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Emergence of an evolutionary innovation: Gene expression differences associated with the transition between oviparity and viviparity

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Original Article

Emergence of an evolutionary innovation: Gene expression differences associated with the transition between oviparity and viviparity

Short running title: Transcriptomics of bimodal reproduction

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Abstract

Our understanding of the evolution of complex biological traits is greatly advanced by examining taxa with intermediate phenotypes. The transition from oviparity (egg-laying) to viviparity (live-bearing) has occurred independently in many animal lineages, but there are few phenotypic intermediates. The lizard *Saiphos equalis* exhibits bimodal reproduction, with some viviparous populations, and other oviparous populations with long-egg-retention, a rare trait where most of embryonic development occurs inside the mother prior to late ovipositioning. We posit that oviparous *S. equalis* represent an intermediate form between "true" oviparity and viviparity. We used transcriptomics to compare uterine gene expression in these two phenotypes, and provide a molecular model for the genetic control and evolution of reproductive mode. Many genes are differentially expressed throughout the reproductive cycle of both phenotypes, which have clearly different gene expression profiles overall. The differentially expressed genes within oviparous and viviparous individuals have broadly similar biological functions putatively important for sustaining embryos, including uterine remodelling, respiratory gas and water exchange, and immune regulation. These functional similarities indicate either that long egg-retention is an exaptation for viviparity, or might reflect parallel evolution of similar gravidity-related changes in gene expression in long egg-retention oviparity. In contrast, gene expression changes across the reproductive cycle of long-egg-retaining oviparous *S. equalis* are dramatically different from those of "true" oviparous skinks (such as *Lampropholis guichenoti*), supporting our assertion that oviparous *S. equalis* exhibit an intermediate phenotype between "true" oviparity and viviparity.

39 **Keywords:**

40 Bimodal reproduction, evolutionary innovation, pregnancy, reproductive mode, transcriptomics,

41 complex traits

1. Introduction

Evolutionary innovations such as camouflage (Stevens and Merilaita 2008), flowers (Sauquet, et al. 2017), wings (Kingsolver and Koehl 1994), and live birth (viviparity) (Blackburn 2015a; Whittington, Griffith, et al. 2015) have allowed organisms to adapt to new environments and shaped their evolutionary trajectories. Determining how such complex traits have evolved is key to understanding the origins of biodiversity. However, in most cases, transitional forms between ancestral and derived states are not available for comparison in these analyses. Here, we use a putative intermediate form between the ancestral egg-laying state and the derived phenotype of live birth to examine the role of gene expression changes in evolutionary innovations.

Among animals with internal fertilisation, oviparous species lay eggs containing developing embryos, whilst viviparous species retain developing embryos internally before giving birth to live young (Blackburn 2015a). Viviparity has evolved from oviparity at least 150 times across a diverse array of vertebrates (Blackburn 2015a), including at least >121 independent origins of viviparity in squamate reptiles (Blackburn 2015a). Transcriptomic analyses of a pair of closely related agamid lizards that differ in parity mode (*Phrynocephalus przewalskii,* oviparous; *P. vlangalii,* viviparous) have been used to investigate the genetic control of viviparity (Gao, et al. 2019). Gene expression in the maternal uterus of both species showed temporal changes in gene expression across the reproductive cycle that reflect many of the morphological, immunological, and physiological differences between oviparous and viviparous species (Gao, et al. 2019). However, cross-species comparisons such as this one risk conflating parity mode-specific adaptation with species-specific adaptations that are unrelated to the evolution of viviparity (Garland Jr and Adolph 1994; Stewart, et al. 2010). Therefore, comparisons between individuals with different reproductive modes

within the same species have a better chance of identifying genetic changes specifically related to the evolution of viviparity.

Here, we examined the three-toed skink *Saiphos equalis* (Squamata: Scincidae), a bimodally reproductive species endemic to Australia with geographic variation in reproductive mode, including some populations that represent an intermediate form between "true" oviparity and viviparity (Smith and Shine 1997; Smith, et al. 2001) (Fig. 1). *Saiphos equalis* from some northern populations are viviparous and give birth to fully developed neonates encased in transparent membranes that are broken at or soon after birth (Smith and Shine 1997). Viviparous *S. equalis* have a simple placenta (Parker, et al. 2010), and therefore probably provide embryonic nutrition via the yolk rather than via substantial maternal provisioning during gravidity (Weekes 1935; Blackburn 2015a). *Saiphos equalis* from populations surrounding Sydney are oviparous, but with a phenotype that is atypical of other oviparous squamates. Most squamates lay eggs containing embryos about a third of the way through development (~stage 30 of 40 stages identified by Dufaure and Hubert (1961)) (Smith and Shine 1997). In contrast to the normal oviparous phenotype for squamates, oviparous *S. equalis* lay eggs with partially calcified eggshells (Stewart, et al. 2010) containing embryos that have completed most of their development (Stage 38-39) (Smith and Shine 1997). This phenotype is called long egg-retention, and the eggs are incubated for only a short time (mean 5.5 d) before hatching (Smith and Shine 1997; Smith, et al. 2001). Parity mode is heritable in this species (Smith 1996).

The intermediate form between "true" oviparity and viviparity exhibited by oviparous *S. equalis* makes this species a powerful model to investigate the transition to viviparity. Given that viviparity probably evolved relatively recently in *S. equalis* (Smith and Shine 1997), differences in behaviour, physiology, gestational tissue morphology or gene expression between populations are likely to be directly related to parity mode. Here, we used the first transcriptome for a bimodally

reproductive animal to compare changes in uterine gene expression in “transitional” oviparous and viviparous *S. equalis.*

2. Materials and Methods

2.1. Animal collection and processing

All animal work was conducted with prior approval from the University of Sydney Animal Ethics Committee (approval number S688; L04/10-2011/3/5607). We collected gravid *S. equalis* females from oviparous (putative transitional) populations in Sydney, NSW, Australia, and from viviparous populations in Mummel Gulf National Park, NSW, Australia (NSW government permit SL100401). We maintained these lizards in captivity according to standard protocols (Linville, et al. 2010) until they reached the desired stage of the reproductive cycle, after which they were then euthanised by injection with 0.1 mL of sodium pentobarbital (6 mg/mL) as previously described (Whittington, Grau, et al. 2015). For viviparous lizards, this corresponded to when embryos were at developmental stages 30–32.5 (early gravid, n=3) and 36–39.5 (late gravid, n=6), and for oviparous lizards stages 30.5 (early gravid, n=1) and 36.5–37 (late gravid, n=3), according to the embryonic staging system of Dufaure and Hubert (1961) (Fig. 2). We also obtained non-reproductive replicates by processing lizards 4–6 weeks after oviposition (n=3) and parturition (n=3). In all cases, uterine tissues were excised and fixed in RNAlater for 24 h at 4°C, then stored at –80 °C.

Total RNA was extracted from uterine tissues using an RNeasy mini kit (Qiagen, Hilden Germany), including an on-column DNAse digestion step (RNase-free DNase set, Qiagen). Following RNA quality assessment using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA), library preparation was conducted with a TruSeq mRNA non-stranded library preparation kit (llumina, San Diego, CA). All samples used for library preparation had an RNA

Integrity Number (RIN) >7.5. Finally, samples were sequenced on the HiSeq 2500 rapid run platform (llumina, San Diego, CA), with 100 bp paired-end sequencing at the Ramaciotti Centre for Genomics, Sydney, Australia.

2.2 Transcriptome assembly and annotation

We assembled a transcriptome for *S. equalis* using RNA-seq data from the uterus of all 19 replicates from both oviparous and viviparous populations throughout the reproductive cycle (Table S1). We sampled uteri containing embryos at both early and late stages of development (early and late-gravid), as well as empty, non-reproductive uteri (non-gravid). *De novo* transcriptome assembly was required because no reference genome is available for *S. equalis*. Prior to assembly, we ensured that the sequencing reads passed standard quality control measures, as assessed with FastQC (Andrews 2010). Residual sequencing adaptors were removed using the BBDuk tool of the BBTools suite (available from https://sourceforge.net/projects/bbmap/). To enable efficient transcriptome assembly, we also normalized the sequencing reads to an average depth of 50x using BBNorm (available from https://sourceforge.net/projects/bbmap/). Given the potential negative impacts on downstream analyses of transcriptomes that can arise from quality trimming (Williams, et al. 2016), we did not quality trim the sequencing reads. We carried out *de novo* assembly using Trinity v2.6.6 (Grabherr, et al. 2011), with all parameters set to default values except for the --no_normalize_reads flag. We assembled the transcriptome using all 278,526,490 normalized sequencing reads in a pooled approach, but maintained separate reads files for downstream expression analyses. We searched for highly conserved orthologues using two different methods of BUSCO v3 (Waterhouse, et al. 2017). Firstly, we included all isoforms per gene, and we then analysed only the longest peptide

sequences per gene that were identified using Transdecoder (available from https://github.com/TransDecoder/TransDecoder/releases).

We annotated the transcriptome using two different approaches. Firstly, we annotated transcripts and their predicted peptide sequences using best NCBI BLASTX and BLASTP (v2.7.1+) hits against a set of reference proteomes in a stepwise approach. The proteomes we used were from *Anolis carolinensis* (AnoCar2.0), *Pogona vitticeps* (GCA_900067755), *Gallus gallus* (GRCg6a), *Mus musculus* (GRCm38.p6), and *Homo sapiens* (GRCh38.p12). Secondly, we used the Trinotate v3.0.0 pipeline (Bryant, et al. 2017), which annotates transcripts based on best hits to the SwissProt database (The UniProt Consortium 2017), protein domain identification using HMMER (Eddy 2018) and PFam (Finn, et al. 2013), prediction of protein signal peptides and transmembrane domains using signalP (Petersen, et al. 2011) and tmHMM (Krogh, et al. 2001), and functional classification using eggNOG (Huerta-Cepas, et al. 2015), Gene Ontology (GO) (Ashburner, et al. 2000), and KEGG Orthology databases (Kanehisa and Goto 2000). Searches using BLAST and HMMER with an E-value ≤ 10^{-3} were considered to be significant.

2.3. Differential expression and functional analysis

We quantified gene expression for all 19 replicates separately by quasi-mapping sequencing reads to our *de novo* transcriptome using the alignment-free approach of Salmon v0.11.0 (Patro, et al. 2017). Transcriptomes assembled by Trinity contain contigs corresponding to different isoforms of genes; therefore, our quantification approach provides expression estimates at both the transcript and gene level. We focused at the level of genes for further analysis, which is appropriate in species lacking a high-quality reference genome (Soneson, et al. 2015).

Using our estimates of expression, we filtered out any genes that did not have sufficiently large counts in a meaningful number of samples using the filterByExpr function from the edgeR

package in R (Robinson, et al. 2010) with the default min.count of 10 and min.total.count of 15. We also used this function on separate counts matrices for viviparous and oviparous individuals in a preliminary search to identify any genes that are putatively uniquely expressed in either parity mode regardless of the stage of gravidity. We identified differential gene expression between non-gravid, early-gravid, and late-gravid oviparous and viviparous *S. equalis* using DESeq2 (Love, et al. 2014). We designed these contrasts to identify changes in gene expression between non-gravid and gravid individuals within each parity mode. This approach allowed us to determine whether oviparous and viviparous *S. equalis* differentially express similar or different genes to meet physiological needs related to embryonic survival. We also directly contrasted oviparous and viviparous *Saiphos* at equivalent reproductive stages (e.g., non-gravid oviparous vs non-gravid viviparous). The single early-gravid oviparous biological replicate was assigned an average dispersion value across all replicates for each gene, as described in the DESeq2 vignette. This approach has less statistical power to identify differentially expressed genes than when including biological replicates for both groups being compared (as we did in all other contrasts), but allowed us to include the early-gravid oviparous stage, without negatively impacting any contrasts not involving this stage. However, the broad similarity in transcriptomes between early gravid and late-gravid replicates led us to ultimately group these two stages together for interpretation of results (see "Results"). To consider genes as being differentially expressed, we used a threshold of an adjusted p-value (FDR) of 0.05 and a log fold change of 1. We also identified any genes that are exclusively differentially expressed in either oviparous or viviparous *S. equalis*.

We tested for functional enrichment of GO terms and KEGG pathways in differentially expressed genes using overrepresentation enrichment analysis in WebGestaltR 2017 (Wang, et al. 2017), with an FDR cutoff of 0.1. We carried out these enrichment analysis based on genes differentially expressed between equivalent stages of gravidity in oviparous and viviparous

individuals, between sequential stages of gravidity within each parity mode, and of genes uniquely differentially expressed within each stage of gravidity for each parity mode.

3. Results

3.1 Transcriptome annotation and analysis

Our assembled transcriptome for *S. equalis* contains 253,397 contigs with a median length of 549 bp, a 93.5 % read alignment rate, and an E90N50 of 2668 bp. When compared to the vertebrate reference lineage of BUSCO v3 (Waterhouse, et al. 2017), our transcriptome is 91.4 % complete (60.1 % duplicated; 4.4 % fragmented) based on analysis of all isoforms per gene, and 86.5 % complete (1.3 % duplicated; 4.3 % fragmented) based on only the longest peptide sequence per gene. Approximately 39.7 % of transcripts in the *S. equalis* transcriptome were successfully annotated based on best BLASTX hits to reference proteomes (Table S2). Similarly low annotation rates are common in *de novo* assemblies from non-model species (e.g., Schunter, et al. 2014; Whittington, Griffith, et al. 2015; Todd, et al. 2018). At the gene level, the *S. equalis* transcriptome mapped to 21,553 unique Ensembl IDs based on similarity to known genes in *A. carolinensis*, *G. gallus*, *M. musculus*, and *H. sapiens* proteomes, and the SwissProt database. Unmapped transcripts may represent non-coding RNAs, spuriously assembled transcripts, or novel genes.

3.2 Distinct gene expression profiles in oviparous and viviparous populations

Oviparous and viviparous *S. equalis* exhibit markedly different uterine gene expression profiles throughout the reproductive cycle. Preliminary data exploration using a filtering approach identified genes that are uniquely expressed in either parity mode at any stage of gravidity. In viviparous *S. equalis*, 36.8% of genes are uniquely expressed, whereas 13% are unique to

oviparous *S. equalis*. Principle components analysis (PCA) of gene expression clearly separates replicates by both parity mode (PC1 = 66 %), and by reproductive stage (gravid/not gravid) (PC2 = 13 %) (Fig. 3). However, early-gravid and late-gravid lizards are not clearly delimited as distinct groups by either component. Therefore, although we provide results for all contrasts between non-gravid, early gravid, and late-gravid groups (Table S3), we have considered early and late-gravidity as a single group in our further discussion of results.

Across differential expression contrasts between non-gravid and gravid *S. equalis*, there were a total of 13,741 differentially expressed genes (Fig. 4, Table S3), of which 27.3 % were annotated to 2,501 unique Ensembl IDs. We examined these differentially expressed genes for functional enrichment of GO terms and KEGG pathways, and a heatmap of the top 1000 most variable differentially expressed genes can be seen in Figure S1 . Genes upregulated during oviparous gravidity compared to the non-gravid stage were functionally enriched for 121 unique GO terms and six unique KEGG pathways, and genes upregulated during viviparous gravidity compared to the non-gravid stage were functionally enriched for 281 unique GO terms and 11 unique KEGG pathways (Tables S4–5). In both oviparous and viviparous *S. equalis*, these upregulated genes were functionally enriched for processes related to the functions of gravidity in squamates, including uterine remodelling and gas exchange (Fig. 5, Tables S4–5). Genes downregulated during oviparous gravidity compared to non-gravidity were not enriched for any GO terms or KEGG pathways. However, genes downregulated during late gravidity in viviparous *S. equalis* relative to non-gravidity were significantly enriched for 16 GO terms, including several terms related to uterine remodelling and regulation of uterine quiescence (Table S6). Differentially expressed genes between oviparous and viviparous *S. equalis* at equivalent reproductive stages were significantly enriched for 183 GO terms and 69 KEGG pathways (Tables S7–8), most of which relate to tissue remodelling processes or metabolism.

We also investigated whether the same or different genes are differentially expressed throughout the reproductive cycle in oviparous and viviparous *S. equalis.* We found that while 893 genes are commonly differentially expressed in both parity modes, a large number are exclusive to a single parity mode: 2278 genes are only differentially expressed during the viviparous reproductive cycle, and 2088 genes are only differentially expressed during the oviparous reproductive cycle (Fig. 6,: Table S9). Additionally, some differentially expressed genes show opposite trends in the the direction of differential expression during gravidity in oviparous and viviparous *S. equalis*. Relative to the non-gravid stage, 153 of the genes that are upregulated during viviparous gravidity are downregulated during oviparous gravidity, and 50 of the genes that are upregulated during viviparous gravidity are downregulated during oviparous gravidity (Fig. 6, Table S9). Functional enrichment results suggest broad functional similarities in the genes exclusively differentially expressed within either parity mode (Tables S10–11).

4. Discussion

Our transcriptome reveals that the evolution of distinct gene expression patterns underpins the evolution of viviparity in *S. equalis* (Figs 3–4). Genes are differentially expressed between gravid and non-gravid uteri in both oviparous long egg-retaining and viviparous *S. equalis*, which indicates dynamic gene regulation to sustain developing embryos. Unfortunately, many highly differentially expressed genes were unannotated, which precludes us from speculating about their functions, although they presumably underpin important uterine processes. Further improvements in our understanding of the evolution of viviparity in *S. equalis* will come through functional studies to understand gene function and interactions, which would be assisted by a genome sequence for this species.

Direct contrasts between gravid viviparous and gravid oviparous individuals revealed 7,210 unique differentially expressed genes. In general, functional differences between equivalent gravid stages of oviparous and viviparous *S. equalis* are related to a variety of physiological functions, including the regulation of metabolism-related genes, enzymes likely involved in uterine remodelling, and genes driving the transport of organic and inorganic ions (Tables S7–8). Our results support previous conclusions that the evolution of viviparity involves the evolution of a distinct "viviparity" gene expression profile associated with gravidity (Griffith, et al. 2016; Gao, et al. 2019). It is also important to consider that non-reproductive female *S. equalis* with different parity modes differ in uterine gene expression patterns, with 3264 genes differentially expressed between oviparous and viviparous *S. equalis* even in the absence of an embryo. The differentially expressed genes between non-gravid oviparous and non-gravid viviparous *S. equalis* are largely involved in processes related to normal organismal function, such as metabolism. This finding suggests that the non-reproductive "resting state" differs between oviparous and viviparous populations, even though the uterine tissues are likely quiescent during this period. These genetic differences align with differences in non-gravid uterine morphology between oviparous and viviparous *S. equalis*: there is a relative absence of shell glands and a thinner uterine epithelium in viviparous individuals (Stewart, et al. 2010). Our transcriptome results suggest that there may be additional differences at the molecular level.

4.1 Similar changes in uterine gene expression throughout the reproductive cycle within egg-layers and live-bearers

Genes upregulated in gravidity within both oviparous and viviparous *S. equalis* are enriched for GO terms and KEGG pathways related to uterine remodelling, including extracellular structure organization, proteolysis, and peptidase activity (Tables S4–5). The genes related to uterine

remodelling in both oviparous and viviparous *S. equalis* are similar to those seen in other viviparous skinks and mammals (Salamonsen and Nie 2002; Brandley, et al. 2012; Gao, et al. 2019). Out of all upregulated genes in gravid *S. equalis* uterus relative to non-gravid uterus, peptidases, proteases and their inhibitors have some of the highest log2-fold changes throughout the reproductive cycle, which indicates dynamic regulation of the proteolysis process during the reproductive cycle. These highly differentially expressed genes include *SPINK5*, *SPINK12* and several unnamed genes with homology to Kunitz-type serine protease inhibitor proteins, similar to changes that occur in the gravid viviparous skink *Chalcides ocellatus* (Brandley, et al. 2012). Both peptidases and proteases facilitate the breakdown of tissues and the extracellular matrix, and, in tandem with their inhibitors, are important for placental and uterine remodelling during pregnancy in other species (McDonald, et al. 1987; Luck and Zhao 1995; Nie, et al. 2003; Chern, et al. 2010).

Other genes differentially expressed in a similar manner throughout the reproductive cycle in both parity modes are related to the transport of respiratory gases and water (Tables S4–5). Genes associated with increased vasculogenesis and angiogenesis assist in meeting embryonic oxygen demands throughout gestation by manipulating blood vessel diameter, or by increasing the number of blood vessels within uterine tissue (Carter 2000; Andrews 2002; Parker, et al. 2010). The *S. equalis* transcriptome contains several vascular endothelial growth factors (VEGFs) (e.g. *VEGFA*, *VEGFC*, *VEGFD*, *VEGFF*, *VEGFRKDRL*). Two of these genes were upregulated in gravidity in *S. equalis* relative to the non-gravid stage (oviparous: *VEGFA*; viviparous: *VEGFRKDRL*), in line with previous qPCR results (Murphy, et al. 2010; Whittington, Grau, et al. 2015). VEGFs are important inducers of angiogenesis through mitogenic activation of endothelial cells (Hoeben, et al. 2004). Increased expression of putative angiogenic and vasculogenic genes during oviparous gravidity relative to the non-gravid stage supports previous morphological work showing increases

295 in uterine vascular density during late gestation in *S. equalis* (Parker, et al. 2010). Other genes
296 upregulated in both oviparous and viviparous gravidity relative to the non-gravid stage are
297 significantly enriched for maintaining water homeostasis, and include several putative aquaporins
298 (*AQP1*, *AQP3*, *AQP12*), which are water channel proteins that play a major role in maintaining the
299 water permeability of cells in animals (Borgnia, et al. 1999).

300 Regulatory processes and homeostasis are also maintained through differential gene
301 expression throughout the reproductive cycle of *S. equalis* (Tables S4–5), and likely represent
302 homeostatic functions shared by both parity modes, such as balancing concentrations of cations,
303 anions, water, and pH. Many genes upregulated in gravid lizards of both parity modes have
304 predicted signal peptides without a predicted downstream transmembrane helix (Table S12),
305 which suggests that these genes are part of secretory pathways rather than coding for membrane-
306 bound proteins (Nielsen 2017). Several of these secretory genes are likely related to the
307 production of peptide hormones to regulate gas exchange, such as arginine vasopressin (*AVPR1A*)
308 and angiotensin (*ACE*) (Roks, et al. 2011) (Table S12).

309 We also recovered evidence of dynamic regulation of genes associated with
310 immunocompetence in both parity modes (Table S13). These genes include complement
311 component (*C3, C9*) genes and genes relating to major histocompatibility complex loci (e.g., *H2-*
312 *EA*), which are differentially regulated throughout the reproductive cycle relative to the non-gravid
313 stage in both oviparous and viviparous *S. equalis* and *C. ocellatus* (Brandley, et al. 2012). The
314 majority of differentially regulated immune genes putatively code for proteins with
315 immunoglobulin receptor binding functions (Table S13). Immunoglobulins are important for
316 antigen recognition and binding (Olivieri, et al. 2016), and prevent rejection of the eutherian fetus
317 by the maternal innate immune system (Alijotas-Reig, et al. 2014). Differential expression of these
318 immune-related genes likely reflects a common need in both long egg-retaining oviparous and

viviparous *S. equalis* to avoid maternal rejection of embryos during prolonged embryo retention (Carter 2012; Van Dyke, et al. 2014). In general, genes downregulated in gravid stages relative to non-gravid stages in both oviparous and viviparous *S. equalis* are related to immunocompetence, or to regulation of uterine quiescence (Table S3; Table S13).

We also investigated the functions of genes that are differentially expressed throughout the reproductive cycle only in oviparous *S. equalis* or viviparous *S. equalis*. Both sets of uniquely differentially expressed genes seem to be important for embryonic development (Tables S10–11), as they are putatively involved in tissue remodelling, biological regulation, angiogenesis, immune regulation, and transport. Thus, oviparous and viviparous *S. equalis* differentially regulate many different genes throughout the reproductive cycle to achieve similar physiological outcomes to support developing embryos.

Collectively, many of the putative genes supporting internal gestation of embryos in both long egg-retaining oviparous and viviparous *S. equalis* are similar to those found in other viviparous squamates and mammals (Salamonsen and Nie 2002; Brandley, et al. 2012; Hendrawan, et al. 2017; Gao, et al. 2019). A key strength of our within-species study is that any similarities or differences in gene expression between oviparous and viviparous *S. equalis* are more likely to be directly associated with parity mode than in a cross-species comparison.

4.2 Differences in uterine gene expression throughout the reproductive cycle within egg-layers and live-bearers

We observed differences in the regulation of genes related to adhesion throughout the reproductive cycle in viviparous and oviparous *S. equalis*. GO term enrichment indicates that many genes related to the regulation of adhesion are upregulated during gravidity in viviparous *S. equalis* relative to the non-gravid stage (Tables S4–5), and the focal adhesion KEGG pathway is also

342 enriched. The regulation of cell-cell adhesion is important during viviparous gravidity (Murphy, et
343 al. 2000; Wu, et al. 2011; Van Dyke, et al. 2014), and potentially aids in controlling paracellular
344 transport of molecules between mothers and embryos (Biazik, et al. 2010). Study of the regulation
345 of adhesion between uterine cells and paracellular permeability in squamates has focused on
346 proteins including occludins and claudins (Van Dyke, et al. 2014). Occludin and claudins-1, -2, -3,
347 and -4 were not detected in uterine tissue of oviparous or viviparous *S. equalis* using
348 immunofluroescence microscopy, but claudin-5 was (Biazik, et al. 2007, 2008). In contrast, we
349 detected expression of occludin in all replicates of *S. equalis*, albeit at low levels (<5 TPM).
350 Occludin was upregulated throughout gravidity in oviparous replicates relative to the non-gravid
351 stage, but not in viviparous replicates. We also detected thirteen different claudins in the *S.*
352 *equalis* transcriptome (*CLDN1*, *CLDN3*, *CLDN4*, *CLDN5*, *CLDN7*, *CLDN9*, *CLDN10*, *CLDN11*, *CLDN12*,
353 *CLDN15*, *CLDN16*, *CLDN20*, *CLDN23*), with expression ranging from very low (<5 TPM) to high
354 (>100 TPM). However, the enrichment of adhesion-related terms in genes upregulated in gravid
355 viviparous *S. equalis* relative to the non-gravid stage is driven by genes other than claudins (Tables
356 S4–5), many of which are important for cell-cell signalling and paracellular transport during
357 adhesion. Dynamic regulation of many of these adhesion genes throughout the reproductive cycle
358 may be important for maintenance of maternal-fetal signalling and paracellular transport during
359 the late gravid stage in viviparity.

360 Many genes associated with calcium transport, including calbindin and several Ca^{2+}-
361 ATPases, are upregulated during the reproductive cycle in viviparous *S. equalis* relative to the non-
362 gravid stage, but not in oviparous *S. equalis* (Tables S4–5). Calbindin-D_{28K} and Ca^{2+}ATPase are
363 associated with embryonic calcium uptake in many snake species, including the viviparous snake
364 *Virginia striatula* (Fregoso, et al. 2012). Other genes driving the enrichment of GO terms related to
365 calcium provision in *S. equalis* include genes that regulate calcium ion transmembrane transport

366 and calcium ion homeostasis (*ADRB1*, *ATP2B2*, *EDNRA*, *FKBP1B*, *ITGB3*, *BHLHA15*, *SLC25A23*,
367 *MCOLN2*, *TMCO1*, *MCOLN3*, *ATP13A4*, *ATP2B3*).

368 In *S. equalis*, embryos of both viviparous and oviparous mothers have access to calcium
369 reserves in yolk, but embryos of oviparous mothers have access to calcified eggshells for an
370 additional calcium source (Linville, et al. 2010). Our results suggest that viviparous *S. equalis* make
371 up the deficit in embryonic calcium supply through active transport of calcium from the mother to
372 developing embryos, a conclusion also reached through analysis of the calcium content of
373 eggshells, yolks, and embryos (Linville, et al. 2010). Calcium reserves in yolk can be sufficient for
374 successful development in squamates, but supplementing this source with eggshell calcium
375 increases hatchling mass (Linville, et al. 2010; Stewart, et al. 2019). Therefore, maternal calcium
376 provision might allow viviparous *S. equalis* to make up the deficit in calcium availability relative to
377 oviparous individuals, and also prevent reduced fitness in viviparous embryos caused by a lack of
378 eggshell calcium. Two GO terms associated with calcium are enriched in genes upregulated in
379 oviparous gravidity in *S. equalis* relative to the non-gravid stage (Table S4), but most genes driving
380 this enrichment are associated with regular cellular responses to calcium, rather than calcium
381 transport. Several genes related to calcium transport, including *ATP2C2* and *ATP13A4*, are also
382 significantly upregulated in gravid viviparous *S. equalis* relative to late gravid oviparous *S. equalis.*
383 Collectively, an increased provision of calcium to embryos is important during development in
384 viviparous *S. equalis*, but not in oviparous *S. equalis*.

385 Further clues to the potential to transition between parity modes might come from
386 focusing on genes that are differentially expressed only in one parity mode or the other, or genes
387 that show opposite trends in the direction of expression throughout gravidity in both parity
388 modes. We identified many genes that fall into these categories (Fig. 6, Table S9). Genes
389 exclusively upregulated in oviparous gravidity are related to processes likely important for normal

uterine homeostatic functions, whereas genes exclusively upregulated in viviparous gravidity can be clearly linked to processes including respiratory gas exchange, response to hormones, and dynamic immune regulation (Table S10). Unfortunately, many genes that show opposite trends in the direction of expression throughout gravidity in both parity modes currently lack annotation, which hinders efforts to determine some of the key differences between oviparous and viviparous *S. equalis*. Those genes in this category that do have annotation are putatively related to uterine remodelling processes (upregulated in viviparous gravidity; downregulated in oviparous gravidity) and dynamic immune regulation (different immune genes up/downregulated in either parity mode) (Table S9). Collectively, our results suggest that a transition between long egg-retention oviparity and viviparity would require prolonged expression of genes necessary for sustaining a developing embryo for the final stages of development.

4.3 Long egg-retention as a transitional form between oviparity and viviparity

Unlike oviparous *S. equalis*, typical oviparous squamates lay eggs a third of the way through embryonic development. In the typical oviparous skinks that have been investigated so far, there are very few differentially expressed genes between gravid and non-gravid uteri: *Lampropholis guichenoti* differentially expresses only two genes, and oviparous *Lerista bougainvillii* has no differentially expressed genes across the reproductive cycle (Griffith, et al. 2016). These species are representatives of two of the major lineages of Australian skinks that contain oviparous members (*Eugongylus* and *Sphenomorphus* groups, respectively), suggesting that this pattern of gene expression (i.e. few differences between non-gravid and gravid uteri) may be the ancestral condition of oviparous skinks. Interestingly, data from oviparous agamid lizards (*Phrynocephalus przewalskii*) show extensive differential expression between gravid and non-gravid uteri (Gao, et al. 2019), suggesting that differences in gene expression during the reproductive cycle is not the

413 case in all oviparous lizards from different lineages; more comparisons are needed to test this
414 hypothesis. In contrast to other oviparous skinks, however, almost 3000 genes are differentially
415 expressed in the uterus of non-gravid and gravid oviparous *S. equalis*, which is comparable to the
416 number differentially expressed in the uterus of viviparous *S. equalis.* The distinct gene expression
417 profiles between uteri with and without embryos likely reflects the fact that the long egg-
418 retention oviparity exhibited by these skinks is a derived form of oviparous reproduction.
419 We postulate that carrying embryos to a very late stage in oviparous *S. equalis* has resulted
420 in changes in the expression of genes associated with functions thought to be important for the
421 evolution of viviparity (Packard, et al. 1977; Guillette 1993; Blackburn 2006; Thompson and Speake
422 2006; Shine 2014; Van Dyke, et al. 2014). The strong dynamic immune response of oviparous *S.*
423 *equalis*, for example, is a clear outlier among transcriptomes of oviparous squamates (Griffith, et
424 al. 2016; Gao, et al. 2019). One of the key steps in the evolution of squamate viviparity is the
425 gradual thinning and loss of a calcified eggshell (Blackburn 2006). In contrast to squamates
426 exhibiting typical oviparity, oviparous *S. equalis* might require strong immune regulation because
427 their eggs have thin, minimally calcified shells and prolonged contact with the uterus until
428 embryos are nearly completely developed (Smith and Shine 1997; Stewart, et al. 2010). The
429 increased expression of genes related to respiratory gas exchange throughout the reproductive
430 cycle in oviparous *S. equalis* is also of particular note. The oxygen demands of embryonic lizards
431 are very low until approximately embryonic stage 30, roughly the stage when most lizard oviposit
432 (Shine and Thompson 2006). Oxygen demands rise exponentially throughout later stages of
433 development (Thompson and Stewart 1997), which requires much more maternal provision of
434 oxygen and removal of CO_2 than in typical oviparous squamates. Maternal expression of genes
435 driving gas exchange with embryos might even need to be higher in a long-egg retaining oviparous
436 species than viviparous species, given the resistance to diffusion of O2 and CO2 across an eggshell

(Deeming and Thompson 1991). Collectively, our results suggest that the dynamic regulation of genes responsible for key requirements of uterine support of embryonic development evolves during the transition to viviparity.

4.4 Could reproductive mode be more labile than previously suspected?

The similarities in differentially expressed gene function between oviparous and viviparous *S. equalis* may provide a molecular explanation for why facultative oviparity may be possible in this species. In the first report of a mixed reproductive mode within a vertebrate clutch, one female from a viviparous population of *S. equalis* laid three eggs (at stage 33, close to the typical stage of oviparous lizards), and then gave birth to a live neonate from the same pregnancy (Laird, et al. 2019). This observation suggested that there may be fewer physiological barriers to transitions between parity modes than previously thought, an assertion that is supported by our gene expression data, in which oviparous and viviparous individuals expressed genes with many of the same functions during the reproductive cycle.

Combined, the apparent facultative oviparity within an individual, and the similarities in differentially expressed genes between parity modes in *S. equalis* suggest that reproductive mode is relatively labile in this species. Whether "reversions" from viviparity back to oviparity are possible is the subject of much current research (Lynch and Wagner 2010; Fenwick, et al. 2012; Pyron and Burbrink 2014; Blackburn 2015b; Griffith, et al. 2015; Esquerré, et al. 2019). Comparisons between closely related oviparous-viviparous agamid reptiles suggest that reversals from viviparity to oviparity might not be as difficult to achieve as previously thought (Gao, et al. 2019) because of the lack of evidence for positive selection on any genes with a clear reproductive role on the viviparous branch of the phylogeny. Differences in parity mode between *Phrynocephalus* spp. seem to be due to changes in gene expression, rather than extensive substitutional changes (Gao, et al. 2019). However, other studies identifying large differences in

gene expression between typical oviparous skinks and viviparous skinks of different genera (Griffith, et al. 2016) suggest that reversions are precluded in lineages in which viviparity evolved in the distant past. Our data suggest that reproductive mode could be a relatively labile trait in cases where viviparity has evolved recently from long-egg retention, although the difficulties of re-evolving egg-shelling machinery cannot be discounted (Griffith, et al. 2015).

4.5 Concluding remarks

Oviparous and viviparous *S. equalis* have distinct gene expression profiles across the reproductive cycle (Fig. 3), although the different genes have putatively similar functions (Tables S4–5). Our results suggest that different populations of *S. equalis* are divergent at the uterine gene expression level. Assembling a *S. equalis* genome will help identify the regulatory elements driving these different patterns of gene expression (Shlyueva, et al. 2014), and, therefore, further explain the molecular mechanisms underpinning uterine function in oviparity and viviparity.

Many *S. equalis* genes are exclusively differentially expressed within a single parity mode. Viviparous lizards upregulate more genes that contribute to important biological processes for gravidity than do oviparous individuals. Nevertheless, there are genetic changes common to both viviparous and oviparous *S. equalis,* including uterine remodelling and the transport of respiratory gases and water, probably because oviparous members incubate developing embryos internally for an unusually long period. These similarities, including in expression of genes related to dynamic immune regulation and respiratory gas exchange, (a) suggest that there are common physiological requirements of sustaining a developing embryo in both long egg-retention and viviparity, (b) may partially explain why it was possible for a viviparous *S. equalis* mother to lay eggs (Laird et al., 2019), and (c) suggest that reversals from recent origins of viviparity back to oviparity might be easier than previously thought. The large number of genes differentially expressed throughout gravidity in oviparous *S. equalis* are dramatically different to the gene

485 expression profiles of typical oviparous skinks. Therefore, the long egg-retention of oviparous *S.*
486 *equalis* probably represents a transitional form between "true" oviparity and viviparity.
487 Since viviparous *S. equalis* were sourced from a northern population and oviparous *S.*
488 *equalis* were sourced from a southern population, reproductive mode and geographic location are
489 confounded within our study. As a result, it is not currently possible to completely disentangle
490 differences in gene expression that are caused by local adaptation to environment from those that
491 are due to differences in reproductive mode. However, there are no existing data that would
492 determine whether there are barriers to gene flow in *S. equalis.* Further landscape genetic studies
493 are needed to determine any possible population-level genetic structuring in *S. equalis*. Future
494 investigations into gene expression throughout gravidity in *S. equalis* should sample from multiple
495 populations per parity mode if they are found to exist.
496 While comparisons of taxa exhibiting ancestral and derived states demonstrate key
497 differences and similarities between traits, they do not directly address changes that occur during
498 the transition to the derived state. Overall, our study emphasises the benefits of studying
499 intermediate phenotypes when investigating evolutionary innovations. Examining taxa with
500 intermediate phenotypes, such as the long-egg retention of oviparous *S. equalis* here, allows us to
501 explore both the key steps in the evolution of new phenotypes, and the potential for reversibility
502 of such traits.

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Data Accessibility

All sequence data have been uploaded to NCBI's GenBank Sequence Read Archive under accession number PRJNA533161. The datasets supporting the conclusions of this article are included within the article and its supporting information. Table S2 is available from Figshare: 10.6084/m9.figshare.8286008

Authors contribution

The study was conceived by MCB and MBT, with components of the study design contributed by CSPF and CMW. Wet lab work was carried out by MCB and CMW. CSPF performed all bioinformatics analyses and wrote the initial manuscript. All authors contributed to drafts of the manuscripts, and read and approved the final manuscript.

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Figure Legends

Figure 1: *Saiphos equalis* and its distribution within Australia. (a) photograph of *S. equalis* from an oviparous population in the Sydney region (credit: Nicholas Wu); (b) the distribution of *S. equalis* throughout eastern Australia, with the oviparous population from this study marked with a diamond, and the viviparous population marked with a star.

Figure 2: Sampling scheme for uterine tissue collection from different stages of the reproductive cycle of *Saiphos equalis*. 'Stage' refers to stage of embryonic development *sensu* Dufaure and Hubert (1961), with stage 0 representing fertilisation and with embryonic development being complete at stage 40.

Figure 3: Principal components analysis (PCA) of cross-sample normalized expression values for oviparous and viviparous *Saiphos equalis* throughout the reproductive cycle. Sample clusters are delimited by parity mode (PC1), and stage of gravidity (PC2). Abbreviations: VNG = viviparous non-gravid; VEG = viviparous early-gravid; VLG = viviparous late gravid; ONG = oviparous non-gravid; OEG = oviparous early-gravid; OLG = oviparous late gravid.

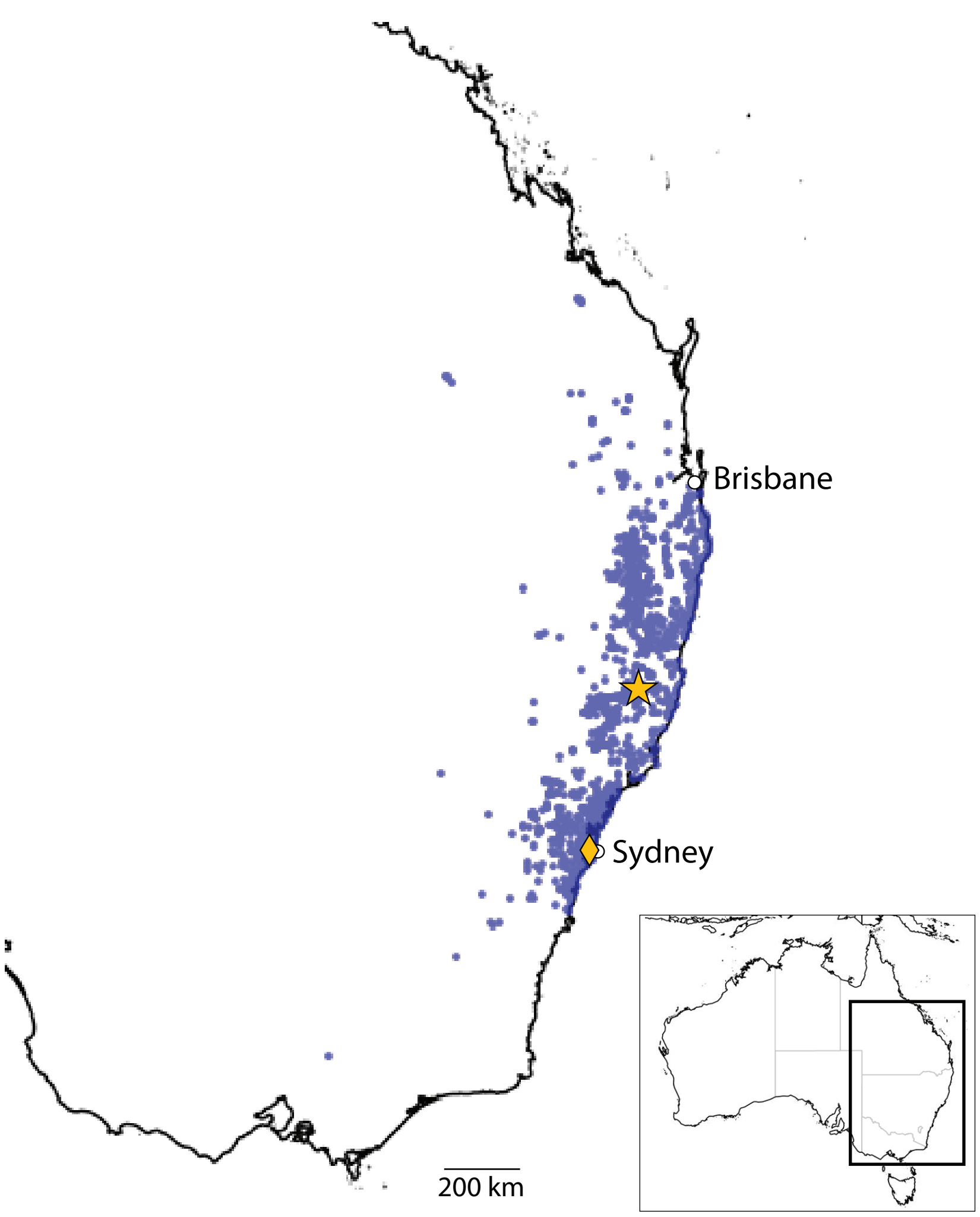
Figure 4: Numbers of differentially expressed genes in pairwise comparisons of gravidity stages in *Saiphos equalis*. Upregulation/downregulation refers to the second reproductive stage for each comparison. Abbreviations: VNG = viviparous non-gravid; VG = viviparous gravid; ONG = oviparous non-gravid; OG = oviparous gravid; OLG = oviparous late gravid.

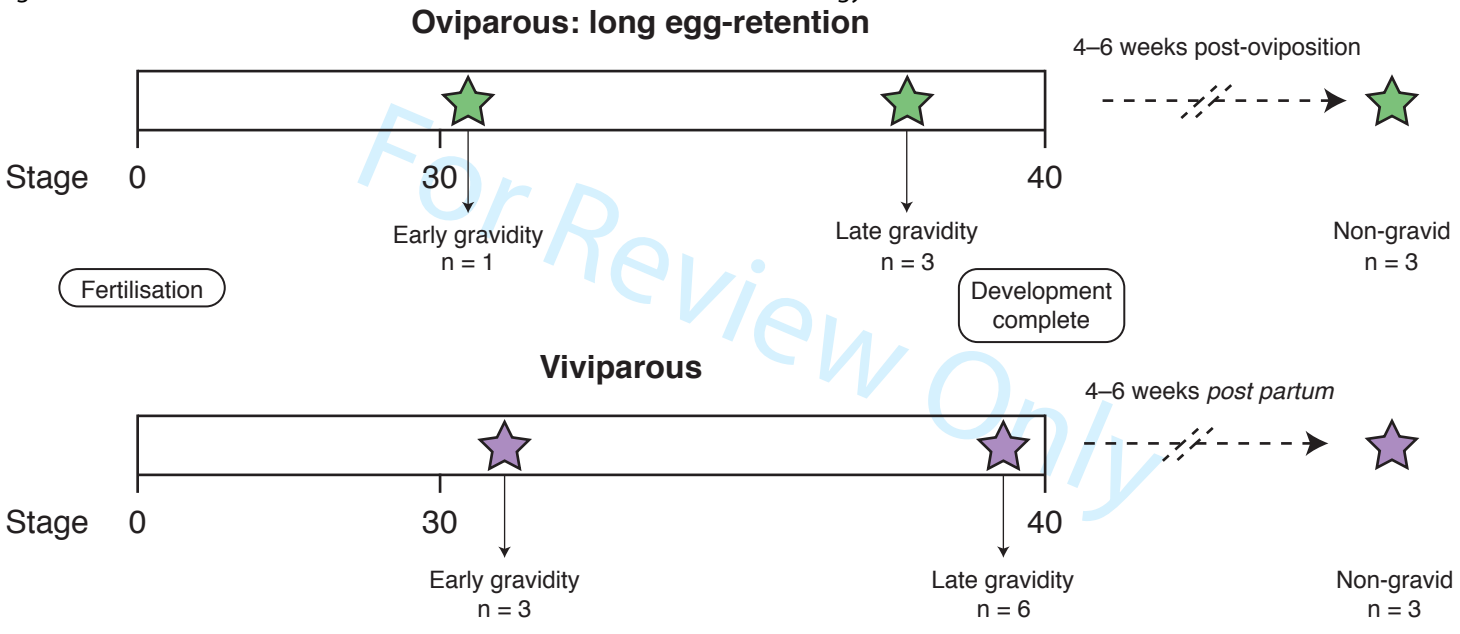
Figure 5: Enrichment map plot of the top 30 biological process GO terms enriched in genes upregulated in gravid stages relative to non-gravid stages in *Saiphos equalis*. Enriched GO terms that are driven by overlapping gene sets are connected by edges, as plotted in the clusterProfiler R package (Yu et al., 2012). **a** Genes upregulated throughout the reproductive cycle in viviparous *S. equalis* are enriched for many GO terms likely related to uterine remodelling, as well as many GO terms related to transport. **b** Genes upregulated throughout the reproductive cycle in oviparous *S. equalis* are similarly enriched for many GO terms related to uterine remodelling. The tight cluster of edge-linked GO terms related to uterine remodelling (functions including peptidase activity, proteolysis) shows that very similar gene sets are driving the enrichment of these GO terms.

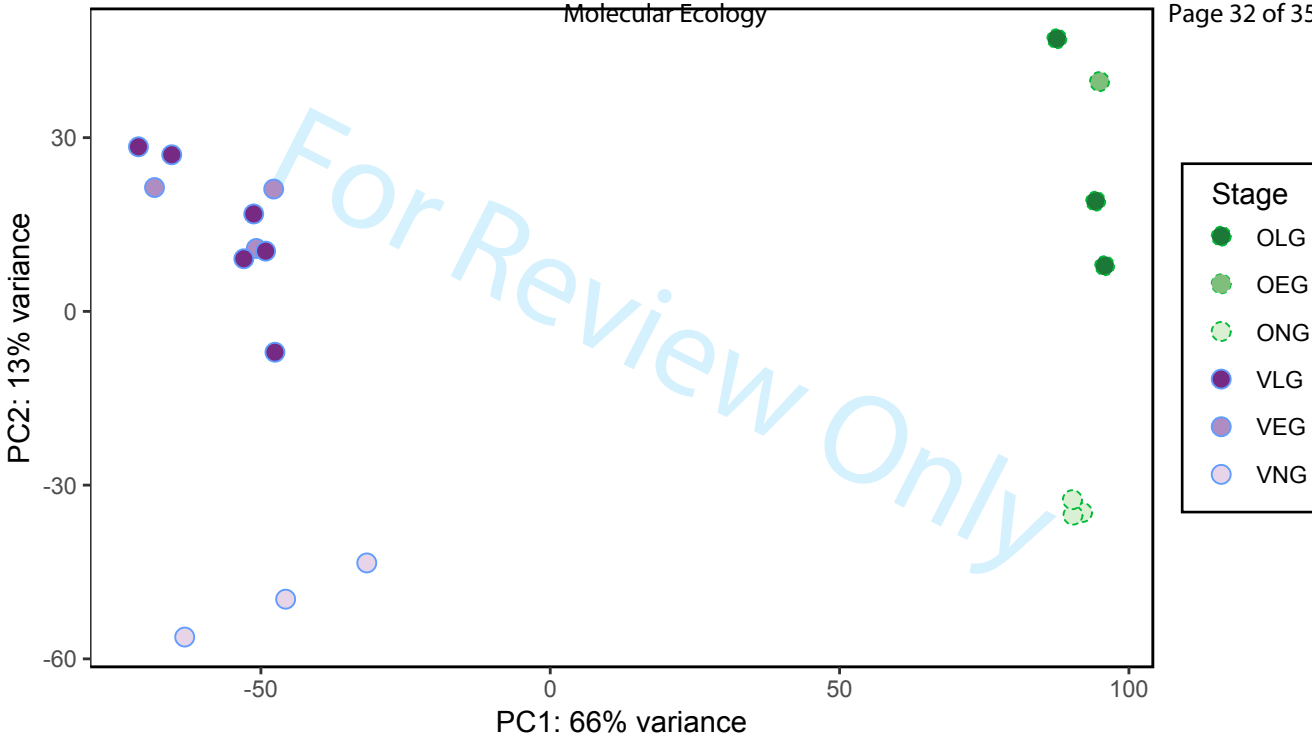
Figure 6: Venn diagram depicting the overlap in patterns of genes that are exclusively differentially regulated throughout gravidity in either oviparous or viviparous *Saiphos equalis.*

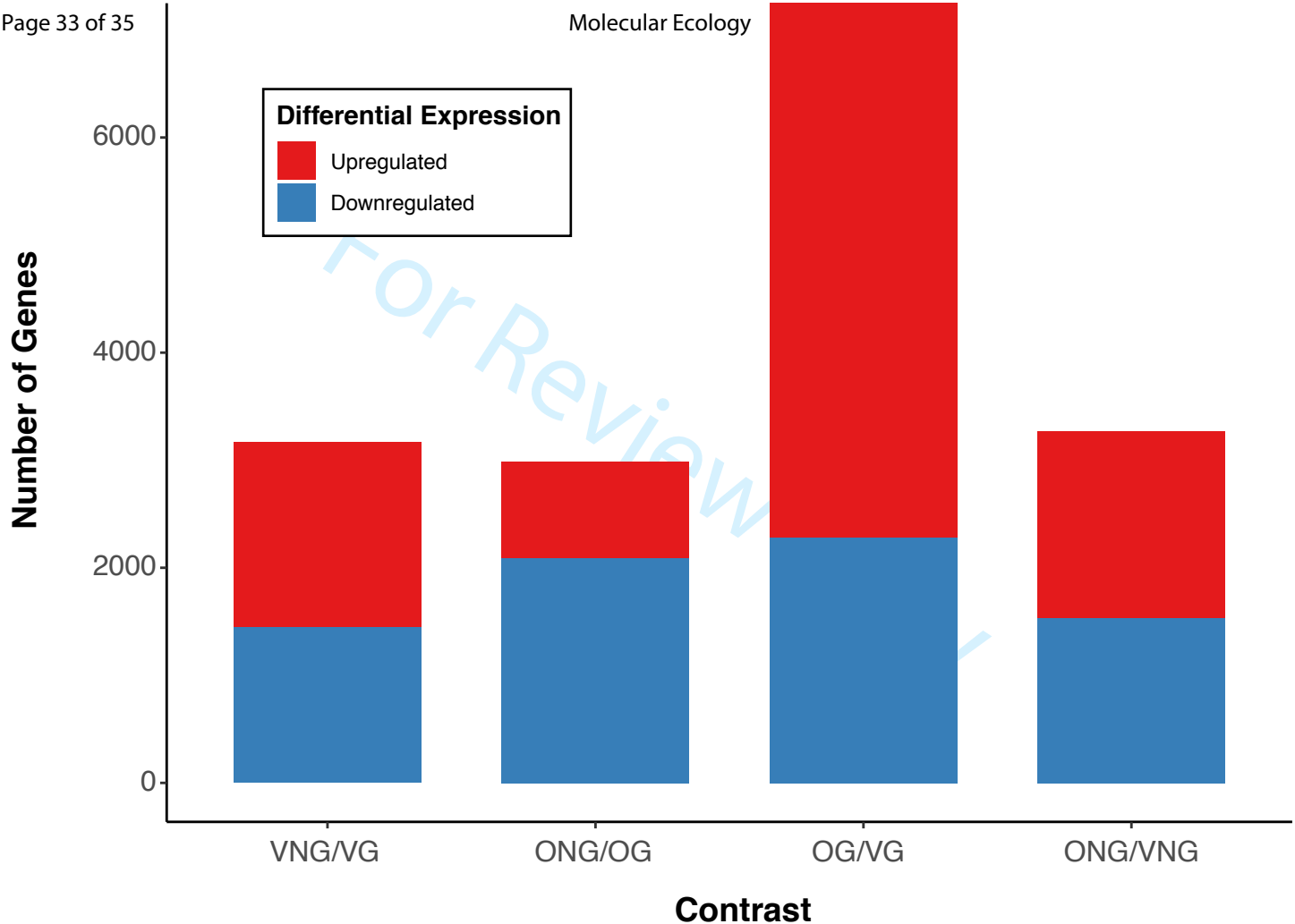
(a)

(b)








Differential Expression
Upregulated
Downregulated
Number of Genes
6000
4000
2000
0
VNG/VG
ONG/OG
OG/VG
ONG/VNG
Contrast