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The Genetic and Cellular Basis of Viviparity

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BSc MSc

Submitted in fulfilment of the requirements for the
Degree of Doctor of Philosophy

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Abstract

Reproductive mode is an important topic in evolutionary biology, as the evolution of viviparity is a major evolutionary transition that has evolved repeatedly in different animal groups. Squamate reptiles are recognised as excellent models for the evolution of viviparity, with over 100 such transitions known from this class of animals, representing a broad range of phenotypes on the oviparity-viviparity continuum, particularly in the case of reproductively bimodal species such as the Eurasian common lizard *Zootoca vivipara*. In this thesis, I present the results of a series of experiments building on and expanding the existing body of work using *Z. vivipara* as a model system. In **Chapter 1** I present a review of the current state of the literature on the evolution of oviparity-viviparity transitions in squamate reptiles across every major squamate group, and discuss the status of *Z. vivipara* as an emerging model organism while summarising previous work on reproductive mode in this and other squamate species. In **Chapters 2-3** I present the results of two new sequencing experiments designed to investigate the uterine transcriptomic changes which accompany pregnancy in viviparous *Z. vivipara*, describing changes in gene expression and alternative-splicing of genes before, during and after pregnancy, as well as exploring gene expression of known viviparity-related genes at the cellular level. In **Chapter 4** I then present a new model for the investigation of reproductive mode in the form of cultured *Z. vivipara* oviduct cells, and characterise and evaluate this system as a potential tool for future research. Finally in **Chapter 5** I discuss the potential for these results to enable a new program of research for the functional validation of candidate genes linked to viviparity, in a new reverse-genetic paradigm for reproductive mode research.

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Additional tables for all significant DEGs and GO terms for **Chapter 4** can be downloaded at the following URL: chapter_4_full_tables.xlsx

Note: sequence read files are permanently archived at the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA) under Bioproject ID PRJNA1069638. All other material listed above is permanently archived on Enlighten: http://dx.doi.org/10.5525/gla.researchdata.1933.

Acknowledgement

I would like to sincerely thank my supervisors Prof Kathryn Elmer, Dr Mark McLaughlin and Prof Maureen Bain for their invaluable support and guidance throughout my time working on this project. Additional thanks are due to Prof Tyler Stevenson for his own and his lab group's assistance with sequencing experiments. I also extend my gratitude to Prof Neil Evans and Dr Lucy Gilbert for pastoral support and advice throughout this challenging few years.

In particular, heartfelt thanks go out to those who assisted with fieldwork throughout the project, without whom none of the experiments described here would have been possible: Darren Hunter for his patient good humour throughout the ups and downs of a long field season; Paula Nagl, Lewis Heriot, Hamish MacDonald, Claudia Acerini, Dr Morgane Bonade, Molly Uzzell, Nic Strowbridge, Dr Bethany Smith, Kiefer Elmer and others for assistance with lizard collecting; Andries van der Haar for his hospitality and assistance at AlpenferienPark Reisach; and Hans Recknagel for invaluable training in the art and science of lizard catching.

This project would not have been feasible without the help and advice of many colleagues and friends within the School of Biodiversity, One Health and Veterinary Medicine and the wider university community. Particular thanks are due to Dr Lauren Black for her patient tuition in the fundamental techniques of tissue culture; to Dr Callum Smith, Dr Hongxin Xie and Dr Li Chao for critical help with sequencing experiments and subsequent analysis; to Dr Arne Jacobs for guidance and advice on alternative splicing and GO-term analysis; to Jenna Nichols, Maria Capstick and Dr Ana Monteiro for their advice, assistance and supervision in laboratory experiments; and to Julie Galbraith, Dr Pawel Herzyk and the team at Glasgow Polyomics as well as Oliver Gibson, Vicky Morrison and other employees at Biomage for their work on single-cell sequencing experiments; to Kate Griffiths and Lorna Kennedy for their tireless work supporting myself and all the students and staff at the Graham Kerr Building with all manner of administrative matters; and many other members of the university community for their kindness and friendship over the last four years.

Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Printed Name: John Laurence Smout

Signature: _____

1 Introduction

1.1 Abstract

Reproduction is fundamental to biology, and the differences in reproductive mode between viviparous and oviparous animals have long interested scientists from a range of disciplines. I first present a review of oviparity-viviparity transitions in amniotes to give context to my own investigation of the molecular and cellular basis of viviparity. I then discuss the specific history of the Eurasian common lizard *Z. vivipara* as a model system for researchers working on reproductive mode, from the first description of viviparity in this species to the investigation of various anatomical, physiological, ecological, life-history and evolutionary questions relating to viviparity and oviparity in this and other species. Finally, I discuss the application of new technologies to the question of reproductive mode in squamates and other animals, summarising significant research to date, and outlining the contributions of this thesis to the ongoing project of uncovering the fundamental processes which determine viviparity and oviparity.

1.2 Evolutionary transitions: reviewing origins of viviparity across the amniote family tree

Some animals lay eggs (oviparity), while others give birth to live young (viviparity). Reproduction is a fundamental aspect of animal biology, and this difference in reproductive mode has knock-on effects on virtually every aspect of animal life, from the microscopic to the ecological to the evolutionary scale (Uller 2003; Kalinka 2015; L. Ma et al. 2022; Andrews 1997; Stewart 2015; Mouton, Flemming, and Stanley 2012). The transition to viviparity is a complex process, generally understood to begin with progressive increases in egg-retention, with the embryo remaining within the oviduct for longer and longer periods before parturition (Packard, Tracy, and Roth 1977; Andrews 1997). This extended egg retention necessitates increased water and oxygen transport to the growing embryo (Michael B. Thompson and Speake 2006), leading to the development of increasingly complex placental structures (Stewart 2015; Michael B. Thompson et al. 1999; M. B. Thompson, Stewart, and Speake 2000; Adams et al. 2005) and progressive reduction of the eggshell to facilitate this,

via reduction in the size and number of shell-glands lining the reproductive tract (Benoit Heulin et al. 2002; 2005). This in turn leads to direct contact between the embryonic and maternal tissues, requiring farther adaptation of the maternal immune response to prevent rejection of the offspring before parturition (Hendrawan et al. 2017; Genebrier and Tarte 2021; Graham et al. 2011a; Samardžija et al. 2020). This progressive evolution of viviparous traits has occurred many times in the history of life, allowing for informative comparisons between taxa at different points on the viviparity-oviparity continuum.

1.2.1 Mammals and non-squamate saurians

For amniotes oviparity is ancestral (Starck, Stewart, and Blackburn 2021), and in most amniote groups transitions to viviparity are rare. While extant therian mammals are universally viviparous, they descend from a single viviparous ancestor which evolved after monotremes diverged from other mammals around 187 MYA (Y. Zhou et al. 2021). While there is considerable variability in reproductive anatomy and physiology within therians, the molecular evidence suggests that the last common ancestor of all eutherian mammals was already highly specialised for viviparity with a complex invasive placenta by 125 MYA (Mika et al. 2022; Archibald 2003). The mammalian clade thus contains only a single origin of viviparity, and one far back in evolutionary history.

Among archosaurs, birds are universally oviparous (Daniel G. Blackburn and Evans 1986), as are all extant crocodilians, although fossil evidence suggests viviparity did evolve in extinct marine archosaurs lineage (J. Liu et al. 2017; Jiang et al. 2023). Indeed, viviparity appears to have evolved repeatedly in ancient marine reptile groups (Cheng, Wu, and Ji 2004; Piñeiro et al. 2012; Maxwell and Caldwell 2003), with the notable exception of marine turtles which are universally oviparous and must return to land for oviposition. However, only one saurian group contains extant viviparous taxa: the squamates. Most of the transitions from oviparity to viviparity are known from this single order, with over 100 independent origins of viviparity (Daniel G. Blackburn 2015a). Virtually all significant squamate clades include multiple oviparity-viviparity transitions, including the lacertoids, geckos, iguanids, anguimorphs, skinks, elapids, colubrids and viperids (**Fig. 1**). What follows is a review of the contemporary literature on oviparity-viviparity transitions within the extant squamate lineages.

Z

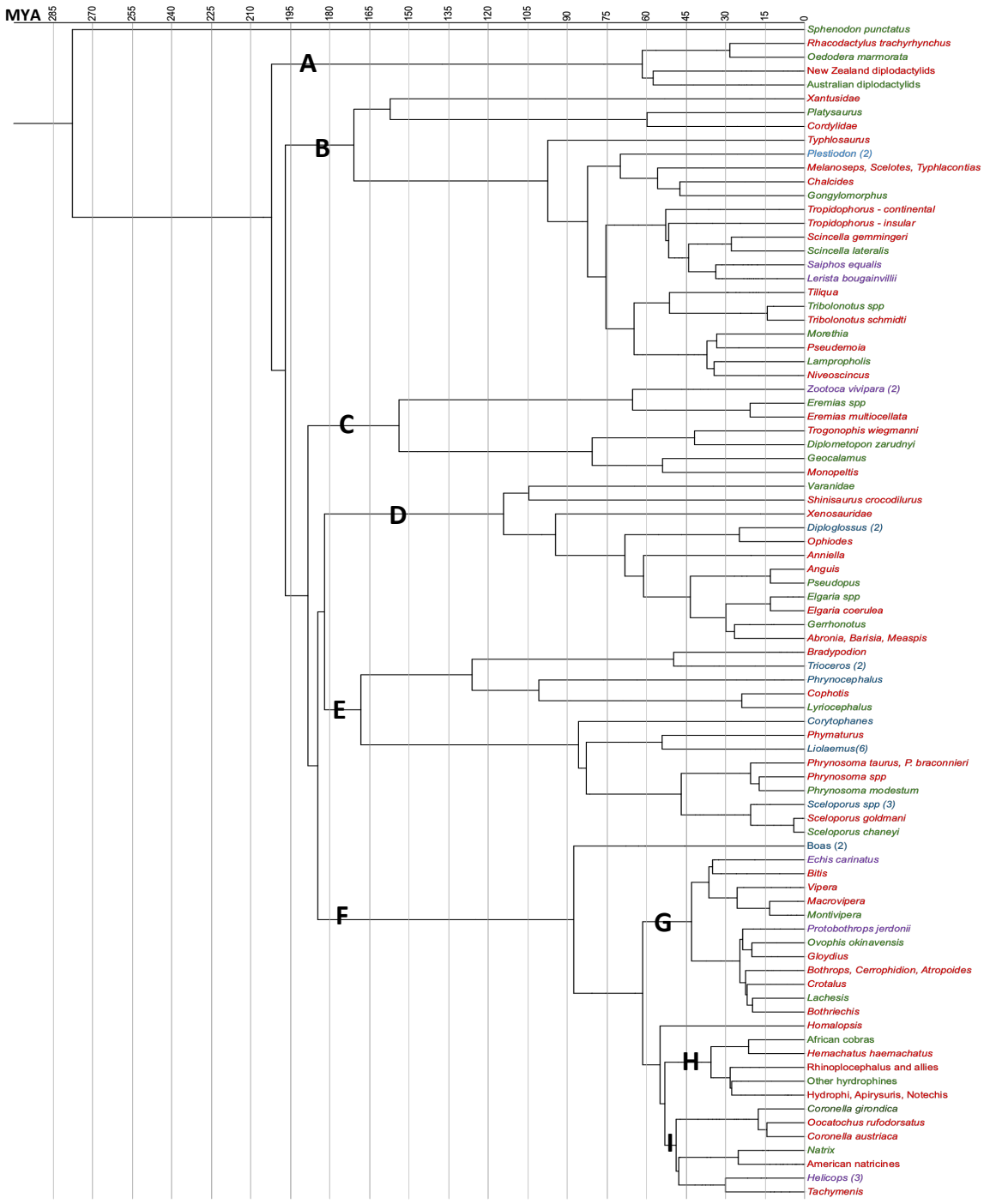

Figure 1-1. Time tree showing selected reproductive mode transitions in squamates.

Oviparous taxa are shown in green, viviparous taxa in red, reproductive bimodality at the genus level in blue, and bimodality at the species level in purple, with numbers in brackets indicating multiple oviparity-viviparity transitions within the taxon indicated. Major clades are labelled as follows: **A:** geckos, **B:** scincoids, **C:** lacertoids, **D:** anguiods, **E:** iguanians, **F:** serpentes, **G:** viperids, **H:** elapoids**, I** colubroids. Divergence dates are from the time-calibrated phylogeny of Zheng and Wiens (2015) in MYA. The tree was constructed using the phylip file from Zheng and Wiens, prumed to include only relevant taxa and annotated using iTOL (Letunic and Bork 2021).

1.2.2 Lacertoids, geckos, iguanids and anguimorphs

Viviparity in lacertoids was first scientifically described in 1820s, in the reproductively bimodal Eurasian common lizard *Zootoca vivipara* (Lichtenstein 1823), which is today perhaps the most extensively studied of all viviparous squamates (see next section). Independent origins of viviparity have since been demonstrated for two more lacertid clades, the Central Asian racerunners *Eremias prezwalskii* and *E. multiocellata* (T. Zhou et al. 2016; Hong Li et al. 2009) and the African lacertid *Tropidosaura essexi* (Nicolau et al. 2022). In all three cases the origin of viviparity is likely relatively recent, estimated at 4.5 MYA in *Z. vivipara* (Luca Cornetti et al. 2014a) and 6.3 MYA in *Eremias* (Guo et al. 2011), and placental anatomy and function is rather simple with the yolk sac providing the bulk of embryonic nutrition (Stewart, Heulin, and Surget-Groba 2004; D. Li et al. 2015). In addition to the fully viviparous taxa, many lacertids exhibit extended egg retention, particularly species endemic to high altitude habitats such as the genus *Iberolacerta* (Braña, Bea, and Jesús Arrayago 1991; Arribas and Galán 2005). Of the other lacertoid groups, viviparity has not been recorded for either teiids or gymnophthalmids, but multiple origins of viviparity are suggested in amphisbaenians. Four viviparous species are known, all from Africa, spanning the clades *Amphisbaenidae* (*Loveridgea ionidesii*), *Rhineuroidea* (*Monopeltis capensis* and *M. ionidesii*) and *Trogonophidae* (*Trogonophis wiegmanni*), suggesting three independent origins for viviparity in amphisbaenians (Andrade, Nascimento, and Abe 2006) and six in lacertoids overall.

Viviparous geckos are known only from the family *Diplodactylidae*, including all the native geckos of New Zealand comprising the families *Hoplodactylus*, *Heteropholis,* and *Naultinus* which seem to have diverged from oviparous Australian geckos around 40.2 MYA (Nielsen et al. 2011). This group is relatively well-studied, with significant literature on their reproductive anatomy and physiology (Girling, Cree, and Guillette 1998; Cree et al. 2003; Cree and Guillette 1995). A second viviparous *Diplodactylid* lineage is composed solely of the New Caledonian species *Rhacodactylus trachyrhynchus* which presumably represents another origin for viviparity in diplodactylids, given that all other known *Rhacodactylus* species are oviparous (D. G. Blackburn 1982). Divergence of members of the *Rhacodactylus* genus has been dated to 7.2 MYA (Nielsen et

al. 2011), so this may represent a much more recent oviparity-viviparity transition, though more recent molecular studies have suggested that *Rhacodactylus* is paraphyletic, with *R. trachyrhynchus* sister to the oviparous *Oedodera marmorata* (Y. Zheng and Wiens 2016).

Iguanids, by contrast, show evidence of many independent transitions to viviparity. The horned lizards of the North American family *Phrynosomatidae* comprises nine genera, two of which include viviparous species: *Phrynosoma* and *Sceloporus*. Ancestral state reconstruction in this group generally supports two origins of viviparity in *Phrynosoma* and no less than four in *Sceloporus* (Hodges 2004; Lambert and Wiens 2013; Benabib, Kjer, and Sites 1997). The highly diverse South American genus *Liolaemus*, containing over 200 species widely distributed across montane and lowland habitats either side of the Andes, shows evidence for as many as six oviparity-viviparity transitions spanning two major clades, which exhibit either lecithotrophic viviparity with relatively simple placentation or oviparity with extended egg-retention (Schulte et al. 2000; Aguilar et al. 2015; Crocco, Ibarguengoytía, and Cussac 2008). The closely related genus *Phrymaturus* is exclusively viviparous, thus implying yet another origin of viviparity connected to this clade (Morando et al. 2013). Other cases from the Americas include the basilisk genus *Corythophanes,* which includes the viviparous *C. percarinatus* (McCoy, 1968) alongside oviparous species. Both the major Afroeurasian iguanid groups, agamas and chameleons, also include multiple examples of oviparity-viviparity transitions. The agamid genus *Phyrnocpehalus* contains a viviparous and an oviparous clade, estimated to have diverged around 9.7 MYA (Y. T. Jin and Brown 2013). The remarkable *Draconinae* subfamily of agamas contains two viviparous genera, *Harpesaurus* and *Cophotis,* which again seem to have evolved independently (Kurita et al. 2020). Chameleons have participated in at least three oviparity-viviparity transitions, one in the South African genus *Bradypodion*, which is entirely viviparous and split from its closest oviparous neighbour over 40 MYA, and two in the genus *Trioceros,* again in Africa, diverging around 30-35 MYA (Hughes and Blackburn 2020), for a total of 20 oviparity-viviparity transitions in all iguanids.

Like their iguanid cousins, anguimorphs also show significant variation in reproductive mode with multiple origins of viviparity. Within the glass lizards

(subfamily *Anguinae*), the slow-worms (genus *Anguis*) are viviparous, diverging from the oviparous sister genus *Pseudophus* at least 18.4 MYA (Lavin and Girman 2019), and have been reported to exhibit parental care (Neaves and Gandola 2021), a trait also found in other anguids (Greene, Rodríguez, and Powell 2006). The other major anguid group *Gerrhonotinae*, the alligator lizards, includes another clade of viviparous lizards comprising the genera *Mesaspis, Abronia* and *Barisia*, which have been shown to exhibit novel forms placentation which may indicate some degree of placentrophy (Stewart and Mendez de la Cruz 2019). The basal gerrhonotine genus *Elgaria* also includes a single viviparous species, *E. coerulea*, diverging from other *Elgaria* approximately 15.2 MYA (Leavitt et al. 2017), representing a third anguid origin for viviparity. The related anguimorph family *Anniellidae*, comprising the single genus *Aniella*, is also viviparous (California, Goldberg, and Miller 1985; D. G. Blackburn 1982), and is now estimated to have branched from the anguid clade as far back as the Cretaceous (Burbrink et al. 2020), indicating another origin of viviparity somewhere in the history of this clade. The Central American galliwasps, superficially skink-like anguimorphs of the family *Diploglossidae*, seem to represent a dizzying array of parity-mode transitions. The genus *Diploglossus* includes oviparous mainland species such as *D. lessonae* and *D. monotrophis,* with derived lineages radiating to the islands of Puerto Rico (*D. pleii*, 13.2 MYA) and Cuba (*D. delasagra, D. nigropunctatus*, and *D. garridoi*, 6.63 MYA) (Schools, Kasprowicz, and Blair Hedges 2022). Viviparity occurs in the Puerto Rican *D. pleii* and the Cuban *D. nigropunctatus* and *D. garridoi*, but the other Cuban species *D. delasagra* is oviparous (Greene, Rodríguez, and Powell 2006), suggesting either three sperate origins for viviparity in this species or a reversal to oviparity in *D. delasagra* after the colonisation of Cuba 6.63 MYA. The sister-group to *Diploglossus, Ophiodes,* diverged from *Diploglossus* more than 25 MYA, and are entirely viviparous (Ortiz, Boretto, and Ibargüengoytía 2017). The closely related Mexican genus *Siderolamprus* includes viviparous species such as *S. rozelae* alongside oviparous species such as *S. legnotus*, and the Hispaniolan and Jamaican clade including the genera *Celestus, Sauresia* and *Wetmorena* are at least partially viviparous (Greene, Rodríguez, and Powell 2006; Schools, Kasprowicz, and Blair Hedges 2022), indicating at least five oviparity-viviparity transitions in *Diploglossidae*. The family *Xenosauridae*, rock-dwelling viviparous anguimorphs from western North America, is estimated to have diverged from the anguid

lineage around 106 MYA (Parks, Harrington, and Thomson 2022), representing another origin of viviparity. The varanids, in contrast to virtually all other significant anguimorph clades, are universally oviparous, and in fact seem to exhibit unusually early-stage oviposition for a squamate (Andrews and Mathies 2000), but the basal anguiform *Shinisaurus crocodilurus*, which is believed to have split from the stem varanid lineage around 100 MYA, is viviparous (Xie, Liang, Chen, et al. 2022; Xie, Liang, Li, et al. 2022), bringing the total number of oviparity-viviparity transitions in anguimorphs to at least 11.

1.2.3 Skinks

Viviparity-oviparity transitions in skinks and their allies have been extensively documented; beginning with the non-skink scincomorphs, the night lizards (family *Xantusiidae*) seem to be universally viviparous (D. G. Blackburn 1982; Ramírez-Bautista et al. 2008; Cortés, Camarillo, and Bezy 1990), presumably representing a single origin of viviparity, and potentially a very ancient one, diverging from the related cordyloid lineage as far back as the mid-Jurassic (Burbrink et al. 2020). The *Cordylidae* themselves are largely viviparous but also include a basal oviparous clade comprising the genus *Platysaurus* (Fras N 2001), and the other cordyloid clade, the family *Gehrosauridae*, seems to be entirely oviparous (Ping and Bates 2022; D. G. Blackburn 1982).

The genus *Chalcides* is viviparous, exhibiting moderately complex placentation (Brandley et al. 2012), and is bracketed by the oviparous genus *Gongylomorphus* (Y. Zheng and Wiens 2016; Zimin et al. 2022). The related genera *Melanoceps, Scelotes* and *Typhlacontias* appear to form a distinct viviparous clade (D. G. Blackburn 1982; Y. Zheng and Wiens 2016). Viviparity-oviparity transitions appear to have evolved at least twice in the widely distributed genus *Plestiodon*, as the oviparous *P. sumichrasti* appears to be nested between the viviparous species *P. gilberti* and *P. brevirostris* (Muñoz-Nolasco et al. 2023; Y. Zheng and Wiens 2016). The genus *Scincella* is also bimodal, including the viviparous *S. gemmingeri* and the closely related oviparous *S. lateralis* (J. Yang et al. 2012; Watson, Makowsky, and Bagley 2014; Y. Zheng and Wiens 2016). The genus *Tropidophorus* appears to be entirely viviparous but is paraphyletic, including a basal clade consisting of species from mainland Southeast Asia and a derived insular clade which is sister to the oviparous *Lipinia vittigera* (and various other

oviparous and viviparous taxa, including *Scincella*) (Honda et al. 2006; Truong et al. 2010), suggesting two origins of viviparity. The genera *Typhlosaurus* and *Acontius* form a wholly viviparous African clade (Huey et al. 1974; D. G. Blackburn 1982) and appear to be one of the most basal skink lineages (Y. Zheng and Wiens 2016). The phylogeny of mabuyid skinks indicates the genus *Trachlepis* includes at least three origins of viviparity, and a putative reversal to oviparity in one viviparous lineage (Weinell et al. 2019).

Australian skinks have been the focus of considerable research on reproductive mode, with at least 11 skink oviparity-viviparity transitions claimed on this continent alone (Fairbairn et al. 1998). Of particular note is the species *Saiphos equalis*, which along with *Z. vivipara* and a handful of other squamates exhibits bimodal reproduction within a single species (Foster et al. 2020; Smith, Austin, and Shine 2001; Stewart et al. 2010) and, according to one report, even within a single individual (Laird, Thompson, and Whittington 2019). Australia in fact is home to not one but three bimodal skink species, the others being *Lerista bougainvillii* (Fairbairn et al. 1998) and *Glaphyromorphus nigricaudis* (Whittington et al. 2022). The tribe *Tiliquini*, including the charismatic blue-tongued skinks, comprises a viviparous clade (Milne, Bull, and Hutchinson 2002; Bull, Pamula, and Schulze 1993) bracketed by the largely oviparous genus *Tribolonotus* which itself contains another origin of viviparity in the single viviparous species *T. schmidti* (Watson, Makowsky, and Bagley 2014). Another oviparity-viviparity transition gave rise to the viviparous genus *Pseudemoia*, including the species *P. entrecasteauxii* which has been the subject of several studies characterising gene expression and placentation, and is one of two Australian genera to demonstrate substantive placentrophy (Stewart and Thompson 2009a; Adams et al. 2005; Griffith et al. 2016; Hendrawan et al. 2017). The other is the related Tasmanian genus *Niveoscincus*, (M. B. Thompson et al., 2001). As both are bracketed by the interstitial oviparous genus *Lampropholis* they represent two independent origins of both viviparity and placentrophy. Other oviparity-viviparity transitions in Australian skinks include the genus *Hemiergis* and its viviparous sister genus *Eremiascincus*; the bimodal genus *Anomalophus* with the viviparous *A. swansoni* and oviparous *A. mackayi* and others; the viviparous genus *Concinnia* bracketed by the oviparous *Calorodius*; and the viviparous *Eulamprus* group diverging from the oviparous

Notoscincus (Michael B. Thompson et al. 2006; Y. Zheng and Wiens 2016; J. Thompson 1982; D. G. Blackburn 1982).

Australia's island neighbours also harbour substantial diversity in skink reproductive mode. In New Zealand skinks have been shown to form a monophyletic clade, and are almost universally viviparous, with the sole exception of *Oligosoma suteri*, indicating at least one oviparity-viviparity transition within the New Zealand skink radiation (Chapple, Ritchie, and Daugherty 2009). New Guinea is home to and the viviparous *Sphenomorphus leptofasciatus,* related to the oviparous *S. fasciatus* of the Phillipines (D. G. Blackburn 1982; Y. Zheng and Wiens 2016), and to the viviparous montane genus *Lobulia* (Greer et al. 2005), which was recently reclassified into five different genera, with new molecular evidence indicating between two and three oviparity-viviparity transitions in this group and its relatives dating between 10-25 MYA. *Prasinohaema semoni*, which forms and outgroup to all other skinks in this group, is viviparous; *Lobulius* forms another viviparous clade; and sister to this group are two clades comprising on the one hand the oviparous genera *Papuascincus, Palaia* and *Alpinoscincus* and on the other a third viviparous group reclassified as *Ornithuroscincus*, (Slavenko et al. 2022), suggesting either three separate origins for viviparity or a single origin of oviparity followed by reversion to oviparity.

1.2.4 Snakes

Like other squamate groups, snakes include multiple oviparity-viviparity transitions, with viviparous members of every significant family, with the sole exception of *Pythonidae*. The *Booidae* are by contrast almost wholly viviparous, with their closest oviparous relative, the misleadingly named Calabar python *Calabria reinhardtii* – with the exception of the oviparous species *Eryx jayakari*, whose position nested between several viviparous clades (see Figueroa et al., 2016) has led some to suggest a potential reversal to oviparity in this lineage (Lynch and Wagner 2010).

Among elapoid snakes, viviparity has evolved several times, mostly within the subfamily *Hydrophinae*. This group includes a diverse clade of viviparous sea snakes, following the previously described trend towards viviparity in marine

reptiles, which some show evidence of placentrophy (Dsouza and Rao 2021; Lettoof, Van Dyke, and Gagnon 2021). Molecular phylogenetic analysis suggests viviparity evolved multiple times in this group, once in the lineage including the viviparous sea snakes (including the genera *Aipysurus, Hydrophis* and others) and the terrestrial viviparous tiger snakes (genus *Notechis*), and once in a wholly terrestrial lineage including cold-climate specialists such as *Rhinoplocephalus bicolor*, with both clades diverging around 8.4 MYA (Sanders et al. 2008). The same study found that the *Acanthophis* genus of death adders, which are also viviparous (Richard Shine, Spencer, and Keogh 2014), diverged 9.1 MYA, branching off from a clade comprising the *Pseudechis*, which are largely oviparous but include the single viviparous species *P. porphyriacus* (Richard Shine 1987), indicating two more related oviparity-viviparity transitions within Australian elapids. Outside of this group, South African elapids also include at least one viviparous species, the rinkhal *Hemachatus haemachatus* (R. Shine et al. 2007), diverging from other African cobras 17.0 MYA (Kazandjian et al. 2021). A second, possibly extinct *Hemachatus* species was recently described from museum specimens, although its reproductive mode is unknown (Major et al. 2023). Also worth mentioning is a small group of African snakes of the family *Pseudaspididae*, which are basal elapoids diverging from other African elapoid lineages 36.0 MYA (Kelly et al. 2009), which includes at least one viviparous member in the mole snake *Pseudaspis cana* (Boycott 1990).

The evolutionary history of colubroids includes a large number of independent viviparity-oviparity transitions. Of particular note are the South American water snakes of the tribe *Hydropsisni* which are characterised by significant diversity in reproductive mode: viviparity appears to have originated independently between three and seven times in different species of the genus *Helicops*, including in the reproductively bimodal species *H. angulatus* (Moraes-da-Silva et al. 2022; Braz, Scartozzoni, and Almeida-Santos 2016). The sister group to the hyrdopsines, the tribe *Tachymenini*, appears to be wholly viviparous, including *Tomodon dorsatus* (Greer 1966), *Thamnodynastes strigatus* (Loebens et al. 2022), and others (Luís et al. 2006), presumably indicating a single transition to viviparity shortly after this lineage diverged from the *Hydropsini*. Within the genus subfamily *Natricinae* the entire North American lineage is viviparous, suggesting a single origin of viviparity preceding the divergence of this clade from the lineage leading to the

genus *Natrix* 26.27 MYA (Deepak et al. 2022). Three other viviparous natricines are known from Asia, including the viviparous island species *Hebius ishigakiensis* (Ota and Iwanaga 1997), diverging from *H. pryeri* around 7.54 MYA (Deepak et al. 2022). *Timerodytes annularis* also seems to represent a recent origin of viviparity, diverging from its oviparous sister species *T. baleteata* just 6.73 MYA (Deepak et al. 2022). The third, *Pseudagkistrodon rudis*, is of uncertain phylogenetic placement, possibly representing remnant of a deeply divergent lineage within natricines (Deepak et al. 2022) or even an outgroup to all other natricine snakes (Kelly, Barker, and Villet 2003), and may thus represent a much more ancient origin for colubrid viviparity. The African grass snake genus *Psammophylax* reportedly contains a single viviparous species (Daniel G. Blackburn 1985) although literature on this group is sparse; similarly poorly documented are the Mexican earth snakes of the genus Conopsis which are also described as viviparous (Greer 1966). At least two additional transitions are indicated in the clade including the widespread viviparous Asian colubrid *Oocatochus rufodorsatus* (H. J. Lee, Lee, and Park 2011; X. Ji et al. 1997) and the smooth snake genus *Coronella* (Figueroa et al. 2016), which includes the viviparous *C. austriaca* and the oviparous *C. girondica*, indicating two independent origins for viviparity (Utiger et al. 2002). The basal colubroid mud snakes (family *Homalopsidae*) seem to be universally viviparous (Junwei et al. 2021; Daniel G. Blackburn 1985; MacK et al. 2021; Y. Du et al. 2015) consistent with a single ancient origin of viviparity in this group as far back as 45.31 MYA (Bernstein et al. 2021).

The viperids have long been known for viviparity, with the very name *Viperidae* referring to the viviparous mode of reproduction common throughout this family. Viviparity has arisen independently on many branches of the viperid family tree (Fenwick, Greene, and Parkinson 2012), including in the two reproductively bimodal species *Echis carinatus* and *Protobothrops jerdonii* (Whittington et al. 2022). Within the viperines, the type genus *Vipera* is of course viviparous, but its sister clade comprises the oviparous *Montivipera* along with the viviparous *Macrovipera* (Fenwick, Greene, and Parkinson 2012; Carrasco et al. 2023; Figueroa et al. 2016) suggesting two more origins for viviparity in viperines. The viviparous genus *Bitis* is sister to the largely oviparous *Echis* (see above); the viviparous *Cerastes vipera* falls within the otherwise oviparous *Cerastes* genus;

the viviparous genera *Bitis* and *Atheris* form another clade; and the viviparous *Proatheris superciliaris* emerges from the lineage leading to the oviparous genus *Causus,* bringing the total implied origins of viviparity within *Viperinae* to at least seven (Figueroa et al. 2016; Carrasco et al. 2023; Fenwick, Greene, and Parkinson 2012). The evolutionary history of reproductive mode in the new world pit vipers is also complex, including five apparent origins of viviparity: one in the clade comprising the viviparous genera *Agkistrodon, Crotalus* and *Sistrurus*; one in the sister lineage which includes the oviparous *Lachesis* and the viviparous *Bothriechis*; one in the lineage leading to the viviparous *Bothrops, Cerrophidion* and *Atropoides*; one after the divergence of the viviparous *Gloydius* genus from the oviparous species *Ovophis okinavensis;* and another in the previously mentioned *P. jerdonii* within the otherwise oviparous *Protobothrops* genus (Fenwick, Greene, and Parkinson 2012; Figueroa et al. 2016). The placement of *Lachesis* nested within three viviparous clades has led some to suggest a reversal to oviparity in the ancestry of this genus, which would result in only four transitions in reproductive mode in *Crotlainae* in total (Fenwick, Greene, and Parkinson 2012).

1.2.5 Reproductive mode transitions in squamates

As demonstrated by the more than 100 viviparity-oviparity transitions reviewed here, squamate reproductive mode is extraordinarily diverse. Around one fifth of extant squamates are viviparous, with both recent and ancient origins of viviparity spread across the squamate family tree (**Fig. 1**). The phylogenetic implication of multiple origins of viviparity for many squamate clades has often led to questions of evolutionary reversibility, as mentioned above for the Boa *Eryx*, the colubrid genus *Helicops*, and the skink genus *Trachylepis* among others (Lynch and Wagner 2010; Weinell et al. 2019; Moraes-da-Silva et al. 2022; Recknagel, Kamenos, and Elmer 2018; Fenwick, Greene, and Parkinson 2012). Such claims have often been controversial, as such evolutionary reversals are believed to be extremely rare because, in general, complex traits such as amniote oviparity do not readily re-evolve once lost, a rule known as Dollo's law (Collin & Miglietta, 2008; Gould, 1970), and claims of widespread reversals to oviparity from viviparity in squamates have been met with considerable skepticism (Blackburn, 1999, 2015b; Griffith et al., 2015; Shine & Lee, 1999). However, in cases where reversal are more parsimonious – that is, require fewer

evolutionary transitions – than scenarios in which reversals are excluded, they may be plausible. The reversals I have discussed above, and the potential evolutionary reversal in *Z. vivipara* (discussed below) all involve oviparous taxa nested within multiple viviparous lineages.

The persistence of divergent modes of reproduction at the genus level is somewhat common in squamates, as I have shown. More unusual are the cases of bimodal reproduction within a single species, as is the case for *Z. vivipara*, *S. equalis*, *H. angulatus* and others mentioned above. Such species are unparalleled models for the study of reproductive mode, as they allow comparison between oviparous and viviparous reproduction in otherwise very similar animals, effectively offering a snapshot of an evolutionary transition in progress (Whittington et al. 2022). In the following section, I will discuss the specific case of *Z. vivipara*, which has emerged as the premier model organism for researchers working on reproductive mode.

1.3 The Eurasian common lizard *Zootoca vivipara*: an emerging model organism for the evolution of reproductive mode

Figure 1-2. The viviparity-oviparity continuum in *Z. vivipara*.

A: Adult female *Z. vivipara* after parturition – note skin folds along flanks, showing reduced body volume after parturition (red arrow). **B:** Oviparous *Z. vivipara* eggs (eastern oviparous lineage). **C:** Viviparous neonates (central viviparous II lineage), not yet emerged from egg sacs. **D, E:** Hybrid eggs (eastern oviparous x central viviparous II) showing different levels of admixture.

Zootoca vivipara (**Fig. 1-2A**) is a small insectivorous lizard of the family *Lacertidae*. It enjoys the broadest geographical distribution of any terrestrial reptile, ranging from the mountains of southern Europe to the shores of the Arctic Ocean, and from the Atlantic coast of Ireland to the islands of Japan (Roitberg et al. 2013). As early as 2003 the species was recognised as an emerging model organism for the study of reproductive mode (Freire, Tennant, and Miyamoto 2003a), and in the past 20 years considerable progress has been made in characterising this important evolutionary transition in this remarkable species. Important questions remain, however, particularly regarding the underlying mechanisms that determine reproductive mode at the molecular and cellular level.

1.3.1 Bimodal reproduction in *Z. vivipara*

The common lizard has long been recognised as an example of viviparity in lizards (Lichtenstein 1823). However, it is now known that common lizards are, in fact, reproductively bimodal: oviparous common lizards were first discovered in southwestern France and northern Spain (Lantz 1927), and more recently oviparous populations of *Z. vivipara* were also found in central Europe (Benoît Heulin et al. 2000). All populations exhibit a seasonal reproductive cycle, hibernating through the winter and mating polygynandrously shortly after emergence in the spring, with males emerging somewhat earlier than females. Female lizards undergo vitellogenesis during the first month after emergence, with ovulation and fertilisation at around the end of May or beginning of June depending on local climate (Bleu et al. 2013). Oviparous females oviposit around one month after ovulation, laying fully calcified eggs (**Fig. 2B**) at around embryo stage 31 (see Hubert, 1962)which then develop externally for around one month more before hatching. In viviparous lineages, on the other hand, development continues internally until around embryo stage 40, at which point the neonates are born enclosed in a non-calcified membranous sac (**Fig. 2C**). Viviparous lineages exhibit lecithotrophic viviparity with the bulk of embryonic nutrition provided by a yolk sac deposited during vitellogenesis (Stewart, Heulin, and Surget-Groba 2004).

Hybridisation between viviparous and oviparous individuals can produce viable offspring: this was first demonstrated in laboratory experiments (Arrayago, Bea,

and Heulin 1996b) and has since been documented in at least one wild population (Lindtke, Mayer, and Bohme 2010; Recknagel et al. 2021), although elsewhere reproductive isolation between sympatric oviparous and viviparous populations has also been observed (Luca Cornetti et al. 2015a). Evidence of more widespread gene flow comes from analysis of introgression between oviparous and viviparous populations of common lizards across Europe (J. L. Horreo et al. 2019a). Hybrid females are phenotypically intermediate between oviparous and viviparous lizards, producing eggs with a reduced eggshell and delayed oviposition (Recknagel et al. 2021; Lindtke, Mayer, and Bohme 2010) **(Fig 2D)**.

1.3.2 The case for evolutionary reversal in *Z. vivipara*

The phylogenetic relationships between oviparous and viviparous populations of *Z. vivipara* are now well understood. Six discrete lineages have been resolved through mitochondrial DNA (mtDNA) analysis: western viviparous, eastern viviparous, central viviparous I, central viviparous II, western oviparous (sometimes defined as its own subspecies, *Z. vivipara louislantzi*) and eastern oviparous (sometimes *Z. vivipara carniolica*) (Surget-Groba et al. 2006). The eastern oviparous lineage (found in central Europe) appears to represent the ancestral state of oviparity, having seemingly diverged from all other lineages between 2.6 – 6.1 MYA (Luca Cornetti et al. 2014a). However, the western oviparous lineage found in southwestern France and in Spain instead appears to be nested within the clade containing all extant viviparous lineages (Surget-Groba et al. 2006; Recknagel, Kamenos, and Elmer 2018), suggesting a reversal to oviparity in this lineage, which would also explain the subtle differences in the oviparous mode of reproduction between these two lineages discussed above.

This additionally resolves a longstanding dilemma over the karyological evolution of *Z. vivipara*. Common lizards possess a unique sex-determination system, dubbed Z_1Z_2W, in which male lizards possess 36 chromosomes (32 autosomes plus the sex chromosomes $Z_1Z_1Z_2Z_2$) whilst females have only 35 (autosomes plus Z_1Z_2W) (Chevalier, Dufaure, and Lecher 1979). This system appears to be a recent evolutionary innovation, and is notably absent in the eastern oviparous lineage, but has been shown to be present in the western oviparous group

(Gaëtano Odierna et al. 2001). While researchers originally assumed that the evolution of the Z_1Z_2W karyotype simply preceded the evolution of viviparity (assuming monophyly for the viviparous lineages) this was discounted when viviparous common lizards possessing the ancestral ZW system were discovered in Hungary (the central viviparous II lineage), leading to the suggestion that the transition to viviparity occurred multiple times in the recent evolutionary history of common lizards (Gaetano Odierna et al. 2004). However, this interpretation requires not only a double origin for viviparity but also a double origin for the Z_1Z_2W karyotype as according to the resulting phylogeny the clade containing the central viviparous II lineage (ZW) separates after the clade containing the eastern and western viviparous lineages (Z_1Z_2W) (Surget-Groba et al. 2006) – a prospect which seems extremely unlikely. The most recent phylogeny instead places central viviparous II as an outgroup to all other lineages except the eastern oviparous (Recknagel, Kamenos, and Elmer 2018), allowing for a single origin of the Z_1Z_2W karyotype after the divergence of the central viviparous II lineage. Future research on the molecular and cellular basis of oviparity in the two oviparous lineages should aid in resolving the controversy over the potential reversal to oviparity in the western oviparous lineage.

1.3.3 Reproductive traits in oviparous and viviparous *Z. vivipara*

Research comparing *Z. vivipara* reproductive traits across different lineages and reproductive modes indicates that offspring size is reduced in viviparous lizards relative to their oviparous cousins (Roitberg et al. 2013; Recknagel and Elmer 2019). Furthermore, in comparing the two oviparous lineages, females of the western oviparous lineage, which retain their eggs within the body until a later developmental stage (Benoît Heulin et al. 2000), have slightly smaller offspring than females of the eastern oviparous lineage, though still in general larger than those born to viviparous parents. This result is in line with the volume constraint hypothesis whereby the limited space available for offspring to develop within the mother's abdominal cavity leads to smaller offspring size at parturition (W. G. Du and Lü 2010). Despite this apparent disadvantage, viviparous lizards predominate across most of the species' range, with oviparous populations restricted to relatively small areas of central and western Europe (Surget-Groba et al. 2006), in line with the predictions of the cold climate hypothesis of squamate viviparity, which holds that the evolution of viviparity in squamates is

driven in large part by selection for extended egg retention to protect developing eggs from low temperatures as squamate species move into cold climates (Richard Shine and Bull 1979). Indeed, it has been suggested that cold temperatures may have been a key factor in the recent evolutionary history of *Z. vivipara*, with the species being reduced to isolated refuge populations during the extensive glaciation of Europe during the Pleistocene, before retreat of the ice sheets allowed the now cold-adapted common lizards to expand to their present, extensive range, achieving in fact the most northerly distribution of any species of lizard (Surget-Groba et al. 2001; Gaetano Odierna et al. 2004).

Z. vivipara has also served as a model for the study of viviparity at the microscopic scale. Comparative histology of oviparous and viviparous *Z. vivipara* oviduct reveals differences throughout the reproductive cycle, beginning during vitellogenesis (**Fig 1-3A, B**). Tubular shell glands, located in the *lamina propria* between the outer *muscularis externa* and the inner epithelial lining of the oviduct, are significantly enlarged in the oviparous oviduct during vitellogenesis, taking up almost the entire thickness of the oviduct wall (**Fig 1-3A**) whereas in the viviparous oviduct they show a far less dramatic increase (**Fig 1-3B**). During gravidity the oviparous oviduct is separated from the fetal tissues by a significant thickness of fibrous eggshell (**Fig 1-3C**) deposited by the shell glands, whereas during pregnancy in viviparous lineages the eggshell is reduced to a thin layer of fibrous material overlying the inner eggshell membrane (**Fig 1-3D**). While most oviparous squamates provision their young with a relatively calcium-rich yolk (as compared to archosaurs such as birds and crocodilians), the calcium content of *Z. vivipara* yolk is comparatively rather poor, and the developing embryo is dependent for calcium either on mobilising calcium from the eggshell (in oviparous lineages) or on calcium secretions provided directly by the uterine epithelium (in viviparous lineages) (Stewart, Ecay, and Heulin 2009). The evolution of viviparity thus involves important changes in the timing of calcium transport in the tissues of the squamate oviduct (Griffith 2021).

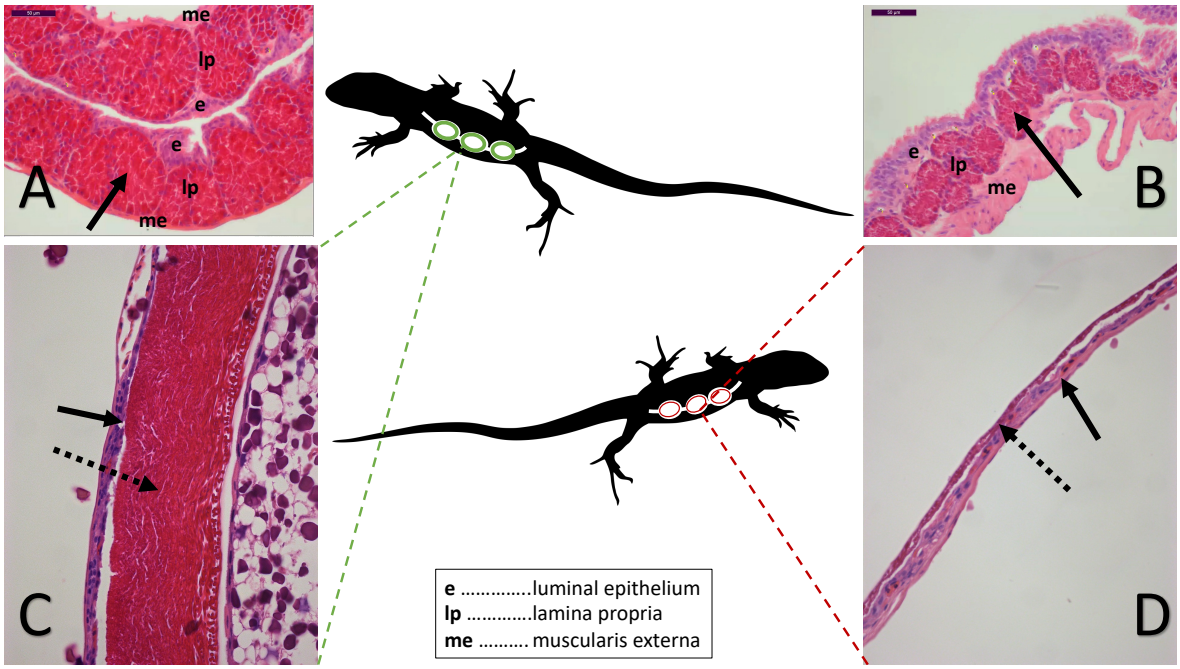

Figure 1-3. The fine structure of viviparity and oviparity in the *Z. vivipara* oviduct.

A: Oviparous oviduct during vitellogenesis (eastern oviparous lineage) with tissues annotated. Note engorged tubular shell glands (solid arrow). **B**: Viviparous oviduct during vitellogenesis (central viviparous I lineage), with smaller tubular shell glands (solid arrow) and tissues annotated. **C**: Maternal-fetal interface in oviparous *Z. vivipara* (eastern oviparous lineage). Note attenuation of the oviduct wall (solid arrow) and relative thickness of the eggshell (dashed arrow). **D**: Maternal-fetal interface in viviparous *Z. vivipara* (central viviparous I lineage). Histological images reproduced with kind permission from Dr Hongxin Xie. Open source *Z. vivipara* artwork by Henadii, iStock (2019) edited by Callan Denhan (2023), edited to add internal oviduct diagrams (license).

As a model system, *Z. vivipara* thus embodies many of the unique features of squamate evolutionary history that make squamates of interest to workers in evolutionary and reproductive biology: recent divergence between oviparous and viviparous lineages, possible evolutionary reversal from viviparous to oviparous reproduction, and most crucially the opportunity to study differences in reproductive mode in otherwise similar organisms at various levels of analysis, as I have reviewed above. Moreover, this species has been at the forefront of the application of modern molecular techniques to the study of viviparity in squamates, as I will discuss in the next section.

1.4 New instruments: reproductive mode in the molecular era

Since the 1990s the introduction of new DNA and RNA sequencing technologies has revolutionized virtually every aspect of biology, and in the past decade these

tools have been brought to bear on the question of reproductive mode evolution. Building on existing anatomical and histological studies of squamate viviparity, attention has turned to the underling changes in gene expression that determine viviparous phenotype. Genomes have now been published for more than 90 squamate species, although this still represents less than 1% of squamate taxa, and the quality of the assemblies and level of annotation varies greatly (Gable et al. 2023). The transcriptomic basis of oviparity and viviparity has been characterized in several squamate organisms, and genes linked to the evolution of reproductive mode across multiple amniote groups have been identified. In this final section, I will summarise previous RNA sequencing experiments in viviparous squamates and outline the new contributions to this field contained in this thesis.

The first squamate species to be the subject of a gene expression study relating to viviparity was the viviparous skink *Chalcides ocellatus* (Brandley et al. 2012), providing the first data on the degree of convergence in gene expression in viviparous squamates and mammals. Two further studies followed comparing gene expression in related oviparous and viviparous squamate taxa, in the skink species *Pseudemoia entrecastauxii, Lampropholis guichenoti* and *Lerista bougainvillii* (Griffith et al. 2016) and in the agamid lizards *Phrynocephalus przewalskii* and *P. vlangalii* (W. Gao et al. 2019). The first study of gene expression and reproductive mode in a bimodal species, *S. equalis,* was soon to follow (Foster et al. 2020). These initial studies paint a complex picture of the transcriptomic landscape of gene expression in viviparous and oviparous squamates, with some themes of convergence in expression of genes such as the vascular endothelial caherin gene CDH5, the Iodothyronine Deiodinase gene DIO2, and the cytochrome gene CYP51A1 which were differentially expressed in all four studies. Parallel to this work on gene expression, a restriction site associated DNA (RAD) sequencing study of viviparous and oviparous lineages of *Z. vivipara* identified 45 candidate genes which potentially involved in determining parity mode, of which 12 were identified as transcriptional regulators, three of which (*DACH2*, *SOX9* and *NOTCH1*) are known to have important regulatory functions in the uteruses of pregnant mammals (Luca Cornetti et al. 2017). Finally, a synthesis of these two approaches came with the publication of a landmark study combining analysis of gene expression in pregnant viviparous and

gravid oviparous *Z. vivipara* with admixture mapping of hybridizing viviparous and oviparous populations of the eastern oviparous and central viviparous II lineage (Recknagel et al. 2021), identifying 38 candidate genes linked to the evolution of viviparity by multiple lines of evidence. Following on from this work, recent analysis of molecular evolution in viviparous snakes has further validated the relevance of these genes to viviparity across the squamate family tree (Maggs 2023).

In this thesis, I continue to build on this existing body of research by applying new sequencing technologies and new experimental approaches to the study of reproductive mode, using *Z. vivipara* as a model system. In **Chapter 2**, I present a new sequencing experiment on the western viviparous lineage of *Z. vivipara*, exploring gene expression before, during and after pregnancy, and leveraging long-read sequencing to describe the role not only of changes in gene expression but also alternative splicing of RNA transcripts associated with pregnancy in this species. In **Chapter 3**, I examine gene expression during pregnancy at the single-cell level, examining expression of these candidate genes in different cell types present in the oviduct during pregnancy, and laying the groundwork for future studies of the cellular basis of viviparity in *Z. vivipara* and other organisms. Finally in **Chapter 4**, I present a new model system for the validation of candidate genes relevant to reproductive mode, in the form of cultured *Z. vivipara* oviduct cells from viviparous and oviparous lizards. I hope that these advances with further the important work of uncovering the genetic, transcriptomic and cellular foundations that underlie the fantastic diversity of reproductive mode across the amniote lineage.

2 Pregnancy in viviparous *Zootoca vivipara* is characterised by changes in expression and alternative splicing of genes linked to pregnancy in other squamates and mammals

2.1 Abstract

Pregnancy in squamates is an important topic in reproductive biology and is of considerable interest to evolutionary biologists due to its repeated evolution in this group. Although recent years have seen considerable progress on describing the role of the uterine transcriptome in pregnancy in *Zootoca vivipara* and other squamates, the role of alternative splicing in squamate pregnancy has not previously been addressed. Furthermore, previous work has focussed on uterine gene expression in viviparous *Z. vivipara* from the Central Viviparous II lineage, while the more widespread Western Viviparous lineage has received comparatively little attention. I sequenced RNA from the oviduct tissue from 8 adult female Western Viviparous *Zootoca vivipara* to analyse differential gene expression and differential transcript usage before, during and after pregnancy. Gene expression analysis showed 1149 differentially expressed genes (DEGs) between pregnant and non-pregnant oviduct tissue, and I was able to assess the functional significance of up and down-regulated genes using gene ontology (GO) term over-representation analysis. I was also able to identify alternative splicing of RNA transcripts during pregnancy, including some with prior relevance to pregnancy in mammals and other squamates.

2.2 Introduction

Pregnancy is the physiological process which defines viviparity: the full development of one or more offspring wholly within the maternal reproductive tract. Pregnancy in amniotes developed from ancestral oviparity, in which animals deposit their young in hard-shelled eggs which complete embryonic development outside the mother's body. This is seen in all extant non-squamate sauropsids (birds, crocodilians, testudines, and Tuataras) as well as a handful of mammals comprising the order Monotremata and the majority of squamate reptiles (Daniel G. Blackburn 2006).

Pregnancy involves significant changes to the structure and function of the reproductive tract including extended retention of the developing embryo (Richard Shine and Guillette 1988; Andrews 1997), an increase in the supply of gasses, water and nutrients to the growing embryo *in utero* (Linville et al. 2010; Mathies and Andrews 1996), elimination of the eggshell (Guillette Jnr 1993), and modulation of the maternal immune response to the developing embryo (Graham et al. 2011b; Saad and El Deeb 1990). In matrotrophic pregnancy, seen in mammals and some derived viviparous squamates, pregnancy entails the delivery of nutrients to the growing embryo from the mother's bloodstream via the placenta (Daniel G Blackburn and Flemming 2010; Daniel G. Blackburn 1992). In most viviparous squamates, however, the embryo still depends on the yolk-sac for all its nutritional requirements, termed lecithotrophic pregnancy (Stewart 2013). The dramatic changes to the reproductive tract required by these physiological adaptations are undergirded by significant transcriptomic changes in the reproductive tract and the accompanying vasculature (Brandley et al., 2012; Girotti & Zingg, 2003; V. L. Hansen et al., 2016; Hendrawan et al., 2017; Kidron et al., 1995; Ma et al., 2006; G.-Y. Nie et al., 2000).

Studies of the uterine transcriptome in pregnancy have understandably placed considerable emphasis on mammalian models of pregnancy: therian mammals demonstrate universal viviparity and sophisticated physiological adaptations to viviparous reproduction, and therian pregnancy has obvious significance to medical interventions to improve reproductive health for the human population. Past studies have characterised changes in uterine gene expression accompanying pregnancy in mice and humans (Bethin et al. 2003), rats (Girotti and Zingg 2003), cows (Binelli et al. 2015), goats (X. Liu et al. 2020), pigs (Samborski et al. 2013), dogs (Zatta et al. 2017), and horses (Merkl et al. 2010) amongst others. However, while viviparity is ubiquitous in therian mammals, it is also widespread in squamate reptiles, with over 100 independent transitions to viviparity in this group (Whittington et al. 2022). The study of pregnancy in squamates is thus of particular interest to evolutionary biologists, as these animals provide an ideal model of a major evolutionary transition occurring many times in parallel within the same group, allowing for comparisons of pregnancy in viviparous organisms to reproduction in closely related oviparous animals (Daniel G. Blackburn 2006). In recent years there has thus been

considerable interest in the transcriptomic changes underpinning pregnancy in reptiles, with significant contributions including gene expression studies in the viviparous skink species *Chalcides ocellatus* (Brandley et al. 2012) and *Pseudemoia entrecasteauxii* (Griffith et al. 2016), a comparison of the viviparous agamid lizard *Phrynocephalus vlangalii* to its oviparous cousin *P. przewalskii* (W. Gao et al. 2019), and comparative studies of oviparous and viviparous individuals of the bimodal lizard species *Zootoca vivipara* and *Saiphos equalis* (Recknagel et al. 2021; Foster et al. 2020). Data from these studies has highlighted intriguing instances of convergence between squamates and therian mammals in uterine gene expression during pregnancy (Mika et al. 2022; Recknagel et al. 2021).

Squamates include a handful of cases of reproductive bimodality (Qualls and Shine 1996), in which viviparous and oviparous taxa are so closely related as to comprise a single species. One such example is the Eurasian common lizard *Zootoca vivipara*, an important emerging model organism for the study of viviparity. Oviparous and viviparous lineages of *Z. vivipara* are thought to have diverged around 4.5 MYA (Luca Cornetti et al. 2014a), and hybridisation and gene-flow between oviparous and viviparous lineages has been well documented (Arrayago et al., 1996a; Horreo et al., 2019b; Lindtke, Mayer, & B??hme, 2010; Recknagel et al., 2021a) although reproductive isolation has also been reported in some cases (Luca Cornetti et al. 2015b; L. Cornetti et al. 2015). Current scholarship divides the species into six distinct lineages, two oviparous and four viviparous (Jose L. Horreo et al. 2018), and their phylogenetic relationships seemingly indicate a single origin of viviparity in this species (Recknagel, Kamenos, and Elmer 2018). To date, RNA sequencing of oviduct tissue from pregnant lizards has been carried out in only one viviparous lineage, the Central Viviparous II lineage (also termed Clade F) (Recknagel et al. 2021).

While the regulation of overall gene expression has long been recognised as vitally important to biological function, it is now understood that for many organisms the majority of genes produce multiple RNA transcripts with alternatively spliced exons (Boue, Letunic, and Bork 2003; Pan et al. 2008; P. Yang, Wang, and Kang 2021). In many cases, this functions as an additional layer of gene regulation, as alternatively spliced transcripts (or the resultant proteins) often show significant differences in stability and may be susceptible to

nonsense-mediated decay (NMD) (Soergel, Lareau, and Brenner 2013) or may lack key functional domains (Resch et al. 2004). This leads to potentially crucially important variance in gene function that may be invisible when considering only the total expression level of a given gene. The patterns of gene expression and alternative splicing tend to target different sets of functional genes, leading to both evolutionary and functional interactions between the two interacting systems of gene regulation (Jacobs and Elmer 2020; Rogers, Palmer, and Wright 2021). Alternative splicing is thus a key area of research for workers in the field of transcriptomics and gene regulation and in evolutionary biology (Verta and Jacobs 2022).

The importance of alternative splicing in pregnancy has been demonstrated many times in mammals, including humans (Ruano et al. 2021; Zeng et al. 2018), mice (Dabertrand et al. 2007; Yatsenko et al. 2004), rats (Gopalakrishnan and Kumar 2020), and rhesus monkeys (Kravitz et al. 2001). However, to date, no published work has investigated the role of alternative splicing in pregnancy in any squamate species nor in a case of lecithotropic viviparity. Although mammalian pregnancy has a distinct evolutionary origin from pregnancy in viviparous squamates, it seems highly likely that alternative splicing also plays a role in squamate pregnancy.

In the present study, I address this deficiency through the application of long-read RNA sequencing to characterise both gene expression and alternative splicing in viviparous *Z. vivipara*. I sequenced RNA from the oviduct tissues of 8 adult female lizards from the widespread Western Viviparous lineage (also called clade C) before, during and after pregnancy (**Fig. 1**) and analysed differential gene expression and differential transcript usage between all conditions. My findings add significant new knowledge to our understanding of genes expressed throughout the reproductive cycle in viviparous squamates, as well as identifying genes undergoing alternative splicing during pregnancy in this species which also play a role in mammalian pregnancy. My results highlight the value of analysing both alternative splicing and gene expression together to understand the transcriptomic underpinnings of complex traits such as pregnancy, and provide new targets for further research on the functional genetics of pregnancy and viviparity in *Z. vivipara* and other species.

2.3 Methods

2.3.1 Fieldwork and animal husbandry

Adult female *Z. vivipara* were collected from a large and stable wild population located at 55° 47′ 33″ – 55° 46′ 0″ N, 4°54′ 5″ – 4° 56′ 4″ W, on the western side of Great Cumbrae, an island off the west coast of Scotland at the northern end of the Firth of Clyde, with permission from NatureScot (licence no. 188744). Lizards were caught by hand by a team of 2-6 fieldworkers between April and September in 2021, 2022 and 2023. Pregnant lizards were identified by body-shape and the presence of one or more mating-bite marks on the ventral side just above the lower leg.

Lizards were housed in naturalistic conditions in secure outdoor enclosures in the courtyard of the Graham Kerr Building at the University of Glasgow, in groups of 1-5 lizards per enclosure. Enclosures were filled with topsoil to a depth of 20 - 30 cm, and included water dishes, live plants, dead wood, rocks and ceramic hides to provide suitable habitat. Enclosures were closed at the top with wire mesh, exposing lizards to natural temperature variations and photoperiod. Enclosures were supplied with fresh dechlorinated water and 1-3 beetle larvae (*Tenebrio molitor*) per individual every 2-3 days.

A total of six pregnant lizards were sacrificed after a period of 4 months in captivity. Time points for lizards sampled during pregnancy were selected based on the ratio of weight to snout-vent length (SVL) to control for pregnancy stage across individual and confirmed by embryo staging. Two postpartum lizards were sacrificed after 5 months in captivity, 2-4 weeks post-parturition. Lizards selected to be sampled pre-pregnancy were overwintered in captivity in a same-sex enclosure to deny any opportunity for mating and were then sampled shortly after emergence in spring, after around 6 months in captivity.

2.3.2 Oviduct sampling and RNA extraction

I selected lizards for sampling at three different stages of the annual reproductive cycle (**Fig 2-1**). Two lizards were sampled pre-pregnancy, after emergence from hibernation in spring (April 2022). Six lizards were sampled

during late pregnancy (embryo stage 39-40 as per Hubert, 1962) in summer (two in July 2022 and four in July 2023), and two were sampled after parturition in late summer (August 2022). The schedule one area was cleaned with 70% alcohol and all dissection tools were autoclaved. Lizards were sacrificed by concussion followed by immediate destruction of the brain in accordance with Schedule 1 of the Animals (Scientific Procedures) Act (ASPA) (Home Office 2013) with the approval of the Senior Named Veterinary Surgeon at the University of Glasgow and of NatureScot. Oviducts were dissected out and either used immediately for RNA extraction in the case of the four 2023 pregnant oviduct samples, or flash-frozen in liquid nitrogen and stored at -70 °C for later use for all other samples.

For RNA extraction, I thawed oviducts on ice (if required) and placed them in TRIzol reagent (Zymo Research, Irvine US) before lysing them using a FastPrep-24 5G lysis system (MP Biomedicals, Irvine US) at a speed of 7 m s^{-1}, for two 30s intervals, at room temperature. I then performed RNA extraction from oviduct samples using either an RNeasy Plus Mini Kit (Qiagen, Venlo NL) in the case of the post-partum and pre-pregnancy samples and the two 2022 pregnancy samples, or a Direct-zol RNA Miniprep Kit (Zymo Research, Irvine US) in the case of the four 2023 pregnancy samples, in accordance with the manufacturer's instructions.

The quantity and purity of the resulting RNA samples was checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham US) and RNA integrity was checked by agarose gel electrophoresis using a 1.5% agarose gel run for 20 minutes at 80 V. RNA extraction from the two pregnant lizards sacrificed in July 2022 unfortunately failed, with nanodrop readings indicating little to no RNA present after purification: this necessitated the collection and sacrifice of the four additional pregnant lizards in 2023. The changes to the RNA extraction protocol for these lizards (use of the Zymo Direct-zol RNA Miniprep Kit and use of fresh rather than flash-frozen tissue) were made to ensure successful extraction for these individuals based on advice from lab colleagues with experience of RNA extraction from difficult tissue types. Following extraction, all RNA samples were stored at -70 °C until library preparation.

2.3.3 Library preparation and sequencing

I prepared barcoded cDNA libraries from oviduct RNA samples with a PCR-cDNA Barcoding Kit (ONT Ltd., Oxford, UK) according to the manufacturer's instructions. RNA samples were thawed and kept on ice up until the reverse-transcription step in the library preparation. Libraries were checked for DNA quantity and purity using a Nanodrop spectrophotometer before adapter annealing and sequencing, confirming a nucleic acid concentration of at least 15 ng μL^{-1} and a 260/280 ratio of at least 1.7 for all samples. I loaded the prepared libraries onto R9.4.1 Flow Cells (ONT Ltd., Oxford UK) and sequenced using either a MinION portable sequencer for pre-pregnancy and postpartum samples, or a GridION benchtop sequencing device for pregnant oviduct samples (both from ONT Ltd., Oxford UK). I performed live basecalling using Guppy (Wick, Judd, and Holt 2019).

After sequencing, I concatenated basecalled reads for each barcode, trimmed adapters using porechop (v0.2.4) applying an extra end trim of 20 bp (Wick et al. 2017) and filtered the trimmed reads with filtlong (v0.2.1) using a mean quality weight of 9 (default 1) and a target base threshold of 5,000,000,000,000 bp ("GitHub - Rrwick/Filtlong: Quality Filtering Tool for Long Reads," n.d.). I then aligned reads to the most recent *Z. vivipara* reference genome (Bioproject accession PRJEB66180) using minimap2 (v2.24) (Heng Li 2018) and quantified transcripts from the aligned reads using salmon (v1.10.1) (Patro et al. 2017) running in ONT mode.

2.3.4 Data analysis and visualisation

I performed all statistical analyses using the statistical programming language R (v4.3.1) (R Core Team 2023). I first imported salmon read quantification data with tximport (v1.30.0) (Soneson, Love, and Robinson 2015), and mapped transcript quantifications to gene counts using the most recent *Z. vivipara* reference genome annotation (NCBI reference GCF_011800845.1) with GenomicFeatures (v1.54.1) and AnnotationDbi (v1.64.1) (Lawrence et al. 2013). I then analysed differential gene expression between all conditions (pre-pregnant, pregnant and post-parturition) using edgeR (v4.0.3) (Y. Chen, Lun, and Smyth 2016). In brief, I first normalised gene counts for library size, then filtered the

resulting matrix of gene-counts to remove lowly expressed genes (fewer than 10 reads per sample group). I then fitted a quasi-likelihood negative binomial generalized log-linear model (GLM) to conduct genewise statistical tests to calculate log fold-change (FC) and false discovery rate (FDR)-adjusted p-values for all genes between all pairs of conditions, and for the pregnant condition compared to the average of pre-pregnant and post-parturition samples. I also performed principal component analysis (PCA) on normalised gene counts.

I then carried out over-representation analysis of biological process (BP) gene ontology (GO) terms using topGO (2.54.0) (Alexa and Rahnenfuhrer 2023), using GO term annotations predicted directly from translated gene protein sequences using the web-based tool eggNOG-mapper (http://eggnog-mapper.embl.de) (Cantalapiedra et al. 2021; Huerta-Cepas et al. 2019). In brief, I tested for over-represented GO-terms using Fishers Exact Test with the "weight" algorithm, using gene lists of all significantly up- and downregulated genes (p-adj < 0.1) for each comparison, against a gene universe of all expressed genes across all samples. Eggnog-mapper was also used to provide annotations for genes which lacked gene-name level annotation in the reference genome, which was incorporated into a custom R script for DGE and DTU analysis.

Finally, I analysed differential transcript usage (DTU) using a two-stage analysis with DEXSeq (v1.48.0) (Anders, Reyes, and Huber 2012) and stageR (v1.24.0) (Van den Berge et al. 2017). I first used DRIMSeq (v1.30.0) (Robinson and Nowicka 2016) to filter out low-expressed transcripts. I then used DEXSeq to fit a GLM model of transcript usage for each gene. Finally, I used StageR to first screen for genes detected as participating in DTU by DEXSeq, and then confirm which transcripts for each of these genes participate in DTU, using an overall false discovery rate (OFDR or alpha) of 0.5. I generated Isoform Fraction (IF) values for all transcripts of all significant genes using IsoformSwitchAnalyseR (v2.2.0) (Vitting-Seerup, Sandelin, and Berger 2019). I used Pfam (https://www.ebi.ac.uk/Tools/hmmer/search/hmmscan) (Mistry et al. 2021) to predict protein domains, SignalP (https://services.healthtech.dtu.dk/services/SignalP-5.0/) (Teufel et al. 2022) to predict signal peptides, IUPred2A (https://iupred2a.elte.hu) (Dosztányi et al. 2005) to predict intrinsically disordered regions (IDRs) within transcripts,

DeepTMHMM (https://dtu.biolib.com/DeepTMHMM/) (Hallgren et al. 2022) to predict protein topology and DeepLoc2.0 (https://services.healthtech.dtu.dk/services/DeepLoc-2.0/) (Thumuluri et al. 2022) to predict protein location within the cell for all transcripts of all significant genes.

Figures were produced using ggplot2 (v3.4.4) (Wickham 2009) along with isoformSwitchAnalyseR for transcript plots and upSetR (v1.4.0) for UpSet plots. Boxplots show the interquartile range (box), median (midline), and range (whiskers) excluding outliers, where outlying points are considered points more than 1.5 times the 1QR outside the box. Unless otherwise stated in the figure or accompanying legend, boxplots show scaled relative expression values calculated by subtracting the mean of the normalised CPM for all samples and dividing by the standard deviation.

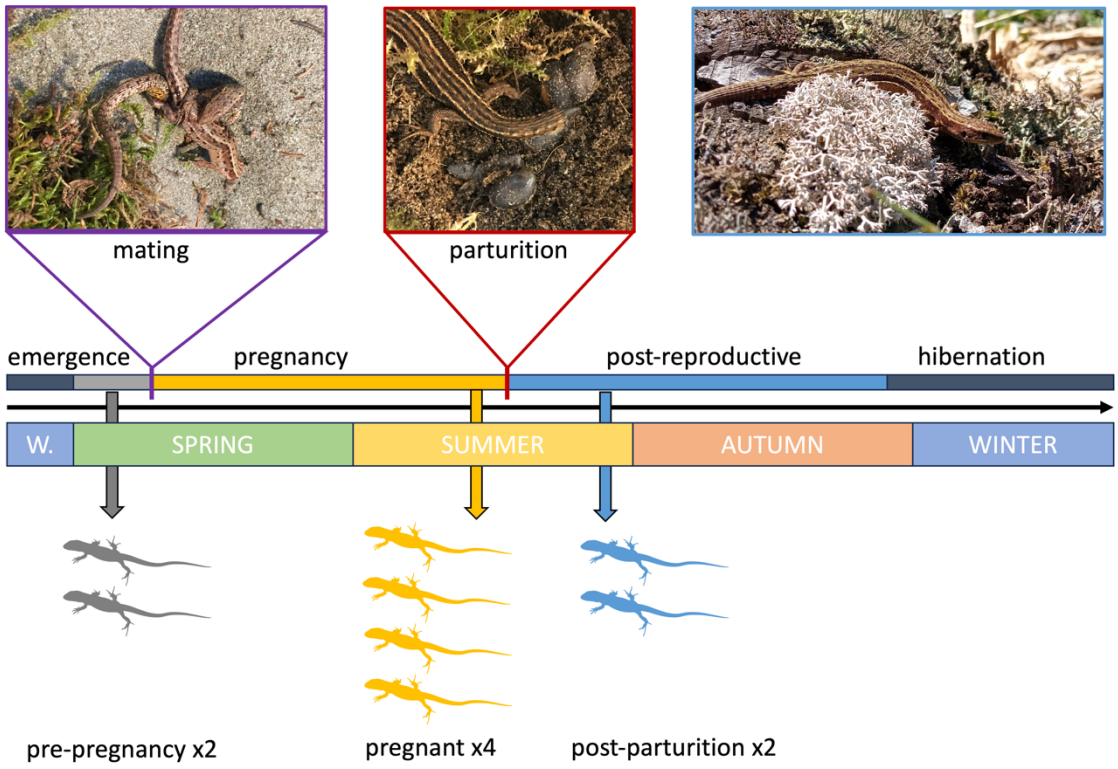

Figure 2-1. Sampling *Zootoca vivipara* oviduct throughout the reproductive cycle.

Diagram showing stages of the annual reproductive cycle in *Z. vivipara* and number of samples sequenced at each point. Adult female lizards were maintained in naturalistic conditions until sampling. Two lizards were sampled pre-pregnancy, after emergence from hibernation but before any opportunity for mating. Four were sampled during late pregnancy (embryo stage 40). Two further lizards were sampled post parturition, 2-4 weeks after giving birth in captivity. Open source *Z. vivipara* artwork by Henadii, iStock (2019) edited by Callan Denhan (2023), colour changed from original (license).

2.4 Results

2.4.1 Gene expression in the pregnant *Z. vivipara* oviduct

For the eight lizards sampled for this study (**Fig. 2-1**) I obtained 626,822 – 2,222,284 reads per sample, with between 84.43 – 94.17% of reads mapping successfully to the *Z. vivipara* reference genome **(Table S2-1)**. Median read length (N50) varied across sequencing runs from 913 bp – 1.52 kbp. Principal component analysis (PCA) showed well defined clustering of pre-pregnancy, pregnant and post-parturition samples, with PC1 (28.62% of variation) clearly separating the pregnant and non-pregnant samples whereas PC2 (22.27% of variation) separated pre-pregnancy and post- parturition oviduct samples with pregnant oviduct occupying an intermediate position between pre-pregnancy and post-parturition samples **(Fig 2-2)**.

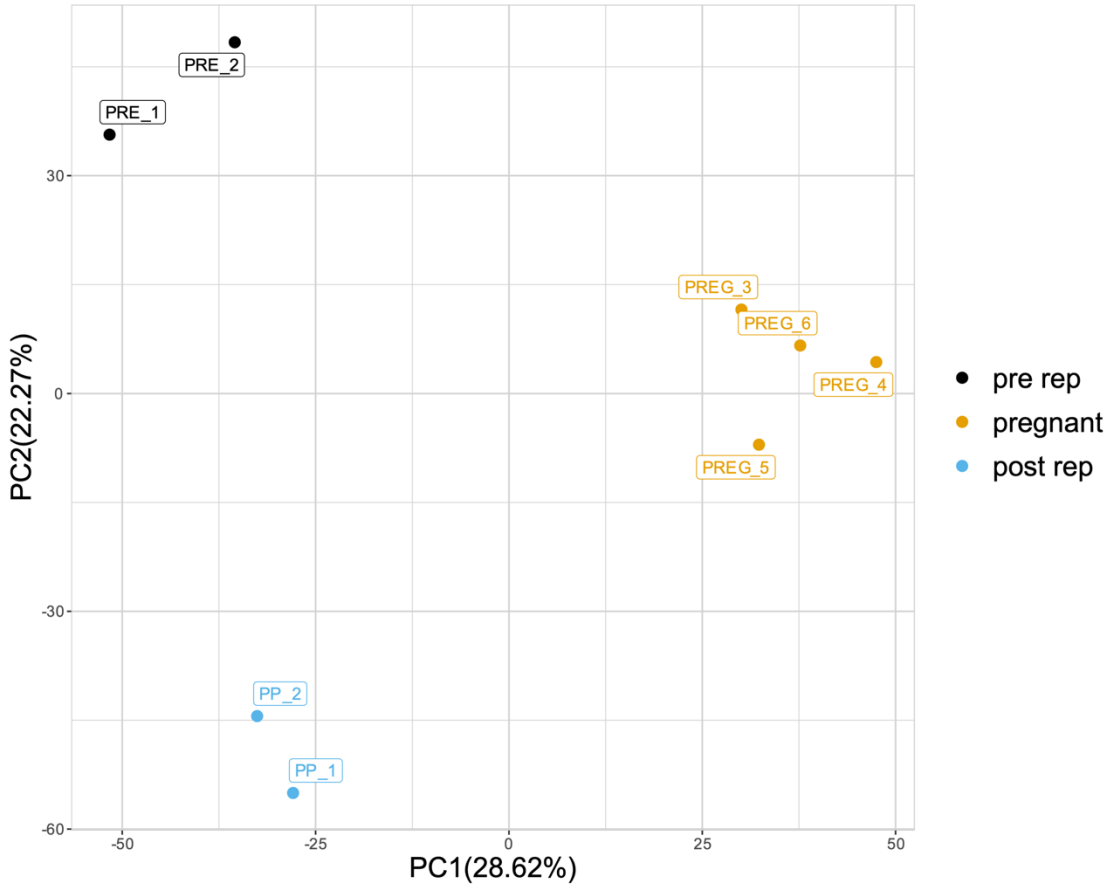

Figure 2-2. Gene expression in the Z. vivipara oviduct throughout the reproductive cycle.
Principle component analysis (PCA) results from gene expression in *Z. vivipara* oviduct tissue before (black), during (gold) and after (blue) pregnancy.

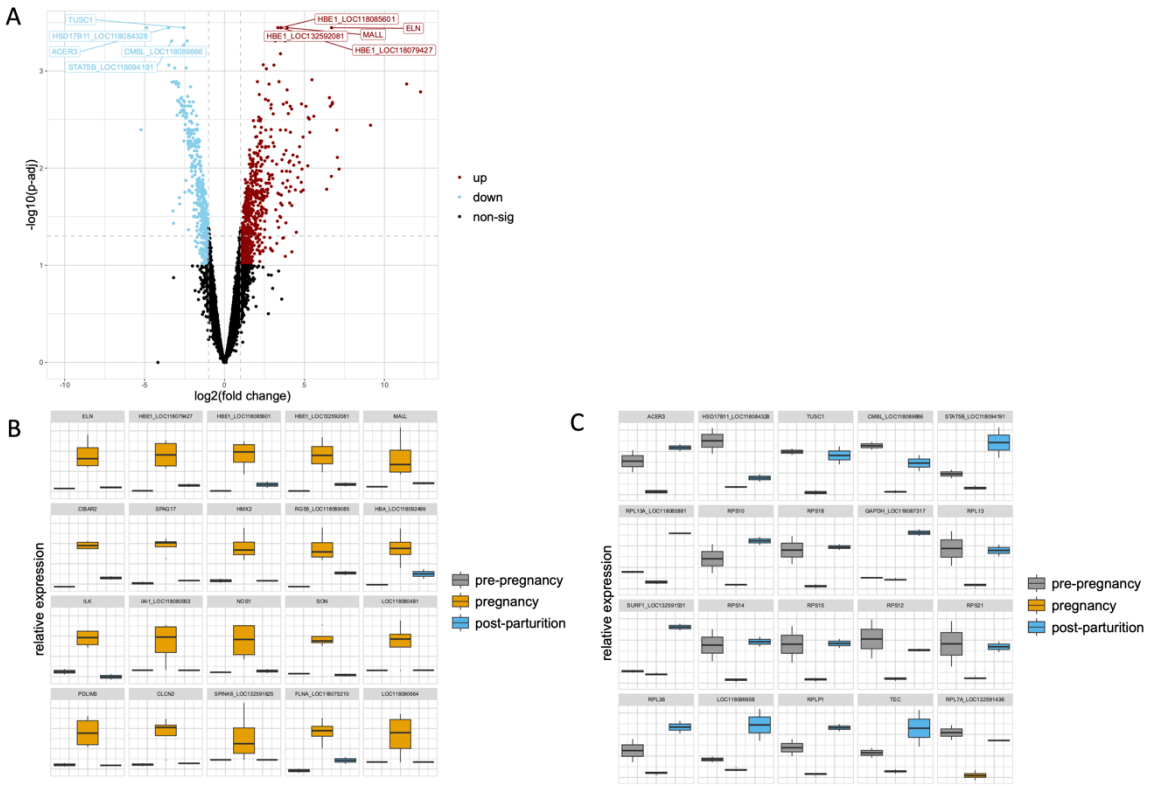

Figure 2-3. Differential gene expression during pregnancy in *Z. vivipara.*

***A*:** Volcano plot showing differentially expressed genes (DEGs) in pregnant Z. vivipara oviduct as compared to non-pregnant states (pre- and post-reproductive). Top 5 most significant up- and downregulated genes are labelled. **B:** Boxplots showing scaled expression values for the top 20 upregulated genes in pregnant vs. non-pregnant oviduct. **C**: Boxplots showing top 20 downregulated genes.

I first analysed gene expression changes both between pregnant and non-pregnant states overall, corresponding to the first component of the PCA which separated pregnant and non-pregnant samples, explaining 28.26% of overall variation in gene expression (**Fig. 2-2**), and allowing for greater statistical power by including 4 samples for each test condition. Comparing pregnant and all non-pregnant samples (**Fig 2-3)**, I found 1149 differentially expressed genes (DEGs) during pregnancy (**Table S2-2**), of which 696 were upregulated and 453 were downregulated. Top up- and downregulated DEGs included genes with a pre-established connection to pregnancy, such as NOS1, MALL and CLCN2 (upregulated) and TUSC1 and RPS14 (downregulated) (Recknagel et al. 2021). Top downregulated DEGs included many ribosomal genes, such as RPS10, RPS18 and RPL13 among others.

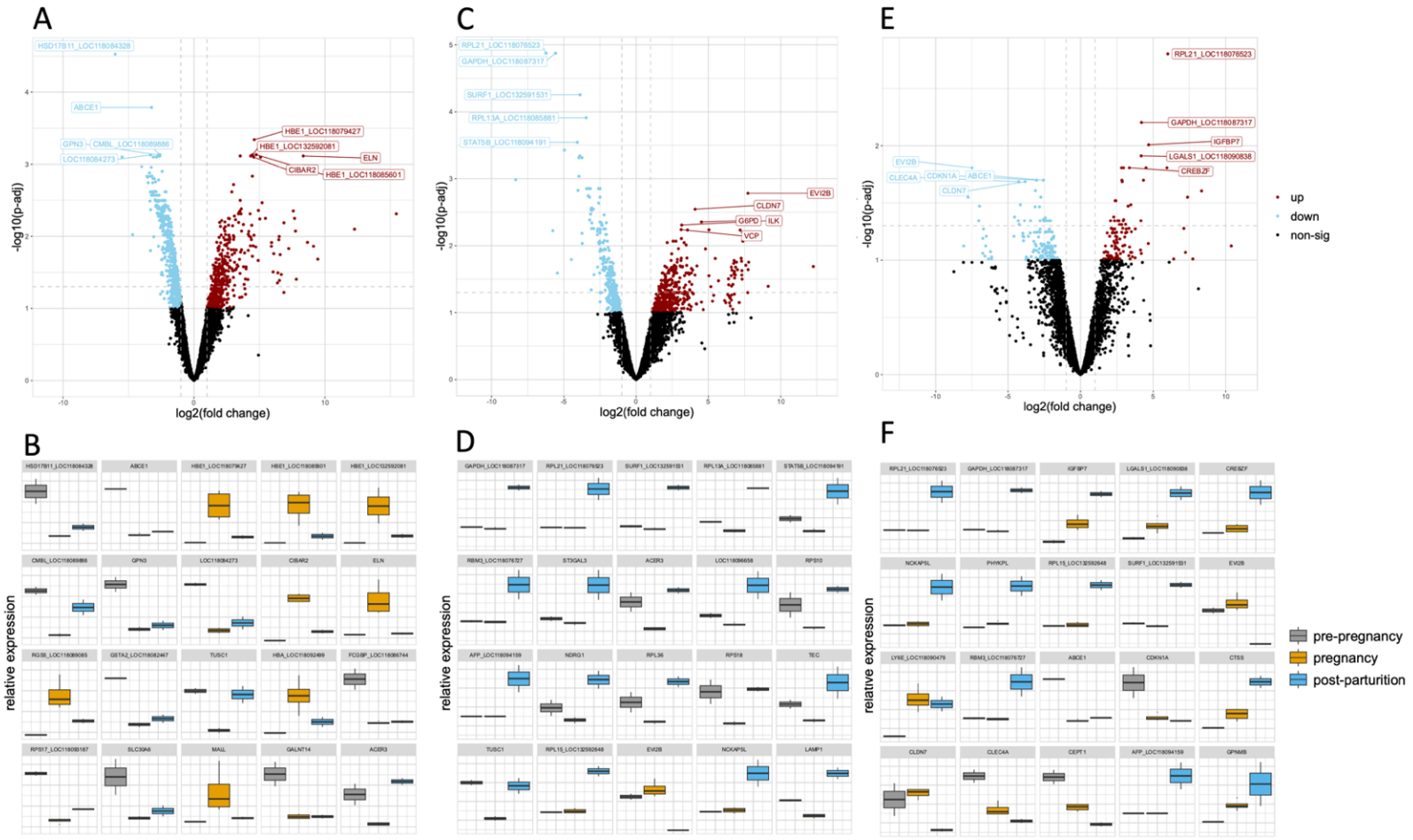

Figure 2-4. Differential gene expression before, during and after pregnancy.

A: Volcano plot showing DGE between pregnant and pre-pregnant oviduct (upregulated genes are upregulated in pregnancy). **B:** Boxplots for top 20 DEGs for pregnant/pre-pregnancy comparison. **C:** Volcano plot showing DGE between pregnant and post-parturition oviduct (upregulated genes are upregulated in pregnancy). **D:** Boxplots for top 20 DEGs for pregnant/post-parturition comparison**. E:** Volcano plot showing DGE between post-parturition and pre-pregnancy oviduct (upregulated genes are upregulated post-parturition. **F:** Boxplots for top 20 DEGs for post-parturition/pre-pregnancy comparison.

Over-representation analysis of DEGs (**Table S2-3**) upregulated during pregnancy found that highly significant GO terms included several connected to features with apparent relevance to pregnancy, for example cardiovascular processes (GO:1903779 and GO:0001525), cilia-related processes (GO:0003341 and GO:0044782), and muscle-related processes (GO:0042490, GO:0006936 and GO:0007517). Top significant GO terms for downregulated DEGs (**Table S2-4**) were dominated by terms relating to ribosomal activity and protein translation (e.g. GO:0002181, GO:0006413, GO:0042254, GO:0006414, etc.) as well as protein transport and localisation (GO:0006614, GO:0015833).

I then went on to specifically assess changes in uterine gene expression between pre-pregnancy, pregnancy, and post-parturition (**Fig 2-4**). 1159 genes were differentially expressed between pre-pregnancy and pregnancy (**Table S2-5**), including 512 upregulated and 647 downregulated genes (**Fig 2-4A**). Top differentially expressed genes are shown in **Fig. 2-4B**. This total included 732 genes identified as DEGs between pregnant and non-pregnant oviduct overall (see above). Among the 512 upregulated genes, over-representation analysis revealed terms relating to interactions with the extracellular matrix (GO:0030198, GO:0007160, and GO:0010811), transmembrane transport of various nutrients (GO:0015671, GO:0015701 and GO:1902476) and again included categories such as cardiovascular-related terms (GO:0001525 and GO:0002576) and muscle-cell related terms (GO:0006936) among the most significantly over-represented GO terms (**Table S2-6)**. For the 647 downregulated genes, the top over-represented GO terms were again dominated by terms relating to protein translation (GO:0002181, GO:0006614, GO:0006413, GO:0042254, GO:0006414 etc.) (**Table S2-7**).

From pregnancy to post-parturition, 784 genes were differentially expressed (**Table S2-8**), including 437 upregulated (during pregnancy) and 347 downregulated **(Fig 2-4C)**. Top differentially expressed genes are shown in **Fig. 2-4D.** This total included 583 genes identified as DEGs between pregnant and non-pregnant oviduct overall, and of these 334 were also differentially expressed between pre-pregnancy and pregnancy. Amongst the 427 upregulated DEGs, over-represented GO terms (**Table S2-9**) included those relating to cell migration and localisation (GO:0051674 and GO:0090131), and once again muscle

cell related processes (GO:0031038, GO:1902905, GO:1901739 and GO:0051153) in the top significant terms, in addition to a number of terms relating to dorsal closure, the morphogenetic process by which embryonic epithelial cells converge at the dorsal midline (GO:0007392, GO:0007395, and GO:0046664). For the 347 downregulated genes highly significant GO terms (**Table S2-10**) showed familiar themes of ribosomal and protein translation related processes (GO:0002181, GO:0006413, GO:0042255, GO:0042273 etc.).

Finally, I compared the pre-pregnancy oviduct to post-parturition oviduct directly, finding 260 DEGs (117 were upregulated and 143 downregulated) (**Fig 2-4E, F, Table S2-11**). Of these, 116 DEGs were also differentially expressed between pre-pregnancy and pregnancy, suggesting maintenance of differential expression of a minority of pregnancy-related genes for some time after parturition. On the other hand, 117 DEGs were shared with the pregnancy to post-parturition comparison, representing specific differences in gene expression after parturition, as distinct from pre-pregnancy or pregnancy. Upregulated genes in the post-parturition oviduct relative to pre-pregnancy were enriched for GO terms with relevance to the immune system (GO:0051138, GO:2000535) and transmembrane transport (GO:0015671, GO:0015701) among others (**Table S2-12**), whereas downregulated genes showed an overrepresentation of terms linked to protein transport (GO:0006606, GO:1904589, GO:0090087) and response to the feminising hormones progesterone (GO:0032570), estrogen (GO:0033148), and estradiol (GO:0032355) **(Table S2-13).**

2.4.2 Differential transcript usage

In addition to differential gene expression, I assessed alternative splicing through differential transcript usage (DTU) between pre-pregnancy, pregnancy and post-parturition oviduct. I found 35 genes with evidence of DTU between pre-pregnancy and pregnancy, of which 11 were also significantly differentially expressed in the same comparison; 91 genes with DTU between pregnancy and post-parturition, of which 16 were also differentially expressed; and 47 genes with DTU between pre-pregnancy and post-parturition, of which a single un-annotated gene (LOC118089512, eggnog mapper prediction: WFDC2) showed both differential expression and differential transcript usage (**Fig. 2-5**).

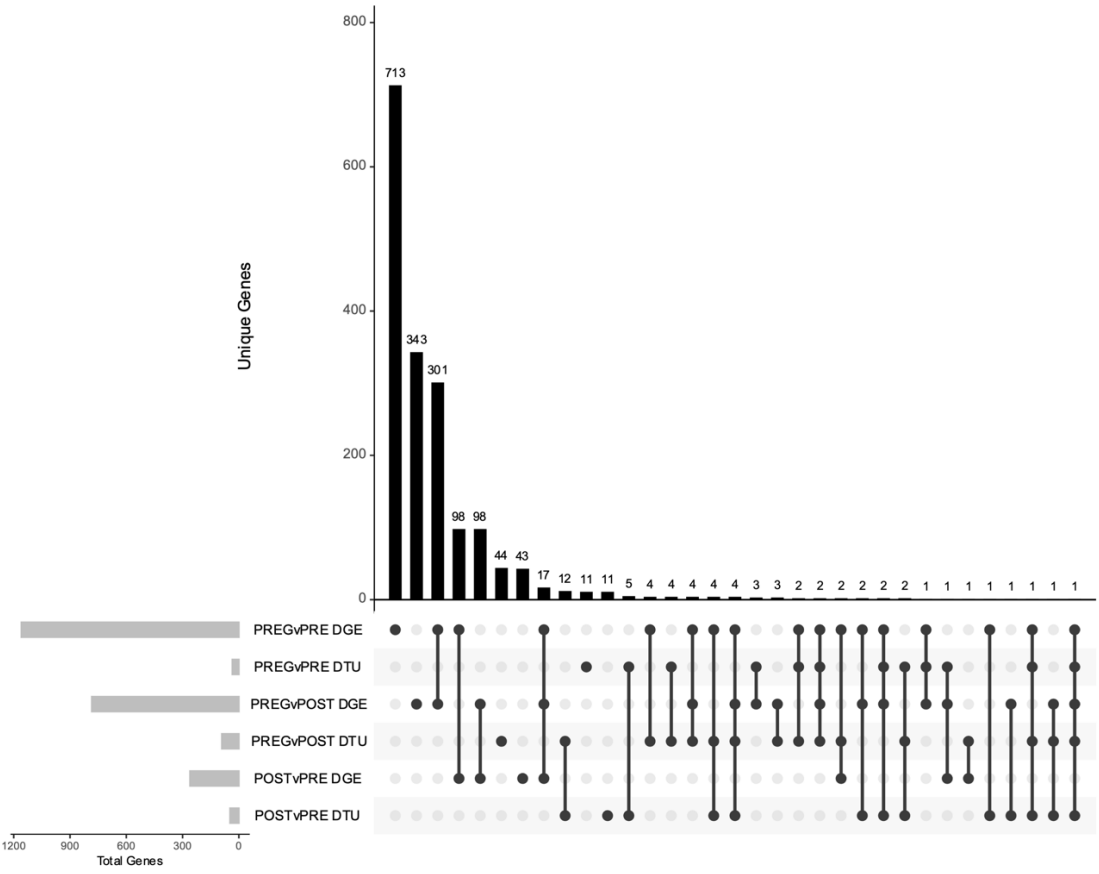

Figure 2-5. Comparison of differential gene expression (DGE) and differential transcript usage (DTU) during pregnancy in *Z. vivipara*.

Bar chart shows unique genes for each comparison across the reproductive cycle which showed significant DTU/DGE and shared genes for each combination of comparisons where one or more genes were significant for more than one comparison.

Of the genes for which significant DTU was detected, 12 genes were shared between both the pre-pregnancy/pregnancy, and post-parturition/pregnancy analyses: FAU, MARCHF3, MYL6, SRSF2, the unannotated gene LOC118083225 (eggnog mapper prediction: H3F3A), PRPS2, NDUFB9, C8H6orf52, ATP5F1C, SRI, BLOC1S2 and LPP. This list represents genes showing differences in isoform usage both between pre-pregnancy and pregnancy, and between pregnancy and post-pregnancy, and so are of particular interest. Of particular note are FAU and SRSF2, genes with an established role in mammalian pregnancy (Gu et al. 2018a; Chwetzoff and D'Andrea 1997; Gu et al. 2015a; G. Y. Nie et al. 2002; Gui Ying Nie, Li, Batten, et al. 2000), and LPP, which was previously shown to be

differentially expressed in pregnancy viviparous *Z. vivipara* as compared to gravid oviparous *Z. vivipara* (Recknagel et al. 2021).

Three isoforms of this relevant pregnancy candidate gene SRSF2 were found at detectable levels in my study: the protein-coding mRNAs XM_035104565.2 and XM_035104567.2, which differ in the conformation of the 3' untranslated region (UTR), and the alternative noncoding isoform XR_009557191.1. The two coding transcripts were both predicted to contain an RNA recognition motif (RRM) followed by an intrinsically disordered region (IDR) (**Fig 2-6A**). Overall, expression of SRSF2 decreased between pre-pregnancy and pregnancy (log FC -0.81, p-adj = 0.144), and again from pregnancy to post-parturition (log FC -0.37, p-adj 0.596) (**Fig 2-6B**), although the changes in expression were not statistically significant after correcting for multiple testing. Of the three isoforms, XM_035104565.2 showed significant participation in DTU during pregnancy when compared to both pre-pregnancy and post-parturition. XM_035104565.2 actually increased during pregnancy despite the overall decrease in expression of SRSF2 (**Fig 2-6C**). The isoform fraction (IF) XM_035104565.2 increased during pregnancy relative to pre-pregnancy (dIF = 0.298, p-adj < 0.001), before falling to undetectable levels post-parturition (dIF = -0.370, p-adj < 0.0001) (**Fig 2-6D**). Variation across reproductive stages in the proportions of the other two isoforms of SRSF was not statistically significant (p-adj > 0.1).

Two isoforms of FAU were detected, XM_035098279.2 and XM_035098280.2. Both are annotated as protein coding, although differing in the structure of the 5' UTR (**Fig. 2-7A**). Overall, FAU showed significant downregulation during pregnancy as compared to pre-pregnancy (log FC = -2.399, p-adj < 0.01), followed by an increase in gene expression post-parturition (log FC = 2.970, p-adj < 0.01) (**Fig. 2-7B**), with both isoforms following this general pattern of expression (**Fig. 2-7C**). However, subtle but significant shifts in the isoform fractions of both XM_035098279.2 and XM_035098280.2 were detected (**Fig. 2-7D**), with the less abundant isoform XM_035098280.2 increasing in proportion between pre-pregnancy and pregnancy (dIF = 0.082, p-adj < 0.0001) before decreasing again post-parturition (dIF -0.050, p-adj < 0.0001).

Four isoforms of LPP were detected, all annotated as protein coding mRNAs. All were predicted to code for intracellular proteins localised to the cytoplasm,

containing an intrinsically disordered region (IDR) followed by three LIM domains (**Fig. 2-7A**). The LPP gene was upregulated during pregnancy, both with respect to pre-pregnancy (log FC = 1.466, p-adj < 0.05) and post-pregnancy (log FC = 1.119, p-adj < 0.1) (**Fig. 2-7B**). Expression of different isoforms, however, differed dramatically from the overall trend (**Fig. 2-7C, D**). Pre-pregnancy, the isoform XM_060274253.1 was the only transcript of LPP detected, during pregnancy a mixture of all four isoforms were found in different proportions, and by post- parturition the isoform XM_060274248.1 had completely replaced all other transcripts of LPP.

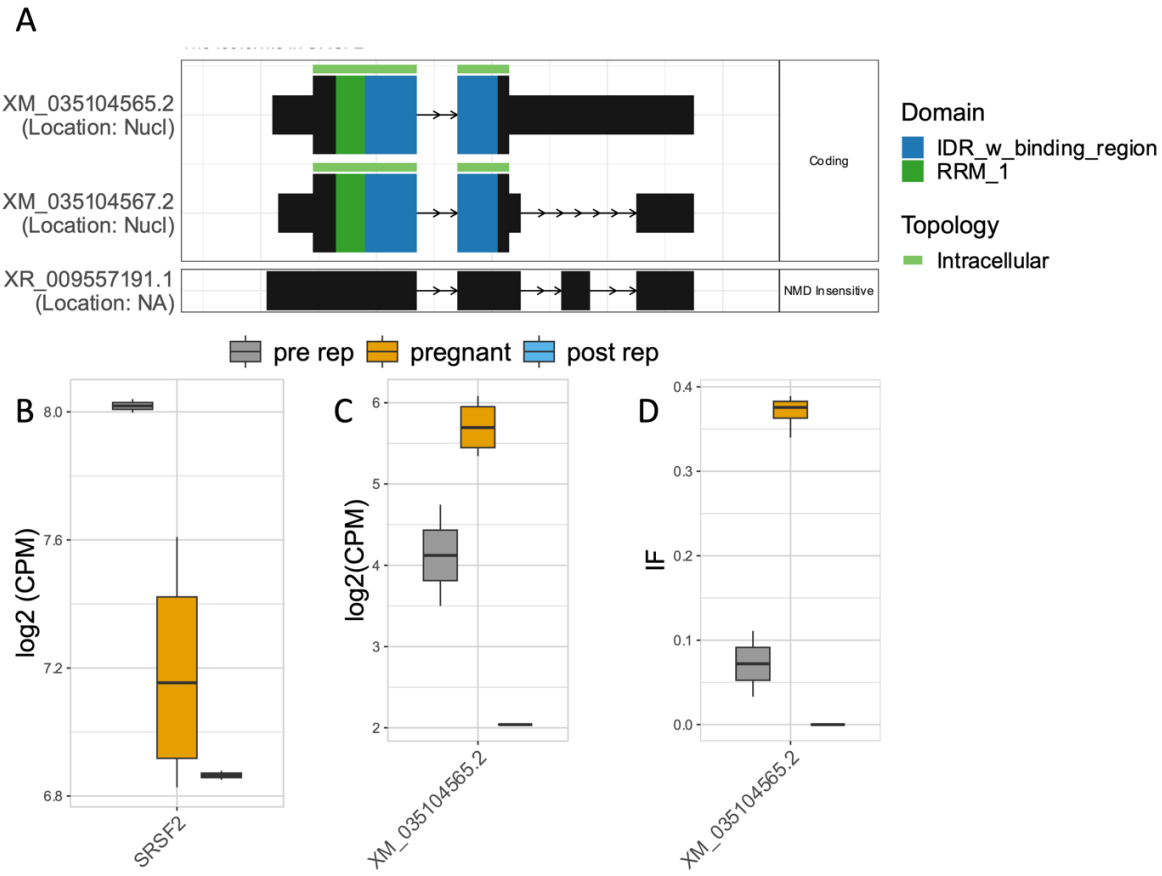

Figure 2-6. Isoform switching in SRSF2.

A: Diagram showing the three alternative transcripts of SRSF2 detected in this study. Coding regions are shown as double thickness with colors showing predicted protein domains. **B:** Overall expression levels of SRSF2 before, during and after pregnancy. **C:** Expression of transcript XM_035104565.2 of SRSF2 before, during and after pregnancy. **D:** Isoform fraction (IF) of transcript XM_035104565.2 before, during and after pregnancy.

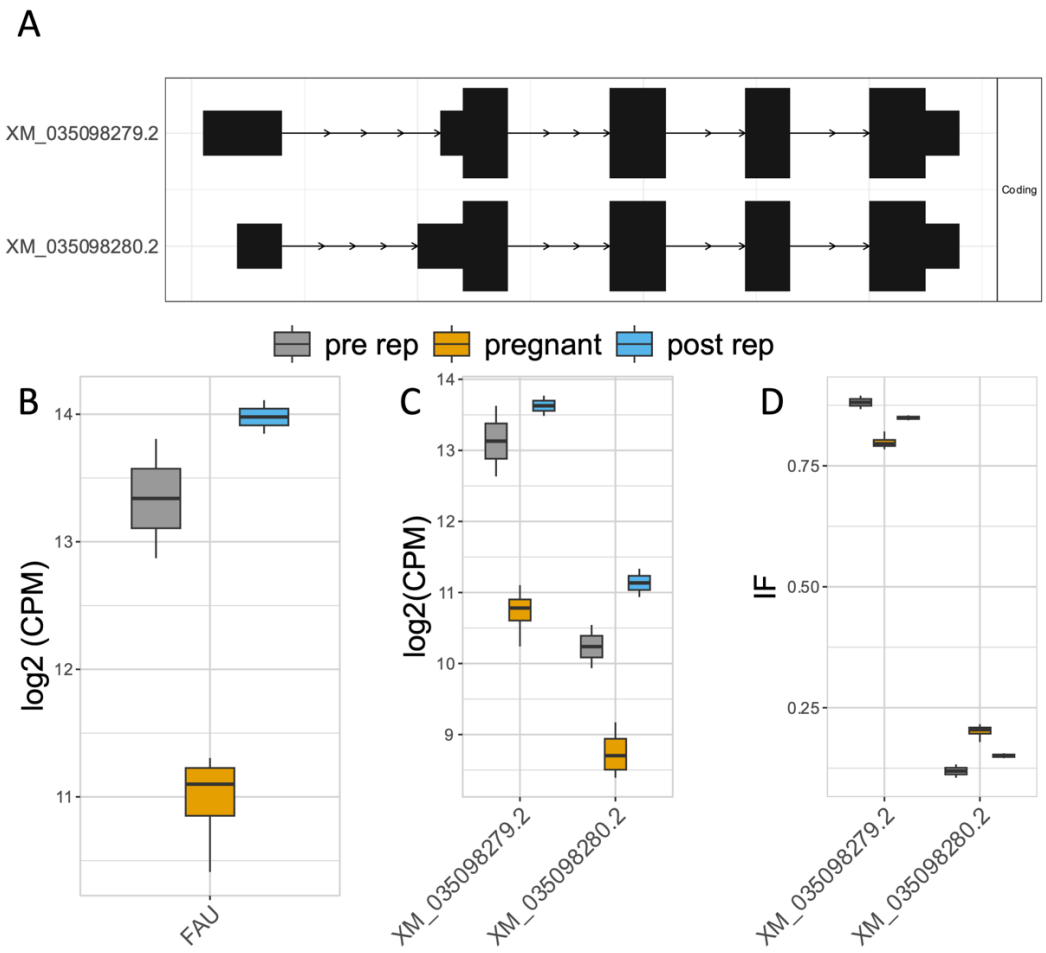

Figure 2-7. Isoform switching in FAU.

A: Diagram showing the two alternative transcripts of FAU detected in this study. Coding regions are shown as double thickness of untranslated regions (UTRs). **B:** Overall gene expression of FAU before, during and after pregnancy. **C:** Expression of each transcript of FAU before, during and after pregnancy. **D:** Isoform fraction (IF) of each transcript of FAU before, during and after pregnancy.

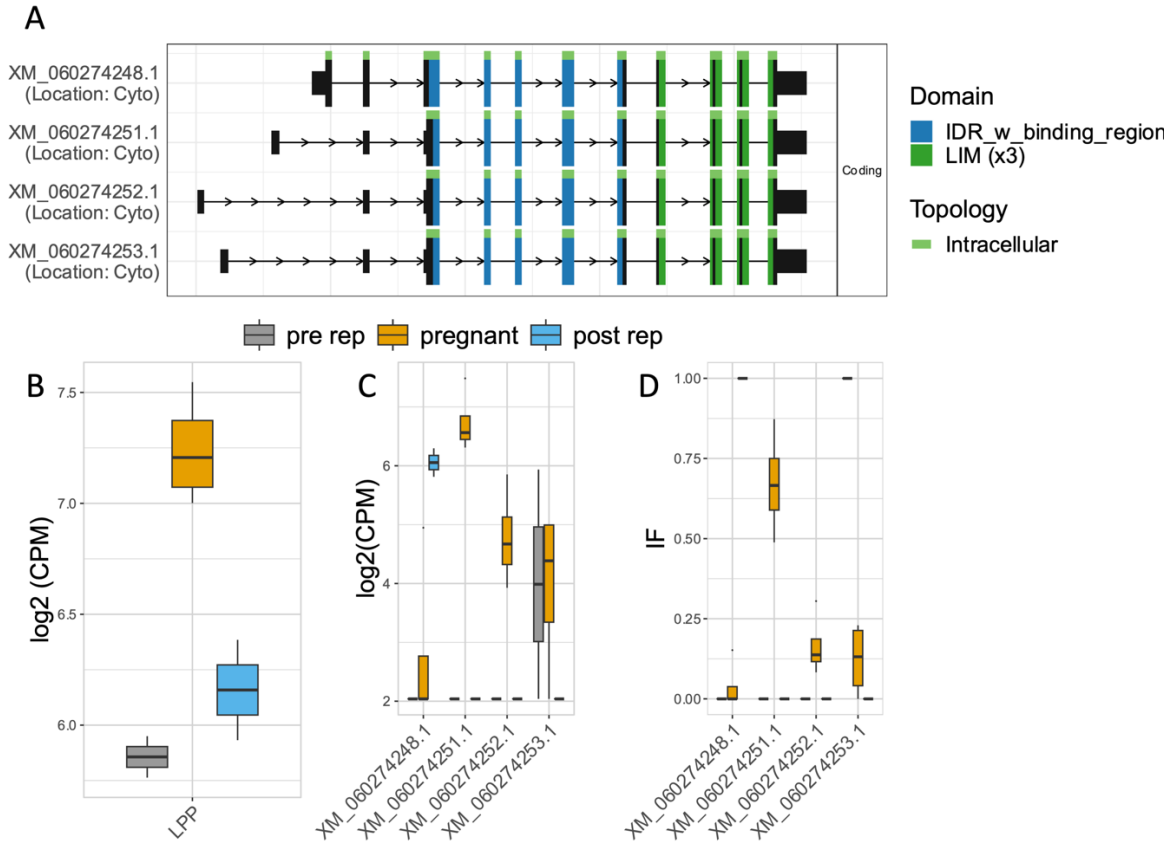

Figure 2-8. Isoform switching in LPP.

A: Diagram showing the four alternative transcripts of LPP detected in this study. Coding regions are shown as double thickness of untranslated regions (UTRs), with colours denoting predicted protein domains. **B:** Overall gene expression of LPP before, during and after pregnancy. **C:** Expression of the four alternative transcripts of LPP before, during and after pregnancy. **D:** Isoform fraction (IF) of the four alternative transcripts of LPP before, during and after pregnancy.

2.5 Discussion

2.5.1 Gene expression suggests shared transcriptomic basis of pregnancy in *Z. vivipara* and other viviparous squamates

I was able to characterise significant shifts in gene expression at different stages of the reproductive cycle in *Z. vivipara*. Previous research on *Z. vivipara* and other lizard species has reported some genes associated with pregnancy in squamates and many of these were also significant in this study. Notably, the 5th most significantly upregulated gene in pregnancy (compared to all non-pregnant states) was the Mal, T Cell Differentiation Protein Like gene (MALL), a member of the MAL proteolipid family. MALL has also been found to be upregulated during pregnancy in the viviparous African skink *Chalcides ocellatus* (Brandley et al. 2012) as well as in the viviparous Australian skink *Pseudemoia*

entrecasteauxii (Griffith et al. 2016). The Nitric Oxide Synthase gene (NOS1), part of the nitric oxide (NO) synthase family of genes, was the 13th most significantly upregulated gene for pregnancy (log FC 3.406, p-adj < 0.01) (**Fig 2-2C**) and has been previously shown to be differentially expressed in pregnant viviparous *Z. vivipara* as compared to gravid oviparous *Z. vivipara* (Recknagel et al. 2021), making this a candidate gene for involvement in the evolution of reproductive mode in this species. NOS1 is particularly known for its role in angiogenesis(Fukumura et al. 2001); given the role of the oviduct vasculature in squamate pregnancy this may well be connected to its upregulation in pregnant viviparous *Z. vivipara*. NO synthases have also been linked to pregnancy in mammals (H. Gao et al. 2009; Welter et al. 2005; Khorram, Garthwaite, and Magness 1999; Yoshiki, Kubota, and Aso 2000; Scott et al. 2007; Batra et al. 2003; Bartlett et al. 1999), possibly suggesting a shared role for NOS1 in the evolution of pregnancy in both mammals and squamates. Another highly upregulated gene in this analysis, the Chloride Voltage-Gated Channel 2 gene (CLCN2) (log FC 3.913, p-adj < 0.01) (**Fig 2-2C**), was also up-regulated during pregnancy in two other viviparous squamates, *C. ocellatus* (Brandley et al. 2012) and the viviparous form of the reproductively bimodal Australian skink *Saiphos equalis* (Foster et al. 2020). The authors of the latter study also found this gene to be significantly differentially expressed during pregnancy or gravidity when comparing viviparous and oviparous forms of *S. equalis,* suggesting a specific role for this gene in viviparous pregnancy. Moreover, chloride channel proteins are also important for mammalian pregnancy: they are abundantly expressed in the human placenta, and expression seems to be increased in response to disease states such as pre-eclampsia (Money et al. 2007; Murthi et al. 2012), possibly relating to their role in secretion (Duran et al. 2010). MALL, NOS1 and CLCN2 are therefore promising targets for future studies of gene function in pregnancy in viviparous squamates.

Top downregulated genes I identified also included genes with possible links to pregnancy in other species. In my dataset the Tumor Suppressor Candidate 1 gene (TUSC1) was the 3rd most strongly downregulated gene in pregnancy as compared to all other states (log FC = -2.546, p-adj < 0.001). TUSC1 is a tumour suppressor gene, but high TUSC1 expression in the endometrium has been linked to miscarriages in humans (Ran et al. 2022). Farther study of the role of TUSC1

downregulation in successful pregnancy is certainly warranted. This result parallels the downregulation of the Tumor Necrosis Factor gene (TNF) seen during pregnancy in *Pseudemoia entrecasteauxii* (Hendrawan et al. 2017). TNF itself was not significantly downregulated during pregnancy in my study, instead showing very low or undetectable levels of expression at all reproductive stages, although the functionally related TNF Alpha Induced Protein 8 gene (TNFAIP8) was downregulated in the pregnant oviduct when compared to pre-pregnancy (log FC -1.341, p-adj < 0.1).

2.5.2 Differential gene expression hints at potentially novel role for haemoglobin orthologues and ribosomal protein genes in *Z. vivipara* pregnancy

Intriguingly three of the most highly up-regulated genes during pregnancy (LOC118079427, LOC118085601 and LOC132592081) were all predicted to be the Hemoglobin Subunit Epsilon 1 gene HBE1 by eggNOG mapper, although none were annotated to gene name in the reference genome. In mammalian models HBE1 is normally expressed in the embryonic tissues very early in development, specifically in the earliest erythrocytes produced in the yolk sac, and is later supplanted first by the foetal haemoglobin genes expressed in the liver, and eventually by adult haemoglobins, in a process known as haemoglobin switching (Vinjamur, Bauer, and Orkin 2018). Little is known of the details of haemoglobin switching in squamate reptiles, although squamate haemoglobin genes are known to differ substantially from those of mammals in terms of both copy number and expression (Hoffmann et al. 2018). The possible expression of "embryonic" type haemoglobin genes in the adult uterus during late-stage pregnancy is unexpected, and certainly deserves further study given the importance of oxygen transport in late-stage pregnancy when the oxygen demands of the embryo are at their highest.

A particularly striking result from my study was the very high number of ribosomal genes which were found to be downregulated during pregnancy. The top 20 most downregulated genes for pregnancy relative to non-pregnancy included at least 9 ribosomal protein genes: Ribosomal Proteins S10, S18, L13, S14, S15, S12, S21, L36 (RPS10, RPS18, RPL13, RPS14, RPS15, RPS12, RPS21 and RPL36) plus the ribosomal protein lateral stalk subunit P1 (RPLP1). Furthermore,

eggNOG mapper results for the un-annotated genes identified two more ribosomal protein genes amongst this set of genes, ribosomal protein L13a (RPL13A, LOC118085881) and ribosomal protein L7a (RPL7A, LOC132591436), for 11 ribosomal protein genes in total – more than half of the top 20 most downregulated genes. The top 7 most significant BP:GO terms for genes downregulated during pregnancy all related to protein translation or ribosome synthesis. Ribosomal proteins are generally highly expressed across many cell types, as they are essential for ribosome synthesis, and thus by extension all of cellular life. However, this puzzling downregulation of ribosomal protein genes is not unique to *Z. vivipara*: in *C. ocelatus,* all but one (RPS18) of the ribosomal protein genes mentioned above were also significantly downregulated. Nor is the phenomenon unique to squamates; ribosomal protein genes also appear to be suppressed in the uterus of the rat *Rattus norvegicus* during pregnancy (Girotti and Zingg 2003). Other reports, however, point to upregulation of ribosomal genes in mammalian pregnancy, especially around early pregnancy and implantation (X.-H. Ma et al. 2006; Kidron et al. 1995). I caution that this evident downregulation of a large number of ribosomal genes does not necessarily indicate a reduction in ribosomes overall in uterine cells during pregnancy; while that is one possible interpretation of the data, other possibilities include changes in the turnover of ribosomes or their protein components, changes in the proportion of ribosomes, or changes in the abundance and characteristics (e.g. size) of cell populations in the oviduct. Further work is required to explore this pattern of ribosomal protein gene downregulation and to investigate its causes and consequences in *Z. vivipara* and other species.

Other genes which were highly differentially expressed which had not previously been linked to pregnancy included the Alkaline Ceramidase 3 gene (ACER3), part of a gene family linked to both cell proliferation and apoptosis (Mao and Obeid 2008) and the most strongly downregulated gene during pregnancy in our analysis (**Fig. 2-3C**); the CBY1 Interacting BAR Domain Containing 2 gene (CIBAR2), a little studied gene with a link to cilia assembly via interaction with the Chibby 1, Beta Catenin Antagonist (CBY1) gene (F.-Q. Li et al. 2016) and the sixth most strongly upregulated gene during pregnancy (**Fig. 2-3B**) and fourth most strongly upregulated gene during pregnancy compared to pre-pregnancy

(**Fig. 3B**); and the Carboxymethylenebutenolidase Homolog gene (CMBL), a cysteine hydrolase gene normally expressed in the human liver and digestive system (Ishizuka et al. 2010), which was the fourth most strongly downregulated gene during pregnancy (**Fig. 2-3**) These and other novel genes may play a unique role in *Z. vivipara* pregnancy or, given the sparse literature on oviduct gene expression in squamate pregnancy, may simply not have been detected in previous studies of gene expression in viviparous squamates.

2.5.3 Differential transcript usage during pregnancy suggests a significant role for alternative splicing and alternate promoter use in pregnancy in *Z. vivipara*

In addition to the role played by gene expression in the process of squamate pregnancy, I was also able to show a significant role for alternative splicing both in the transition from pre-pregnancy to pregnancy and from pregnancy to post-parturitio. Given the significance of alternative splicing in mammalian pregnancy (Ruano et al. 2021; Gopalakrishnan and Kumar 2020; Gui Ying Nie, Li, Batten, et al. 2000) these results are not unexpected, but they do help to fill in an important part of the picture of the transcriptomic regulation of pregnancy in viviparous squamates.

My analysis of differential transcript usage in *Z. vivipara* before, during and after pregnancy produced contrasting results to my analysis of gene expression. Whereas the largest difference in gene expression was observed between pre-pregnancy and pregnancy, this comparison saw the fewest genes with DTU, whereas the highest rates of DTU were seen in the pregnancy/post-parturition comparison (**Fig 5A**). Furthermore, many genes undergoing DTU were not recovered as significant by the DGE analysis (**Fig. 5B**). This result highlights the importance of considering both DGE and DTU when investigating the transcriptomic basis of complex traits, where regulation of many genes is implicated in determining phenotype, and the power of long-read RNA sequencing in describing a fuller range of gene regulation through both differential expression and alternative splicing (Jacobs and Elmer 2020). Although previous studies of squamate pregnancy have omitted to address alternative splicing directly, I note that one gene that was found to exhibit a complex pattern of both differential expression and alternative splicing during

pregnancy with respect to both post-parturition and pre-pregnancy states, was previously reported as differentially expressed between viviparous and oviparous *Z. vivipara* (Recknagel et al. 2021), evidencing a specific role in pregnancy in this species: the LIM Domain Containing Preferred Translocation Partner In Lipoma gene (LPP).

LPP is a gene with a complex cellular role. First characterised in connection with lipomas (Petit et al. 1996), LPP has been suggested as a mediator of cell-cell adhesion through interaction with cell junctions and the cytoskeleton (M. D. H. Hansen and Beckerle 2006; 2008), as a scaffold protein for the assembly of other protein complexes, and as a potential regulator of gene expression (Petit et al. 2000). Alternative splicing of LPP has been reported previously in connection with disease states such as gastric cancer and hypoxic-ischemic encephalopathy (HIE) (Y. Jin et al. 2022; Xue et al. 2020). Gastric cancer cells showed alternative splicing of LPP leading to 3rd exon-skipping and the loss of the triple LIM domain in the resultant protein product. On the other hand, rats with HIE showed evidence of usage of an alternative first exon of LPP. Notably, a study of LPP deficient mice found that female mice deficient in LPP showed a dramatic reduction in fertility, implying a possible role for LPP in mammalian pregnancy as well (Vervenne et al. 2009). The authors also found an alternative transcript of LPP, produced by an alternate promoter site immediately upstream of exon 7b, expressed specifically in the testes of male mice and localised entirely to the cell nucleus, as it lacked the nuclear export signal present on exon 7a.

My study also found both short (XM_060274251.1, XM_060274252.1 and XM_060274253.1) and long (XM_060274248.1) variants of LPP, although all four variant transcripts recovered from my analysis were predicted to be cytoplasmic, rather than nuclear, proteins by DeepLoc2.0 (**Fig. 2-8A**). As in the case in mice, these alternative transcripts appear to result from alternate promoter use. Although they do not seem to show the same pattern of alternative exon usage seen in the mouse study, the short variants do show a truncated version of exon 3 in which the coding sequence starts part of the way through the exon (**Fig. 2-8A**). These short forms of LPP appear to predominate before and during pregnancy, whereas post-parturition only the longer form is expressed (**Fig. 2-8C, D**), so this truncation of exon 3 may have functional

significance linked to pregnancy. All transcript variants also had different 5' UTR sequences, which, given the role of the 5' UTR in downstream regulation of mRNA translation (Ryczek, Łyś, and Makałowska 2023; Van Der Velden and Thomas 1999; Araujo et al. 2012), may have significant implications for LPP protein expression levels at different stages of the reproductive cycle. To my knowledge this is the first time that isoform switching in LPP has been implicated in pregnancy for any animal, and further investigation of the role of alternate isoforms of this gene in pregnancy in *Z. vivipara* is strongly indicated.

2.5.4 Alternative splicing of implantation genes SRSF2 and FAU implicated in *Z. vivipara* pregnancy

Among the candidates showing differential transcript usage before, during and after pregnancy in *Z. vivipara*, two genes had been previously linked to pregnancy in mammals: the Serine And Arginine Rich Splicing Factor 2 gene (SRSF2) and the FAU Ubiquitin Like And Ribosomal Protein S30 Fusion gene (FAU).

SRSF2 is itself a key component of the spliceosome (Kramer 1996), and an important regulator of alternative splicing in a range of biological contexts (Lei et al. 2023; Komeno et al. 2013; H.-H. Chen, Wang, and Fann 2006; H. Y. Wang et al. 2001). SRSF2 is ubiquitously expressed in most tissues (Fagerberg et al. 2014) – however, it also plays a critical role in early pregnancy in both primates and mice, specifically in implantation (G. Y. Nie et al. 2002; Gui Ying Nie, Li, Batten, et al. 2000; Salamonsen, Nie, and Findlay 2002). Implantation is a complex, multi-stage process involving the attachment of the mammalian blastocyst to the endometrium, and, in rodents, primates, and certain other placental mammals, the invasion of the embryo into the uterine wall (McGowen et al. 2014; Chavatte-Palmer and Guillomot 2007). Implantation of the blastocyst involves significant changes to both gene expression as well as alternative splicing in the mammalian uterus, for which SRSF2 is theorised to be a key regulator. SRSF2 in the uterus is strongly upregulated at implantation sites during early pregnancy in mice (Gui Ying Nie, Li, Batten, et al. 2000), as well as in primates during the secretory phase of the oestrous cycle (G. Y. Nie et al. 2002), when the mammalian uterus becomes receptive to embryo implantation.

Alternative splicing of SRSF2 is implicated in its role in mammalian pregnancy. Four distinct transcripts of the gene have been identified in the murine uterus, with distinct conformations of the 3' UTR (Gui Ying Nie, Li, Batten, et al. 2000). Changes to the 3' UTR of SRSF2 transcripts has been shown to alter the half-life, translational efficiency, and localisation of mRNAs (Sureau and Perbal 1994; Tushev et al. 2018; Tanguay and Gallie 1996), with potential ramifications for splicing of a host of other transcripts acted on by the spliceosome. The isoform XM_035104565.2, which showed significant variance in usage in this study, differed from the alternative coding mRNA XM_035104567.2 specifically in the 3' UTR (**Fig. 2-6A**), suggesting a role for alternative splicing of the 3' UTR sequence of this gene in the overall regulation of transcript splicing in pregnancy in *Z. vivipara* as well.

FAU is another gene which has been linked to mammalian pregnancy, and specifically implantation (Gu et al. 2018b; Gui Ying Nie, Li, Hampton, et al. 2000; Gu et al. 2015b; Chwetzoff and D'Andrea 1997). The protein encoded by FAU is a fusion protein comprising the ubiquitin-like protein FUBI and the ribosomal protein S30, with the resulting protein product cleaved to yield both proteins (Kas, Michiels, and Merregaert 1992; van den Heuvel et al. 2021). The FUBI protein is a subunit of the MNSF protein complex, which functions as a nonspecific suppressor of B- and T-cell-mediated immune response (Nakamura, Ogawa, and Tsunematsu 1987), and its role in implantation and pregnancy in mammals is likely linked to modulation of the maternal immune response in the endometrium to prevent rejection of the embryo. Like SRSF2, it has been shown to be downregulated at implantation sites in the murine uterus (Gui Ying Nie, Li, Hampton, et al. 2000).

FAU appears strongly downregulated in the pregnant *Z. vivipara* oviduct (log FC -2.684, p-adj < 0.01) (**Fig. 4-6B**), consistent with the downregulation of other ribosomal protein genes (**Fig. 2-3C**) given that the fusion protein product of FAU includes RPS30. Additionally, this downregulation is accompanied by a subtle but significant shift in the IF of the two alternatively spliced transcripts of FAU, with the less abundant transcripts XM_035098280.2, containing a different conformation of the 5' UTR, showing a slight but consistent increase in isoform fraction. Given that the translated FAU fusion protein includes the FUBI protein

at the N-terminus, this isoform switch likely primarily affects FUBI, even if the pattern of whole gene expression may simply reflect overall downregulation of ribosomal protein genes acting on the RPS30 product. It is notable that mouse model studies of the role of FAU in the endometrium during implantation also recovered isoforms of FAU that differed in the 5' UTR (Gui Ying Nie, Li, Hampton, et al. 2000), suggesting that changes to this region may also be functionally relevant to pregnancy in mammals.

Invasive implantation as seen in mice and primates is a derived process which is largely specific to mammalian pregnancy (though see Blackburn & Flemming, 2010), and the patterns of overall gene expression for SRSF2 and FAU observed in this study contrast to those seen at implantation sites in mouse models of pregnancy. Furthermore, these expression patterns in mice were observed at the start of pregnancy, during initial attachment of the embryo to the implantation site, whereas my study looked at late-stage pregnancy in *Z. vivipara*. Given these differences in expression patterns, the separate evolution of pregnancy in *Z. vivipara* and mammals, and the different physiological processes underpinning pregnancy in mice and common lizards, I expect that these genes likely play a somewhat different role in pregnancy in *Z. vivipara* as compared to mice or other mammals. However, given their significance for pregnancy in both *Z. vivipara* and in several mammals, the role of alternative splicing of these genes during pregnancy in *Z. vivipara* other species should be a target for future research.

2.5.5 Conclusions

I present the first report on the role of alternative splicing and alternate promotor use in pregnancy in a viviparous squamate, and the first study of gene expression during pregnancy in the widespread Western Viviparous lineage of *Z. vivipara*, an important emerging model organism for researchers working on the evolution of pregnancy. My results underline previous findings showing convergence in the molecular basis of pregnancy in squamates and other groups (Brandley et al. 2012; Recknagel et al. 2021), while also suggesting the possibility of novel functions in squamate pregnancy for genes which are also involved in pregnancy in other amniotes (e.g. FAU). These results further reinforce the importance of alternative splicing in pregnancy, already well

evidenced in mammals but previously unknown in squamates. It is my hope that these findings lay the groundwork for future experiments to characterise the function of pregnancy-related genes in pregnancy in *Z. vivipara* and further deepen our understanding of the molecular basis of pregnancy in this species by giving insight into the whole picture of transcriptomic regulation, including not just whole-gene expression but also isoform switching (alternate promoter use and alternative splicing) as key regulatory processes that act on different genes throughout the reproductive cycle.

3 Single-cell RNA sequencing of pregnant *Z. vivipara* oviduct offers insights into expression of candidate genes for the evolution of viviparity

3.1 Abstract

The evolution of viviparity is an important topic of research and RNA sequencing has yielded significant insights into the evolution of viviparity in several squamate species, including recent work linking expression of specific candidate genes to the evolution of viviparity in multiple squamate species and mammals (Recknagel et al. 2021). I characterised expression of these candidate genes during pregnancy in different cell types present in the squamate oviduct by conducting the first single-cell RNA sequencing study of the squamate oviduct, in the Eurasian common lizard *Zootoca vivipara*. I sequenced RNA from 13,369 cells across one pregnant and one post-parturition oviduct sample to explore how changes in gene expression during pregnancy are realised at the cellular level. I was able to tentatively identify several cell types present in the oviduct, characterise changes in cell populations during and after pregnancy, and provide single-cell resolution of the expression of candidate genes for viviparity. These results provide a foundation for future research on the cellular basis of viviparity in *Z. vivipara* and other species.

3.2 Introduction

Viviparity is the carrying of offspring within the mother's body cavity until full development, contrasting with oviparity, in which offspring are deposited in eggs to complete their development externally. This mode of reproduction has evolved many times in vertebrates and most frequently in squamate reptiles, with over 100 independent transitions known (Mika et al. 2022), making squamate viviparity an area of active research for evolutionary biologists working on major evolutionary transitions. Viviparity involves important structural and functional changes to the reproductive tract to facilitate extended retention of the embryo within the oviduct, such as modified vasculature to supply sufficient water and oxygen to the developing embryo as it grows (Adams et al. 2005; Michael B. Thompson et al. 2004), modulation of the innate immune response to avoid rejection of the embryo (Graham et al. 2011b),

and reduction in the number and size of shell glands (Stewart et al. 2010; Benoit Heulin et al. 2005). In some derived squamate lineages, these adaptations also include placentrophy: the provision of energy to the developing embryo directly from the mother's blood via complex placental structures analogous to those in placental mammals (Daniel G. Blackburn 1992). However, the majority of viviparous squamates are lecithotrophic, with the developing embryo reliant on its own yolk sac for all of its energy needs (Daniel G. Blackburn 2015b; Stewart 2013).

The squamate oviduct at the microscopic level is composed of three tissue layers, the outermost of which is the *muscularis externa*, an outer muscular sheath composed of smooth muscle cells (SMCs). Below the *muscular externa* lies the *lamina propria*, a complex layer of connective tissue containing diverse cell types, such as fibroblasts, macrophages, and mast cells, as well as cells composing the shell glands and vasculature. Finally lining the inner surface of the oviduct is a layer of epithelial cells (Uribe et al. 1988). The specific composition and structure of the oviduct varies between species and throughout the reproductive cycle; for a full review and detailed description of oviduct structure and function in squamates, see Blackburn (1998).

The significant structural and functional adaptations to viviparity in the oviduct suggest equally dramatic changes in gene expression. While the morphology and histology of the viviparous oviduct have been studied since the 19th century (Daniel G Blackburn, Avanzati, and Paulesu 2015; Daniel G. Blackburn 2016), the last decade has seen the publication of several landmark studies on the transcriptomic basis of viviparity (Griffith et al. 2016; Hendrawan et al. 2017; Foster et al. 2020; Recknagel et al. 2021; Brandley et al. 2012). This expanding body of work has yielded intriguing clues about the evolutionary convergence at the level of gene expression between viviparous squamates and viviparous mammals, and identified candidate genes for the evolution of viviparity across diverse evolutionary lineages (Recknagel et al. 2021), such as the legumain gene LGMN or the Acyl-protein thioesterase gene LYPLA1. However, to date all previous studies of viviparity in squamates have depended on traditional bulk RNA sequencing, that is, measuring gene expression at the level of the oviduct as a whole.

Single-cell RNA sequencing is an emerging technology enabled by recent advances in microfluidics and next-generation nucleotide sequencing, which enables the analysis of gene expression at the level of individual cells. Single cells are first dissociated and suspended in solution, and then isolated in microfluidic droplets through limiting dilution. Nucleic acids from each cell are then barcoded by PCR primers containing droplet-specific barcodes, and the resulting libraries can then be recombined and sequenced normally, with the barcodes allowing for identification of specific reads to individual cells (Jovic et al. 2022). Such detailed resolution allows for the detection of subtle changes in gene expression in specific cell types, and the characterisation of broader patterns of gene expression in different cell types within tissues, and has already seen extensive application in the mammalian reproductive system, yielding important insights into the cellular mechanisms of pregnancy in health and disease (e.g. Fitzgerald et al., 2019; Garcia-Flores et al., 2023; He et al., 2022; Ji et al., 2024; Rytkönen et al., 2022).

Here I report the first application of single-cell RNA sequencing to the reproductive system in a squamate reptile, specifically in the viviparous Eurasian common lizard *Zootoca vivipara*, a small insectivorous lizard with a broad distribution across northern Eurasia (Jose L. Horreo et al. 2018). *Z. vivipara* is a key emerging model organism for the evolution of viviparity, as it is one of a handful of squamate species which is reproductively bimodal, with extant viviparous and oviparous lineages thought to have diverged as recently as 4.5 MYA (Luca Cornetti et al. 2014b). Viviparous *Z. vivipara* exhibit lecithotrophic viviparity, with pregnancy lasting around two months, and the fully developed young born enclosed in a non-calcified membrane (Stewart, Heulin, and Surget-Groba 2004). Recent work on this species has revealed key genes linked to the determination of parity mode through admixture mapping of naturally hybridising viviparous and oviparous populations and bulk RNA sequencing of pregnant viviparous and gravid oviparous lizards (Recknagel et al. 2021). I was able to build on this work by providing the first single-cell resolution data on expression of these candidate genes both during pregnancy and post-parturition, in what I hope will provide a foundation for future research on the evolution of viviparity at the cellular level. By identifying the cell types in which particular

candidate genes are expressed, I shed fresh light on the functional role such genes may play in determining reproductive phenotype.

3.3 Methods

3.3.1 Fieldwork and animal husbandry

Pregnant female *Zootoca vivipara* were caught by hand by a team of 2-6 fieldworkers from a wild population on the island of Great Cumbrae in the Firth of Clyde (55° 47′ 33″ – 55° 46′ 0″ N, 4°54′ 5″ – 4° 56′ 4″ W) in April and May 2021 with permission from NatureScot (licence no. 188744). Pregnant females were identified by body-shape and the presence of one or more crescent shaped mating-bite marks on the lower ventral surface of the body.

Lizards were housed in naturalistic conditions in secure outdoor enclosures in the courtyard of the Graham Kerr Building at the University of Glasgow, in groups of 1-5 lizards per enclosure. Enclosures were filled with topsoil to a depth of 20 - 30 cm, and included water dishes, live plants, dead wood, rocks and ceramic hides to provide suitable habitat. Enclosures were closed at the top with wire mesh, exposing lizards to natural temperature variations and photoperiod. Enclosures were supplied with fresh dechlorinated water and 1-3 beetle larvae (*Tenebrio molitor*) per individual every 2-3 days.

3.3.2 Oviduct single-cell sample preparation and optimisation

To prepare suspensions of single cells for library preparation using the 10X Chromium single cell protocol I first carried out dummy preparations using two pregnant female lizards sacrificed in June 2021 after about 3 months in captivity. Library preparation requires a suspension of individual cells at a concentration of between 500-1000 cells μL^{-1} in a volume of at least 100 µL, for a total of 50,000 cells. Additionally, cell death must be minimised as lysed cells will leak RNA into solution, leading to contamination of other cell barcodes with transcripts from apoptotic cells. The dummy preparations were therefore carried out to optimise my protocol to obtain sufficient cell yields and cell viability from processed oviduct tissues.

The schedule one area was first cleaned with 70% alcohol and all dissection tools were autoclaved. The two lizards selected for the dummy preparations were sacrificed by concussion followed by destruction of the brain in accordance with the Animals (Scientific Procedures) Act 1986 (Home Office 2013) with the approval of the Senior Named Veterinary Surgeon and of NatureScot. Oviducts were dissected out and washed in a solution of phosphate buffered saline (PBS) with 0.75 μg mL^{-1} amphotericin B (Thermo Fisher Scientific, Waltham USA), 10 mL L^{-1} penicillin-streptomycin (Thermo Fisher Scientific, Waltham USA) and 1 mg mL^{-1} Primocin (Invivogen, Toulouse France) to eliminate fungal or bacterial contamination. Oviducts were first processed manually with sterile scalpel blades to break up the tissue, and fragments were then digested in 1 mg mL^{-1} collagenase P (Sigma-Aldrich Company Limited, St Louis USA) dissolved in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham USA). For the first dummy preparation the oviduct tissue was digested for 12 hours at 30 ºC; for the second for 2 hours at 37.5 ºC. Digested samples were pelleted by centrifugation for 5 minutes at 400 RCF at room temperature and resuspended in a solution of 0.4 mg mL^{-1} bovine serum albumen (BSA) dissolved in PBS. This centrifugation step was repeated to remove extracellular RNA, and the resulting cell suspension was then strained to remove clumps of non-disassociated cells using 40 μm Flowmi cell strainers (SP Scienceware, Warminster USA). Cell concentration was estimated using a Neubauer counting chamber, and was within the target range for both samples. Cell viability was confirmed using a LIVE/DEAD viability cytotoxicity kit (Thermo Fisher Scientific, Waltham USA) via fluorescence microscopy. 10 μL of cell suspension was combined with the LIVE/DEAD reagents and a single droplet of the resulting mixture was placed on a microscope slide. 3x fields were then captured using the red (fluorescing in dead cells) and green (fluorescing in live cells) fluorescent channels in a haphazard fashion. Cells present in each image were counted automatically using ImageJ, and the proportion of live cells was calculated as a percentage of total cells and averaged across all three fields. For the first dummy preparation, viability was below recommended levels; I therefore performed the second dummy preparation with a shorter incubation step carried out at a higher temperature which succeeded in reducing cell death during sample preparation to below 10% of cells in the final solution.

Ultimately, one lizard was selected for sampling during pregnancy in June and one 7 days after parturition in July 2021, after 3 and 4 months in captivity respectively. Lizards were sacrificed by the same method described for the dummy preparations and oviducts were dissected out and washed as described above using the same procedure and reagents. Oviducts were processed manually with sterile scalpel blades and then digested in 1 mg mL^{-1} collagenase P dissolved in DMEM for 2 hours at 37.5 ºC. Digested samples were pelleted by centrifugation for 5 minutes at 400 RCF at room temperature and resuspended in a solution of 0.4 mg mL^{-1} BSA dissolved in PBS. This centrifugation step was repeated to remove extracellular RNA, and the resulting cell suspension was then strained to remove clumps of non-disassociated cells using 40 µm Flowmi cell strainers as previously described. Cell concentration was estimated using a Neubauer counting chamber. For the second, post-parturition sample the cells were further concentrated by centrifugation at 400 RCF at room temperature and resuspension to ensure cell concentrations between 500-1000 cells μL^{-1}, as initial cell concentration after straining fell slightly below this threshold. Cell viability was confirmed using a LIVE/DEAD viability cytotoxicity kit as previously described for the dummy preparations.

3.3.3 Library preparation and sequencing

Libraries were prepared by Glasgow Polyomics (J Galbraith). Library preparation was performed using Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (10X Genomics Inc., Pleasanton US) in accordance with the manufacturer's protocol (see: https://www.10xgenomics.com/support/single-cell-gene-expression/documentation/steps/library-prep/chromium-single-cell-3-reagent-kits-user-guide-v-3-1-chemistry). 50,000 cells per sample were sequenced using the NextSeq 500 System (Illumina, San Diego USA) for 28 bp for the initial index read for 28 cycles, and 130 bp for the second read for 91 cycles at Glasgow Polyomics.

3.3.4 Data analysis and visualisation

Raw reads were first processed into counts using 10x Genomics Cell Ranger (v7.2.0) (G. X. Y. Zheng et al. 2017) with standard settings. As a reference I used the latest *Z. vivipara* reference genome (NCBI accession GCF_963506605.1) –

however, as this genome lacked mitochondrial genome in its archive, I combined this genome with the mitochondrial genome from a previous *Z. vivipara* reference genome (NCBI accession GCF_011800845.1). Following Cell Ranger counts, the features file was further updated to fix annotation of mitochondrial genes to facilitate downstream filtering.

Data processing, analysis and visualisation were done using Cellenics community instance (https://scp.biomage.net/) hosted by Biomage (https://biomage.net/). Filtering and data processing was performed using standard settings, with the following exceptions. Firstly, the mitochondrial content filter was manually set to 25% after reviewing the mt-gene content of in-cell reads, as the default setting was extremely lax, especially for the pregnant sample which contained a high proportion of cells with over 25% mt-genes. Secondly, mitochondrial genes were excluded from the data integration step, due to the overall high mt-gene content. Thirdly, Louvain clustering resolution was reduced to 0.4 to better accord with uniform manifold approximation and projection for dimension reduction (UMAP) embedding (Becht et al., 2018). Higher resolution clustering produced clusters that were poorly defined with respect to UMAP embedding, and a resolution setting of 0.4 is within the range recommended by benchmarks for standard single cell datasets (Seth et al. 2022). I then explored differential gene expression between different clusters and groups of clusters and all other cells using presto (Korsunsky et al. 2019) as implemented in Cellenics. Figures shown were produced using the Cellenics data visualisation tools.

Tables of differentially expressed genes were downloaded from Cellenics in .csv format. To identify genes not annotated by gene symbol in the official NCBI annotation, I used eggNOG mapper (http://eggnog-mapper.embl.de) (Cantalapiedra et al. 2021) to predict the nearest orthologue for each gene based on the protein sequence from the NCBI reference genome and combined this data with the gene tables using a custom R script. I then manually searched highly expressed genes using the human protein atlas (https://www.proteinatlas.org/) (Karlsson et al. 2021) to identify cell types associated with high levels of expression for each gene.

3.4 Results & discussion

3.4.1 Sequencing QC and filtering

For the pregnant oviduct sample, Cell Ranger recovered an estimated 38,240 cells after initial processing, with a mean of 3,286 reads per cell and a median of 150 genes per cell. Cells showed a somewhat low fraction of valid unique molecular indices (UMIs) per cell, at 68.4% (vs. an ideal figure of >75%) and also a somewhat low fraction of reads judged to be in cells (66% vs. an ideal figure of >70%). For the post-parturition sample, Cell Ranger recovered an estimated 62,539 cells after initial processing, with a mean of 1,157 reads per cell and a median of 36 genes per cell. Read quality scores suggested a higher fraction of reads in cells as compared to the pregnant sample (89.5%) and a somewhat higher though still sub-optimal fraction of valid UMIs (67.7% vs. an ideal of >75%). Cellenics filtering steps removed a large number of cells recovered as non-viable based on the classifier filter and mt content filter, as well as a significant number of doublets, that is, droplets containing two (or more) cells in the case of the pregnant sample, indicating that cell counts may have underestimated the concentration of cells in this case. After all filtering steps, the number of cells per sample was reduced by 87.2% for the pregnant sample and 86.4% for the post-parturition sample. For full details see **Table 3-1**.

The number of reads per cell was very low in both cases, falling well below recommended levels (10x genomics recommends sequencing to a depth of 20,000 reads per cell). This is a significant limitation for this study given that such low read depth can impact accurate measurement of gene expression levels in cells (Tung et al. 2017; Rizzetto et al. 2017). However, I note that even ultra low coverage single cell datasets can allow for transcriptional profiling and identification of cell types (Pollen et al. 2014; Heimberg et al. 2016). The following results should thus be treated with caution, but it is my hope that they are still informative in providing a basis for future work.

Table 3-1: Single cell filtering and QC results

		Cell Ranger count	*Classifier filter*	*Mitochondrial content filter*	*Genes vs. UMIs filter*	*Double filter*	*% change*
Pregnant	*Cells*	38,240	-	7527	7525	4903	-87.2
	Median genes/cell	150	-	472	472	421	+180.7
	Median UMIs/cell	470	-	1106	1106	952	+102.6
Post-parturition	*Cells*	62,465	9630	8620	8612	8466	-86.4
	Median genes/cell	36	103	106	106	106	+194.4
	Median UMIs/cell	65	153	157	157	156	+140.0

3.4.2 Sample embedding and Louvain clustering

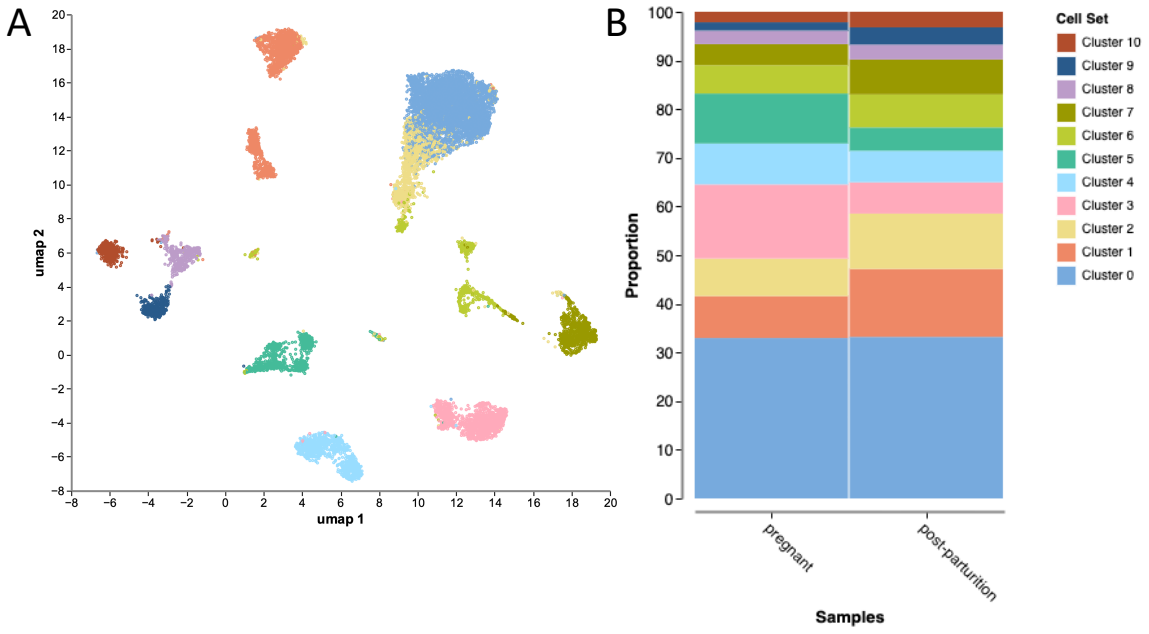

Figure 3-1. UMAP embedding and Louvain clustering of cells from pregnant and post-parturition *Z. vivipara* oviduct.

A: Integration of pregnant and post-parturition samples using Harmony with 30 principal components explaining 30% of variation. Embedding performed with UMAP using default settings (minimum distance 0.3 and distance metric Cosine), and Louvain clustering with a resolution of 0.4 recovered 10 well-defined clusters congruent with UMAP embedding. **B:** Relative proportions cells assigned to different cell clusters in pregnant and post-parturition samples.

After filtering out low-quality reads I was left with a total of 13368 cells, 4903 from the pregnant sample and 8466 from the post-parturition sample. Louvain clustering returned 10 clusters which were largely congruent with UMAP

embedding, although cluster 0 and cluster 2 showed considerable overlap and most clusters contained at least a handful of cells which were grouped with different clusters under UMAP embedding (**Fig. 3-1A**). Samples appeared generally well integrated, with every cluster present in both the pregnant and post-parturition sample, although proportions varied between the two samples (**Fig. 3-1B**).

3.4.3 Characterisation of cell types

Due to the lack of existing single-cell resolution data for the squamate oviduct, I employed an exploratory approach to the classification of cell types, first searching for expression of markers for expected cell types and then comparing differentially expressed genes between the remaining clusters to attempt to characterise other cell types present in my samples.

Firstly, I compared relative expression of cell type specific markers for two cell types known to compose a significant portion of the oviduct, specifically epithelial cells and smooth muscle cells (SMCs), in all 10 clusters across both samples. Cluster 6 showed the highest expression of the epithelial cytokeratin genes keratin 7, keratin 8 & keratin 18 (KRT7, KRT8 and KRT18) and the epithelial gene mucin 1 (MUC1). Cluster 6 also expressed the cell-cell adhesion protein Cadherin 1 gene (CDH1); however, this was more strongly expressed in Cluster 8 (**Fig. 3-2A**). For the SMC markers Transgelin (TAGLN), Calponin 1 (CNN1), Actin Alpha 2 (ACTA2) and Caldesmon 1 (CALD1) the strongest signal was seen in cluster 5, while for the Myocardin Related Transcription Facotr A gene (MRTFA) expression was highest in cluster 10 (**Fig. 3-2B**). I therefore assigned cluster 6 as epithelial cells, and cluster 5 as consisting of SMCs.

Secondly, I attempted to categorise the remaining clusters based on analysis of upregulated differentially expressed genes (DEGs) in each cluster as compared to all other clusters. Significant DEGs (log FC > 1, p-adj < 0.1) with annotation were checked against the Human Protein Atlas (Karlsson et al. 2021) to identify cell-type specific marker genes for each cluster, which are summarised in **Table 3-2** (a full list of DEGs for all clusters can be found in **Tables S3-1** to **S3-11**). Cluster 0 had 20 upregulated DEGs, which included the Haemoglobin Subunit Mu gene (HBM, log FC 5.073, p-adj = 2.225×10^{-308}), the Solute Carrier Family 4 Member 1

gene (SLC4A1, log FC 1.57, p-adj = 2.225 x 10^{-308}) and the 5'-Aminolevulinate Synthase 2 gene (ALAS2, log FC 1.03, p-adj 2.225 x 10^{-308}), all three of which are highly specific to erythroid cells. This was the largest cluster, with 4426 cells across both samples, and made up a similar proportion of cells in both pregnant and post-parturition samples (**Fig. 3-1B**).

Clusters 1 and 2 contained no upregulated DEGs. Cluster 3 had 21 upregulated DEGs, the most strongly upregulated of which was the Coagulation Factor X gene (F10, log FC 2.26, p-adj = 2.225 x 10^{-308}) which is associated with SMCs. However, other highly upregulated genes including the Aquaporin 1 gene (AQP1, log FC 1.73, p-adj = 2.225x10^{-308}), the Von Willebrand Factor gene (VWF, log FC 1.55, p-adj = 2.225 x 10^{-308}) and the Plasmalemma Vesicle Associated Protein gene (PLVAP, log FC 1.54, p-ad 2.225 x 10^{-308}) are specifically enriched in endothelial cells, and given that clusters 3 showed only moderate expression of SMC markers with the exception of CALD1 (**Fig. 3-2B**) I suggest that this cluster more likely represents endothelial cells associated with the uterine vasculature. Cluster 4 also differentially expressed PLVAP (log FC 3.06, p-adj = 2.225x10^{-308}) and AQP1 (log FC 2.84, p-adj = 2.225x10^{-308}), among other genes suggestive of endothelial cells, and may represent a distinct population of vascular endothelial cells.

As discussed above, I found elevated expression of SMC markers in cluster 5 (**Fig. 3-2B**). However, differential expression analysis found only a single upregulated DEG with a link to SMCs, the Collagen type III alpha 1 chain gene (COL3A1, log FC 1.073, p-adj = 2.225x10^{-308}) out of 8 total upregulated genes. Other genes in upregulated in this group were linked to a broad range of cell types, including hepatocytes and cell types related to connective tissue such as fibroblasts and different stromal cell types (see **Table 3-2**). This may indicate that this cluster in fact predominantly comprises connective tissue cells, or includes a mix of different cell types.

Cluster 6 showed upregulation of two genes, one of which was the S100 calcium binding protein A6 (S100A6, log FC 1.389, p-adj 3.655x10^{-80}), linked to paneth cells and distal enterocytes, two epithelial cell types found in the intenstine and associated with secretion and nutrient absorption respectively. Cluster 7 included 5 upregulated genes linked to ciliated cells: the RP1 Axonemal

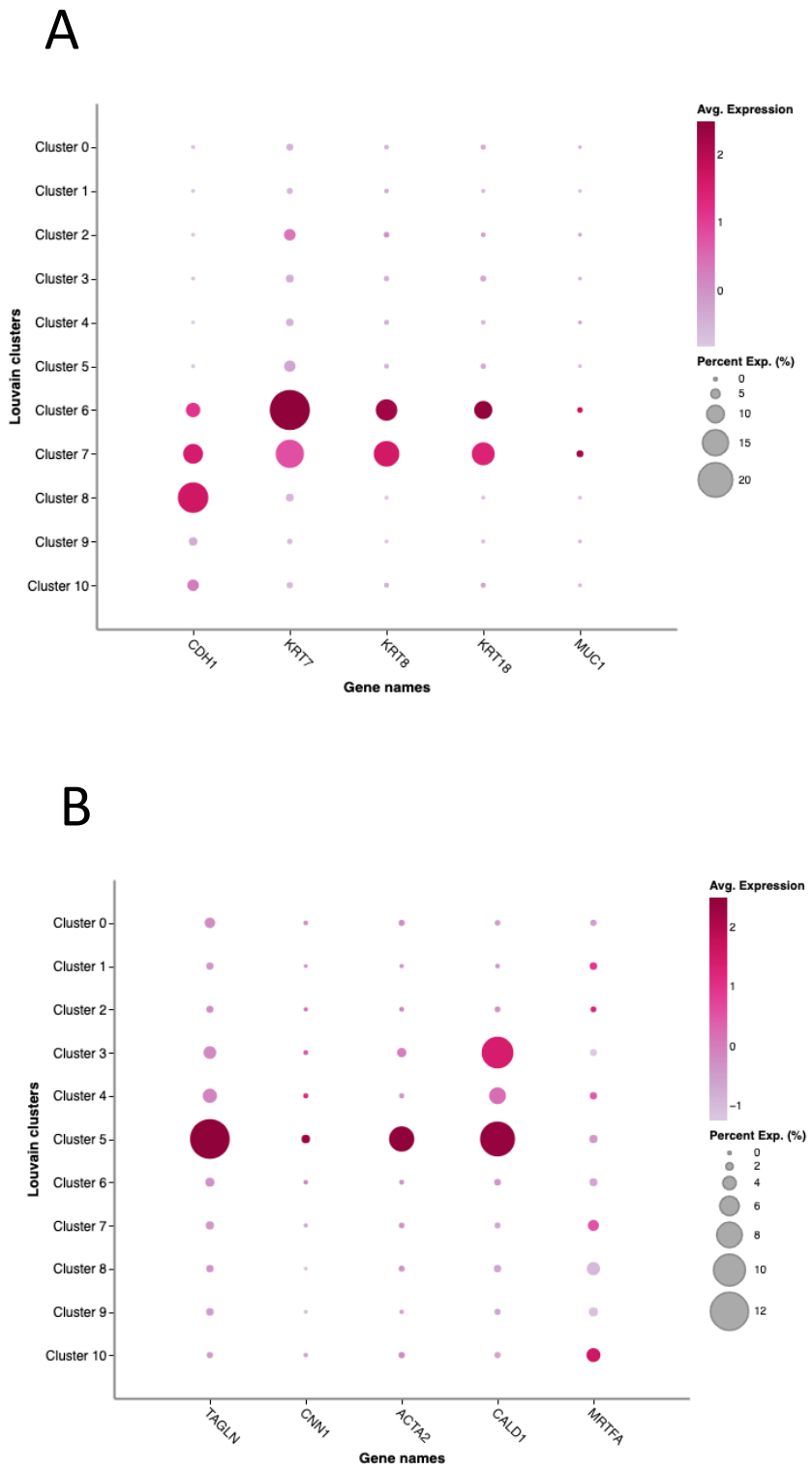

Figure 3-2. Expression of cell type specific marker genes.

A: The epithelial cell markers CDH1, KRT7, KRT8, KRT18, and MUC1 across all Louvain clusters and. **B:** The smooth muscle cell (SMC) markers TAGLN, CNN1, ACTA2, CALD1 and MRTFA.

Microtubule Associated gene (RP1, log FC 1.30, p-adj = $2.225x10^{-308}$), the Dynein Axonemal Heavy Chain 5 gene (DNAH5, log FC 1.20, p-adj = $2.225x10^{-308}$), the Sentan, Cilia Apical Structure Protein gene (SNTN, log FC 1.15, p-adj = $2.225x10^{-308}$), the Tetraspanin 1 gene (TSPAN1, log FC 1.05, p-adj = $2.225x10^{-308}$) and the WAP four-disulfide core domain 2 (WFDC2, log FC 1.009, p-adj = $2.225x10^{-308}$). Cluster 7 showed a relatively strong signal of expression for cytokeratins and other epithelial cell markers (**Fig. 3-2A**), and UMAP embedding placed this cluster adjacent to cluster 6 **(Fig. 3-1A**), which also showed a strong signal for epithelial markers (**Fig. 3-2A**). There is thus reasonable evidence to indicate that this group includes ciliated epithelial cells, which are known to occur in the squamate oviduct (Daniel G. Blackburn 1998a), whereas cluster 6 perhaps consists of non-ciliated epithelial cells.

Clusters 8, 9 and 10 all showed upregulation of markers associated with phagocytotic cells such as macrophages, monocytes and Hofbauer cells. These genes included the Actin Beta gene (ACTB, upregulated in all 3 clusters, log FC > 1.6, p-adj < $1x10^{-55}$), the Cathepsin D gene (CTSD, upregulated in cluster 8 and 9 only, log FC > 1.2, p-adj < $1x10^{-99}$) or the Cathepsin B gene (CTSB, upregulated in cluster 9 and 10 only, log FC > 1.1, p-adj < $1x10^{-212}$). Cluster 10 in particular showed upregulation of genes strongly linked to macrophages, Kupffer cells and Hofbauer cells, specifically the Complement C1q B Chain gene (C1QB, log FC 2.22, p-adj = 2.225×10^{-308}), the Complement C1q C Chain gene (C1QC, log FC 1.78, p-adj = 2.225×10^{-308}) and the Complement C1q A Chain gene (C1QA, log FC 1.23, p-adj 2.225×10^{-308}). However, I also note that ACTB and some other upregulated genes for these clusters, such as the CCAAT enhancer binding protein beta gene (CEBPB, log FC 1.283, p-adj 7.735×10^{-297}) in cluster 8 or the legumain gene (LGMN, log FC 1.471 p-adj = 2.772E-214) in cluster 9, are also associated with trophoblasts. Trophoblasts are a cell type in derived therian mammals which are embryonic in origin and play an important role in forming the placenta (Y. Wang and Zhao 2010), and are not found in squamates; however, convergent gene expression between mammalian trophoblast cells and cell populations in the squamate uterus has been suggested in *Pseudemoia entrecasteauxii* (Griffith et al. 2016).

Table 3-2: Selected highly expressed genes and associated cell types by cluster

Louvain cluster	*Gene symbol*	*Gene log FC*	*Gene p-adj*	*Associated cell types (**bold** indicates at least 4-fold enrichment)*
0	HBM	5.073	2.225 E-308	**Erythroid cells**
	SLC4A1	1.560	2.225 E-308	**Erythroid cells**
	ALAS2	1.034	2.225 E-308	**Erythroid cells**
1	*None: all DEGs downregulated*			
2	*None: all DEGs downregulated*			
3	F10	2.193	2.225 E-308	Peritubular cells, Leydig cells, Hepatocytes, Fibroblasts, Smooth muscle cells
	AQP1	1.731	2.225 E-308	**Adipocytes, Endothelial cells, Mesothelial cells, Alveolar cells type 2, Alveolar cells type 1, Cholangiocytes, Proximal tubular cells**
	VWF	1.547	2.225 E-308	**Adipocytes, Endothelial cells**
	PLVAP	1.539	2.225 E-308	**Adipocytes, Endothelial cells**
4	PLVAP	3.062	2.225 E-308	**Adipocytes, Endothelial cells**
	AQP1	2.838	2.225 E-308	**Adipocytes, Endothelial cells, Mesothelial cells, Alveolar cells type 2, Alveolar cells type 1, Cholangiocytes, Proximal tubular cells**
	PECAM1	2.030	2.225 E-308	Adipocytes, Endothelial cells, Monocytes, Lymphatic endothelial cells, Plasma cells
	SELE	1.554	2.225 E-308	**Endothelial cells**
5	APOC1	2.299	2.225 E-308	**Hepatocytes**
	NR4A1	1.315	2.225 E-308	Ovarian stromal cells
	COL3A1	1.073	2.225 E-308	Endometrial stromal cells, Fibroblasts, Smooth muscle cells
	DCN	1.014	2.225 E-308	Fibroblasts, Leydig cells, Ovarian stromal cells, Peritubular cells, Endometrial stromal cells
	STAR	1.004	2.225 E-308	**Ovarian stromal cells, Sertoli cells**
6	S100A6	1.389	3.665 E-80	Distal enterocytes, Paneth cells

Louvain cluster	*Gene symbol*	*Gene log FC*	*Gene p-adj*	*Associated cell types (**bold** indicates at least 4-fold enrichment)*
7	RP1	1.297	2.225 E-308	Rod photoreceptor cells, Cone photoreceptor cells, Ciliated cells, Bipolar cells
	DNAH5	1.203	2.225 E-308	**Ciliated cells**
	SNTN	1.148	2.225 E-308	**Ciliated cells**
	TSPAN1	1.051	2.225 E-308	Distal enterocytes, Ionocytes, Ciliated cells, Paneth cells, Late spermatids, Intestinal goblet cells, Club cells
	WFDC2	1.009	2.225 E-308	Salivary duct cells, Club cells, Ionocytes, Glandular and luminal cells, Basal respiratory cells, Mucus glandular cells, Secretory cells, Ciliated cells, Serous glandular cells
8	ACTB	1.965	3.18E-81	Hofbauer cells, Extravillous trophoblasts
	CEBPB	1.283	7.735 E-297	Monocytes, Syncytiotrophoblasts, Macrophages
	TMSB4X	1.232	1.014 E-35	Langerhans cells, Hofbauer cells
	HSPA2	1.212	5.868 E-28	Extravillous trophoblasts, Spermatocytes, Basal squamous epithelial cells
	CTSD	1.211	4.409 E-99	Hofbauer cells, Proximal enterocytes, Distal enterocytes, Macrophages
	FTH1	1.173	1.76E-32	Langerhans cells, Monocytes, Proximal enterocytes
9	APOE	4.511	2.225 E-308	Muller glia cells, Hepatocytes, Proximal tubular cells, Hofbauer cells, Peritubular cells, Melanocytes, Leydig cells
	CTSB	2.463	2.225 E-308	Hofbauer cells, Kupffer cells, Macrophages
	GRN	2.309	2.225 E-308	Hofbauer cells, Kupffer cells
	FTL	2.250	7.678 E-116	Hofbauer cells, Proximal tubular cells, Kupffer cells, Macrophages, Hepatocytes, Monocytes
	ACTB	2.157	1.873 E-92	Hofbauer cells, Extravillous trophoblasts
	CD68	1.549	2.225 E-308	Kupffer cells, Hofbauer cells, Proximal enterocytes, Macrophages, Langerhans cells
	CTSD	1.515	1.516 E-135	Hofbauer cells, Proximal enterocytes, Distal enterocytes, Macrophages
	LGMN	1.471	2.772 E-214	Hofbauer cells, Syncytiotrophoblasts

Louvain cluster	*Gene symbol*	*Gene log FC*	*Gene p-adj*	*Associated cell types (**bold** indicates at least 4-fold enrichment)*
10	C1QB	2.222	2.225 E-308	**Kupffer cells, Macrophages, Hofbauer cells**
	APOE	1.994	2.441 E-256	Muller glia cells, Hepatocytes, Proximal tubular cells, Hofbauer cells, Peritubular cells, Melanocytes, Leydig cells
	C1QC	1.776	2.225 E-308	**Kupffer cells, Hofbauer cells, Macrophages**
	ACTB	1.696	9.652 E-56	Hofbauer cells, Extravillous trophoblasts
	GRN	1.440	5.49E-170	Hofbauer cells, Kupffer cells
	C1QA	1.227	2.225 E-308	**Kupffer cells, Macrophages, Hofbauer cells**
	MAFB	1.150	1.131 E-213	Monocytes, Macrophages, Kupffer cells, Hofbauer cells, Squamous epithelial cells
	CTSB	1.137	1.131 E-213	Hofbauer cells, Kupffer cells, Macrophages
	CTSC	1.077	4.562 E-308	Hofbauer cells, Basal respiratory cells, Monocytes, Ionocytes, Club cells, Macrophages
	LY86	1.056	2.225 E-308	Hofbauer cells, Kupffer cells, B-cells, Macrophages, Langerhans cells, Microglial cells, Schwann cells
	LTA4H	1.008	1.161 E-284	Hofbauer cells, Kupffer cells, Monocytes

Other cell types reported to be present in the *lamina propria* of the oviduct including mast cells, neurons, and fibroblasts, (Daniel G. Blackburn 1998a) could not be identified in my analysis. It is possible that these cell types were not resolved as distinct clusters during the analysis, and may be present within other clusters – as discussed above, cluster 6 does show signals that could be congruent with fibroblasts for example. Alternatively, it's possible that during single cell sequencing these cells were disproportionately lost due to clumping, cell death or cell filtration and so were excluded from sequencing. Further refinements of the methods described here may be required to establish a fuller picture of gene expression throughout all cell types known to comprise the oviduct tissues in *Z. vivipara*.

I also noted changes in gene expression for each putative cell type between the pregnant and post-parturition samples; top annotated genes by fold-change for each type are summarised in **Table 3-3**. Differential expression could not be

calculated for these genes as I only had samples from two individuals. In the past, some workers using single-cell expression data have treated individual cells as replicates in order to perform statistical analyses on comparisons where the sample size is one (or is very small) – but this approach is not sound (Squair et al. 2021).

Table 3-3: Top genes by fold-change between pregnancy and post-parturition

Putative cell type	*Expression during pregnancy*	*Top annotated genes*	*log FC*
Erythroid cells	Upregulated	ASB1	6.613
	Downregulated	MPV17L	-4.853
	Downregulated	CDS1	-4.627
	Upregulated	SAMHD1	4.425
	Downregulated	EXOC3L2	-4.299
Endothelial cells	Downregulated	CCDC80	-4.795
	Downregulated	NECAB1	-4.663
	Downregulated	EMP1	-4.597
	Downregulated	ACTG2	-4.555
	Downregulated	WIF1	-4.398
SMCs	Upregulated	ERBB4	7.596
	Upregulated	HMGA1	7.226
	Upregulated	STAR	7.149
	Upregulated	OIT3	6.998
	Upregulated	RUNDC3A	6.913
Epithelial cells	Downregulated	SLC4A2	-6.001
	Downregulated	ATP1B2	-5.966
	Upregulated	IQCM	5.697
	Downregulated	TRIB2	-5.427
	Downregulated	FGFBP1	-5.399
Phagocytotic cells	Downregulated	CRYAB	-5.19
	Downregulated	SLC5A5	-4.819
	Downregulated	TOM1L1	-4.317
	Downregulated	AHRR	-4.279
	Downregulated	MMP9	-4.257

3.4.4 Comparing cell types in the pregnant and post-parturition oviduct

I examined differences in cell populations between the pregnant and post-parturition oviduct samples. As I had only one sample for each condition, no statistical tests were possible. I also note that my characterisation of cell types (see previous section) is very tentative due to both small sample size and the absence of existing single-cell resolution data in similar systems. The following

results are thus purely descriptive, but I hope they may inform future research on single-cell gene expression in the squamate oviduct.

I note that cell clusters varied in proportion between the pregnant and post-parturition samples (**Fig. 3-1B**). If individual clusters are combined into the putative cell-types identified in the previous section, the proportion of putative erythroid cells is relatively constant between the two conditions; putative endothelial cells and SMCs decreased in relative proportion in the post-parturition sample; and putative epithelial cells and phagocytotic cells increased in proportion (**Fig. 3-3**).

Hypervascularisation of the uterus during pregnancy is well established in squamates, and likely an essential adaptation to viviparity due to the increased water and oxygen requirements of the embryo during late-stage pregnancy (Michael B. Thompson et al. 2004; Murphy, Belov, and Thompson 2010; Daniel G. Blackburn 1998a). An increase in the proportion of clusters 3 & 4 is thus in line with expectations for endothelial cells. An increase in the number of SMCs throughout pregnancy might also be expected, both to strengthen the wall of the oviduct as the volume of the embryos contained within increases, and to prepare for the muscle contractions required during parturition. On the other hand, attenuation of the epithelial layer and of the *lamina propria*, where resident macrophages are found, has been suggested in the pregnant squamate oviduct (Daniel G. Blackburn 1998a), in line with the proportional reduction of these cell types in the pregnant sample.

3.4.5 Single cell resolution of expression of viviparity-associated candidate genes

A recent study combining admixture mapping of hybridising oviparous and viviparous *Z. vivipara* and analysis of differential gene expression during pregnancy across more than a dozen viviparous vertebrates suggested 38 candidate genes that may be key to the functional genetic architecture of viviparity (Recknagel et al. 2021). The majority of these candidates showed low levels of expression or no particular pattern of expression between different cell types in the single-cell expression data, but several appeared to show distinctive

patterns of gene expression. However, for seven candidate genes I saw localised expression in one or more clusters which I will describe below.

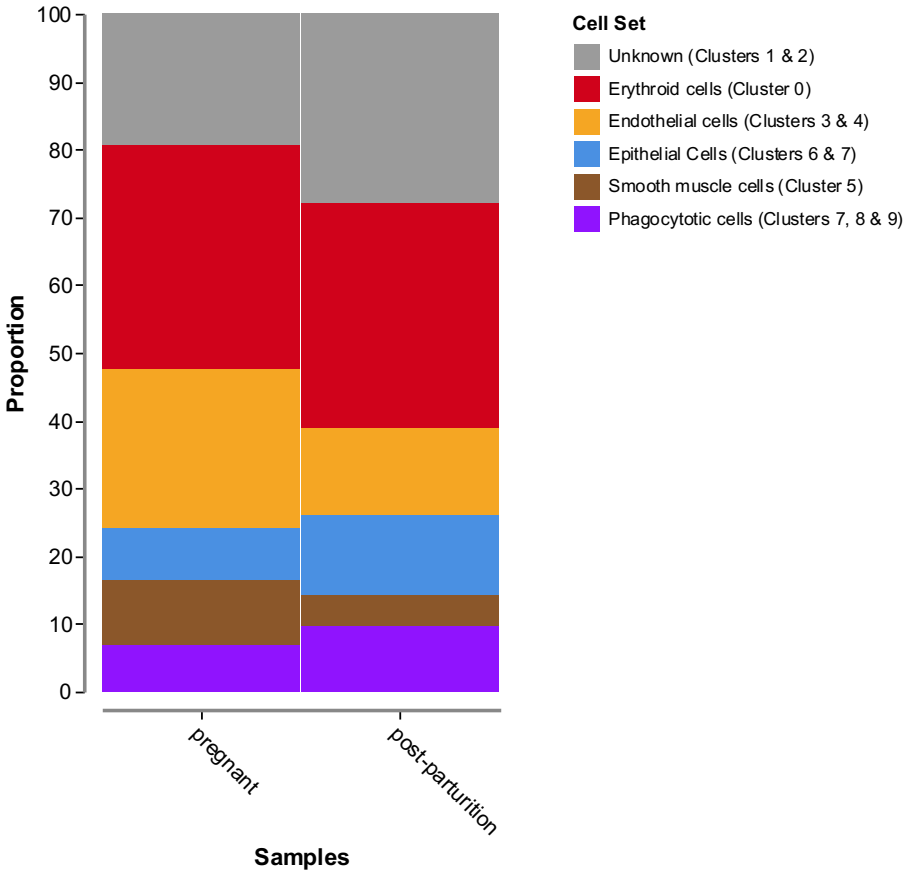

Figure 3-3. Relative proportions of putative cell-types in pregnant and post-pregnant samples.

Louvain cluster 5 was annotated as smooth muscle cells (SMCs) and clusters 6 and 7 as epithelial cells based on expression of preselected markers, while cluster 0 was annotated as erythroid cells, clusters 3 and 4 as endothelial cells, and clusters 8, 9 and 10 as phagocytotic cells based on differential expression of genes enriched in these cell types. Cell types shown are speculative and provisional given the lack of single-cell resolution data on gene expression in the squamate oviduct.

LGMN, a gene linked to pregnancy through it's role in implantation and tissue remodelling of the maternal-foetal interface in mammals (Evans et al. 2020; Shim et al. 2013; Barraza et al. 2020), was one of the genes differentially expressed between cluster 10 and all other Louvain clusters, but looking at expression throughout all samples the gene also appears upregulated in the two adjacent clusters also identified as phagocytotic cells (clusters 8 and 9) (**Fig. 3-4A**). Indeed, I also found this gene was differentially expressed when comparing

the phagocytotic cells group to all other clusters during pregnancy (log FC 1.34, p-adj = $2.225x10^{-308}$) although not after parturition (log FC 0.95). This suggests that expression of LGMN during pregnancy is linked specifically to this group of cells. The legumain protein is a highly specific protease, with an established role in regulating many aspects of tissue homeostasis, with unique roles in different species (Solberg et al. 2022; Dall and Brandstetter 2016). Phagocytotic cells are best known as a part of the innate immune system, but they are also important in tissue remodelling (Wynn and Vannella 2016; Bohaud et al. 2021). I speculate that there may be some link between expression of protease genes such as LGMN in viviparous amniotes and the extensive tissue remodelling necessitated by the physiological demands of pregnancy. Further research on the function of LGMN in the pregnant oviduct and its expression profile at the single-cell level should be a priority for researchers working on squamate viviparity.

Other genes showed an apparent bias for expression in particular cell types, although they were not significantly differentially expressed (log FC < 1). The KN Motif And Ankyrin Repeat Domains 3 gene (KANK3), a gene encoding one of the Kank family of proteins which are important players in the organisation of the actin cytoskeleton (Kakinuma et al. 2009), was expressed predominantly in clusters 3 and 4 **(Fig. 3-4B**), which I propose as possible endothelial cells. The log fold-change in expression between these clusters and all other cells was lower than 1, but was notably higher during pregnancy (log FC 0.662) as compared to post-parturition (log FC 0.177), and given the previous link to embryo retention in *Z. vivipara* and differential expression in other viviparous squamates and mammals (Recknagel et al. 2021), the unique role of the actin cytoskeleton in the construction of the endothelial barrier (Bogatcheva and Verin 2008), and the overall importance of changes to the vascular system in pregnancy (Osol and Mandala 2009), the functional role of KANK3 in pregnancy in *Z. viviparity* likely also deserves further attention.

Following a similar pattern, expression of the integrin-associated protein gene CD47, a gene which has been linked to pregnancy outcomes in humans, was localised to cluster 6 and cluster 7 **(Fig. 3-4C)**, which I annotated as likely epithelial cells. Again, expression was more elevated in this group during pregnancy (log FC 0.61) than post-parturtion (log FC 0.12), although not rising to

the level of differential expression. Integrin associated proteins are associated with polarity in epithelial cells (J. L. Lee and Streuli 2014), and given previous work on the significance of this gene and the importance of the epithelial cell layer in the oviduct as the point of contact between the maternal body and the developing embryo, this too should be considered a candidate gene for further work on the cellular mechanics of viviparity in this species and others.

In addition, I also saw some degree of cell-type specific expression of the apoptosis-inducing HtrA Serine Peptidase 3 gene (HTRA3), linked to implantation in mammals (Bowden et al. 2008), localised within cluster 5 **(Fig. 3-4D**), which I suggest to be potential SMCs. Once again, the slight increase in expression in this cluster was greater for the pregnant sample (log FC 0.46) as opposed to post-parturition (log FC 0.04). I have no hypothesis as to what role this protein might potentially play in SMCs specifically. Aside from its role in apoptosis, it has well established significance for implantation in mammalian pregnancy (Gui Ying Nie et al. 2003; G. Nie et al. 2006), but the embryos of viviparous *Z. vivipara* do not implant themselves deeply within the uterine wall as occurs in derived therian mammals, and certainly not within the *muscularis externa*, the only portion of the oviduct where SMCs are expected to be found (Daniel G. Blackburn 1998a). This may indicate that my putative identification of cluster 5 as SMCs is erroneous, or that HTRA3 plays a different role in pregnancy in squamates such as *Z. vivipara* to that demonstrated for species exhibiting more complex implantation.

Other genes that showed localised expression patterns in particular cell clusters include the N-Acylsphingosine Amidohydrolase 1 gene (ASAH1) (**Supplementary Fig. 1A**) and the LYN Proto-Oncogene, Src Family Tyrosine Kinase gene (LYN) (**Supplementary Fig. 1B**) in the putative phagocytotic group (clusters 8 – 10) and the Endothelial PAS Domain Protein 1 gene (EPAS1) (**Supplementary Fig. 1C**) which was expressed in cluster 0 (possible erythroid cells) cluster 4 (possible endothelial cells) and cluster 5 (possible SMCs). The fold changes involved here are extremely small, but I note them here due to the prior evidence of convergent changes in gene expression during pregnancy linking them to viviparity in *Z. vivipara* and other squamates and mammals (Recknagel et al. 2021).

3.4.6 Summary and conclusions

Here I report the first single cell RNA sequencing experiment targeting the squamate reproductive system. I was able to pinpoint expression of an important candidate gene for viviparity, LGMN, in specific clusters of cells, and link these to specific cell types known to be present in the oviduct. Other candidate genes also showed non-uniform distribution, although not significant differential expression. My results are preliminary and based on only two samples, but give intriguing clues as to the roles specific cell types play in determining viviparous phenotype, and offer many potential avenues for future research. In particular, I highlight the potential importance of phagocytotic cells to pregnancy in squamates, as has been demonstrated in other viviparous animals (Faas, Spaans, and De Vos 2014; True et al. 2022), and the potential role expression of the legumain gene LGMN in such cells may play in viviparity. I also provide further context to the expression of other important viviparity-associated genes, including KANK3, CD47 and HTRA3. I hope that these results will provide the basis for further studies on the molecular and cellular basis of viviparity in *Z. vivipara* and other viviparous squamates, and the role of these and other important genes in determining viviparity at the cellular level.

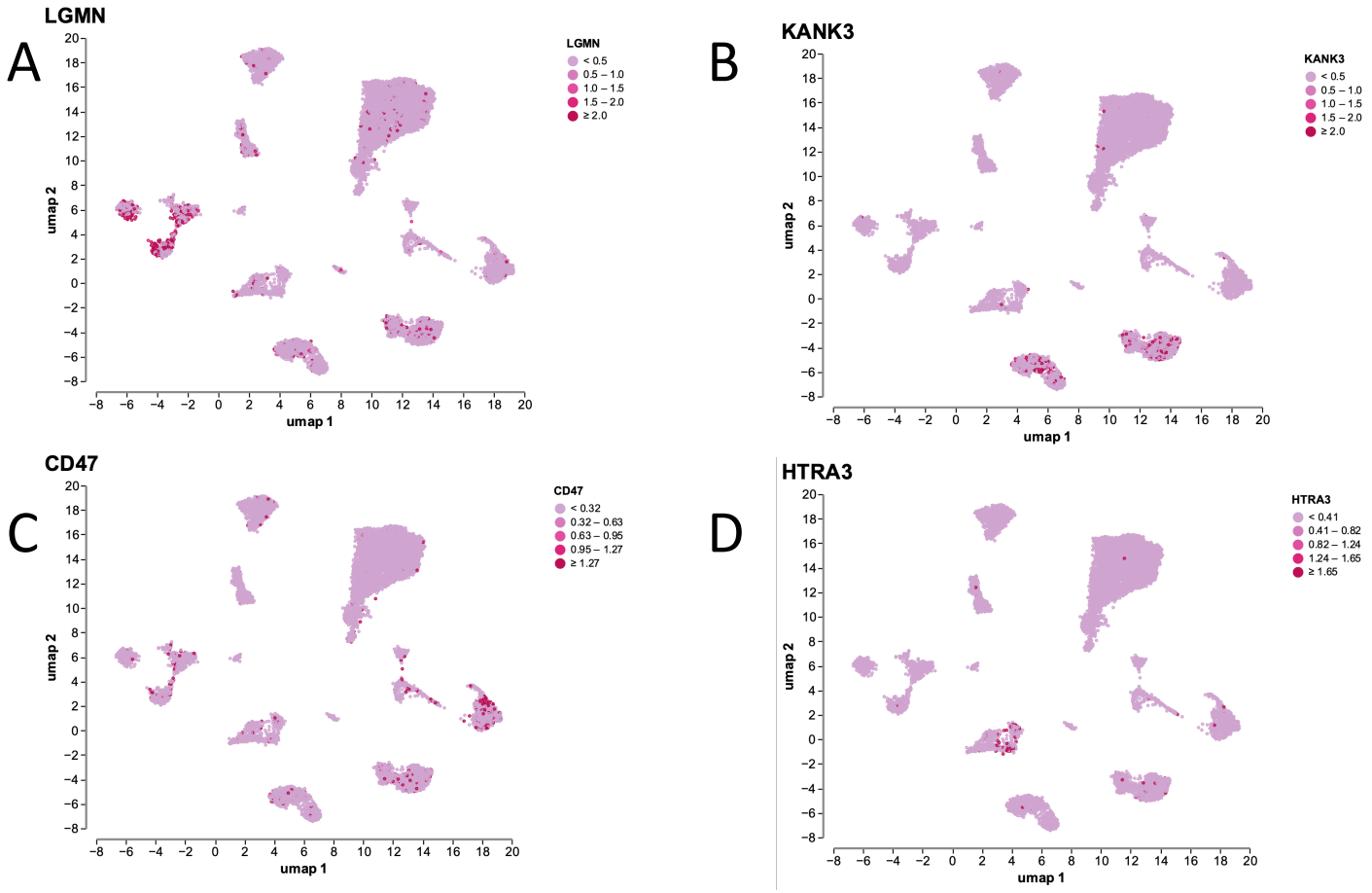

Figure 3-4. Expression of viviparity-associated genes in the *Z. vivipara* oviduct at single-cell resolution.

Expression shown across both pregnant and post-parturition samples for **(A)** the legumain gene LGMN, **(B)** the Kank family cytoskeletal regulation gene KANK3, **(C)** the integrin-associated protein gene CD47 and **(D)** The apoptosis inducing protease gene HTRA3.

4 *Zootoca vivipara* primary oviduct cell culture: a model system for the genetic and cellular basis of viviparity

4.1 Abstract

I report the successful primary culture of *Z. vivipara* oviduct cells from both oviparous and viviparous lizards, which I sought to develop as a model for viviparity research and as a platform for testing hypotheses about cellular and molecular mechanisms underpinning viviparity. I was able to maintain and expand these cultures for a period of over 100 days, including subculturing of the initial primary cultures and cryopreservation followed by successful revival of cells. I carried out preliminary immunocytochemical investigation of cultured cells to characterise the cells present in culture, which showed evidence of both epithelial and fibroblast-like cytoskeletal proteins. I also performed RNA sequencing of cultured cells and *in vivo* oviduct tissue to assess changes in gene expression in response to the cell culture environment, finding 1186 differentially expressed genes. Despite the significant impact of tissue culture conditions on the cellular transcriptome, I confirmed the maintenance of distinct gene expression patterns in viviparous and oviparous cells after long periods (60+ days), finding 354 differentially expressed genes between viviparous and oviparous cells even after two months in culture. I also confirmed the expression of 15 viviparity-associated candidate genes in cells maintained for up to 60+ days in culture.

4.2 Introduction

Viviparity, the bearing of live young, has evolved many times in vertebrates, including once in mammals, 8 times in amphibians, 13 times in bony fishes, and over 100 times in squamate reptiles (Daniel G. Blackburn 2015a). Viviparity is a complex trait involving many physiological adaptations such as increased embryo retention, provisioning of the developing embryo via matrotrophy(Daniel G. Blackburn 2015b; Stewart 2013), and modulation of the maternal immune response to the developing embryo (Genebrier and Tarte 2021; Graham et al. 2011b; Samardžija et al. 2020), all underpinned by complex transcriptomic changes (Mika et al. 2022). The repeated evolution of viviparity in divergent

animal groups is of considerable interest to workers in evolutionary biology studying major evolutionary transitions (W. Gao et al. 2019). Additionally, the study of the cellular and molecular basis of viviparity has significance to human health and disease: humans are viviparous amniotes, and complications of pregnancy account for hundreds of thousands of maternal deaths annually, in addition to non-fatal injuries and neonatal mortality (Van Den Broek and Falconer 2011). Insights from cell and molecular biology have the potential to deepen our understanding of these medical issues - for example, work in the emerging field of evolutionary transcriptomics has identified genes and pathways relating to adverse pregnancy outcomes in humans (Mika et al. 2021).

Zootoca vivipara, the Eurasian common lizard, has been described as an emerging model organism for the study of viviparity (Freire, Tennant, and Miyamoto 2003b). This species is reproductively bimodal, with extant viviparous and oviparous lineages diverging as recently as the 500,000 years ago (B. Heulin et al. 1999; Recknagel, Kamenos, and Elmer 2017). Viviparous *Z. vivipara*, prevalent across most of the species' range, give birth to fully developed young (embryo stage 40) contained in a non-calcified membrane, from which they emerge hours after parturition (Hubert 1962; Recknagel et al. 2021). Oviparous *Z. vivipara* are restricted to the southern fringes of the species range in western and central Europe, and lay eggs with embryos at stage 30-34, enclosed in a fully calcified eggshell, which then continue to develop externally for up to 30 days before hatching (Benoit Heulin et al. 2002; Braña, Bea, and Jesús Arrayago 1991; Recknagel et al. 2021). Recent research has shown the significance of changes in gene expression to the evolution of viviparity in squamates (Foster et al. 2020; W. Gao et al. 2019; Griffith et al. 2016; Recknagel et al. 2021) as well as shedding light on the underlying genetic architecture of viviparity and oviparity in this species, revealing candidate genes implicated in the evolution of viviparity including the legumain gene LGMN and the Acyl-protein thioesterase gene LYPLA1 amongst others (Recknagel et al. 2021). However, the functional role of these genes in determining reproductive phenotype has not been established.

Experimental study of reproductive traits in *Z. vivipara* is constrained by the fact that reproduction in this species is seasonal, with females dependent on

winter hibernation for normal reproductive function (Gavaud 1983). Additionally, individual lizards typically reach sexual maturity in their second year, thus requiring long experimental timeframes. As *Z. vivipara* are not widely bred in captivity, wild-caught individuals are generally required for experiments, entailing time consuming fieldwork to collect the necessary specimens. Studying the transcriptomic changes associated with pregnancy has advanced our understanding of viviparity in *Z. vivipara* and other squamates (W. Gao et al. 2019; Recknagel et al. 2021), but requires the sacrifice of multiple female lizards to extract reproductive tissue for analysis. Finally, while successful targeted gene editing of embryos has been demonstrated in two species of lizard (Abe, Kaneko, and Kiyonari 2023; Rasys et al. 2019), validation of gene function in live animal experiments involves overcoming significant practical hurdles and potential ethical considerations.

Primary cell culture as a model of the lizard oviduct potentially overcomes many of these limitations and allows for novel experimental approaches which are challenging to achieve with live animal experiments. These include testing the impact of factors that influence growth rates, differentiation and cellular pathways and editing the expression of selected genes over various time periods in a controlled environment (T. K. Kim and Eberwine 2010). Additionally, the use of cultured cells for experiments which would otherwise involve the use of multiple live lizards allows for the partial replacement of experimental animals, in accordance with the '3 Rs' (replacement, reduction and refinement) in relation to animal experiments.

The most developed oviduct cell culture model from a saurian animal is that of the domestic chicken *Gallus gallus*. The study of cultured chicken oviduct epithelial cells (COECs) has included the characterisation and optimisation of COEC primary cultures (Jung et al. 2011; Kasperczyk et al. 2012); quantification of gene expression in such cultures (Stadnicka et al. 2018); transfection of oviduct cells with exogenous DNA via both gene gun (Ochiai et al. 1999) and polyethyleneimine transfection (B. Gao et al., 2005); and gene editing using CRISPR/Cas9 delivered via viral vector (Qin et al. 2019). In contrast, the development of cell culture in squamates has been comparatively limited. There have been several cell culture studies of the regenerative properties of lizard

tails, as a more or less (though see Xu et al., 2020) squamate-specific feature among amniotes (Simpson and Cox 1967; Moghanjoghi et al. 2018; Sun et al. 2018; Palade et al. 2018). Other applications include the study of ecdysis, another distinctive feature of squamate biology, via skin cell cultures derived from *Podarcis muralis*, a relatively close relative of *Z. vivipara* (Polazzi and Alibardi 2011) and the successful culture of glial cells derived from *Eublepharis macularius* central nervous system tissue (Grzesiak et al. 2013). In contrast, the squamate oviduct has been the subject of only a handful of studies using intact or homogenised oviduct tissue maintained *in vitro* for up to 24 hours (Guillette, Masson, and DeMarco 1991; Guillette et al. 1990).

The development of a cell culture model of the squamate oviduct as a functional tissue thus poses significant challenges. Cultured cells will be a restricted representation of cell phenotype *in vivo*, as cells change in response to the artificial environment of the culture vessel. The two main factors which drive divergence of primary cell cultures from their tissue of origin are dedifferentiation of cells, in which cells exposed to a cell culture environment revert of an undifferentiated stem-like state, and differential proliferation rates of cell types (Y. Liu et al. 2009; 2010; Zhuang, Duan, and Yan 2012; Ogata, Yokoyama, and Iwabuchi 2012). The variation in cell growth rate in a heterogeneous population means that fast-growing cell types will dominate and overcome the initial mixed population of primary cells present in the tissue of origin. For example, fibroblasts are putative mesenchymal cells which play key role in the creation and maintenance of the extracellular matrix, wound healing, and diseases including fibrosis and cancers (Sahai et al. 2020; Tommelein et al. 2015; Fries et al. 1994; Chiquet, Katsaros, and Kletsas 2015; Knoedler et al. 2023). Fibroblasts exist in most tissues and their precursors, fibrocytes, circulate throughout the body in the bloodstream (Zhang et al. 2018; Bucala 2008). Due to their multifunctional roles, they are perhaps uniquely vigorous in primary cell culture, adapting quickly to life *in vitro* and proliferating rapidly, often swamping other cell types (Pal and Grover 1983; Kisselbach et al. 2009a).

In the present study, I aimed to develop primary cell culture of *Z. vivipara* oviduct from both oviparous and viviparous lizards as a model for viviparity research and a platform for testing hypotheses about cellular and molecular

mechanisms. I investigated the biological features of the model, firstly at the level of gene expression, to facilitate future comparisons and evaluate its utility and informativeness as a model of reproductive mode. This builds on prior work using gene expression to characterise and validate cell culture models (Prpar Mihevc, Ogorevc, and Dovc 2014; Ogata, Yokoyama, and Iwabuchi 2012). I supplemented this approach with immunocytochemical visualisation of key cytoskeletal proteins vimentin and cytokeratin to aid in characterising cellular anatomy. Both vimentin and cytokeratin are structural components of the cytoskeleton, specifically intermediate filaments (IF) which are found throughout the cytoplasm of eukaryotic cells. IF containing vimentin are associated with mesenchymal cells and fibroblasts (Ivaska et al. 2007), whereas those composed of keratins are typical of epithelial cells (Fuchs and Weber 2003). This study shows that primary cell culture of *Z. vivipara* oviduct cells is a viable and informative model for parity mode research.

4.3 Methods

4.3.1 Collection of viviparous and oviparous *Z. vivipara*

Adult female lizards of the Western Viviparous lineage were collected from an established sampling site on Great Cumbrae (55° 47′ 33″ – 55° 46′ 0″ N, 4°54′ 5″ – 4° 56′ 4″ W) from April-September between 2019 and 2023 with permission from NatureScot (licence no. 188744). Adult female lizards of the Eastern Oviparous lineage were collected from a woodland site in the Gail Valley, Austria (46° 39′ 14″ N, 13° 9’ 8” W) from May-June 2022 with permission from the local government in Hermagor (permit no. HE3-NS-036/2019). In both cases live lizards were caught by hand by a team of 2 – 6 fieldworkers.

Oviparous lizards collected in Austria were temporarily housed in small individual terraria (dimensions: 280 x 200 x 140 mm) stored in large tents adjacent to the sampling area for a period of 1-2 months, exposed to natural outdoor temperature and with sunlight provided by a mesh screen. Terraria were furnished with a water dish and children’s play sand to a depth of approx. 5 cm, along with sterilised rocks to create a hide and basking area. During this time, they were provided with fresh water and 1-2 beetle larvae (*Tenebrio molitor*) daily for sustenance. Offspring born in captivity were released at the site of

capture of their mother. Lizards were then transported to Glasgow by car in their terraria before being transferred to larger outdoor enclosures described below.

On arrival at Glasgow (either from the Austrian field site or from Great Cumbrae) Lizards were housed in naturalistic conditions in secure outdoor enclosures in the courtyard of the Graham Kerr Building at the University of Glasgow, in groups of 1-5 lizards per enclosure, with oviparous and viviparous lizards housed separately. Enclosures were filled with topsoil to a depth of 20 - 30 cm, and included water dishes, live plants, dead wood, rocks and ceramic hides to provide suitable habitat. Enclosures were closed at the top with wire mesh, exposing lizards to natural temperature variations and photoperiod. Enclosures were supplied with fresh dechlorinated water and 1-3 beetle larvae (*Tenebrio molitor*) per individual every 2-3 days.

4.3.2 Optimisation of tissue preparation and primary cell culture

To optimise my primary cell culture methodology, oviduct tissue was prepared from 2 viviparous individuals in November 2019 during the post-reproductive phase of the annual reproductive cycle after 2 months in captivity. The schedule one area was cleaned with 70% alcohol and all dissection tools were autoclaved before sacrifices were carried out. Lizards were sacrificed by concussion followed by immediate destruction of the brain in accordance with Schedule 1 of the Animals (Scientific Procedures) Act (ASPA) (Home Office 2013) with the approval of the Senior Named Veterinary Surgeon and of NatureScot. The lizards were then dissected, and the oviducts were removed and transferred to a wash solution consisting of phosphate-buffered saline (PBS) containing the antifungal amphotericin B (Fisher Scientific, UK) at 0.75 µg mL^{-1} along with the antibiotics penicillin-streptomycin (Fisher Scientific, UK) at 10 mL L^{-1} and Primocin (Invivogen, France) at 1 mg mL^{-1}. Oviducts were first processed manually using sterile scalpel blades to fragment the tissue to facilitate enzymatic digestion. Oviduct fragments were then transferred to a digestion mix consisting of DMEM (Fisher Scientific, UK) containing 1 mg mL^{-1} collagenase P (Sigma-Aldrich Company Limited, UK) and digested.

Each oviduct was digested for 18 hours at either 25 ºC or 30 ºC to compare cell yield and viability at each temperature. The resulting digestate was gently triturated first with a 1 mL pipette. For cell counts, a 50 µL aliquot of the cell suspension was combined with Trypan Blue Solution, 0.4% (Thermo Fisher Scientific, Waltham US) at a 1:1 ratio and mixed by pipetting. Cells were then counted with a Neubauer haemocytometer with Trypan Blue staining used to distinguish non-viable cells.

Remaining digestate was then transferred to 60 mm cell culture dishes containing complete culture media composed of 56.94% DMEM, 25.90% Ham's F10 nutrient mix (Thermo Fisher Scientific, Waltham US), 6.21% Foetal Calf Serum (FCS) (Thermo Fisher Scientific, Waltham US), 6.21% Chicken Serum (ChS) (Thermo Fisher Scientific, Waltham US), and 1.04% Insulin-Transferrin-Selenium (ITS-G) (Thermo Fisher Scientific, Waltham US) with the addition of the antifungal amphoteracin B (Thermo Fisher Scientific, Waltham US) at 0.75 µg mL^{-1} and the antibiotics penicillin-streptomycin at 100 µg mL^{-1}, Gentamicin at 40 µg mL^{-1} and Primocin at 1 mg $µL^{-1}$. Cells were then transferred to an incubator at either 25 ºC or 30 ºC with 5% CO_2 for 48 hours to allow cells to adhere to the culture vessel. Culture dishes were then maintained at the same temperature for up to 4 weeks with culture medium replaced every 48-72 hours. These initial cell cultures showed relatively poor growth rates, particularly at 25 ºC, and did not yield enough material for experiments. All future preparations and cultures were thus incubated at 30 ºC

4.3.3 Tissue preparation for experimental primary cell cultures

The tissue cultures which were used for the data presented below were prepared from one viviparous lizard sacrificed in October 2020 (after 5 months in captivity) and an additional viviparous lizard sacrificed in September 2022 (after 4 months in captivity) along with two oviparous lizards sacrificed in September and November 2022 (after 4 and 6 months in captivity respectively). Sacrifices were carried out by concussion and destruction of the brain as described above, and oviducts were dissected, washed and manually processed as before. Digestion with collagenase P was carried out at 30 ºC as this resulted in greater viability and higher cell counts in the initial test preparations. After trituration of the digestate with a 1 mL pipette, samples were triturated again with a 21G

(0.8 mm) needle to further dissociate cells, before being trtansferred to transferred to 60 mm cell culture dishes containing complete culture media containing the same ingredients as before, and then incubated at 30 ºC 5% CO_2 for 24 hours to allow cells to adhere.

After 24h medium was transferred to a new culture dish and the original dish was replenished with fresh complete culture medium. The new dish was then incubated for a further 24h to allow any remaining cells in suspension to adhere to the dish. This was repeated 2-4 times per sample to produce 2-4 primary oviduct cultures per preparation. These cultures were then maintained at 30 ºC 5% CO_2 with culture medium replaced every 48-72 hours depending on cell density and growth rate. In contrast to the previous cultures, these preparations began to show significant growth of cells after the first few weeks in culture (see **Results**).

4.3.4 Subculturing, cryopreservation, and revival of cells

At approx. 80% confluency, cells were subcultured to allow for continuous growth and expansion. Old culture medium was removed and cells were first washed three times with sterile PBS (pre-warmed to 30 ºC), then incubated with 400 µL 0.025% 50 mM Trypsin-EDTA (or 600 µL for larger cell culture flasks) for 2 minutes at 30 ºC. Cells were then manually dislodged by tapping the side of the cell culture vessel, which was confirmed by phase contrast microscopy, with the whole process repeated until the majority of cells were observed to detach from the surface. The cells were then suspended in complete medium (recipe described in above) and transferred to a 10 mL centrifuge tube and pelleted by centrifugation at room temperature at 800 RCF for 5 minutes. Supernatant containing trypsin-EDTA was then discarded, and the cells were resuspended in 5-10 mL complete medium and divided between 1-4 new culture vessels to allow for continued growth and development, with additional complete medium added as needed, and returned to incubation at 30 ºC 5% CO_2.

To cryopreserve excess cells for future experiments the following method was used. As described above, old medium was removed and cells were washed with PBS before being trypsinised to remove them from the culture vessel. Cells were then pelleted through centrifugation as previously described and contaminated

medium was discarded. Cells were then resuspended in 1-2 mL of high serum cryopreservation medium composed of 46.53% DMEM, 21.15% Ham's F10, 10.15% FCS, 10.15% ChS, 10% dimethyl sulfoxide (DMSO) and 0.85% ITS-G with the addition of amphoteracin B at 0.75 μg mL^{-1} and the antibiotics penicillin-streptomycin at 100 μg mL^{-1}, Gentamicin at 40 μg mL^{-1} and Primocin at 1 mg $μL^{-1}$. The resulting suspension of cells in freezing medium was transferred to 1-4 cryotubes which were transferred to a cryotube holder filled with isopropyl alcohol for gradual cooling. The cryotube holder containing the tubes with cell culture samples was then cooled for 24h at -20 ºC before being transferred to medium term storage at -70 ºC. Cryovials were transferred to liquid nitrogen storage at -196 ºC for long-term preservation (12 months or longer).

For revival of cryopreserved cells, cryotubes were first allowed to gradually thaw at room temperature. The thawed cell suspension from each cryotube was then transferred to a 10 mL centrifuge tube. 5 mL of complete medium (recipe described above) was pre-warmed to 30 ºC and was then added dropwise to prevent osmotic shock. Cells were centrifuged at room temperature at 800 RCF for 5 minutes to produce a pellet, and supernatant containing DMSO was discarded. Cells were then resuspended in 5-mL complete medium and transferred to a 60 mm cell culture dish, which was incubated at 30 ºC 5% CO_2 for 48 hours to allow cells to adhere to the surface of the cell culture vessel.

4.3.5 Immunocytochemistry and image capture

For immunocytochemistry and other applications requiring fixing and staining of cells on coverslips, cultured cells were first trypsinised as described in the previous section and then seeded onto glass coverslips treated with poly-L-lysine housed in 4-well plates, in densities of approximately 20,000 cells per coverslip (approx. 105 cells mm^{-2}), with 500 μL complete medium per coverslip, and cultured at 30 ºC 5% CO_2 until around 50% confluent (usually 24-72 hours). Ceoverslips were then washed with sterile PBS and fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature. Cells were then again washed three times with sterile PBS and then permeabilized by immersion in chilled Methanol for 10 minutes at -20 ºC.

For immunocytochemistry, cells were again washed three times with sterile PBS and then incubated at 4 ºC for 18 hours in a solution of either vimentin monoclonal (Sigma V6630) at1:500 in 1% volm/volm normal goat serum (NGS) in PBS or pan-cytokeratin monoclonal (Agilent M082101-2) at 1:100 in NGS. Cells were then once again washed three times with sterile PBS and incubated for 2-4 hours with FITC conjugated (1:100 in NGS). Coverslips were washed three more times in PBS and immersed in a solution of 5 µg mL^{-2} 4′,6-diamidino-2-phenylindole (DAPI) to stain cell nuclei. Coverslips were then finally washed three more times in distilled water to remove salts from the PBS solution before being mounted on a microscope slide and imaged using an Olympus IX70 microscope as described previously by Qi et al. (2019), with the green channel used to detect the antibody fluorescence and the blue channel to detect DAPI-stained nuclei.

4.3.6 Transfection of cultured cells with GFP

To test transfection of cultured *Z. vivipara* oviduct cells with exogenous DNA, while simultaneously testing for the presence of autophagic vesicles, I first seeded cultured oviduct cells from one oviparous and one viviparous lizard after 47 days in culture (passage number: P5) onto 24 coverslips (as described in the previous section). To test transfection efficiency at different cell densities, 4 coverslips each for the oviparous and viviparous derived cells were prepared using 4000, 20,000 or 40,000 cells per coverslip (for cell densities of 21, 105 and 211 cells mm^{-2} respectively). Cultures were then incubated for at 30 ºC 5% CO_2 for 24 hours to allow cells to attach to the coverslips before attempting lipofection.

I performed lipofection of cell cultures with a DNA construct containing green fluorescent protein (GFP) plasmid (pmax) conjugated to LC3, a protein which localises to autophagic vesicles within living cells (Runwal et al. 2019). I first prepared a solution of Lipofectamine 3000 reagent (Thermo Fisher Scientific, Waltham USA) in accordance with the manufacturer's instructions, preparing two solutions with different concentrations of Lipofectamine 3000 reagent (3% or 6%) to which I added the aforementioned LC3-GFP construct. For each coverslip, I added 50 µL of either the 3% or 6% Lipofectamine solution, which I then to the live cell cultures for a final concentration of either 0.3 or 0.6% (accounting for

approx 500 µL of medium per well). Control coverslips for each 4-well plate were either treated with 3% Lipofectamine only or with no treatment. Coverslips were then incubated for a further 48 hours at 30 ºC 5% CO_2 to allow time for cells to take up and express the plasmid construct.

Cells were then washed and fixed in 4% PFA and permeabilised with chilled methanol as described in the previous section, before proceeding directly to DAPI staining and mounting as described above. Cells were again imaged using an Olympus IX70 microscope, with the green channel used to detect GFP fluorescence and the blue channel to detect DAPI-stained cell nuclei.

4.3.7 RNA extraction

Cells culture dishes selected for RNA sequencing were first flash-frozen by placing near-confluent cell culture dishes on either dry ice or liquid nitrogen, and then stored at -70 ºC until RNA extraction could be performed.

Three batches of viviparous-derived cells (comprising two dishes i.e. two technical replicates each) from the culture line established in late 2020 (WV1) were flash frozen after 36 days (passage number: P3), 61 days (passage number: P5) and 69 days (passage number: P6) in culture. For the viviparous-derived cell culture line established in 2022 (WV2), two dishes were snap frozen after 30 days in culture (passage number: P2) and a further two dishes were snap frozen after 61 days in culture (passage number: P3). For the first of the oviparous-derived cell lines (EO1), two dishes were snap frozen after 30 days in culture (passage number: P3) and a further two after 61 days in culture (passage number: P8). For the final oviparous-derived cell line (EO2), two dishes were snap frozen after 34 days in culture (passage number: P2), and two more after 62 days in culture (passage number: P8).

RNA extraction was attempted using either an RNeasy Plus Mini Kit (Qiagen, Venlo NL) according to the manufactuer's instructions for all cell cultures except for the last two (62 day in culture) samples obtained from the EO2 cell line. The quantity and purity of the resulting RNA samples was checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham US) to ensure an A260/A280 ratio of at least 1.8, and RNA integrity was checked by agarose gel

electrophoresis, using a 1.5% agarose gel run for 20 minutes at 80V. While RNA extraction was successful for the majority of samples, no RNA was obtained for either of the WV1 samples frozen at 30 days and one of the EO1 samples frozen at the same timepoint also failed to yield any RNA. Therefore, an aliquot of frozen EO1 cells was revived (as described in **Methods 4.3.4**) and then snap frozen after 41 days in culture (passage number: P6). Unfortunately, no cryopreserved WV1 cells from the first 30 days in culture were available to replace these samples. To ensure sufficient RNA was obtained for analysis, the RNA extraction method for these tissues as well as the final two EO2 samples (snap frozen at 62 days/P8) was modified to use a Direct-zol RNA Miniprep Kit (Zymo Research, Irvine US) in place of the Qiagen kit previously used, which resulted in increased RNA yield.

Following extraction, purified RNA samples were stored at -70 °C until library preparation.

4.3.8 Library preparation and sequencing

RNA samples were reverse-transcribed into cDNA libraries which were then barcoded with a PCR-cDNA Barcoding Kit (ONT Ltd., Oxford, UK) according to the manufacturer's instructions. RNA samples were thawed and kept on ice up until the reverse-transcription step in the library preparation. Libraries were checked for DNA quantity and purity using a Nanodrop spectrophotometer, ensuring an A260/A280 ratio of at least 1.7, before adapter annealing and sequencing. I loaded the prepared libraries onto R9.4.1 Flow Cells (ONT Ltd., Oxford UK) and sequenced using either a MinION portable sequencer in the case of the samples derived from the initial viviparous cell culture line (WV1) or a GridION benchtop sequencing device for all other samples (both from ONT Ltd., Oxford UK). In all cases I, performed live basecalling using Guppy (Wick, Judd, and Holt 2019). After sequencing, I concatenated basecalled reads for each barcode, trimmed adapters using porechop (v0.2.4) applying an extra end trim of 20 bp (Wick et al. 2017) and filtered the trimmed reads with filtlong (v0.2.1) using a mean quality weight of 9 (default 1) and a target base threshold of 5,000,000,000,000 bp (https://github.com/rrwick/Filtlong). I then aligned reads to the most recent *Z. vivipara* reference genome (Bioproject accession PRJEB66180) using minimap2

(v2.24) (Heng Li 2018) and quantified transcripts from the aligned reads using salmon (v1.10.1) (Patro et al. 2017) running in ONT mode.

4.3.9 Data processing and analysis

All statistical analyses were performed using the statistical programming language R (v4.3.1) (R Core Team 2023). The salmon read quantification data was imported with tximport (v1.30.0) (Soneson, Love, and Robinson 2015), and transcript quantifications were mapped to gene counts using the latest reference genome annotation (NCBI accession GCF_011800845.1) with GenomicFeatures (v1.54.1) and AnnotationDbi (v1.64.1) (Lawrence et al. 2013; Pagès et al. 2023). Differential gene expression between oviparous and viviparous cultures and between viviparous cell cultures and live viviparous oviduct was analysed using edgeR (v4.0.3) (Y. Chen, Lun, and Smyth 2016). In brief, gene counts were normalised for library size, then filtered the resulting matrix of gene-counts to remove lowly expressed genes (fewer than 10 reads per sample group). A quasi-likelihood negative binomial generalized log-linear model (GLM) was fitted in order to conduct genewise statistical tests to calculate log fold-change (FC) and p-values for all genes in all comparisons. edgeR was used to produce a batch-corrected matrix of log2(CPM) for all cell culture samples, which was used to calculate the proportion of variance in gene expression attributable to between-individual variation, time in culture and lineage (viviparous or oviparous) using a generalised linear model with the R package variancePartition (Hoffman and Schadt 2016). Additionally, the R package WGCNA was used to search for gene co-expression models using weighted network correlation analysis (Langfelder and Horvath 2012). Over-representation analysis of biological process (BP) gene ontology (GO) terms was performed using topGO (2.54.0) (Alexa and Rahnenfuhrer 2023), using GO term annotations predicted directly from the translated gene protein sequences using the web-based tool eggNOG-mapper (http://eggnog-mapper.embl.de) (Cantalapiedra et al. 2021; Huerta-Cepas et al. 2019). In brief, over-represented GO-terms were tested for using Fishers Exact Test with the “weight” algorithm, using gene lists of significantly up- and downregulated genes (p-adj < 0.1) for each comparison, and for genes associated with WGCNA modules, against a gene universe of all expressed genes across all samples. Figures were produced using ggplot2 (v3.4.4) (Wickham 2009). Boxplots show the interquartile range (box), median (midline), and range

(whiskers) excluding outliers, where outlying points are considered points more than 1.5 times the IQR outside the box. Unless otherwise stated in the figure or accompanying legend, boxplots show scaled relative expression values calculated by subtracting the mean of the normalised CPM for all samples and dividing by the standard deviation.

4.4 Results

4.4.1 Oviduct primary cell morphology, growth & development

Oviparous- and viviparous-derived oviduct cell cultures from all four individuals showed essentially identical patterns of growth and development, beginning with round or oval undifferentiated cells ~15 µm in length attaching to the surface of the cell culture vessel and differentiating within the first 24 hours in culture into small elongated bipolar cells around 30 µm long, which appeared dark under phase contrast microscopy (PC) (**Fig. 4-1A, D**). After around 10 days in culture, the cells became much larger (~250 µm) and more diverse in appearance, including a mixture of bipolar and multipolar cells frequently growing in close or loose associations, often with clusters of bright internal vesicles visible under phase contrast microscopy (**Fig. 4-1B, E**). This morphology was maintained for around 90 days in culture with few noticeable changes. After 90 days some cells began to exhibit very large internal vesicles distinct form the small bright vesicles seen earlier and progressed to a rounded, sheet-like morphology (**Fig. 4-1C, F**).

In addition, in all primary cell preparations, following the plating out of the homogenised tissue, cells were also observed growing outwards in a monolayer from 3-dimensional granules or spheres of cells adhering to the surface of cell-culture vessels (**Fig 4-2**). These cells showed generally fibrocyte- or fibroblast-like morphology, flattened and elongated in shape with processes extending outwards, similar to the individually adhering cells (**Fig. 4-1A and E**). These 3-dimensional granules or spheres gradually disappeared from cell culture lines over time, generally after 45 days in culture, indicating they are most likely remnants of un-disassociated cells from my initial tissue preparations.

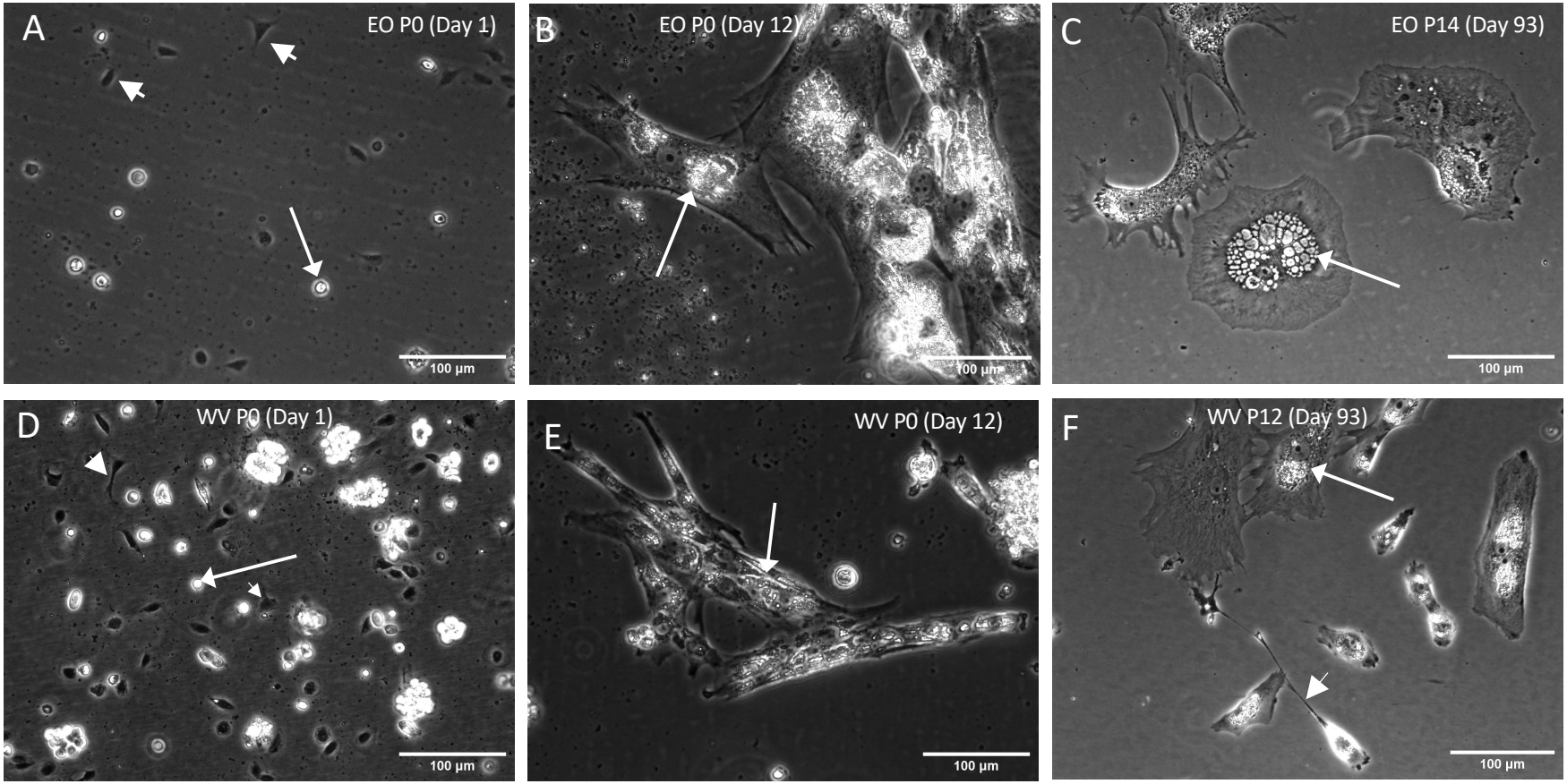

Figure 4-1. Cells derived from oviparous (A-C) and viviparous (D-F) oviduct and maintained in culture for over 90 days.

After 24h small elliptical cells with no visible differentiation (**A** and **D** arrow) began to progress to small flattened cells (arrowhead) with a dark appearance and two or more processes. After 12 days (**B** and **E**) these cells had enlarged substantially and exhibited bright internal vesicles (arrow), with complex and variable morphology including sheet like associations and long processes. After 90 days in culture (**C** and **F**), very large vesicles appeared in some cells (**D**) and increasingly flat and sheet-like morphology. Some fields would display cells with long processes (**F** arrowhead). All images captured via phase-contrast microscopy of live cell cultures using an Olympus IX70 microscope.

Proliferation of cells was generally variable, especially in the early stages of cell culture, with cells forming loose associations or densely packed monolayers in some areas while others showed few or no adherent cells. Initial primary cell cultures reached confluency after around 15 days in culture, at which point they were subcultured to allow for continued growth and proliferation. These P1 subcultures reached confluency after a further 10 days. The growth rate of cultured oviduct cells increased gradually with time in culture and passage number, with doubling time estimated to be around 3.5 days after 45 days in culture (~P5), reaching P14 after around 90 days.

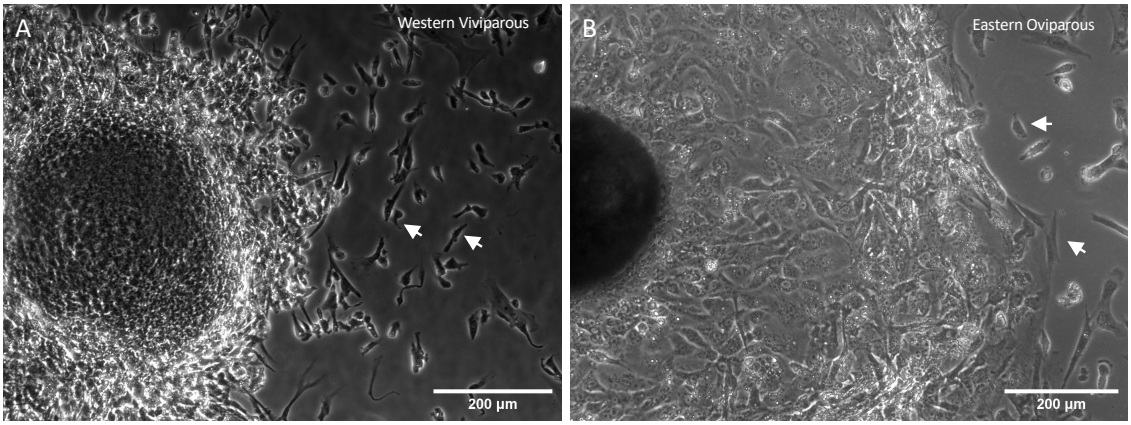

Figure 4-2 . Differentiated *Z. vivipara* oviduct cells spreading outwards from tissue granules adhered to the cell culture surface.

During the first 45 days of culture, cells derived from both viviparous (**A**) and oviparous (**B**) demonstrated a characteristic growth pattern of spreading outwards in a monolayer from small, dense granules or spheres of cells. Cells forming a monolayer (arrowhead) showed evidence of differentiation, often with fibrocyte- or fibroblast-like characteristics.

4.4.2 Immunocytochemical characterisation of oviduct cells

I tested oviparous and viviparous derived oviduct primary cells for epithelial markers using a pan-cytokeratin antibody cocktail, and for the mesenchymal marker vimentin. Immunocomplexes were visible under fluorescence microscopy as a dispersed network throughout the cytoplasm, as expected for cytoskeletal proteins (**Fig. 4-3**). Both oviparous- and viviparous-derived oviduct cell cultures showed a positive signal for both pan-cytokeratin and vimentin. Virtually all cells imaged (as identified by the presence of a cell nucleus, visualised by DAPI staining) were positive for the marker protein targeted in each case. Cultured *Z. vivipara* oviduct cells thus appear to express both classes of intermediate filament proteins, a condition associated with cells undergoing the epithelial-

mesenchymal transition (EMT) (Kuburich et al. 2022; Mendez, Kojima, and Goldman 2010).

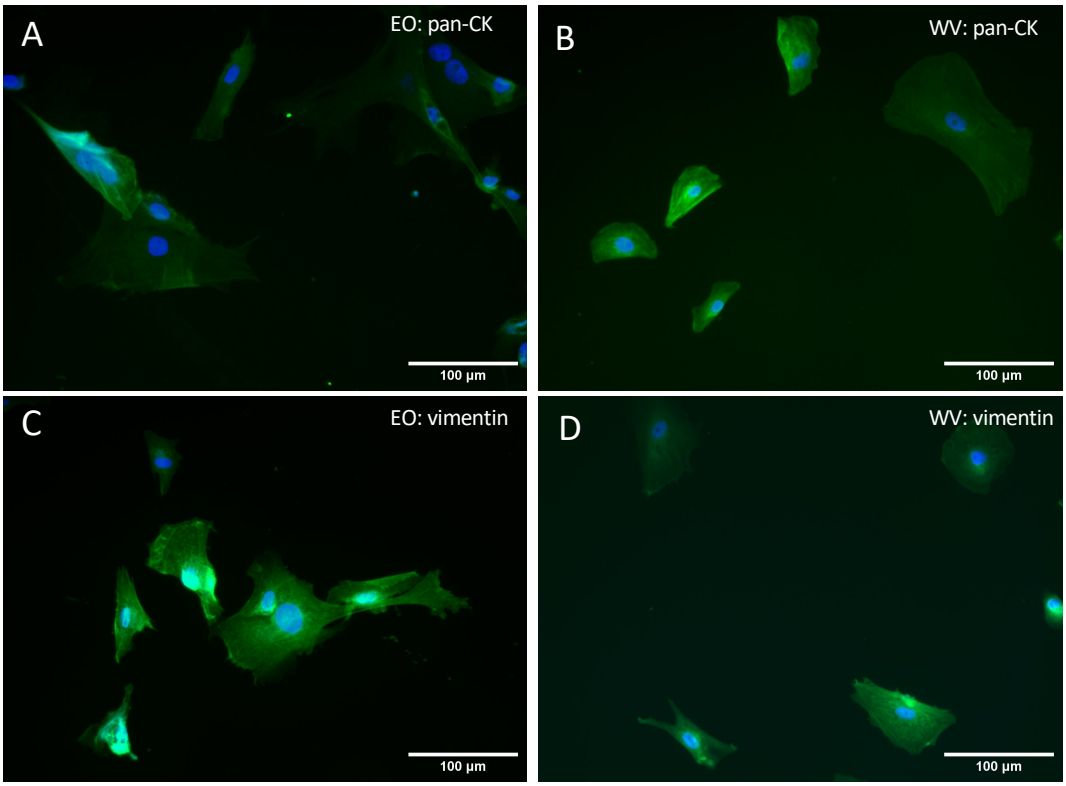

Figure 4-3. Immunocytochemistry staining of epithelial and mesenchymal cell markers in *Z. vivipara* primary oviduct tissue cultures.

Oviparous (**A** and **C**) and viviparous (**B** and **D**) derived cultures were fixed after 119 days in culture and treated with antibodies for either the epithelial cell marker pan-Cytokeratin (**A** and **B**) or the mesenchymal cell marker vimentin (**C** and **D**). Nucleic acids were stained with DAPI, shown in blue. Intermediate filaments containing either pan-CK (A and **B**) or vimentin (**C** and **D**) are shown in green. For both oviparous and viviparous cell lines, virtually all cells showed a positive signal for both epithelial and mesenchymal elements in the cytoskeleton. Images were captured via fluorescence microscopy using an Olympus IX70 microscope.

4.4.3 Transfection of *Z. vivipara* oviduct primary cell cultures with LC3-GFP

I transfected both oviparous and viviparous cells with a DNA construct incorporating DNA and the autophagic vesicle associated protein LC3. Imaging showed a clear signal of green fluorescence in transfected cells when compared to controls. In general, cells showed a broadly homogenous distribution of LC3 throughout the cytoplasm **(Supplementary Fig. 2A)** although a few cells showed a more punctate appearance **(Supplementary Fig. 2B)** which could indicate LC3

recruited to the membranes of autophagic vesicles. However, there was no clear association between LC3 localisation and the distinctive bright vesicles visible under phase contrast microscopy.

To optimise lipofection efficiency, I seeded cultured oviduct cells onto coverslips at three different densities (21 cells mm^{-1}, 105 cells mm^{-1} and 211 cells mm^{-1}) and attempted lipofection with the two different concentrations of Lipofectamine 3000 reagent **(Supplementary Fig. 3A)** I then captured fields from each coverslip in haphazard fashion, and calculated transfection efficiency for each treatment as the proportion of visibly fluorescing cells (indicated by green fluorescence) out of all visible cells (indicated by DAPI staining of the cell nucleus). Transfection efficiencies were similar for EO and WV cells, whereas efficiency was greater both at higher concentrations of Lipofectamine 3000 reagent, and at lower cell densities **(Supplementary Fig. 3B)**.

4.4.4 RNA sequencing

I successfully sequenced a total of 18 samples, 2 *ex vivo* oviduct samples and 16 cell culture samples, across four sequencing runs. The cell culture samples included cultured cells from 4 individuals (biological replicates) at different timepoints in culture with at least 2 cultures (technical replicates) for each timepoint. Median read length (N50) varied between 913 bp – 1.52 kb, with 197,044 - 2,086,214 reads per sample of which 72.18 - 89.83% mapped successfully to the reference genome (**Table S4-1**).

4.4.5 Assessment of cell culture induced changes in gene expression

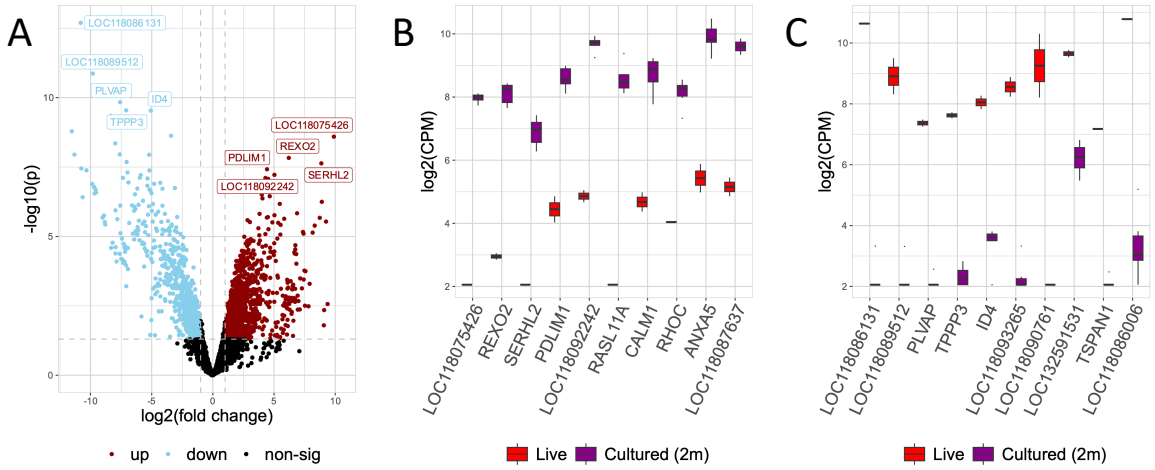

Figure 4-4. Differential gene expression in live and cultured viviparous *Z. vivipara* oviduct cells.

A: Volcano plot showing genes expressed in cultured viviparous oviduct cells 2 months as compared to live viviparous oviduct. **B:** Top 10 upregulated genes in cells cultured for 2 months as compared to live oviduct. **C:** Top 10 downregulated genes in cells cultured for 2 months as compared to live oviduct.

To examine the stability of gene expression in cultured oviduct cells, I performed differential gene expression (DGE) analysis comparing gene expression in the oviduct tissue to oviduct cells derived from lizards from the same population and reproductive stage (post-parition), and maintained in culture for 60+ days. 1800 genes were significantly differentially expressed between cultured cells and the live oviduct (abs LogFC > 1, $p < 0.05$) (**Fig. 4-4A, Table S4-2).** 1186 differentially expressed genes (DEGs) were upregulated in cultured cells, with top upregulated genes including REXO2, SERHL2, PDLIM1, RASL11A, CALM1, RHOC and ANXA5 **(Fig. 4-4B)**. Upregulated genes were highly significantly enriched (Fisher's Exact Test, $p < 0.01$) for 55 biological process (BP) gene ontology (GO) terms (**Table S4-3**). The top 5 enriched BP:GO terms were GO:0046034, GO:0009167 and GO:0009206 which relate to purine ribonucleoside or ATP metabolism, and GO:0070125 and GO:0070126 which relate to mitochondrial translation, suggesting significant changes to cellular energy metabolism in response to the cell culture environment. 614 DEGs were downregulated; top downregulated genes included PLVAP, TPPP3, ID4 and TSPAN1 **(Fig. 4-4C**). Downregulated genes were significantly enriched for 97 GO terms (**Table S4-4**). The top 5 BP:GO terms were strikingly diverse; including

GO:0001817 (regulation of cytokine production), GO:0006357 (regulation of transcription by RNA polymerase II), GO:0007613 (memory), GO:0001655 (urogenital system development) and GO:2000117 (negative regulation of cysteine-type endopeptidase activity). Broadly similar results were obtained when comparing the live oviduct to cells cultured for 30-45 days (see **Tables S4-5, S4-6, S4-7**).

4.4.6 Comparison of the gene expression profile of cultured oviparous and viviparous cells

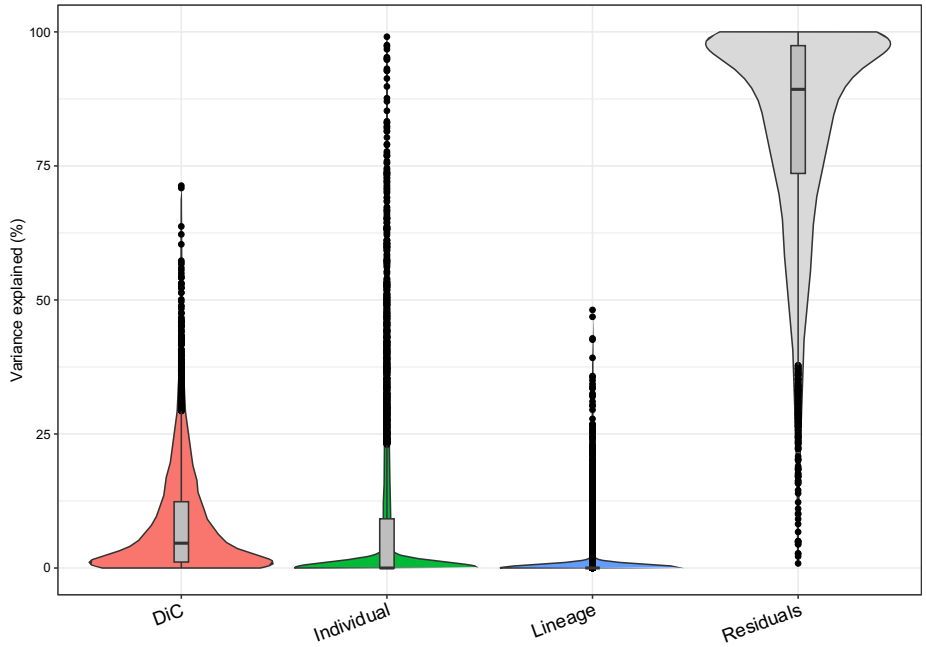

Figure 4-5. Contribution of days in culture (DiC), individual and lineage to variance in gene expression in cultured oviduct cells.

Results from linear mixed model of gene expression in 16 cell culture samples derived from 4 individual lizards from two lineages (oviparous and viviparous). Batch effects were removed using edgeR prior to analysis. “Residuals” plot includes all variance in gene expression not explained by these three factors.

To assess transcriptomic differences between oviparous- and viviparous-derived cell cultures under equivalent conditions, I compared gene expression at a 30 and 60 days in culture period. The model showed days in culture (DiC) to be the most significant variable, explaining a median of 4.67% of variance, whereas the contribution of individual and lineage were negligeable overall. The majority of variance was attributed to residual factors, indicating significant variance in expression driven by factors outwith the model **(Fig. 4-5)**.

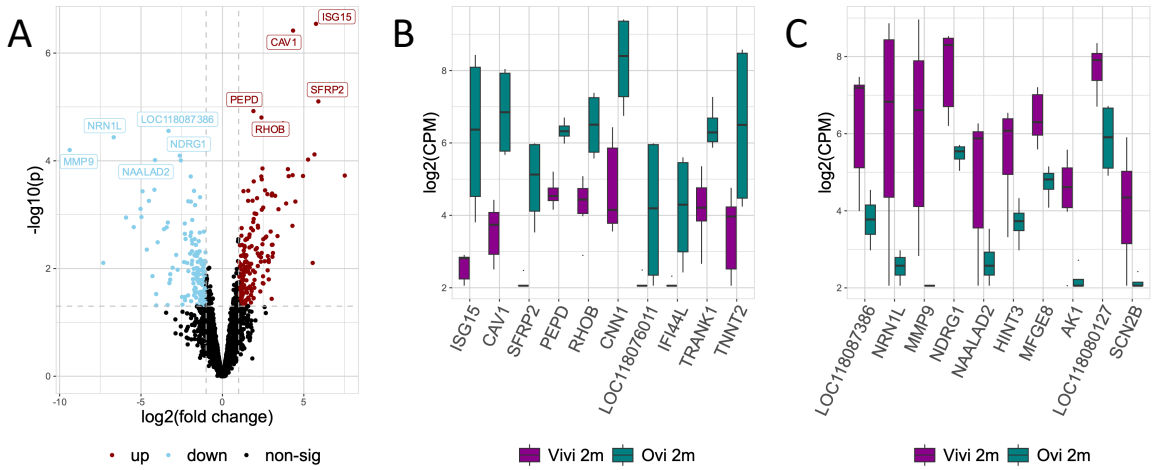

Figure 4-6. Differential gene expression in cultured viviparous and oviparous *Z. vivipara* oviduct cells.

A: Volcano plot shows differentially expressed genes (DEGs) for oviparous vs viviparous cultured oviduct cells after 60+ days in culture. **B:** Boxplot showing top upregulated DEGs in oviparous as compared to viviparous cells **C)** Boxplot showing top downregulated DEGs in oviparous as compared to viviparous cells (i.e. upregulated DEGs in viviparous as compared to oviparous).

Given the impact of the duration of cell culture on gene expression, I performed DGE analysis of oviparous and viviparous oviduct cell cultures specifically at 60+ days, to control for gene expression changes driven by DiC. I found 354 significant DEGs between cells from oviparous and viviparous lizards **(Fig 4-6A, Table S4-8)**. Of the 185 DEGs upregulated in oviparous cells, the top significant genes included ISG15, CAV1, SFRP2, PEPD, RHOB, CNN1 IFI44L, TRANK1, and TNNT2 **(Fig. 4-6B)**. Testing for over-representation of BP:GO amongst these upregulated genes revealed 35 highly significant terms **(Table S4-9)**, with the top 5 including GO:0019730 (antimicrobial humoral response), GO:0010543 (regulation of platelet activation), GO:1903792 (negative regulation of monoatomic anion transport), GO:0050806 (positive regulation of synaptic transmission) and GO:1990748 (cellular detoxification). Of the 169 DEGs downregulated in oviparous cells (i.e. upregulated in viviparous cells), top significant genes included NRN1L, MMP9, NDRG1, NAALAD2, HINT3, MFGE8, AK1, and SCN2B **(Fig 4-6C)**. There were 20 highly significant over-represented GO terms (**Table S4-10)** with the top 5 including GO:0033631 (cell-cell adhesion mediated by integrin), GO:0006024 (glycosaminoglycan biosynthetic process), GO:0006027 (glycosaminoglycan catabolic process), GO:0042475 (odontogenesis of dentin-containing tooth) and GO:0006284 (base-excision repair). I also found

differential expression between oviparous and viviparous cell cultures at 30-45 days **(Tables S4-11, S4-12, S4-13)**; 108 DEGs were significant in both analyses.

4.4.7 Weighted gene network correlation analysis of cultured and live oviduct tissue

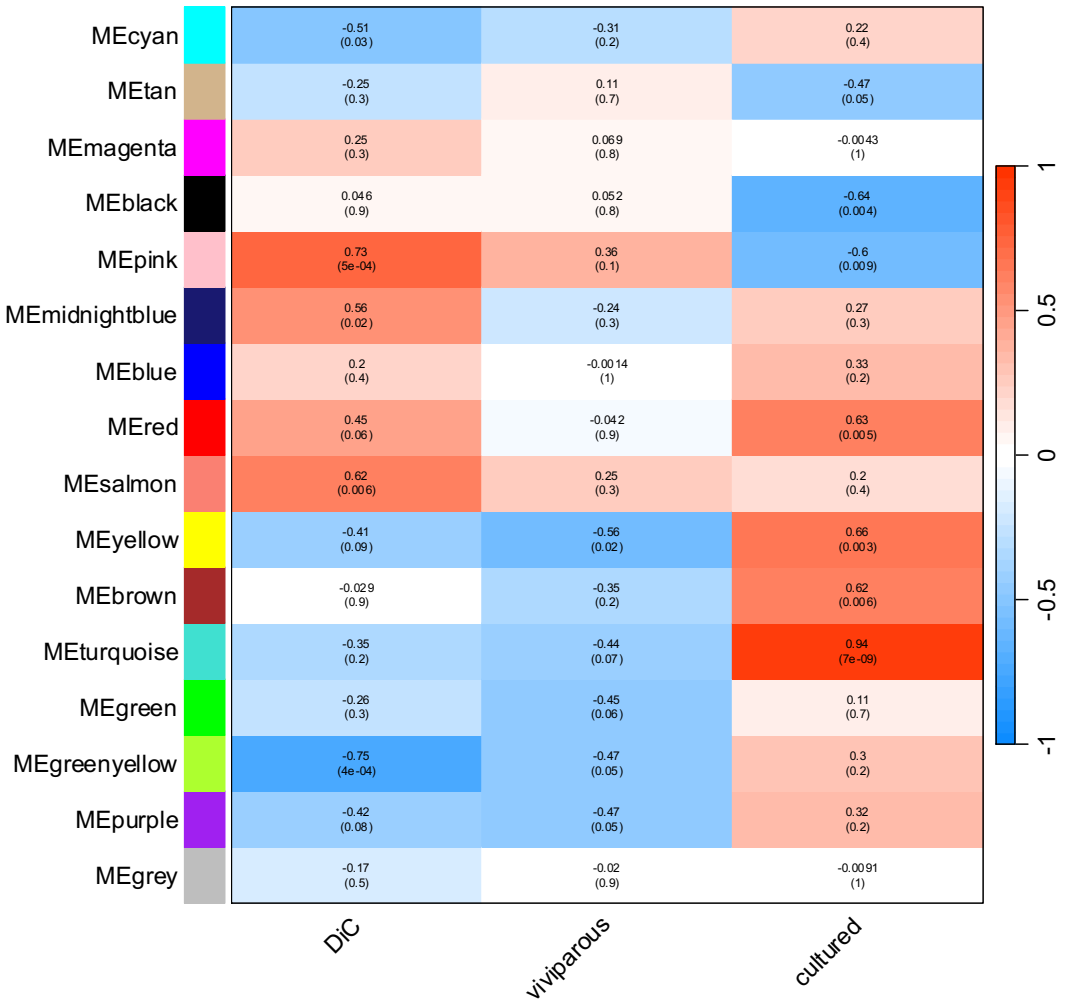

Figure 4-7. Module-trait relationships for co-expressed gene modules in oviduct cell cultures and live oviduct samples.

16 gene co-expression modules were recovered using weighted-gene network correlation analysis (WGCNA) of gene expression in 16 cell culture samples from two lineages (oviparous and viviparous) and two live oviduct samples (viviparous) using a soft-thresholding power of 7. Rows show module eigengenes for each gene co-expression module, columns show correlation of each eigengene with days in culture (DiC), viviparous lineage (as opposed to oviparous lineage) of the cells, and cultured (as opposed to *in vivo*) cells. Legend indicates strength and direction of correlation, with red indicating positive correlation and blue negative correlation. Numbers in cells show the strength of the correlation with p-values in brackets indicating the probability of the apparent correlation occurring purely by chance.

I performed weighted gene network co-expression analysis (WGCNA) to search for module of co-expressed genes correlated with days in culture (DiC), parity mode (oviparous or viviparous) and condition (cell culture vs. *in vivo*), using a single co-expression network which clustered into 18 modules **(Fig. 4-7**). A single module was significant for parity mode, while 7 modules were significant for condition (cultured vs. *in vivo*) and 7 were significant for DiC. Of these, a single module was significant for all three factors, and one other module was significant for both DiC and condition.

The co-expression module with significance to viviparity (as well as condition and DiC) contained 438 genes. The module was downregulated in viviparous cells (as opposed to oviparous), and also downregulated with increasing days in culture, but upregulated in cultured as opposed to *in vivo* tissues. Among genes in this module, 15 BP:GO terms were highly significantly overrepresented (**Table S4-14**). The top 5 BP:GO terms included GO:0006890 (retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum), GO:0051155 (positive regulation of striated muscle cell differentiation) and GO:0045851 (pH reduction), as well as the immune-system related GO terms GO:0045579 and GO:0002478.

The module with the highest significance for condition was a large module containing 1258 genes, and was positively correlated with cultured cells. 22 BP:GO terms were highly significantly overrepresented for genes in this module (**Table S4-15**). The most significant GO terms included two related to regulation of calcium ion concentration (GO:0007204 and GO:0051480) and two related to signalling (GO:0019932 and GO:0031664). By contrast, the most significant module negatively correlated with the cell culture condition was a comparatively small module containing 196 genes. 30 BP:GO terms were highly significant for this module, with three of the most significant terms relating to RNA metabolism (GO:0051254, GO:0051253 and GO:0006357).

4.5 Discussion

I report the first successful primary culture of cells isolated from lizard oviduct tissue. Microscopy of developing cell cultures demonstrated a broadly stable cell morphology between 12 – 90 days in culture (**Fig. 4-1),** during which time cell

cultures underwent steady expansion for over a dozen passages. Obtaining a proliferative primary culture that can be frozen and regrown and is susceptible to transfection with exogenous DNA has significant value as a tool to investigate the basis of reproduction differences. In this study, I was able to show distinct transcriptomic features of viviparous and oviparous cells even after significant time in culture, as well as confirm the presence of specific candidate genes with relevance to viviparity in this system.

4.5.1 Cultured *Z. vivipara* cells show both epithelial and fibroblast-like characteristics and cytoskeletal markers

The overall pattern of growth and development observed in my *Z. vivipara* oviduct cell cultures was indicative of fibroblast-like cells. The progression seen in all cultures from small bipolar cells to large morphologically diverse cells growing in close and loose associations resembles the development of fibrocytes into fibroblasts. Upregulation of genes relating to collagen synthesis processes (GO:0032964) in cultured cells as compared to *in vivo* expression is also suggestive of fibroblasts. However, I also note the appearance of cell granules **(Fig. 4-2)** resembling those reported in chicken epithelial oviduct cell cultures (Stadnicka et al. 2018), and of bright vesicles similar to those seen in lizard keratinocyte cultures (Polazzi and Alibardi 2011), both of which may indicate an epithelial origin for at least some cells. Fibroblasts play an important role in the structure of the squamate oviduct, forming the intermediate layer of tissue known as the *lamina propria* which separates the inner epithelium from the muscular outer sheath (Daniel G. Blackburn 1998b). Fibroblasts likely play a role in viviparity, given their role in tissue remodelling and the dramatic changes in the structure of the oviduct throughout pregnancy. However, in my view it is likely that the most important differences between oviparous and viviparous lizards are expressed in epithelial cells, which make up the inner surface of the oviduct and thus form the interface between the mother and the developing egg or neonate (Daniel G. Blackburn 1998b; Uribe et al. 1988).

The results of my immunocytochemical investigation of my primary cells were also somewhat contradictory. While virtually all cells appeared positive for the mesenchymal intermediate filament (IF) protein vimentin, as would be typical of fibroblast-like cells, I also detected many cells which were positive for

cytokeratin, the IF protein typical of epithelial cells **(Fig. 4-3**). The expression of both vimentin and cytokeratin is characteristic of cells undergoing the epithelial-mesenchymal transition, in which epithelial cells differentiate into fibroblast-like cells (Kuburich et al. 2022; Mendez, Kojima, and Goldman 2010). It is now understood that many fibroblast-like cells in fact have their origin in epithelial cell layers (K. K. Kim et al. 2006; Iwano et al. 2002). The presence of EGF, which I used in my cell culture medium, may promote EMT, as has been previously shown in breast cancer cells (J. Kim et al. 2016). The reverse process, mesenchymal-epithelial transition, has also been recognised as playing an important role in organismal development and disease states (Pei et al. 2019), and might also explain the contradictory signals observed in my immunocytochemical testing. RNA sequencing confirmed that all cell cultures expressed epithelial genes including the Epithelial Membrane Protein 1 gene (EMP1), the keratin 1 gene (KRT8) and the keratin 18 gene (KRT18). In general, it appears that cell culture triggers dramatic changes in the composition of cytoskeletal proteins (and gene expression more broadly) but that cells do continue to express some typical epithelial genes, potentially including genes relevant to reproductive mode.

4.5.2 Transcriptomic profiles differ between oviduct tissue and cultured cells and between oviparous and viviparous cells

My analyses suggest oviduct cell cultures showed dramatic differences in gene expression when compared to the oviduct *in vivo*. This result are consistent with prior studies on gene expression in cultured cells (Ogata, Yokoyama, and Iwabuchi 2012). In this study, comparison of live and cultured cells yielded the largest number of differentially expressed genes (DEGs) (**Fig. 4-4**) and was also associated with a number of co-expression modules **(Fig. 4-7**). Time in culture significantly impacted gene expression (**Fig. 4-5**) suggesting continuing changes in cell cultures over time after transfer to the cell culture environment. In particular, I noted several cell-cycle and cancer-related genes amongst highly upregulated DEGs in cultured cells. The RNA Exonuclease 2 gene (REXO2, log FC 6.208, $p = 1.48 \times 10^{-8}$) is an exonuclease which plays a role in degradation of small mitochondrial RNAs and has been linked to poor prognosis in various cancers (H. Wang et al. 2021). The Ras Homolog Family Member C gene (RHOC, log FC 4.519, $p = 8.49 \times 10^{-8}$) is part of a family of genes known to be involved in

pathways related to cytoskeletal rearrangement and cell motility and is implicated in tumour cell proliferation and metastasis (Horiuchi et al. 2003). The Calmodulin 1 gene (CALM1, log FC 4.296, $p = 8.49 \times 10^{-8}$) encodes a calmodulin protein responsible for regulating a range of cellular pathways mediated by calcium ion signalling pathways, including cytokinesis (Tsang et al. 2006). Upregulation of genes relating to cell motility and proliferation makes sense as an adaptation to life in the cell culture environment. Additionally, GO term analysis of DEGs between live and cultured cells, and gene modules associated with the cell culture condition, suggests significant changes to cellular metabolism, especially in the mitochondria, as well as dramatic changes to the regulation of the cell cycle, RNA metabolism, and cell-cell communication, further reinforcing the breadth of the changes in gene expression in cultured cells relative to normal *in vivo* expression.

Nonetheless, even after long-term cell culture for over 60 days, at which point cultured cells had undergone significant expansion and apparent de-differentiation, transcriptional differences between oviparous and viviparous cell cultures were present, both in the differential expression of 354 genes (**Fig. 4-6**) and in a significant module of co-expressed genes (**Fig. 4-7**). These differences are subtle compared to the changes induced by primary cell culture, but their persistence even after long-term culture of oviduct primary cells shows the validity of the model for the study of reproductive mode, provided confounding factors such as time in culture are accounted for. Highly upregulated genes in oviparous cells included immune-related genes, such as the ISG15 Ubiquitin Like Modifier gene (ISG15, log FC 5.749, $p = 2.86 \times 10^{-7}$) which encodes a member of the ubiquitin family of proteins which plays a role in antiviral immune responses (Perng and Lenschow 2018), and the Caveolin 1 gene (CAV1, log FC 4.338, $p = 3.82 \times 10^{-7}$) which has been linked to T-cell proliferation (Ohnuma et al. 2007). Suppression of the maternal immune response to the developing embryo is an important aspect of the evolution of viviparity (Samardžija et al. 2020; Keverne 2014; Genebrier and Tarte 2021), suggesting a possible link between downregulation of these genes and viviparity. Intriguingly, highly downregulated genes in oviparous cells (i.e. highly upregulated genes in viviparous) included two genes linked to the nervous system: the Neuritin 1 Like gene (NRN1L, log FC -6.676, $p = 3.66 \times 10^{-5}$) is an extracellular protein which promotes growth and

survival in neuronal cells (Fujino et al., 2008), and the Matrix Metallopeptidase 9 gene (MMP9, log FC -9.378, p = 6.31 x 10^{-5}) is part of the matrix metalloproteinase gene family which has been linked to neurological illness in humans (Dickerson et al. 2023; Seitz-Holland et al. 2021). The potential significance of such genes to reproductive mode is obscure, although matrix metalloproteinases in general are connected to the breakdown of the extracellular matrix during tissue remodelling and embryonic development, processes with a more direct link to the biology of viviparity.

4.5.3 Cultured oviduct cells express candidate genes linked to the evolution of viviparity

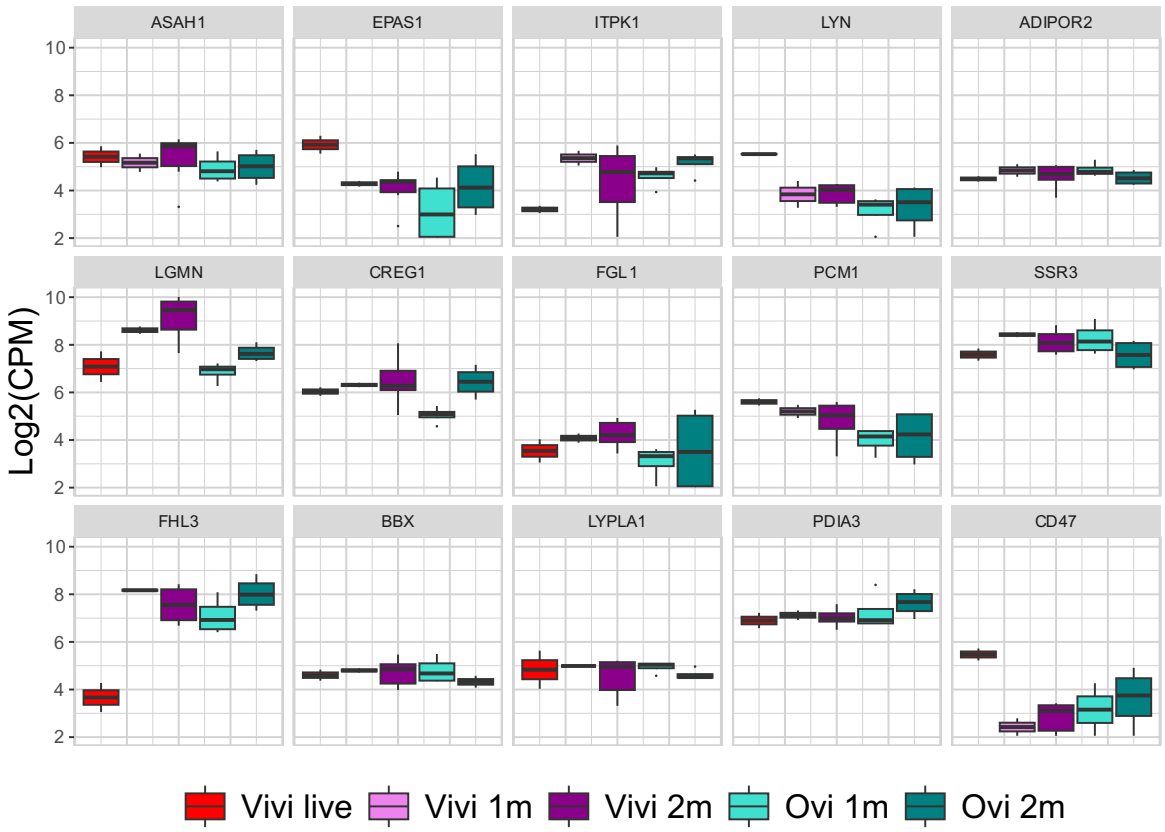

Figure 4-8. Candidate genes for viviparity expressed in cultured Z. vivipara oviduct cells.

Of 38 potential candidate genes previously identified in common lizards from admixture mapping and transcriptomics (Recknagel et al., 2021), 15 were significantly expressed in both oviparous and viviparous cell cultures, based on a threshold of ~10 CPM in at least one sample group after library size correction.

In addition to characterising patterns of differential expression and gene co-expression modules under tissue culture conditions, RNA-sequencing also confirmed the continued expression of several key genes with significance to

reproductive mode. Of 38 candidate genes previously identified through admixture mapping of hybridised *Z. vivipara* and differential expression during pregnancy in four or more viviparous vertebrates (Recknagel et al. 2021), 15 were expressed at significant levels at both time points and across both lineages **(Fig. 4-8)**. The candidate genes include the Lysophospholipase 1 gene (LYPLA1), the Legumain gene (LGMN) and the LYN Proto-Oncogene, Src Family Tyrosine Kinase gene (LYN), all three of which were strongly associated with both eggshell traits and embryo retention in the previously published admixture mapping analysis, showing strong relevance to reproductive traits in *Z. vivipara*. The remaining genes were significant for embryo retention only, but all genes showed differential expression in multiple viviparous squamates and/or mammals during pregnancy (Recknagel et al. 2021).

Significantly, LGMN also showed differential expression between oviparous and viviparous cells after 60 days in culture (log FC -1.68, p = 0.000665), making this a strong candidate for future study in this system. Expression of these genes is an important confirmation of the utility of the primary cell culture model for the analysis of the functional genetics of reproductive mode, as these represent important potential targets for future gene knockout studies.

4.5.4 Conclusion

I developed a new biological model for the study of the genetic and cellular basis of reproductive mode consisting of cultured *Z. vivipara* oviduct tissue, with potential applications including the functional validation of candidate genes underpinning viviparity in *Z. vivipara* and other species. Although I demonstrated significant changes in gene expression in *Z. vivipara* oviduct cells exposed to the tissue culture environment, I nonetheless found significant differences in expression of oviparous and viviparous cells were maintained for more than 60 days in culture, and confirmed the expression of over a dozen important candidate genes for viviparity, confirming the validity of this model system for the study of reproductive mode. Future work will further characterise the behaviour of *Z. vivipara* oviduct cells in culture, refine the cell culture conditions and medium to provide a valuable tool for future experimental studies of the genetic and cellular basis of viviparity.

5 General discussion

5.1 Abstract

In the preceding chapters I presented a number of studies that offer new insights into the question of the biological foundations of viviparity and oviparity, and the evolution of reproductive mode in *Zootoca vivipara* and other animals. I first summarise the key findings below, and go on to discuss their potential impact on the field of study of reproductive mode evolution and squamate pregnancy. I then go on to discuss key avenues for future research on these topics, concluding by outlining an experimental approach to the validation of gene function for candidate genes associated with reproductive mode, using the model described in **Chapter 4** informed by the results from **Chapter 2** and **Chapter 3** and by previous research in the fields of reproductive mode and primary cell culture technique.

5.2 Summary of key findings

A graphical summary of the key findings for all data chapters is shown in **Fig. 5-1**.

In the first part of this thesis (**Chapters 2** and **3**) I report the results of two RNA sequencing experiment aimed at building upon existing work on the transcriptomic basis of viviparity in *Z. vivipara* and other animals, integrating previous work on pregnancy in both squamates and mammals. The goal of this section was to characterise transcriptional regulation in the squamate oviduct before, during and after pregnancy; link established patterns at the scale of whole oviduct tissue to expression in specific cell types, especially with regard to established parity-mode-related genes; and investigate the potential role of alternative splicing alongside changes in overall gene expression by leveraging long-read sequencing technology. Given the large body of research pointing to the importance of alternative splicing in pregnancy in other viviparous animals (G. Y. Nie et al. 2002; Ruano et al. 2021; Gopalakrishnan and Kumar 2020; Gui Ying Nie, Li, Batten, et al. 2000; Zeng et al. 2018) and the lack of any published studies on alternative splicing in pregnancy in squamates, my results bridge an important gap between the growing, but still relatively small literature on gene

regulation in the squamate reproductive system (Hendrawan et al. 2017; Brandley et al. 2012; W. Gao et al. 2019; Foster et al. 2020; Recknagel et al. 2021) and the much more detailed picture of the importance of these different elements of transcriptional regulation to mammalian pregnancy.

In **Chapter 3** I reported the first single-cell sequencing study of the reproductive system of a squamate, with a focus on describing the expression patterns of previously described viviparity-associated candidate genes at single-cell resolution, and characterising changes in expression in pregnant and non-pregnant states. I attempted to characterise cell types present in the sample on the basis of previously described gene expression markers for human cell types, given the extremely limited single-cell resolution data on gene expression in reptile tissues, and the lack of a comprehensive squamate equivalent to the cell atlases available for humans, mice and certain other taxa (Garcia-Flores et al. 2023; Travaglini et al. 2020). In this exploratory study I was able to confirm differential expression of one candidate gene for viviparity, LGMN, (Recknagel et al. 2021) in a set of cells expressing markers which in humans are associated with phagocytotic cells of the innate immune system. I suggested a possible role for such cells in the processes of tissue remodelling that are required for successful pregnancy, as has been shown for other viviparous species. I also reported some evidence of elevated expression of other markers in specific cell types, although the effect sizes were below the threshold for differential expression. I also commented on apparent proportional changes in cell populations during and after pregnancy, and their potential relevance to observed changes in the tissues of the viviparous squamate oviduct in pregnancy. These results show the potential of single cell resolution gene expression analysis to give important context to gene expression changes seen in tissue-level gene expression analysis, as many important processes specific to viviparity will involve expression of genes in specific cell types.

In **Chapter 4** I presented a new model system for research on the molecular and cellular basis of reproductive mode, consisting of primary cell cultures derived from viviparous and oviparous *Z. vivipara*. I was successful in establishing proliferating cell cultures derived from *Z. vivipara* oviduct tissue from both viviparous and oviparous lizards, maintaining them in culture for long periods,

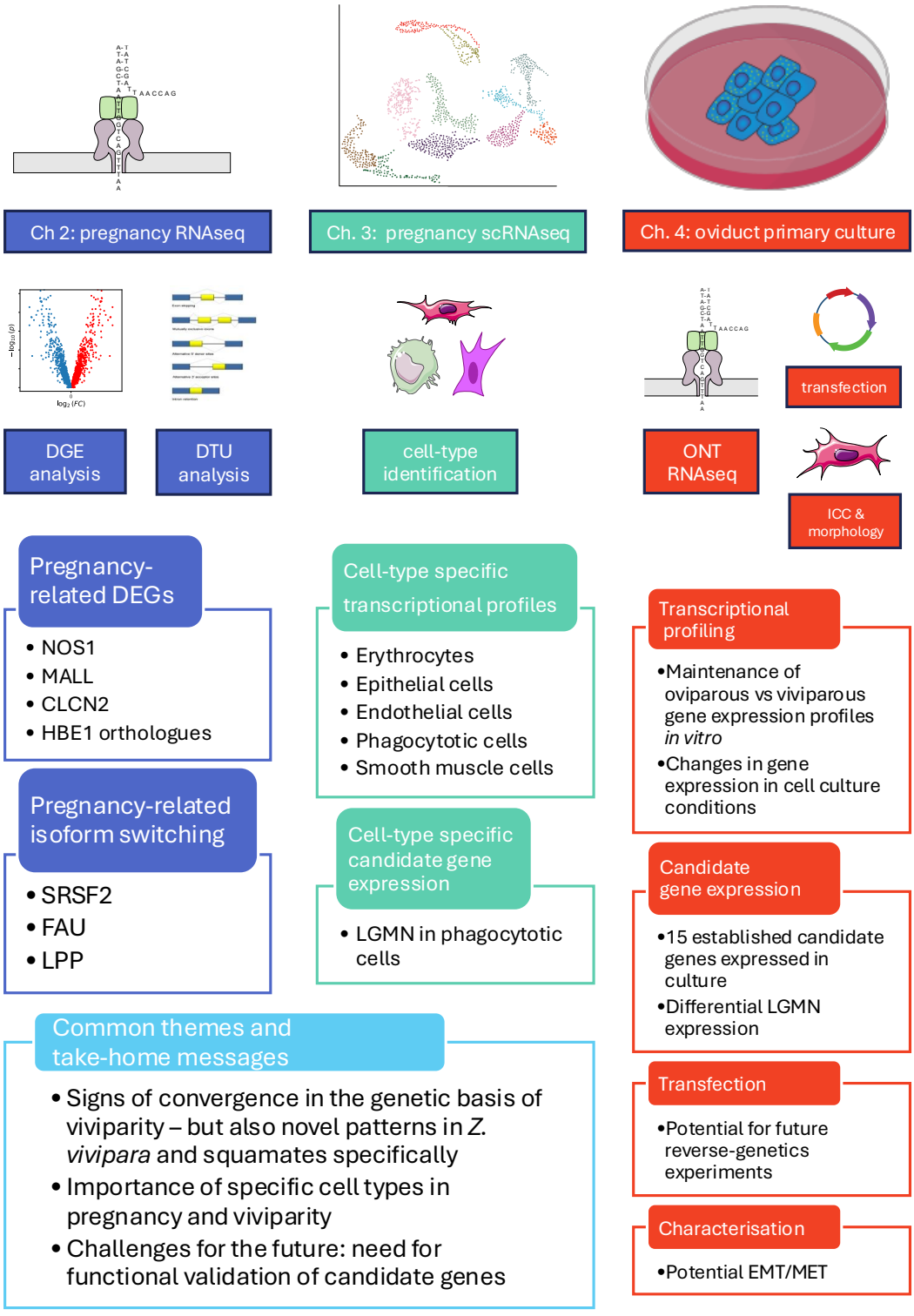

Figure 5-1: Schematic representation and summary of key findings from chapters 2, 3 and 4.

freezing and reviving cells, characterising cytoskeletal markers present in cell cultures, and transfecting cells with exogenous DNA. I also reported the results of experiments characterising differences in gene expression resulting from the cell culture environment and the underlying reproductive mode of the cells, by comparing gene expression in oviduct tissue and cultured cells, cultured cells at different time points, and cells from oviparous and viviparous individuals, using a range of statistical analyses of gene expression. As expected, the impact of the *in vitro* environment on gene expression was substantial, but distinctive oviparous and viviparous gene expression profiles were nonetheless maintained, even after substantial periods of primary cell culture during which cells continued to proliferate. This supports the validity of cell culture as a putative model for the investigation of differences between oviparous and viviparous lineages and the genetic, transcriptomic, and cellular level. Furthermore, I was able to confirm the expression of 15 candidate genes previously established as relevant to the evolution of reproductive mode, which are potential targets for future experiments described below. Additionally, I was able to successfully transfect the cultured cells with an exogenous DNA construct, laying the groundwork for potential future reverse genetic experiments using this cell culture model.

5.3 Study limitations

As with any study requiring lethal sampling of wild vertebrates, there were limitations imposed by the logistics of fieldwork, legal protections and permitting requirements, and ethical obligations under the 3Rs, which limited the number of samples which could be used for experiments. Experiments requiring lizards to be sacrificed at specific points in the reproductive cycle required maintaining lizards in captivity until that point, given the seasonal nature of *Z. vivipara* reproduction – while care was taken to simulate naturalistic conditions as far as possible for lizards housed in captivity at the University of Glasgow, it is possible this period of captivity could have exerted an influence on the features of reproductive biology under study through diet, social environment, or other stressors or factors outwish my control.

A good deal of the work presented here hinges on analysing gene expression to try to investigate the molecular basis of viviparity. I note that analyses of gene

expression data alone are limited in terms of establishing any causal relationship between genes and biological processes. For example gene up- or down-regulation may be the cause of physiological changes in the oviduct which occur during pregnancy, or may be triggered by those physiological changes, or (as is perhaps more usual for any biological system) may be subject to more complex patterns of cause and effect involving positive and negative feedback loops, intricate molecular pathways, and other mechanisms of gene regulation and action. Unpicking such complex interactions for even a single gene is beyond the scope of the studies presented here (but see **Section 5.5** below for proposed experimental approaches to these questions), so the results of these studies should be taken as highlighting viviparity-associated, not necessarily viviparity-causing, changes in gene expression (or for that matter alternative splicing and alternate promotor use).

The work reported in all three chapters makes use of oviduct tissue for either sequencing or as a basis for cell culture experiments. However, the squamate oviduct is comprised of different tissues (chiefly the luminal epithelium, *lamina propria* and *muscularis externa*) and regions (such as the infundibulum, uterine tube, uterus, and vagina) which have distinct functions and are expected to show differences in function, gene expression and presence or absence of different cell types. Since the experiments described here are performed on the whole oviduct, this means I am unable to distinguish in my results any changes related to these different tissues. Although the oviducts of *Z. vivipara* are relatively small and thus different tissues and regions may be difficult to distinguish and separate during sample processing and preparation, separate analyses of these different regions would almost certainly offer additional insight.

Also, data on oviduct length and weight were not collected while sampling oviducts for the experiments in the preceding chapters. Priority was given to rapid processing of tissue to maintain cell viability and RNA integrity respectively, and no significant differences in oviduct size were visually apparent beyond those explicable by differences in overall body size, but changes in the mass or length of the oviduct at different time points during the

reproductive cycle or between different reproductive modes could have offered insight into both the underlying biology and variation in experimental results.

I further note that for my gene expression analyses in **Chapter 2** and the *in vivo* comparisons in **Chapter 4**, sample sizes for some conditions were quite low, (n = 2) for post-parturition and pre-pregnancy. Gene expression analyses lose statistical power at low sample sizes, increasing the probability of errors (Maleki et al. 2019; Hart et al. 2013). I believe that the differential gene expression results are still worth taking seriously but follow up studies with additional biological replicates would increase confidence in the result and may reveal additional differentially expressed genes which could not be detected in this experiment.

Cell ranger quality control checks for the single-cell sequencing study gave values somewhat below the optimal range of valid UMIs per cell for both samples, and for the pregnant sample also a somewhat low fraction of reads in cells, and although the droplet filtering techniques applied by the Cellenics program should correct for this to some extent, results in **Chapter 3** should be interpreted with caution until confirmed by follow-up experiments, particularly with the extremely low sequencing depth obtained for both samples. This is generally true in any case, given the exploratory nature of this study, and I note that comparison of pregnant and non-pregnant states in this section are purely descriptive given that I had only one sample per condition.

Additionally, as I note in **Chapter 4**, for any cell culture study there are inherent limitations to cell culture as a model of living tissue. Cultured cells will always be an imperfect representation of cell phenotype and genotype *in vivo*, as cells change in response to the artificial environment of the culture vessel. The two main factors which drive divergence of primary cell cultures from their tissue of origin are dedifferentiation of cells, and differential proliferation of cell types (Zhuang, Duan, and Yan 2012; Y. Liu et al. 2010; 2009; Ogata, Yokoyama, and Iwabuchi 2012). The possible co-expression of epithelial and mesenchymal cell proteins in my cultures is a potential sign of dedifferentiation, and the fibroblast-like forms which I observed may indicate overgrowth of cells atypical in the living oviduct. These challenges should be borne in mind when

interpreting my results and planning future experiments making use of the cell culture model I describe in this thesis.

5.4 A note on the impact of the COVID-19 pandemic

I began my PhD programme in October of 2019, and the global coronavirus disease 2019 (COVID-19) pandemic which began officially in March 2020 had far-reaching impacts on virtually every aspect of the projects presented here.

Initial plans for the PhD discussed with supervisors would have included 2-3 field seasons spent at the hybrid zone in Austria collecting lizards for use in cell culture and RNA sequencing experiments. However, due to legal restrictions, safety concerns and uncertainties around international travel international fieldwork was delayed until 2022. Given the limitations on international travel, I adapted the projects presented in **Chapter 2** and **Chapter 3** to use only lizards collected from the UK. The common lizard populations present in the UK are universally viviparous: this meant that planned comparative analyses between oviparous and viviparous reproductive tissues were delayed, leaving limited time for the experiments and analysis presented in **Chapter 4**. A planned extension of the tissue culture project, involving targeted gene knockouts of viviparity-related genes carried out using cultured cells, was thus not possible within the timeframe of the PhD.

In addition to legal limitations on international travel, the pandemic had significant impacts on other aspects of my research throughout the PhD. Access to lab space and equipment was highly constrained during the first months of the pandemic, after which lab work was allowed to resume but with limitations on occupancy of labs which slowed the pace of experiments. Additional limitations on the use of office space throughout 2020 – 2022 necessitated extensive home working, which limited opportunities for learning and knowledge and skills transfer through working directly alongside more senior colleagues.

5.5 Oviparity and viviparity at the cellular level: expectations, challenges and possibilities

As with previous studies of the uterine transcriptome of viviparous squamates, my results showed a mixed picture, including some common patterns but also considerable novelty with respect to previous studies, underlining the significant gaps in our current knowledge of this field of study. Gene expression and splicing analysis indicated significant roles for key genes with a broad relevance to pregnancy in other, independently derived viviparous lineages, such as mammals and the other squamates. For example, I detected splicing of the splicing factor SRSF2, which is also differentially expressed at implantation sites in mammals and also shows differences in alternative splicing related to pregnancy in these organisms (G. Y. Nie et al. 2002; Gui Ying Nie, Li, Batten, et al. 2000); and I found differential expression of the legumain gene LGMN in cell cultures derived from oviparous and viviparous cells, which has previously been linked to both eggshell traits and embryo retention in a hybridising population of oviparous and viviparous common lizards (Recknagel et al. 2021). However, the specific patterns of expression and splicing I detected did not map clearly onto results from mammalian model systems. The changes to SRSF2 were detected in late rather than early pregnancy suggesting that the same genes may be being used in different ways to solve the unique problems posed by squamate pregnancy, with processes invoked in early pregnancy in mammals taking place late in squamate gestation.

Expression of LGMN in the uterine tissue in mammals is expected in late pregnancy, but in the epithelium – my results from **Chapter 3** suggest it is not strongly expressed in epithelial-like cells in *Z. vivipara* (Shim et al. 2013). Other results, such as the downregulation of ribosomal protein genes during pregnancy, may parallel results in some mammalian systems (Girotti and Zingg 2003), but was generally unexpected. Indeed, very high levels of convergence in patterns of transcriptional regulation would be surprising given the different evolutionary history **(**see **Chapter 1**) and the different physiology and anatomy of pregnancy in squamates as opposed to mammalian models of pregnancy (or indeed between different viviparous squamates) in terms of placentation and embryonic nutrition (Stewart 2015; 2013), and overall structure and function of the reproductive system (Daniel G. Blackburn 1998a).

The cellular processes underlying pregnancy have so far been described in great detail only in viviparous mammals, particularly by biomedical researchers aiming to better understand health outcomes for human pregnancies (and by veterinary researchers interested in the health and welfare of commercially important domesticated animals) employing a range of approaches including histology (Roach, Guderian, and Brewer 1960; McCartney 1969; Zhemkova and Topchieva 1964; Laguens and Lagrutta 1964; Nelson and Greene 1958), primary cell culture (Cho et al. 2021; Feinberg 1995; Ciccone, Trousdell, and dos Santos 2020; Ho et al. 2001; Chitham, Quayle, and Hill 1973; Roberts, Hubel, and Taylor 1995; Sanyal et al. 1989), and in recent years also single-cell sequencing (Bunis et al. 2022; Whettlock et al. 2022; Q. Chen et al. 2023). By contrast, our knowledge of the role of specific cell types in the pregnant squamate oviduct is limited to a modest number of histological studies (Uribe et al. 1988; D. G. Blackburn et al. 2017; Daniel G. Blackburn 1998a). The studies collected in this thesis thus represent significant progress in improving our knowledge of the cellular basis of pregnancy in squamates, building up the toolkit available to researchers in this field, and thus unlocking the potential for comparative analysis of oviparity and viviparity at the cellular level.

The immediate task following from the results described here is the further characterisation and refinement of the cell culture model of the squamate oviduct. I was successful in maintaining and expanding *Z. vivipara* oviduct cells for over 90 days in culture and over multiple passages, and in showing that differences in reproductive mode can be sustained in cell culture for significant time periods, but considerable work remains to fully characterise the cell types present in the resulting cultures, and further immunocytochemical testing of a range of markers, preferably using different secondary antibodies to allow co-visualisation of multiple markers in the same cell sample, would be invaluable in following the development of processes such as the epithelial-mesenchymal transition and in determining other cell-type specific properties of cultured cells. In particular, testing of protein markers which were differentially expressed in my single-cell sequencing results would potentially allow mapping of the different cell types in the living oviduct which may persist in culture in some recognisable form. If successful, this approach could also help to confirm the identity of cell clusters putatively identified from gene expression alone by

visual inspection of cell morphology and behaviour, and the localisation of specific proteins.

An additional refinement of the protocols described in **Chapter 4** could allow for the isolation of particular cell types from mixed cultures recovered from whole oviduct digestate. Potential approaches to achieve this such as addition of growth factors to promote the differentiation of cells into the desired type or to inhibit the growth of unwanted cell types (Cunha et al., 2008; Hirao et al., 2023; Mizushima et al., 2009; Zhao & Guo, 2023) (Eisinger 1982), use of coatings such as laminin, collagen or hydrogel coatings on culture vessels, 3-dimensional cell culture vessels to promote particular patterns of cell growth (Jensen and Teng 2020; S. Chen, Einspanier, and Schoen 2013), and the "passaging out" of rapidly proliferating fibroblast type cells by cold trypsinisation (Kisselbach et al. 2009b). The current mixed cell culture population seemingly exhibits a generally mesenchymal phenotype, though with some unusual characteristics, and if this characterisation is correct then they may also show potential for multilineage differentiation (pluripotency) (Lorenz et al. 2008; Pittenger et al. 1999), potentially providing a source for many different cell types if appropriate conditions can be found to induce differentiation. If identifiable, differentiated cell types such as for e.g. epithelial cells, endothelial cells or phagocytotic cells can be isolated through such techniques, and their gene expression profiles characterised by RNA sequencing, this would be a powerful tool to inform future single-cell RNA and bulk sequencing studies in *Z. vivipara* and other squamates, as well as providing the basis for a programme of functional validation of candidate genes (see later section).

5.6 The future of reproductive mode research: towards functional gene validation

The ultimate test of the role of any given candidate gene, such as the legumain gene LGMN (see **Chapter 3**) or the splicing factor SRSF2 (see **Chapter 2**) in determining reproductive mode would be controlled reverse-genetic experiments in live animals: gene knock-downs, knock-outs, or knock-ins using model organisms such as *Z. vivipara* and direct observation of the resulting effect on uterine gene expression profile and reproductive phenotype. Such an approach represents the ideal method to evaluate the function of a given gene

(Adolfi et al. 2019; Rohde et al. 2018; Lu et al. 2007; Boettcher and Simons 2022); however, such experiments are generally challenging and resource-intensive to carry out, even when working with model organisms which are readily available as captive-bred experimental lines with consistent characteristics and well established and straightforward methods of husbandry (Boettcher and Simons 2022). In a nonmodel (or emerging model) organism such as *Z. vivipara*, or indeed any squamate, the challenges are that much greater. I therefore see a significant role for cell culture studies of viviparity as preceding any such experiment in live animals, and thus both streamlining the experimental programme and minimising unnecessary use of experimental animals in line with the three R's (Hubrecht and Carter 2019).

With further refinement of the model as described above, particularly if supported by single-cell resolution gene expression data from both reproductive modes at different stages of pregnancy, it should be possible to identify expression of a given candidate gene to a particular cell type, and to generate a primary cell culture consisting predominantly of the target cell type. Using established transfection techniques such as Lipofection as described in **Chapter 4**, accompanied by methods for selecting successfully transfected cells such as fluorescence-activated cell sorting (FACS), I could then produce targeted gene knockouts for the gene of interest by introducing a CRISPR-Cas9 gene editing construct (Reuven and Shaul 2022; Giuliano et al. 2019). Genes regulated through alternative splicing may require more sophisticated approaches, either knocking-out the splicing regulator of the spliced gene if this is known, or by knocking-in mutations at conserved splicing signals (Gapinske et al. 2018), or by knocking-in artificial splicing factors to artificially produce desired splice variants (M. Du et al. 2020). One important point given the high residual variance observed in our linear mixed model of cell cultures under different conditions is that such experiments would require a high number of technical replicates to ensure that any biological signal is not lost in the noise, but this should be possible given the relatively rapid proliferation of cells in culture after the initial lag period, allowing for many technical replicates to be produced from a single cell culture preparation.

If the gene manipulation produces results worthy of further investigation, for example changes to cell phenotype or growth patterns, or significant changes in gene expression of other genes relevant to viviparity, then live animal experiments can then be undertaken with substantially greater probability of obtaining meaningful results.

5.7 Conclusions: unanswered questions and future horizons

This thesis represents the latest addition to an expanding body of research on the evolution of reproductive mode in squamates and other animals. The study of the molecular and cellular basis of this complex trait has the potential to shed light on many important questions in evolutionary biology; such as convergence vs. parallelism in the genetic basis of homoplasies (Pickersgill 2018; Conte et al. 2012; Storz 2016; Arendt and Reznick 2008); the mechanisms that drive the evolution of new organs and tissues (Griffith and Wagner 2017; Pankey et al. 2014; Gregory 2008; Griffith 2021); and the possibility of, and barriers to, evolutionary reversals (Collin & Miglietta, 2008; Dollo, 1893; Gould, 1970; Lynch & Wagner, 2010; Recknagel et al., 2018).

The question of whether convergence at the level of phenotype (homoplasy) is generally the product of convergence at the molecular level has a long history, and like many important questions in evolutionary biology can be difficult to study experimentally, at least in complex organisms. The evolution of viviparity in squamates and other vertebrate groups, represents a striking case of homoplasy, and the picture that has emerged as genomic and transcriptomic data is published is an interesting one. Recent work using signatures of selection to test for convergence in the evolution of viviparity in Cyprinodontiformes found only a handful of candidate genes showing convincing evidence of convergence in two viviparous lineages (Yusuf et al. 2023); however, a similar study of selection in viviparous snakes showed that candidate genes identified from studies in lizards were also under selection in viviparous snakes, suggesting some degree of parallel evolution (Maggs 2023). Viviparity in amniotes involves specific adaptations, for example relating to eggshell-loss and specific interactions between maternal and embryonic membranes, as opposed to anamniote taxa(Griffith 2021), so it may be that convergence in squamates, but

not in fishes, reflects this underlying biological reality. However, until more is known about the molecular and cellular basis of viviparity in more squamate lineages, this question remains open.

The evolution of complex organs has been a core issue in evolutionary biology going back even farther than the advent of the modern synthesis, going all the way back to Charles Darwin and the earliest debates around natural selection (Gregory 2008). Most complex organs are extremely ancient in origin in, many being older than the divergence of the vertebrate lineage, which makes drawing inferences about their evolution from modern organisms challenging. A notable exception however is the placenta, which is both a complex organ and a recent evolutionary innovation, leading some to propose the placenta as a model for organ evolution in vertebrates (Griffith and Wagner 2017). The key focus for such research is exploring how existing tissues gain new functions in response to evolutionary pressures, through changes in genetics and gene regulation, and in particular changes to signalling pathways between maternal and embryonic cells (Griffith 2021). The further development of cell culture models of the squamate oviduct could offer unique tools to help answer such questions.

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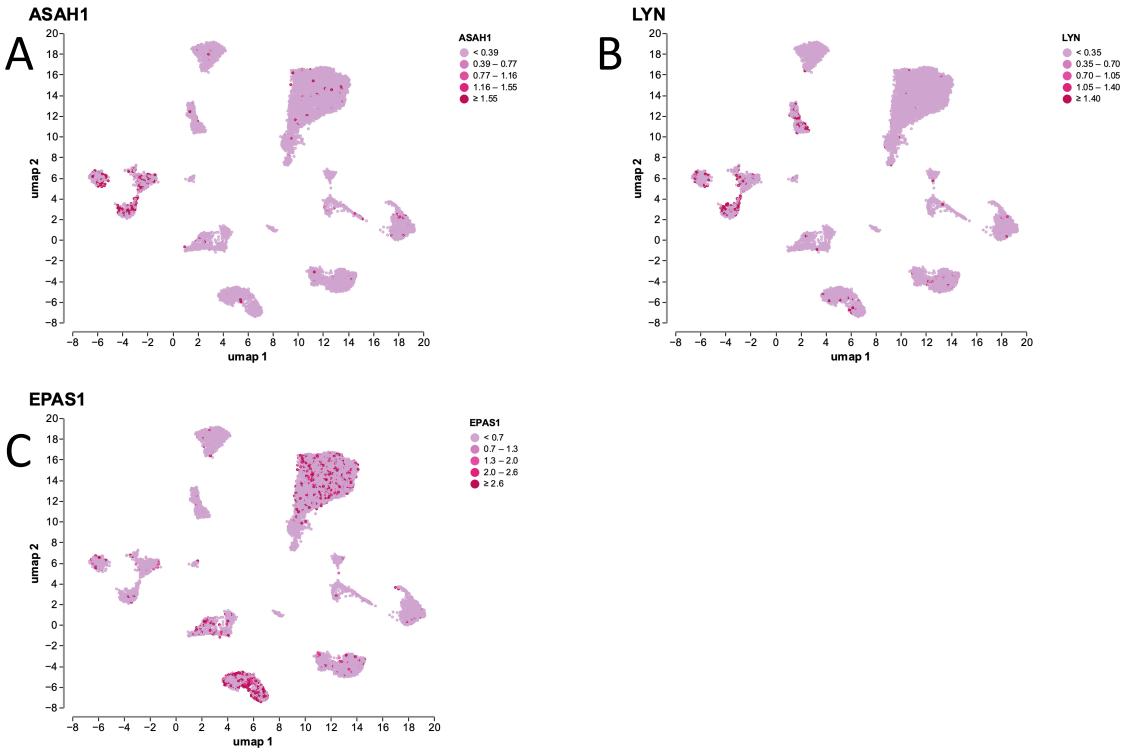
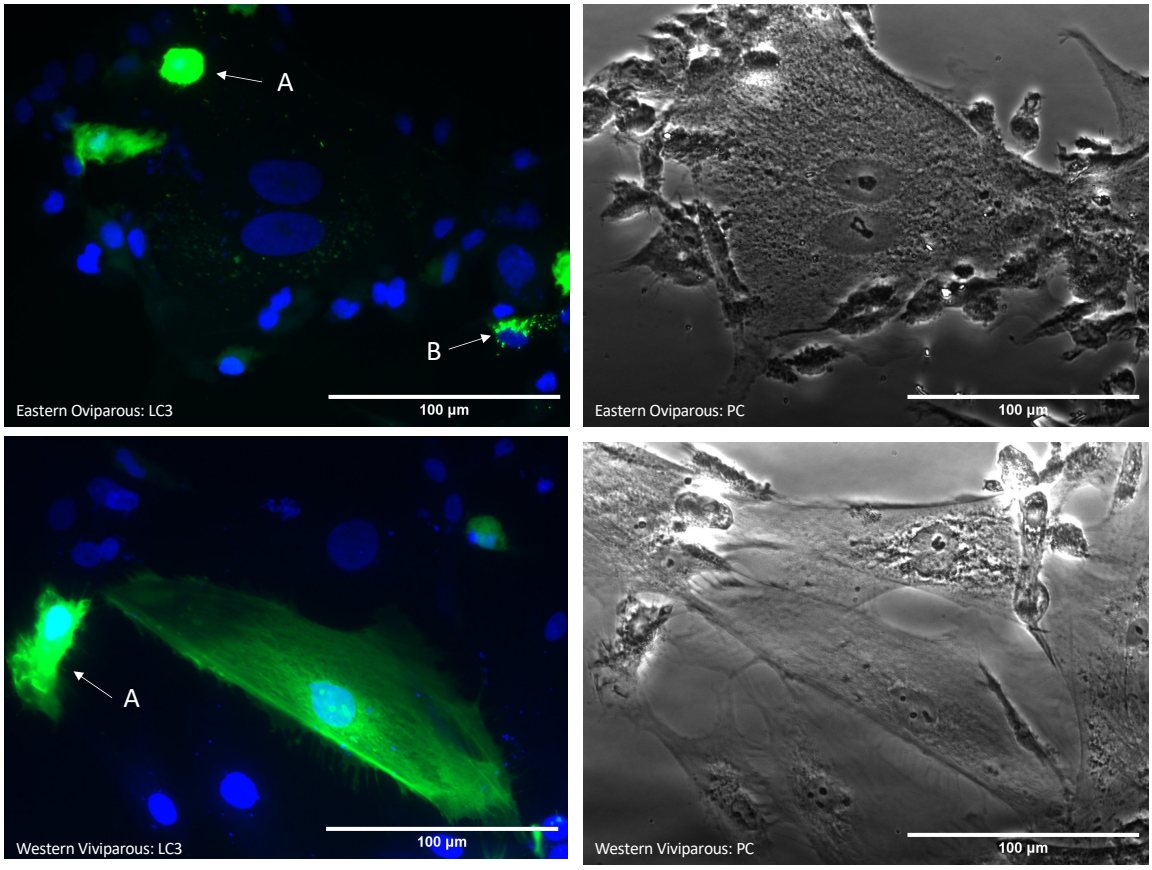
Appendices

Appendix A: Supplementary Figures



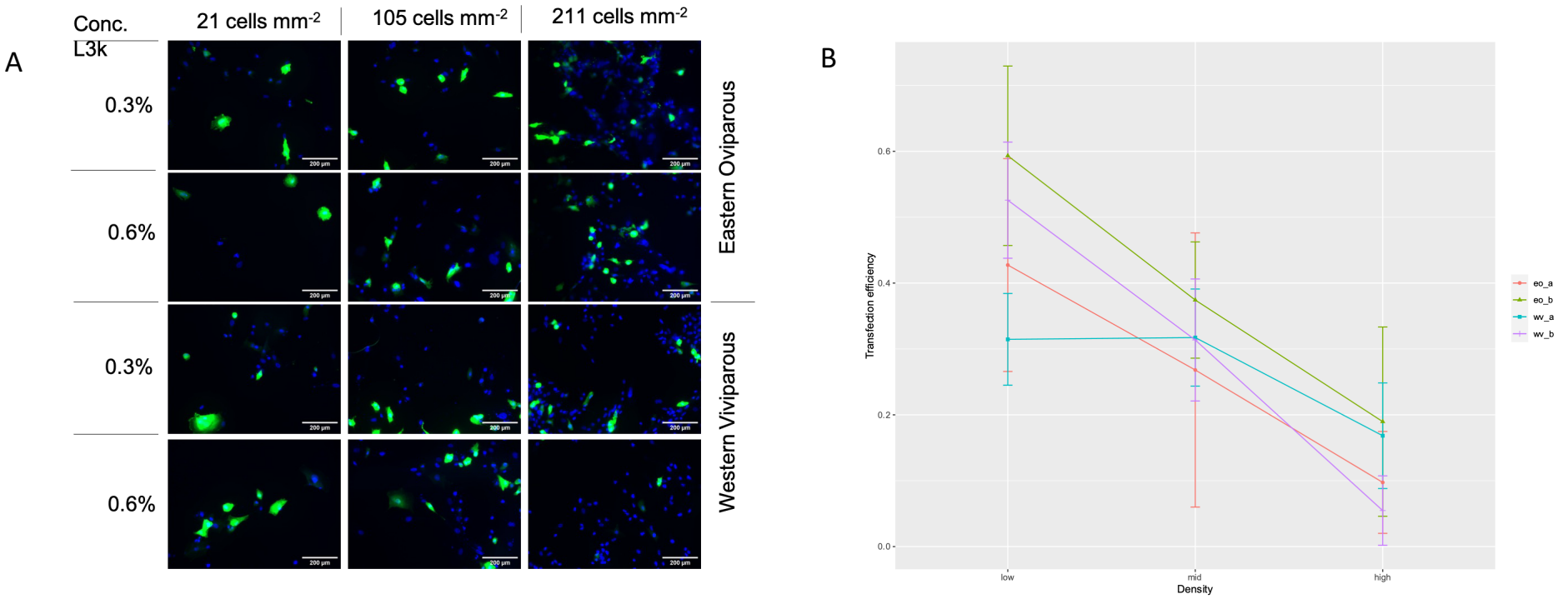
Supplementary Figure 1. Expression of viviparity-associated genes in the *Z. vivipara* oviduct at single-cell resolution.

Expression shown across both pregnant and post-parturition samples for (**A**) ASAH1 (**B**) LYN (**C**) EPAS1



Supplementary Figure 2. Transfection of cultured *Z. vivipara* oviduct cells shows distribution of LC3 in the cytoplasm.

Oviduct cells were isolated from oviparous (Eastern Oviparous) and viviparous (Western Viviparous) were seeded onto coverslips and transfected with a GFP-LC3 plasmid construct (green), before fixing and staining with DAPI (blue) to visualise cell nuclei. Images were captured using an Olympus IX70 microscope in both fluorescence (Left) and phase-contrast (Right) mode. Cells showed varying distribution of LC3, including cytoplasm-wide **(A)** and more punctate distribution **(B)** perhaps indicating the presence of autophagic vesicles.



Supplementary Figure 3. Transfection efficiency in cultured *Z. vivipara* oviduct cells varies with density of cell cultures.

Oviduct cells isolated from oviparous (Eastern Oviparous) and viviparous (Western Viviparous) *Z. vivipara* were seeded onto coverslips at densities of 21, 105 and 211 cells mm $^{-2}$ and transfection was attempted with the GFP-LC3 using Lipofectamine 3000 reagent at two different concentrations. After overnight incubation cells were fixed and stained with DAPI for imaging. Five fields were captured per treatment and parity mode - representative fields from each condition for each parity mode are shown **(A).** Fluorescing cells are shown in green; cell nuclei are shown in blue. Transfection efficiencies tended to decrease with increasing densities **(B)**

Appendix B: Supplementary Tables

Table S2-1: Oviduct libraries prepared for long-read RNA sequencing

SampleID	*Individual*	*Condition*	*Reads*	*Mapping*
O1	ELT13768	pre rep	1788746	90.28%
O2	ELT13769	pre rep	821220	94.17%
PREG3	ELT13777	pregnant	1994634	84.43%
PREG4	ELT13778	pregnant	2222284	86.34%
PREG5	ELT13779	pregnant	277789	87.39%
PREG6	ELT13780	pregnant	601866	86.25%
PP1A	ELT13078	post rep	726071	72.18%
PP2B	ELT13080	post rep	449962	79.52%

Table S2-2: DEGs (pregnant vs. all non-pregnant)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
ACER3	-3.5059654	8.90E-07	0.0003584
ELN	6.6918342	3.42E-07	0.0003584
HBE1_LOC118079427	3.53435762	2.52E-07	0.0003584
HBE1_LOC118085601	-4.8823074	2.68E-07	0.0003584
HBE1_LOC132592081	3.51288364	7.15E-07	0.0003584
HSD17B11_LOC118084328	3.33470759	8.33E-07	0.0003584
MALL	3.91547594	1.01E-06	0.0003584
TUSC1	-2.5459737	9.91E-07	0.0003584
CIBAR2	3.17639615	1.80E-06	0.00048992
CMBL_LOC118089886	-2.3341528	1.62E-06	0.00048992
SPAG17	-3.3049295	1.81E-06	0.00048992
STAT5B_LOC118094191	3.79667913	1.74E-06	0.00048992
RPL13A_LOC118085881	-2.5422261	2.10E-06	0.00054219
HMX2	3.49848339	3.04E-06	0.00066497
RPS10	-3.6158817	2.76E-06	0.00066497
RPS18	-3.3791147	2.89E-06	0.00066497
RGS5_LOC118089085	2.42084907	4.29E-06	0.00086367
GAPDH_LOC118087317	-3.4805834	4.75E-06	0.00086997
HBA_LOC118092499	3.09036766	4.61E-06	0.00086997
RPL13	-2.4047673	5.40E-06	0.00093044
SURF1_LOC132591531	-3.1155558	5.40E-06	0.00093044
ILK	2.61812405	5.84E-06	0.00094853
il4i1_LOC118080933	5.46778304	7.79E-06	0.00123039
NOS1	3.40649327	9.40E-06	0.00128836
RPS14	-3.0152388	9.26E-06	0.00128836
RPS15	-3.1402782	9.51E-06	0.00128836
SON	2.05479354	9.19E-06	0.00128836
RPS12	-3.2860073	1.01E-05	0.00130087
RPS21	-3.2744412	1.01E-05	0.00130087
RPL36	-2.8611044	1.11E-05	0.00134572
LOC118080481	11.3927524	1.17E-05	0.00136172
LOC118096658	-3.0324302	1.17E-05	0.00136172
PDLIM3	2.78217884	1.20E-05	0.00136493
RPLP1	-2.8820301	1.34E-05	0.00145189
TEC	-2.1274015	1.34E-05	0.00145189
CLCN2	3.91263973	1.49E-05	0.00156544
SPINK6_LOC132591625	12.2621023	1.59E-05	0.00163955
FLNA_LOC118075210	2.17183487	1.72E-05	0.00174587
RPL7A_LOC132591436	-2.3147727	1.84E-05	0.00183165
FAU	-2.6842154	1.91E-05	0.0018744
LOC118090664	6.55349067	1.95E-05	0.00188306
RPL31	-2.7463634	2.04E-05	0.00193497
ITGB1	2.55463024	2.10E-05	0.00195321
HSPB6	2.73242567	2.27E-05	0.00201503
RPL8	-2.5650157	2.27E-05	0.00201503
For full table, see chapter_2_full_tables.xlsx			

Table S2-3: Upregulated GO terms (pregnant vs non-pregnant)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0042060	53	25.1	6.70E-07	wound healing
GO:0051674	82	50.44	2.20E-06	localization of cell
GO:0035152	18	5.79	5.00E-06	regulation of tube architecture, open trac…
GO:0003341	22	8.21	6.60E-06	cilium movement
GO:1903779	11	2.65	1.40E-05	regulation of cardiac conduction
GO:0001525	39	19.31	2.90E-05	angiogenesis
GO:0042490	11	2.65	6.10E-05	mechanoreceptor differentiation
GO:0044782	42	22.69	6.40E-05	cilium organization
GO:0120031	58	34.03	7.00E-05	plasma membrane bounded cell projectio…
GO:0007391	19	7.84	0.00015	dorsal closure
GO:0015671	6	1.09	0.00018	oxygen transport
GO:0006936	38	13.88	0.00019	muscle contraction
GO:0007349	18	7.36	0.0002	cellularization
GO:0007298	21	9.41	0.00025	border follicle cell migration
GO:0021549	15	4.95	0.00026	cerebellum development
GO:0007517	42	22.69	0.00027	muscle organ development
GO:0060972	22	10.14	0.00027	left/right pattern formation
GO:0045214	14	5.19	0.00033	sarcomere organization
GO:1902905	25	12.31	0.00033	positive regulation of supramolecular fibe…
GO:0010830	10	3.02	0.00036	regulation of myotube differentiation
GO:0048260	12	4.1	0.00038	positive regulation of receptor-mediated …
GO:1901739	8	2.05	0.00038	regulation of myoblast fusion
GO:0010761	10	2.65	0.00039	fibroblast migration
GO:0008362	5	0.84	0.00043	chitin-based embryonic cuticle biosynthet…
GO:0009855	22	9.77	0.00047	determination of bilateral symmetry
GO:0007186	37	17.74	0.00052	G protein-coupled receptor signaling path…
GO:0030198	30	14.36	0.00055	extracellular matrix organization
GO:0031424	8	2.17	0.00062	keratinization
GO:0060041	19	7.48	0.00063	retina development in camera-type eye
GO:0051291	19	8.69	0.00064	protein heterooligomerization
GO:0050878	34	17.5	0.00065	regulation of body fluid levels
GO:0002065	36	21.6	0.00087	columnar/cuboidal epithelial cell differenti…
GO:0031036	4	0.6	0.00095	myosin II filament assembly
GO:0007443	8	2.29	0.00096	Malpighian tubule morphogenesis
GO:0048731	288	221.43	0.00104	system development
GO:0007009	15	4.95	0.00109	plasma membrane organization
GO:0010469	20	9.41	0.00128	regulation of signaling receptor activity
GO:0042472	13	4.22	0.00138	inner ear morphogenesis
GO:0035277	8	2.41	0.00143	spiracle morphogenesis, open tracheal sys…
GO:0090101	13	5.31	0.00148	negative regulation of transmembrane rec…
GO:0015701	7	1.93	0.00154	bicarbonate transport
GO:0006811	84	54.06	0.00174	monoatomic ion transport
For full table, see chapter_2_full_tables.xlsx				

Table S2-4: Downregulated GO terms (pregnant vs non-pregnant)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0002181	76	8.42	< 1e-30	cytoplasmic translation
GO:0006614	59	5.89	< 1e-30	SRP-dependent cotranslational protein…
GO:0000184	62	6.79	< 1e-30	nuclear-transcribed mRNA catabolic pr…
GO:0006413	70	10.55	< 1e-30	translational initiation
GO:0042254	59	13	1.00E-22	ribosome biogenesis
GO:0006414	26	8.18	1.20E-07	translational elongation
GO:0015833	100	62.97	3.70E-07	peptide transport
GO:0097421	10	1.96	8.20E-06	liver regeneration
GO:0006415	20	6.87	8.80E-06	translational termination
GO:1904401	4	0.33	4.40E-05	cellular response to Thyroid stimulatin…
GO:1904667	4	0.41	0.00021	negative regulation of ubiquitin protei…
GO:1901798	5	0.82	0.00064	positive regulation of signal transducti…
GO:1990928	8	2.29	0.00134	response to amino acid starvation
GO:0071479	9	2.94	0.00191	cellular response to ionizing radiation
GO:0097428	3	0.33	0.00204	protein maturation by iron-sulfur clust…
GO:0051029	3	0.33	0.00204	rRNA transport
GO:0071493	4	0.65	0.00236	cellular response to UV-B
GO:0006839	19	9.98	0.00348	mitochondrial transport
GO:2000059	8	2.86	0.00612	negative regulation of ubiquitin-depen…
GO:0035606	2	0.16	0.00667	peptidyl-cysteine S-trans-nitrosylation
GO:0000740	2	0.16	0.00667	nuclear membrane fusion
GO:0043335	2	0.16	0.00667	protein unfolding
GO:0035444	2	0.16	0.00667	nickel cation transmembrane transport
GO:0035434	2	0.16	0.00667	copper ion transmembrane transport
GO:0044571	2	0.16	0.00667	[2Fe-2S] cluster assembly
GO:1901194	2	0.16	0.00667	negative regulation of formation of tra…
GO:0045585	2	0.16	0.00667	positive regulation of cytotoxic T cell d…
GO:0070670	5	1.31	0.0073	response to interleukin-4
GO:0033033	3	0.49	0.009	negative regulation of myeloid cell apo…
GO:0031167	3	0.49	0.009	rRNA methylation
GO:0007007	7	2.45	0.00904	inner mitochondrial membrane organi…
GO:0006654	4	1.06	0.01738	phosphatidic acid biosynthetic process
GO:0006824	2	0.25	0.01893	cobalt ion transport
GO:0006610	2	0.25	0.01893	ribosomal protein import into nucleus
GO:1902626	2	0.25	0.01893	assembly of large subunit precursor of…
GO:1904751	2	0.25	0.01893	positive regulation of protein localizati…
GO:0060699	2	0.25	0.01893	regulation of endoribonuclease activity
GO:0002864	2	0.25	0.01893	regulation of acute inflammatory resp…
GO:0034137	2	0.25	0.01893	positive regulation of toll-like receptor…
For full table, see chapter_2_full_tables.xlsx				

Table S2-5: DEGs (pregnant vs. pre-pregnancy)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
HSD17B11_LOC118084328	-6.0155054	1.57E-08	2.97E-05
ABCE1	-3.2393541	2.09E-07	0.00016273
HBE1_LOC118079427	4.61256752	8.85E-07	0.00045698
CMBL_LOC118089886	4.76208795	1.70E-06	0.00073749
HBE1_LOC118085601	-2.6009403	1.67E-06	0.00073749
HBE1_LOC132592081	4.48439866	1.95E-06	0.00073749
GPN3	-2.7199398	2.17E-06	0.00074333
LOC118084273	-3.3146457	2.22E-06	0.00074333
CIBAR2	4.33803649	3.06E-06	0.00076805
ELN	8.34563637	3.11E-06	0.00076805
GSTA2_LOC118082447	-2.7170692	2.98E-06	0.00076805
RGS5_LOC118089085	3.53543589	2.81E-06	0.00076805
TUSC1	-2.5987127	3.11E-06	0.00076805
FCGBP_LOC118086744	-5.4891779	3.70E-06	0.00079522
HBA_LOC118092499	4.57071511	3.92E-06	0.00079522
RPS17_LOC118093187	-2.8597673	3.85E-06	0.00079522
SLC30A6	-3.0960395	3.58E-06	0.00079522
MALL	5.08863296	4.07E-06	0.00079799
GALNT14	-3.9888229	5.71E-06	0.0010814
ACER3	-3.2666331	6.02E-06	0.00110338
METAP1	-2.4257232	7.09E-06	0.0012585
PLD6	-3.3937034	7.90E-06	0.00131967
CH25H	-3.5754065	9.59E-06	0.00146406
RPL22L1	-3.2021386	9.77E-06	0.00146406
RPS18	-3.3272727	1.00E-05	0.00146406
SPAG17	4.46976159	9.86E-06	0.00146406
LOC118092881	-3.5353829	1.06E-05	0.00151268
G0S2	-3.126911	1.14E-05	0.00157781
NFKBIA	-3.0287771	1.17E-05	0.00158724
EEF1E1	-2.7001847	1.25E-05	0.00165062
MMGT1_LOC118084223	-2.9284403	1.36E-05	0.00175518
RPL13	-3.1499603	1.46E-05	0.00180545
RPS12	-3.4975667	1.45E-05	0.00180545
LY6E_LOC118090479	3.02976225	1.58E-05	0.00191606
RPL7	-3.0126247	2.00E-05	0.0023718
RPL8	-2.8921583	2.08E-05	0.00240756
FLNA_LOC118075210	2.89391313	2.26E-05	0.00241254
MRPL57	-2.1363834	2.31E-05	0.00241254
RIDA	-2.2058435	2.23E-05	0.00241254
RPS10	-3.2727556	2.24E-05	0.00241254
RPS21	-3.33106	2.37E-05	0.00241254
RPSA	-3.1830135	2.38E-05	0.00241254
RPL27A	-3.1372846	2.56E-05	0.00255459
RPL31	-2.9703098	2.81E-05	0.00269885
RPS15A	-3.2528969	2.79E-05	0.00269885
For full table, see chapter_2_full_tables.xlsx			

Table S2-6: Upregulated GO terms (pregnant vs. pre-pregnancy)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0030198	40	10.44	5.20E-12	extracellular matrix organization
GO:0001525	44	14.03	4.90E-11	angiogenesis
GO:0007160	21	6.05	2.00E-07	cell-matrix adhesion
GO:0010811	18	4.74	3.30E-07	positive regulation of cell-substrate ad…
GO:0015671	7	0.79	1.20E-06	oxygen transport
GO:0010469	21	6.84	1.90E-06	regulation of signaling receptor activity
GO:0002576	16	4.39	2.80E-06	platelet degranulation
GO:0051674	64	36.66	3.30E-06	localization of cell
GO:0070372	20	6.93	9.30E-06	regulation of ERK1 and ERK2 cascade
GO:0007475	9	1.67	1.20E-05	apposition of dorsal and ventral imagi…
GO:0015701	8	1.4	2.20E-05	bicarbonate transport
GO:0006936	34	10.09	2.60E-05	muscle contraction
GO:0001700	21	8.16	3.70E-05	embryonic development via the syncyt…
GO:1902476	9	1.93	5.00E-05	chloride transmembrane transport
GO:0090101	13	3.86	6.20E-05	negative regulation of transmembrane…
GO:0071773	14	4.39	6.30E-05	cellular response to BMP stimulus
GO:0035160	6	0.88	6.80E-05	maintenance of epithelial integrity, op…
GO:0003151	11	2.63	7.30E-05	outflow tract morphogenesis
GO:0090288	15	5	7.60E-05	negative regulation of cellular respons…
GO:0007298	18	6.84	9.80E-05	border follicle cell migration
GO:0017015	13	4.03	0.0001	regulation of transforming growth fact…
GO:0032570	10	2.63	0.00015	response to progesterone
GO:0010830	9	2.19	0.00016	regulation of myotube differentiation
GO:0008277	15	4.12	0.00016	regulation of G protein-coupled recept…
GO:2000095	5	0.53	0.00027	regulation of Wnt signaling pathway, p…
GO:0003206	13	3.51	0.00027	cardiac chamber morphogenesis
GO:0051216	18	5.96	0.00027	cartilage development
GO:0050930	4	0.44	0.00027	induction of positive chemotaxis
GO:0003384	4	0.44	0.00027	apical constriction involved in gastrula…
GO:1901739	7	1.49	0.00034	regulation of myoblast fusion
GO:1903779	8	1.93	0.00035	regulation of cardiac conduction
GO:0030334	52	26.49	0.00035	regulation of cell migration
GO:0051291	16	6.31	0.00038	protein heterooligomerization
GO:0050678	25	10.7	0.00039	regulation of epithelial cell proliferation
GO:0048639	25	11.4	0.0004	positive regulation of developmental g…
GO:0051930	6	1.14	0.00044	regulation of sensory perception of pain
GO:0044849	5	0.79	0.00047	estrous cycle
GO:0030177	15	5.44	0.0005	positive regulation of Wnt signaling pa…
For full table, see chapter_2_full_tables.xlsx				

Table S2-7: Downregulated GO terms (pregnant vs. pre-pregnancy)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0002181	77	12.21	< 1e-30	cytoplasmic translation
GO:0006614	59	8.54	< 1e-30	SRP-dependent cotranslational protein…
GO:0000184	63	9.84	< 1e-30	nuclear-transcribed mRNA catabolic pr…
GO:0006413	77	15.29	< 1e-30	translational initiation
GO:0042254	66	18.85	1.00E-18	ribosome biogenesis
GO:0006414	34	11.86	1.70E-08	translational elongation
GO:0042886	136	92.83	1.20E-07	amide transport
GO:0032543	30	11.38	2.70E-07	mitochondrial translation
GO:0006415	27	9.96	5.70E-07	translational termination
GO:0097421	11	2.85	3.40E-05	liver regeneration
GO:1904401	4	0.47	0.0002	cellular response to Thyroid stimulatin…
GO:0000055	4	0.59	0.00089	ribosomal large subunit export from n…
GO:1990928	10	3.32	0.00091	response to amino acid starvation
GO:0071493	5	0.95	0.00095	cellular response to UV-B
GO:0046031	9	2.85	0.00109	ADP metabolic process
GO:2000059	12	4.15	0.0028	negative regulation of ubiquitin-depen…
GO:0009168	15	6.99	0.0029	purine ribonucleoside monophosphat…
GO:0046039	6	1.66	0.00351	GTP metabolic process
GO:0006370	8	2.73	0.00358	7-methylguanosine mRNA capping
GO:0071480	7	2.25	0.00444	cellular response to gamma radiation
GO:0090305	21	11.62	0.00455	nucleic acid phosphodiester bond hydr…
GO:0006417	46	26.67	0.0046	regulation of translation
GO:0046033	5	1.3	0.00576	AMP metabolic process
GO:0051029	3	0.47	0.00605	rRNA transport
GO:0097428	3	0.47	0.00605	protein maturation by iron-sulfur clust…
GO:1902236	4	0.95	0.00924	negative regulation of endoplasmic ret…
GO:0050821	22	13.16	0.00977	protein stabilization
GO:0034498	3	0.59	0.01379	early endosome to Golgi transport
GO:0007228	3	0.59	0.01379	positive regulation of hh target transcr...
GO:1904667	3	0.59	0.01379	negative regulation of ubiquitin protei…
GO:0033387	2	0.24	0.01403	putrescine biosynthetic process from …
GO:0043335	2	0.24	0.01403	protein unfolding
GO:0046051	2	0.24	0.01403	UTP metabolic process
GO:0044571	2	0.24	0.01403	[2Fe-2S] cluster assembly
GO:2000255	2	0.24	0.01403	negative regulation of male germ cell …
GO:0046495	2	0.24	0.01403	nicotinamide riboside metabolic process
GO:0035444	2	0.24	0.01403	nickel cation transmembrane transport
GO:0035434	2	0.24	0.01403	copper ion transmembrane transport
For full table, see chapter_2_full_tables.xlsx				

Table S2-8: DEGs (pregnancy vs post-parturition)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
GAPDH_LOC118087317	-6.2309592	9.38E-09	1.33E-05
RPL21_LOC118076523	-5.5758145	8.62E-09	1.33E-05
SURF1_LOC132591531	-3.8818176	4.90E-08	5.57E-05
RPL13A_LOC118085881	-3.4609896	1.52E-07	0.0001232
STAT5B_LOC118094191	-4.0697151	4.71E-07	0.00028677
RBM3_LOC118076727	-4.9676099	7.66E-07	0.00037402
ST3GAL3	-3.9001112	1.04E-06	0.00045677
ACER3	-3.7452976	1.23E-06	0.00048557
LOC118096658	-3.9637252	1.28E-06	0.00048557
RPS10	-3.9590077	2.75E-06	0.00091996
AFP_LOC118094159	-8.3378462	3.28E-06	0.00103471
NDRG1	-3.1673283	6.73E-06	0.00140285
RPL36	-3.3052515	7.17E-06	0.00140285
RPS18	-3.4309568	7.14E-06	0.00140285
TEC	-2.5971711	6.03E-06	0.00140285
TUSC1	-2.4932346	6.48E-06	0.00140285
RPL15_LOC132592648	-4.0246078	8.41E-06	0.00149307
EVI2B	7.73262451	9.87E-06	0.0016501
NCKAP5L	-3.3237766	1.11E-05	0.00180372
LAMP1	-2.4961551	1.23E-05	0.00188847
RPLP1	-3.2311055	1.20E-05	0.00188847
SLIRP	-2.5889648	1.55E-05	0.00231795
RPL13	-3.0811514	1.88E-05	0.00273804
CLDN7	4.07435687	2.11E-05	0.00285632
FAU	-2.9695797	2.10E-05	0.00285632
RPS7	-3.1195324	2.07E-05	0.00285632
LAPTM4A	-3.1229898	2.21E-05	0.00291474
RPS14	-3.0740656	2.30E-05	0.00297154
RPS15	-3.1543648	2.67E-05	0.00337602
PHYKPL	-2.2145337	2.80E-05	0.00346456
RPS21	-3.2178225	3.43E-05	0.00414189
CREBZF	-2.4946105	3.90E-05	0.00442863
G6PD	4.5130259	3.86E-05	0.00442863
CMBL_LOC118089886	-2.0673653	4.10E-05	0.00456629
RPS27A	-3.0720816	4.21E-05	0.00459304
SLC44A3	-2.4007365	4.28E-05	0.00459304
ILK	3.15335793	4.69E-05	0.00493569
GAPDH	-2.3762096	4.88E-05	0.00495505
YBX1	-2.3475286	4.83E-05	0.00495505
RPL12_LOC118084173	-2.9415868	5.57E-05	0.0055542
RPS12	-3.0744478	5.67E-05	0.0055549
METTL23	-2.5002105	6.34E-05	0.00562806
NAP1L1	-2.2382747	5.91E-05	0.00562806
PDRG1	-2.1348066	6.25E-05	0.00562806
RPL28	-2.6652827	5.95E-05	0.00562806
For full table, see chapter_2_full_tables.xlsx			

Table S2-9: Upregulated GOs (pregnant vs post-parturition)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0031038	7	1.06	2.80E-05	myosin II filament organization
GO:0006048	5	0.53	4.40E-05	UDP-N-acetylglucosamine biosynthetic…
GO:1902905	20	7.7	5.50E-05	positive regulation of supramolecular …
GO:0051674	53	31.54	7.40E-05	localization of cell
GO:0051345	38	20.37	0.00011	positive regulation of hydrolase activity
GO:1901739	7	1.28	0.00013	regulation of myoblast fusion
GO:0006041	4	0.38	0.00015	glucosamine metabolic process
GO:0007392	6	0.98	0.00019	initiation of dorsal closure
GO:0007395	6	0.98	0.00019	dorsal closure, spreading of leading ed…
GO:0051153	12	3.55	0.00019	regulation of striated muscle cell differ…
GO:0035026	5	0.68	0.00023	leading edge cell differentiation
GO:0051291	15	5.43	0.00024	protein heterooligomerization
GO:0046664	6	1.06	0.00032	dorsal closure, amnioserosa morpholo…
GO:1901073	3	0.23	0.00043	glucosamine-containing compound b…
GO:0060744	3	0.23	0.00043	mammary gland branching involved in …
GO:0090131	3	0.23	0.00043	mesenchyme migration
GO:0035151	7	1.58	0.0006	regulation of tube size, open tracheal s…
GO:0045851	7	1.58	0.0006	pH reduction
GO:0032868	23	10.64	0.00065	response to insulin
GO:0032873	10	3.09	0.00071	negative regulation of stress-activated …
GO:0051495	22	8.45	0.00079	positive regulation of cytoskeleton org…
GO:0042742	18	7.92	0.00088	defense response to bacterium
GO:0010763	4	0.53	0.00093	positive regulation of fibroblast migrat…
GO:0045214	10	3.24	0.00106	sarcomere organization
GO:0048259	10	3.39	0.00154	regulation of receptor-mediated endo…
GO:0030048	13	4.45	0.00155	actin filament-based movement
GO:0090315	3	0.3	0.00161	negative regulation of protein targetin…
GO:0048251	3	0.3	0.00161	elastic fiber assembly
GO:0030521	8	2.34	0.00164	androgen receptor signaling pathway
GO:0010592	5	0.98	0.00184	positive regulation of lamellipodium a…
GO:0030588	5	0.98	0.00184	pseudocleavage
GO:0045104	7	1.58	0.00203	intermediate filament cytoskeleton or…
GO:0007443	6	1.43	0.00206	Malpighian tubule morphogenesis
GO:0007169	41	24.75	0.00223	transmembrane receptor protein tyros…
GO:0071248	14	5.58	0.0024	cellular response to metal ion
GO:0030952	9	3.02	0.0024	establishment or maintenance of cyto…
GO:0051653	11	4.22	0.00246	spindle localization
GO:0035006	5	1.06	0.00269	melanization defense response
For full table, see chapter_2_full_tables.xlsx				

Table S2-10: Downregulated GOs (pregnant vs. post-parturition)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0002181	65	6.46	< 1e-30	cytoplasmic translation
GO:0006614	52	4.52	< 1e-30	SRP-dependent cotranslational protein…
GO:0000184	53	5.21	< 1e-30	nuclear-transcribed mRNA catabolic …
GO:0006413	58	8.1	< 1e-30	translational initiation
GO:0042255	19	3.08	2.80E-11	ribosome assembly
GO:0015833	78	48.33	1.90E-06	peptide transport
GO:0042273	14	3.33	2.80E-06	ribosomal large subunit biogenesis
GO:0006364	25	6.78	1.20E-05	rRNA processing
GO:1904401	4	0.25	1.50E-05	cellular response to Thyroid stimulatin…
GO:0000462	8	1.44	4.70E-05	maturation of SSU-rRNA from tricistro…
GO:0097421	8	1.51	6.60E-05	liver regeneration
GO:1904667	3	0.31	0.0022	negative regulation of ubiquitin protei…
GO:1901798	4	0.63	0.0024	positive regulation of signal transducti…
GO:0033108	11	4.39	0.0038	mitochondrial respiratory chain compl…
GO:0035606	2	0.13	0.0039	peptidyl-cysteine S-trans-nitrosylation
GO:0000740	2	0.13	0.0039	nuclear membrane fusion
GO:0035444	2	0.13	0.0039	nickel cation transmembrane transport
GO:0035434	2	0.13	0.0039	copper ion transmembrane transport
GO:1901194	2	0.13	0.0039	negative regulation of formation of tra…
GO:0019731	5	1.13	0.0041	antibacterial humoral response
GO:0071480	5	1.19	0.0053	cellular response to gamma radiation
GO:0018993	3	0.5	0.0108	somatic sex determination
GO:0019646	10	4.39	0.0112	aerobic electron transport chain
GO:0006824	2	0.19	0.0113	cobalt ion transport
GO:0006610	2	0.19	0.0113	ribosomal protein import into nucleus
GO:1902626	2	0.19	0.0113	assembly of large subunit precursor of…
GO:0003149	2	0.19	0.0113	membranous septum morphogenesis
GO:0034137	2	0.19	0.0113	positive regulation of toll-like receptor…
GO:1990145	2	0.19	0.0113	maintenance of translational fidelity
GO:0006241	2	0.19	0.0113	CTP biosynthetic process
GO:0001912	3	0.56	0.0155	positive regulation of leukocyte media…
GO:0006414	13	6.28	0.017	translational elongation
GO:0071320	5	1.63	0.0208	cellular response to cAMP
GO:0071421	2	0.25	0.0216	manganese ion transmembrane transp…
GO:0048541	2	0.25	0.0216	Peyer's patch development
GO:0002227	2	0.25	0.0216	innate immune response in mucosa
GO:0051029	2	0.25	0.0216	rRNA transport
GO:0034063	3	0.69	0.0277	stress granule assembly
For full table, see chapter_2_full_tables.xlsx				

Table S2-11: DEGs (post-parturition vs pre-pregnancy)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
RPL21_LOC118076523	6.00948898	1.10E-06	0.00156833
GAPDH_LOC118087317	4.19046215	5.50E-06	0.00624789
IGFBP7	4.6842116	1.03E-05	0.00979986
LGALS1_LOC118090838	4.17181517	1.51E-05	0.01223595
CREBZF	3.35285709	3.83E-05	0.01556442
EVI2B	-7.4867135	3.46E-05	0.01556442
NCKAP5L	2.95410073	2.74E-05	0.01556442
PHYKPL	5.95124754	2.61E-05	0.01556442
RPL15_LOC132592648	4.50871107	3.08E-05	0.01556442
SURF1_LOC132591531	2.8151572	3.86E-05	0.01556442
ABCE1	-2.5649754	6.71E-05	0.01997627
CDKN1A	-3.0422393	6.55E-05	0.01997627
LY6E_LOC118090479	4.33028746	7.03E-05	0.01997627
RBM3_LOC118076727	2.84323976	6.46E-05	0.01997627
CLDN7	-3.8157376	8.69E-05	0.02058124
CLEC4A	-4.260573	8.14E-05	0.02058124
CTSS	2.86379594	8.56E-05	0.02058124
CEPT1	-3.1229431	0.00010827	0.02461121
AFP_LOC118094159	8.33784617	0.00011325	0.02475488
CLPX	-7.7617303	0.00014771	0.02814773
EPRS1	-2.7483875	0.00014076	0.02814773
GPNMB	7.37763963	0.00015482	0.02814773
NDFIP2	-2.735488	0.0001349	0.02814773
AHNAK	2.38632714	0.0001796	0.0301906
CUTA	2.42966565	0.00018062	0.0301906
BCL2L10	3.45558113	0.00020323	0.03278289
HSPB9_LOC118087112	-4.1525234	0.00020767	0.03278289
HGS	-6.9653165	0.00023424	0.03369255
SULF2	3.26865908	0.000226	0.03369255
TUBA4A_LOC118090764	-2.8370557	0.00024831	0.03416465
ZMYM2	-3.1394266	0.00025249	0.03416465
FCN2_LOC118088874	-4.5518355	0.00033161	0.04187852
WTAP	2.28691904	0.00032568	0.04187852
TXNIP	-2.4443408	0.00034472	0.04258753
RNF11_LOC118080004	-2.5633115	0.00036503	0.04413794
HBA_LOC118092499	2.96069491	0.00037368	0.04422562
ABHD2	2.33856472	0.0004541	0.04519068
ADIPOR1	-2.7974739	0.00049971	0.04519068
AGR2	2.64278029	0.00043901	0.04519068
G6PD	-3.87129	0.00053761	0.04519068
GALNT14	-4.2990724	0.00044249	0.04519068
GPN3	-2.1277406	0.000503	0.04519068
HNRNPUL1	2.60629453	0.00049362	0.04519068
ITPR3	4.15250037	0.00047773	0.04519068
LOC118088270	-2.4647171	0.00053287	0.04519068
For full table, see chapter_2_full_tables.xlsx			

Table S2-12: Upregulated GO terms (post-parturition vs. pre-pregnancy)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0015671	5	0.19	4.70E-07	oxygen transport
GO:0043062	15	2.92	2.10E-06	extracellular structure organization
GO:0030574	4	0.26	8.50E-05	collagen catabolic process
GO:0051930	4	0.28	0.00012	regulation of sensory perception of pain
GO:0042698	5	0.62	0.00032	ovulation cycle
GO:0051138	2	0.04	0.00045	positive regulation of NK T cell differen…
GO:0051291	7	1.54	0.00077	protein heterooligomerization
GO:0050817	8	1.9	0.00088	coagulation
GO:0007599	8	1.92	0.00095	hemostasis
GO:0007494	4	0.47	0.00106	midgut development
GO:0098742	5	0.81	0.00115	cell-cell adhesion via plasma-membra…
GO:0045453	4	0.53	0.00175	bone resorption
GO:0042058	6	1.37	0.00224	regulation of epidermal growth factor …
GO:2000535	2	0.09	0.00263	regulation of entry of bacterium into h…
GO:0070934	2	0.09	0.00263	CRD-mediated mRNA stabilization
GO:0015701	3	0.34	0.00432	bicarbonate transport
GO:0007414	2	0.11	0.00433	axonal defasciculation
GO:0010469	6	1.66	0.00605	regulation of signaling receptor activity
GO:0048149	3	0.38	0.0061	behavioral response to ethanol
GO:0071407	14	5.53	0.00613	cellular response to organic cyclic com…
GO:0006935	13	5.42	0.00613	chemotaxis
GO:0050913	2	0.13	0.0064	sensory perception of bitter taste
GO:0018146	2	0.13	0.0064	keratan sulfate biosynthetic process
GO:0021551	2	0.13	0.0064	central nervous system morphogenesis
GO:2000345	2	0.13	0.0064	regulation of hepatocyte proliferation
GO:0035634	2	0.15	0.00883	response to stilbenoid
GO:0048240	2	0.15	0.00883	sperm capacitation
GO:0032743	2	0.15	0.00883	positive regulation of interleukin-2 pro…
GO:0072327	2	0.15	0.00883	vulval cell fate specification
GO:0048839	5	1.34	0.01059	inner ear development
GO:1901076	2	0.17	0.01161	positive regulation of engulfment of a…
GO:0060707	2	0.17	0.01161	trophoblast giant cell differentiation
GO:0001701	10	3.61	0.01711	in utero embryonic development
GO:0007566	3	0.55	0.01719	embryo implantation
GO:2000027	7	2.2	0.01772	regulation of animal organ morphogen…
GO:0001755	2	0.21	0.01815	neural crest cell migration
GO:0002479	2	0.21	0.01815	antigen processing and presentation o …
GO:0046464	2	0.21	0.01815	acylglycerol catabolic process
For full table, see chapter_2_full_tables.xlsx				

Table S2-13: Downregulated GO terms (post-parturition vs pre-pregnancy)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0060744	3	0.08	1.80E-05	mammary gland branching involved in …
GO:0006606	10	2.46	0.00015	protein import into nucleus
GO:1904589	7	1.37	0.00038	regulation of protein import
GO:0010039	4	0.58	0.00235	response to iron ion
GO:0016082	3	0.29	0.00254	synaptic vesicle priming
GO:0090087	15	7.45	0.00358	regulation of peptide transport
GO:0006591	2	0.11	0.00401	ornithine metabolic process
GO:0043249	2	0.11	0.00401	erythrocyte maturation
GO:0003406	2	0.11	0.00401	retinal pigment epithelium developme…
GO:0030888	3	0.4	0.00649	regulation of B cell proliferation
GO:0045618	2	0.13	0.00657	positive regulation of keratinocyte diff…
GO:0032570	4	0.79	0.00747	response to progesterone
GO:0009636	14	6.47	0.00753	response to toxic substance
GO:0008209	3	0.42	0.00784	androgen metabolic process
GO:0030521	4	0.82	0.00841	androgen receptor signaling pathway
GO:0045471	8	2.43	0.00938	response to ethanol
GO:0050830	4	0.85	0.00941	defense response to Gram-positive ba…
GO:0033599	2	0.16	0.00969	regulation of mammary gland epitheli…
GO:0032354	2	0.16	0.00969	response to follicle-stimulating hormo…
GO:0033148	2	0.16	0.00969	positive regulation of intracellular estr…
GO:0046839	2	0.16	0.00969	phospholipid dephosphorylation
GO:0043312	12	5.63	0.00999	neutrophil degranulation
GO:0035914	3	0.48	0.01099	skeletal muscle cell differentiation
GO:0032355	6	1.9	0.01134	response to estradiol
GO:0044829	2	0.18	0.01333	positive regulation by host of viral gen…
GO:0006544	2	0.18	0.01333	glycine metabolic process
GO:0060074	2	0.18	0.01333	synapse maturation
GO:0048190	3	0.53	0.01477	wing disc dorsal/ventral pattern forma…
GO:0031647	10	4.54	0.01487	regulation of protein stability
GO:0034472	2	0.21	0.01746	snRNA 3'-end processing
GO:0043697	2	0.21	0.01746	cell dedifferentiation
GO:0046688	3	0.61	0.02167	response to copper ion
GO:0010881	2	0.24	0.02207	regulation of cardiac muscle contracti…
GO:0008293	2	0.24	0.02207	torso signaling pathway
GO:0010906	4	1.08	0.02211	regulation of glucose metabolic process
GO:0043547	7	2.91	0.02552	positive regulation of GTPase activity
GO:0006307	1	0.03	0.02642	DNA dealkylation involved in DNA repair
GO:1904145	1	0.03	0.02642	negative regulation of meiotic cell cycl…
For full table, see chapter_2_full_tables.xlsx				

Table S3-1: Cluster 0 differentially expressed genes

og Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p val_adj*	*Log FC*
LOC118092498	HBM	0	0.00E+00	5.073
LOC118092499	HBA	0	0.00E+00	5.047
LOC118079427	HBE1	0	0.00E+00	4.821
LOC132592081	HBE1	0	0.00E+00	4.626
LOC118093265	HBA	0	0.00E+00	4.562
LOC118093251	HBM	0	0.00E+00	4.301
LOC118085601	HBE1	0	0.00E+00	4.257
LOC118085062	HBE1	0	0.00E+00	3.152
LOC118094239	FTL	0	0.00E+00	2.232
LOC118090305	CA2	0	0.00E+00	1.614
LOC118093266	HBA	0	0.00E+00	1.57
SLC4A1	SLC4A1	0	0.00E+00	1.56
LOC118092188	SAMD9L	0	0.00E+00	1.493
GPX1	GPX1	0	0.00E+00	1.363
LOC118092668	Nrk1	0	0.00E+00	1.306
ANK1	ANK1	0	0.00E+00	1.283
LOC132593050	-	0	0.00E+00	1.066
ALAS2	ALAS2	0	0.00E+00	1.034
FTH1	FTH1	1.02E-195	5.68E-193	1.021
LOC118076446	S100A6	2.84E-210	1.71E-207	-1.153
TMSB4X	TMSB4X	2.14E-254	1.77E-251	-1.183
ACTB	ACTB	4.25E-229	3.16E-226	-1.206

Table S3-2: Cluster 1 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
LOC118085062	HBE1	1.60E-113	1.11E-110	-1.279
LOC118094239	FTL	1.30E-124	1.08E-121	-1.501
LOC118085601	HBE1	3.50E-140	3.72E-137	-1.716
LOC118093251	HBM	1.70E-141	1.90E-138	-1.752
LOC132592081	HBE1	1.07E-163	1.83E-160	-1.986
LOC118093265	HBA	5.39E-164	1.00E-160	-1.988
LOC118079427	HBE1	1.37E-165	2.79E-162	-2.079
LOC118092499	HBA	8.05E-167	1.80E-163	-2.193
LOC118092498	HBM	5.32E-174	1.70E-170	-2.279

Table S3-3: Cluster 2 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
LOC118085062	HBE1	2.94E-68	3.28E-64	-1.055
LOC118093251	HBM	4.81E-76	1.07E-71	-1.347

Table S3-4: Cluster 3 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
F10	F10	0	0.00E+00	2.193
LOC118080966	-	0	0.00E+00	2.054
AQP1	AQP1	0	0.00E+00	1.731
SEMA6A	SEMA6A	0	0.00E+00	1.698
VWF	VWF	0	0.00E+00	1.547
PLVAP	PLVAP	0	0.00E+00	1.539
SPTBN1	SPTBN1	0	0.00E+00	1.525
LOC118092250	-	0	0.00E+00	1.454
ZNF521	ZNF521	0	0.00E+00	1.382
CAVIN2	SDPR	0	0.00E+00	1.329
LOC118091810	MRC1	0	0.00E+00	1.321
TGM2	TGM2	0	0.00E+00	1.302
LOC118076446	S100A6	5.72E-111	8.64E-109	1.301
RELN	RELN	0	0.00E+00	1.286
KLF6	KLF6	2.69E-257	1.40E-254	1.263
TMSB4X	TMSB4X	5.03E-115	8.08E-113	1.243
S100A10	S100A10	7.81E-252	3.79E-249	1.185
ANXA2	ANXA2	5.10E-187	1.54E-184	1.079
CD63	CD63	5.54E-191	1.70E-188	1.053
AHNAK	AHNAK	4.80E-137	9.84E-135	1.016
LOC118094239	FTL	3.11E-63	2.43E-61	-1.042
LOC118085062	HBE1	9.15E-64	7.30E-62	-1.066
LOC118093251	HBM	7.02E-84	7.76E-82	-1.499
LOC118085601	HBE1	4.36E-99	5.76E-97	-1.596
LOC132592081	HBE1	5.74E-103	7.96E-101	-1.74
LOC118093265	HBA	2.35E-107	3.45E-105	-1.793
LOC118079427	HBE1	3.26E-111	5.01E-109	-1.908
LOC118092499	HBA	3.15E-100	4.24E-98	-1.912
LOC118092498	HBM	1.67E-102	2.29E-100	-1.952

Table S3-5: Cluster 4 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
PLVAP	PLVAP	0	0.00E+00	3.062
AQP1	AQP1	0	0.00E+00	2.838
PECAM1	PECAM1	0	0.00E+00	2.03
FOS	FOS	1.03E-220	6.06E-218	2.02
SOCS3	SOCS3	0	0.00E+00	1.891
NR4A1	NR4A1	3.74E-168	1.37E-165	1.716
BTG2	BTG2	3.92E-208	1.99E-205	1.698
S1PR1	-	0	0.00E+00	1.689
SELE	SELE	0	0.00E+00	1.554
SLC38A2	SLC38A2	0	0.00E+00	1.384
JUNB	JUNB	2.88E-130	6.83E-128	1.36
FLT1	FLT1	0	0.00E+00	1.348
FOSB	FOSB	6.41E-210	3.41E-207	1.143
CAVIN2	SDPR	2.06E-202	9.79E-200	1.124
SPP1	SPP1	0	0.00E+00	1.066
EPAS1	EPAS1	1.09E-160	3.64E-158	1.019
LOC118084151	FLT1	0	0.00E+00	1.013
LOC118085062	HBE1	7.19E-53	5.64E-51	-1.104
LOC118094239	FTL	9.14E-84	1.25E-81	-1.511
LOC118093251	HBM	1.82E-66	1.78E-64	-1.523
LOC118085601	HBE1	5.07E-80	6.22E-78	-1.648
LOC118093265	HBA	6.59E-86	9.15E-84	-1.823
LOC118079427	HBE1	3.46E-83	4.63E-81	-1.867
LOC132592081	HBE1	5.28E-91	7.70E-89	-1.884
LOC118092499	HBA	1.61E-86	2.25E-84	-2.023
LOC118092498	HBM	1.48E-89	2.15E-87	-2.092

Table S3-6: Cluster 5 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
APOC1	APOC1	0	0.00E+00	2.299
NR4A1	NR4A1	1.70E-94	2.84E-92	1.315
C1H11orf96	C11orf96	0	0.00E+00	1.261
EGR1	EGR1	3.53E-153	1.27E-150	1.133
THBS1	THBS1	8.14E-307	8.27E-304	1.071
DCN	DCN	0	0.00E+00	1.014
LOC118086005	MT2A	6.74E-57	6.54E-55	1.004
STAR	STAR	0	0.00E+00	1.004
B2M	B2M	8.30E-53	7.22E-51	-1.013
LOC118085062	HBE1	4.50E-56	4.31E-54	-1.153
LOC118094239	FTL	7.59E-75	9.75E-73	-1.398
LOC118093251	HBM	6.13E-66	6.91E-64	-1.533
LOC118085601	HBE1	4.80E-74	5.99E-72	-1.618
LOC132592081	HBE1	2.11E-79	2.98E-77	-1.792
LOC118093265	HBA	4.77E-84	7.29E-82	-1.86
LOC118079427	HBE1	6.28E-83	9.35E-81	-1.912
LOC118092499	HBA	1.75E-82	2.59E-80	-2.008
LOC118092498	HBM	3.65E-83	5.50E-81	-2.06

Table S3-7: Cluster 6 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
LOC118076446	S100A6	2.95E-83	3.66E-80	1.389
LOC118080966	-	1.25E-86	1.64E-83	1.132
LOC118085062	HBE1	1.54E-59	9.83E-57	-1.221
LOC118094239	FTL	4.25E-59	2.57E-56	-1.309
LOC118093251	HBM	2.05E-76	2.09E-73	-1.704
LOC118085601	HBE1	3.20E-80	3.58E-77	-1.725
LOC118093265	HBA	2.72E-91	4.05E-88	-1.981
LOC132592081	HBE1	2.09E-93	3.59E-90	-2.013
LOC118079427	HBE1	3.04E-91	4.24E-88	-2.072
LOC118092499	HBA	3.63E-93	5.79E-90	-2.193
LOC118092498	HBM	1.11E-107	2.77E-104	-2.426

Table S3-8: Cluster 7 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
LOC118086005	MT2A	1.92E-75	1.47E-73	1.4
RP1	RP1	0	0.00E+00	1.297
DNAH5	DNAH5	0	0.00E+00	1.203
SNTN	SNTN	2.52E-292	2.35E-289	1.148
TSPAN1	TSPAN1	0	0.00E+00	1.051
LOC118089512	WFDC2	0	0.00E+00	1.009
LOC118085062	HBE1	2.47E-42	1.01E-40	-1.054
LOC118094239	FTL	2.88E-47	1.33E-45	-1.2
LOC118093251	HBM	1.98E-55	1.05E-53	-1.46
LOC118085601	HBE1	2.19E-59	1.26E-57	-1.513
LOC132592081	HBE1	1.24E-61	7.49E-60	-1.622
LOC118093265	HBA	1.09E-61	6.60E-60	-1.657
LOC118079427	HBE1	3.48E-62	2.12E-60	-1.705
LOC118092499	HBA	3.69E-63	2.28E-61	-1.805
LOC118092498	HBM	1.79E-69	1.23E-67	-1.934

Table S3-9: Cluster 8 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
LOC118077197	LYSC6	0	0.00E+00	2.612
LOC118091898	CAMP	0	0.00E+00	2.398
ACTB	ACTB	1.75E-83	3.18E-81	1.965
LOC118094983	CXCL8	5.20E-270	6.84E-267	1.765
ACTG1	ACTG1	1.77E-77	2.86E-75	1.717
LOC118096176	PSAP	1.45E-130	5.04E-128	1.691
LOC118097010	CCL4	8.45E-143	3.25E-140	1.608
LOC118092073	CAMP	0	0.00E+00	1.582
LOC118081051	-	0	0.00E+00	1.331
CEBPB	CEBPB	4.50E-300	7.74E-297	1.283
HSP90AA1	HSP90AA1	2.45E-32	1.35E-30	1.259
LOC118076126	ECM1	4.61E-194	3.21E-191	1.247
TMSB4X	TMSB4X	1.55E-37	1.01E-35	1.232
HSPA2	HSPA2	1.15E-29	5.87E-28	1.212
CTSD	CTSD	1.76E-101	4.41E-99	1.211
FTH1	FTH1	3.02E-34	1.76E-32	1.173
DUSP1	DUSP1	1.44E-70	2.03E-68	1.17
SDC4	SDC4	2.15E-97	5.00E-95	1.119
AHNAK	AHNAK	2.21E-49	1.94E-47	1.101
NUAK2	NUAK2	2.36E-278	3.52E-275	1.026
LOC118085062	HBE1	2.58E-25	1.13E-23	-1.156
LOC118093251	HBM	7.19E-30	3.70E-28	-1.571
LOC118085601	HBE1	1.24E-34	7.31E-33	-1.633
LOC118093265	HBA	1.48E-39	1.01E-37	-1.897
LOC132592081	HBE1	1.06E-41	7.81E-40	-1.953
LOC118079427	HBE1	4.73E-42	3.53E-40	-2.046
LOC118092499	HBA	2.24E-42	1.71E-40	-2.189
LOC118092498	HBM	4.00E-46	3.34E-44	-2.341

Table S3-10: Cluster 9 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
APOE	APOE	0	0.00E+00	4.511
CTSB	CTSB	0	0.00E+00	2.463
LOC118096176	PSAP	1.28E-218	1.91E-215	2.4
GRN	GRN	0	0.00E+00	2.309
FTL	FTL	1.75E-118	7.68E-116	2.25
CLU	CLU	0	0.00E+00	2.199
ACTB	ACTB	6.80E-95	1.87E-92	2.157
LOC118090838	LGALS1	3.19E-222	5.93E-219	1.936
LOC118094239	FTL	1.71E-62	2.67E-60	1.934
LOC118076126	ECM1	0	0.00E+00	1.893
LOC118097010	CCL4	2.32E-145	1.36E-142	1.64
CD68	CD68	0	0.00E+00	1.549
LOC118077197	LYSC6	9.76E-167	8.07E-164	1.523
CTSD	CTSD	2.78E-138	1.52E-135	1.515
LGMN	LGMN	1.99E-217	2.77E-214	1.471
GPNMB	GPNMB	0	0.00E+00	1.436
LOC118075447	CSTA	2.58E-223	5.23E-220	1.387
LOC118086005	MT2A	6.42E-42	6.29E-40	1.371
CST3	CST3	4.89E-55	6.62E-53	1.273
CLDN5	CLDN5	1.20E-149	7.63E-147	1.247
TMSB4X	TMSB4X	8.58E-37	6.99E-35	1.24
LOC118094983	CXCL8	4.15E-113	1.60E-110	1.152
CTSL	CTSL	1.57E-112	5.85E-110	1.133
NPC2	NPC2	2.39E-114	9.53E-112	1.103
LOC118075388	CSTB	1.66E-96	4.69E-94	1.097
CTSS	CTSS	1.01E-175	9.01E-173	1.052
LOC118076446	S100A6	1.76E-22	7.71E-21	1.008
LOC118085062	HBE1	3.95E-24	1.93E-22	-1.149
LOC118093251	HBM	1.34E-28	7.80E-27	-1.521
LOC118085601	HBE1	3.29E-30	2.05E-28	-1.551
LOC132592081	HBE1	9.29E-35	6.87E-33	-1.777
LOC118079427	HBE1	1.83E-36	1.45E-34	-1.934
LOC118093265	HBA	1.60E-40	1.48E-38	-1.972
LOC118092499	HBA	1.92E-35	1.46E-33	-2.027
LOC118092498	HBM	3.35E-39	2.99E-37	-2.155

Table S3-11: Cluster 10 differentially expressed genes

Gene (NCBI)	*Gene (eggnog)*	*p_val*	*p_val_adj*	*Log FC*
LOC118077197	LYSC6	0	0.00E+00	3.843
CST3	CST3	6.59E-185	6.40E-182	2.577
LOC118087819	PINLYP	0	0.00E+00	2.386
C1QB	C1QB	0	0.00E+00	2.222
LOC118080967	C4A	0	0.00E+00	2.164
LOC118096176	PSAP	4.84E-160	3.60E-157	2.098
APOE	APOE	1.75E-259	2.44E-256	1.994
C1QC	C1QC	0	0.00E+00	1.776
ACTB	ACTB	3.73E-58	9.46E-56	1.696
LOC118091915	MRC1	0	0.00E+00	1.691
LOC118090838	LGALS1	1.92E-162	1.53E-159	1.634
GRN	GRN	6.15E-173	5.49E-170	1.44
LOC118091898	CAMP	6.01E-211	6.10E-208	1.297
C1QA	C1QA	0	0.00E+00	1.227
CFH	CFH	0	0.00E+00	1.165
MAFB	MAFB	1.01E-216	1.13E-213	1.15
CTSB	CTSB	7.33E-94	2.87E-91	1.137
CTSC	CTSC	5.93E-162	4.56E-159	1.077
LY86	LY86	0	0.00E+00	1.056
LTA4H	LTA4H	6.76E-288	1.16E-284	1.008
LOC118081368	HLA-DPB1	3.17E-99	1.36E-96	1.004
LOC118085062	HBE1	1.56E-20	1.38E-18	-1.046
LOC118093251	HBM	1.50E-29	1.76E-27	-1.542
LOC118085601	HBE1	2.48E-33	3.29E-31	-1.622
LOC132592081	HBE1	4.59E-37	6.83E-35	-1.835
LOC118093265	HBA	1.57E-36	2.30E-34	-1.857
LOC118092499	HBA	1.30E-34	1.81E-32	-1.96
LOC118079427	HBE1	2.47E-38	3.80E-36	-1.969
LOC118092498	HBM	4.05E-40	6.70E-38	-2.146

Table S4-1: Oviduct and tissue culture libraries prepared for long-read RNA sequencing

SampleID	*Individual*	*Lineage*	*Condition*	*Passage*	*DiC*	*Reads*	*Mapping*
PP1A	ELT13078	WV	post rep	P3	NA	726071	72.18%
PP2B	ELT13080	WV	post rep	P3	NA	449962	79.52%
WV1SA	Lisa-Lisa	WV	cultured	P5	36	889208	88.74%
WV1SB	Lisa-Lisa	WV	cultured	P5	36	1123529	86.84%
WV1LA	Lisa-Lisa	WV	cultured	P6	61	1238536	89.04%
WV1LB	Lisa-Lisa	WV	cultured	P6	61	558343	86.71%
WV1LC	Lisa-Lisa	WV	cultured	P3	69	1179552	87.32%
WV1LD	Lisa-Lisa	WV	cultured	P2	69	2086214	86.58%
EO1SB	ELT13774	EO	cultured	P2	30	660698	84.48%
EO2SA	ELT13775	EO	cultured	P8	34	866957	81.64%
EO2SB	ELT13775	EO	cultured	P8	34	197044	83.34%
EO1LA	ELT13774	EO	cultured	P6	61	1118263	78.26%
EO1LB	ELT13774	EO	cultured	P6	61	1698588	82.87%
WV2LA	ELT13776	WV	cultured	P6	61	890975	87.43%
WV2LB	ELT13776	WV	cultured	P8	61	668106	86.42%
EO1SC	ELT13774	EO	cultured	P8	41	930138	87.34%
EO2LA	ELT13775	EO	cultured	P3	62	254734	89.83%
EO2LB	ELT13775	EO	cultured	P3	62	342319	88.38%

Table S4-2: DEGs (2 month cell culture vs live)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
LOC118086131	-10.8023	2.01E-13	9.70E-10
LOC118089512	-9.81959	1.35E-11	3.26E-08
PLVAP	-7.57955	1.46E-10	2.34E-07
TPPP3	-7.08916	2.87E-10	2.80E-07
ID4	-5.07355	2.91E-10	2.80E-07
LOC118093265	-8.35672	4.61E-10	3.70E-07
LOC118090761	-11.5378	1.64E-09	9.86E-07
LOC132591531	-3.42258	2.35E-09	1.22E-06
LOC118075426	9.894345	2.54E-09	1.22E-06
TSPAN1	-7.98001	4.45E-09	1.95E-06
LOC118086006	-7.61587	1.11E-08	3.92E-06
AGR2	-11.3182	1.12E-08	3.92E-06
MORN2	-5.20669	1.14E-08	3.92E-06
REXO2	6.208332	1.48E-08	4.75E-06
LOC118095080	-7.05161	2.08E-08	6.27E-06
SERHL2	8.866376	2.33E-08	6.59E-06
LOC118092499	-10.7549	3.54E-08	9.45E-06
PDLIM1	4.426125	3.80E-08	9.64E-06
LOC132592081	-10.3331	4.16E-08	9.65E-06
TEC	-5.18734	4.21E-08	9.65E-06
RAMP2	-6.98386	4.96E-08	1.09E-05
LOC118092242	5.030708	6.05E-08	1.23E-05
APOE	-5.89691	6.13E-08	1.23E-05
RASL11A	10.57411	7.42E-08	1.35E-05
PACRG	-5.97492	7.65E-08	1.35E-05
MYH11	-7.35342	7.78E-08	1.35E-05
CALM1	4.295785	7.84E-08	1.35E-05
SPTBN1	-5.33955	8.16E-08	1.35E-05
RHOC	4.518739	8.49E-08	1.36E-05
LOC118085601	-6.89749	1.14E-07	1.77E-05
RSRP1	-4.30221	1.18E-07	1.77E-05
EPCAM	-4.79557	1.27E-07	1.78E-05
LOC118077197	-9.8585	1.30E-07	1.78E-05
CREBZF	-5.35505	1.49E-07	1.99E-05
PCP4	-9.7134	1.66E-07	2.16E-05
WTAP	-3.1027	1.81E-07	2.24E-05
LOC118094079	-6.17702	1.89E-07	2.24E-05
LOC132592311	-4.12223	1.90E-07	2.24E-05
LOC118079427	-9.59636	2.03E-07	2.31E-05
CDH1	-5.18184	2.06E-07	2.31E-05
CRY1	-5.11007	2.16E-07	2.32E-05
ANXA5	4.589439	2.17E-07	2.32E-05
LOC118087637	4.565742	2.50E-07	2.62E-05
SNTN	-9.54176	2.61E-07	2.67E-05
LOC118081368	-5.02232	2.77E-07	2.67E-05
For full table, see chapter_4_full_tables.xlsx			

Table S4-3: Upregulated GO terms (2m vs live)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0046034	75	42.2	1.30E-08	ATP metabolic process
GO:0009167	66	38.62	1.40E-06	purine ribonucleoside monophosphat…
GO:0009206	37	18.41	2.00E-06	purine ribonucleoside triphosphate …
GO:0070125	38	20.46	1.60E-05	mitochondrial translational elongation
GO:0070126	37	20.21	3.10E-05	mitochondrial translational termination
GO:0010469	36	18.93	4.70E-05	regulation of signaling receptor activity
GO:0070482	71	46.04	0.0001	response to oxygen levels
GO:0032964	12	4.35	0.00012	collagen biosynthetic process
GO:0006734	15	6.14	0.00014	NADH metabolic process
GO:0006839	50	29.92	0.00025	mitochondrial transport
GO:0030833	32	18.16	0.00026	regulation of actin filament polymeriza…
GO:1902600	34	18.16	0.00032	proton transmembrane transport
GO:1902905	36	21.48	0.00037	positive regulation of supramolecular f…
GO:0061621	10	3.58	0.00041	canonical glycolysis
GO:0006887	113	83.89	0.00062	exocytosis
GO:1990542	24	13.04	0.0007	mitochondrial transmembrane transp…
GO:0002444	76	54.99	0.00079	myeloid leukocyte mediated immunity
GO:0051495	38	23.27	0.00081	positive regulation of cytoskeleton org…
GO:0019359	14	6.39	0.00113	nicotinamide nucleotide biosynthetic…
GO:0007007	17	8.44	0.0012	inner mitochondrial membrane organi…
GO:1904059	6	1.79	0.00152	regulation of locomotor rhythm
GO:0046129	7	2.3	0.00154	purine ribonucleoside biosynthetic pro…
GO:0014912	7	2.3	0.00154	negative regulation of smooth muscle …
GO:0070584	10	4.09	0.00194	mitochondrion morphogenesis
GO:0032868	47	31.46	0.00222	response to insulin
GO:0034383	11	4.86	0.00272	low-density lipoprotein particle cleara…
GO:0042866	11	4.86	0.00272	pyruvate biosynthetic process
GO:0006094	16	7.93	0.00296	gluconeogenesis
GO:0006023	15	7.42	0.00298	aminoglycan biosynthetic process
GO:0043280	22	12.79	0.00336	positive regulation of cysteine-type …
GO:0046031	12	5.63	0.00344	ADP metabolic process
GO:0009611	86	64.2	0.00399	response to wounding
GO:0010880	7	2.56	0.00399	regulation of release of sequestered c…
GO:0043096	4	1.02	0.00426	purine nucleobase salvage
GO:0070358	4	1.02	0.00426	actin polymerization-dependent cell m…
GO:0007488	4	1.02	0.00426	histoblast morphogenesis
GO:0007527	6	2.05	0.00475	adult somatic muscle development
GO:0051384	31	20.21	0.00489	response to glucocorticoid
For full table, see chapter_4_full_tables.xlsx				

Table S4-4: Downregulated GO terms (2m vs live)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0001817	43	19.07	1.50E-05	regulation of cytokine production
GO:0006357	108	74.89	1.60E-05	regulation of transcription by RNA …
GO:0007613	23	9.22	1.60E-05	Memory
GO:0001655	34	15.66	1.70E-05	urogenital system development
GO:2000117	17	5.81	2.10E-05	negative regulation of cysteine-type …
GO:0052652	6	0.88	2.50E-05	cyclic purine nucleotide metabolic pro…
GO:0051674	67	41.67	2.60E-05	localization of cell
GO:1902679	72	45.59	3.00E-05	negative regulation of RNA biosyntheti…
GO:0140962	12	3.16	3.70E-05	multicellular organismal-level chemica…
GO:0038083	8	1.64	4.40E-05	peptidyl-tyrosine autophosphorylation
GO:0048659	17	6.57	5.70E-05	smooth muscle cell proliferation
GO:0010628	121	85.12	0.00013	positive regulation of gene expression
GO:0015669	6	0.88	0.00017	gas transport
GO:0007420	56	30.69	0.00022	brain development
GO:0001710	6	1.14	0.00024	mesodermal cell fate commitment
GO:0015701	4	0.51	0.00025	bicarbonate transport
GO:0010629	122	86.38	0.00026	negative regulation of gene expression
GO:0009611	58	31.7	0.0003	response to wounding
GO:0008258	8	2.02	0.00031	head involution
GO:0033138	12	4.29	0.00057	positive regulation of peptidyl-serine …
GO:2000736	12	4.04	0.00066	regulation of stem cell differentiation
GO:0032570	11	3.79	0.00067	response to progesterone
GO:0032496	24	12.25	0.00071	response to lipopolysaccharide
GO:0050851	15	5.3	0.00072	antigen receptor-mediated signaling …
GO:0060259	9	2.78	0.00083	regulation of feeding behavior
GO:0051607	20	7.7	0.00102	defense response to virus
GO:0042311	6	1.39	0.00104	vasodilation
GO:0019934	4	0.63	0.00113	cGMP-mediated signaling
GO:0042246	10	3.03	0.00119	tissue regeneration
GO:0071549	7	1.89	0.00127	cellular response to dexamethasone st…
GO:0010543	5	1.01	0.00127	regulation of platelet activation
GO:0050672	9	2.53	0.00128	negative regulation of lymphocyte prol…
GO:0045638	10	3.54	0.00148	negative regulation of myeloid cell diff…
GO:0071466	18	7.7	0.00163	cellular response to xenobiotic stimulus
GO:0140888	12	4.55	0.00164	interferon-mediated signaling pathway
GO:0009617	52	26.39	0.0018	response to bacterium
GO:0014003	6	1.52	0.00186	oligodendrocyte development
GO:0061067	3	0.38	0.002	negative regulation of dauer larval dev…
For full table, see chapter_4_full_tables.xlsx				

Table S4-5: DEGs (1 month vs live)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
LOC118086131	-12.578318	7.98E-10	3.84E-06
LOC118075426	10.302051	2.12E-09	5.10E-06
REXO2	6.8664035	6.47E-09	1.04E-05
LOC118089512	-10.954895	1.20E-08	1.44E-05
LOC118092242	5.8590515	1.81E-08	1.74E-05
PDLIM1	4.8581306	2.65E-08	2.13E-05
ID4	-6.5858884	3.24E-08	2.23E-05
CALM1	4.8242289	4.12E-08	2.48E-05
TPPP3	-9.5392973	5.32E-08	2.63E-05
RHOC	4.9769677	5.46E-08	2.63E-05
SERHL2	8.7747882	6.77E-08	2.96E-05
PLVAP	-9.282087	8.03E-08	3.22E-05
RASL11A	10.626934	1.20E-07	4.46E-05
LOC118093265	-8.4664291	1.91E-07	6.57E-05
LOC132591531	-3.9514839	2.19E-07	6.98E-05
CFL2	4.9113332	4.14E-07	0.0001172
TIMM17A	4.403027	5.09E-07	0.0001301
ANXA5	4.5870661	5.21E-07	0.0001301
LDHA	4.2723675	5.65E-07	0.0001301
ABRACL	5.2892209	5.68E-07	0.0001301
TK1	9.4030848	6.75E-07	0.0001476
LOC118097010	7.5859896	7.44E-07	0.0001557
LOC118090761	-9.4322813	8.89E-07	0.0001783
HSPB6	5.6423923	9.39E-07	0.0001808
MPZL2	5.3984624	1.12E-06	0.0001992
PPA1	3.9405464	1.14E-06	0.0001992
TSPAN1	-9.0800979	1.16E-06	0.0001992
SNX3	3.5062745	1.35E-06	0.0002245
LOC118076433	6.4469849	1.42E-06	0.0002273
VIM	4.8163915	1.73E-06	0.0002608
LOC118091338	6.4851071	1.73E-06	0.0002608
LOC118087637	4.1858978	1.82E-06	0.000265
AGR2	-11.318222	2.24E-06	0.0003171
EMP3	3.5651554	2.37E-06	0.0003178
LOC118092499	-10.754883	2.38E-06	0.0003178
LOC132592081	-10.333063	2.60E-06	0.0003349
CDK1	5.1735282	2.69E-06	0.0003349
LOC118087112	8.0189295	2.71E-06	0.0003349
MORN2	-5.3107461	3.12E-06	0.0003758
RAMP2	-9.1058296	3.31E-06	0.000389
APOE	-8.136341	3.50E-06	0.0004013
ENO1	4.1190178	3.91E-06	0.0004321
LOC118095080	-9.0986664	3.95E-06	0.0004321
TMEM70	4.3058009	5.20E-06	0.0005495
LTB4R2	9.2742232	5.25E-06	0.0005495
For full table, see chapter_4_full_tables.xlsx			

Table S4-6: Upregulated GO terms (1m vs live)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0070125	39	20.58	6.60E-06	mitochondrial translational elongation
GO:0009167	65	38.84	1.10E-05	purine ribonucleoside monophosphat…
GO:0070126	38	20.32	1.30E-05	mitochondrial translational termination
GO:1990542	27	13.12	2.70E-05	mitochondrial transmembrane transp…
GO:0046034	71	42.44	3.60E-05	ATP metabolic process
GO:0030833	34	18.26	4.10E-05	regulation of actin filament polymeriza…
GO:0051495	41	23.41	5.10E-05	positive regulation of cytoskeleton org…
GO:0009206	34	18.52	5.90E-05	purine ribonucleoside triphosphate …
GO:1902905	38	21.61	9.40E-05	positive regulation of supramolecular …
GO:0009156	30	15.95	0.00012	ribonucleoside monophosphate biosy…
GO:0002446	77	53.76	0.00018	neutrophil mediated immunity
GO:0006839	51	30.1	0.00028	mitochondrial transport
GO:0043039	16	6.69	0.00029	tRNA aminoacylation
GO:0006949	21	11.06	0.0008	syncytium formation
GO:1902600	33	18.26	0.00087	proton transmembrane transport
GO:0010469	33	19.04	0.0009	regulation of signaling receptor activity
GO:0046364	22	11.32	0.00106	monosaccharide biosynthetic process
GO:0007377	5	1.29	0.00112	germ-band extension
GO:1902237	5	1.29	0.00112	positive regulation of endoplasmic reti…
GO:0034314	13	4.89	0.00119	Arp2/3 complex-mediated actin nucle…
GO:0030588	9	3.34	0.00121	pseudocleavage
GO:1904059	6	1.8	0.00157	regulation of locomotor rhythm
GO:0046129	7	2.32	0.00159	purine ribonucleoside biosynthetic pro…
GO:0061621	9	3.6	0.00263	canonical glycolysis
GO:0006122	9	3.6	0.00263	mitochondrial electron transport, ubiq…
GO:0002433	14	6.95	0.00317	immune response-regulating cell surfa…
GO:1904031	7	2.57	0.00413	positive regulation of cyclin-dependen…
GO:0019359	13	6.43	0.00429	nicotinamide nucleotide biosynthetic …
GO:0043096	4	1.03	0.00436	purine nucleobase salvage
GO:0048013	14	7.2	0.00486	ephrin receptor signaling pathway
GO:0043174	6	2.06	0.0049	nucleoside salvage
GO:0055015	5	1.54	0.00528	ventricular cardiac muscle cell develop…
GO:0007588	9	5.14	0.00677	excretion
GO:0000724	15	8.23	0.00768	double-strand break repair via homolo…
GO:0007229	15	8.23	0.00768	integrin-mediated signaling pathway
GO:0007525	15	8.23	0.00768	somatic muscle development
GO:0032869	38	25.98	0.00769	cellular response to insulin stimulus
GO:0006293	7	2.83	0.00886	nucleotide-excision repair, preincision …
For full table, see chapter_4_full_tables.xlsx				

Table S4-7: Downregulated GO terms (1m vs live)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0006357	104	66.16	3.80E-07	regulation of transcription by RNA …
GO:0001817	42	16.85	4.50E-06	regulation of cytokine production
GO:1902679	69	40.28	4.60E-06	negative regulation of RNA biosyntheti…
GO:0052652	6	0.78	1.20E-05	cyclic purine nucleotide metabolic pro…
GO:0007613	21	8.14	2.70E-05	memory
GO:0019935	15	4.13	4.00E-05	cyclic-nucleotide-mediated signaling
GO:1903707	16	5.02	6.90E-05	negative regulation of hemopoiesis
GO:0015669	6	0.78	9.10E-05	gas transport
GO:0001710	6	1	0.00012	mesodermal cell fate commitment
GO:0010628	111	75.2	0.00013	positive regulation of gene expression
GO:0015701	4	0.45	0.00015	bicarbonate transport
GO:0050878	30	13.72	0.00015	regulation of body fluid levels
GO:0045861	28	13.84	0.00016	negative regulation of proteolysis
GO:0001776	9	2.01	0.00019	leukocyte homeostasis
GO:0050817	21	8.59	0.00022	coagulation
GO:0050851	15	4.69	0.00026	antigen receptor-mediated signaling …
GO:0032496	23	10.82	0.00029	response to lipopolysaccharide
GO:0060259	9	2.45	0.00032	regulation of feeding behavior
GO:0006959	23	8.7	0.00035	humoral immune response
GO:0007189	7	1.45	0.00052	adenylate cyclase-activating G protein …
GO:0045638	10	3.12	0.00055	negative regulation of myeloid cell diff…
GO:0071549	7	1.67	0.00059	cellular response to dexamethasone st…
GO:0002697	26	11.49	0.00061	regulation of immune effector process
GO:0001655	28	13.84	0.00065	urogenital system development
GO:0097028	5	0.67	0.00069	dendritic cell differentiation
GO:0060192	4	0.56	0.0007	negative regulation of lipase activity
GO:0050672	8	2.23	0.00084	negative regulation of lymphocyte prol…
GO:0038083	7	1.45	0.00095	peptidyl-tyrosine autophosphorylation
GO:1902036	6	1.34	0.00096	regulation of hematopoietic stem cell …
GO:0010629	110	76.32	0.001	negative regulation of gene expression
GO:0071466	17	6.81	0.00117	cellular response to xenobiotic stimulus
GO:0048660	14	5.58	0.0012	regulation of smooth muscle cell prolif…
GO:0140888	11	4.02	0.00132	interferon-mediated signaling pathway
GO:1902107	11	3.46	0.00132	positive regulation of leukocyte differe…
GO:0007187	9	2.9	0.00135	G protein-coupled receptor signaling …
GO:0007449	3	0.33	0.00138	proximal/distal pattern formation, ima…
GO:0002503	3	0.33	0.00138	peptide antigen assembly with MHC …
GO:0002505	3	0.33	0.00138	antigen processing and presentation o…
For full table, see chapter_4_full_tables.xlsx				

Table S4-8: DEGs (2 month cell cultures oviparous vs viviparous)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
ISG15	5.7486844	2.86E-07	0.0009206
CAV1	4.3383862	3.82E-07	0.0009206
SFRP2	5.8916031	7.89E-06	0.0095009
PEPD	1.9037739	1.19E-05	0.0102528
RHOB	2.3970566	1.57E-05	0.0108048
CNN1	3.7197329	2.02E-05	0.0121481
LOC118087386	-3.3122411	2.78E-05	0.0148846
NRN1L	-6.6761195	3.66E-05	0.0176217
MMP9	-9.3778697	6.31E-05	0.0275975
LOC118076011	5.6543163	7.62E-05	0.0295384
NDRG1	-2.6181687	8.03E-05	0.0295384
IFI44L	5.2618731	9.49E-05	0.0295384
NAALAD2	-4.1400039	9.71E-05	0.0295384
HINT3	-2.5533983	9.82E-05	0.0295384
TRANK1	2.4598299	0.0001387	0.0362406
TNNT2	4.0221641	0.000143	0.0362406
LOC118080481	4.3135629	0.0001849	0.037681
COL8A1	7.5148498	0.000188	0.037681
MYO5B	4.948626	0.0001914	0.037681
TMEM100	4.0682583	0.000193	0.037681
CAV2	2.387775	0.0001953	0.037681
MFGE8	-1.9456964	0.0001957	0.037681
LOC118080190	2.4206645	0.0002215	0.041018
EIF4A1	1.9234475	0.0002416	0.0430744
AK1	-4.1777157	0.0003452	0.0536078
IGFBP5	2.6212502	0.0003536	0.0536078
SDHC	1.584693	0.0003596	0.0536078
LOC118080127	-1.7454527	0.0003617	0.0536078
SLC22A18	1.291922	0.0003665	0.0536078
SCN2B	-4.9085296	0.0003675	0.0536078
LOC118080169	3.0508809	0.000405	0.0565659
LOC118081368	2.7635911	0.0004161	0.0565659
LOC118081394	3.0285045	0.0004398	0.0565659
MATCAP2	3.2398999	0.0004465	0.0565659
LOC118077192	-1.3754183	0.000474	0.0585143
FHL2	2.126002	0.0004943	0.0594878
LOC118097081	1.8524753	0.0005508	0.0599225
AKIRIN2	1.5779531	0.0005532	0.0599225
CFAP210	-4.5152785	0.0005543	0.0599225
ATP6V1C2	-2.0948315	0.000565	0.0599225
CHL1	4.4807617	0.0005711	0.0599225
LOC118080966	-1.9319432	0.0005726	0.0599225
MGAT3	3.9129392	0.0006201	0.0635173
LGMN	-1.6782466	0.0006648	0.0666763
NBL1	2.5031006	0.000739	0.0726031
For full table, see chapter_4_full_tables.xlsx			

Table S4-9: Upregulated GO terms (2m ovi vs vivi)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0019730	10	2.29	9.30E-05	antimicrobial humoral response
GO:0010543	4	0.39	0.00033	regulation of platelet activation
GO:1903792	4	0.39	0.00033	negative regulation of monoatomic ani…
GO:0050806	8	1.9	0.00046	positive regulation of synaptic transmi…
GO:1990748	10	2.29	0.00074	cellular detoxification
GO:0030449	5	0.83	0.00101	regulation of complement activation
GO:2000257	5	0.83	0.00101	regulation of protein activation cascade
GO:0033630	3	0.24	0.00106	positive regulation of cell adhesion me…
GO:0019934	3	0.24	0.00106	cGMP-mediated signaling
GO:0010715	3	0.29	0.00205	regulation of extracellular matrix disas…
GO:1902998	2	0.1	0.00237	positive regulation of neurofibrillary ta…
GO:0090361	2	0.1	0.00237	regulation of platelet-derived growth f…
GO:0031016	6	1.46	0.00278	pancreas development
GO:1901687	4	0.63	0.00278	glutathione derivative biosynthetic pro…
GO:0010874	3	0.34	0.00346	regulation of cholesterol efflux
GO:0035640	3	0.34	0.00346	exploration behavior
GO:2001024	4	0.73	0.00491	negative regulation of response to drug
GO:0002673	7	1.42	0.00504	regulation of acute inflammatory resp…
GO:0007501	3	0.39	0.00534	mesodermal cell fate specification
GO:2000117	7	2.24	0.00627	negative regulation of cysteine-type …
GO:0051956	2	0.15	0.00688	negative regulation of amino acid tran…
GO:0036289	2	0.15	0.00688	peptidyl-serine autophosphorylation
GO:2000766	2	0.15	0.00688	negative regulation of cytoplasmic tra…
GO:1901194	2	0.15	0.00688	negative regulation of formation of tra…
GO:0002503	2	0.15	0.00688	peptide antigen assembly with MHC …
GO:0002505	2	0.15	0.00688	antigen processing and presentation …
GO:0071823	2	0.15	0.00688	protein-carbohydrate complex subunit…
GO:1905908	2	0.15	0.00688	positive regulation of amyloid fibril …
GO:0045986	2	0.15	0.00688	negative regulation of smooth muscle …
GO:0007263	2	0.15	0.00688	nitric oxide mediated signal transducti…
GO:0002455	3	0.44	0.00773	humoral immune response mediated …
GO:1900745	3	0.44	0.00773	positive regulation of p38MAPK cascade
GO:0033280	4	0.83	0.00793	response to vitamin D
GO:0098742	6	1.85	0.00934	cell-cell adhesion via plasma-membra…
GO:0048662	4	0.88	0.00981	negative regulation of smooth muscle …
GO:0032462	3	0.49	0.01065	regulation of protein homooligomeriza…
GO:0030901	4	0.93	0.01196	midbrain development
GO:0010637	2	0.2	0.01332	negative regulation of mitochondrial …
For full table, see chapter_4_full_tables.xlsx				

Table S4-10: Downregulated GO terms (2m ovi vs vivi)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0033631	4	0.47	0.00049	cell-cell adhesion mediated by integrin
GO:0006024	8	2.12	0.00078	glycosaminoglycan biosynthetic process
GO:0006027	7	1.73	0.00104	glycosaminoglycan catabolic process
GO:0042475	6	1.33	0.00131	odontogenesis of dentin-containing to…
GO:0006284	5	0.94	0.00144	base-excision repair
GO:0043137	3	0.31	0.0018	DNA replication, removal of RNA primer
GO:0033567	3	0.31	0.0018	DNA replication, Okazaki fragment pro…
GO:0071377	3	0.31	0.0018	cellular response to glucagon stimulus
GO:0045737	4	0.63	0.00202	positive regulation of cyclin-dependen…
GO:0048311	4	0.63	0.00202	mitochondrion distribution
GO:0001953	4	0.71	0.00341	negative regulation of cell-matrix adhe…
GO:0090023	3	0.39	0.00425	positive regulation of neutrophil chem…
GO:0000727	2	0.16	0.00614	double-strand break repair via break-i…
GO:1901165	2	0.16	0.00614	positive regulation of trophoblast cell …
GO:0006780	2	0.16	0.00614	uroporphyrinogen III biosynthetic proc…
GO:0006784	2	0.16	0.00614	heme A biosynthetic process
GO:1904046	2	0.16	0.00614	negative regulation of vascular endoth…
GO:0006777	2	0.16	0.00614	Mo-molybdopterin cofactor biosynthe…
GO:0010635	3	0.47	0.00801	regulation of mitochondrial fusion
GO:1903671	3	0.47	0.00801	negative regulation of sprouting angio…
GO:0050900	18	8.24	0.01143	leukocyte migration
GO:0001843	7	2.59	0.01234	neural tube closure
GO:0033260	5	1.1	0.01492	nuclear DNA replication
GO:0015721	2	0.24	0.01746	bile acid and bile salt transport
GO:0007448	2	0.24	0.01746	anterior/posterior pattern specificatio…
GO:0033629	2	0.24	0.01746	negative regulation of cell adhesion m…
GO:1901621	2	0.24	0.01746	negative regulation of smoothened sig…
GO:0008359	2	0.24	0.01746	regulation of bicoid mRNA localization
GO:0090399	2	0.24	0.01746	replicative senescence
GO:0043103	2	0.24	0.01746	hypoxanthine salvage
GO:0051549	2	0.24	0.01746	positive regulation of keratinocyte mig…
GO:0097647	2	0.24	0.01746	amylin receptor signaling pathway
GO:0032287	2	0.24	0.01746	peripheral nervous system myelin mai…
GO:0070344	2	0.24	0.01746	regulation of fat cell proliferation
GO:0032927	2	0.24	0.01746	positive regulation of activin receptor …
GO:0006824	2	0.24	0.01746	cobalt ion transport
GO:0061053	6	2.2	0.01914	somite development
GO:0006271	6	0.94	0.01946	DNA strand elongation involved in DN…
For full table, see chapter_4_full_tables.xlsx				

Table S4-11: DEGs (1 month cell culture oviparous vs viviparous)

Gene symbol	*Log FC*	*P value*	*P-adj (FDR)*
EIF3F	-3.403574	1.78E-06	0.0053129
EMP1	-3.1546699	2.21E-06	0.0053129
RAB21	-2.4324413	4.96E-06	0.0076244
LOC132593055	-4.5049279	6.34E-06	0.0076244
PFDN6	-2.7524471	8.49E-06	0.0081733
LOC118091338	-2.9798956	1.47E-05	0.0117753
INO80C	-4.305933	1.78E-05	0.0122148
LOC118094065	8.2328107	2.07E-05	0.0124827
LOC118085881	5.3993196	3.47E-05	0.0185371
LOC118082368	-3.0749855	3.92E-05	0.0188741
LAMTOR1	2.9750086	4.37E-05	0.0191254
ITGA5	-2.2511857	6.75E-05	0.0234295
GCHFR	-4.1061616	6.79E-05	0.0234295
LOC118096596	8.6323504	6.86E-05	0.0234295
TMEM185A	-3.0757172	7.59E-05	0.0234295
LOC118087386	-4.2531053	7.79E-05	0.0234295
ACER3	-3.8895844	0.0001036	0.0293388
LUM	-3.8215299	0.0001106	0.0295859
LOC118093187	-2.9539944	0.0001168	0.0295973
RPRM	4.7336501	0.000137	0.0312918
LOC118088605	2.2980541	0.0001524	0.0312918
NT5C1B	-1.7255647	0.0001554	0.0312918
GALNS	-2.8731323	0.0001581	0.0312918
LOC118095131	-3.0840969	0.0001604	0.0312918
RASSF3	-3.9738135	0.0001646	0.0312918
PDS5A	-2.2673713	0.0001775	0.0312918
CNOT6L	-2.7622313	0.0001799	0.0312918
NDRG1	-2.9246041	0.000183	0.0312918
LOC118084393	-2.1132119	0.0001885	0.0312918
CHST14	-3.3445312	0.0002135	0.0342653
DAGLB	6.2739687	0.000233	0.0361858
ADCYAP1	3.0370075	0.0002431	0.0365765
PDSS1	-2.0715486	0.0002539	0.0370365
TNNT2	8.0396903	0.0002648	0.0374861
SPTY2D1	3.0149463	0.0003036	0.041754
LOC118085695	7.1792626	0.0003334	0.0421112
BGN	3.6381232	0.0003477	0.0421112
NT5C	2.6310179	0.0003478	0.0421112
ITPR3	3.0495075	0.0003484	0.0421112
VSIR	-3.4238186	0.0003499	0.0421112
RAD51AP1	-1.9680081	0.0003677	0.0423703
LOC118078057	5.5106509	0.0003798	0.0423703
SPRYD7	-2.5659746	0.0003884	0.0423703
DNAJC25	-2.2643402	0.0003895	0.0423703
CCND3	-2.2997361	0.0003961	0.0423703
For full table, see chapter_4_full_tables.xlsx			

Table S4-12: Upregulated GO terms (1m ovi vs vivi)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0019730	10	2.29	9.30E-05	antimicrobial humoral response
GO:0010543	4	0.39	0.00033	regulation of platelet activation
GO:1903792	4	0.39	0.00033	negative regulation of monoatomic ani…
GO:0050806	8	1.9	0.00046	positive regulation of synaptic transmi…
GO:1990748	10	2.29	0.00074	cellular detoxification
GO:0030449	5	0.83	0.00101	regulation of complement activation
GO:2000257	5	0.83	0.00101	regulation of protein activation cascade
GO:0019934	3	0.24	0.00106	cGMP-mediated signaling
GO:0033630	3	0.24	0.00106	positive regulation of cell adhesion me…
GO:0010715	3	0.29	0.00205	regulation of extracellular matrix disas…
GO:0090361	2	0.1	0.00237	regulation of platelet-derived growth f…
GO:1902998	2	0.1	0.00237	positive regulation of neurofibrillary ta…
GO:1901687	4	0.63	0.00278	glutathione derivative biosynthetic pro…
GO:0031016	6	1.46	0.00278	pancreas development
GO:0035640	3	0.34	0.00346	exploration behavior
GO:0010874	3	0.34	0.00346	regulation of cholesterol efflux
GO:2001024	4	0.73	0.00491	negative regulation of response to drug
GO:0002673	7	1.42	0.00504	regulation of acute inflammatory resp…
GO:0007501	3	0.39	0.00534	mesodermal cell fate specification
GO:2000117	7	2.24	0.00627	negative regulation of cysteine-type e…
GO:0051956	2	0.15	0.00688	negative regulation of amino acid tran…
GO:0045986	2	0.15	0.00688	negative regulation of smooth muscle …
GO:1905908	2	0.15	0.00688	positive regulation of amyloid fibril fo…
GO:2000766	2	0.15	0.00688	negative regulation of cytoplasmic tra…
GO:0007263	2	0.15	0.00688	nitric oxide mediated signal transducti…
GO:0036289	2	0.15	0.00688	peptidyl-serine autophosphorylation
GO:1901194	2	0.15	0.00688	negative regulation of formation of tra…
GO:0002503	2	0.15	0.00688	peptide antigen assembly with MHC cl…
GO:0002505	2	0.15	0.00688	antigen processing and presentation o…
GO:0071823	2	0.15	0.00688	protein-carbohydrate complex subunit…
GO:0002455	3	0.44	0.00773	humoral immune response mediated …
GO:1900745	3	0.44	0.00773	positive regulation of p38MAPK cascade
GO:0033280	4	0.83	0.00793	response to vitamin D
GO:0098742	6	1.85	0.00934	cell-cell adhesion via plasma-membra…
GO:0048662	4	0.88	0.00981	negative regulation of smooth muscle …
GO:0032462	3	0.49	0.01065	regulation of protein homooligomeriza…
GO:0030901	4	0.93	0.01196	midbrain development
GO:0010637	2	0.2	0.01332	negative regulation of mitochondrial f…
For full table, see chapter_4_full_tables.xlsx				

Table S4-13: Downregulated GO terms (1m ovi vs vivi)

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0033631	4	0.47	0.00049	cell-cell adhesion mediated by integrin
GO:0006024	8	2.12	0.00078	glycosaminoglycan biosynthetic process
GO:0006027	7	1.73	0.00104	glycosaminoglycan catabolic process
GO:0042475	6	1.33	0.00131	odontogenesis of dentin-containing to…
GO:0006284	5	0.94	0.00144	base-excision repair
GO:0043137	3	0.31	0.0018	DNA replication, removal of RNA primer
GO:0071377	3	0.31	0.0018	cellular response to glucagon stimulus
GO:0033567	3	0.31	0.0018	DNA replication, Okazaki fragment pro…
GO:0048311	4	0.63	0.00202	mitochondrion distribution
GO:0045737	4	0.63	0.00202	positive regulation of cyclin-dependen…
GO:0001953	4	0.71	0.00341	negative regulation of cell-matrix adhe…
GO:0090023	3	0.39	0.00425	positive regulation of neutrophil chem…
GO:0000727	2	0.16	0.00614	double-strand break repair via break-i…
GO:1904046	2	0.16	0.00614	negative regulation of vascular endoth…
GO:1901165	2	0.16	0.00614	positive regulation of trophoblast cell…
GO:0006780	2	0.16	0.00614	uroporphyrinogen III biosynthetic proc…
GO:0006784	2	0.16	0.00614	heme A biosynthetic process
GO:0006777	2	0.16	0.00614	Mo-molybdopterin cofactor biosynthe…
GO:0010635	3	0.47	0.00801	regulation of mitochondrial fusion
GO:1903671	3	0.47	0.00801	negative regulation of sprouting angio…
GO:0050900	18	8.24	0.01143	leukocyte migration
GO:0001843	7	2.59	0.01234	neural tube closure
GO:0033260	5	1.1	0.01492	nuclear DNA replication
GO:1901621	2	0.24	0.01746	negative regulation of smoothened sig…
GO:0070344	2	0.24	0.01746	regulation of fat cell proliferation
GO:0043103	2	0.24	0.01746	hypoxanthine salvage
GO:0008359	2	0.24	0.01746	regulation of bicoid mRNA localization
GO:0032287	2	0.24	0.01746	peripheral nervous system myelin mai…
GO:0007448	2	0.24	0.01746	anterior/posterior pattern specificatio…
GO:0033629	2	0.24	0.01746	negative regulation of cell adhesion m…
GO:0015721	2	0.24	0.01746	bile acid and bile salt transport
GO:0051549	2	0.24	0.01746	positive regulation of keratinocyte mig…
GO:0032927	2	0.24	0.01746	positive regulation of activin receptor …
GO:0006824	2	0.24	0.01746	cobalt ion transport
GO:0097647	2	0.24	0.01746	amylin receptor signaling pathway
GO:0090399	2	0.24	0.01746	replicative senescence
GO:0061053	6	2.2	0.01914	somite development
GO:0006271	6	0.94	0.01946	DNA strand elongation involved in DN…
For full table, see chapter_4_full_tables.xlsx				

Table S4-14: GO terms "yellow" gene co-expression module

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0006890	11	3.84	0.001	retrograde vesicle-mediated transport…
GO:0051155	6	1.37	0.0014	positive regulation of striated muscle c…
GO:0045851	7	2.01	0.0026	pH reduction
GO:0045579	3	0.37	0.0028	positive regulation of B cell differentiat…
GO:0002478	12	5.12	0.0039	antigen processing and presentation o…
GO:0043648	10	3.75	0.0041	dicarboxylic acid metabolic process
GO:0030220	3	0.46	0.0066	platelet formation
GO:0060716	3	0.46	0.0066	labyrinthine layer blood vessel develo…
GO:0051295	3	0.46	0.0066	establishment of meiotic spindle locali…
GO:1905165	2	0.18	0.0083	regulation of lysosomal protein catabo…
GO:0010886	2	0.18	0.0083	positive regulation of cholesterol stora…
GO:0033140	2	0.18	0.0083	negative regulation of peptidyl-serine …
GO:0071499	2	0.18	0.0083	cellular response to laminar fluid shea…
GO:0007161	2	0.18	0.0083	calcium-independent cell-matrix adhe…
GO:0006701	2	0.18	0.0083	progesterone biosynthetic process
GO:0002066	20	11.71	0.0113	columnar/cuboidal epithelial cell devel…
GO:0010830	7	2.2	0.0117	regulation of myotube differentiation
GO:0007035	5	1.46	0.0117	vacuolar acidification
GO:0035159	5	1.46	0.0117	regulation of tube length, open trache…
GO:0031647	23	14.09	0.0118	regulation of protein stability
GO:1901998	6	2.01	0.0118	toxin transport
GO:0055015	3	0.55	0.0123	ventricular cardiac muscle cell develop…
GO:2001014	3	0.55	0.0123	regulation of skeletal muscle cell differ…
GO:0007298	11	5.21	0.0126	border follicle cell migration
GO:0031034	4	1.01	0.0135	myosin filament assembly
GO:0002446	29	19.12	0.0137	neutrophil mediated immunity
GO:0061572	13	6.13	0.0186	actin filament bundle organization
GO:0007443	4	1.1	0.0188	Malpighian tubule morphogenesis
GO:0015988	5	1.65	0.0196	energy coupled proton transmembran…
GO:0035988	3	0.64	0.0201	chondrocyte proliferation
GO:0099563	3	0.64	0.0201	modification of synaptic structure
GO:0061050	3	0.64	0.0201	regulation of cell growth involved in ca…
GO:0007386	3	0.64	0.0201	compartment pattern specification
GO:0046663	3	0.64	0.0201	dorsal closure, leading edge cell differ…
GO:0051489	9	4.21	0.0212	regulation of filopodium assembly
GO:0015833	78	64.03	0.0218	peptide transport
GO:0006487	7	2.93	0.0228	protein N-linked glycosylation
GO:1990255	2	0.27	0.0235	subsynaptic reticulum organization
For full table, see chapter_4_full_tables.xlsx				

Table S4-15: GO terms "purple" gene co-expression module

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0007204	25	13.03	0.00023	positive regulation of cytosolic calcium…
GO:0042542	31	18.25	0.00067	response to hydrogen peroxide
GO:0019932	33	20.59	0.00111	second-messenger-mediated signaling
GO:0051480	23	12.77	0.00125	regulation of cytosolic calcium ion con…
GO:0031664	6	1.82	0.00169	regulation of lipopolysaccharide-medi…
GO:0040040	6	1.82	0.00169	thermosensory behavior
GO:0033762	6	1.82	0.00169	response to glucagon
GO:0002429	38	21.64	0.00173	immune response-activating cell surfa…
GO:1902036	8	3.13	0.00365	regulation of hematopoietic stem cell …
GO:0002762	10	4.43	0.00424	negative regulation of myeloid leukocy…
GO:0032273	23	13.82	0.0044	positive regulation of protein polymeri…
GO:0006968	7	2.61	0.00448	cellular defense response
GO:0015701	4	1.04	0.0046	bicarbonate transport
GO:1903430	4	1.04	0.0046	negative regulation of cell maturation
GO:0045838	6	2.09	0.00526	positive regulation of membrane pote…
GO:1902905	36	21.9	0.00537	positive regulation of supramolecular f…
GO:0015669	6	1.82	0.0056	gas transport
GO:0006606	30	20.07	0.00841	protein import into nucleus
GO:0046688	12	5.73	0.00878	response to copper ion
GO:0007599	31	20.59	0.00913	hemostasis
GO:2001039	7	2.87	0.00957	negative regulation of cellular respons…
GO:1901032	7	2.87	0.00957	negative regulation of response to rea…
GO:0007200	6	2.35	0.01232	phospholipase C-activating G protein-…
GO:0010569	6	2.35	0.01232	regulation of double-strand break repa…
GO:0030727	6	2.35	0.01232	germarium-derived female germ-line …
GO:0002455	6	2.35	0.01232	humoral immune response mediated …
GO:0051900	6	2.35	0.01232	regulation of mitochondrial depolariza…
GO:0048581	8	3.65	0.01326	negative regulation of post-embryonic …
GO:0051156	8	3.65	0.01326	glucose 6-phosphate metabolic process
GO:2001169	12	6.52	0.01481	regulation of ATP biosynthetic process
GO:0030851	6	2.09	0.01539	granulocyte differentiation
GO:2000352	5	1.82	0.01544	negative regulation of endothelial cell …
GO:0090325	5	1.82	0.01544	regulation of locomotion involved in …
GO:0014855	11	5.73	0.01611	striated muscle cell proliferation
GO:0033138	15	8.86	0.01677	positive regulation of peptidyl-serine …
GO:0046329	16	9.64	0.017	negative regulation of JNK cascade
GO:0050817	30	20.07	0.01737	coagulation
GO:0010421	4	1.56	0.01766	hydrogen peroxide-mediated program…
For full table, see chapter_4_full_tables.xlsx				

Table S4-16 GO terms "black" gene co-expression module

GO.ID	*Sig genes*	*Expected genes*	*P-value*	*Term*
GO:0051254	41	20.94	4.40E-05	positive regulation of RNA metabolic …
GO:0046500	4	0.4	0.00043	S-adenosylmethionine metabolic proc…
GO:0051253	34	16.15	0.00054	negative regulation of RNA metabolic …
GO:0006357	39	23.7	0.00107	regulation of transcription by RNA pol…
GO:0016360	3	0.24	0.00115	sensory organ precursor cell fate deter…
GO:0060235	2	0.08	0.00159	lens induction in camera-type eye
GO:0043968	3	0.28	0.00195	histone H2A acetylation
GO:0048841	3	0.32	0.00303	regulation of axon extension involved i…
GO:0031065	3	0.32	0.00303	positive regulation of histone deacetyl…
GO:0038128	3	0.32	0.00303	ERBB2 signaling pathway
GO:0008586	4	0.64	0.00307	imaginal disc-derived wing vein morph…
GO:0048255	5	0.72	0.00381	mRNA stabilization
GO:0008587	6	1.56	0.00405	imaginal disc-derived wing margin mo…
GO:0051081	3	0.36	0.00441	nuclear membrane disassembly
GO:0045161	2	0.12	0.00464	neuronal ion channel clustering
GO:0016330	2	0.12	0.00464	second mitotic wave involved in comp…
GO:0035814	2	0.12	0.00464	negative regulation of renal sodium ex…
GO:1902275	9	3.32	0.00541	regulation of chromatin organization
GO:0009952	13	5.24	0.00723	anterior/posterior pattern specification
GO:0007392	3	0.44	0.00816	initiation of dorsal closure
GO:0001956	3	0.44	0.00816	positive regulation of neurotransmitte…
GO:0034334	3	0.44	0.00816	adherens junction maintenance
GO:0008582	7	2.36	0.00848	regulation of synaptic assembly at neu…
GO:1903432	4	0.84	0.00864	regulation of TORC1 signaling
GO:0061072	2	0.16	0.00903	iris morphogenesis
GO:0023019	2	0.16	0.00903	signal transduction involved in regulati…
GO:0035865	2	0.16	0.00903	cellular response to potassium ion
GO:0060059	2	0.16	0.00903	embryonic retina morphogenesis in ca…
GO:0035563	2	0.16	0.00903	positive regulation of chromatin binding
GO:0046831	3	0.48	0.01057	regulation of RNA export from nucleus
GO:0043967	5	1	0.01179	histone H4 acetylation
GO:0090336	2	0.2	0.01466	positive regulation of brown fat cell dif…
GO:0007319	2	0.2	0.01466	negative regulation of oskar mRNA tra…
GO:0051255	2	0.2	0.01466	spindle midzone assembly
GO:0033033	2	0.2	0.01466	negative regulation of myeloid cell apo…
GO:0010842	2	0.2	0.01466	retina layer formation
GO:0010628	43	26.94	0.01477	positive regulation of gene expression
GO:0048024	8	3.28	0.01579	regulation of mRNA splicing, via splice…
For full table, see chapter_4_full_tables.xlsx				