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ORIGINAL ARTICLE

Allelic expression of mammalian imprinted genes in a matrotrophic lizard, *Pseudemoia entrecasteauxii*

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Abstract Genomic imprinting is a process that results in the differential expression of genes depending on their parent of origin. It occurs in both plants and live-bearing mammals, with imprinted genes typically regulating the ability of an embryo to manipulate the maternal provision of nutrients. Genomic imprinting increases the potential for selection to act separately on paternally and maternally expressed genes, which increases the number of opportunities that selection can facilitate embryonic control over maternal nutrient provision. By looking for imprinting in an independent matrotrophic lineage, the viviparous lizard *Pseudemoia entrecasteauxii* (Scincidae), we test the hypothesis that genomic imprinting facilitates the evolution of substantial placental nutrient transport to embryos (matrotrophy). We sequenced transcriptomes from the embryonic component of lizard placentae to determine whether there are parent-of-origin differences in expression of genes that are imprinted in mammals. Of these genes, 19 had sufficiently high expression in the lizard to identify polymorphisms in transcribed sequences. We identified biallelic expression in 17 genes (including *insulin-like growth factor 2*), indicating that neither allele was imprinted. These data suggest that either genomic imprinting has not evolved in this matrotrophic skink or, if it has, it has evolved in different genes to mammals. We outline how these hypotheses can be tested. This study highlights important differences between mammalian and reptile pregnancy and the absence of any shared imprinting genes reflects fundamental differences in the way that pregnancy has evolved in these two lineages.

Keywords Viviparity · Parent-offspring conflict · *Pseudemoia* · Placenta · Placentotrophy · Lizard · Scincidae · Genomic imprinting

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Background

The principles of Mendelian inheritance posit that offspring inherit one allele from each parent and that the phenotype of offspring is the product of the expression of both of these genes. Genomic imprinting subverts Mendelian inheritance and results in genes being expressed from only one of the two parental chromosomes, depending on its parent of origin (Wilkins and Haig 2003). The evolution of genomic imprinting is puzzling because it negates the heterozygosity benefit of being diploid by only expressing a single copy of each imprinted gene. Despite the potential costs, genomic imprinting has evolved independently in flowering plants and live-bearing (viviparous) mammals, but is apparently absent in egg-laying (oviparous) birds and the platypus (Killian et al. 2001; Feil and Berger 2007; Pask et al. 2009; Frésard et al. 2014). *Insulin-like growth factor 2* (IGF2) is the most widely studied imprinted gene, and is imprinted in a range of eutherian and marsupial mammals, but it is not imprinted in two live-bearing fish species (Lawton et al. 2005).

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In vertebrates, genomic imprinting occurs exclusively in viviparous mammals (Renfree et al. 2008). While most imprinted genes are predominantly expressed in placental tissues (Prickett and Oakey 2012), genomic imprinting occurs in fewer marsupial genes than eutherian genes, even though imprinting likely evolved in their common ancestor (Das et al. 2012; Graves and Renfree 2013). Genomic imprinting has not been found in the egg-laying mammals (monotremes) despite the formation of a short-lived placenta prior to egg deposition, suggesting that genomic imprinting is associated with the evolution of viviparity and not a placenta per se (Renfree et al. 2008). Further, several imprinted genes have significant expression in mammary tissue, showing that imprinting as a process may be important for regulating both post- and prenatal nutrition (Stringer et al. 2014).

Multiple studies have proposed selection pressures that could have resulted in the evolution of genomic imprinting (Wolf and Hager 2006; Holman and Kokko 2014). A prominent hypothesis is that genomic imprinting evolves as a result of ongoing genetic conflict between mothers and offspring, the latter of whom have inherited half of their genetic material from their father (Haig 2000; Wilkins and Haig 2003; Crespi and Semeniuk 2004; Holman and Kokko 2014). Embryos are connected to the mother by a placenta or placenta-like structure in both mammals (via the fetal placenta and maternal uterine tissue) and flowering plants (via the embryonic endosperm and maternal seed coat). A placenta typically allows for maternal transport of nutrients to the embryo (matrotrophy), and differences in the "preferred" amount of nutrient transport results in conflict between the maternal and paternal genome (Moore and Haig 1991).

Under the conflict theory, selection maintains maternal imprinting of genes that facilitate embryonic manipulation of maternal nutrient transport (such as *IGF2*), limiting the embryo's ability to control maternal nutrient transfer. Similarly, selection will maintain paternal imprinting in genes that encode proteins that decrease embryonic manipulation (such as insulin-like growth factor 2 receptor, which deactivates IGF2 activity in mammals), ultimately increasing the potential for embryonic regulation of matrotrophy (Haig and Graham 1991; Renfree et al. 2013).

Imprinting of genes that regulate matrotrophy is common to both plants and viviparous mammals, which can be observed when the system of imprinting is subverted. In plants, seed endosperm formation is impacted by parental genome dosage, with a double dose of the paternal genome resulting in larger endosperm and a larger embryo, while a double dose of the maternal genome results in both smaller endosperm and embryos (Haig and Westoby 1991; Scott et al. 1998). Similar phenotypic differences occur in mammals; offspring with two maternal copies of the genome have poor placental development and develop poorly after implantation, further knockouts to paternally imprinted genes typically result in placental and fetal growth restriction, and knockouts of maternally imprinted genes results in increases in placental or fetal growth (Surani et al. 1984; Angiolini et al. 2006).

Parent-offspring conflict can still result in evolution in genes that do not exhibit parent-of-origin differences in gene expression, but gene variants that offer fitness benefits to offspring at the expense of the maternal genome will have negative fitness effects on female offspring when they reproduce, resulting in antagonistic selection (O'Neill et al. 2007). This phenomenon can be illustrated by considering the effects of a hypothetical gene that increases the nutrients received by an embryo from its mother. This gene will offer a fitness advantage to any offspring that carries it, but the gene will later impose a cost on female offspring, because when they reproduce their offspring will carry the gene resulting in her providing more resources to the embryo than is in her interest. By maternal silencing of this gene, female offspring can receive the benefit of the gene when they inherit it from their fathers, but do not receive the fitness cost associated with it functioning when they reproduce. In this way, genomic imprinting increases maternal-offspring conflict because it allows genes that facilitate conflict to be freed from antagonistic selection, increasing the rate at which they can evolve.

The ancestral mode of embryonic nutrition in vertebrates is lecithotrophy, where nutrition is provided as yolk prior to ovulation and fertilization (Yu et al. 1981; Blackburn 2014). There is the potential for embryonic nutrition to be provided across a placenta or similar structure as development occurs (matrotrophy) in organisms where embryonic development occurs inside the mother. Incipient transfer of nutrients occurs in all viviparous organisms that have been examined, with a net uptake of nutrients by the embryo through pregnancy (substantial matrotrophy) in some species (most notably in eutherian mammals) (Hoffman 1970; Van Dyke and Beaupre 2012; Blackburn 2014; Whittington et al. 2015). Substantial matrotrophy, where there is a net increase in mass of offspring relative to eggs at ovulation, has evolved only seven times in the ~122 viviparous amniote lineages. Unlike the transition to simple viviparity, substantial matrotrophy is either evolutionarily "difficult" (i.e., requires many inter-dependent mutations that have not occurred frequently in viviparous squamates) or offers little selective advantage (Griffith et al. 2015).

To extend our understanding of the role of imprinting in the evolution of placental nutrient transfer in general, it is necessary to determine whether or not genomic imprinting is present in other non-mammalian organisms that independently evolved matrotrophy. Reptiles are an ideal study system because placentae that transport nutrients to developing offspring have evolved independently in multiple lineages (Van Dyke et al. 2014a; Wright et al. 2015). The placentae of both reptiles and mammals are convergently evolved in both function and the genes used to fulfill these functions (Thompson and Speake 2006; Brandley et al. 2012; Griffith 2015).

Moreover, the mammal and reptile placentae are structurally homologous, with the maternal half of the placenta composed of uterine tissue, and embryonic half from the chorioallantoic and yolk sac membranes.

Using RNA-Seq methods, we tested the association between the evolution of genomic imprinting and matrotrophy, and determined if the genomic imprinting that occurs in mammals also exists in a matrotrophic viviparous reptile, the southern grass skink (*Pseudemoia entrecasteauxii*). *P. entrecasteauxii* is an ideal study organism because viviparity in this lizard genus evolved independently from mammals. Moreover, it has a non-invasive placenta that contributes large quantities of nutrients to offspring and it has high rates of multiple paternity (Stewart and Thompson 1993; Stapley et al. 2003; Speake et al. 2004; Griffith et al. 2013a, b). Multiple paternity heightens the potential for conflict as offspring have lower relatedness with each other and therefore, maternal fitness measured by lifetime reproductive success is de-coupled from the fitness of any one sire (Haig 1999). Pregnant *P. entrecasteauxii* provide nutrients to offspring even when nutrient limitation results in maternal weight loss, suggesting nutrient transport favors the embryo's preferred transfer rate, providing evidence of parent-offspring conflict over resources (Van Dyke et al. 2014b). Furthermore, when corticosterone concentration (a modulator of stress) is raised, mothers behave selfishly and allocated greater resources to their own body condition (Itonaga et al. 2012).

Methods

Transcriptome sequencing

We collected gravid *P. entrecasteauxii* from Kanangra Boyd National Park, NSW, Australia, between October and November in 2011 and 2012. Lizards were housed individually until late pregnancy. To collect placental tissues, we removed developing eggs from the uterus and dissected away the chorioallantoic membrane and yolk sac membrane. There is no embryonic invasion of placental tissues in this species, so the uterus can be cleanly dissected away from the embryonic tissues (Fig. 1). Yolk was scraped away from the yolk sac membrane. Tissues were fixed in RNAlater (Ambion, 24 h, 4 °C) and stored (−80 °C). Chorioallantoic and yolk sac membranes were collected from one embryo from each of six females. Embryos were at developmental stages 36 ($n=1$), 37 ($n=1$), or 40 ($n=4$) of the Dufaure and Hubert (1961) 40 stage staging scheme.

To extract RNA, tissues were macerated using a mechanical homogenizer in 600 μL of Buffer RLT (QIAGEN) then homogenized using a QIAshredder spin column (QIAGEN). Total RNA was extracted using the RNeasy Mini Kit (QIAGEN). Extracted RNA was treated with Amplification Grade DNase 1 (Sigma-Aldrich). RNA quality was measured on the Agilent 2000 Bioanalyzer (Agilent Technologies) and was only used for transcriptome analysis if the RNA integrity number was greater than 8 (a scale from 1 to 10, where values above seven are considered good quality for downstream analyses). Sequencing libraries were generated in house using the TruSeq RNA Sample Preperation kit (Illumina, inc.). Libraries were pooled into lanes containing ten samples and were sequenced on the HiSeq2000 (Illumina, Inc.). We collected a mean of $2.4\times10^7\pm3.8\times10^6$ sequenced reads per sample. Raw transcriptome reads are available in the Sequenced Read Archive (accession on acceptance).

We assembled transcriptomes for *P. entrecasteauxii* de novo from a range of tissues, including the whole uterine tissue of early ($n=1$) and late ($n=2$) pregnant females, the embryonic chorioallantoic membrane ($n=2$) and yolk sac membranes ($n=2$) of late developing embryos and adult brain tissue ($n=1$). In all cases, transcriptomes were analyzed from single tissues only, and were not pooled prior to sequencing. Transcriptomes were assembled with ABySS 1.3.4 (Simpson et al. 2009) from tissues of a single individual at a time. Once each transcriptome was assembled, they were pooled to build a reference transcriptome. In the reference transcriptome contigs smaller than 100 bp and redundant contigs were removed using

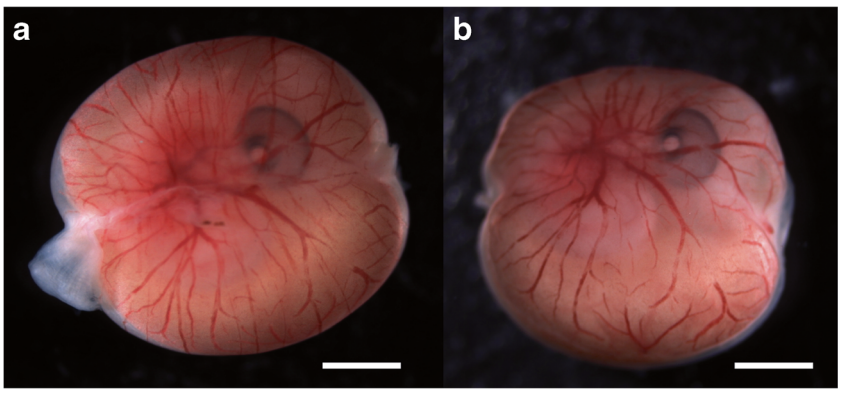

Fig. 1 Image of uterus encasing a developing egg (**a**) and then the same tissue with the uterus dissected away (**b**). No maternal tissue remains following removal of the uterus. *Scale bars* are 3 mm. Embryo is at developmental stage 32 of the Dufaure and Hubert (1961) 40 stage staging scheme

CD-HIT-EST (Huang et al. 2010) using default options. We annotated the reference transcriptome by aligning contigs against the *Anolis* proteome (Ensembl Build 70) using BlastX with an *e* value of 10–5. The alignment rate of the raw reads to the assembled transcriptome was >90 % for all samples. Sixty thousand seven hundred seventy-three assembled transcripts were identified following blast to the *Anolis* proteome, which equates to 74 % sequence coverage of protein coding genes in the published *Anolis* proteome. A further 27,431 transcripts were identified after aligning unidentified contigs to a composite of the proteomes of *Homo sapiens* (human), *Gallus gallus* (chicken), *Monodelphis domestica* (opossum), *Ornithorhynchus anatinus* (platypus), *Taeniopygia guttata* (zebra finch), and *Pelodiscus sinensis* (Chinese soft-shell turtle) (Ensembl Build 70).

Heterozygosity in candidate genes

We collated a list of all imprinted genes from *H. sapiens* (human), *Mus musculus* (mouse), *Mo. domestica* (opossum), and *Macropus eugenii* (tammar wallaby) in the Geneimprint database (Jirtle 2006). In total, 213 mammalian imprinted genes were used as candidates for our analysis. Of these candidates, only genes with a minimum of 15-fold coverage across the coding region of the messenger RNA (mRNA) could be used to examine imprinting, leaving 19 genes for which genomic imprinting results in the expression of only one of the two inherited alleles for each gene. Genomic imprinting can be excluded for any locus with bi-allelic expression. We examined our transcriptomes for evidence of bi-allelic expression in all candidate genes in the chorioallantoic and yolk sac placental tissues of *P. entrecasteauxii*. Bi-allelic expression was identified by the presence of heterozygous single nucleotide polymorphisms (SNPs) in the coding regions of the candidate genes. The coding region of sequences was defined as the region of the alignment between the candidate gene and the protein of interest when aligned to all proteins in the NCBI protein database with tBLASTn (Altschul et al. 1997). We identified heterozygosity by examining the alignments of the raw sequencing reads from each placental sample to the genes of interest in the assembled *P. entrecasteauxii* transcriptome. Heterozygosity was assumed for a sample if it had a minimum of 15-fold sequencing coverage, and at least six sequenced copies of each variant.

Lab confirmation of findings from transcriptome sequencing

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

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

Heterozygosity of SNPs and allele expression rates were confirmed by pyrosequencing

To show that heterozygosity and homozygosity in expressed genes reflect underlying allelic differences in individuals, we performed pyrosequencing on PCR products of *RB1* and *UBE3A* generated from paired gDNA and cDNA samples. gDNA and RNA were extracted simultaneously from embryonic chorioallantoic placenta sample using the All Prep DNA/RNA mini kit (QIAGEN) following manufacturer's instructions. PCRs and pyrosequencing were performed as mentioned above.

Results

Nineteen candidate genes had sufficiently high expression to evaluate imprinting status. Seventeen genes had at least one individual with heterozygous polymorphisms in the sequenced transcriptomes, ruling out genomic imprinting of these genes in placental tissues (Table 1). Two genes lacked any polymorphisms in all samples studied (Table 1).

In all cases, there was concordance between allelic expression in the transcriptomic and pyrosequencing methods, confirming the use of transcriptomics for detecting bi-allelic expression of genes (Supp. Table 2). In all cases,

Table 1 Polymorphisms in genes as determined by highthroughput mRNA sequencing

Highly expressed genes expressing two distinct alleles (non-imprinted genes)	Highly expressed genes without polymorphisms
AMPD3, *COPG2*, *DHCR7*, *DIO3*, *DLK1*, *DNMT1*, *EPHA4*, *GAB1*, *GATM*, *IGF2*, *NAA60*, *RB1*, *RBP5*, *SHCE*, *SLC38A4*, *TSSC4*, *UBE3A*	*DCN*, *LIN28B*

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

Discussion and conclusions

This study is the first to seek evidence of GENOMIC imprinting in a non-mammalian viviparous amniote. Bi-allelic expression (the expression of both maternal and paternally inherited allele) of mammalian imprinted genes in the lizard *P. entrecasteauxii* suggests that genomic imprinting and differential parent-of-origin gene expression has not evolved in the same genes in reptiles and mammals.

Two genes (*DCN*, *LIN28B*) in this study did not contain any polymorphism in any individual. Without the presence of polymorphisms in these genes between any samples, it is not possible to identify genomic imprinting as there are no genetic markers that would allow differentiation between bi- and mono-allelic expression. Although we cannot rule out genomic imprinting for these genes, they typically had lower expression of the coding region than other genes for which polymorphisms were identified, suggesting they play a minor role in placental functions.

The proximate mechanisms for genomic imprinting are not completely understood, but several molecular processes are involved, including methylation of CpG sites on the genome, chromatin structure, and histone modifications (Reik and Walter 1998; Feil and Khosla 1999; Lawton et al. 2008; Pask et al. 2009). Imprinting at some loci has evolved following the incorporation of novel CpG islands, which are stretches of DNA with a high frequency of CpG sites that can be methylated (Renfree et al. 2013; Rademacher et al. 2014). CpG islands have been incorporated into the genome at varying points, including in the stem eutherians (resulting in imprinting in *PEG3* and *MEG1*), in the stem therians (resulting in imprinting of *PEG10* and *SNRPN*) and in the stem mammals (resulting in imprinting of SLC38A4) (Renfree et al. 2013). However, imprinting of these loci sometimes evolves much later than the origin of these CpG islands, for example a CpG island arose near SLC38A4 in the stem mammals, but it is only imprinted in eutherian mammals (Renfree et al. 2013). CpG islands between the *H19* and *IGF2* genes facilitate imprinting at this locus in therians (Thorvaldsen et al. 1998). Multiple CpG islands (longer than 200 bp, with C+G to CpG ratio greater than 0.6 and greater than 50 % C+G content, calculated by http://www.ebi.ac.uk/emboss/cpgplot) are present in the homologous stretch of DNA in *Anolis carolinensis*, suggesting that the CpG island responsible for imprinting in IGF2 could be conserved in amniotes (Cunningham et al. 2015).

IGF2 has been the candidate gene of choice to examine genomic imprinting outside of eutherian mammals because it is one of the few genes imprinted in both eutherian and marsupial mammals (O'Neill et al. 2000; Killian et al. 2001; Lawton et al. 2005). Imprinting of IGF2 is regulated in part by a CpG island, which acts to insulate the IGF2 gene from nearby enhancers on the paternal chromosome (Sasaki et al. 2000). This CpG-rich region appears to be a conserved feature of amniotes, and therefore imprinting of this gene is not constrained by the genome. Rather, the absence of imprinting in this gene may reflect differences in the proteins that support placental functions in reptiles and mammals, or the absence of selection pressures for the evolution of imprinting in this species.

The lack of genomic imprinting of genes such as *IGF2* in *P. entrecasteauxii* shows that imprinting is not essential for the evolution of matrotrophy. Given the parent-offspring conflict hypothesis for the evolution of genomic imprinting, we expect imprinting to evolve in species that exhibit viviparity, mechanisms of nutrient provision to offspring after fertilization (e.g., placentotrophy), and high rates of multiple paternity (Haig 1999; Haig 2000; Crespi and Semeniuk 2004; Blackburn 2015). As *P. entrecasteauxii* exhibits these characteristics, but we failed to find evidence of imprinting, we conclude that either genomic imprinting has not evolved in *P. entrecasteauxii* or that imprinting has evolved, but in different genes from mammals. For imprinting to be selected for under the conflict model, embryos must have mechanisms for manipulating the amount of resources provided to themselves through pregnancy. While mothers transfer nutrients to offspring even when this results in a loss of stored nutrients through pregnancy (Van Dyke et al. 2014b) and embryos of other viviparous skinks are able to manipulate the development of uterine vasculature through pregnancy (Murphy et al. 2010), embryonic control of placental nutrient transfer has not been documented in reptiles. Without a mechanism for embryos to manipulate placental transfer, we would not expect imprinting to evolve. However, if embryos did have a mechanism for manipulating placental transport, but this mechanism used different genetic pathway from those in mammals (e.g., *IGF2* was not involved), then we would expect genomic imprinting to evolve in different genes. To separate these hypotheses, it is necessary to systematically assess imprinting across the genome, which would require both testing for mono-allelic expression of all genes, using a hybridization approach such as the one used by Wang et al (2013), or by looking for consistent differences between the methylation signatures in the genomes of male and female gametes, and then assessing if these methylation signatures are conserved through development.

If genomic imprinting is absent from this viviparous matrotrophic lizard, then this suggests that mammalian genomic imprinting has evolved not simply as a result of parent offspring conflict but as a result of other selection pressures. A variety of theoretical explanations for the evolution of

genomic imprinting have been proposed (Wolf and Hager 2006; Holman and Kokko 2014), but many of these theories lack empirical study in part because the literature has been dominated by the conflict hypothesis (Spencer and Clark 2014). We hope that our findings act as a stimulant for more empirical studies into the proximate and ultimate causes for the evolution of genomic imprinting in organisms.

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

Compliance with ethical standards Animal work was conducted under University of 441 Sydney Animal Ethic approval.
Conflict of interest The authors declare that they have no conflict of interest.

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