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Title

A comparison of uterine contractile responsiveness to arginine vasopressin in oviparous and viviparous lizards

Running Title

Uterine contractility and parity mode

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Competing interests

The authors have no competing interests to declare.

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Abstract

Nonapeptides and their receptors regulate a diverse range of physiological processes. We assessed the contractile responsiveness of uteri from the squamate viviparous-oviparous species pair, *Pseudemoia entrecasteauxii* and *Lampropholis guichenoti*, as well as the bimodally reproductive species, *Saiphos equalis*, to arginine vasopressin (AVP). We assessed the resulting uterine contractility as a function of pregnancy status, species and parity mode. We also measured mRNA abundance for the nonapeptide receptor, oxytocin receptor (*oxtr*), in uteri from *P. entrecasteauxii* and *L. guichenoti* and compared expression across pregnancy status and parity mode. We found that pregnant uteri exhibited a significantly greater contractile response to AVP than non-pregnant uteri in all three lizard species studied. Cross-species comparisons revealed that uteri from viviparous *P. entrecasteauxii* were significantly more responsive to AVP than uteri from oviparous *L. guichenoti* during both pregnant and non-pregnant states. Conversely, for non-pregnant *S. equalis*, uteri from viviparous individuals were significantly less responsive to AVP than uteri from oviparous individuals, while during pregnancy, there was no difference in AVP contractile responsiveness. There was no difference in expression of *oxtr* between *L. guichenoti* and *P. entrecasteauxii*, or between pregnant and non-pregnant individuals within each species. We found no significant correlation between *oxtr* expression and AVP contractile responsiveness. These findings indicate that there are differences in nonapeptide signalling across parity mode and suggest that in these lizards, labour may be triggered either by an increase in plasma nonapeptide concentration, or by an increase in expression of a different nonapeptide receptor from the vasopressin-like receptor family.

Introduction

Viviparity (livebearing reproduction) has evolved independently from the ancestral state of oviparity (egg-laying reproduction) in more than 150 vertebrate lineages, with 115 independent origins in squamate reptiles, 22 in fish, eight in amphibians and a single origin in mammals (Blackburn 2015). The single mammalian transition to viviparity is ancient, and as such, it is unlikely that extant mammals have retained morphological or genetic mechanisms associated with this transition (Van Dyke et al. 2014). In contrast, squamate reptiles allow comparisons to be made between multiple independent origins of live birth, some of which are quite recent (Smith et al. 2001), making squamate reptiles excellent models for understanding the transition to viviparity (Van Dyke et al. 2014; Blackburn 2006). A fundamental question has arisen as to whether the same suite, or 'toolkit', of genes is implicated across independent origins of viviparity (Thompson and Speake 2006). This concept of a common toolkit of genes underpinning viviparity is supported by origins of viviparity in other vertebrates, including anamniotes, where similar genes may have been recruited to both support pregnancy and trigger parition (oviposition or parturition) (Brandley et al. 2012; Griffith et al. 2016; Whittington et al. 2015b; Whittington et al. 2018).

Scincid lizards are ideal models to study the evolution of viviparity as they allow comparisons between closely related taxa displaying different parity modes (Van Dyke et al. 2014). For example, comparative studies across oviparous-viviparous species pairs have confirmed that the transition to viviparity is associated with reduced eggshell thickness (Guillette 1993; Heulin et al. 2005; Packard et al. 1977), delayed oviposition (Guillette 1993; Murphy and Thompson 2011; Thompson and Speake 2006), placental development facilitating water supply, nutrient

exchange and gas exchange with the mother (Guillette and Jones 1985; Murphy and Thompson 2011; Thompson and Speake 2006; Thompson et al. 2000; Van Dyke et al. 2014), and modulation of the maternal immune system (Graham et al. 2011; Hendrawan et al. 2017). *Lampropholis guichenoti* (oviparous) and *Pseudemoia entrecasteauxii* (viviparous) are closely related taxa exhibiting different parity modes, whereas *Saiphos equalis* is a reproductively bimodal skink that has both oviparous and viviparous populations. These Scincid lizards are thus excellent models for further interrogating the evolution of viviparity.

Oviparous skinks, such as *L. guichenoti*, oviposit at approximately embryonic stage 30 (Qualls and Shine 2000), with embryonic eyes beginning to become pigmented and fringed stumps of limbs. In contrast, viviparous skinks, such as *P. entrecasteauxii,* give birth at Dufaure and Hubert's embryonic stage 40 (Dufaure and Hubert 1961; Smith and Shine 1997), with embryos having fully developed organs, scales and pigmentation. The significantly increased duration of embryo retention, which produces neonates at later stages of development, is, therefore, a defining aspect of vivparity, and raises questions as to how regulation of the timing of labour is achieved.

Interestingly, oviparous squamates retain their eggs for much longer than most oviparous reptiles (up to the limb bud stage). This trait of longer egg retention may be an exaptation for viviparity that helps explain the relatively high incidence of independent origins of viviparity among squamates, compared to other vertebrate species (Blackburn 2006). There are only a few 'transitional' species that retain eggs for an intermediate period, with most squamate reptiles exhibiting either 'normal' oviparity (oviposition around stage 30) (Blackburn 1995; Shine 1983), or 'normal' viviparity (parturition at stage 40) (Smith and Shine 1997). The evolution of viviparity is therefore likely to be associated with distinct differences in the timing of expression of key genes involved in triggering labour. Indeed, in their genomic analysis of a closely related oviparous-viviparous lizard pair (*Phrynocephalus przewalskii* and

Phrynocephalus vlangalii), Gao *et al*. concluded that temporal and spatial changes in gene expression account for the major physiological, morphological and immunological aspects of the transition from oviparity to viviparity (Gao et al. 2019). Prime candidates that could underpin delayed parition include receptors that mediate the effects of 'nonapeptide' hormones. Nonapeptide hormones, which are nine amino acids long in their mature form, fulfil important and diverse functions in vertebrates, affecting behaviour, osmoregulation and reproduction (Banerjee et al. 2017; Wircer et al. 2016). These hormones are divided into two family groups: the vasopressin-like family and the oxytocin-like family (Banerjee et al. 2017; Goodson 2008), and differ by only one or two amino acids. The nonapeptides are primarily produced in the hypothalamus and secreted from the posterior pituitary gland, as well as being produced locally in reproductive tissues, including the ovary, corpus luteum and the uterus (Blanks and Thornton 2003; Fuchs et al. 1982; Vrachnis et al. 2011). Across vertebrates, these hormones are potentially a trigger or mediator of labour (Gimpl and Fahrenholz 2001; Blanks and Thornton 2003; Fergusson and Bradshaw 1991) in that they are potent stimulators of contractions in smooth muscle, including in the uterus or oviduct (Banerjee et al. 2017; Freund-Mercier and Richard 1981; Mitchell and Schmid 2001). These pro-contractile properties are mediated through nonapeptides binding to the specific 7-transmembrane domain of G-protein-coupled cell surface receptors (Wircer et al. 2016; Kota et al. 2013). Binding to the receptors activates intracellular phospholipase C (PLC), which hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors on the sarcoplasmic reticulum, causing the release of intracellular calcium stores, while DAG activates protein kinase C (PKC). PKC and elevated intracellular calcium then activate a multitude of pro-contraction signalling pathways that ultimately converge at the initiation of actin-myosin cross-bridge cycling, which causes the smooth muscle cells to contract (Smith 2007).

Like the nonapeptide hormones, the nonapeptide receptors are structurally similar, and as such, both the oxytocin and vasopressin family nonapeptides can bind to all receptors with different affinities (Wircer et al. 2016). The vertebrate receptor repertoire consists of a total of 6 possible nonapeptide receptors with two, OXTR and V1A, likely mediating smooth muscle contractility (Banerjee et al. 2017), although not all species possess all 6 receptors.

While the role of oxytocin signalling and mechanisms behind the induction of labour in humans are still not well-understood (Mitchell and Schmid 2001; Mitchell and Taggart 2009; Smith 2007), the physiological response of the uterus (contraction) to the nonapeptides is directly correlated with the concentration of the receptors (Fuchs et al. 1983), suggesting a role in either the establishment or augmentation of contractions of labour. In lizards, the oviduct of viviparous *Phrynocephalus vlangalii*, progression from embryonic stage 34-36 to late stage 40 is associated with a significant upregulation of the oxytocin signalling pathway (transcriptomic analyses) (Gao et al. 2019), however, studies are yet to examine uterine contractile responsiveness to nonapeptides across an oviparous-viviparous species pair. Common triggers of labour may also operate in pregnant anamniotes, as several genes seem to play a similar role in parition in seahorses (Whittington et al. 2015b).

In this study, we compare the nonapeptide-induced contractile response of the uterus of oviparous and viviparous species pairs. We hypothesised that different parity modes in skinks would be associated with different levels of uterine contractile responsiveness to nonapeptides between oviparous and viviparous individuals, and that this difference would be reflected in different levels of expression of the genes encoding nonapeptide receptors in the uteri. To test this hypothesis, we compared the contractile responsiveness of uteri to the nonapeptide hormone, arginine vasopressin (AVP), between non-pregnant and pregnant skinks within a species, as well as between oviparous and viviparous individuals (*P. entrecasteauxii* versus *L. guichenoti,* and across bimodal *S. equalis*). Additionally, we quantified oxytocin receptor (*oxtr*)

mRNA abundance in the uteri of *P. entrecasteauxii* and *L. guichenoti* to determine whether *oxtr* expression is higher in pregnant/gravid individuals than in non-pregnant individuals, and whether *oxtr* expression differs between parity modes.

Materials and Methods

Study species and tissue collection

Study species

Lizards were collected under New South Wales National Parks and Wildlife Licence 6L100401. We collected uteri from pregnant/gravid and non-pregnant/non-gravid *P. entrecasteauxii* (Kanangra Boyd National Park, NSW), *L. guichenoti* (University of Sydney, Camperdown campus), viviparous *S. equalis* (Mummel Gulf National Park, NSW) and oviparous *S. equalis* (Sydney, NSW). All procedures were approved by the University of Sydney Animal Ethics Committee (permit number 2016/1039) and the University of Newcastle Animal Care and Ethics Committee (permit number A-2016-620). Animals were housed in cages with conditions appropriate for each species; lizards were fed 3 – 4 small crickets three times per week, provided with water *ad libitum* and received seven hours of heat per day. The lizards were transported to the University of Newcastle for processing when parition was determined to be imminent.

Stages of pregnancy

Embryonic development was determined using the 40-stage protocol of Dufaure and Hubert (Dufaure and Hubert 1961). Viviparous skinks (*P. entrecasteauxii* and *S. equalis*, from Mummel Gulf National Park) give birth at embryonic stage 40 (Smith and Shine 1997) (embryos have fully developed organs, scales and pigmentation). We used pregnant individuals at stages 39-40. Oviparous skinks, such as *L. guichenoti*, generally oviposit at approximately embryonic stage 30 (Qualls and Shine 2000) (embryonic eyes beginning to become pigmented,

stumps of limbs fringed). In our study, we sampled gravid *L. guichenoti* with embryos at stages 25-30. Oviparous *S. equalis* (from Sydney) are long egg-retainers that oviposit at approximately embryonic stage 38 – 39 (Smith and Shine 1997). In our study, we sampled gravid oviparous *S. equalis* with embryos at stage 39. For the non-pregnant samples, we used lizards that were either non-pregnant at capture, or were pregnant at capture, gave birth, and were then held in captivity for at least three weeks post-parition before being processed. Waiting three weeks ensured that the uteri had involuted and returned to the non-pregnant state (Biazik et al. 2007).

Tissue harvesting and processing

Lizards were euthanised by decapitation and pithing, after which the animals were dissected and the two uteri were excised under stereo microscope. Embryos, if present, were excised from the uteri and fixed in 10% neutral-buffered formalin (NBF) for staging (Table 1). For each lizard, the two uteri were collected into different buffers. For the contraction assay, one uterus was immediately placed into Munsick's solution (113 mM NaCl, 6 mM KCl, 0.5 mM $CaCl_2$, 0.5 M $MgCl_2$, 30 mM $NaHCO_3$, 0.8 mM Na_2HPO_4, 0.18 mM NaH_2PO_4, 2.77 mM glucose) (pH 7.4) (Munsick 1960) at room temperature. These uteri were utilised within 60 min to conduct the contraction assays. The other uterus was preserved in RNAlater (Qiagen, Hilden Germany) at 4°C for 24 h (as per the manufacturer's instructions), then stored at -80°C for subsequent RNA extraction. The liver was also preserved in RNAlater for use as a non-reproductive tissue control during real-time quantitative polymerase chain reaction (RT-qPCR) analyses.

Uterine contraction assays

Uterine strips (~1 cm) (*P. entrecasteauxii*: n=8 pregnant, n=7 non-pregnant; *L. guichenoti*: n=7 gravid, n=8 non-gravid) were connected to Grass FT03C force transducers (Grass Instruments,

Quincy, MA) using nylon thread. Each uterine strip was then lowered into a drainable organ bath containing 30 mL of Munsick's solution (pH 7.4), which was continuously gassed with 95% O_2, 5% CO_2. Passive tension (stretch) of 0.5 g was applied to each uterine strip by adjusting the transducer micrometers (0.5 g calibrated to equal 1.0 V) as previously described (Paul et al. 2017; Paul et al. 2011). The Munsick's solution was replaced with fresh solution 3 times at 10 min intervals, with uterine strips re-tensioned to 0.5 g after each wash. The temperature of the organ bath was maintained at the mean selected body temperature for each species: *L. guichenoti*: 33.7 °C (Greer 1989), *P. entrecasteauxii:* 32°C (Greer 1989) and *S. equalis:* 22.1°C (Wu et al. 2009). Mean selected body temperature was chosen as this is the temperature at which the uterus displays maximal sensitivity to nonapeptides (La Pointe 1977). After the final re-tensioning, uterine strips were incubated for a further 1 h to allow spontaneous contractions to develop *ex vivo*. At the mean selected body temperatures and under continual gassing (95% O_2, 5% CO_2), Munsick's pH was 7.6 – 7.7. Data were digitised using a MacLab/8E data-acquisition system and the contractility generated by each uterine strip was visualised in real-time as a contraction trace using LabChart software (ADInstruments, Melbourne, Australia).

Dose-response

Synthetic arginine vasopressin (AVP; amino acid sequence: CYFQNCPRG, NovoPro Biosciences), dissolved in Munsick's solution, was used to elicit a contractile response. AVP has previously been demonstrated to elicit contractions in the oviduct of the viviparous lizard, *Xantusia* (*Klauberina*) *riversiana* (Heller 1969). For each uterine strip, a contraction baseline (measurement of spontaneous contractions) was obtained to serve as a reference for contractile activity prior to AVP administration. To generate dose-response curves, cumulative doses of AVP were injected into the organ baths (Munsick's solution) at final concentrations of 100 pM (10^{-10} M), 1 nM (10^{-9} M), 10 nM (10^{-8} M), 100 nM (10^{-7} M) and 1 μM (10^{-6} M). Tissue

contractile responses were recorded for at least 10 min before the next cumulative dose was applied.

Dose-response statistical analysis

To ascertain the responsiveness of the uteri to AVP, LabChart software was used to calculate the area under the curve (AUC) (*g* tension x seconds) for each contraction trace. Measurement of AUC commenced 3 min after application of each AVP treatment (to allow time for the tissue to respond) and terminated 3 minutes later (i.e. a measurement period of 3 min per dose). AUC was normalised to baseline (baseline = 1.0) and expressed as percentage increase above baseline (% of baseline). As saturation doses were not reached (in terms of stimulating uterine contractility), traditional sigmoidal dose-response curves were not produced. As such, data were checked for normality using Shapiro-Wilks before being fitted with a centred second order polynomial (quadratic) with least squares fitting (non-linear regression). To compare AVP contractile responsiveness across species (reproductive mode) and pregnancy status, a Comparison of Fits was performed to assess whether 3 parameters were the same for both plotted data sets being compared. Data were analysed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA), with p-value <0.05 considered significant.

RT-qPCR

RNA extraction

RNA was extracted from uterine (*P. entrecasteauxii*: n=5 pregnant, n=3 non-pregnant; *L. guichenoti*: n=5 pregnant, n=5 non-pregnant) and liver (*P. entrecasteauxii*: n=5 pregnant, n=3 non-pregnant; *L. guichenoti*: n=3 pregnant, n=4 non-pregnant) samples preserved in RNAlater by homogenising tissue samples in lysis buffer using the steel bead TissueLyser II system (Qiagen, Hilden, Germany) and QiaShredders (Qiagen, Hilden, Germany). Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany), which included an in-

built DNAse treatment. RNA concentration and integrity were assessed using a Bioanalyzer (Agilent, Santa Clara, CA, USA) and only high-quality RNA (RIN >7) was used for qPCR analysis. For each sample, 500 ng of RNA was reverse-transcribed into cDNA then amplified using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) and combined $Oligo_{dT}$ and random hexamer primers, as per the manufacturer's instructions for 20 μL reaction volumes. cDNA was stored at -20˚C.

Primer design and validation

Primers for *oxtr* were designed based upon the *oxtr* sequence of *P. entrecaustauxii*, which were obtained through local BLAST searches of a uterine transcriptome (Griffith et al. 2016) using the *oxtr* sequence of *Anolis carolinensis*. This sequence was aligned with predicted *oxtr* sequences of other non-mammalian amniotes, obtained from NCBI's GenBank (https://www.ncbi.nlm.nih.gov/genbank/, accessed 17/09/2017), including *A. carolinensis* (XM_008117007.2), *Pogona vitticeps* (XM_020791431.1), *Crocodylus porosus* (XM_019536582.1), *Python bivittatus* (XM_007425483.2), *Gekko japonicus* (XM_015405585.1), *Thamnophis sirtalis* (XM_014068477.1), *Alligator mississippiensis* (XM_006272653.3) and *Gallus gallus* (NM_001031569.1), to determine the boundaries of each intron and exon. Primers were then designed using Primer Blast (Ye et al. 2012) to span at least one exon-exon boundary of the *P. entrecasteauxii oxtr* sequence. The Sequence Manipulation Suites PCR Primer Stats software (Stothard 2000) was used to determine the suitability of these primers for qPCR. Any primers that displayed primer dimers (self-annealing) or secondary structures (hairpins) were excluded. The *oxtr* primers were: Sense 5'-TTTCCCGAGTCAGCAGTGTC-3' in Exon 2; 5'-GAGGTGATGACGAACGGCAA-3' in Exon 3. These primers produced a 175 bp amplicon.

Primers were purchased in desalted, powdered form from Sigma Aldrich (St Louis, MO, USA). Each set of primers, including those for the RT-qPCR reference genes outlined below, was

validated by checking for the expected amplicon size in the qPCR product. To do this, PCR products were subjected to gel electrophoresis (100 V for 30 min in 1 % TBE agarose gels) alongside a 100 bp – 1.5 kb DNA ladder as a size standard (Bio Basic, Canada). Gels were stained with SybrSafe (Life Technologies) and visualised using a blue light illumination system (Maestrogen, Las Vegas, NV, USA). For the newly designed *oxtr* primers, the resulting bands were excised and purified using QIAquick Gel Extraction Kit (Qiagen, Hilden Germany). The purified PCR product was then sequenced by dye-termination sequencing at the Australian Genome Research Facility (Sydney, Australia). Resulting partial sequences were aligned and concatenated using BioEdit (Hall 1999) and checked against the expected gene sequence. Genes encoding hypoxanthine phosphoribosyltransferase 1 (*hprt1*) and β-actin (*actb*) were used as the reference genes and have been validated in previous qPCR analyses of lizard uterine tissue across the reproductive cycle (Griffith et al. 2013; Whittington et al. 2015a).

RT-qPCR

Gene expression was quantified by RT-qPCR analyses using the Quantifast SYBR green protocol (Qiagen) and a Rotor-Gene Q machine (Qiagen). PCR reactions were set up manually as 20 μL volumes, with 1.56 ng of cDNA equivalence per reaction for *hprt1* and *actb*, and 25 ng of cDNA equivalence for *oxtr*. The thermal cycling profile started with an enzyme activation step (95°C for 10 min), then 45 cycles of amplification (95°C for 15 sec, 60°C for 30 sec). A melt curve analysis (temperature ramping from 65 – 95°C at 1°C per sec) was carried out at the end of each run to confirm the amplification of a single amplicon (i.e. primer specificity). Samples were run in triplicate and all runs included triplicate no template controls and standards representing a known point on the standard curve, as well as reverse transcriptase negative control reactions in duplicate to confirm that genomic DNA was not being amplified. All reverse transcriptase negative controls with a peak in the melt curve at the expected size were below the linear dynamic range of the assay.

The standard curve for the reference genes, *hprt1* and *actb,* was constructed using a mix of 24 randomly selected liver and uterus cDNA samples across the two skink species. Due to the lower expression of *oxtr* than the reference genes, the *oxtr* standard curve was generated from the serial dilution of PCR products, which is standard procedure for genes or splice variants with low expression (Whittington et al. 2017). All dilutions for each standard curve were run in triplicate. Standard curves had an R^2 value >0.985, contained at least 6 dilutions and had a PCR efficiency within the acceptable range of 0.9 - 1.2 (Table). In each run, samples generated values known as quantification cycles (Cq). Cq values were adjusted for inter-run variation by comparing the known included standard in each run with the relative concentrations calculated by comparing the Cq to the appropriate standard curve.

For each sample, *oxtr* gene expression was normalised using the geometric mean of the expression of the two reference genes (*hprt1* and *actb)*. As there were insufficient quantities of RNAs to allow qPCR of both reference genes for some samples, *oxtr* expression for each sample was also normalised against expression of *hprt1* alone, for which we had expression data for all samples.

Table 1. Characteristics of the standard curves of each gene.

Gene	R^2	Slope	y-intercept	PCR efficiency
oxtr	0.99603	-3.237	13.361	1.04
hprt1	0.99710	-3.020	29.599	1.14
actb	0.99000	-3.318	30.044	1.00

Statistical Analyses

qPCR statistical analysis

Following normalisation to the reference gene(s), uterine and liver *oxtr* expression values were $\log_2$ transformed then checked for normality (Shapiro–Wilk normality test; $p > 0.05$) and equivalent variance among groups (Brown-Forsythe test; $p > 0.05$). For multiple comparisons (*L. guichenoti*: non-gravid vs gravid; *P. entrecasteauxii*: non-pregnant vs pregnant; Non-gravid/non-pregnant: *L. guichenoti* vs *P. entrecasteauxii*; Gravid/pregnant: *L. guichenoti* vs *P. entrecasteauxii*) a one-way analysis of variance (ANOVA), followed by post-hoc test of Sidak multiple comparisons, was performed. *P*-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism 7 software (La Jolla, CA, USA).

Uterine oxtr expression versus contractile responsiveness

For individuals where matched uterine *oxtr* expression and contractile responsiveness to AVP (10^{-6} M) data were available, Pearson correlation (two-tailed) was performed where data were normally distributed, or nonparametric Spearman correlation (two-tailed) was performed where data were not normally distributed (GraphPad Prism). Uterine contractile responsiveness was correlated against (i) pooled *L. guichenoti* samples (non-gravid + gravid individuals), (ii) pooled *P. entrecasteauxii* samples (non-pregnant + pregnant individuals) and (iii) all samples across both species and pregnancy status.

Results

Contractility

Spontaneous contractility was observed in uteri as ongoing irregular contractions of fluctuating amplitude (contraction baseline). Upon treatment with cumulative doses of AVP, enhanced uterine contractility was evident for pregnant and non-pregnant *P. entrecasteauxii* and gravid

L. guichenoti at AVP doses of 10^{-8} M and above, whereas no AVP responsiveness was evident for non-gravid *L. guichenoti* up to the maximum doses of 10^{-6} M (Figure 1).

For bimodal *S. equalis*, AVP contractile responsiveness was evident at AVP doses of 10^{-8} M and above for viviparous pregnant, oviparous gravid and oviparous non-gravid individuals. For viviparous non-pregnant individuals, however, no AVP contractile responsiveness was evident up to the maximum doses of 10^{-6} M (Figure 2).

Pregnant vs Non-Pregnant: For viviparous *P. entrecasteauxii*, pregnant uteri (n=8) were significantly more responsive to AVP than non-pregnant uteri (n=7) ($F_{3,4}$ =76.97, p=0.0005, Figure 3a). A similar response was found for oviparous *L. guichenoti* in that pregnant uteri (n=7) were significantly more responsive to AVP than non-pregnant uteri (n=8) ($F_{3,4}$ =18.15, p=0.0086, Figure 3b).

For viviparous *S. equalis* results were similar, in that pregnant uteri (n=9) were significantly more responsive to AVP than non-pregnant uteri (n=5) ($F_{3,4}$ =38.54, p=0.0021, Figure 3c). For oviparous *S. equalis*, the sample size for pregnant individuals was limited (n=2), however, analysis suggests that pregnant uteri are more responsive to AVP than non-pregnant uteri (n=7) ($F_{3,4}$ =22.01, p=0.006, Figure 3d).

Viviparous vs Oviparous: Uteri from pregnant viviparous *P. entrecasteauxii* (n=8) were significantly more responsive to AVP than uteri from gravid oviparous *L. guichenoti* (n=7) ($F_{3,4}$ =84.41, p=0.0005, Figure 4a). Similarly, uteri from non-pregnant *P. entrecasteauxii* (n=7) were significantly more responsive to AVP than uteri from non-gravid *L. guichenoti* (n=8) ($F_{3,4}$ =67.13, p=0.0007, Figure 4b).

For pregnant/gravid *S. equalis,* the sample size for oviparous pregnant individuals was limited, however, data suggest there is no difference in AVP contractile responsiveness between pregnant viviparous individuals (n=9) and gravid oviparous individuals (n=2) ($F_{3,4}$ =0.5707,

p<0.664, Figure 4c). For non-pregnant/non-gravid *S. equalis*, uteri from oviparous individuals (n=7) were significantly more responsive to AVP than uteri from viviparous individuals (n=5) ($F_{3,4}$ =304.6, p<0.0001, Figure 4d).

Sequencing of *oxtr*

We identified the full sequence of *oxtr* in *P. entrecasteauxii* using BLAST searches of uterine transcriptome (GenBank MK761220). We also identified a partial *oxtr* sequence for *L. guichenoti* using this method. After RT-qPCR, the product was sequenced and yielded a 177 bp region of *oxtr*, representing 58 amino acids of the encoded protein, confirming that the qPCR primers targeted the appropriate gene (Figure 5).

Expression of *oxtr*

Expression of *oxtr* (mRNA abundance) was measured in the uteri (tissue of interest) and livers (control tissue, where no difference was expected) of *P. entrecasteauxii* (pregnant and non-pregnant) and *L. guichenoti* (gravid and non-gravid).There was no significant difference in liver *oxtr* expression across species or pregnancy status, regardless of whether *oxtr* expression was normalised to *hprt1* expression alone (*L. guichenoti*: non-gravid (n=4) vs gravid (n=3) (*p*=0.932); *P. entrecasteauxii*: non-pregnant (n=3) vs pregnant (n=5) (*p*>0.999); Non-gravid/non-pregnant: *L. guichenoti* (n=4) vs *P. entrecasteauxii* (n=3)(*p*=0.993); Gravid/pregnant: *L. guichenoti* (n=3) vs *P. entrecasteauxii* (n=5) (*p*=0.992)) or to the geometric mean of *hprt1* and *actb* expression (*L. guichenoti*: non-gravid (n=4) vs gravid (n=3) (*p*=0.999); *P. entrecasteauxii*: non-pregnant (n=3) vs pregnant (n=5) (*p*>0.984); Non-gravid/non-pregnant: *L. guichenoti* (n=4) vs *P. entrecasteauxii* (n=3)(*p*=0.838); Gravid/pregnant: *L. guichenoti* (n=3) vs *P. entrecasteauxii* (n=5) (*p*=0.998)).

No significant differences were detected for uterine *oxtr* expression between species or pregnancy status when *oxtr* expression was normalised to *hprt1* expression (*L. guichenoti*: non-gravid (n=5) vs gravid (n=5)(p=0.997); *P. entrecasteauxii*: non-pregnant (n=3) vs pregnant (n=5)(p=0.994); Non-gravid/non-pregnant: *L. guichenoti* (n=5) vs *P. entrecasteauxii* (n=3)(p=0.743); Gravid/pregnant: *L. guichenoti* (n=5) vs *P. entrecasteauxii* (n=5)(p=0.560; Figure 6a). Similarly, no significant differences were detected when uterine *oxtr* expression was normalised to the geometric mean of uterine expression for the two reference genes (*hprt1* and *actb*) (*L. guichenoti*: non-gravid (n=5) vs gravid (n=5)(p=0.892); *P. entrecasteauxii*: non-pregnant (n=3) vs pregnant (n=5)(p=0.872); Non-gravid/non-pregnant: *L. guichenoti* (n=5) vs *P. entrecasteauxii* (n=3)(p=0.890); Gravid/pregnant: *L. guichenoti* (n=5) vs *P. entrecasteauxii* (n=5)(p=0.946)). These analyses had approximately 0.8 power (80%) to detect a 4-fold difference in gene expression between non-gravid and gravid *L. guichenoti*, and a 5-fold difference in gene expression between non-pregnant and pregnant *P. entrecasteauxii* (post-hoc power calculation).

Furthermore, no significant correlations were detected upon correlating contractile responsiveness to AVP (AUC at 10^{-6} M dose) against uterine *oxtr* expression for; (i) all *L. guichenoti* samples (pooled non-gravid + gravid individuals; Pearson correlation R=0.1425, p=0.694), (ii) all *P. entrecasteauxii* samples (pooled non-pregnant + pregnant individuals; Pearson correlation R=0.6761, p=0.324) or (iii) all samples across both species and pregnancy status (Spearman correlation R=0.3495, p=0.221) (Figure 6b).

Discussion

We examined uterine contractile responsiveness to AVP across pregnancy status in two independent origins of viviparity: viviparous *P. entrecasteauxii* and oviparous *L. guichenoti*,

as well as vivi- and oviparous (long-egg retaining) populations of bimodal *S. equalis*. In each of the four lizard populations studied, uterine tissue from the pregnant/gravid individuals elicited a significantly greater contractile response to AVP than uterine tissue from non-pregnant/non-gravid individuals (Figure 3). Similar results have been reported in other oviparous and viviparous reptiles. For example, uterine tissue from pregnant viviparous *Liolaemus gravenhorti* (an iguanian lizard) is more sensitive to oxytocin *in vitro* than uterine tissue from non-pregnant females, and oviparous *Liolaemus tenuis* exhibit a similar response, with increased contractility of the uterine tissue in gravid individuals compared to non-gravid individuals (Lemus et al. 1970). However, in viviparous *Tiliqua rugosa*, a skink representing an independent origin of viviparity, the strength of arginine vasotocin (AVT)-induced contractions *in vitro* does not differ between pregnant and non-pregnant individuals (Fergusson and Bradshaw 1992). Rather, spontaneous rhythmic contractions only occur in pregnant individuals, suggesting a qualitatively different response in contractile activity between the reproductive stages (Fergusson and Bradshaw 1992). Such differences between species suggest that the mechanisms underpinning delayed embryo deposition and then parition may be different in independent origins of viviparity.

Comparing across reproductive mode, we found that *P. entrecasteauxii* uteri were significantly more responsive to AVP than *L. guichenoti* uteri during both pregnant and non-pregnant states (Figure 4a and 4b). Both species were sampled at reproductive stages when the uteri should be primed for the contractility required for successful parition. Given that these species are closely related, we speculate that viviparous species may be more reliant on nonapeptide hormones for parition than oviparous species. This hypothesis is supported by the previously outlined study in *Liolaemus sp*, an independent origin of viviparity, which demonstrated that uterine tissue of the viviparous species was more responsive to nonapeptide hormone than uterine tissue from the oviparous species of the pair (Lemus et al. 1970). Furthermore, in the non-pregnant/non-

gravid individuals, uteri from viviparous *P. entrecasteauxii* were again significantly more responsive to AVP than uteri from oviparous *L. guichenoti* (Figure 4b), suggesting that the underlying response of the uterus to nonapeptides is indeed linked to parity mode. Although unlikely, we note that we cannot exclude the possibility that the differences in *P. entrecasteauxii* and *L. guichenoti* contractile response may be the result of lineage-specific change, rather than parity mode differences. Future studies examining additional species pairs will help to address this possibility. Notwithstanding the above caveat, the question remains as to why, within a species pair, viviparous individuals generate greater uterine contractility than oviparous individuals (Figure 4). The reason is currently unclear, however, one speculative explanation is that lizards may experience intrapartum mortality, as many mammals do. If so, increased contractility may shorten the time taken to deliver the neonates and reduce the likelihood of intrapartum death. Such a consideration may not be relevant during oviposition.

Our data for bimodal *S. equalis* represents a slightly different comparison, because oviparous *S. equalis* are long egg-retainers that deposit eggs at a very late stage of development compared to oviparous species, such as *L. guichenoti*. There is a comparatively minor temporal separation of parition in oviparous compared to viviparous *S. equalis* (Smith and Shine 1997). As such, despite displaying different parity modes (viviparous individuals produce neonates enclosed in transparent membranes, while oviparous individuals produce partially shelled eggs, and eggs from oviparous individuals have longer incubation periods), ovi- and viviparous *S. equalis* still undergo labour at similar embryonic stages (Smith and Shine 1997), and there may be facultative switches in parity mode in this species (Laird et al. 2019). These facts seem to be reflected in our contraction data, which suggest there is no difference in AVP contractile responsiveness between vivi- and oviparous individuals (Figure 4c), while for non-pregnant *S. equalis*, uteri from oviparous individuals exhibited greater contractile responsiveness to AVP than uteri from viviparous individuals (Figure 4d). With parition occurring within close

temporal proximity between ovi- and viviparous individuals, the contractile responsiveness of uteri to AVP in pregnant/gravid individuals may be consistent across parity mode when parition is imminent. This hypothesis is consistent with a report that the structure of the uteri from individuals with different parity modes does not differ (Stewart et al. 2010), which is likely attributable to the transition in reproductive mode having occurred quite recently in reproductively bimodal species.

Unexpectedly, we found that for non-pregnant *S. equalis*, uteri from oviparous individuals exhibited greater contractile responsiveness to AVP than viviparous individuals. This finding is in contrast to the results in our *P. entrecasteauxii*/*L. guichenoti* species pair, where viviparous uteri were more responsive to AVP than oviparous uteri. This difference was attributable to an almost complete lack of AVP contractile responsiveness in uteri from non-gravid viviparous *S. equalis* (Figure 2). This result may be due to the fact that viviparous *S. equalis* were processed later in the year than oviparous individuals. At the time of processing, the ovaries of the non-pregnant viviparous *S. equalis* were vitellogenic and had started to develop yolking follicles, in contrast to the non-gravid oviparous *S. equalis*, which did not have yolking follicles. Vitellogenesis causes changes to the hormonal environment in the oviduct (Callard et al. 1978; Edwards and Jones 2001) and is associated with increased levels of progesterone (Moore et al. 1985). Since progesterone is a potent inhibitor of uterine contractions and can reduce the effectiveness of nonapeptide hormones in stimulating contractions (Callard et al. 1992), it is plausible that non-pregnant viviparous *S. equalis* may have had elevated progesterone levels at the time of processing, which may have attenuated the *in vitro* uterine responsiveness to AVP in these individuals.

To understand the differences in AVP contractile responsiveness between pregnancy status and parity mode, we compared *oxtr* expression across *P. entrecasteauxii* and oviparous *L. guichenoti*, for which we had sufficient sample sizes for such analyses. We anticipated that

differing responsiveness to nonapeptide hormones between pregnancy status and parity mode may be attributable to differences in the expression of nonapeptide receptors within the uteri. However, we found that *oxtr* mRNA abundance was not significantly different between viviparous *P. entrecasteauxii* and oviparous *L. guichenoti*, or between non-pregnant/non-gravid and pregnant/gravid individuals (Figure 6a). Furthermore, we found no significant correlation between *oxtr* expression and AVP contractile responsiveness (Figure 6b). In the myometrium of the rat, the abundance of OXTR on uterine myocytes increases across pregnancy and peaks just prior to parturition (Alexandrova and Soloff 1980b), while in the guinea pig, OXTR abundance peaks 9 days prior to parturition (Alexandrova and Soloff 1980a). In contrast, we found that *oxtr* mRNA abundance did not change with skink pregnancy status or parity mode. We note, however, that little is known about nonapeptide receptor expression during pregnancy in non-mammalian vertebrates. Although all nonapeptide receptors can bind to all nonapeptide hormones, the affinity of the receptor-ligand interaction determines the magnitude of the response (Wircer et al. 2016). For example, mesotocin, oxytocin, vasotocin and vasopressin, which are homologous nonapeptides, all cause contractions in uteri from the viviparous lizard, *Xantusia riversiana*, however, AVT was found to be 10 times more potent than oxytocin and 16 times more potent than mesotocin at stimulating contractions (La Pointe 1977). This suggests that OXTR may not be the receptor involved in the contractile response to AVP, thus accounting for the lack of correlation between AVP contractile responsiveness and *oxtr* expression in the skinks examined here. Instead, the contractile mechanisms may rely on receptors for the ancestral nonapeptide, vasotocin, as opposed to the receptor for oxytocin. In reptiles, five nonapeptide receptors have been identified (Ocampo Daza et al. 2012). Future studies should measure the expression of the full suite of nonapeptide receptors to determine which receptors exhibit expression changes across pregnancy and may, therefore, play a role in parition. We note that while changes in *oxtr*

expression do not appear to be the major mechanism underpinning parity mode differences in the timing of labour in these species, this receptor may still play a role in the initiation or maintenance of parition in combination with other mechanisms.

It is also possible that labour in the species examined here is triggered by changes in plasma nonapeptide hormone concentration, rather than expression changes in any nonapeptide receptors. Across vertebrates, an increase in plasma nonapeptide hormone concentration occurs towards the end of pregnancy. For example, circulating plasma AVT concentration in pregnant *T. rugosa* increases 30 days prior to parturition (Fergusson and Bradshaw 1991), and plasma AVT concentration increases in sea turtles at the time of oviposition (Figler et al. 1989). Moreover, oxytocin uterine plasma concentrations rise at some stage during the process of labour in all eutherian mammals (Blanks and Thornton 2003). In our contraction assays, increasing nonapeptide concentration was associated with greater uterine contractility, and so it is plausible that changes in AVT concentration, but not receptor abundance, mediate labour in these species. To examine this hypothesis, future studies should track plasma nonapeptide levels across pregnancy in both species to confirm whether a significant change in AVT concentration triggers labour, and whether there is a difference in AVT concentration and the timing of its release between oviparous and viviparous animals.

Conclusions

This study is the first to compare the uterine responsiveness to AVP of closely related reptiles with differing parity modes and to relate the outcome to the expression of a nonapeptide hormone receptor. Consistent with previously studied vertebrates, pregnant/gravid uteri from *P. entrecasteauxii*, *L. guichenoti* and *S. equalis* exhibit greater contractile responsiveness to AVP than non-pregnant uteri. Where there is a significant temporal separation of oviposition and birth between the species pairs, uteri from viviparous *P. entrecasteauxii* had a greater contractile response to the nonapeptide hormone than oviparous *L. guichenoti*, consistent with

findings in iguanian lizards representing an independent origin of viviparity (Lemus et al. 1970). However, in a long egg-retaining bimodal species, where oviposition and birth are in close temporal proximity, there was no difference in contractile responsiveness to the nonapeptide when parition was imminent. Finally, for *P. entrecasteauxii* and *L. guichenoti*, the expression of the nonapeptide receptor *oxtr* did not differ with pregnancy status or parity mode, suggesting that in these skinks, parition may be triggered by either increasing concentrations of nonapeptide hormones or by upregulated expression of one of the other nonapeptide receptors. Future studies should focus on measuring plasma hormone concentrations in all 3 species across parity modes as pregnancy progresses, as well as measuring the expression of the full suite of nonapeptide receptors in the uterus. Comparative studies examining ovi- and viviparous individuals from another bimodal species, such as *Lerista bougainvilli* (Qualla et al. 1995), would also be particularly valuable.

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Figure Legends

Figure 1. Representative traces of *ex vivo* uterine contractility recorded for *P. entrecasteauxii* and *L. guichenoti* during AVP dose-response studies. Examples of contraction traces recorded for a) pregnant *P. entrecasteauxii* (n=8), b) non-pregnant *P. entrecasteauxii* (n=7), c) gravid *L. guichenoti* (n=7) and d) non-gravid *L. guichenoti* (n=8). All traces are displayed with consistent ranges (tension) of 1.5 g. Dotted red lines indicate the points at which cumulative AVP treatments were added to the organ baths.

Figure 2. Representative traces of *ex vivo* uterine contractility recorded for viviparous and oviparous *S. equalis* during AVP dose-response studies. Examples of contraction traces recorded for a) viviparous pregnant *S. equalis* (n=9), b) viviparous non-pregnant *S. equalis* (n=5), c) oviparous gravid *S. equalis* (n=2) and d) oviparous non-gravid *S. equalis* (n=5). All traces are displayed with consistent ranges (tension) of 1.5 g. Dotted red lines indicate the points at which cumulative AVP treatments were added to the organ baths.

Figure 3. Effect of pregnancy status on uterine contractile responsiveness to AVP. a) *Pseudemoia entrecasteauxii:* ■ pregnant (n=8) and □ non-pregnant (n=7), b) *L. guichenoti:* ● gravid (n=7) and ○ non-gravid (n=8), c) Viviparous *S. equalis:* ▲ pregnant (n=9) and △ non-pregnant (n=5), d) Oviparous *S. equalis:* ◆ gravid (n=2) and ◇ non-gravid (n=7). Contraction responses (area under curve) were normalised to the pre-treatment baseline and expressed as percentage increase above the baseline. Dose-response curves (centred second-order polynomial) were compared by 3-parameter Comparison of Fit. Data are mean ± SEM. Error bars are not visible for some points due to being shorter than the height of the symbol. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. Comparison of uterine contractile responsiveness to AVP between species pairs with differing parity mode. a) ■ pregnant viviparous *P. entrecasteauxii* (n=8) and ● gravid oviparous *L. guichenoti* (n=7); b) □ non-pregnant viviparous *P. entrecasteauxii* (n=7) and ○ non-gravid oviparous *L. guichenoti* (n=8); c) ▲ pregnant viviparous *S. equalis* (n=9) and ◆ gravid oviparous *S. equalis* (n=2); d) △ non-pregnant viviparous *S. equalis* (n=5) and ◇ non-gravid oviparous *S. equalis* (n=7). Contraction responses (area under curve) were normalised to the pre-treatment baseline and expressed as percentage increase above the baseline. Dose-response curves (centred second order polynomial) were compared by 3-parameter Comparison of Fit. Data are mean ± SEM. Error bars are not visible for some points due to being shorter than the height of the symbol. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. Alignment of OXTR amino acid sequences. The newly identified *P. entrecasteauxii* OXTR amino acid sequence, confirmed by PCR and Sanger sequencing (deposited in GenBank, accession MK761220), and fragmented *L. guichenoti* sequences. 'X' indicates missing sequence (an artefact of transcriptome sequencing and assembly). Sequence accession numbers are: *Gekko japonicus* (XP_015261071.1), *Pogona vitticeps* (XP_020647090.1), *Python bivittatus* (XP_007425545.1), *Thamnophis sirtalis* (XP_013923952.1), *Crocodylus porosus* (XP_019392127.1), *Alligator mississippiensis* (XP_006272715.1), *Danio rerio* (NP_001186299.1), *Gallus gallus* (NP_001026740.1), *Homo sapiens* (NP_000907.2), *Mus musculus* (NP_001074616.1) and *Anolis carolinensis* (XP_016851486.1). Alignment generated using T-Coffee v11.00 (Di Tommaso et al. 2011; Notredame et al. 2000) and BOXSHADE v3.21. (Hofmann and Baron 2019).

Figure 6. Uterine expression of *oxtr* across species pair (*P. entrecasteauxii* and *L. guichenoti*) and pregnancy status. a) Expression of *oxtr* in uteri from *L. guichenoti* (non-gravid, n=5; gravid, n=5) and *P. entrecasteauxii* (non-pregnant, n=3; pregnant, n=5), normalised to *hprt1* uterine expression. b) Uterine contractile responsiveness to AVP (at 10^{-6} M) against uterine *oxtr* expression for all samples where matched uterine *oxtr* expression and contractile responsiveness data were available (*L. guichenoti:* non-gravid, n=5; gravid, n=5; *P. entrecasteauxii:* non-pregnant, n=2; pregnant, n=2). Data in panel (a) are mean ± SEM. One-way ANOVA with multiple comparisons was performed; no significant differences were detected. Panel (b) is an XY plot (regression line not applicable).