology or evo-devo. This diversity encompasses morphologies which have evolved numerous, independent times within a clade (evolutionary convergence) and others which have evolved only once within a clade (i.e. evolutionary novelty). Though many of these diverse morphologies exhibit functional capabilities and are intertwined with the ecology and diversification of the evolutionary lineage in question, their origins are largely unknown. Robust phylogenetic analysis, genomic information, and detailed developmental data are critical lines of evidence to explain the evolutionary and ontogenetic origins of morphological novelty and convergence. The central goal of my dissertation is to understand how development has changed over evolutionary time to produce convergence within the morphologically diverse and species-rich clade of gecko lizards (Infraorder Gekkota). To address this goal, I first characterized the embryonic development of the mourning gecko (*Lepidodactylus lugubris*): a parthenogenetic species which exhibits a unique compliment of derived and ancestral character states. Second, I investigated embryonic pigment development between gecko species with different activity times. Third I investigated the development of morphological novelty, the adhesive pads on the tails of New Caledonian crested geckos (*Correlophus ciliatus*), which has converged in function with a different gecko structure, the adhesive pads found on the digits of many gecko species. Finally I investigated the developmental processes which result in morphological convergence: repeated independent evolution of interdigital webbing retention in southeast Asian gliding geckos (Gekkonidae). Collectively, these data demonstrate how developmental patterns are altered to produce convergently evolved morphologies.
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Aaron Harp Griffing

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“The eggs of lizards do kill speedily, except there come a remedy from falcon’s dung and pure wine.”

Edward Topsell,

*The History of Serpents* (1608)
I. INTRODUCTION: THE GEKKOTA AS A MODEL CLADE TO STUDY MORPHOLOGICAL DIVERSITY

The immense morphological diversity exhibited by animals has captivated biologists for centuries (e.g. Cuvier 1816; Owen 1848; Darwin 1859; Osborn 1902; Alberch 1980; Carroll 2001). Despite this, the numerous patterns and processes which give rise to “endless forms most beautiful” *sensu* Darwin 1859) remain poorly understood. As organisms evolve and diverge from one another, morphological structures are expected to also diversify; however, morphological phenotypes sometimes evolve independently, numerous times (evolutionary convergence, homoplasy; Wake et al. 2011). These repeated events provide the opportunity to investigate parameters of adaptation and selection in a natural, evolutionary “experiment” and how development changes in an evolutionary time scale (Losos 2011; Mahler et al. 2017). Examination and identification of developmental patterns which underlie a morphological character in question is required for understanding the origins of this diversity (Raff 2000). By studying replicated evolutionary events, we can begin to understand how phenotypic diversity is limited (i.e. constrained) through either conservation or derivation of spatial, temporal, and genetic pathways in development (Alberch 1982; Maynard-Smith et al. 1985; Hall 1992). This evolutionary developmental (evo-devo) approach to studying morphology involves three phases: 1) quantification of morphological variation of adult organisms, 2) identification of candidate developmental causes, and 3) functional studies of genes and pathways (Mallarino and Abzhanov 2012). Through integrative exploration of these three phases, one can understand organismal evolution of a phenotype in
question, its genotypic background, and how the phenotype is selected upon by extrinsic pressures (Riedl 1978; Gould and Lewontin 1979; Cheverud 1982).

Evo-devo has been in practice for well over 50 years and has focused on innumerable species and evolutionary lineages (e.g. de Beer 1951; Gould 1977; Alberch et al. 1979). However, foundations of modern vertebrate evo-devo were built on a limited handful of “traditional model” species in developmental biology. Due to their tractability in laboratory settings and devoted communities of scientists, zebrafish (Danio rerio), clawed frogs (Xenopus laevis), chickens (Gallus gallus), and laboratory mice (Mus musculus) have served as the core species in developing vertebrate evo-devo theory and methods (Hopwood 2011; Bolker 2014; Minelli and Baedke 2014). More recently, these theories and methods created for “traditional models” have been co-opted to study other vertebrate species in an evo-devo context, such as threespined sticklebacks (Gasterosteus aculeatus; Cresko et al. 2006), Galapagos finches (Geospiza spp.; Mallarino et al. 2011) and anole lizards (Anolis spp.; Sanger and Kircher 2017). An inherent setback to these well-studied models is the massive evolutionary scale in which comparisons can be made. A comparison between a chicken, a mouse, and a zebrafish model would allow identification of conserved developmental patterns across broad evolutionary scales, such as all vertebrates or restricted to amniotes. Conversely, identifying derivations from developmental patterns using this taxon sampling would be much more difficult. Ideally, when investigating an evo-devo question, an ideal comparative framework would involve several species, preferably in a much younger evolutionary lineage (clade) which exhibit a range of phenotypic diversity (Sanger and Rajakumar 2019). This type of “model clade” approach allows for polarization of ancestral character states and interrogation of
subtle differences in development (i.e. tinkering) that can produce a range of downstream morphological changes (Jacob 1977; Müller 1990; Sanger and Rajakumar 2019). With a comparative framework such as this, coupled with robust phylogenetic hypotheses, genomic information, and detailed developmental data, one can create a high-resolution investigation into the evolution and development of morphological diversity. Herein, I argue gekkotan lizards represent an ideal model clade and propose employing a “model clade” approach to study morphological diversity in lizards.

Life in the Trees, on the Rocks, on the Ground, and in the Leaves

Gekkotan lizards (commonly referred to as geckos) are a clade of over 2,100 described species of squamate reptiles (lizards and snakes) that are distributed across every continent, excluding Antarctica (Kluge 1967a; Uetz et al., 2021). Geckos are the sister clade to all other lizards and snakes, with the possible exception of the poorly known, limbless, dibamids, whose phylogenetic position remains unresolved (Wiens et al. 2012; Pyron et al. 2013; Reeder et al. 2015; Zheng and Wiens 2016; Burbrink et al. 2020; Signhal et al. 2021; Fig. 1a). Within the Gekkota, seven families are recognized: the Gekkonidae, Phyllodactylidae, Sphaerodactylidae, Eublepharidae, Diplodactylidae, Carphodactylidae, and Pygopodidae (Gamble et al. 2008, 2015a; Fig 1b). These lineages share a most recent common ancestor approximately 120 million years ago (Gamble et al. 2015a) and since that time, evolved a suite of diverse morphologies, ecologies, and physiologies. Geckos are found in both mesic and xeric environments in the tropics (e.g. Dial and Grismer 1992). In these environments, they inhabit the gamut of terrestrial niches: terrestrial, detriticolous, rupicolous, fossorial, and arboreal lifestyles (e.g. How et al. 1986; Shine 1986; Vitt et al. 2005; Russell and Delaugerre 2017).
Figure 1.1. Phylogenetic hypotheses for the Gekkota. A) Time-calibrated phylogeny of the Squamata following the hypothesis of Zheng and Wiens (2016). Vertical width of each clade corresponds to relative species-richness. B) Genus-level phylogeny of the Gekkota following the hypothesis of Gamble et al. (2015a). Each gekkotan family is highlighted by a different shade of purple or orange and is represented by a single photographed gecko demonstrating morphological diversity. Photo representatives courtesy of Dr. Stuart Nielsen. MYA, millions of years.
Although the general gecko body plan is considered similar to the ancestral squamate form, there are many specialized derivations which make geckos a fascinating clade to study (Conrad 2008). With their diversity of ecologies comes a suite of morphological adaptations. To facilitate the navigation through complex environments, geckos range in size from some of the smallest known tetrapod vertebrates, such as the Jaragua sphaero (*Sphaerodactylus ariasae*, 17mm snout–vent length [SVL]; Hedges and Thomas 2001) to over 14-times larger in the New Caledonian Giant Gecko (*Rhacodactylus leachianus*, 240 mm SVL; Bauer and Russell 1986). Depending on their habitat, geckos may evolve longer or shorter limbs to aid in climbing and perching on substrate (e.g. Higham and Russell 2010; Grismer et al. 2015). Some arboreal taxa use specialized prehensile tails to grasp branches and some evolved flappy, parachute-like skin to glide from tree to tree (e.g. Bauer 1998; Russell et al. 2001; Young et al. 2002; Griffing et al. 2021). One lineage has even evolved elongate, limb-reduced, snake-like body forms to enable fossorial, “grass-swimming,” and “sand-swimming” locomotion (Kluge 1976a). However, arguably the most note-worthy aspect of gecko locomotion is that of adhesive toe pads, which were gained and lost independently at least 14 and six times, respectively, across gecko evolutionary history (Gamble et al. 2012; Russell and Gamble 2019).

How geckos move through their environments is not their only selective pressure for morphological diversity. The ancestor to extant geckos was likely nocturnal, necessitating derived morphologies for inhabiting low-light environments, such as trachea enabling acoustic communication (Rohtla et al. 2019), large olfactory bulbs (Schwenk 1993), and a suite of internal and size modifications to the eyes (Röll 2000a,
Additionally, several lineages have secondarily evolved diurnal activity patterns (Gamble et al. 2015a; Pinto et al. 2019a). This is accompanied by further derivation of eye morphology and accumulation of excess pigment to protect from UV exposure (Röll 2001a; Guerra-Fuentes et al. 2014; Griffing et al. 2020).

**Leveraging Robust Comparisons on Different Evolutionary Scales**

With their morphological diversity and their penchant for convergent evolution both within and outside the Gekkota, geckos offer the unique opportunity to leverage robust comparisons at the several evolutionary scales (e.g. Gamble et al. 2012, 2015a, 2015b; Griffing et al. 2018a, 2022; Gamble 2019; Pinto et al. 2019a; Glynne et al. 2020; Ramm 2020). These contrasts involve comparing gecko morphological development with that of other lizards or even other vertebrates (macroevolutionary; e.g. Griffing et al. 2022), comparing morphological development between two or more distantly related clades of gecko (mesoevolutionary; e.g. Griffing et al. 2020, 2021, 2022), or comparing morphological development between individuals of the same species or same genus (microevolutionary; e.g. Griffing et al. 2018a). These investigations into gecko morphological development are aided by recent advances in phylogenetic, genomic, and developmental resources. Gecko microcomputed tomography (μCT) data are robust new data are being generated regularly (e.g. Paluh et al. 2017; Bauer et al. 2018; Laver et al. 2020; Gurgis et al. 2021). By characterizing morphology across the Gekkota, coupled with a robust phylogenetic hypothesis serving as a comparative framework (Gamble et al. 2015a), we can begin quantifying morphological diversity at the evolutionary scale we choose (phase 1 *sensu* Mallarino and Abzhanov 2012). With a diversity of species
available in the pet trade, protocols for laboratory husbandry and embryo collection (Noro et al. 2008; Griffing et al. 2018b), and embryonic staging tables (Noro et al. 2008; Wise et al. 2009; Griffing et al. 2019, 2021), we can begin to identify candidate developmental patterns underlying morphological diversity (phase 2 sensu Mallarino and Abzhanov 2012). Finally, by using newly generated genomic and transcriptomic data (Xiong et al. 2016; Hara et al. 2018; Griffing et al. 2019; Pinto et al. 2019a), we can use molecular tools to attempt to tease apart developmental pathways and mechanisms underlying developmental patterns (phase 3 sensu Mallarino and Abzhanov 2012).

In the following work, I will use this comparative framework to investigate developmental patterns underlying several aspects of convergently evolved morphology in geckos. In chapter II, we characterized the in ovo embryonic development of the mourning gecko (Lepidodactylus lugubris), which serves as foundational reference material for the embryological work in each remaining chapter. We characterized embryos via light microscopy, µCT, and generated a high-quality embryonic transcriptome. In chapter III, we investigate the relationship between pigmentation and diel activity in geckos. We describe both the adult pigment phenotypes of several species and show that the gliding gecko (Gekko kuhli) have converged on hyperpigmented phenotypes through a derived developmental pattern. In chapter IV, we investigate the morphology and development of the adhesive tail pads of the New Caledonian crested gecko (Correlophus ciliatus). We show support for the putative serial homology of diplodactylid gecko toe pads and tail pads. Finally, in chapter V, we investigate the developmental patterns of gliding morphologies in gekkonid geckos. We use light microscopy and fluorescent apoptosis imaging to describe both conserved and distinct
patterns between convergently evolved gliding geckos. Taken together, we employ the study of adult morphology, embryonic patterns, and developmental pathways to lay the foundation of studying evo-devo of morphological convergence in geckos.
II. EMBRYONIC DEVELOPMENT OF A PARTHENOGENETIC VERTEBRATE, THE MOURNING GECKO (*LEPIDODACTYLUS LUGUBRIS*)

*Peer-reviewed and published as:*

**Introduction**

A central goal of evolutionary-developmental biology (evo-devo) is to understand the developmental changes that result in phenotypic novelty and convergence. Interest in how developmental processes have influenced morphological diversity has led to the recent investigation of squamate (lizard, snake, and amphisbaenian) development. These studies have demonstrated the immense utility of squamates as models for the development of amniote *bauplans* (e.g. Cohn and Tickle 1999; Noro et al. 2009; Nomura et al. 2015; Diaz Jr. et al. 2017; Sanger and Kircher, 2017; Infante et al. 2018; Leal and Cohn 2018). Ideally, model clades or species in evo-devo will meet several criteria (reviewed by Jenner and Wills 2007). First, the primitive character states and subsequent evolution of derived character states should be known in a phylogenetic framework. Second, the study of this clade, or species, should reveal some combination of unique, derived patterns and broad, conserved patterns. Finally, the clade or species should be practical to rear in a laboratory setting and have available resources to facilitate the investigation of evo-devo questions.

Gecko lizards (Gekkota) are a clade that exemplifies these criteria. The gecko *bauplan* is considered similar to the ancestral squamate form (Conrad 2008) while also exhibiting several derived morphologies, such as adhesive toepads, which have independently evolved numerous times within gecko evolutionary history (Russell 1979a;
Gamble et al. 2012; Russell et al. 2015; Russell and Gamble, 2019). Homoplasies (convergent character states), such as this, have been uncovered with robust phylogenetic analyses of gecko relationships (Gamble et al. 2012, 2015a, 2015b). Recent phylogenetic studies using molecular data find high support for geckos, with the exception of dibamid lizards, as the sister clade to remaining squamates (Reeder et al. 2015; Zheng and Wiens, 2016). Therefore, geckos are an integral component for studies exploring the full range of comparative squamate evo-devo. This combination of the ancestral bauplan, complex, derived morphologies, rampant convergence on numerous character states, and robust phylogenetic hypotheses make geckos the ideal group to study gecko-specific derived patterns, as well as enlighten squamate- or vertebrate-wide conserved patterns.

Logistically, many gecko species have simple protocols for their husbandry, can be reared in space-efficient enclosures, and have an increasing number of genomic resources that are available or in development (e.g. Thorogood and Whimster, 1979; Koeptli et al. 2015; Liu et al. 2015; Xiong et al. 2016; Griffing et al. 2018b; Hara et al. 2018; Vickaryous and Gilbert, 2019).

Of more than 1,800 described species of gecko (Uetz et al. 2018), the Mourning Gecko (*Lepidodactylus lugubris*) stands out as an ideal model to study developmental questions. *Lepidodactylus lugubris* is a small-bodied gecko native to Southeast Asia and nearly all Pacific islands (Bauer and Henle 1994; Röll 2002; Griffing et al. 2018b). This species belongs to the pan-Asian *Gekko* clade of gekkonid lizards, including the charismatic gliding geckos (*Ptychozoon*), flap-legged geckos (*Luperosaurus*), true geckos (*Gekko*), and slender geckos (*Pseudogekko*; Kluge 1968; Brown et al. 2012a; Gamble et al. 2015a). Like many other species in this clade, *L. lugubris* exhibits distally divided
basal toe pads, which facilitate digital adhesion (Russell 1979a). Gekkonid lizards, such as *L. lugubris*, are oviparous; they reproduce by laying hard-shelled eggs, and have a fixed clutch-size of two eggs (Bustard 1968). Arguably, the most interesting aspect of *L. lugubris* biology is parthenogenesis. This species is composed almost entirely of females which reproduce successfully without male gametes, resulting in clonal daughters that are genetically identical to mothers (i.e. obligate parthenogenesis; Cuellar and Kluge 1972). Male phenotypes are occasionally encountered in the wild or in captivity, however these individuals are extremely rare and typically infertile (Cuellar and Kluge 1972; Brown and Murphy-Walker 1996; Röll and von Düring 2008). Numerous clonal lineages have been described for *L. lugubris*, each of which are considered to have been derived from separate hybridization events between *Lepidodactylus moestus* and an as of yet undescribed *Lepidodactylus* species from the South Pacific (Radtkey et al. 1995). Interestingly, backcrosses between diploid (*2n=44*) *L. lugubris* lineages and one of the parental species can result in triploid (*3n=66*) clonal lineages (Moritz et al. 1993; Radtkey et al. 1995). Thus, *L. lugubris* is a suitable model to study morphological development, phenotypic plasticity, sexual development, and the evolution of changes in ploidy.

Parthenogenetic organisms are ideal study-systems for developmental studies for several reasons: 1) every individual in a laboratory colony after reaching sexual maturity is reproductively active (i.e. no “two-fold” cost of sex; Maynard-Smith 1971), 2) there is no need for mate-pairing, and 3) individuals within a clonal lineage should theoretically be genetically identical. Despite these factors, few parthenogenetic vertebrates are routinely maintained in laboratory settings (Maslin 1971; Cole and Townsend 1977;
Several additional characteristics make *L. lugubris* a desirable model organism, including reproductive output (they are highly fecund, laying eggs year round; Brown and Sakai 1988), there are published protocols for husbandry and embryo collection (Griffing et al. 2018b), they can easily be targeted for field collection (including from populations in the Hawaiian Islands; Bauer and Henle 1994) or commercial purchase, and, unlike the majority of squamates, have hard-shelled eggs which make embryological dissection and manipulation substantially easier (Nomura et al. 2015).

Embryonic staging series, or normal plates, are valuable foundational tools for the study of new and diverse developmental model systems (Hamburger and Hamilton 1951; Schreckenberg and Jacobson 1975; Greenbaum 2002; Hopwood 2007; Sanger et al. 2008). Detailed descriptions of embryonic development facilitate effective experimental design and allow for an understanding of the timing of major developmental events. Since the first squamate embryonic staging series was generated almost 115 years ago (*Lacerta agilis*; Peter 1904), another 34 complete squamate embryonic staging series have been published (Lima et al. 2018; reviewed in Ollonen et al. 2018; van der Vos et al. 2018; Jungman et al. 2019), thus providing ample comparative material for broad investigations into trends of squamate evolution and development.

Staging tables are not the only resource needed by modern developmental biologists. Genomic and transcriptomic resources are necessary for studying spatial and temporal aspects of gene expression during development. Having sequence information for gene families, such as bone morphogenetic proteins (BMPs), which are critical for myriad functions during embryogenesis (e.g. mesoderm formation and patterning, neural
patterning, skeletal development, etc.; reviewed in Hogan 1996), will allow for the designing and generation of qPCR primers and in situ hybridization probes. Other tools, such as micro-computed tomography (μCT), have facilitated new view of staging series that can, for example, accurately trace ossification sequence of skeletal elements (Polachowski and Werneburg 2013; Werneburg et al. 2015; Ollonen et al. 2018; van der Vos et al. 2018). Despite what seems to be a renaissance of embryological work, few embryonic staging series of reptiles have utilized soft-tissue μCT imaging (Boughner et al. 2007). Using soft-tissue μCT techniques for embryological characterization provides the opportunity to non-destructively track ossification sequence, as well as, detail the subtleties of visceral, limb, craniofacial, or neural development (Gignac et al. 2016; Wong et al. 2012).

Combining light microscopy with soft-tissue μCT imaging, we herein describe the embryonic development of an emerging evo-devo model species, *L. lugubris*, and provide an annotated de novo transcriptome of a late-stage *L. lugubris* embryo as a resource for future evolutionary and developmental investigations. Data generated by this study, particularly the open-access transcriptomic and μCT data, taken together, provide ample resources for the continued study of *L. lugubris* as an evo-devo model and sets the foundation for the expansion of integrative developmental studies in squamates.

**Materials and Methods**

*Embryo Collection and Visualization*

We collected a post-ovipositional ontogenetic series of 242 embryos from a captive colony of *Lepidodactylus lugubris*, housed at Marquette University (Milwaukee, WI USA; IACUC protocol AR-279). The colony includes A and B clonal lineages as
well as both diploid and triploid clones (Griffing et al. 2018b) and we do not distinguish among them here. Incubation times of *L. lugubris* are known to range between 65 dpo and 103 dpo when incubated at 25.5°C and 22.0°C, respectively (Brown and Murphy-Walker 1992). The embryonic series in this study was incubated at approximately 26.0°C (room temperature in the Marquette University live-lizard facility) and spans 0 dpo to 77 dpo (Fig. 2.1; R Core Team 2014). Captive animals were cared for and embryos were collected following protocols described by Griffing et al. (2018b). To summarize, we checked animal enclosures daily for eggs. We dissected embryos out of eggs using #5 watchmaker’s forceps while immersed in diethyl pyrocarbonate (DEPC) treated, RNase-free 1% phosphate-buffered saline (PBS). We visualized the external morphology of embryos under a Nikon® SMZ 74ST stereoscope and a Zeiss V16 with Axiocam 305 camera. We visualized three embryos from stages 30, 31, and 32 using whole-mount scanning electron microscopy (SEM) to verify choroid fissure closure, apical ectodermal ridge formation, and pharyngeal arch fusion. Additionally, we visualized a subset of embryos (N=13), spanning embryonic stages 30–43, using soft-tissue µCT. To demonstrate the utility of the µCT data for evo-devo research, we describe the prenatal development of the *L. lugubris* brain — a structure that is difficult to visualize three-dimensionally in embryos without careful microdissection. Voucher specimens utilized in this study are housed at Marquette University (Milwaukee, WI USA) or the Florida Museum of Natural History (Gainesville, FL USA).
Figure 2.1. The observed ranges of post-ovipositional stages defined in this study when incubated at 26°C. Black circles correspond to outliers.
Computed Tomography

We imaged a series of 29 specimens (stages 30–43) using contrast-enhanced microcomputed tomography at the University of Florida’s Nanoscale Research Facility. We soaked specimens from stages 30–41 in 0.3% Phosphotungstic Acid for one to three weeks, and 42–43 in 2.5% aqueous Lugol’s Iodine (IKI) for 4 days, following modified protocols from Metscher (2009). Prior to scanning, we embedded specimens in low-melt agar, carefully excised them into in close fitting blocks, placed the blocks into a low-density resealable plastic bag, which we then wrapped around a 2 mm diameter carbon fiber rod and placed in a drill chuck inside the computer numerical controlled stage of a dual tube GE Phoenix V|tome|X M. We scanned the specimens using the 180kV Nanofocus tube, with voltage, current, detector capture time, and rotation angles modified to optimize contrast and signal and minimize artifacts (Supplemental Material 2.1). We converted the radiographs to tomograms using Phoenix DatosOS|X reconstruction software and then segmented various regions of interest from the volumes using VGStudioMax 3.3 (VolumeGraphics, Heidelberg Germany). Tomogram stacks and metadata for all scans are available via www.morphosource.org (see Supplemental Material 2.1 for DOIs).

Diagnosing Developmental Stages

We discretized and assigned sixteen developmental stages based on external morphology using the embryonic staging series of *Eublepharis macularius* (Wise et al. 2009), *Anolis sagrei* (Sanger et al. 2008), and *Gallus gallus* (Hamburger and Hamilton 1951) as guides. We primarily diagnosed stages by qualitative traits with the exception of
somite number and mean snout-to-vent length (SVL). We calculated SVL for a sample of 118 embryos using Fiji v2.0.0 image processing software (Schindelin et al. 2012) by measuring from the tip of the craniofacial region, to hindbrain, down through the thorax, and ending immediately posterior of the hindlimb or hindlimb bud. Further investigation into development of different clone lineages may be needed to identify lineage-specific differences in development. However, in this investigation, ontogenetic variation between different clonal lineages of *L. lugubris* appears negligible; therefore, these stage diagnoses may be applied to both A and B clones as well as diploid and triploid lineages, and potentially other *L. lugubris* clone lineages (Moritz et al. 1993; Radtkey et al. 1995).

**Transcriptome Sequencing, Assembly, and Annotation**

To further enable the utility of *L. lugubris* as a developmental model, we sequenced and annotated a transcriptome from a late stage embryo. Transcriptomic resources are vital for investigations in developmental biology as they facilitate use of a wide array of methodologies, including: PCR primer design for qPCR; a reference for mapping RNAseq reads; and facilitating the design of probes for *in situ* hybridization.

We extracted RNA from a whole *L. lugubris* embryo (specimen ID: “BJP18”) at 59 dpo (Stage 42) and followed a modified protocol for extracting RNA from TRIzol™ preserved tissue (Zumbo 2011). RNAseq library preparation was identical to methods used by Pinto et al. (2019b). Briefly, we used the KAPA Stranded mRNA-Seq Kit for Illumina® (KR0960 [v5.17]) using oligo-dT beads for mRNA enrichment. We sequenced this RNAseq library on an Illumina® HiSeq 2500 at the Medical College of Wisconsin (Milwaukee, WI, USA; paired-end 125 bp reads). These reads were submitted to the NCBI Sequence Read Archive (SRA; Bioproject: PRJNA476550; Accession number:
SRP150730). We calculated quality statistics and scores from these raw data using FastQC (v0.11.6; Andrews 2010).

We assembled a de novo transcriptome using the de novo RNA-Seq Assembly Pipeline (DRAP [v1.91]; Cabau et al. 2017), which is a compilation of assembly and quality control scripts using several software packages. Briefly, raw Illumina paired-end reads were trimmed, normalized, and assembled into contigs using Trinity (v2.4.0; Grabherr et al. 2011). DRAP then filters, maps, compacts, and quality-assesses the initial Trinity assembly using a series of integrated tools. Overall, DRAP generates an assembled transcriptome with less redundancy, without compromising the completeness, or quality, of the transcriptome assembly (Cabau et al. 2017). Reference peptide sequences provided for reference mapping in all assemblies and assessment reports were from Gekko japonicus (Liu et al. 2015; GenBank 2693898, NCBI RefSeq Assembly v1.1).

We identified candidate open reading frames (ORFs; coding-regions) using TransDecoder (v5.0.2; Haas 2015) within the de novo transcripts we assembled. Next, we determined the identity of each transcript by assessing homology between the predicted gene region and annotated proteins from the SwissProt database (The UniProt Consortium 2017) and Pfam (v31.0; Finn et al. 2016) databases using BLASTp (v2.7.1; Altschul et al. 1990) and Hmmer (v3.1b2; Finn et al. 2011), respectively. Finally, we associated these homology searches and gene predictions with each transcript using TransDecoder ([v5.0.2], Haas et al. 2013) and appended the final gene predictions to the FASTA headers in the final transcriptome file using SeqKit software package (v0.7.2; Shen et al. 2016). We assessed our final transcriptome assembly using two transcriptome
benchmarking methods: TransRate (v1.01; Smith-Unna et al. 2016) and Benchmarking Universal Single-Copy Orthologs (BUSCO, [v3.0]; Simão et al. 2015) against two different databases (tetrapoda_odb9 and CVG). BUSCO analyses were conducted via the gVolante web server (Nishimura et al. 2017).

To illustrate the utility of this transcriptomic assembly we used BLAST to identify paralogous sequences in the transcriptome from a subset of a well-studied gene family with important developmental functions, the bone morphogenetic proteins (bmp). We downloaded all annotated peptide sequences (BMPs 2, 4–8) from published genomes within the major amniote lineages (mammals: Homo sapiens and Mus musculus; birds: Gallus gallus, and Taeniopygia guttata; crocodilians: Crocodylus porosus; turtles: Pelodiscus sinensis; and (non-gecko) squamate reptiles: Anolis carolinensis and Pogona vittatus) via the Ensembl database [v96]. For geckos, we queried Anolis sequences to GenBank to identify the predicted sequence for each BMP gene in Gekko japonicus. We then used these Gekko sequences to query the de novo Lepidodactylus transcriptome, the Paroedura picta genome (Hara et al. 2018), and the Eublepharis macularius genome (Xiong et al. 2016). To further increase the sampling of squamate reptiles, we also incorporated Chamaeleo calyptratus BMP sequences (Pinto et al. 2019b). We aligned each BMP molecule separately using MAFFT [v7.388] implemented in Geneious® [v11.1.2] (Katoh et al. 2002; Kearse et al. 2012). Then, used the Geneious® consensus alignment function to generate an alignment of all 6 BMP paralogs. We generated gene trees using the RAxML Blackbox [v8.2.10] (Stamatakis 2014), under the BLOSUM62 protein substitution matrix, implemented on the CIPRES portal (Miller et al. 2010). We
rooted the gene tree at the split between the clade containing BMP2 and BMP4 as sister to the clade containing BMPs 5–8 (sensu Ducy and Karsenty 2000).

Results

Characterization of External Morphology

We diagnosed 16 developmental stages using a numbering scheme commonly used for squamates (e.g. Dufaure and Hubert 1961; Wise et al. 2009; Khannoon 2015). Stage 28 corresponds to stage at oviposition and Stage 43 corresponds to a stage which directly precedes hatching. Though temperature can significantly affect the length of incubation (Brown and Murphy-Walker 1992), our sample of 242 embryos ranged from 0 days post-oviposition (dpo) to 77 dpo (Fig. 2.1).

Stage 28 (Fig. 2.2a–b)

Mean SVL: 4.11mm (standard deviation [sd]=0.18, n=3). Neural: There is little cephalic bulging (Fig. 2.2a). The mesencephalon is distinct from the metencephalon and diencephalon, and the telencephalon is distinct from the diencephalon (Fig. 2.2b). The margin of the otic capsule is faintly visible and translucent. The neural tube is open along the dorsum, starting anteriorly at the metencephalon and open most posteriorly at the first 3 somites. Pharyngeal arches and facial prominences: Pharyngeal clefts 1–2 are distinct (Fig. 2.2b). Pharyngeal arches I–III are distinct (Fig. 2.2b). Facial primordia are present but unfused. Eye: The eye is completely unpigmented (Fig. 2.2b). The optic cup is circular in shape and the margin of the lens is round but irregular in shape. The choroid fissure margins are in contact and in the process of fusing. Limbs: Limb buds are only present as small, barely visible swellings. Thorax and tail: 30–32 somites. The endocardial tube is prominent and beating. Liver formation is indicated by condensed
Figure 2.2. Stages 28–31 of *L. lugubris* embryonic development. **Stage 28**: lateral view of the whole embryo (a.) with closer view of the cranial region (b.). **Stage 29**: lateral view of the whole embryo and illustration of the limbs (c.), closer view of the cranial and pharyngeal regions (d.), and closer view of the thorax (e.). **Stage 30**: lateral view of the whole embryo and illustration of the limbs (f.), closer view of the cranial and pharyngeal regions (g.), SEM image of the eye (h.), and dorsal view of the neural tube of a preserved embryo (i.). **Stage 31**: lateral view of the whole embryo and illustration of the limbs (j.), closer view of the pharyngeal and thoracic regions (k.), and closer views of a SEM forelimb (l.) and hindlimb (m.). Scale bars = 1mm. 1–4, pharyngeal clefts 1–4; I–V, pharyngeal arches I–V; AER, apical ectodermal ridge; At, atrium CF, choroid fissure; Die, diencephalon; ED, endolymphatic duct; Eph, epiphysis; ET, endocardial tube; FNP, frontonasal prominences; FL, forelimb; HL, hindlimb; Li, liver; Lu, lungs; MA, mandibular arch; Mes, mesencephalon; Met, metencephalon; Mph, mesonephros; Mye, myelencephalon; NgM, nephrogenous mesenchyme; NP, nasal pits; OpC, optic cup; Otc, otic capsule; RPE, retinal pigmented epithelium; S, somites; T, tail; Tel, telencephalon; Ve, ventricle.
tissue directly posterior to the endocardial tube (Fig. 2.2b). The nephrogenous mesenchyme, which currently lacks mesonephric tubules and gives rise to the mesonephros, is visible. *Flexures and rotation:* The cranial flexure is underway with the axes of the hind- and forebrains at an acute angle. The dorsal contour from the mesencephalon to the tail is curved, with the lumbo-sacral region exhibiting a more extreme curve (Fig. 2.2a).

*Stage 29 (Fig. 2.2c–e)*

*Mean SVL:* 6.30mm (sd=0.42, n=5). *Neural:* A dorsal mesencephalic bulge (i.e. the developing optic tectum) is prominent (Fig. 2.2c). The myelencephalic, metencephalic, and diencephalic bulges are present but less distinct than mesencephalon (Fig. 2.2d). The diencephalic-telencephalic boundary is visible. The otic capsule is still translucent but the margin is much more distinct than the previous stage. *Eye:* The retinal pigmented epithelium (RPE) is beginning to pigment. The optic cup is more ovoid in shape, expanding posteriorly. The lens is less irregular in shape than the previous stage. The choroid fissure is closing, but not yet fully fused (Fig. 2.2d). *Pharyngeal arches and facial prominences:* Pharyngeal clefts 1–3 are present and distinct. Pharyngeal arch I is separated into the maxillary arch and the mandibular arch. Pharyngeal arch II now obscures the majority pharyngeal arch III and pharyngeal cleft 2. Pharyngeal arch IV is visible. The maxillary arch fused anteriorly to the cranial region. The nasal pits are present (Fig. 2.2d). Facial primordia have yet to fuse. *Limbs:* Both hindlimb and forelimb buds are present and are similar sizes (Fig. 2.2c,e). *Thorax and tail:* 40–41 somites extending into the tail. The liver is now opaque and completely visible posterior to the endocardial tube. The lungs are now visible as opaque tissue condensations dorsally
adjacent to the endocardial tube. The mesonephros and its tubules are visible directly posterior to the forelimb buds (Fig. 2.2e). Flexures and rotation: The axes of the hind- and forebrains are at a nearly right angle. The dorsal contour from the mesencephalon to the tail is similar to the previous stage (Fig. 2.2c).

Stage 30 (Fig. 2.2f–i)

Mean SVL: 7.76mm (sd=0.81, n=17). Neural: The dorsal bulge of the optic tectum is more exaggerated than the previous stage (Fig. 2.2f). The telencephalic bulges are paired and beginning to fuse in the frontal region. The margins of the otic capsule are distinct with the fluid-filled inside remaining translucent (Fig. 2.2f,g). The opening of the neural tube is closing from posterior to anterior (Fig. 2.2i). Eye: The optic cup remains an ovoid shape (Fig. 2.2g). Pigment has now spread to the anterior portion of the eye, though still heaviest at the posterior. The choroid fissure remains only as a faint groove (Fig. 2.2h, Fig. 2.3a). Pharyngeal arches and facial prominences: The frontonasal prominences are paired and visible. Pharyngeal arch V and pharyngeal cleft 4 is now present (Fig. 2.2g). Limbs: Both hindlimbs and forelimbs are paddle-shaped and similar sizes (Fig. 2.2f, Fig. 2.3b). Thorax and tail: 45–47 somites. The heart now has a distinct unified atrium and ventricle. The liver is completely opaque white (Fig. 2.2f). Flexures and rotation: The axes of the cervical and thoracic regions are nearly at a right angle. The contour of the thoracic region to the tail is similar to the previous stage (Fig. 2.2f).

Stage 31 (Fig. 2.2j–m)

Mean SVL: 9.69mm (sd=0.71, n=23). Neural: The dorsal bulge of the optic tectum is more exaggerated than the previous stage (Fig. 2.2j). The endolymphatic ducts are now opaque and separated (Fig. 2.2j–k). The opening of the neural tube is now restricted to its
Figure 2.3. Scanning electron micrographs of *L. lugubris* embryos at Stages 30 (A, B), 31 (C, D), and 32 (E, F). Lateral views of the eye (A, C), fusing facial primordia (E), and limb development (B, D, F). Scale bars = 100µm. CF, Choroid fissure; AER, apical ectodermal ridge; FP, facial primordia.
most anterior portion. The otic capsule is more opaque than the previous stage (Fig.
2.2k). The epiphysis (pineal gland) is prominent and located between the frontonasal
prominences (Fig. 2.2k). **Eye:** The choroid fissure is fused (Fig. 2.3c). The optic cup
remains ovoid in shape but has extended slightly along the antero-posterior axis (Fig.
2.2j). The eye is more pigmented, particularly at the equator near the lens and in the
dorsal margin of the eye. **Pharyngeal arches and facial prominences:** There is fusion of
the anterior three pharyngeal arches and clefts (Fig. 2.2k). The mandibular arch spans
halfway along the ventral length of the cranium (Fig. 2.2k). **Limbs:** The autopodia of the
forelimbs, but not the hindlimbs, are paddle-shaped with obvious constriction
distinguishing them from the zeugopodium from the rest of the limb (Fig. 2.2j). The
apical ectodermal ridge (AER) is present in both fore- and hindlimbs (Fig. 2.2l,m, Fig.
2.3d). **Thorax and tail:** The somites are now present along the majority of the tail (Fig.
2.2j). The tail is looping and exhibits a blunt distal tip. The cloaca is clearly visible
proximal to the tail and exhibits subtle swellings of adjacent tissue. The heart is less
bulbous and exhibits two atria. The liver has grown in size from the previous stage (Fig.
2.2k). The lungs and mesonephros are more opaque than previous stages (Fig. 2.2k). The
mesonephros visibly spans from the posterior portion of the liver to the hindlimb.

**Stage 32 (Fig. 2.4a–d)**

*Mean SVL:* 10.12mm (sd=0.72, n=18). **Neural:** The optic tectum has grown (Fig. 2.4a,d).
The endolymphatic ducts are positioned more medially than the previous stage. **Eye:**
There is more diffuse pigment, spread equally across the majority of the eye (Fig. 2.4b).
Iris development is underway superficial to the RPE and appears as condensed pigment
along the margin of the lens. **Pharyngeal arches and facial prominences:** The mandibular
**Figure 2.4. Stages 32–35 of *L. lugubris* embryonic development.** 
**Stage 32:** lateral view of the whole embryo and illustration of the limbs (a.), closer view of the craniofacial region (b.), closer view of the thorax (c.), and dorsal view of the closed neural tube of a formalin-fixed embryo (d.). **Stage 33:** lateral view of the whole embryo and illustration of the limbs (e.), closer view of the limbs and the thorax (f.), view of the developing cloacal region (g.). **Stage 34:** lateral view of the whole embryo and illustration of the limbs (h.), closer view of the craniofacial region (i.), and closer view of a hindlimb and the cloacal region (j.). **Stage 35:** lateral view of the whole embryo and illustration of the limbs (k.) with closer view of the limbs (l). Scale bars = 1mm. AER, apical ectodermal ridge; Aut, autopodium; FL, forelimb; GB, gallbladder; H, heart; HL, hindlimb; Hp, hemiphallus; Li, liver; M, mandible; Mes, mesencephalon; MP, mandibular prominence; Mph, mesonephros; Met, metencephalon; Sty, stylopodium; Zeu, zeugopodium. White arrows indicate interdigital webbing recession.
prominences nearly meet the medial nasal processes (Fig. 2.2e, Fig. 2.4b). The region of the craniofacial prominences does not project far past the anterior margin of the eye. 

**Limbs:** The podial elements (autopodia, zeugopodia, and stylopodia) are distinct in the forelimbs (Fig. 2.2f, Fig. 2.4a). The autopodia are asymmetrical. The autopodia of the hindlimbs are distinct but stylopodium and zeugopodium are not. The AER is a solid, irregular line. The digits have not condensed yet but the blood vessels mark the position where digits will form. **Thorax and tail:** The tail has narrowed to a sharp tip (Fig. 2.4a). The heart is less bulbous than the previous stage (Fig. 2.4c). The liver now exhibits distinct lobes, is more pigmented, and is more vascularized (Fig. 2.4a). The gallbladder is visible as a small green-brown spot on the ventral side of the liver (Fig. 2.4c). The mesonephros is more pigmented and vascularized than the previous stage. Paired cloacal swellings are visible, indicating the onset of cloacal and hemiphallic development.

**Stage 33 (Fig. 2.4e–g)**

*Mean SVL:* 11.45mm (sd=0.57, n=11). **Neural:** The optic tectum is now its largest relative to the cranial region of the embryo (Fig. 2.4e). The endolymphatic ducts are nearly touching medially. **Eye:** The eye exhibits much denser pigment. The pupil is now readily defined based on pigmentation of the iris (Fig. 2.4e). **Pharyngeal arches and facial prominences:** All pharyngeal clefts are fused. The maxillary prominence has fused with the medial-nasal. The facial region has elongated anterior to the eye. The mandibular prominence does not extend to the tip of the face (Fig. 2.4f). **Limbs:** Digital condensations are visible and the irregular margins of the autopodia mark where the distal tips of the digits will form and that the reduction of interdigital webbing has begun (Fig. 2.4e,f). The AER is more distinct on digit tips rather than between digits (Fig. 2.4f).
All podial elements are distinct in the hindlimbs. The autopodia of both hind- and forelimbs are asymmetrical (Fig. 2.4e). **Thorax and tail:** The majority of the viscera, with the exception of the posterior portion of the liver and gallbladder, is enclosed within the body wall and less visible (Fig. 2.4f). The gallbladder exhibits darker color than the previous stage, indicating accumulation of bile. The tail is less coiled than the previous stages. Cloacal swellings and hemiphallus are prominent and easily distinguished using light microscopy (Fig. 2.4g).

**Stage 34 (Fig. 2.4h–j)**

*Mean SVL:* 12.46mm (sd=0.69, n=8). **Neural:** There are no obvious changes in neural morphology (fig. 2.4h). **Eye:** The eye is heavily pigmented. The outer margin of the iris exhibits a band of lighter coloration and is expanded from the previous stage (Fig. 2.4h,i). **Craniofacial:** The embryo has distinct snout which comes in contact with the mandible (Fig. 2.4i). The prominences are no longer visible. **Limbs:** All podial elements are elongated (Fig. 2.4h). Interdigital webbing has recessed to the point where the distal tips of the digits are free (Fig. 2.4j). The digits themselves have expanded laterally. **Thorax and tail:** The body wall is completely closed, covering and obscuring the view of the heart and remaining viscera (Fig. 2.4h). The hemiphallic bulges are more bulbous than the previous stage (Fig. 2.4j). These bulges exhibit morphology more similar to developing hemipenes than hemiclitores in other squamates and will be henceforth be referred to as hemipenes (Gredler et al. 2015; Whiteley et al. 2018).

**Stage 35 (Fig. 2.4k–l)**

*Mean SVL:* 12.21mm (sd=0.69, n=2). **Neural:** The areas of the brain adjacent to the optic tectum are relatively larger (Fig. 2.4k). The endolympathic ducts have met medially at the
posterior portion of the mesencephalon. Eye: The overall shape of the eye is still ovoid, with the first signs of the upper and lower eyelid appearing where the eye meets the lateral portion of the face. The overall collection of eye pigment appears darker (Fig. 2.4k). The light band of iris pigment from the previous stage has expanded in size.

Craniofacial: The craniofacial region anterior to the eye (i.e. snout) is further elongated (Fig. 2.4k). Limbs: All podial elements are further elongated (Fig. 2.4k). Interdigital webbing has recessed further, resulting in a pointed digital condensation (Fig. 2.4l). The digital condensations are wider than the previous stage. Thorax and tail: The vertebrae are faintly visible through the thorax and the tail (Fig. 2.4k).

Stage 36 (Fig. 2.5a–b)

Mean SVL: 13.98mm (sd=1.40, n=3). Neural: The other regions of the brain, especially the telencephalon, have increased in size relative to the mesencephalon — the telencephalic bulge and bulge of the optic tectum appear similar in size (Fig. 2.5a). Eye: The overall shape of the eye remains ovoid on the antero-posterior axis while shape of the iris is circular (Fig. 2.5a). The upper and lower eyelids are visible, overlaying a small portion of iris pigment. Craniofacial: Craniofacial region more elongate than the previous stage (Fig. 2.5a). The ear is visible and open. Limbs: Reduction of interdigital webbing is complete (Fig. 2.5b). Skeletal elements of the digits and limbs are visible. Thorax and tail: The ribs are faintly visible. Each of the two hemipenes are bilobed.

Stage 37 (Fig. 2.5c–d)

Mean SVL: 14.98mm (sd=0.84, n=5). Neural: The optic tectum is less distinct due to the relative increase in size of the embryo (Fig. 2.5c). Eye: The eye is superficially similar to the previous stage with the exception that the iris and the pupil have increased in size and
Figure 2.5. Stages 36–39 of *L. lugubris* embryonic development. Stage 36: lateral view of the whole embryo (a.) with closer view of the limbs (b.). Stage 37: lateral view of the whole embryo (c.) with closer view of the limbs (d.). Stage 38: lateral view of the whole embryo (e.), closer view of the manus (f.), closer view of pigmentation density on dorsum (g.), and ventral view of the cloacal region exhibiting everted hemiphallus (h.). Stage 39: lateral view of the whole embryo (i.), closer view of the thorax (j.), closer view of the manus (k.), and closer view of the eye (l.). Scale bars = 1mm. E, ear; R, ribs. White arrows indicate tapering of the digit to form the claw. Red arrows indicate lateral expansion of the toepad.
the upper and lower eyelids appear fused (Fig. 2.5c). Craniofacial: The craniofacial region is more elongate (Fig. 2.5c). The ear is distinct and well-developed. Limbs: The digits are more elongate than the previous stage. The distal tip of each digit is and beginning to taper, which will eventually form the claw (Fig. 2.5d). The first subdigital lamellar ridges of the toepads are visible. Thorax and tail: The ribs are more visible through the body wall than the previous stage. The lobes of each hemipenis are more prominent than the previous stage. Scales and pigment: Besides the subdigital lamellae, the scales of the hind- and forelimbs are the first visible.

Stage 38 (Fig. 2.5e–h)

Mean SVL: 15.51 mm (sd=1.13, n=4). Neural: The mesencephalon and telencephalon are less distinct (Fig. 2.5e). Eye: The eye is more rounded and darker in color than the previous stage (Fig. 5e). The pupil is rounded and, with the fusion of the eyelids, the brille (i.e. spectacle) is apparent. Craniofacial: The craniofacial region is more elongate (Fig. 2.5e). The nostrils are faintly visible. Limbs: Toepad development is well underway with more subdigital lamellae forming and the pads themselves expanding laterally. The claws are now distinct from the rest of the digits and beginning to curve down towards the plantar side of the autopodia (Fig. 2.5f). Thorax and tail: The hemipenes are completely forked and engorged (Fig. 2.5h). Scales and pigment: The hind- and forelimb scales are more distinct. The first signs of scales and sparse pigment appear along the dorsal surface of the thorax and head (Fig. 2.5g).

Stage 39 (Fig. 2.5i–l)

Mean SVL: 16.09 mm (sd=1.39, n=4). Neural: The mesencephalon and telencephalon are less distinct (Fig. 2.5i). Eye: The chromatophores (xanthophores and melanophores) of
the iris are visible (Fig. 2.5l). The pupil is ovoid. Craniofacial: The snout is more elongate (Fig. 2.5i). The first signs of labial scales are present. Limbs: The toepads continue to expand laterally (Fig. 2.5k). The claw is now fully developed. Thorax and tail: The ribs are well developed and distinct (Fig. 2.5j). The body wall is more opaque, beginning to obstruct the view of the liver and gallbladder. Scales and pigment: The dorsal scales are more distinct than the previous stage. The ventral and caudal scales are visible for the first time. Pigment is more widespread along the dorsum (Fig. 2.5j).

Stage 40 (Fig. 2.6a–b)

Mean SVL: 17.60mm (sd=1.07, n=3). Neural: The mesencephalon and telencephalon are less distinct (Fig. 2.6a). Eye: Yellow coloration, presumably from xanthophores, is denser. The pupil remains ovoid but is thinner than the previous stage (Fig. 2.6b). Craniofacial: There is no noticeable change in craniofacial region from the previous stage. Limbs: The toepads continue to expand laterally (Fig. 2.6a). Thorax and tail: The previous 90° angle of head to the thorax is beginning to open, becoming more obtuse (Fig. 2.6a). Scales and pigment: The external naris is visible (Fig. 2.6b). The pigment is more widespread across to the body. Pigment is denser in areas which will eventually become the postnatal coloration patterns (e.g. dorsolateral streak from the snout to posterior of the eye).

Stage 41 (Fig. 2.6c–e)

Mean SVL: 19.24mm (sd=4.31, n=2). Eye: The pupil is narrower than the previous stage and its vertical slit-shape has become irregular (Fig. 2.6d). Limbs: The toepads are now completely developed, taking a bulbous shape approximately half-way up the length of the digit and tapering off again at the claw. The claw is now opaque (Fig. 2.6e). Thorax
Figure 26. Stages 40–43 of *L. lugubris* embryonic development. **Stage 40**: lateral view of the whole embryo (a.) with closer view of the head (b.). **Stage 41**: lateral view of the whole embryo (c.), closer view of the head (d.), and closer view of the thorax (e.). **Stage 42**: lateral view of the whole embryo (f.), closer view of the head (g.), and closer view of the cloacal region (h.). **Stage 43**: lateral view of the whole embryo (i.), with a closer view of the eye (j.), medial view of the mouth (k.), and ventral view of the cloacal region exhibiting recessed hemiphallus (l.). Scale bars = 1mm. Hp, hemiphallus; N, external naris.
and tail: The hemipenes remain everted and forked. Each individual hemipenis lobe is thicker than previous stages (Fig. 2.6e). Scales and pigment: The labial scales are well defined and the external naris is closed (Fig. 2.6d). Scales and associated pigments have made the dorsal surface of the embryo essentially opaque, thus obscuring the view of the vertebral column, epaxial muscle, and the brain (Fig. 2.6c). The ventral surface remains somewhat translucent, with the liver, gallbladder, and some bones (e.g. ribs, femora) remaining visible (Fig. 2.6c).

Stage 42 (Fig. 2.6f–h)
Mean SVL: 18.31mm (sd=1.39, n=4). Eye: The pupil is narrower and the irregular shape is forming, that when fully contracted, creates the multiple-pinhole slit pupil (sensu Roth et al. 2009), which is typical of nocturnal gekkotans (Fig. 2.6g). Thorax and tail: The hemipenes remain everted, yet have stopped growing with the rest of the body, giving the appearance of being smaller (0.75mm hemipenes, 17.59mm SVL; Stage 42) than the previous stage (0.77mm hemipenes, 16.50mm SVL; Stage 41) indicating recession into the cloacal region (Fig. 2.6h). Scales and pigment: The scales are fully developed. The external naris is open. The pigment is denser along the ventrolateral region of the thorax and the ventral surface of the tail (Fig. 2.6f). The ventrum remains somewhat translucent medially (Fig. 2.6f).

Stage 43 (Fig. 2.6i–l)
Mean SVL: 19.54mm (sd=1.52, n=6). Eye: The pupil fully contracted, creating a multiple-pinhole slit pupil (Fig. 2.6j). Craniofacial: There are no externally visible egg teeth (Fig. 2.6k; Fig. 2.7). Thorax and tail: The hemipenes are receded (Fig. 2.6l). Scales
Figure 2.7. Egg teeth (ET) are not externally visible (A, C) but are easily identified using μCT volume rendering (B, D). Mediolateral (A, B) and ventral (C, D) views of a Stage 42 *Lepidodactylus lugubris* rostrum. Scale bars = 500µm.
and pigment: The scales are opaque, obscuring the view of the viscera, and fully pigmented (Fig. 2.6i).

**Brain and CNS Development**

The five secondary vesicles of the embryonic neural tube (i.e. the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon) are often used to define embryonic stages (Arey 1974; Bellairs and Osmond 2005; Sanger et al. 2008; Noro et al. 2009; Wise et al. 2009). The five vesicles give rise to the following brain structures: the telencephalon gives rise to the cerebral hemispheres and olfactory region; the diencephalon gives rise to the epithalamus, thalamus, and hypothalamus; the mesencephalon gives rise to the optic tectum and tegmentum; the metencephalon gives rise to the tegmentum and the cerebellum; finally, the myelencephalon gives rise to the medulla oblongata (Arey 1974; Senn 1979; Bellairs and Osmond 2005). Following previous brain developmental series of *Podarcis siculus* (Senn 1979), we produced an approximate sequence of gross morphological changes in four of the main regions of the developing *L. lugubris* brain: telencephalon + diencephalon region (TD), optic tectum, cerebellum, and the remainder of the hindbrain. Although the identification of these structures is approximate, we believe the overall descriptions of these main regions might facilitate understanding of large regions of the brain, but we mention some important regions that are easy to visualize in tomography. The µCT reconstructed series of brain development is illustrated in Figure 2.8.

At Stage 30, the TD shows a clear anterior region that corresponds to the cerebral hemisphere, a ventrally directed region or thalamus, and a superior region or epithalamus.
Figure 2.8. Embryonic development of the *L. lugubris* brain visualized through soft-tissue µCT. Whole-embryo µCT images are pictured below the corresponding isolated brain. The four distinct regions of the brain are color-coded as follows: Telencephalon + diencephalon, purple; Optic Tectum, green; Cerebellum, blue; Hindbrain, yellow. Scale bars = 1mm. Cb, cerebellum; Cr, cerebrum; HB, hindbrain; OB, olfactory bulbs; ON, optic nerve; OT, optic tectum; pg, pineal gland; ptg, pituitary gland.
One of the most prominent regions is the optic tectum, which protrudes on the dorsal surface of the head. The optic tectum lies between the telencephalon and the cerebellum. The cerebellum at this state is elongated and runs parallel to the rest of the hindbrain. The hindbrain is nearly straight, having a slight flexure and not wider than the spinal cord. Dorsal to the TD, a small knob projects dorsally representing the pineal gland. At Stage 31, the cerebral hemisphere grows proportionally larger, reaching a similar size to the tectum. On the ventral surface of the TD, the pituitary gland is exhibited as a small projection. At Stage 32 the optic tectum becomes the most prominent structure of the brain and the cerebellum folds. At Stage 33, the olfactory bulbs appear and project anteroventrally. The cerebellum begins expanding towards the hindbrain. The pineal gland is distinct on the dorsal surface of the TD. At Stage 34, the olfactory bulbs elongate, the hindbrain becomes slightly larger in diameter than the spinal cord. At Stage 35, the olfactory bulbs extend approximately four times the size in the previous stage and the optic nerve extends anteroventrally from the TD. The hindbrain curvature is more marked, to the point that this structure is mostly horizontal, followed by a cervical flexure that indicates the limit with the spinal cord. At Stage 36, the cerebral hemisphere expands dorsally. The cerebellum and optic tectum are relatively smaller than the cerebral hemisphere. The cerebellum is embedded on the hindbrain. At Stage 37, the size of the optic tectum is sub-equal to the size of the cerebral hemispheres. At Stage 38, the anterior portion of the olfactory bulbs start expanding. Between Stages 40–43, the expansion of the olfactory bulbs is more evident. The proportions of all the brain parts remain equal, but the brain increases its size.

3.3 Transcriptome Assembly
We generated our final \textit{de novo} transcriptome assembly from 18,394,074 raw read pairs. This assembly contained 115,656 transcripts with a total length of 129,611,368 bases (bp), with transcripts ranging from 201bp to 15,792bp in length. The TransRate assembly score attempts to assess the reliability and completeness of the assembly by calculating the geometric mean of contig scores and multiplying by the ratio of mapped/unmapped raw reads (Smith-Unna et al. 2016). Our assembly queried a total of 58% of the reference \textit{Gekko japonicus} peptides, which provided 32,823 conditional Reciprocal Best BLAST (RBB) hits, to generate a modest TransRate assembly score of 0.0926. We assessed the completeness of this transcriptome by comparing its content against databases of conserved orthologs for tetrapods (tetrapoda\_odb9; 3950 genes) and Core Vertebrate Genes (CVG; 233 genes). Against the database of conserved tetrapod genes, our assembly contained a total of 2029 (51.4%) complete and single-copy orthologs, 1248 (31.6%) complete and duplicated orthologs, 388 (9.8%) fragmented orthologs, and 285 (7.2%) missing genes; while against the CVG database, our assembly contained 209 (89.70%) complete orthologs, 226 (97.0%) complete and partial orthologs, and was missing only 7 (3.00%) of genes. Indeed, our assembly possesses at least a partial assembly of 92.8% conserved tetrapod orthologs (tetrapoda\_odb9) and 97.0% conserved vertebrate genes (CVG).

There are ten Bone Morphogenetic Proteins (BMPs) annotated in the \textit{G. japonicus} genome, and we were able to confirm nine in our \textit{L. lugubris} transcriptome assembly (BMPs: 1–8, and 11). We used a subset of paralogous BMP genes that are hypothesized to be recently diverged (Ducy and Karsenty 2000) to construct a gene tree to validate the accuracy of assigning these paralogs to their respective ortholog (Fig. 2.9). Indeed, we
Figure 2.9. Maximum-likelihood gene tree of paralogous BMP sequences. Each paralogous BMP gene clade is highlighted in alternating colors/shades. All short, terminal nodal support values were removed for clarity, while at deeper nodes, only bootstrap support >70 are shown. Tree was rooted at the divergence between BMP2/4 and BMP5–8. Scale bar is in substitutions per-site.
found that each BMP paralog was present, correctly annotated, and the topology of the tree was similar to a Ducy and Karsenty’s (2000) gene tree at well-supported nodes.

**Discussion**

This description of *Lepidodactylus lugubris* embryonic development is the sixth complete staging series of a gecko (Wise et al. 2009; Noro et al. 2009; Khannoon 2015; Zhao et al. 2017; van der Vos et al. 2018) and the first staging series of an obligate parthenogenetic vertebrate. Similar to all other geckos investigated to date, eggs of *L. lugubris* are oviposited during embryo organogenesis (Andrews 2004). However, the precise developmental stage at oviposition varies between examined gekkotans without apparent phylogenetic specificity. *Tarentola annularis* (Phyllodactylidae) are oviposited at stage 29 and *Paroedura picta* (Gekkonidae) are oviposited approximately at stage 24 (*sensu* Dufaure and Hubert 1961; Noro et al. 2009; Khannoon 2015). The remainder of examined geckos, *L. lugubris* (Gekkonidae), *Gekko japonicus* (Gekkonidae), *Hemidactylus* sp. (Gekkonidae), and *Eublepharis macularius* (Eublepharidae) oviposit eggs at stage 28 (Wise et al. 2009; Zhao et al. 2017; van der Vos et al. 2018). Indeed, more taxonomic sampling is required to identify any trends in oviposition stage across geckos. However, the average developmental time between oviposition and hatching is longer for *L. lugubris* than most non-gekkotan squamates studied, but similar to other hard-shelled gekkotan eggs (60–65 dpo). For example, the time between oviposition and hatching for *Anolis sagrei* (Pleurodonta) is 22–27 dpo (Sanger et al. 2008). The longer incubation time of hard-shelled gecko eggs is likely due to lower amounts of gas exchange across the eggshell and thus lower amounts of oxygen available to the embryo (Pike et al. 2012; Andrews et al. 2013a).
Prior to this study, little was known about *L. lugubris* development. Digital ossification of *L. lugubris* is discussed in-depth by Rieppel (1994). In short, phalangeal ossification occurs in a typical proximo-distal direction; however, *L. lugubris* and *Gehyra oceanica* (Gekkonidae) exhibit delayed ossification in the shortened intermediate phalanges of digits III and IV of both the manus and pes. Delayed ossification of the 2nd phalanx of digit IV is hypothesized to be a consequence of gekkotan digital paedomorphosis, another aspect that departs from the ancestral squamate bauplan (Rieppel 1984, 1994). This developmental delay often results in the loss of this element in some gecko lineages (e.g. *Asaccus*; Rieppel 1984). Gekkonids develop mineralized egg teeth between stages 39–40 (Andrews 2012). Although egg teeth are not externally visible in any embryonic stages of *L. lugubris* (Fig. 2.6k), µCT data along with cleared and stained data from Kluge (1967a) corroborates their presence in the pre-hatchling stages. The egg teeth are paired, erupting from the premaxilla, each directed medially to form a single point (Fig. 2.7), a trait shared by several other gekkonid species (Rösler 2001).

A striking result of our investigation of *L. lugubris* is the presence and persistence of hemipenis-like structures throughout embryonic development in an all-female species. In the lizard *Anolis carolinensis* and *Anguis fragilis*, paired phalluses develop in both sexes; however, hemipenes are large in males and hemiclitores are small in females (Gredler et al. 2014; Gredler et al. 2015; Raynaud and Pieau 1985). However, other lizards, such as *Pogona vitticeps* and *Barisia imbricata*, have female embryos with large, hemipenis-like organs that are equal in size and shape with males, that subsequently regress prior to and just after hatching, respectively (Martinez-Torres et al. 2015;
Whiteley et al. 2018). Our results, coupled with results from the recent literature, make it clear that many aspects of squamate sexual development warrant more detailed examination. Further investigation of external genital development in *L. lugubris* will be discussed in-detail separately.

Gekkotan development has been characterized by the following: later appearance of the paired hemiphallic bulges when compared to other squamates (excluding gymnophthalmids; Andrews et al. 2013b), appearance of the three podial elements later than acrodonts but earlier than pleurodonts (Andrews et al. 2013b), larger relative pupil diameter than pleurodonts and lacertids (Py-Daniel et al. 2017), and earlier fusion of facial primordia than pleurodonts (Py-Daniel et al. 2017). Indeed, pupil diameter of embryonic gekkotans, including *L. lugubris*, is relatively larger than pleurodont and lacertid pupils (e.g. Hubert 1985; Sanger et al. 2008). However, *L. lugubris* exhibit deviations from the other three gecko-specific character states. Paired hemiphallic bulges of *L. lugubris* appear at Stage 32, which is later than the gecko *P. picta*, the same timing as some pleurodonts, acrodonts, and gymnophthalmids, but earlier than anguimorphs, and later than other pleurodonts and lacertid (Dufaure and Hubert, 1961; Muthukkaruppan et al. 1970; Noro et al. 2009; Gregorovicova et al. 2012; Roscito and Rodrigues 2012; Py-Daniel et al. 2017; Whiteley et al. 2017; Lima et al. 2019). All three podial elements appear in the forelimbs at *L. lugubris* Stage 32, which matches the timing of most geckos, gymnophthalmids, lacertids, some acrodonts, and anguimorphs, but is earlier than other acrodonts and pleurodonts, and later than other pleurodonts. Finally, fusion of the facial primordia occurs at Stage 33 which is the same timing as other geckos, some acrodonts, and anguimorphs, but earlier than lacertids, other acrodonts, and pleurodonts, and later
than gymnophthalmids. Along with other squamate taxa, the deviations from these sequences observed in *L. lugubris* in a mere three characters, which are considered by some to have gecko-specific character states, suggests widespread heterochrony in squamate development. Further investigations into normal embryonic development of additional taxa are required to determine which developmental characteristics actually are clade-specific.

Squamates are becoming increasingly used as models in evolutionary and developmental neurobiology (Nomura et al. 2013; Desfilis et al. 2018; Hoops 2018). However, the general difficulty of gross dissection of delicate, soft tissues like the embryonic brain is challenging, especially in small species; this task is facilitated by means of non-destructive imaging methods, such as magnetic resonance imaging (MRI; Hoops et al. 2018) or diffusible iodine-based contrast-enhanced computed tomography (diceCT; Gignac et al. 2016). Among amniotes, mammals and avian reptiles exhibit a well-developed telencephalon that possesses derived internal architectures (Nomura et al. 2013). Alternatively, non-avian reptiles (and squamates in particular) possess relatively smaller telencephalic regions that exhibit the ancestral amniote internal architecture, expanded olfactory regions, and small cerebella (Bruce 2007; Nomura et al. 2013). Gekkotan brains exhibit average “brain mass:body mass” ratios on par with limbed non-Gekkotan squamates (De Meester et al. 2019) and exhibit further elaboration in size of the main olfactory bulbs (Smeets et al. 1986). Indeed, the large relative size and cellular structure of gekkotan olfactory bulbs, when compared to other squamate lineages, suggests geckos are olfactory specialists with derived forebrain morphologies (Smeets et al. 1986; Schwenk 1993; Rehorek et al. 2000). Our investigation into the development of
*L. lugubris* brain development demonstrates that anterior extension of the forebrain, and the resulting appearance of olfactory bulbs, occurs at approximately Stage 33 (Fig. 2.8). Furthermore, the olfactory peduncles, which link the olfactory bulbs with the telencephalon, do not appear to extend much past Stage 35, suggesting that elongation and further gross morphological development of the olfactory region occurs postnatally. The cerebellum, which is extremely small in squamates when compared to birds or mammals, does not superficially appear to increase in volume relative to the other regions of the brain. The most substantial change in appearance that occurs is the movement of the pontine flexure, where the cerebellum folds in on itself between Stages 30–33 (Fig. 8). Alternatively, in birds, which have large cerebella that comprise approximately one quarter of the brain (Nomura et al. 2013), the cerebellum is distinguishable by MRI at 9 dpo (Stage 35; Hamburger and Hamilton 1954) and continues to grow rapidly within the next 10 days (Stages 35–45; Hamburger and Hamilton 1954; Bellairs and Osmond 2005; Zhou et al. 2015). As non-destructive visualization techniques such as soft-tissue µCT are relatively new, we expect development of resources for additional taxa that will facilitate robust comparative investigations of developmental neuroanatomy.

The annotated transcriptome we presented here provides a description of transcripts expressed in a late-stage *L. lugubris* embryo (i.e. BUSCO score of 83.0%). The presence of most BMPs annotated in the *G. japonicus* genome is evidence of its completeness and utility. Our gene tree, using a subset of recently diverged paralogous BMP genes validated the accuracy of our ortholog assignment. We also corroborate eutherian mammal-specific duplication of BMP8 (i.e. BMP8A and BMP8B; Zhao and Hogan 1996; Carson and Scherer 2009). Our characterization of the *L. lugubris*
embryonic transcriptome will be useful in future investigations of the evolution and
development of this species, such as mapping RNAseq reads for differential expression
analysis or designing probes for in situ hybridization. Furthermore, the transcriptome
may be useful for broader comparative analyses, particularly since there are few genomic
resources available for geckos (G. japonicus, E. macularius, P. picta; Liu et al. 2015;
Xiong et al. 2016; Hara et al. et al. 2018), and even fewer transcriptomic resources (E.
macularius, P. picta; Hara et al. 2015; Tzika et al. 2015).

Robust phylogenetic analysis, genomic information, and detailed developmental
data are critical tools to investigate the origins of morphological novelty and
convergence. Ideally, by employing a model clade approach, an integrated comparison
between closely related taxa with variable phenotypes allows for polarization of ancestral
character states and fine-scale investigations into morphological evolution and
development (Sanger and Rajakumar 2019). By including another gecko species to the
growing number of squamate embryonic staging series, we hope to promote model clade
approaches to squamate and vertebrate evo-devo. For example, comparative squamate
evo-devo studies that include a gecko and any other non-gekkotan squamate species
allows the investigators to sample the phylogenetic breadth of squamates. Furthermore,
using geckos themselves as a model clade is becoming more feasible. Currently, normal
stages of development are characterized for six gecko species, with varying morphologies
and ecologies (Wise et al. 2009; Noro et al. 2009; Khannoon 2015; Zhao et al. 2017; van
der Vos et al. 2018). Access to additional resources — protocols for husbandry and
embryo collection (Griffing et al. 2018b; Vickaryous and Gilbert 2019), genomic and
transcriptomic resources (Liu et al. 2015; Tzika et al. 2015; Xiong et al. 2016; Hara et al.
2015, 2018), and now manipulatable soft-tissue μCT data across development — sets the foundation for geckos, and specifically *L. lugubris*, to be powerful evo-devo models.
III. DISTINCT PATTERNS OF PIGMENT DEVELOPMENT UNDERLIE CONVERGENT HYPERPIGMENTATION BETWEEN NOCTURNAL AND DIURNAL GECKOS (SQUAMATA: GEKKOTA)

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Introduction

Temporal niche, also known as diel activity niche, is an important aspect of the biology of an organism, necessitating the evolution of specialized morphology, physiology, ecology, and behavior (e.g. Duellman and Pianka 1990; Röll 2001b; Valeix et al. 2007; Levy et al. 2012). For example, many diurnal ectotherms thermoregulate through basking behavior (i.e. heliothermy), whereas nocturnal ectotherms thermoregulate through contact with surfaces of different temperatures (i.e. thigmothermy; Cowles and Bogert 1944; Pough and Gans 1982 Adolph and Porter 1993). Temporal niche appears to be phylogenetically conserved across major tetrapod clades (Anderson and Wiens 2017) and thus many adaptations to specific temporal niches (diurnal, nocturnal, crepuscular, or cathemeral) are shared among closely related species. Despite its conservation in tetrapod evolutionary history (Anderson and Wiens 2017), several squamate clades do exhibit temporal niche turnover. The crown group of geckos (Infraorder Gekkota) are hypothesized to be ancestrally nocturnal, with reversals to diurnality occurring in at least 10 lineages (Walls 1942; Gamble et al. 2015a; Anderson and Wiens 2017). Many of these lineages exhibit an array of diurnal-specialized adaptations, most notably eye morphologies, with oil droplets which aid in light filtering and spectral tuning (Walls 1942; Underwood 1951; Bowmaker and Knowles 1977; Röll 2000b), concavicilicate temporal fovea to aid in binocular vision (Underwood 1970; Röll
2001a), and ovoid retinal pigmented epithelia (RPE) to aid in light filtering and absorption (Strauss 2005; Guerra-Fuentes et al. 2014).

Another phenotype that is typically correlated with diurnal temporal niche in vertebrates is the hyperpigmentation of internal structures, such as the overlaying connective tissues of the brain, gonads, subcutaneous dorsum, and peritoneum (Krüger and Kern 1924; Hill 1942; Collette 1960; Porter 1967). These dense collections of melanophores are hypothesized to protect internal structures from injurious and mutagenic UV radiation, which heliotherms encounter more frequently than thigmotherms (Klauber 1939; Cole 1943; Collette 1961; Porter 1967; Pough et al. 1978). Though heliothermy is correlated with hyperpigmentation of internal structures, some gecko species exhibit a disconnect between thermoregulatory behavior and temporal niche. For example, *Sphaerodactylus* geckos (Sphaerodactylidae) are primarily diurnal, but are active underneath leaf-litter and are thus thigmothermic (Henderson and Powell 2009). Alternatively, *Strophurus* geckos (Diplodactylidae) are primarily nocturnal, yet occasionally bask during daylight hours (Greer 1989). This “occasionally-heliothermic” classification is supported by *Strophurus* exhibiting hyperpigmented peritonea (Greer 1989).

Parachute geckos (Subgenus *Ptychozoon*) of the genus *Gekko* comprise 12 described species which inhabit dipterocarp forests of southeast Asia (Brown et al. 2012b; Heinicke et al. 2012; Uetz et al. 2019; Wood et al. 2020). This clade is characterized, in part, by a suite of specialized traits, including expanded trunk folds, expanded caudolateral folds, and elaborate interdigital webbing, which allow for a gliding predator escape behavior (Boulenger 1890). Following Russell’s (1979b) step-
wise hypothesis, gliding behavior through these elaborate cutaneous folds was exapted from use of the folds to reduce shadows (i.e. cryptic behavior) and thus, in conjunction with cryptic coloration, conceal the animal from predators (Barbour 1912; do Rooij 1915; Smith 1935; Tweedie 1950; Pong 1974; Vetter and Brodie 1977; Grismer 2004, 2011a, 2011b). Though chiefly nocturnal, Gekko (Ptychozoon) kuhli can occasionally be found on exposed tree trunks and branches during the day (Taylor 1963; Grismer, 2004, 2011a, 2011b). This is likely a byproduct of cryptic behavior, as remaining motionless on tree trunks and branches throughout the day may result in exposure to direct sunlight and suggests an occasionally-heliothermic thermoregulatory classification. As mentioned previously, prolonged exposure to direct sunlight necessitates adaptations to tolerate higher temperatures and increased UV. We therefore hypothesize that behavioral crypsis, as implemented by G. kuhli, can lead to occasional heliothermy and the correlated phenotypic changes despite exhibiting a nocturnal temporal niche. To further investigate this hypothesized association between temporal niche, behavior, and pigment phenotype, we qualitatively characterized subcutaneous (fascial, visceral, and peritoneal) pigment for eight gecko species exhibiting diverse temporal niche and thermoregulatory behaviors. We predicted that nocturnal/potentially-heliothermic G. kuhli would exhibit hyperpigmentation of internal structures like that of diurnal/heliothermic geckos. Furthermore, to characterize patterns of pigment accumulation through embryonic development, we examined embryos at various stages of development from four gecko species exhibiting all combinations of temporal niche and thermoregulatory character states. We predicted that embryonic pigment accumulation of G. kuhli should also resemble that of diurnal/heliothermic as opposed to nocturnal/thigmothermic geckos.
Materials and Methods

We qualitatively characterized subcutaneous (fascial, visceral, and peritoneal) pigment for six gekkonid gecko species exhibiting a diversity of temporal niche and thermoregulatory behaviors: Gekko kuhli (nocturnal/potentially-heliothermic), Gekko badenii (nocturnal/thigmothermic), Lepidodactylus lugubris (nocturnal/thigmothermic), Hemidactylus frenatus (nocturnal/thigmothermic), Hemidactylus platyurus (nocturnal/thigmothermic), and Phelsuma laticauda (diurnal/heliothermic). This taxon sampling allows us to compare dorsal fascial pigmentation of 3 of the 4 possible character state combinations and spanning the diversity of the Gekkonidae (Gamble et al. 2015a). We also compare two outgroups: one sphaerodactylid (Sphaerodactylus leonardovaldesi) and one eublepharid (Coleonyx brevis) which exhibit diurnal/thigmothermic and nocturnal/thigmothermic charter states, respectively. Each individual (N=8) was euthanized humanely using MS222 following Conroy et al. (2009), skinned and eviscerated to reveal subcutaneous pigment, and finally observed and photographed using a Nikon SMZ 74ST stereoscope. We characterized degree of pigmentation following Bauer (1997): no melanophores (no pigment), scattered melanophores present (lightly pigmented), many melanophores present (darkly pigmented), and complete opaque coating of melanophores present (black).

We collected eggs from captive colonies of four gecko species exhibiting all combinations of character states to observe embryonic patterns of pigment development: 49 embryos of G. kuhli (nocturnal/potentially-heliothermic), 141 embryos of L. lugubris (nocturnal/thigmothermic), 13 embryos of P. laticauda (diurnal/heliothermic), and 26 embryos of Sphaerodactylus macrolepis (diurnal/thigmothermic). Because embryos of S.
Leonardoveldesi were unavailable, we collected embryos of *S. macrolepis* as a congeneric proxy. We collected embryos (N=229) following protocols described by Griffing et al. (2018b). To briefly summarize, we removed embryos from eggs using #5 watchmaker’s forceps while immersed in diethyl pyrocarbonate (DEPC) treated, RNase free 1% phosphate-buffered saline, and visualized and photographed using a Nikon SMZ 74ST stereoscope. As geckos exhibit interspecific variation between the precise time points (days post-oviposition; DPO) of developmental stages (Noro et al. 2009; Wise et al. 2009; Kahnoon et al. 2015; Zhao et al. 2017; Vos et al. 2018; Griffing et al. 2019), we discretized and assigned developmental stages based on external morphology using previous embryonic staging series of geckos rather than characterizing by DPO (Wise et al. 2009; Griffing et al. 2019).

**Results**

*Adult Morphology*

Adult nocturnal/thigmothermic species exhibited no pigment on the subcutaneous dorsal fascial surface (Fig. 3.1). Of these five species, only *Hemidactylus platyurus* exhibits pigment on the inside of the body cavity — the gonadal serosa is lightly pigmented, the peritoneum is lightly pigmented, and the intestinal serosa is black (Fig. 3.2; Table 3.1). The only diurnal/thigmothermic species, *Sphaerodactylus leonardoveldesi*, exhibits no pigment on the subcutaneous dorsal fascial surface, with the exception of a lightly pigmented area posterior to the parietals (Fig. 3.1). Internally, *S. leonardoveldesi* exhibits a lightly pigmented peritoneum and liver (Fig. 3.2; Table 3.1). The diurnal/heliothermic *Phelsuma laticauda* exhibits a black subcutaneous dorsal fascia surface along the skull, through the parietal region and along the trunk, shifting from
Figure 3.1. Convergent evolution of subcutaneous dorsal hyperpigmentation in geckos. Phylogenetic relationships of eight gekkotan taxa, exhibiting a variety of temporal niche and basking behavior character states, following the topology of Gamble et al. (2015a). Dorsal views of the skinned parietal region (brown) and the mid trunk region (green) correspond to adjacent tips of the phylogeny. Gecko photographs: Stuart Nielsen.
Figure 3.2. Diversity of pigmented visceral serosae and peritonea in geckos. A) ovaries and lightly pigmented peritoneum of *G. kuhli*. B) Black intestines of *H. platyrurus*. C) Lightly pigmented ovaries and peritoneum of *H. platyrurus*. D) Black and lightly pigmented peritoneum of *P. laticauda*. E) Lightly pigmented liver of *P. laticauda*. F) Black intestines and darkly pigmented testes of *P. laticauda*. G) Lightly pigmented liver of *S. leonardovaldesi*. H) Ovaries and lightly pigmented peritoneum of *S. leonardovaldesi*. I) Completely unpigmented viscera and peritoneum of *C. brevis* which is identical to all other species investigated lacking internal melanophores. i, intestines; li, liver; o, ovaries; p, peritoneum; t, testes. Scale bars =1mm.
Table 3.1. Hyperpigmentation in geckos. Pigment levels are coded as follows: 0, no melanophores or no pigment; 1, scattered melanophores or lightly pigmented; 2, many melanophores or darkly pigmented; and 3, opaque coating of melanophores or black. D, diurnal; H, heliothermic; N, nocturnal; T, thigmothermic.
Names of organs are listed with their associated serosal pigment level.

<table>
<thead>
<tr>
<th>Species</th>
<th>Temporal niche / thermoregulatory behavior</th>
<th>Fascial Pigment (Anterior, Posterior)</th>
<th>Peritoneal Pigment</th>
<th>Visceral Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>G. kuhli</td>
<td>N/H</td>
<td>2, 3</td>
<td>1</td>
<td>liver (0), stomach/intestines (0), gonads (0)</td>
</tr>
<tr>
<td>G. badenii</td>
<td>N/T</td>
<td>0, 0</td>
<td>0</td>
<td>liver (0), stomach/intestines (0), gonads (0)</td>
</tr>
<tr>
<td>L. lugubris</td>
<td>N/T</td>
<td>0, 0</td>
<td>0</td>
<td>liver (0), stomach/intestines (0), gonads (0)</td>
</tr>
<tr>
<td>H. frenatus</td>
<td>N/T</td>
<td>0, 0</td>
<td>0</td>
<td>liver (0), stomach/intestines (0), gonads (0)</td>
</tr>
<tr>
<td>H. platyurus</td>
<td>N/T</td>
<td>0, 0</td>
<td>1</td>
<td>liver (0), stomach/intestines (3), gonads (1)</td>
</tr>
<tr>
<td>P. laticauda</td>
<td>D/H</td>
<td>3, 3</td>
<td>1–3</td>
<td>liver (1), stomach/intestines (3), gonads (2)</td>
</tr>
<tr>
<td>S. leonardovaldesi</td>
<td>D/T</td>
<td>1, 0</td>
<td>1</td>
<td>liver (1), stomach/intestines (0), gonads (0)</td>
</tr>
<tr>
<td>C. brevis</td>
<td>N/T</td>
<td>0, 0</td>
<td>0</td>
<td>liver (0), stomach/intestines (0), gonads (0)</td>
</tr>
</tbody>
</table>
black to dark pigmentation near the pelvic region (Fig. 3.1). Internally, *P. laticauda* exhibits a lightly pigmented liver, darkly pigmented gonadal serosa, both light and black areas of the peritoneum, and black intestinal serosa (Fig. 3.2; Table 3.1). Finally, the nocturnal/potentially-heliothermic *Gekko kuhli* exhibits a black subcutaneous dorsal fascial surface along the trunk and a darkly pigmented parietal region and remaining skull (Fig. 3.1). Internally, *G. kuhli* exhibits a lightly pigmented peritoneum and no pigment on the remaining viscera (Fig. 3.2; Table 3.1).

**Embryonic Morphology**

The first external pigment cells to accumulate in all gecko embryos are restricted to the RPE (Fig. 3.3; Guerra-Fuentes et al. 2014). Accumulation of melanophores, outside of the RPE, during embryonic development of *G. kuhli* begins shortly after oviposition Stage 29 (i.e. mid-limb bud stage; Fig. 3.3a,b). These initial sparse accumulations are located in the epidermis along the dorsum, outside of the developing optic tectum, and adjacent to the eye (Fig. 3.3a,b). At Stage 30, sparse melanophore accumulation spreads over the pharyngeal arches and the majority of the craniofacial region (Fig. 3.3a,b). By Stage 31, sparse accumulation has reached the forelimbs and the pigment accumulation along the dorsum and craniofacial region is more dense (Fig. 3.3a,b). From Stage 31 to Stage 36, melanophore accumulation becomes denser and covers the entire surface of the embryo and begins to resemble the color pattern of near-hatchling *G. kuhli*: little pigment on the ventral surface, dense pigment on the dorsum creating faint chevron patterns, and dense pigment adjacent to the eye forming a dorsolateral stripe (Fig. 3.3a–c). By comparison, embryos of *L. lugubris* do not exhibit visible melanophores outside of the RPE during equivalent stages of development (Stages
29–36; Fig. 3.3d–f). Indeed, regardless of temporal niche or basking behavior, all gecko embryos examined, with the exception of *G. kuhli*, lacked visible accumulation of pigment outside of the RPE until Stage 38–39 (Fig. 3.4). Sparse pigment accumulates along the center of the dorsum in Stage 38, and eventually spreads to the craniofacial region in Stage 39 (Fig. 3.4). During Stage 39, the pigment faintly resembles the eventual pattern of the near-hatchling animal and is colocalized with the epidermal papillae that will give rise to scales (i.e. Stage 42; Fig. 3.4).

**Discussion**

As predicted, *G. kuhli* exhibits darkly pigmented to black subcutaneous dorsal fascia while none of the other nocturnal gecko species examined exhibit dorsal fascia pigmentation (Fig. 3.1). As expected, and similar to *G. kuhli*, the diurnal/heliothermal gecko, *P. laticauda*, also exhibits black dorsal fascia pigmentation (Fig. 3.1). Furthermore, the diurnal/thigmothermic gecko, *S. leonardovaldesi* exhibits an intermediate phenotype: light dorsal fascia pigmentation near the braincase (Fig. 3.1).

The only previous in-depth investigations into gecko subcutaneous pigmentation was performed by Duncker (1965a,b, 1968), who examined 20 species. Duncker, who noted the extreme pigmentation of *Phelsuma* spp., also described fascial pigmentation in the largely nocturnal but often heliothermal *Tarentola* spp. (Schleich et al. 1996) as well as pigmented nervous and vascular tissue of the largely nocturnal but heliothermal *Ptyodactylus hasselquistii* (Werner and Goldblatt 1978; Arad et al. 1989). The peritonea and the serosa of various visceral elements are pigmented in *G. kuhli*, *P. laticauda*, and *S. leonardovaldesi*. Duncker (1965b) reported pigmented intestine of *G. kuhli*, though we did not find this. There are multiple explanations for this discrepancy. First, Duncker’s *G.
Figure 3.4. Three embryonic stages of four gecko species showcasing lack of dorsal and craniofacial pigment (stage 36), early visible accumulation of dorsal and craniofacial pigment (stage 39), and near-hatching dorsal and craniofacial pigment (stage 42). *Gekko kuhli* stages 36 (A), 39 (B), and 42 (C). *Lepidodactylus lugubris* stages 36 (D), 39 (E), and 42 (F). *Phelsuma laticauda* stages 36 (G), 39 (H), and 42 (I). *Sphaerodactylus macrolepis* stages 36 (J), 39 (K), and 42 (L). Scale bars = 2mm.
*kuhli* specimens may represent a different species from the *G. kuhli* specimens we examined, and *G. kuhli*, like many other species in the genus, may be a species complex comprised of multiple undescribed taxa (Das and Vijayakumar 2009; Brown et al. 2012b). Second, *G. kuhli* is a widespread species in Southeast Asia (Brown et al. 2012b) and there may be intraspecific, regional variation. Interestingly, the gonads, intestines, and peritoneum of *H. platyurus* are pigmented. *Hemidactylus platyurus*, similar to *G. kuhli*, is known to parachute, use elaborate body folds to aid in cryptic behavior, and is occasionally known to bask (Smith 1935; Russell 1979b; Ulber and Ulber 2001; Honda et al. 1997; de Silva et al. 2016), supporting the hypothesis that nocturnal geckos with cryptic diurnal behavior are exposed to ultraviolet radiation more frequently than other nocturnal gecko species and therefore require specialized protection. Indeed, the nocturnal and behaviorally cryptic, *Uroplatus fimbriatus* exhibits pigmentation in the digestive tract and the cloaca (Werner 1912). These hyperpigmented patterns represent similar evolutionary routes to protect the various internal delicate organs from UV and suggests species can take similar evolutionary paths to achieve similar functional goals in different structures (Wake et al. 2011; Natarajan et al. 2014; Blount et al. 2018). When compared to *G. kuhli*, the lower degree of subcutaneous pigmentation exhibited by *H. platyurus* may be explained by behavioral differences between the species. Though *H. platyurus* is indeed behaviorally cryptic, anecdotal evidence suggests its behavioral crypsis is less effective than that of *G. kuhli* (Smith 1935). Taylor (1963) noted that *Gekko (Ptychozoon) lionotum* can be reluctant to move from their cryptic positions and will flee only following “considerable disturbance,” whereas *H. platyurus* flee from similar positions with little disturbance (de Silva et al. 2016). Field observations also
suggest that *H. platyurus* regularly use crevices in trees, rocks, gardens, and houses near human activity as day-time hiding locations (Smith 1935; Taylor 1963; Brown and Alcala 1978; pers. observation in Philippines by AHG and TG) and are less likely to be exposed during the day compared to *G. kuhli*. This preliminary association between cryptic behavior and hyperpigmented phenotype, though promising, requires further corroboration through robust taxon sampling.

Vertebrate pigment cells are ultimately derived from neural crest cells, which begin migrating from the neural tube during the 6–9 somite stage in *Chamaeleo calyptatus* (Le Douarin 1999; Diaz et al. 2019). In avian reptiles and mammals, these unpigmented precursor cells migrate to the epidermis where mature melanocytes synthesize pigment which can then be deposited to epidermal appendages such as hair or feathers (Slominski et al. 2005; Yamaguchi et al. 2007). Alternatively, non-avian reptiles, amphibians, and fishes produce three common types of chromatophores (xanthophores, iridophores, or melanophores) as well as more phylogenetically restricted pigment cell types (e.g. cyanophores, leucophores), for which developmental trajectories are still not well understood (Bagnara et al. 1968; Parichy et al. 2006; Kelsh 2004; Fujii 2000). Despite this diversity, there is considerable conservation in molecular pathways responsible for melanocyte and chromatophore development (Parichy et al. 2006; Cooper and Raible 2008; Parichy and Spiewak 2015). The overall spatial pattern of pigment accumulation exhibited by *G. kuhli* appears similar the other gecko species examined — pigment accumulates along the epidermis overlaying the developing brain and the dorsum, adjacent to the anterior portion of the neural tube. However, the early onset temporal pattern of pigment development exhibited by *G. kuhli* has not been described in
any other gecko species to date (Mahendra 1936; Werner 1971; Noro et al. 2009; Wise et al. 2009; Guerra-Fuentes et al. 2014; Khanoon et al. 2015; Zhao et al. 2017; van der Vos et al. 2018; Griffing et al. 2019), let alone other lizard species (e.g. Py-Daniel et al. 2017; Ollonen et al. 2018; Diaz et al. 2019; Lima et al. 2019). Heterochrony, specifically an early onset of melanophore migration, maturation, or pigment production, may explain the hyperpigmented adult phenotype of *G. kuhli* (Fig. 3.3). However, the same cannot be said for the hyperpigmented adult phenotype of *P. laticauda* or the intermediate pigmented phenotype of *S. macrolepis* (Fig. 3.4) highlighting how distinct developmental programs can lead to convergent phenotypes (Wake et al. 2011; Sanger et al. 2013). Further studies of squamate neural crest development are necessary to investigate interspecific variation in melanophore migration, specifically with regards to hyperpigmented peritonea or dorsal fascia (Reyes et al. 2010; Diaz et al. 2019).

Herein we propose the hypothesis that behavioral crypsis can lead to situations which require heliothermic adaptation. *Gekko kuhli*, a nocturnal gliding gecko with behavioral crypsis, exhibits a degree of subcutaneous pigmentation that is typically only seen in diurnal/heliothermic geckos such as *Phelsuma* spp. Another behaviorally cryptic, nocturnal gecko, *H. platyurus*, exhibits similar elaborate pigmentation on some viscera but not the dorsal fascia. Further investigations into this connection between thermoregulatory behavior and pigment phenotypes should test whether *G. kuhli* and *H. platyurus* can tolerate higher temperatures and are exposed to less ultraviolet damage than sister taxa with less pigment. Furthermore, *G. kuhli* appears to exhibit hyperpigmentation throughout most of postovipositional embryonic development, a developmental pattern which differs from other geckos, including heliothermic species.
Due to this unique pattern, we suggest *G. kuhli* as a model to study temporal changes to typical reptilian patterns of neural crest derivative migration.
Introduction

The adhesive capabilities of lizards have captivated naturalists since Aristotle, over 2000 years ago (e.g. Aristotle 1918; Schneider 1812; Hora 1923; Maderson 1964; Russell 1972, 1986; Irschick et al. 1996; Gamble et al. 2012). The lamellae and scansors (sensu Russell 1972) of gecko and Anolis adhesive toe pads are highly specialized. Adhesive ability is facilitated through hair-like, hypertrophied elaborations of the epidermis known as setae (Maderson 1970; Ruibal and Ernst 1965). At a gross morphological level scansors possess tendinous connections to the digits and either reticular vascular networks or adipose pads to facilitate control while lamellae lack these structures (Russell 1986; Russell et al. 2019). Geckos exhibit a spectrum of digital morphologies, including toe pads with scanners, toe pads with a combination of scanners and lamellae, or no adhesive structures at all (Russell 1972; Russell and Gamble 2019; Russell et al. 2019), whereas Anolis only have toe pads with adhesive lamellae (Russell 2017). Excluding an analogous, yet poorly understood, evolutionary origin of digital adhesion in scincid lizards (Williams and Peterson 1982), adhesive toe pads are hypothesized to have evolved independently approximately 15 times (~14 gains in gekkotans and one gain in Anolis; Losos 2009; Gamble et al. 2012; Russell and Gamble, 2019). However, toe pads are not the only adhesive, setae-bearing structures of lizards. Several gecko lineages exhibit setae-bearing, adhesive scanners at the venterodistal tip of
the tail. These lineages are geographically and phylogenetically disparate and comprise species in 21 genera in three of the seven gecko families (Supplemental Material 4.1, 4.2). While no phylogenetic analyses of adhesive tail evolution have been done, it appears these structures evolved independently at least five times — once each in the families Sphaerodactylidae and Diplodactylidae and three times in the Gekkonidae (Gamble et al. 2015a). The adhesive tail pads of diplodactylid geckos, henceforth called tail pads, are perhaps the most well-studied and are hypothesized to be serial homologs of adhesive toe pads (Bauer 1998). Serial homologs are morphological structures that are present as multiple copies in the same organism and share a set of developmental constraints, such as fore- and hindlimbs of tetrapods (Wagner 1989, 2014; Hall 1995; Ruvinsky and Gibson-Brown 2000). Bauer's (1998) hypothesis is based upon striking morphological similarities between adult adhesive toes and tails: reticular networks of blood vessels in addition to muscle fibers attaching directly to the dermal cores of scansors to provide control of the adhesive apparatus, adipose tissue to function as a cushion for the scansors, and of course, fields of setae covering the distal subcaudal tip (Bauer 1998). Further evidence for the serial homology between tail and toe pads is their apparent evolutionary coupling. The absence of taxa exhibiting tail pads, but no toe pads, suggests that the evolution of toe pads is a prerequisite for evolving tail pads (Bauer 1998; Nussbaum et al. 1998). Corroborating the identity of a character as a serial homolog requires developmental data. The only developmental data available to Bauer (1998) were a small post-natal series of *Rhacodactylus auriculatus*, preventing any further corroboration of serial homology.
The development of lizard toe pads, in general, is poorly known. In some geckos, the first scansorial ridges form at the distal half of the digit and then develop along the entire length of the digit while becoming more asymmetrical in the proximal-distal direction (*Tarentola, Ptyodactylus*; Rosenberg et al. 1992; Khannoon 2015; Khannoon et al. 2015; Alturk and Khannoon, 2020). In *Anolis*, the beginning of lamella development follows similar patterns of lepidosaurian scale development (Maderson 1985); however, the epidermis subsequently undulates, giving rise to asymmetrical lamellae (Alibardi 1997b). Alternatively, previous studies suggest that all other body scales, with the exception of tail scales, arise from individual, dome-like epidermal papillae (i.e. placodes; Djouailly and Maderson, 1984; Alibardi 1996). This suggests that the developmental program that gives rise to adhesive toe pads is derived. With the exception of a handful of works (Tornier 1899; Underwood 1954; Maderson 1971; Vitt and Ballinger 1982; Bauer 1998; Nussbaum et al. 1998), the evolutionary morphology of adhesive tail pads has been largely ignored and remains enigmatic and the function and development of these pads have not yet been investigated. The combination of developmental and functional data will provide a robust test of the serial homology hypothesis posited by Bauer (1998). Here we: 1) characterize the anatomy and microanatomy of *Correlophus ciliatus* (Diplodactyloidae) tail pads, 2) characterize the functional capability of an adhesive tail pad in relation to its toe pads, and 3) reinvestigate Bauer's (1998) serial homology hypothesis by using developmental data to identify potential developmental constraint in the evolution of adhesive digits and tails.

**Materials and Methods**
Correlophus ciliatus are large-bodied (108 mm average Snout–Vent Length [SVL]), arboreal geckos native to New Caledonia (Bauer and Sadlier 2000; Seipp and Henkel 2000; Bauer et al. 2012). Like all other New Caledonian diplodactylids, C. ciliatus exhibit not only adhesive toe pads, but also an adhesive tail pad at the tip of a robust, prehensile tail (Fig. 4.1a). This species was not included in Bauer’s (1998) investigation because it was thought to be extinct and only "re-discovered" in the mid-1990's (Bauer 1990; Seipp and Klemmer 1994). To compare tail development, we collected embryos of C. ciliatus and embryos of a digital pad-bearing, but non-adhesive tailed gecko (Lepidodactylus lugubris) following protocols of Sanger et al. (2008) and Griffing et al. (2018b), respectively. Embryos from both species were collected from captive colonies housed at Marquette University. Protocols for gecko husbandry are detailed elsewhere (Seipp and Henkel 2000; Griffing et al. 2018b). Using 286 collected embryos, we produced an embryonic staging series for C. ciliatus, the first staging series for any pygopodoid gecko, using published gecko staging series as a reference (Wise et al. 2009; Griffing et al. 2019).

**Morphology**

We examined tails and feet of pre- and post-natal specimens using Scanning Electron Microscopy (SEM). Through a separate investigation of embryonic apoptosis, we also identified areas of substantial apoptotic activity within early tail pad development (stage 35 embryo) using Lysotracker Red DND-99 (Fogel et al. 2012). Setal densities of SEMs were estimated, following Bauer (1998). We investigated the relationship between setal density and maximum seta height in a phylogenetic context combining our C. ciliatus data with data from Bauer (1998) and Schleich and Kästle (1986) using the
Figure 4.1. Adult caudal morphology of *Correlophus ciliatus*. **A**) A time-lapse of a subadult *C. ciliatus* using its prehensile tail with adhesive tail pad to climb down a branch. **B**) Scanning electron micrographs in ventral view of an adult *C. ciliatus* tail pad. Magnified images of different areas of the tail tip are framed by solid white boxes (distal), dashed white boxes (middle), and dotted white boxes (proximal). Each column is equally magnified relative to the three regions of the tail. Note that the distal tip of the pad exhibits dense fields of setae and more proximal regions have increasingly shorter spinules. **C**) Caudal osteology of *C. ciliatus* depicted through μCT. Ventral view of caudal and sacral vertebrae and portion of pelvic girdle. Dashed white box illustrates autotomic vertebrae magnified in lateral view (1) and transverse view (2). **D**) Hall-Brunt Quadruple stained sagittal section of the *C. ciliatus* adhesive tail pad. Scansor rows 7–12. a, adipose tissue; d, dermis; e, epidermis; h, hypodermis; hm, hypaxial muscle; s, scansorial unit; sf, setal field. **E**) Average heights of a non-pad bearing gecko (*Nactus*) spinules, *Anolis* toe pad setae, *C. ciliatus* tail pad setae, and *Gekko* toe pad setae. Figure adapted from Russell (1976) and Peattie (2008).
phylogeny from Skipwith et al. (2019). We also examined internal anatomy of post-natal tails using computed tomography (Rawson et al. 2020), clearing and staining (Hanken and Wassersug 1981), and histology (Kerney et al. 2009).

Adhesive Performance Measurements and Scaling

We measured frictional adhesive performance from the tails and forelimbs of 10 C. ciliatus over a range of body sizes (6.0 – 41.3 g) following the methods of Higham et al. (2017), in which peak tensile force (Newtons) is obtained by placing the animal's adhesive pad(s) onto a pristine section of acrylic and slowly pulling the animal (or autotomized tail) in parallel opposition to an attached portable force gauge until pad slipping occurs; a single maximum force value was taken after multiple trials for each forelimb and tail. Obtaining reliable measurements often required the tail be autotomized. Once disconnected from the body, the adhesive strength of the tail was measured as above. Adhesive performance in geckos does not require active control (Stewart and Higham 2014), and removing the tail first avoided any variation due to behavior or motivation (i.e. prehension). That said, we obtained tail adhesive performance before and after autotomy for most geckos.

We quantified the scaling relationships between adhesive force and body mass using linear regressions. Variables were first log-transformed to linearize the data. The slope of the regression represents the scaling exponent, with a slope of one representing the expected relationship from previous studies of inter-specific scaling (Higham et al. 2017; Irschick et al. 1996). Scaling relationships were obtained for both the manus and the tail. Additionally, we calculated the potential for the adhesive tail tip to support the entire mass of the animal in a vertical orientation, as might occur when hanging from a
branch. To do this we calculated tail adhesive safety factor, the ratio of maximum adhesive force of the tail to the force due to gravity (body mass x acceleration due to gravity). A value greater than one indicates the tail alone could support the body.

We investigated scaling of toe pad and tail pad area with relation to SVL using 24 formalin-fixed specimens, ranging from hatchlings to adult (36.8–108.3 mm). After log-transformation, we tested for differences between toe pad and tail pad area versus SVL scaling using linear regressions and analysis of variation (R Core Team 2019).

**Results**

*Adult Morphology of the Adhesive Tail Pads*

The tail of *Correlophus ciliatus* comprises 27 amphicoelous caudal vertebrae with reduced transverse vertebral processes, similar to other functionally prehensile-tailed geckos (e.g. *Aleuroscalabotes felinus*; Koppetsch et al. 2020; Fig. 4.1c; Supplemental Materials 4.3). Unlike most geckos, no autotomy planes are visible distal to the eighth vertebra. Hypaxial muscle bundles are larger than epaxial muscle bundles (Supplemental Materials 4.3). Fields of long, branching setae cover the ventrodistal tip of the adult tail (Fig. 4.1b, d). This field occupies the distal most 13–16 scale/scansor rows, with proximal scale rows exhibiting shorter setae, and ultimately, non-branched spinules (Fig. 4.1b). Mean height of tail setae = 20.0µm (N=30 setae, measured from a ventrolateral tail scansor), the longest measuring 26.6µm (Fig. 4.1b, e; Supplemental Materials 4.4). Mean height of toe setae = 23.8µm (N=30), the longest measuring 32.7µm (Supplemental Materials 4.5). Setal densities between tail and toe pads are close to each other: tail = 32,950 setae/mm²; and toe = 30,000 setae/mm². There is an inverse relationship between maximum setal height and setal density in toe pads ($r^2 = 0.2889, F = 5.468, P = 0.0414$),
but not tail pads ($r^2 = 0.2909$, $F = 3.282$, $P = 0.1076$; Supplemental Materials 4.6). Distal scansors/scales are more imbricate and asymmetrical than proximal scales or the scales on the dorsal surface of the tail (Fig. 4.1d; Supplemental Materials 4.3). Unlike the dorsal scales, the dermal core of scansors sits above a thick hypodermal layer of adipose tissue (Fig. 4.1d; Supplemental Materials 4.3).

*Adhesive Performance Measurements and Scaling*

The adhesive performance of the manus ranged from 1.33 N to 8.12 N, with larger geckos clinging with greater force ($r^2 = 0.95$, $P<0.001$; Fig. 4.2a). Adhesive performance of the manus scaled with mass$^{1.04}$. The adhesive performance of the tail pad ranged from 0.22 N to 1.19 N, and also increased with body size ($r^2 = 0.88$, $P<0.001$; Fig. 4.2a). Adhesive performance of the tail scaled with mass$^{0.97}$. The safety factors for the tail pad ranged from 2.45 (largest gecko) to 5.57 (moderately-sized gecko). Given an expected scaling exponent of 2 under isometry, the toepad area scaled with isometry (scaled with SVL$^{1.88 \pm 0.183}$), whereas the tail pad area scaled with negative allometry (scaled with SVL$^{1.54 \pm 0.298}$; Fig. 4.2b).

*Pre-natal Development of Adhesive Structures*

*Correlophus ciliatus* toe pad development begins shortly after interdigital webbing recession (stage 36; Fig. 4.3; Supplemental Materials 4.7). Four subdigital scansorial ridges initially form in the widest, distal portion of the digit. Shortly after, a small number of new ridges form distally and many more ridges form proximally, all while simultaneously expanding laterally (Fig. 4.3). Individual scansors become more imbricate with one another until toe pad development is complete at stage 42 (Fig. 4.3).
Figure 4.2. Adhesion and scaling of the *Correlophus ciliatus* tail pad. A) Adhesive performance of *C. ciliatus* tail pads and forelimbs. Points to the right of the dashed arrow denotes adults from juveniles/subadults. B) Isometric scaling of toe pad area and negative allometric scaling of tail pad area with respect to SVL.
Tail tip shape changes drastically during *C. ciliatus* embryonic development. The tail tip is initially pointed and subsequently sculpted away into a wide, blunt end through apoptosis (Fig. 4.3; Supplemental Material 4.8). Tail pad development occurs immediately after toe pad development begins. Prior to any signs of scansor development, a subcaudal sulcus forms along nearly the entire length of the tail (stage 36). This sulcus is associated with enlarged hypaxial muscle bundles found in other prehensile-tailed geckos (Bauer 1998). The distal-most portion of the tail exhibits lateral outgrowths, creating a distal pad which is somewhat wider than the rest of the tail (Fig. 4.3; early stage 36). Shortly after (mid stage 36), large lateral scansorial ridges form in the distal portion of the pad before forming new ridges in a distoproximal direction (stage early–mid stage 37). At stage 38, the distal scansorial ridges begin subdividing into numerous, raised units within a scansorial ridge (Fig. 4.3). We henceforth refer to this process as granularization. Subsequently (stage 39), the distal portion of the tail expands further laterally, forming a spatulate pad. The pad expands laterally and scales granularize in a distoproximal direction until tail pad development is complete by stage 42 (Fig. 4.3). Embryonic development of the non-padded tail of *L. lugubris* is noticeably different from *C. ciliatus*. *Lepidodactylus lugubris* does not exhibit a subcaudal sulcus and the first signs of tail scale development occur at stage 39 when approximately eight evenly-spaced annular scale rows form simultaneously along the length of the tail (Fig. 4.3). Further annular scale rows form in between these initial rows by stage 40 and then granularize simultaneously by stage 41 (Fig. 4.3).

**Discussion**

*Adhesive Tail Tip Structure and Homoplasy*
Correlophus ciliatus has been considered unique among adhesive-tailed geckos in having a paddle-shaped distal tail tip, thus expanding the adhesive field compared to tail pads of other species (Guichenot 1866; Bauer 1998). However, despite this unique paddle-shape, the surface morphology and histomorphology of the C. ciliatus adhesive pad is largely similar to other gecko tail pads (Bauer 1998; Alibardi and Meyer-Rochow 2017; Alibardi and Bonfitto 2019). Setae are branched and of comparable height to toe setae, hypaxial muscle bundles are enlarged to presumably assist with prehension, and subdermal adipose tissue likely plays a role in cushioning scanners against surfaces (Fig. 4.1b,e; Supplemental Material 4.3,4.4; Russell 1972; Russell and Bauer 1988; Hale 1996; Bauer 1998; Zippel et al. 1999).

Setal heights and densities are largely similar between toe and tail pads (Supplemental Material 4.2). We demonstrate a significant inverse relationship between setal height and density in diplodactylid toe pads. The lack of this relationship in tail pads is likely due to a small sample size. In the gekkonid gecko genus Gekko, Bauer and Good (1986) hypothesized that as body size increases between species, setal height and density increase and decrease, respectively. This relationship generally holds true for New Caledonian diplodactylids, but not New Zealand diplodactylids (Supp. Info. 2; Bauer 1998). Small-bodied Bavavia exhibit moderate setal heights (29–32 µm) with extremely dense fields of setae (35,600–42,900 setae/mm$^2$) while the large-bodied Rhacodactylus exhibit larger setal heights (34–38 µm) with less dense fields of setae (13,700–18,000 setae/mm$^2$). The microanatomy of C. ciliatus tail pads deviates from these trends. Although they exhibit setae of a comparable height to other large-bodied New Caledonian diplodactylids, setal densities of C. ciliatus tail and toe pads are much higher,
on par with densities exhibited by *Bavayia sauvagii* toes and tails (Supplemental Material 4.3, 4.4). Further, the setal tip width (i.e. the amount of branching) is larger than any other studied diplodactylids (Supplemental Material 4.4), with both toe and tail setae having a setal tip that is 2–3 times wider than the closely related *Correlophus sarasinorum*. The increased density of tail setae, coupled with large setal tip width, may provide *C. ciliatus* with adhesive ability which exceeds that of other diplodactylid geckos. However, it should be noted that setal densities can vary on gecko toe pads depending on location of measurement (Autumn 2007; Russell and Johnson 2007; Russell and Garner 2021).

**Functional Significance of Tail Adhesion**

Digital adhesion in *C. ciliatus* is similar to some of the highest absolute adhesive forces recorded for geckos (see Irschick et al. 1996 and Higham et al. 2017 for comparative values). *Gekko gecko* is the only species with higher recorded forces (Irschick et al. 1996; Stewart and Higham 2014). *Correlophus ciliatus* tail adhesion is substantial; forces frequently exceeded 1 N, which, in those cases, represented up to 80.2% of the force estimated to be produced by a single digit. *Correlophus ciliatus* tail adhesion values far exceed digital adhesion of *Anolis*, another pad-bearing group of lizards often studied in the context of digital adhesion. With both forelimbs engaged, *A. carolinensis* and *A. sagrei* generate 1.5 and 1.3 N of adhesive force, respectively (Irschick et al. 1996). Thus, crested gecko tail adhesive force often exceeds the forces that these anoles can generate with a single manus.

Safety factors for tail adhesion are, in all cases, sufficient to support the mass of the entire body in a vertical orientation, which means that *C. ciliatus* could potentially
hang from a branch using only their tail. In fact, the maximum value of safety factor exceeds five, indicating that a single tail could theoretically hold up to five *C. ciliatus* without losing grip. Additionally, the tails are capable of prehension, adding yet another component to their clinging ability. Although it is unclear how much grasping force the tail could exert on a perch, some lizards (e.g. chameleons) can exert up to 35 times their own body mass in grip force with their tail (Herrel et al. 2012).

The scaling factors of digital and tail adhesion with respect to body mass in *C. ciliatus* were not significantly different from 1, which is comparable with previous research on geckos and other pad-bearing lizards (Higham et al. 2017; Irschick et al. 1996), as well as leaf-cutting ants (Labonte and Federle 2015). This ‘functional similarity’ is not found when examining the relationship between toepad area and adhesive force (Irschick et al. 1996), where it largely follows the predicted scaling factor of 0.67 with respect to body mass. Therefore, other aspects of adhesive morphology, such as setal dimensions or density, are likely driving the functional similarity. Although the scaling exponents of adhesive pad area of the tail and digits (with respect to SVL) are not significantly different from each other, only toepad area scaled with isometry. In contrast, the tail pad scaled with negative allometry, possibly indicating that tails are more important for clinging in smaller geckos. This could be attributed to a shift in habitat use through ontogeny, although little is known about substrate use in nature.

*Revisiting Serial Homology of Adhesive Tail Tips to Adhesive Digits*

*Correlophus ciliatus* toe and tail pads exhibit strikingly similar patterns of pad subdivision and extension during development (Fig. 4.3). Additionally, the onset of pad formation and subdivision occurs near synchronously in both structures. The
Figure 4.3. Scanning electron micrographs of digital and caudal development in *Correlophus ciliatus* and caudal development in *Lepidodactylus lugubris*. Embryonic stages 36–42. Plantar views of left manus, digit IV and ventral views of the distal tail tip. In *Correlophus ciliatus*, both toe pads and tail pads exhibit a distal-to-proximal development of scansion rows, which subsequently subdivide into what will become the adult scansion. In *L. lugubris*, scale annuli form synchronously along the entire length of the tail and wrap around the circumference of the structure (Stage 38, white arrows). Initial annuli become more distinct (Stage 39, black arrow) with annuli appearing near the distal tail tip (Stage 39, white arrows). Eventually additional annuli form in between the initial annuli (Stage 40, white arrows) and subsequently become granular (Stages 41–42). Scale bars = 500µm.
development of adhesive toe pads in *C. ciliatus* is similar to *Tarentola* geckos (Khannoon 2015; Khannoon et al. 2015; Alturk and Khannoon 2020) suggesting comparable developmental mechanisms underly the formation of adhesive structures in both species. Consequently, our data provide further support to Bauer’s (1998) hypothesis that the adhesive tail pads of some diplodactylid geckos are serially homologous to their toe pads. Our results only support this conclusion for the single origin of adhesive tail pads which includes *C. ciliatus* (clade comprising *Pseudothecadactylus* + New Caledonian geckos; Skipwith et al. 2019). A comparative developmental investigation of other groups (e.g. New Zealand geckos, *Lygodactylus*, *Euleptes*, etc.) is required to corroborate serial homology in other tail pad-bearing taxa. Further evidence of serial homology between tail and toe pads comes from similarities of setal development on the digits and regenerating tail of the New Zealand diplodactylid, *Woodworthia maculata* (Alibardi and Meyer-Rochow 2017). Such an experiment is impossible with *C. ciliatus* as they do no regenerate a full tail after autotomy, nor amputation (Griffing, in preparation). Although there are underlying differences between toe and tail pads in skeletal, muscular, and tendinous morphologies, these likely reflect the distinct ancestry upon which the adhesive pads evolved (Bauer 1990, 1998) and do not detract from the serial nature of the tail and toe adhesive apparatus.

The degree to which tail pads and toe pads are evolving independently (paramorphs; sensu Wagner 2014) or in tandem (homomorphs; sensu Wagner 2014) is unclear. Although the presence of tail pads appears linked to the presence of adhesive toe pads, anecdotal evidence suggests toe pad shape and size does not predict tail pad shape and size (Bauer 1986, 1998), suggesting some degree of independent evolution. Further
investigations into other tail pad bearing taxa may determine whether they exhibit different ranges of covariation between tail and toe pad shape and size (Young and Hallgrímsson 2005).

Following pad subdivision and extension, tail pads deviate from the developmental pattern seen in toe pads and begin to exhibit granularization of the individual scansors (Fig. 4.3). We posit the adhesive scanner developmental program, via homeosis, was supplanted onto the tail tip, resulting in markedly similar development of scannerial ridges. Soon after, the scanners granularize, creating numerous placode-like structures, which resemble typical reptile body scale development (Di-Poï and Milinkovitch 2016). Unlike *C. ciliatus*, other adult gecko tail pads, like those of *Pseudothecadactylus* and *Lygodactylus*, exhibit mediolaterally broad scanners with few to no granular scanners (Loveridge 1947; Bauer 1998). To our knowledge, this derived, granularizing pattern has not been documented in other studies of amniote integumentary development (Di-Poï and Milinkovitch 2016; Cooper et al. 2019). Identifying the patterns of activator and inhibitor morphogens in tail pad development is required to determine whether this is a two-step process of lateral inhibition to form tail scanners (Noramly and Morgan 1998). These derivations further demonstrate that our current understanding of epidermal development is incredibly simplified and requires further descriptive embryology to fully characterize the diversity of epidermal developmental patterns (Cooper et al. 2019). Further investigation into the molecular patterns and processes that produce both digital and tail adhesive pads is necessary to definitively determine the degree of homology the two structures share.
Tail pads of *C. ciliatus*, but not the non-adhesive-tailed *L. lugubris*, appear to pass through the three main stages exhibited by developing toe pads (Fig. 4.3; Supplemental Material 4.8). Adhesive toe pads of *C. ciliatus* and *Tarentola* (Kahnoon 2015; Kahnoon et al. 2015) exhibit these stages following digital webbing reduction: 1) pad formation, 2) distal scansorial/lamellar ridge formation, and 3) distal-to-proximal and lateral ridge extension. By contrast, non-padded lizards of the genus *Pogona* develop all plantar scales synchronously across the length of the digit (Cooper et al. 2019). Following these stages, the scansorial rows of developing tail pads begin to granularize, presumably being released from previous constraint (Fig. 4.3). Further developmental research is required to determine: 1) if the pattern exhibited by *Pogona* is the ancestral state, and 2) if there are biases in the production of morphological variation during pad morphogenesis (i.e. developmental constraint; Maynard-Smith et al. 1985).

Our in-depth investigation into the structure, function, and development of *C. ciliatus* reveals their tail pads are largely similar to other diplodactylid lizards (Bauer 1998; Alibardi and Meyer-Rochow 2017), with the exception of their extraordinarily dense fields of setae for their body size and large branching setal tips. The adhesive tail pad of *C. ciliatus* is highly functional, with adhesive capabilities on par with an entire *Anolis* manus. Paradoxically, the highly functional *C. ciliatus* tails do not regenerate, unlike nearly all other gecko species which autotomize their tails, including their close relatives, *Correlophus sarasinorum*. Finally, we add evidence that toe pads and tail pads are serial homologs. Investigation into the molecular underpinnings of toe and tail pad development are required to definitively corroborate serial homology and identify the
degree to which developmental constraint has affected the evolution of these enigmatic structures.
V. HOW TO BUILD A PARACHUTE: INTERDIGITAL WEBBING RETENTION AND THE EVOLUTION OF GLIDING STRUCTURES IN GECKOS (GEKKONIDAE)

Introduction

The evolution of specialized phenotypes allows organisms to interact with environments in novel ways (Simpson 1944, 1953). The evolution of aerial locomotion (flying, gliding, or parachuting) is an example of morphological innovation that facilitates occupation of new niches (e.g. Savile 1962; Norberg 1994; Ord et al. 2020). Structures used for aerial locomotion (flying, gliding, or parachuting) are arguably the most well-known examples of convergently evolved structural analogy, with flight and gliding having evolved at least 30 times across tetrapod evolutionary history (Savile 1962; Norberg 1990; Rayner 1988). Parachuting and gliding, in particular, has evolved in diverse lineages, including, frogs (e.g. Davis 1965), mammals (e.g. Thorton et al. 1998; Jackson 1999), snakes (e.g. Socha 2011), and lizards (e.g. Colbert 1967; Arnold 2002; Young et al. 2002). This behavioral phenotype is associated with a suite of derived morphologies that increase surface area of the individual and, in tandem, slow vertical descent (Norberg 1990). This includes retention of webbing in the interdigital spaces (e.g. Rhacophorus frogs), widening of the lateral trunk through lateral extension of the ribs (e.g. Draco lizards, Holaspis lizards, Chrysopelea snakes) or membranous patagia (e.g. Glaucomys squirrels, Petaurus gliders), or a combination of these (e.g. Gekko kuhli geckos; Fig. 5.1a).

Gecko lizards have independently evolved gliding or parachuting behavior in at least seven lineages (Boulenger 1890; Honda et al. 1997; Vitt and Zani 1997; Brown et al. 1997, 2007, 2012; Bauer et al. 2010; Wood et al. 2020). These consist of one
Figure 5.1. Gliding morphology in geckos. A) Ventral view of *G. kuhli* illustrating elaborate gliding morphology. B) Evolutionary relationships of species investigated in this study. Taxon names correspond to adjacent photographs of ventral aspects of the pes. Characters at tree tips include gliding behavior (GB) and interdigital webbing (IW). Character states include extreme elaboration (black), intermediate elaboration (grey), and little to no elaboration of the trait (white). Phylogeny and divergence times (millions of years ago; MYA) based on Gamble et al. (2015a). Photos: S. Nielsen, T. Gamble.
Neotropical phylloactylid lineage (*Thecadactylus*) and six Southeast Asian gekkonid lineages (*Hemidactylus platyurus* + *H. craspedotus* clade, *Gekko* [*Ptychozoon*, *Rhacogekko*, *Lamatodactylus*, and *Balwangekko* clades], and *Luperosaurus sensu stricto*). The gliding or parachuting behavioral phenotypes are accompanied by interdigital webbing (IW), expanded lateral trunk folds (i.e. patagia), and expanded lateral caudal folds (Russell 1979). Russell (1979) hypothesized a step-wise exaptive process to patagia evolution in gekkonids. First, small lateral body folds, exhibited by many gecko taxa, serve a fat storage function. These small lateral folds are expanded and co-opted to aid in crypsis (avoiding detection by flattening the body against a surface and eliminating shadows outlining the animal; de Rooij 1915), and when coupled with predator-escape behavior, leads to parachuting. What could now be considered patagia are then expanded further, like those of *Gekko kuhli* and *Hemidactylus platyurus*, and co-opted for gliding. Interestingly, closely related gekkonid taxa (e.g. *Lepidodactylus lugubris*, *Hemidactylus turcicus*) exhibit both intermediate morphologies and non-gliding morphologies. This makes gekkonids the ideal clade to study morphological diversity, development, and exaptation of gliding morphologies.

Although little is known about the development of vertebrate gliding patagia, the patterns underlying development and retention of interdigital webbing (IW) is known for diverse vertebrate taxa (Saunders and Fallon 1967; Ballard and Holt 1968; Hincheliffe 1974; Fallon and Cameron 1977; Kimura and Shiota 1996; Weatherbee et al. 2006; Tokita et al. 2020). Amphibians develop free digits through outgrowth and very little apoptosis (programmed cell death; e.g. Cameron and Fallon 1977; Shimizu-Nishikawa et al. 2012). Conversely, free digits of mammals, birds, and non-avian reptiles develop
through apoptosis of the interdigital space (Saunders and Fallon 1967; Ballard and Holt 1968; Hincheliffe 1974; Fallon and Cameron 1977). In the latter scenario, IW recession occurs after digital condensations are visible during embryonic development (Wanek et al. 1989). In most free digit species, such as mice (Mus musculus), chickens (Gallus gallus), and skinks (Plestiodon fasciatus), apoptotic activity occurs between digital condensations and “sculpts” away IW (Hincheliffe 1974; Fallon and Cameron 1977; Wanek et al. 1989). Alternatively, in species that exhibit IW as adults, such as ducks (Anas platyrhynchos), bats (Corollia perspicillata), or turtles (Chelydra serpentina), apoptosis is limited to the distal regions of IW, resulting in varying degrees of webbed digits (Fallon and Cameron 1977; Merino et al. 1999; Weatherbee et al. 2006). Despite these previous characterizations, patterns of apoptosis in the developing autopodia of lizards, particularly geckos, remain largely unknown. We aimed to investigate embryonic patterns of IW recession in gliding geckos using a model clade comparative framework (Sanger and Rajakumar 2019). By studying the convergent evolution of gliding morphology in southeast Asian gliding geckos and their non-gliding relatives, we sought to determine whether gliding morphologies evolve in a predictable way, through similar alterations to the presumed ancestral pattern of IW recession in geckos. We hypothesized that similar developmental patterns and processes result in the retention of IW in gliding geckos. We predicted that apoptotic activity would be limited to the distal interdigital spaces in taxa with intermediate to extreme IW. Furthermore, we predicted the intensity of apoptotic activity would be significantly greater in taxa with no IW.

**Materials and Methods**

*Taxon Sampling and Embryology*
We investigated six species of gecko: two gliding taxa (*Gekko kuhli*, *Hemidactylus platyurus*), two non-gliding close relatives (*Lepidodactylus lugubris*, *Hemidactylus turcicus*), and two outgroups (*Eublepharis macularius*, *Correlophus ciliatus*). These taxa exhibit a range of IW, with *G. kuhli* and *H. platyurus* exhibiting extreme IW, *L. lugubris* and *C. ciliatus* exhibiting intermediate IW, and *H. turcicus* and *E. macularius* exhibiting little-to-no IW (Fig. 5.1b). We chose these species based on their range of gliding phenotypes, commercial availability, and ability to reliably breed in captivity. *Gekko kuhli*, in particular, exhibits some of the most extreme gliding morphology of any gecko (Fig. 5.1a). We collected embryos from captive colonies of all six species housed at Marquette University (Milwaukee, WI USA). We harvested embryos (N=1,135) following the protocols of Griffing et al. (2018b) and subsequently characterized complete postovipositional embryonic staging series, following previous characterizations (Dufaure and Hubert 1961; Wise et al. 2009; Griffing et al. 2019, 2021, in prep.). These stages are diagnosed by combinations of morphological characters to discretize comparable developmental windows between species. Full staging series of previously unpublished species can be found in elsewhere (Supplemental Material 5.1, 5.2).

**Visualizing Interdigital Webbing**

Using our established embryonic staging series, we characterized external morphology of IW by imaging fresh embryos under Nikon® SMZ 74ST stereoscope and identified the timing of digital condensation formation in the paddle-shaped limb, the onset of IW reduction, and the completion of IW reduction. To visualize apoptotic activity, we treated a subset of whole-mount harvested embryos (N=72) with
Lysotracker® DND-99 following a protocol modified from Fogel et al. (2012; Supplemental Material 5.3). Fluorescent Lysotracker® probes serve as a proxy for apoptotic activity by binding to lysosomes. We imaged these whole-mount specimens using a Nikon SMZ1500 stereoscope. Due to dehydration and distortion of the tissue through Lysotracker® processing, we quantified fluorescence between manual digits III and IV. Digits III and IV are generally the longest digits of geckos and allowed a clear view of IW. In two cases (H. turcicus, early stage 35 and H. platyurus late stage 35), where tissue distortion was severe between digits III and IV, we measured fluorescence between manual digits II and III. We measured fluorescence by two methods using Fiji v2.0.0 image processing software (Schindelin et al. 2012). First, we measured the percentage of fluorescent area in the total interdigital space. Second, we calculated corrected total cell fluorescence (CTCF) by subtracting the product of IW area and mean background fluorescence from IW integrated density (McCloy et al. 2014). We determined that phylogenetic signal from our taxon sampling was substantial by performing a phylogenetic generalized least squares (PGLS; $\lambda = 1.737409$; Grafen 1989; R Core Team 2021) using the phylogenetic branch lengths of Gamble et al. (2015a) and the R v4.0.5 packages ape (v5.3; Paradis and Schleip 2019), nlme (Pinheiro et al. 2020), and geiger (Harmon et al. 2008). Using the same packages, we then corrected measurement for phylogenetic signal using phylogenetic independent contrasts (PICs; Felsenstein 1985).

Results

Light Microscopy
Digital condensations are first visible via light microscopy between stages 32 and 33 (Fig. 5.2). Subsequently, IW recesses from stages 33 to 36. Manual developmental sequence occurs slightly earlier than pedal, meaning the IW of pes is the last to recede. However, in late stage 36 and early stage 37, when claw formation is visible by light microscopy, IW recession is complete in both the manus and pes. The ratios of IW to digit length seen in adult specimens of each species are established during this window of embryonic development: by the end of IW recession, IW occupies over half of the interdigital space in *G. kuhli* and over a third of the interdigital space in *H. platyurus* (Fig. 5.2).

On average, the temporal window when IW recession occurs was shorter in taxa with extreme IW (6 days, *G. kuhli*; 7 days, *H. platyurus*). The period of IW recession was longer in taxa with intermediate IW (8 days, *L. lugubris* and *C. ciliatus*) and taxa with little to no IW (10 days, *H. turcicus*; 9 days, *E. macularius*). When corrected for phylogeny, taxa with intermediate-to-extreme IW exhibit a significantly shorter time frame of IW recession (stages 33–36) than the two taxa without IW (p=6.8 x 10^{-5}; PIC ANOVA).

**Apoptotic Activity**

Apoptotic activity is first visible in early stage 32 and is localized the apical ectodermal ridge (AER) adjacent to where digit I will form (Fig. 5.3). Both species of *Hemidactylus* deviated from this pattern: no signal was visible in the AER of *H. turcicus*, whereas signal was visible in the AER adjacent to both digits I and V in *H. platyurus*. Beginning in stage 33, small areas of apoptotic activity are visible in the distal most regions of some interdigital spaces (Fig. 5.3). In *G. kuhli*, apoptosis is limited to the AER
Figure 5.2. Light microscopy of the interdigital webbing (IW) through gecko embryonic development. Lateral views of the trunks and limbs of embryos from embryonic stage 32 to stage 36. Yellow arrowheads point to IW between the manual digits.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Gekko kuhli</th>
<th>Lepidodactylus lugubris</th>
<th>Hemidactylus turcicus</th>
<th>Hemidactylus platyurus</th>
<th>Eublepharis macularius</th>
<th>Correlophus ciliatus</th>
</tr>
</thead>
</table>

**Figure 5.3.** Apoptotic signal in embryonic gecko interdigital space. Anteplantar views of the right manus of geckos from embryonic stage 32 to stage 37. From left to right, digits are numbered I–V. Brighter areas correspond to areas of higher apoptotic activity.
adjacent to digits I and V and the distal interdigital space between digits IV and V. In *L. lugubris*, faint apoptosis is visible in the distal most portions of the interdigital spaces between digits II, III, IV, and V. In *H. turcicus*, apoptosis is clearly visible deep between digital condensations in the IW, but not in the distal portions. Little to no apoptosis is visible in *H. platyurus* at this stage. In *E. macularius*, apoptosis is localized in the entire AER, adjacent to all digits and interdigital spaces. Furthermore, apoptosis is visible deep in the interdigital spaces between digits I and II as well as IV and V. *Correlophus ciliatus* exhibits a pattern similar to *L. lugubris*.

Apoptotic activity is visible at stage 34 in all species examined (Fig. 5.3). In *G. kuhli*, apoptosis is limited to the distal regions of all interdigital spaces. In *L. lugubris*, apoptosis is visible in the entire interdigital space between all digits except digits I and II, where it is limited to the distal portion of the IW. In *H. turcicus*, apoptosis is visible in the entire interdigital space between all digits. In *H. platyurus*, apoptosis is limited to the distal regions of all interdigital spaces. *Eublepharis macularius* exhibits similar patterns to *H. platyurus* at this stage. In *C. ciliatus*, strong apoptotic signal is present in the distal half of the IW between all digits.

The majority of IW recession occurs in stage 35 (Fig. 5.3). In early stage 35, *G. kuhli* exhibits apoptosis in a slim portion of the proximal IW with the majority of activity is still restricted to the distal interdigital space. By late stage 35, *G. kuhli* exhibits apoptosis only in a thin margin of the digits and the distal IW. Throughout all of stage 35, *L. lugubris* exhibits apoptosis in both proximal and distal portions of the interdigital space. Small regions without apoptotic activity are adjacent to the distal portion of the digital condensation. This appears to create the wider digital surface where the toe pad
will eventually form (Griffing et al. 2022). Throughout all of stage 35, *H. turcicus* exhibits apoptosis in the entire interdigital region. There are also regions of apoptosis in distal tips of the free digits at this stage. Throughout all of stage 35, *H. platyurus* exhibits apoptosis only in a thin margin of the digits and distal IW. In early stage 35, *E. macularius* exhibits apoptosis in both proximal and distal portions of the interdigital space. Similar to *L. lugubris* at this stage, regions without apoptotic activity are adjacent to the distal portion of the digital condensation. In late stage 35, *E. macularius* strong apoptotic signal is in the distal margins of the IW, with noticeably less signal in the proximal interdigital space. Throughout all of stage 35, *C. ciliatus* exhibits strong apoptotic signal in both proximal and distal portions of the interdigital space. Similar to *L. lugubris* at this stage, regions without apoptotic activity are adjacent to the distal portion of the digital condensation. These regions are more pronounced in late stage 35.

Very little apoptosis is visible in stages 36 and 37 and the proportion of digital webbing to free digit is largely similar to ratios seen in postnatal specimens (Fig. 5.3). *Gekko kuhli, L. lugubris,* and *H. platyurus* exhibit very faint apoptosis in a thin margin of the distal IW and digits at stage 26. No apoptosis is visible at stage 37. At stage 36, *H. turcicus* exhibits fluorescent activity in a thin margin of the digits and IW which is still faintly visible at stage 37. This same pattern is seen in *E. macularius* and *C. ciliatus.* Outside of the manus, apoptotic signal is present in the cheek patagia and trunk patagium of stage 37 *G. kuhli,* but not the tail patagium. There is no apoptotic signal in the patagia of *H. platyurus* at stage 37 or earlier (Fig. 5.5).

At the onset of IW recession (stage 33), taxa with little to no IW (*H. turcicus, E. macularius*) exhibited significantly higher percentages of apoptotic IW area than those
with intermediate or extreme IW (p=1.64 x 10^{-3}, no IW vs. intermediate and extreme IW; p=1.02 x 10^{-2}, no IW vs. intermediate IW vs. extreme IW; Fig. 5.4; Supplemental Material 5.4). This was the only statistically significant relationship between apoptotic IW areas. The highest percentage of apoptotic IW area was achieved between stages 34 and 35 by *H. turcicus* (Fig. 5.4; Supplemental Material 5.4). *Lepidodactylus lugubris* and *Correlophus ciliatus* exhibited similarly high apoptotic IW area in these stages. *Gekko kuhli*, *H. platyurus*, and *E. macularius* exhibited similarly low apoptotic IW area in these stages. Apoptotic IW areas decrease in all species after stage 36. There were no significant relationships between CTCF measurements either between species of between classes of IW (Supplemental Material 5.4).
Figure 5.4. Apoptotic activity in some, but not all, developing gliding patagia. A) Lateral view of a stage 37 *G. kuhli* embryo. B) Fluorescent apoptosis imaging of the lateral cheek fold patagium of stage 37 *G. kuhli* embryo. C) Fluorescent apoptosis imaging in the trunk fold patagium of stage 37 *G. kuhli* embryo. D) Fluorescent apoptosis imaging of caudal fold patagia of stage 37 *G. kuhli* embryo. E) Fluorescent apoptosis imaging of trunk fold patagium of stage 37 *H. platyurus*. Yellow arrows point to substantial apoptotic signal while yellow circles indicate a lack of apoptotic signal.
Figure 5.5. Percentage of fluorescent apoptotic signal in the interdigital space between manual digits III and IV from embryonic stage 33 to stage 37. All measurements represented are prior to correction for phylogenetic distance. Slopes for each species: *C. ciliatus*, 0.28100; *E. macularius*, 1.00507; *H. turcicus*, 0.08047; *H. platyurus*, -3.02302; *L. lugubris*, -1.09719; *G. kuhli*, 1.08450.
Discussion

Most lizards do not exhibit IW as adults (Pianka and Vitt 2006). Despite this, the degree of IW which geckos exhibit is extremely variable, even between closely related species (e.g. Bauer and Sadlier 2000; Brown et al. 2011; Higham et al. 2014; Wood et al. 2020). We demonstrate that in our taxon sampling scheme, the period of IW recession is significantly shorter in the four species with intermediate-to-extreme IW. Temporal changes in development related to shape and size, such as this, has long been hypothesized to be a drivers of morphological diversity (i.e. heterochrony; Alberch et al. 1979). However, further taxon sampling is required to determine if this temporal shift is a widespread in geckos with IW and how constrained the process of IW retention is.

Apoptosis is the primary mechanism in sculpting the free digits of amniotes (Saunders and Fallon 1967; Ballard and Holt 1968; Hincheliffe 1974; Fallon and Cameron 1977). We found geckos with little to no IW (H. turcicus, E. macularius) exhibited a significantly greater area of interdigital apoptotic activity when compared to those with intermediate-to-extreme IW at stage 33 (Figs. 5.3, 5.5). The spatial profiles of high apoptotic activity differed between the two species. Apoptosis occurred only in the proximal IW of H. turcicus and only in the distal IW of E. macularius. Although the precise locations of the apoptotic activity are not conserved between the two species, these spatial pattern suggests widespread apoptotic activity early in development is important to reduce IW and free the embryonic digits. No other relationships with fluorescent area were significant. Generally, species with intermediate IW exhibited apoptosis in the entire distoproximal interdigital space (Fig. 5.3). Interestingly, both of these species (C. ciliatus, L. lugubris) lacked apoptosis in the immediate adjacent areas of
the digital condensations. This may be a mechanism to expand the digital space to accommodate the soon-to-develop adhesive pad (Griffing et al. 2022). In species we examined with extreme IW (G. kuhli, H. platyurus) exhibited apoptosis limited in the distal portions of the interdigital space (Fig. 5.3). This pattern is seen in other amniote taxa which have substantial IW (Merino et al. 1999; Weatherbee et al. 2006; Kaltcheva et al. 2016; Tokita et al. 2020). In these cases, at the onset of IW recession, BMPR1A inhibits FGF8, allowing retinoic acid production and turning on a BMPs (BMP4, 2, and 7) pathway which results in IW apoptosis, and thus, free digits. Birds retain IW on their hindlimbs through proximal expression of Gremlin, which in turn blocks the BMP pathway leading to apoptosis (Merino et al. 1999; Tokita et al. 2020). Bats have evolved a similar pattern of forelimb IW retention in which Gremlin and increased and widespread FGF8 expression prohibit the IW apoptosis (Weatherbee et al. 2006). The expression of BMPs, FGFs, and Gremlin likely also controls the amount of IW in gliding geckos. However, nothing is known of the spatial or temporal expression profiles of these genes of interests in geckos. Future investigations into these gene families will likely uncover the mechanism of IW retention in geckos and how conserved or derived it is from previous amniote models.

We found no significant differences in apoptotic intensity (CTCF) between webbed and non-webbed geckos we investigated. This may be the result of substantial background noise in fluorescent samples. Furthermore, G. kuhli exhibits large numbers of melanocytes accumulated on the limbs by early stage 32 and beyond (Griffing et al. 2020). This likely affected the measured background mean fluorescence. Relative width of developing toe pads, which exhibited no apoptotic signal may have also affected
fluorescent measurements. Our lack of significant results when comparing this measurement suggests it may not be an informative measurement when comparing across species which exhibit drastically different embryonic sizes or digital morphologies.

As a product of our fluorescent assays, we found apoptotic signal in some of the patagia of *G. kuhli* (Fig. 5.4). Beginning at stage 37, apoptotic signal was present in the cheek and trunk folds, but not the caudal folds. There was no apoptotic signal in the patagia of *H. platyurus* at stage 37 or earlier. These differences suggest that multiple developmental mechanisms underlie different patagia within the same individual and between species. This also suggests that apoptosis plays an important role in “sculpting” excess tissue and flattening some patagia. Future investigations into the evolution and development of gliding structures should work to characterize the underlying cellular and transcriptomic profiles of patagium development to better elucidate how conserved or derived these patterns are between convergently evolved structures.

Gliding taxa exhibit shorter stages of IW recession and have apoptotic activity limited to the distal portions of the interdigital space. Although our fluorescent intensity data were inconclusive, taken together, we find support that similar developmental patterns result in the convergent retention of IW in gliding geckos. Future studies may focus on increasing taxon sampling and investigating underlying molecular mechanisms for the retention of IW and outgrowth of patagial gliding membranes.
VI. CONCLUSIONS AND PROSPECTUS

Understanding how morphological diversity is attained across the tree of life is a central goal of evolutionary biology (Foote 1997). Studying underlying developmental processes can uncover how adult organisms achieve this diversity of shapes and sizes (Alberch 1982; Raff 2000). The study of morphological evo-devo begins with the quantification of adult morphologies (phase 1), moves on to study the developmental patterns which underlie that morphology (phase 2), and ultimately tests underlying genes and pathways mechanistically (phase 3; Mallarino and Abzhanov 2012). Characterizing patterns of adult gecko morphology (i.e. phase 1) has been well-underway for decades (e.g. Kluge 1967; Russell 1976; Bauer 1990; Gamble et al. 2012); however, upon beginning this dissertation in 2016, investigations into gecko development were limited to only a handful of reports (Sewertzoff 1908; Werner 1971; Rieppel 1992, 1994; Rosenberg et al. 1992; Noro et al. 2009; Wise et al. 2009; Guerra-Fuentes et al. 2014; Khannoon 2015; Khannoon et al. 2015).

In chapter II, I employed the second phase by characterizing the embryonic development of *Lepidodactylus lugubris*. Using light microscopy, µCT, and transcriptomics, we laid a foundation for comparative gecko embryology, gecko brain development, and gecko developmental genetics. Prior to this work, common leopard geckos (*Eublepharis macularius*) and Madagascar ground geckos (*Paroedura picta*) were gaining traction as laboratory models (Noro et al. 2009; Wise et al. 2009). Both of these species, however, exhibit derived, “non-typical” gecko morphologies, such as robust bodies, distal leaf-toe pads, or moveable eyelids. Establishing *L. lugubris* as our primary
gecko model had clear advantages: protocols for laboratory husbandry, rapid asexual reproduction, and “typical” gekkonid morphology (Griffing et al. 2018b). Indeed, this species became a focal taxon in each subsequent chapter. Ongoing projects using *L. lugubris* as an evo-devo model include investigations into gekkotan skeletal development, the development of hemipenes in all-female gekkotans, evolution and development of adhesive toe pads, and lung development. Future avenues for *L. lugubris* as an evo-devo model are myriad.

In chapter III, I employed phases 1 and 2 by characterizing adult and embryonic hyperpigmentation phenotypes in geckos with variable diel activities. A potential next step in this research would be expanding on the first phase and characterizing the presence of pigment phenotypes in the peritonea and viscera of other extremely cryptic geckos. Bark-mimicking taxa such as Madagascan *Uroplatus* or Australian *Saltuarius* are ideal taxa which have converged on the cryptic behavior of *Gekko kuhli*. These taxa exhibiting hyperpigmentation in their internal structures would provide further support for our hypothesis that cryptic animals are exposed to greater UV than other nocturnal geckos. The next step in understanding the mechanistic origins (phase 3) of convergently evolved hyperpigmentation is to investigate how the neural crest differentiates into melanocytes earlier in *G. kuhli* when compared to other species. Furthermore, characterizing embryonic development of other species of *Gekko* may elucidate how widespread this derived developmental pattern is and when it evolved in this clade.

Development of reptile pigment cells and their neural crest precursors is still a nascent field; however, including *G. kuhli* embryos as comparative material to future
investigations with emerging neural crest models (e.g. *Chamaeleo calyptratus*; Diaz et al. 2019) may yield insight into the mechanisms of neural crest differentiation.

In chapter IV, we employed phases 1 and 2 by characterizing the adult and embryonic morphology of adhesive toe pads and tail pads of *Correlophus ciliatus*. This work, in tandem with characterizations of anole and gecko toe pad development, sets the foundation for studying the origins of lizard adhesion (Griffing et al. 2022). Expanding upon phase 2 is the next step for this research. We aim to characterize digital development from taxa that represent additional origins of toe pads, secondarily padless taxa, and ancestrally padless taxa. Furthermore, we also aim to characterize caudal development of an unambiguous convergently evolved tail pad (i.e. *Lygodactylus* spp.; Loveridge 1947). This additional taxon sampling will uncover how conserved the patterns we see in adhesive lizard pad development are. This, in tandem, will reveal how much developmental constraint is involved in the evolution of lizard adhesion.

Proceeding to phase 3 of understanding the evolution of adhesive pads will require an integrative approach. The next step is identifying the role bone morphogenetic proteins (BMPs) in determining scensor identity and boundaries, and how they compare to general body scale development step (Di-Poï and Milinkovitch 2016; Cooper et al. 2019). Additionally, through a combination of methods such as RNAseq and ATACseq, we can begin to understand both the transcriptional and regulatory profiles that underlie the formation of adhesive toe and tail pads (Lowe et al. 2019). From there, candidate genes and regulatory networks can be experimentally disrupted using small molecular inhibitors or newly developed gene editing technology (Rasys et al. 2019).
In chapter V, we employed phase 2 by investigating the role of apoptosis in the retention of interdigital webbing in gliding geckos. The next obvious step in this research is to employ phase 3 and investigate the molecular mechanisms of interdigital webbing retention. Using *in situ* hybridization, we can identify the spatial and temporal profiles of BMP4, BMP2, and BMP7 and how they compare to the profiles of Gremlin and fibroblast growth factor (FGF) 8. Understanding these patterns will determine if different evolutionary origins of gliding geckos have converged with respect to their underlying developmental pattern as well. Furthermore, we would understand if their mechanisms for retaining interdigital webbing are similar or different to established models for interdigital webbing retention (i.e. ducks and bats; Merino et al. 1999; Weatherbee et al. 2006). Additional work is required to understand the developmental basis of patagium formation. At this point, nothing is known about the molecular mechanisms which result formation of vertebrate gliding membranes. A similar exploratory framework we propose for continuing work in chapter IV (i.e. RNAseq and ATACseq) is required to identify candidate genes and regulatory networks which result in membranous outgrowths.

Since beginning this dissertation, over a dozen studies have been published, laying a substantial foundation to investigate several avenues of gecko evo-devo to the phase 3 level of understanding (Zhao et al. 2017; Griffing et al. 2018a,b, 2019, 2020, 2021, 2022; Paluh et al. 2018; van der Vos et al. 2018; Andrews 2019; Alturk and Khannoon 2020; Khannoon and Evans 2020; Laver et al. 2020; Brink et al. 2021; Smith-Paredes et al. 2022). Although pursuing the final phase in each of these chapters could constitute a life’s work, this dissertation serves as a foundation for each. The burgeoning field of gecko evo-devo will only grow from here.


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## APPENDICES

### I: SUPPLEMENTAL MATERIALS ASSOCIATED WITH CHAPTER II

**Supplemental Material 2.1.** Specifications of microcomputed tomography of *Lepidodactylus lugubris* embryonic specimens. Units for the various measurements are as follows: voxel size, millimeters (mm); voltage (V), kilovolts (kV); amperage (A), microangstroms (µA); exposure time, seconds (s). No filter was used, projections = 2200, and frame averaging = 3. Digital Object Identifiers (DOI) provided for each specimen.

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II: SUPPLEMENTAL MATERIALS ASSOCIATED WITH CHAPTER IV

Supplemental Material 4.1. Gekkotan genera known to exhibit subcaudal adhesive pads. Number of species allocated to each genus, not the number of species investigated, is listed. D, Diplodactylidae; G, Gekkonidae; S, Sphaerodactylidae.

<table>
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Supplemental Material 4.2. Phylogeny of gecko genera demonstrating phylogenetic diversity of adhesive pads. Taxa with tail pads are bolded. Phylogeny and character state information modified from Gamble et al. (2015) and Russell & Gamble (2019), respectively.
**Supplemental Material 4.3.** Histology of an adult *Correlophus ciliatus* tail. Hall-Brunt Quadruple stain. 

**A)** Sagittal section of distal most tip of adhesive tail pad. 

**B)** Magnified dorsal portion of tail tip exhibiting dome-shaped scales with thin Oberhäutchen layer. 

**C)** Magnified ventral portion of tail tip exhibiting imbricate adhesive scanners with setae-bearing Oberhäutchen layer. 

**D)** Transverse section through tail at mid-length exhibiting enlarged hypaxial muscle bundles. 

- d, dermis; 
- e, epidermis; 
- em, epaxial muscle; 
- h, hypodermis; 
- hm, hypaxial muscle; 
- nc, nerve cord; 
- o, non-setal spinulate Oberhäutchen; 
- sf, setal field; 
- vc, vertebral centrum.
Supplemental Material 4.4. Features of diplodactylid gecko scale microstructures. Updated from Bauer (1998). BSW, basal setal width (µm); MSL, maximum setal length (µm); NCP, New Caledonian + \textit{Pseudothecadactylus} Clade; NZ, New Zealand Clade; SD, setal density (10^2 setae/mm^2); STW, setal tip width (µm). \textit{Dactylocnemis pacificus} data from Schleich & Kästle (1986). *Publishing error in Bauer (1998) as “(10^3 setal stalks/mm^2)” — correct unit is 10^2 setal stalks/mm^2.

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Supplemental Material 4.5. Scanning electron micrographs in ventral view of an adult *Correlophus ciliatus* toe pad (manus, digit IV). Magnified images of the distal portion of the toe tip are framed by solid white boxes.
Supplemental Material 4.6. Features of *Correlophus ciliatus* toe and tail setae. A) Relationship between maximum setal length and setal density. B) Raw data for setal length and setal density with fitted lines from phylogenetic independent contrasts (PIC). Significant relationship between setal length and density exists for toe pads but not tail pads. *Correlophus ciliatus* data are colored as a red square (tail data) and red circle (toe data).
Supplemental Material 4.7. Stages 30–43 of *Correlophus ciliatus* embryonic development. Lateral views of whole embryos. Scale bars = 2mm. When incubated at 25.6°C, *Correlophus ciliatus* hatched approximately 60 days post-oviposition. **Stage 30:** *Correlophus ciliatus* are oviposited at this stage, a later stage at oviposition than any other gecko, and exhibit 43–44 somites, fewer somites at stage 30 than *L. lugubris* (Griffing et al., 2019). The eyes are ovoid, with choroid fissure visible, and exhibiting condensed pigment in both the anterior and posterior regions of the retinal pigmented epithelium (RPE). The developing optic tectum, the telencephalon, and translucent otic capsules are visible. The heart is subdivided into a unified atrium and ventricle. Both fore- and hindlimbs are paddle-shaped and exhibit an apical ectodermal ridge (AER). **Stage 31:** Somites are present along the full length of the tail. The eye exhibits dense pigment in the RPE. The optic tectum is bulging dorsally. The mandibular arch is spans halfway along the length of the cranium. The endolymphatic ducts are opaque. The lungs, liver, and mesonephros are visible and the heart now exhibits two atria. The autopodia are distinct from the rest of both the fore- and hindlimbs. **Stage 32:** Iris development is underway and visible as dense pigment along the rim of the lens. The choroid fissure of the eye is still visible. The mandibular arch has grown anteriorly but does not meet the unfused maxillary process and medial nasal processes (i.e. facial primordia). The gallbladder is now visible ventral to the liver. Paired cloacal swellings are visible, indicating the onset of cloacal and genital development. The autopodia, zeugopodia, and stylopodia are distinct in the forelimbs while only the autopodia is distinct from the remaining forelimb. **Stage 33:** The areas of the brain adjacent to the optic tectum are relatively larger, giving the optic tectum a smaller appearance. The choroid fissure is not visible via light microscopy. The facial primordia are fused, forming a snout, and the mandibular arch has grown anteriorly. The autopodia, zeugopodia, and stylopodia are now distinct in the hindlimbs and both fore- and hindlimbs exhibit digital condensations. **Stage 34:** The mandible nearly meets the snout. The autopodia have grown in size. Cloacal swellings, or developing genital buds, of all embryos examined now resemble developing hemipenes more than squamate hemiclitores (Gredler et al., 2015). Digital webbing recession is underway; however, the distal tip of each digit is not yet free. **Stage 35:** The areas of the brain adjacent to the optic tectum have grown further, giving the optic tectum a smaller appearance than previous stages. The iris has doubled in size and is darker than previous stages. An external ear is visible. The mandible meets the snout. Digital webbing recession continues and the distal tip of each digit is free. The tissue directly adjacent to digital condensations does not recess to the same degree as seen in other gecko species (Gredler et al., 2019). The tip of the tail is slightly laterally expanded, obscuring its initial pointed tip appearance. **Stage 36:** The upper and lower eyelids are visible and appear to be fused with the spectacle. The snout and mandible are more elongate than the previous stages. Digital webbing recession is complete despite much of the webbing remaining. The overall lesser degree of apoptosis at this stage, when compared to other gecko species (Wise et al., 2009; Griffing et al., 2019), is a precursor for the adult *C. ciliatus* phenotype of wide toes and intermediate interdigital webbing. Toe pad and tail pad development begins shortly after digital webbing recession is complete. **Stage 37:** Fleshy papillae are visible on the dorsal margin of the fused eyelid where the adult *C. ciliatus* will exhibit its characteristic superciliary “crests.” The snout and mandible are more elongate than the previous stage. Translucent claws on the
developing digits are distinct and visible via light microscopy. **Stage 38:** Little to no dorsal bulging of the optic tectum is visible. The chromatophores (xanthophores and melanophores) of the iris are faintly visible in a thin strip, marking the boundary of the circular pupil. Nares are faintly visible. The snout and mandible are more elongate than the previous stage. The fleshy papillae which will constitute the adult *C. ciliatus* dorsolateral crest is faintly visible along the cervical region. Ribs are visible in the trunk as well as a fleshy, opaque strip that will become the lateral fold. In all embryos examined at this stage, the hemipenes are fully everted. Claws are well-developed and opaque white. The tail is more robust than previous stages. **Stage 39:** The iris occupies more of the eye, creating an ovoid pupil. The post-cranial body has grown substantially compared to the cranial region. The body wall is less translucent, beginning to obscure the view of viscera. Some of the larger tubercular scales of the dorsum are visible. **Stage 40:** The brain is not visible through the opaque skin. The iris occupies more of the eye and the snout and mandible are more elongate than previous stages. The body wall is more opaque. Tubercular scales and “crests” are more distinct than previous stages. The first pigment patterns are present on the dorsum of the trunk and tail. **Stage 41:** The iris occupies more of the eye than the previous shape, making an almond-shaped pupil. The body wall completely opaque with the exception of the ventral surface of the trunk which remains faintly translucent. Pigment is now wide-spread on the dorsal and lateral surfaces. **Stage 42:** The body wall completely opaque, scales full developed, and pigment development complete. Toe pad and tail pad development is complete. Hemipenes remain everted. **Stage 43:** The scales are noticeably hydrophobic when the embryo is submerged in PBS. The hemipenes are inverted. The embryo is ready to hatch.
Supplemental Material 4.8. Fluorescent image of a stage 35 *Correlophus ciliatus* in lateral view. Brighter areas indicate areas of intense apoptotic activity. Yellow dashed-line box corresponds to a magnified image of the developing tail tip. Yellow arrow points toward area of high apoptotic activity in the tail. Interdigital webbing also exhibits high-levels of apoptosis. Scale bar = 2mm.
III: SUPPLEMENTAL MATERIALS ASSOCIATED WITH CHAPTER V


![Embryonic Development Stages](image)
Supplemental Material 5.2. Stages 28–43 of *Gekko kuhli* embryonic development. Lateral views of whole embryos. Scale bars = 2 mm.

**Solutions Needed:**
1x DEPC-treated Phosphate buffered saline (PBS)
- 1x PBS buffer tablet
- 100µL of DEPC
- 100mL of dH₂O
- *Autoclave*
  *Other buffers, such as Hanks BSS, are acceptable (Fogel et al. 2012).*

Lysotracker Probe Solution (1:100 dilution)
- 50µL probe stock solution
- 5mL 1x DEPC-treated PBS

4% Paraformaldehyde (PFA)
- 800ml of 1x DEPC-treated PBS
- *Heat to approximately 60°C in a glass container on a heat plate, under a hood. Do not boil.*
- 40g of paraformaldehyde
- *Raise pH with NaOH until paraformaldehyde goes into solution*
- *Cool and filter. Then adjust volume to 1L with 1x DEPC-treated PBS.*
- *Adjust pH to approximately 6.9 using small amounts of HCl.*
- *Store solution in fridge.*

Methanol (MeOH) Series
- 50%, 75%, 80%, 100% dilutions with dH₂O

**Embryo Collection**
1. Collect egg of interest based on species and time since oviposition (dpo).
2. Following Griffing et al. (2018), dissect embryo out of egg using assorted dissection tools in a sterile petri dish with 1X DEPC-treated PBS. Be sure to remove any membranes.
3. Transfer embryo to a new petri dish with 1X DEPC-treated PBS and photograph using dissecting scope. Be sure to zoom in and focus on structures of interest (e.g. digits). *This step should be completed quickly — embryos must be fresh for proper Lysotracker binding.*

**LysoTracker Staining and Fixation**
1. Preheat Lysotracker probe solution to 30°C.
2. Add embryo(s) to microfuge tubes or vials with preheated Lysotracker probe solution. *Be sure to keep embryos in their own separate, labeled containers. Keep light exposure at a minimum.*
3. Incubate embryos in Lysotracker probe solution at 30°C for 60–70 minutes. *If feasible, gently rock tubes during incubation to ensure proper infiltration of probes in all tissues.*
4. Rinse gently in 1X DEPC-treated PBS for 5min, repeat 3 times (N=4) on rocker.
5. Add embryo to container with 4% PFA and let fix overnight at 8°C.
6. Rinse in 1X DEPC-treated PBS for 10min on rocker.
7. Dehydrate through a MeOH series (to achieve proper signal-noise ratio) on rocker
   a. 50% MeOH in dH2O, 5min
   b. 75% MeOH in dH2O, 5min
   c. 80% MeOH in dH2O, 5min
   d. 100% MeOH in dH2O, 5min
8. Store embryo indefinitely in 100% MeOH at -20°C. Keep container wrapped in tin foil to protect fluorophores from light degradation.

**Visualizing LysoTracker and Analysis**

1. Place embryo in small petri dish with shallow layer of 100% MeOH to prevent tissue distortion and fluorophore degradation.
2. Visualize using fluorescent stereoscope with rhodamine or Texas Red filter (excitation/emission = 577/590nm). Staining should be very bright so long exposure may not be necessary.
Supplemental Material 5.4. Apoptosis measurements of the developing manus. Area and fluorescent units are pixels² and grey values calculated by Fiji, respectively. Values for fluorescent area were listed as “0” if undetectable. Cc, *Correlophus ciliatus*; CTCF, corrected total cell fluorescence; e, early; Em, *Eublepharis macularius*; Fluor., fluorescence; Hp, *Hemidactylus platyurus*; Ht, *Hemidactylus turcicus*; ID, integrated density; l, late; Ll, *Lepidodactylus lugubris*; MBF, mean background fluorescence.

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IV: LITERATURE CITED IN APPENDICES


