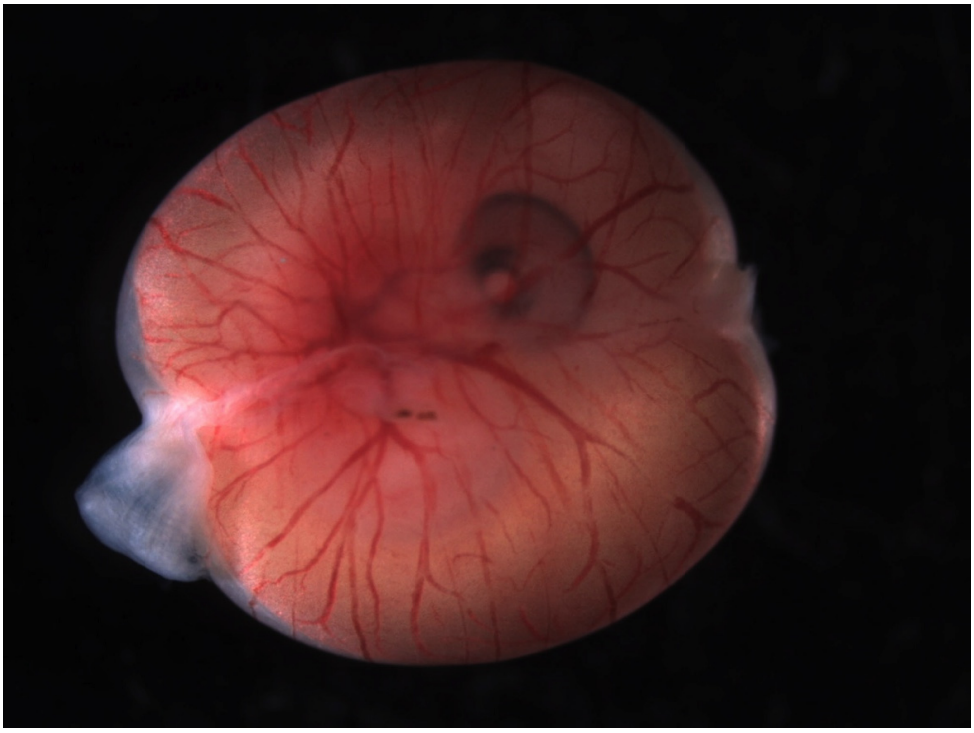
MECHANISMS OF PLACENTAL EVOLUTION: THE GENETICS AND PHYSIOLOGY OF PREGNANCY IN LIZARDS



Oliver William Griffith

July 2015

A thesis submitted in fulfilment of the requirements for the degree of Doctorate of Philosophy in the Faculty of Science at the University of Sydney

THE UNIVERSITY OF SYDNEY

Contents

Thesis Summary

The placenta is a unique example of a complex organ that has evolved independently more than 115 times in amniotes (birds, reptiles, and mammals). Placentae exchange materials including respiratory gasses, nutrients, and hormones, between the mother and embryo. The evolution of placentation is an excellent model for understanding the evolution of complex structures because the evolution of placental functions requires integrated changes to many genes and their expression. I aimed to identify the physiological and ecological factors that contribute to the evolution of placentation in vertebrates, focusing on the genetic mechanisms underpinning the evolution of placental functions, and the role of parent-offspring conflict in the evolution of placental traits.

Recent phylogenetic analyses have re-evaluated the number and frequency of transitions from oviparity to viviparity and the reverse. This re-evaluation has led to conclusions that are at odds with previous studies of the biology of squamates. I used data on the physiology and ecology of squamates to evaluate hypotheses about the number of transitions between reproductive modes in squamates and whether transitions from oviparity to viviparity have occurred as frequently as reversions from viviparity to oviparity (Chapter 2). The confluence of biological data clearly supports oviparity as the ancestral reproductive mode for squamates, with many independent origins of viviparity in this group. In this study I suggest that model-based phylogenetic analyses can produce well supported but inaccurate results, and describe how biological data can be used to test hypotheses generated by these models.

Given placentation has evolved independently many times, I assessed whether convergent evolution of placental functions is underpinned by the same genes in mammals and reptiles

(Chapter 3). I used high throughput sequencing to identify the gene expression patterns that facilitate placental functions in the placentotrophic southern grass skink (*Pseudemoia entrecasteauxii*). The grass skink has partitioned placental functions to different placental regions, with the chorioallantoic placenta being a major site of nutrient transport via membrane bound transport proteins, whilst the yolk sac placenta transports nutrients using alternative mechanisms such as the secretion of nutrient packed vesicles (Chapter 3). These placental functions are underpinned by complex gene expression patterns converging on the eutherian condition, with protein transport to embryos having evolved using the same transport proteins in both lineages. I developed a statistical approach to assess parallel evolution, which shows that similar use of genes in human and skink placenta is not the result of stochastic selection of genes but is the outcome of other yet identified biological constraints, such as, ancestral patterns of gene expression or pleiotropic effects of altering gene regulation.

Direct contact between maternal and embryonic tissue during pregnancy provides an opportunity for embryos to manipulate the rates that mothers provide resources to offspring. Given that embryos have inherited their genome from both their mother and father, there is potential for the preferred resource transfer rates to differ between mother and offspring resulting in conflict. The impacts of parent offspring conflict during pregnancy have been long known, but under-explored as a driver for the evolution of placental functions. The central assumption of the parent offspring conflict hypothesis is that embryos are capable of manipulating the supply of resources from mothers to offspring through pregnancy.

Mammals with non-invasive placentation have extra-embryonic membranes that are capable of invading maternal tissue but are prevented from doing so during pregnancy by the

properties of the uterine epithelium, which provides strong evidence for persistent conflict in mammalian placentation. I investigated whether invasive embryonic membranes were a common feature of amniotes by examining an extra-uterine pregnancy in the southern grass skink (Chapter 4). Invasive placentation is neither ancestral to amniotes nor necessary for viviparity, but is derived in therian mammals, because the extra-embryonic membranes do not attach or invade maternal tissue outside the uterus in the southern grass skink.

I used transcriptomics to identify the expression of hormones and hormone metabolic processes in the placental tissues of a range of oviparous and viviparous amniotes (Chapter 5). In this chapter I show that hormone production occurs in the embryonic membranes of amniotes, and the production of growth factors by embryonic placental tissues is an exaptation of ancestrally expressed genes. Given that embryonic membranes produce a diversity and abundance of hormones that could manipulate how mothers provide resources to offspring, I show that embryonic hormone production is a mechanism by which embryos can manipulate the function of the placenta, and regulate placental nutrient transport. This study provides fundamental support to the assumption of the parent offspring conflict hypothesis, which states that embryos must be capable of manipulating maternal tissue for conflict to lead to evolutionary innovations.

Conflict during pregnancy ultimately results in antagonistic selection on genes that are able to manipulate the outcome of the provision of resources to the embryo from the mother. Genomic imprinting is a mechanism present in viviparous mammals that prevents antagonistic selection on some genes. I tested to see if mammalian imprinted genes were also imprinted in the southern grass skink by examining polymorphisms in the transcriptomes of placental tissues (Chapter 6). I showed that the 17 most highly expressed mammalian imprinted genes exhibited bi-allelic expression in the skink and therefore cannot be

imprinted. Thus, genomic imprinting in these genes is not necessary for viviparity in amniotes, but may be the result of other features of viviparous mammal biology such as invasive placentation.

By investigating the physiology and genetic underpinning of placental functions in reptiles, I provide fundamental data for understanding the evolution of viviparity and placentation in a lineage independent of viviparous mammals. I identify key similarities and differences between reptile and mammal pregnancy that outline the limits to which comparisons between the two lineages can be made. Finally, I show that parent offspring conflict is unlikely to play a role in the evolution of nutrient transport mechanisms in the placenta, but may be a driving force in the shift from lecithotrophic (a reliance on egg yolk for embryonic development) to placentotrophic nutrient provisioning through pregnancy.

Declaration

I hereby declare that this work is my own, except where otherwise acknowledged. It has not been submitted in any form for another degree or diploma at any university or other institution. I consent to this thesis being made available for photocopying and loan under the appropriate Australian copyright laws.

Oliver Griffith

April 2015

Author contributions

Griffith, O.W., Blackburn, D.G., Brandley, M.C., Van Dyke, J.U., Whittington, C.M., Thompson, M.B. (2015) Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: A case study examining the evolution of reproductive mode in squamate reptiles. Journal of Experimental Zoology Part B: Molecular and Developmental Evolution: http://dx.doi.org/10.1002/jez.b.22614.

MB Brandley, DG Blackburn, MB Thompson, JU Van Dyke and CM Whittington contributed to initial discussion on, contributed revisions to and approved the final version of the manuscript.

Griffith, O.W., Van Dyke, J.U., Thompson, M.B. (2013) No implantation in an extrauterine pregnancy of a placentotrophic reptile. Placenta 34 (6), 510-511.

JU Van Dyke collected lizards and helped with dissections. MB Thompson and JU Van Dyke contributed revisions to and approved the final version of the manuscript.

Griffith OW, Brandley MC, Belov K, Thompson MB. Reptile pregnancy is underpinned by complex changes in uterine gene expression that converge on the eutherian condition.

MC Brandley, K Belov, and MB Thompson contributed to experimental design. MCB helped in collection of the lizards, aided with sequencing library generation and taught me to do differential gene expression analysis. All authors contributed revisions to and approved the final version of the manuscript.

Griffith, O.W., Brandley, M.C., Belov, K., Thompson, M.B.. Genomic imprinting is not necessary for the evolution of matrotrophy.

MC Brandley, K Belov, and MB Thompson contributed to experimental design. MCB helped in collection of the lizards, and aided with sequencing library generation. All authors contributed revisions to and approved the final version of the manuscript.

Griffith, O.W., , Brandley, M.C., , Whittington, C.M., Belov, K., Thompson, M.B. The evolution of embryonic hormonal signaling in amniotes.

MC Brandley, K Belov, CM Whittington, and MB Thompson contributed to experimental design. MCB helped in collection of the lizards, and aided with sequencing library generation. Furthermore MCB performed all tissue collection, processing an analysis on *Lerista bougainvillii* data. All authors contributed revisions to and approved the final version of the manuscript.

Co-author signatures

The co-authors below certify that they have contributed to publications and manuscripts in this thesis as outlined on the previous page.

Katherine Belov, Faculty of Veterinary Science, University of Sydney, NSW, 2006, Australia

Signed: _____

Daniel G Blackburn, Department of Biology, Trinity College, Hartford, CT 06106, USA.

Signed: _____

Matthew C. Brandley, School of Biological Sciences, University of Sydney, NSW, 2006, Australia

Signed: _____

viii

Mike B. Thompson, School of Biological Sciences, University of Sydney, NSW, 2006, Australia

Signed: _____

James U. Van Dyke, School of Biological Sciences, University of Sydney, NSW, 2006, Australia

Signed: _____

Camilla M. Whittington, School of Biological Sciences, University of Sydney, NSW, 2006, Australia

Signed: _____

Acknowledgements

I would like to begin by giving a huge thankyou to Mike Thompson for his support through my scientific apprenticeship. He gave me un-bridled permission to address the questions I found interesting, and found the resources that allowed me to do some really cool projects. He was a great mentor that always managed to be available when I needed advice.

Kathy Belov my second supervisor has been a huge support throughout my years as a student. Kathy has been a great mentor and has given me good guidance, challenged me to network, find funding, and publish my work. Kathy is an inspiration in her ability to collaborate and develop a comprehensive research program.

I would like to thank all the members of the Thompson lab, including Fran van den Berg, Jacquie Herbert, James Van Dyke, Matthew Brandley, Melanie Laird, Jessica Dudley, Jessie McKenna, Jess McGlashan, Camilla Whittington, Erin McDonald, and Kevin Hendrawan for supporting me through the years and for providing valuable comments on various drafts of the manuscripts in this thesis. In particular Jacquie provided great proof reading help and painstakingly checked every reference for mistakes. A special mention must go to Fran, her rigorous approach to science and statistical enquiry and her critical comments on my writing, have made it all the better. Another special mention must go to the postdocs of the Thompson lab for facilitating exciting and insightful debates about evolutionary biology, much of which has inspired the research questions I have addressed and will continue to address in the future.

I must also thank the members of the Belov lab, including Mette Lillie and Katrina Morris for teaching me molecular techniques, and Belinda Wright, for teaching me several bioinformatics tools that allowed me to think differently about my data.

I need to thank my parents for supporting me through the many years of my education. Both my parents are a huge inspiration that have shown me that the secret to success is perseverance. My mum, Dorothy Griffith, has a huge passion for creating works of art, which has contributed to my ability to think laterally and creatively. My dad, Robert Griffith, has always been fascinated by the natural world, and his fondness for the bush has been a huge part of what led me to biology in the first place.

I must also thank my brother, Dylan Griffith. Dylan was my go to person when I had any programing issues, and helped me debug many scripts. Dylan has been more of an influence on my life than he probably knows. Although our interests differ substantially, we both value the truth, and knowledge.

I need to thank various sources of funding through my PhD including, the University of Sydney Postgraduate Research Support Scheme, and The Sydney University Graduates' Union of North America for funding to attend conferences and the Australian Society of Herpetologists for funding my research directly. I also need to thank EMBL Australia for running and funding my place at the EMBL Australia PhD course, this course gave me strong footings in the cross disciplinary nature of molecular biology.

I cannot finish these acknowledgements without thanking my partner Tommy Wallace. He has been a rock that has supported my long hours in the lab, my emotional trauma when things didn't go to plan, and has been supportive on my many trips away from home to

conduct fieldwork or attend conferences. He has also made many sacrifices to support me, helped me on many field trips through the PhD and has agreed to follow me in the next stage of my career to the USA. For all of this I am incredibly grateful.

Chapter 1: General Introduction

General Introduction

Structural and physiological complexity in organisms is central to organismal evolutionary biology (Schwenk, et al. 2009). Understanding how complex structures such as organs evolve is a question fundamental to evolutionary biology (Gould 1980). Complex traits offer an intriguing puzzle to evolutionary biologists because they require integrated changes to multiple tissues that must be underpinned by multiple genetic changes (Gregory 2008). Furthermore, the evolution of complex structures typically occurs over long time-spans, with many complex structures evolving only once. To identify the mechanisms underpinning the evolution of complex phenotypes, ideally one needs a model system that includes: repeated evolution of a novel phenotype; instances where the trait has evolved relatively recently; and extant species with intermediate forms.

The placenta is an ideal model for the study of complex innovations because placentae have evolved independently multiple times, evolved relatively recently in some lineages, and are present in intermediate forms in extant taxa (Reznick, et al. 2002; Van Dyke, et al. 2014a). A placenta is an organ formed by close apposition of maternal and embryonic tissue that facilitates exchange between mother and offspring (Mossman 1937). Placentae are necessary in live bearing amniotes (reptiles and mammals) to supply respiratory gasses and water to offspring during development. For this reason, we know placentae have evolved concurrently with the evolution of live birth (viviparity) more than 115 times in amniotes (Blackburn 2014). In some lineages placentae have evolved relatively recently (such as in the skink lizard *Lerista bougainvillii*, which has both oviparous and viviparous populations) (Fairbairn, et al. 1998; Qualls and Shine 1998). Placental diversity also includes a variety of intermediate

forms in extant taxa. Most viviparous lizards have almost complete reliance on egg yolk for embryonic nutrition (lecithotrophic), but placentae that supplement embryonic nutrition through pregnancy by the transfer of nutrients have evolved in some taxa (placentotrophy) (Thompson, et al. 2000). The lizard family Scincidae contains species with a complete reliance on lecithotrophy, complete reliance on placentotrophy and species with intermediate reliance on both forms of embryonic nutrition (Ramírez-Pinilla, et al. 2011; Thompson et al. 2000; Van Dyke, et al. 2014b).

Studies on the evolution of the placentae have been dominated by two major questions: *How do new placental functions evolve?* and *Why do particular traits evolve in placental tissues?* (Enders and Carter 2004; Lynch, et al. 2015; Murphy and Thompson 2011; Stewart and Thompson 2000; Thompson, et al. 2002; Van Dyke et al. 2014a; Wilkins and Haig 2003). Lizards and snakes (squamates) represent the ideal model system to answer these fundamental questions because in this group placentation has evolved multiple times independently, evolved relatively recently in some taxa, and contains species with intermediate placental forms (Thompson and Speake 2006). Furthermore all squamates develop placentae from homologous tissues, the uterine tissue of the mother and extra-embryonic membranes of the embryo, including the chorioallantoic and yolk sac membranes (Fig. 1; Van Dyke et al. 2014a). The traditional understanding of the evolution of reproductive mode asserts that oviparity is the ancestral reproductive mode for lizards and snakes (squamates) and that viviparity has evolved independently in many squamate lineages (Blackburn 1999; Blackburn 2006; Blackburn 2014; Lee and Shine 1998).

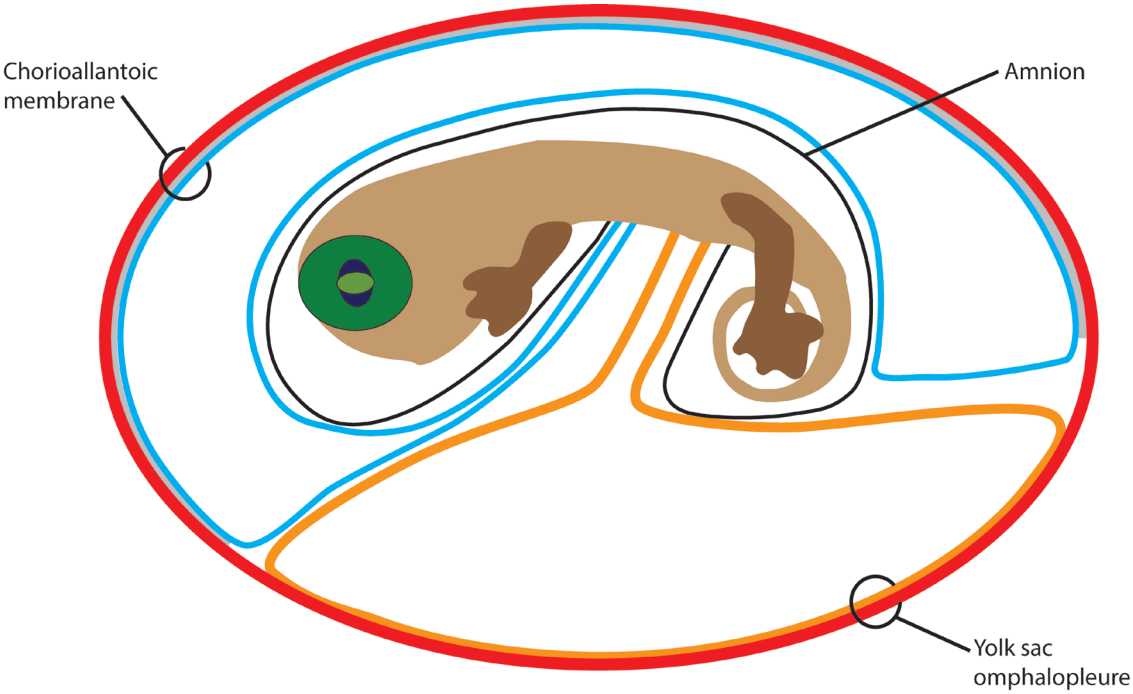


Figure 1. Simplified diagram showing the generic layout of embryonic membranes in reptiles, modified from Ferner and Mess (2011). In oviparous taxa, these structures sit inside the eggshell membrane, in viviparous species the egg is maintained in the uterus. Red – embryonic ectoderm; grey –embryonic endoderm; blue – allantoic membrane; yellow – yolk sac membrane.

Physiology of the placenta and mechanisms of nutrient transfer

The placenta has been described as one of the most diverse organs in mammals (Arnaud, et al. 2008; Gundling and Wildman 2015; Mess and Carter 2007). This diversity stems from a range of innovations that alter either the capacity for, or the regulation of, nutrient transport between the mother and embryo. The mechanisms that underpin the evolution of novel functions in the placenta are likely to be similar to those that underpin the evolution of novel traits more broadly in organisms. This makes understanding how novel traits evolve in the placenta a tool for understanding the processes that underpin the evolution of complex structures generally.

Substantial transfer of nutrients across the placenta has evolved as few as six times in the more than 115 live bearing squamate lineages (Blackburn 2014). The small number of origins of placentotrophy suggests that it is relatively difficult to evolve. Embryos require a range of nutrients to complete development including amino acids, lipids, and a large number of inorganic ions, with each nutrient requiring its own mechanism of transport through the placenta (Carter 2012; Enders and Carter 2006; Jones, et al. 2007; Knipp, et al. 1999). Comparative analyses suggest that placentotrophy supplies relatively the same ratio of nutrients as is provided to eggs at ovulation (Thompson et al. 2002), so the benefits of placentotrophy are unlikely to manifest until mechanisms for transporting each nutrient have evolved.

In viviparous skinks, there is direct contact between uterine and trophoblast epithelial cells. All nutrients transported to offspring must move across the uterine epithelium (Thompson

and Speake 2006; Van Dyke et al. 2014a). In viviparous skinks, uterine epithelial cells become more tightly "connected" by increasing the presence of tight junction molecules, which means that transport to offspring must occur through epithelial cells rather than between them (Biazik, et al. 2007; Biazik, et al. 2008). Thus, the mothers have greater control over nutrient transfer to offspring and over the chemical environment of the uterine lumen (Biazik, et al. 2010). The processes that facilitate nutrient transport are likely to vary widely with each nutrient; some nutrients, such as water, are likely to transfer relatively easily via diffusion whereas other nutrients may be mobilized by the presence of nutrient transport pumps, which mobilize nutrients between cell layers from the maternal blood to the uterine lumen. Finally nutrients that are not readily transmitted may need to be packaged and released in vesicles for uptake by the embryo (Fig. 2) (Dutta-Roy 2000; Moe 1995; Shennan and Boyd 1987; Verkman, et al. 1996). Diffusion of nutrients (Fig. 2a) occurs in all viviparous taxa, which is likely the major mechanism for transport of respiratory gasses and facilitates nutrient transfer in species that have no net uptake of nutrients through pregnancy (Stewart 2013; Stewart, et al. 1990; Van Dyke and Beaupre 2012). Both active transport mechanisms (Fig. 2b,c) are probably necessary for substantial placentotrophy in species that do not allow embryonic tissue direct access to the maternal blood supply (Adams, et al. 2005; Carter 2012). Membrane bound transport proteins are likely to be involved in the transfer of inorganic ions and small organic molecules (Fig. 2b), whilst large organic and hydrophobic nutrients are probably transferred by the packaging of materials into vesicles and then secretion of vesicles into the uterine lumen for embryonic uptake (apocrine secretion) (Fig. 2c). Given these mechanisms, I predict that the evolution of placentotrophy would require the expression of genes that facilitate each of these functions through pregnancy. To test this

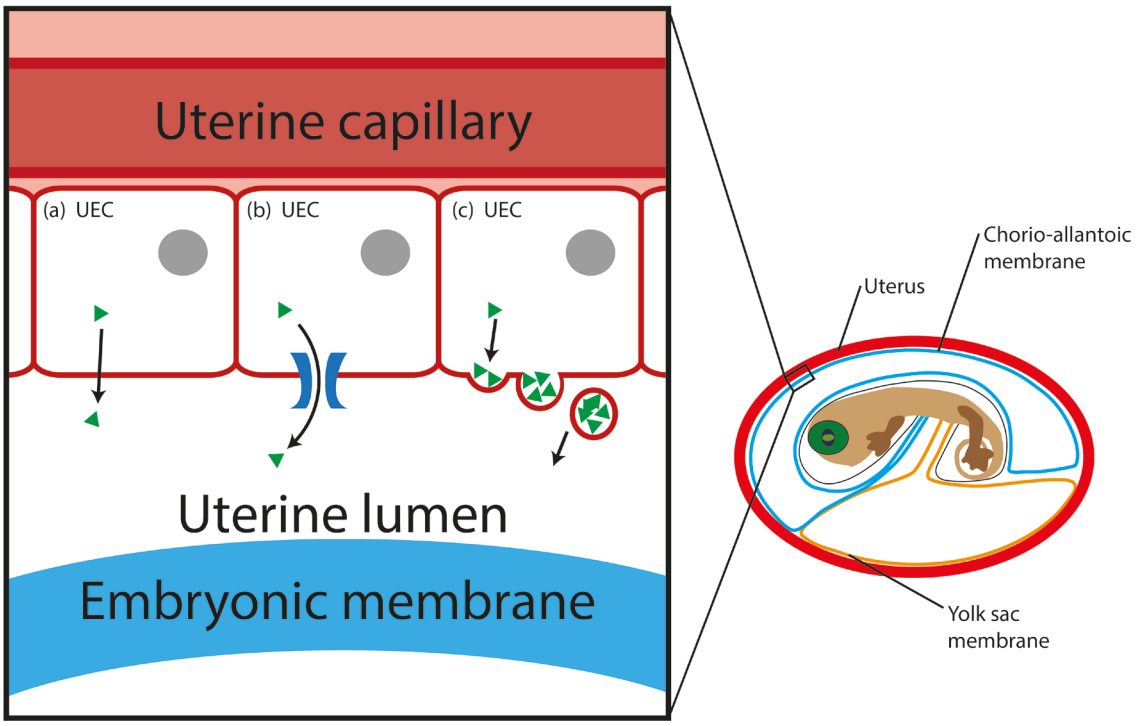


Figure 2. Diagrammatic representation of nutrient transport mechanisms from uterine epithelial cells (UEC) to the uterine lumen in a placenta during pregnancy. (a) Simple diffusion of molecules from the uterine epithelial cells, across the plasma membrane into the uterine lumen. (b) Transport mediated by membrane bound transport proteins. Nutrients inside the epithelium are actively transported across the plasma membrane requiring energy, typically in the form of ATP. (c) Nutrient transfer via apocrine secretion. Identified transport mechanisms in placental tissues taken from Carter (2012), diagrammatic representation of transport processes modified from Ladiges, et al. (2005).

prediction, I identify the genes expressed in the uterus of pregnant and non-pregnant lizards, and identify differentially expressed genes that could support nutrient transport (Chapter 3).

To understand the mechanisms underpinning the evolution of placentotrophy, it is necessary to identify the proteins that facilitate nutrient transport. Whilst the genes responsible for the mechanisms of apocrine secretion are likely to be difficult to identify, the genes that encode membrane bound nutrient transport proteins are well characterized in vertebrates, are conserved among taxa, and due to the discrete number of transporters for any particular nutrient, they are amenable to statistical analysis (He, et al. 2009; Hediger, et al. 2004; Höglund, et al. 2011). I identified the gene expression patterns that underpin several uterine functions of pregnancy in the southern grass skink by examining the expression of all the genes simultaneously (Chapter 3). I also compared uterine gene expression in the morphologically distinct, chorioallantoic and yolk sac placentas to identify if regional morphological specializations of the placenta are facilitated by different gene expression patterns. Finally, by focusing on the genes responsible for amino acid transport, I tested whether placental amino acid transport has evolved by regulation of the same genes in the placenta of the lizard *Pseudemoia entrecasteauxii* and the human placenta.

Parent offspring conflict and the function of the placenta

During pregnancy, mothers provide nutrients to developing embryos, with the goal of producing reproductively fit offspring. Conflict between parents and offspring can arise when offspring seek to obtain additional resources to increase their inclusive fitness at a cost to their mother's life time reproductive output (Crespi and Semeniuk 2004; Trivers 1974). In effect, selective pressures on embryos are likely to maximise individual fitness, but selective

pressures on mothers are likely to favour the maximisation of fitness across all current and future offspring (Trivers 1974).

Viviparity and the presence of a placenta during pregnancy facilitates parent-offspring conflict because offspring and mothers have tissues that come into direct contact, giving embryos a medium to manipulate maternal resource provisioning (Zeh and Zeh 2008). The transfer of nutrients to embryos is likely to be a major source of maternal-offspring conflict, where we expect to see adaptations in embryos such that they are provided with additional nutrients, allowing them to be born at a larger size or retain greater fat reserves for later use (Chapple, et al. 2002). If individual embryos obtain extra nutrients, mothers may bear a cost to future reproductive success, overall decreasing her fitness (Bernardo and Agosta 2005). In effect, the different pressures on mothers and embryos may result in the evolution of different and opposing mechanisms to gain control over the activity of the placenta (Crespi and Semeniuk 2004).

Parent-offspring conflict facilitates an evolutionary arms race between mother and offspring with iterative adaptations in embryos to manipulate maternal resources, and counter adaptations in mothers to reduce embryonic control over maternal resource provisioning. This arms race is expected to drive the evolution of a range of placental innovations, including changes in placental intimacy, mechanisms of embryonic communication and manipulation of mothers, as well as maternal and paternal mechanism of regulating offspring phenotype such as genomic imprinting (Crespi and Semeniuk 2004; Haig 1996a; Haig 1996b; Haig 2000).

The parent offspring conflict hypothesis has three key assumptions: 1) the relatedness between siblings is less than one; 2) the evolution of placental functions such as

placentotrophy involves potential benefits to offspring and potential costs to mothers; and 3) embryos exhibit the ability to manipulate how mothers provide resources (Crespi and Semeniuk 2004). Relatedness between siblings in any litter is always less than one in sexually reproducing organisms, and is lowered by multiple paternity (Haig 2000). Multiple paternity is normal in squamate reptiles and has been observed in many viviparous lizards and snakes, which suggests this assumption is usually met (Friesen, et al. 2014; Stapley, et al. 2003; Uller and Olsson 2008). The benefits to offspring from increased resource provisioning are well documented, as larger offspring have fitness advantages over their smaller siblings (Sinervo 1990). The costs of providing additional resources to offspring in viviparous lizards are clear, as placentotrophic organisms can endure significant costs to their own body condition during nutrient limitation (Van Dyke et al. 2014b). Furthermore, viviparity alone imposes costs on mothers as pregnant females are less mobile and have greater risk of predation than females capable of depositing eggs in the environment (Schultz, et al. 2008; Shine 2003). Hence, assumptions 1 and 2 are well supported by the literature, whilst assumption 3 has less support, primarily due to limited studies on mechanisms of embryonic regulation of placental functions outside of mammals. Identifying the mechanisms that facilitate embryonic control of placental functions is necessary to test if parent offspring conflict can facilitate the evolution of those functions.

Placental intimacy

The major categorization of placental types in mammals is the degree to which embryonic tissue breaches the maternal uterine tissue. In amniote vertebrates, placentae are composed of six ancestral tissue layers (Fig. 3) (Carter 2001; Ferner and Mess 2011; Mess and Carter 2007; Vogel 2005). In species with non-invasive placentation, all six tissue layers are left

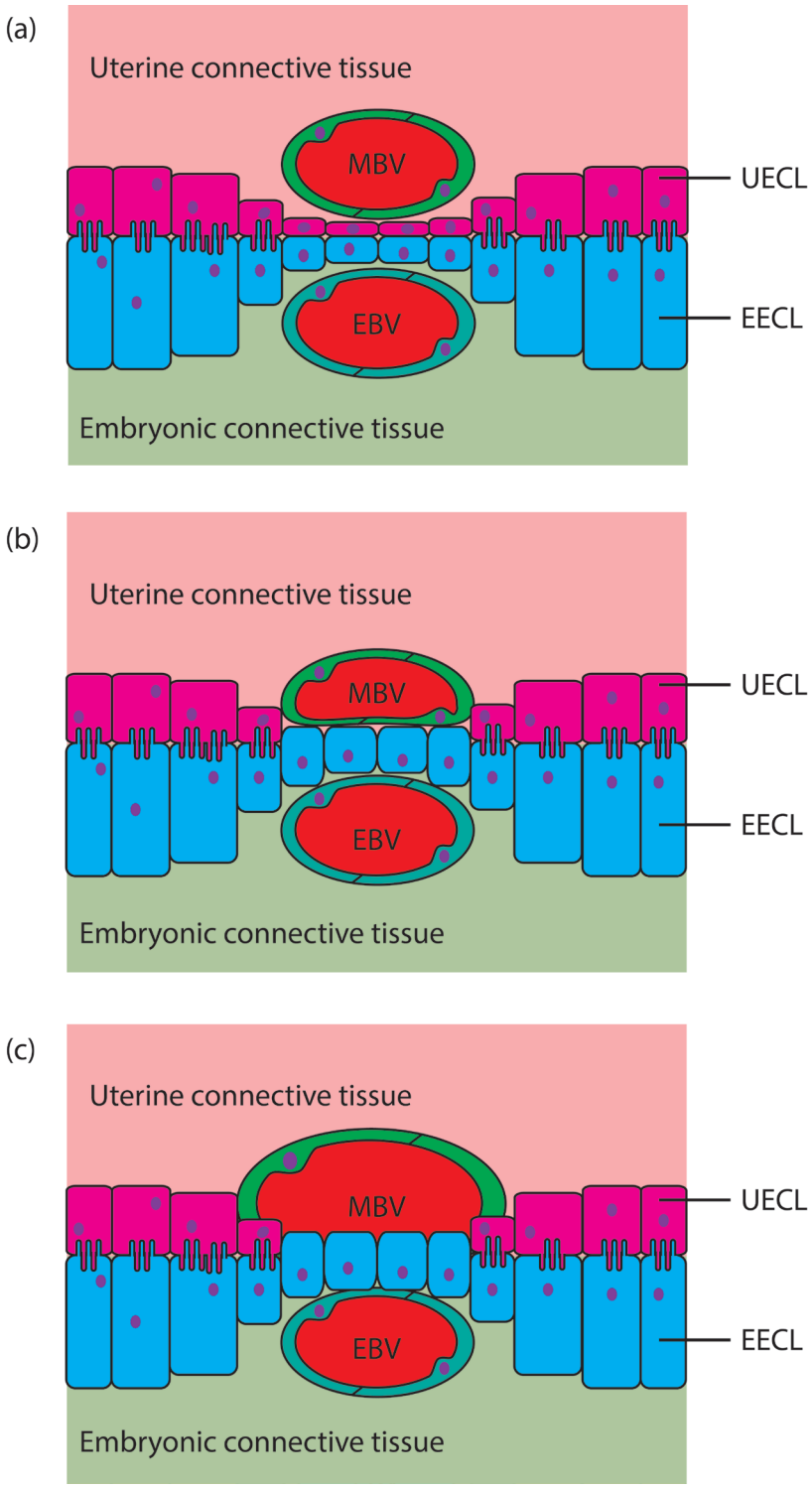


Figure 3. Diagrammatic representation of the different forms of placentation, adapted from Carter (2001), Mess & Carter (2007) and Vogel (2005). (a) Non-invasive epitheliochorial placentation; the embryonic membranes meet the uterine epithelium with no invasion. (b) Invasive endotheliochorial placentation; embryonic membranes invade through the epithelial lining of the uterus so they are in contact with the uterine endothelium. (c) Invasive haemochorial placentation; the extra embryonic membranes of the embryo invade through the uterine epithelium and endothelium, such that embryonic tissue is bathed in maternal blood. UECL: uterine epithelial cell layer, EECL: Embryonic epithelial cell layer, EBV: Embryonic blood vessel, MBV: maternal blood vessel.

intact and any nutrients transported to offspring must be mobilized across each cell layer (epitheliochorial placentation; Fig. 3a; Carter and Enders 2013). In some species, the embryonic epithelium invades maternal tissues, which decreases the number of cell layers nutrients must be transported through (Enders and Carter 2004; Wildman, et al. 2006). In some mammals, the chorioallantoic membrane invades through the uterine epithelium, so that embryonic tissue sits against the endothelial cells of the uterine blood vessels (endotheliochorial placentation, Fig. 3b). In species with the most invasive forms (haemochorial placentation Fig. 3c), embryonic epithelium is mobilized through the uterine epithelium and endothelium, and is bathed in maternal blood.

Understanding the nature of placental intimacy is fundamental to understanding the evolution of placentation. Changes in placental invasiveness completely alter how placentae function, including changes to the processes of nutrient transfer and the mechanisms of maternal-embryo communication (Bell and Ehrhardt 2002; Enders and Carter 2006; Fowden, et al. 2009). Placental invasiveness also has large effects on other pregnancy traits such as immune-recognition of the embryo and the physiology of tissues that regulate invasion (Croy, et al. 2009; D'Souza and Wagner 2014).

Phylogenetic analyses suggest the ancestral eutherian placental type was invasive haemochorial placentation (Elliot and Crespi 2009; Vogel 2005; Wildman et al. 2006). There is a diversity of placental morphologies across the eutherian phylogeny, with several independent origins of less invasive endotheliochorial and epitheliochorial forms (Garratt, et al. 2013). Furthermore evidence for hemochorial placentation being ancestral to eutherian mammals comes from the nature of the extra-embryonic membranes of eutherians outside the uterine environment. Pigs have non-invasive epitheliochorial placentation, but pig embryos

that are transplanted to ectopic sites readily invade non-uterine tissues (Samuel and Perry 1972). Therefore, although pigs exhibit non-invasive placentation, the trophoblast acts to invade maternal tissue but is prevented from doing so in the uterine environment. Even in species with invasive placentation, embryonic invasion is regulated by the uterine environment, as the mouse trophoblast is more invasive at ectopic sites than in the uterus (Cross, et al. 1994).

The evolutionary significance of placental types is still a matter of debate. No studies have been able to correlate placental invasiveness in mammals with ecological conditions or aspects of life history (Murphy 2010), but recent discussions have focused on parent offspring conflict as a driver for varying degrees of intimacy between placental species (Garratt et al. 2013). The conflict hypothesis for transitions in placental invasiveness suggests that invasive placentation evolves to give embryos greater control over nutrient provisioning as nutrient transfer occurs across fewer or no maternal tissue layers, whilst non-invasive placentation evolves from invasive forms to return control over nutrient provisioning to mothers (Garratt et al. 2013).

The vast majority of squamates exhibit non-invasive epitheliochorial placentation. However, invasive placentation has been observed in two lizard species, *Mabuya* sp. and *Trachylepis ivensi* (Blackburn and Flemming 2011; Vieira, et al. 2007). It is not clear if the embryonic membranes of all viviparous squamates are capable of invading maternal tissue but are prevented from doing so by features of the uterus as is seen in mammals with non-invasive placentation. Furthermore it is possible that invasive properties of extra-embryonic membranes are ancestral to amniotes. To test these hypotheses it is necessary to examine the

behavior of extra-embryonic membranes of reptiles when development occurs outside of the uterus, as identified in ectopic-pregnancies of pigs (Chapter 4; Samuel and Perry 1972).

Embryonic regulation of pregnancy and placental activity

Hormones are signaling molecules that are produced by tissues to alter the physiology of distant tissues or organs. In mammals, embryonic production of hormones plays a fundamental role in regulating both pregnancy and the activity of the placenta (Mendelson 2009). Placental hormone production regulates the maternal recognition of pregnancy and regulates the physiology of the placenta including the regulation of nutrient transport to offspring (Fowden and Forhead 2009; Fowden, et al. 2006; Stocco, et al. 2007; Tuckey 2005). Hormone production has been identified in the embryonic component of placental tissues in viviparous lizards, and may be a necessary function of the placenta in viviparous amniotes (Guarino, et al. 1998). Hence, the evolution of signaling by the embryonic placenta represents an ideal system to identify the mechanisms underpinning the evolution of novel phenotypes.

Embryonically produced hormones also play a key role in parent offspring conflict during pregnancy, as they are the primary mechanism by which embryos can manipulate the physiology of maternal tissues (Moore 2012; Murphy, et al. 2006). By identifying the hormones that are produced by embryonic placental tissues, and how this expression has changed during the evolution of viviparity and placentotrophy, it is possible to identify if increased placental hormone production has been an important process in the evolution of placental innovations such as nutrient transport. This question is fundamental to our understanding of parent offspring conflict and its role in the evolution of substantial placentotrophy.

I used a comparative transcriptomic approach to identify the genes that encode enzymes that produce or interact with hormones in the chorioallantois (the embryonic membrane that forms the definitive placenta in eutherian mammals and viviparous squamates) of viviparous and oviparous amniotes (Chapter 5). By using a transcriptomic approach, I was able to examine the expression of both peptide hormones, and the proteins responsible for steroid hormone synthesis. The comparative aspect of these analyses allowed identification which genes have been recruited to placental tissues during the evolution viviparity or placentotrophy, and which genes are ancestrally expressed in these tissues.

Genomic imprinting

Genomic imprinting is a process that results in genes being differentially expressed depending on the parent from which they are inherited (Holman and Kokko 2014). In effect, offspring express exclusively the maternal or paternal copy of the gene. Genomic imprinting is fundamental to the function and physiology of placentation in eutherian mammals, and the loss of genomic imprinting results in a variety of pregnancy and developmental diseases (Falls, et al. 1999). People have looked for genomic imprinting in specific genes of egg laying birds and platypus, and have extensively surveyed for imprinted genes in the chicken, but no imprinted genes have been identified (Frésard, et al. 2014; Killian, et al. 2001; Lawton, et al. 2005). Furthermore, no imprinting has been identified in the insulin like growth factor 2 (IGF2) gene in a viviparous fish (Lawton et al. 2005).

In typical non-imprinted genes, gene variants will be selected for during the embryonic phase of life that allow embryos to obtain additional resources from the mother, but in adults the same gene variant is likely to be selected against in mothers because it results in the mother bearing entire litters that have offspring that are able to obtain more resources from her than

she is willing to provide (Haig 1997). These two antagonistic selection pressures on genes associated with conflict are likely to reduce the rate at which genes can evolve to facilitate embryonic manipulation of the mother. Genomic imprinting increases the rate of maternal-offspring conflict through pregnancy, because it results in selection only acting on genes when they are inherited from a single parent. For example, only the paternally inherited IGF2 allele is expressed in embryos, which means that variations in IGF2 that are of benefit to the paternal genome will be selected for, whilst variation that could be beneficial for the maternal genome cannot be selected for as the gene is not expressed (Smith, et al. 2006). Hence, imprinted genes are likely to evolve to be beneficial to either the maternal or paternal genome.

The evolution of genomic imprinting is likely to change the rate of evolution of placental innovations as it increases the evolutionary potential of genes in the parent-offspring conflict arms race. I use a transcriptomics approach to assess for genomic imprinting in all candidate genes in the placentotrophic *P. entrecasteauxii* (Chapter 6). By testing for imprinting in this lizard we can identify if the conflict associated with imprinting is involved in the evolution of substantial placentotrophy and the evolution of placental functions more broadly.

A lizard model for the evolution of placentae and placental functions

Through this thesis, I use the Australian southern grass skink (*Pseudemoia entrecasteauxii*) as a model species to address key hypotheses regarding the physiology and evolution of placentae in reptiles. More is known about the reproductive biology of *Pseudemoia entrecasteauxii* than any other placentotrophic viviparous skink (Van Dyke et al. 2014a).

Pseudemoia entrecasteauxii has high rates of multiple paternity, which heightens parent-offspring conflict, as siblings have lower relatedness and decreased inclusive fitness (Haig 1997; Haig 1999; Stapley et al. 2003). The placental morphology of *P. entrecasteauxii* is well characterized to the ultra-structure level (Adams et al. 2005; Stewart and Thompson 1996). Both uterine and embryonic placental tissues have morphological specializations that support gas and nutrient exchange (Adams et al. 2005). Studies using a candidate gene approach have suggested several nutrient transport mechanisms and identified enzymes responsible for lipid, calcium, and macromolecule transport (Biazik, et al. 2009; Griffith, et al. 2013; Herbert, et al. 2006; Herbert, et al. 2010). *Pseudemoia entrecasteauxii* embryos have a substantial reliance on placentotrophic nourishment, with approximately half of embryonic nutrition coming from the mother via the placenta. However, as egg yolk is still a major source of embryonic nutrition, this species allows us to test hypotheses regarding the physiology of taxa with intermediate reliance on lecithotrophic and placentotrophic resource provisioning strategies (Stewart and Thompson 1993; Van Dyke et al. 2014b).

Thesis structure

This thesis is set out as a series of five manuscripts. Due to the manuscript format there may be some repetition of content between chapters. Chapter 2 is a published paper that details the biological evidence for the traditional model of reproductive mode evolution in lizards and snakes. In Chapter 3 I compare the gene expression changes that occur in the uterus of a placentotrophic lizard during pregnancy, to identify which genes may play a role in key placental functions. Chapter 4 is a published paper that presents the discovery of an extra-uterine pregnancy in a placental lizard, and discusses its significance for the evolution of

placental complexity in reptiles. In Chapter 5 I compare the gene expression of the chorioallantoic membrane of oviparous and viviparous amniotes, to identify how embryonic signaling changes during the evolution of pregnancy and placental nutrient transfer. In Chapter 6 I show that genomic imprinting does not occur in candidate genes of model viviparous lizard, and discuss the significance of this for the evolution of placental functions such as nutrient transfer. Finally in Chapter 7 I present a general discussion of my findings to draw conclusions on the evolution of placentae in reptiles.

References

Adams SM, Biazik JM, Thompson MB, Murphy CR 2005. Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: a new placental morphotype. J Morphol 264: 264-276.

Arnaud F, Varela M, Spencer TE, Palmarini M 2008. Coevolution of endogenous Betaretroviruses of sheep and their host. Cell Mol Life Sci 65: 3422-3432.

Bell AW, Ehrhardt RA 2002. Regulation of placental nutrient transport and implications for fetal growth. Nutr Res Rev 15: 211-230.

Bernardo J, Agosta SJ 2005. Evolutionary implications of hierarchical impacts of nonlethal injury on reproduction, including maternal effects. Biol J Linn Soc 86: 309-331.

Biazik JM, Thompson MB, Murphy CR 2007. The tight junctional protein occludin is found in the uterine epithelium of squamate reptiles. J Comp Physiol 177B: 935-943.

Biazik JM, Thompson MB, Murphy CR 2008. Claudin-5 is restricted to the tight junction region of uterine epithelial cells in the uterus of pregnant/gravid squamate reptiles. Anat Rec 291: 547-556.

Biazik JM, Thompson MB, Murphy CR 2009. Lysosomal and alkaline phosphatase activity indicate macromolecule transport across the uterine epithelium in two viviparous skinks with complex placenta. J Exp Zool 312B: 817-826.

Biazik JM, Thompson MB, Murphy CR 2010. Paracellular and transcellular transport across the squamate uterine epithelium. Herpetol Conserv Biol 5: 257-262.

Blackburn DG 1999. Are viviparity and egg-guarding evolutionarily labile in squamates? Herpetologica 55: 556-573.

Blackburn DG 2006. Squamate reptiles as model organisms for the evolution of viviparity. Herpetol Monogr 20: 131-146.

Blackburn DG 2014. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative Analysis. J Morphol: http://dx.doi.org/10.1002/jmor.20272.

Blackburn DG, Flemming AF 2011. Invasive implantation and intimate placental associations in a placentotrophic african lizard, *Trachylepis ivensi* (Scincidae). J Morphol 273: 137-159.
Carter AM 2001. Evolution of the placenta and fetal membranes seen in the light of molecular phylogenetics. Placenta 22: 800-807.
Carter AM 2012. Evolution of placental function in mammals: the molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. Physiol Rev 92: 1543-1576.
Carter AM, Enders AC 2013. The evolution of epitheliochorial placentation. Annu Rev Anim Biosci 1: 443-467.
Chapple DG, McCoull CJ, Swain R 2002. Changes in reproductive investment following caudal autotomy in viviparous skinks (*Niveoscincus metallicus*): lipid depletion or energetic diversion? J Herpetol 36: 480-486.
Crespi B, Semeniuk C 2004. Parent-offspring conflict in the evolution of vertebrate reproductive mode. Am Nat 163: 635-653.
Cross J, Werb Z, Fisher S 1994. Implantation and the placenta: key pieces of the development puzzle. Science 266: 1508-1518.
Croy BA, Wessels J, Linton N, Tayade C 2009. Comparison of immune cell Recruitment and function in endometrium during development of epitheliochorial (pig) and hemochorial (mouse and human) placentas. Placenta 30, Supplement: 26-31.
D'Souza AW, Wagner GP 2014. Malignant cancer and invasive placentation: A case for positive pleiotropy between endometrial and malignancy phenotypes. Evol Med Public Health 2014: 136-145.
Dutta-Roy AK 2000. Transport mechanisms for long-chain polyunsaturated fatty acids in the human placenta. Am J Clin Nutr 71: 315S-322S.
Elliot MG, Crespi BJ 2009. Phylogenetic evidence for early hemochorial placentation in Eutheria. Placenta 30: 949-967.
Enders AC, Carter AM 2004. What can comparative studies of placental structure tell us?--A Review. Placenta 25: S3-S9.
Enders AC, Carter AM 2006. Comparative placentation: Some interesting modifications for histotrophic nutrition – a review. Placenta 27, Supplement: 11-16.
Fairbairn J, Shine R, Moritz C, Frommer M 1998. Phylogenetic relationships between oviparous and viviparous populations of an Australian lizard (*Lerista bougainvillii*, Scincidae). Mol Phylogenet Evol 10: 95-103.
Falls JG, Pulford DJ, Wylie AA, Jirtle RL 1999. Genomic Imprinting: Implications for Human Disease. Am J Pathol 154: 635-647.
Ferner K, Mess A 2011. Evolution and development of fetal membranes and placentation in amniote vertebrates. Res Physiol Neurobi 178: 39-50.
Fowden AL, Forhead AJ 2009. Endocrine regulation of feto-placental growth. Horm Res 72: 257-265.
Fowden AL, Sferruzzi-Perri AN, Coan PM, Constancia M, Burton GJ 2009. Placental efficiency and adaptation: endocrine regulation. J Physiol 587: 3459-3472.
Fowden AL, Ward JW, Wooding FPB, Forhead AJ, Constancia M 2006. Programming placental nutrient transport capacity. J Physiol 572: 5-15.
Frésard L, Leroux S, Servin B, Gourichon D, Dehais P, Cristobal MS, Marsaud N, Vignoles F, Bed'hom B, Coville J-L, Hormozdiari F, Beaumont C, Zerjal T, Vignal A, Morisson

M, Lagarrigue S, Pitel F 2014. Transcriptome-wide investigation of genomic imprinting in chicken. Nucleic Acids Res 42: 3768-3782.
Friesen C, Kerns A, Mason R 2014. Factors influencing paternity in multiply mated female red-sided garter snakes and the persistent use of sperm stored over winter. Behav Ecol Sociobiol 68: 1419-1430.
Garratt M, Gaillard J-M, Brooks RC, Lemaître J-F 2013. Diversification of the eutherian placenta is associated with changes in the pace of life. P Natl Acad Sci USA 110: 7760-7765.
Gould SJ 1980. The evolutionary biology of constraint. Daedalus 109: 39-52.
Gregory TR 2008. The evolution of complex organs. Evo Edu Outreach 1: 358-389.
Griffith OW, Ujvari B, Belov K, Thompson MB 2013. Placental lipoprotein lipase (LPL) gene expression in a placentotrophic lizard, *Pseudemoia entrecasteauxii* J Exp Zool Part B 320: 465-470.
Guarino FM, Paulesu L, Cardone A, Bellini L, Ghiara G, Angelini F 1998. Endocrine activity of the corpus luteum and placenta during pregnancy in *Chalcides chalcides* (Reptilia, Squamata). Gen Comp Endocr 111: 261-270.
Gundling WE, Wildman DE 2015. A review of inter- and intraspecific variation in the eutherian placenta. 370: http://dx.doi.org/10.1098/rstb.2014.0072.
Haig D 1996a. Altercation of generations: genetic conflicts of pregnancy. Am J Reprod Immunol 35: 226-232.
Haig D 1996b. Placental hormones, genomic imprinting, and maternal—fetal communication. J Evol Biol 9: 357-380.
Haig D 1997. Parental antagonism, relatedness asymmetries, and genomic imprinting. Proc R Soc B 264: 1657-1662.
Haig D 1999. Multiple paternity and genomic imprinting. Genetics 151: 1229-1231.
Haig D 2000. The kinship theory of genomic imprinting. Annu Rev Ecol Syst 31: 9-32.
He L, Vasiliou K, Nebert DW 2009. Analysis and update of the human solute carrier (SLC) gene superfamily. Hum Genomics 3: 195-205.
Hediger M, Romero M, Peng J-B, Rolfs A, Takanaga H, Bruford E 2004. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. Pflugers Arch - Eur J Physiol 447: 465-468.
Herbert JF, Lindsay LA, Murphy CR, Thompson MB 2006. Calcium transport across the uterine epithelium of pregnant lizards. Herpetol Monogr 20: 205-211.
Herbert JF, Murphy CR, Thompson MB 2010. Calcium ATPase localization in the uterus of two species of *Pseudemoia* (Lacertilia: Scincidae) with complex placentae. Herpetol Conserv Biol 5: 290-296.
Höglund PJ, Nordström KJV, Schiöth HB, Fredriksson R 2011. The solute carrier families have a remarkably long evolutionary history with the majority of the human families present before divergence of bilaterian species. Mol Biol Evol 28: 1531-1541.
Holman L, Kokko H 2014. The evolution of genomic imprinting: costs, benefits and long-term consequences. Biol Rev 89: 568-587.
Jones HN, Powell TL, Jansson T 2007. Regulation of placental nutrient transport – A review. Placenta 28: 763-774.
Killian JK, Nolan CM, Stewart N, Munday BL, Andersen NA, Nicol S, Jirtle RL 2001. Monotreme IGF2 expression and ancestral origin of genomic imprinting. J Exp Zool 291: 205-212.

Knipp GT, Audus KL, Soares MJ 1999. Nutrient transport across the placenta. Adv Drug Deliv Reg 38: 41-58.
Ladiges P, Evans B, Saint R, Knox B. 2005. *Biology an Australian Focus*. McGraw-Hill Australia, North Ryde, Australia.
Lawton BR, Sevigny L, Obergfell C, Reznick D, O'Neill RJ, O'Neill MJ 2005. Allelic expression of *IGF2*, in live-bearing, matrotrophic fishes. Dev Genes Evol 215: 207-212.
Lee MSY, Shine R 1998. Reptilian Viviparity and Dollo's Law. Evolution 52: 1441-1450.
Lynch Vincent J, Nnamani Mauris C, Kapusta A, Brayer K, Plaza Silvia L, Mazur Erik C, Emera D, Sheikh Shehzad Z, Grützner F, Bauersachs S, Graf A, Young Steven L, Lieb Jason D, DeMayo Francesco J, Feschotte C, Wagner Günter P 2015. Ancient transposable elements transformed the uterine regulatory landscape and transcriptome during the evolution of mammalian pregnancy. Cell Rep 10: 551-561.
Mendelson CR 2009. Minireview: Fetal-maternal hormonal signaling in pregnancy and labor. Mol Endocrinol 23: 947-954.
Mess A, Carter AM 2007. Evolution of the placenta during the early radiation of placental mammals. Comp Biochem Phys A 148A: 769-779.
Moe AJ 1995. Placental amino acid transport. Am J Physiol-Cell Ph 268: C1321-C1331.
Moore T 2012. Review: parent-offspring conflict and the control of placental function. Placenta 33, Supplement: S33-S36.
Mossman H. 1937. *Comparative Morphogenesis of the Fetal Membranes and Accessory Uterine Structures.* Carnegie Institution of Washington Publication.
Murphy B, Thompson M 2011. A review of the evolution of viviparity in squamate reptiles: the past, present and future role of molecular biology and genomics. J Comp Physiol 181B: 575-594.
Murphy CR 2010. Comparative placentation, epithelial plasticity and the 'efficient barrier hypothesis'. Herpetol Conserv Biol 5: 320-323.
Murphy VE, Smith R, Giles WB, Clifton VL 2006. Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocr Rev 27: 141-169.
Qualls CP, Shine R 1998. *Lerista bougainvillii*, a case study for the evolution of viviparity in reptiles. J Evol Biol 11: 63-78.
Ramírez-Pinilla MP, Rueda ED, Stashenko E 2011. Transplacental nutrient transfer during gestation in the Andean lizard *Mabuya* sp (Squamata, Scincidae). J Comp Physiol 181B: 249-268.
Reznick DN, Mateos M, Springer MS 2002. Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. Science 298: 1018-1020.
Samuel CA, Perry JS 1972. Ultrastructure of pig trophoblast transplanted to an ectopic site in uterine wall. J Anat 113: 139-149.
Schultz TJ, Webb JK, Christian KA 2008. The physiological cost of pregnancy in a tropical viviparous snake. Copeia 2008: 637-642.
Schwenk K, Padilla DK, Bakken GS, Full RJ 2009. Grand challenges in organismal biology. Integr Comp Biol 49: 7-14.
Shennan DB, Boyd CAR 1987. Ion transport by the placenta: a review of membrane transport systems. Biochim Biophys Acta 906: 437-457.
Shine R 2003. Effects of pregnancy on locomotor performance: an experimental study on lizards. Oecologia 136: 450-456.

Sinervo B 1990. The evolution of maternal investment in lizards: an experimental and comparative analysis of egg size and its effects on offspring performance. Evolution 44: 279-294.
Smith FM, Garfield AS, Ward A 2006. Regulation of growth and metabolism by imprinted genes. Cytogenet Genome Res 113: 279-291.
Stapley J, Hayes CM, Scott Keogh J 2003. Population genetic differentiation and multiple paternity determined by novel microsatellite markers from the mountain log skink (*Pseudemoia entrecasteauxii*). Mol Ecol Notes 3: 291-293.
Stewart JR 2013. Fetal nutrition in lecithotrophic squamate reptiles: toward a comprehensive model for evolution of viviparity and placentation. J Morphol 274: 824-843.
Stewart JR, Blackburn DG, Baxter DC, Hoffman LH 1990. Nutritional provision to embryos in a predominantly lecithotrophic placental reptile, *Thamnophis ordinoides* (Squamata: Serpentes). Physiol Zool 63: 722-734.
Stewart JR, Thompson MB 1993. A novel pattern of embryonic nutrition in a viviparous reptile. J Evolution Biol 174: 97-108.
Stewart JR, Thompson MB 1996. Evolution of reptilian placentation: development of extraembryonic membranes of the Australian scincid lizards, *Bassiana duperreyi* (oviparous) and *Pseudemoia entrecasteauxii* (viviparous). J Morph 227: 349-370.
Stewart JR, Thompson MB 2000. Evolution of placentation among squamate reptiles: recent research and future directions. Comp Biochem Phys A 127A: 411-431.
Stocco C, Telleria C, Gibori G 2007. The molecular control of corpus luteum formation, function, and regression. Endocr Rev 28: 117-149.
Thompson MB, Speake BK 2006. A review of the evolution of viviparity in lizards: structure, function and physiology of the placenta. J Comp Physiol 176B: 179-189.
Thompson MB, Stewart JR, Speake BK 2000. Comparison of nutrient transport across the placenta of lizards differing in placental complexity. Comp Biochem Phys 127A: 469-479.
Thompson MB, Stewart JR, Speake BK, Hosie MJ, Murphy CR 2002. Evolution of viviparity: what can Australian lizards tell us? Comp Biochem Phys 131B: 631-643.
Trivers RL 1974. Parent-offspring conflict. Am Zool 14: 249-264.
Tuckey RC 2005. Progesterone synthesis by the human placenta. Placenta 26: 273-281.
Uller T, Olsson M 2008. Multiple paternity in reptiles: patterns and processes. Mol Ecol 17: 2566-2580.
Van Dyke JU, Beaupre SJ 2012. Stable isotope tracer reveals that viviparous snakes transport amino acids to offspring during gestation. J Exp Biol 215: 760-765.
Van Dyke JU, Brandley MC, Thompson MB 2014a. The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. Reproduction 147: R15-R26.
Van Dyke JU, Griffith OW, Thompson MB 2014b. High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. Am Nat 184: 198-210.
Verkman AS, van Hoek AN, Ma T, Frigeri A, Skach WR, Mitra A, Tamarappoo BK, Farinas J 1996. Water transport across mammalian cell membranes. Am J Physiol 270: C12-30.
Vieira S, de Perez G, Ramírez-Pinilla MP 2007. Invasive cells in the pacentome of Andean populations of *Mabuya*: an endotheliochorial contribution to the placenta? Anat Rec 290: 1508-1518.

Vogel P 2005. The current molecular phylogeny of eutherian mammals challenges previous interpretations of placental evolution. Placenta 26: 591-596.
Wildman DE, Chen C, Erez O, Grossman LI, Goodman M, Romero R 2006. Evolution of the mammalian placenta revealed by phylogenetic analysis. Proc Natl Acad Sci USA 103: 3203-3208.
Wilkins JF, Haig D 2003. What good is genomic imprinting: the function of parent-specific gene expression. Nat Rev Genet 4: 359-368.
Zeh JA, Zeh DW 2008. Viviparity-driven conflict. Ann NY Acad Sci 1133: 126-148.

Chapter 2: Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: A case study examining the evolution of reproductive mode in squamate reptiles

Published manuscript:

Griffith OW, Blackburn DG, Brandley MC, Van Dyke JU, Whittington CM, Thompson MB 2015. Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: a case study examining the evolution of reproductive mode in squamate reptiles. J Exp Zool Part B: http://dx.doi.org/10.1002/jez.b.22614.

Title: **Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: A case study examining the evolution of reproductive mode in squamate reptiles**

Oliver W. Griffith[1]*, Daniel G. Blackburn[2], Matthew C. Brandley[1], James U. Van Dyke[1], Camilla M. Whittington[1], Michael B. Thompson[1]

[1]School of Biological Sciences, Heydon Laurence Building (A08), University of Sydney, Camperdown, NSW 2006, Australia

[2]Department of Biology, Trinity College, Hartford, CT 06106, USA.

Correspondence t:o Oliver Griffith; School of Biological Sciences, Heydon Laurence Building (A08), University of Sydney, Camperdown, NSW 2006, Australia; email: oli.griffith@gmail.com; Phone: +614 3209 3216; Fax: +61 2 9351 4119

Running title: Evolution of squamate reproductive mode

Accepted for publication in the *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*

Abstract

To understand evolutionary transformations it is necessary to identify the character states of extinct ancestors. Ancestral character state reconstruction is inherently difficult because it requires an accurate phylogeny, character state data, and a statistical model of transition rates and is fundamentally constrained by missing data such as extinct taxa. We argue that model based ancestral character state reconstruction should be used to generate hypotheses but should not be considered an analytical endpoint. Using the evolution of viviparity and reversals to oviparity in squamates as a case study, we show how anatomical, physiological, and ecological data can be used to evaluate hypotheses about evolutionary transitions. The evolution of squamate viviparity requires changes to the timing of reproductive events and the successive loss of features responsible for building an eggshell. A reversal to oviparity requires that those lost traits re-evolve. We argue that the re-evolution of oviparity is inherently more difficult than the reverse. We outline how the inviability of intermediate phenotypes might present physiological barriers to reversals from viviparity to oviparity. Finally, we show that ecological data supports an oviparous ancestral state for squamates and multiple transitions to viviparity. In summary, we conclude that the first squamates were oviparous, that frequent transitions to viviparity have occurred, and that reversals to oviparity in viviparous lineages either have not occurred or are exceedingly rare. As this evidence supports conclusions that differ from previous ancestral state reconstructions, our paper highlights the importance of incorporating biological evidence to evaluate model-generated hypotheses.

Key words: phylogenetic, viviparity, placenta, live birth, parity mode, Squamata, eggshell

Introduction

Ancestral character state reconstruction is a powerful tool for modelling the evolution of traits in a phylogenetic context. Predicting ancestral states is fundamentally constrained by missing data, including the absence of extinct (or undiscovered) taxa, appropriate estimates of transition rates between character states, knowledge of initial character states, and accuracy of phylogenetic trees (Goldberg and Igić 2008; Litsios and Salamin 2012; Wright et al., In Press). These analyses have produced some surprising results including the re-evolution of insect wings (Whiting et al 2003) and vertebrate limbs (Kohlsdorf and Wagner 2006, Brandley et al 2008, Kohlsdorf et al 2010). We argue that ancestral state reconstructions are powerful for generating hypotheses of trait evolution, but they should not be interpreted as analytical end points. Prior to becoming new evolutionary paradigms, these hypotheses require interpretation in light of existing knowledge about the biology of the traits examined.

Here, we use the evolution of parity mode in squamate reptiles (lizards and snakes) as a case study to demonstrate how biological data, in particular morphology and physiology, can be used to evaluate hypotheses of character state evolution generated via ancestral state reconstruction. Modern statistical methods of ancestral state reconstruction require three pieces of information: a phylogeny, character state data for taxa in the study (e.g. oviparity and viviparity), and a statistical model of evolution that incorporates parameters such as the rate of transition between character states. With this information, one can estimate the character state of hypothetical ancestors of the sampled taxa, quantify how often transitions between each state occur across the phylogeny, and calculate the relative rate at which they occur.

Pyron and Burbrink (2014; P&B hereafter) used phylogenetic ancestral state reconstruction to evaluate how reproductive mode has evolved in squamate reptiles. The traditional conceptual view of the evolution of reproductive mode in squamates posits that oviparity is the ancestral state from which viviparity evolved independently, perhaps up to 115 times (Blackburn '99, 2006, 2014, Lee and Shine '98). Moreover, this view assumes that evolutionary reversals from viviparity to the ancestral oviparous state are impossible or exceedingly rare (Neill '64; Tinkle and Gibbons '77; Lee and Shine '98; Lynch and Wagner 2010). In contrast to the traditional view of parity mode evolution, P&B's analyses support viviparity as the ancestral reproductive mode for the most recent common ancestor of all squamates, and suggest that oviparity evolved from viviparous ancestry more frequently than the traditional opposite scenario. For example, one of their models suggests 34 origins of viviparity and 59 viviparity-to-oviparity reversals. The hypothesis that oviparity evolves from a viviparous ancestor is not new (de Fraipont et al., '99; Lynch and Wagner, 2010; Fenwick et al., 2012), but the number of transitions from viviparity to oviparity estimated by P&B are radically different from those proposed in any previous study.

In the course of estimating ancestral states, P&B statistically evaluated multiple models of evolution using the Akaike Information Criterion (AIC; Akaike, '74) as an indicator of model fit, including models that assume no reversals to oviparity. All models that constrained any parameter were statistically worse fits to the data than the model where all parameters vary, including the rate of evolution between oviparity and viviparity. This unconstrained model therefore underlies the major conclusions of P&B's study. We do not dispute that the unconstrained model is a statistically better fit than a model that assumes the impossibility of

reversals. Rather, we contend that the model is flawed because the anatomy, physiology, ecology, behaviour, and environment do constrain the viability of transitions between reproductive modes.

Here, we re-evaluate P&B's provocative hypotheses that reversals from viviparity to oviparity are common in squamate reptiles, and that the most recent common ancestor of this group was viviparous. We first discuss the influence of phylogenetic error, particularly in relation placental evolution. We then review the existing biological evidence putatively supporting reversals from viviparity to oviparity. We discuss morphological and physiological factors that would likely prevent transitions from viviparity to oviparity. Finally, we evaluate the ecological factors that influence the likelihood of transitions between viviparity and oviparity.

Phylogenetic accuracy

The results of ancestral state reconstruction analyses are fundamentally reliant on the underlying phylogeny (or phylogenies) used in the analysis. For their phylogenetic framework, P&B used the single maximum likelihood tree inferred by Pyron et al (2014). Thus, any instance of phylogenetic error could artificially inflate the number of evolutionary transitions between both reproductive states (see Duchene and Lanfear, In Press). We do not present an exhaustive list of potential phylogenetic errors, but instead focus on a particularly striking example of how the biology of an organism can be used to evaluate viviparous to oviparous transitions inferred by ancestral state reconstruction.

The skink genus *Pseudemoia* is an important model for studying the evolution of viviparity and placentation in squamate reptiles (Stewart and Thompson, 2003, 2009a; Thompson et al.,

2000; Griffith et al., 2013a; Van Dyke et al., 2014a). All *Pseudemoia* are viviparous, and all species in the genus that have been examined have a complex placenta that transports substantial amounts of nutrients to offspring during pregnancy (Stewart and Thompson, '93; Thompson and Stewart, '94; Thompson et al., '99; Thompson et al., 2000; Adams et al., 2005; Itonaga et al., 2012). Transport in the *Pseudemoia* placenta requires complex transport mechanisms that involve changes in morphology and gene expression (Griffith et al., 2013b; Van Dyke et al., 2014a). Specifically, each species examined so far has a specialized region of the chorioallantoic placenta, known as the placentome, that is highly folded, lined with secretory and absorptive cells, and likely facilitates nutrient transport (Stewart et al., 2006; Biazik et al., 2009; Stewart and Blackburn, 2014). The evolution of a complex placenta and substantial placentotrophy is rare in squamates, occurring approximately six times in extant squamate reptiles (Blackburn 2014).

In the phylogeny used by P&B, *Pseudemoia* is not monophyletic with respect to *Bassiana duperreyi*; the latter being the sister taxon to *P. entrecasteauxii*. *Bassiana duperreyi* is oviparous, and P&B's ancestral state reconstruction analyses identify this as a case of reversal from viviparity to oviparity. If oviparity did re-evolve from a lineage that had extensive modifications to support complex placentation, we would expect some remnants of this history to be present in the morphology of the uterus and fetal membranes. However, *B. duperreyi* lacks a placentome, and both the uterus and extra-embryonic membrane resemble those of other oviparous squamates (Stewart and Thompson, '96). The alternative less parsimonious scenario is that the complex placenta evolved twice in *Pseudemoia*. We interpret the rarity of the complex placenta amongst squamates as evidence that it is evolutionarily "difficult", and thus it is exceedingly unlikely that the morphological and physiological changes associated with complex placentation evolve independently in the

same genus. The positioning of *B. duperreyi* within *Pseudemoia* is incongruent with recent phylogenies of these taxa (Haines et al. 2014; Brandley et al, In Press), and therefore an example of how phylogenetic error may result in provocative but erroneous, conclusions.

Given the size of the phylogeny used in P&B's ancestral state reconstruction analyses, it may be unreasonable to expect authors using any large phylogenetic tree to validate each estimated transition using the primary literature. Rather, we argue that the minimum due diligence when inferring patterns of evolution using ancestral state reconstruction is to incorporate phylogenetic uncertainty. For example, it is commonplace to estimate ancestral states over a distribution of trees from non-parametric bootstrap or Bayesian analyses (although the latter may not be feasible because the P&B data set is so large); this would have the effect of minimizing the influence of poorly supported parts of the tree and provide at least a crude measure of precision when estimating the number and rate of evolutionary transitions.

Egg teeth and evidence for reversals from viviparity to oviparity

The principle line of biological evidence that has been presented repeatedly to support the putative reversals to oviparity from viviparity is the reported absence of an egg tooth in the oviparous Arabian Sand Boa *Eryx jayakari* (Lynch and Wagner, 2010; Pyron and Burbrink, 2014; Pyron and Burbrink, In Press). Egg teeth are structures used by oviparous species to break through the eggshell at hatching. The egg tooth has been proposed to be lost in viviparous forms that lack an eggshell because it is not needed to break through embryonic membranes alone (Lynch and Wagner, 2010). The absence of an egg tooth in an oviparous

squamate could indicate that oviparity has re-evolved from a viviparous ancestor that did not need to hatch from an egg.

The absence of an egg-tooth in the oviparous *E. jayakari* was reported based on observations of hatching behaviour and gross morphology of live hatchlings (Staub and Emberton, 2002). However, egg teeth are deciduous and rapidly shed after birth and therefore only a temporary feature of neonatal squamates. Thus, the reported absence of an egg tooth in neonates is not sufficient evidence that an egg tooth is never present during development in this species. In fact, egg teeth are present in some viviparous squamates, including *Boa constrictor* (Figure 1), a congener of *E. jayakari*, *Eryx colubrinus* (Anan'eva and Orlov, 2013), and in a wide variety of viviparous pit vipers, although often in reduced form (Fitch, '60; Klauber, '72; Anan'eva and Orlov, 2013). Thus, there is limited evidence that egg teeth have been lost in viviparous squamate taxa. Furthermore, the presence of egg teeth in related viviparous taxa means that the absence of egg teeth in oviparous *E. jayakari* is evolutionarily uninformative. Even if the lack of an egg tooth in *E. jayakari* is eventually confirmed, more data are required on the egg teeth of other oviparous taxa that are suspected to have evolved from viviparous ancestors before this line of evidence can be regarded as definitively supporting a reversal to oviparity.

Eggshell complexity and barriers to reversals

The evolution of viviparity occurs by adjusting the timing of reproductive events, increasing uterine vasculature, and by the loss of features such as eggshell and the uterine glands that construct it, as well as changes to nesting behaviour (Jones et al., '91; Blackburn, 2006; Thompson and Speake, 2006; Stewart, 2013; Stewart and Blackburn, 2014; Whittington et

Figure 1. Newborn *Boa constrictor* with egg tooth (blue arrow) below the rostral scale. Photo provided by Mitchell Kranz, Better Boa Inc, Calgary Alberta.

al., 2015). Reversal to oviparity would not simply involve reversing the order of these events (summarized in Figure 2). The re-evolution of the eggshell is not trivial and would require the re-evolution of a host of correlated changes:

(i) development of uterine glands to synthesize and secrete shell fibres;

(ii) careful timing of shell gland activity to ensure the eggshell is deposited with the correct thickness;

(iii) turning of the fertilized egg by oviductal musculature to wrap shell fibres around it; and

(iv) activation of uterine epithelial cells to secrete calcium soon after ovulation rather than late in gestation (Herbert et al., 2006, 2010).

Due to their cost of maintenance in the absence of fitness benefits, lineages that have been viviparous for millions of years would have likely lost the genetic machinery that underlies oviparous physiological processes. Even if not under the influence of natural selection, we would still expect changes due to genetic drift. For example, genome sequences indicate that oviparous amniotes typically have three vitellogenin genes, whilst the oviparous platypus retains one vitellogenin gene plus a pseudogene, and viviparous therians have lost all copies of this gene (Warren et al., 2008). Progressive gene loss during mammalian evolution (rather than simple loss of gene expression alone) has occurred through the mutations and indels that now encode premature stop codons and frame-shifts (Brawand et al., 2008). The degradation of oviparity-specific genes during the evolution of viviparity means that the genes themselves would need to be re-evolved (not just re-expressed) to allow re-evolution of oviparity. The loss of these complex physiological features responsible for oviparity suggests that transitions

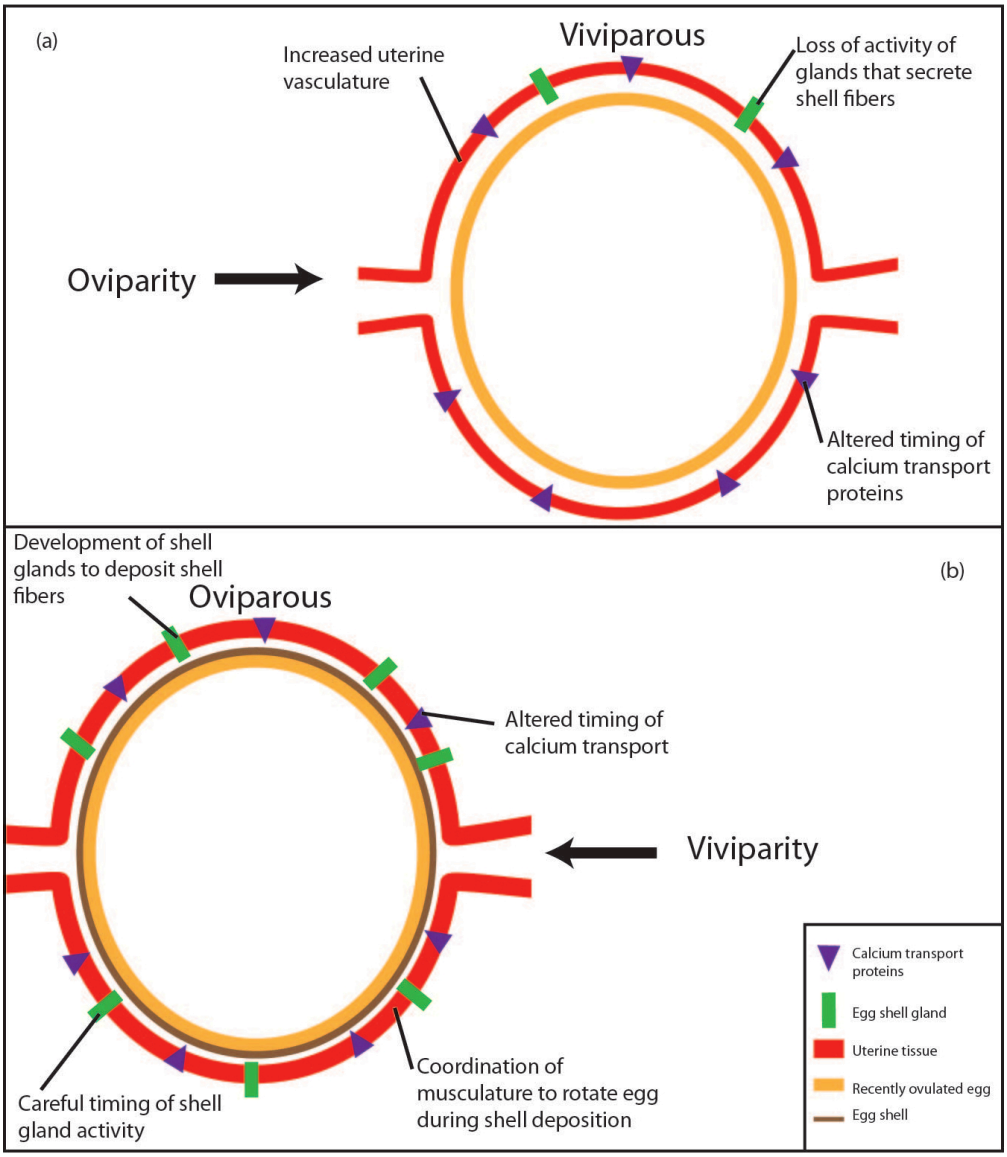


Figure 2. Diagram illustrating changes that are necessary for transitions from oviparity to viviparity (a) and reversals from viviparity to oviparity (b).

to oviparity would require the evolution of new genetic pathways. However, eggshell structure in oviparous squamates shows little evidence of multiple origins. With the exception of three gekkotan families (Gekkonidae, Phyllodactylidae, Sphaerodactylidae), which have highly calcified eggshells (Bustard, '68; Packard et al., '77, '82), most oviparous squamates have very similar parchment-like eggshells, regardless of phylogenetic placement. The phylogenetic distribution of shell types in squamates is therefore not consistent with the hypothesis that eggshells have re-evolved multiple times across the Squamata.

For reversals to oviparity to be successful, intermediate stages would need to be physiologically viable. A major function of the calcified eggshell is to protect terrestrial eggs from desiccation during embryogenesis (Packard et al., '77; Packard et al., '82; Shine and Thompson, 2006). In contrast, a necessary step in the transition from oviparity to viviparity is an increase in egg retention to the point at which a full term embryo is deposited into the environment (e.g. Packard et al., '77). The transition from oviparity to viviparity eliminates desiccation as a risk to developing embryos as long as the mother is sufficiently hydrated (Shine and Thompson 2006; Dupoué et al., In Press). However, the retention of embryos to full term within the mother requires that the mother facilitate gas exchange across the uterus. In viviparous taxa, gas exchange is facilitated by the close apposition of maternal and uterine capillary beds, in which both uterine and embryonic capillaries are separated by only very thin epithelia and (at most) a vestige of the eggshell (Parker et al., 2010). In contrast, the presence of any calcified eggshell in between maternal and embryonic tissues is likely to greatly impede gas exchange (Yaron '85; Blackburn, '95, '98; Qualls, '96; Heulin et al., 2002; Thompson et al., 2004). Thus, viviparous squamate embryos often develop surrounded by only a very thin eggshell membrane, with little or no calcification (Hoffman, '70; Blackburn and Lorenz, 2003; Stewart and Brasch, 2003; Blackburn et al., 2009). In some

cases, the eggshell is entirely lost during development (Guillette and Jones, '85; Blackburn, '93a; Stewart and Thompson, '98, 2009b; Murphy et al., 2011; Blackburn and Flemming, 2012), as confirmed by electron microscopy (Ghiara et al., '87; Adams et al., 2005; Ramírez-Pinilla et al., 2006; Blackburn et al., 2010; Anderson et al., 2011). Studies of *Zootoca vivipara* show that egg retention time and shell thickness are inversely related, with eggshell thickness being substantially reduced in oviparous forms with extended egg- retention and in viviparous populations (e.g. Heulin et al., 2002; Stewart et al., 2004). The same pattern has also been reported in other reproductively bimodal squamates (e.g. Qualls, '96; Stewart et al., 2010).

An eggshell would need to re-evolve before oviparity could evolve from viviparity because it would again be necessary to prevent oviparous embryos from desiccating. However, the evolution of a calcified eggshell prior to the transition to oviparity would result in greatly restricted gas exchange that could result in developmental failure in the viviparous condition (Mathies and Andrews '99; Parker and Andrews 2006). These constraints suggest that viviparity is a fitness valley, where selection may prevent the re-emergence of intermediate forms between viviparity and oviparity (Blackburn '95). We suggest that the incompatibility of these two drivers of selection make reversal from viviparity back to oviparity extremely unlikely.

Ecological influences on transitions between viviparity and oviparity

Ecological factors are likely to strongly alter the frequency of transitions between oviparity and viviparity. One ecological factor that affects the transition rates between reproductive

modes is in aquatic systems, where reversals to oviparity are fundamentally constrained if organisms are incapable of moving onto land to deposit eggs. Viviparous sea snakes (Hydrophiidae) and file snakes (Acrochordidae) are constrained to aquatic environments due to adaptions for underwater life (Cogger, 2014). As a result, both groups are entirely viviparous. A second factor is the association of viviparity with cold climates; cold temperatures limit developmental success in oviparous squamates because egg temperature equilibrates to nest temperature, whilst in viviparous squamates developmental temperature can be increased by maternal basking (Shine ‘85, 2014). Indeed, the cold-climate hypothesis suggests that viviparity allows females to regulate the thermal conditions experienced by developing embryos in cold climates, while oviparity forces eggs to develop in potentially unsuitable thermal conditions in cold climates (Weekes, ‘35; Sergeev, ‘40; Shine, 2014). The evolution of viviparity in response to cold climates is well supported by both the biogeography of squamates and incubation experiments (e.g. Qualls et al., ‘95; Andrews, 2000; Shine, 2004). Therefore, viviparity provides a selective advantage over oviparity in cold climates. Pyron and Burbrink (2014) invoke a modification of the cold-climate hypothesis for the evolution of viviparity to explain how oviparity might evolve from viviparity. In warm climates, oviparous embryos do not face the potentially deleterious effects of sub-optimal temperatures, and therefore P&B suggest that the selective benefits of viviparity would be lost. Furthermore, they argue that metabolic costs of pregnancy and embryonic development, as well as fitness costs of thermoregulation and predator avoidance, should be higher in viviparous species than in oviparous species. Thus, viviparity should, in total, be more costly than oviparity in warm climates, and viviparous species that enter warm climates should encounter selective pressures that promote transitions to oviparity (Pyron and

Burbrink, 2014). For simplicity, we will call this the warm-climate hypothesis for reversions to oviparity.

If the warm-climate hypothesis is true, we would expect both the proportion and number of species that were viviparous to decrease as temperature increases, because viviparity would be a less successful strategy than oviparity in warm climates. In contrast, P&B's data from lizards and snakes (their Figure 3) show that although the proportion of species that are viviparous decreases with increasing temperature (because there is a greater number of oviparous species in warm climates), the absolute number of viviparous species does not change. In contrast, their Figure 3 clearly shows that the absolute number and proportion of oviparous species both decrease with decreasing mean annual temperature. These data show that oviparity is less successful than viviparity in cold climates, rather than supporting the hypothesis that viviparity is less successful than oviparity in warm climates. Thus, they support the cold-climate hypothesis for the evolution of viviparity, not the warm-climate hypothesis for reversions to oviparity.

Although life history theory predicts that viviparity should be more costly than oviparity in warm climates, experimental tests of this prediction have been equivocal. The greatest theoretical cost associated with viviparity is probably a reduction in reproductive frequency (Shine, In Press). As viviparous females must carry litters throughout development, they have less time available to invest in multiple clutches in a single year (Tinkle and Gibbons, '77). However, viviparous squamates may be capable of producing multiple litters in a single year under captive conditions with abundant food (Naulleau, '73), which suggests that the ability to produce multiple clutches is at least partially constrained by environmental conditions like food abundance or length of active season rather than the physiological differences between

viviparity and oviparity. In addition, oviparous lizards that produce multiple clutches each year are usually smaller as adults, produce fewer eggs, and have shorter lifespans than species that are viviparous or produce only single clutches (Tinkle et al., ‘70). Furthermore, viviparity allows increased protection from predators in comparison to oviparity, regardless of climate (Tinkle and Gibbons, ‘77). Taken together, these lines of reasoning suggest that the theoretical reproductive frequency benefits of oviparity in warm climates may not apply to wild populations, and lifelong estimates of reproductive output and offspring survival in both oviparous and viviparous taxa are needed to test this hypothesis. Furthermore, viviparity allows maternal manipulation of offspring phenotypes in general, in addition to maternal optimization of incubation temperature (Shine, ‘95). The ability to manipulate offspring phenotype could provide a fitness advantage to viviparity over oviparity even in warm climates.

Pyron and Burbrink (2014) suggest that the physiological costs of reproduction are greater in viviparous taxa than in oviparous taxa. However, the metabolic costs associated with viviparity are not necessarily higher than those associated with oviparity. All oviparous squamates and the vast majority of viviparous squamates are lecithotrophic, and allocate nearly all of the energy and nutrients required for embryogenesis prior to ovulation, during vitellogenesis (Blackburn, ‘92, ‘98; Stewart and Thompson, 2000; Thompson et al., 2000; Thompson and Speake, 2003, 2006; Blackburn and Stewart, 2011; Stewart and Blackburn, 2014). Therefore, the metabolic costs of embryogenesis are “paid” during vitellogenesis in both oviparous and most viviparous species, and should not contribute to the metabolic costs of pregnancy in viviparous species. In addition, the metabolic costs of maintaining the “state” of pregnancy in gestating females appear to be relatively low or even undetectable in most lecithotrophic viviparous species (Birchard et al., ‘84; Demarco and Guillette, ‘92; Robert

and Thompson, 2000; Schultz et al., 2008; Van Dyke and Beaupre, 2011). Even if the costs of pregnancy are detectable in viviparous species, oviparous species may also experience similar costs prior to oviposition (Angilletta and Sears, 2000). Indeed, metabolic costs of reproduction do not differ between oviparous and viviparous *Zootoca vivipara* during vitellogenesis and early pregnancy, and the only difference is that viviparous females continue to respire oxygen and carbon dioxide for their offspring after oviparous females have oviposited (Foucart et al., 2014).

Pyron and Burbrink (2014) also suggest that viviparous taxa may incur greater energetic or fitness costs of predator avoidance and thermoregulation. Although viviparous squamates can suffer reduced locomotor performance and increased predation during pregnancy (Shine, ‘80), this cost has not been demonstrated in wild populations. Instead, viviparous reptiles often change their behaviour to reduce the potential fitness costs of locomotion. Specifically, they reduce their reliance on locomotion for predator avoidance, and instead rely on crypsis (Bauwens and Thoen, ‘81; Brodie, ‘89; Braña, ‘93; Kissner et al., ‘97). Viviparous squamates are also capable of storing large energy reserves to avoid starvation as a result of the effects of locomotor impairment on foraging success, or due to the metabolic demands of carrying a litter of offspring (Bonnet et al., ‘98; Gignac and Gregory, 2005). Ambush-foraging species in particular may not suffer detrimental effects of viviparity on either predation or foraging success (Schuett et al., 2013). Viviparous reptiles can also cannibalize non-viable offspring to quickly recoup energy after reproduction (Lourdais et al., 2005; Mocino-Deloya et al., 2009; Van Dyke et al., 2014b). Finally, even oviparous species face effects of locomotor impairment throughout the active season due to factors other than reproduction, including cool temperatures, large meals, and tail autotomy (Shine, 2003). Viviparous squamates can avoid the fitness costs of thermoregulation using many of the same strategies used to avoid

costs of locomotion. They can avoid predation by relying on crypsis during thermoregulation, and by thermoregulating close to easily-accessible shelter (Stewart, '84; Graves and Duvall, '93; Ladyman et al., 2003; Wittenberg, 2012). If thermoregulation precludes them from foraging or is metabolically costly, they may be able to use stored energy reserves and post-partum cannibalism to avoid starvation.

We feel it is important to re-state that many of these arguments regarding relative reproductive costs of viviparity and oviparity remain to be experimentally tested. The dogmatic view that viviparity is necessarily more costly than oviparity, in either fitness or energetic terms, is pervasive in the literature (Tinkle, '77; Shine, In Press). Indeed, the cold-climate hypothesis for the evolution of viviparity is popular in the literature partly because it suggests that cold climates are the one environmental condition where viviparity might be less costly than oviparity. Thus, part of our goal in offering an alternative viewpoint here is to suggest possible areas for future research that will further test the implicit assumptions underlying the cold-climate hypothesis itself.

Squamates are ancestrally oviparous

We argue that the "ease" of transitioning from oviparity to viviparity, and the comparative "difficulty" of the opposite transition, suggests that the evolutionary transition from oviparity to viviparity has occurred far more frequently than the reverse. Indeed, we suggest that the barriers to reversals -- anatomical, physiological, behavioural, and ecological -- pose significant constraints that might prevent them altogether. Existing morphological and physiological data do not support reversions from viviparity to oviparity. Conversely, the large number of transitions from oviparity to viviparity is supported by the morphological

diversity of viviparous squamates. For example, despite placentae arising from homologous tissues in every known viviparous squamate, there are extensive morphological differences in placentae. These morphological differences are most noticeable in species that have evolved complex placentation, and include a vessel-dense elliptical area in *Eulamprus tympanum* (Murphy et al., 2011), interdigitating regions of the chorioallantois and uterus in *Niveoscincus* species (Stewart and Thompson, '94, 2009b; Wu et al. In Press), invasive placentae in *Trachylepis ivensi* (Blackburn and Flemming, 2012), and elaborate placentome structures in *Pseudemoia* species (Stewart and Thompson, '96, '98; Adams et al., 2005), *Chalcides chalcides* (Blackburn, '93b; Blackburn and Callard, '97), and *Mabuya* species (Jerez and Ramírez-Pinilla, 2001; Blackburn and Vitt, 2002; Ramírez-Pinilla et al., 2006), as well as mechanisms of nutrient transport (Brandley et al., 2013; Griffith et al., 2013b). The diversity in placental structures in squamates, but lack of morphological diversity in the features responsible for egg production and oviparity strongly supports an oviparous ancestor for the squamates.

Although oviparity is the ancestral character state for squamates, this does not mean that viviparity is a recent phenomenon, as discussed in Pyron and Burbrink (In Press). If viviparity has evolved frequently across the squamate phylogeny, it is reasonable to expect that viviparity evolved frequently in ancient squamates too. Therefore, the discovery of fossilized viviparous pleisiosaurs, ichthyosaurs, and lizards (Maxwell and Caldwell, 2003; O'Keefe and Chiappe, 2011; Wang and Evans, 2011) is not evidence for a viviparous ancestral squamate, but is expected if the evolution of viviparity occurs frequently.

Conclusions

In the course of their ancestral state reconstruction analyses, Pyron and Burbrink (2014) tested different evolutionary models and found that the most general, parameter-rich model is significantly better fit to the parity data, and that the models with the worst scores were those that assume reversals from viviparity to oviparity are impossible. This is unsurprising; with no information about the biology of viviparity, simply visually inspecting the distribution of parity states across the squamate tree could convince someone that transitions from viviparity to oviparity are common. Our contention is that the statistical results are irrelevant if the underlying model of evolution is not biologically possible. No statistical model is perfect, and there will always be sources of error that a model cannot incorporate. We nonetheless contend that "biological reality" must play a subjective role in evaluating bold hypotheses generated from ancestral state reconstruction analyses. Moreover, ancestral state reconstruction analyses must incorporate phylogenetic uncertainty – this is especially relevant in phylogenetic analyses of 1000s of taxa and those using supermatrices with large amounts of missing data.

Although methods for ancestral character state reconstruction are likely to improve in the future, these analyses cannot be utilized to test evolutionary hypotheses on their own. In particular, the discrepancies we describe here suggest that transitions and reversals among character states are not equally likely, but are instead differentially limited by anatomical, physiological, or environmental constraints. Relative strengths of these biophysical constraints, determined using biological evidence, must be incorporated into transition probabilities in ancestral state reconstruction models. In cases where this is impossible, we propose that anatomical, physiological, and ecological evidence be used to evaluate

hypotheses drawn from ancestral state reconstruction analyses developed using only phylogenetic data.

Acknowledgments

This project was supported by ARC Discovery grants to M.B.T. and Discovery Early Career Research Award to M.C.B. J.U.V. was supported by an NSF International Research Fellowship (#1064803). We are grateful to two anonymous reviewers for extensive comments on the manuscript.

Literature Cited

Adams SM, Biazik JM, Thompson MB, Murphy CR. 2005. Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: A new placental morphotype. J Morphol 264:264-276.

Akaike H. 1974. A new look at the statistical model identification. IEEE T Automat Cont, 19:716-723.

Anan'eva NB, Orlov NL. 2013. Egg teeth of squamate reptiles and their phylogenetic significance. Biol Bull Russ Acad Sci 40:600-605.

Anderson KE, Blackburn DG, Dunlap KD. 2011. Scanning EM of the placental interface in the mountain spiny lizard, *Sceloporus jarrovi.* J Morphol 272:465- 484.

Andrews RM. 2000. Evolution of viviparity in squamate reptiles (*Sceloporus* spp.): a variant of the cold-climate model. J Zool 250:243-253.

Angilletta MJ, Sears MW. 2000. The metabolic cost of reproduction in an oviparous lizard. Funct Ecol 14:39-45.

Bauwens D, Thoen C. 1981. Escape tactics and vulnerability to predation associated with reproduction in the lizard *Lacerta vivipara*. J Anim Ecol 50:733-743.

Biazik JM, Thompson MB, Murphy CR. 2009. Lysosomal and alkaline phosphatase activity indicate macromolecule transport across the uterine epithelium in two viviparous skinks with complex placenta. J Exp Zool 312B:817-826.

Birchard GF, Black CP, Schuett GW, Black V. 1984. Influence of pregnancy on oxygen consumption, heart rate and hematology in the garter snake: Implications for the "cost of reproduction" in live bearing reptiles. Comp Biochem Phys A 77:519-523.

Blackburn DG. 1992. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. Am Zool 32:313-321.

Blackburn DG. 1993a. Chorioallantoic placentation in squamate reptiles: structure, function, development, and evolution. J Exp Zool 266:414-430.

Blackburn DG. 1993b. Histology of the late-stage placentae of the matrotrophic skink *Chalcides chalcides* (Lacertilia: Scincidae). J Morphol 216:179-195.
Blackburn DG. 1995. Saltationist and punctuated equilibrium models for the evolution of viviparity and placentation. J Theor Biol 174:199-216.
Blackburn DG. 1998. Structure, function, and evolution of the oviducts of squamate reptiles, with special reference to viviparity and placentation. J Exp Zool 282:560-617.
Blackburn DG. 1999. Are viviparity and egg-guarding evolutionarily labile? Herpetologica 55:556-573.
Blackburn DG. 2006. Squamate reptiles as model organisms for the evolution of viviparity. Herpetol Monogr 20:131-146.
Blackburn DG. 2014. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative analysis. J Morphol: http://dx.doi.org/10.1002/jmor.20272
Blackburn DG, Callard IP. 1997. Morphogenesis of the placental membranes in the viviparous, placentotrophic lizard *Chalcides chalcides* (Squamata: Scincidae). J Morphol 232:35-55.
Blackburn DG, Flemming AF. 2012. Invasive ovo-implantation and intimate placental associations in a placentotrophic African lizard, *Trachylepis ivensi* (Scincidae). J Morphol 273:137-159.
Blackburn DG, Lorenz R. 2003. Placentation in garter snakes. Part II. Transmission EM of the chorioallantoic placenta of *Thamnophis radix* and *T. sirtalis.* J Morphol 256:171-186.
Blackburn DG, Stewart JR. 2011. Viviparity and placentation in snakes. In: Sever D, Aldridge R, editors. Reproductive biology and phylogeny of snakes. Enfield, New Hampshire: CRC Press. p 119-181.
Blackburn DG, Vitt LJ. 2002. Specializations of the chorioallantoic placenta in the Brazilian scincid lizard, *Mabuya heathi*: a new placental morphotype for reptiles. J Morphol 254:121-131.
Blackburn DG, Anderson KE, Johnson AR, Knight SR, Gavelis GS. 2009. Histology and ultrastructure of the placental membranes of the viviparous brown snake, *Storeria dekayi* (Colubridae: Natricinae). J Morphol 270:1137-1154.
Blackburn DG, Gavelis GS, Anderson KE, Johnson AR, Dunlap KD. 2010. Placental specializations in the mountain spiny lizard *Sceloporus jarrovi*. J Morphol 271:1153–1175.
Bonnet X, Don B, Shine R. 1998. Capital versus income breeding: An ectothermic perspective. Oikos 83:333-342.
Braña F. 1993. Shifts in body temperature and escape behaviour of female *Podarcis muralis* during pregnancy. Oikos 66:216-222.
Brandley MC, Bragg JG, Chapple DG, Jennings CK, Lemmon AR, Lemmon EM, Singhal S, Thompson MB, Moritz C. In Press. Evaluating the phylogenetic informativeness of anchored enrichment loci at the tips of the tree of life: an analysis using *Eugongylus* group scincid lizards. BMC Evol Biol.
Brandley MC, Huelsenbeck JP, Wiens JJ. 2008. Rates and patterns in the evolution of snake-like body form in squamate reptiles: evidence for repeated re-evolution of lost digits and long-term persistence of intermediate body forms. Evolution 62:2042-2064.
Brandley MC, Young RL, Warren, DL, Thompson MB, Wagner GP. 2012. Uterine gene expression in the live-bearing lizard, *Chalcides ocellatus*, reveals convergence of

squamate reptile and mammalian pregnancy mechanisms. Genome Biol Evol 4:394-411.
Brawand D, Wahli W, Kaessmann H. 2008. Loss of egg yolk genes in mammals and the origin of lactation and placentation. PLoS Biol 6:e63.
Brodie ED. 1989. Behavioral modification as a means of reducing the cost of reproduction. Am Nat 134:225-238.
Bustard HR. 1968. The egg-shell of gekkonid lizards: A taxonomic adjunct. Copeia:162-164.
Cogger HG. 2014. Reptiles and amphibians of Australia. Collingswood, Victoria, Australia: CSIRO Publishing.
Demarco V, Guillette LJ. 1992. Physiological cost of pregnancy in a viviparous lizard *(Sceloporus jarrovi)*. J Exp Zool 262:383-390.
Duchene S, Lanfear R. In Press. Phylogenetic uncertainty can bias ancestral state reconstructions. J Exp Zool Part B.
Dupoué A, Brischoux F, Angelier F, DeNardo DF, Wright CD, Lourdais O. (In Press) Intergenerational trade-off for water may induce a mother-offspring conflict in favour of embryos in a viviparous snake. Funct Ecol. DOI: 10.1111/1365-2435.12349
Fenwick AM, Greene HW, Parkinson CL. 2012. The serpent and the egg: Unidirectional evolution of reproductive mode in vipers? J Zool Syst Evol Res 50:59-66.
Fitch HS. 1960. Autecology of the copperhead. Univ Kansas Publ Mus Nat Hist 13:85-288.
Foucart T, Lourdais O, DeNardo DF, Heulin B. 2014. Influence of reproductive mode on metabolic costs of reproduction: Insight from a bimodal lizard, *Zootoca vivipara*. J Exp Biol 217:4049-4056.
de Fraipont M, Clobert J, Meylan S, Barbault R. 1999. On the evolution of viviparity and egg-guarding in squamate reptiles: A reply to R. Shine and M. S. Y. Lee. Herpetologica 55:550-555.
Ghiara G, Angelini F, Zerani M, Gobbetti A, Cafiero G, Caputo V. 1987. Evolution of viviparity in Scincidae (Reptilia, Lacertilia). Acta Embryol Exper n.s. 8:187–201.
Gignac A, Gregory PT. 2005. The effects of body size, age, and food intake during pregnancy on reproductive traits of a viviparous snake, *Thamnophis ordinoides*. Ecoscience 12:236-243.
Goldberg EE, Igić B. 2008. On phylogenetic tests of irreversible evolution. Evolution 62:2727-2741.
Graves BM, Duvall D. 1993. Reproduction, rookery use, and thermoregulation in free-ranging, pregnant *Crotalus v. viridis*. J Herpetol 27:33-41.
Griffith OW, Van Dyke JU, Thompson MB. 2013a. No implantation in an extra-uterine pregnancy of a placentotrophic reptile. Placenta 34:510-511.
Griffith OW, Ujvari B, Belov K, Thompson MB. 2013b. Placental lipoprotein lipase (lpl) gene expression in a placentotrophic lizard, *Pseudemoia entrecasteauxii* J Exp Zool Part B 320:465-470.
Guillette LJ, Jones RE. 1985. Ovarian, oviductal, and placental morphology of the reproductively bimodal lizard, *Sceloporus aeneus.* J Morphol 184:85-98.
Haines ML, Moussalli A, Stuart-Fox D, Clemann N, Melville J. 2014. Phylogenetic evidence of historic mitochondrial introgression and cryptic diversity in the genus *Pseudemoia* (Squamata: Scincidae). Mol Phylogenet Evol 81:86-95.
Heulin B, Ghielmi S, Vogrin N, Surget-Groba Y, Guillaume CP. 2002. Variation in eggshell characteristics and in intrauterine egg retention between two oviparous clades of the

lizard *Lacerta vivipara*: Insight into the oviparity–viviparity continuum in squamates. J Morphol 252:255-262.
Herbert JF, Lindsay LA, Thompson MB, Murphy CR. 2006. Calcium transport across the uterine epithelium of pregnant lizards. Herpetol Monogr 20: 205-211.
Herbert JF, Murphy CR, Thompson MB. 2010. Calcium ATPase localization in the uterus of two species of *Pseudemoia* (Lacertilia: Scincidae) with complex placentae. Herpetol Conserv Bio 5: 290-296.
Hoffman LH. 1970. Placentation in the garter snake, *Thamnophis sirtalis.* J Morphol 131: 57-88.
Itonaga K, Wapstra E, Jones SM. 2012. A novel pattern of placental leucine transfer during mid to late gestation in a highly placentotrophic viviparous lizard. J Exp Zool B (Mol Dev Evol) 318:308-315.
Jerez A, Ramírez-Pinilla MP. 2003. Morphogenesis of extraembryonic membranes and placentation in *Mabuya mabuya* (Squamata, Scincidae). J Morphol 258:158-178.
Jones RE, Propper CR, Rand MS, Austin HB. 1991. Loss of nesting behavior and the evolution of viviparity in reptiles. Ethology 88:331-341.
Kissner KJ, Forbes MR, Secoy DM. 1997. Rattling behavior of prairie rattlesnakes (*Crotalus viridis viridis,* Viperidae) in relation to sex, reproductive status, body size, and body temperature. Ethology 103:1042-1050.
Klauber LM. 1972. Rattlesnakes: Their habits, life histories, and influence on mankind. Berkely, CA: University of California Press.
Kohlsdorf T, Wagner GP. 2006. Evidence for the reversibility of digit loss: a phylogenetic study of limb evolution in *Bachia* (Gymnophthalmidae: Squamata). Evolution 60:1896-1912.
Kohlsdorf T, Lynch VJ, Rodrigues M, Brandley MC, Wagner GP. 2010. Data and data-interpretation in the study of limb evolution: a reply to Galis et al., on the re-evolution of digits in the lizard genus *Bachia*. Evolution 64:2477–2485.
Ladyman M, Bonnet X, Lourdais O, Bradshaw D, Naulleau G. 2003. Gestation, thermoregulation, and metabolism in a viviparous snake, *Vipera aspis*: Evidence for fecundity-independent costs. Physiol Biochem Zool 76:497-510.
Lee, MS, Shine, R. 1998. Reptilian viviparity and Dollo's law. Evolution 52:1441-1450.
Litsios G, Salamin N. 2012. Effects of phylogenetic signal on ancestral state reconstruction. Syst Biol 61:533-538
Lourdais O, Brischoux F, Shine R, Bonnet X. 2005. Adaptive maternal cannibalism in snakes (*Epicrates cenchria maurus*, Boidae). Biol J Linn Soc 84:767-774.
Lynch VJ, Wagner GP. 2010. Did egg-laying boas break Dollo's law? Phylogenetic evidence for reversal to oviparity in sand boas (*Eryx:* Boidae). Evolution 64:207-216.
Mathies T, Andrews RM. 1999. Determinants of embryonic stage at oviposition in the lizard *Urosaurus ornatus*. *Physiol Biochem Zool* 72:645-655.
Maxwell EE, Caldwell MW. 2003. First record of live birth in Cretaceous ichthyosaurs: closing an 80 million year gap. Philos T Roy Soc B, 270:S104-S107.
Mocino-Deloya E, Setser K, Pleguezuelos JM, Kardon A, Lazcano D. 2009. Cannibalism of nonviable offspring by postparturient Mexican lance-headed rattlesnakes, *Crotalus polystictus*. Anim Behav 77:145-150.
Murphy BF, Parker SL, Murphy CR, Thompson MB. 2011. Placentation in the eastern water skink (*Eulamprus quoyii*): A placentome-like structure in a lecithotrophic lizard. J Anat 218:678-689.

Naulleau G. 1973. Reproduction twice in one year in a captive viper (*Vipera aspis*). Brit J Herpetol 5:353-357.
Neill, WT. 1964. Viviparity in snakes: some ecological and zoogeographical considerations. Am Nat 98:35-55.
O'Keefe FR, Chiappe LM. 2011. Viviparity and K-selected life history in a Mesozoic marine plesiosaur (Reptilia, Sauropterygia). Science 333:870-873.
Packard GC, Tracy CR, Roth JJ. 1977. The physiological ecology of reptilian eggs and embryos, and the evolution of viviparity within the Class Reptilia. Biol Rev 52:71-105.
Packard MJ, Packard GC, Boardman TJ. 1982. Structure of eggshells and water relations of reptilian eggs. Herpetologica 38:136-155.
Parker SL, Andrews RM. 2006. Evolution of viviparity in sceloporine lizards: in utero PO2 as a developmental constraint during egg retention. Physiol Biochem Zool 79:581-592.
Parker SL, Manconi F, Murphy CR, Thompson MB. 2010. Uterine and placental angiogenesis in the Australian skinks, *Ctenotus taeniolatus* and *Saiphos equalis*. Anat Record 293: 829–838.
Pyron RA, Burbrink FT. 2014. Early origin of viviparity and multiple reversions to oviparity in squamate reptiles. Ecol Lett 17:13-21.
Pyron RA, Burbrink FT. In Press. Contrasting models of parity-mode evolution in squamate reptiles. J Exp Zool B.
Qualls CP. 1996. Influence of the evolution of viviparity on eggshell morphology in the lizard *Lerista bougainvillii*. J Morphol 228:119-125.
Qualls CP, Shine R, Donnellan S, Hutchinson M. 1995. The evolution of viviparity within the Australian scincid lizard *Lerista bougainvillii*. J Zool 237:13–26.
Ramírez-Pinilla MP, De Pérez G, Carreño-Escobar JF. 2006. Allantoplacental ultrastructure of an Andean population of *Mabuya* (Squamata, Scincidae). J Morphol 267:1227-1247.
Robert KA, Thompson MB. 2000. Energy consumption by embryos of a viviparous lizard, *Eulamprus tympanum*, during development. Comp Biochem Phys A 127:481-486.
Schuett GW, Repp RA, Amarello M, Smith CF. 2013. Unlike most vipers, female rattlesnakes (*Crotalus atrox*) continue to hunt and feed throughout pregnancy. J Zool 289:101-110.
Schultz TJ, Webb JK, Christian KA. 2008. The physiological cost of pregnancy in a tropical viviparous snake. Copeia 2008:637-642.
Sergeev AM. 1940. Researches on the viviparity of reptiles. Moscow Society of Naturalists 13:1-34.
Shine R. 1980. "Costs" of reproduction in reptiles. Oecologia 46:92-100.
Shine R. 1985. The evolution of viviparity in reptiles: an ecological analysis. In: Gans C, Billet F, editors. Biology of the Reptilia, vol. 15. New York: John Wiley. p 605-694.
Shine R. 1995. A new hypothesis for the evolution of viviparity in reptiles. Am Nat 145:809-823.
Shine R. 2003. Locomotor speeds of gravid lizards: Placing "costs" of reproduction within an ecological context. Funct Ecol 17:526-533.
Shine R. 2004. Does viviparity evolve in cold climate reptiles because pregnant females maintain stable (not high) body temperatures? Evolution 58:1809-1818.
Shine R. 2014. Evolution of an evolutionary hypothesis: a history of changing ideas about the adaptive significance of viviparity in reptiles. J Herpetol 48:147-161.
Shine R. In Press. The evolution of oviparity in squamate reptiles: An adaptationist perspective. J Exp Zool B.

Shine R, Thompson MB. 2006. Did embryonic responses to incubation conditions drive the evolution of reproductive modes in squamate reptiles? Herpetol Monog 20:159-171.
Staub RE, Emberton J. 2002. *Eryx jayakari* (Arabian sand boa) reproduction. Herpetol Rev 33:214.
Stewart JR. 1984. Thermal biology of the live bearing lizard *Gerrhonotus coeruleus.* Herpetologica 40:349-355.
Stewart JR. 2013. Fetal nutrition in lecithotrophic squamate reptiles: toward a comprehensive model for evolution of viviparity and placentation. J Morphol 274:824-831.
Stewart JR, Blackburn DG. 2014. Viviparity and placentation in lizards. In: Rheubert JL, Siegel DS, Trauth SE, editors. Reproductive biology and phylogeny of lizards and tuatara. Boca Raton, Florida: CRC Press. p. 448-563.
Stewart JR, Brasch KR. 2003. Ultrastructure of the placentae of the natricine snake, *Virginia striatula* (Reptilia: Squamata). J Morphol 255:177-201.
Stewart JR, Thompson MB. 1993. A novel pattern of embryonic nutrition in a viviparous reptile. J Evolution Biol 174:97-108.
Stewart JR, Thompson MB. 1994. Placental structure of the australian lizard, *Niveoscincus metallicus* (Squamata: Scincidae). J Morphol 220:223-236.
Stewart JR, Thompson MB. 1996. Evolution of reptilian placentation: Development of extraembryonic membranes of the Australian scincid lizards, *Bassiana duperreyi* (oviparous) and *Pseudemoia entrecasteauxii* (viviparous). J Morphol 227:349-370.
Stewart JR, Thompson MB. 1998. Placental ontogeny of the Australian scincid lizards *Niveoscincus coventryi* and *Pseudemoia spenceri.* J Exp Zool 282: 535-559.
Stewart JR, Thompson MB. 2000. Evolution of placentation among squamate reptiles: Recent research and future directions. Comp Biochem Phys A 127A:411-431.
Stewart JR, Thompson MB. 2003. Evolutionary transformations of the fetal membranes of viviparous reptiles: a case study in two lineages. J Exp Zool 299A:13-32.
Stewart JR, Thompson MB. 2009a. Parallel evolution of placentation in Australian scincid lizards. J Exp Zool B (Molec Devel Evol) 312:590-602.
Stewart JR, Thompson MB. 2009b**.** Placental ontogeny in Tasmanian snow skinks (genus *Niveoscincus*) (Lacertilia: Scincidae). J Morphol 270: 485-516.
Stewart JR, Heulin B, Surget-Groba Y. 2004. Extraembryonic membrane development in a reproductively bimodal lizard, *Lacerta (Zootoca) vivipara.* Zoology 107:289-314.
Stewart JR, Thompson MB, Attaway MB, Herbert JF, Murphy, CR. 2006. Uptake of dextran-FITC by epithelial cells of the chorioallantoic placentome and the omphalopleure of the placentotrophic lizard, *Pseudemoia entrecasteauxii*. J Exp Zool A (Comp Exp Biol) 305:883-889.
Stewart JR, Mathieson AN, Ecay TW, Herbert JF, Parker SL, Thompson MB. 2010. Uterine and eggshell structure and histochemistry in a lizard with prolonged uterine egg retention (Lacertilia, Scincidae, *Saiphos*). J Morphol 271:1342-1351.
Thompson MB, Speake BK. 2003. Energy and nutrient utilisation by embryonic reptiles. Comp Biochem Physiol A: Mol Integr Physiol 133:529-538.
Thompson MB, Speake BK. 2006. A review of the evolution of viviparity in lizards: Structure, function and physiology of the placenta. J Comp Physiol 176B:179-189.
Thompson MB, Stewart JR. 1994. Egg and clutch size of the viviparous australian skink, *Pseudemoia pagenstecheri* and the identity of species with type iii allantoplacentae. J Herpetol 28:519-521.

Thompson MB, Stewart JR, Speake BK, Russell KJ, McCartney RJ, Surai PF. 1999. Placental nutrition in a viviparous lizard (*Pseudemoia pagenstecheri*) with a complex placenta. J Zool 248:295-305.
Thompson MB, Stewart JR, Speake BK. 2000. Comparison of nutrient transport across the placenta of lizards differing in placental complexity. Comp Biochem Phys 127A:469-479.
Thompson MB, Adams SM, Herbert JF, Biazik JM, Murphy CR. 2004. Placental function in lizards. Internat Cong Ser 12785:218-225.
Tinkle DW, Gibbons JW. 1977. The distribution and evolution of viviparity in reptiles. Misc Publ Mus Zool, Univ Mich 154: 1–55.
Tinkle DW, Wilbur HM, Tilley SG. 1970. Evolutionary strategies in lizard reproduction. Evolution 24:55-74.
Van Dyke JU, Beaupre SJ. 2011. Bioenergetic components of reproductive effort in viviparous snakes: Costs of vitellogenesis exceed costs of pregnancy. Comp Biochem Phys A 160:504-515.
Van Dyke JU, Brandley MC, Thompson MB. 2014a. The evolution of viviparity: Molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. Reproduction 147:R15-R26.
Van Dyke JU, Griffith OW, Thompson MB. 2014b. High food abundance permits the evolution of placentotrophy: Evidence from a placental lizard, *Pseudemoia entrecasteauxii*. Am Nat 184:198-210.
Wang Y, Evans SE. 2011. A gravid lizard from the Cretaceous of China and the early history of squamate viviparity. Naturwissenschaften 98:739-743.
Warren WC, Hillier LW, Graves JAM, Birney E, Ponting CP, Grützner F, Belov, K et al.. 2008. Genome analysis of the platypus reveals unique signatures of evolution. Nature 453:175-183.
Weekes CH. 1935. A review of placentation among reptiles with particular regard to the function and evolution of the placenta. Proc Zool Soc Lond 2:625-645.
Whiting MF, Bradler S, Maxwell T. 2003. Loss and recovery of wings in stick insects. Nature 421(6920): 264-267.
Whittington CM, Grau GE, Murphy CR, Thompson MB. In Press. Unusual angiogenic factor plays a role in lizard pregnancy but is not unique to viviparity. J Exp Zool B.
Wittenberg RD. 2012. Foraging ecology of the timber rattlesnake *(Crotalus horridus)* in a fragmented agricultural landscape. Herpetol Conserv Bio 7:449-461.
Wright A, Lyons K, Brandley MC, Hillis DM. In Press. Which Came First: The Lizard or the Egg? J Exp Zool Part B.
Wu Q, Fong CK, Thompson MB, Murphy CR. In Press. Changes to the uterine epithelium during the reproductive cycle of two viviparous lizard species (*Niveoscincus* spp.) Acta Zool.
Yaron, Z. 1985. Reptile placentation and gestation: structure, function, and endocrine control. In: Gans C, Billet F, editors. Biology of the Reptilia, vol. 15. New York: John Wiley. p. 527-603.

Chapter 3: Reptile pregnancy is underpinned by complex changes in uterine gene expression that converge on the eutherian condition

Chapter formatted for submission to *Molecular Biology and Evolution*

Title: Reptile pregnancy is underpinned by complex changes in uterine gene expression that converge on the eutherian condition

Running title: Uterine gene expression in a placentotrophic reptile

Authors: Oliver W. Griffith[1*], Matthew C. Brandley[1], Katherine Belov[2], Michael B. Thompson[1]

Affiliations:

[1]School of Biological Sciences, University of Sydney, Camperdown, NSW 2006, Australia

[2] Faculty of Veterinary Science, University of Sydney, Camperdown, NSW 2006, Australia

* Correspondence to Oliver Griffith; School of Biological Sciences, Heydon Laurence Building (A08), University of Sydney, Camperdown, NSW 2006, Australia; email: oli.griffith@gmail.com

Keywords: Placenta, complex trait, RNA-seq, *Pseudemoia entrecasteauxii*, viviparity, placentotrophy

Abstract:

The evolution of new organs and organ systems is extremely difficult to study because most vertebrate organs evolved only once, at least 500 million years ago. An ideal model for understanding complex organ evolution is the placenta, a structure that is common in most live bearing (viviparous) vertebrates in which it has evolved independently more than 115 times. We used transcriptomics to identify genes important for placental functions in a squamate reptile, the southern grass skink (*Pseudemoia entrecasteauxii*). *Pseudemoia entrecasteauxii* has two distinct placental regions, the chorioallantoic placenta and yolk sac placenta. The chorioallantoic and yolk sac regions of *P. entrecasteauxii* perform distinct placental functions, converging on the eutherian viviparous condition. Functional gene annotation of genes that are differentially expressed between placentae suggest that nutrient transport occurs in both placental regions, but that this transport occurs by different mechanisms. In the chorioallantoic placenta, transport is facilitated by the presence of membrane bound nutrient transport proteins, whilst the yolk sac placenta facilitates transport by apocrine secretion. By comparing the expression of genes to known eutherian systems, we show that complex placental functions have evolved using the same set of genes in deeply divergent lineages (mammals and squamate reptiles). We show that constraints imposed by ancestral gene expression, pleiotropy, functional properties of genes, and underlying gene regulatory networks, can explain why repeated convergent evolution of complex traits are common. These findings suggest that if the evolutionary clock was rewound hundreds of millions of years and replayed, similar complex features of vertebrates would evolve repeatedly.

INTRODUCTION

Comparison of organs that have evolved convergently in different lineages provides a unique opportunity to identify the genetic mechanisms that have resulted in the evolution of complex structures (Stern 2013; Wake, et al. 2011). The evolution of new functions in pre-existing tissues occurs by three broad processes, the recruitment of genes expressed elsewhere in the organism (gene expression recruitment), the modification of expressed genes so that they now perform novel functions (gene neofunctionalization), and the introduction of new genes (typically introduced by viruses, retro-transposons, or gene duplication) (Cross, et al. 2003; Long, et al. 2003; True and Carroll 2002; Weake and Workman 2010; Zhang 2003). The evolution of new functions in pre-existing tissues typically occurs by recruiting genes expressed elsewhere in the organism, rather than altering the function of genes already expressed (Knox and Baker 2008). Therefore, the evolution of functional innovations in tissues is constrained by the availability of genes that can be recruited.

The evolution of novelty can also be constrained by restrictions or limitations on the outcomes of evolutionary events, which can be imposed by inheritance, development, selection, and design limitations (Arnold 1992). Biological constraints can result in novel phenotypes evolving convergently in different lineages using the same genes (parallel evolution) despite alternative genetic pathways that could result in the same trait (Fong, et al. 2005; Stern 2013). In this way, biological constraints are fundamental to the evolution of novelty as they constrain the available phenotypes that are likely to arise. Novel functional traits typically evolve via changes in gene regulation, which requires specific mutations in the gene regulatory networks. Unlike single nucleotide substitutions, which are unpredictable (Soria-Carrasco, et al. 2014), we propose that mutations that result in changes to gene

regulation are prone to biological constraint, as gene regulation is controlled by the activity of many interacting enzymes. In total these biological constraints change the probability of any particular novel trait evolving, ultimately shaping the given phenotypes that will evolve, and increasing the probability of particular traits evolving convergently in multiple lineages.

Placentae are an ideal model for understanding organ evolution as they have evolved many times independently, have evolved relatively recently in some lineages, and they involve complex functions that require changes in the expression of suites of genes (Blackburn 2014; Brandley, et al. 2012; Griffith, et al. 2015; Hou, et al. 2012; Reznick, et al. 2002; Van Dyke, et al. 2014a). A placenta is an organ formed by both maternal and embryonic tissue that function together to exchange materials between mother and embryo during development (Mossman 1937). Placentae are necessary in most live bearing (viviparous) vertebrates as they facilitate the exchange of respiratory gasses and water between mother and offspring and have been independently derived in the more than 115 viviparous amniote lineages (Blackburn 2014; Stewart 2013). In amniotes, placentation always evolves by modifications to maternal uterine tissue and extra-embryonic membranes such as the chorioallantois and yolk sac (Van Dyke et al. 2014a). The evolution of placentotrophy (embryonic nutrition provided via a placenta during development) has occurred seven times in viviparous amniote vertebrates, once in therian mammals and six times in lizards (Blackburn 2014; Murphy and Thompson 2011). As placentotrophy has evolved so few times in the more than 115 viviparous amniote lineages, mechanisms of placental nutrient transport are likely to be complex and relatively difficult to evolve (Murphy and Thompson 2011).

In *P. entrecasteauxii* and humans, the placental tissues responsible for nutrient uptake during pregnancy are not homologous. Humans exhibit highly invasive hemochorial placentation,

which has resulted in embryonic tissue invading through multiple layers of maternal cells, to be bathed in maternal blood (Wildman, et al. 2006). As fetal tissue has direct access to maternal blood, the embryonic cells of the trophoblast are directly responsible for nutrient uptake (Lager and Powell 2012). Most reptiles such as the Australian southern grass skink (*Pseudemoia entrecasteauxii*) exhibit non-invasive epitheliochorial placentation, so nutrients must be transported from the maternal blood to the embryo by the maternal uterine epithelium (Adams, et al. 2005; Griffith, et al. 2013a). Whilst the tissues which facilitate nutrient uptake from the mother differ between species, the function which is required of these two tissues is fundamentally the same; in the skink, nutrients are absorbed from the maternal blood by the uterine endothelium, are transported across several layers of uterine tissue and then released into the uterine lumen by the uterine epithelium. In humans, nutrients are absorbed from the maternal blood by the trophoblast epithelium, are transported across several layers of trophoblast tissue and then released into the embryonic blood supply by placental endothelial cells. Whilst convergent evolution has been noted in the genes facilitating placental functions before (Brandley et al. 2012; Van Dyke et al. 2014a), typically this has been documented in homologous tissues, where homology may be the primary reason for the observed similar use of genes in both lineages. Studying the convergent evolution of placental transport of nutrients in non-homologous tissues is ideal to test if the convergent evolution of complex traits is constrained in the absence of shared developmental histories.

The placenta of the southern grass skink has regional specialization that allows for regions to perform discrete functional tasks. The morphological differences in these regions are most notable in uterine epithelial cells (the cells lining the uterus in direct contact with the embryo). Non-reproductive uterus is uniformly flat without folding and epithelial cells are either ciliated or non-ciliated. During pregnancy, however, there are substantial changes to

the uterine epithelium and these changes vary in relation to the orientation of the embryo (Fig. 1). The embryonic hemisphere is comprised of a placenta formed between uterine tissue and the chorioallantois of the embryo; this placenta contains two morphologically distinct placental regions, the placentome and the paraplacentome. The placentome is a tightly folded placental region, where the uterus contains large cuboidal epithelial cells, and it is likely associated with nutrient transfer but not via secretion of nutrient containing vesicles (Fig. 1a; Adams et al. 2005; Biazik, et al. 2009; Biazik, et al. 2010). In contrast, the epithelial cells of the paraplacentome (a region that sits around the placentome) are extremely thin and attenuated, a morphology that has the minimum diffusion distance and likely facilitates gas exchange by diffusion (Fig. 1b; Adams et al. 2005; Stewart and Thompson 1996). In the yolk sac placenta, uterine epithelial cells are low to tall columnar, contain electron dense granules, and have characteristic vesicles budding from the apical surface, which is consistent with nutrient transfer via vesicle mediated transport or apocrine secretion (Fig. 1c; Adams et al. 2005). Although the morphology of the uterine epithelium is suggestive of placental functions in these discrete regions, there is little physiological or molecular data that allows us to identify what cellular processes are occurring in the different placental regions during pregnancy.

By comparing the expression of genes in uterine tissue of the chorioallantoic placenta, the yolk sac placenta, and tissue from non-reproductive females, we identify which genes are up-regulated during pregnancy in the uterine component of the placenta. Furthermore, we test if morphological and functional differences in different regions of the uterus are underpinned by differential gene expression. A third aim of this project is to identify if placental functions in reptiles and mammals are facilitated by the same genes. To meet this aim we specifically

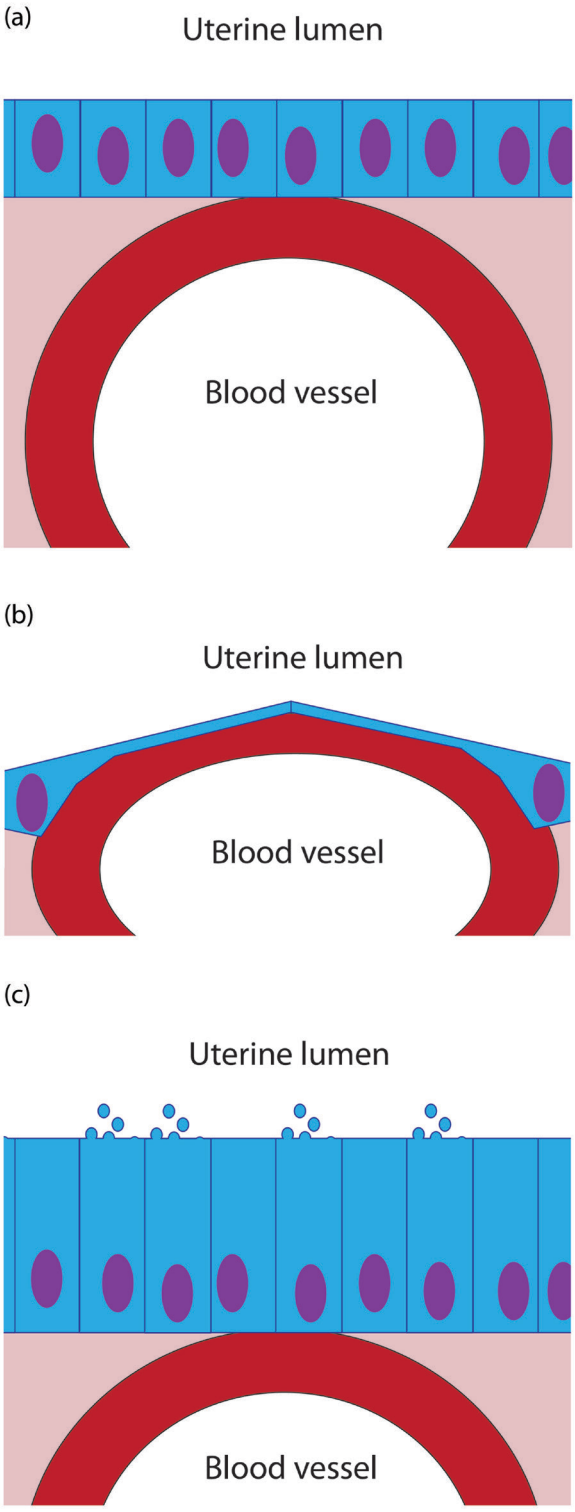


Figure 1. Illustration highlighting the morphological differences between the uterine tissue of the placentome (a), paraplacentome (b), and yolk sac placenta (c).Uterine epithelial cells are indicated in blue, epithelial cell nuclei are purple, the endothelium of a uterine blood vessel is indicated in red and the uterine stroma is indicated in pink.

examine the expression of amino acid transport proteins in the southern grass skink and human trophoblast then test if gene use is significantly different to random.

RESULTS

Differential gene expression in the skink placenta

We sequenced a mean of $5.04 \times 10^7 \pm 2.8 \times 10^6$ one hundred and one base pair (bp) paired end reads per sample. Our assembled transcriptome contained 367 722 contigs with a mean contig length of 465 bp. Alignment of assembled contigs to proteomes of the anole lizard, human, chicken, opposum, platypus, zebra finch and Chinese tortoise allowed us to identify a total of 88 204 contigs. These contigs mapped to 16 942 unique genes.

The uterus of the chorioallantoic placenta and yolk sac placenta when compared to non-pregnant uterine tissue had 2555 and 3861 differentially expressed genes respectively. Pairwise comparisons between the uterus of the chorioallantoic placenta and yolk sac placenta found 282 differentially expressed genes.

Gene ontology analysis on genes that are differentially expressed between the uterus of the chorioallantoic and yolk sac placentas supports the hypothesis that these tissues are regionally specialized to support different pregnancy associated functions (Table 1, 2; Fig. 2).

Convergent use of genes between lineages

We tested for non-random use of amino acid transport protein genes between human trophoblast tissue (Carter 2012) and uterine placental tissues from the southern grass skink,

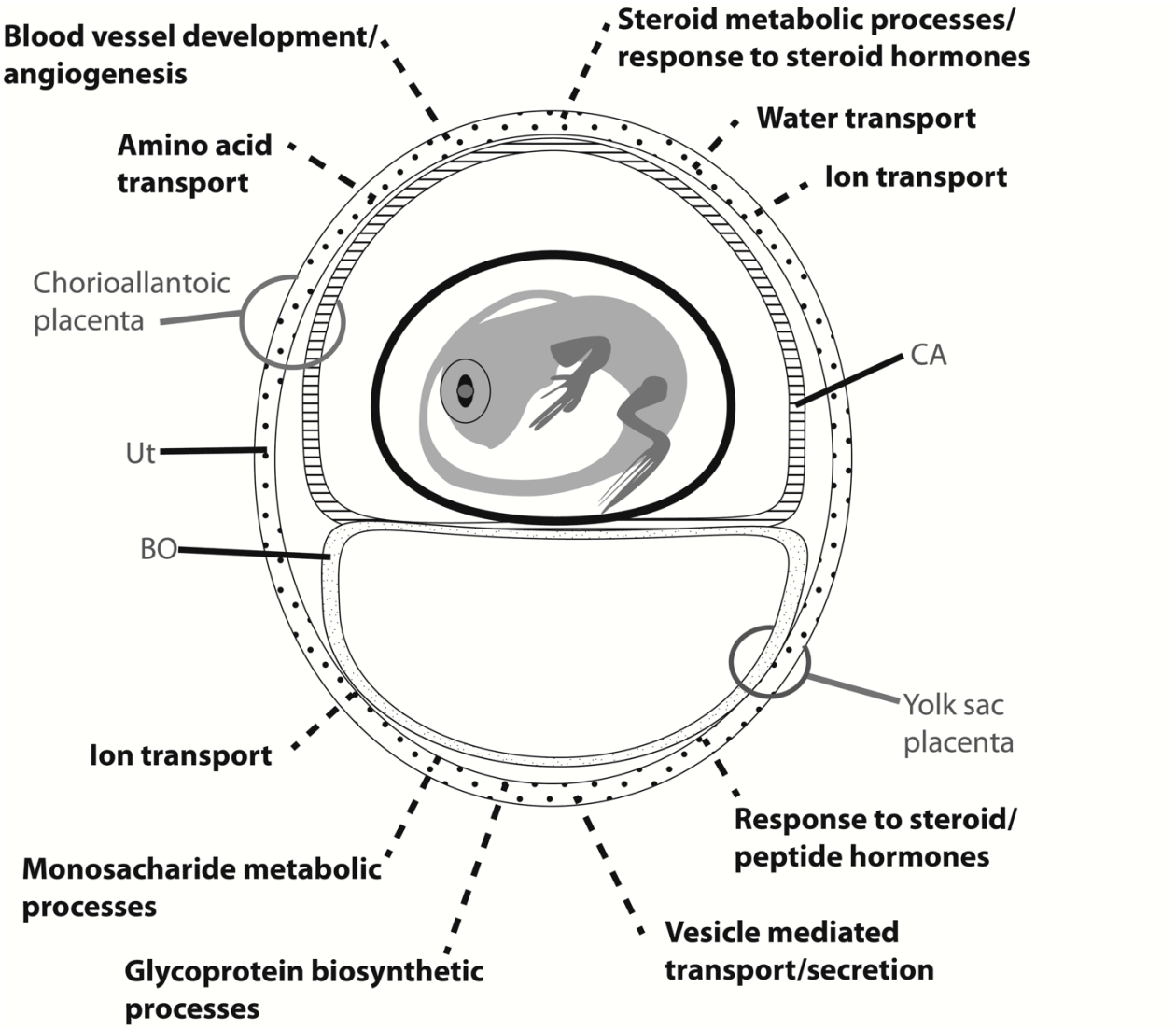


Figure 2. Processes occurring in the two placentae of the grass skink. Stylized figure (modified from Stewart and Thompson 2003) outlining the processes (dashed lines) occurring in the uterine tissue of each placenta in grass skinks as identified by functional annotation analysis of differentially expressed genes between chorioallantoic and yolk sac placental samples. Ut – uterus, BO – bilaminar omphalopleure, CA – chorioallantois.

Table 1. Gene ontology (GO) annotation for genes significantly more highly expressed in the uterus of the chorioallantoic placenta relative to the yolk sac placenta during pregnancy.

GO Term (biological processes)	P Value[a]	Benjamini corrected P Value	Fold Enrichment	Gene symbols in cluster
Organic acid transport (Cluster 1)	**Enrichment Score: 4.22**			
GO:0015837~amine transport	0.0000	0.00	17.37	*SLC38A3*, *AQP9*, *SLC6A2*, *SLC6A4*, *SLC7A8*, *SLC3A2*, *SLC22A3*, *SLC7A5*, *PDZK1*, *CRYM*
GO:0046942~carboxylic acid transport	0.0000	0.00	11.15	*SLC38A3*, *SLC16A1*, *AQP9*, *AQP8*, *SLC7A8*, *SLC3A2*, *SLC7A5*, *PDZK1*
GO:0015849~organic acid transport	0.0000	0.00	11.08	*SLC38A3*, *SLC16A1*, *AQP9*, *AQP8*, *SLC7A8*, *SLC3A2*, *SLC7A5*, *PDZK1*
GO:0015804~neutral amino acid transport	0.0001	0.02	39.04	*SLC38A3*, *SLC7A8*, *SLC3A2*, *SLC7A5*
GO:0006865~amino acid transport	0.0009	0.10	11.39	*SLC38A3*, *SLC7A8*, *SLC3A2*, *SLC7A5*, *PDZK1*
GO:0015807~L-amino acid transport	0.0054	0.26	26.74	*SLC38A3*, *SLC7A8*, *SLC7A5*
GO:0015718~monocarboxylic acid transport	0.024	0.58	12.30	*SLC16A1*, *AQP9*, *AQP8*
Neuron related transport (Cluster 2)	**Enrichment Score: 2.36**			
GO:0015844~monoamine transport	0.0001	0.01	48.23	*SLC6A2*, *SLC6A4*, *SLC22A3*, *CRYM*
GO:0019226~transmission of nerve impulse	0.0014	0.13	4.69	*UNC119*, *CLDN19*, *SLC6A2*, *GALR3*, *SLC6A4*, *LPAR3*, *SLC22A3*, *VIPR1*
GO:0007268~synaptic transmission	0.0030	0.22	4.81	*UNC119*, *SLC6A2*, *GALR3*, *SLC6A4*, *LPAR3*, *SLC22A3*, *VIPR1*
GO:0006836~neurotransmitter transport	0.0074	0.29	9.88	*SLC6A2*, *SLC6A4*, *SLC22A3*, *SLC6A17*
GO:0007267~cell-cell signaling	0.025	0.57	2.73	*UNC119*, *SLC6A2*, *GALR3*, *SLC6A4*, *LPAR3*, *SLC22A3*, *VIPR1*, *IHH*
Lipid metabolism (Cluster 3)	**Enrichment Score: 2.09**			

GO:0008202~steroid metabolic process	0.0004	0.05	7.10	*SDR42E2*, *NPC1*, *OSBPL3*, *HSD17B2*, *SDR42E1*, *SORL1*, *NPC1L1*
GO:0006869~lipid transport	0.033	0.63	5.65	*NPC1*, *OSBPL3*, *SORL1*, *NPC1L1*
GO:0010876~lipid localization	0.040	0.68	5.22	*NPC1*, *OSBPL3*, *SORL1*, *NPC1L1*
Ion transport (Cluster 4)	**Enrichment Score: 1.95**			
GO:0006811~ion transport	0.0034	0.21	2.94	*SLC12A7*, *SLC38A3*, *SLC16A1*, *SLC20A2*, *SLC9A3*, *CLIC6*, *SLC3A2*, *SLC22A3*, *ABCC4*, *SLC13A4*, *PDZK1*
GO:0006814~sodium ion transport	0.0035	0.20	7.88	*SLC12A7*, *SLC38A3*, *SLC20A2*, *SLC9A3*, *SLC13A4*
GO:0006820~anion transport	0.0049	0.25	7.17	*SLC12A7*, *SLC16A1*, *SLC20A2*, *CLIC6*, *SLC13A4*
GO:0055085~transmembrane transport	0.0057	0.25	3.24	*SLC12A7*, *SLC16A1*, *SLC25A31*, *AQP9*, *SLC9A3*, *SLC22A3*, *ABCC4*, *SLC13A4*, *PDZK1*
GO:0015698~inorganic anion transport	0.010	0.34	8.82	*SLC12A7*, *SLC20A2*, *CLIC6*, *SLC13A4*
GO:0006812~cation transport	0.016	0.48	2.97	*SLC12A7*, *SLC38A3*, *SLC20A2*, *SLC9A3*, *SLC3A2*, *SLC22A3*, *SLC13A4*, *PDZK1*
Steroid metabolism (Cluster 5)	**Enrichment Score: 1.65**			
GO:0008202~steroid metabolic process	0.0004	0.05	7.10	*SDR42E2*, *NPC1*, *OSBPL3*, *HSD17B2*, *SDR42E1*, *SORL1*, *NPC1L1*
GO:0006694~steroid biosynthetic process	0.0079	0.29	9.65	*SDR42E2*, *HSD17B2*, *SDR42E1*, *NPC1L1*
Cell homeostasis (Cluster 6)	**Enrichment Score: 1.07**			
GO:0042592~homeostatic process	0.0029	0.23	3.00	*SLC12A7*, *NPC1*, *GCLC*, *AQP9*, *FGGY*, *SLC9A3*, *VEGFA*, *TRHR*, *SLC7A8*, *LPAR3*, *NPC1L1*
GO:0048878~chemical homeostasis	0.0030	0.20	3.60	*NPC1*, *GCLC*, *AQP9*, *SLC9A3*, *VEGFA*, *TRHR*, *SLC7A8*, *LPAR3*, *NPC1L1*
Response to steroid hormones (Cluster 7)	**Enrichment Score: 0.99**			
GO:0006790~sulfur metabolic process	0.018	0.49	7.13	*GCLC*, *GPX4*, *SULF1*, *GHR*

GO:0032355~response to estradiol stimulus	0.028	0.59	11.39	*GPX4, GHR, IHH*
Endocytosis (Cluster 8)	**Enrichment Score: 0.90**			
GO:0006898~receptor-mediated endocytosis	0.027	0.59	11.60	*SLC9A3, SORL1, GHR*

[a]For brevity, only go terms with P< 0.05 are displayed.

Table 2. Gene ontology (GO) annotation for genes significantly more highly expressed in the uterus of the yolk sac placenta relative to the chorioallantoic placenta during pregnancy.

GO Term (biological processes)	P Value[a]	Benjamini corrected P Value	Fold Enrichment	Gene symbols in cluster
Metabolic Processing (Cluster 1)	**Enrichment Score: 2.16**			
GO:0006041~glucosamine metabolic process	0.0055	0.60	26.35	*PGM3*, *GNE*, *CHST4*
GO:0006044~N-acetylglucosamine metabolic process	0.0055	0.60	26.35	*PGM3*, *GNE*, *CHST4*
GO:0005996~monosaccharide metabolic process	0.0082	0.59	4.75	*PGM3*, *GNE*, *ALDOB*, *GFPT2*, *HK2*, *CHST4*
GO:0006040~amino sugar metabolic process	0.0086	0.57	21.08	*PGM3*, *GNE*, *CHST4*
Regulation of transport (Cluster 2)	**Enrichment Score: 1.64**			
GO:0051048~negative regulation of secretion	0.0036	0.51	12.78	*FAM3D*, *ERBB3*, *EDN2*, *INHA*
GO:0048511~rhythmic process	0.0058	0.56	6.86	*EGR2*, *ERBB3*, *EDN2*, *TIMP4*, *INHA*
GO:0051051~negative regulation of transport	0.0070	0.58	6.51	*PTGS2*, *FAM3D*, *ERBB3*, *EDN2*, *INHA*
GO:0046888~negative regulation of hormone secretion	0.0093	0.53	20.27	*FAM3D*, *EDN2*, *INHA*
GO:0051046~regulation of secretion	0.027	0.77	4.35	*UNC13D*, *FAM3D*, *ERBB3*, *EDN2*, *INHA*
GO:0051050~positive regulation of transport	0.037	0.80	3.94	*UNC13D*, *ERBB3*, *EDN2*, *P2RY1*, *INHA*
organic acid transport (Cluster 3)	**Enrichment Score: 1.36**			
GO:0015718~monocarboxylic acid transport	0.032	0.78	10.54	*SLC16A3*, *PPARG*, *ANXA1*
GO:0046942~carboxylic acid transport	0.0498	0.87	4.78	*SLC16A3*, *PPARG*, *ANXA1*, *SLC22A5*
response to hormones (Cluster 4)	**Enrichment Score: 1.25**			

GO:0042493~response to drug	0.0013	0.35	5.69	*PTGS2*, *ERBB3*, *PPARG*, *TIMP4*, *SLC22A5*, *SLC46A2*, *MVP*
GO:0048511~rhythmic process	0.0058	0.56	6.86	*EGR2*, *ERBB3*, *EDN2*, *TIMP4*, *INHA*
GO:0043434~response to peptide hormone stimulus	0.011	0.57	5.70	*EGR2*, *ERBB3*, *ALDOB*, *PPARG*, *TIMP4*
Regulation of nerve activity (Cluster 5)	**Enrichment Score: 1.23**			
GO:0044057~regulation of system process	0.0017	0.34	4.55	*SRI*, *EGR2*, *PTGS2*, *EDN2*, *EPHX2*, *BHLHE40*, *INHA*, *SLC22A5*
Carbohydrate metabolism (Cluster 6)	**Enrichment Score: 1.15**			
GO:0005996~monosaccharide metabolic process	0.0082	0.59	4.75	*PGM3*, *GNE*, *ALDOB*, *GFPT2*, *HK2*, *CHST4*
GO:0016051~carbohydrate biosynthetic process	0.022	0.75	6.57	*PGM3*, *GNE*, *ALDOB*, *GFPT2*
Cell homeostasis (Cluster 7)	**Enrichment Score: 1.11**			
GO:0044057~regulation of system process	0.0017	0.34	4.55	*SRI*, *EGR2*, *PTGS2*, *EDN2*, *EPHX2*, *BHLHE40*, *INHA*, *SLC22A5*
GO:0042592~homeostatic process	0.0090	0.55	2.57	*SRI*, *SLC26A4*, *EGR2*, *EDN2*, *PPARG*, *SLC9A2*, *EPHX2*, *CLDN1*, *HEPH*, *INHA*, *SLC22A5*
GO:0048878~chemical homeostasis	0.025	0.76	2.75	*SRI*, *SLC26A4*, *EGR2*, *EDN2*, *PPARG*, *SLC9A2*, *EPHX2*, *CLDN1*
GO:0050801~ion homeostasis	0.027	0.76	3.01	*SRI*, *SLC26A4*, *EGR2*, *EDN2*, *SLC9A2*, *EPHX2*, *CLDN1*
Fatty acid metabolism (Cluster 8)	**Enrichment Score: 1.04**			
GO:0008217~regulation of blood pressure	0.019	0.71	7.03	*PTGS2*, *EDN2*, *PPARG*, *EPHX2*
GO:0033559~unsaturated fatty acid metabolic process	0.033	0.78	10.33	*PTGS2*, *EDN2*, *EPHX2*
Inflamation response (Cluster 9)	**Enrichment Score: 1.02**			
GO:0009611~response to wounding	0.0291	0.76	2.65	*IRAK2*, *UNC13D*, *ERBB3*, *P2RY1*, *ANXA1*, *EPHX2*, *CHST4*, *GRHL3*

Regulation of transport (Cluster 17)	**Enrichment Score: 0.5159817951223741**			
GO:0051050~positive regulation of transport	0.037	0.79721324	3.939199814	*UNC13D, ERBB3, EDN2, P2RY1, INHA*

[a]For brevity, only go terms with P< 0.05 are displayed.

and found a non-random use of amino acid transport genes between lineages ($P < 0.001$, Table 3, Fig. 3).

Non-random use of amino acid transport proteins to the placenta of both lineages could be explained by non-independent recruitment of amino acid transporter genes or differences in the probability of inducing the expression of each gene to the placenta. Non-independent selection of genes would require that multiple genes are recruited simultaneously by the activation of a similar set of cis- or trans-acting regulatory networks (Davidson and Erwin 2006). This hypothesis predicts that selected genes have correlated expression across tissues. We tested this prediction by clustering amino acid transport protein gene expression across a human tissue gene expression database. Selected genes did not show correlated expression across tissues (Fig. 4), rejecting the hypothesis of non-independent selection. Hence, non-random use of amino acid transport proteins in the placenta is constrained such that only a subset of these genes are utilized, even though multiple genes are available to be co-opted to facilitate placental protein transport.

DISCUSSION

Lizard placentation converges on eutherian condition

Growth and re-modelling of the uterus

During pregnancy it is necessary to extensively re-model uterine tissue to support placental functions. Uterine remodelling requires additional folding of uterine tissue to increase the area for material exchange, and modifications to cell morphology to facilitate the new functions of the uterine tissue (Thompson, et al. 2006; Thompson and Speake 2006).

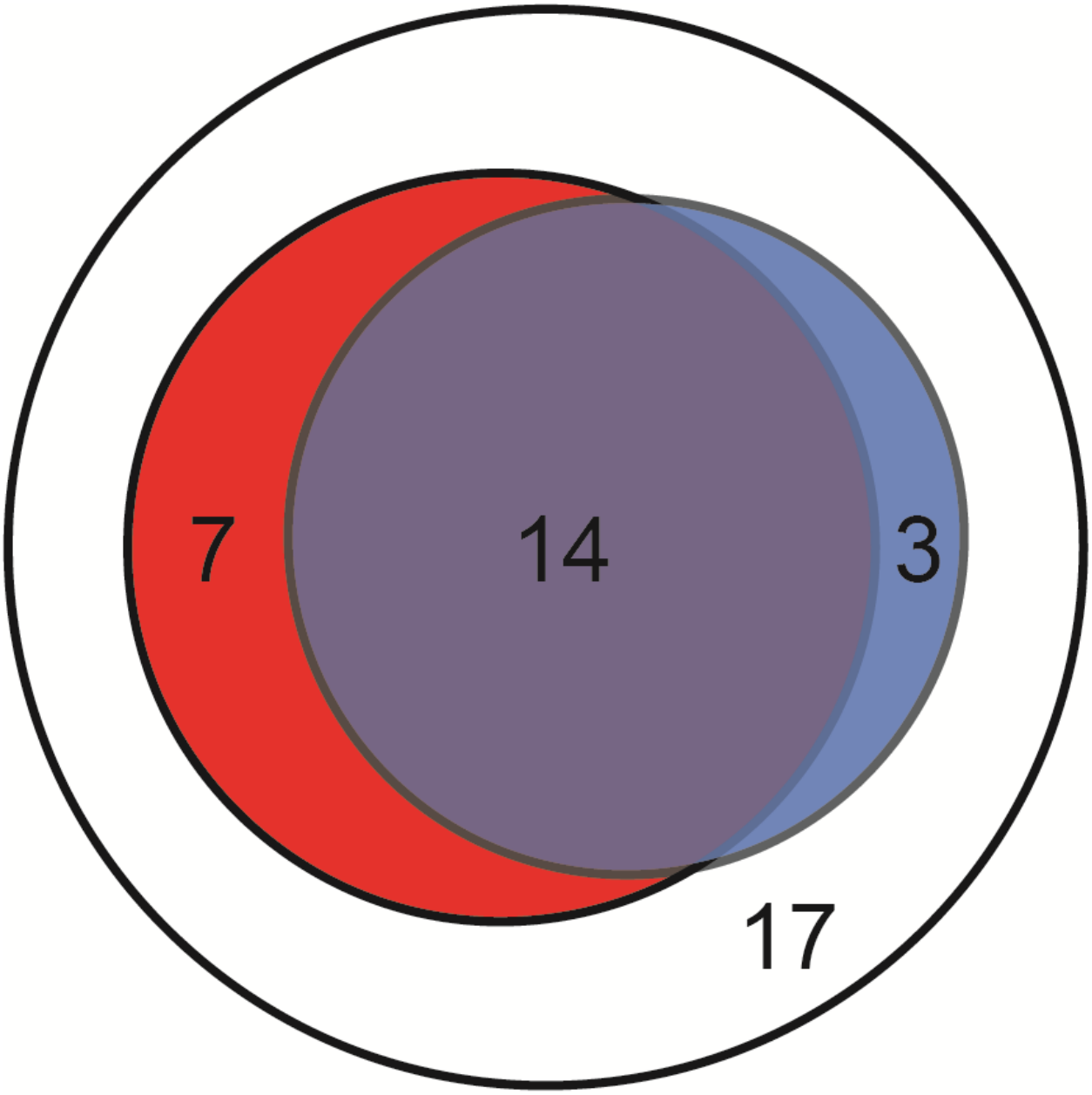


Figure 3. Overlap in the use of orthologous amino acid transport protein genes by human trophoblast (red) and grass skink uterine tissue (blue) during pregnancy. 41 orthologous amino acid transport proteins are shared by both lineages, 21 are utilized in human placenta, whilst 17 are used in grass skinks. 17 genes are not utilized in the placental tissues of either species (white). Lineages show a significantly non-random use of genes ($P < 0.001$).

Table 1. Gene ontology (GO) annotation for genes significantly more highly expressed in the uterus of the chorioallantoic placenta relative to the yolk sac placenta during pregnancy.

GO Term (biological processes)	P Value[a]	Benjamini corrected P Value	Fold Enrichment	Gene symbols in cluster
Organic acid transport (Cluster 1)	**Enrichment Score: 4.22**			
GO:0015837~amine transport	0.0000	0.00	17.37	*SLC38A3*, *AQP9*, *SLC6A2*, *SLC6A4*, *SLC7A8*, *SLC3A2*, *SLC22A3*, *SLC7A5*, *PDZK1*, *CRYM*
GO:0046942~carboxylic acid transport	0.0000	0.00	11.15	*SLC38A3*, *SLC16A1*, *AQP9*, *AQP8*, *SLC7A8*, *SLC3A2*, *SLC7A5*, *PDZK1*
GO:0015849~organic acid transport	0.0000	0.00	11.08	*SLC38A3*, *SLC16A1*, *AQP9*, *AQP8*, *SLC7A8*, *SLC3A2*, *SLC7A5*, *PDZK1*
GO:0015804~neutral amino acid transport	0.0001	0.02	39.04	*SLC38A3*, *SLC7A8*, *SLC3A2*, *SLC7A5*
GO:0006865~amino acid transport	0.0009	0.10	11.39	*SLC38A3*, *SLC7A8*, *SLC3A2*, *SLC7A5*, *PDZK1*
GO:0015807~L-amino acid transport	0.0054	0.26	26.74	*SLC38A3*, *SLC7A8*, *SLC7A5*
GO:0015718~monocarboxylic acid transport	0.024	0.58	12.30	*SLC16A1*, *AQP9*, *AQP8*
Neuron related transport (Cluster 2)	**Enrichment Score: 2.36**			
GO:0015844~monoamine transport	0.0001	0.01	48.23	*SLC6A2*, *SLC6A4*, *SLC22A3*, *CRYM*
GO:0019226~transmission of nerve impulse	0.0014	0.13	4.69	*UNC119*, *CLDN19*, *SLC6A2*, *GALR3*, *SLC6A4*, *LPAR3*, *SLC22A3*, *VIPR1*
GO:0007268~synaptic transmission	0.0030	0.22	4.81	*UNC119*, *SLC6A2*, *GALR3*, *SLC6A4*, *LPAR3*, *SLC22A3*, *VIPR1*
GO:0006836~neurotransmitter transport	0.0074	0.29	9.88	*SLC6A2*, *SLC6A4*, *SLC22A3*, *SLC6A17*
GO:0007267~cell-cell signaling	0.025	0.57	2.73	*UNC119*, *SLC6A2*, *GALR3*, *SLC6A4*, *LPAR3*, *SLC22A3*, *VIPR1*, *IHH*
Lipid metabolism (Cluster 3)	**Enrichment Score: 2.09**			

GO:0008202~steroid metabolic process	0.0004	0.05	7.10	*SDR42E2*, *NPC1*, *OSBPL3*, *HSD17B2*, *SDR42E1*, *SORL1*, *NPC1L1*
GO:0006869~lipid transport	0.033	0.63	5.65	*NPC1*, *OSBPL3*, *SORL1*, *NPC1L1*
GO:0010876~lipid localization	0.040	0.68	5.22	*NPC1*, *OSBPL3*, *SORL1*, *NPC1L1*
Ion transport (Cluster 4)	**Enrichment Score: 1.95**			
GO:0006811~ion transport	0.0034	0.21	2.94	*SLC12A7*, *SLC38A3*, *SLC16A1*, *SLC20A2*, *SLC9A3*, *CLIC6*, *SLC3A2*, *SLC22A3*, *ABCC4*, *SLC13A4*, *PDZK1*
GO:0006814~sodium ion transport	0.0035	0.20	7.88	*SLC12A7*, *SLC38A3*, *SLC20A2*, *SLC9A3*, *SLC13A4*
GO:0006820~anion transport	0.0049	0.25	7.17	*SLC12A7*, *SLC16A1*, *SLC20A2*, *CLIC6*, *SLC13A4*
GO:0055085~transmembrane transport	0.0057	0.25	3.24	*SLC12A7*, *SLC16A1*, *SLC25A31*, *AQP9*, *SLC9A3*, *SLC22A3*, *ABCC4*, *SLC13A4*, *PDZK1*
GO:0015698~inorganic anion transport	0.010	0.34	8.82	*SLC12A7*, *SLC20A2*, *CLIC6*, *SLC13A4*
GO:0006812~cation transport	0.016	0.48	2.97	*SLC12A7*, *SLC38A3*, *SLC20A2*, *SLC9A3*, *SLC3A2*, *SLC22A3*, *SLC13A4*, *PDZK1*
Steroid metabolism (Cluster 5)	**Enrichment Score: 1.65**			
GO:0008202~steroid metabolic process	0.0004	0.05	7.10	*SDR42E2*, *NPC1*, *OSBPL3*, *HSD17B2*, *SDR42E1*, *SORL1*, *NPC1L1*
GO:0006694~steroid biosynthetic process	0.0079	0.29	9.65	*SDR42E2*, *HSD17B2*, *SDR42E1*, *NPC1L1*
Cell homeostasis (Cluster 6)	**Enrichment Score: 1.07**			
GO:0042592~homeostatic process	0.0029	0.23	3.00	*SLC12A7*, *NPC1*, *GCLC*, *AQP9*, *FGGY*, *SLC9A3*, *VEGFA*, *TRHR*, *SLC7A8*, *LPAR3*, *NPC1L1*
GO:0048878~chemical homeostasis	0.0030	0.20	3.60	*NPC1*, *GCLC*, *AQP9*, *SLC9A3*, *VEGFA*, *TRHR*, *SLC7A8*, *LPAR3*, *NPC1L1*
Response to steroid hormones (Cluster 7)	**Enrichment Score: 0.99**			
GO:0006790~sulfur metabolic process	0.018	0.49	7.13	*GCLC*, *GPX4*, *SULF1*, *GHR*

GO:0032355~response to estradiol stimulus	0.028	0.59	11.39	*GPX4, GHR, IHH*
Endocytosis (Cluster 8)	**Enrichment Score: 0.90**			
GO:0006898~receptor-mediated endocytosis	0.027	0.59	11.60	*SLC9A3, SORL1, GHR*

[a]For brevity, only go terms with P< 0.05 are displayed.

Table 2. Gene ontology (GO) annotation for genes significantly more highly expressed in the uterus of the yolk sac placenta relative to the chorioallantoic placenta during pregnancy.

GO Term (biological processes)	P Value[a]	Benjamini corrected P Value	Fold Enrichment	Gene symbols in cluster
Metabolic Processing (Cluster 1)	**Enrichment Score: 2.16**			
GO:0006041~glucosamine metabolic process	0.0055	0.60	26.35	*PGM3*, *GNE*, *CHST4*
GO:0006044~N-acetylglucosamine metabolic process	0.0055	0.60	26.35	*PGM3*, *GNE*, *CHST4*
GO:0005996~monosaccharide metabolic process	0.0082	0.59	4.75	*PGM3*, *GNE*, *ALDOB*, *GFPT2*, *HK2*, *CHST4*
GO:0006040~amino sugar metabolic process	0.0086	0.57	21.08	*PGM3*, *GNE*, *CHST4*
Regulation of transport (Cluster 2)	**Enrichment Score: 1.64**			
GO:0051048~negative regulation of secretion	0.0036	0.51	12.78	*FAM3D*, *ERBB3*, *EDN2*, *INHA*
GO:0048511~rhythmic process	0.0058	0.56	6.86	*EGR2*, *ERBB3*, *EDN2*, *TIMP4*, *INHA*
GO:0051051~negative regulation of transport	0.0070	0.58	6.51	*PTGS2*, *FAM3D*, *ERBB3*, *EDN2*, *INHA*
GO:0046888~negative regulation of hormone secretion	0.0093	0.53	20.27	*FAM3D*, *EDN2*, *INHA*
GO:0051046~regulation of secretion	0.027	0.77	4.35	*UNC13D*, *FAM3D*, *ERBB3*, *EDN2*, *INHA*
GO:0051050~positive regulation of transport	0.037	0.80	3.94	*UNC13D*, *ERBB3*, *EDN2*, *P2RY1*, *INHA*
organic acid transport (Cluster 3)	**Enrichment Score: 1.36**			
GO:0015718~monocarboxylic acid transport	0.032	0.78	10.54	*SLC16A3*, *PPARG*, *ANXA1*
GO:0046942~carboxylic acid transport	0.0498	0.87	4.78	*SLC16A3*, *PPARG*, *ANXA1*, *SLC22A5*
response to hormones (Cluster 4)	**Enrichment Score: 1.25**			

GO:0042493~response to drug	0.0013	0.35	5.69	*PTGS2*, *ERBB3*, *PPARG*, *TIMP4*, *SLC22A5*, *SLC46A2*, *MVP*
GO:0048511~rhythmic process	0.0058	0.56	6.86	*EGR2*, *ERBB3*, *EDN2*, *TIMP4*, *INHA*
GO:0043434~response to peptide hormone stimulus	0.011	0.57	5.70	*EGR2*, *ERBB3*, *ALDOB*, *PPARG*, *TIMP4*
Regulation of nerve activity (Cluster 5)	**Enrichment Score: 1.23**			
GO:0044057~regulation of system process	0.0017	0.34	4.55	*SRI*, *EGR2*, *PTGS2*, *EDN2*, *EPHX2*, *BHLHE40*, *INHA*, *SLC22A5*
Carbohydrate metabolism (Cluster 6)	**Enrichment Score: 1.15**			
GO:0005996~monosaccharide metabolic process	0.0082	0.59	4.75	*PGM3*, *GNE*, *ALDOB*, *GFPT2*, *HK2*, *CHST4*
GO:0016051~carbohydrate biosynthetic process	0.022	0.75	6.57	*PGM3*, *GNE*, *ALDOB*, *GFPT2*
Cell homeostasis (Cluster 7)	**Enrichment Score: 1.11**			
GO:0044057~regulation of system process	0.0017	0.34	4.55	*SRI*, *EGR2*, *PTGS2*, *EDN2*, *EPHX2*, *BHLHE40*, *INHA*, *SLC22A5*
GO:0042592~homeostatic process	0.0090	0.55	2.57	*SRI*, *SLC26A4*, *EGR2*, *EDN2*, *PPARG*, *SLC9A2*, *EPHX2*, *CLDN1*, *HEPH*, *INHA*, *SLC22A5*
GO:0048878~chemical homeostasis	0.025	0.76	2.75	*SRI*, *SLC26A4*, *EGR2*, *EDN2*, *PPARG*, *SLC9A2*, *EPHX2*, *CLDN1*
GO:0050801~ion homeostasis	0.027	0.76	3.01	*SRI*, *SLC26A4*, *EGR2*, *EDN2*, *SLC9A2*, *EPHX2*, *CLDN1*
Fatty acid metabolism (Cluster 8)	**Enrichment Score: 1.04**			
GO:0008217~regulation of blood pressure	0.019	0.71	7.03	*PTGS2*, *EDN2*, *PPARG*, *EPHX2*
GO:0033559~unsaturated fatty acid metabolic process	0.033	0.78	10.33	*PTGS2*, *EDN2*, *EPHX2*
Inflamation response (Cluster 9)	**Enrichment Score: 1.02**			
GO:0009611~response to wounding	0.0291	0.76	2.65	*IRAK2*, *UNC13D*, *ERBB3*, *P2RY1*, *ANXA1*, *EPHX2*, *CHST4*, *GRHL3*

Regulation of transport (Cluster 17)	**Enrichment Score: 0.5159817951223741**			
GO:0051050~positive regulation of transport	0.037	0.79721324	3.939199814	*UNC13D, ERBB3, EDN2, P2RY1, INHA*

[a]For brevity, only go terms with P< 0.05 are displayed.

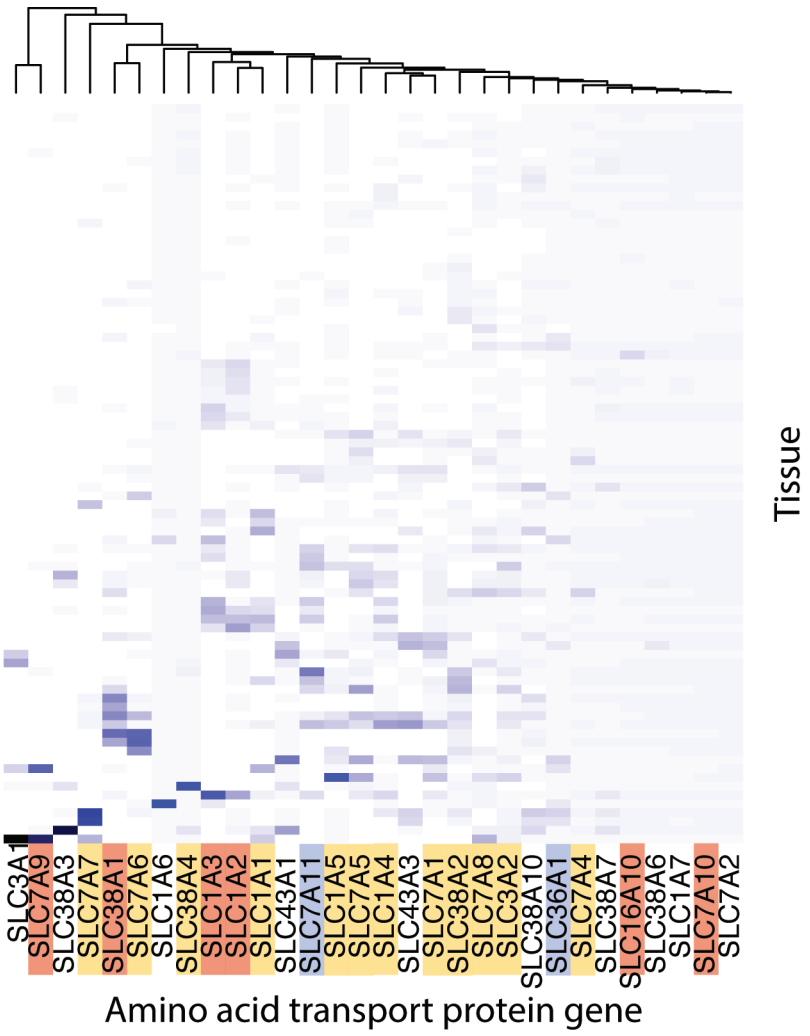


Figure 4. Heat map of amino acid transport protein gene expression across human tissues; as measured by microarray analysis (Freeman et al. 2012; Su et al. 2004). Gene symbols are highlighted based on whether they are expressed solely in human trophoblast (red), grass skink uterus (blue) or both (yellow), non-highlighted genes are not used in the placentae of either lineage. Although multiple genes are co-expressed in some human tissues, no genes show correlated expression across all tissue. Further co-expressed genes are in most cases not co-opted as a batch into placental tissues of humans and grass skinks.

Pseudemoia entrecasteauxii has morphologically distinct placental regions which support different placental functions (Fig. 1). Tissue re-modelling requires a series of simultaneous processes, the promotion of cell death and cell proliferation, and the production of transcription factors/hormones that direct cell differentiation so that the cells have the appropriate form.

Proteases are enzymes that digest other proteins and are essential for tissue re-modelling because they promote apoptosis and recycle proteins, allowing tissues to be re-formed from the products of cell degeneration (Green and Lund 2005; Kirschke, et al. 1998). Cathepsins (CTSs) are a group of cysteine proteases that can initiate cell death, degrade extra-cellular proteins and catabolize intracellular proteins (Kirschke et al. 1998). Four CTS proteins are up-regulated in uterine tissue during pregnancy in *P. entrecasteauxii* (CTSA, CTSL, CTSZ, and CTSO). The viviparous lizard *Chalcides chalcides*, pigs, and sheep all produce CTSB and CTSL in endometrial tissue suggesting that tissue re-modelling by cathepsins is a strategy used widely for tissue re-modelling during pregnancy in amniotes (Brandley et al. 2012; Song, et al. 2010; Song, et al. 2005). We found that cathepsins are not differentially expressed between the chorioallantoic and yolk sac placental regions of the uterus suggesting that they function to initiate and sustain placental remodelling during pregnancy but do not function specifically to produce the morphological and functionally distinct regions of the placenta.

Regional differentiation of uterine tissue in the placenta is likely facilitated by specific transcription factors that regulate cellular differentiation. The uterus of the chorioallantoic and yolk sac placenta differentially express several transcription factors and hormone receptors between placental regions (Table 1, 2). To better understand the role of these genes

in the evolution of regional specialization in the uterus, it is necessary to both identify the cell types that express these transcription factors through pregnancy, and identify their function in closely related oviparous taxa. These genes are the best candidate genes for regulators of cellular differences between placental regions and may be key to understanding the evolution of innovative placental morphologies and functions in squamates.

Gas exchange and blood vessel proliferation

As embryonic development progresses, the embryonic demand for oxygen and the production of metabolic waste products such as carbon dioxide increases. Demand for exchange of materials between offspring and mother requires an increase in the exchange rate of respiratory gasses between mother and embryo, which is achieved by increasing the vascular density and blood vessel diameter in the uterus (Parker, et al. 2010). In some lizards this vasculature is likely regulated by both maternal and embryonic cues (Murphy, et al. 2010b). The expression of genes involved in blood vessel development such as *VEGFA* may provide mechanisms for vascular proliferation (Table 1, Adams et al. 2005). *VEGFA* is a highly potent angiogenic factor that has been found in reptile uterine tissue in the past (Ferrara, et al. 2003; Murphy, et al. 2010a). Expression of *VEGFA* correlates with embryonic development in the viviparous lizard *Saiphos equalis* (Whittington, et al. 2015). *VEGFA* exhibits greater uterine expression in the chorioallantoic placenta than the yolk sac placenta (Table 1). The chorioallantoic placenta forms two distinct regions, the placentome and paraplacentome. Both regions are highly vascularized, but for different reasons. Vascularization in the paraplacentome supports the exchange of respiratory gasses, whilst the placentome supports nutrient transport to embryos (Biazik et al. 2009). Increased expression of VEGFA in the chorioallantoic placenta may support blood vessel proliferation for gas exchange in the

paraplacentome, and be involved in nutrient transport to embryos from the placentome or both. To determine its site of activity, future studies should localise VEGFA to either of these two regions.

Nutrient transport

We propose that the chorioallantoic placenta transports nutrients to the embryo via membrane bound nutrient transport proteins, whilst the yolk sac placenta transports packaged vesicles to embryos containing lipids and other nutrients (Fig. 2), as previously suggested (Adams et al. 2005; Griffith, et al. 2013b). Differences in gene expression show that functional specializations of these tissues are underpinned by complex changes in gene regulation, on par with the complex mechanisms of placental function in eutherians (Sood, et al. 2006). The morphological and gene expression changes necessary for placental nutrient transport may explain why placentotrophy has evolved so few times in amniote vertebrates (Thompson and Speake 2006).

Chorioallantoic placenta

The chorioallantoic placenta has been assumed to play a role in gas exchange and the transport of some organic and inorganic solutes (Biazik et al. 2009; Herbert, et al. 2010; Stewart, et al. 2006). Our gene ontology (GO) analysis identified that the chorioallantoic placenta contains membrane bound transport proteins for the transport of a range of molecules, including amino acids, carboxylic acids, sulphates, inorganic ions, and water (Table 1). The large number and diversity of nutrient transport proteins expressed in the chorioallantoic placenta is strong evidence the area transports a large range of nutrients to developing embryos. Given that amino acid transport proteins represent the largest and most over-represented group of genes in the list of genes upregulated in the chorioallantoic

placenta, this region is likely to be a major site of protein processing and transport to embryos. This finding is at odds with conclusions drawn from Itonaga, et al. (2012), where they suggested the correlation between leucine transport rates through pregnancy and vascularization of the yolk sac splanchnopleure implicated the yolk sac placenta as the site of amino acid transport. However, the patterns of leucine transport presented by Itonaga et al. (2012) are also correlated with development of the placentome (a tightly folded region of the chorioallantoic placenta with large uterine secretory epithelial cells) in the chorioallantoic placenta (Stewart and Thompson 1996). The embryonic tissues in the placentome are capable of taking up organic molecules, including dextran, suggesting any maternally secreted molecules could be absorbed by the embryo (Stewart et al. 2006). Combined these data support the placentome as a major site of amino acid/protein transport to embryos, further highlighting the similarities of reptile placentomes with the discoid placentomes found in ruminants (Fowden, et al. 2006; Wildman et al. 2006).

Yolk sac placenta

Transcriptomic data support the yolk-sac placenta as a transporter of organic nutrients during pregnancy, with the second most enriched cluster of GO terms including genes involved in transport regulation and secretion, whilst other clusters including terms for fatty acid metabolism and organic acid transport (Table 2). Unlike the chorioallantoic placenta there are few up-regulated membrane bound transport proteins, reflecting the different ways in which nutrients are transported in these different placental regions. Nutrient transport in the yolk sac placenta likely occurs via membrane bound vesicles that actively bud from the uterine surface rather than secretion of nutrients directly into the lumen (Adams et al. 2005; Biazik et al. 2009; Biazik et al. 2010).

Fatty acid binding proteins (FABPs) are intracellular proteins that facilitate intracellular fatty acid transport (Storch and Thumser 2000). *Pseudemoia entrecasteauxii* expresses significantly more FABP1 and FABP9 in the uterine tissue of the yolk sac placenta than in non-pregnant uterine tissue. FABP1 and FABP9 have different specificity for particular lipid molecules (Vogel Hertzel and Bernlohr 2000) suggesting they perform discrete roles in the transport of lipids to offspring. In the human placenta FABP1 and FABP3 appear to participate in lipid mobilization from mother to embryo, with each protein probably having a distinct role; FABP3 only binds long chain fatty acids whilst FABP1 binds a range of different ligands (Campbell, et al. 1998; Das, et al. 1993). In ruminants FABP3 is expressed in the endometrium of pregnant cows and, FABP3 expression correlates with progesterone levels and embryonic elongation (Forde, et al. 2011). In pigs, low expression of fatty acid binding proteins has been proposed to limit the supply of fatty acids to the fetal pig (Ramsay, et al. 1991).

Shared gene use between reptile and mammalian placentae

An aim of this project was to identify whether reptilian and mammalian placental functions were underpinned by the same genes. To meet this goal we focused on the differential expression of amino acid transport proteins in the uterus between pregnant and non-pregnant states. Embryos are essentially built from proteins/amino acids during pregnancy, so changes in the mechanisms of protein allocation are likely to be drivers in the evolution of placentotrophy (Van Dyke, et al. 2014b). Furthermore, amino acids have a diversity of chemistries and many are hydrophilic so they do not readily diffuse across cell membranes. Thus, the transport of amino acids from mother to embryo requires a complex array of transport proteins (Palacín, et al. 1998). Amino acid transport proteins are highly conserved

across vertebrates; and one to one orthology has been identified in existing squamate genomes for almost all mammalian amino acid transport protein genes (Flicek, et al. 2014; Fujita, et al. 2011). For these reasons, the repeated evolution of amino acid transport in placentae provides a unique opportunity to determine the role of the genome in the evolution of complex functions and complex organs.

By examining the amino acid transport proteins used in the uterus of the southern grass skink and in the trophoblast of humans we show that the complex function of amino acid transport to embryos have evolved in these two structures using a non-random selection of the amino acid transport proteins encoded in the genomes of each species (Fig. 3). Two explanations for the similar expression of these genes in the placenta exist, either ancestral expression of these genes occurred prior to the evolution of pregnancy or these genes were recruited to these tissues during the evolution of placentation. By comparing the expression of amino acid transport proteins in the tissues from which these placentae are derived in oviparous/non-placentotrophic taxa, future research will identify if the parallel use of genes is the result of ancestral expression or independent recruitment of the same sets of genes between the two lineages.

Tissues that share a developmental history are likely to share patterns of gene expression due to underlying gene regulatory networks (Arendt 2008; Kin, et al. 2015). However, shared developmental history of placental tissues cannot account for common use of amino acid transport proteins in skinks and humans, because the skink uterus and human trophoblast share little developmental history. The human trophoblast (a spherical coat of cells which will form the embryonic component of the placenta) separates from the embryoblast (the cells that will differentiate and form the embryo proper) at day five of development (Moore, et al.

2013). After day five, the trophoblast follows a different developmental trajectory than the tissue that ultimately gives rise to the uterus. If the amino acid transport proteins are ancestral to the tissues that comprise the placenta, then parallel use of genes between these two structures must be because of an alternate functional role that these genes play in the physiology of these tissues. One reasonable hypothesis is that these genes are expressed to facilitate nutrient uptake by cells to meet the metabolic demands of the uterus during gravidity or in the chorioallantoic membrane of oviparous eggs. To test this hypothesis, it is essential to first identify if these genes show the same patterns of expression in oviparous amniotes. Furthermore it would be interesting to identify if these genes show consistent expression patterns in other metabolically active tissues, such as the gut of the Burmese python. Burmese pythons typically undergo periods of fasting and then eat large meals. Pythons allow their gut to atrophy during periods of fasting to reduce metabolic demand (Secor 2008). This process affects the expression of suites of genes in the python heart, but researchers are yet to study the effect on the gut tissue itself (Wall, et al. 2011). The uterus, like the gut, goes through periods of activity and inactivity. Identifying which amino acid transport proteins are up-regulated in the python gut during feeding would allow us to test if there is a common set of amino acid transport proteins that are up-regulated in metabolically active tissues independent of reproduction.

Convergent recruitment of genes

Evolution occurs by two broad processes. First, mutations give rise to variation in organisms, and then selection acts to fix specific phenotypes (Streisfeld and Rausher 2011). If the parallel use of genes between the skink and human placenta (Fig. 3) is the result of independent recruitment of genes, then biological constraints have imposed limits on the

evolution of placental functions. To understand why convergent evolution occurs at the genetic level in lineages that diverged 320 million years ago, we need to investigate how evolutionary processes may bias evolutionary outcomes. Constraints that occur during the process of mutation are likely to arise from differences in the underlying gene regulatory networks of the gene of interest (Davidson and Erwin 2006), the developmental history for the tissue for which gene expression is being induced, and the rates of mutation in the gene networks being recruited (Stern 2013; Wagner and Misof 1993). Constraints during the process of selection will arise from differences in the fitness associated with each mutation, including effects in the placental tissues and pleiotropic effects of changes in gene expression outside the placenta (Streisfeld and Rausher 2011). We investigated one of these constraints, using available datasets to see if they could explain the observed convergent use of genes.

One explanation for the observed parallel use of genes between lineages is that multiple genes were recruited to each placenta simultaneously by similar cis- or trans-regulatory elements. If this explanation is true, then recruited genes should have correlated expression across different tissues due to similar mechanisms of regulation. Correlated expression was not found (Fig. 4), suggesting that these genes are not regulated by the same pathways and that the genes were unlikely to have been recruited by inducing a single regulatory gene.

Unexplored constraints that could explain the observed convergent use of genes include unique functional attributes of amino acid transport proteins that are ancestral to amniotes, pleiotropic effects of recruitment of genes to the placenta, mutational biases (features of the genome that result in different mutation rates between genes) in the regulatory networks of these genes; or other features present in the genomes of these organisms such as the locations of genes on chromosomes (Lynch, et al. 2011).

Conclusions

Differential gene expression between the uterus of the chorioallantoic and yolk sac placenta suggests these regions perform different functions during pregnancy. The chorioallantoic placenta has high expression of a range of membrane bound nutrient transport proteins that transfer a diversity of materials including amino acids, water, and inorganic ions. In contrast, the yolk sac placenta does not have greater expression of membrane bound transport proteins, but expresses genes involved in vesicle mediated transport. These findings support morphological data that suggests that nutrient transport occurs via apocrine secretion in the yolk sac placenta and by membrane bound transport in the placentome.

There is a significant overlap in the amino acid transport proteins that are used by the uterine tissue of the southern grass skink and those used in the trophoblast of humans. Future work is needed to identify if the expression of these genes is ancestral to the tissues from which each placenta is derived, but the parallel use of genes between lineages suggests some form of biological constraint that has increased the likelihood of specific genes being utilized in placental functions. Furthermore, the convergent evolution of placental traits, using the same underlying genes suggests that if the evolutionary process over the last 320 million years was repeated, we would expect to see many of the same complex features of vertebrates evolve predictably.

MATERIALS AND METHODS

Tissue collection and storage

Gravid female *Pseudemoia entrecasteauxii* were collected in Kanangra Boyd National Park, NSW, Australia in November 2011. Lizards were housed individually and fed three times weekly on crickets dusted with calcium gluconate powder. Once lizards had reached the appropriate period of the reproductive cycle, they were euthanized by injection with 0.1mL of sodium pentobarbital (6mg/mL). Non-pregnant lizards were collected during pregnancy and housed for an additional two months after giving birth before processing. The egg chamber of the uterus in gravid females was excised and the uterus was cut along the boundary of the chorioallantoic membrane and yolk-sac prior to removing the egg (Girling 2002). In non-gravid females, egg chambers constituting stretched regions of the uterus, were excised individually (Girling 2002). After excision, uterine tissues was fixed in RNAlater (24hrs) and stored at -80^{o}C. After tissue processing embryos were staged using the 40 stage staging scheme for the lizard, *Zootoca vivipara* (Dufaure and Hubert 1961).

RNA extraction, purification and sequencing

To extract RNA, tissue was macerated using a mechanical homogenizer in 600µL of Buffer RLT (QIAGEN) then homogenized using a QIAshredder spin column (QIAGEN). Total RNA was then extracted using the RNeasy Mini Kit (QIAGEN). Extracted RNA was treated with Amplification Grade DNase 1 (Sigma-Aldrich). RNA quality was measured on the Agilent 2000 Bioanalyzer (Agilent Technologies) and was only used for transcriptome analysis if the RIN was greater than 8. Sequencing libraries were generated in house using the

TruSeq RNA Sample Preperation kit (Illumina,inc.), were pooled into lanes containing ten samples and were sequenced on the HiSeq2000 (Illumina, Inc.).

Generation of reference transcriptome

An assembled reference transcriptome was built from the uterine tissue of early (n=1) and late (n=2) pregnant females, the embryonic membranes of late developing embryos (n=2) and adult brain tissue (n=1). Transcriptomes were assembled with ABySS 1.3.4 (Simpson, et al. 2009) from tissues of a single individual at a time. Once each transcriptome was assembled they were pooled, contigs smaller than 100bp and redundant contigs were removed with CD-HIT-EST (Huang, et al. 2010) using default options. Attributes of assembled transcriptome are summarised in Table 4. Contigs were identified by aligning against the Anolis proteome (Ensembl Build 70) using BlastX with an e-value of 10^{-5}. The alignment rate of the raw reads to the assembled transcriptome was > 90% for all samples. 60, 773 assembled transcripts were identified following blast to the *Anolis* proteome, which equates to 74% sequence coverage of protein coding genes in the published *Anolis* proteome. Un-identified contigs were further screened by aligning to a composite of the proteomes of human, chicken, opposum, platypus, zebra finch and Chinese tortoise (Ensembl Build 70) using the same criteria as above. A further 27, 431 transcripts were identified after blasting against additional proteomes.

Measuring gene expression.

Expression of each contig in the reference transcriptome was measured for each sample by aligning the raw reads of each sequenced transcriptome against the reference transcriptome with Bowtie2 (version 2.0.0-beta7) (Langmead and Salzberg 2012). The number of unique

Table 4. Attributes of assembled transcriptome

Number of transcripts	367722
Maximum contig length	21636
Mean contig length	465
Median contig length	186
N50	1130

alignments to each contig were counted using samtools view (version 0.1.18) (Li, et al. 2009). Counts from contigs that aligned to the same gene were summed, and only counts for contigs that could be identified were used in statistical analyses.

Experimental design and statistics

The transcriptomes of the uterus of the chorioallantoic placenta (n=3) and yolk sac placenta (n=3) were sequenced for pregnant females as well as the uterus of non-reproductive females (n=2). Comparisons were done in a pair wise fashion, that is each pregnant placental tissue was compared to non-reproductive tissue as well as compared with each other. Differentially expressed genes were identified by fitting normalized transcript counts to a negative binomial distribution and testing for deviations from the model with the DESeq package (Anders and Huber 2010). The uterus of the chorioallantoic placenta had 1087 up and 1468 down regulated genes when compared to non-reproductive uterus, while the uterus of the yolk-sac placenta had 1849 up and 2012 down regulated genes when compared to non-reproductive tissue . When compared with each other the uterus of the chorioallantoic placenta had greater expression of 152 genes, while the yolk-sac placenta had greater expression of 130.

Functional annotation analysis

Gene-ontology (GO) annotation was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resources 6.7 (Huang, et al. 2009). Gene symbol lists for up and down regulated genes from each of the three comparisons were entered and converted to DAVID ID's. Genes were annotated with GO terms corresponding to biological processes (BP-FAT). Up-regulated genes in the

chorioallantoic placenta fell into 16 clusters of gene ontology terms, whilst genes up-regulated in the yolk sac placenta fell into 25 clusters.

qPCR confirmation

To ensure the observed gene expression patterns were real, we confirmed the expression of five genes (Table 5) that showed differential expression between sample groups. RNA was extracted from the uterus of non-pregnant (n=4), and the uterine component of the chorioallantoic (n=7) and omphalopleuric placenta (n=7) of pregnant individuals as above. cDNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (QIAGEN) with 461ng of input RNA for all samples. Realtime-PCR analysis was conducted with the QuantiFast SYBR Green PCR protocol (QIAGEN) in 15uL reaction volumes with a cDNA equivalence of 6 ng of RNA and reaction conditions described in Table 5. All samples were run in triplicate, non-template controls were run in triplicate in every run. Reactions were set-up manually. The specificity of the PCR reaction was assured by confirming a single PCR product was observed in every reaction by melt curve analysis (50^{o}C - 99^{o}C) and the product was confirmed to be the right size by running PCR products from each primer combination on an agarose gel (3% TBE agarose, 110V, 20min) and comparing to the DNA standard HyperLadder IV (Bioline). Standard curves were generated using serial 1:4 dilutions of a composite sample containing equal parts of 17 uterine cDNA samples. All dilutions were run in triplicate. Standard curves had an $R^2 \geq 0.99$ and contained at least four dilutions from the dilution series with a linear dynamic range of at least three orders of magnitude and had PCR efficiencies between 0.9 and 1.05. All Cq values for unknowns fall within the linear quantifiable range of the appropriate standard curves. The calculated relative concentrations were normalized by dividing by the geometric mean of 18S rRNA and β-actin expression,

Table 5 – Primers and reaction conditions for qPCR analysis

Gene Symbol	Fragment Size (bp)	Primer Concentration (μM)	Primer Sequence
18S ribosomal RNA	154	0.4	5’ TCAGATACCGTCGTAGTTCC 3’
			5’ TTCCGTCAATTCCTTTAAGTT 3’
ACTB	65	0.5	5’ CTGGCCTCACTGTCCACCTT 3’
			5’ GGGCCGGACTCATCGTACT 3’
AQP33	89	0.5	5’ ATCAGAACACTTGAACACC 3’
			5’ GATCCACAATAGCCAGAA 3’
CTSA	115	0.1	5’ ACAACACCAACCTGAACT 3’
			5’ CATCCCATTATCGTACCT 3’
SLC22A5	100	0.4	5’ GGACCTTGTGAGAACCCGAA 3’
			5’ AGTGTCCAGCGAAAGACCAA 3’
RARRES1	134	0.5	5’ GAATGGGAGAAATCAACCGAGGA 3’
			5’ TCCAAGGCATCATTACCAAGCA 3’
PINLYP	113	0.5	5’ ACTGTCGTGGATGGCAAGAA 3’
			5’ GCACTCCGCTTTCGCTTTG 3’

which have been confirmed as good reference genes in these tissues of this species (Griffith, et al. 2013b). Normalized relative gene expression was compared between uterus of the chorioallantoic and yolk sac placenta with a paired-student's t-test. Whilst gene expression in non-reproductive tissue was compared with the reproductive tissue of each placenta individually. All genes that were identified as significantly differentially expressed in the transcriptomes were also significantly differentially expressed when measured by qPCR.

Gene family member identification

Identified amino acid transport protein genes in humans (Carter 2012; Hediger, et al. 2004) were extracted from the *P. entrecasteauxii* transcriptome. For non-identified genes in *Pseudemoia*, we aligned the amino acid sequence for that gene from *Anolis* against the assembled transcriptome with tblastn. We then re-aligned the top hits against all proteins in the NCBI database with blastn to confirm orthology. Due to absence in the *Anolis* and Chicken genomes, SLC7A12, SLC36A2 and SLC36A3 were assumed to be absent from the genome of *Pseudemoia* and excluded from the analyses. SLC1A7 was not identified by the alignment approach but due to its presence in related genomes, this was assumed to be due to low expression in sequenced tissues. The origins of genes were calculated by identifying the clade for which 1-1 orthology is shared for the gene across taxa using data from Ensembl build 75 (Flicek, et al. 2013).

Null model for overlap in gene use between lineages

We calculated the probability of using equal number or more overlapping amino acid transport protein genes to the placentae of both lineages. To assess this probability we made two assumptions; (a) gene use was independent of the other genes used in the placental tissue

and (b) genes were selected randomly from those present in the genome of both species. The probability was calculated using equation (1), where x is the number of genes utilized by *Pseudemoia*, y is the number of genes utilized by *Homo,* z= total number of genes available in the group, w is the number of genes in common between *Pseudemoia* and *Homo*, n = x – w, a = z – x, b_i = y – x + i, and d_i = x - i. If the probability of a selected gene family was less than 0.05 we rejected the assumptions of our analysis, suggesting either gene use is not independent of the other used genes or genes are not selected randomly for use in the placental tissue.

$$\frac{\sum_{i=0}^{n}\binom{x}{d_i}\binom{a}{b_i}}{\binom{z}{y}} \quad (1)$$

Clustering of amino acid transport protein gene expression

To identify if amino acid transport protein used in the placentae of humans and skinks showed clustered amino acid transport protein expression across human tissues, we collected publicly available microarray gene expression data [17]. The expression of all genes that had gene expression data were included. To identify if amino acid transport proteins that were not used in the placental tissues of humans and skinks showed constrained tissue specific expression patterns, we performed clustering analysis of amino acid transport protein gene

expression across human, mouse and pig tissues (Freeman, et al. 2012; Su, et al. 2004). Tissues were only included in clustering analysis if they were present in two or more data sets, whilst genes were included if they had been measured for all three species. Heat maps of the expression of each amino acid transport protein across tissues were built using the package "gplots" in R (Team 2012). Expression was clustered by gene and by tissue using the 'McQuitty' clustering method.

DATA ACCESS

Raw transcriptome reads are available for download from the NCBI Sequence Read Archive (accession: SRP040433).

ACKNOWLEDGEMENTS AND FUNDING INFORMATION

This project was supported by ARC Discovery grants to M.B.T., and Discovery Early Career Research Award to M.C.B. All animal work was conducted with University of Sydney Animal Ethics approval. Lizards were collected under New South Wales National Parks and Wildlife License to M.B.T. (SL100401). Library sequencing was performed at the Ramaciotti Centre. RNA integrity analysis was carried out in the Bosch MBF, and qPCR analysis was performed in the M. Olsson Lab at the University of Sydney.

REFERENCES

Adams SM, Biazik JM, Thompson MB, Murphy CR 2005. Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: a new placental morphotype. J Morphol 264: 264-276.

Anders S, Huber W 2010. Differential expression analysis for sequence count data. Genome Biol 11: R106.

Arendt D 2008. The evolution of cell types in animals: emerging principles from molecular studies. Nat Rev Genet 9: 868-882.

Arnold SJ 1992. Constraints on phenotypic evolution. Am Nat 140: S85-S107.

Biazik JM, Thompson MB, Murphy CR 2009. Lysosomal and alkaline phosphatase activity indicate macromolecule transport across the uterine epithelium in two viviparous skinks with complex placenta. J Exp Zool 312B: 817-826.

Biazik JM, Thompson MB, Murphy CR 2010. Paracellular and transcellular transport across the squamate uterine epithelium. Herpetol Conserv Biol 5: 257-262.

Blackburn DG 2014. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative Analysis. J Morphol. http://dx.doi.org/10.1002/jmor.20272

Brandley MC, Young RL, Warren DL, Thompson MB, Wagner GP 2012. Uterine gene expression in the live-bearing lizard, *Chalcides ocellatus*, reveals convergence of squamate reptile and mammalian pregnancy mechanisms. Genome Biol Evol 4: 394-411.

Campbell FM, Bush PG, Veerkamp JH, Dutta-Roy AK 1998. Detection and cellular localization of plasma membrane-associated and cytoplasmic fatty acid-binding proteins in human placenta. Placenta 19: 409-415.

Carter AM 2012. Evolution of placental function in mammals: the molecular basis of gas and nutrient transfer, hormone secretion, and immune responses. Physiol Rev 92: 1543-1576.

Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG, Yamamoto H, Kingdom JCP 2003. Genes, development and evolution of the placenta. Placenta 24: 123-130.

Das T, Sa G, Mukherjea M 1993. Characterization of cardiac fatty-acid-binding protein from human placenta. Eur J Biochem 211: 725-730.

Davidson EH, Erwin DH 2006. Gene regulatory networks and the evolution of animal body plans. Science 311: 796-800.

Dufaure JP, Hubert J 1961. Table de developpement du lezard vivipare *Lacerta (Zootoca) vivipara* Jacquin. Arch Anat Micr Morph Exp 50: 309-328.

Ferrara N, Gerber H-P, LeCouter J 2003. The biology of VEGF and its receptors. Nat Med 9: 669-676.

Flicek P, Amode MR, Barrell D, Beal K, Billis K, Brent S, Carvalho-Silva D, Clapham P, Coates G, Fitzgerald S, Gil L, Girón CG, Gordon L, Hourlier T, Hunt S, Johnson N, Juettemann T, Kähäri AK, Keenan S, Kulesha E, Martin FJ, Maurel T, McLaren WM, Murphy DN, Nag R, Overduin B, Pignatelli M, Pritchard B, Pritchard E, Riat HS, Ruffier M, Sheppard D, Taylor K, Thormann A, Trevanion SJ, Vullo A, Wilder SP, Wilson M, Zadissa A, Aken BL, Birney E, Cunningham F, Harrow J, Herrero J,

Hubbard TJP, Kinsella R, Muffato M, Parker A, Spudich G, Yates A, Zerbino DR, Searle SMJ 2014. Ensembl 2014. Nucleic Acids Res 42: D749-D755.

Fong SS, Joyce AR, Palsson BØ 2005. Parallel adaptive evolution cultures of *Escherichia coli* lead to convergent growth phenotypes with different gene expression states. Genome Res 15: 1365-1372.

Forde N, Beltman ME, Duffy GB, Duffy P, Mehta JP, Ó'Gaora P, Roche JF, Lonergan P, Crowe MA 2011. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. Biol Repro 84: 266-278.

Fowden AL, Ward JW, Wooding FPB, Forhead AJ, Constancia M 2006. Programming placental nutrient transport capacity. J Physiol 572: 5-15.

Freeman T, Ivens A, Baillie JK, Beraldi D, Barnett M, Dorward D, Downing A, Fairbairn L, Kapetanovic R, Raza S, Tomoiu A, Alberio R, Wu C, Su A, Summers K, Tuggle C, Archibald A, Hume D 2012. A gene expression atlas of the domestic pig. BMC Biol 10: 90.

Fujita MK, Edwards SV, Ponting CP 2011. The anolis lizard genome: an amniote genome without isochores. Genome Biol Evol 3: 974-984.

Girling JE 2002. The reptilian oviduct: a review of structure and function and directions for future research. J Exp Zool 293: 141-170.

Green KA, Lund LR 2005. ECM degrading proteases and tissue remodelling in the mammary gland. BioEssays 27: 894-903.

Griffith OW, Blackburn DG, Brandley MC, Van Dyke JU, Whittington CM, Thompson MB 2015. Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: A case study examining the evolution of reproductive mode in squamate reptiles. J Exp Zool Part B. http://dx.doi.org/10.1002/jez.b.22614

Griffith OW, Ujvari B, Belov K, Thompson MB 2013a. Placental lipoprotein lipase (LPL) gene expression in a placentotrophic lizard, *Pseudemoia entrecasteauxii* J Exp Zool Part B 320: 465-470.

Griffith OW, Van Dyke JU, Thompson MB 2013b. No implantation in an extra-uterine pregnancy of a placentotrophic reptile. Placenta 34: 510-511.

Hediger M, Romero M, Peng J-B, Rolfs A, Takanaga H, Bruford E 2004. The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteins. Pflügers Archiv 447: 465-468.

Herbert JF, Murphy CR, Thompson MB 2010. Calcium ATPase localization in the uterus of two species of *Pseudemoia* (Lacertilia: Scincidae) with complex placentae. Herpetol Conserv Biol 5: 290-296.

Hou Z-C, Sterner KN, Romero R, Than NG, Gonzalez JM, Weckle A, Xing J, Benirschke K, Goodman M, Wildman DE 2012. Elephant transcriptome provides insights into the evolution of eutherian placentation. Genome Biol Evol 4: 713-725.

Huang DW, Sherman BT, Lempicki RA 2009. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 37: 1-13.

Itonaga K, Wapstra E, Jones SM 2012. A novel pattern of placental leucine transfer during mid to late gestation in a highly placentotrophic viviparous lizard. J Exp Zool Part B 318: 308-315.

Kin K, Nnamani Mauris C, Lynch Vincent J, Michaelides E, Wagner Günter P 2015. Cell-type Phylogenetics and the Origin of Endometrial Stromal Cells. Cell Rep 10: 1398-1409.

Kirschke H, Barrett AJ, Rawlings ND. 1998. *Lysosomal Cysteine Proteases*. Oxford University Press, Oxford.

Knox K, Baker JC 2008. Genomic evolution of the placenta using co-option and duplication and divergence. Genome Res 18: 695-705.

Lager S, Powell TL 2012. Regulation of nutrient transport across the placenta. J Pregnancy 2012: 179827.

Langmead B, Salzberg SL 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9: 357-359.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing Subgroup 2009. The Sequence Alignment/Map format and SAMtools. Bioinformatics 25: 2078-2079.

Long M, Betran E, Thornton K, Wang W 2003. The origin of new genes: glimpses from the young and old. Nat Rev Genet 4: 865-875.

Lynch VJ, Leclerc RD, May G, Wagner GP 2011. Transposon-mediated rewiring of gene regulatory networks contributed to the evolution of pregnancy in mammals. Nat Genet 43: 1154-U1158.

Moore KL, Persaud TVN, Torchia MG. 2013. *The Developing Human: Clinically Oriented Embryology, 9th Edition*. Elsevier Saunders, Philadelphia, PA.

Mossman H. 1937. *Comparative Morphogenesis of the Fetal Membranes and Accessory Uterine Structures.* Carnegie Institution of Washington Publication.

Murphy B, Thompson M 2011. A review of the evolution of viviparity in squamate reptiles: the past, present and future role of molecular biology and genomics. J Comp Physiol 181B: 575-594.

Murphy BF, Belov K, Thompson MB 2010a. Evolution of viviparity and uterine angiogenesis: vascular endothelial growth factor (VEGF) in oviparous and viviparous skinks. J Exp Zool 314B: 148-156.

Murphy BF, Parker SL, Murphy CR, Thompson MB 2010b. Angiogenesis of the uterus and chorioallantois in the eastern water skink *Eulamprus quoyii*. J Exp Bio 213: 3340-3347.

Palacín M, Estévez R, Bertran J, Zorzano A 1998. Molecular biology of mammalian plasma membrane amino acid transporters. Physiol Rev 78: 969-1054.

Parker SL, Manconi F, Murphy CR, Thompson MB 2010. Uterine and placental angiogenesis in the Australian skinks, *Ctenotus taeniolatus,* and *Saiphos equalis*. Anat Rec 293: 829-838.

Ramsay TG, Karousis J, White ME, Wolverton CK 1991. Fatty acid metabolism by the porcine placenta. J Anim Sci 69: 3645-3654.

Reznick DN, Mateos M, Springer MS 2002. Independent origins and rapid evolution of the placenta in the fish genus *Poeciliopsis*. Science 298: 1018-1020.

Secor SM 2008. Digestive physiology of the Burmese python: broad regulation of integrated performance. J Exp Biol 211: 3767-3774.

Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJM, Birol İ 2009. ABySS: A parallel assembler for short read sequence data. Genome Res 19: 1117-1123.

Song G, Bailey DW, Dunlap KA, Burghardt RC, Spencer TE, Bazer FW, Johnson GA 2010. Cathepsin B, cathepsin L, and cystatin C in the porcine uterus and placenta: potential roles in endometrial/placental remodeling and in fluid-phase transport of proteins secreted by uterine epithelia across placental areolae. Biol Reprod 82: 854-864.

Song G, Spencer TE, Bazer FW 2005. Cathepsins in the ovine uterus: regulation by pregnancy, progesterone, and interferon tau. Endocrinology 146: 4825-4833.

Sood R, Zehnder JL, Druzin ML, Brown PO 2006. Gene expression patterns in human placenta. Proc Natl Acad Sci USA 103: 5478-5483.

Soria-Carrasco V, Gompert Z, Comeault AA, Farkas TE, Parchman TL, Johnston JS, Buerkle CA, Feder JL, Bast J, Schwander T, Egan SP, Crespi BJ, Nosil P 2014. Stick insect genomes reveal natural selection's role in parallel speciation. Science 344: 738-742.

Stern DL 2013. The genetic causes of convergent evolution. Nat Rev Genet 14: 751-764.

Stewart JR 2013. Fetal nutrition in lecithotrophic squamate reptiles: toward a comprehensive model for evolution of viviparity and placentation. J Morphol 274: 824-843.

Stewart JR, Thompson MB 1996. Evolution of reptilian placentation: development of extraembryonic membranes of the Australian scincid lizards, *Bassiana duperreyi* (oviparous) and *Pseudemoia entrecasteauxii* (viviparous). J Morph 227: 349-370.

Stewart JR, Thompson MB 2003. Evolutionary transformations of the fetal membranes of viviparous reptiles: A case study of two lineages. J Exp Zool Part A 299A: 13-32.

Stewart JR, Thompson MB, Attaway MB, Herbert JF, Murphy CR 2006. Uptake of dextran-FITC by epithelial cells of the chorioallantoic placentome and the omphalopleure of the placentotrophic lizard, *Pseudemoia entrecasteauxii*. J Exp Zool 305A: 883-889.

Storch J, Thumser AEA 2000. The fatty acid transport function of fatty acid-binding proteins. Biochim Biophys Acta 1486: 28-44.

Streisfeld MA, Rausher MD 2011. Population genetics, pleiotropy, and the preferential fixation of mutations during adaptive evolution. Evolution 65: 629-642.

Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB 2004. A gene atlas of the mouse and human protein-encoding transcriptomes. Proc Natl Acad Sci USA 101: 6062-6067.

R Core Team. 2012. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Thompson MB, Biazik JB, Lui S, Adams SM, Murphy CR 2006. Morphological and functional changes to the uterus of lizards with different placental complexities. Herpetol Monogr 20: 178-185.

Thompson MB, Speake BK 2006. A review of the evolution of viviparity in lizards: structure, function and physiology of the placenta. J Comp Physiol 176B: 179-189.

True JR, Carroll SB 2002. Gene co-option in physiological and morphological evolution. Annu Rev Cell Dev Bi 18: 53-80.

Van Dyke JU, Brandley MC, Thompson MB 2014a. The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. Reproduction 147: R15-R26.

Van Dyke JU, Griffith OW, Thompson MB 2014b. High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. Am Nat 184: 198-210.

Vogel Hertzel A, Bernlohr DA 2000. The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. Trends Endocrin Met 11: 175-180.

Wagner GP, Misof BY 1993. How can a character be developmentally constrained despite variation in developmental pathways? J Evolution Biol 6: 449-455.

Wake DB, Wake MH, Specht CD 2011. Homoplasy: From detecting pattern to determining process and mechanism of evolution. Science 331: 1032-1035.

Wall CE, Cozza S, Riquelme CA, McCombie WR, Heimiller JK, Marr TG, Leinwand LA 2011. Whole transcriptome analysis of the fasting and fed Burmese python heart: insights into extreme physiological cardiac adaptation. Physiol Genomics 43: 69-76.

Weake VM, Workman JL 2010. Inducible gene expression: diverse regulatory mechanisms. Nat Rev Genet 11: 426-437.

Whittington CM, Grau GE, Murphy CR, Thompson MB 2015. Unusual angiogenic factor plays a role in lizard pregnancy but is not unique to viviparity. J Exp Zool Part B. http://dx.doi.org/10.1002/jez.b.22615

Wildman DE, Chen C, Erez O, Grossman LI, Goodman M, Romero R 2006. Evolution of the mammalian placenta revealed by phylogenetic analysis. Proc Natl Acad Sci USA 103: 3203-3208.

Zhang JZ 2003. Evolution by gene duplication: an update. Trends Ecol Evol 18: 292-298.

Chapter 4: No implantation in an extrauterine pregnancy of a placentotrophic reptile

Published manuscript:

Griffith OW, Van Dyke JU, Thompson MB (2013) No implantation in an extrauterine pregnancy of a placentotrophic reptile. *Placenta*. 34(6) 510-511. http://dx.doi.org/10.1016/j.placenta.2013.03.002

No implantation in an extrauterine pregnancy of a placentotrophic reptile

Oliver W. Griffith[1]*, James U. Van Dyke[1], Michael B. Thompson[1]

[1]School of Biological Sciences, Heydon Laurence Building (A08), University of Sydney, Camperdown, NSW 2006, Australia

Correspondence t:o Oliver Griffith; School of Biological Sciences, Heydon Laurence Building (A08), University of Sydney, Camperdown, NSW 2006, Australia; email: ogri8521@uni.sydney.edu.au; Phone: +614 3209 3216; Fax: +61 2 9351 4119

Published in the journal *Placenta*

Abstract

Placentation is a common feature of live-bearing reptiles and mammals. Placentae are variable between species and can be classified by the extent that embryonic tissue breaches (invades) the uterus. Non-invasive placentation in eutherians is maternally imposed as extrauterine embryos of species with epitheliochorial placentation will readily invade non-uterine tissues. This study documents the first observation of an extrauterine pregnancy in a reptile; *Pseudemoia entrecasteauxii*, which *in-utero* exhibits non-invasive epitheliochorial placentation. The extrauterine embryo did not invade maternal tissue suggesting fundamental differences between the nature and evolution of placentation in *P. entrecasteauxii* and eutherian mammals.

Keywords: placenta; squamate; invasion; epitheliochorial; Pseudemoia

INTRODUCTION

Live birth (viviparity) has frequently evolved in amniote vertebrates (reptiles, birds and mammals), where pregnancy occurs by retention of the egg *in-utero* until development is complete [1]. In viviparous amniotes, direct contact between the uterus and embryonic membranes facilitates the exchange of respiratory gasses, water and some nutrients, forming a placenta [2]. Placental types are primarily characterized by the degree to which embryonic tissue breaches uterine tissue and decreases the distance between embryonic tissue and maternal blood [2, 3]. Hemochorial placentation is the most invasive, with embryonic trophoblast breaching both uterine epithelium and endothelium so that embryonic tissue is bathed in maternal blood. By contrast, epitheliochorial placentation is non-invasive because there is no breaching of maternal tissue by the conceptus [4].

Invasive placentation is ancestral in eutherian mammals, with epitheliochorial placentation subsequently evolving three times independently [5]. In mammals, epitheliochorial placentation probably evolved from more invasive placental types as a result of conflict between maternal and paternal genes over the maternal provisioning of resources, with epitheliochorial placentation restricting fetal influence on nutrient allocation [4]. Embryonic membranes of species with non-invasive placental types can still invade maternal tissues but are unable to do so *in-utero* due to physical barriers to invasion and maternal secretions that hinder invasive mechanisms of the embryo [6].

Viviparity and placentation have evolved in squamate reptiles (lizards and snakes) more frequently than in any other vertebrate group [7]. Most viviparous squamates exhibit epitheliochorial placentation, but two examples of invasive placentae have been documented in placentotrophic species [8, 9].

Given that epitheliochorial placentation can exist in mammals that exhibit invasive embryonic tissues when ectopic [6], it is unclear if embryonic tissues of squamates with non-invasive placentas have the ability to invade maternal tissues but are prevented from doing so by the uterus. This paper describes the serendipitous discovery of an extra-uterine pregnancy in the viviparous lizard, *Pseudemoia entrecasteauxii*. This lizard species is placentotrophic, with approximately 50% of nutrient provisions coming from the placenta and the rest from the ovulated ovum [10, 11]. The placenta of *P. entrecasteauxii* has a complex epitheliochorial placenta with specializations for gas and nutrient exchange [12, 13]. We used this discovery to test if the embryonic membranes of *P. entrecasteauxii* have the ability to invade non-uterine maternal tissues.

MATERIALS AND METHODS

Gravid female *Pseudemoia entrecasteauxii* were collected in Kanangra Boyd National Park, NSW, Australia in October 2012. Lizards were euthanized by injection with 0.1mL of sodium pentobarbital (6mg/mL). The uterine eggs of each lizard where examined by cutting along the ventral midline and laterally across the pelvis and mid region of the rib cage. On inspection of one lizard a "growth" was located in the abdominal cavity proximal to the right ovary. This "growth" was not attached to any tissue of the lizard and was picked up with forceps and fixed in 10% neutral buffered formalin for 24 h and then transferred to 70% ethanol. The lizard from which the growth was removed was pregnant with 3 uterine eggs (less developed than embryonic stage 21 of Dufaure and Hubert's [14] 40 stage developmental scheme), with three corpora lutea in the ovaries. The growth was photographed under a calibrated Leica EZ4D microscope and camera (Leica Microsystems, Germany) and was identified to be a partially yet unusually developed embryo. Body and eye length were measured using ImageJ [15] on photographed images and developmental features were noted.

RESULTS AND DISCUSSION

Total body length of the extra-uterine embryo is 3.5mm (Fig. 1). There is no differentiation of limb buds or tail, but there is substantial eye development and many pigmented spots on the epidermis; development has clearly not followed the usual progression [14]. Given that the number of corpora lutea matches the number of uterine eggs, and that the extra-uterine embryo is substantially more developed than uterine eggs, the extra-uterine embryo is likely from a previous reproductive cycle. The extra-uterine embryo is contained within two layers of embryonic membranes, assumed to be the chorioallantoic and amniotic membranes. The yolk sac is located laterally and has been mostly depleted. Given that this is the only example

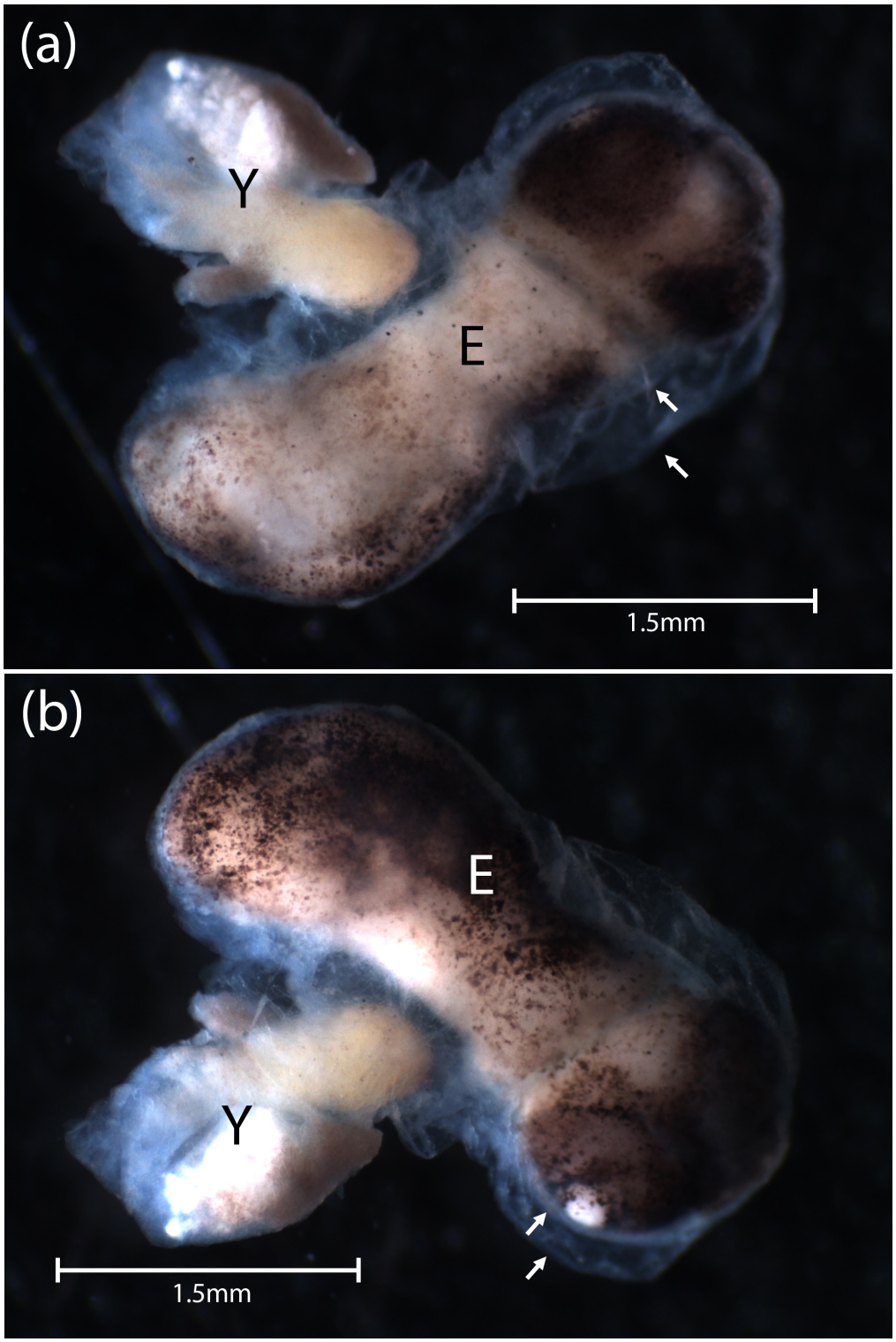


Figure 1. Dorsal (a) and ventral (b) view of the extra-uterine embryo (E). Yolk sac (Y) and additional embryonic membranes (white arrows) can be seen. The embryo was fixed in 10% NBF and photographed under 25 x magnifications with the Leica EZ4D microscope and camera (Leica Microsystems, Wetzlar, Germany).

of an extra-uterine pregnancy in this species, the inferences drawn from this study should be considered tentative, until more examples of this phenomenon are observed.

In squamate pregnancy, the embryonic membranes do not shift relative to the uterus, allowing uterine and embryonic tissue to form specialized regions that remain proximate [16]. The lack of attachment of the egg to non-uterine tissues in this study suggests that embryonic implantation is not driven by the embryo alone but relies on properties of the uterus. Mechanisms to prevent egg rotation may include the elasticity of the uterus and specific cell-adhesion molecules, such as cadherins [17] and likely requires hormonal queues [18].

Epitheliochorial placentation in eutherian mammals is maintained by either a physical barrier to invasion in the uterus or the secretion of compounds that inhibit embryonic tissue from invading [5, 6]. Embryos of eutherian species with non-invasive placental types will readily invade maternal tissue if they implant outside the uterus [19]. The lack of ectopic invasion reported here supports the hypothesis that epitheliochorial placentation in *P. entrecasteauxii* and eutherian mammals have evolved convergently via fundamentally different mechanisms. Whilst epitheliochorial placentation has evolved as a result of parent offspring conflict in eutherian mammals, in *P. entrecasteauxii* it appears to have evolved by alternative selective pressures, and may be an important point in the evolution of more invasive placental types. This finding suggests that epitheliochorial placentation likely evolved in all ancestral viviparous amniotes and that epitheliochorial placentation is a reversion to the ancestral condition when present in eutherian mammals.

Acknowledgements

Animals were collected with NSW scientific license number SL100401 and ethics was approved by University of Sydney Animal Ethics committee (number L04/10-2012/2/5827). J.U.V. was supported by an international fellowship from the US National Science Foundation (IRFP #1064803). We thank Murray Thomson for the use of his microscope and digital camera.

Bibliography

[1] Blackburn DG. Convergent evolution of viviparity, matrotrophy, and specializations for fetal nutrition in reptiles and other vertebrates. Am Zool. 1992;32(2):313-21.
[2] Mossman HW. Vertebrate Fetal Membranes: Comparative Ontogeny and Morphology; Evolution; Phylogenetic significance; basic functions; research opportunities 1987; New Brunswick, USA: Rutgers University Press.
[3] Wildman DE, Chen C, Erez O, Grossman LI, Goodman M and Romero R. Evolution of the mammalian placenta revealed by phylogenetic analysis. P Natl Acad Sci USA. 2006;103(9):3203-8.
[4] Mess A and Carter AM. Evolution of the placenta during the early radiation of placental mammals. Comp Biochem Phys A. 2007;148A(4):769-79.
[5] Vogel P. The current molecular phylogeny of eutherian mammals challenges previous interpretations of placental evolution. Placenta. 2005;26(8-9):591-6.
[6] Samuel CA and Perry JS. Ultrastructure of pig trophoblast transplanted to an ectopic site in uterine wall. J Anat. 1972;113(1):139-49.
[7] Blackburn DG. Are viviparity and egg-guarding evolutionarily labile in squamates? Herpetologica. 1999;55(4):556-73.
[8] Vieira S, de Perez G and Ramírez-Pinilla MP. Invasive cells in the pacentome of Andean populations of *Mabuya*: an endotheliochorial contribution to the placenta? Anat Rec. 2007;290(12):1508-18.
[9] Blackburn DG and Flemming AF. Invasive implantation and intimate placental associations in a placentotrophic african lizard, *Trachylepis ivensi* (scincidae). J Morphol. 2011;273(2):137-59.
[10] Speake BK, Herbert JF and Thompson MB. Evidence for placental transfer of lipids during gestation in the viviparous lizard, *Pseudemoia entrecasteauxii*. Comp Biochem Phys A. 2004;139A(2):213-20.
[11] Stewart JR and Thompson MB. A novel pattern of embryonic nutrition in a viviparous reptile. J Evolution Biol. 1993;174:97-108.

[12] Adams SM, Biazik JM, Thompson MB and Murphy CR. Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: a new placental morphotype. J Morphol. 2005;264(3):264-76.
[13] Stewart JR and Thompson MB. Parallel evolution of placentation in Australian scincid lizards. J Exp Zool B. 2009;312B(6):590-602.
[14] Dufaure JP and Hubert J. Table de developpement du lezard vivipare *Lacerta (Zootoca) vivipara* Jacquin. Arch Anat Micr Morph Exp. 1961;50:309-28.
[15] Schneider CA, Rasband WS and Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671-5.
[16] Murphy BF, Parker SL, Murphy CR and Thompson MB. Angiogenesis of the uterus and chorioallantois in the eastern water skink *Eulamprus quoyii*. J Exp Bio. 2010;213(19):3340-7.
[17] Wu Q, Thompson MB and Murphy CR. Changing distribution of cadherins during gestation in the uterine epithelium of lizards. J Exp Zool Part B 2011;316B(6):440-50.
[18] Biazik JM, Parker SL, Murphy CR and Thompson MB. Uterine Epithelial Morphology and Progesterone Receptors in a Mifepristone-treated Viviparous Lizard *Pseudemoia entrecasteauxii* (Squamata: Scincidae) During Gestation. J Exp Zool Part B. 2012;318(2):148-58.
[19] Corpa JM. Ectopic pregnancy in animals and humans. Reproduction. 2006;131(4):631-40.

Chapter 5: The evolution of embryonic hormonal signaling in amniotes

Chapter formatted for submission to *General and Comparative Endocrinology*

Title: The evolution of embryonic hormonal signaling in amniotes

Authors:

Oliver W. Griffith[1]*, Matthew C. Brandley[1,2], Camilla M. Whittington[1], Katherine Belov[3], Michael B. Thompson[1]

Addresses:

[1] School of Biological Sciences, Heydon-Laurence Building, University of Sydney, Sydney, NSW 2006, Australia

[2] New York University – Sydney, The Rocks, NSW 2000, Australia.

[3] Faculty of Veterinary Science, University of Sydney, Sydney, NSW 2006, Australia

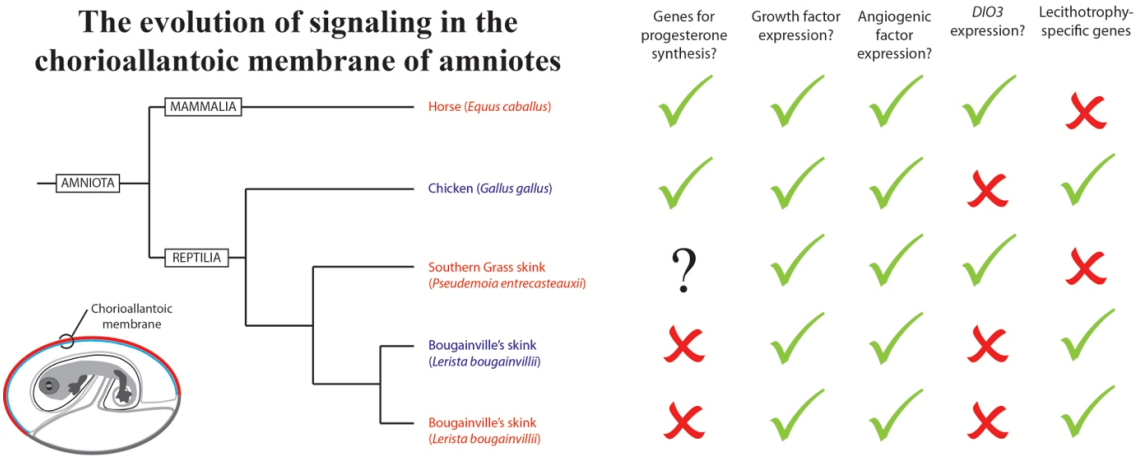
* Author for correspondence, email: oli.griffith@gmail.com, phone: +61 4 3209 3216, fax: +61 2 9351 4119

Abstract

In live bearing amniotes (reptiles, birds, mammals) communication during embryonic development occurs across placental tissues, which form between the uterine tissue of the mother and the chorioallantoic membrane (CAM) of the embryo. Embryonic communication to the mother can be achieved by the production of hormones to coordinate the development of specific structures such as placental vascular beds, and to regulate maternal transport of nutrients to offspring (Murphy et al., 2010). To understand the mechanisms involved in the evolution of placental signaling, we examined the expression of genes involved in hormone synthesis, hormone metabolism, and hormone receptivity in the chorioallantoic membrane of species across the amniote phylogeny. We collected transcriptome data for the chorioallantoic membranes of the chicken (oviparous), and the lizards *Lerista bougainvillii* (both oviparous and viviparous populations) and *Pseudemoia entrecasteauxii* (viviparous). To these data we added transcriptome data from the horse, *Equus caballus* (viviparous). The viviparous taxa differ in their mechanisms of nutrient provisioning; *L. bougainvillii* is lecithotrophic, but *P. entrecasteauxii* and the horse are placentotrophic (embryos are nourished via placental transport). Of the 423 hormone-related genes that we examined, 91 genes are expressed in all studied species, suggesting that the chorioallantoic membrane ancestrally had an endocrine function, and that hormone-related processes have been retained during the evolution of viviparity from oviparity. No genes are expressed only in viviparous species, suggesting that the evolution of viviparity has not required the recruitment of any specific hormone-related genes to the chorioallantoic membrane. Finally, we found that the expression of at least 10 hormone-related genes has been lost in species that exhibit substantial nutrient transport across the placenta, which suggests that the loss of expression of genes might be an important

mechanism that occurs during the evolution of novel phenotypes such as placentotrophy. By evaluating the genes involved in the synthesis and metabolism of steroid, growth, and thyroid hormones in the chorioallantoic membrane, we show that the evolution of maternal-offspring communication in amniotes involves both maintenance of ancestral hormone synthesis and gene expression loss.

Graphical abstract



Keywords:

Viviparity, placenta, placentotrophy, hormone, endocrinology, transcriptome

1. INTRODUCTION

Reproduction is fundamental for organisms because it is the only mechanism that allows genes to be transmitted through generations. The ancestral reproductive mode for vertebrates is egg-laying (oviparity), but live birth (viviparity) has evolved independently multiple times in fishes, amphibians, reptiles, and once in mammals (Blackburn, 2014; Griffith et al., 2015). Embryonic development requires a suitable supply of respiratory gases and water; in viviparous species where the embryo is not exposed to the external environment adequate structures are required to facilitate the exchange of materials between mother and offspring. In viviparous amniotes (reptiles, birds, and mammals), transfer of materials between mother and offspring occurs in a placenta composed of both maternal (uterine) and embryonic (chorioallantoic or yolk sac membranes) tissues (see Fig. 1 for summary of embryonic membranes; Blackburn, 2006; Van Dyke et al., 2014; Wake, 1992). In eutherian mammals, the definitive placenta is produced by the uterus and the embryonic chorioallantoic membrane (CAM). Chorioallantoic placentae are also found in all viviparous squamate reptiles, with the placental region in some species having morphological and physiological specializations for gas exchange and nutrient transport (Adams et al., 2005; Biazik et al., 2010; Blackburn, 1993; Griffith et al., 2013a). In oviparous species, the chorioallantois is an embryonic membrane that lines most of the internal surface of the eggshell late in incubation, where it is the primary gas exchange organ between the egg and the external environment (Piiper et al., 1980). Although they are derived from homologous tissues in viviparous amniote vertebrates, placentae are extremely diverse in structure and function, with some placentae transporting respiratory gases, water, and small amounts of other molecules, and others transporting all classes of nutrients required for embryonic development (placentotrophy) (Ferner and Mess,

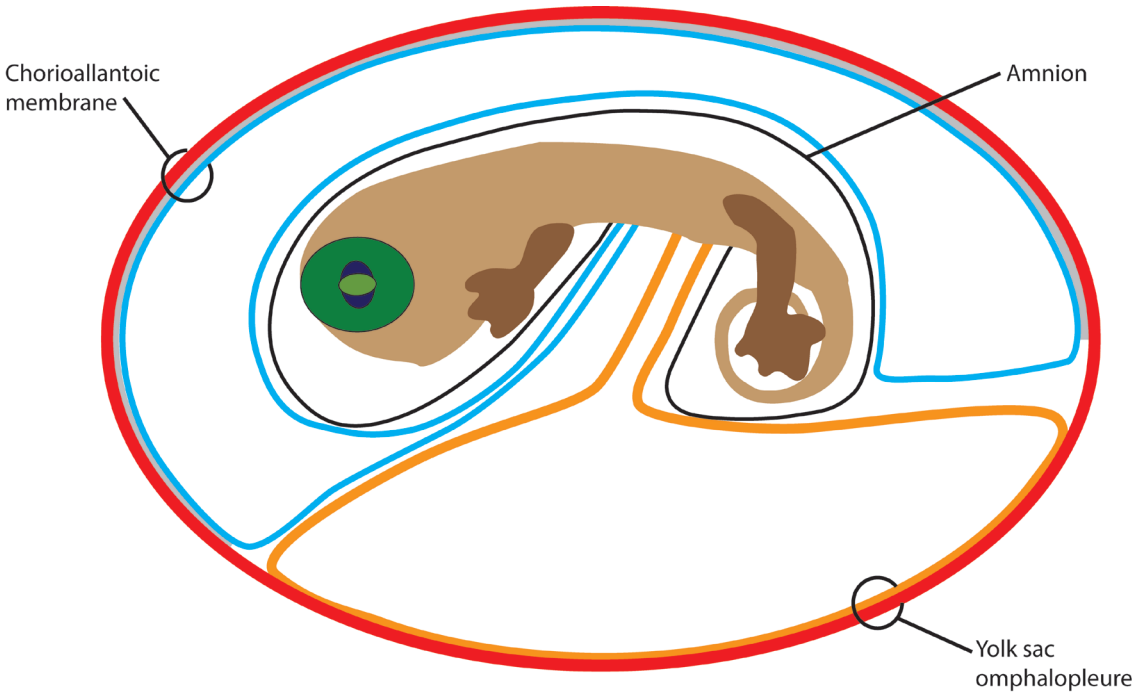


Figure 1. Simplified diagram showing the generic layout of embryonic membranes in amniotes, modified from Ferner and Mess (2011). In oviparous taxa, these structures sit inside the eggshell membrane; in viviparous species the egg is maintained in the uterus. The shape, size, and layout of each membrane differs between amniotes (see Ferner and Mess (2011) for comparisons between major amniote groups). Red – embryonic ectoderm; grey – embryonic endoderm; blue – allantoic membrane; orange – yolk sac membrane.

2011; Thompson and Speake, 2002; Thompson et al., 2002). In amniotes placentotrophy has evolved relatively few times, and exists along a continuum, with complete reliance on egg yolk resources (lecithotrophy, in most squamates) at one end, complete reliance on placental nutrient transfer on the other end (as in eutherian mammals and some lizards), and species with intermediate reliance on both placental transfer and ovulated yolk resources (occurring in some squamates such as the lizard *Pseudemoia entrecasteauxii*) (Ferner and Mess, 2011; Thompson et al., 2000).

To understand how viviparity evolves, it is essential to understand the processes that regulate key pregnancy functions and how these processes have been modified during evolutionary transitions (Thompson and Speake, 2006). Hormones play an essential role in almost every step of amniote reproduction. Hormonal signals are responsible for egg production, vitellogenesis, ovulation, maintenance of the egg *in utero*, and oviposition/parturition (Callard et al., 1992; Custodia-Lora and Callard, 2002; Licht, 1979). Furthermore, hormones are required to induce tissue level changes that facilitate pregnancy, including, for example, cellular changes in the uterus for nutrient transport to offspring. Finally, hormones facilitate communication between maternal and embryonic tissue throughout gestation, which is important for coordination of developmental features such as apposition of vascular beds within the placenta (Murphy et al., 2011).

In oviparous species, eggs are retained *in utero* for a short period and then oviposited into the external environment. The fundamental difference between oviparous and viviparous species is retention of the egg *in utero* until development is completed. Embryonic development in ectotherms is temperature rather than time dependent, so mothers cannot precisely determine the developmental state of offspring without relying on embryonic cues. Hormone production

by embryos represents one possible way for mothers to maintain a state of pregnancy. Progesterone is a key regulator of pregnancy/gravidity in both mammals and reptiles (Custodia-Lora and Callard, 2002). Genes involved in progesterone biosynthesis have been identified in the chorioallantoic tissue of the chicken, suggesting that this tissue may ancestrally produce progesterone (Albergotti et al., 2009). Production of progesterone by the chorioallantoic placenta could play a significant role in the evolution of viviparity, because egg retention could be achieved in part, by simply increasing the transfer of progesterone from embryo to mother by, for example, reducing eggshell thickness.

As well as facilitating maternal-offspring communication, embryonic production of hormones allows embryos to manipulate maternal physiology, which can facilitate parent-offspring conflict. Parent-offspring conflict is a major driver of the evolution of viviparity and placental functions in vertebrates (Crespi and Semeniuk, 2004; Zeh and Zeh, 2008). Conflict arises when offspring demand more resources than is optimal for the life time reproductive success of the parent (Trivers, 1974). For maternal-offspring conflict to occur during pregnancy, embryos must be able to manipulate maternal reproductive behavior such as nutrient transport across the placenta. This manipulation can be achieved either by gaining direct access to maternal resources through invasive placentation, or through embryonic secretions that change the physiology of maternal tissue. In species with non-invasive epitheliochorial placentation, nutrients must be actively transported across maternal tissue to the embryo, restricting embryonic control. However, embryonic control of transport is not precluded in species with non-invasive placentation (Fowden et al., 2006). In the horse, a species with epitheliochorial placentation, embryos actively manipulate placental nutrient supply, likely by the production of hormones such as insulin like growth factor 2 (Allen et al., 2002).

In species with non-invasive placentation, for conflict to occur offspring must be able to manipulate the activity of maternal tissues by the secretion of hormones. Extensive embryonic production of growth factors occurs in the embryonic trophoblast of mammals (Fowden and Forhead, 2009; Fowden et al., 2006). Understanding how the production of growth factors correlates with different reproductive modes and the evolution of placentotrophy will allow us to understand if embryonic manipulation, through growth factor production, is a novel function of the chorioallantois in placentotrophic lineages. Alternatively, the production of growth factors may be the result of modifications to processes that occurred ancestrally in chorioallantoic tissue, with growth factors expressed in the chorioallantois of oviparous species.

We identified gene expression in the embryonic chorioallantoic tissue of species across the amniote phylogeny to correlate changes in reproductive mode and placental nutrition with changes in hormone production and metabolism. We quantified the expression of hormone genes to identify if hormone production by the chorioallantoic placenta is widespread in viviparous amniotes, and if the expression of hormones has evolved by recruitment in viviparous lineages of hormone-synthesizing genes, or by ancestral hormone production of these genes in the chorioallantoic membrane of egg laying amniotes. Furthermore, we identify whether genes involved in hormone synthesis and metabolism have been lost and/or gained during the evolution of viviparity or placentotrophy.

2. MATERIALS AND METHODS

Species selection

Recent advances in next-generation sequencing technology make it possible to simultaneously measure the expression of most genes in a given tissue (transcriptome). We evaluated the expression of hormone-related genes in multiple species using newly-generated and published transcriptome data for the chorioallantoic membrane (Fig. 2). We sampled two oviparous taxa including the chicken (*Gallus gallus*) and an oviparous population of the skink *Lerista bougainvillii.* We sampled two separate origins of lizard viviparity in the skink *Pseudemoia entrecasteauxii* and a viviparous population of *L. bougainvillii.* The viviparous taxa differ in their mode of embryonic nutrition. *Lerista bougainvillii* is lecithotrophic—embryos rely on yolk for a vast majority of nutrients, and the simple placenta instead facilitates flux of water, ions, and gasses. In contrast, *P. entrecasteauxii* is placentotrophic—embryos receive nutrients supplied by the placenta (and reduced yolk) via a relatively complex placenta with specializations for nutrient transport (Adams et al., 2005; Griffith et al., 2013a). Like most viviparous squamates, *P. entrecasteauxii* has non-invasive epitheliochorial placentation, meaning that the maternal and embryonic epithelia directly contact, but there is no breaching of the maternal tissue (Adams et al., 2005; Griffith et al., 2013b). To these data we added published mammalian transcriptome data for the CAM of the horse (Wang et al., 2012), thereby representing a third origin of viviparity and a second origin placentotrophy in amniotes. The horse also possesses a non-invasive epitheliochorial placenta, making it possible to evaluate gene expression in two independent derivations of the same placental type in mammals and reptiles.

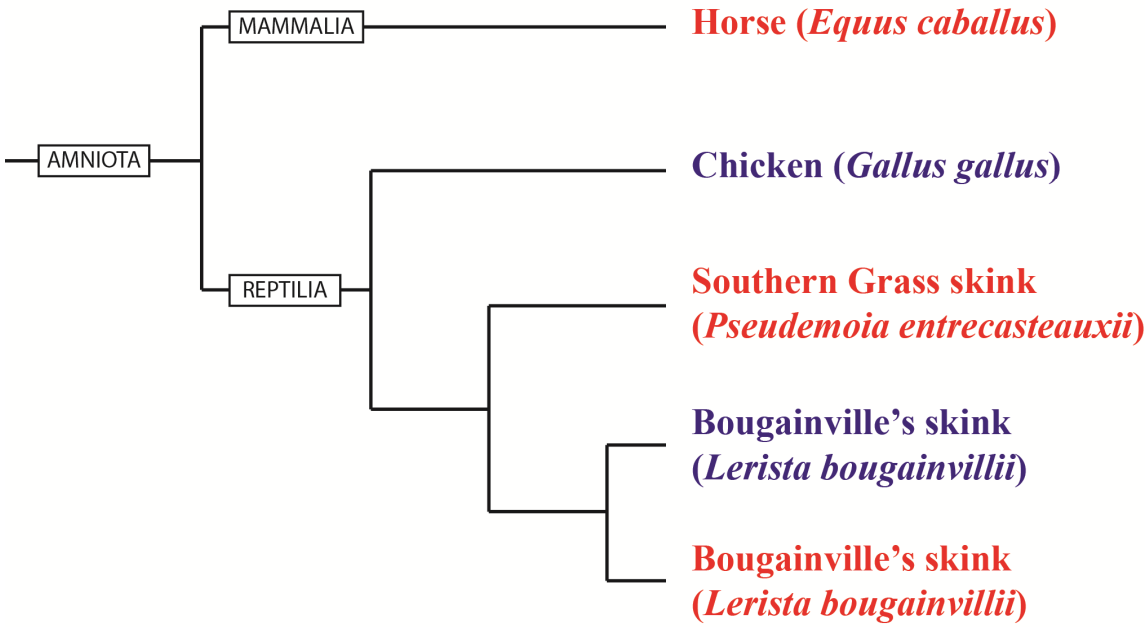


Figure 2. Phylogenetic relationship of amniotes species used in this study. Viviparous taxa are denoted in red text, whilst oviparous taxa are denoted in blue.

Measuring gene expression in chorioallantoic tissue

Chicken, *Gallus gallus*

We dissected chorioallantoic tissue from day 8 (n=2) and day 18 (n=3) chicken eggs in May 2012. To extract RNA, tissue was macerated using a mechanical homogenizer in 600μL of Buffer RLT (QIAGEN) then homogenized using a QIAshredder spin column (QIAGEN). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Extracted RNA was treated with Amplification Grade DNase 1 (Sigma-Aldrich). RNA quality was measured on the Agilent 2000 Bioanalyzer (Agilent Technologies) and was only used for transcriptome analysis if the RIN was greater than 8. Sequencing libraries were generated in house using the TruSeq RNA Sample Preperation kit (Illumina,inc.), were pooled into lanes containing ten samples, and were sequenced on the HiSeq2000 (Illumina, Inc.). Raw transcriptome reads are available in the Sequenced Read Archive (Accession on publication). Gene counts for each gene were calculated by aligning raw sequencing reads to the complete cDNA library for *Gallus gallus* from Ensembl Release 78 (Cunningham et al., 2015) with Bowtie2 (version 2.0.0-beta7) (Langmead and Salzberg, 2012). We counted the number of transcripts that aligned to each cDNA contig with Samtools view (Li et al., 2009).

Southern grass skink, *Pseudemoia entrecasteauxii*

We collected chorioallantoic placental tissues from mid (embryonic stage 36/37 of the staging scheme used for *Zootoca vivipara*, n=2) and late pregnant females (embryonic stage 40, which is the final developmental stage, n=4, Dufaure and Hubert, 1961). RNA purification and sequencing was performed using the same methodology as the chicken. We collected a mean of $2.4 \times 10^7 \pm 3.8 \times 10^6$ sequenced reads per sample. Data analysis was

performed using the same approach as the chicken except raw sequencing reads were aligned to the transcriptome for *P. entrecasteauxii* that was previously assembled *de novo* (Chapter 3).

Bougainville's Skink, *Lerista bougainvillii*

We collected a gravid oviparous *Lerista bougainvillii* from the Burra Region of South Australia in 2011. This lizard was maintained in captivity until it laid eggs, and the CAM was dissected from one egg. Pregnant viviparous *L. bougainvillii* were collected on Kangaroo Island, South Australia in December of 2011 and 2012. Lizards were housed and then euthanized during late pregnancy, chorioallantoic placental tissue was dissected. All embryos were between developmental stage 35 and 40. RNA extraction and transcriptome sequencing was performed the same as for the chicken. We collected a mean of $2.9 \times 10^7 \pm 2.2 \times 10^6$ one hundered and one bp sequenced reads per sample. We used ABySS 1.3.4 to assemble all individual raw Illumina reads into larger contigs. Contigs in the transcriptome smaller than 100bp and redundant contigs were removed with CD-HIT-EST (Huang, et al. 2010) using default options. We identified the contigs using BLASTX with ten vertebrate reference genomes (Ensembl build 72). Finally, we used the short-read aligner Bowtie 0.12.9 (Langmead et al. 2009) to align the original raw Illumina reads to the identified contigs.

Horse, *Equus callabus*

Raw 40bp single end Illumina reads from horse chorioallantoic girdle were taken from Wang et al. (2012). Four samples sequenced on the Illumina Genome Analyzer IIx were included, and had a mean of $3.1 \times 10^7 \pm 6.2 \times 10^5$ sequenced reads per transcriptome. We aligned raw sequenced reads to the complete *Equus callabus* cDNA collection in Ensembl Release 78 (Cunningham et al., 2015) with Bowtie2 (version 2.0.0-beta7) (Langmead and Salzberg,

2012). We counted the number of transcripts that aligned uniquely to each cDNA contig with Samtools view (version 0.1.18) (Li et al., 2009).

Identifying expressed genes

Gene expression requires copying genes from the genome into mRNA (transcription). Although transcription occurs in specific parts of the genome, deep sequencing by RNA-seq will also identify small numbers of transcripts from genes that are actively repressed (see Wagner et al. (2013) for more details). We used the transcript per million (TPM) metric to separate "expressed genes" from genes which had some sequenced transcripts but are unlikely to be genuinely regulated (Wagner et al., 2012, 2013). In cases where multiple assembled cDNA sequences existed for a single gene, gene counts from these transcripts were summed and this summed transcript number was divided by the summed contig length. Genes that had a mean TPM value greater than four were considered expressed; this cut off is consistent with the chromatin state of each gene (Hebenstreit et al., 2011). Our cut off is conservative and can confidently identify genes that are transcriptionally active, and not actively repressed (Wagner et al., 2013). We report all gene expression values in TPM.

Hormone-related genes

We compiled a gene list from the DAVID bioinformatics resource, the GeneCards Human Gene Database, and additional genes from readings in the literature to identify hormone-related genes for this analysis. Gene ontology terms (GO terms) for biological processes were downloaded from the DAVID bioinformatics resources 6.7 (Huang et al., 2009). All GO terms for biological processes containing a set of keywords (Supplementary Table 1) were collated. Gene symbols associated with these GO terms were extracted using a custom perl

script using the data associated with DAVID IDs. Gene symbols were extracted from the GeneCards Human Gene Database (Version: 3.12.316 2 Feb 2015), by searching for genes that contained the word 'hormone' or the word 'growth factor' in their symbol, alias or identifier. In addition, a list of all Cytochrome P450 genes collected from GeneCards Human Gene Database (Version: 3.12.316 2 Feb 2015) were included, as this group of genes facilitates many cellular chemical processes including steroid metabolism (Lewis, 1996). Other genes were added if they were reported in the literature to be of importance to the regulation of hormone production or metabolism.

3. RESULTS AND DISCUSSION

3.1 CAM hormone gene expression

The CAM is a hormone-producing organ in amniotes, of the 423 hormone-related genes examined, 321 genes are expressed in the CAM of at least one of the studied species. Expression of hormone-related genes is not confined to viviparous taxa, which confirms findings previously reported in oviparous birds, crocodiles, and tortoises (Albergotti et al., 2009; Cruze et al., 2013; Cruze et al., 2012). Ninety-three genes show conserved expression across all studied species, suggesting they may perform essential functions of the chorioallantoic tissues in amniotes. However, it is possible that the physiological function of these proteins has been modified in viviparous lineages to perform novel pregnancy associated roles. No genes were expressed only in viviparous species, suggesting the evolution of viviparity does not require *de novo* recruitment of any particular hormone-related genes to placental tissues. From our survey, only one gene exhibited expression specific to placentotrophic species, although ten genes are not expressed in placentotrophic

species, suggesting a role of gene expression loss in the evolution of placentotrophy. In the remainder of the discussion, we concentrate on expression of genes related to specific groups of hormones that are important in amniote reproduction, and discuss the significance of gene loss during the evolution of placentotrophy.

3.2 Steroid hormone synthesis

We examined the expression of 47 genes involved in steroid hormone synthesis and metabolism (Table 1). Of these genes, six are expressed in all species, with four of these belonging to the cytochrome P450 family of proteins (CYP). CYPs are a diverse group of enzymes that are involved in cholesterol and steroid synthesis and metabolization (Werck-Reichhart and Feyereisen, 2000). Although the specific functions of the encoded proteins of these genes are largely unknown, they may play essential roles in the CAM. The newly-sequenced reptile species differ from the alligator, which expresses specific genes in the CAM involved in androgen and estrogen synthesis (*CYP17A1* and *CYP19A1* respectively) (Cruze et al., 2012).

3.2.1 Progesterone

The defining feature of a viviparous species is that embryos are maintained in the mother until development is complete (Blackburn, 2006). Progesterone is a regulator of gravidity/pregnancy in all studied amniotes and so was a hormone of interest in our study. In turtles increased progesterone production is associated with ovulation (Guillette et al., 1991; Licht et al., 1982) and prevents oviposition (Klicka and Mahmoud, 1977; Mahmoud et al., 1988). In the relatively short reproductive cycle of chickens (26 h), progesterone peaks just prior to ovulation (Laguë et al., 1975). In oviparous squamates, progesterone is produced by

Table 1. Expression patterns of genes involved in steroid synthesis and metabolisation in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n=5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n=6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n=1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n=4)	Horse, *Equus caballus* (n=4)	Pattern
GENES INVOLVED IN PROGESTERONE SYNTHESIS						
STARD3	**15.5 ± 1.4**	**22 ± 2**	**22.9**	**21.1 ± 1.1**	**10.9 ± 0.6**	All
CYP11A1	**20.3 ± 2.6**	-	0	0.2 ± 0.2	**115.9 ± 79.7**	
HSD3B1	**44.6 ± 15.9**	-	0.2	0.2 ± 0.1	**74 ± 9.9**	
STAR	0.9 ± 0.1	0.4 ± 0.2	**44.8**	3.4 ± 1.3	**72.9 ± 34**	
STEROID HORMONE RECEPTORS						
AR	**22.7 ± 6.3**	0.9 ± 0.3	**107.5**	**140.3 ± 15.2**	0.3 ± 0.2	Lecithotrophic
PGR	1.1 ± 0.6	3.1 ± 0.3	**12.4**	**14.2 ± 1.5**	0.2 ± 0.1	
ESR1	0.2 ± 0.1	0.2 ± 0.1	1.6	2.2 ± 0.1	-	
ESR2	0.1 ± 0	-	0	0.6 ± 0.3	-	
CYP20A1	**24.6 ± 2.1**	**89.3 ± 7.5**	**19**	**19.7 ± 2.1**	**14.4 ± 3.3**	All
CYP27A1	**9.1 ± 1.4**	**711.4 ± 79.4**	**19.9**	**13.1 ± 1.9**	**5.1 ± 2.9**	All
CYP39A1	**15.1 ± 2.7**	**5.1 ± 2.1**	**24.7**	**22.5 ± 2.3**	**13.6 ± 2.4**	All
CYP51A1	**25.5 ± 5.5**	**385.4 ± 103.3**	**29**	**84.4 ± 6.3**	**102.1 ± 16.3**	All
NR3C1	**21.2 ± 2.2**	**39.4 ± 6**	**75.5**	**77.7 ± 10.4**	**7.5 ± 1.2**	All
CYP26B1	**4.9 ± 0.5**	0.3 ± 0.3	**18.3**	**33.7 ± 11.5**	0.2 ± 0.1	Lecithotrophic
CYP46A1	**18.5 ± 3.8**	-	**39.6**	**28.2 ± 8.3**	0.1 ± 0.1	Lecithotrophic
CYP4F22	**30.7 ± 4.3**	-	**164.4**	**62.5 ± 9**	0.1 ± 0.1	Lecithotrophic
CYP2U1	2.9 ± 0.3	**5.3 ± 0.8**	**14.6**	**21.9 ± 5.5**	1.2 ± 0.5	Lizard
CYP7B1	3.4 ± 0.4	**30.3 ± 12.5**	**18.4**	**25.8 ± 5.8**	0.2 ± 0.1	Lizard
CYP2D6	**22.5 ± 2.2**	**28.8 ± 3.5**	**65.9**	**120.6 ± 22.1**	-	Reptile
CYP2R1	**5.2 ± 0.5**	**7.5 ± 1.4**	**7**	**7.6 ± 1.3**	0.2 ± 0.1	Reptile
CYP4V2	**7.4 ± 1.9**	**5.5 ± 1.2**	**68.2**	**63.6 ± 15.9**	0 ± 0	Reptile
CYP17A1	0.4 ± 0.1	1.7 ± 0.5	1	0.3 ± 0.2	**1918.6 ± 1310.5**	
CYP19A1	0.7 ± 0.1	1 ± 0.7	-	-	**1572 ± 574.7**	
CYP1A1	0.5 ± 0.1	-	**87.2**	**114.3 ± 15.2**	**4.3 ± 4.3**	
CYP1A2	-	-	-	-	0.3 ± 0.3	
CYP1B1	3.9 ± 0.9	**5.4 ± 1**	**94.5**	**38.7 ± 2.1**	**29.6 ± 14.7**	
CYP21A2	2.2 ± 0.8	-	-	-	0.4 ± 0.3	
CYP24A1	0.8 ± 0.4	-	**4.4**	**55.7 ± 21.4**	3.2 ± 1.3	
CYP26A1	1.1 ± 0.5	0.3 ± 0.2	-	-	**6.8 ± 2.4**	
CYP26C1	0.3 ± 0.1	**4.5 ± 0.8**	**5.8**	3.5 ± 1.2	1.4 ± 0.6	
CYP27B1	-	1.7 ± 0.4	-	-	0 ± 0	
CYP27C1	1.5 ± 0.5	-	-	-	0.2 ± 0.1	
CYP2A13	-	-	**1129.7**	**517.5 ± 151.8**	-	
CYP2A6	-	-	**396.9**	**31.3 ± 17.6**	-	
CYP2A7	-	-	**317.6**	**16.1 ± 7**	-	

CYP2B6	-	-	**4**	**14.6 ± 5.1**	-
CYP2F1	-	1.2 ± 0.2	**11.8**	**9.3 ± 4.4**	0.1 ± 0.1
CYP2J2	-	**18.9 ± 1.7**	**49.1**	**166.2 ± 41.9**	**4.7 ± 2.2**
CYP2S1	-	-	-	-	**6.8 ± 0.7**
CYP2W1	-	-	**7.2**	**47.7 ± 29.3**	0.7 ± 0.4
CYP3A7	-	-	**208**	**11.5 ± 4.9**	-
CYP4F3	-	-	**766.9**	**279.8 ± 47.7**	-
CYP4F8	-	-	-	-	1.1 ± 0.7
CYP4X1	-	-	-	-	0 ± 0
CYP7A1	2.8 ± 0.2	-	-	-	0.5 ± 0.2
CYP8B1	-	-	**16.7**	**18.4 ± 2.4**	0 ± 0
NR5A1	0.2 ± 0.1	0.1 ± 0.1	-	-	-

the corpus luteum and hormone concentration is correlated with gravidity (the holding of eggs) and typically declines prior to oviposition (Díaz et al., 1994; Fox and Guillette, 1987). Typically in viviparous amniotes, progesterone is maintained at relatively high concentrations and decreases prior to parturition (Bazer, 1992; Fergusson and Bradshaw, 1991; Murphy and Thompson, 2011). Whilst in some taxa (e.g. mice, pigs, and oviparous squamates) progesterone is produced by corpora lutea through pregnancy, during pregnancy in humans and the horse, progesterone is produced by ovarian *corpora lutea* until the embryonic placenta has developed sufficiently to take over hormone production (Meyer, 1994; Stocco et al., 2007; Tuckey, 2005). The pattern of shifting progesterone production from the *corpus luteum* to the chorioallantois during pregnancy also occurs in the viviparous lizard, *Chalcides chalcides* (Guarino et al., 1998). Uterine receptivity to progesterone occurs via the progesterone receptor (*PGR)*, which is expressed in the uterus of the live bearing lizards *Chalcides ocellatus* and *P. entrecasteauxii* (Chapter 3; Brandley et al., 2012). Uterine PGR production has occurred in amniote reproduction since before the split of these two amniote lineages ~300 million years ago.

Progesterone production occurs by first transporting cholesterol to the mitochondrion (typically by steroidogenic acute regulatory protein; StAR), in the mitochondrion it is transported to the inner mitochondrial membrane by StAR-related lipid transfer domain protein 3 (StARD3), and converted to pregnenolone by cytochrome P450, family 11, subfamily A, polypeptide 1(CYP11A1). Finally pregnenolone is converted to progesterone by 3 beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I (HSD3B1) (Fig. 3; Payne and Hales, 2004).

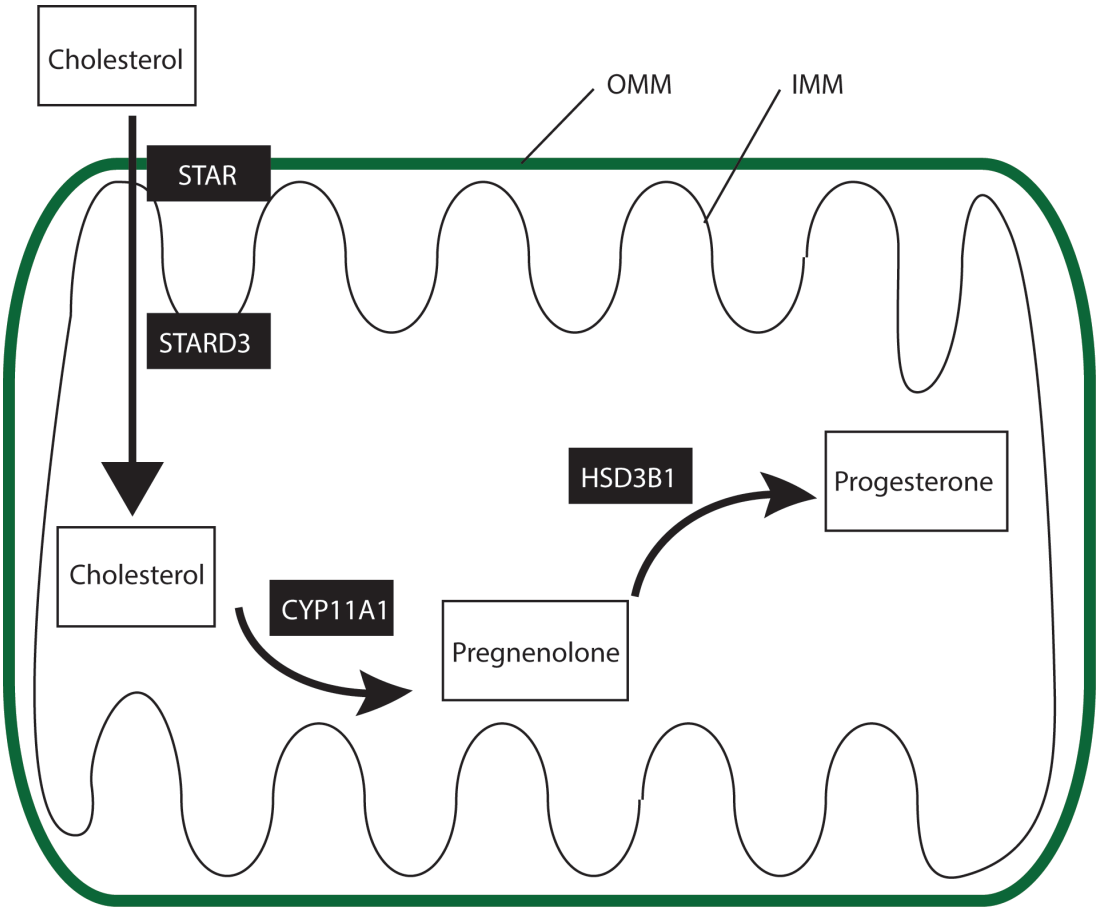


Figure 3. Progesterone synthesis pathway, which occurs inside the mitochondrion pictured (green). Arrows indicate movement or modification of steroid hormones (empty boxes). Black boxes indicate enzymes responsible for mobilization or movement of hormones at each step. OMM - outer mitochondrial membrane; IMM - inner mitochondrial membrane; STAR - steroidogenic acute regulatory protein; STARD3 - StAR-related lipid transfer domain containing 3; CYP11A1 - cytochrome P450, family 11, subfamily A, polypeptide 1; HSD3B1 - 3 beta-hydroxysteroid dehydrogenase/delta(5)-delta(4)isomerase type I.

Expression of genes encoding each enzyme in the progesterone synthesis pathway occurs in the horse placenta (Table 1), but not all are expressed in the chorioallantoic tissues of the other studied species. *STARD3* is expressed in all examined taxa; as STARD3 is important for mobilization of cholesterol in the mitochondria, this step in the progesterone synthesis pathway is common in the synthesis and metabolisation of many steroid hormones (Payne and Hales, 2004). In humans (and potentially chickens) production of STARD3 is sufficient to facilitate cholesterol mobilization for progesterone synthesis (Tuckey, 2005). Chickens lack expression of *STAR*, which facilitates mobilization of cholesterol to the mitochondria, similarly STAR is not produced in the human trophoblast despite production of progesterone in this tissue (Strauss et al., 1996). CYP11A1 and HSD3B1 are necessary for the production of progesterone from cholesterol (Payne and Hales, 2004). The presence of both enzymes in chickens is consistent with progesterone production during development (Albergotti et al., 2009). However, progesterone is an intermediate in the production of other steroid hormones including estrogen and androgens. So the presence of genes responsible for progesterone synthesis might reflect the production of other steroid hormones rather than progesterone proper. In *P. entrecasteauxii HSD3B1*and *CYP11A1* are absent from the reference transcriptome of this species, so it is not possible to confidently say these genes are not expressed. In *L. bougainvillii*, both *CYP11A1* and *HSD3B1* are present in the reference transcriptome (so our approach could reliably detect the expression of these genes) but are not expressed in the chorioallantoic membrane of either oviparous or viviparous populations. This result strongly suggests that in some viviparous lizards the embryonic component of the placenta is not producing progesterone via any known synthesis pathway. As expression of these genes in the CAM is absent in *L. bougainvillii*, embryonic progesterone production by

this pathway is not simply an ancestral trait of amniotes that has been utilized during the evolution of all instances of viviparity.

3.2.2 Steroid hormone receptors

Androgen receptor (AR) binds steroid hormones, including testosterone, and participates in the development of male characteristics, but it is also important to female physiology (Cloke and Christian, 2012). AR is expressed in female reproductive tissues, and in humans its expression fluctuates through cycling of the endometrium (Barad et al., 2007). Androgens have been identified in mammalian trophoblast, and increased AR levels are associated with pre-eclampsia (Hsu et al., 2009). Androgen receptor is highly expressed in all lecithotrophic taxa we examined, but is not expressed in horse and grass skinks (Table 1). The presence of maternally produced hormones (such as androgens) could negatively impact offspring development, in particular by affecting sexual characteristics (Uller and Olsson, 2003). In experiments where testosterone concentrations have been artificially increased during development, offspring have reduced embryonic growth rate, compromised immune function, and altered behavior (Sinervo et al., 2000; Uller and Olsson, 2003). Our results suggest that by decreasing the expression of androgen receptor in species with high levels of placental transport, embryos may decrease the effect of maternally derived androgens on embryonic development.

The progesterone receptor gene (*PGR*) is only expressed in the CAM of both oviparous and viviparous populations of *L. bougainvillii* (Table 1), suggesting that receptivity to progesterone in the CAM may be absent in most non-mammalian amniotes and is not necessary for viviparity. As the CAM of *L. bougainvillii* expresses *PGR*, but does not express the genes necessary for progesterone synthesis, we suggest progesterone may play a

different role during reproduction in these lizards. Further work is necessary to identify if progesterone is necessary for the maintenance of pregnancy in *P. entrecasteauxii* and *L. bougainvillii*, and to confirm where it is produced.

Estrogen is an important hormone in for the regulation of the female reproductive system in reptiles and mammals (Callard et al., 1972; Katzenellenbogen and Greger, 1974; Wang et al., 2000). Estrogen receptors are not expressed in the CAM of all studied species (Table 1), suggesting that estrogen does not regulate growth of the chorioallantoic membrane in oviparous or viviparous species.

3.3 Growth factors and peptide hormones

3.3.1 IGF2

Insulin like growth factor 2 (IGF2) is a key regulator of placental growth and function in mammals. Under-expression of IGF2 results in decreased growth of mouse embryonic and placental tissues, whilst increased IGF2 abundance *in utero* results in offspring overgrowth in humans, mice and sheep (Coan et al., 2008; Fowden and Forhead, 2009). IGF2 works as a signal of fetal demand, regulates fetal growth, and interacts with the expression of nutrient transport proteins in placental tissues (Constância et al., 2005; Reik et al., 2003; Sibley et al., 2004). The maternal allele of *IGF2* is silenced by genomic imprinting in live bearing therian mammals. Imprinting of this gene occurs because of conflict between genes of maternal and paternal origin within embryos, where mother and embryo differ in their preferred rate of nutrient transfer to embryos (Haig, 2000). The production of IGF2 by offspring during gestation also occurs in live-bearing teleost fishes, where ongoing parent-offspring conflict

has driven selection on the gene, likely because of its ability to regulate placentotrophy (O'Neill et al., 2007).

IGF2 is expressed in the CAM of all studied species, regardless of reproductive mode (Table 2). Ancestral expression of *IGF2* in the CAM suggests that this protein may be a regulator of growth and proliferation of the embryo in oviparous taxa. Uterine expression of the receptor for IGF2, the insulin growth factor like receptor 1, is ancestral to mammals and present in the uterine tissue of *P. entrecasteauxii* (Chapter 3; Lynch et al., 2015). During the evolution of viviparity, reduced eggshell thickness and the apposition of maternal and fetal tissues would allow IGF2 to affect the physiology of the placenta, which suggests that placental production of IGF2 is actually an exaptation that has been utilized to manipulate maternal tissues during the evolution of viviparity.

3.3.2 Angiogenic factors

In conjunction with its ability to regulate placental nutrient transfer, IGF2 can regulate endothelial cell migration (Herr et al., 2003). Changes in uterine angiogenesis are essential for the evolution of viviparity to support increasing embryonic oxygen demand during pregnancy (Parker et al., 2010a). Increased vascularization during pregnancy is a gross morphological change that is necessary for the evolution of viviparity (Parker et al., 2010a; Parker et al., 2010b). Embryonic regulation of vascularization occurs in some viviparous squamates, where the vascular bed of the mother is aligned with the position of the embryo inside the egg, even in cases where the embryo is oriented obliquely to the uterus (Murphy et al., 2010). A number of genes encoding proteins with angiogenic potential show consistent expression in the CAM across all studied species (Table 3). Of particular note are VEGFA and PGF, which are the most well studied genes of uterine angiogenesis (Autiero et al., 2003;

Table 2. Expression patterns of growth factors and growth factor receptors in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n=5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n=6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n=1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n=4)	Horse, *Equus caballus* (n=4)	Pattern
IGF1R	**14.8 ± 1.8**	**29.8 ± 4.2**	**25.7**	**19.1 ± 3.4**	**38.8 ± 16.1**	All
IGF2	**59.6 ± 7.3**	**15.1 ± 2.3**	**17.9**	**34.3 ± 8.2**	**1901.3 ±**	All
PDGFB	**60.5 ± 14.6**	**20 ± 2.7**	**8.5**	**17.2 ± 3.7**	**6.4 ± 5.2**	All
PDGFC	**9.6 ± 0.6**	**39.2 ± 6.9**	**10.5**	**18.8 ± 3.6**	**31.9 ± 6.6**	All
PPAP2B	**117.7 ± 18.4**	**37.3 ± 3.3**	**33.4**	**56 ± 4.5**	**16.7 ± 1.9**	All
PSIP1	**29.7 ± 3**	**37 ± 2.7**	**19.6**	**29.1 ± 4.4**	**43 ± 9**	All
FGF20	**4.8 ± 0.6**	-	-	-	3.9 ± 1.3	Chicken
FGF22	**11.9 ± 4.3**	-	-	-	-	Chicken
FGF23	**17.8 ± 1.7**	-	-	-	0 ± 0	Chicken
INHBA	**17.9 ± 2.4**	0.5 ± 0.2	**4.2**	**10 ± 4.7**	0.7 ± 0.2	Lecithotrophic
KITLG	**6.1 ± 0.7**	-	**23.9**	**14.5 ± 1.6**	0.9 ± 0.4	Lecithotrophic
HGF	-	**5.9 ± 0.8**	**17.9**	**19.9 ± 1.7**	0.4 ± 0	Lizard
NGF	2.4 ± 0.5	**14.5 ± 3.3**	**10**	**6.2 ± 0.9**	0 ± 0	Lizard
AREG	**71.1 ± 17.6**	-	**5.1**	3.2 ± 0.7	1.4 ± 0.9	Oviparous
AMH	1 ± 0.3	-	-	-	-	
BMP15	2.6 ± 0.5	3.6 ± 0.8	-	-	-	
BMP6	**153.2 ± 29.1**	**21.5 ± 1.8**	**24.9**	**38.9 ± 3.5**	1.3 ± 0.4	
CLEC11A	-	1 ± 0.2	-	-	-	
FGF11	-	-	-	-	0.2 ± 0.1	
FGF12	**29.5 ± 3.8**	2.5 ± 0.3	2.5	**45.5 ± 38.8**	0 ± 0	
FGF13	0.6 ± 0.1	-	**40.4**	**127.9 ± 22.5**	0.4 ± 0.1	
FGF14	0.6 ± 0.2	0.7 ± 0.2	-	-	-	
FGF16	1.3 ± 0.7	1 ± 0.6	2.3	0.7 ± 0.2	0.1 ± 0.1	
FGF19	0.7 ± 0.2	1 ± 0.7	**8.7**	**34.2 ± 10**	0.2 ± 0.2	
FGF21	-	0.6 ± 0.6	**33.5**	1.2 ± 0.6	0 ± 0	
FGF3	0.3 ± 0.1	-	-	-	0.2 ± 0.2	
FGF4	0 ± 0	-	-	-	0 ± 0	
FGF5	2.5 ± 0.2	-	-	-	-	
FGF7	2.3 ± 0.3	3.7 ± 1.1	2.8	**18.4 ± 6**	**55.3 ± 17.7**	
GDF10	3.9 ± 1.5	-	**7.3**	**33.7 ± 6.8**	0 ± 0	
GDF11	-	0.2 ± 0.2	-	1.7 ± 0.3	**13.7 ± 4.2**	
GDF15	-	-	**26.6**	**62.7 ± 17.3**	-	
GDF2	-	-	-	-	0.1 ± 0.1	
GDF5	3.3 ± 0.9	**17.9 ± 2.3**	-	-	0.1 ± 0.1	
GDF6	-	-	**21**	**26.8 ± 4.2**	**4.2 ± 1.1**	
GDF7	-	**12.4 ± 2.5**	1.4	**5 ± 1.2**	-	
GDF9	-	-	-	-	0.1 ± 0.1	

HBEGF	**43.4 ± 15.9**	**46.1 ± 1.8**	3.8	**6.6 ± 0.8**	2.8 ± 0.8
HDGF	-	-	**1335.8**	**1187.1 ± 289.9**	**179.6 ± 22.1**
HDGFRP3	-	-	**38.1**	**65.4 ± 5.6**	-
IGF1	-	0.2 ± 0.1	-	-	0.2 ± 0.1
IGF2R	**13.3 ± 2.6**	-	**318.7**	**209.7 ± 27.3**	**70.2 ± 6.2**
IL4	0 ± 0	-	-	-	-
IL6	3.7 ± 1.1	-	-	-	0 ± 0
IL9	1.8 ± 0.9	-	-	-	-
LEFTY2	0 ± 0	-	**74.2**	**256.4 ± 37.7**	-
MDK	**900.5 ± 268.6**	**128.7 ± 18.5**	-	-	-
MST1	**16.3 ± 1.6**	-	-	-	**109.3 ± 18.7**
MSTN	-	-	-	-	0.1 ± 0.1
NODAL	0.2 ± 0.1	0.6 ± 0.2	-	-	0.2 ± 0.1
NOV	**28.7 ± 6.8**	0.9 ± 0.4	**2823.2**	**2664 ± 385.9**	**20.3 ± 12.1**
NRG1	0.5 ± 0.1	0.5 ± 0.1	**11.8**	**9.2 ± 1.1**	0.8 ± 0.2
NTF3	**30.1 ± 5.5**	**11.5 ± 1.5**	**29.3**	**17 ± 2.1**	1.1 ± 0.4
PDGFD	**62 ± 10.6**	**30.4 ± 8.6**	33	**25.7 ± 9.3**	1.8 ± 0.6
PTN	**39.4 ± 9.7**	0.5 ± 0.2	3.4	**6.3 ± 3.5**	**35 ± 11.5**
TDGF1	-	-	-	-	0.1 ± 0.1
TGFB1	-	**23.6 ± 9.2**	**14.7**	**14.2 ± 1.9**	**12.2 ± 4.4**
TGFB3	**176.8 ± 25.7**	-	3.5	**7 ± 1.1**	0.5 ± 0.2
VEGFB	-	-	-	-	**71.4 ± 14.8**
VGF	-	0.2 ± 0.1	-	-	-

Table 3. Expression patterns of genes involved in angiogenesis in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n=5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n=6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n=1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n=4)	Horse, *Equus caballus* (n=4)	Pattern
CTGF	**113.2 ± 27**	**57.4 ± 8**	**59**	**90.3 ± 6.9**	**40.4 ± 16.1**	All
CYR61	**75.8 ± 5**	**64.6 ± 5.9**	**40.5**	**57.3 ± 6.5**	**56.1 ± 24.9**	All
EGFL7	**190.4 ± 26.9**	**74.1 ± 4.4**	**227.6**	**405 ± 51**	**4.7 ± 1.3**	All
MAP3K7	**69.6 ± 4.3**	**45.2 ± 3.1**	**117.4**	**103 ± 2.7**	**11.9 ± 1.9**	All
NRP1	**50.9 ± 4.3**	**22.6 ± 1.8**	**43**	**59.4 ± 12**	**6.4 ± 0.7**	All
NRP2	**55.4 ± 5.8**	**19 ± 0.8**	**18.2**	**24 ± 3**	**6.6 ± 1.1**	All
PGF	**108 ± 41.7**	**43 ± 1.6**	**35.1**	**68.8 ± 10.7**	**6.1 ± 5.5**	All
VEGFA	**33.5 ± 6.7**	**1433.8 ± 168.3**	**146.1**	**134.4 ± 11**	**49 ± 20.7**	All
FGF9	**19.4 ± 1.8**	3.2 ± 1	0.6	2.6 ± 0.5	-	Chicken
FGF2	-	**7.4 ± 0.9**	**4.9**	**6.1 ± 0.8**	0.1 ± 0.1	Lizard
PDGFA	-	**15.5 ± 2.3**	**45.2**	**11.2 ± 2.8**	-	Lizard
ANGPTL6	0 ± 0	**68.6 ± 6**	**9.5**	**45.4 ± 11.2**	**12.8 ± 10.8**	
CXCL12	**390.5 ± 69.1**	**19.9 ± 8.4**	**20.7**	**25.8 ± 5.5**	0.9 ± 0.4	
EGF	0.1 ± 0	0.2 ± 0.1	0	0.2 ± 0	0.1 ± 0	
FGF1	**4.5 ± 1.6**	1.7 ± 0.4	**4.5**	3.8 ± 1.2	**4.4 ± 0.8**	
FGF10	1.3 ± 0.3	-	-	-	1.8 ± 0.4	
FGF18	0.1 ± 0	0.3 ± 0.1	1	3.7 ± 1.1	0.1 ± 0.1	
FGF6	0 ± 0	-	-	-	-	
FIGF	**28 ± 9.3**	**39 ± 8.7**	**8.8**	**31.2 ± 4**	0.1 ± 0.1	
FLT1	**6.4 ± 0.6**	**67.4 ± 9**	**12.5**	**9.2 ± 1.8**	0.5 ± 0.1	
KDR	**32.2 ± 4.6**	**57.3 ± 8.1**	**13.3**	**22.2 ± 3**	1.5 ± 0.1	
PROK1	3.3 ± 0.5	**68.6 ± 8.6**	-	-	-	
SHH	1.3 ± 0.2	0.3 ± 0.1	-	-	0.4 ± 0.2	
TGFA	3.2 ± 0.4	-	**47.2**	**44.7 ± 9.8**	1 ± 0.2	
TGFB2	**26 ± 3**	-	**6**	**12 ± 1.7**	**4.7 ± 0.9**	
TGFBR2	**42.8 ± 4.5**	**92.1 ± 12.4**	**77.5**	**95.9 ± 6.2**	-	
TNFRSF12A	-	**35.9 ± 7.5**	**33.2**	**48.3 ± 8.1**	**77.6 ± 14.9**	
TYMP	-	-	**142.4**	**139.2 ± 20.5**	**35.4 ± 3.3**	
VEGFC	**18.9 ± 1.4**	**10.7 ± 1.1**	**28.5**	**39.4 ± 4.5**	1.2 ± 0.6	

Danastas et al., 2015; Murphy et al., 2010; Torry et al., 2003; Whittington et al., 2015). No genes show a viviparity-specific expression pattern, suggesting that uterine angiogenesis during pregnancy has not evolved by recruiting gene expression to the embryonic membranes. Embryonic signals to increase uterine vasculature may occur by simply utilising proteins already expressed in these tissues.

3.4 Thyroid hormones

Thyroid hormones are essential for normal embryonic development, especially of brain tissue, and are regulators of metabolism and developmental rate (Burrow et al., 1994; Morreale de Escobar et al., 2004). Type 3 iodothyronine deiodinase (DIO3) metabolizes thyroid hormones in the placental tissues of mammals and is required for thyroid hormone transport to embryos (Chan et al., 2009; Roti et al., 1981). Of the 463 genes examined, only *DIO3* is present in the reference transcriptomes of all studied species, and is uniquely expressed in the CAM of placentotrophic species (Table 4). If thyroid hormone is transported to embryos in placentotrophic species, expression of *DIO3* in the CAM may be an embryonically controlled mechanism to regulate thyroid hormone uptake. Decreases in thyroid hormone abundance, by a lack of dietary iodine or thyroid removal, typically cause significant neurological developments in the brain (Bernal, 2002). Uptake of thyroid hormones by offspring could be essential for maintenance of normal development, but increased uptake may increase the growth rate of offspring directly or indirectly by increasing the growth and metabolic activity of placental tissues. Alternatively, however, it is possible that DIO3 acts to limit maternal thyroid hormone transport to embryos during development.

Type 3 iodothyronine deiodinase is a paternally expressed gene in therian mammals, suggesting that its expression imposes different fitness implications depending on the parent

Table 4. Expression patterns genes involved in thyroid hormone metabolism and receptor binding in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for per gene, corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n=5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n=6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n=1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n=4)	Horse, *Equus caballus* (n=4)	Pattern
Thyroid hormone metabolic process						
DIO3	0	**1310.5 ± 330**	0.60	2.3 ± 2.1	**16.8 ± 6.1**	Placentotrophic
MED1	**22.2 ± 1.5**	**10.5 ± 1.5**	**24.40**	**20.2 ± 2.1**	**6 ± 1.2**	All
DIO1	**24.3 ± 4.3**	-	-	-	-	Chicken
CGA	-	1.3 ± 1	-	-	**8432.2 ±**	Horse
CRYM	**26.2 ± 2.9**	**50.9 ± 5.9**	**111.70**	**126.7 ± 13.4**	1.5 ± 0.4	Reptile
SULT1B1	**82.3 ± 11.6**	**4.7 ± 1.5**	**33.60**	**48.8 ± 2.6**	-	Reptile
DIO2	**22.7 ± 4.2**	**402.5 ± 73**	1.10	2.7 ± 0.7	0 ± 0	
Thyroid hormone receptor binding						
HMGN3	**562.8 ± 18**	**75 ± 4.1**	**323.40**	**322.2 ± 19.2**	**23.1 ± 4.6**	All
JMJD1C	**13.4 ± 2**	**16.9 ± 2.9**	**23.30**	**18.4 ± 3.8**	**7.4 ± 1.7**	All
MED1	**22.2 ± 1.5**	**10.5 ± 1.5**	**24.40**	**20.2 ± 2.1**	**6 ± 1.2**	All
MED12	**13.2 ± 1.1**	**24.9 ± 3.9**	**23.00**	**14.8 ± 2.2**	**28.2 ± 6.8**	All
MED13	**9 ± 0.3**	**16.7 ± 2.6**	**19.20**	**22.5 ± 3.7**	**7.5 ± 2.2**	All
MED14	**15.5 ± 0.8**	**41.7 ± 2.6**	**11.80**	**12.2 ± 1.4**	**7.9 ± 1.7**	All
MED16	**14.3 ± 1.1**	**26.3 ± 3.4**	**10.40**	**12.5 ± 1.4**	**17.1 ± 2.2**	All
MED17	**23.4 ± 1**	**16.8 ± 1.3**	**100.90**	**125.8 ± 15.2**	**7.1 ± 0.7**	All
MED24	**20.5 ± 1.6**	**14.1 ± 1.1**	**21.90**	**18.3 ± 0.7**	**7.7 ± 1.1**	All
NCOA3	**8.4 ± 2**	**36.6 ± 5**	**17.40**	**17.7 ± 2**	**17.4 ± 5.8**	All
NCOA6	**14.9 ± 2.1**	**32.1 ± 4**	**21.70**	**13.8 ± 2.2**	**11.5 ± 3.3**	All
THRAP3	**84.8 ± 4.3**	**63.5 ± 4.5**	**104.30**	**84.2 ± 6.5**	**79.8 ± 13.1**	All
TRIP12	**30.5 ± 2.8**	**78.4 ± 11.3**	**156.80**	**115 ± 16.1**	**8.2 ± 1.7**	All
ZNHIT3	**57.2 ± 2.6**	**13.7 ± 2.1**	**24.60**	**34 ± 5.8**	**37 ± 2.3**	All
TRIP6	-	-	-	-	**4.5 ± 1.2**	Horse
MED30	**39.4 ± 1.8**	**15.7 ± 1.6**	-	-	**30.1 ± 1.2**	

from which it is inherited (Haig, 2000; Rocha et al., 2008). Typically this is the result of conflict over resources, whereby fathers (by means of the paternally inherited genome) manipulate mothers into providing more nutrients to the embryos than is optimal for the mother's life time reproductive success (Crespi and Semeniuk, 2004). During pregnancy, *DIO3* expression by embryos can manipulate mothers in two ways, by metabolizing and increasing uptake of thyroid hormones for embryonic use (this may be in conflict with mothers, if the components of thyroid hormone such as iodine are limited), or by increasing embryonic and placental growth allowing offspring to uptake extra nutrient resources from the mother. The observed expression of genes involved in thyroid hormone receptor binding in oviparous and viviparous species (Table 3) suggests that the chorioallantoic tissue is ancestrally responsive to thyroid hormones.

The role of thyroid hormone in the placentae of mammals and reptiles is poorly understood. The expression of *DIO3* in the chorioallantoic tissue of placentotrophic species but no other taxa, suggests that thyroid hormone may be important for the evolution of placental nutrient transport, but further work is required to identify the functional significance of these findings. In particular it is necessary to identify if thyroid hormone is transported across the placenta to offspring during development in non-mammalian viviparous amniotes. Secondly, it is important to identify the role of *DIO3* in pregnancy, which is poorly understood in both reptiles and mammals.

3.5 Lecithotrophy-specific gene expression

3.5.1 Parent offspring conflict and gene expression loss in placentotrophic species

Parent offspring conflict theory predicts that selection will act on offspring phenotype to maximize the resources they can obtain from their mother (Crespi and Semeniuk, 2004; Trivers, 1974). If some hormones that are ancestrally expressed in the CAM act to limit the amount of resources that are acquired in placentotrophic species, we would expect selection to act to reduce their expression. The expression of the 10 genes expressed solely in lecithotrophic taxa may be selected against in placentotrophic lineages. Sufficient functional information on two of these genes is available for us to evaluate how conflict may have resulted in them not being expressed.

Endothelin proteins are potent vasoconstrictors and play an important role in cell proliferation, migration, and differentiation (Bagnato et al., 2011; Smollich and Wulfing, 2007). Endothelin 3 (EDN3) is expressed in lecithotrophic but not placentotrophic species. Silencing of EDN3 frequently occurs in human breast cancer, where it is believed to be a natural tumor suppressor (Wiesmann et al., 2009). Expression of EDN3 in the CAM of placentotrophic species may inhibit placental cell proliferation, and may thus be selected against during the evolution of placentotrophy. In *P. entrecasteauxii*, *EDN3* is significantly up-regulated through pregnancy in uterine tissue (Chapter 3), suggesting that maternal tissue may produce EDN3 to suppress placental growth. As the expression of this gene has been lost in embryonic tissue but the gene is expressed in the maternal component of the placenta,

understanding the functional role of this gene during pregnancy may be significant for understanding the evolution of parent offspring conflict during pregnancy.

Inhibin beta A (INHBA) is a protein that can both promote or inhibit cell proliferation depending on the ligands to which it binds (Lewis et al., 2000). INHBA has tumor suppressive properties in some cancer types (Matzuk et al., 1992). Despite the antagonistic role of inhibin, placental expression of INHBA in humans is associated with intrauterine growth restriction (McMinn et al., 2006). As INHBA is not expressed in the placentotrophic species of this study, we predict that it may have acted to either reduce embryonic or maternal placental growth, thereby reducing embryonic uptake of nutrients.

3.5.2 The role of gene expression loss in the evolution of placentotrophy

In our data set, ten genes are present in all lecithotrophic taxa, present in the reference transcriptomes of all species, and not expressed in placentotrophic species (Table 5). Whilst the statistical significance of this lost gene expression cannot be tested empirically due to the number of species studied, our data presents an over-representation of genes that follow this expression pattern across taxa when compared to all genes that are exclusively expressed in only three taxa. Of the 176 genes that can be quantified across all taxa in this study, 24 genes are expressed in three groups and absent from the remaining two lineages. Whilst there are ten available patterns to which genes could be assigned 10 out of the 24 genes are expressed in a lecithotrophic expression pattern. The large number of genes expressed in this specific profile suggests it is a pattern that is worthy of further investigation.

Gene expression loss is a potential driver of many phenotypes, including pregnancy and placentotrophy (Olson, 1999). In rats, prolactin silences the uterine expression of genes that have deleterious effects on pregnancy, including 20α-HSD, which when expressed inactivates

Table 5. Expression of genes that are actively transcribed only in lecithotrophic taxa in the chorioallantoic membrane. Gene counts have been transformed to reflect the number of transcripts for each gene corrected for contig length, per million sequenced reads (TPM). Genes with a TPM greater than the expression threshold, which is correlated with active cellular gene expression, are indicated in bold. Dashes indicate genes that are absent from the reference transcriptomes of the relevant species.

Official gene symbol	Chicken, *Gallus gallus* (n=5)	Southern grass skink, *Pseudemoia entrecasteauxii* (n=6)	Bougainville's skink, *Lerista bougainvillii* (oviparous, n=1)	Bougainville's skink, *Lerista bougainvillii* (vivip, n=4)	Horse, *Equus caballus* (n=4)	Pattern
NELL2	**100.8 ± 15.5**	0.3 ± 0	**11.9**	**6.7 ± 1.4**	1.9 ± 0.1	Lecithotrophic
AR	**22.7 ± 6.3**	0.9 ± 0.3	**107.5**	**140.3 ± 15.2**	0.3 ± 0.2	Lecithotrophic
CYP26B1	**4.9 ± 0.5**	0.3 ± 0.3	**18.3**	**33.7 ± 11.5**	0.2 ± 0.1	Lecithotrophic
DLK2	**29.8 ± 11.5**	2.2 ± 0.7	**60.7**	**47.3 ± 6.4**	2.4 ± 0.7	Lecithotrophic
INHBA	**17.9 ± 2.4**	0.5 ± 0.2	**4.2**	**10 ± 4.7**	0.7 ± 0.2	Lecithotrophic
CD40	**14 ± 0.8**	2 ± 0.8	**7.6**	**6 ± 2**	0.7 ± 0.3	Lecithotrophic
FAT2	**9 ± 2.5**	3 ± 0.7	**39.8**	**19.1 ± 3.5**	2.2 ± 0.7	Lecithotrophic
LTBP2	**6.8 ± 1.8**	3.7 ± 1.1	**8.2**	**6.7 ± 0.6**	0.2 ± 0.1	Lecithotrophic
DDX11	**4.3 ± 0.4**	1.4 ± 0.2	**19.9**	**22.4 ± 2.2**	1.5 ± 0.3	Lecithotrophic
EDN3	**26 ± 8.2**	3 ± 0.5	**49.1**	**75.9 ± 13**	2.3 ± 1.6	Lecithotrophic

progesterone and reduces the possibility of a successful pregnancy (Bao et al., 2007). Loss of expression of many genes, particularly ion transporters, is also associated with the origin of mammalian viviparity (Lynch et al., 2015). The complete inactivation of genes has also occurred during the evolution of viviparity and substantial placentotrophy from oviparity in mammals, with egg yolk genes converting into pseudogenes in therian mammals (Brawand et al., 2008). Two mechanisms may explain the observed loss of expression of 10 genes in placentotrophic lineages: either their expression was neutral and expression loss occurred due to random mutations in specific regulators of these genes in the CAM; or, selection has acted in placentotrophic lineages to decrease the expression of these genes.

4. Conclusions

Conserved expression of hormone producing genes suggests that the chorioallantois is ancestrally an endocrine organ. Ancestral expression of several hormones including the angiogenic factors VEGFA and PGF are examples of genes that facilitate embryonic regulation of pregnancy and were present in the ancestral amniote. Differences in the CAM expression of specific genes between taxa allow us to understand how the endocrine properties of this tissue have changed with respect to the evolution of viviparity and placentotrophy. Given that no genes are solely expressed in viviparous species, we can conclude that the evolution of viviparity has not required new recruitment of the expression of any particular hormone-related gene. Instead, viviparity is likely to have evolved using genes that were ancestrally expressed in the CAM. Only one gene, *DIO3*, shows a placentotrophy-specific pattern of gene expression. *DIO3* is a paternally expressed imprinted gene in mammals and its expression likely acts to increase offspring resource uptake during

pregnancy, which may explain why is has also been recruited to the placental tissues of *P. entrecasteauxii* and the horse. Whilst only one gene shows a placentotrophy-specific expression pattern, many genes have lost their expression in placentotrophic lineages. Thus, the loss of gene expression may be an important mechanism in the evolution of novel tissue-specific functions such as placental nutrient transport.

5. Acknowledgements

Lizards were collected under New South Wales National Parks and Wildlife licence to MBT (SL100401). All animal work was conducted with University of Sydney Animal Ethics approval. I thank James Van Dyke, Fran van den Berg, Melanie Laird, and Jessica Dudley for comments on the manuscript. We thank Intersect Australia Ltd. for supercomputing resources and Bosch MBF for laboratory resources. Project supported by The Australian Society of Herpetologist's student research grant to OWG, Australian Research Council Discovery Early Career Research Award to MCB (DE120101615), and ARC Discovery Project funding to MBT (DP120100649).

References

Adams, S.M., Biazik, J.M., Thompson, M.B., Murphy, C.R., 2005. Cyto-epitheliochorial placenta of the viviparous lizard *Pseudemoia entrecasteauxii*: a new placental morphotype. J Morphol 264, 264-276.

Albergotti, L.C., Hamlin, H.J., McCoy, M.W., Guillette, L.J., Jr., 2009. Endocrine activity of extraembryonic membranes extends beyond placental amniotes. PLoS ONE 4, e5452.

Allen, W., Wilsher, S., Turnbull, C., Stewart, F., Ousey, J., Rossdale, P., Fowden, A., 2002. Influence of maternal size on placental, fetal and postnatal growth in the horse. I. Development in utero. Reproduction 123, 445-453.

Autiero, M., Waltenberger, J., Communi, D., Kranz, A., Moons, L., Lambrechts, D., Kroll, J., Plaisance, S., De Mol, M., Bono, F., Kliche, S., Fellbrich, G., Ballmer-Hofer, K., Maglione, D., Mayr-Beyrle, U., Dewerchin, M., Dombrowski, S., Stanimirovic, D.,

Van Hummelen, P., Dehio, C., Hicklin, D.J., Persico, G., Herbert, J.-M., Communi, D., Shibuya, M., Collen, D., Conway, E.M., Carmeliet, P., 2003. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med 9, 936-943.

Bagnato, A., Loizidou, M., Pflug, B.R., Curwen, J., Growcott, J., 2011. Role of the endothelin axis and its antagonists in the treatment of cancer. Brit J Pharmacol 163, 220-233.

Bao, L., Tessier, C., Prigent-Tessier, A., Li, F., Buzzio, O.L., Callegari, E.A., Horseman, N.D., Gibori, G., 2007. Decidual prolactin silences the expression of genes detrimental to pregnancy. Endocrinology 148, 2326-2334.

Barad, D., Brill, H., Gleicher, N., 2007. Update on the use of dehydroepiandrosterone supplementation among women with diminished ovarian function. J Assist Reprod Genet 24, 629-634.

Bazer, F.W., 1992. Mediators of Maternal Recognition of Pregnancy in Mammals. Exp Biol M 199, 373-384.

Bernal, J., 2002. Action of thyroid hormone in brain. J Endocrinol Invest 25, 268-288.

Biazik, J.M., Thompson, M.B., Murphy, C.R., 2010. Paracellular and transcellular transport across the squamate uterine epithelium. Herpetol Conserv Biol 5, 257-262.

Blackburn, D.G., 1993. Chorioallantoic placentation in squamate reptiles: structure, function, development, and evolution. J Exp Zool 266, 414-430.

Blackburn, D.G., 2006. Squamate reptiles as model organisms for the evolution of viviparity. Herpetol Monogr 20, 131-146.

Blackburn, D.G., 2014. Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative Analysis. J Morphol: http://dx.doi.org/10.1002/jmor.20272

Brandley, M.C., Young, R.L., Warren, D.L., Thompson, M.B., Wagner, G.P., 2012. Uterine gene expression in the live-bearing lizard, *Chalcides ocellatus*, reveals convergence of squamate reptile and mammalian pregnancy mechanisms. Genome Biol Evol 4, 394-411.

Brawand, D., Wahli, W., Kaessmann, H., 2008. Loss of egg yolk genes in mammals and the origin of lactation and placentation. PLoS Biol 6, e63.

Burrow, G.N., Fisher, D.A., Larsen, P.R., 1994. Maternal and fetal thyroid function. New Engl J Med 331, 1072-1078.

Callard, I.P., Bayne, C.G., McConnell, W.F., 1972. Hormones and reproduction in the female lizard *Sceloporus cyanogenys*. Gen Comp Endocr 18, 175-194.

Callard, I.P., Fileti, L.A., Perez, L.E., Sorbera, L.A., Giannoukos, G., Klosterman, L.L., Paul, T., Mccracken, J.A., 1992. Role of the corpus luteum and progesterone in the evolution of vertebrate viviparity. Am Zool 32, 264-275.

Chan, S.Y., Vasilopoulou, E., Kilby, M.D., 2009. The role of the placenta in thyroid hormone delivery to the fetus. Nat Clin Pract End Met 5, 45-54.

Cloke, B., Christian, M., 2012. The role of androgens and the androgen receptor in cycling endometrium. 358, 166-175.

Coan, P.M., Fowden, A.L., Constancia, M., Ferguson-Smith, A.C., Burton, G.J., Sibley, C.P., 2008. Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. J Physiol 586, 5023-5032.

Constância, M., Angiolini, E., Sandovici, I., Smith, P., Smith, R., Kelsey, G., Dean, W., Ferguson-Smith, A., Sibley, C.P., Reik, W., Fowden, A., 2005. Adaptation of nutrient

supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. P Natl Acad Sci USA 102, 19219-19224.
Crespi, B., Semeniuk, C., 2004. Parent-offspring conflict in the evolution of vertebrate reproductive mode. Am Nat 163, 635-653.
Cruze, L., Hamlin, H.J., Kohno, S., McCoy, M.W., Guillette Jr, L.J., 2013. Evidence of steroid hormone activity in the chorioallantoic membrane of a Turtle (*Pseudemys nelsoni*). Gen Comp Endocr 186, 50-57.
Cruze, L., Kohno, S., McCoy, M.W., Guillette, L.J., 2012. Towards an understanding of the evolution of the chorioallantoic placenta: steroid biosynthesis and steroid hormone signaling in the chorioallantoic membrane of an oviparous reptile. Biol Reprod 87, 71.
Cunningham, F., Amode, M.R., Barrell, D., Beal, K., Billis, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fitzgerald, S., Gil, L., Girón, C.G., Gordon, L., Hourlier, T., Hunt, S.E., Janacek, S.H., Johnson, N., Juettemann, T., Kähäri, A.K., Keenan, S., Martin, F.J., Maurel, T., McLaren, W., Murphy, D.N., Nag, R., Overduin, B., Parker, A., Patricio, M., Perry, E., Pignatelli, M., Riat, H.S., Sheppard, D., Taylor, K., Thormann, A., Vullo, A., Wilder, S.P., Zadissa, A., Aken, B.L., Birney, E., Harrow, J., Kinsella, R., Muffato, M., Ruffier, M., Searle, S.M.J., Spudich, G., Trevanion, S.J., Yates, A., Zerbino, D.R., Flicek, P., 2015. Ensembl 2015. Nucleic Acids Res 43, D662-D669.
Custodia-Lora, N., Callard, I.P., 2002. Progesterone and progesterone receptors in reptiles. Gen Comp Endocr 127, 1-7.
Danastas, K., Combes, V., Lindsay, L.A., Grau, G.E.R., Thompson, M.B., Murphy, C.R., 2015. VEGF111: new insights in tissue invasion. Frontiers Physiol 6.
Díaz, J.A., Alonso-Gómez, A.L., Delgado, M.J., 1994. Seasonal variation of gonadal development, sexual steroids, and lipid reserves in a population of the lizard *Psammodromus algirus*. J Herpetol 28, 199-205.
Dufaure, J.P., Hubert, J., 1961. Table de developpement du lezard vivipare *Lacerta (Zootoca) vivipara* Jacquin. Arch Anat Micr Morph Exp 50, 309-328.
Fergusson, B., Bradshaw, S.D., 1991. Plasma arginine vasotocin, progesterone, and luteal development during pregnancy in the viviparous lizard *Tiliqua rugosa*. Gen Comp Endocr 82, 140-151.
Ferner, K., Mess, A., 2011. Evolution and development of fetal membranes and placentation in amniote vertebrates. Res Physiol Neurobi 178, 39-50.
Fowden, A.L., Forhead, A.J., 2009. Endocrine regulation of feto-placental growth. Horm Res 72, 257-265.
Fowden, A.L., Ward, J.W., Wooding, F.P.B., Forhead, A.J., Constancia, M., 2006. Programming placental nutrient transport capacity. J Physiol 572, 5-15.
Fox, S.L., Guillette, L.J., 1987. Luteal morphology, atresia, and plasma progesterone concentrations during the reproductive cycle of two oviparous lizards, Crotaphytus collaris and Eumeces obsoletus. Am J Anat 179, 324-332.
Griffith, O.W., Blackburn, D.G., Brandley, M.C., Van Dyke, J.U., Whittington, C.M., Thompson, M.B., 2015. Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: a case study examining the evolution of reproductive mode in squamate reptiles. J Exp Zool Part B: http://dx.doi.org/10.1002/jez.b.22614
Griffith, O.W., Ujvari, B., Belov, K., Thompson, M.B., 2013a. Placental lipoprotein lipase (LPL) gene expression in a placentotrophic lizard, *Pseudemoia entrecasteauxii* J Exp Zool Part B 320, 465-470.

Griffith, O.W., Van Dyke, J.U., Thompson, M.B., 2013b. No implantation in an extra-uterine pregnancy of a placentotrophic reptile. Placenta 34, 510-511.
Guarino, F.M., Paulesu, L., Cardone, A., Bellini, L., Ghiara, G., Angelini, F., 1998. Endocrine activity of the corpus luteum and placenta during pregnancy in *Chalcides chalcides* (Reptilia, Squamata). Gen Comp Endocr 111, 261-270.
Guillette, L.J., Jr., Bjorndal, K.A., Bolten, A.B., Gross, T.S., Palmer, B.D., Witherington, B.E., Matter, J.M., 1991. Plasma estradiol-17β, progesterone, prostaglandin F, and prostaglandin E2 concentrations during natural oviposition in the loggerhead turtle (*Caretta caretta*). Gen Comp Endocr 82, 121-130.
Haig, D., 2000. The kinship theory of genomic imprinting. Annu Rev Ecol Syst 31, 9-32.
Hebenstreit, D., Fang, M., Gu, M., Charoensawan, V., van Oudenaarden, A., Teichmann, S.A., 2011. RNA sequencing reveals two major classes of gene expression levels in metazoan cells. Mol Syst Biol: http://dx.doi.org/10.1038/msb.2011.28
Herr, F., Liang, O.D., Herrero, J., Lang, U., Preissner, K.T., Han, V.K.M., Zygmunt, M., 2003. Possible angiogenic roles of insulin-like growth factor II and its receptors in uterine vascular adaptation to pregnancy. J Clin Endocr Metab 88, 4811-4817.
Hsu, T.-Y., Lan, K.-C., Tsai, C.-C., Ou, C.-Y., Cheng, B.-H., Tsai, M.-Y., Kang, H.-Y., Tung, Y.-H., Wong, Y.-H., Huang, K.-E., 2009. Expression of androgen receptor in human placentas from normal and preeclamptic pregnancies. Taiwan J Obstet Gyne 48, 262-267.
Huang, D.W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protocols 4, 44-57.
Katzenellenbogen, B.S., Greger, N.G., 1974. Ontogeny of uterine responsiveness to estrogen during early development in the rat. Mol Cell Endocrinol 2, 31-42.
Klicka, J., Mahmoud, I.Y., 1977. The effects of hormones on the reproductive physiology of the painted turtle, *Chrysemys picta*. Gen Comp Endocr 31, 407-413.
Laguë, P.C., Van Tienhoven, A., Cunningham, F.J., 1975. Concentrations of estrogens, progesterone and LH during the ovulatory cycle of the laying chicken [*Gallus domesticus*]. Biol Reprod 12, 590-598.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with Bowtie 2. Nat Meth 9, 357-359.
Lewis, D.F.E., 1996. Guide to Cytochromes: Structure and Function, 3 ed. CRC Press, London.
Lewis, K.A., Gray, P.C., Blount, A.L., MacConell, L.A., Wiater, E., Bilezikjian, L.M., Vale, W., 2000. Betaglycan binds inhibin and can mediate functional antagonism of activin signalling. Nature 404, 411-414.
Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., Durbin, R., Subgroup, G.P.D.P., 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25, 2078-2079.
Licht, P., 1979. Reproductive endocrinology of reptiles and amphibians: Gonadotropins. Ann. Rev. Physiol. 41, 337-351.
Licht, P., Owens, D.W., Cliffton, K., Penaflores, C., 1982. Changes in LH and progesterone associated with the nesting cycle and ovulation in the olive ridley sea turtle, Lepidochelys olivacea. Gen Comp Endocr 48, 247-253.
Lynch, Vincent J., Nnamani, Mauris C., Kapusta, A., Brayer, K., Plaza, Silvia L., Mazur, Erik C., Emera, D., Sheikh, Shehzad Z., Grützner, F., Bauersachs, S., Graf, A., Young, Steven L., Lieb, Jason D., DeMayo, Francesco J., Feschotte, C., Wagner,

Günter P., 2015. Ancient transposable elements transformed the uterine regulatory landscape and transcriptome during the evolution of mammalian pregnancy. Cell Rep 10, 551-561.
Mahmoud, I.Y., Cyrus, R.V., McAsey, M.E., Cady, C., Woller, M.J., 1988. The role of arginine vasotocin and prostaglandin F2α on oviposition and luteolysis in the common snapping turtle *Chelydra serpentina*. Gen Comp Endocr 69, 56-64.
Matzuk, M.M., Finegold, M.J., Su, J.-G.J., Hsueh, A.J.W., Bradley, A., 1992. α-lnhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature 360, 313-319.
McMinn, J., Wei, M., Schupf, N., Cusmai, J., Johnson, E.B., Smith, A.C., Weksberg, R., Thaker, H.M., Tycko, B., 2006. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 27, 540-549.
Meyer, H.H.D., 1994. Luteal versus placental progesterone: the situation in the cow, pig and bitch. Exp Clin Endocrinol 102, 190-192.
Morreale de Escobar, G., Obregon, M., Escobar del Rey, F., 2004. Role of thyroid hormone during early brain development. Eur J Endocrinol 151, U25-U37.
Murphy, B., Thompson, M., 2011. A review of the evolution of viviparity in squamate reptiles: the past, present and future role of molecular biology and genomics. J Comp Physiol 181B, 575-594.
Murphy, B.F., Parker, S.L., Murphy, C.R., Thompson, M.B., 2010. Angiogenesis of the uterus and chorioallantois in the eastern water skink *Eulamprus quoyii*. J Exp Bio 213, 3340-3347.
Murphy, B.F., Parker, S.L., Murphy, C.R., Thompson, M.B., 2011. Placentation in the eastern water skink (*Eulamprus quoyii*): a placentome-like structure in a lecithotrophic lizard. J Anat 218, 678-689.
O'Neill, M.J., Lawton, B.R., Mateos, M., Carone, D.M., Ferreri, G.C., Hrbek, T., Meredith, R.W., Reznick, D.N., O'Neill, R.J., 2007. Ancient and continuing Darwinian selection on insulin-like growth factor II in placental fishes. Proc Natl Acad Sci USA 104, 12404-12409.
Olson, M.V., 1999. When Less Is More: Gene Loss as an Engine of Evolutionary Change. Am J Hum Genet 64, 18-23.
Parker, S.L., Manconi, F., Murphy, C.R., Thompson, M.B., 2010a. Uterine and placental angiogenesis in the Australian skinks, *Ctenotus taeniolatus,* and *Saiphos equalis*. Anat Rec 293, 829-838.
Parker, S.L., Murphy, C.R., Thompson, M.B., 2010b. Uterine angiogenesis in squamate reptiles: implications for the evolution of viviparity. Herpetol Conserv Biol 5, 330-334.
Payne, A.H., Hales, D.B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active seroid hormones. Endocr Rev 25, 947-970.
Piiper, J., Tazawa, H., Ar, A., Rahn, H., 1980. Analysis of chorioallantoic gas exchange in the chick embryo. Resp Physiol 39, 273-284.
Reik, W., Constância, M., Fowden, A., Anderson, N., Dean, W., Ferguson-Smith, A., Tycko, B., Sibley, C., 2003. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. J Physiol 547, 35-44.
Rocha, S.T.d., Edwards, C.A., Ito, M., Ogata, T., Ferguson-Smith, A.C., 2008. Genomic imprinting at the mammalian Dlk1-Dio3 domain. Trends Genet 24, 306-316.

Roti, E., Fang, S.L., Green, K., Emerson, C.H., Braverman, L.E., 1981. Human placenta is an active site of thyroxine and 3,3',5-triiodothyronine tyrosyl ring deiodination. J Clin Endocr Metab 53, 498-501.
Sibley, C.P., Coan, P.M., Ferguson-Smith, A.C., Dean, W., Hughes, J., Smith, P., Reik, W., Burton, G.J., Fowden, A.L., Constância, M., 2004. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. Proc Natl Acad Sci USA 101, 8204-8208.
Sinervo, B., Miles, D.B., Frankino, W.A., Klukowski, M., DeNardo, D.F., 2000. Testosterone, endurance, and darwinian fitness: Natural and sexual selection on the physiological bases of alternative male behaviors in side-blotched lizards. Horm Behav 38, 222-233.
Smollich, M., Wulfing, P., 2007. The endothelin axis: a novel target for pharmacotherapy of female malignancies. Curr Vasc Pharmacol 5, 239-248.
Stocco, C., Telleria, C., Gibori, G., 2007. The molecular control of corpus luteum formation, function, and regression. Endocr Rev 28, 117-149.
Strauss, J.F., Martinez, F., Kiriakidou, M., 1996. Placental steroid hormone synthesis: unique features and unanswered questions. Biol Reprod 54, 303-311.
Thompson, M.B., Speake, B.K., 2002. Energy and nutrient utilisation by embryonic reptiles. Comp Biochem Phys A 133, 529-538.
Thompson, M.B., Speake, B.K., 2006. A review of the evolution of viviparity in lizards: structure, function and physiology of the placenta. J Comp Physiol 176B, 179-189.
Thompson, M.B., Stewart, J.R., Speake, B.K., 2000. Comparison of nutrient transport across the placenta of lizards differing in placental complexity. Comp Biochem Phys 127A, 469-479.
Thompson, M.B., Stewart, J.R., Speake, B.K., Hosie, M.J., Murphy, C.R., 2002. Evolution of viviparity: what can Australian lizards tell us? Comp Biochem Phys 131B, 631-643.
Torry, D.S., Mukherjea, D., Arroyo, J., Torry, R.J., 2003. Expression and Function of Placenta Growth Factor: Implications for Abnormal Placentation. J Soc Gynecol Invest 10, 178-188.
Trivers, R.L., 1974. Parent-offspring conflict. Am Zool 14, 249-264.
Tuckey, R.C., 2005. Progesterone synthesis by the human placenta. Placenta 26, 273-281.
Uller, T., Olsson, M., 2003. Prenatal exposure to testosterone increases ectoparasite susceptibility in the common lizard (*Lacerta vivipara*). P Roy Soc Lond B Biol 270, 1867-1870.
Van Dyke, J.U., Brandley, M.C., Thompson, M.B., 2014. The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. Reproduction 147, R15-R26.
Wagner, G., Kin, K., Lynch, V., 2012. Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory Biosci. 131, 281-285.
Wagner, G., Kin, K., Lynch, V., 2013. A model based criterion for gene expression calls using RNA-seq data. Theory Biosci. 132, 159-164.
Wake, M.H., 1992. Evolutionary scenarios, homology and convergence of structural specializations for vertebrate viviparity. Am Zool 32, 256-263.
Wang, H., Eriksson, H., Sahlin, L., 2000. Estrogen receptors α and β in the female reproductive tract of the rat during the estrous cycle. Biol Reprod 63, 1331-1340.
Wang, X., Miller, D.C., Clark, A.G., Antczak, D.F., 2012. Random X inactivation in the mule and horse placenta. Genome Res 22, 1855-1863.

Werck-Reichhart, D., Feyereisen, R., 2000. Cytochromes P450: a success story. Genome Biol 1, 1-9.
Whittington, C.M., Grau, G.E., Murphy, C.R., Thompson, M.B., 2015. Unusual angiogenic factor plays a role in lizard pregnancy but is not unique to viviparity. J Exp Zool Part B, http://dx.doi.org/10.1002/jez.b.22615.
Wiesmann, F., Veeck, J., Galm, O., Hartmann, A., Esteller, M., Knuchel, R., Dahl, E., 2009. Frequent loss of endothelin-3 (EDN3) expression due to epigenetic inactivation in human breast cancer. Breast Cancer Res 11, R34.
Zeh, J.A., Zeh, D.W., 2008. Viviparity-driven Conflict. Ann NY Acad Sci 1133, 126-148.

Supplementary table 1. List of key words used to extract GO terms relevant to hormone synthesis, metabolism and hormone receptivity.

Keywords		
hormone	calcitonin	melanocyte
epinephrine	cholecystokinin	motilin
melatonin	corticotropin	orexin
triiodothyronine	cortistatin	oxytocin
thyroxine	enkephalin	pancreatic polypeptide
prostaglandins	endothelin	parathyroid hormone
leukotrienes	erythropoietin	pituitary adenylate cyclase-activating peptide
prostacyclin	follicle-stimulating	prolactin
thromboxane	follicle stimulating	relaxin
amylin	galanin	renin
müllerian	gastrin	secretin
adiponectin	ghrelin	somatostatin
adrenocorticotropic	glucagon	thrombopoietin
corticotropin	gonadotropin	thyroid stimulating
angiotensinogen	growth hormone	thyrotropin
andangiotensin	hepcidin	androgen
antidiuretic	lactogen	mineralocorticoid
vasopressin	inhibin	estrogen
arginine	insulin	glucocorticoid
vasopressin	leptin	progestogen
atriopeptin	lipotropin	secosteroid
natriuretic	luteinizing hormone	

Chapter 6: Genomic imprinting is not necessary for the evolution of substantial matrotrophy

Chapter formatted for submission to *Proceedings of the Royal Society B*

Title: Genomic imprinting is not necessary for the evolution of substantial matrotrophy

Running title: No genomic imprinting in a placental lizard

Oliver W. Griffith[1*], Matthew C. Brandley[1,2], Katherine Belov[3], Michael B. Thompson[1]

1. School of Biological Sciences, University of Sydney, NSW 2006, Australia

2. New York University – Sydney, The Rocks, NSW 2000, Australia.

3. Faculty of Veterinary Science, University of Sydney, NSW 2006, Australia

*Author for correspondence, email: oliver.griffith@sydney.edu.au, phone: +61 432093216, fax: +61 293514119

Abstract

Genomic imprinting is a process that results in the differential expression of genes depending on their parent-of-origin. It occurs in both flowering plants and live bearing mammals, with imprinted genes typically regulating the ability of an embryo to manipulate the maternal provision of nutrients during development. Genomic imprinting increases the potential for selection to act separately on paternally and maternally expressed genes resulting in greater embryonic control over maternal nutrient provision. We test the hypothesis that matrotrophy evolves as a result of genomic imprinting by looking for imprinting in another matrotrophic lineage, the viviparous lizard, *Pseudemoia entrecasteauxii* (Scincidae). We sequenced transcriptomes from the embryonic component of the lizard placenta to determine bi-allelic expression in known mammalian-imprinted genes. Of these genes, 19 had sufficiently high expression in the lizard to identify polymorphisms in coding sequences. Of these, 17 genes (including *insulin like growth factor 2*) have heterozygous individuals that express two different alleles in placental tissues showing they are not imprinted. We show that matrotrophy can evolve without imprinting of key candidate genes, that genomic conflict generated by viviparity is not sufficient for the evolution of imprinting, and propose that the evolution of imprinting is likely the result of multiple evolutionary processes.

Keywords: Viviparity, Parent-offspring conflict, *Pseudemoia*, placenta, placentotrophy, lizard

BACKGROUND

The principles of Mendelian inheritance posit that offspring inherit one allele of each gene from each parent and the phenotype of offspring is the product of the expression of both of these genes. Genomic imprinting subverts Mendelian inheritance and results in genes being expressed from only one of the two parental chromosomes depending on its parent-of-origin [1]. The evolution of genomic imprinting is puzzling because it negates the heterozygosity benefit of being diploid by only expressing a single copy of each imprinted gene. Despite the potential costs, genomic imprinting has evolved independently in live bearing (viviparous) mammals and in flowering plants, but is apparently absent in egg-laying birds and the platypus [2-4]. *Insulin like growth factor 2* is also bi-allelically expressed in two live-bearing fish species [5].

The best supported hypothesis for the evolution of genomic imprinting, is that it evolves as a result of ongoing genetic conflict between mothers and offspring, resulting from the inheritance of half of the offspring's genetic material from their fathers [1, 6-8]. In both mammals (via the fetal placenta and maternal uterine tissue) and flowering plants (via the embryonic endosperm and maternal seed coat) embryos are connected to the mother inside reproductive tissue. This connection typically allows for maternal transport of nutrients to the embryo (matrotrophy), and differences in the 'preferred' amount of nutrient transport results in conflict between the maternal and paternal genome [9]. Under the conflict theory, selection will maintain maternal imprinting (switching genes off in the maternal gamete) in genes that encode proteins that facilitate embryonic manipulation of maternal nutrient transport (such as *insulin like growth factor 2*), limiting the embryo's ability to control maternal nutrient transfer. Similarly selection will maintain paternal imprinting in genes that encode proteins

which decrease embryonic manipulation (such as *insulin like growth factor 2 receptor,* which deactivates IGF2 activity in mammals), ultimately increasing the potential for embryonic regulation of matrotrophy [10, 11]. Imprinting of genes that regulate matrotrophy is common to both plants and viviparous mammals, which can be observed when the system of imprinting is subverted. In plants, parental genome dosage impacts seed endosperm formation, with a double dose of the paternal genome resulting in larger endosperm and a larger embryo, whilst a double dose of the maternal genome results in both smaller endosperm and embryos [12, 13]. In mammals similar phenotypic differences are observed; knockouts to paternally imprinted genes typically result in placental and fetal growth restriction and knockouts of maternally imprinted genes results in increases in placental or fetal growth [14] .

Parent-offspring conflict can still result in evolution in genes that do not exhibit genomic imprinting, however, gene variants that offer fitness benefits to offspring at the expense of the maternal genome will have negative fitness effects on female offspring when they reproduce. This can be illustrated by a hypothetical gene which in embryos increases the nutrients received from the mother. This gene will offer a fitness advantage to any offspring that carries it, but the gene will later impose a cost on female offspring, because when they reproduce their offspring will carry the gene resulting in her provisioning more resources than is in her interest. By maternal silencing of this gene, female offspring can receive the benefit of the gene when they inherit it from their fathers, but do not receive the fitness cost associated with it functioning when they reproduce. In this way genomic imprinting increases maternal-offspring conflict because it allows genes that facilitate conflict to be freed from antagonistic selection, increasing the rate which they can evolve.

The ancestral mode of embryonic nutrition in vertebrates is lecithotrophy, where nutrition is provided as yolk prior to ovulation and fertilization [15, 16]. In organisms where embryonic development occurs inside the mother there is the potential for embryonic nutrition to be provided across a placenta or similar structure as development occurs (matrotrophy). In all viviparous organisms which have been observed there is exchange of both organic and inorganic material between offspring, suggesting that once viviparity evolves there is potential for exchange between the mother and embryo [17-19]. However, in some species (most notably in eutherian mammals) there is a net uptake of nutrients by the embryo through pregnancy (substantial matrotrophy), this has evolved in as few as seven of the 122 viviparous lineages of amniote vertebrates [16].

The evolution of substantial matrotrophy requires two key steps, an increase in the mass of nutrients transported to embryos through development, and a decrease in the amount of yolk deposited into eggs prior to ovulation (see Fig. 1 for summary of reproductive cycle). In species with non-invasive placentation placental nutrient transport is fundamentally regulated by maternal tissues, but embryonic secretion of growth factors and other hormones can leverage some control over nutrient transport to offspring. In this system matrotrophy can be regulated by both the mother's and the embryo's genome, whilst the size of ovulated eggs is regulated solely by the mother. If variations were to arise in a population that allowed offspring to increase the transport of nutrients across the placenta, offspring would be born at a larger size giving them a fitness advantage. If the fitness advantage is large, we would expect these genes to readily spread through the population, which would result in mothers transferring more nutrients to their offspring than is in her interest, decreasing her life time reproductive success, placing a fitness burden on the mother. This fitness burden may act to select two different traits in mothers, variants that limit placental transport may be selected to

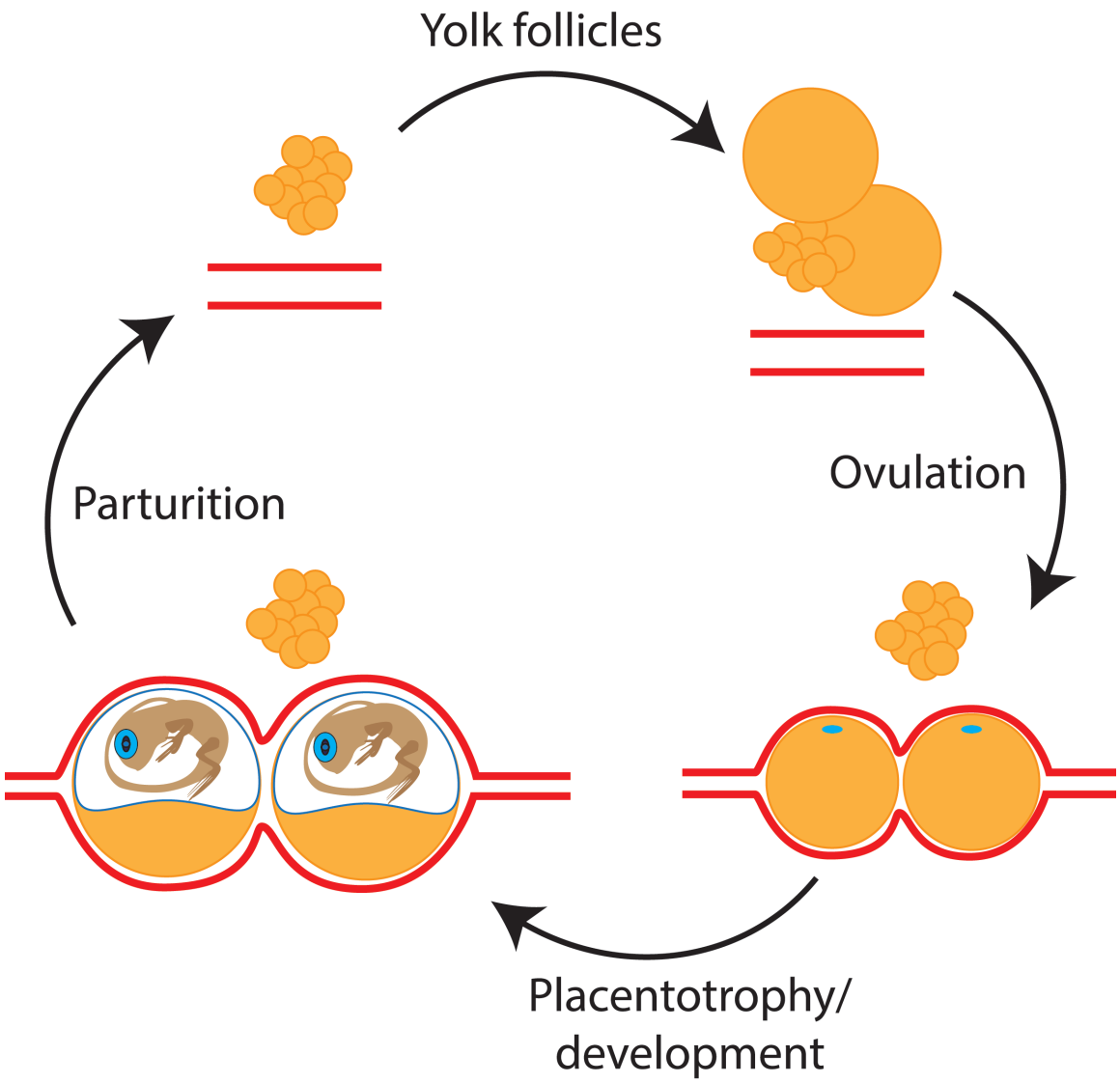


Figure 1. Typical reproductive cycle of viviparous amniote vertebrates. Firstly, ovarian follicles are recruited for reproduction and yolk is deposited into recruited follicles (vitellogenesis). After follicles are sufficiently developed they are released from the ovary and are fertilized then passed into the uterus (ovulation). In the uterus development is completed, and if matrotrophy occurs, there is a net uptake of nutrients from the embryo through the placenta. Once development is complete, the mother gives birth (parturition).

offset the increased transport to the embryos, or selection may act on mothers to decrease the size of eggs at ovulation. Both selective paradigms would have the same fitness outcome for mothers and offspring because they would equally limit the total amount of nutrients provided to embryos before birth, but selection for a decrease in the size of eggs at ovulation would ultimately act to decrease the reliance of embryos on lecithotrophy and increase the reliance on matrotrophy. Hence, substantial matrotrophy may evolve as a result of iterative adaptations in embryos to manipulate maternal transport of nutrients through gestation and counter adaptations in mothers that decrease the size of eggs at ovulation; we call this the conflict driven matrotrophy hypothesis. The conflict driven matrotrophy hypothesis, is not mutually exclusive with other theories that aim to understand the evolution of matrotrophy, and does not aim to explain how the mechanisms that facilitate matrotrophy evolve, rather it explains why organisms switch from a lecithotrophic mode of embryonic nutrition to a matrotrophic one.

The correlation between the evolution of genomic imprinting and matrotrophy in both plants and mammals suggests that the two traits may be linked. Genomic imprinting allows selection to act separately on genes of either maternal or paternal origin by removing antagonistic selection on genes when they are inherited from the other parent. Once genes are freed from antagonistic selection their evolution is likely to occur more rapidly to align with the preferred reproductive strategy of the parent of origin. By this mechanism genomic imprinting allows parent offspring conflict to facilitate the evolution of greater degrees of matrotrophy, by further leveraging embryonic control over nutrient provisioning. To test this hypothesis, it is necessary to look for genomic imprinting in other organisms in which matrotrophy has evolved and identify if genomic imprinting is or is not present. Reptiles represent an ideal system for testing the association between genomic imprinting and

matrotrophy because placentas that transport a diversity of nutrients to developing offspring have evolved independently in multiple lineages [17]. The placentae formed by reptiles and mammals are convergent in function, convergent in the genes used to fulfil these functions, and are formed from homologous tissues (maternal uterine tissue, and embryonic chorioallantoic and yolk sac membranes) [Chapter 3, 20, 21]. Furthermore, the genomic regions that facilitate imprinting in some genes (such as the methylation sites in the introns of *igf2*) are ancestral to both reptiles and mammals and are present in fishes [22]. However, no published studies have aimed to identify if genomic imprinting is also a feature of reptiles.

To test the association between the evolution of genomic imprinting and the evolution of matrotrophy we assessed whether genomic imprinting was present in mammalian imprinted genes in the viviparous southern grass skink (*Pseudemoia entrecasteauxii*). *Pseudemoia entrecasteauxii* is the ideal model to test for genomic imprinting because it is viviparous (independently from mammals), has a placenta that contributes large quantities of nutrients to offspring, and has high rates of multiple paternity [23-25]. Multiple paternity heightens the potential for conflict as offspring have lower relatedness with each other and therefore maternal fitness measured by life time reproductive success is de-coupled from the fitness of any one sire [26]. Furthermore, pregnant *P. entrecasteauxii* provide nutrients to offspring even when nutrient limitation results in maternal weight loss, suggesting nutrient transport favors the embryo's 'preferred' transfer rate, providing evidence of conflict over resources [27].

METHODS

Transcriptome sequencing of skink extra-embryonic membranes

We collected gravid *P. entrecasteauxii* from Kanangra Boyd National Park between October and November in 2011 and 2012, lizards were housed individually until late pregnancy. To collect placental tissues we removed developing eggs from the uterus and dissected away the chorioallantoic membrane and yolk sac membrane. Yolk was scraped away from the yolk sac membrane. Tissues were fixed in RNAlater (Ambion, 24 h, 4 °C) and stored (-80 °C). In total there were paired six paired embryonic placental tissues composed of the chorioallantoic membrane and yolk sac membrane (each pair of samples was collected from a different female).

To extract RNA, tissue was macerated using a mechanical homogenizer in 600µL of Buffer RLT (QIAGEN) then homogenized using a QIAshredder spin column (QIAGEN). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Extracted RNA was treated with Amplification Grade DNase 1 (Sigma-Aldrich). RNA quality was measured on the Agilent 2000 Bioanalyzer (Agilent Technologies) and was only used for transcriptome analysis if the RIN was greater than 8. Sequencing libraries were generated in house using the TruSeq RNA Sample Preperation kit (Illumina,inc.), were pooled into lanes containing ten samples and were sequenced on the HiSeq2000 (Illumina, Inc.). Raw transcriptome reads are available in the Sequenced Read Archive (Accession on acceptance).

We collected a mean of $2.4 \times 10^7 \pm 3.8 \times 10^6$ sequenced reads per sample. Data analysis was performed using the same approach as the chicken except raw sequencing reads were aligned to a transcriptome for *P. entrecasteauxii* that was assembled *de-novo*.

We assembled reference transcriptome for *P. entrecasteauxii de-novo* from the uterine tissue of early (n=1) and late (n=2) pregnant females, the embryonic chorioallantoic membrane (n=2) and yolk sac membranes (n=2) of late developing embryos, and adult brain tissue (n=1). Transcriptomes were assembled with ABySS 1.3.4 (Simpson, et al. 2009) from tissues of a single individual at a time. Once each transcriptome was assembled they were pooled, contigs smaller than 100bp and redundant contigs were removed with CD-HIT-EST (Huang, et al. 2010) using default options. Attributes of assembled transcriptome are summarised in supplementary table 1. Contigs were identified by aligning against the Anolis proteome (Ensembl Build 70) using BlastX with an e-value of 10-5. The alignment rate of the raw reads to the assembled transcriptome was > 90% for all samples. 60, 773 assembled transcripts were identified following blast to the *Anolis* proteome, which equates to 74% sequence coverage of protein coding genes in the published *Anolis* proteome. A further 27, 431 transcripts were identified after aligning un-identified contigs to a composite of the proteomes of human, chicken, opposum, platypus, zebra finch and Chinese tortoise (Ensembl Build 70).

Heterozygosity in candidate genes

We collated a list of all imprinted genes from *Homo sapiens* (human), *Mus musculus* (mouse), *Monodelphis domestica* (opossum), and *Macropus eugenii* (tammar wallaby) in the Geneimprint database [28]. Genomic imprinting results in the expression of only one of the two inherited alleles for each gene, so to rule out genomic imprinting, we tested for bi-allelic

expression (the expression of two distinct alleles) in all candidate genes in the chorioallantoic and yolk sac placental tissues of *P. entrecasteauxii*. Bi-allelic expression of genes was identified by examining heterozygous single nucleotide polymorphisms (SNPs) in the coding regions of the candidate genes. The coding region of sequences were defined as the length of the alignment between the candidate gene and the protein of interest when aligned to all proteins in the NCBI protein database with tblastn [29]. We identified heterozygosity by examining the alignments of the raw sequencing reads from each placental sample to the genes of interest in the assembled *P. entrecasteauxii* transcriptome (Table 1). Heterozygosity was noted in a sample if it had a minimum of 15 fold sequencing coverage, and at least six sequenced copies of each variant. To validate the transcriptomics methods for identifying heterozygous SNPs in mRNA samples, we performed pyrosequencing of selected polymorphisms in three genes (see supplementary methods for more details). To confirm alleleic differences between individuals were due to genetic differences, we performed pyrosequencing on paired placental mRNA and gDNA samples (see supplementary methods for more details).

RESULTS

Nineteen candidate genes had sufficiently high expression to be examined. Seventeen genes had individuals with heterozygous polymorphisms in the sequenced transcriptomes, ruling out genomic imprinting of these genes in placental tissues. Polymorphisms were not found in two genes (Table 1). In all cases, there was concordance between the allelic expression in the transcriptomics and pyrosequencing methods, confirming the use of transcriptomics for detecting bi-allelic expression of genes (Sup. Table 2). In all cases heterozygosity in genomic

Table 1 – Genes with polymorphisms as determined by high-throughput mRNA sequencing.

Highly expressed genes expressing two distinct alleles (non-imprinted genes)	Highly expressed genes without polymorphisms
ampd3, copg2, dhcr7, dio3, dlk1, dnmt1, epha4, gab1, gatm, igf2, naa60, rb1, rbP5, shce, slc38A4, tssc4, ube3A	*dcn, lin28b*

DNA corresponded to heterozygosity of mRNA, furthermore confirming bi-allelic expression (Sup. Table 3).

DISCUSSION AND CONCLUSIONS

This study is the first to look for genomic imprinting in any non-mammalian viviparous amniote. Bi-allelic expression (the expression of both maternal and paternally inherited allele) of mammalian imprinted genes in *Pseudemoia entrecasteauxii* suggests genomic imprinting has not evolved in the same genes in reptiles and mammals. Two genes in the study did not contain any polymorphisms between all individuals included in the transcriptomics study. This is not evidence for imprinting in these genes, and although we cannot rule-out genomic imprinting, they typically had lower expression of the coding region than other genes for which polymorphisms could be identified, which likely indicates they play a minor role in placental functions.

We show that genomic imprinting in genes such as *igf2* is not essential for the evolution of matrotrophy. The absence of imprinting is a significant advance because it allows us to identify the sequence of events that led to the evolution of our own reproductive strategy. In any reproductive mode that facilitates close contact of maternal and embryonic tissue, there is the potential for matrotrophy and genomic imprinting [30, 31]. Given the absence of imprinting in a matrotrophic reptile, genomic imprinting is neither necessary nor likely to play a major part in the evolution of matrotrophy in other organisms. Alternatively, we suggest that imprinting is selected for by genomic conflict that unfolds in species with substantial reliance on matrotrophy.

Given the parent-offspring conflict hypothesis for the evolution of genomic imprinting, we would expect imprinting to evolve in species that exhibit viviparity, mechanisms of provisioning to offspring after fertilization (e.g. placentotrophy), and high rates of multiple paternity [7, 8, 26]. *Pseudemoia entrecasteauxii* meets all of these criteria and is therefore a good species to asses whether these traits are sufficient to drive the evolution of imprinting. The lack of genomic imprinting in *P. entrecasteauxii* suggests that the conflict hypothesis is not sufficient to explain the presence/absence of imprinting in any specific taxa. Whilst the conflict hypothesis, may explain why there is uniparental silencing, which parental allele is silenced, and the likely physiological effects of uniparental silencing, imprinting does not evolve by single nucleotide mutations, and the processes that result in a particular locus being imprinted in any taxa are complex. We propose that the evolution of imprinting requires first a mutation that infers imprinting status to nearby genes, and then selection must act on this imprinting status for it to spread through the population.

One explanation for the origins of imprinted genes in mammals is the "host defence hypothesis", which proposes that genomic imprinting results from the insertion of foreign DNA (such as retroviruses and retrotransposons) into the genome, and that methylation of this foreign DNA led to imprinting in nearby genes [32]. Evidence for the "host defence hypothesis" comes from the molecular mechanisms of genomic imprinting, which uses the same molecular machines as those used to silence foreign DNA, and the close proximity between imprinted genes and retroviral and retrotransposon sequences [32, 33]. Although foreign DNA (from retroviral insertions) is widespread in reptiles [34], given the absence of genomic imprinting in *P. entrecasteauxii*, we expect retroviral insertions have not occurred in sites that could lead to selection described by the parent-offspring conflict hypothesis (i.e. they have not inserted near genes that facilitate embryonic manipulation of the mother). Thus,

we argue that, whilst the genomic conflict hypothesis explains the adaptive significance of the presence of imprinting in organisms, the evolution of imprinting is unlikely to occur in organisms if mechanisms to induce imprinting at specific loci do not arise. Therefore, we conclude that to understand the evolution of imprinting it is essential to identify the mechanisms that led to imprinting at specific loci, and the selective pressures that resulted in these loci spreading to fixation in organisms.

Ethics statement

Animal work was conducted under University of Sydney Animal Ethic approval.

Data Access

Raw transcriptome reads are available for download from the NCBI Sequence Read Archive (accession number upon acceptance).

Competing interests

We declare no competing interests.

Author contributions

OWG performed, pyrosequencing, data analyses, and wrote the manuscript. OWG and MCB collected lizards and constructed RNA-seq libraries. MBT, MCB, KB, and OWG contributed to experimental design and the editing of this manuscript.

Acknowledgements

Lizards collected under New South Wales National Parks and Wildlife Licence to MBT (SL100401). We thank Intersect Australia Ltd. for supercomputing resources as well as Bosch MBF and M. Olsson for laboratory resources.

Funding

Project supported by The Australian Society of Herpetologist's student research grant to OWG, Australian Research Council Discovery Early Career Research Award to MCB (DE120101615), and ARC Discovery Project funding to MBT (DP120100649).

Bibliography

1. Wilkins J.F., Haig D. 2003 What good is genomic imprinting: the function of parent-specific gene expression. Nat Rev Genet **4**(5), 359-368.
2. Frésard L., Leroux S., Servin B., Gourichon D., Dehais P., Cristobal M.S., Marsaud N., Vignoles F., Bed'hom B., Coville J.-L., et al. 2014 Transcriptome-wide investigation of genomic imprinting in chicken. Nucleic Acids Res **42**(6), 3768-3782.
3. Killian J.K., Nolan C.M., Stewart N., Munday B.L., Andersen N.A., Nicol S., Jirtle R.L. 2001 Monotreme IGF2 expression and ancestral origin of genomic imprinting. J Exp Zool **291**(2), 205-212.
4. Feil R., Berger F. 2007 Convergent evolution of genomic imprinting in plants and mammals. Trends Genet **23**(4), 192-199.
5. Lawton B.R., Sevigny L., Obergfell C., Reznick D., O'Neill R.J., O'Neill M.J. 2005 Allelic expression of *IGF2*, in live-bearing, matrotrophic fishes. Dev Genes Evol **215**(4), 207-212.
6. Holman L., Kokko H. 2014 The evolution of genomic imprinting: costs, benefits and long-term consequences. Biol Rev **89**(3), 568-587.
7. Crespi B., Semeniuk C. 2004 Parent-offspring conflict in the evolution of vertebrate reproductive mode. Am Nat **163**(5), 635-653.
8. Haig D. 2000 The kinship theory of genomic imprinting. Annu Rev Ecol Syst **31**, 9-32.
9. Moore T., Haig D. 1991 Genomic imprinting in mammalian development - a parental tug-of-war. Trends Genet **7**(2), 45-49.
10. Haig D., Graham C. 1991 Genomic imprinting and the strange case of the insulin-like growth factor II receptor. Cell **64**(6), 1045-1046.
11. Renfree M.B., Suzuki S., Kaneko-Ishino T. 2013 The origin and evolution of genomic imprinting and viviparity in mammals. Phil Trans R Soc B **368**(1609).
12. Scott R.J., Spielman M., Bailey J., Dickinson H.G. 1998 Parent-of-origin effects on seed development in *Arabidopsis thaliana*. Development **125**(17), 3329-3341.
13. Haig D., Westoby M. 1991 Genomic imprinting in endosperm - its effect on seed development in crosses between species, and between different ploidies of the same species, and its implications for the evolution of apomixis. Philos T Roy Soc B **333B**(1266), 1-13.
14. Angiolini E., Fowden A., Coan P., Sandovici I., Smith P., Dean W., Burton G., Tycko B., Reik W., Sibley C., et al. 2006 Regulation of Placental Efficiency for Nutrient Transport by Imprinted Genes. Placenta **27, Supplement**, 98-102.

15. Yu J.Y.L., Dickhoff W.W., Swanson P., Gorbman A. 1981 Vitellogenesis and its hormonal regulation in the Pacific hagfish, *Eptatretus stouti* L. Gen Comp Endocr **43**(4), 492-502.
16. Blackburn D.G. 2014 Evolution of vertebrate viviparity and specializations for fetal nutrition: a quantitative and qualitative Analysis. J Morphol, http://dx.doi.org/10.1002/jmor.20272.
17. Van Dyke J.U., Brandley M.C., Thompson M.B. 2014 The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. Reproduction **147**(1), R15-R26.
18. Hoffman L.H. 1970 Placentation in the garter snake, *Thamnophis sirtalis*. **131**(1), 57-87.
19. Van Dyke J.U., Beaupre S.J. 2012 Stable isotope tracer reveals that viviparous snakes transport amino acids to offspring during gestation. J Exp Biol **215**(5), 760-765.
20. Thompson M.B., Speake B.K. 2006 A review of the evolution of viviparity in lizards: structure, function and physiology of the placenta. J Comp Physiol **176B**(3), 179-189.
21. Brandley M.C., Young R.L., Warren D.L., Thompson M.B., Wagner G.P. 2012 Uterine gene expression in the live-bearing lizard, *Chalcides ocellatus*, reveals convergence of squamate reptile and mammalian pregnancy mechanisms. Genome Biol Evol **4**(3), 394-411.
22. Xie B.H., Zhang L., Zheng K., Luo C. 2009 The evolutionary foundation of genomic imprinting in lower vertebrates. Chinese Sci Bull **54**(8), 1354-1360.
23. Griffith O.W., Blackburn D.G., Brandley M.C., Van Dyke J.U., Whittington C.M., Thompson M.B. 2015 Ancestral state reconstructions require biological evidence to test evolutionary hypotheses: a case study examining the evolution of reproductive mode in squamate reptiles. J Exp Zool Part B, http://dx.doi.org/10.1002/jez.b.22614.
24. Stapley J., Hayes C.M., Scott Keogh J. 2003 Population genetic differentiation and multiple paternity determined by novel microsatellite markers from the mountain log skink (*Pseudemoia entrecasteauxii*). Mol Ecol Notes **3**(2), 291-293.
25. Griffith O.W., Ujvari B., Belov K., Thompson M.B. 2013 Placental lipoprotein lipase (LPL) gene expression in a placentotrophic lizard, *Pseudemoia entrecasteauxii* J Exp Zool Part B **320**(7), 465-470.
26. Haig D. 1999 Multiple paternity and genomic imprinting. Genetics **151**(3), 1229-1231.
27. Van Dyke J.U., Griffith O.W., Thompson M.B. 2014 High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. Am Nat **184**(2), 198-210.
28. Jirtle R.L. Geneimprint. www.geneimprint.com [Accessed on 2 February 2015]
29. Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25**(17), 3389-3402.
30. Stewart J.R. 2013 Fetal nutrition in lecithotrophic squamate reptiles: toward a comprehensive model for evolution of viviparity and placentation. J Morphol **274**(7), 824-843.
31. Coan P.M., Burton G.J., Ferguson-Smith A.C. 2005 Imprinted genes in the placenta – A review. Placenta **26, Supplement**, S10-S20.

32. McDonald J.F., Matzke M.A., Matzke A.J. 2005 Host defenses to transposable elements and the evolution of genomic imprinting. Cytogenet Genome Res **110**(1-4), 242-249.
33. Pask A., Papenfuss A., Ager E., McColl K., Speed T., Renfree M. 2009 Analysis of the platypus genome suggests a transposon origin for mammalian imprinting. Genome Biol **10**(1), http://dx.doi.org/10.1186/gb-2009-1110-1181-r1181.
34. Walsh A.M., Kortschak R.D., Gardner M.G., Bertozzi T., Adelson D.L. 2013 Widespread horizontal transfer of retrotransposons. Proc Natl Acad Sci USA **110**(3), 1012-1016.

SUPPLEMENTARY METHODS

Lab confirmation of findings from transcriptome sequencing

RNA used for transcriptome sequencing from the chorioallantoic membrane was reverse transcribed with *SuperScript III First Strand Synthesis* (Invitrogen). Genes were amplified with HotStar Taq DNA polymerase (0.1 units.μL^{-1}, QIAGEN) with 1x reaction buffer, gene specific forward and reverse primers (1 μM, Sup. Table 1 for sequence) in 20 μL reaction volume with an initial denaturation (95 ºC, 4 m), followed by 40 cycles of denaturation (95 ºC, 30 s), annealing (see supplementary table 1 for temperature, 30 s), and extension (72 ºC, 30 s), with a final extension step (72 ºC, 7 min).

Pyrosequencing was performed using Pyro Gold Q24 Reagents (QIAGEN), Streptavidin Sepharose beads (GE Healthcare), and gene specific sequencing primers (supplementary table 1), on the PyroMark Q24 (QIAGEN) using manufacturer's instructions.

Heterozygosity of SNPs and allele expression rates were confirmed by pyrosequencing.

To show that heterozygosity and homozygosity in expressed genes reflect underlying allelic differences in individuals, we performed pyrosequencing on PCR products of *RB1* and

UBE3A generated from paired gDNA and cDNA samples. gDNA and RNA were extracted simultaneously from embryonic chorioallantoic placenta sample using the All Prep DNA/RNA mini kit (QIAGEN) following manufacturer's instructions. PCRs and pyrosequencing were performed as above.

Supplementary Table 1 – Primers used for amplification of genomic and reverse-transcribed DNA, [btn] indicates biotinylation of adjacent nucleotide.

Gene	Primer Type	Sequence	Fragment Size	Annealing temperature
IGF2	Forward	5'GGACCTCTCATCATCTTCTCTTGT3'	114	53
	Reverse	5'[btn]TGTGAGCTTTTCTTCTGCCATAT3'		
	Sequencing	5'CCAGCATTAAACAAGGATA3'		
RB1	Forward	5'[btn]CATACAAATCTATGAATGCAATGC3'	137	53
	Reverse	5'CCCGTGTTCTTTACAAAGTTCTTC3'		
	Sequencing	5'TGTTCTTGTTGAACCG3'		
UBE3A	Forward	5'GGCTTTTCGAAGAGGATTCCA3'	80	56
	Reverse	5' [btn]GCAATTCAATCTCCTCAGGTCTAA3'		
	Sequencing	5'TCCACATGGTGACAAA3'		

Supplementary Table 2 – Expressed alleles inferred from transcriptomic (trans) and pyrosequencing (pyro) techniques for *retinablastoma 1* (*rb1*), *insulin like growth factor 2* (*igf2*), and *ubiquitin protein ligase E3A* (*ube3A*).

	rb1		*igf2*		*ube3A*	
Lizard number	trans	trans	trans	pyro	trans	pyro
1	A/G	A/G	C	C	C/T	C/T
2	A/G	A/G	C	C	C/T	C/T
3	A/G	A/G	C	C	C	C
4	A	A	C	C	C	C
5	A	A	C/T	C/T	C/T	C/T
6	A	A	C	C	T	T

Supplementary Table 3 – Genotype and expressed alleles for the *retinablastoma 1* (*rb1*) and *ubiquitin protein ligase E3A* (*ube3A*) genes inferred by pyrosequencing in the chorioallantoic tissue of seven developing *P. entrecasteauxii*.

	rb1		*ube3A*	
Lizard number	Genotype	Expressed alleles	Genotype	Expressed alleles
7	A	A	C/T	C/T
8	A	A	C/T	C/T
9	A	A	T	T
10	A	A	C/T	C/T
11	A/G	A/G	C	C
12	A	A	T	T
13	A/G	A/G	T	T

Chapter 7: General Discussion

GENERAL DISCUSSION

Mechanisms underpinning the evolution of placental functions

Innovative functions in tissues evolve from three distinct processes, modifications to genes expressed in the ancestral tissue, recruiting the expression of genes typically expressed in other tissues, or the introduction of new genetic material (from either introduction of foreign DNA such as viral genes, or from the duplication of genes) (Knox and Baker 2008; Lynch, et al. 2008; Zhang 2003). The role of these genetic processes in the evolution of complex phenotypes is poorly understood. Analysis of the roles of these mechanisms in the evolution of placental traits in independent origins of placentation is a powerful tool for understanding how these processes facilitate the evolution of complex traits more broadly. In particular, comparisons between the genes that underpin placental functions in independent viviparous lineages enable assessment of whether complex phenotypic traits evolve using the same genes. I aimed to identify the gene expression patterns that are correlated with placental functions and identify the mechanisms that have resulted in the expression of these genes in placental tissues. Furthermore, by comparing the genes expressed in the placental tissues of organisms in which placental structures have evolved independently, I assessed whether placental innovations evolve in predictable ways.

Homology – placental functions that result from ancestral properties of tissues

Placentae in amniotes evolve from pre-existing tissues. Hence, to understand the evolution of placental functions, it is important to identify the role of homology in the gene expression that occurs in the placenta. Viviparity evolves from oviparity and the structures that support pregnancy must evolve from pre-existing tissues in squamates and other amniotes (Chapter 2). The uterus of the most recent common ancestor of extant amniotes had two prominent functions, to transport the fertilized egg from the ovaries to the external environment and to deposit the eggshell around the embryonic membranes (Thompson and Speake 2006). The extra-embryonic membranes of this ancestor exchanged respiratory gases with the external environment, absorbed calcium from the eggshell, and played some role in hormonal signaling for embryonic development (Albergotti, et al. 2009; Cruze, et al. 2013; Gabrielli and Accili 2010; Thompson and Speake 2006). Conservation of the processes that facilitated these ancestral functions is an important process that under pinned the evolution of novel functions in placental tissues.

The vast majority of viviparous squamates have non-invasive placental morphologies (Van Dyke, et al. 2014a). The ancestral embryonic membranes of amniotes did not exhibit invasive properties (Chapter 4). Hence, the structural and functional innovations that occur in placental tissues of squamates must result from the modification of non-invasive placental morphologies. The maintenance of non-invasive embryonic membranes in most squamates is an example of a phenotypic trait that has been retained during the evolution of viviparity, which is in contrast to the evolution of non-invasive placentation in eutherian mammals,

where the embryonic membranes exhibit invasive properties, yet properties of the uterus prevent invasion (Samuel and Perry 1972).

Calcium is an essential nutrient for vertebrate embryonic development (Stewart 2013). In oviparous amniotes calcium can be derived from both ovulated yolk reserves and by removing calcium from the eggshell (Stewart and Ecay 2010). Hence, ancestrally the embryonic membranes of amniotes are capable of absorbing calcium (Ecay, et al. 2004; Mossman 1987). In amniotes, eggshell is deposited around embryonic membranes in the uterus, where specialized structures such as shell glands secrete calcium and other shell constituents (Adams, et al. 2004; Girling 2002). These tissues are therefore capable of calcium transport, and viviparous lineages use the same calcium transport mechanisms that supports egg-shell deposition in oviparous species (Stewart and Ecay 2010; Stewart, et al. 2011). Uterine transport of calcium in squamates occurs in specialized shell glands and is facilitated by a plasma membrane bound Ca^{2+}- ATPase (PMCA)(Herbert, et al. 2006; Herbert, et al. 2010; Thompson, et al. 2007). *Pseudemoia entrecasteauxii* has altered the timing of PMCA expression such that calcium is transported late in pregnancy when it is utilized by the embryo for growth (Chapter 3; Herbert et al. 2010). The utilization of calcium transport structures in the uterine tissue of viviparous squamates is an example of a placentotrophic trait that is ancestral to the tissues that are derived to form placentae in viviparous amniotes.

The extra-embryonic membranes of oviparous amniotes were capable of producing both steroid and peptide hormones (Chapter 5; Albergotti et al. 2009; Cruze et al. 2013; Cruze, et al. 2012). The functional significance of hormone production in the embryonic membranes of oviparous taxa is not understood. One explanation is that the embryonic membranes produce

hormones in response to environmental cues. Signaling of this sort may be necessary to allow plasticity in embryonic development and allow for regulation of properties of the egg, such as regulation of water transfer across the eggshell in different environmental conditions. Whatever the functional significance, hormone production of extra-embryonic membranes has been retained in viviparous lineages, where it can facilitate various pregnancy-specific functions (Chapter 5). Insulin like growth factor 2 (IGF2) is a peptide hormone that increases the growth of tissues and organs (Wolf, et al. 1998). In mammals IGF2 is produced by the trophoblast to facilitate placental growth and plays an important role in manipulating nutrient transport across placental tissues (Sibley, et al. 2004). The expression of IGF2 in many oviparous and viviparous amniotes (Chapter 5), suggests that its expression is ancestral to the amniote chorioallantoic membrane. In therian mammals, and potentially other viviparous amniotes, conservation of IGF2 expression has resulted in a unique pregnancy-associated function of the embryonic placenta.

Recruitment – placental functions that derive from recruitment of genes expressed elsewhere in organisms

During the evolution of novel complex phenotypes, novel traits can evolve by expressing genes typically expressed elsewhere in the organism (Knox and Baker 2008; True and Carroll 2002). Gene expression recruitment typically occurs to changes in regulatory regions of the DNA proximate to the gene (cis-regulatory element) or changes to other genes that regulate gene expression (trans-regulatory elements) (Lynch et al. 2008; Wittkopp and Kalay 2012). Identifying genes that are recruited to facilitate placental functions, and the processes that resulted in their recruitment is fundamental to understanding how new placental functions evolve.

Transposon mediated changes to gene regulation have been fundamental to the evolution of the mammalian reproductive system (Lynch, et al. 2011; Lynch, et al. 2015). Transposons are short sequences of DNA that, when transcribed, are capable of copying themselves and inserting themselves in another region of the genome (Kidwell and Lisch 2000). Transposons can contain their own regulatory elements, and once inserted near other genes they can induce the expression of that gene, ultimately resulting in the gene being expressed at times when it would not normally be expressed (Feschotte 2008; Feschotte and Pritham 2007). Transposons occur in the genomes of most eukaryotes and can be hyper abundant in the genomes of squamates and birds (Feschotte 2008; Gilbert, et al. 2011; Kordis 2009). Multiple retrotransposon genes are expressed in the uterine tissue of *P. entrecasteauxii*, but none that were identified are up or down-regulated during pregnancy (Chapter 3). The role of transposons in the recruitment of placental functions in reptiles is not clear, but transposons have been implicated in the evolution of other complex traits in reptiles, including venom and tail regeneration (Hutchins, et al. 2014; Ikeda, et al. 2010). Identifying the role of retrotransposons in the evolution of placental functions in reptiles should be an area of active research as it is likely to be an important mechanism for the evolution of complex novel traits.

In mammals, viviparity evolved by the recruitment of thousands of genes (Lynch et al. 2015). Whilst, thousands of genes are significantly up-regulated during pregnancy in skinks (Chapter 3), it is not clear if these changes in regulation are due to recruitment of these genes to the uterus during pregnancy, or because they exhibit ancestral expression in these tissues. Further work is needed to answer this question, but it is interesting to note that largely the same amino acid transport proteins are expressed in the trophoblast of humans and are upregulated in the uterus of *P. entrecasteauxii* during pregnancy (Chapter 3). If the

expression of these genes in either tissue is the result of gene expression recruitment, then convergent gene use suggests that there are constraints on the genes that can be recruited to derive novel placental traits. An alternative explanation is that the amino acid transport protein genes expressed in both tissues are induced by the expression of a single regulatory protein that can be mobilized between embryonic and maternal placental tissues, such as a growth factor expressed in the embryonic membrane (Chapter 5).

The evolution of viviparity in amniotes does not require the recruitment of any single hormone or hormone related gene (Chapter 5). However, differences in gene expression show that some genes have been recruited to each viviparous lineage, albeit not consistently to viviparous lineages. One protein responsible for thyroid hormone metabolism, deiodinase iodothyronine type III (DIO3), was recruited to the placental tissues of two matrotrophic amniotes (Chapter 5). The functional significance of this gene in the placenta of both taxa is yet to be understood, but the encoded protein facilitates the metabolisation of thyroid hormones, and the gene is genomically imprinted in eutherian mammals, but not in *P. entrecasteauxii* (Chapter 6; Chan, et al. 2009; Edwards, et al. 2008; Roti, et al. 1981). The expression of DIO3 in the placentae of matrotrophic species is an example of how gene expression recruitment can facilitate the evolution of novel placental functions in both mammals and reptiles. By identifying the cis- and trans-regulatory elements responsible for DIO3 expression in matrotrophic and closely related lecithotrophic taxa, it would be possible to identify the proximate mechanisms responsible for recruitment of this gene to the placenta.

Gene expression loss

Whilst the evolution of innovative functions in tissues typically requires a gain in function, gene expression is an important process in the evolution of novel phenotypes. The expression

of 10 hormone related genes has been lost in matrotrophic amniotes, suggesting that gene loss might be important for the evolution of matrotrophy (Chapter 5). The loss of gene expression appears to have occurred in the evolution of viviparity in mammals too, where several genes responsible for shell production are not expressed in the endometrium of viviparous mammals (Lynch et al. 2015). The processes that underpin the loss of gene expression are likely to be similar to those that result in the recruitment of expression, changes to cis- and trans-regulatory elements (Prud'homme, et al. 2007; Wray 2007). In rats, endometrial prolactin expression results in down-regulation of genes that are detrimental to pregnancy (Bao, et al. 2007). Gene expression loss is likely to be fundamentally important for the evolution of complex phenotypic traits, because the rate of gene expression loss in organisms is far higher than the rate of gene expression recruitment (Oakley, et al. 2006).

Parent offspring conflict and the evolution of placentotrophy

Conflict and the origins of matrotrophy

The fundamental assumption of the viviparity-conflict model for the evolution of placental innovations is that offspring are capable of manipulating maternal investment in themselves (Crespi and Semeniuk 2004). In viviparous amniotes embryonic placental tissues express a range of signaling molecules such as growth factors that can potentially alter the physiology of maternal tissue (Chapter 5). Understanding how hormone production by embryos could manipulate the provision of maternal resources requires an understanding of the mechanisms of nutrient transport to embryos across the placenta.

During pregnancy, the uterus of *P. entrecasteauxii* increases the expression of many genes that encoded nutrient transport proteins, suggesting that mechanisms of nutrient transport to

offspring are complex (Chapter 3). Offspring must produce hormones that can regulate the expression of these suites of genes for conflict to be the primary driver for the evolution of matrotrophy. Whilst I identified the expression of multiple growth factors in the embryonic component of the placenta in *P. entrecasteauxii*, it is unlikely that these genes are sufficient to regulate complex mechanisms of transport on their own, because growth factors typically induce general growth/development pathways, and are not known to induce the expression of novel genes to perform innovative cellular functions (Carter-Su, et al. 1996). Furthermore, it is unlikely that these mechanisms of transport could evolve by selection on offspring alone and not selection on mothers to optimize the processes of transport.

The extra-embryonic membranes of *P. entrecasteauxii* differ from those of eutherians with similar placental morphology because they lack mechanisms to invade maternal tissues (Chapter 4). Eutherians have ancestrally invasive placentation, which gives embryos greater power to manipulate maternal provision of nutrients (Elliot and Crespi 2009; Mess and Carter 2007; Wildman, et al. 2006). In eutherian mammals, non-invasive placentation (and hence mechanisms of histotrophy) may have evolved due to selection on mothers to regain control over placental nutrient transfer during pregnancy (Garratt, et al. 2013). In *P. entrecasteauxii*, the extra-embryonic membranes of offspring are not ancestrally invasive (Chapter 4). Without ancestrally invasive tissues, parent-offspring conflict does not predict that mechanisms of placentotrophy are likely to evolve in response to selection on mothers. Together, these results strongly suggest that the evolution of mechanisms for matrotrophy, and that matrotrophy itself, must have non-conflict driven selective advantages, i.e. advantages that are of mutual benefit to maternal and paternal genomes in offspring.

Several alternative hypotheses for the evolution of placentotrophy in vertebrates exist, but more data are required to support these models. One explanation is the facultative placentotrophy hypothesis, which suggests matrotrophy allows mothers to be plastic in the amount of nutrients provided to offspring depending on the availability of resources in the environment during reproduction. Although facultative placentotrophy has been documented in placentotrophic lizards, food shortage can lead to developmental failure, small offspring, and infanticidal cannibalism, which means placentotrophy is likely to only be a successful strategy when food is abundant (Pollux and Reznick 2011; Swain and Jones 2000; Van Dyke, et al. 2014b). Alternatively, under the life history model, placentotrophy may allow mothers to increase reproductive output when food abundance is both high and predictable (Trexler and DeAngelis 2003). An assumption of this hypothesis, is that mothers are capable of aborting and absorbing resources from inviable offspring (Trexler and DeAngelis 2003). Whilst there is limited evidence of maternal recouping of resources in fish, high food abundance appears to be necessary in placentotrophic fish and reptiles (Banet and Reznick 2008; Riesch, et al. 2013; Van Dyke et al. 2014b).

Conflict and obligate placentotrophy

Although the nutrient transport mechanisms that support matrotrophy are unlikely to have evolved as a result of parent offspring conflict that does not mean that parent offspring conflict has not contributed to the evolution of other traits of viviparous taxa. The selective pressures that led to the evolution of matrotrophy need not be the same as the pressures that drove the evolution of obligate placentotrophy. Given that the embryo is likely to have the ability to influence the maternal provision of nutrients, but not complete control over it, the role of conflict in the evolution of placentotrophy may be restricted to shifting the reliance

from lecithotrophic to placentotrophic nutrition. Under this model, nutrient transport mechanisms must first evolve in mothers under a non-conflict associated selection program, such as to facilitate facultative nutrient supply to offspring. After placental nutrient transport mechanisms have evolved, strategies may evolve in embryos to manipulate maternal nutrient transfer. Embryos that are able to gain additional nutrients from mothers through gestation are likely to be fitter than siblings that are not (Sinervo 1990). If mothers bear children that are capable of taking more nutrients than is in the interest of her life time reproductive success, then selection will act to decrease the supply of nutrient to offspring through the reproductive cycle. Selection may then act on mothers to either decrease placental transport of nutrients or decrease the size of eggs at ovulation. In lineages with the highest mass of nutrient transport (eutherian mammals and *Mabuya* sp.) mothers ovulate microlecithal eggs with very limited yolk resources (Ramírez-Pinilla, 2006). Furthermore, in the lizard genus *Pseudemoia,* species with increasing placental transport rates correlate with decreasing size of eggs at ovulation (Thompson et al. 2000). Combined this evidence supports the idea that the evolution of placentotrophy has occurred concurrently with a decrease in ovulated yolk mass.

With smaller eggs being ovulated, it is easy to see how iterative adaptations for embryonic mechanisms to increase nutrient transfer and selection on mothers to decrease the size of eggs at ovulation would result in a shift from lecithotrophic to matrotrophic nutrition. This model makes two assumptions, first that embryos are capable of altering the rate of nutrient transfer across the placenta in maternal tissue and secondly that the size of eggs at ovulation is both variable and heritable. Once mechanisms of nutrient transport have evolved, the embryo may have the capability of manipulating the activity of placental transporters. The embryo could further manipulate the size and surface area for transport by increasing the folding of the

chorioallantoic membrane, and the production of hormones/growth factors may increase expression of transporters in the mother. Finally, the embryo could increase the vascularization of the uterine tissue by the production of angiogenic factors that can be transferred to uterine tissue and promote blood vessel growth and development.

I propose that regulation of angiogenesis is an important mechanism by which embryos can manipulate the capacity for transport of the placenta. The assumptions of this model requires that embryos are capable of regulating angiogenesis in the uterine tissue of mothers, and that increasing angiogenesis can result in changes to nutrient transport capacity. Reptile embryos can manipulate uterine angiogenesis because embryonic signaling contributes to increased vascular development in the uterus of the viviparous skink *Eulamprus quoyii* (Murphy, et al. 2010b). Furthermore, production of angiogenic factors is ancestral to the embryonic membranes of amniotes (Chapter 5; Murphy, et al. 2010a; Whittington, et al. 2015), suggesting that the capacity for embryos to induce angiogenesis in maternal tissues is widespread in viviparous amniotes. Increasing angiogenesis without mechanisms for nutrient transport is not sufficient to increase nutrient transfer across the placenta through pregnancy, because variability in uterine blood vessel density exists in lecithotrophic species without observable placentotrophy (Murphy, et al. 2012; Thompson, et al. 2000). However, in species with mechanisms for transport in place, increased vascularization could result in increased rate of transfer because it increases the rate of supply of nutrients to the uterus. An analogous mechanism for increased nutrient supply happens in cancers (Vaupel, et al. 1989). Cancer cells actively secrete angiogenic factors such as VEGF into their micro-environment to increase vascular development around the tumor site (Carmeliet 2005; Folkman 2002). A range of drugs have been identified to either inhibit or promote angiogenesis in tissues through research to fight angiogenesis in cancer (Carmeliet and Jain 2000). The use of these

drugs in the uterus of placentotrophic reptiles provide a way to experimentally test if changes in angiogenesis alter the rates of nutrient transfer across the placenta.

The second assumption of the conflict model for the evolution of obligate placentotrophy is that heritable variation in egg size existed to be selected on. Geographic variation in egg size at ovulation has been identified in lizards including *Takydromus septentrionalis* and *Uta stansburiana* under common garden experiments (Du, et al. 2005; Sinervo and Licht 1991), suggesting that size of eggs at ovulation is variable and probably genetically controlled. *Pseudemoia* is a genus of Australian skinks that exhibits varying degrees of placentotrophy and differences in the size of eggs at ovulation among its species (Haines, et al. 2014; Stewart and Thompson 1993; Thompson, et al. 1999a; Thompson, et al. 1999b); given that size of eggs at ovulation is species-specific, it is likely to be a trait with a genetic basis.

Since the assumptions of the conflict model for the evolution of obligate placentotrophy hold, I argue that the hypothesis needs further investigation as a model for understanding why taxa shift from a lecithotrophic to matrotrophic nutrient provisioning strategy. In particular, further work is needed to identify to what degree embryonic manipulation of maternal tissues can increase the rate of nutrient transport across the placenta through pregnancy.

Concluding remarks

Discussion of reptilian placentation in the context of mammalian pregnancy is important for cross taxa comparisons that seek to understand how placental traits evolve broadly and is essential for understanding what reptilian viviparity can tell us about the evolution of pregnancy and placentation in amniotes generally. I have made major advances in our understanding of the genetics and evolution of pregnancy in reptiles, by generating and

annotating the first transcriptome of the most widely studied viviparous and placentotrophic reptile, *Pseudemoia entrecasteauxii*. I used whole transcriptome sequencing to describe the gene expression profiles of embryonic and maternal placental tissues. My focus on genes that are important for key placental functions, such as embryonic signaling during pregnancy and the mechanisms by which these functions evolved at the genetic level, provide a significant advanced our understanding of the genetic mechanisms of complex trait evolution. I provide strong evidence that placental functions in mammals and reptiles have evolved by the same genetic mechanisms, i.e. utilization of ancestrally expressed genes, and recruiting the expression of genes to perform novel placental functions. Furthermore, I show that the mechanisms that facilitate placental amino acid transport in the reptile uterus significantly overlap with those used by the human trophoblast. This provides the first evidence that the evolution of complex traits do not occur by the random recruitment of functionally equivalent genes, but instead other processes result in the recruitment of the same specific genes. That the same genes facilitate placental functions in independently derived placental structures in lineages that shared a common ancestor more than 300 million years ago suggests that, if time was rewound and replayed even hundreds of millions of years, we would expect the same complex structures of vertebrates to evolve predictably.

Genomic imprinting has been central to debates regarding the role of parent offspring conflict in the evolution of placentation, as it is correlated with the evolution of viviparity in mammals and plants. I provide the first evidence that genomic imprinting is not essential for the evolution of placentotrophy in organisms as it is not present in all candidate genes in a placentotrophic reptile. This finding resolves the debate regarding the role of genomic imprinting as a driving force in the evolution of substantial matrotrophy, and suggests that instead imprinting is best understood as an outcome of conflict between genes of maternal

and paternal origin in viviparous organisms. Once matrotrophy and imprinted genes are both present, the imprinted genes may have an important role in the evolution of placental innovations.

The central assumption to the parent-offspring conflict hypothesis for the evolution of placental functions is that embryos are capable of manipulating how mothers provide resources to themselves. Evidence for mechanisms of embryonic control over resource provisioning has been lacking outside of mammalian studies, primarily because of a lack of research on the signaling processes occurring in the placental tissues of reptiles. In this thesis I identify a suite of embryonically produced hormones and growth factors, providing the first evidence for mechanisms of embryonic regulation of the placenta.

This thesis allows us to conclude that parent offspring conflict is a poor explanation for the evolution of mechanism of placental nutrient transport, but a potentially good explanation for a shift from lecithotrophic to placentotrophic nutrition. The parent-offspring conflict hypothesis for the evolution of obligate placentotrophy is a new explanation for why obligate placentotrophy evolves that can be tested broadly across animals. The identification of hormones expressed in placental tissues provides the foundations for future research to test the parent-offspring conflict hypothesis for the evolution of obligate placentotrophy, which will allow us to understand why different reproductive strategies evolve in organisms.

References

Adams SM, Hosie MJ, Murphy CR, Thompson MB 2004. Changes in oviductal morphology of the skink, *Lampropholis guichenoti*, associated with egg production. J Morphol 262: 536-544.

Albergotti LC, Hamlin HJ, McCoy MW, Guillette LJ, Jr. 2009. Endocrine activity of extraembryonic membranes extends beyond placental amniotes. PLoS ONE 4: e5452.
Banet AI, Reznick DN 2008. Do placental species abort offspring? Testing an assumption of the Trexler-DeAngelis model. Funct Ecol 22: 323-331.
Bao L, Tessier C, Prigent-Tessier A, Li F, Buzzio OL, Callegari EA, Horseman ND, Gibori G 2007. Decidual prolactin silences the expression of genes detrimental to pregnancy. Endocrinology 148: 2326-2334.
Carmeliet P 2005. VEGF as a key mediator of angiogenesis in cancer. Oncology 69 Suppl 3: 4-10.
Carmeliet P, Jain RK 2000. Angiogenesis in cancer and other diseases. Nature 407: 249-257.
Carter-Su C, Schwartz J, Smit LJ 1996. Molecular mechanism of growth hormone action. Annu Rev Physiol 58: 187-207.
Chan SY, Vasilopoulou E, Kilby MD 2009. The role of the placenta in thyroid hormone delivery to the fetus. Nat Clin Pract End Met 5: 45-54.
Crespi B, Semeniuk C 2004. Parent-offspring conflict in the evolution of vertebrate reproductive mode. Am Nat 163: 635-653.
Cruze L, Hamlin HJ, Kohno S, McCoy MW, Guillette Jr LJ 2013. Evidence of steroid hormone activity in the chorioallantoic membrane of a Turtle (*Pseudemys nelsoni*). Gen Comp Endocr 186: 50-57.
Cruze L, Kohno S, McCoy MW, Guillette LJ 2012. Towards an understanding of the evolution of the chorioallantoic placenta: steroid biosynthesis and steroid hormone signaling in the chorioallantoic membrane of an oviparous reptile. Biol Reprod 87: 71.
Du WG, Ji X, Zhang YP, Xu XF, Shine R 2005. Identifying sources of variation in reproductive and life-history traits among five populations of a Chinese lizard (*Takydromus septentrionalis*, Lacertidae). Biol J Linnean Soc 85: 443-453.
Ecay TW, Stewart JR, Blackburn DG 2004. Expression of calbindin-D28K by yolk sac and chorioallantoic membranes of the corn snake, *Elaphe guttata*. J Exp Zool Part B 302B: 517-525.
Edwards CA, Mungall AJ, Matthews L, Ryder E, Gray DJ, Pask AJ, Shaw G, Graves JAM, Rogers J, Dunham I, Renfree MB, Ferguson-Smith AC, The Savoir Consortium. 2008. The evolution of the *DLK1-DIO3* imprinted domain in mammals. In *PLoS Biol*, Vol 6, p. e135. Public Library of Science.
Elliot MG, Crespi BJ 2009. Phylogenetic evidence for early hemochorial placentation in Eutheria. Placenta 30: 949-967.
Feschotte C 2008. Transposable elements and the evolution of regulatory networks. Nat Rev Genet 9: 397-405.
Feschotte C, Pritham EJ 2007. DNA transposons and the evolution of eukaryotic genomes. Annu Rev Genet 41: 331-368.
Folkman J 2002. Role of angiogenesis in tumor growth and metastasis. Semin Cancer Biol 29: 15-18.
Gabrielli MG, Accili D 2010. The chick chorioallantoic membrane: a model of molecular, structural, and functional adaptation to transepithelial Ion transport and barrier function during embryonic development. J Biomedicine Biotech 2010: 940741.
Garratt M, Gaillard J-M, Brooks RC, Lemaître J-F 2013. Diversification of the eutherian placenta is associated with changes in the pace of life. Proc Natl Acad Sci USA 110: 7760-7765.

Gilbert C, Hernandez SS, Flores-Benabib J, Smith EN, Feschotte C 2011. Rampant horizontal transfer of SPIN transposons in squamate reptiles. Mol Biol Evol: http://dx.doi.org/10.1093/molbev/msr1181.

Girling JE 2002. The reptilian oviduct: a review of structure and function and directions for future research. J Exp Zool 293: 141-170.

Haines ML, Moussalli A, Stuart-Fox D, Clemann N, Melville J 2014. Phylogenetic evidence of historic mitochondrial introgression and cryptic diversity in the genus *Pseudemoia* (Squamata: Scincidae). Mol Phylogenet Evol 81: 86-95.

Herbert JF, Lindsay LA, Murphy CR, Thompson MB 2006. Calcium transport across the uterine epithelium of pregnant lizards. Herpetol Monogr 20: 205-211.

Herbert JF, Murphy CR, Thompson MB 2010. Calcium ATPase localization in the uterus of two species of *Pseudemoia* (Lacertilia: Scincidae) with complex placentae. Herpetol Conserv Biol 5: 290-296.

Hutchins ED, Markov GJ, Eckalbar WL, George RM, King JM, Tokuyama MA, Geiger LA, Emmert N, Ammar MJ, Allen AN, Siniard AL, Corneveaux JJ, Fisher RE, Wade J, DeNardo DF, Rawls JA, Huentelman MJ, Wilson-Rawls J, Kusumi K 2014. Transcriptomic analysis of tail regeneration in the lizard *Anolis carolinensis* reveals activation of conserved vertebrate developmental and repair mechanisms. PLoS ONE 9: e105004.

Ikeda N, Chijiwa T, Matsubara K, Oda-Ueda N, Hattori S, Matsuda Y, Ohno M 2010. Unique structural characteristics and evolution of a cluster of venom phospholipase A2 isozyme genes of Protobothrops flavoviridis snake. Gene 461: 15-25.

Kidwell MG, Lisch DR 2000. Transposable elements and host genome evolution. Trends Ecol Evol 15: 95-99.

Knox K, Baker JC 2008. Genomic evolution of the placenta using co-option and duplication and divergence. Genome Res 18: 695-705.

Kordis D 2009. Transposable elements in reptilian and avian (sauropsida) genomes. Cytogenet Genome Res 127: 94-111.

Lynch VJ, Leclerc RD, May G, Wagner GP 2011. Transposon-mediated rewiring of gene regulatory networks contributed to the evolution of pregnancy in mammals. Nat Genet 43: 1154-1158.

Lynch Vincent J, Nnamani Mauris C, Kapusta A, Brayer K, Plaza Silvia L, Mazur Erik C, Emera D, Sheikh Shehzad Z, Grützner F, Bauersachs S, Graf A, Young Steven L, Lieb Jason D, DeMayo Francesco J, Feschotte C, Wagner Günter P 2015. Ancient transposable elements transformed the uterine regulatory landscape and transcriptome during the evolution of mammalian pregnancy. Cell Rep 10: 551-561.

Lynch VJ, Tanzer A, Wang Y, Leung FC, Gellersen B, Emera D, Wagner GP 2008. Adaptive changes in the transcription factor HoxA-11 are essential for the evolution of pregnancy in mammals. Proc Natl Acad Sci USA 105: 14928-14933.

Mess A, Carter AM 2007. Evolution of the placenta during the early radiation of placental mammals. Comp Biochem Phys A 148A: 769-779.

Mossman HW. 1987. *Vertebrate Fetal Membranes: Comparative Ontogeny and Morphology; Evolution; Phylogenetic Significance; Basic Functions; Research Opportunities*. Rutgers University Press, New Brunswick, USA.

Murphy BF, Belov K, Thompson MB 2010a. Evolution of viviparity and uterine angiogenesis: vascular endothelial growth factor (VEGF) in oviparous and viviparous skinks. J Exp Zool 314B: 148-156.

Murphy BF, Brandley MC, Murphy CR, Thompson MB 2012. Morphology and development of the placentae in *Eulamprus quoyii* group skinks (Squamata: Scincidae). J Anat 220: 454-471.
Murphy BF, Parker SL, Murphy CR, Thompson MB 2010b. Angiogenesis of the uterus and chorioallantois in the eastern water skink *Eulamprus quoyii*. J Exp Bio 213: 3340-3347.
Oakley TH, Østman B, Wilson ACV 2006. Repression and loss of gene expression outpaces activation and gain in recently duplicated fly genes. Proc Natl Acad Sci USA 103: 11637-11641.
Pollux BJA, Reznick DN 2011. Matrotrophy limits a female's ability to adaptively adjust offspring size and fecundity in fluctuating environments. Funct Ecol 25: 747-756.
Prud'homme B, Gompel N, Carroll SB 2007. Emerging principles of regulatory evolution. Proc Natl Acad Sci USA 104: 8605-8612.
Ramírez-Pinilla MP (2006) Placental transfer of nutrients during gestation in an Andean population of the highly matrotrophic lizard genus *Mabuya* (Squamata: Scincidae). Herpetol Monogr 20: 194-204.
Riesch R, Martin RA, Langerhans RB 2013. Predation's role in life-history evolution of a livebearing fish and a test of the Texler-DeAngelis model of maternal provisioning. Am Nat 181: 78-93.
Roti E, Fang SL, Green K, Emerson CH, Braverman LE 1981. Human placenta is an active site of thyroxine and 3,3',5-triiodothyronine tyrosyl ring deiodination. J Clin Endocr Metab 53: 498-501.
Samuel CA, Perry JS 1972. Ultrastructure of pig trophoblast transplanted to an ectopic site in uterine wall. J Anat 113: 139-149.
Sibley CP, Coan PM, Ferguson-Smith AC, Dean W, Hughes J, Smith P, Reik W, Burton GJ, Fowden AL, Constância M 2004. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. Proc Natl Acad Sci USA 101: 8204-8208.
Sinervo B 1990. The evolution of maternal investment in lizards: an experimental and comparative analysis of egg size and its effects on offspring performance. Evolution 44: 279-294.
Sinervo B, Licht P 1991. Hormonal and physiological control of clutch size, egg size, and egg shape in side-blotched lizards (*Uta stansburiana*): constraints on the evolution of lizard life histories. J Exp Zool 257: 252-264.
Stewart JR 2013. Fetal nutrition in lecithotrophic squamate reptiles: toward a comprehensive model for evolution of viviparity and placentation. J Morphol 274: 824-843.
Stewart JR, Ecay TW 2010. Patterns of maternal provision and embryonic mobilization of calcium in oviparous and viviparous squamate reptiles. Herpetol Conserv Biol 5: 341-359.
Stewart JR, Ecay TW, Heulin B, Fregoso SP, Linville BJ 2011. Developmental expression of calcium transport proteins in extraembryonic membranes of oviparous and viviparous *Zootoca vivipara* (Lacertilia, Lacertidae). J Exp Biol 214: 2999-3004.
Stewart JR, Thompson MB 1993. A novel pattern of embryonic nutrition in a viviparous reptile. J Evolution Biol 174: 97-108.
Swain R, Jones SM 2000. Facultative placentotrophy: half-way house or strategic solution? Comp Biochem Phys 127A: 441-451.

Thompson MB, Lindsay LA, Herbert JF, Murphy CR 2007. Calcium ATPase expression in the oviducts of the skink, *Lampropholis guichenoti*. Comp Biochem Phys A 147: 1090-1094.
Thompson MB, Speake BK 2006. A review of the evolution of viviparity in lizards: structure, function and physiology of the placenta. J Comp Physiol 176B: 179-189.
Thompson MB, Stewart JR, Speake BK 2000. Comparison of nutrient transport across the placenta of lizards differing in placental complexity. Comp Biochem Phys 127A: 469-479.
Thompson MB, Stewart JR, Speake BK, Russell KJ, McCartney RJ 1999a. Placental transfer of nutrients during gestation in the viviparous lizard, *Pseudemoia spenceri*. J Comp Physiol B 169B: 319-328.
Thompson MB, Stewart JR, Speake BK, Russell KJ, McCartney RJ, Surai PF 1999b. Placental nutrition in a viviparous lizard (*Pseudemoia pagenstecheri*) with a complex placenta. J Zool 248: 295-305.
Trexler JC, DeAngelis DL 2003. Resource allocation in offspring provisioning: an evaluation of the conditions favoring the evolution of matrotrophy. Am Nat 162: 574-585.
True JR, Carroll SB 2002. Gene co-option in physiological and morphological evolution. Annu Rev Cell Dev Biol 18: 53-80.
Van Dyke JU, Brandley MC, Thompson MB 2014a. The evolution of viviparity: molecular and genomic data from squamate reptiles advance understanding of live birth in amniotes. Reproduction 147: R15-R26.
Van Dyke JU, Griffith OW, Thompson MB 2014b. High food abundance permits the evolution of placentotrophy: evidence from a placental lizard, *Pseudemoia entrecasteauxii*. Am Nat 184: 198-210.
Vaupel P, Kallinowski F, Okunieff P 1989. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer Res 49: 6449-6465.
Whittington CM, Grau GE, Murphy CR, Thompson MB 2015. Unusual angiogenic factor plays a role in lizard pregnancy but is not unique to viviparity. J Exp Zool Part B: http://dx.doi.org/10.1002/jez.b.22615.
Wildman DE, Chen C, Erez O, Grossman LI, Goodman M, Romero R 2006. Evolution of the mammalian placenta revealed by phylogenetic analysis. Proc Natl Acad Sci USA 103: 3203-3208.
Wittkopp PJ, Kalay G 2012. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. Nat Rev Genet 13: 59-69.
Wolf E, Hoeflich A, Lahm H 1998. What is the function of IGF-II in postnatal life? Answers from transgenic mouse models. Growth Horm IGF Res 8: 185-193.
Wray GA 2007. The evolutionary significance of cis-regulatory mutations. Nat Rev Genet 8: 206-216.
Zhang JZ 2003. Evolution by gene duplication: an update. Trends Ecol Evol 18: 292-298.