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REVIEW

A review of the evolution of viviparity in squamate reptiles: the past, present and future role of molecular biology and genomics

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Abstract Squamate reptiles (lizards and snakes) offer a unique model system for testing hypotheses about the evolutionary transition from oviparity (egg-laying) to viviparity (live-bearing) in amniote vertebrates. The evolution of squamate viviparity has occurred remarkably frequently (>108 times) and has resulted in major changes in reproductive physiology. Such frequent changes in reproductive strategy pose two questions: (1) what are the molecular mechanisms responsible for the evolution of squamate viviparity? (2) Are these molecular mechanisms the same for separate origins of viviparity? Molecular approaches, such as RT-PCR, in situ hybridisation, Western blotting and immunofluorescence, have been invaluable for identifying genes and proteins that are involved in squamate placental development, materno-foetal immunotolerance, placental transport, placental angiogenesis, hormone synthesis and hormone receptor expression. However, the candidate-gene or -protein approach that has been used until now does not allow for de novo gene/protein discovery; results to date suggest that the reproductive physiologies of mammals and squamate reptiles are very similar, but this conclusion may simply be due to a limited capacity to study the subset of genes and proteins that are unique to reptiles. Progress has also been slowed by the lack of appropriate molecular and genomic resources for squamate reptiles. The advent of next-generation sequencing provides a relatively inexpensive way to conduct rapid high-throughput sequencing of genomes and transcriptomes. We discuss the potential use of next-generation sequencing technologies to analyse differences in gene expression between oviparous and viviparous squamates, provide important sequence information for reptiles, and generate testable hypotheses for the evolution of viviparity.

Keywords Molecular biology · Genomics · Squamate · Viviparity · Next-generation sequencing

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Introduction

The evolution of viviparity has profound consequences for the life-history strategy of a species, and has resulted in a fascinating diversity of placental structures, placental functions and modes of embryonic nutrition (Blackburn 1993; 1995; 1998; Stewart and Thompson 2000; Thompson and Speake 2006). Frequent evolutionary shifts in reproductive mode among squamate reptiles (lizards and snakes) offer a unique model system, which can be used to provide robust tests of hypotheses about the evolution of viviparity (Stewart and Thompson 2000; Thompson et al. 2002; Blackburn 2006).

An understanding of the physiology and genetics of reptilian reproduction is a necessary prerequisite for studying the differences between oviparous and viviparous squamates. Molecular biology and genomics, both powerful approaches for investigating modes of inheritance, evolution and system function, have been used previously to study the potential role of genes and proteins in squamate reproduction. With few genomic resources available for squamate reptiles, current molecular approaches for studying squamate viviparity consist of a candidate-gene (or candidate-protein) approach, a slow process that does

not allow for de novo gene discovery. Traditional methods of DNA sequencing, such as Sanger sequencing (Sanger et al. 1977), are expensive and labour-intensive option for improving genomic resources for squamate reptiles. Genome projects were becoming increasingly driven by international research consortia and conducted by large industrialised sequencing centres (Hall 2007), and were drifting further out of reach for smaller research groups. Next-generation sequencing technologies are now faster and more affordable than Sanger sequencing, employing massively parallel sequencing to produce millions of small DNA reads at once (Hall 2007). Using bench-top equipment rather than sequencing centres, next-generation sequencing has the potential to sequence billions of base pairs in a matter of a few days (Ansorge 2009), identify both unique and evolutionarily conserved genes, and provide powerful analyses of global gene expression in an organ or even a single cell (Tang et al. 2009). These technologies are used routinely to study model organisms, such as humans (e.g. Sultan et al. 2008), mice (e.g. Mortazavi et al. 2008) and zebrafish (e.g. Hegedus et al. 2009), but are only just starting to be used in reptiles (e.g. Casewell et al. 2009; Gracheva et al. 2010; Schwartz et al. 2010; Wall et al. 2011).

The purpose of this review is to explore the potential of molecular biology and genomics as tools for investigating the evolution of viviparity in squamates. We discuss some of the challenges of using molecular biology and genomics in reptiles and outline recent studies that have used the candidate-gene or -protein approach to improve our understanding of squamate reproductive physiology. We also highlight the caveats of the candidate-gene or -protein approach and explain how new sequencing technologies will be invaluable for constructing and testing hypotheses about the evolution of viviparity in squamates.

A complex but frequent change

The evolution of viviparity has occurred far more frequently in squamate reptiles than in any other amniote vertebrate lineage; there are more than 108 independent origins of viviparity in squamates (Blackburn 2006), compared with just a single evolutionary origin in mammals, and there are no viviparous birds, crocodilians or turtles. Viviparity in squamates is a heritable trait; crosses between oviparous and viviparous populations of *Zootoca* (*Lacerta*) *vivipara* produce an F_1 generation with an "intermediate" phenotype (Arrayago et al. 1996), in which females retain embryos in utero for longer than oviparous females and embryos are surrounded by a thin, transparent shell. The physiological and structural characteristics of viviparous squamates must, therefore, be genetically or epigenetically encoded. Thus, the physiological and structural changes that happen during the transition from oviparity to viviparity are the result of mutations in the genetic or epigenetic elements that control these traits.

There are four major structural and physiological changes that must occur during the transition from oviparity to viviparity (Thompson and Speake 2006). (1) By definition, viviparous animals retain embryos in utero until they are fully developed (Fitch 1970; Packard et al. 1977), so the ability to physically delay oviposition of the embryo is a necessary step in the evolution of viviparity (Thompson and Speake 2006). (2) The thickness of the eggshell membrane must be reduced and the uterus must become more vascular in the final stages of embryonic development to allow sufficient diffusion of oxygen between the uterus and the growing embryo (Thompson and Speake 2006). (3) Mechanisms must develop that allow both the transport of water, normally absorbed from the external environment through the eggshell in oviparous species, and the transport of calcium to the embryo, since embryos of oviparous species mobilise significant amounts of calcium from the eggshell (Stewart and Thompson 2000; Stewart and Ecay 2010). (4) Mechanisms that protect or "hide" the embryo from the maternal immune system must also evolve in viviparous species in which the eggshell membrane disintegrates during pregnancy, bringing fetal and maternal tissues into direct contact (Guillette 1993; Paulesu 1997; Murphy et al. 2009).

The evolutionary transition from embryonic lecithotrophy to embryonic placentotrophy requires another physiological change. Embryos of oviparous squamates are lecithotrophic, as the egg contains sufficient yolk and nutrients to sustain the development of the embryo to full term, and there is no transfer of ions or nutrients between the embryo and the mother while the egg is in utero. In contrast, viviparous squamates display varying degrees of placentotrophy, the process by which nutrients are transferred across the placenta and taken up by the embryo. For placentotrophy to evolve, the uterus must be able to actively transport nutrients to the embryo. Similarly, the embryo must also possess the ability to capture and utilise these nutrients.

The evolution of squamate viviparity has resulted in a diversity of placental morphologies. Traditionally, the morphology of the chorioallantoic placenta has been used to classify squamate placentae as one of four "types" (Weekes 1935; Blackburn and Vitt 2002). A Type I chorioallantoic placenta, found in most viviparous squamates, comprises apposing chorioallantoic and uterine tissue that both exhibit squamous epithelia (Weekes 1935; Blackburn 1993). Type II chorioallantoic placentae exhibit bulging uterine capillaries, producing a ridged pattern at the interface between the uterus and the chorioallantois (Weekes

1930; 1935; Adams et al. 2007; Stewart and Thompson 2009). Squamates with a Type III chorioallantoic placenta display a placentome, a central elliptical region of the chorioallantoic placenta in which the uterine epithelium is hypertrophied and thrown into folds (Weekes 1929; 1930; 1935; Stewart and Thompson 1996; Adams et al. 2005). A Type IV chorioallantoic placenta, found only in the scincid genus *Mabuya*, exhibits chorionic epithelium that interdigitates deeply with the folded uterine epithelium of the placentome (Blackburn and Vitt 2002; Jerez and Ramírez-Pinilla 2003). While the Weekes' classification scheme (Weekes 1935) is still used in the literature, it does not adequately describe the chorioallantoic placentae of some species (e.g. Corso et al. 2000; Murphy et al. 2011), nor does it address the wide diversity of yolk sac placentae in squamates.

The number of physiological and structural changes required for the evolution of viviparity suggests that this transition is complex and potentially involves the polygenic control of several biochemical pathways. Major and complex shifts in life-history traits such as reproductive mode are generally rare (Qualls et al. 1995), but the number of independent origins of viviparity in squamate reptiles indicates that the mechanism(s) controlling this change is particularly evolutionarily labile (and potentially less labile in other types of reptiles). Such frequent and major changes in reproductive strategy pose the question: What are these proximate mechanisms for the evolution of viviparity, and are they the same for separate origins of viviparity? In other words, has the same mutation been selected during each evolutionary transition, or did changes occur by many different pathways, producing similar outcomes?

Molecular biology and genomics: the potential

Using just a few model organisms, molecular biology and genomics have produced a wealth of knowledge about fundamental cellular processes and genomic organisation. As the fields of molecular biology and genomics have advanced, however, the costs and technical difficulty of the associated technology increased, putting it out of the reach of single-investigator research programs, groups with smaller budgets or those working with non-model organisms (Jenner and Wills 2007; Ellegren 2008; Rokas and Abbot 2009). Unfortunately, groups currently studying the evolution of squamate viviparity often fall more than one of these categories.

Furthermore, the most powerful model species for testing hypotheses about the evolution of viviparity, bimodally reproductive lizards (Heulin et al. 1991; 1993; Qualls et al. 1995; Smith and Shine 1997), are only distantly related to any model organisms used in molecular biology. Sequenced genomes, genetic linkage maps, commercially available antibodies and gene sequences are generally not available for lizards and snakes. Until recently, the chicken genome provided the closest point of reference for studies of squamate molecular biology, but the comparison was less than ideal; birds and squamates last shared a common ancestor nearly 300 million years ago (Kumazawa 2007). The first sequenced genome for a squamate, and the first for a non-avian reptile, is from the oviparous iguanid lizard *Anolis carolinensis* (Losos et al. 2005; Janes et al. 2010), and has been available for download from Ensembl since February 2009. While *A. carolinensis* provides valuable sequence information for iguanids, and lizards in general, the divergence dates for the modern lizard families are very ancient (110–250 million years ago) (Kumazawa 2007) and there are likely to be important genomic and sequence differences between squamate families, and in particular, between *A. carolinensis* (Iguanidae) and bimodally reproductive lizards (Scincidae and Lacertidae). To put the depauperate state of reptilian genomics into perspective, divergence dates for lizard families are far more ancient than those for mammalian orders (75–85 million years ago) (Bininda-Emond et al. 2007), but while at least one representative from each mammalian order has been sequenced (Broad Institute Mammalian Genome Project website, Ensembl websites) only one lizard genome is available. Previous sequencing efforts have not been spread evenly across the vertebrate evolutionary tree; genomes of 32 of the 5,200 extant mammal species have been sequenced and are available, compared with only one (*A. carolinensis*) sequenced genome from approximately 7,500 non-avian reptiles (Janes et al. 2010).

The possibility of elucidating elements of inheritance, evolution and system function across the entire genome is a highly attractive prospect for researchers studying the evolution of viviparity in squamates. However, a lack of appropriate genome resources for squamates has hampered the use of large-scale molecular and genomic approaches. Nevertheless, some research groups have successfully investigated single genes and proteins that are associated with reproduction and the evolution of viviparity in squamates. The majority of these studies have used techniques such Western blotting (immunoblotting) to detect expressed proteins (e.g. Ecay et al. 2004; Thomson et al. 2005; 2006; Biazik et al. 2007; 2008; 2010), immunohistochemistry to localise expressed proteins to particular cells within a tissue sample (e.g. Paulesu et al. 1995; Herbert et al. 2006; Biazik et al. 2007; 2008; 2010; Khambaty et al. 2008), and radioimmunoassay (Table 1) or ELISA (e.g. Jessop et al. 2009) to quantify expressed proteins and hormones. Others have investigated mRNA expression, using reverse transcriptase polymerase chain reaction

Table 1 Studies that have used radioimmunoassay to measure the concentration of hormones in maternal plasma during reproduction

Species	Lizard/ Snake	Parity mode	Hormones assayed	Reference
Amblyrhychus cristatus	Lizard	Oviparous	Corticosterone, estrogen, progesterone, testosterone	Rubenstein and Wikelski (2005)
Anolis carolinensis	Lizard	Oviparous	Estrogen, progesterone	Jones and Guillette (1982)
			Estrogen, progesterone	Jones et al. (1983)
			Testosterone	Lovern and Wade (2001)
			Estrogen, testosterone	Lovern and Wade (2003)
Barisia imbricata imbricata	Lizard	Viviparous	Progesterone	Martinez-Torres et al. (2003)
Calotes versicolor	Lizard	Oviparous	Estrogen, progesterone	Radder et al. (2001)
			Progesterone	Shanbhag et al. (2001)
Chalcides chalcides	Lizard	Viviparous	Progesterone	Guarino et al. (1998)
Chamaeleo pumilus pumilus	Lizard	Viviparous	Progesterone	Veith (1974)
Cnemidophorus inornatus	Lizard	Oviparous	Estrogen, progesterone	Moore and Crews (1986)
Cnemidophorus sexlineatus	Lizard	Oviparous	Corticosterone	Grassman and Hess (1992)
Cnemidophorus uniparens	Lizard	Oviparous	Estrogen, progesterone, testosterone	Moore et al. (1985)
			Corticosterone, estrogen, progesterone	Grassman and Crews (1989)
Cordylus giganteus	Lizard	Viviparous	Estrogen, progesterone	van Wyk (1994)
Cordylus polyzonus polyzonus	Lizard	Viviparous	Estrogen, progesterone	Flemming (1994)
Crotaphytus collaris	Lizard	Oviparous	Progesterone	Fox and Guillette (1987), Masson and Guillette (1987)
Ctenophorus ornatus	Lizard	Oviparous	Corticosterone	Baverstock and Bradshaw (1975)
Cyclura carinata carinata	Lizard	Oviparous	Corticosterone	MacDonald et al. (2007)
Egernia whitii	Lizard	Viviparous	Corticosterone, progesterone	Cartledge et al. (2005)
			Corticosterone	Cartledge and Jones (2006)
Eublepharis macularius	Lizard	Oviparous	Estrogen, testosterone	Tousignant et al. (1995)
			Estrogen, progesterone, testosterone	Rhen et al. (2000)
Eumeces obsoletus	Lizard	Oviparous	Progesterone	Fox and Guillette (1987), Masson and Guillette (1987)
Hemidactylus frenatus	Lizard	Oviparous	Corticosterone	Hanley et al. (1998)
Hoplodactylus duvaucelii	Lizard	Viviparous	Corticosterone	Barry et al. (2010)
Hoplodactylus maculatus	Lizard	Viviparous	Corticosterone	Girling and Cree (1995)
Lepidodactylus lugubris	Lizard	Oviparous	Corticosterone	Hanley et al. (1998)
Niveoscincus microlepidotus	Lizard	Viviparous	Estrogen, progesterone	Girling et al. (2002),
			Progesterone	Girling and Jones (2003)
Niveoscincus metallicus	Lizard	Viviparous	Estrogen, progesterone	Jones and Swain (1996)
			Progesterone	Bennett and Jones (2002)
Niveoscincus ocellatus	Lizard	Viviparous	Estrogen, progesterone	Jones et al. (1997)
Oligosoma maccanni	Lizard	Viviparous	Progesterone	Holmes and Cree (2006)
Phrynosoma cornutum	Lizard	Oviparous	Corticosterone, estrogen, progesterone, testosterone	Wack et al. (2008)
Podarcis sicula sicula	Lizard	Oviparous	Estrogen	Carnevali et al. (1991)
			Adrenaline, corticosterone	DeFalco et al. (2004)
Pogona barbata	Lizard	Oviparous	Corticosterone, estrogen, progesterone, total androgen	Amey and Whittier (2000)
Psammodrommus algirus	Lizard	Oviparous	Estrogen, progesterone	Diaz et al. (1994)
Tiliqua nigrolutea	Lizard	Viviparous	Estrogen, progesterone, testosterone	Edwards and Jones (2001)

Table 1 continued

Species	Lizard/ Snake	Parity mode	Hormones assayed	Reference
Tiliqua rugosa	Lizard	Viviparous	Progesterone	Bourne et al. (1986)
			Arginine vasotocin, progesterone	Fergusson and Bradshaw (1991)
Sceloporus cyanogenys	Lizard	Viviparous	Progesterone	Callard et al. (1972)
Sceloporus jarrovi	Lizard	Viviparous	Progesterone	Guillette et al. (1981)
			Corticosterone, estrogen, testosterone	Woodley and Moore (1998)
			Corticosterone, progesterone, testosterone	Painter et al. (2002)
Sceloporus occidentalis	Lizard	Oviparous	Corticosterone	Dunlap (1995)
Sceloporus undulatus	Lizard	Oviparous	Progesterone	Masson and Guillette (1987)
Sceloporus virgatus	Lizard	Oviparous	Androstenedione, corticosterone, estrogen, progesterone, testosterone	Abell (1998)
			Estrogen, progesterone, testosterone	Weiss et al. (2002)
Uromastix hardwicki	Lizard	Oviparous	Estrogen, progesterone, testosterone	Arslan et al. (1977)
Urosaurus ornatus	Lizard	Oviparous	Corticosterone, estrogen, progesterone	Woodley and Moore (2002)
Uta stansburiana	Lizard	Oviparous	Corticosterone	Wilson and Wingfield (1992, 1994)
Varanus albigularis	Lizard	Oviparous	Estrogen, testosterone	Phillips and Millar (1998)
Zootoca (Lacerta) vivipara	Lizard	Viviparous	Progesterone	Xavier (1982)
			Estrogen	Gavaud (1986)
			Aldosterone, corticosterone	Dauphin-Villemant and Xavier (1987)
			Aldosterone, corticosterone, progesterone	Dauphin-Villemant et al. (1990)
			Estrogen	Heulin et al. (2008)
		Oviparous	Estrogen	Heulin et al. (2008)
Acrochordus granulatus	Snake	Viviparous	Estrogen, progesterone, testosterone	Gorman et al. (1981)
Cerberus rhynchops	Snake	Viviparous	Estrogen, progesterone, testosterone	Gorman et al. (1981)
Crotalus atrox	Snake	Viviparous	Corticosterone, estrogen, progesterone, testosterone	Schuett et al. (2004), Taylor et al. (2004)
Crotalus horridus	Snake	Viviparous	Corticosterone, progesterone, testosterone	Lutterschmidt et al. (2009)
Crotalus oreganus	Snake	Viviparous	Corticosterone, estrogen, progesterone, testosterone	Lind (2009), Lind et al. (2010)
Lamprophis fuliginosus	Snake	Oviparous	Insulin-like growth factor-1	Sparkman et al. (2010)
Laticauda colubrina	Snake	Viviparous	Estrogen, progesterone, testosterone	Gorman et al. (1981)
Naja naja	Snake	Oviparous	Estrogen, gonadotrophin, progesterone, thyroxine	Bona-Gallo et al. (1985)
Nerodia sp.	Snake	Viviparous	Estrogen	Kleis-San Francisco and Callard (1986)
Nerodia (Natrix) sipedon pictiventris	Snake	Viviparous	Progesterone	Chan et al. (1973)
Thamnophis elegans	Snake	Viviparous	Progesterone	Highfill and Mead (1975)
			Insulin-like growth factor-1	Sparkman et al. (2009)
Thamnophis sirtalis parietalis	Snake	Viviparous	Corticosterone, estrogen, progesterone, testosterone	Whittier et al. (1987)
Trimeresurus stejnegeri stejnegeri	Snake	Viviparous	Estrogen, progesterone	Tsai and Tu (2001)
Vipera aspis	Snake	Viviparous	Estrogen	Bonnet et al. (1994)
			Progesterone	Naulleau and Fleury (1990), Bonnet et al. (2001)

(RT-PCR) to detect and obtain cDNA sequences of expressed genes (e.g. Young et al. 1995a; Murphy et al. 2009; 2010b), and in situ hybridisation to localise the expression of mRNA to particular cell types (e.g. Paulesu et al. 2001; Endo and Park 2005). The use of molecular and genomic techniques have significantly advanced our fundamental knowledge about aspects of squamate reproductive physiology, including processes of placental development (e.g. Paulesu et al. 2001; Thomson et al. 2005; 2006; Biazik et al. 2010), immunotolerance of the uterus

during pregnancy (Paulesu et al. 1995; 2005b; Paulesu 1997; Romagnoli et al. 2003; Murphy et al. 2009), placental transport (e.g. Ecay et al. 2004; 2010; Herbert et al. 2006; Biazik et al. 2007; 2008; 2009; Thompson et al. 2007), placental angiogenesis (Parker et al. 2010; Murphy et al. 2010a, b, 2011), hormone synthesis (e.g. Polzonetti-Magni et al. 1994; Simpson et al. 1994; Endo and Park 2005; Hammouche et al. 2009; Table 1) and hormone receptivity (e.g. Whittier et al. 1991; Young et al. 1995a; Rhen and Crews 2001; Singh et al. 2007; 2008; Katsu et al. 2010).

Placental development

In many mammals, blastocysts not only attach but continue to invade and breach the lumenal epithelium of the uterus when they contact the uterine wall during implantation. Invasion results in the development of an endotheliochorial placental structure if the uterine capillaries remain intact. If blastocysts also breach the endothelium of the maternal capillaries during implantation, as occurs in rodents and primates (Pijnenborg et al. 1981), the resultant placenta is haemochorial. When blastocysts of viviparous squamates and many mammals come into contact with the uterine wall, they do not breach the uterine epithelium but simply attach to it, resulting in the development of a non-invasive epitheliochorial placenta (see Moffett and Loke 2006 for schematic diagrams of haemochorial, endotheliochorial and epitheliochorial placentation). An exception occurs in the highly specialised chorioallantoic placenta of an Andean population of a species of *Mabuya* skink, in which some chorionic cells send cytoplasmic projections through the syncitial cells of the uterine epithelium to form a structural relationship with uterine capillaries (see Vieira et al. 2007 for diagram).

While the uterine epithelium remains intact in viviparous squamates, the uterus still undergoes dramatic remodelling during pregnancy as the placenta develops (e.g. Weekes 1929; Stewart and Thompson 1994, 1996). Some molecular mechanisms that control placental development in mammals have also been detected in the placenta of viviparous squamates, such as the Hβ58 gene, which is essential for the normal formation of the chorioallantoic placenta in mice (Radice et al. 1991; Lee et al. 1992; Rider et al. 2000). Both the mRNA and protein expression of Hβ58 increases during the period of placental development in the viviparous lizard *Chalcides chalcides*, and both the mRNA and protein are localised in the foetal chorion and the uterine epithelial components of the chorioallantoic placenta (Paulesu et al. 2001).

HoxA10, which is required for the formation of uterodomes (cell-surface protrusions of the uterine epithelium) in mice (Bagot et al. 2001), and is associated with their uterine receptivity (Taylor et al. 1997), is another gene that may be involved in the placental development of both mammals and reptiles. HoxA10 proteins occur in the uterus of both *Eulamprus tympanum*, a viviparous lizard that displays uterodomes, and *Lampropholis guichenoti*, an oviparous lizard that has no uterodomes (Thomson et al. 2005). While the amount of HoxA10 protein does not change in the uterus of either lizard during the reproductive cycle, these species may express different isoforms of HoxA10 in their uterus (Thomson et al. 2005).

Uterine plasticity in humans and rodents is driven in part by estrogen, which triggers cellular signalling systems that phosphorylate and dephosphorylate the tyrosine residues of key effector proteins, such as insulin-like growth factor I (IGF-I) (Richards et al. 1996; Adesanya et al. 1999). This process of tyrosine phosphorylation and dephosphorylation effectively switches the function of key proteins "on" and "off", resulting in a changing uterine morphology during the reproductive cycle. Different profiles of phosphotyrosine proteins in the uterus of *L. guichenoti* and *E. tympanum* were observed using Western blotting, indicating that tyrosine phosphorylation may contribute to different uterine morphologies in these two species (Thomson et al. 2006). Examining changes in the phosphotyrosine profile of the uterus during pregnancy can also be used to identify individual proteins that drive changes in uterine morphology; for example, the intensity of one protein band in *E. tympanum* samples varies significantly during pregnancy, peaking in early pregnancy and decreasing for the remainder of gestation (Thomson et al. 2006). Protein bands such as these could be purified and identified using mass spectrometry in future studies.

Some molecular systems previously thought to be associated with the invasive properties of mammalian placentae are also present in the non-invasive placentae of viviparous lizards. Desmosomes are part of the junctional complex that helps a cell adhere to laterally adjoining cells, and the number of desmosomes between uterine epithelial cells decreases during the implantation period in mice and rats (Enders and Schlafke 1971), which is likely to assist the invasion of the blastocyst (Illingworth et al. 2000). In lizards, both the number of desmosomes between uterine epithelial cells and the expression of desmoglein-2, a protein found in desmosomes, remains the same during pregnancy in lizards with simple Type I chorioallantoic placentae. In contrast, the number of desmosomes and desmoglein-2 expression decreases during pregnancy in lizards that have complex Type III chorioallantoic placentae (Biazik et al. 2010). Therefore, while a decrease in the number of desmosomes in mammalian uterine epithelium is associated with invasion of the blastocyst, a decrease in desmosome number in lizards may allow deformation and remodelling of the uterus necessary to

produce the morphological characteristics of a structurally complex and placentotrophic (Type III) chorioallantoic placenta in squamates (Biazik et al. 2010). Similarly, adherens junctions, and the cadherin proteins that comprise them, undergo specific redistribution during the period of blastocyst invasion in mammals, disappearing from the lateral plasma membrane but remaining on the apical plasma membrane of uterine epithelial cells (Takeichi 1990; Hyland et al. 1998). The same redistribution of cadherins occurs in the uterus of two viviparous lizards *Niveoscincus metallicus* and *N. ocellatus*, but this change is not associated with blastocyst invasion as lizard placentae are non-invasive. Instead, these changes are likely to allow deformation, restructuring and stretching of the lizard uterus to accommodate the rapidly growing embryo (Wu et al. 2011). Cadherin does not redistribute during pregnancy in the uterine epithelium of barren uteri, indicating that the changes in the junctional complex are not solely driven by maternal hormones, but are stimulated by the presence of an embryo (Wu et al. 2011).

Immunological interactions between mother and foetus

The evolution of viviparity poses a major immunological hurdle for mother and foetus (Medawar 1953). The shell membrane that surrounds the developing embryo in the oviparous condition becomes significantly thinner during the evolution of viviparity in squamate reptiles (Qualls 1997; Heulin et al. 2002; Stewart et al. 2010a, b). The thin shell membrane disintegrates during pregnancy in the majority of viviparous squamates (Blackburn 1993), and maternal and embryonic tissues come into contact to form a placenta. While the majority of pregnancies are successful, classical immunology theory predicts that contact between antigenically different cells, in situations such as the placenta, would elicit a major response from the maternal immune system and result in a rejection of the foetus. However, components of the maternal immune system such as cytokines and leukocytes, may actually aid rather than hinder blastocyst implantation (Saito 2001). Mechanisms of maternal-foetal tolerance, such as combinations of cytokines and chemokines that allow cross-talk between mother and foetus (Vigano et al. 2003) and reduced antigen presentation by the foetus during pregnancy (Bainbridge 2000; Huddleston and Schust 2004; Petroff 2005; Trowsdale and Betz 2006), are well studied in mammals. Mechanisms of materno-foetal tolerance in mammals are likely to play a similar role in viviparous squamates (Paulesu et al. 1995, 2005b; Paulesu 1997; Romagnoli et al. 2003; Murphy et al. 2009).

Cytokines accumulate at the maternal-foetal interface during pregnancy, influence the activity of immune cells and growth factors, and help modulate maternal-foetal tolerance (Saito 2001; Paria et al. 2002; Schafer-Somi 2003). However, it is the balance of different cytokines, rather than the expression of any single cytokine, that facilitates successful implantation of the blastocyst and placental development (Zourbas et al. 2001; Chaouat et al. 2002; Paulesu et al. 2005a). Interleukin-1 and transforming growth factor-β (TGF-β) proteins, key cytokines in mammalian reproduction (Simon et al. 1994; Jones et al. 2006), are expressed in both the maternal and foetal components of the chorioallantoic placenta of the viviparous lizard *Chalcides chalcides* (Paulesu et al. 1995; Paulesu 1997). The entire interleukin-1 system, comprising interleukin -1α (IL-1α), IL-1β and the IL-1 receptor (IL-1R tI), is present in the uterus of *C. chalcides* both before and after ovulation (Romagnoli et al. 2003). The IL-1 system is also expressed in the uterus of oviparous and viviparous populations of the bimodally reproductive lizard *Zootoca vivipara*, and expression does not differ during pregnancy or between oviparous and viviparous females (Paulesu et al. 2005b). Detection of the IL-1 system in *C. chalcides* and in both oviparous and viviparous *Z. vivipara* indicate that its presence is not associated with placental complexity or even the presence of a placenta (Paulesu et al. 2005b), but potential quantitative changes in the expression of the IL-1 system during pregnancy should be investigated and would best be measured using Western blotting or qPCR.

Mammalian embryos also evade detection by the maternal immune system by regulating the expression of genes in the major histocompatibility complex (MHC) (Moffett and Loke 2006), a region of the genome that encodes many components of the immune system (Benacerraf 1981; Snell 1981). Classical MHC class I genes encode highly polymorphic proteins that are expressed on the surface of all cells and present antigens to T cells (Klein 1986). In contrast, non-classical MHC class I genes have tissue-specific expression and show little or no polymorphism (Geraghty 1993; Stroynowski and Lindahl 1994; Gouin et al. 2006). Embryos of most mammals display decreased expression of classical MHC class I genes on the surface of the trophoblast (embryonic tissue that contributes to the placenta), allowing the foetus to "hide" from the maternal immune system (Moffett and Loke 2006). Embryos of many mammals also show increased expression of non-classical MHC class I genes in trophoblast tissue, which may result in localised suppression of the maternal immune system; HLA-G, for example, is a non-classical class I gene in humans that suppresses T-lymphocyte function and reduces cell lysis by natural killer (NK) cells (Lin et al. 2007; Selmani et al. 2008).

Class I genes are expressed in the uterus of both oviparous and viviparous lizards, during both pregnancy and non-reproductive periods (Murphy et al. 2009).

A more detailed analysis of the class I genes in the uterus of *Pseudemoia entrecasteauxii*, which is a viviparous lizard with a complex placenta, revealed that some of these genes resemble the classical class I type while others display characteristics of non-classical class I genes. Thus, *P. entrecasteauxii* encodes and expresses the MHC genes that contribute to materno-foetal tolerance during pregnancy in mammals, and the same mechanism may also function in viviparous squamates to allow the embryo to "hide" from the maternal immune system (Murphy et al. 2009).

Placental vascularisation

Viviparous squamates retain embryos in utero during the final stages of embryonic development, when embryonic mass and metabolism increase rapidly. The embryo's increasing demand for oxygen as it develops is satisfied by the placenta, which becomes more vascular during pregnancy (Guillette and Jones 1985; Masson and Guillette 1987). While major blood vessels in the uterus and embryonic membranes are visible with the naked eye, it is the microvasculature of these tissues that changes during pregnancy and facilitates the diffusion of respiratory gases between mother and embryo (Guillette and Jones 1985; Masson and Guillette 1987).

Recently, immunofluorescence and confocal microscopy have been used to quantify placental vascular density and morphology in detail (Murphy et al. 2010a, 2011; Parker et al. 2010). This technique was used to demonstrate that the uterus of *Saiphos equalis*, a lizard displaying incipient viviparity (Stewart et al. 2010a, b), is more vascular than the uterus of a closely related oviparous lizard *Ctenotus taeniolatus* (Parker et al. 2010). The viviparous lizard *Eulamprus quoyii* also has a very vascular uterus, but uterine blood vessels are organised and distributed in a very different pattern to *S. equalis* (Murphy et al. 2010a, 2011). In both *E. quoyii* and *S. equalis*, increases in uterine vascular density occur in concert with increases in embryonic mass and metabolism (Murphy et al. 2010a; Parker et al. 2010), which suggests that the oxygen demand of the embryo triggers feedback mechanisms to stimulate the growth of blood vessels in the adjacent uterus. This feedback mechanism is likely to involve vascular endothelial growth factor (VEGF); mRNA of the VEGF gene is expressed in the uterus of both oviparous and viviparous skinks, and the mRNA expression of three VEGF transcripts in the uterus increases during pregnancy in *S. equalis* (Murphy et al. 2010b). One of these VEGF transcripts had been found previously only in cultured human skin cells containing DNA damaged with UV light and other genotoxic agents (Mineur et al. 2007; Murphy et al. 2010b). Obtaining full nucleotide sequences for squamate genes, as has been done for VEGF (Murphy et al. 2010b), would allow the construction of oligonucleotide primers for PCR that are specific for a species or for a particular transcript of the gene. Full-length gene sequences would also allow us to predict whether squamate genes have a similar or different function to related genes in other animals.

Placental transport

An obvious discrepancy between the size of the ovulated egg and the size of the neonate indicates a net transfer of material into the egg during development. Much of the increase is due to substantial water uptake by the embryo, which occurs in both oviparous and viviparous species (Thompson 1981; Packard et al. 1985). Embryos of oviparous species absorb water through the eggshell after oviposition (Packard 1991). In contrast, embryos of viviparous species gain significantly more water than their oviparous counterparts during development (Thompson et al. 2000) and receive this water via transport across the placenta (Thompson and Speake 2006). The mechanisms of placental water transport in viviparous squamates are not known, but it is possible that water channel proteins such as aquaporins are involved. Aquaporins are membrane-bound proteins that selectively move water molecules in and out of cells; they are present in placentae of humans, sheep and mice (Liu and Wintour 2005) and are upregulated in rat uterus during blastocyst implantation (Lindsay and Murphy 2007). Antibodies generated to detect rat aquaporins have been used to determine a putative distribution for aquaporins in the placenta of the viviparous lizard *Mabuya* sp. (Wooding et al. 2010). Other transport proteins, including calcium binding proteins and glucose transporters, were also identified using antibodies generated for rats, but the specificity of these antibodies in lizards has not yet been confirmed with Western blots (Wooding et al. 2010).

Dissolved ions, including calcium, magnesium, potassium and sodium, are also transported to the embryo across the placenta in viviparous squamates and the amount of ion transport differs among species (Thompson et al. 2000). For example, the proportion of total neonatal calcium that is supplied by the placenta is species-specific and varies from 0 to 100% (Stewart and Thompson 2000; Thompson et al. 2000; Ramirez-Pinilla 2006). While all oviparous squamates obtain some calcium from the yolk and some from the eggshell (Packard et al. 1984, 1985), calcium provision to viviparous squamates may occur in one of three patterns: (1) all of the calcium needed for embryonic development is provided in the yolk, (2) some calcium is provided by the yolk and the rest is supplied across the

placenta, and (3) no calcium is provided in the yolk and the placenta supplies the embryo with all its calcium requirements (Stewart and Ecay 2010). Therefore, the transition from oviparity to viviparity requires that either more calcium be deposited in the yolk of the ovulated egg or that the placenta has the capacity to transport significant amounts of calcium to the embryo during pregnancy.

Some molecular components of the processes controlling calcium mobilisation, egg-shelling and placental calcium transport in squamates have been successfully identified (Ecay et al. 2004, 2010; Herbert et al. 2006, 2010; Thompson et al. 2007; Khambaty et al. 2008). Calbindin-D_{28K}, involved in the process of calcium mobilisation in the yolk sac splanchnopleure of chickens, is present in both the yolk sac splanchnopleure and chorioallantois of the oviparous snake *Pantherophis guttatus*. Expression of calbindin-D_{28K} increases in both the yolk sac splanchnopleure and the chorioallantois of *P. guttatus* during incubation, coinciding with increases in calcium transport to the embryo during the later stages of embryonic development (Ecay et al. 2004).

Carbonic anhydrase II (CAII) is expressed in the chorioallantois of chicken embryos late in development and is thought to pump protons out of the chorionic epithelium, acidifying the inner surface of the eggshell and dissolving calcium carbonate, making it available for absorption by the embryo (Ecay et al. 2010). CAII is present in the chorioallantois of *P. guttatus* but is not detectable in the yolk sac splanchnopleure, and chorioallantoic expression of CAII increases during the late stages of embryonic development, similar to the expression profile of calbindin-D_{28K} (Khambaty et al. 2008). Chorioallantoic expression of CAII also increases in the viviparous snake *Virginia striatula*, which suggests that CAII may have functions other than dissolving eggshell calcium and may be involved in gas exchange or acid–base balance (Ecay et al. 2010).

Calcium ATPase (Ca^{2+}-ATPase) pumps, which control the transport of calcium ions out of cells, are present in the epithelium of uterine glands in the oviparous lizard *Lampropholis guichenoti* (Thompson et al. 2007). Ca^{2+}-ATPase is absent from uterine glands until eggs have been ovulated and are being calcified, suggesting that Ca^{2+}-ATPase pumps contribute to the deposition of calcium during eggshell calcification (Thompson et al. 2007). Ca^{2+}-ATPase is also expressed in uterine and glandular epithelium of four viviparous lizards, *Pseudemoia entrecasteauxii*, *P. spenceri*, *Niveoscincus metallicus* and *N. ocellatus* (Herbert et al. 2006, 2010). The timing of Ca^{2+}-ATPase expression in these species is lineage-specific; both *Pseudemoia* species exhibit prolonged Ca^{2+}-ATPase expression that persists from early pregnancy until birth, while both *Niveoscincus* species display a narrower window of Ca^{2+}-ATPase expression during vitellogenesis and early pregnancy (Herbert et al. 2006, 2010). It is possible that a prolonged period of Ca^{2+}-ATPase occurs in highly placentotrophic species while a shorter period of Ca^{2+}-ATPase expression is characteristic of species that are less placentotrophic, but previous studies are too limited phylogenetically to confirm this conclusion (Herbert et al. 2010).

Dissolved ions may also pass from mother to embryo via paracellular transport, in which ions pass between uterine epithelial cells and into the uterine lumen for embryonic absorption (Biazik et al. 2007, 2008). The ion selectivity and "leakiness" of the paracellular pathway is determined by the tight junction, a complex of proteins that forms an adhesion point between laterally-adjoining cells (Anderson 2001). Occludin, one of the proteins in the tight junction, is present in the uterus of both oviparous and viviparous lizards (Biazik et al. 2007). The expression of occludin in the tight junctions between uterine epithelial cells increases during pregnancy in viviparous species, making tight junctions less permeable and reducing paracellular flux through the placenta.

Claudins, a family of proteins also found in tight junctions, show similar changes in expression during pregnancy to occludin (Biazik et al. 2008). There are at least 24 different claudins in mammals, each with different tissue and cell type specificities, and combinations of different claudins vary the permeability properties of tight junctions (Van Itallie and Anderson 2006). A survey using antibodies directed against claudins-1, -2, -3, -4 and -5 detected claudin-5 in the uterus of both oviparous and viviparous skinks. Claudin-5 is present in the apical, lateral and basal plasma membranes of uterine epithelial cells in non-reproductive females, but becomes redistributed by late pregnancy, by which time claudin-5 is detectable only in the tight junctions of the uterine epithelium (Biazik et al. 2008). Redistribution of claudin-5 to the tight junctions late in pregnancy suggests that the paracellular pathway is highly regulated and may become more ion selective as gestation proceeds (Biazik et al. 2008). Other mechanisms of placental transport, such as histotrophy, may compensate for decreased paracellular transport during late pregnancy in viviparous species (Biazik et al. 2009).

The volume of placental transport and the types of nutrients that pass between the mother and foetus varies among squamates. The structure of the placenta is usually indicative of the degree of placental transport from mother to foetus (Thompson and Speake 2006); species with simple chorioallantoic placentae (Type I and II) transport mainly water and ions across the placenta, while the energy requirements of the embryo are sustained by the yolk. In addition to water and ions, species with complex chorioallantoic placentae (Type III and IV) also secrete lipids and amino acids from the uterine epithelium for uptake by the

embryo (Thompson and Speake 2006). However, an increasing number of exceptions to the "rule" suggest that placental type does not necessarily indicate placental function. For example, *Sceloporus jarrovi* exhibits ultrastructural specialisations of the omphaloplacenta that have functional implications that were unexpected for a species with a Type I chorioallantoic placenta (Blackburn et al. 2010). *Eulamprus quoyii* displays a placentome-like structure in its chorioallantoic placenta (Murphy et al. 2011), but unlike other species with placentomes, it is lecithotrophic rather than placentotrophic (Thompson 1977, 1981). Species in the genus *Niveoscincus* display different degrees of placentotrophy and placental structural complexity, but placental structure is not a reliable predictor of placental function (Stewart and Thompson 2009); *N. ocellatus*, the most highly placentotrophic species in the genus, exhibits the same placental structures as a predominantly lecithotrophic species, *N. microlepidotus* (Stewart and Thompson 2009). Future investigations of the molecular mechanisms of placental nutrient transport will help elucidate the relationship between structure and function in squamate placentae.

Steroid hormones

Steroid hormones play an integral role in reptilian reproduction; hormones affect the timing of vitellogenesis, ovulation and embryo expulsion, and contribute to the hypertrophy and morphological changes that occur in the uterus during pregnancy (see Girling 2002 for a review). Hormones are also key upstream transcriptional regulators for many genes and biochemical pathways that affect reproductive processes. The potential role of hormones during the evolution of viviparity in squamates has been studied using many species and is discussed in several reviews (Jones and Guillette 1982; Callard et al. 1992; Guillette 1993; Girling 2002). Our discussion, therefore, focuses on how molecular and genetic techniques have been used to investigate the role of hormones in the evolution of viviparity.

Radioimmunoassay, the most widely used form of molecular analysis to study female reproduction in squamates, uses a radiolabelled antibody (Murphy 1964) to quantify changes in plasma hormone levels (Table 1) and to quantify hormone synthesis by different reproductive organs during the female reproductive cycle (e.g. Guarino et al. 1998; Bennett and Jones 2002; Girling and Jones 2003; Painter and Moore 2005). More recently, hormone concentrations in squamates have been quantified using enzyme-linked immunosorbent assay (ELISA), using antibodies that are labelled with a fluorescent tag (e.g. Jessop et al. 2009).

Steroidogenesis of tissues in squamates has been investigated traditionally using enzyme histochemistry to identify the distribution and activity of 3-β hydroxysteroid dehydrogenase (3β-HSD) (Hoyer and Andersen 1977; Robertson 1979), an enzyme that catalyses the conversion of pregnenolone to progesterone, 17-hydroxypregnenolone to 17-hydroxyprogesterone, and dehydroepiandrosterone to androstenedione. Enzyme histochemistry has been used to demonstrate steroid synthesis by the corpora lutea (*Lacerta sicula*, Botte and Del Rio 1965; *Chamaeleo pumilus pumilus*, Veith 1974; *Nerodia* sp., Kleis-San Francisco and Callard 1986; *Chalcides chalcides*, Guarino et al. 1998; *Barisia imbricata imbricata*, Martinez-Torres et al. 2003) and placenta (*Chalcides chalcides*, Guarino et al. 1998) of squamates.

More recently, molecular approaches have been used to identify and characterise steroidogenesic enzymes in female squamates. P450 aromatase catalyses the conversion of androgens to estrogens and is a key enzyme in estrogen biosynthesis (Simpson et al. 1994). The cDNA of P450 aromatase has been cloned and sequenced in the leopard gecko (*Eublepharis macularius*), and P450 aromatase mRNA is expressed in both the thecal and granulosa cell layers in the ovary (Endo and Park 2005). P450 aromatase protein is also present in the ovary of *Uromastyx acanthinura*, where it is abundant during the period of peak estrogen production in vitellogenic females but produces minimal staining during the rest of the breeding season and in non-reproductive females (Hammouche et al. 2009). Ovarian expression of β-endorphin, which has an inhibitory effect on estrogen synthesis in reproductive tissues, is inversely correlated with the expression of P450 aromatase in *U. acanthinura* (Hammouche et al. 2009). β-Endorphin has been identified immunohistochemically in ovaries of the oviparous lizard *Podarcis sicula sicula*. As predicted, β-endorphin is more abundant in the ovaries of non-reproductive females, which produce little estrogen, than in ovaries of reproductive females, when ovarian production of estrogen is high (Polzonetti-Magni et al. 1994). Depauperate genomic and molecular resources for squamates has made the identification and localisation of steroidogenic enzymes in squamates a slow and laborious process, trialling antibodies designed for mammalian species in the hope that they will detect the homologous protein in snakes and lizards.

Much of what we understand about hormone concentrations in blood plasma and sites of hormone synthesis during reptilian reproduction comes from studies of single species. Previous inter-species comparisons of plasma hormone profiles during reproduction reveal some generalised patterns and consistent differences between oviparous and viviparous squamates, but there is also substantial variation and notable outliers (e.g. Callard et al. 1992).

Comparisons of hormone profiles in bimodally reproductive lizards will provide a robust test of current hypotheses about the role of hormones in the evolution of viviparity while minimising variation due to species differences.

Hormone receptors

The first sex steroid hormone receptors were identified using protein-binding analyses (Tokarz et al. 1981; Kleis-San Francisco and Callard 1986; Riley et al. 1988). Recently, antibodies have been used to detect estrogen receptors in the oviducts of the garter snake *Thamnophis sirtalis parietalis* (Whittier et al. 1991), progesterone receptors in the oviduct of the oviparous lizard *Podarcis sicula* (Paolucci and Di Cristo 2002), and both estrogen and progesterone receptors in the ovary of the lizard *Uromastyx acanthinura* (Hammouche et al. 2007). Gonadotrophin releasing hormone I (GnRH I), bradykinin, and their respective receptors are present in the ovary of the oviparous lizard *Calotes versicolor* (Singh et al. 2007). Immunohistochemistry and Western blotting revealed that the concentration of these hormones and their receptors fluctuate during vitellogenesis and interaction between these hormones may regulate follicular development and oocyte maturation (Singh et al. 2008).

Hormone receptors in the reproductive organs of reptiles have been identified using PCR-based methods. The first partial cDNA sequences for estrogen, progesterone and androgen receptors in a reptile were obtained from a whiptail lizard *Cnemidophorus uniparens*, and ribonuclease protection assays revealed that the mRNA expression of both estrogen and progesterone receptors varies in the oviduct of *C. uniparens* during the reproductive cycle (Young et al. 1995a). Partial cDNA sequences are also available for estrogen and androgen receptors in the leopard gecko (*Eublepharis macularius*) (Rhen and Crews 2001) and for two estrogen receptors (ESR1 and ESR2) in two snake species, *Protobothrops flavoviridis* and *Elaphe quadrivirgata* (Katsu et al. 2010).

In addition to the reproductive organs, the reptilian brain plays an important role in production and detection of sex steroids during reproduction. Oxytocin-like proteins are present in the subcommisural organ of the brain in both the oviparous lizard *Podarcis sicula* and the viviparous lizard *Chalcides chalcides*. Secretion of these oxytocin-like proteins from the subcommisural organ varies during the reproductive cycle and differs between the two species examined (Limatola D'Uva et al. 1997). Changes in the distribution and expression of hormone receptor mRNA in the brain of squamates during reproduction are also well documented. For example, the distribution of mRNA encoding estrogen and progesterone receptors in the brain changes during the reproductive cycle of two lizards, *Cnemidophorus inornatus* and *C. uniparens* (Young et al. 1995b). The expression of androgen receptors in the forebrain of leopard geckos (*Eublepharis macularius*) also changes during the female reproductive cycle (Rhen et al. 2003). Changing patterns of hormone receptor mRNAs in the brain most likely reflect the influence of fluctuations in plasma hormone concentrations on breeding behaviour (Crews and Moore 2005). The molecular tools developed to study hormone receptors in the reptilian brain will be more appropriate than tools developed for mammalian systems for investigating the role of these receptors in the evolution of squamate viviparity.

Limitations of the current molecular and genomic approaches used to study squamate viviparity

So far, we have taken a gene-by-gene, protein-by-protein approach to studying the molecular and genomic aspects of the evolution of viviparity in squamates. This tack has uncovered a wealth of information about the basic physiology and molecular biology of reproduction in squamates, but progress is frustratingly slow when compared with advances made in mammals and in model organisms. Most candidate-gene or -protein studies suggest that the molecular mechanisms underpinning reproductive physiology are shared between reptiles and mammals, but it is likely to be the differences that are the most informative when it comes to understanding the evolution of viviparity. Unfortunately, current antibody- and PCR-based techniques makes finding reproductive similarities between mammals and reptiles a self-fulfilling prophesy; particular genes and proteins are often selected for investigation because they play an important role in mammalian reproduction and they are evolutionarily conserved across vertebrates, making them easier to detect using antibodies originally designed for mammals and birds or using consensus PCR primers constructed from alignments of highly similar gene sequences from other vertebrates.

Highly divergent, rapidly evolving genes and their protein products, are logistically difficult to study using antibody- and PCR-based methods; mammalian- or avian-based antibodies cannot detect them and it is difficult to design consensus primers for genes that have low sequence similarities across vertebrates. Studies that fail to detect a mammalian homologue of a gene or protein in reptiles are common but are rarely published, but it is these "non-results" that may indicate elements of reproductive physiology that are unique to reptiles.

Limited nucleotide sequence information for squamate reptiles presents major problems for researchers aiming not only to identify genes but also to quantify gene expression.

The 'touchstone' methodology for quantifying gene expression (Bustin et al. 2009) is fluorescence-based reverse transcription quantitative real-time PCR (RT-qPCR) (Higuchi et al. 1992, 1993; Wittwer et al. 1997). Most data from RT-qPCR experiments are not absolute, but are relative measurements of gene expression in samples normalised using reference genes (e.g. Livak and Schmittgen 2001). Sample normalisation is crucial for reliable RT-qPCR as it controls for variations in RNA quality, reverse-transcription efficiency and PCR efficiency, all of which can affect quantification of the target mRNA in a sample (Bustin et al. 2009). The minimum requirement guidelines for RT-qPCR stipulate that: (1) multiple reference genes should be used to normalise samples, and (2) reference genes must show invariable expression in the different experimental conditions used (Bustin et al. 2009). Reference genes for RT-qPCR have been evaluated for many model species in molecular biology (e.g. Czechowski et al. 2005; Sindelka et al. 2006; Tang et al. 2007), a process expedited by the availability of large archives of nucleotide sequence data for these organisms. Appropriate reference genes are necessary before we can accurately quantify gene expression in the uterus or other reproductive organs during squamate pregnancy. However, a lack of sequence information for reptiles seriously hinders the identification of reference genes to conduct accurate RT-qPCR assays.

Next-generation sequencing: new discoveries and new hypotheses for studying squamate viviparity

Until recently, the main tools for DNA sequencing and de novo identification of genes were Sanger-sequenced cDNA or EST (expressed sequence tag) libraries, which are expensive, relatively low-throughput and have only a limited capability to quantify transcripts (Wang et al. 2009). Hybridisation approaches, whereby fluorescently-labelled cDNAs are incubated with custom-made microarrays, have been previously proposed as a method for studying differences in gene expression between oviparous and viviparous individuals of the bimodally reproductive lizard *Zootoca vivipara* (Freire et al. 2003). However, microarrays require a priori knowledge of sequence information to detect and quantify expressed genes (Marioni et al. 2008), which make microarrays impractical, given the lack of genomic tools and the small number of sequenced genes for reptiles.

Next-generation sequencing provides a new, high-throughput and less expensive method for sequencing genomes and transcriptomes, and can detect, identify and quantify transcript abundance within a tissue. RNA-Seq, the use of next-generation sequencing to sequence transcriptomes, is highly sensitive, detecting transcripts expressed at very low or at very high levels, and can identify multiple isoforms and splice variants of genes (Wang et al. 2009). Sequence reads can be assembled de novo without the use of an existing genome for reference (Wang et al. 2009), making this method highly attractive for research groups studying non-model organisms such as reptiles. RNA-Seq is not limited to detecting transcripts that correspond to known sequences and has the potential to discover molecular and genomic aspects of reproduction that are truly unique to reptiles. Transcriptome assembly is easier if reads can be aligned to a reference genome or reference transcript, and a new genome initiative means these genomic tools could soon be available for many reptile species. The initiative, Genome 10 K, aims to take advantage of rapid improvements in the efficiency and affordability of sequencing technologies to sequence the genome of more than 10,000 vertebrate species (Haussler et al. 2009), facilitating comparative biology on a huge scale and potentially providing genomes for more than 3,000 species of reptiles.

Several reviews provide comprehensive explanations of next-generation sequencing and the different sequencing platforms available (such as Illumina, 454 and SOLiD) (e.g. Mardis 2008; Wang et al. 2009; Wilhelm and Landry 2009), but here we discuss briefly two studies that have used RNA-seq to identify expressed genes in reptiles. Next-generation sequencing has been used to construct a large-scale multi-organ transcriptome for the garter snake (*Thamnophis elegans*), the first for an ectothermic reptile (Schwartz et al. 2010). Using a 454 sequencing platform, 1.24 million sequence reads with an average length of 366 bp were obtained from sex-specific pools of RNA that incorporated seven different tissue types from 35 individuals (Schwartz et al. 2010). The short reads were assembled into 96, 379 larger contiguous sequences, which were mined for open-reading frames and genes homologous to those in other species. Approximately 13,000 genes with homologues in other species and more than 66,000 transcripts for unidentified genes were found in the transcriptome of the garter snake, as well as nearly 100,000 single nucleotide polymorphisms (SNPs) and 190 sex-specific genes (Schwartz et al. 2010). Next-generation sequencing has also been used to determine the molecular mechanisms of cardiac hypertrophy and regression during feeding and fasting in the Burmese python (*Python molurus*) (Wall et al. 2011). Between 15 and 30 million sequence reads, 36 bp in length, were obtained from each heart sample, and reads were assembled into contigs at least 100 bp in length (Wall et al. 2011). Using basic local alignment seach tool (BLAST), these contigs were compared with genes identified from the *Anolis carolinensis* and *Gallus gallus* genomes. Of the approximately 2,774 mRNAs analysed,

464 genes were differentially expressed in the hearts of fed and fasted pythons and qPCR was used to validate differences in expression for 10 selected genes (Wall et al. 2011). These two transcriptome studies highlight the wealth of information that can be gleaned by using next-generation sequencing to investigate squamates.

RNA-Seq has the potential to identify and quantify every gene expressed in the reproductive tissues of a squamate reptile during the reproductive cycle. This technology will provide crucial gene sequence data that will help identify and reconstruct the potential evolutionary steps involved in the transition from oviparity to viviparity in squamates. A transcriptome-based approach will allow the detection of genes that are regulated during the reproductive cycle or exhibit different expression profiles in oviparous and viviparous species, potentially indicating the specific genetic or epigenetic controls that underpin the evolution of viviparity.

Next-generation sequencing technologies will also be integral in determining the genetic and epigenetic basis for the evolution of viviparity. Genome-wide comparison is a particularly powerful approach for identifying gross differences between two closely related genomes. Bimodally-reproductive squamates are ideal candidates for genome-wide comparisons; consistent differences in the sequence (genetic) or methylation pattern (epigenetic) of the genome between oviparous and viviparous individuals of bimodally reproductive species may represent regions of the genome (or epigenome) that encode the heritability of reproductive mode. Traditional subtractive methods for genome-wide comparisons, such as genomic representational difference analysis (gRDA) (Lisitsyn et al. 1993), are currently being adapted for use with next-generation sequencing, allowing greater genome coverage and higher sample throughput (Laird 2010). Sequences that are present in viviparous individuals and not oviparous ones (or vice versa) could be identified using basic local alignment search tool (BLAST) searches against either the chicken genome or the genome of *Anolis carolinensis*. An alternative, more sensitive approach to identifying differences between the genomes of oviparous and viviparous individuals might be restriction site-associated DNA (RAD) tag sequencing, a technique that yields large numbers of single nucleotide polymorphism (SNP) markers that are useful for follow up analyses in organisms without a reference genome (Baird et al. 2008). Single nucleotide polymorphism markers generated from RAD tag sequencing of oviparous and viviparous individuals of a bimodally reproductive species could be screened for possible linkage with loci that determine reproductive mode. Markers associated with reproductive mode could be identified using BLAST or used as starting points for deeper sequencing projects.

Differences in gene expression between oviparous and viviparous squamates are likely controlled at least in part by epigenetic changes in the genome, such as DNA methylation. There are now many methods of genome-wide methylation analysis which, like gRDA, are being used in combination with next-generation sequencing (Laird 2010). Differences in genome methylation patterns between oviparous and viviparous individuals of bimodally reproductive species could be detected using methylation-sensitive representational difference analysis (msRDA) (Toyota et al. 1999; Kaneda et al. 2003) in conjunction with next-generation sequencing (Laird 2010). Post-translational modifications, such as protein phosphorylation, hydrolysis and sulfation, modulate the activity and function of proteins (see Mann and Jensen 2003 for review) and are likely to contribute to differences between oviparous and viviparous squamates. Mass spectroscopy can now be used in conjunction with proteomic analyses to determine which proteins in a tissue exhibit post-translational modifications (e.g. Pang et al. 2010; Danielsen et al. 2011; Zhao et al. 2011). As we begin to accumulate sufficient genomic and proteomic information about squamate reptiles, it will become more feasible to apply these types of analyses to study the evolution of squamate viviparity.

Next-generation sequencing by no means renders antibody- and PCR-based approaches obsolete. Instead, it is likely that next-generation sequencing will become the discovery and hypothesis-generating tool of choice for studying the evolution of squamate viviparity, while techniques such as qPCR, in situ hybridisation, Western blotting, ELISA and immunohistochemistry will be continued to be necessary to rigorously test these hypotheses. There are also limitations associated with next-generation sequencing that pose unique challenges to researchers pioneering its use in squamate reptiles. Sequencing projects require access to sufficient computing resources that are able to store and analyse large amounts of data. Bioinformatics programs for genome, transcriptome and proteome analyses are constantly changing and evolving. While the number of researchers with the expertise to conduct these analyses is growing, it may be difficult to entice them to work on squamate sequencing projects rather than in more lucrative research fields. Finally, interpretation of the genes identified and their differential expression is likely to be a daunting task; while bioinformatics programs and genomic and protein databases will be able to sort some of the data, significant human interpretation of the results will also be needed due to the large number of different biological processes that are likely to be involved in the evolution of viviparity. Nevertheless, next-generation sequencing is an exciting new frontier that will help provide the basic nucleotide sequence data that is so sorely lacking for

squamate reptiles and will facilitate major advances in the molecular and genomic studies of squamate viviparity.

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