The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes

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Abstract

Squamate reptiles (lizards and snakes) are an ideal model system for testing hypotheses regarding the evolution of viviparity (live birth) in amniote vertebrates. Viviparity has evolved over 100 times in squamates, resulting in major changes in reproductive physiology. At a minimum, all viviparous squamates exhibit placentae formed by the appositions of maternal and embryonic tissues, which are homologous in origin with the tissues that form the placenta in therian mammals. These placentae facilitate adhesion of the conceptus to the uterus as well as exchange of oxygen, carbon dioxide, water, sodium, and calcium. However, most viviparous squamates continue to rely on yolk for nearly all of their organic nutrition. In contrast, some species, which rely on the placenta for at least a portion of organic nutrition, exhibit complex placental specializations associated with the transport of amino acids and fatty acids. Some viviparous squamates also exhibit reduced immunocompetence during pregnancy, which could be the result of immunosuppression to protect developing embryos. Recent molecular studies using both candidate-gene and next-generation sequencing approaches have suggested that at least some of the genes and gene families underlying these phenomena play similar roles in the uterus and placenta of viviparous mammals and squamates. Therefore, studies of the evolution of viviparity in squamates should inform hypotheses of the evolution of viviparity in all amniotes, including mammals.

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Introduction

Reproduction is perhaps the single most important biological function as it is the means by which organisms transmit genes to the next generation. Consequently, modifications of reproductive mode will directly impact fitness and are subject to intense natural selection pressure. Transitions in reproductive mode, between oviparity (egg laying) and viviparity (live birth), require dramatic changes to reproductive morphology and physiology, and are among the most fundamental transitions in vertebrate evolution.

Although viviparity has evolved independently in multiple lineages of vertebrates (Anderson *et al.* 1972, Frick 1998, Farley 1999), chondrichthyan fish (Wourms 1977), actinopterygian fish (Wourms 1981), mammals, and squamate reptiles (e.g. lizards and snakes; Blackburn 1982), the majority of the morphological, physiological, and evolutionary studies of viviparous animals has focused on mammals. While mammals represent an excellent model for understanding the evolution of placental complexity, they are a poor model to study the transition from oviparity to viviparity, because it likely occurred only once in the ancestor to

therian mammals (Lillegraven 1979). Moreover, the transition to mammalian viviparity occurred 191–124 million years ago (the estimated time period between the maximum age of crown Mammalia and minimum age of crown Theria; dos Reis *et al.* 2012). Therefore, it is unlikely that extant mammals retain identifiable ancestral morphological or genetic signatures of the early stages of this transition because they have been modified over 100 million years of evolution.

On the other hand, the transition to viviparity has occurred independently over 100 times in squamates (Blackburn 1985, 1992, 2000b, 2006). Compared with other taxa, oviparous squamates may even be considered ‘preadapted’ for viviparity because most species retain eggs for at least the first third of gestation (Blackburn 2006, Blackburn & Stewart 2011). Within Squamata, viviparity has evolved recently in a number of clades, including at least three lizard species that possess both oviparous and viviparous populations (*Zootoca* (*Lacerta*) *vivipara* (Heulin 1990), *Lerista bougainvillii* (Heulin 1990, Smith & Shine 1997, Qualls & Shine 1998), and *Saiphos equalis* (Smith & Shine 1997)). These three species thus represent particularly excellent

models for studying the general mechanisms by which the transition to viviparity evolved in amniote vertebrates.

Squamates are amniote vertebrates and thus possess the same extraembryonic membranes as mammals, including the amnion, allantois, chorion, and yolk sac (Blackburn 1992, 2000b, 2006). As in viviparous mammals, these membranes interact with adjacent uterine tissues to develop placentae responsible for adhesion to the uterus, respiratory gas exchange, water transport, and nutrient transport. As a result, all live-bearing reptiles, even if they rely primarily on yolk for embryonic nutrition (i.e. lecithotrophy), are truly viviparous, rather than ovoviviparous (Blackburn 2000a). Because placentae are formed from homologous tissues in both mammals and squamates, we can directly compare placental structures not only across the squamate phylogeny but also amongst all amniotes. In other words, mechanisms of squamate viviparity may help us to understand the transition to viviparity in amniotes in general, including the transition in early therian mammals. Finally, advances in molecular techniques now allow detailed analysis of gene expression in 'non-model' organisms (e.g. Brandley *et al.* (2012)). Perhaps the greatest advance has been next-generation transcriptome sequencing, which provides the snapshots of nearly all the genes expressed in a tissue and allows direct comparisons of molecular function amongst squamate lineages and between squamates and mammals.

Here, we review the proximate changes necessary for the transition from oviparity to viviparity in squamates. Specifically, we discuss the development of mechanisms for embryonic implantation and placentation, exchange of respiratory gases, water transport, nutrient transport, and regulation of the maternal immune system. We highlight recent discoveries of molecular and genetic mechanisms involved in the transition because they provide the best opportunity for comparing the viviparous conditions of squamates and mammals.

Implantation and placentation

By definition, viviparous animals must retain embryos *in utero* until embryogenesis is complete, which means that viviparity must evolve coincident with mechanisms to keep developing embryos inside the uterus during development. If specialized placental structures transport water, gases, and nutrients between mothers and offspring, then adhesion mechanisms are necessary to keep the uterine and embryonic components of these structures adjacent throughout development. Uterine plasma membrane transformations similar to those that facilitate implantation in the rat, including flattening of the apical plasma membrane of uterine epithelial cells, occur during pregnancy in the skink genera *Niveoscincus* and *Eulamprus* (Murphy *et al.* 2000, Hosie *et al.*

2003). Throughout pregnancy, uterine tissues opposite the yolk sac of viviparous skinks of the genera *Pseudemoia*, but not *Saiphos*, exhibit decreases in desmosome expression (Biazik *et al.* 2010a). During pregnancy, occludin expression increases in the uterine tissues of *Pseudemoia*, but not in *Saiphos* or *Eulamprus* skinks (Biazik *et al.* 2007). In addition, claudin 5 expression increases throughout pregnancy in the uterine tissues of *Eulamprus*, *Pseudemoia*, and *Saiphos* (Biazik *et al.* 2008). Interestingly, decreasing desmosome density reduces adhesion between uterine cells, while increasing density of occludin and claudin-5 both increase adhesion between uterine cells. Desmosomes, occludin, and claudins are all involved in the modifications of uterine tissues before blastocyst implantation in rats (Nicholson & Murphy 2005, Preston *et al.* 2006), so could serve similar purposes in squamates. Alternatively, they could regulate paracellular permeability to the molecules transported between maternal and fetal tissues during pregnancy (Biazik *et al.* 2010b).

Unlike eutherian mammals, most squamate embryos exhibit both a chorioallantoic placenta on the embryonic pole (Fig. 1A and B) and some form of yolk-sac placenta on the abembryonic pole (Fig. 1C; Weekes 1935, Hoffman 1970, Guillelte *et al.* 1981, Blackburn 1992, Stewart 1992, 1993). Both placentae are formed via close appositions of uterine and embryonic tissues, and are defined as epitheliochorial in most viviparous reptiles (Blackburn & Vitt 2002, Adams *et al.* 2005) because the embryonic tissues do not breach or invade uterine epithelia. In extant mammals, epitheliochorial placentation has likely evolved secondarily from more-invasive hemochorial or endotheliochorial placentation (Mess & Carter 2007). The uteri of epitheliochorial mammals exhibit physical barriers to invasion and produce secretions that hinder invasion mechanisms of the embryos, but the embryos themselves are capable of invading other maternal tissues in extra-uterine pregnancies (Samuel & Perry 1972). In contrast, extra-uterine embryos in the viviparous skink, *Pseudemoia entrecasteauxii*, which exhibits complex placentation and substantial placentotrophy, are not capable of invading maternal tissues (Griffith *et al.* 2013b). Thus, epitheliochorial placentation in reptiles is probably not maintained by maternal resistance to uterine invasion, but occurs because reptile embryos lack the ability to invade maternal tissues. Exceptions may occur in skinks of the genera *Mabuya* and *Trachylepis*, both of which exhibit invasive placental specializations that may facilitate attachment to uterine tissues. In *Mabuya*, embryonic cells from the chorioallantois send cytoplasmic projections through the syncytial cells of the uterine epithelium to interact with uterine capillaries (Vieira *et al.* 2007). In *Trachylepis*, embryonic epithelial cells from the chorioallantois invade, and ultimately replace, uterine epithelial tissue, and lie in direct contact with the uterine capillaries (Blackburn & Flemming 2012).

Figure 1 Hypothetical schematic illustration of the placenta in viviparous squamates based on the best available information from multiple species (nearly all scincid lizards). Embryo-maternal communication likely facilitates the regional diversification of placental tissues described in (A, B and C), but the mechanisms underlying communication remain unknown. (A) Chorioallantoic placenta: in species that have a placentome, this illustration represents the paraplacentome region of the chorioallantoic placenta only. Both uterine and embryonic membranes are typically made up of simple squamous cells, and are attenuated near capillaries to facilitate respiratory gas exchange. Nutrient transport may occur in the paraplacentome/chorioallantoic placenta, but only transport of Na, K, and water has been documented in nonplacentotrophic species, and the mechanisms of transport for most nutrients are unknown in most species. (B) Placentome region of the chorioallantoic placenta, which is found only in viviparous squamates that exhibit a placentome. Uterine and embryonic membranes of the placentome are usually composed of columnar or cuboidal cells. Nutrient transfer might occur in the placentome via histotrophy, secretion, or both. Limited gas exchange may also occur. (C) Yolk-sac placenta. Uterine and embryonic membranes of the yolk-sac placenta are usually composed of columnar or cuboidal cells, and the yolk-sac membrane may only include a single cell layer. Histotrophic nutrient transport may not occur in species that are not placentotrophic. Other mechanisms of nutrient transfer, including alkaline phosphatase, lipoprotein lipase, and lysosomes, have only been studied in *Pseudemoia* species. In all placentae, desmosomes, claudin-5, and/or occludin may regulate cellular adherence in the uterine epithelium, but have only been investigated in a few species of Scincid lizards. Similarly, cadherins may play a role in adhering embryonic and maternal tissues during implantation, but have only been studied in *Niveoscincus* species. Genes potentially upregulated in the uterus, but not localized to any particular region, include apolipoproteins, aquaporins, cysteine and serine proteases, cystatins, fatty acid-binding proteins, ion transporters, protease inhibitors, solute carrier proteins, and genes involved in angiogenesis (e.g. *VEGFs* and *EPAS1*).

Except in species whose placental tissues exhibit any embryonic invasion of uterine epithelium (i.e. *Mabuya* and *Trachylepis*), mechanisms that allow embryonic attachment to uterine tissues remain largely unknown in viviparous reptiles (Murphy & Thompson 2011). In mammals that exhibit epitheliochorial placentation, embryonic attachment to the uterus is accomplished via interdigitation of embryonic and uterine tissues, as well as proliferation of integrins (Jones *et al.* 2000,

Burghardt *et al.* 2009). Cadherins mediate changes in uterine epithelial cells that are necessary before implantation in mammals with hemochorial placentation (Hyland *et al.* 1998, Dey *et al.* 2004). Few squamate placentae exhibit interdigitation of maternal and embryonic tissues (Blackburn 1992) and, in the skink genus *Niveoscincus*, cadherins are a potential mechanism of embryonic attachment rather than integrins (Wu *et al.* 2011).

Genes putatively used in the growth and remodelling of the placenta are by far the most highly upregulated genes in the uterus of the skink, *Chalcides ocellatus*, during pregnancy (Brandley *et al.* 2012). In mammals, proteases and their inhibitors continuously remodel the placenta by destroying and building new tissue (Salamonsen & Nie 2002). The cathepsin family of cysteine proteases play a major role in this process in multiple lineages of mammals (Afonso *et al.* 1997, Divya *et al.* 2002, Song *et al.* 2006, 2010, Varanou *et al.* 2006). Proteases are also some of the most highly expressed genes in the pregnant *C. ocellatus* uterus; cathepsins B, L, and V mRNAs comprise almost 8% of all the mRNAs of all genes expressed in the pregnant uterus (Brandley *et al.* 2012). Moreover, because tissue remodelling is achieved by the reciprocal actions of proteases and their inhibitors (Afonso *et al.* 1997, Salamonsen & Nie 2002, Song *et al.* 2006, 2007, 2010), it is notable that there is also significant upregulation of cysteine and serine protease inhibitors such as cystatins, tissue factor pathway inhibitor 2 (TFPI2), and serine peptidase inhibitor, Kunitz types 1 and 2 (SPINT1 and 2) in the pregnant *C. ocellatus* uterus. It is unclear whether similar genes are used by all viviparous squamates for placental growth and remodelling, or whether the strength of their expression is linked to placental complexity.

Most viviparous squamates are lecithotrophic (i.e. rely on yolk for nutrition) and exhibit simple chorio-allantoic (Fig. 1A) and yolk-sac placentae (Fig. 1C) with little morphological specialization (Weekes 1935, Bellairs *et al.* 1955, Hoffman 1970, Yaron 1977, Guillette *et al.* 1981, Stewart 1990, 1992, 1993). In contrast, viviparous skinks of the genera *Niveoscincus*, *Pseudemoia*, *Chalcides*, *Eumecia*, *Mabuya*, and *Trachylepis* exhibit complex chorioallantoic placentae and increasing reliance on placentotrophy (Weekes 1935, Blackburn *et al.* 1984, Ghiara *et al.* 1987, Blackburn 1992, Flemming & Branch 2001, Blackburn & Vitt 2002, Stewart & Thompson 2004, 2009a, Adams *et al.* 2005, Blackburn & Flemming 2012). The chorioallantoic placenta is differentiated into a paraplacentome (Fig. 1A) and a placentome (Fig. 1B) in *Pseudemoia*, *Chalcides*, and *Mabuya* (Blackburn 1993b, Stewart & Thompson 1996, Blackburn & Vitt 2002), but not in *Niveoscincus*, *Eumecia*, or *Trachylepis* (Flemming & Branch 2001, Stewart & Thompson 2004, Blackburn & Flemming 2012). The placentome is a region in the dorsal center of the chorioallantoic placenta, which is defined by thickened, occasionally interdigitating uterine and chorioallantoic tissues directly beneath the uterine mesometrium (Weekes 1935, Blackburn *et al.* 1984, Blackburn 1993b). The paraplacentome surrounds the placentome and exhibits little thickening or interdigitating of uterine and chorioallantoic tissues, but capillaries in both tissues are closely apposed (Fig. 1A; Blackburn 1993b). The uterine component of the chorioallantoic placenta in the lecithotrophic skink *Eulamprus quoyii* also develops a

placentome-like structure late in development, but its function remains unclear (Murphy *et al.* 2011) and is absent from other *Eulamprus* skinks (Murphy *et al.* 2012). Yolk-sac placental structure also varies among viviparous squamates (Stewart & Blackburn 1988), and varying degrees of specialization occur among species regardless of reliance on placentotrophy (Stewart 1992, Stewart & Thompson 2004, 2009a, 2009b, Thompson *et al.* 2006).

Respiratory gas exchange

Gestating female squamates flux oxygen and carbon dioxide with the environment for developing embryos (Beuchat & Vleck 1990, Schultz *et al.* 2008, Van Dyke & Beaupre 2011), so the uterus must transport respiratory gases to and from developing embryos. Possibly to facilitate the flux of oxygen and carbon dioxide, squamates exhibit progressive thinning of the eggshell the longer eggs are retained before oviposition (Heulin 1990, Qualls 1999, Heulin *et al.* 2002). The eggshell and shell membrane are thinned via reductions in either the number of shell glands present in the oviduct (Guillette 1993), or a reduction in the size of the shell gland which presumably impedes function (Heulin *et al.* 2005). Most viviparous species retain a reduced eggshell membrane (Blackburn 1998), but it is lost during gestation in placentotrophic species (Blackburn *et al.* 1984, Stewart & Thompson 2009a). Although shell membranes are present in most viviparous squamates, embryos develop in close contact with heavily vascularized uterine tissues that provide avenues of transport for oxygen, carbon dioxide, water, and other materials.

Oviparous embryos exchange oxygen and carbon dioxide across the chorioallantoic membrane, a capillary-dense structure that lines most of the inner surface of the eggshell (Stewart & Thompson 1996, Stewart & Florian 2000, Blackburn *et al.* 2003, Stewart *et al.* 2012). In viviparous species, uterine and chorioallantoic capillaries are closely apposed in the chorioallantoic placenta (Fig. 1A), which likely facilitates the exchange of oxygen and carbon dioxide (Hoffman 1970, Blackburn *et al.* 1984, 2010, Stewart 1990, 1992, Blackburn 1993a, Blackburn & Vitt 2002, Murphy *et al.* 2010b, Blackburn & Stewart 2011). The epithelia of uterine and chorioallantoic tissues are also attenuated in some viviparous reptiles to bring capillaries closer together and reduce diffusion distances for respiratory gases (Fig. 1A; Adams *et al.* 2005, Blackburn *et al.* 2010). In taxa with chorioallantoic placentae that differentiate into a placentome and paraplacentome, the paraplacentome is the region of the chorioallantoic placenta that is primarily responsible for gas exchange (Fig. 1A; Thompson *et al.* 2004, 2006, Adams *et al.* 2005, Thompson & Speake 2006, Wooding *et al.* 2010).

In viviparous skinks, uterine capillary density increases as embryonic mass and oxygen demand

increase (Murphy *et al.* 2010b, Parker *et al.* 2010), which suggests that embryos might influence the upregulation of uterine angiogenesis (Murphy & Thompson 2011). Expression of vascular endothelial growth factors (VEGFs) increases in the uterine tissues of *S. equalis* and *E. quoyii* during pregnancy (Murphy *et al.* 2010a), so VEGFs are involved in the angiogenic pathway in some species (Murphy & Thompson 2011). In contrast, VEGF genes are not highly expressed in the pregnant uterus of viviparous *C. ocellatus* skinks. Instead, the angiogenic-promoting gene, endothelial PAS domain protein 1 (EPAS1), is massively upregulated (Brandley *et al.* 2012). Therefore, similar functions, such as uterine angiogenesis, may have evolved via different genetic pathways in different squamate lineages. Both VEGFs and EPAS1 also regulate uterine angiogenesis in mammals (Sharkey *et al.* 1993, Song *et al.* 2008).

In viviparous squamates that exhibit epitheliochorial placentation (i.e. most species; Blackburn & Vitt 2002, Adams *et al.* 2005), respiratory gases must cross the uterine and embryonic epithelial tissues to reach apposing capillaries. Respiratory gas transport may differ in *Mabuya* and *Trachylepis*, both of which exhibit specializations of embryonic tissues that interact with uterine capillaries (Vieira *et al.* 2007, Blackburn & Flemming 2012). As in mammals, fetal hemoglobins of viviparous squamates exhibit higher oxygen affinity than do maternal hemoglobins, which should maximize oxygen diffusion from maternal to fetal circulation (Blackburn 1993a). The mechanism(s) of carbon dioxide excretion from the fetal to maternal circulation are less known. As in mammals, carbonic anhydrases in reptilian erythrocytes convert carbon dioxide to carbonic acid for transport in blood plasma, and re-convert carbonic acid to carbon dioxide in the lung for atmospheric release (Stabenau & Vietti 2002). A similar mechanism may transport carbon dioxide from the fetal tissues to the chorioallantoic membrane for release to the uterine membrane (Ecay *et al.* 2010). Alternatively, carbonic acid might not be reconverted to carbon dioxide before transport to the uterine membrane. In the mammalian placenta, carbonic anhydrase is primarily active in the maternal tissues in species with epitheliochorial placentation and in fetal tissues in species with endotheliochorial and hemochorial placentation (Ridderstråle *et al.* 1997). Thus, because the chorioallantoic placenta of viviparous squamates exhibit epitheliochorial placentation, carbonic anhydrase may be more abundant in the uterine component than in the embryonic component of the placenta.

Water transport

Eggs of oviparous squamates must uptake water from the environment or resist desiccation (Andrews & Sexton 1981). Because embryos of viviparous species cannot regulate water balance and gas exchange with the

external environment, the evolution of viviparity must include the development of maternal mechanisms to complete these functions *in utero* (Shine & Thompson 2006). Viviparous embryos must absorb water while in the uterus, but intrauterine development provides an incubation environment that is water saturated (Shine & Thompson 2006). Thus, embryonic mechanism(s) for water absorption might change along with the evolutionary transition from oviparity to viviparity. Embryonic water absorption is likely regulated by aquaporins in viviparous species (Wooding *et al.* 2010). Transcriptomic analysis of a pregnant uterus of the *C. ocellatus* skink revealed significant upregulation of multiple members of the aquaporin gene family, including AQ3, AQ5, and AQ11 (Brandley *et al.* 2012). In contrast, the primary aquaporins involved in placental water transport in mammals are AQ3, AQ8, and AQ9 (Wang *et al.* 2001, 2004, Beall *et al.* 2007). Future transcriptomic analyses of oviparous species will reveal whether the upregulation of aquaporins is specific to viviparous species, but functional studies are also needed to determine the functions of each aquaporin. For example in sheep, placental AQ3 also transports fetal urea to the mother (Johnston *et al.* 2000), but this role has not been explored in reptiles.

Mechanisms of placental nutrient transport

In addition to water and respiratory gases, the placenta of viviparous squamates transport inorganic and organic nutrients to varying degrees. Because oviparous squamates utilize the eggshell as a source for 20–80% of their calcium demand (Packard & Packard 1988, Shadrix *et al.* 1994, Stewart *et al.* 2004), the loss of the eggshell in viviparous species compromises an important component of embryonic nutrition. As a result, viviparous squamates, even those that are primarily lecithotrophic, transport significant quantities of calcium across placenta during development (Thompson *et al.* 1999b, 1999c, Ramirez-Pinilla 2006, Stewart *et al.* 2009a, 2009b, Stewart & Ecay 2010, Ramirez-Pinilla *et al.* 2011, Stewart 2013). In oviparous species, calcium ATPase is upregulated in the uterus only during eggshell deposition (Thompson *et al.* 2007), but is upregulated throughout pregnancy in the viviparous skink, *Pseudemoia spenceri* (Fig. 1A, B and C), presumably to provide calcium to the embryo throughout development (Herbert *et al.* 2006). In addition, the calcium transporter calbindin-D_{28K} is expressed in the embryonic membranes of both oviparous and viviparous squamates (Ecay *et al.* 2004, Fergoso *et al.* 2012), suggesting that trans-placental calcium transport remains important after the evolution of viviparity (Fig. 1A, B and C). To underscore the importance of calcium placentotrophy in the evolution of squamate viviparity, Stewart (2013) has even suggested that mechanisms of calcium transport, likely co-opted from eggshell deposition, must evolve

before, or at least concurrently with viviparity for viviparous embryos to receive sufficient calcium to complete embryogenesis. Therefore, structural complexes necessary to accomplish placental calcium transport might provide important foundations for selection to promote greater dependence on placental transport of other nutrients.

Other inorganic nutrients, particularly sodium, potassium, iron, and magnesium, may also be transported across the placenta of viviparous squamates (Fig. 1A, B and C). Placental transport of sodium and potassium has been reported from every viviparous species studied, regardless of reliance on placentotrophy (Hoffman 1970, Thompson 1982, Stewart 1989, Stewart *et al.* 1990, Thompson *et al.* 2000, Ramirez-Pinilla 2006, Ramirez-Pinilla *et al.* 2011). In contrast, the only lecithotrophic species reported to exhibit magnesium transport thus far is *Eulamprus tympanum* (Thompson *et al.* 2000, 2001a). Placental transport of iron has only been reported in highly placentotrophic *Mabuya* skinks (Ramirez-Pinilla 2006, Ramirez-Pinilla *et al.* 2011), does not occur in lecithotrophic *Thamnophis* snakes (Hoffman 1970), but has not been investigated in other species (Thompson 1982, Thompson *et al.* 2000). The mechanisms of inorganic ion transport are largely unknown in viviparous squamates, but in snakes of the genus *Thamnophis*, sodium may be transported by the yolk-sac placenta to increase osmolarity of the embryo and to facilitate water absorption (Blackburn *et al.* 2002, Blackburn & Lorenz 2003). Gene expression profiles of the pregnant uterus of the skink, *C. ocellatus*, reveal 136 genes in 35 families of solute carrier proteins including transporters of inorganic ions (Ca^{2+} , Cl^- , CO_3^{2-} , H^+ , HCO_3^- , K^+ , Na^+ , and PO_4^{3-}) and metals (Cu^{2+} , $\text{Fe}^{2/3+}$, Mg^{2+} , and Zn^{2+} ; Brandley *et al.* 2012). Thus, nutrient transport is controlled by a large suite of genes, yet we still lack information whether these same genes are also used by other viviparous or oviparous squamates.

Much research has focused on placental provisioning of lipids and amino acids (but not carbohydrates; Blackburn 1994) in viviparous squamates. Radioactive and stable isotope tracer studies have documented transport of amino acids, fatty acids, or both, to the developing offspring in species with (Swain & Jones 1997, Jones & Swain 2006, Itonaga *et al.* 2012) and without (Hoffman 1970, Veith 1974, Thompson 1977, Yaron 1977, Van Dyke & Beaupre 2012) complex placentation. In contrast, comparisons of mass composition between eggs and newborn offspring suggest that only species with complex placentae exhibit biologically meaningful transport of amino acids and lipids to offspring (Thompson *et al.* 1999b, 1999c, 2000, 2001b, Flemming & Branch 2001, Ramirez-Pinilla 2006, Ramirez-Pinilla *et al.* 2011), while species with simple placentae exhibit net reductions in amino acids and lipids during development (Thompson 1981, Stewart 1989, 1992, Stewart *et al.* 1990, Blackburn 1994,

Thompson *et al.* 2000). Paradoxically, species with simple placentae exhibit placental transport of labelled organic nutrient tracers and yet they sustain a net loss of organic nutrients during development. Thus, Blackburn (1994) criticized tracer studies for being unable to distinguish between obligate and incipient placentotrophy, particularly in species that are highly lecithotrophic. Conversely, chemical composition comparisons between eggs and offspring, because they ignore any mass lost as metabolic waste (Blackburn 1994), only measure the net change in the quantity of a given nutrient. As a result, if embryos transport wastes to the mother for excretion, then chemical composition comparisons may underestimate gross placental nutrient transport during development. Respirometric studies clearly show that embryos transport carbon dioxide to mothers during development (Beuchat & Vleck 1990, Schultz *et al.* 2008, Van Dyke & Beaupre 2011). Whether viviparous embryos are also capable of transporting nitrogenous wastes, e.g. urea (Packard *et al.* 1977), to mothers remains largely untested (Clark & Sissen 1956). Phylogenetic comparisons of tracer transport among placentotrophic and lecithotrophic species, together with compositional studies that measure metabolic waste production in addition to egg and newborn content, could be useful to determine whether lecithotrophic species with simple placentae are capable of transporting biologically meaningful quantities of organic nutrients to offspring.

Although amino acids and lipids are transported across the placenta of at least some species of viviparous squamates, the mechanisms of transport are poorly understood (Fig. 1A, B and C). In species that exhibit a placenta and paraplacenta, such as the skink genera *Mabuya* and *Pseudemoia*, the placenta appears to be a site of histotrophic transport (Fig. 1B; Jerez & Ramirez-Pinilla 2003, Thompson *et al.* 2004) or nutrient secretion (Fig. 1B; Thompson *et al.* 2006). In the skink genus *Niveoscincus*, *N. ocellatus* exhibits significantly greater reliance on placentotrophy than does *N. metallicus* (Thompson *et al.* 1999a, 2001b), but both species exhibit similar placental morphologies that are less structurally complex than those of *Pseudemoia* sp. (Stewart & Thompson 2004, 2009a, 2009b). Although they lack a placenta, the highly placentotrophic skinks of the genera *Eumecia* and *Trachylepis* appear to transport nutrients across both chorioallantoic and yolk-sac placentae (Flemming & Branch 2001, Blackburn & Flemming 2009).

Species in the skink genera *Eulamprus*, *Niveoscincus*, and *Pseudemoia*, as well as the snake genera *Thamnophis* and *Virginia*, exhibit hypertrophied uterine epithelial cells, which appear to be secretory, in the yolk-sac placenta (Fig. 1C; Stewart 1992, Blackburn & Lorenz 2003, Stewart & Thompson 2004, Thompson *et al.* 2006, Stewart & Thompson 2009a, 2009b). The yolk-sac placenta has been associated with histotrophic nutrient

transport in *Pseudemoia* sp. and in some but not all (Thompson *et al.* 1999a); species of *Niveoscincus* (Thompson *et al.* 2001b, 2006, Itonaga *et al.* 2012). In *Pseudemoia* sp., the uterine component of the yolk-sac placenta secretes electron-dense vesicles assumed to be rich in lipids (Fig. 1C; Adams *et al.* 2005), but, which could also be responsible for the transport of amino acids (Itonaga *et al.* 2012). The epithelium of the yolk-sac placenta of *P. entrecasteauxii* and *P. spenceri* also contains lysosomes that may supply fetal nutrition via histotrophy in the maternal epithelium (Fig. 1C; Biazik *et al.* 2009). The highly placentotrophic *Eumecia* sp., *Mabuya* sp., and *Trachylepis* sp. also appear to transport nutrients across yolk-sac placentae (Flemming & Branch 2001, Jerez & Ramirez-Pinilla 2001, Blackburn & Flemming 2009). In contrast, the genera *Eulamprus*, *Thamnophis*, and *Virginia* are not placentotrophic, which suggests that uterine epithelial hypertrophy in the yolk-sac placenta is not always associated with organic nutrient transport (Stewart *et al.* 1990, Thompson *et al.* 2001a).

Molecular mechanisms of nutrient transport have only been investigated in the placentotrophic skinks *P. entrecasteauxii*, *P. spenceri*, and *C. ocellatus*. In *Pseudemoia* sp., lysosomes and alkaline phosphatase are upregulated in the uterine epithelium of the yolk-sac placenta during pregnancy (Fig. 1C; Biazik *et al.* 2009). Lysosomes are involved in apocrine secretion, while alkaline phosphatase is usually indicative of glucose and/or lipid transport. Lipoprotein lipase is also heavily expressed in the uterine component of the yolk-sac placenta in pregnant *P. entrecasteauxii* (Fig. 1C; Griffith *et al.* 2013a). Lipoprotein lipase is responsible for hydrolysis of triglycerides, and in the placenta could function to break down triglycerides into their component fatty acids for repackaging in the vesicles for apocrine secretion to the developing embryo. Furthermore, numerous genes involved in amino acid and lipid packaging and transport are upregulated in the pregnant uterus of *C. ocellatus* (Brandley *et al.* 2012). These genes included fatty acid-binding proteins (*FABP1–5,7*) that facilitate intracellular transport of fatty acids (Chmurzyńska 2006) and apolipoproteins (APOA, APOE, and APOM), transporters of fatty acids and phospholipids. Moreover, multiple solute carrier protein transporters for glucose, amino acids, and fatty acids are upregulated in the pregnant uterus of *C. ocellatus*. Importantly, many of the same amino acid transporters found in *C. ocellatus* are present in the placentae of eutherian mammals (Verrey *et al.* 2004).

Regulation of the maternal immune system

Embryos are allografts of maternal and paternal tissues and are therefore potentially at risk of attack by the maternal immune system. A variety of mechanisms downregulate the immune system in pregnant eutherian mammals to mitigate this risk (see Moffett & Loke (2006)).

Analyses of immunocompetence and reproduction in viviparous and oviparous squamate reptiles have yielded mixed results. In *Agkistrodon piscivorus*, a lecithotrophic viviparous snake with a simple placenta, the blood serum of pregnant females has a ~20% decreased *ex-vivo* bacterial lysis ability (Graham *et al.* 2011). Conversely, pregnant females of the lecithotrophic lizard, *Z. vivipara*, decrease reproductive and physiological performance when immunologically challenged (Meylan *et al.* 2013), and the placentotrophic viviparous skink, *C. ocellatus*, exhibits reduced immune function during pregnancy (Saad & El Deeb 1990). On the other hand, the reduced immune function in a gravid oviparous lizard species, *Urosaurus ornatus*, in the laboratory is related to the amount of food available rather than the reproductive state, *per se* (French *et al.* 2007). As previous assessments of immune function in viviparous *A. piscivorus*, *C. ocellatus*, and *Z. vivipara* did not vary diet in a systematic way, it is unclear whether the demonstrated decreased immune function in pregnant females was due to active downregulation or level of food resources (but see discussion of *C. ocellatus* later).

During pregnancy in the placentotrophic skink, *C. ocellatus*, multiple important innate immune system genes are downregulated, including complement component 3 (C3), the primary activator of the immune complement system (Brandley *et al.* 2012). In addition, pregnancy in *C. ocellatus* is associated with downregulation of proinflammatory cytokines and upregulation of anti-inflammatory cytokines (inflammation of the uterus is particularly harmful to maintaining pregnancy; Challis *et al.* 2009). Mammals also regulate C3 activity in the uterus and placental tissues via the production of complement regulators (Niederhorn 2006, Baek *et al.* 2007). Thus, the innate immune system is downregulated during pregnancy in *C. ocellatus*, possibly by a means similar to those of mammals. It is still unclear whether immune downregulation has practical implications for the health of female squamates.

Future research

Squamate reptiles are an ideal model system for studying the evolution of amniote viviparity because their uterine, embryonic, and placental structures are homologous to those of mammals and other viviparous amniotes. Furthermore, viviparity has evolved over 100 times in squamates, producing multiple independent 'natural experiments' through which to study this transition. Recent advances in next-generation sequencing has been poised to significantly advance our understanding of the oviparity to viviparity transition in squamates (e.g. Brandley *et al.* (2012)) because we now have the ability to uncover the genetic mechanisms that underlie the morphological and physiological changes associated with viviparity and complex placentation.

Transcriptomic studies of nonmammalian viviparity and placental development will continue to reveal suites of genes associated with placental functions, including the transport of respiratory gases, water, ions, and, in some species, organic nutrients (Blackburn 1998, Thompson *et al.* 2000). Furthermore, the specific structural differences on both maternal and uterine components of the chorioallantoic placenta, yolk-sac placenta, placentome, and paraplacentome must be coordinated by embryonic–maternal communication, the mechanism of which remains unknown. In mammals, this communication is facilitated by a number of hormones, including gonadotropins, interleukin 1 β , and insulin-like growth factor (Herrier *et al.* 2003). There is limited evidence of progesterone production in squamate placentas (Girling & Jones 2003), and whether squamates use signaling factors similar to those in mammals remains unknown. In addition, there exist multiple examples of immune suppression associated with squamate pregnancy (Saad & El Deeb 1990, Graham *et al.* 2011), but it is unclear whether the strength of this suppression is similar across squamates or is instead directly related to increased materno-fetal interaction in highly placentotrophic species. Uterine and embryonic tissues should adhere to one another, both to hold embryos in place and to keep capillary beds aligned to facilitate transport, but the mechanisms of adherence or ‘implantation’ in most species are unknown (Murphy & Thompson 2011). Transcriptomic studies are critical to understanding the mechanisms underlying these phenomena, all of which must occur for the transition to viviparity to be successful.

Unfortunately, the small number of sequenced, annotated genomes of squamate species hinders the study of viviparity in the genomics era (the only published squamate genome to date is the oviparous lizard *Anolis carolinensis*). Increasing the number of sequenced genomes will allow investigation of genome-wide signatures of convergent evolution of viviparity amongst squamates. Also, more genomic information is critical to increase the number of genes identified in sequenced transcriptomes. We also have yet to identify the elements regulating viviparity-associated gene expression; more genomic information will permit evaluation of potential gene expression promoters and the influence of transposable elements on gene expression (e.g. Emera & Wagner (2012) and Jacques *et al.* (2013)).

While continuing these gene discovery studies, squamate viviparity researchers must begin to experimentally test the specific functions of genes hypothesized to be important to the evolution of viviparity in reptiles. Unfortunately, a variety of factors, including difficulty of breeding squamates in captivity and the low number of reproductive cycles per year (one to two), make the laboratory production of gene knockout lineages unfeasible in the short-term. Instead, gene functions should be inferred experimentally with

techniques using synthetic gene expression inhibitors in concert with transcriptomic sequencing. For example, transcriptomic studies reveal that proteases are highly expressed in the pregnant uterus of placentotrophic squamates (Brandley *et al.* 2012), and the function of proteases could be assessed by blocking their actions using chemical inhibitors (Barrett *et al.* 1982). Gene functions may also be inferred using diet manipulations and subsequent effects on nutrient transporter and packaging genes (e.g. Geay *et al.* (2011) and Hamill *et al.* (2013)).

All future studies of gene expression and function must be conducted in a comparative phylogenetic framework, including multiple lecithotrophic, placentotrophic, and oviparous lineages, to assess the evolution of the genetic regulators behind viviparity and placental function. For example, what roles do fetal and maternal hormones play in aligning uterine and embryonic tissues for attachment and material exchange? Do placental nutrient transporters evolve *de novo* in placentotrophic species, or are they co-opted from pre-existing mechanisms in lecithotrophic species? Do the chorioallantoic and yolk sac placentae serve different functions in different lineages of viviparous squamates, and if so, are these functions governed by the expression of the same genes?

Ultimately, these studies will inform perhaps the most basic question in amniote viviparity: is the evolution of viviparity associated with the same suites of genes in both mammals and reptiles? If the 100+ independent transitions to viviparity in amniotes use different suites of genes to maintain pregnancy, it would be an astounding example of the diversity of ways that natural selection can evolve similar, yet complex phenotypes. If the repeated transition to viviparity is associated with similar suites of genes, implying that there are relatively few ways for viviparity to evolve in amniotes, it would be an equally remarkable example of repeated convergent evolution of both phenotype and genotype. The most recent transcriptomic data available suggest that at least some genes and gene families play similar roles in the uterus and placenta of viviparous mammals and squamates (Brandley *et al.* 2012). Establishing the diversity (or lack thereof) of genetic mechanisms that underlie the transition to viviparity in squamates will also inform how early therian mammals made this transition. If similar genetic changes occurred during the repeated evolution of viviparity in multiple viviparous squamate lineages and in therian mammals, it will reveal how amniotes use the same elements of the shared ancestral genetic ‘toolkit’ to make one of the most drastic evolutionary shifts in reproductive morphology and physiology.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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