A synthetic review: natural history of amniote reproductive modes in light of comparative evolutionary genomics

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ABSTRACT

There is a current lack of consensus on whether the ancestral parity mode was oviparity (egg-laying) or viviparity (live-birth) in amniotes and particularly in squamates (snakes, lizards, and amphisbaenids). How transitions between parity modes occur at the genomic level has primary importance for how science conceptualizes the origin of amniotes, and highly variable parity modes in Squamata. Synthesizing literature from medicine, poultry science, reproductive biology, and evolutionary biology, I review the genomics and physiology of five broad processes (here termed the 'Main Five') expected to change during transitions between parity modes: eggshell formation, embryonic retention, placentation, calcium transport, and maternal–fetal immune dynamics. Throughout, I offer alternative perspectives and testable hypotheses regarding proximate causes of parity mode evolution in amniotes and squamates. If viviparity did evolve early in the history of lepidosaurs, I offer the nucleation site hypothesis as a proximate explanation. The framework of this hypothesis can be extended to amniotes to infer their ancestral state. I also provide a mechanism and hypothesis on how

squamates may transition from viviparity to oviparity and make predictions about the directionality of transitions in three species. After considering evidence for differing perspectives on amniote origins, I offer a framework that unifies (1) the extended embryonic retention model and (2) the traditional model which describes the amniote egg as an adaptation to the terrestrial environment. Additionally, this review contextualizes the origin of amniotes and parity mode evolution within Medawar's paradigm. Medawar posited that pregnancy could be supported by immunosuppression, inertness, evasion, or immunological barriers. I demonstrate that this does not support gestation or gravidity across most amniotes but may be an adequate paradigm to explain how the first amniote tolerated internal fertilization and delayed egg deposition. In this context, the eggshell can be thought of as an immunological barrier. If serving as a barrier underpins the origin of the amniote eggshell, there should be evidence that oviparous gravidity can be met with a lack of immunological responses in utero. Rare examples of two species that differentially express very few genes during gravidity, suggestive of an absent immunologically reaction to oviparous gravidity, are two skinks *Lampropholis guichenoti* and *Lerista bougainvillii*. These species may serve as good models for the original amniote egg. Overall, this review grounds itself in the historical literature while offering a modern perspective on the origin of amniotes. I encourage the scientific community to utilize this review as a resource in evolutionary and comparative genomics studies, embrace the complexity of the system, and thoughtfully consider the frameworks proposed.

Key words: reproductive mode, parity modes, oviparity, squamates, eggshell deposition, embryonic retention, embryonic calcium transport, maternal–fetal interface, comparative evolutionary physiology.

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X. Supporting information

I. INTRODUCTION

A synthetic review is needed to improve the conceptual framework used to research the evolution of oviparity (egg-laying) and viviparity (live-birth) in amniotes (birds, non-avian reptiles, and mammals). Squamates (snakes, lizards and amphisbaenids) are particularly unique amongst amniotes because they have highly variable parity modes (Fig. 1B, C). Beginning with the first phylogenetic analyses on the subject, heated scientific disagreement persisted over the labile nature of evolutionary transitions between parity modes (Blackburn, 1999, de Fraipont, Clobert & Barbault, 1996; Griffith *et al*., 2015; Harrington & Reeder, 2017; Lee & Shine, 1998; Pyron, 2015; Pyron & Burbrink, 2014; Recknagel, Kamenos & Elmer, 2018; Recknagel *et al*., 2021*b*). A growing number of transcriptomic and genomic studies analysing the molecular underpinnings of reproductive mode evolution in squamates (e.g. Brandley *et al*., 2012; Cornetti *et al*., 2018; Gao *et al*., 2019; Griffith *et al*., 2016, 2017*a*; Foster *et al*., 2020, 2022; Recknagel *et al*., 2021*a*; Yurchenko, Recknagel & Elmer, 2020; Xie *et al*., 2022) and recent advances in palaeontology contribute to the discussion (Jiang *et al*., 2023; Norell *et al*., 2020). It is prudent to acknowledge that the relative difficulty of a phenotypic change cannot be determined from morphology alone or by unidentified physiological mechanisms. At least theoretically, any phenotypic change could be facilitated by simple genomic changes [e.g. a single nucleotide polymorphism (SNP)] or any combination of multi-omic changes to any number of loci. As research begins to reveal the molecular networks involved with parity mode evolution, it is important to avoid bias that could be introduced by prior assumptions on the feasibility of transitions. This reality brings weight to ancestral state reconstructions that identify highly labile transitions in squamates (Pyron & Burbrink, 2014) and an early origin of viviparity in amniotes (Jiang *et al*., 2023).

The earliest estimates predicted that viviparity evolved independently between 91 and 97 times in squamates (Blackburn, 1982, 1985, 1992). These estimates assumed that oviparity was the ancestral state and, based on the theoretical arguments of Dollo's law, that reversals back to oviparity should be exceedingly rare (Blackburn, 1992; Fitch, 1970; Neill, 1964; Tinkle & Gibbons, 1977). An intermediate phenotype when re-evolving an eggshell has been considered as physiologically unviable, preventing reversals (Blackburn, 1995; Griffith *et al*., 2015). This was demonstrated when experimentally induced extended egg retention in phrynosomatid lizards resulted in adverse embryonic development attributed to impeded gas exchange imposed by the eggshell (Mathies & Andrews, 1999, 2000; Parker & Andrews, 2006). However, assuming this fitness valley applies to all clades is presumptive.

Intermediate phenotypes as fitness valleys assumes that (1) eggshells inherently impede gas exchange and (2) an eggshell must re-evolve before a reversal back to oviparity is possible (Griffith *et al*., 2015). By contrast, eggshells are considered a component of the placenta in viviparous rough earth snakes (*Haldea striatula*) and in viviparous reproductively bimodal European common lizards (*Zootoca vivipara*) and yellow-bellied three-toed skinks (*Saiphos equalis*) (Stewart, 2013). Additionally, *Saiphos equalis* is a reproductively bimodal skink that has an oviparous population with incubation times as short as 5 days, thus embryos spend a significant time *in utero* within an eggshell (Smith, Austin & Shine, 2001). A surprising example of eggshells being compatible with full embryonic development includes a report of a captive tortoise that retained viable eggs until the hatching stage (Kuchling & Hofmeyr, 2022).

Several studies predict early origins of viviparity in squamates (Jiang *et al*., 2023; Pyron & Burbrink, 2014) and reversals back to oviparity (de Fraipont *et al*., 1996; Fenwick, Greene & Parkinson, 2011; Harrington & Reeder, 2017; Lee & Shine, 1998; Pyron & Burbrink, 2014; Recknagel *et al*., 2018). *Saiphos equalis* proved the possibility of reversals

when a viviparous individual oviposited an egg prior to birthing fully developed young within the same litter (Laird, Thompson & Whittington, 2019). The unusual absence of an egg-tooth in oviparous Arabian sand boas (*Eryx jayakari*) (Lynch & Wagner, 2010; Staub & Emberton, 2002) serves as additional biological evidence of a reversal, although this has been challenged (Griffith *et al*., 2015). Importantly, extended embryonic retention (EER), characteristic of oviparous squamates compared to birds, is viewed as compatible with labile transitions (Jiang *et al*., 2023). Current expectations are that oviparity may re-evolve more easily in squamate lineages that recently evolved viviparity and which have not lost specific avian eggshell-matrix proteins (Laird *et al*., 2019; Xie *et al*., 2022).

Although models that restrict parity mode evolution to be unidirectional (from oviparity to viviparity) are shown to be poor fits for squamates (Pyron & Burbrink, 2014; Recknagel *et al*., 2021*b*), there is resistance to the proposition that viviparity originated early in Squamata (e.g. Griffith *et al*., 2015). The most recent ancestral state reconstruction, built from biomineralization and parity mode data across 80 extinct and extant amniotes using a single structured Markov model, inferred viviparity with EER in the first amniotes and in the most recent common ancestor of lepidosaurs (squamates and sphenodontians) (Jiang *et al*., 2023). A testable hypothesis regarding a molecular mechanism that may have supported a transition to viviparity at the base of lepidosaurs and EER at the base of amniotes (Sections III.3 and VII) may help conclude decades-long debates.

Discoveries of viviparity in ancient amniotes are numerous, dating back to the Early Permian (Chuliver, Scanferla & Smith, 2022; Motani *et al*., 2014; Piñeiro *et al*., 2012; Jiang *et al*., 2023). EER and/or viviparity in the last common ancestor of amniotes may not be unreasonable. A compelling example is the report that *Ikechosaurus* sp., a basal archosauromorph, reached an articulated stage of embryonic development inside a parchment-shelled egg (Jiang *et al*., 2023).

The ecological drivers of parity mode evolution are beyond the scope of this review. However, it is generally proposed that viviparity increases protection from adverse environmental conditions (Ma *et al*., 2018; Pincheira-Donoso *et al*., 2017), and a general trend that supports this is the higher frequency of viviparous squamates, relative to oviparous, observed at increasing distances from the equator. The cold-climate hypothesis suggests that viviparity is an adaptation to cold climates, and this is generally accepted by the scientific community (e.g. Ma *et al*., 2018; Zimin *et al*., 2022). Consistent with the cold-climate hypothesis, a recent study that utilized 65 million years of global paleoclimate data, squamate phylogeny and parity data for over 3,000 taxa showed that persistent, stable cold climates are correlated with transitions to viviparity (Recknagel *et al*., 2021*b*). Less focus has been given to the adaptive nature of oviparity, which should incur less of a maternal burden given the shortened length of embryonic retention. Higher offspring mass and reproductive output is associated with oviparity in laboratory-housed *Zootoca vivipara*, leading authors to propose that oviparity may be advantageous when individuals live at optimal temperatures for embryonic development (Recknagel & Elmer, 2019).

Regarding the evolutionary genomics of parity mode evolution, two recent studies reached alternate conclusions when they compared differential gene expression during pregnancy across viviparous vertebrates, ranging from sea horses (*Hippocampus abdominalis*) to humans (*Homo sapiens*) (Foster *et al*., 2022; Recknagel *et al*., 2021*a*). Recknagel *et al.* (2021*a*) highlighted the overlap of differentially expressed genes during gestation across viviparous amniotes and vertebrates, whereas Foster *et al*. (2022) concluded that different genes with similar functions are recruited to the placenta and uterus to support independent origins of viviparity. Improved contiguity of assemblies, more comprehensive annotations, analysis of total RNA rather than messenger RNA (mRNA), and advanced

approaches to comparative transcriptomics may clarify the level of molecular convergence across independent origins of viviparity.

The recent ancestral state reconstruction that estimated EER in the most recent common ancestor of amniotes (Jiang *et al*., 2023) brings support to the EER model of amniote origins (Hubrecht, 1910). The EER model postulates that amniote extraembryonic membranes (including the chorion, allantois, and amnion) arose through pressure to support survival of the embryo in the uterine environment for an extended period of time compared to anamniotes [see Laurin (2005) for a summary of earlier ancestral reconstructions of EER]. Interestingly, delayed egg deposition is ancestral to amniotes (Starck, Stewart & Blackburn, 2021). Essentially, when the terms ‘delayed egg deposition’ or ‘extended embryonic retention’ are applied to amniotes they both describe longer embryonic retention relative to anamniotes. Therefore, it is prudent to consider how EER may have influenced the origin of amniotes. I emphasize that the EER model is agnostic to the parity mode of the first amniote.

The EER model juxtaposes the traditional perspective that the original amniote egg washed ashore and adapted to the terrestrial environment over evolutionary time (e.g. Romer, 1957). Importantly, regardless of where the first amniote egg was deposited (i.e. dry land or in water), the EER model offers an explanation for how the eggshell and extraembryonic membranes originated. For example, the origin of delayed egg deposition with internal fertilization should have been met with adaptations to support uterine exposure to foreign tissue (see Section VI). The eggshell, as an immunological barrier, may represent that adaptation. The environmental context of an adaptation matters. The eggshell for example develops early *in utero*, and amniotes are the only vertebrates with uterine secreted shell coats (reviewed in Menkhorst & Selwood, 2008). Extraembryonic membranes, interestingly, begin development when the ectoderm first emerges, during the earliest stages of gastrulation (Chuva de Sousa Lopes *et al.*, 2022). In birds, reptiles and mice, the emergence of these

membranes begins with the formation of amniochorionic fold(s) that develop into the chorion and amnion (e.g. Chuva de Sousa Lopes *et al.*, 2022). The amnion develops from the epiblast while the egg is *in utero* (Chuva de Sousa Lopes *et al.*, 2022). Across amniotes, the egg stays *in utero* until at least the primitive streak stage or early somite stage (Starck *et al.*, 2021). Ferner & Mess (2011) describe the development of the amnion as a prerequisite for survival of the egg on land. This is further explored by Starck *et al*. (2021) who describe the myogenic contractility of the amnion as a potential adaptation to maintain separation of the embryo from extraembryonic materials. Cataloguing where the chorion and allantois (jointly called the chorioallantois) complete development (*in utero* or *ex utero*) across the amniote phylogeny would provide further evolutionary insights.

The scientific community has been hesitant to infer that extraembryonic membranes originated in response to EER. The chorioallantois and amnion may have originated to support embryonic gas and water supply given the deficits of these resources *in utero*. Without substantial amounts of water, converting yolk nutrients to somatic tissue is impossible (Thompson & Speake, 2003). Water is the primary resource provisioned by the mother of viviparous squamates and it is stored in extraembryonic membranes (Lourdais *et al*., 2015). Improper water and gas exchange are associated with poor chorioallantoic blood flow (Wootton, McFadyen & Cooper, 1977). If extraembryonic membranes developed as an adaptation to the uterine environment, this translates to an egg arriving on land with exaptations to support gas and water exchange. I highlight that the EER model does not necessarily oppose the traditional paradigm, but rather adds context. Ultimately, I provide a list of evolutionary events that unify the traditional paradigm with the EER model to explain the origin of amniotes more holistically (see Section VII).

Throughout this review, considering viviparity as the most extreme form of EER, I hope I engage readers with thinking about the EER model in a new light. With a deep review

of interdisciplinary literature and associated supplementary materials across amniotes, I explore genomic and physiological features of gestation and gravidity, including those that could be exploited to support labile shifts, and those that may facilitate or impede reversals. I provide the nucleation site hypothesis to describe how lepidosaurs may have transitioned to viviparity early in their evolutionary history (Section III.3), a phylogenetic framework to infer ancestral states based on mechanisms of maternal–embryonic calcium provisioning (Section V.2), evolutionary pathways that may support transitions between parity modes (see Section VII), and a unified framework to understand the origin of amniotes (see Section VII). I advocate for using squamates as a model to understand the ancestral state of the amniote egg. Future work should consider this thoughtfully and embrace the complexity of the system. I hope this review serves as a foundation for further research on the evolutionary history of the amniote egg and reproductive mode evolution.

(1) Terminology

I use the conventional definition of viviparity as retention of eggs until the stage when the embryo is fully developed (Blackburn & Stewart, 2021; van Dyke, Brandley & Thompson, 2014). Oviparity is defined by embryos that develop outside the mother. I use the terms gravidity and gestation to describe the period of internal retention of the embryo in oviparous and viviparous taxa, respectively. Vertebrate placentas are conventionally defined by apposition of parental and fetal tissues (Mossman, 1937). It is accepted that all viviparous squamates have a chorioallantoic placenta under this definition (Blackburn & Stewart, 2021; Stewart & Blackburn, 1988). The avian chorioallantoic membrane and mammalian chorioallantoic placenta are homologous (Metcalfe & Stock, 1993). I sometimes refer to this organ as the chorioallantoic tissue to describe it for both parity modes. Oviposition refers to the process and act of egg-laying, while parturition refers to the process and act of giving

birth to live young. Parition refers to both oviposition and parturition (Blackburn, 1992; Smith, 1975).

(2) Main Five physiological changes of parity mode transitions

Several physiological features are expected to change during transitions between oviparity and viviparity (Fig. 1). I consider herein five physiological features (hereafter the 'Main Five'): (1) length of embryonic retention (Murphy & Thompson, 2011; Packard, Tracy & Roth, 1977) – only viviparous mothers retain the embryo for the entirety of development; (2) eggshell formation (Heulin *et al*., 2005; Packard *et al*., 1977; van Dyke *et al*., 2014) – viviparous embryos generally do not have an eggshell; (3) exchange of water, gas and/or nutrients (Blackburn, 1992, 2015*a*; Thompson, Stewart & Speake, 2000; Thompson & Speake, 2006); (4) embryonic calcium provisioning (Packard *et al*., 1985; Shadrix *et al*., 1994; Thompson & Speake, 2006) – sources of embryonic calcium and timing of uterine calcium secretions generally differ between oviparous and viviparous reproduction; and (5) maternal–fetal immune dynamics (e.g. Graham *et al*., 2011; Hendrawan *et al*., 2017; Foster *et al*., 2020) – viviparous reproduction is associated with maternal and embryonic exposure to foreign tissues, which is likely to require enhanced regulation of maternal–fetal immune systems.

II. LENGTH OF EMBRYONIC RETENTION

Viviparous amniotes retain the embryo until it is fully developed, but oviparous amniotes retain the embryo for a fraction of that time. Rather than using precocious hatching and parturition (PH&P), like that of opossums and early viviparous mammals (Wagner *et al*., 2014), squamates evolve viviparity through extended egg retention (García-Collazo *et al*.,

2012; Shine, 1983). Thus, processes affecting the length of embryonic retention are expected to change to support transitions between parity modes (van Dyke *et al*., 2014).

(1) Parturition and oviposition

The genes and hormones involved with initiating and ending gestation may provide insights into the tools squamates can co-opt to change the length of embryonic retention during parity mode transitions. Parition terminates embryonic retention. Parturition can be divided into four parts (Terzidou, 2007; Vannuccini *et al*., 2016): quiescence (Phase 0); activation (Phase 1); stimulation (Phase 2); and involution (Phase 3). In eutherian mammals, several processes contribute to the initiation and termination of gestation including inflammation (Challis *et al*., 2009; Hansen *et al*., 2017), maternal recognition of pregnancy (MRP), mechanical stretch of uterine tissues (Sooranna *et al*., 2004; Shynlova *et al*., 2008), and fluctuating concentrations of corticotropin-releasing hormone (CRH), progesterone, and oestrogen (Challis *et al*., 2000).

(*a*) *Quiescence and sustained progesterone production in reproductive tissues*

EER could be achieved by triggering mechanisms that extend uterine quiescence, i.e. inactivity of the uterus. Inhibition of myometrial contractions through sustained progesterone production supports quiescence across different viviparous amniotes (Bazer, 1992; Casey & MacDonald, 1997; Fergusson & Bradshaw, 1991; Ilicic *et al*., 2017; Murphy & Thompson, 2011; Putnam *et al*., 1991; Soloff *et al*., 2011). The corpus luteum, a transient progesterone-producing organ, releases progesterone during gestation. Extended lifespan of the corpus luteum likely aided the evolution of viviparity in mammals (Amoroso, 1968; Callard *et al*., 1992; Stouffer & Hennebold, 2015). Thus, early research on squamate viviparity also explored the influence of corpus luteum lifespan. The lifespan of corpora lutea is associated

with oviparous egg retention and oviposition (Diaz, Alonso-Gomez & Delgado, 1994; Fox & Guillette 1987; Jones & Guillette, 1982). Eggshell formation in oviparous whiptail lizards (*Cnemidophorus uniparens*) is even disrupted by experimental removal of corpora lutea (Cuellar, 1979). However, the lifespan of corpora lutea does not consistently correlate with length of embryonic retention in viviparous squamates like it does in mammals (Albergotti & Guillette, 2011; Callard *et al*., 1992).

Maternal recognition of pregnancy (MRP) refers to the early signalling of the embryo to prevent luteolysis (Thatcher, Meyer & Danet-Desnoyers, 1995), i.e. degradation of the corpus luteum, which takes place in the absence of pregnancy. MRP enables continued progesterone production by the corpus luteum to support uterine quiescence during early gestation. An independent evolution of MRP is reported for Macropodidae, a lineage of marsupial mammals (Freyer, Zeller & Renfree, 2003), and endometrial recognition of pregnancy is known in the opossum (Griffith *et al*., 2019). MRP has not been explicitly studied in squamates, but is assuredly present: the corpora lutea are not degraded in the earliest stages of gravidity/gestation in oviparous or viviparous squamates (Callard *et al*., 1992; Albergotti & Guillette, 2011).

Different genes are signalled by embryos for MRP across mammals. Human chorionic gonadotropin hormone (hCG) establishes MRP (Ross, 1979; Behrman *et al*., 1993; Duncan, McNeilly & Illingworth, 1998; Duncan, 2000; Ticconi *et al*., 2007). In pigs, MRP is hypothesized to be triggered by collaborative signalling of oestradiol (E2) and prostaglandins (PGs) (Geisert *et al*., 2023). Similarly, glycoproteins, E2 and prostaglandin E2 (PGE2) have been implicated in signalling MRP in horses (*Equus caballus*) (Klein & Troedsson, 2011; Klein, 2016). In ruminants, embryonic signalling of interferon tau (IFN-τ) establishes MRP (Bazer, 2013; Bazer, Spencer & Ott, 1997; Thatcher *et al*., 1995). During gestation in the uterus of viviparous African ocellated skinks (*Chalcides ocellatus*), four receptors for IFN-α,

IFN-β, IFN-ω, and IFN-γ are differentially expressed but no expression of IFN-τ was detected compared to non-gestational uterine tissue (Brandley *et al*., 2012). I was unable to find expression patterns of MRP signalling homologs in other squamate reproductive tissues. Should MRP occur in squamates, it may be signalled by genes that are clade specific, as in mammals. This makes comparative evaluation of the influence of MRP on the evolution of viviparity an interesting avenue for future research.

The evolution of viviparous EER may be sufficiently supported by maintenance of chorioallantoic progesterone production coupled with eggshell loss (Griffith *et al*., 2017*a*). This theory may be broadly applicable across amniotes given that the most recent common ancestor of amniotes likely had a chorioallantois with an endocrine function (Griffith *et al*., 2017*a*). Following death of the corpus luteum during gestation, placental progesterone production supports EER in eutherian mammals (Castracane & Goldzieher, 1986; Rothchild, 2003; Spencer & Bazer, 2004). Viviparous Italian three-toed skinks (*Chalcides chalcides*) shift to chorioallantoic progesterone production following degradation of corpora lutea during gestation (Guarino *et al*., 1998). The placenta of viviparous southern snow skinks (*Carinascincus microlepidotus*) produces minimal progesterone but has a strong capacity to convert pregnenolone to progesterone (Girling & Jones, 2003). Whereas all genes involved with a known biosynthesis pathway for progesterone production are expressed in the placenta of horses, only some of these genes were detected in the chorioallantois of chickens (*Gallus gallus*), viviparous southern grass skinks (*Pseudemoia entrecasteauxii*), and oviparous and viviparous southeastern sliders (*Lerista bougainvillii*) (Griffith *et al*., 2017*a*). Thus, if chorioallantoic progesterone production has supported multiple origins of viviparity in amniotes, it is not evidenced by a conserved ancestral gene expression pattern for the biosynthesis of progesterone (Griffith *et al*., 2017*a*). Nonetheless, parity trait genes in a reproductively bimodal lizard, *Zootoca vivipara*, are associated with progesterone-binding

functions (Recknagel *et al*., 2021*a*), highlighting the role of progesterone in squamate reproduction.

Other female reproductive tissues in squamates express genes involved with progesterone biosynthesis: StAR-related lipid transfer domain protein 3 (*StARD3*) and hydroxy-delta-5-steroid dehydrogenase (*HSD3B1*). *STARD3* is significantly upregulated in the uterine tissue during pregnancy in viviparous *Chalcides ocellatus*, along with significant differential expression of seven paralogs (Brandley *et al*., 2012). While StARD3 is expressed during gestation in *Zootoca vivipara*, it is not significantly differentially expressed compared to oviparous counterparts; *HSD3B1*, on the other hand, is significantly upregulated during mid-gestation (Recknagel *et al*., 2021*a*). Compared to non-gestational samples, *HSD3B1* is significantly upregulated in the uterus during early and late gestation in viviparous individuals of reproductively bimodal *Saiphos equalis* (Foster *et al*., 2020). Oviparous individuals from the same species did not exhibit this expression pattern (Foster *et al*., 2020). Activity of *HSD3B1* was detected in the mucosal epithelium of oviparous eastern garden lizards (*Calotes versicolor*) (Kumari, Sarkar & Shivanandappa, 1992), and in the uterine glands of oviparous keeled Indian mabuya (*Eutropis carinata*) (Mundkur & Sarkar, 1982). Other genes involved with the biosynthesis of progesterone (e.g. steroidogenic acute regulatory protein or cytochrome-P450-family-11-subfamily-A-polypeptide-1) serve as further candidates for exploring the relationship between organ-specific patterns of progesterone production and the evolution of EER in viviparous squamates.

For progesterone to prevent myometrial contractions and support quiescence, there must be progesterone receptors (PGRs) in the uterus (Mesiano, Wang & Norwitz, 2011; Young *et al*., 2011). In humans, progesterone responsiveness is related to specific ratios of the PGRs, PR-A and PR-B in myometrial cells (Young *et al*., 2011). Minimal research exists on PGR expression in squamate reproductive tissues. One study found that in the uterus of the yolk

sac in viviparous *Pseudemoia entrecasteauxii*, one progesterone receptor gene, *PGRMC2*, is upregulated compared to non-gestational uterine tissue (Griffith *et al*., 2016). Another progesterone receptor gene, *PGR*, is downregulated in the uterus of the chorioallantoic placenta and yolk sac placenta compared to non-gestational uterine tissue (Griffith *et al*., 2016). Downregulation of both *PGR* and *PGRMC2* in the uterus during gestation was detected in viviparous *Chalcides ocellatus* (Brandley *et al*., 2012). While *PGR* is differentially expressed at mid-gestation in viviparous compared to oviparous individuals, *PGRMC1* and *PGRMC2* are not differentially expressed (Recknagel *et al*., 2021*a*). However, admixture mapping revealed that the three SNPs most highly associated with gestation length in *Zootoca vivipara* are in close proximity to *PGRMC1* (Recknagel *et al*., 2021*a*). Measuring expression of PGRs and their ratios in uteruses of oviparous and viviparous squamates will help elucidate the receptors needed to support progesterone responsiveness in squamate uteruses and their relationship to EER.

(*b*) *Activation and progesterone withdrawal*

The activation stage of parturition is marked by the withdrawal, or functional withdrawal, of progesterone leading to an oestrogen-dominated response during the next stage: stimulation (Bakker, Pierce & Myers, 2017; Fergusson & Bradshaw, 1991). Progesterone may be withdrawn in response to environmental stimuli in reptiles during parturition (Shine & Guillette, 1988). In mammals, activation is marked by increasing concentrations of CRH and contraction-associated proteins (CAPs) including connexin-43, prostaglandins, oxytocin receptors, prostanoid receptors and cell signalling proteins (Bakker *et al*., 2017; Ilicic *et al*., 2017; Leadon *et al*., 1982; Pashen & Allen, 1979; Whittle *et al*., 2000). Pro-inflammatory cytokines and chemokines, prostaglandin synthase-2 (COX-2, also referred to as PTGS-2), and nuclear factor kappa B (NF-κB) also influence activation in mammals (Christiaens *et al*.,

2008; Lappas *et al*., 2002; Lappas & Rice, 2007; Lindström & Bennett, 2005; Olson, 2003; Terzidou, 2007).

Some similar patterns are associated with oviposition in birds. In chickens, prostaglandin F (PGF) concentrations increase in the hours leading up to oviposition (Takahashi *et al*., 2004). Experimental injection of oxytocin and arginine vasotocin (AVT), which are similar neurohypophyseal peptides, revealed that uterine tissues of chickens maintain responsiveness to oxytocin but are more sensitive to AVT (Ewy, 1970). Murphy & Thompson (2011) provide an extensive list of resources on progesterone and oestrogen assays across oviparous and viviparous squamates. Future research should consider exploring parallels between mechanisms of activation in mammals and squamates. Any process that can trigger or stall activation could be exploited over evolutionary time to influence EER.

(*c*) *Stimulation and electrical gradients, inflammation, and hormonal regulation*

Mechanical stretch, electrical gradients, inflammatory processes, and hormonal regulation contribute to stimulation, the phase when contractions, cervical ripening and dilation occur. Stimulation involves contributions from maternal and fetal tissues. As early as 460 BC there was uncertainty over the proportional influence of mother or fetus on the initiation of parturition. Hippocrates proposed that the fetus initiates parturition by pushing its feet on the fundus of the uterus. Although the reality is not so cartoonish, mechanical stretch of the uterus from the growing embryo does play a role in parturition (Lefebvre *et al*., 1995; Tamizian & Arulkumaran, 2004; Wray *et al*., 2015).

Physical stretching of the uterus causes an influx of calcium and Na^+, altering the muscle action potential and enabling contractions (Kao & McCullough, 1975). Calcium further activates voltage-gated calcium channels on myometrial cell membranes, enhancing the influx of calcium ions and mediating the force and speed of myometrial contractility

(Arrowsmith & Wray, 2014; Wray *et al*., 2015). The influence of uterine overdistension on parition in birds and non-avian reptiles has not yet been examined to my knowledge. However, differentially expressed genes functionally enriched the gene ontology (GO) term for 'voltage-gated calcium channel activity' in uterine tissues during gravidity and gestation in *Saiphos equalis* (Foster *et al*., 2020). A uterine response to overdistension is among the many possible explanations for this. It may be important to consider the influence of uterine overdistension on squamate parity mode transitions, because if bioelectrical responses to uterine overdistension are a common feature of vertebrate parturition, reduced distension may be a barrier to reversals back to oviparity. Uterine overdistension may influence parturition by triggering an 'inflammatory pulse' that activates further myometrial contractility, which leads to preterm birth in primates (Adams Waldorf *et al*., 2015).

During parturition, there is an influx of uterine and embryonic pro-inflammatory genes and immune cells (Adams Waldorf *et al*., 2015; Charpigny *et al*., 2003; Mesiano *et al*., 2002; Park *et al*., 2005). Uterine contractions in humans involve actions of PGs, oxytocin, CRH, cytokines, and neutrophils (Adams Waldorf *et al*., 2015; De Rensis *et al*., 2012; Olson & Hertelendy, 1983; Park *et al*., 2005; Sykes *et al*., 2014; Terzidou, 2007).

The cycling concentrations of the neuropeptide CRH support parturition in humans. This has been compared to a biological clock that is initiated at early stages of gestation (Lockwood, 2004; McLean & Smith, 2001). Increased production of CRH facilitates parturition by interacting with the CRH receptors, CRH-R1 and CRH-R2, which are suggested to promote myometrial relaxation or contractility, respectively (Hillhouse & Grammatopoulos, 2001). Altered regulation, phenotype or function of hormones that act as biological clocks, like CRH, may have a particularly strong influence on evolutionary changes to length of embryonic retention, a trait inherently related to time.

Placental CRH production has only been identified in primates thus far (Challis *et al*., 2005; Emanuel *et al*., 1994; Florio *et al*., 2002; Hillhouse & Grammatopoulos, 2001; Karteris *et al*., 1998; Mendelson, 2009; Robinson *et al*., 1989). Placental CRH production may, therefore, be unique to primates. However, the amino acid sequence of CRH is highly conserved in vertebrates (Noy *et al*., 2017), indicating there is a possibility for shared function across diverse taxa. Like CRH cycling in mammals, timely fluctuations of AVT stimulate uterine contractions, enabling oviposition in birds, turtles, and lizards (Ewy, 1970; Fergusson & Bradshaw, 1991; Guillette & Jones, 1980; Jones *et al*., 1987; Rzasa, 1978; Wu *et al*., 2019).

PGE2 and prostaglandin F2α (PGF2α) influence uterine contractions and cervical relaxation for parition across many amniotes, including humans (Terzidou, 2007), domestic pigs (De Rensis *et al*., 2012), chickens (Hertelendy, Yeh & Biellier, 1974; Olson, Shimada & Etches, 1986), and loggerhead turtles (*Caretta caretta*) (Guillette *et al*., 1991). Injections of PGF2α and PGE2 induce parturition in viviparous Yarrow's spiny lizards (*Sceloporus jarrovi*) and raukawa geckos (*Woodworthia maculatus*) (Cree & Guillette, 1991; Guillette *et al*., 1992). However, no injected dosages of PGF2α or PGE2 induced oviposition in oviparous collard lizards (*Crotaphytus collarus*), eastern fence lizards (*Sceloporus undulatus*), six-lined racerunners (*Aspidoscelis sexlineatus*), or striped plateau lizards (*Sceloporus virgatus*) (Guillette *et al*., 1991). It is interesting that injections of PGF2α and PGE2 induced parturition in viviparous lizards but did not induce oviposition in the oviparous lizards studied. Therefore, it is plausible that regulatory or functional changes to PGF2α and/or PGE2 in squamates could facilitate changes to the length of embryonic retention to support transitions between reproductive modes. However, induction of parturition with PGF2α in viviparous *Woodworthia maculatus* only worked following injection of β-adrenoceptor (Cree & Guillette, 1991).

PGF2α decreases progesterone concentrations during phase 2 of parturition, stimulation (De Rensis *et al*., 2012). In humans, biosynthesis of PGs is driven largely by the enzyme COX-2 (= PTGS-2) rather than COX-1 (Slater *et al*., 1995, 1999). This helps maintain the decreased progesterone/oestrogen ratio of phase 2, stimulation. In ovariectomized viviparous garter snakes (*Thamnophis elegans*), higher oestrogen levels stimulated increased thickness of uterine epithelial cells and glandular activity, whereas administration of progesterone had little effect on uterine histology (Mead, Eroschenko & Highfill, 1981). Uterine pig models revealed that oestrogen stimulates involuntary contraction and relaxation (peristalsis) of the uterus (Mueller *et al*., 2006).

The softening of the cervix is important during the stimulation stage of parturition. A hormone related to insulin, relaxin, promotes myometrial softening in humans, domestic pigs, and turtles (Mercado-Simmen *et al*., 1982; Sorbera, Giannoukos & Collard, 1988; Weiss & Goldsmith, 2001). The cervix also softens in response to actions of PGE2. PGE2 activates pro-inflammatory cytokines, interleukin (IL)-8 and tumour necrosis factor (TNF)-α, resulting in activation of the collagenases and matrix metalloproteinases involved in cervical softening (Bakker *et al*., 2017). This causes a positive feedback loop between IL-8 and PGE2 synthesis (Denison *et al*., 1998; Denison, Calder & Kelly, 1999*a*; Terzidou, 2007; Li *et al*., 2010). Upregulation of IL-8 is also promoted by the protein complex NF-kB during parturition in humans (Elliott, 2001). Stimulated by fetal signalling of surfactant protein A (SP-A), increased production of NF-kB and IL-1 is associated with parturition in mice (*Mus musculus*) (Mendelson & Condon, 2005; Mendelson, 2009).

A few studies focus on the role of cytokines on squamate reproduction but not explicitly during oviposition or parturition (Hendrawan *et al*., 2017; Paulesu *et al*., 1995, 2005*a*, 2008; Paulesu, Romagnoli & Bigliardi, 2005*b*). Some studies detected expression of cytokines during late gestation (Foster *et al*., 2020; Gao *et al*., 2019; Recknagel *et al*., 2021*a*). TNF-α

related activity was only detected at this time in viviparous tussock cool-skinks (*Pseudemoia entrecasteauxii*) which were found to downregulate TNF-α-induced genes (*TNFAIP6* and *TNFAIP8L2*) in the 'uterus of the chorioallantoic placenta' and *TNFAIP6*, *TNFAIP1*, and *TNFAIP2* in the 'uterus of the yolk-sac placenta' compared to non-gestational uterine tissues (Griffith *et al*., 2016).

Altered expression or phenotype of contractility agonists, oxytocin receptors and oestrogen receptors, and contractility antagonists, progesterone receptors and β-adrenergic receptors (Ravanos *et al*., 2015) may also change the length of embryonic retention to support transitions between parity modes. Differences in length of embryonic retention in the oviparous and viviparous agamas, *Phrynocephalus przewalskii* and *Phrynocephalus vlangalii*, respectively, appears to be driven by regulatory differences in prostaglandins, COX-2, an AVT receptor (MTR), β-adrenergic receptors, and oestrogen receptors. During oviposition, *P. przewalskii* exhibited promotion of contractions through downregulation of genes associated with the β-adrenergic receptor (*ADRB2*), and upregulation of *COX-2* and prostaglandin, and absent (potentially lost) expression of genes for two oestrogen receptors (*ESR1* and *ESR2*) and the AVT receptor (*MTR*) (Gao *et al*., 2019). During the stage of gestation corresponding to oviposition, its viviparous sister species, *P. vlangalii*, exhibited a different pattern: inhibition of contractions caused by upregulation of *ADRB2* and downregulation of genes for two oestrogen receptors (*ESR1*, *ESR2*), *MTR*, *COX-2*, and prostaglandin (Gao *et al*., 2019). Three viviparous squamates, *Saiphos equalis*, *Chalcides ocellatus*, and *Pseudemoia entrecasteauxii*, share some of these expression patterns (*COX-2*, *MTR*, and *ADRB*, respectively) thought to be involved with EER in viviparous *P. vlangalii* (Brandley *et al*., 2012; Foster *et al*., 2020; Gao *et al*., 2019; Griffith *et al*., 2016), and *ADRB2* is upregulated at mid-gestation in viviparous *Zootoca vivipara* compared to oviparous individuals of this species (Recknagel *et al*., 2021*a*). Overexpressed genes in viviparous

uterine tissues of *Zootoca vivipara* also functionally enriched beta 1 and beta 2 adrenergic receptor signalling pathways (Recknagel *et al*., 2021*a*). The latter study, which compared uterine expression profiles during gestation across viviparous species of squamates, rodents, canines, ungulates, and humans, concluded that shared regulatory networks are recruited to support viviparity (Reckangel *et al*., 2021*a*).

Recently, in humans, the only classical major histocompatibility antigen (C-MHC) expressed by trophoblasts (specialized placental cells) was found to be associated with parturition: human leukocyte antigen (HLA)-C is significantly increased during labour in term and preterm placentas compared to non-labouring placentas (Hackmon *et al*., 2017). The authors suggested a mechanism whereby fetal HLA-C open conformers on the placenta provoke inflammation of maternal tissues, leading to parturition (Hackmon *et al*., 2017). Expression of MHC alloantigens, i.e. foreign antigens to the host, by fetal cells is also associated with parturition in cows and horses (Benedictusa, Koets & Ruttena, 2015; Davies *et al*., 2004; Joosten, Sanders & Hensen, 1991; Rapacz-Leonard *et al*., 2018). Around one month prior to parturition in cows, the endometrial epithelium thins and eventually disappears completely, putting the antigen-presenting trophoblasts (Adams *et al*., 2007) in contact with maternal connective tissue of the endometrium (Podhalicz-Dzięgielewska *et al*., 2000). Fetal MHC alloantigens are proposed to promote the loosening of contact between maternal and fetal tissues (Benedictusa *et al*., 2015). MHC molecules are expressed during gestation in some squamates (Murphy, Thompson & Belov, 2009) but their role in oviposition or parturition has not yet been considered to my knowledge. Identifying the presence or absence of MHC alloantigens on embryonic tissues before and during parition across more diverse taxa may reveal how ubiquitous the influence of embryonic MHC molecules is.

Involution (phase 3) occurs after the embryo(s) is released. In eutherian involution, the placenta detaches, and the uterus shrinks. This is supported by actions of prostaglandins (Husslein, 1984) and oxytocin (Terzidou, 2007). It seems unlikely that processes of involution are related to evolutionary changes to the length of embryonic retention.

(2) Unique qualities of oviposition and parturition in sauropsids

The physiology of avian oviposition is dependent on a circadian schedule (Williams, 2012), with the general model of an 'open period' when eggs are laid, separated by 'laying gaps'. Chicken ovulation and oviposition cycles have an 8-h open period where luteinizing hormone (LH) and progesterone levels increase, initiating ovulation. At the extreme, the ancient murrelet (*Synthliboramphus antiquus*), oviposits a two-egg clutch at seven-day intervals (Williams, 2012). Longer laying intervals have been associated with longer intervals between initiations of yolk development (Astheimer & Grau, 1990). In contrast to birds, oviparous squamates retain eggs for longer than the ovarian cycle (Tinkle & Gibbons, 1977).

Non-avian reptiles are unique in that they are the only ectothermic amniotes. This makes them uniquely reliant on temperature for embryonic retention and associated embryonic signalling to indicate the stage of embryonic development. Additionally, females are the heterogametic sex in several squamates, leading some researchers to suggest that chromosome linkage evolution may increase the speed of evolution in genes associated with gestation length (Recknagel *et al*., 2021*a*). Admixture mapping, made possible by the natural hybridization of oviparous and viviparous populations of *Zootoca vivipara*, revealed 439 candidate genes associated with embryonic retention (Recknagel *et al*., 2021*a*). Eleven of these genes were also associated with eggshell traits (Recknagel *et al*., 2021*a*), underscoring the pleiotropic roles of some genes putatively involved in squamate parity mode evolution.

(3) Pre-term birth and embryonic retention mechanisms

The literature on the genetics of pre-term birth may be a fruitful avenue of research to inform understanding on the evolutionary genomics of embryonic retention length. A genome-wide association study (GWAS) investigating preterm birth across over 43,000 women identified variants in six genes (*EBF1*, *EEFSEC*, *AGTR2*, *WNT4*, *ADCY5* and *RAP2C*) associated with gestation length (Zhang *et al.*, 2017). These likely act at the level of the maternal genome (Zhang *et al*., 2017). Whole-genome sequencing across family trios revealed 160 variants associated with eight clinical phenotypes of preterm birth in non-coding regions of 66 genes, intergenic regions, and long intergenic non-coding RNAs (Knijnenburg *et al.*, 2019). Many differentially expressed genes and methylation patterns of genes associated with very early pre-term birth (< 28 weeks) are involved with growth factor signalling, inflammation- and immunity-related pathways (Knijnenburg *et al.*, 2019). Slower increases of CRH (Ellis *et al*., 2002) and higher expression of neurokinin B are also associated with pre-term birth in humans (Torricelli *et al*., 2007). Performing similar integrative studies, and examining homologs of genes involved with human pre-term birth in squamates may provide further candidate genes that could impact the length of embryonic retention across amniotes. Some evolutionary studies are taking implications of pre-term birth into account. For example, a comparative evolutionary transcriptomics study across therians, monotremes, squamates, and an amphibian recently associated *HAND2* with preterm birth in eutherian mammals (Marinić *et al*., 2021).

In humans, pregnancy loss from infection follows distorted ratios of immune factors at the maternal–fetal interface (Arenas-Hernandez *et al*., 2016; Chaturvedi *et al*., 2015; Chattopadhyay *et al*., 2010). Future research on the evolution of lengthened embryonic retention to support viviparity may benefit from exploring ratios of immune cells in the uterus and embryonic tissues during term and pre-term pregnancy in squamates. I direct

researchers to the literature on the reptile immune system and on immune cell ratios at the maternal–fetal interface during term and pre-term mammalian pregnancy for further exploration (Yang, Zheng & Lin, 2019; Zimmerman, Vogel & Bowden, 2010; Zimmerman, 2020).

(4) Discussion and future directions – embryonic retention and parity mode evolution

The physiological processes involved at the start of gestation (MRP) and the end of gestation (parition) in birds and mammals provide insights into the genes and hormones squamates may co-opt to alter length of embryonic retention during transitions between parity modes. Unsurprisingly, hormones like oestrogen and progesterone play important roles in parition across amniotes. Further processes to be examined in squamates include signalling of homologous genes for MRP, placental progesterone production, novel pathways for biosynthesis of progesterone, the role of beta 1 and beta 2 adrenergic receptor signalling pathways, fluctuating ratios of progesterone receptors, the lifespan of the corpus luteum across a broader range of taxa, production and circulation of homologs for AVT and CRH or other similarly structured genes, expression of fetal alloantigens and inflammatory cytokines *in utero*, and the influence of uterine overdistension on contractions. Regarding squamate parity mode transitions, the role of uterine overdistension in mammalian parturition suggests that a lack of uterine overdistension may be a hurdle for reversals back to oviparity. Understanding the evolutionary physiology and genomics of embryonic retention in oviparous and viviparous squamates will benefit from focused attention on reproductively bimodal species (Whittington *et al*., 2022) and from genomics/physiological research across more taxa that vary in reproductive modes.

III. EGGSHELL FORMATION

Oviparous amniotic embryos develop within an eggshell that is at least partially mineralized, whereas viviparous embryos generally do not. Evolutionary transitions between parity modes therefore require changes to the process of eggshell formation. Primarily, the eggshell serves as physical protection and as a calcium reserve. The eggshell matrix contains immune properties and pores that enable gas exchange and water uptake, though the extent of this is variable across species (Attard *et al*., 2021; Packard *et al*., 1977). The history of research on the evolutionary morphology of the amniote egg is important for future comparative research (Blackburn & Stewart, 2021). Controversially, some have suggested that the amniote eggshell originated multiple times (Aoki, 1993). Interestingly, across vertebrates, only amniotes have uterine-secreted shell coats (Menkhorst & Selwood, 2008). Is this evidence of a single common ancestor? Or evidence of competition with the amniote embryo (with extraembryonic membranes that compete for resources) in utero? Science has yet to reveal the answer.

Birds have hard calcareous eggshells. Other than two lineages of geckos with hard shells, oviparous squamates have parchment-shelled eggs with a thin layer of calcium deposits on the outer surface of the shell membrane (Blackburn & Stewart, 2021; Choi *et al*., 2018). Monotremata (egg-laying mammals) have an eggshell but far less has been documented about its structure compared to other amniotes (e.g. Legendre, Choi & Clarke, 2022). The structure and physiological mechanisms involved in eggshell calcification are best resolved in birds (Choi *et al*., 2018; Francesch *et al*., 1997; Jonchère *et al*., 2010, 2012; Rose-Martel, Du & Hincke, 2012). Eggshell deposition in tuatara, *Sphenodon punctatus*, and squamates differs dramatically from birds (Choi *et al*., 2018). Viviparous squamates lack an eggshell, absorb the eggshell during gestation, or have a shell with only a thin layer of calcium deposits.

The earliest records of amniote eggshells have features characteristic of archosaur eggshells, including the mammillary layer (Stein *et al*., 2019; Legendre *et al*., 2022). Recent reconstructions are consistent with a thin eggshell in ancestral dinosaurs (Norell *et al*., 2020; Stein *et al*., 2019). It is important to consider that the semi-rigid shells of lepidosaurs and testudines are not homologous (Legendre *et al*., 2022); the microstructure of Archelosauria (birds, crocodiles, turtles and dinosaurs) and lepidosaur eggshells is remarkably different (Choi *et al*., 2018); and recent reconstructions of the composition and ultrastructure of dinosaur eggshells revealed that a calcified hard eggshell originated three times in dinosaurs (Norell *et al*., 2020). In the remainder of this section, I consider how structural, mineral, genomic/transcriptomic, and proteomic information on amniote eggshells can inform scientific understanding of the ancestral eggshell of amniotes and lepidosaurs.

The genetic drivers of eggshell formation are not resolved in squamates. Two oviparous lizards, *Lerista bougainvillii* and *Lampropholis guichenoti*, differentially express either two or zero genes, respectively, *in utero* in gravid *versus* non-gravid comparisons (Griffith *et al*., 2016). However, the study only measured gene expression at one developmental stage, making it difficult to infer if regulatory changes influence eggshell formation. A subsequent reanalysis of the data for *L. guichenoti* revealed 269 differentially expressed genes during gravidity, markedly few compared to other vertebrates in the study (Foster *et al*., 2022). Furthermore, the expression profile was not consistent across biological replicates (Foster *et al*., 2022). By contrast, oviparous *Saiphos equalis* and *Phrynocephalus przewalskii* have over 1,800 differentially expressed genes during gravidity compared to the non-gravid state (Foster *et al*., 2020; Gao *et al*., 2019). It is interesting to see drastically different uterine gene expression profiles associated with oviparity.

Some genetically determined traits are known to be evolutionarily labile in squamates, such as venom and limb reduction (Camaiti *et al*., 2021; Sites, Reeder & Wiens, 2011). In

Saiphos equalis, shell characteristics of facultatively paritioned oviparous and viviparous embryos are similar, leading the authors to infer that both parity modes utilize the same machinery to produce egg coverings (Laird *et al*., 2019). In this species, environmental influences on gestation length, rather than genetic influences on eggshell thickness, may play a more dominant role in parity mode evolution (Laird *et al*., 2019). In *Zootoca vivipara*, Recknagel *et al*. (2021*a*) identified 38 candidate genes associated with eggshell traits and concluded that the genetic architecture of eggshell traits is simpler than that of gestation length.

(1) Mineral composition of eggshells

The different mineral compositions of eggshells across amniotes may provide insights into the differing physiological conditions and evolutionary histories under which they formed (Table 1). Taxa use a polymorph of calcium carbonate – calcite, aragonite or vaterite – to construct the eggshell (Hincke *et al*., 2012). Amorphous calcium carbonate (ACC) is a transient non-crystalline precursor of calcite and aragonite that is important in many calcification processes in invertebrates (Hincke *et al*., 2012). It was recently shown to control avian eggshell mineralization (Rodríguez-Navarro *et al*., 2015).

In birds, the organic components of uterine fluid promote the formation of calcite (Hernández-Hernández *et al*., 2008*a*,*b*,*c*). Most amniotes use this polymorph (Hernández-Hernández *et al*., 2008*a*,*b*; Legendre *et al*., 2022). However, turtle eggshells are predominately developed with aragonite (Choi *et al*., 2022; Mikhailov, 1997). The eggshell of most squamates consists of an inner fibrous protein layer overlain by calcium carbonate that can be a single layer or scattered crystals (Choi *et al*., 2018; Packard & DeMarco, 1991; Stewart *et al*., 2010).

There are differing accounts of the microstructure of monotreme eggshells, however conceptus coats include three layers including a zona pellucida, mucoid coat and shell coat (Frankenberg & Renfree, 2018). Further studies are needed to test for secondary homology. Monotreme shells are described as proteinaceous, permeable, and flexible (Hughes, 1984). Marsupials lack an eggshell but have an eggshell coat, similar to that of monotremes (Frankenberg & Renfree, 2018), that is secreted by the epithelial cells and endometrial glands early in embryonic development prior to implantation (Roberts, Breed & Mayrhofer, 1994; Roberts & Breed, 1996). Upon hatching of the shell coat and attachment of the embryo, a cooperative inflammatory response ensues (Stadtmauer & Wagner, 2020*a*,*b*).

(2) Uterine glands and the evolution of parity modes

Eggshell formation occurs in the uterus where the uterine glands secrete precursors of the eggshell (Girling, 2002; Guillette, Fox & Palmer, 1989; Jonchère *et al*., 2010; Nys *et al*., 2004; Picariello, Ciarcia & Angelini, 1989; Stewart & Ecay, 2010). Uterine glands are critical for gravidity/gestation in both oviparous and viviparous amniotes (Braz *et al*., 2018; Burton *et al*., 2002; Cooke *et al*., 2013). For example, in humans, uterine glands provide histiotrophic nutrition to the early embryo (Burton *et al*., 2002). In reptiles, precursors for the proteinaceous eggshell membrane are secreted by the uterine glands (Corso, Delitala & Carcupino, 2000; Heulin *et al*., 2005; Palmer, Demarco & Guillette, 1993). Calcium secretion can also involve uterine epithelial cells (Herbert, Thompson & Lindsay, 2006; Thompson *et al*., 2007). The uterine epithelium of the soft-shelled turtle (*Lissemys punctata punctata*) and the eastern collared skink (*Chrotaphytus collaris*) stain positive for calcium (Guillette *et al*., 1989; Sarkar, Sarkar & Maiti, 1995).

Viviparous squamates have an absent or reduced eggshell membrane to facilitate gas exchange (Blackburn, 1993; Braz *et al*., 2018). Some viviparous squamates are encased in the

744 thin membrane, with or without detectable calcium (Stewart, 2013), throughout development.
745 Others have the membrane only in the early stages of embryonic development as in the garter
746 snakes *Thamnophis radix* and *T. sirtalis* (Blackburn & Lorenz, 2003).
747 Reduced number or size of eggshell glands leads to reduced eggshell membrane thickness
748 in viviparous squamates. In chickens, variation in size and spacing of eggshell glands may
749 also be important for eggshell structure (Guillette & Jones, 1985). In the reproductively
750 bimodal *Saiphos equalis*, the density of eggshell glands plays a role in eggshell thickness
751 (Stewart *et al.*, 2010). In the reproductively bimodal lizard *Zootoca vivipara*, viviparous
752 individuals have a uterine glandular layer that is less developed during the stage of eggshell
753 formation compared to oviparous individuals (Heulin *et al.*, 2005). Additionally, in *Lerista*
754 *fragilis*, which lays eggs that hatch within just hours of oviposition, the uterus contains very
755 few mucosal glands (Guillette, 1992). In the fence lizard (*Sceloporus a. aeneus*), the irregular
756 surface of the eggshell was attributed to the irregular spacing of shell glands (Guillette &
757 Jones, 1985). In an oviparous gecko *Hemidactylus turcicu*s, the eggshell glands have loosely
758 packed secretory granules that produce a hard, calcareous shell (Girling, Cree & Guillette,
759 1998). In a comparison of oviparous and viviparous water snakes from the genus *Helicops*,
760 viviparous embryos have thinner shell membranes which are associated with reduced size of
761 eggshell glands (Braz *et al.*, 2018). In an oviparous gecko *Saltuarius wyberba*, their secretory
762 granules are tightly packed, and their shell is soft and parchment-like (Girling *et al.*, 1998). In
763 a viviparous relative *Hoplodactylus maculatus*, there are far fewer eggshell glands, and where
764 there are glands, the secretory granules are smaller and more electron dense (Girling, Cree &
765 Guillette, 1997; Girling *et al.*, 1998). Smaller eggshell gland size during or after
766 vitellogenesis is also found in other viviparous squamates compared to oviparous
767 counterparts (Braz *et al.*, 2018; Gao *et al.*, 2019; Heulin *et al.*, 2005). To my knowledge, in

768 monotremes the relationship between eggshell thickness and shell gland size, density or
769 compaction of secretory granules has not been explored.
770 In the oviparous Przewalski's toadhead agama lizard (*Phrynocephalus przewalskii*), 148
771 genes are highly expressed in the uterus during the stage of eggshell gland development (Gao
772 *et al*., 2019). Only three of these are highly expressed in *P. vlangalii*, a viviparous close
773 relative, at this time, suggesting that differences between oviparous and viviparous eggshell
774 gland development require regulatory changes to dozens of genes (Gao *et al*., 2019). In the
775 grey short-tailed opossum (*Monodelphis domestica*), a marsupial, proliferation of uterine
776 glands is not induced by the conceptus (Griffith *et al*., 2019).
777
778 **(3) Evolutionary implications of the physiology of eggshell formation**
779 Presumably because of the influence it has on food production, the process of eggshell
780 formation has been studied most extensively in chickens (Hincke *et al*., 2012). During
781 eggshell formation in birds, uterine fluid containing a supersaturation of ionized calcium and
782 bicarbonate ions surrounds the egg (Nys *et al*., 1991). Transport of calcium in the uterus
783 correlates with plasma membrane Ca^{2+}-ATPase (PMCA) activity and with concentrations of
784 calbindin-D28K within shell gland epithelial cells (Herbert *et al*., 2006; Wasserman *et al*.,
785 1991). This leads to the spontaneous precipitation of calcium carbonate into calcite (Hincke
786 *et al*., 2012). In the oviparous lizard *Lampropholis guichenoti*, immunofluorescence
787 microscopy revealed activity of PMCA in the uterus at the time of eggshell calcification
788 (Thompson *et al*., 2007).
789 Eggshell formation begins with the eggshell membrane. Two unciliated cell types in the
790 uterus contribute to eggshell membrane formation in a viviparous skink *Chalcides ocellatus*
791 *tiligugu* (Corso *et al*., 2000). One secretes sulfated glycosaminoglycans to form the inner
792 shell membrane, and the other secretes acidic glycoproteins to form the outer layers (Corso *et*

793 *al*., 2000). Simple alveolar glands in the lamina propria secrete collagen fibres (Corso *et al*.,
794 2000). Inhibition of fibre formation or cross-linking, typically caused by aminoproprionitrile
795 or a copper deficiency, causes distorted formations of the eggshell membrane in birds (Arias
796 *et al*., 1997; Chowdhury & Davis, 1995; Hincke *et al*., 2012).

797 In characteristic archosaur eggshells (Choi *et al*., 2018; Legendre *et al*., 2022), organic
798 aggregates are deposited onto the shell membrane creating mammillary knobs, which are
799 absent in lepidosaur shells (Choi *et al*., 2018). Mammillary knobs are a distinct layer between
800 the outer eggshell membrane and the calcified shell matrix layer (Hamilton, 1986). Part of the
801 mammillary knobs, called basal caps, are embedded into the outer eggshell membrane fibres
802 (Tyler, 1965). Mammillary knobs serve as regions of crystal initiation where ACC is
803 deposited (Gautron *et al*., 2021) and converted into calcite crystals with no intermediate
804 phase (Rodríguez-Navarro *et al*., 2015). Cones are formed that radiate in all upward
805 directions, extending up to the shell matrix layer (Tyler, 1965). Despite the direct relationship
806 between mammillary knobs and calcium carbonate crystallization (Rao *et al*., 2015), the
807 protein comprising mammillary knobs remains uncharacterized. A keratan sulfate (KS)-
808 proteoglycan, ‘mammillan’, has been implicated in the composition of mammillary knobs
809 (Fernandez *et al*., 2001; Hincke *et al*., 2012). Any given proteoglycan is a product of multiple
810 coding genes and biosynthesis of KS-proteoglycans is non-trivial (Caterson & Melrose, 2018;
811 Funderburgh, 2002; Iozzo & Schaefer, 2015). However, investigations into the keratan
812 sulfate proteoglycan proposed as ‘mammillan’ and identifying its properties that facilitate (or
813 regulate) calcium deposition (P-FCD) has far-reaching implications given that KS-
814 proteoglycans are proving to be important players in neurological and cancer research
815 (Leiphrakpam *et al*., 2019). The role of homologs of ‘mammillan’ in eggshell formation in
816 squamates may reveal more about the evolutionary history of the eggshell in amniotes.

817 Perhaps presence of homologous proteoglycans in the eggshell can reveal whether the
818 eggshell is truly a synapomorphy or if it is a convergently evolved trait.
819 Parsimony would suggest that all oviparous amniotes shared an ancestral process of
820 eggshell formation. In archosaurs, the process of eggshell formation relies on mammillary
821 knobs and upward growth of calcite, as described above. In lepidosaur eggshells, which have
822 substantially less calcite growth, calcium is deposited on the surface of the eggshell
823 membrane and, in the case of gekkonids and the tuatara, crystal growth proceeds inward
824 toward the centre (Choi *et al*., 2018). The strikingly divergent structure and directionality of
825 eggshell formation between Archosauria and Lepidosauria suggests that the dissimilar
826 processes of eggshell formation are a result of genetic drift (e.g. Schiffman & Ralph, 2022),
827 selection for specific eggshell traits, or, in the case of an early origin of viviparity in amniotes
828 (Jiang *et al*., 2023) and/or lepidosaurs (Pyron & Burbrink, 2014), eggshells evolved
829 convergently.
830 Hypothetically, if a version of the avian eggshell was the microstructure for basal
831 lepidosaurs, loss of mammillan may have prevented calcium deposition because this is the
832 site at which calcium carbonate spontaneously precipitates into calcite. Given that embryonic
833 signalling supports at least two main differences between oviparous and viviparous
834 squamates – the timing of calcium secretions and the length of embryonic retention (Griffith
835 *et al*., 2015, 2017*a*; Stewart & Ecay, 2010) – the loss of mammillan may have supported an
836 early origin of viviparity in squamates. It would have theoretically facilitated (1) an early loss
837 of the eggshell, (2) enhanced contact between maternal and embryonic tissues and (3)
838 enhanced signalling from the embryo to support both altered timing of calcium secretions and
839 hormonal signalling for EER. This potential mechanism for an early origin of viviparity in
840 squamates is proposed here, for the first time, as the nucleation site hypothesis. The
841 evolutionary timing for when calcite crystal growth became associated with mammillan at

nucleation sites is important to this hypothesis, and inferences that can be gained from applying it to the evolution of oviparity and viviparity. If calcite was deposited on top of mammillan in early amniotes the loss of it could disrupt eggshell formation and result in a relatively fast transition to viviparity or EER. Disruption to the formation of the hypothesized KS-proteoglycan that forms mammillan could be relatively easy to achieve given that proteoglycans are formed by multiple genes.

Extending to the ancestral state of amniotes (e.g. Jiang *et al.*, 2023; Laurin, 2005; Romer, 1957), absence of functional 'mammillan' with P-FCD in squamates and mammals would be consistent with a derived state of calcified eggshells in archosaurs. Absence of functional 'mammillan' with P-FCD exclusively in lepidosaurs would be consistent with the nucleation site hypothesis. Presence of functional 'mammillan' with P-FCD across Amniota, especially if it is identified in the eggshell, would provide a homologous product through which amniotes may have originally deposited eggshell calcium (regardless of there being a mammillary layer). Overall, identifying the evolutionary trajectories of the biosynthetic pathway of 'mammillan' across amniotes is likely to create a better picture of the evolution of the amniote egg. However, investigating the ultrastructure of the monotreme eggshell is likely to provide faster insights than attempts to identify mammillan across amniotes. If the monotreme eggshell has nucleation sites, like Archelosaurs, then it would be most parsimonious to conclude that nucleation sites were lost in Lepidosaurs.

New recommendations for estimating the ancestral microstructure of amniote eggshells have recently been put forth, which abandon the traditional classification of hard/soft/semi-rigid shells (Legendre *et al.*, 2022). Including the structure of eggshell membranes in oviparous and viviparous amniotes (e.g. Corso *et al.*, 2000) would also improve phylogenetic reconstructions of the amniote eggshell.

Several pieces of biological evidence lend themselves to an early origin of viviparity in lepidosaurs and the nucleation site hypothesis including the lack of homology between the semi-rigid shells of testudines and lepidosaurs (Legendre *et al*., 2022), the later stage of embryonic development when eggs are commonly oviposited in squamates (Blackburn, 1995), and the more predominant reliance on yolk calcium rather than eggshell calcium in squamates compared to archelosaurs (Packard, 1994; Stewart & Ecay, 2010). Viviparity in the most recent common ancestor of lepidosaurs may provide clear evolutionary insights on these phenomena.

Other features of eggshells are also worth consideration. In chickens, ovotransferrin is present in the eggshell membrane and basal cap layer (Gautron *et al*., 2001*b*). Ovotransferrin promotes the development of elongated crystals (Gautron *et al*., 2001*b*). The resulting shell matrix is made up of the crystal layer and cuticle (Hamilton, 1986). On the inner portion of the avian eggshell, it is unclear what prevents growing crystalized cones from extending into the inner membrane or the albumen. Collagen type X has been implicated (Arias *et al*., 1993, 1997; Hincke *et al*., 2012). The role of collagen type X in creating a boundary that prevents calcite from passing through the eggshell membrane could inform squamate eggshell deposition (as discussed, they deposit calcium only on the outer surface, or crystals grow inwards).

Over 500 proteins are found in the chicken eggshell matrix (Mann, Maček & Olsen, 2006; Mikšík *et al*., 2007, 2010). Ovocleidin-116 (OC-116), ovocalyxin-36 (OCX-36 or BPIFB4), ovocalyxin-21 (OCX-21), and ovocleidin-17 (OC-17) are important for avian eggshell formation (Hernández-Hernández *et al*., 2008*a*; Jonchère *et al*., 2010; Tian *et al*., 2010). *OC-116*, *OC-36*, *OCX-21*, and *OC-17* are some of the most differentially expressed genes during eggshell calcification in chickens (Gautron *et al*., 2007; Hincke *et al*., 1999, 2012; Jonchère *et al*., 2010). OCX-21 may serve as a chaperone protein along with the

protein endoplasmin (ENPL) to facilitate proper folding of the avian eggshell matrix (Jonchère *et al*., 2010). In birds, OC-17 is concentrated in the inner mammillary cone layer, it interacts strongly with ACC, and is implicated in early stages of biomineralization of the eggshell (Gautron *et al*., 2021). The only non-avian eggshell matrix protein, pelovaterin, was identified in the Chinese soft-shell turtle (*Pelodiscus sinensis*) (Lakshminarayanan *et al*., 2005).

Originally considered avian specific, several homologs of avian eggshell matrix proteins have now been identified in non-avian reptiles and mammals (Le Roy *et al*., 2021). A recent study found a significantly reduced number of intact avian eggshell matrix proteins in viviparous squamates compared to oviparous squamates, a pattern that was especially apparent in snakes (Xie *et al*., 2022). This study also found that *OC-17* was absent in viviparous squamates but was always present in the oviparous species in the data set (Xie *et al*., 2022). Due to this, and to the central role of *OC-17* in avian eggshell formation in birds, they ascribe losing intact *OC-17* to the prevention of reversal back to oviparity (Xie *et al*., 2022). However, given that *OC-17* is implicated in initiation of mineralization in the mammillary cone layer, which is absent in squamates, the necessity of *OC-17* for squamate eggshell formation requires further investigation. Other genes, like osteopontin (*OPN* or *SPP1*), also play a central role in biomineralization of the avian eggshell and should be investigated in squamates before conclusions about fixed states are made.

OCX-36 and other bactericidal/permeability-increasing (BPI) family B proteins (also called LPLUNCs) are now thought to have a common origin in vertebrates with multiple duplication events (Gautron *et al*., 2007; Tian *et al*., 2010). Orthologs of *OCX-36* are found in Archelosauria and Monotremata (Le Roy *et al*., 2021). In birds, OCX-36 plays a role in innate immune responses and is found in high concentrations in the inner eggshell membrane (Gautron *et al*., 2007, 2011; Tian *et al*., 2010).

916 *OC-116* is homologous to mammalian *MEPE*, which plays a role in bone and teeth
917 mineralization (Bardet, Delgado & Sire, 2010*a*; Bardet *et al*., 2010*b*). In birds, *OC-116*
918 influences shell thickness, elastic modulus, and egg shape (Le Roy *et al*., 2021). *OC-116* was
919 identified in a crocodile, *Crocodylus siamensis*, proteome (Le Roy *et al*., 2021; Mikšík *et al*.,
920 2018). Synteny analysis across seven turtle species and platypus (*Ornithorhynchus anatinus*)
921 revealed absence of *MEPE/OC116* (Le Roy *et al*., 2021). Other genes and long non-coding
922 RNAs (lncRNAs) are purported to be important for the quality of eggshell formation in hens;
923 these include *FGF14*, *COL25A1*, *GPX8*, and several members of the solute carrier protein
924 (*SLC*) gene family (Yang *et al*., 2020). Research into lncRNAs activity in squamate
925 reproductive tissues during embryonic development represents another valuable avenue for
926 research.
927 Various evolutionary genomics studies have revealed candidate genes for shell formation
928 in squamates (e.g. Recknagel *et al*., 2021*a*; Gao *et al*., 2019. Many candidates have deep
929 evolutionary origins. Seven of the genes expressed during eggshell gland development in
930 *Phrynocephalus przewalskii* (*HYPOU1*, *KCNMA1*, *P4HB*, *PRDX4*, *PTN*, *RRBP1* and
931 *TRAM1*) are purported to be important for eggshell calcification in chickens (Brionne *et al*.,
932 2014). Given this overlap across species that diverged over 300 million years ago (Shen *et*
933 *al*., 2011), these are excellent candidates for further exploration.
934 A functional genomics study harnessed hybridizations of oviparous and viviparous
935 individuals of *Zootoca vivipara* to reveal 17 SNPs and 38 genes associated with eggshell
936 traits (Recknagel *et al*., 2021*a*). These genes enriched terms related to cell communication
937 and the immune system, while differentially expressed genes during gravidity enriched
938 pathways for transforming growth factor (TGF) (Recknagel *et al*., 2021*a*). The three loci with
939 the strongest association with eggshell traits mapped closely to *LGMN*, *LYPLA1*, and *CRTC1*
940 (Recknagel *et al*., 2021*a*). The association of these genes with eggshell traits is particularly

interesting. *LGMN*, for example, is involved with the cadherin pathway. Cadherins have an established role in squamate reproduction, where they influence embryonic attachment in viviparous taxa (Wu, Thompson & Murphy, 2011). *LGMN* is also differentially expressed across many viviparous squamates and mammals (Recknagel *et al*., 2021*a*). Thus, *LGMN* appears to support both oviparous and viviparous gestation in different ways. There are a number of ways to approach exploring how *LGMN* may support both maternal–fetal interconnectivity (viviparous individuals) and eggshell formation (oviparous individuals). Cell-to-cell communication analysis using single-cell data on uteruses of a reproductively bimodal species would enable researchers to identify different interaction networks of *LGMN* and associated cells in oviparous *versus* viviparous individuals.

During gravidity in *Saiphos equalis*, two GO terms associated with calcium homeostasis are enriched by the set of upregulated genes (Foster *et al*., 2020). However, most of these genes are associated with regular cellular responses to calcium and even those associated with calcium transport are upregulated in both early and late stages of gravidity (Foster *et al*., 2020). Their role in eggshell formation in this uniquely labile species is therefore ambiguous.

In oviparous individuals of another reproductively bimodal skink, *Lerista bougainvillii*, only two genes are significantly differentially expressed in gravid uterine tissue compared to non-gravid uterine tissue (Griffith *et al*., 2016). Few genes are differentially expressed in gravid uterine tissue of the oviparous *Lampropholis guichenoti*, compared to non-gravid uterine tissue (Foster *et al*., 2022; Griffith *et al*., 2016). The genes involved in the shelling process in these species may not involve changes in expression from the non-gravid state. The dissimilarity in uterine gene expression profiles across lizards during gravidity suggests there may be multiple ways in which oviparous squamates shell their eggs. Given the variation already observed, eggshell deposition in squamates should be considered in a phylogenetic context and under the different evolutionary histories inferred by ancestral state

reconstructions (Harrington & Reeder, 2017; Pyron & Burbrink, 2014). Table S1 (see online Supporting Information) compares candidate genes associated with eggshell formation and shell gland development in squamates to those of birds.

(4) Pleiotropy of genes and proteins involved with eggshell formation

Substantial pleiotropy of genes involved with eggshell formation would imply that, regardless of parity mode, taxa have innately conserved toolkits that can be readily exploited to form an eggshell for oviparous gestation. In addition to the candidate genes associated with both gestation length and eggshell traits in *Zootoca vivipara* (Reckagel *et al*., 2021*a*), several genes associated with eggshell deposition have pleiotropic effects within species or have different effects in oviparous *versus* viviparous amniotes. Osteopontin (*SPP1* or *OPN*) is found in bone and kidneys, and transports calcium to other tissues in the body (Pines, Knopov & Bar, 1995). It plays an important role in calcium carbonate biomineralization of the avian eggshell (Gautron *et al*., 2021). It is highly expressed in the chicken uterus during calcification (Jonchère *et al*., 2010) but supports pregnancy recognition and implantation in sheep (Bazer *et al*., 2011). Improper functioning of SPP1 in the uterus leads to cracked and abnormal shells in birds (Arazi *et al*., 2009; Hincke *et al*., 2008).

When expressed in the uterus, some bone morphogenic protein-coding genes (*BMP*s) aid eggshell calcification (Jonchère *et al*., 2010). BMPs are part of the TGF-β superfamily and are involved with the formation of new cartilage and bone, and with biomineralization in corals and molluscs (Canalis, Economides & Gazzero, 2003; Lelong, Mathieu & Favrel, 2000; Zoccola *et al*., 2009). Chordin (CHRD) is an antagonist of the BMP pathway. BMP-binding endothelial regulatory protein (*BMPER*) and *CHRD* are expressed in the chicken uterus during the stage of eggshell calcification (Jonchère *et al*., 2010). Regulation of BMPs by *CHRD* is essential for early embryogenesis and adult homoeostasis.

BMPER and seven *BMP*s are expressed during gestation in *Chalcides ocellatus*, a viviparous skink (Brandley *et al*., 2012). Most of these are upregulated (Brandley *et al*., 2012). *BMP* genes are expressed during both gravidity and non-gravidity in oviparous *Lerista bougainvillii* and *Lampropholis guichenoti* (Griffith *et al*., 2016). *BMP2* is upregulated in oviparous late gestation compared to viviparous late gestation in the reproductively bimodal lizard *Saiphos equalis* (Foster *et al*., 2020).

Differential expression of *BMPR1B* is associated with differences in eggshell quality in chickens (Yang *et al*., 2020). Another study associated stage-specific high expression of *BMPR1B* with the stage corresponding to EER and placentation in *Phrynocephalus vlangalii* (Gao *et al*., 2019). They identified a co-expression network of highly expressed genes, including *BMPR1B*, that they associated with placentation (Gao *et al*., 2019). *BMPR1B* also reaches significant levels of differential expression in uterine tissues of two other gestating viviparous lizards, *Chalcides ocellatus* and *Pseudemoia entrecasteauxii*, compared to non-gestational uterine tissue (Brandley *et al*., 2012; Griffith *et al*., 2016). Receptors for BMPs are also expressed in the uterus during gestation in two other viviparous lizards, *Phrynocephalus vlangalii* and *Pseudemoia entrecasteauxii* (Gao *et al*., 2019; Griffith *et al*., 2016). Perhaps unsurprisingly, *BMPR1B* is also differentially expressed in the uterus of viviparous *Zootoca vivipara* compared to oviparous individuals during gestation.

The potential role of these genes in squamate eggshell formation remains unclear. In vertebrates, BMPs influence dorsal–ventral axis patterning during early embryogenesis and growth of skeletal structures in post-natal tissues (Medeiros & Crump, 2012). It therefore may be difficult to disentangle their roles in embryonic development, placental development, and eggshell deposition. Future research on them may inform scientific understanding of parity mode evolution.

SLIT genes are purported to be involved with folding the eggshell matrix in chickens (Jonchère *et al*., 2010). The *SLIT2* gene functions across birds and mammals in diverse organs, and encodes a protein that provides a structural framework for protein–protein interactions (Jonchère *et al*., 2010; Marillat *et al*., 2002). In a functional genomics study, *SLIT2* was identified as an important gene for eggshell traits in *Zootoca vivipara* (Recknagel *et al*., 2021*a*). *SLIT2* is among the 50 most downregulated genes in the uterus during pregnancy in the viviparous *Chalcides ocellatus* compared to non-pregnancy (Brandley *et al*., 2012). However, in the uterus of the yolk-sac placenta in the viviparous skink *Pseudemoia entrecasteauxii*, *SLIT2* is upregulated compared to non-reproductive uterine tissue (Griffith *et al*., 2016). *SLIT3* is differentially expressed during the stage of placentation in the viviparous agama lizard *Phrynocephalus vlangalii* (Gao *et al*., 2019). *SLIT* genes also play a role in axonal pathfinding and neuronal migration in rats (Marillat *et al*., 2002). *SLIT2* was associated with reproduction in humans (Chen *et al*., 2015).

Podocalyxin (PODXL) is a sialoprotein associated with eggshell calcification in chickens (Jonchère *et al*., 2010). In the viviparous Qinghai toad-headed agama lizard (*Phrynocephalus vlangalii*), a weighted gene correlation network analysis associated *PODXL* with uterine structural changes (Gao *et al*., 2019). The gene may play a role in placentation in these species given that it was also differentially expressed in the uterus during the stage of placentation (Gao *et al*., 2019). Interestingly, *PODXL* is downregulated in the uterus of the yolk-sac placenta in another viviparous skink *Pseudemoia entrecasteauxii* (Griffith *et al*., 2016). Based on its role in chickens and *P. vlangalii*, *PODXL* is a good candidate for further research on the molecular evolution of eggshell formation and placentation in squamates.

(5) Eggshell formation termination

When eggshell formation is terminated, the egg is still bathed in the supersaturated calcium and bicarbonate ion fluid (Hincke *et al*., 2012). Some component(s) of the terminal uterine fluid may prevent precipitation of calcium carbonate (Gautron, Hincke & Nys, 1997), such as phosphate anions (Lin & Singer, 2005). The presence of phosphorus in the superficial layers of the chicken shell suggest that it may be a factor preventing deposition of calcite crystals in the terminal stage. Additionally, the high concentration of OCX-32 in the outer eggshell and cuticle suggest that OCX-32 may inhibit proteinaceous crystal growth in the terminal stage of eggshell calcification (Gautron *et al*., 2001*a*). It is informative to viviparous reproduction and consistent with the nucleation site hypothesis that exposure to precursors of the eggshell does not necessitate eggshell deposition. The influence of phosphate anions and OCX-32 on inhibition of calcium carbonate precipitation on the eggshell membrane of viviparous squamate embryos has not been examined to my knowledge.

(6) Rotating the egg for eggshell formation

Oviparous amniotes rotate the egg for calcium formation and viviparous mammals rotate the embryos for parturition. One hurdle to reversing back to oviparity may be re-evolving rotation of the egg for shell formation early in gravidity (Griffith *et al*., 2015). Given the complex musculature of the uterus across taxa, which allows for multidirectional force application for parturition and eggshell formation, it is difficult to determine the degree of difficulty of re-evolving appropriate timing of egg rotation. Cadherins and hormonal signalling support embryonic attachment (Wu *et al*., 2011; Biazik *et al*., 2012), which could theoretically prevent rotation of the egg. Oviparous taxa lack embryonic attachment, enabling the uterus to rotate the egg for eggshell formation. This rotation does not happen until later in gestation for eutherian mammals when, for example, the embryo detaches and cadherins become less concentrated (Wu *et al*., 2011). A possible candidate gene for studying this is

CDH5, the only gene that is differentially expressed in all viviparous squamates studied thus far (Recknagel *et al*., 2021*a*). Genes that enrich the GO term for 'voltage-gated calcium channel activity' are also useful candidates for investigating uterine rotation associated with eggshell formation because voltage-gated calcium channels are involved in creating the action potential of cells and in muscle contractions.

(7) Discussion and future directions – eggshell formation and parity mode evolution

The process of eggshell formation is more resolved in birds than in non-avian reptiles and monotremes (Choi *et al*., 2018; Frankenberg & Renfree, 2018). Table S1 presents overlaps gleaned from the literature which are candidates for further research. Of particular interest are avian eggshell matrix proteins (Alföldi *et al*., 2011; Le Roy *et al*., 2021; Tian *et al*., 2010; Xie *et al*., 2022), genes with biomineralization functions, candidate genes associated with eggshell traits in *Zootoca vivipara* (Recknagel *et al*., 2021*a*), and homologs for avian eggshell matrix proteins identified in the *Anolis carolinensis* genome (Alföldi *et al*., 2011; Tian *et al*., 2010). Additionally, genes purported to be important for eggshell calcification in chickens that are also associated with eggshell gland formation in an oviparous lizard, *Phrynocephalus przewalskii*, are relevant: *HYPOU1*, *KCNMA1*, *P4HB*, *PRDX4*, *PTN*, *RRBP1* and *TRAM1* (Brionne *et al*., 2014; Gao *et al*., 2019). Overlaps between the genes associated with gestation length and eggshell traits in *Zootoca vivipara* (Recknagel *et al*., 2021*a*) hint at the potential for single genes to affect multiple traits relevant to parity mode transitions. The nucleation site hypothesis also offers a simple evolutionary mechanism to investigate the evolutionary history of amniote parity mode evolution (see Section III.3). Complementary to the nucleation site hypothesis are that dissimilar eggshells and eggshell deposition processes evolved through selective pressure, genetic drift, or both. Fortunately, the nucleation site hypothesis can be utilized to ascertain the likelihood of this.

IV. PLACENTATION AND TRANSPORT OF EMBRYONIC WATER, GAS, AND NUTRIENTS

The evolutionary pressures on fluid allocation, gas exchange and nutrient transport should differ between oviparous and viviparous taxa because their sources of all or some of these resources differ (Blackburn, 1992; Bonnet *et al*., 2001; Bonnet, Naulleau & Shine, 2017; van Dyke *et al*., 2014). In viviparity, maternal gas and water are accessed through the chorioallantois, which is especially important in the latter half of development (van Dyke *et al*., 2014; Carter, 2012). Nutrients can be available from the yolk, maternal transfer, or both yolk and maternal transfer. As such, changes to the uterus, yolk sac, and chorioallantois are possible during transitions between parity modes. Interestingly, whereas other amniotes can rely on the albumen for fluid allocation, squamates lack an albumen (Blackburn & Stewart, 2021). Instead, the eggshells of various squamates support uptake of water from the environment (Blackburn & Stewart, 2021). The evolutionary implications of this have not been documented to my knowledge.

(1) Anatomy and methods of water, gas and nutrient provisioning

The embryonic membranes regulate embryonic fluid transport, nutrient supply, respiration, immunity, and waste (Brace, 1997; Burton & Tullett, 1985; Ferner & Mess, 2011; Packard & Packard, 1980). Fluids are important for the developing embryo because they prevent desiccation and compression (Ferner & Mess, 2011; Packard & Packard, 1980). Over- or under-abundance of embryonic sac fluids leads to reproductive failure (Chamberlain *et al*., 1984; Fedakâr, Semiz & Peker, 2016; Hadi, Hodson & Strickland, 1994; Mercer *et al*., 1984). Water is the predominant resource provisioned by the mother in most viviparous squamates (Lourdais *et al*., 2015).

Oxygen flux in embryonic mammals is largely determined by oxygen-diffusing capacity of the placenta, the rates of blood flow in the umbilical and uterine arteries, and the oxygen capacities and affinities of fetal and maternal blood (Carter, 2009). Reptilian and mammalian blood vessels differ in basic characteristics such as capillary density, capillary surface, and oxygen diffusion gradients (Pough, 1980). Oviparous taxa regulate gas exchange through pores in their eggshells.

Patterns of embryonic nutrient exchange can be broadly categorized into lecithotrophy, i.e. obtaining nutrients from the yolk, and placentotrophy or matrotrophy, where nutrients are obtained from the mother. Taxa belonging to Archelosauridae are generally lecithotrophic. The ancestral state of mammals was most likely oviparous matrotrophy that later evolved into viviparous matrotrophy in therians (Blackburn, 2005). The ancestral state of reptiles was likely lecithotrophy (Blackburn, 2005). Most viviparous squamates are lecithotrophic, some are lecithotrophic and matrotrophic, and a few have specializations for substantial matrotrophy (e.g. Blackburn, 2015*a*; Stewart & Thompson, 1993; Thompson *et al.*, 1999; van Dyke *et al.*, 2014). Even in lecithotrophic viviparous squamates some organic or inorganic nutrients pass through the chorioallantoic placenta (Blackburn, 2005; Swain & Jones, 1997, 2000; Stewart & Ecay, 2010; Thompson *et al.*, 1999; Thompson & Speake, 2002). Reversals may be most unlikely in lineages that have specialized placentas for substantial nutrient exchange because they would need to re-evolve lecithotrophy. Highly matrotrophic squamates are extremely rare (Blackburn, 2015*a*).

(2) Evolutionary history of yolk-sac formation and yolk processing

Vitellogenesis is the process of yolk formation in the oocyte, providing the embryo with a valuable source of nutrients, primarily through the accumulation of the precursor proteins to yolk: vitellogenins. Vitellogenin is produced in the liver, in a process called hepatic

vitellogenesis, and is transported to the maturing ovum (Ho, 1987). Vitellogenins were lost in all mammals except monotremes (Brawand, Wahli & Kaessmann, 2008). They are a primary source of nutrition for other amniotes. Functionally similar to vitellogenin, caseins have persisted in all mammalian milks (Brawand *et al.*, 2008). Active functioning of the yolk sac is restricted to the first trimester in placental mammals (Kuzima, 2023), where it plays an essential role in early nutrient supply (Shibata, Makihara & Iwasawa, 2023). The detection of glycodelin in the yolk-sac epithelium also supports this (Burton *et al*., 2002). In the yolk sac of bats, dogs, and non-human primates the mesoderm-derived layer is absorptive and may transfer substances from the exocoelomic cavity where the yolk sac is located (Enders, Wimsatt & King, 1976; Freyer & Renfree, 2009; King & Wilson, 1983; Lee *et al*., 1983).

The morphology of the yolk sac and process of vitellogenesis differs between birds and non-avian reptiles. In birds, during the process of meroblastic cleavage, the zygote's cells divide while the yolk component does not. The yolk forms a large, fluid, non-cellularized mass surrounded by the extraembryonic yolk sac. The formation of the yolk-sac placenta in birds has the following pattern: first the bilaminar omphalopleure forms, followed by the trilaminar omphalopleure; blood vessels move into folds of the extraembryonic endoderm, becoming stratified epithelium; and finally, the folds carrying the blood vessels reach the peripheral regions of the yolk only with the centre of the yolk mass remaining uncellularized (Starck, 2021). Intensive development of haemopoietic tissue surrounding the blood vessels during most of embryonic development, thus far, appears to be unique to birds (Starck, 2021). Compared to non-avian sauropsids, the unique pattern of yolk processing in birds facilitates faster embryonic development (Blackburn, 2021).

The yolk sac characteristic of non-avian reptilian eggs may serve as a model for the transition between the egg of anamniotes and that of amniotes (Blackburn, 2021; Elinson *et al.*, 2014). A series of recent papers, covering species of snakes, lizards, crocodiles, and

turtles, indicate that these taxa utilize similar developmental pathways of yolk-sac formation and yolk processing that differ from birds (Blackburn, 2021; Blackburn *et al*., 2019; Elinson *et al*., 2014; Elinson & Stewart, 2014; Stinnett *et al*., 2011). Across these taxa, a bilaminar/trilaminar omphalopleure overgrows the yolk mass, and the yolk mass is invaded by proliferating endodermal cells that phagocytose the yolk material. These cells form clumps, progressively filling the yolk mass. Small blood vessels derived from yolk-sac vasculature invade the yolk-sac cavity and the endodermal cells arrange in monolayers around these vessels, forming "spaghetti bands" (Blackburn, 2021). The yolk sac of *Pantherophis guttatus* is one suitable model for studying the transition of the yolk sac from anamniotes to amniotes (Elinson & Stewart, 2014; Elinson *et al*., 2014).

A major difference between avian and non-avian reptilian yolk-sac formation is the morphology and extent of vascularization and cellularization in the yolk sac cavity (Starck, 2021). Birds have a yolk sac with an absorptive endodermal lining that digests nutrients and sends them into blood circulation (Starck, 2021) whereas snakes, lizards, turtles, and crocodilians have a yolk sac that becomes invaded by endodermal cells that proliferate and phagocytose yolk material (Blackburn, 2021). In these taxa, yolk material becomes cellularized, digested, and transported by vitelline vessels to the developing embryo (Blackburn, 2021). Factors involved with cellularization of the yolk sac are proposed to include cell cycle regulators and structural proteins (Elinson *et al*., 2014). Generation of these cells is suspected to be reliant on processes of angiogenesis (Elinson *et al*., 2014). Few transcriptomic profiles of yolk-sac placentas in reptiles have been documented to my knowledge (Griffith *et al*., 2016). Significant overlaps in the yolk-sac transcriptomes of human, mouse, and chicken, including apoliproteins and SLC transporters, however, suggest functional conservation (Cindrova-Davies *et al*., 2017).

As discussed in Section II.1, progesterone inhibits myometrial contractility, but it also inhibits oestrogen-induced hepatic vitellogenin synthesis (Custodia-Lora, Novillo & Callard, 2004; Callard *et al*., 1992). Variable progesterone concentrations in circulation throughout gestation in viviparous squamates may reflect a trade-off to allow oestrogen expression to support hepatic vitellogenin synthesis during embryonic development, thus supporting nutrient provisioning during the lengthened embryonic retention. Although hepatic vitellogenesis usually ceases during gestation, vitellogenin synthesis and mother-to-embryo transfer was detected in one viviparous fish, *Xenotoca eiseni*, during gestation (Iida *et al*., 2019). Future research should consider the timing of vitellogenin synthesis throughout the reproductive cycle in gestating and non-gestating viviparous squamates to investigate this further.

(3) Evolutionary history of placentotrophy in mammals and squamates

Traditionally, it was thought that placentotrophy evolved after viviparity in squamates (Packard *et al*., 1977; Shine & Bull, 1979). Further research demonstrated that matrotrophy preceded the evolution of viviparity in mammals, whereas in squamates the potential for both incipient matrotrophy and evolution of placentotrophy after viviparity is supported (Stewart & Ecay, 2010). The incipient matrotrophy model relies on evidence that (1) uterine provisioning of nutrients pre-dates the origin of viviparity (Blackburn, 1985, 1992, 2006), (2) uterine and embryonic tissues have a close anatomical and physiological association in viviparous taxa, and (3) some degree of placental transfer of organic or inorganic molecules occurs in viviparous taxa (Stewart & Ecay, 2010).

Placentation and implantation are not homologous in mammals compared to squamates (Griffith, van Dyke & Thompson, 2013*b*). Several placental specializations for gas and nutrient exchange are unique to mammals, including erosion of the uterine mucosa,

extensively invasive implantation, haemochorial contact, retention of a vascularized choriovitelline membrane, and countercurrent patterns of blood flow (Blackburn, 2005). This enables extensive exchange of nutrients in addition to water and gas. The vast majority of viviparous squamates have the most superficial type of chorioallantoic placenta called an epitheliochorial placenta (Blackburn, 1993).

Nutrient provisioning through placentotrophy is obligate for embryonic development in only five lineages of squamates, all of which are scincid lizards (Blackburn, 2000; Flemming & Blackburn, 2003; Ramírez-Pinilla, Rueda & Stashenko, 2011; van Dyke *et al*., 2014). For example, *Pseudemoia entrecasteauxii* is a moderately matrotrophic viviparous skink, with roughly half of embryonic nutrient uptake from the yolk and half through a specialized cyto-epitheliochorial placenta (Adams *et al*., 2005; Speake, Herbert & Thompson, 2004; Stewart & Thompson, 1993, 2009).

Specializations of the chorioallantoic placenta for nutrient provisioning in some squamates include elaborate structures for uterine secretion and absorption, including placentomes, chorionic areolae, hypertrophied uterine mucosa, and chorionic epithelia modified for absorption (Blackburn, 2005). In squamates, specializations for gas exchange across the chorioallantoic placenta include decreased diffusion distance between maternal and fetal capillaries, uterine vascularity, shell membrane deterioration, and modifications of both fetal and maternal blood properties (Blackburn, 1998, 2005; Blackburn & Lorenz, 2003; Blackburn & Vitt, 2002).

Mammalian placenta-specific genes have deep origins in vertebrates (Rawn & Cross, 2008). One study that looked at placentation and gene expression across a small sample of divergent amniotes found only one gene with a placentotrophy-specific pattern of gene expression, *DIO3* (Griffith *et al*., 2017*a*). In mammals, *DIO3* is an imprinted gene and preferentially paternally expressed. The authors suggest that the gene may increase offspring

resource uptake during pregnancy in the horse and a viviparous lizard, *Pseudemoia entrecasteauxii*, where it is recruited to the placenta (Griffith *et al*., 2017*a*).

(4) Genes involved with embryonic water, gas, and nutrient transport

Water transport in animals is regulated by a family of molecular water channels called aquaporins (AQs or AQPs) (Borgnia *et al*., 1999). In humans, AQP1, AQP3, AQP4, AQP8 and AQP9 are found in the placenta but further research is needed to understand how these influence water fluxes between maternal and fetal tissues (Damiano, 2011). Transcriptomic analysis on uterine tissue of the gestating viviparous skink *Chalcides ocellatus* revealed differential expression of *AQP1*, *AQP3*, *AQP5*, *AQP6*, *AQP8*, *AQP9* and *AQP11* when compared to non-gestating uteruses (Brandley *et al*., 2012). In birds, *AQP1* is expressed in the chorioallantoic membrane, and it is suggested to influence angiogenesis throughout embryonic development (Ribatti *et al*., 2002). In a viviparous lizard, *Pseudemoia entrecasteauxii*, *AQP8* and *AQP9* were more highly expressed in the chorioallantoic placenta compared to the yolk-sac placenta (Griffith *et al*., 2016). During gestation in both oviparous and viviparous populations of the reproductively bimodal skink *Saiphos equalis*, several genes involved with water homeostasis are upregulated in the uterus including *AQP1*, *AQP3* and *AQP12B* (Foster *et al*., 2020). In uteruses of *Saiphos equalis*, *AQP5* and *AQP8* are upregulated during oviparous late gestation compared to viviparous late gestation. In sheep, *AQP3* is differentially expressed during gestation, where it serves a dual role of water transport to the embryo and fetal urea export (Johnston *et al*., 2000). This is similar to the function of *AQP9* in humans (Damiano, 2011). Immunocytochemistry reveals that *AQP1* and *AQP3* are expressed in the uterus of the highly placentotrophic South American scincid lizard, *Mabuya* sp. (Wooding, Ramirez-Pinilla & Forhead, 2010). In *Zootoca vivipara*, *AQP9* is upregulated at mid-gestation (Recknagel *et al*., 2021*a*).

Some molecules are implicated in the regulation of aquaporins including insulin (INS), hCG, cyclic AMP (cAMP) and cystic fibrosis transmembrane conductance regulator (CFTR) (Damiano, 2011). Genes predicted to be involved with reproduction in *Anolis carolinensis* are enriched for the GO term for cAMP-mediated signalling (Alföldi *et al*., 2011). Further comparative research could elucidate the functional differences of aquaporins in oviparous and viviparous amniotes and how they relate to the differing conditions under which these embryos develop.

Genes involved with embryonic oxygen transport pre-date the origin of amniotes. Haemoproteins arose in evolutionary history well before they were used for placental oxygen transfer (Hardison, 1998). In mammals, adult [alpha (HBA); beta (HBB, HBD)] and embryonic haemoglobins [alpha (HBZ, HBA); beta (HBE, HBG, and HBH)] are involved with oxygen transport (Carter, 2012). Some of these are unique to eutherian mammals following a series of duplication events (Opazo, Hoffmann & Storz, 2008). However, fetal haemoglobins are found in turtles, lizards, and snakes (Pough, 1980). HBA, HBB and HBM are all significantly downregulated in the uterine tissue of the viviparous *Chalcides ocellatus* during gestation compared to non-gestation (Brandley *et al*., 2012). The oxygen demands of reptile embryos are relatively low until stage 30, when most oviparous squamates oviposit (Shine & Thompson, 2006). In viviparous and oviparous species with long egg retention, embryonic demand for maternal provision of oxygen and removal of CO_2 increases at this stage.

Improper water, gas and nutrient exchange can occur due to poor chorioallantoic blood flow (Wootton *et al*., 1977). Thus, viviparous taxa require greater degrees of vascularization and vasodilation to facilitate enhanced requirements for maternal resources compared to oviparous taxa. Rather than increasing the size of the placenta, increasingly dense blood vessels can support fetal growth without compromising space for embryonic growth as

occurs in some pigs (Ford, 1997; Vonnahme, Wilson & Ford, 2002). In populations of oviparous individuals of *Saiphos equalis* with extended egg retention, there is expansion of the uterine vascular bed and thickening of the chorioallantoic tissue that supports increased embryonic growth in the later portion of oviparous gravidity (Parker *et al*., 2010). In the viviparous scincid lizard *Eulamprus quoyii*, angiogenesis, the formation of new blood vessels, and expansion of the vessel-dense elliptical area of the uterus is associated with supporting increased embryonic oxygen demand (Murphy *et al*., 2010).

Several protein-coding genes are known to be involved with angiogenesis, vascularization, and vasodilation *in utero*. Differential gene expression analyses on oviparous and viviparous individuals of *Zootoca vivipara* revealed pathways for angiogenesis were enriched in viviparous female reproductive tissues; and pathways for angiogenesis were enriched across genes under divergent selection in oviparous and viviparous *Z. vivipara* individuals (Recknagel *et al*., 2021*a*). However, a study that examined expression patterns across chickens (oviparous), horses (viviparous), two viviparous squamates, and one oviparous squamate found that no examined genes for angiogenesis showed a viviparity-specific expression pattern, based on differentially expressed genes between pregnant and non-pregnant state (Griffith *et al*., 2017*a*). Other than the chicken, the only oviparous taxa included in this study was a reproductively bimodal skink, *Lerista bougainvillii* (Griffith *et al*., 2017*a*).

In the uterine tissue of gestating viviparous skinks and rats, several genes for angiogenesis are upregulated: *EPAS1*, *HIF1A* and *VEGFA* (Brandley *et al*., 2012; Whittington *et al*., 2015, 2017). Proteins involved in vascularization and vasodilation *in utero* include members of the vascular endothelial growth factor (*VEGF*) gene family, VEGF receptors (VEGFRs), placental growth factor (PGF) and nitric oxide synthase (NOS) (Blomberg *et al*., 2010; Reynolds *et al*., 2006; Risau, 1997; Torry *et al*., 2003; Vonnahme,

Wilson & Ford, 2001). In *Saiphos equalis*, different homologs of *NOS* experience different patterns of gene expression across the oviparous and viviparous stages of gestation/gravidity (Foster *et al*., 2020). One homolog of *NOS* is upregulated during oviparous late gestation, and another is upregulated during viviparous late gestation (Foster *et al*., 2020). Several genes involved with angiogenesis and vascular morphogenesis are downregulated in the pre-implantation uterus of a marsupial, the fat-tailed dunnart (*Sminthopsis crassicaudata*): *ADGRA2*, *ADGRB2*, *ANGPTL1*, *EPHB4*, *ISM1*, *PDZRN3*, *RHOJ*, *TNMD* and *VEGFD* (Whittington *et al*., 2018).

In humans, immune factors are also responsible for increasing embryonic blood supply. Embryonic non-classical MHC class I molecule, HLA-G, and uterine natural killer (uNK) cells support an increased embryonic blood supply (Moffett & Loke, 2006; Rajagopalan *et al*., 2006). A similar pattern of utilizing immune properties to support embryonic blood supply has not been identified yet in squamates.

Lipids are a main energy source for embryos. Lipoprotein lipase (LPL) is an important enzyme in lipid transport. LPL is significantly expressed on the syncytiotrophoblasts, which are specialized placental cells, of humans (Lindegaard *et al*., 2005), and in the endometrium of cows (Forde *et al*., 2011), and pigs (Ramsay *et al*., 1991), where it plays a role in lipid mobilization. A viviparous lizard, *Pseudemoia entrecasteauxii*, increases capacity for lipid transport towards the end of pregnancy (Griffith *et al*., 2013*a*). The uterine tissue of the yolk-sac placenta in this species had significantly higher expression of LPL than the uterine tissues of the chorioallantoic placenta (Griffith *et al*., 2013*a*), leading the authors to suggest that the yolk-sac placenta is the major site of lipid transport. LPL expression was not detected during pregnancy in the viviparous skink *Chalcides ocellatus* (Blackburn, 1992; Brandley *et al*., 2012). Instead, lipid transport may be facilitated by fatty acid binding proteins in this species

(Chmurzyńska, 2006; Brandley *et al*., 2012). These are also active in the mammalian placenta (Haggarty, 2002).

Apolipoproteins are also suitable candidates for transport of fatty acids, cholesterol, and phospholipids. Five apolipoprotein genes (*APOA1*, *APOA2*, *APOA4*, *APOE* and *APOM*) and *APOA1BP* are significantly upregulated in the pregnant uterus of the viviparous skink *Chalcides ocellatus* (Brandley *et al*., 2012). *APOA1BP* is also upregulated in the uterus of the chorioallantoic placenta and yolk-sac placenta compared to non-gestational uterine tissues in *Pseudemoia entrecasteauxii* (Griffith *et al*., 2016). Additionally, upregulation of 136 genes that encode SLCs in the pregnant uterus of *Chalcides ocellatus* are associated with transport of inorganic ions, metals, glucose, amino acids, peptides, fatty acids, and carboxylic acids (Brandley *et al*., 2012).

A supply of amino acids is required for embryonic development. SLCs have important transport functions, including the transport of amino acids, and thus they are considered to be important for gestation (Foster *et al*., 2022). However, a recent study found no overlap in the amino-acid-transporting SLCs upregulated in placentas of the viviparous placentotrophic vertebrates studied, which included eight representatives from Mammalia, Reptilia, and Chondrichthyes (Foster *et al*., 2022). However, *SLC38A3* was upregulated in all viviparous species except *Rattus norvegicus* (Foster *et al*., 2022).

Cathepsins and phospholipases are important for uterine secretions for embryonic development in horses, pigs, sheep, and cattle (Bazer, 1975; Satterfield *et al*., 2007; Song *et al*., 2010). Cathepsins are present in yolk sacs of humans and mice. They function to degrade proteins to free amino acids (Cindrova-Davies *et al*., 2017). Two genes for cathepsin L (*CTSL1* and *CTSL2*) are upregulated in the uterus during gestation in *Chalcides ocellatus* (Brandley *et al*., 2012). *CTSL* is also upregulated in the uterus during the pre-implantation phase in the marsupial *Sminthopsis crassicaudata* (Whittington *et al*., 2018), and in the uterus

of the chorioallantoic placenta and uterus of the yolk-sac placenta during gestation in *Pseudemoia entrecasteauxii* (Griffith *et al*., 2016).

In viviparous individuals of the reproductively bimodal lizard *Saiphos equalis*, many genes for cellular adhesion are upregulated during late gestation (Foster *et al*., 2020). The authors postulated that this helps facilitate maternal–fetal signalling and paracellular transport (Foster *et al*., 2020). Gao *et al*. (2019) identified a set of genes in *Phrynocephalus vlangalii* that were differentially expressed in the uterus during the placentation stage and these enriched GO terms were functionally related to the process of placentation. This included an oestrogen receptor (*ESR1*) and two growth factor receptors (*GHR* and *IGF1R*) (Gao *et al*., 2019).

Finally, the proteomes of the ovary and placenta from obligately placentotrophic *Mabuya* sp. lizards can serve as a useful resource for examining nutrient provisioning in squamates (Hernández-Díaz, Torres & Ramírez-Pinilla, 2017). In the placenta they found protein expression involved with nutrient metabolism, transport, protein synthesis, and embryonic development (Hernández-Díaz *et al*., 2017).

(5) Uterine glands: adenogenesis, placenta development and histotrophy

In addition to their role in eggshell deposition in oviparous taxa, uterine glands also secrete growth factors and cytokines that support placental development in mammals. In humans, these include TGF-β, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF, and leukemia inhibitory factor (LIF) (Hempstock *et al*., 2004). In eutherians, TGF-β supports placental development by regulating proliferation and invasion rates of placental cell lines (Caniggia *et al*., 2000; Hempstock *et al*., 2004; Lafontaine *et al*., 2011).

Histotrophy (also called histiotrophy) occurs when nutrients are secreted into the uterine lumen from vesicles of the columnar epithelial cells of the uterus and taken up by the

1387 embryo. Histotrophic nutrient provisioning is documented across amniotes including
1388 marsupials (Whittington *et al*., 2018), several ungulate taxa (Bazer *et al*., 2011; Han *et al*.,
1389 2016; Gao *et al*., 2009), and humans (Burton *et al*., 2002), and appear to occur in some
1390 viviparous squamates (van Dyke *et al*., 2014). In humans, histotrophic nutrient provisioning
1391 occurs during the first trimester. The intervillous space is filled with fluid containing uterine
1392 gland secretions that are phagocytosed by the syncytiotrophoblasts and represent the initial
1393 nutrient source for the fetus (Burton *et al*., 2002). Two of these glycoproteins are epithelial
1394 mucin (MUC1) and glycodelin A (GdA) (Burton *et al*., 2002). Interestingly, the *MUC15* gene
1395 is upregulated during pregnancy in the uterus of oviparous and viviparous *Saiphos equalis*
1396 individuals (Foster *et al*., 2020). This also occurs in the chorioallantoic placenta of
1397 *Pseudemoia entrecasteauxii* during gestation (Griffith *et al*., 2016). Several mucins are
1398 expressed in the uterus in non-gravid and gravid samples from oviparous individuals of
1399 *Lerista bougainvillii* and *Lampropholis guichenoti* (Griffith *et al*., 2016).
1400 A survey of viviparous squamates with modest to extensive placentotrophy revealed a
1401 prevalence of histotrophic nutrient provisioning rather than haemotrophy, i.e. transfer of
1402 nutrients between maternal and fetal blood streams (Blackburn, 2015*b*). Embryos of
1403 *Chalcides chalcides* have extensive placentotrophy that supports substantial maternal nutrient
1404 provisioning and histotrophy (Blackburn, 2015*a*). Histotrophy may reduce parent–offspring
1405 conflict and give the mother control over nutrient provisioning compared to haemotrophy
1406 (Blackburn, 2015*b*).
1407 *Chalcides ocellatus* has less extensive placentotrophy than *C. chalcides* but the gestating
1408 uterus still illustrates expression of many genes associated with organic and inorganic
1409 nutrient transport (Blackburn, 2015*a*). Multiple *TGF-β* genes are differentially expressed in
1410 the uterus during gestation in *C. ocellatus*, however most these are downregulated compared
1411 to non-gestational uterine tissue (Murphy *et al*., 2012). The influence of *TGF-β* on placental

development and nutrient provisioning in *Chalcides* spp. remains to be explored. A TGF-β receptor (TGFBR1) was associated with placental development in *Phrynocephalus vlangalii* (Gao *et al*., 2019).

Essential to histotrophy is adenogenesis, i.e. the generation of endometrial glands. Adenogenesis allows for the secretion of histotrophs. The period of early development during which adenogenesis occurs is highly variable among vertebrates but it is required for embryonic survival (Gray *et al*., 2001, 2002; Spencer & Bazer, 2004). Genes involved with adenogenesis in sheep include insulin-like growth factor 1 (*IGF-1*), *IGF-2*, *PAX2*, *LHX1* (also known as *LIM1*) and *EMX2*, genes in the abdominal-B *HOXA* cluster, members of both *Wnt* and Hedgehog (*Hh*) gene families (Fazleabas, Kim & Strakova, 2004), prolactin (*PRL*), fibroblast growth factor 7 (*FGF7*), *FGF10*, *FGFR2IIIb*, hepatocyte growth factor (*HGF*), a receptor tyrosine kinase (*c-Met)*, and cadherins (Fazleabas, 2007).

In the gestating uterus of *Chalcides ocellatus*, insulin-like growth factor-binding protein 5 (*IGFBP5*) is one of the most significantly downregulated genes compared to non-gestational uterine tissue (Brandley *et al*., 2012). *IGFBP5* is evolutionarily conserved and multifunctional, with an important role in regulating IGF signalling, including that of IGF-1 and IGF-2 (Duan & Allard, 2020). Other than adenogenesis in sheep, IGFs serve an important role in the growth of fetal and maternal tissues in mammals (Gibson *et al*., 2001; Kampmann *et al*., 2019).

Genes involved with histotrophic secretion in the marsupial *Sminthopsis crassicaudata* include *AP4S1*, *HYOU1*, and *SRPRA* (Whittington *et al*., 2018). Genes for nutrient transporters significantly upregulated at this time are *APOL6* (cholesterol transport; Baardman *et al*., 2013), *PLA2G10* (hydrolysis of fatty acids during pregnancy; Miele, Cordella-Miele & Mukherjee, 1987) and a wealth of SLCs (for transport of sugar, ions, anions, glucose, fatty acids, calcium and zinc; Whittington *et al*., 2018). Subsequent research

has identified downregulation of *HYOU1* at early and mid-gestation; and downregulation of *SRPRA* at mid-gestation in viviparous *Zootoca vivipara* compared to oviparous individuals (Recknagel *et al*., 2021*a*). In a reproductively bimodal skink, *Saiphos equalis*, *PLA2G10* is upregulated during viviparous late gestation compared to oviparous late gestation (Foster *et al*., 2020). Upregulation of SLCs also occurs in the viviparous skink *Chalcides ocellatus* (Brandley *et al*., 2012; Van Dyke *et al*., 2014) and in the uterus during pregnancy in the marsupial *Monodelphis domestica* (Hansen, Schilkey & Miller, 2016).

Uterine glands are also important for secretions of eggshell precursors. It is possible that genes involved with adenogenesis of uterine glands may be similarly used to support histotrophic nutrient provisioning during transitions to viviparity, but further research is necessary. Specialized uterine areolar glands are found in some *Mabuya* lizards, a genus with oviparous species and viviparous species that utilize placentotrophy and histotrophy (Corso *et al*., 1988, 2000; Jerez & Ramírez-Pinilla, 2001; Ramírez-Pinilla, 2006; Vieira, De Perez & Ramírez-Pinilla, 2007; Visser, 1975). Transcriptomic research focused on histotrophic nutrient provisioning, placental development, and secretions of eggshell precursors in oviparous and viviparous *Mabuya* spp. would complement the literature on this genus.

(6) Discussion and future directions – embryonic nutrients, gas and water supply

Many genes for placental functions in mammals have deep origins in vertebrates (Rawn & Cross, 2008). In pairwise comparisons of different viviparous amniotes, there is overlap in hormones and proteins (SLC superfamily, insulin-like growth factors, aquaporins and solute carrier proteins, etc.) involved in uterine remodelling, placentation, and placental transport. The relationship of these observations to embryonic nutrient provisioning and the evolution of the amniotic egg requires further investigation. Table S2 illustrates how genes mentioned

above for water, gas, and nutrient transport are expressed in reproductive tissues of squamates during gravidity and gestation.

If specific genes or physiological processes impact more than one of the Main Five categories, this could have a disproportionate influence on transitions. Such an overlap has already been identified in *Zootoca vivipara*, where 11 genes are associated with both eggshell traits and gestation length (Recknagel *et al*., 2021*a*). The *SLC* gene superfamily is involved with both nutrient transport (Brandley *et al*., 2012; Whittington *et al*., 2018) and eggshell deposition (Yang *et al*., 2020). Adenogenesis is essential for histotrophic nutrient provisioning and secretion of eggshell precursors. Additionally, progesterone production influences both uterine quiescence, which is an important state to maintain in lengthened embryonic retention, and also inhibits hepatic vitellogenesis, an important process for lecithotrophic nutrient provisioning. Thus, examining the role of *SLC* gene superfamily members, processes of adenogenesis, and progesterone production during embryonic development in oviparous and viviparous squamates may reveal how interconnected the Main Five are.

V. EMBRYONIC CALCIUM PROVISIONING

The embryonic growth stage represents the greatest demand for calcium (Ecay *et al*., 2017; Packard & Packard, 1984; Stewart & Ecay, 2010). To support this, peak uterine concentrations of calcium are highest either during eggshell deposition or later in development, presumably during the embryonic growth phase, in oviparous and viviparous taxa, respectively (Linville *et al*., 2010; Stewart *et al*., 2009*a*). Regardless of parity mode, embryonic metabolism drives calcium uptake (Packard & Packard, 1984). The calcium source(s) utilized have clade-specific implications on the genomic and/or physiological changes required to transition between parity modes.

(1) Phylogenetic context of embryonic calcium sources

Calcium can be acquired by the embryo in three forms: calcium carbonate in the eggshell, calcium bound to proteins and lipids in the yolk, and/or free ionic calcium from maternal delivery through the placenta (Stewart & Ecay, 2010). These correspond with five calcium mobilization patterns: (1) birds, turtles and crocodiles predominantly depend on the eggshell; (2) most squamates, regardless of parity mode, predominantly depend on the yolk; (3) some squamate species are reliant on both the eggshell and yolk; (4) some viviparous squamate species are reliant on both the yolk and placenta; and (5) therian mammals and rare viviparous squamates predominantly depend on the placenta (Blackburn, 2015*a*; Hoenderop, Nilius & Bindels, 2005; Jenkins & Simkiss, 1968; Kovacs, 2015; Packard, 1994; Stewart *et al*., 2009*a*; Stewart, Ecay & Heulin, 2009*b*; Stewart & Ecay, 2010; Thompson *et al*., 1999, 2000; Ramírez-Pinilla, 2006).

From an evolutionary perspective, squamate eggs might serve as the best models of the ancestral amniote egg. Unlike birds, oviparous squamates generally rely on yolk calcium rather than eggshell calcium. The yolk sac of non-avian reptiles is argued to be a good model for the transition between the egg of anamniotes and amniotes (Blackburn, 2021). Taken together, and given that hard calcified eggshells of archosaurs are likely derived (as discussed in Section III.3), squamate eggs may have the closest resemblance to the ancestral amniote egg. Interestingly, to my knowledge, oviparous squamates do not sequester calcium from the eggshell into the yolk during incubation (Packard, 1994).

(2) Hypotheses on calcium mobilization and the evolution of parity modes

It was hypothesized that a predominant reliance on eggshell calcium should constrain lineages to oviparity because the evolution of viviparity would result in a lost calcium source

(hereafter the eggshell calcium constraint hypothesis) (Stewart & Ecay, 2010; Packard *et al*., 1977; Packard & Packard, 1984). This hypothesis suggested that viviparity should only evolve in lineages predominately reliant on yolk calcium (Packard *et al*., 1977; Packard & Packard, 1984). Fittingly, birds, turtles and crocodilians generally rely on eggshell calcium, and they are constrained to oviparity (Anderson, Stoyan & Ricklefs, 1987). Consistent with the eggshell calcium constraint hypothesis, most viviparous squamates rely predominantly on yolk calcium (Stewart & Castillo, 1984; Stewart & Ecay, 2010; van Dyke *et al*., 2014).

Subsequent research revealed that viviparity is not constrained by a prerequisite reliance on yolk calcium. Oviparous scincid skinks studied thus far rely on both eggshell and yolk calcium (Linville *et al*., 2010; Shadrix *et al*., 1994; Stewart *et al*., 2009*a*,*b*; Stewart & Thompson, 1993). Calcium placentotrophy contributes substantially to embryonic development in several viviparous squamates including *Pseudemoia entrecasteauxii*, *Eulamprus quoyi*, *Zootoca vivipara*, *Saiphos equalis*, and a species of *Mabuya* lizard (Ecay *et al*., 2017; Linville *et al*., 2010; Ramírez-Pinilla, 2006; Ramírez-Pinilla *et al*., 2011; Stewart & Thompson, 1993). These taxa, with the exception of *Zootoca vivipara*, are in the family Scincidae (Burbrink *et al*., 2020), which is also the family with the most independent origins of viviparity in squamates according to most estimates (Blackburn, 1982, 1985, 1999*a*; Pyron & Burbrink, 2014).

To understand the breadth of physiological conditions from which oviparity and viviparity evolve in squamates, future research should examine calcium transport in other lineages. Studies focused on snakes would be particularly informative given the sparse literature on them. *Helicops angulatus*, a reproductively bimodal water snake from South America, is an ideal model for this (Braz, Scartozzoni & Almeida-Santos, 2016). Thus far, many oviparous snakes are known to show intermediate reliance on yolk and eggshell calcium. This has not precluded viviparity from evolving in these lineages.

The presence of embryos during EER may trigger positive feedback stimuli for continued uterine calcium secretions which may support placental calcium transport, and thus incipient calcium matrotrophy (Stewart & Ecay, 2010). This is postulated to resemble the hormonal and mechanical stress mechanisms implicated in avian eggshell formation and uterine calcium secretions (Bar, 2009*a*; Stewart & Ecay, 2010). The influx of calcium late in viviparous gestation may be triggered in part by embryonic growth that over distends the uterus. This is seen in studies on myometrial stretch in mammals when uterine overdistension triggers spikes in calcium (Kao & McCullough, 1975; e.g. Wray *et al*., 2015).

Dramatic changes to activity in the chorioallantois should not be required during parity mode transitions because these homologous tissues (Metcalfe & Stock, 1993) transport calcium regardless of parity mode (Ecay, Stewart & Blackburn, 2004; Tuan & Scott, 1977; Tuan & Knowles, 1984; Tuan, Scott & Cohn, 1978; Tuan *et al*., 1986). Specialized placental structures in some viviparous squamates enhance calcium provisioning but specialization is not required for placental calcium transport (Stewart *et al*., 2009*a,b*; Stewart & Ecay, 2010; Thompson *et al*., 2000). Loss of chorioallantoic calcium transport capacity would be disadvantageous to either parity mode. Growing research reveals that, like mammals, placentotrophy and viviparity can evolve concurrently in squamates (Blackburn, 2015*a*; Ecay *et al*., 2017; Stewart & Ecay, 2010).

Placing these previously proposed models in a phylogenetic context, the calcium transport method of oviparous ancestors likely has an influence on the method of calcium transport used for viviparous taxa, i.e. matrotrophic calcium provisioning, lecithotrophic calcium provisioning, or a combination of the two. Consistent with the nucleation site hypothesis, when viviparity arises from oviparous ancestors with embryos that depended predominantly on eggshell calcium, this should favour a transition to viviparity *via* incipient calcium matrotrophy because the chorioallantois already plays the major role in transporting

calcium from the eggshell to the embryo. Since the reproductive mode and calcium provisioning of oviparous ancestors are essentially unknown, researchers can use the closest oviparous relatives as proxies. Similarly, viviparous taxa that are in close phylogenetic proximity to oviparous taxa that depend on lecithotrophic calcium provisioning should remain reliant on yolk calcium. Together, these guidelines provide a framework from which researchers can form hypotheses about the calcium provisioning method of a viviparous lineage if the calcium provisioning method of oviparous close relatives is known, or *vice versa*. Measurements of the proportional contribution of different calcium sources during development have only been reported for select taxa (e.g. Packard, 1994; Stewart, 2013; Stewart & Ecay, 2010; Stewart, Ecay & Blackburn, 2004). Once validated, the framework (i.e. the calcium provisioning method of close relatives) could help increase the speed at which science measures and infers the evolutionary history of calcium provisioning across amniotes and squamates. Collection of these data across the squamate phylogeny may enable assignment of these hypotheses to specific clades.

Embryonic calcium source could have implications on the physiological changes required to transition between parity modes. Reliance on yolk calcium should require essentially no mechanistic changes for calcium transport. On the other hand, calcium matrotrophy requires regulatory changes in the uterus, like timing of calcium secretions (Griffith *et al.*, 2015). However, regardless of parity mode (1) the uterus secretes calcium, (2) the chorioallantois transports calcium, and (3) embryonic metabolism drives uptake of calcium. Assuming maternal tissue remains responsive to embryonic metabolism, the joint evolution of matrotrophic calcium provisioning with viviparity may require little to no physiological adjustments.

The diversity of embryonic calcium provisioning patterns in viviparous squamates may not be fully explained by the eggshell calcium constraint hypothesis (Packard *et al.*, 1977;

Packard & Packard, 1984) or incipient calcium matrotrophy (Stewart & Ecay, 2010). Both hypotheses implicitly assume that viviparity equates to a lost eggshell. In one viviparous squamate, *Haldea striatula*, and in viviparous populations of two reproductively bimodal lizards, *Zootoca vivipara* and *Saiphos equalis*, the calcified eggshell is considered as a component of the placenta (Stewart, 2013). Some other viviparous squamates have transient calcified patches on their embryonic membranes (Blackburn, 1998; Heulin, 1990; Qualls, 1996) suggesting that uterine calcium-secreting capabilities in early gestation may be retained in some viviparous lineages. In the case of reversals, it remains unknown how the uterus shifts back to early calcium secretions after ovulation (Blackburn, 2015*b*; Griffith *et al*., 2015).

(3) Embryonic calcium-provisioning mechanisms

In vertebrates, specialized tissues that recover environmental calcium and transport it into blood circulation maintain conserved mechanisms for intracellular calcium transport (Bronner, 2003; Hoenderop *et al*., 2005). These include the uterus, chorioallantoic tissues, and yolk splanchnopleure (Bronner, 2003; Hoenderop *et al*., 2005; Stewart, 2013). Therefore, uterine and embryonic tissues may be pre-adapted for maternal and embryonic calcium provisioning.

In birds, a sub-compartment of the mammillary layer of the eggshell is the calcium reserve body (Chien, Hincke & McKee, 2009), which contains microcrystals of calcite that are dissolved and transported as calcium to the embryo (Chien *et al*., 2009). Calcium is eroded from the eggshell by acid released from villus cavity cells (VCCs) in the chorioallantoic membrane (Anderson, Gay & Schraer, 1981; Narbaitz, Kacew & Sitwell, 1981; Packard & Lohmiller, 2002; Simkiss, 1980). This increases the carbonic anhydrase activity of the cells enabling calcium to be released into the cavity between the eggshell and

the chorionic epithelium, where it is taken up by capillary covering cells (CCCs) in the chorioallantoic membrane (Coleman & Terepka, 1972). In some species this erosion leads to a gradual weakening of the eggshell that facilitates hatching (Chien, Hincke & McKee, 2008). In chickens, transcalcin, a calcium binding protein (CaBP), is credited for the calcium-transporting capacity of the chorioallantoic membrane (Tuan & Knowles, 1984; Tuan & Ono, 1986; Tuan & Scott, 1977; Tuan *et al*., 1978, 1986). The presence of VCCs and CCCs in the chorioallantois of viviparous squamates would indicate a known route through which calcium can be absorbed.

Transcellular calcium transport has been modelled as a three-step process involving the proteins calbindin-D9K and calbindin-D28K, and the highly calcium-specific ion channels of the transient receptor potential vanilloid gene family (TRPV5 and TRPV6) (Stewart & Ecay, 2010). Across vertebrates, this machinery is shared in epithelial tissues with significant roles in calcium transport (Hoenderop *et al*., 2005). Oestrogen and vitamin D3 have regulatory roles in this process.

Calbindin-D9K, calbindin-D28K, TRPV5, and TRPV6 are involved with calcium exchange in multiple organs of birds, squamates, and mammals. Broadly, activity of calbindin-D9K and/or calbindin-D28K is associated with patterns of calcium absorption in the mammalian kidney and uterus (Bindels, 1993; Luu *et al*., 2004), murine uterus and placenta (Lafond & Simoneau, 2006; Koo *et al*., 2012), and chicken duodenum and uterus (Bar, 2009*b*; Yang *et al*., 2013). In humans, calbindin-D9K and calbindin-D28K are critical to the active transport of Ca^{2+} across placental cells (Faulk & McIntyre, 1983; Belkacemi, Simoneau & Lafond, 2002; Belkacemi *et al*., 2004). A study on rats suggests that calbindin-D9K increases by over 100-fold in the last 7 days of gestation (Glazier *et al*., 1992), when the embryo gains the majority of calcium. TRPV6 is involved with maternal–fetal calcium transport in mice (Suzuki *et al*., 2008). Increased TRPV6 and calbindin-D28K expression

occurs during eggshell formation in chickens (Yang *et al*., 2013). Given the involvement of these genes in both eggshell deposition and embryonic calcium transport, squamates may have exploited this pathway to support transitions. Expression of these genes during gestation or gravidity in squamates has been detected (e.g. calbindin-d9K in *Saiphos equalis* and calbindin-d28k in *Zootoca vivipara*) (Foster *et al*., 2020; Recknagel *et al*., 2021*a*).

In several viviparous lizards, embryonic uptake of calcium is associated with placental expression of calbindin-D28K (Stewart *et al*., 2011; Stinnett *et al*., 2011). In both oviparous and viviparous embryos of *Zootoca vivipara*, a sharp increase in calcium uptake in late development coincides with increased calbindin-D28K and PMCA production by the chorioallantois (Stewart *et al*., 2011). In oviparous corn snakes (*Pantherophis guttatus*), expression of calbindin-D28K in the yolk sac and chorioallantoic membrane coincides with growth of these tissues and calcium transport activity (Ecay *et al*., 2004).

Viviparous embryos of *Zootoca vivipara*, a reproductively bimodal lizard, incubated *ex utero* respond to availability of calcium by increasing expression of calbindin-D28K (Ecay *et al*., 2017). In this species, embryonic recognition of environmental calcium stimulates a transcellular calcium-transporting mechanism and may also alter chorioallantoic membrane paracellular permeability to calcium (Ecay *et al*., 2017). The authors proposed that there is a calcium sensing receptor (CaSR) on chorionic epithelial cells to support this in both oviparous and viviparous *Zootoca vivipara* embryos (Ecay *et al*., 2017), similar to the CaSRs expressed by vertebrate cells involved in calcium homeostasis (Brennan *et al*., 2013).

As mentioned in Section III.2, PMCA activity is associated with eggshell deposition in birds and oviparous squamates (Bar, Rosenberg & Hurwitz, 1984; Hincke *et al*., 2012; Wasserman *et al*., 1991). PMCA is also crucial for calcium transport in late embryonic development in rats (Glazier *et al*., 1992). In the viviparous scincid lizards *Niveoscincus metallicus*, *N. ocellatus*, and *Pseudemoia spenceri*, PMCA was expressed in uterine glandular

and surface epithelia during pregnancy but only *P. spenceri* expressed it throughout gestation (Herbert *et al*., 2006). Na^{+}/Ca^{2+} exchangers (NCXs), are also important for placental calcium transport in humans (Belkacemi *et al*., 2005).

Calciotropic hormones (involved with calcium transport), and phosphotropic hormones (involved with phosphorus transport) operate *via* an interconnected pathway (Andrukhova *et al*., 2016; Biber, Hernando & Forster, 2013; Blaine, Chonchol & Levi, 2015; Erben & Andrukhova, 2015). Phospho- and calciotropic hormones are important regulators of fetal serum mineral concentrations (Kovacs, 2015). Evidence from viviparous amniotes suggests that these are suitable candidates for embryonic calcium provisioning. In mice, genes encoding parathyroid hormone (*PTH*) and PTH-related peptide (*PTHrP*) are important regulators of placental calcium transport (Kovacs *et al*., 1996; Simmonds *et al*., 2010). A non-exhaustive list of additional candidates for embryonic calcium provisioning includes fibroblast growth factor 23 (Bar, 2009*a*; Erben & Andrukhova, 2015; Stewart & Ecay, 2010), the annexin gene family (Matschke *et al*., 2006), carbonic anhydrase (Narbaitz *et al*., 1981; Tuan & Knowles, 1984), and CaBPs.

(4) Discussion and future directions – calcium provisioning and parity mode evolution

Phylogenetic frameworks enable researchers to make broader testable hypotheses about the evolutionary history of traits in specific clades. Such a framework is proposed in Section V.2 to infer ancestral parity modes in the context of calcium provisioning in amniotes. Implications gleaned from taxon-specific studies then can be explored in distantly related analogous groups.

Genes involved with calcium transport in uterine and embryonic tissues have been described across mammals, birds, and reptiles. Like other amniotes, activity of calbindin-D28K and PMCA supports embryonic calcium provisioning across diverse oviparous and

viviparous squamates. Their involvement with both eggshell deposition and embryonic calcium provisioning makes these particularly interesting candidates for parity mode evolution. The regulatory influence of other molecules in calcium transport, like PTH, PTHrP and NCXs has not been evaluated thoroughly in squamates. Additional reviews on mechanisms of embryonic calcium provisioning in squamates can be found in the literature (Stewart, 2013; Stewart & Ecay, 2010).

Additionally, I add a speculation. Perhaps lineages with incipient calcium matrotrophy could more feasibly revert to oviparity because of the continued role of the uterus in calcium provisioning. However, this hypothesis only holds if maternal provisioning of calcium is not synonymous with maternal provisioning of all nutrients.

VI. MATERNAL–FETAL IMMUNE DYNAMICS

Medawar (1953) pointed out a paradigm between the peripheral body's normal attack response to allografts (foreign tissue) and uterine tolerance to embryos. This was inspired by earlier work by Owen (1945). Stricter regulation of the maternal and fetal immune systems is expected for viviparous reproduction because of contact between uterine and embryonic tissues. Oviparity may pose less of an immunological challenge. Medawar suggested that barriers, inertness and/or immunosuppression enable pregnancy. This formed the foundation of decades of medical research on immune dynamics between maternal, embryonic, and paternal immune factors.

In recent years, there have been calls for a reappraisal of Medawar's paradigm (Chaouat, 2016; Moffett & Loke, 2004, 2006; Mor *et al.*, 2011; Stadtmauer & Wagner, 2020*b*; Yoshizawa, 2016). Moffett & Loke (2006) caution against conceptualizing embryos as analogues of allografts. To my knowledge, this perspective has yet to reach the evolutionary literature on squamate parity mode evolution (Foster *et al.*, 2020; Graham *et al.*, 2011; Gao *et*

al., 2019; Murphy & Thompson, 2011; van Dyke *et al*., 2014; Murphy *et al*., 2009; Recknagel *et al*., 2021*a*). Importantly, challenges to Medawar's paradigm do not preclude immunological responses to viviparity. They simply suggest that the immune environment of the uterus is uniquely evolved to support exposure to foreign tissue. My perspective is that Medawar's paradigm is an excellent hypothesis to describe explicitly the origin of the first amniote (e.g. lack of immune response *in utero* made possible by the evolution of the eggshell).

The uterine immune system has a distinct evolutionary history from the periphery. It enables cooperative dynamics with foreign tissues. It supports fertilization and early embryonic development. This should have started evolving, distinct from the periphery, since internal fertilization first originated. To contextualize this, I discuss the changing landscape of immunological research at the maternal–fetal interface and what it means in the context of amniote parity mode evolution. Overall, I hope readers consider how the uterus evolved to support internal gestation, and which model systems may be appropriate to investigate this.

(1) Comparing amniote immune systems

Cellular components of the innate immune system are conserved across jawed vertebrates (Uribe *et al*., 2011; Zimmerman, Vogel & Bowden, 2010). The general machinery of the adaptive immune system is ancient despite divergences and convergences across all domains of life (Ghosh *et al*., 2011; Morales *et al*., 2017; Müller *et al*., 2018; Rimer, Cohen & Friedman, 2014). Diversification of antigen receptor genes likely occurred independently in a lineage-specific fashion (Boehm *et al*., 2018). Compared to mammals, the avian immune system requires less antigen (Larsson, Carlander & Wilhelmsson, 1998). Birds also have faster but shorter antibody responses, potentially due to their higher body temperatures (Zimmerman *et al*., 2010).

Reptiles have the same general components as the mammalian immune system (Zimmerman, 2020). However, the reptilian immune system may not fit neatly into the two arms of mammalian immune systems: innate and adaptive (Zimmerman *et al.*, 2010; Zimmerman, 2020). Expanding upon this is beyond the scope of this review, but it is worth considering in future evolutionary research. Squamates may serve as a better comparative model for understanding the evolution of the uterine immune system. Active research on the peripheral reptilian immune system (Zimmerman *et al.*, 2010; Zimmerman, 2020) and uterine immune activity in squamates (Graham *et al.*, 2011; Hendrawan *et al.*, 2017; Murphy *et al.*, 2009; Paulesu *et al.*, 1995, 2008, 2005*b*) will support future insights. I refer readers to articles by Zimmerman *et al.* (2010), Zimmerman (2020), Ghorai & Priyam (2018), and a book by Williams (2012) for more information on the reptilian and avian immune systems.

(2) Medawar's paradigm

Tolerance towards the foreign fetus was postulated to occur through immunological inertness, immunosuppression or immunotolerance mechanisms (Medawar, 1953). Theoretically, immunotolerance could be established if there are relatively small quantities of alloantigens present, resulting in regulatory responses rather than activating responses. Contradicting this, the larger the alloantigen difference between the mother and embryo the bigger and healthier the placenta is in rats (Chaouat *et al.*, 2010). In humans, divergent HLA profiles between mother and embryo do not lead to detrimental immune responses (Tilburgs, Scherjon & Claas, 2010). Instead, cooperative inflammatory responses between maternal and fetal tissues support reproduction (Stadtmauer & Wagner, 2020*a*). In humans, microchimeric cell populations, i.e. the presence of cells from one individual in another genetically distinct individual, are now considered a normal expectation of pregnancy (Nelson, 2012).

In his 1991 Nobel Lecture, Medawar acknowledged that maternal and embryonic tissues have regular exposure to alloantigens (Medawar, 1991). It has become clear that the maternal immune system actively responds to fetal alloantigen rather than responding solely with ignorance or anergy (Arck & Hecher, 2013). Neither maternal immunosuppression/privilege nor embryonic inertness/immaturity fully explain immune dynamics during gestation in mammals, including those with simple epitheliochorial placentation (Chaouat *et al*., 2010; Chavan, Griffith & Wagner, 2017; Moffett & Loke, 2004, 2006; Stadtmauer & Wagner, 2020*a*).

(3) Perspectives on the evolution of the uterine immune system

Viviparous reproduction existed long before the origin of mammals and, to my knowledge, no evidence suggests there was immune conflict within these taxa (Chaouat, 2016). Placentotrophy existed as far back as the invertebrate clade Bryozoa (Ostrovsky, 2013; Schwaha *et al*., 2019), suggesting an ancient history for supportive maternal–fetal immune dynamics. Differing from Medawar's paradigm, Matzinger, who proposed the 'danger model' for the immune system (Matzinger, 2007), stated "Reproduction cannot be a danger. It does not make evolutionary sense" (Chaouat, 2016, p. 48).

In mammals, self–non-self discrimination as a framework to describe the functioning of the immune system has been challenged (Pradeu & Vitanza, 2011). Immune interactions at the maternal–fetal interface may be more nuanced (e.g. Chaouat, 2016; Moffett & Loke, 2004, 2006). The 'maternal–fetal interface' may be better conceptualized as 'maternal–fetal intra-action' given the dynamics between maternal and fetal immune systems in mammals (Yoshizawa, 2016). It is unclear if these insights apply to other viviparous amniotes.

In mammals, immune factors in the uterus and placenta appear to be specifically evolved to support maternal–fetal immune dynamics. Several cell types have unique functions and/or

phenotypes *in utero*: uNK cells, uterine macrophages, and uterine T regulatory cells (Faas & de Vos, 2017; Mold *et al*., 2008, 2010; Mold & McCune, 2011). An immunosuppressive antigen, HLA-G, is almost exclusively expressed by trophoblasts (Faulk & Temple, 1976; Kovats *et al*., 1990; Rajagopalan & Long, 2012; Rouas-Freiss *et al*., 1997). Taken from an evolutionary perspective, this suggests that the uterine immune system in viviparous mammals evolved unique responses to allogenic tissues that differ from those in the periphery. Whether the evolution of this system pre-dates mammals remains to be explored.

It is suggested that viviparous reproduction is immunologically compatible in species with a less-active adaptive immune system, like sharks (Chaouat, 2016). In these clades, innate immune cells, like uNK cells, may be sufficient to regulate immune responses during pregnancy (Moffett & Loke, 2004; Chaouat, 2016). Given that there is an unclear distinction between the innate and adaptive immune system in reptiles (Zimmerman, 2020), determining the immunological difficulty of evolving viviparity in squamates requires further investigation.

In uterine tissue of oviparous and viviparous skinks maternal antigens are expressed prior to and during pregnancy (Murphy *et al*., 2009), but the viviparous species in that study have a unique expression profile of MHC antigens which may 'hide' the embryo from the maternal immune system. Similarly, in a reproductively bimodal skink, *Saiphos equalis*, both oviparous and viviparous gravidity/gestation is associated with expression of MHC genes (Foster *et al*., 2020). Regardless of parity mode, *S. equalis* expresses genes associated with immunocompetence, including MHC genes such as *H2-EA* (Foster *et al*., 2020). The similar profile between the oviparous and viviparous state is attributed to the very long egg retention utilized by oviparous *S. equalis* (Foster *et al*., 2020). This highlights that EER is generally accompanied by immunological responses *in utero*, which is relevant to the EER model on amniote origins.

Some of these genes expressed by *S. equalis* are also expressed in viviparous *Chalcides ocellatus* during gestation, including complement component (C3, C9) genes and MHC genes (Brandley *et al*., 2012; Foster *et al*., 2020). The majority of immune genes expressed during pregnancy in *S. equalis* have immunoglobulin receptor binding functions (Foster *et al*., 2020), an important feature of eutherian pregnancy that prevents rejection of the fetus through actions of the maternal innate immune system (Alijotas-Reig, Llurba & Gris, 2014). In another reproductively bimodal skink, *Zootoca vivipara*, immune system response genes are enriched in the set of genes under divergent selection in oviparous and viviparous genomes (Recknagel *et al*., 2021*a*).

(4) Implications of the reptilian immune system and morphology on parity mode evolution

Ectothermic reptiles may inherently have a more tolerogenic uterine environment compared to mammals due to their slower antibody response. It can take up to six weeks to reach peak concentrations (Ingram & Molyneux, 1983; Grey, 1963; Marchalonis, Ealey & Diener, 1969; Pye *et al*., 2001; Origgi *et al*., 2001; Work *et al*., 2000). A slower metabolism also makes several reptiles more tolerogenic to pathogens (Ghorai & Priyam, 2018).

During pregnancy in the viviparous skink *Chalcides ocellatus*, there is a reduced response to *in vitro* exposure to the mitogens concanavalin A (Con A), phytohemagglutinin (PHA), and *Escherichia coli* lipopolysaccharide (LPS) (Saad & El Deeb, 1990). Oviparous lizards exhibit immune activation trade-offs during reproductive cycles (Cox, Peaden & Cox, 2015; Durso & French, 2018; French, Johnston & Moore, 2007; Uller, Isaksson & Olsson, 2006).

In the majority of viviparous squamates, the eggshell membrane is absorbed during pregnancy (Blackburn, 1993). In mammals, epitheliochorial placentation (the most superficial and non-invasive placenta type) is sufficient to cause immunorecognition from the

mother. In mammals, trophoblasts are antigen presenting and actively participate in maternal–fetal immune dynamics. A gene with fusogenic properties characteristic of trophoblast syncytins was identified in the *Mabuya* sp. lizard placenta (Cornelis *et al*., 2017).

A few viviparous squamates have placentas with characteristics similar to placentas found in eutherian mammals, i.e. syncytialized cell layers, specialized zones such as areolae and placentomes, or cellular invasion of maternal tissues by the fetus (Blackburn & Flemming, 2012; Jerez & Ramírez-Pinilla, 2001; Vieira *et al*., 2007). The increased contact here may require more tightly regulated immune dynamics at the maternal–fetal interface compared to other viviparous squamates.

(5) The inflammation paradox

In mammals, implantation evolved from an ancestral inflammatory attachment reaction (Griffith *et al*., 2017*b*). Inflammation is the most crucial system to support implantation, but it is also the greatest threat to the continuation of pregnancy (Chavan *et al*., 2017). This phenomenon is called the inflammation paradox. In humans, numbers of immune cells including uterine macrophages, T cells of multiple subtypes, uNK cells, dendritic cells, and natural killer T (NKT) cells increase until implantation and remain abundant in the uterus throughout the first trimester (Bulmer *et al*., 1991; Bulmer, Williams & Lash, 2010). Early implantation in humans is characterized by high numbers of pro-inflammatory T helper (Th)-1 cells and levels of cytokines (IL-6, IL-8, and TNF-α) (Yoshinaga, 2008). The exploitation of inflammatory mechanisms for eutherian implantation and the shift towards non-inflammatory activity to maintain pregnancy may have been key in enabling EER in eutherians (Griffith *et al*., 2017*b*).

How the inflammation paradox applies to viviparous squamates is unclear, given that placentation in squamates and mammals in not homologous (Griffith *et al*., 2013*b*). In

extrauterine pregnancies of mammals with non-invasive placentas, the embryo will invade extrauterine tissue because it is not inhibited by uterine secretions (Vogel, 2005; Samuel & Perry, 1972). However, in *Pseudemoia entrecasteauxii*, a viviparous skink that also has a non-invasive placenta, extrauterine pregnancy does not result in invasive implantation of extrauterine tissues (Griffith *et al*., 2013*b*). The inherent invasive nature of mammalian embryos outside of the uterus, compared to the non-invasive nature of viviparous squamate embryos studied thus far, suggests that the parent–offspring conflict and the inflammation paradox may be less pronounced in viviparous squamates compared to viviparous mammals.

(6) Inertness and barriers at the maternal-fetal interface

The uterine environment is not inert or sterile (Agostinis *et al*., 2019; Erlebacher, 2013; Moffett & Loke, 2006; Munoz-Suano, Hamilton & Betz, 2011; Murphy *et al*., 2009; Yoshimura, Okamoto & Tamura, 1997). In humans, the decidual layer of the uterus during pregnancy is comprised of ~40% leukocytes (Ander, Diamond & Coyne, 2019; Manaster & Mandelboim, 2010). This cellular subpopulation has 70% uNK cells, 10–20% antigen-presenting cells (APCs) including macrophages and dendritic cells, and 3–10% T cells of several subtypes (Abrahams *et al*., 2004; Hanna *et al*., 2006; Kämmerer *et al*., 2006; Le Bouteiller & Piccinni, 2008; Liu *et al*., 2017; Manaster & Mandelboim, 2010; Moffett-King, 2002; Moffett & Loke, 2006; Roussev *et al*., 2008). There is an abundance of decidual large granular lymphocytes (LGLs), CD3-NK cells and CD3+ activated cytotoxic T cells in the human uterus that have cytotoxic properties and produce cytokines, and these are affected by fetal MHC molecules (Rieger, 2002).

Birds also have immunocompetent cells in their oviducts. T and B cells are present in the chicken ovary where they are stimulated by oestrogen (Barua & Yoshimura, 1999; Withanage *et al*., 2003; Zettergren & Cutlan, 1992). Other immunocompetent cells in the

chicken oviduct include IgG+, IgA+ and CD3+ (Yoshimura *et al.*, 1997). Immune-competent cells located throughout the mucosal tissue of avian oviductal segments include macrophages, APCs expressing MHC class II antigens, helper T cells and cytotoxic T cells, and premature B cells (Das, Isobe & Yoshimura, 2008).

Inert barriers between maternal and fetal tissues may 'hide' the embryo. In oviparous taxa, the eggshell may serve as a barrier. However, the antimicrobial properties of the eggshell matrix in birds demonstrate that even the eggshell is not inert. The FAS (FS-7 associated) ligand, also called APO-1 or CD95, is a type II membrane protein belonging to the TNF superfamily that was proposed to serve as a barrier in humans and rodent embryonic tissue because it causes apoptosis of surrounding maternal immune cells (Kayisli *et al.*, 2003; Makrigiannakis *et al.*, 2008).

Medawar (1991) suggested that an impermeable placenta strictly regulates molecular exchanges, preventing rejection of the embryo. Synctiotrophoblasts lack cellular junctions and thus were postulated to serve as this barrier (Ander *et al.*, 2019). However, the growing data on bidirectional cellular traffic of APCs, even in mammals with non-invasive placentas, rejected this hypothesis (Bakkour *et al.*, 2014; Burlingham & Bracamonte-Baran, 2015; Fujiki *et al.*, 2008; Turin *et al.*, 2007).

(7) T cell populations and mammalian viviparity

In mammals, immune dynamics at the maternal–fetal interface are established through innate and adaptive immune responses. There is a delicate balance between ratios of T helper type 1 (Th1), Th2, Th17, Treg (regulatory T cells) and memory T cells at the maternal–fetal interface in eutherian mammals during gestation (Chaouat *et al.*, 1997; Kieffer *et al.*, 2019; Peck & Mellins, 2010; Saito *et al.*, 2010; Wu *et al.*, 2014). A shift *in utero* from Th1 cells to Th2 cells during gestation in mammals equates to a shift from pro-inflammation to anti-

inflammation. The galectin proteins GAL-13 and GAL-14 expressed by syncytiotrophoblasts, bind to T cells where they inhibit activation, induce apoptosis, and enhance IL-8 production (Balogh *et al*., 2019).

Growing research is revealing the central role of Tregs at the maternal–fetal interface during pregnancy in mammals (Teles *et al*., 2013; Wienke *et al*., 2020). Tregs play a central role in immunosuppression in mammals (Attias, Al-Aubodah & Piccirillo, 2019). Differentiation of Tregs is governed by the transcription factor forkhead box P3 (FOXP3) (Ramsdell & Rudensky, 2020). Alloantigen-dependent, uterine T cell signalling, and immunocompetent embryonic cells and their products facilitate overall enhanced regulatory phenotypes of immune cells (Ander *et al*., 2019).

The T-cell-dependent adaptive immune system of mammals is unique. This may have prompted their intricate balance of Treg mediators of immunotolerance at the maternal–fetal interface (Chaouat, 2016). Birds rely more heavily on B cells. In non-avian reptiles, T helper cells are functional, but the presence and function of other T cell subsets is unclear (Zimmerman, 2020; Zimmerman *et al*., 2010). The potential role of T cells and Tregs in viviparous squamate gestation should not be discounted. Treg-like cells have been identified in a pufferfish, *Tetraodon nigroviridis* (Wen *et al*., 2011), suggesting that Tregs may have an ancient evolutionary history.

(8) Progesterone, cytokines, and maternal-fetal immune dynamics

In addition to the role of progesterone in uterine quiescence (embryonic retention) and hepatic vitellogenesis (nutrient provisioning), it also plays a role in maternal–fetal immune dynamics. In the uterus of pregnant mammals, progesterone concentrations are associated with altered B cell immunoglobin secretion, inhibition of NK-cell-mediated cytotoxicity and the shift from Th1- (pro-inflammatory) to Th2- (anti-inflammatory) dominated immune

responses (Druckmann & Druckmann, 2005). Progesterone is also associated with immunomodulatory effects (Ortega Brown *et al*., 1990). During gestation in *Agkistrodon piscivorus*, a viviparous pit viper, progesterone concentrations are associated with decreased complement performance (Graham *et al*., 2011), a portion of the immune system that promotes inflammation, among other immune functions.

In humans, progesterone-induced protein (PIBF) is transported by placental extravillous trophoblasts to maternal lymphocytes causing the induction of IL-10 production, and thereby contributing to Th2-dominant responses (Szekeres-Bartho, Šućurović & Mulac-Jeričević, 2018). IL-10 is a potent anti-inflammatory cytokine that is produced by multiple cell types (Zimmerman, Bowden & Vogel, 2014). It is associated with the Th2 response, and inhibits Th1 responses. The phenotype of uterine macrophages is affected by trophoblasts when they secrete IL-10 and macrophage colony-stimulating factor (M-CSF) (Svensson-Arvelund *et al*., 2021). IL-10 inhibits IFN-γ and increases in response to infection in chickens (Giansanti, Giardi & Botti, 2006; Rothwell *et al*., 2004). In the uterus of the oviparous skink *Lampropholis guichenoti* during gravidity and non-gravidity, IL-10 is expressed (Griffith *et al*., 2016).

Proinflammatory cytokines may be downregulated during reproductive periods to limit maladaptive immune responses to the foreign fetus (Zimmerman *et al*., 2010). In mammals, IL-1 allows release of hormones in human trophoblasts (Petraglia *et al*., 1990; Masuhiro *et al*., 1990; Yagel *et al*., 1989), facilitates implantation (Haimovici, Hill & Anderson, 1991; Hill, 1992; Tartakovsky & Ben-Yair, 1991), and influences the initiation of labour (Romero *et al*., 1989, 1992). Regulation of the proinflammatory cytokines TNF and IL-1β is of particular importance in eutherian pregnancy (Haider & Knöfler, 2009; Paulesu *et al*., 2005*b*; Saito *et al*., 2010; Tayade *et al*., 2006).

The uterine tissue of two reproductively bimodal squamates – viviparous individuals of *Chalcides chalcides* and oviparous and viviparous individuals of *Zootoca vivipara* – express IL-1β (Paulesu *et al*., 1995, 2005*a*; Romagnoli *et al*., 2003). In the uterus of the viviparous skink *Pseudemoia entrecasteauxii*, regulation during gestation of TNF and IL-1β at the transcriptional and post-translation levels, respectively, may reduce inflammation (Hendrawan *et al*., 2017). The pro-inflammatory function of IL-1β in *Pseudemoia entrecasteauxii* may play a role in the development of a more complex placenta (Hendrawan *et al*., 2017). The placenta of *Chalcides chalcides* expresses pro-inflammatory cytokines, IL-1α and IL-1β, at specific times during gestation (Paulesu *et al*., 1995). During gestation, *Chalcides ocellatus* also differentially expresses 27 other interleukins and interleukin-related products (Brandley *et al*., 2012).

The expression of IL-34 in a marsupial the fat-tailed dunnart *Sminthopsis crassicaudata*, during pre-implantation (Whittington *et al*., 2018) may have an immunosuppressive function to help tolerate potential contact of maternal and fetal tissues when the embryonic shell coat disintegrates (Lindau *et al*., 2015). In chickens, IL-34 regulates Th1 and Th17 cytokine production (Truong *et al*., 2018). During gestation in *Pseudemoia entrecasteauxii,* IL-16 and IL-1α are expressed in addition to three receptors for Th17 family cytokines: IL-17RA, IL-17RC, and IL-17RA (Griffith *et al*., 2016). In the yolk sac of *Pseudemoia entrecasteauxii* during pregnancy the interleukin-related genes *ILDR1*, *IRAK1* and *SIGIRR*, are differentially expressed (Griffith *et al*., 2016). This profile suggests the presence of tricellular tight junctions and/or tricellulin (Higashi *et al*., 2013; Ikenouchi *et al*., 2005), and regulation of Toll-like receptors (TLRs) and/or IL-1R signalling (Kawagoe *et al*., 2008; Lin, Lo & Wu, 2010; Muzio *et al*., 1997).

(9) The major histocompatibility complex and maternal–fetal immune dynamics

A substantial amount of literature on maternal–fetal immune dynamics has focused on uNK cells. These cells have a distinct phenotype and function from peripheral NK cells. They have several activating receptors (Manaster & Mandelboim, 2010) but do not exert cytolytic functions on embryonic trophoblasts that they are in contact with (King, Birkby & Loke, 1989). Allorecognition of embryonic placental cells by uNK cells is a key regulator of the maternal–fetal immune mechanisms that support placentation in mammals (Moffett & Colucci, 2014). When cells lose their ability to express any HLAs, uNK cells kill them (Hunt *et al.*, 2005; Ishitani *et al.*, 2003; King *et al.*, 2000*a*).

In humans, expression of the C-MHCI molecule HLA-C, and non-classical MHC class I (NC-MHCI) molecules HLA-E, HLA-F and HLA-G on trophoblasts inhibit uNK-cell-mediated cytotoxicity (Hunt *et al.*, 2003; King *et al.*, 2000*b*). Differing from this, antigenic mismatches of HLA-C can lead to graft-versus-host diseases in organ transplantation (e.g. Petersdorf *et al.*, 2014). Selection for balanced polymorphisms in HLA-C alleles and their killer immunoglobin receptors (KIRs) is proposed to be driven by reproductive success, rather than immune recognition of pathogens (Trowsdale & Betz, 2006). Dimorphisms of HLA-C emerged recently within primates (Adams & Parham, 2001).

Similar patterns in MHC profiles have been explored in other viviparous amniotes. The C-MHCI antigen H2-K is expressed on giant trophoblast cells of mice, and this is attributed to trophoblast-induced uterine vasculature transformation (Arcellana-Panlilio & Schultz, 1994; ChatterJee-Hasrouni & Lala, 1982; Hedley *et al.*, 1989; King *et al.*, 1987; Sellens, Jenkinson & Billington, 1978). H2-D antigen is co-expressed with H2-K in virtually all their other nucleated cells (Madeja *et al.*, 2011). However, H2-K-expressing trophoblasts lack H2-D expression. This parallels the expression patterns of C-MHC molecules at the maternal–fetal interface in humans and may be an evolutionarily conserved pattern (Madeja *et al.*, 2011).

In humans, the NC-MHCI molecule HLA-G is especially tolerogenic (Carosella *et al.*, 2015; González *et al.*, 2012; Hviid *et al.*, 2004; Kovats *et al.*, 1990). HLA-G is almost exclusively expressed by fetal trophoblasts compared to adult cells (Faulk & Temple, 1976; King *et al.*, 2000*a*; Kovats *et al.*, 1990; Rajagopalan & Long, 2012; Rouas-Freiss *et al.*, 1997). It supports immunotolerance at the maternal–fetal interface (Rebmann *et al.*, 2014). The role of HLA-G in supporting tolerogenic responses to organ transplants appears to be an exploitation of its role in immunotolerance *in utero* during pregnancy (Rebmann *et al.*, 2014). HLA-G is upregulated by several molecules that serve essential roles during gestation including progesterone (Yie, Xiao & Librach, 2006*b*; Yie *et al.*, 2006*a*), IFN-α, IFN-β, and IFN-γ (Rebmann *et al.*, 2003; Lefebvre *et al.*, 2001; Ugurel *et al.*, 2001; Yang, Geraghty & Hunt, 1995), and IL-10 and TGF-β (Cadet *et al.*, 1995; Moreau *et al.*, 1999).

A similar NC-MHCI gene to HLA-G exists in horses (Davies *et al.*, 2006) where it likely functions to protect the embryo from NK-cell-mediated attack (Ott *et al.*, 2014). Non-homologous NC-MHC molecules with similar properties to HLA-G are also found in rhesus monkeys (*Macaca mulatta*) (Boyson *et al.*, 1997) and baboons (*Papio anubis*) (Langat *et al.*, 2004; Stern *et al.*, 1987). Mice have two NC-MHCI genes that are expressed on the surface of their placentas and on pre-implanted embryos (Sipes *et al.*, 1996).

In the gestating uterus of the viviparous skink *Pseudemoia entrecasteauxii*, four putative C-MHCI and two putative NC-MHCI molecules are expressed (Murphy *et al.*, 2009). This pattern resembles the C-MHCI and NC-MHCI expression profiles of mammals, suggesting that this viviparous skink utilizes a similar physiological mechanism to ‘hide’ the embryo (Murphy *et al.*, 2009). One of the putative NC-MHCI genes (*Psen-160Ut*/*Psen-78G*) has a substitution at position 150 where a tryptophan is substituted for a leucine (Murphy *et al.*, 2009). Tryptophan is conserved at position 150 in all NC-MHCI genes of vertebrates ranging from fish to eutherian mammals, except in *Psen-160Ut*/*Psen-78G* and *HLA-G* (Murphy *et al.*,

2009). Whether this substitution reflects an evolutionary history associated with immune tolerance at the maternal–fetal interface in *Pseudemoia entrecasteauxii* requires further investigation.

MHCI genes are also expressed in reproductive tissues of the oviparous skinks *Ctenotus taeniolatus* and *Lampropholis guichenoti* during non-reproductive periods and during late gravidity (Murphy *et al*., 2009). A similar pattern is found in the viviparous skinks *Eulamprus tympanum*, *Niveoscincus metallicus*, and *Pseudemoia entrecasteauxii* and in the reproductively bimodal skink *Saiphos equalis*, which all express MHCI genes during non-reproductive periods and in late pregnancy (Murphy *et al*., 2009). MHC gene *H2-EA* is also expressed during long egg retention in oviparous *Saiphos equalis*.

The butyrophilin subfamily 1 member A (*BTN1A1*) is located in the MHCI region of the genome in mammals (Trowsdale, 2011). *BTN1A1* is differentially expressed in the uterus during gestation in a viviparous lizard, *Chalcides ocellatus* (Brandley *et al*., 2012). *BTN1A1* may have important antimicrobial properties in chicken eggshells (Mann *et al*., 2006). In mammals *BTN1A1* is the major protein associated with fat droplets in milk (Jeong *et al*., 2009).

(10) Microchimerism and maternal–fetal immune dynamics

Billingham, Brent & Medawar (1953) first suggested the concept of actively acquired immunologic tolerance during pregnancy 70 years ago (Ribatti, 2015). Subsequent research over the following decades revealed that substantial transfer of proteins, parasites and even immunologically active cells occurs between mother and embryo, at least in mammals (Adams & Nelson, 2004; Axiak-Bechtel *et al*., 2013; Bakkour *et al*., 2014; Burlingham, 2010; Fujiki *et al*., 2008; Gitlin *et al*., 1965; Khosrotehrani *et al*., 2005; Owen, 1945; Turin *et al*., 2007).

Microchimerism, where there is <0.1% donor chimeras in host tissue, is relatively pervasive among eutherians during pregnancy. It plays a role in establishing tolerance to non-inherited antigens. For example, cell populations from the mother that are transferred into embryonic lymph nodes enable the establishment of embryonic Tregs that are tolerogenic towards non-inherited maternal antigens (Mold *et al.*, 2008).

Microchimeric cellular populations are transferred across all placental types in mammals (Axiak-Bechtel *et al.*, 2013; Bakkour *et al.*, 2014; Fujiki *et al.*, 2008; Khosrotehrani *et al.*, 2005; Turin *et al.*, 2007). Fetal and maternal cells persist for decades after birth across a range of tissues in mother and offspring, respectively (Adams & Nelson, 2004; Bakkour *et al.*, 2014; Bayes-Genis *et al.*, 2005; Bianchi *et al.*, 1996; Evans *et al.*, 1999; Jonsson *et al.*, 2008; Stevens *et al.*, 2004). There is even a call in the immunology literature to shift from the conventional paradigm of 'self *versus* other' to instead consider the 'self' as inherently chimeric (Nelson, 2012). Given that epitheliochorial placentation is sufficient to elicit microchimeric cell populations, the occurrence of similar bidirectional cellular traffic is a reasonable possibility in viviparous squamates.

(11) Paternal alloantigens

Under tenets gleaned from transplant medicine, the maternal immune system should mount a response as early as insemination when maternal tissues are exposed to and aware of paternal alloantigens (Borziak *et al.*, 2016; Schumacher & Zenclussen, 2015; Seavey & Mosmann, 2006). Instead, the dynamic is more complex. In mammals, paternal alloantigens and cytokines in seminal fluid drive immune tolerance ultimately (Schjenken & Robertson, 2014). Treg expansion, a process with major influence on maternal–fetal immunotolerance in mammals, is proposed to be driven by several different factors found in seminal plasma (Baratelli *et al.*, 2005; Teles *et al.*, 2013). Mothers may maintain fetal-specific Tregs *via*

memory of the paternal alloantigens (Zenclussen *et al.*, 2010), expediting Treg responses in future pregnancies with the same father (Rowe *et al.*, 2012).

Alloantigen exposure at the time of insemination is not restricted to mammals. Seminal fluid of chickens contains two MHCI paternal alloantigens and one MHCII alloantigen (Borziak *et al.*, 2016). It also contains proteins involved in immunity and antimicrobial defences (Borziak *et al.*, 2016). In hens, evidence suggests that a protective local immunity to pathogens is established after exposure to semen but the mechanisms for this remain unclear (Reiber & Conner, 1995; Reiber, Conner & Bilgili, 1995).

Immune properties of mammalian seminal plasma play a role in fertility. Immune factors detected in human seminal plasma include over 50 detected cytokines (Lyons *et al.*, 2023), PGE2 and 19-hydroxyprostaglandin E (19-hydroxy PGE) (Denison *et al.*, 1999*b*), soluble TNF receptors (Liabakk *et al.*, 1993), receptors for the Fc portion of γ-globulin, spermine (Evans, Lee & Flugelman, 1995), and complement inhibitors (Kelly, 1995). In horses and pigs, respectively, the protein cysteine rich secretory protein 3 (CRISP3) prevents spermatozoa from binding to polymorphonuclear neutrophils (PMNs) (Doty *et al.*, 2011, 2024), and the porcine sperm adhesions (PSP)-I/PSP-II heterodimer triggers recruitment of PMNs to the uterus (Rodriguez-Martinez *et al.*, 2010).

Secretion of growth factors, cytokines and chemokines from cervical and endometrial tissues immediately following insemination generates a proinflammatory environment that likely aids in implantation. In the utero-vaginal junction of chickens and the utero-tubal junction of pigs, expression of several genes was shared following mating compared to non-mating and these genes were involved with immune modulation (*IFIT5*, *IFI16*, *MMP27*, *ADAMTS3*, *MMP3*, *MMP12*) and pH regulation (*SLC16A2*, *SLC4A9*, *SLC13A1*, *SLC35F1*, *ATP8B3*, *ATP13A3*), a process essential for implantation (Atikuzzaman *et al.*, 2017, 2015). Instead of mounting an attack, it appears that the uterine immune system and paternal genes

work cooperatively to support pregnancy in mammals and gravidity in birds. Whether this applies to reptiles, and how it may influence immune dynamics involved with squamate parity mode evolution, deserves investigation.

(12) Discussion and future directions – maternal–fetal immune dynamics and the evolution of parity modes

Immune processes appear to be important for both oviparity and viviparity, as evidenced here, in part, by overlapping expression profiles of immune genes in female reproductive tissues of chickens and pigs, expression of paternal antigens in avian seminal fluid, and uterine expression of maternal antigens in oviparous and viviparous skinks. This highlights the scientific advances made since Medawar's paradigm, when embryos were treated as analogues to allografts. Viviparity is associated with complex immune dynamics between maternal, fetal, and paternal tissues. But are there any species that do not exhibit an immune response to fertilization, reminiscent of Medawar's paradigm? Oviparous *Lampropholis guichenoti* and *Lerista bougainvillii* differentially express remarkably few genes during gravidity, suggesting that they lack an immune response (Foster *et al*., 2022; Griffith *et al*., 2016). While the expectations of Medawar's paradigm are met by exceedingly few amniotes, it may still be an appropriate framework when applied to the origin of amniotes. *L. guichenoti* and *L. bougainvillii* may represent the most suitable models for the original amniotes (given this, and other evidence presented throughout the review justifying squamates as, broadly, the best model).

Overall, evolving appropriate immunological responses is one hurdle for transitions to viviparity in squamates. This is evidenced by the unique MHC expression profiles identified in some viviparous skinks compared to oviparous relatives (Murphy *et al*., 2009); and the detection of divergent selection in immune response genes in viviparous *versus* oviparous

Zootoca vivipara (Recknagel *et al*., 2021*a*). Labile parity modes in squamates may be supported if they are more heavily reliant on the innate immune system for reproduction. Testing this is difficult given that reptiles may not have separate innate and adaptive immune systems (Zimmerman *et al*., 2010; Zimmerman, 2020).

Changes to genes that serve overlapping functions across the Main Five may have a disproportionate influence on transitions between parity modes. I reviewed two molecules, TGF-β and progesterone, that exert influences on multiple Main Five categories. Progesterone influences uterine quiescence (embryonic retention), hepatic vitellogenesis (nutrient provisioning) and regulation of inflammatory responses *in utero* (maternal–fetal immune dynamics). Genes in the TGF-β family play a role in placental development and maternal–fetal immune dynamics and are implicated in placental development in eutherians (Hempstock *et al*., 2004; Caniggia *et al*., 2000; Lafontaine *et al*., 2011). A TGF-β receptor protein (TGFBR1) was associated with placental development in *Phrynocephalus vlangalii* (Gao *et al*., 2019). In humans TGF-β upregulates tolerogenic HLA-G *in utero* and is an immune factor in mammalian seminal fluid. Multiple genes in the TGF-β family are also differentially expressed during gestation in the viviparous lizards *Pseudemoia entrecasteauxii* and *Saiphos equalis* (Foster *et al*., 2020; Griffith *et al*., 2016). Examining the functions of TGF-β and progesterone across other amniotes may reveal insights into how these molecules influence the evolution of parity modes.

In mammals, inflammation appears to be involved with two of the Main Five processes: regulation of maternal–fetal immune dynamics and embryonic retention. It is intriguing to consider the implications of this for the interconnectedness of the Main Five. Greater interconnectedness would suggest that changes to a few genes involved with the Main Five could cause cascading effects to support more labile transitions between parity modes.

Implantation and parturition in therian mammals evolved from a shared inflammatory attachment reaction (Hansen *et al*., 2017). The process of implantation has important implications for maternal–fetal exchanges of inorganic and organic material and maternal–fetal immune dynamics. The association of inflammation with implantation and parturition implicates it in gas, water, and nutrient (including calcium) provisioning, maternal–fetal immune dynamics and length of embryonic retention. However, implantation in mammals and viviparous squamates is not homologous (Griffith *et al*., 2013*b*). Therefore, it is difficult to make inferences about how substantial the influence of inflammation is on the evolution of parity modes in squamates. Nonetheless, the abundant literature on uterine inflammatory processes during human pregnancy and the evolution of inflammatory processes that supported the evolution of viviparity in mammals (Challis *et al*., 2009; Chavan *et al*., 2017; Mor *et al*., 2011; Griffith *et al*., 2017*b*; Stadtmauer & Wagner, 2020*a*) serve as indispensable resources for exploring the role of inflammation in squamate viviparity. I suspect that the immune system plays a central role in dictating the degree of lability of parity modes, however, further work is necessary.

VII. CONCLUSIONS

(1) Through holistic consideration of the unique complexity of parity mode evolution, within the context of genomic and transcriptomic studies across interdisciplinary fields, this review provides a new perspective on the history of parity mode transitions in amniotes and squamates. The overlapping activity of immune genes *in utero*, and of genes for calcium transport, placentation, and hormonal regulation across mammals, birds, and reptiles hint at discoveries to be made. There is a fascinating history to the evolutionary physiology and genomics of reproduction in amniotes that is ripe for further research.

(2) Changes to gene(s) or physiological processes associated with more than one of the Main Five should disproportionately influence parity mode evolution. Possible examples include the *SLC* gene superfamily, TGF-β, *BMPR1B*, progesterone, *PMCA*, calbindin-D28K, *SPP1*, sustained functioning of the corpora lutea and inflammation, and the genes associated with both gestation length and eggshell traits in *Zootoca vivipara* (Recknagel *et al*., 2021*a*).

(3) The medical and agriculture literature exemplify how interactions of immune systems at the maternal–fetal interface is not known to occur simply through immunotolerance, evasion, immunosuppression, or immunological barriers (Chaouat, 2016; Chavan *et al*., 2017; Moffett & Loke, 2004, 2006). Instead, maternal–fetal immune dynamics have a deep evolutionary history that enables both embryo and mother to interact cooperatively (Yoshizawa, 2016). Even oviparous birds and squamates are known to have immunological activity *in utero* during gravidity and differentially express an abundance of genes, with two exceptions to my knowledge, *Lampropholis guichenoti* and *Lerista bougainvillii* (Foster *et al.*, 2022; Griffith *et al*., 2016). Although Medawar's paradigm was originally created to explain viviparous gestation, the absence of uterine immunological responses to oviparous gravidity in these species suggests that the eggshell serves as adequate barrier to prevent the maternal immune system from negatively reacting to the developing embryo. Therefore, the role of the eggshell as an immunological barrier may explain why it originally evolved. *L. guichenoti* and *L. bougainvillii* may therefore serve as good models of the first amniote egg.

(4) Compared to viviparous endothermic amniotes, ectothermy likely influences parity mode evolution differently because it entails slower antibody responses and a greater reliance on climatic conditions for embryonic development. This and the cold climate hypothesis may be relevant to squamate parity mode evolution and the origin of the amniote egg.

(5) Synthesizing the EER model with the traditional paradigm, I offer the following list of evolutionary events that may have supported the origin of the amniote egg: (*a*) the ancestral

state of internal fertilization originated (Starck *et al.*, 2021); (*b*) the ancestral state of delayed egg deposition originated (Starck *et al.*, 2021); (*c*) uterine secreted coats originated (Menkhorst & Selwood, 2008) which ultimately led to the origin of the eggshell; (*d*) the amnion evolved as a water resource during delayed egg deposition and thus served as a pre-adaptation to land (Ferner & Mess, 2011); (*e*) over evolutionary time, the chorion and allantois originated *in utero* as immature organs that improved gas exchange and waste allocation, respectively, during EER; (*f*) additionally, or alternatively, the chorioallantois may have originated to support delayed egg deposition in amniotes given the endocrine function of the chorioallantois is likely ancestral (Griffith *et al.*, 2017*a*) and progesterone extends the length of embryonic retention; (*g*) the egg arrived on shore; (*h*) the chorioallantois and eggshell became specialized to the terrestrial environment; and (*i*) finally, the amniote egg and its developmental trajectory resembled what is seen today. I anticipate continued scientific engagement will lead to improved synthesis of the traditional paradigm and EER model.

(6) Two new mechanisms for transitions between oviparity and viviparity, without necessitating intermediate stages, stand out from the cumulative research on the Main Five. These are presented below (points 7 and 8) as tools to be broadened and challenged with the goal of advancing scientific insight.

(7) The genomics and physiology of amniote parity mode evolution does not preclude an origin of viviparity in the most recent common ancestor (MRCA) of lepidosaurs. I propose the following possible mechanism: a change to the phenotype or function of mammillary knobs occurred in the MRCA of lepidosaurs, preventing calcium carbonate deposition (nucleation site hypothesis); the resulting eggshell loss enabled uterine exposure to chorioallantoic progesterone production (extending embryonic retention) and incipient calcium matrotrophy (supporting embryonic development); parturition occurred *via* (*a*)

placental progesterone withdrawal or (*b*) overdistension of the uterus triggering contractions. To test this hypothesis, research could investigate whether the genes that code for the KS-proteoglycan 'mammillan', which makes up mammillary knobs, are absent or non-functional across amniotes (see Section III.3). First, the genes that code for 'mammillan' must be identified in avian genomes. Additionally, ancestral state reconstructions on the eggshell and eggshell membrane should be generated across oviparous and viviparous amniotes, utilizing current recommendations for characterizing eggshell microstructure (Legendre *et al*., 2022). This will require the development of a system to characterize eggshell membranes accurately.

(8) The calcium-secreting capacity of the uterus is maintained in oviparous and viviparous squamates. Therefore, a reversal back to oviparity may evolve through the following sequence of events: calcium secretions *in utero* adhere to the eggshell membrane instead of being absorbed by the chorioallantois; oviposition can then occur early in embryonic development in one of two ways (*a*) the death of corpora lutea or (*b*) the calcified eggshell blocks a threshold of chorioallantoic progesterone production from reaching uterine tissue; the calcified eggshell then provides embryonic calcium that is transported upon embryonic metabolic demand. To test this hypothesis, consider that recent reversals should have physiological or genomic remnants of a viviparous past. Given that viviparous squamates have more active uterine immune systems to support gestation, oviparous reversals should (*a*) have more immune genes expressed *in utero* than ancestrally oviparous squamates, and (*b*) these immune genes should have stronger signatures of relaxed selection than immune genes expressed in a close relative during viviparous gestation.

(9) Given the above, the substantial number of genes that are differentially expressed during gravidity in oviparous populations of *Saiphos equalis* and *Zootoca vivipara* (Foster *et al*., 2020; Recknagel *et al*., 2021*a*) is consistent with reversals back to oviparity. The absence of substantial differential gene expression in oviparous *Lerista bougainvillii* (a reproductively

bimodal species) and oviparous *Lampropholis guichenoti* (Foster et al., 2022; Griffith *et al.*, 2016) is consistent with ancestral oviparity.

(10) While I am agnostic about whether the first amniote was oviparous or viviparous, it makes logical sense that the early embryo was first ensheathed in an eggshell membrane and later accumulated calcium deposits like those observed in squamates today. I hope this review is evidence that interdisciplinary work has the power to influence deep questions in evolutionary biology. Sometimes looking at the same thing from a different perspective can shape scientific understanding in profound ways.

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X. SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

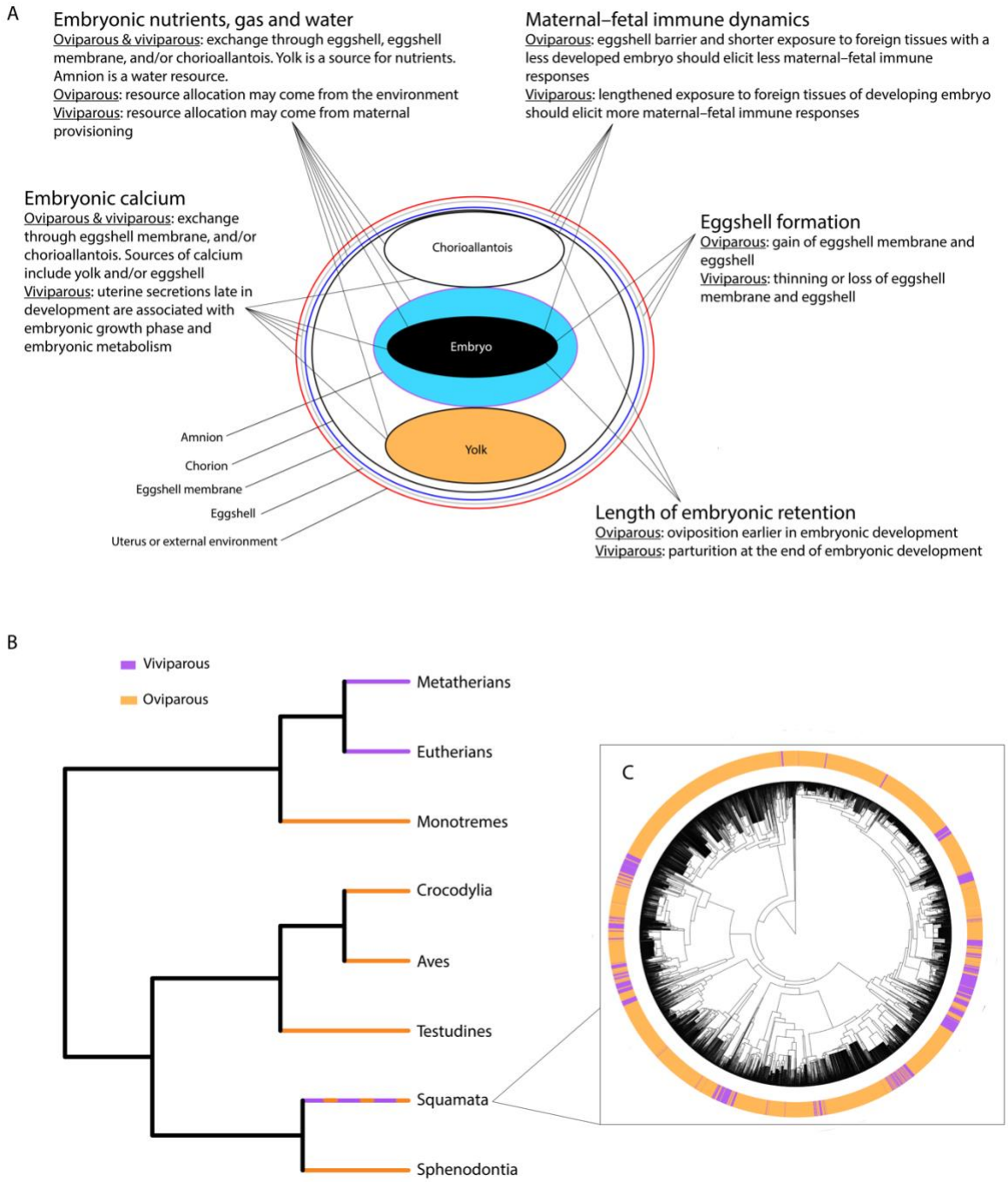
Table S1. Genes associated with eggshell deposition.

Table S2. Differential expression of genes associated with water, gas, and nutrient transport during gravidity and gestation in squamates.

4128 Table 1. Amniote eggshell ultrastructures.

Taxon	**Eggshell ultrastructure**
Testudoid	Radial aragonite with organic core at base
Crocodiloid	Tabular, arranged in wedges of calcite with no organic core
Squamate	Two types: • rigid-shelled eggs with well-developed crystalline layer in dibamid and gekkonid lizards; stem-like crystals grow downwards making a rigid shell • flexible-shelled eggs with parchment-like shell of fibrils overlaid with thin crystal caps or no crystalline material (other squamates)
Ornithoid (avian)	Calcite with a clear boundary between lower and upper parts; mammillary layer defines the lower portion of the shell, with calcite crystals that radiate upwards
Monotreme	Distensible, permeable and highly proteinaceous

4129 Adapted from Choi *et al*. (2018); Frankenberg & Renfree (2018); Hallmann & Griebeler (2015); Hincke *et al*.
4130 (2012); Trauth & Fagerberg (1984).
4131



4132

4133 **Fig. 1.** (A) Schematic demonstrating the anticipated processes that change during transitions
4134 between oviparity and viviparity, and the organs associated with those changes. Lines from
4135 the process to features of the egg indicate those putatively involved with evolutionary shifts
4136 between parity modes. (B) Relationships between major amniote clades and their associated
4137 reproductive mode. (C) Variation in reproductive modes across squamates. The squamate
4138 phylogeny and reproductive modes is adapted from Pyron & Burbrink (2014).