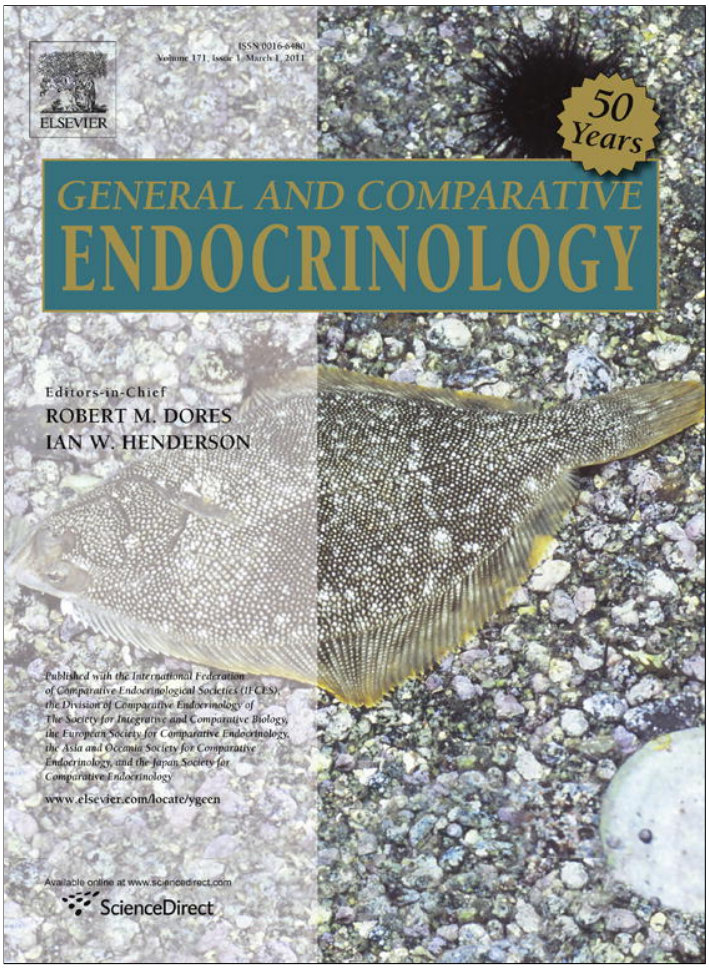

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Molecular characterization and brain distribution of the progesterone receptor in whiptail lizards

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ABSTRACT

Progesterone and its nuclear receptor are critical in modulating reproductive physiology and behavior in female and male vertebrates. Whiptail lizards (genus *Cnemidophorus*) are an excellent model system in which to study the evolution of sexual behavior, as both the ancestral and descendent species exist. Male-typical sexual behavior is mediated by progesterone in both the ancestral species and the descendant all-female species, although the molecular characterization and distribution of the progesterone receptor protein throughout the reptilian brain is not well understood. To better understand the gene targets and ligand binding properties of the progesterone receptor in whiptails, we cloned the promoter and coding sequence of the progesterone receptor and analyzed the predicted protein structure. We next determined the distribution of the progesterone receptor protein and mRNA throughout the brain of *Cnemidophorus inornatus* and *Cnemidophorus uniparens* by immunohistochemistry and *in situ* hybridization. We found the progesterone receptor to be present in many brain regions known to regulate social behavior and processing of stimulus salience across many vertebrates, including the ventral tegmental area, amygdala, nucleus accumbens and several hypothalamic nuclei. Additionally, we quantified immunoreactive cells in the preoptic area and ventromedial hypothalamus in females of both species and males of the ancestral species. We found differences between both species and across ovarian states. Our results significantly extend our understanding of progesterone modulation in the reptilian brain and support the important role of the nuclear progesterone receptor in modulating sexual behavior in reptiles and across vertebrates.

1. Introduction

An individual's ability to produce an adaptive behavioral response requires the integration of external stimuli and internal physiological cues into a molecular signal processed by brain neural networks into gene expression changes. Steroid hormones are excellent candidates for mediating this information processing through altering neural circuit function and properties [4,5,33]. Since classical steroid hormone receptors act as transcription regulators, these pathways are good candidates for integrating social signals into changes in the transcriptome, which is a key channel for affecting such long-term modifications in behavior [3,9,25,44,43].

Progesterone has been found to regulate a diverse array of social behavior, such as male and female sexual behavior, parental behavior, and aggression in many vertebrates [11,17,27,60,67,71]. Despite a research bias towards investigating the role of progesterone in females, substantial evidence indicates that progesterone and its receptor are also involved in the regulation of sexual behavior in males [67,71]. Work in both reptiles and rats reveal that exogenous progesterone at physiological levels stimulate copulatory behavior in castrated males [13,36,35,34,71,72,76]. Testosterone and progesterone can also synergize in promoting male-typical sexual behavior [36,35,71,72,76], in a similar manner to estradiol and progesterone in stimulating female-like sexual behavior in rats [53]. Although progesterone is important in modulating social behavior across vertebrates, relatively little is known about the functional biochemistry and distribution of the progesterone receptor in non-mammalian vertebrates.

Whiptail lizards (genus *Cnemidophorus*) consist of both an ancestral and descendant species. The ancestral *Cnemidophorus inornatus* species consists of both males and females that perform sex-specific roles in mating behavior. The hormonal regulation of these behaviors and downstream gene expression has been extensively studied [11]. Castrated males do not mount or copulate with females, but will do so after androgen treatment [34], and some

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List of Abbreviations

AC	anterior commissure
ACC	nucleus accumbens
AH	anterior hypothalamus
AMC	nucleus centralis amygdalae
AME	nucleus externus amygdalae
AML	nucleus lateralis amygdalae
BST	bed nucleus of the stria terminalis
Cb	cerebellum
CG	central grey
CXD	cortex dorsalis
CXL	cortex lateralis
CXM	cortex medialis
DH	nucleus dorsalis hypothalami
DL	nucleus dorsolateralis anterior
DM	nucleus dorsomedialis
DVR	dorsal ventricular ridge
I	nucleus interstitialis
lb	longitudinal bundle
LCo	locus coeruleus
LFB	lateral forebrain bundle
LHA	lateral hypothalamic area
Ls	lateral septal nucleus
LPA	lateral preoptic area
LTP	lentiformis thalami pars plicta
MAM	mammillary nucleus
mPOA	medial preoptic area
NS	nucleus sphericus
NSA	nucleus sphericus anterior
NSL	nucleus sphericus lateralis
NSM	nucleus sphericus medialis
OC	optic chiasm
OT	optic tract
PC	posterior commissure
PH	nucleus periventricularis hypothalami
PR	nucleus premammillaris
pvPOA	nucleus periventricularis preopticus
R	raphe nucleus
SC	nucleus suprachiasmaticus
SO	nucleus supraopticus
STR	striatum
SUM	supramammillary nucleus
TECT	optic tectum
Tor	torus semicircularis
VE	ventricular ependymal organ
VMH	nucleus ventromedialis hypothalami
VP	ventral pallidum
VTA	ventral tegmental area

also respond to exogenous progesterone with both mounting and copulation [35,34]. Female *C. inornatus* are receptive to a mounting male during an increase in circulating estradiol during the preovulatory (follicular) phase. Progesterone levels, which are low during most of the preovulatory (PreOv) phase, are at their highest postovulation (PostOv) when females are not receptive to males. Androgens are not detectable in the circulation at any time of the ovarian cycle [40].

The all-female whiptail lizard, *Cnemidophorus uniparens*, consists of clonal descendants [8], and displays both male-like mounting behavior and female-like receptivity during discrete phases of the ovarian cycle [12,41,42]. A PostOv 'male-like' lizard will mount and pseudocopulate with a PreOv individual. The pattern of circulating steroid hormones in the unisexual species is similar to that of female *C. inornatus* with the exception of lower estradiol levels [40,41,42,74]. Like in *C. inornatus* females, androgens are not detectable at any time of the ovarian cycle in *C. uniparens* [40].

Progesterone plays a unique role in whiptail lizards depending on species, sex, and individual. In female whiptails, there are species differences in the role progesterone plays in mediating sexual behavior. Female *C. inornatus* will show decreased receptivity when given progesterone, but do not display male-typical mounting behavior [20]. On the other hand, *C. uniparens* individuals show male-typical mounting behavior during the progesterone surge in the ovarian cycle and will display mounting behavior when given exogenous progesterone [23,41,42]. Interestingly, this male-typical behavioral response in the all-female descendant species to progesterone has an ancestral antecedent. In *C. inornatus* males, there is individual variation in response to progesterone administration after castration, as some males will respond with mounting behavior while others will not. This effect of progesterone in males is mimicked by R5020, a non-metabolizable PR agonist, suggesting that progestins mediate this behavior at the level of PR rather than via progestin metabolites such as androgens [35]. Although the distribution of PR mRNA has been described in a few forebrain and hypothalamic regions [77], the understanding of how and where PR acts in the brain to modulate these behaviors in whiptail is not well understood.

Neural networks, rather than genes, modulate behavior. Work in reptiles, mammals, birds and teleosts suggest that there are two neural networks that encode the salience of (social) stimuli and/or regulate social behavior. First, the neural substrates of social behavior in mammals have been proposed by Newman [47] to form a "social behavior network". The six nodes of this network are reciprocally connected, are involved in multiple forms of social behavior, and – by definition – contain sex steroid hormone receptors. This framework has since been expanded to reptiles, birds, and teleosts [10,22]. Secondly, many studies indicate that the mesolimbic "reward" system (including the mid-brain dopaminergic system) is the neural network where the evaluation of stimulus salience takes place [14,69,75]. Although well studied in mammals, identifying brain regions involved in the dopaminergic reward system and Newman's social behavior network in other amniotes remains a challenge, especially regarding the basal ganglia and amygdaloid complex [7]. However, a consensus is emerging from neurochemical, hodological, lesion/stimulation and developmental studies that provide support for putative homologies for most of the relevant areas in the reptilian brain [7,48,62,64,63]. The combination of these two neural networks can be used as a framework for understanding the neural underpinnings of sexual behavior in reptiles and in other vertebrates.

The main aim of this study is to test the hypothesis that PR protein is present in brain regions important for the regulation of social behavior and evaluation of stimulus salience. Towards this aim, we chose to first describe the molecular characteristics of the whiptail PR including analysis of sequence, promoter, and structure, because this information will give us a better understanding of how PR is regulated and how the receptor itself functions. We then determined the distribution of PR mRNA and protein in the brain of two species of whiptail lizards, *C. inornatus* and *C. uniparens*. As progesterone plays an important role in sexual behavior in both species, we quantified cells immunoreactive to PR

in males and females of both species, as species comparisons in this genus offer a unique opportunity to understand the evolution of sexual behavior.

2. Materials and methods

2.1. Animals

Adult *C. inornatus* were captured in the vicinity of Sanderson, TX in May 2008 and transported to the University of Texas at Austin campus, where they were individually housed in environmentally controlled chambers in terraria with *ad libitum* water and food in the form of crickets as in Lindzey and Crews [34]. Adult *C. uniparens* were captured in the vicinity of Portal, Arizona in June 2008, transported to the University of Texas at Austin and housed in environmentally controlled chambers as described previously [73].

Where possible, we adopted the neuroanatomical nomenclature of Young et al. [77]. We followed the nomenclature of Font et al. [16] for septal nuclei, and Smeets [61] for the mid-brain and hindbrain. All procedures were approved by the University of Texas IACUC.

2.2. Behavioral tests

2.2.1. Male C. inornatus

After one to two weeks to acclimatize to the laboratory environment, sexually active males were identified by successfully mounting a receptive female in at least three out of five ten-minute tests as previously described [58]. Only sexually active males were used for this study ($n = 5$).

2.2.2. C. uniparens and C. inornatus females

Female preovulatory (PreOv) and postovulatory (PostOv) ovarian states were determined as previously described [6] using abdominal palpation. All PreOv animals were characterized by the presence of developing follicles and receptive behavior. PostOv animals had corpora lutea and were not receptive. Follicles were measured post-mortem and were significantly larger in PostOv females (*t*-test: *C. uniparens*: $t = 10.8$, df = 26, $p = 4.1 \times 10^{-11}$; *C. inornatus*: $n = 12$; $t = 15.8$, df = 19, $p = 2.2 \times 10^{-12}$). The PostOv follicle size mean was 1.42 cm ± 0.05 (SE) in *C. uniparens* ($n = 16$) and 1.32 cm ± 0.04 (SE) in *C. inornatus* ($n = 12$). The mean follicle size for PreOv females was 0.49 cm ± 0.07 (SE) in *C. uniparens* ($n = 12$) and 0.37 cm ± 0.05 (SE) in *C. inornatus* ($n = 9$).

2.3. Cloning the full length Cnemidophorus progesterone receptor mRNA

A radioactive subclone of a 1045 bp *C. uniparens* PR sequence (Genbank S79937) was prepared by PCR in the presence of 32-P labeled dCTP. This transcript was used to probe the cDNA library prepared from pooled cDNA from whole brains of *Cnemidophorus* lizards of both sexes by a commercial company (Lambda ZAP II, Stratagene, La Jolla, CA, USA). Plaque-lifts and probing were performed according to the library manufacturer's instructions, yielding an 1832-bp fragment. This fragment was sequenced on both strands beginning with primers matching the M13 sites present in the cloning vector, and then using internal primers designed from the resulting sequence. The remainder of the full length sequence corresponding to the 5′ and 3′ end of the coding region and the 5′ and 3′ untranslated region was cloned by RACE (Clontech, Palo Alto, CA, USA) into the pCRII-TOPO vector (Invitrogen) according to the manufacturer's instructions (See Table 1 for primer sequences). The additional fragment was sequenced on both strands using M13 primers, and the entire sequence submitted to GenBank (accession number FJ425273).

Table 1
Primers used for cloning the PR coding and promoter sequences.

Primer name	Primer sequence
5′RACE outer primer	5′- GGGAAAGACGAGCGGCTGGTAATG
5′RACE nested primer	5′- GTTCTTGGGCTGGGGGAGAGCAG
3′RACE outer primer	5′- CCCACAGTCCTTCCCAATGAACACC
3′RACE nested primer	5′- CATTGGGTTGCGGGCGAAGGGAG
Promoter outer primer	5′- GCTCTTGTTTGCTCCTGGACACAGTATTTG
Promoter nested primer	5′- AGCCGTTTTAATGATGCCTTGTCAGTCAG

2.4. PR sequence and phylogenetic analysis

The whiptail progesterone receptor translated protein sequence based on the gene sequence was compared with 9 additional progesterone receptor sequences and aligned using ClustalW in Mega 4 (Mega http://www.megasoftware.net/m_con_select.html). Phylogenetic trees were generated using the Mega4 program and included PR protein sequences of multiple species; the human androgen receptor was included as an outgroup. For comparative purposes, we used the following species and Genbank accession numbers: *H. sapiens* AR NP_000035; *H. sapiens* PR NP_000917; *M. musculus* PR NP_032855; *D. rerio* PR XP_001923769; *G. gallus* PR NP_990593; *O. cuniculus* PR NP_001075736, *X. laevis* PR NP_001079100; *P. nelsoni* PR BAF91193; *A. mississippiensis* PR BAD08350; *C. siamensis* PR AAB81722.

2.5. PR structural homology analysis

We used Swiss-Model's online automated protein structure homology modeling server [1,30,52] to predict the structure of the whiptail PR. The structure search predicted a match to the human PR (2w8y) with an *E*-value of 2.30e-114. The predicted model was visualized and overlayed with the human PR for comparison using Pymol [15].

2.6. PR promoter cloning and analysis

Genomic DNA from whiptail livers was isolated by phenol/chloroform extraction. The whiptail PR promoter was cloned using GenomeWalker (Clontech, Palo Alto, CA, USA) following the manufacturer's instructions. The promoter sequence was submitted to Genbank (GU206828). Putative steroid hormone receptor binding sites were identified using MatInspector (Genomatix). The PR promoter was scanned for putative CpG islands using Emboss CpGPlot (http://emboss.bioinformatics.nl/cgi-bin/emboss/cpgplot).

2.7. PR immunohistochemistry (IHC)

Brains were fixed in 4% paraformaldehyde in PBS for 24 h, cryoprotected overnight in 30% sucrose, embedded in OCT (Tissue-Tek), and stored in −80 °C for 2–6 weeks before sectioning. Brains were sectioned at 30 μm onto Super-Frost Plus slides (Fisher Scientific, Itasca, IL) and stored at −80 °C until processing for immunohistochemistry as previously described [50]. Briefly, slides were dried and then rinsed in PBS, and incubated in 3% hydrogen peroxide in PBS for 20 min. Antigen retrieval was performed by incubating in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). After 2 min, the boiling citrate buffer was replaced two times and incubated for 5 min each, followed by two PBS washes. After blocking for 1 h in blocking solution (5% normal goat serum and 0.3% TritonX-100 in PBS), sections were incubated in primary antibody (PR 1:500, abcam 2767, monoclonal antibody raised against

chicken PR) in PBS with 2% normal goat serum and 0.3% Triton-X at room temperature overnight. Sections were then rinsed, incubated for 2 h in a biotinylated goat anti-mouse secondary antibody (1:200, Vector Laboratories), rinsed again and, after treatment with the ABC peroxidase staining kit (Vector Laboratories) according to the manufacturer's instructions, immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) substrate (Vector Labs). Sections were then dehydrated and cover-slipped with Permount (Fisher Scientific, Itasca, IL). For control sections, all procedures were the same except that primary antibody is omitted. This antibody has been confirmed to bind the appropriate lizard antigens by western blot in O'Connell et al. [50].

2.8. PR in situ hybridization (ISH)

Brains of *C. uniparens* ($n = 6$) were removed, embedded in OCT and stored at −80 °C until sectioning. Brains were then sectioned on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) in three series. Riboprobes were reverse-transcribed in the presence of Fluorescein-labeled UTP (Roche, Indianapolis, IN) using a T7/SP6 Maxiscript in vitro transcription kit (Ambion, Austin, TX) to produce anti-sense or sense Fluorescein-labeled riboprobes. The template used to make the PR probe was the same as in Young et al. [77]. Sections were fixed and processed for *in situ* hybridization staining for mRNA as previously described [50]. Briefly, Slides were pre-equilibrated in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt's solution, 125 mg/mL Baker's yeast tRNA, 250 mg/mL denatured herring sperm DNA) for 2 h at 60 °C and then incubated in riboprobe overnight at the same temperature. Experimental slides were exposed to anti-sense Fluorescein-labeled probe, whereas control slides were incubated with sense Fluorescein-labeled probe. After RNase A treatment at 37 °C, sections were washed in a decreasing series of SSC and equilibrated in 150 mM NaCl/100 mM Tris (pH 7.5) at room temperature before incubation in 1:1000 anti-Fluorescein-alkaline phosphatase Fab fragments (Roche) in 0.5% Tween 20/PBS for 2 h at room temperature. Sections were then washed in 100 mM Tris (pH 7.5). Chromogenic product was formed using BM Purple (Roche) at room temperature until desired darkness was achieved and was terminated simultaneously for all slides. The sections were then dehydrated, delipidated, and coversliped under Permount (Fisher).

2.9. Photomicroscopy

Brightfield optics were used to visualize ISH and IHC staining throughout the brain at low (5×) and high magnification (20×). Photographs were taken with a digital camera (AxioCam MRc, Zeiss) attached to a Zeiss AxioImager.A1 AX10 microscope using the AxioVision (Zeiss) image acquisition and processing software. Images were compiled and brightness- and contrast-enhanced in Adobe Photoshop CS2.

2.10. Cell counting

Cells labeled by PR IHC were counted using the Fractionator routine of the Stereo Investigator software package (Microbrightfield, Williston, VT, USA) as in Sanderson et al. [58]. A hypothalamic region of interest was defined under low power (4×), and then, under higher magnification (20×), positive cells were counted that fell within 75-μm-square counting frames. The computer placed counting frames systematically every 80 μm (i.e. with 5 μm between each counting frame) within the region, after a randomly chosen start-site. Cell nuclei containing PR protein were clearly marked by dark brown staining. For each brain region, reported cell numbers are calculated as the average number of positive cells per counting frame. Slides were coded and processed by a blinded observer.

2.11. Statistical analysis

All statistical analysis was conducted using SPSS for Mac with significance set at $p < 0.05$. As PR cell counts were normally distributed, a 2-way ANOVA was conducted using cell number as dependent variable and with both species and reproductive state (PreOv, PostOv or male) as independent variables.

3. Results

3.1. Analysis of the PR gene sequence and promoter region

We cloned the full coding region of the whiptail PR, and the predicted amino acid sequence was compared to other vertebrate PR protein sequences and compiled in a phylogenetic tree (Fig. 1A), which demonstrates that whiptail PR is very similar to turtle PR and is at the node in the tree where mammals and other amniotes form different clades. We also compared the similarity of the different whiptail PR domains to the turtle, chicken, human, and mouse sequences (Fig. 1B). We found that the DNA-binding domain (DBD) and ligand-binding domain (LBD) sequences are highly conserved across these vertebrates, while the transactivation domains and hinge region are less conserved.

To examine the potential regulatory elements governing expression of PR, we cloned the promoter region of PR and analyzed the sequence for CpG islands and putative transcription factor binding sites. We found a putative CpG island along the translational start site (Fig. 2), suggesting that PR could be under epigenetic control depending on methylation patterns. We also screened the promoter for potential regulation by steroid hormone receptors. We found putative sites for estrogen, androgen, progestin, and corticoid regulation of PR expression (Table 2), suggesting that PR expression can be modulated by all these hormone receptors directly.

3.2. Structural analysis of PR

We analyzed the predicted protein structure of the whiptail PR protein using SWISS-MODEL, and compared it to human PR (Fig. 3). The ligand-binding domain (LBD) is highly conserved (>80% identity) between whiptail and human (Fig. 3A). The amino acids that form the hydrogen bond network essential for specific recognition of the 3-keto group on the A-ring of progesterone (instead of the 3-hydroxyl group on the A-ring of estradiol) are entirely conserved [70]. Fifteen of the sixteen amino acids whose packing defines the hormone-binding pocket are also predicted to be identical. The only striking difference in the LBD is the replacement of G722 with a cysteine, which may sterically occlude the binding of RU486 [70], a PR antagonist.

The whiptail DNA-binding domain (DBD) is also highly conserved (>95% identity) (Fig. 3B), with complete conservation of the amino acids responsible for DNA binding suggesting the whiptail PR binds similar promoter motifs compared to human PR.

3.3. Neural distribution of PR

The distribution of PR mRNA and protein in the brain of whiptail lizards, along with photomicrographs of representative brain areas is depicted in Figs. 4 and 5. As we did not observe any brain regions that had mRNA but not protein (and vice versa), we conclude that protein and mRNA distribution show high concordance. For each representative section of the map, the nomenclature is

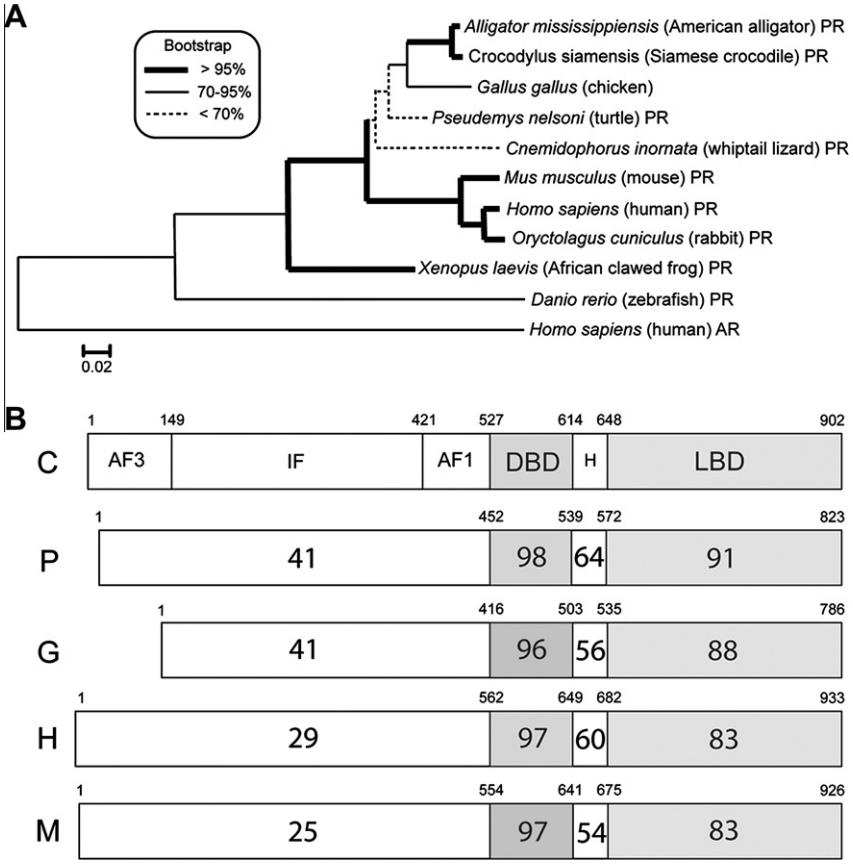

Fig. 1. Analysis of the PR coding region. (A) A phylogenetic tree with neighbor-joining clustering shows the whiptail PR compared to the PR protein sequences in other vertebrates. (B) The *Cnemidophorus* progesterone receptor protein sequence was compared to turtle (P), chicken (G), human (H), and mouse (M). Numbers indicate the percent similarity within each domain: third transactivation domain (AF3), inhibitory function (IF), first transactivation domain (AF1), DNA-binding domain (DBD), hinge domain (H), and the ligand-binding domain (LBD).

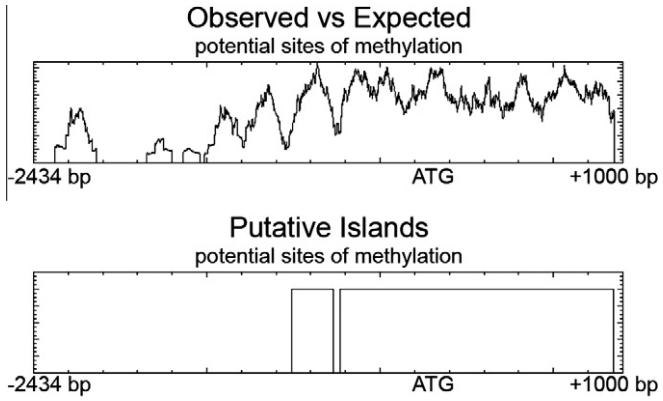

Fig. 2. Analysis of potential methylation sites in the PR promoter region. Top panel illustrates regions of the PR promoter that has potential sites of methylation by measured the observed/expected number of CpG repeats. The bottom panel represents location of putative CpG islands.

displayed on the right side while mRNA and protein signal as determined by *in situ* hybridization and immunohistochemistry is represented by dots on the left side. The density of dots representing PR mRNA and protein indicates qualitatively the density of cells positive for PR. The general distribution pattern shown here is representative of both *C. uniparens* and *C. inornatus* males and females, as there were no qualitative differences (total absence or presence) between species or sex. Control slides that omitted primary antibody for immunohistochemistry showed no signal, and control slides for the *in situ* hybridization that included pretreatment with RNAse A or incubation with the sense riboprobe showed no signal.

3.3.1. Forebrain

PR mRNA and protein is widely distributed throughout the forebrain of whiptail lizards. There is an abundance of PR in the medial and dorsal cortex (CXM and CXD, respectively; Fig. 4A). There is also PR mRNA and protein in the dorsal ventricular ridge (DVR;

Table 2
Putative steroid hormone receptor binding sites in the whiptail PR promoter.

	Location relative to ATG	Within CpG island
ER	-2401 (5′-CTAGGTCAATAGTACTTGT), -1448 (5′-CTAGCCCAAGGTCACCCAG)	No
GR	-1502 (5′-ACAGAACACCCTGTGATGT),+100 (3′-CAGGGACAGATCGATCGCC)	Yes
PR	-2396 (5′-TCAATAGTACTTGTTCTCA), -1924 (3′-TGAGGACTGTGTCTTCTGT)	No
AR	-2129 (5′-TTGGAGCGTTCTGGTCTGT), -1108 (3′-TTGGAGCGCTTCGTTCTTT)	Yes

Putative steroid hormone binding sites are shown with its location relative to the ATG start site and the putative binding sequence in parentheses. Location of the putative response elements in a CpG island is noted in the last column.

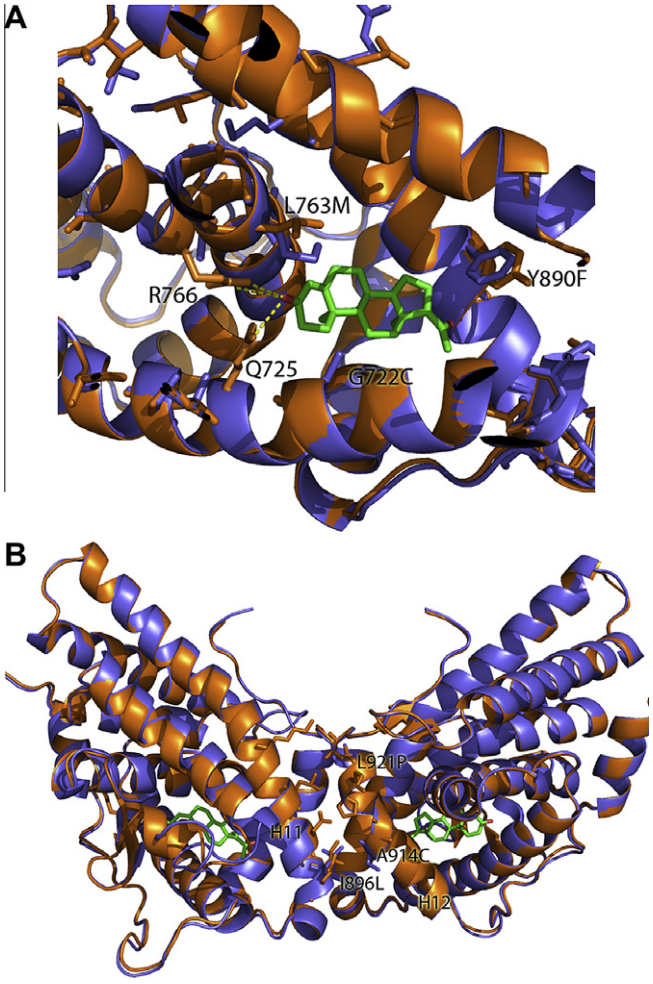

Fig. 3. PR structure analysis. (A) An overlay of the backbone of human (orange) and whiptail PR (blue) side chains for residues that differ. Residues 90 through 103 are hidden to reveal the hormone-binding pocket. Polar bonds between the 3-oxy group of progesterone (green) and the conserved R766 and Q725 are shown in yellow. (B) Dimerization domains of the PR LBD. Side chains for residues involved in dimerization are shown along H11 or H12 for each half of the dimer. (Pymol, PDB: 1a28). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4A1–2) and along the ventral portion of the subpallium. Further caudal as the basal ganglia emerge, PR is present in the striatum (STR), ventral pallidum (VP), and nucleus accumbens (ACC; Fig. 4B1–2). Dorsal of the basal ganglia, there is PR mRNA and protein in the anterior sphericus nucleus (NSA) and lateral septum (Ls). More caudally, the anterior sphericus nucleus splits into the lateral and medial sphericus nucleus (NSL and NSM, respectively), both of which contain cells immunoreactive for PR protein and mRNA (Fig. 4C). The third ventricle begins to appear at this level of the brain. The reptilian homologue of the rat anteroventral periventricular nucleus, the periventricular preoptic area (pvPOA), and the medial preoptic area (mPOA) surround the third ventricle and contain an abundance of PR mRNA and protein (Fig. 4C1–2). PR mRNA and protein is also present in the externus amygdala (AME; Fig. 4D1–2) and cells that appear to wrap around the lateral forebrain bundle (LFB) also contain PR mRNA and protein. Further caudal, the central amygdala (AMC) emerges and contains PR mRNA and protein (Fig. 4D). Dorsolateral to the central amygdala, the sphericus nucleus (NS) also contains cell immunoreactive to PR. The lateral preoptic area (LPA), which is lateral to the mPOA, does not contain PR protein or mRNA. More caudally, the bed nucleus of the stria terminalis (BST) emerges between the anterior commissure (AC) and lateral forebrain bundle (LFB) and contains an abundance of PR mRNA and protein. The suprachiasmatic nucleus (SC), which is ventral to the mPOA, also contains PR expression of mRNA and protein.

PR is less abundant in the diencephalon compared to the telencephalon (Fig. 5). PR is present within the lateral hypothalamic area (LHA) and the dorsomedial nucleus (DM; Fig. 5A). More caudally, PR is abundant in the ventromedial hypothalamus (VMH; Fig. 5A1–2) and periventricular hypothalamic nucleus (PH), as well as the dorsal hypothalamus (DH) and more sparsely in the lentiformis thalami pars plicta (LTP, Fig. 5B).

3.3.2. Mid-brain and hindbrain

The distribution of PR protein was further investigated in the mid-brain and hindbrain. PR protein is abundant within the optic tectum (TECT). PR protein is also present in the torus semicircularis (Tor), supramammillary nucleus (SUM), and the mammillary nucleus (MAM; Fig. 5C). More caudally, PR protein is present along the longitudinal bundle (lb) and the ventral tegmental area (VTA, Fig. 5D). Finally in the hindbrain, PR protein is present within the raphe nucleus (R), central grey (CG), locus coeruleus (LCo), and cerebellum (cb; Fig. 5E).

3.4. Species differences in PR immunoreactivity

In order to determine how the number of cells expressing PR varied between species, sex, and ovarian state, we quantified PR-immunoreactivity in the mPOA, pvPOA, and VMH in *C. uniparens* and *C. inornatus* males and females (Fig. 6). We chose these regions as they have been shown to regulate male- or female-typical sexual behaviors in whiptails [29,31]. We found species differences in the pvPOA, with *C. uniparens* having less PR-immunoreactive cells than *C. inornatus* ($F = 13.693$, df = 1, $p = 0.001$). There was no effect of reproductive state ($F = 0.475$, df = 2, $p = 0.625$). In the mPOA, we found both an effect of reproductive state ($F = 3.840$, df = 2, $p = 0.03$) and species ($F = 19.725$, df = 1, $p = 7.17 \times 10^{-5}$). Finally, we found an effect of species ($F = 50.271$, df = 1, $p = 1.34 \times 10^{-8}$) but no effect of reproductive state ($F = 2.553$, df = 2, $p = 0.09$) in the VMH. There was no interaction of species and reproductive state in any brain region. Overall, *C. inornatus* have more PR-immunoreactive cells than *C. uniparens* in each of these three hypothalamic regions important in regulating behavior.

4. Discussion

We have provided evidence that the whiptail PR likely acts in a similar manner to the human PR by structural similarity and have confirmed that there are potential sites of hormonal and epigenetic modulation in the whiptail PR promoter. Further, we have determined that PR is widely distributed throughout the brain of these reptiles. We find PR in brain regions that are known across vertebrates to modulate social behaviors and/or encode stimulus salience. Finally, we find sex, species, and ovarian state differences in PR-immunoreactive cell counts in brain regions known to mediate sexual behaviors in these species.

4.1. PR sequence, structure, function and regulation

The whiptail PR predicted protein sequence is highly conserved in the DNA-binding domain and ligand-binding domain. The structural analysis provides evidence that the ligand-binding domain and the DNA-binding domain are very similar to the human PR, suggesting that the whiptail PR binds a similar promoter motif.

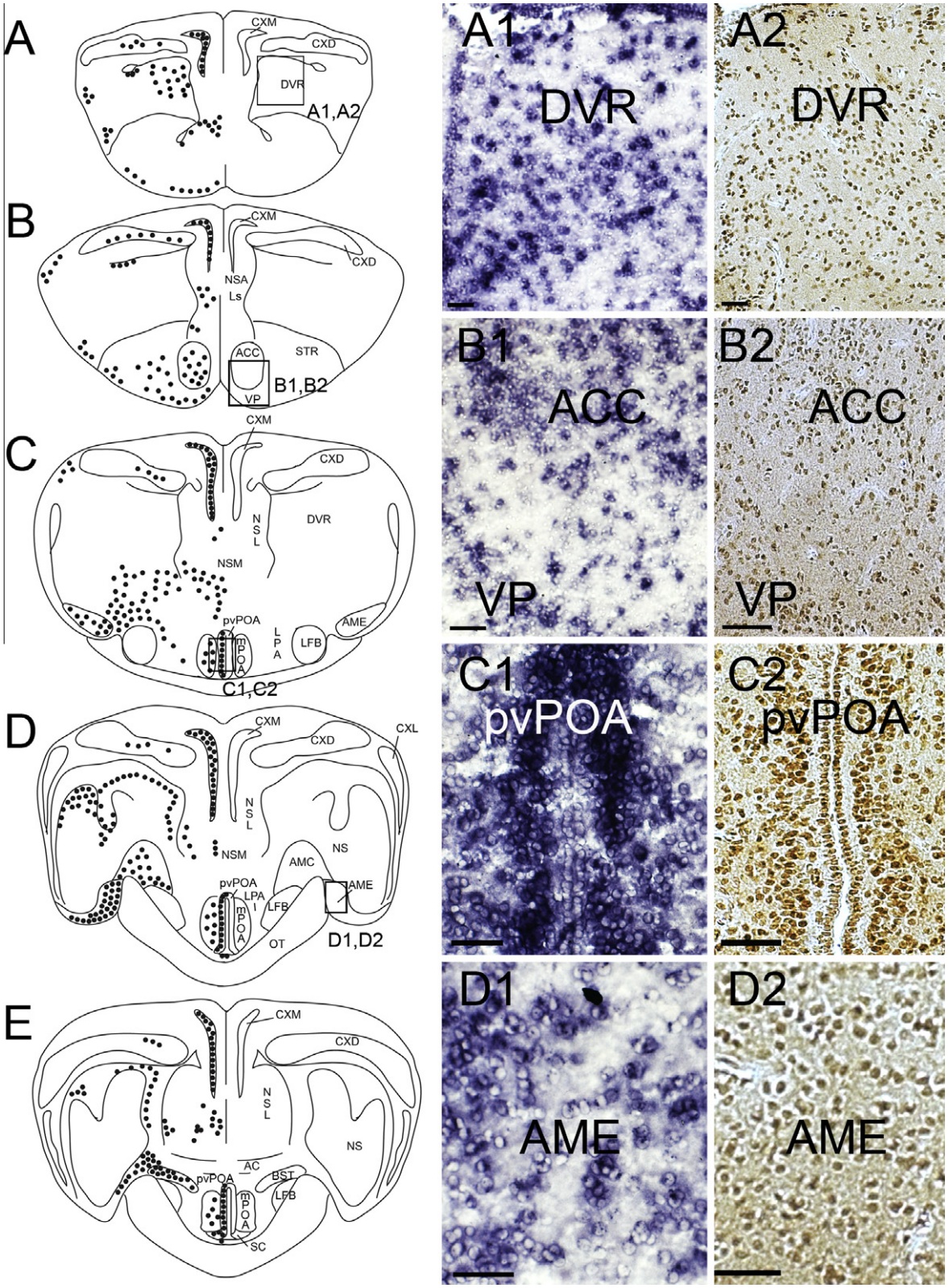

Fig. 4. PR distribution in the forebrain of whiptails (genus *Cnemidophorus*). Representative brain sections depicts PR distribution as dots on the left and neuroanatomical landmarks on the right. Density of dots qualitatively represents density of PR-positive cells. Locations of micrographs are represented by squares on representative sections. Micrograph in the top row shows PR mRNA (A1) and protein (A2) containing cells in the dorsal ventricular ridge (DVR). The second micrograph shows PR mRNA (B1) and protein (B2) containing cells in the nucleus accumbens (ACC). The third micrograph shows PR mRNA (C1) and protein (C2) in the periventricular preoptic area (pvPOA). The fourth micrograph shows PR mRNA (D1) and protein (D2) in the external amygdala (AME). All scale bars are 50 μm.

However, this does not imply that the regulatory network of the whiptail and human PR are identical. It is much more likely that differences between the regulatory networks of the whiptail and human PR have occurred at the level of the DNA sequence (gaining or losing the progesterone response element in the promoter region) than a change in the active sites of the protein itself [57,66].

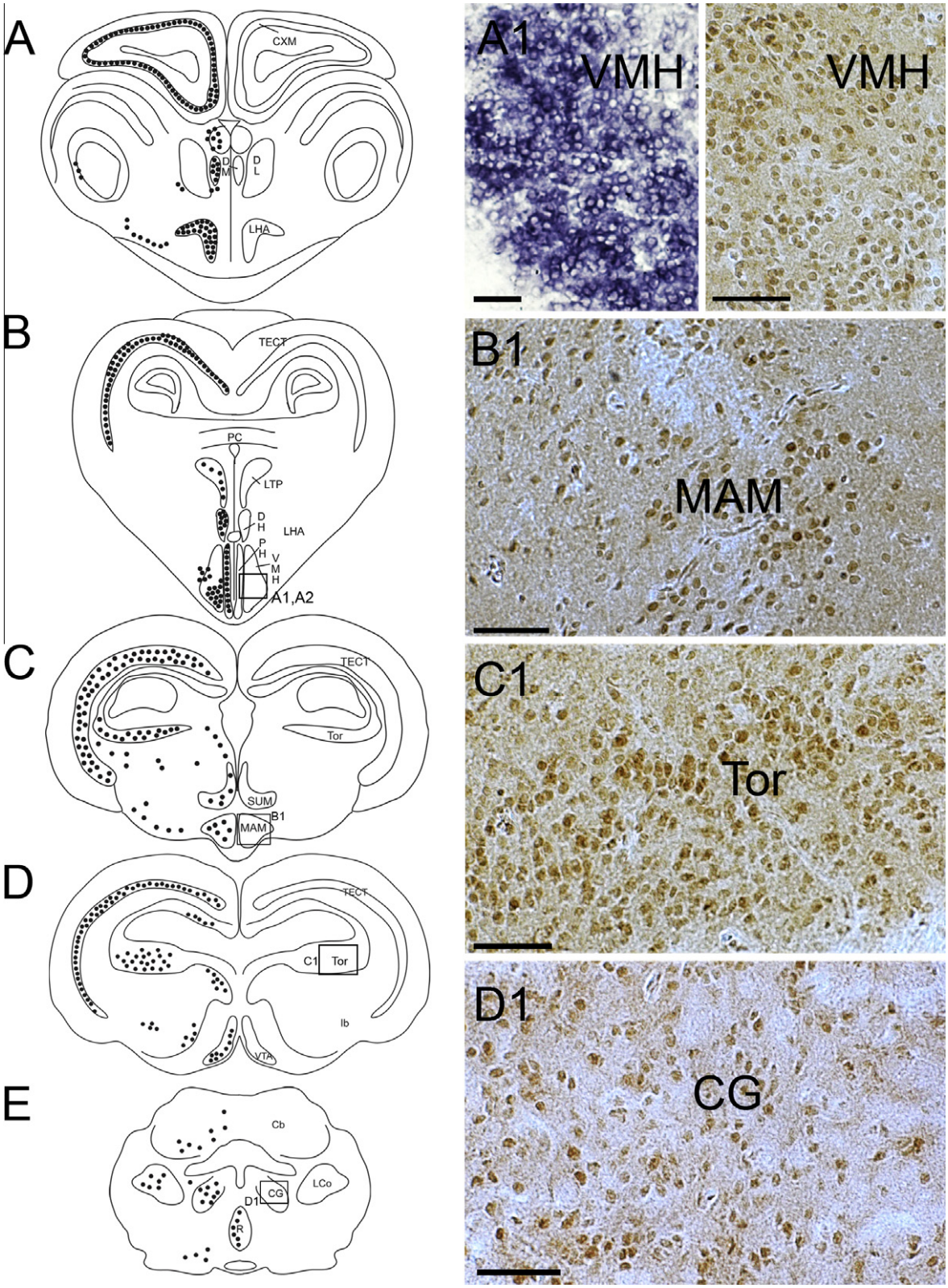

Fig. 5. PR distribution in the caudal forebrain, mid-brain and hindbrain of whiptails (genus *Cnemidophorus*). Representative brain sections depict PR distribution as dots on the left and neuroanatomical landmarks on the right. Density of dots qualitatively represents density of PR-positive cells. Locations of micrographs are represented by squares on representative sections. Micrograph in the top row shows PR mRNA (A1) and protein (A2) containing cells in the ventromedial hypothalamus (VMH). The second micrograph shows PR-immunoreactive cells in the mammillary body (MAM; B1). The third micrograph shows PR protein in the torus semicircularis (Tor; C1). The fourth micrograph shows PR protein in the central grey (CG; D1). All scale bars are 50 μm.

We have also found putative steroid hormone receptor binding sites in the PR promoter. In whiptails, expression of PR is regulated by estrogens [19], progestins [20], and androgens [21] in both the ancestral and descendant species. In mammals, the regulation of PR by estrogens is well established [46,51,55]. Although these putative steroid hormone response elements need to be validated

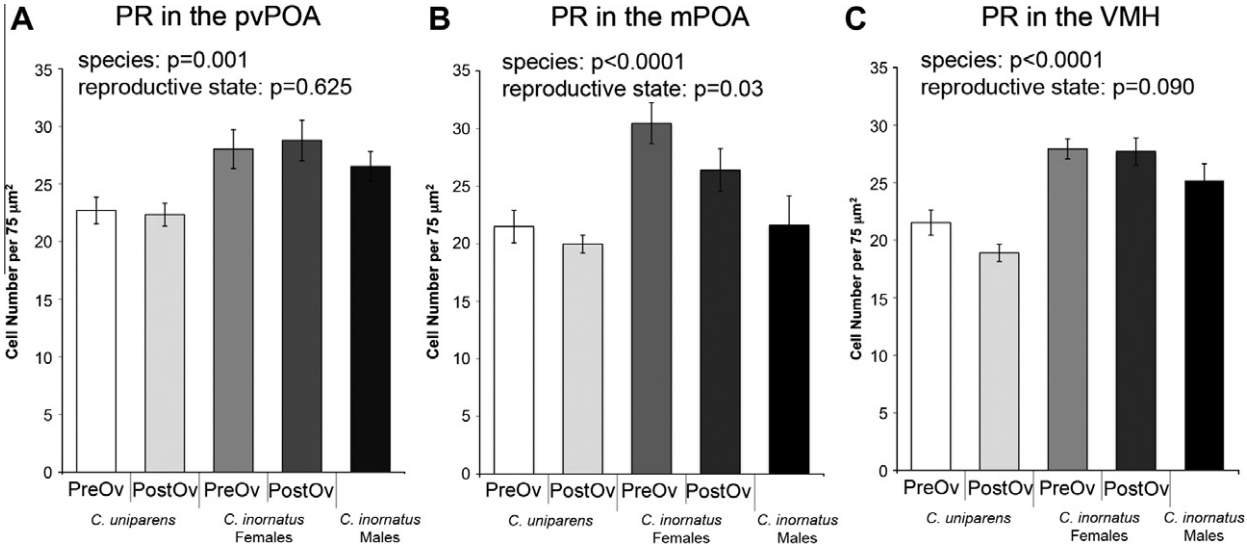

Fig. 6. Species and sex differences in PR cell-immunoreactivity. Species and reproductive state (PostOv, PreOv, and male conditions) in PR immunoreactivity are shown in the (A) pvPOA, (B) mPOA, and (C) VMH. Error bars are ±SEM.

by reporter assays in a future study, the promoter analysis suggests that the all steroid hormone receptors can potentially regulate the transcription of PR. More studies on the transcriptional regulation of PR in other vertebrates will provide useful comparative information on conserved promoter binding sites as well as variation in promoter regulation in various brain regions.

4.2. Comparison of PR mRNA and protein distribution to mRNA in reptiles

To our knowledge there are no descriptions of PR protein distribution in a reptile. However, there is a description of PR mRNA in the whiptail brain [77], where we find some discrepancies that may be due to differences in technique sensitivity of radioactive *in situ* hybridization [77] and non-radioactive, non-fluorescent *in situ* hybridization and immunohistochemistry (present study). There may also be signal amplification difference, as non-radioactive non-fluorescent *in situ* hybridization relies on signal amplification for visualization. Additionally, as many proteins can be made from a single mRNA and proteins are more stable than mRNA, their detection is more likely. Young and colleagues [77] did not describe the distribution throughout the whole brain, but only the fore- and some mid-brain regions. We therefore discuss similarities and differences in brain regions here that are reported in Young et al. In the forebrain, we have found PR mRNA and protein in many rostral forebrain regions including the cortex, dorsal ventricular ridge and striatum, where Young et al. reported no mRNA. We have also found PR mRNA and protein in the sphericus nuclei (NS), although Young et al. did not report PR mRNA expression in this region. Additionally, we found abundant PR mRNA and protein present in the external amygdala (AME), although Young et al. did not report any PR mRNA here.

4.3. Comparison of PR distribution to other vertebrates

Here, we compare the distribution of the progesterone receptor in whiptail lizards to other vertebrates, specifically focusing on two neural networks that likely regulate social decision-making in vertebrates: the mesolimbic dopamine system [14] and the social behavior network [47]. Within these two neural networks, the distribution of PR is remarkably conserved across the major vertebrate lineages.

The mesolimbic reward system is founded on the dopaminergic ventral tegmental area that projects to many forebrain nuclei and is important for reinforcement of behavior [75]. In mammals, many forebrain regions receive projections from this dopaminergic group including the nucleus accumbens, ventral pallidum, basolateral amygdala, striatum, bed nucleus of the stria terminalis, the lateral septum, and the hippocampus. Many of these brain nuclei contain the PR in teleosts [24,45], amphibians [49], birds [2,18,65], and mammals [28,37,56]. A consensus has emerged as to the putative reptilian homologies to these forebrain nuclei, but some are still considered contentious, such as the amygdaloid and some basal ganglia regions. The medial cortex (CXM), a putative homologue to the mammalian hippocampus [7], and the caudodorsal dorsal ventricular ridge (DVR), a putative homologue of the mammalian lateral amygdala [7] both express PR in whiptails. Other forebrain regions, such as the nucleus accumbens and the striatum also express PR in whiptail lizards, suggesting that progesterone may play important roles in modulating this neural network involved in evaluating the salience of social and other stimuli.

The second neural network important for the regulation of social behavior is the "social behavior network". Originally proposed for mammals [47], this network contains mostly hypothalamic regions that express steroid hormone receptors and are reciprocally connected. This network has more recently been applied to other vertebrate classes including reptiles, teleosts, and birds [10,22]. The nodes of this network include the medial amygdala/bed nucleus of the stria terminalis, lateral septum, preoptic area, anterior hypothalamus, ventromedial hypothalamus, and periaqueductal grey/central grey. The brain regions in the social behavior network contain PR in every vertebrate class studied including teleosts [24,45], amphibians [49], birds [2,18,65], and mammals [28,37,56]. We have shown here that PR is expressed in each of these brain regions in whiptail lizards, providing further neurochemical evidence in support of these reptilian homologies in the social behavior network.

4.4. Functional implications of progesterone in regulating sexual behavior

In male and female vertebrates, progesterone is important in the regulation of reproduction, including male- and female-typical sexual behavior. In males, progesterone seems to facilitate

male-typical behaviors at physiological concentrations [13,36,35,34,71,72], and PR knockout mice show a decrease in male-typical sexual behavior [54]. In females, the effects of progesterone on receptivity vary across vertebrate classes as exogenous progesterone increases receptivity in mammals [68] and amphibians [59], but decreases female-typical behavior in reptiles [20] and birds [32]. The molecular mechanisms of this differential action of progesterone in female vertebrates is not well understood and further comparative work into the distribution and quantitative expression of the PR is needed to increase our understanding of how and where PR acts to either facilitate or inhibit female-typical behavior.

The mechanisms of PR action are particularly interesting in PostOv female *C. inornatus* and *C. uniparens*, as the latter descendant species responds to the postovulatory progesterone surge with male-like mounting behavior while the ancestral females do not. In the mPOA, pvPOA, and VMH, PostOv *C. inornatus* females have higher PR cell counts than *C. uniparens*. This may be due to regulation of PR by estrogens, as *C. inornatus* have higher estradiol levels in the preovulatory phase than *C. uniparens*, which may result in more PR-immunoreactive cells. The levels of progesterone between female of the two species are relatively equivalent [40]. Although we did not find differences in PR immunoreactivity between ovulatory states in females of either species, mRNA levels have been previously found to be different in some regions of the hypothalamus [74]. Given the extensive amount of post-translation regulation of proteins, as well as higher stability of proteins compared to mRNA, it is possible that the effects of reproductive state on transcription of PR mRNAs do not lead to (detectable) changes in the levels of PR proteins. Additionally, PR seems to have different transcriptional activity in *C. uniparens* and *C. inornatus* females, as PR upregulates neuronal nitric oxide synthase to facilitate male-typical mounting behavior in *C. uniparens* but not *C. inornatus* females [50]. Future studies will focus on the gene regulatory network of PR, as several gene networks may be differentially regulated by PR in *C. uniparens* in response to progesterone that facilitate male-like behavior compared to *C. inornatus* females.

In mammals, it is well established that the molecular pathways of progesterone and dopamine act in concert to facilitate female sexual behavior [39]. Mani and colleagues [38] have shown that PR is required for progesterone- and dopamine-facilitated lordosis in female rats. Similarly, dopamine action in the preoptic area is important for the display of male sexual behavior in rodents [26], although the involvement of progesterone and dopamine crosstalk has not been established in males. Given that progesterone facilitates male-typical sexual behavior in whiptail lizards and is present in many regions of the mesolimbic reward system, progesterone and dopamine may facilitate this behavior in male whiptails in a similar manner to the facilitation of sexual behavior in female rodents.

5. Conclusions

In the present study, we have described the molecular and structural characteristics of the whiptail PR and have also demonstrated that PR is expressed in brain regions whose homologues regulate social behavior and evaluation of stimulus salience in other vertebrates. These findings suggest that progesterone might play an important role not only in the regulation sexual behavior, but also other complex social behaviors and the evaluation of potentially rewarding stimuli. Future experiments will require further quantitative detection methods and pharmacological manipulations to further dissect the role PR may play in regulating behavior in whiptail lizards, either on its own or in concert with dopaminergic modulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygcen.2010.12.010.

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