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Species Differences in Estrogen Receptor and Progesterone Receptor-mRNA Expression in the Brain of Sexual and Unisexual Whiptail Lizards

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Abstract

Circulating concentrations of gonadal steroid hormones and reproductive behavior in female vertebrates vary as a function of ovarian state. Steroids secreted by the ovary, specifically estrogen and progesterone, influence the expression of behaviors associated with reproduction by interacting with intracellular sex steroid receptors located in specific regions of the brain. Using *in situ* hybridization, we analyzed estrogen receptor and progesterone receptor messenger RNA expression in several brain regions of ovariectomized, vitellogenic, and postovulatory individuals from two species of whiptail lizards (*Cnemidophorus uniparens* and *C. inornatus*). Although these species are genetically very similar, they differ in two aspects of their reproductive biology: (i) the unisexual *C. uniparens* alternate between expressing female-typical and male-like pseudosexual behaviors while female *C. inornatus* normally express only female receptive behavior, and (ii) circulating estradiol concentrations in reproductively active female *C. uniparens* are approximately five-fold lower than in reproductively active female *C. inornatus*. We found that the regulation of sex steroid receptor gene expression was region specific, with receptor-mRNA expression being increased, unchanged, or decreased during vitellogenesis depending on the area. Furthermore, several species differences in the amount of sex steroid receptor-mRNA were found that may be relevant to the species differences in circulating estrogen concentrations and sexual behavior.

Gonadal steroid hormones modulate reproductive behavior and physiology by interacting with steroid-specific receptor proteins localized in specific neuronal groups in the vertebrate brain (1). Sex steroid receptors are members of a superfamily of ligand-dependent transcription factors which mediate the genomic effects of gonadal steroids by altering the rate of gene expression (2). Sex steroid hormone-induced changes in gene expression in certain brain nuclei are thought to influence directly the display of reproductive behaviors. For example, estrogenic induction of the neuropeptide oxytocin and its receptor in the ventromedial nucleus of the hypothalamus is thought to be crucial to the expression of lordosis behavior in female rats (3). Although a great deal of information is available on the relationship between circulating steroid hormone concentration, gonadal development, and reproductive behavior in several small mammal species (4), little is known regarding the dynamics of sex steroid receptor expression and its relation to behavior in the brain of non-rodents.

Whiptail lizards (genus *Cnemidophorus*) provide a model system for studying the evolution of hormone-brain-behavior relationship (5). *Cnemidophorus uniparens* is an all-female species of lizard descended from a hybridization event between *C. inornatus* and another *Cnemidophorus* species that resulted in a triploid genome, two-thirds of which is derived from *C. inornatus* (6). The sexual ancestor and unisexual descendant species differ in reproductive behavior patterns and normal circulating concentrations of estrogen. Investigations into the hormonal and neuronal control of reproductive behavior of both species reveal that the expression of reproductive behavior in female whiptail lizards varies with ovarian condition (7, 8) and is controlled by ovarian steroids which act on certain hypothalamic brain regions (9–11).

Recently, *in situ* hybridization studies have revealed the neuroanatomical distribution of estrogen receptor (ER), progesterone receptor (PR), and androgen receptor (AR)-mRNA expression in *C. uniparens* and *C. inornatus* (12). Information on the estrogenic regulation of ER- and PR-mRNA expression in the brain has also been reported (13). The primary goal of this study was to use *in situ* hybridization assays to determine (i) if sex steroid receptor-mRNA expression in the lizard brain varies as a function of ovarian development, and (ii) whether species differences in sex steroid receptor-mRNA expression exist which may be relevant to species differences in reproductive behavior and circulating estradiol concentrations.

The data presented here provides the first information on sex steroid receptor gene expression in relation to the ovarian cycle

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of nonmammalian vertebrates and offers insights into the evolution of hormone-brain-behavior relationships.

Results

Hybridization with the radiolabeled antisense cRNA probes (Fig. 1) resulted in specific labeling of neurons (Fig. 2) in a pattern consistent with previous studies (12). The neuroanatomical positions of the brain regions analyzed are illustrated in Fig. 3. The results of the analysis of the silver grain density in brains hybridized to ER and PR antisense probes are presented in Figs 4 and 5, respectively.

Sex steroid receptor-mRNA expression changes with ovarian state in some, but not in all, brain regions. The relative abundance of ER-mRNA varied as a function of ovarian condition only in the periventricular nucleus of the preoptic area and the ventromedial hypothalamus. In the periventricular nucleus of the preoptic area, ER-mRNA levels were lowest during vitellogenesis when estradiol concentrations are elevated. In the ventromedial hypothalamus, ER-mRNA levels were highest in ovariectomized and lowest in postovulatory animals. When ER-mRNA expression in the ventromedial hypothalamus of vitellogenic animals was correlated with the stage of ovarian development, it was found that as a group, *C. uniparens* with medium sized follicles (MPRE, 4–7 mm diameter) had lower ER-mRNA concentrations than animals in earlier (EPRE) or later (LPRE) stages of vitellogenesis. Although the significance of this difference is unclear (a similar correlation was not found in female *C. inornatus*), analysis of estradiol concentrations in the pooled plasmas from these groups indicated that animals with medium sized follicles also had a lower estradiol concentration than the other groups (Fig. 6).

Species differences in ER-mRNA expression were found in the periventricular nucleus of the preoptic area and the medial preoptic area. In both areas, ER-mRNA expression was higher in *C. uniparens* compared to female *C. inornatus*.

Significant effects of ovarian condition on PR-mRNA expression were found in the ventromedial hypothalamus, lentiformis thalami pars plicta, periventricular nucleus of the hypothalamus, and the torus semicircularis (Fig. 5). In all of these areas, except for the lentiformis thalami pars plicta, silver grain density was highest in vitellogenic animals; in this area, silver grain density was highest in postovulatory animals.

Species differences in PR-mRNA expression were found in the medial preoptic area, nucleus centralis amygdalae, ventromedial hypothalamus, and dorsal hypothalamus. In the medial preoptic area, amygdala, and dorsal hypothalamus, PR-mRNA expression was higher in *C. uniparens*. In contrast, silver grain density in the ventromedial hypothalamus was highest in female *C. inornatus*.

Discussion

Plasma estradiol and progesterone levels change significantly with ovarian development in *C. uniparens* and *C. inornatus* (14, 15). Estradiol levels are elevated during vitellogenesis, and decline rapidly after ovulation. Progesterone levels are low during vitellogenesis and rise around the time of ovulation. This experiment

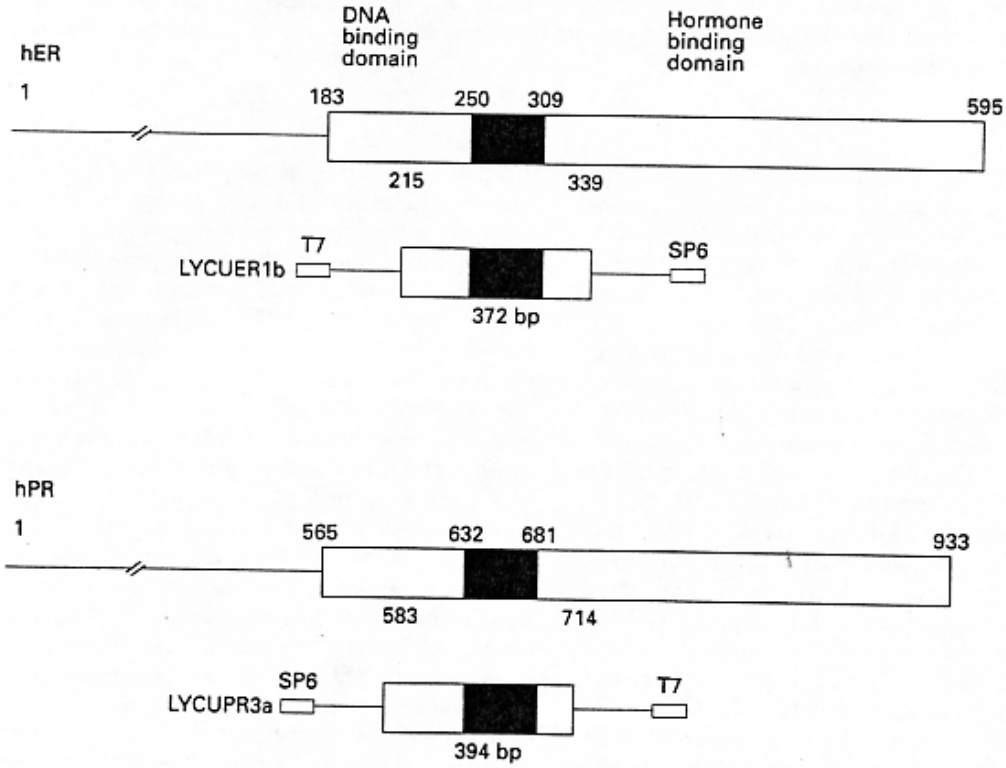


FIG. 1. Schematic diagrams showing the size and position of the *Cnemidophorus* estrogen receptor (LYCUER1b) and progesterone receptor (LYCUPR3a) clones relative to the full length human sequences. The structures of the human estrogen receptor (hER), and progesterone receptor (hPR) are illustrated above the lizard clones to demonstrate the relative position of the sequences used to generate the radioactive probes used in the present study. The numbers positioned above and below the human receptor structures are amino acid sequence positions for the hER (47) and the hPR (48).

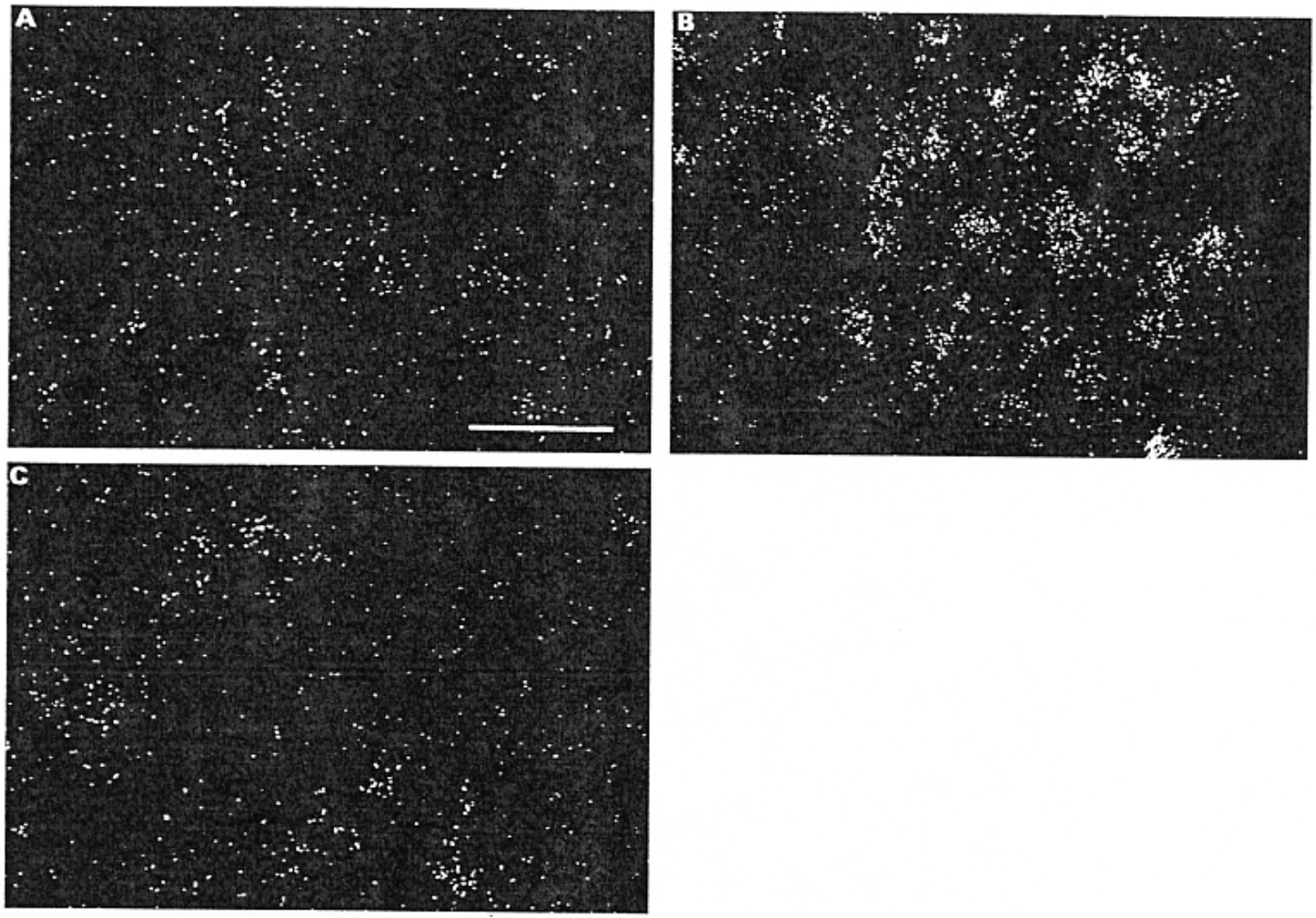
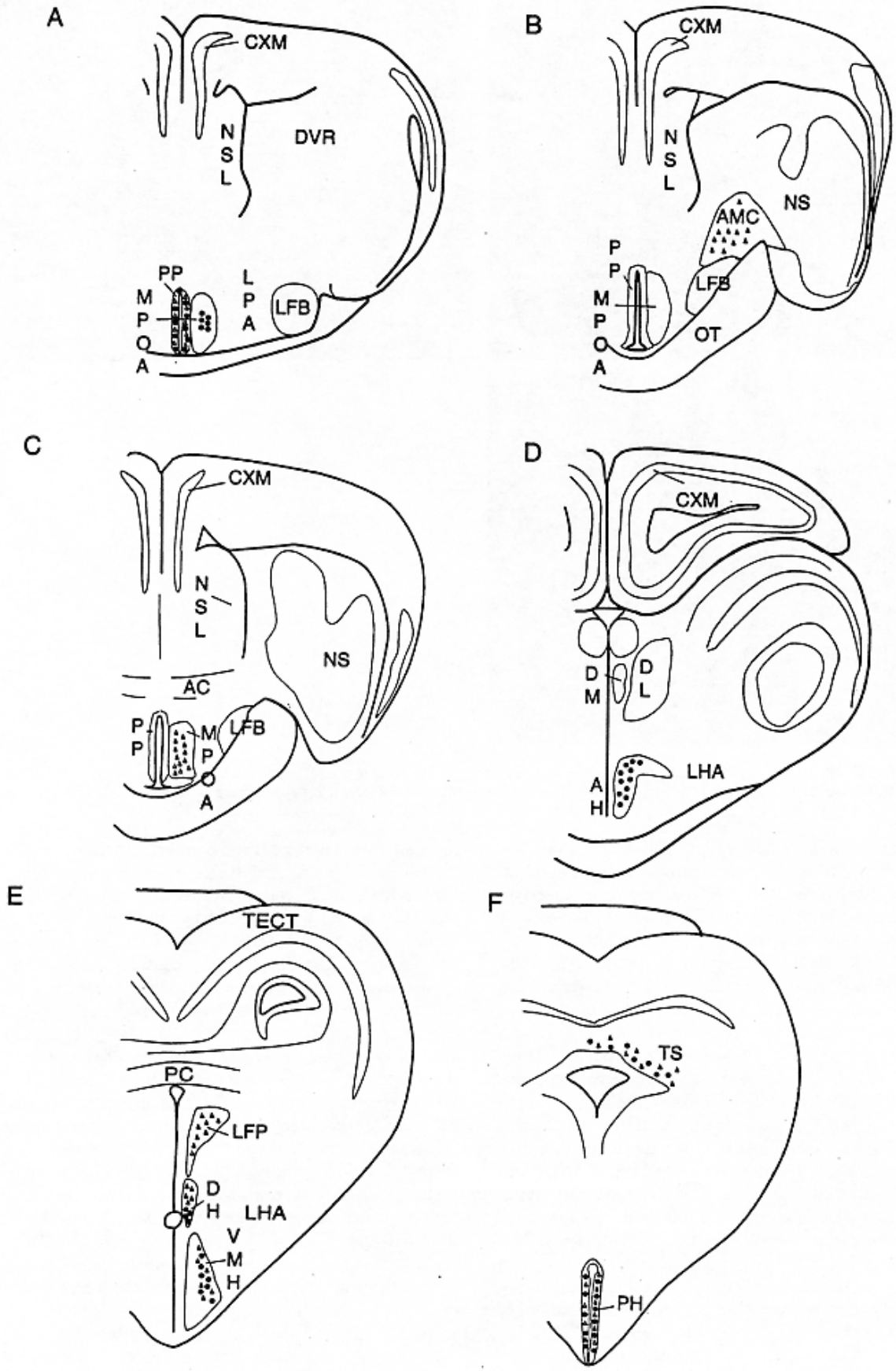


FIG. 2. Photomicrograph illustrating silver grain density over cells in the ventromedial hypothalamus of (A) ovariectomized, (B) vitellogenic, and (C) postovulatory *Cnemidophorus uniparens* following *in situ* hybridization with the antisense progesterone receptor probe. Scale bar = 50 μm.

demonstrates that in some brain regions ER- and PR-mRNA expression also change over the course of ovarian development. Furthermore, the changes in sex steroid receptor gene expression occur in a region specific manner and are most likely mediated by the changes in ovarian sex steroid hormone secretion.

The changes in ER-mRNA expression in the periventricular nucleus of the preoptic area are consistent with the hypothesis that ER-mRNA expression in that area is suppressed by estradiol. Silver grain density was elevated in ovariectomized and post-ovulatory animals compared to vitellogenic animals. The results obtained for ER-mRNA expression in the ventromedial hypothalamus are more difficult to interpret. Unlike in the pattern found in the female rat, ER-mRNA expression in ovariectomized lizards is clearly increased with estradiol benzoate treatment (13, 16). Yet, the concentration of ER-mRNA in the present study was highest in ovariectomized animals. This paradoxical result prompted us to compare ER-mRNA expression in the ventromedial hypothalamus with plasma estradiol concentrations. This comparison suggests that in intact animals, ER-mRNA expression appears to be positively correlated with estradiol concentrations, consistent with the earlier experiments with administration of exogenous estrogen (13). Estrogen receptor-mRNA concentration appears to be higher in vitellogenic animals with higher estradiol concentrations (Fig. 6). Upon ovariectomy, however, ER-mRNA expression increases, while exogenous EB treatment increases ER-mRNA expression even further (13). Estrogen receptor gene expression in the ventromedial hypothalamus of the rat is also elevated upon ovariectomy (17). It is possible that some factor, whether it be chemical (18) or a neuronal connection (see (19) for a review) originating from the ovary suppresses ER-mRNA expression. Removal of the ovary relieves the suppression, thus increasing ER-mRNA expression. If this hypothesis is correct, ovariectomy would remove the influence of this factor, resulting in an increase in the basal rate of ER gene expression. We are currently investigating the effects of unilateral ovariectomy on ER-mRNA expression in the ventromedial hypothalamus.

Estradiol stimulates hypothalamic PR expression in a number of species, including the whiptail lizard (13, 20–25). In the present study, PR-mRNA expression in the ventromedial hypothalamus, periventricular nucleus of the hypothalamus, and the torus semicircularis was elevated in vitellogenic animals. This result is consistent with the previous work which demonstrated that in the ventromedial hypothalamus and the torus semicircularis, PR-mRNA expression is estrogen-sensitive (13). In each of these areas, ER-mRNA appears to be co-distributed with PR-mRNA (12). In the lentiformis thalami pars plicta, however, PR-mRNA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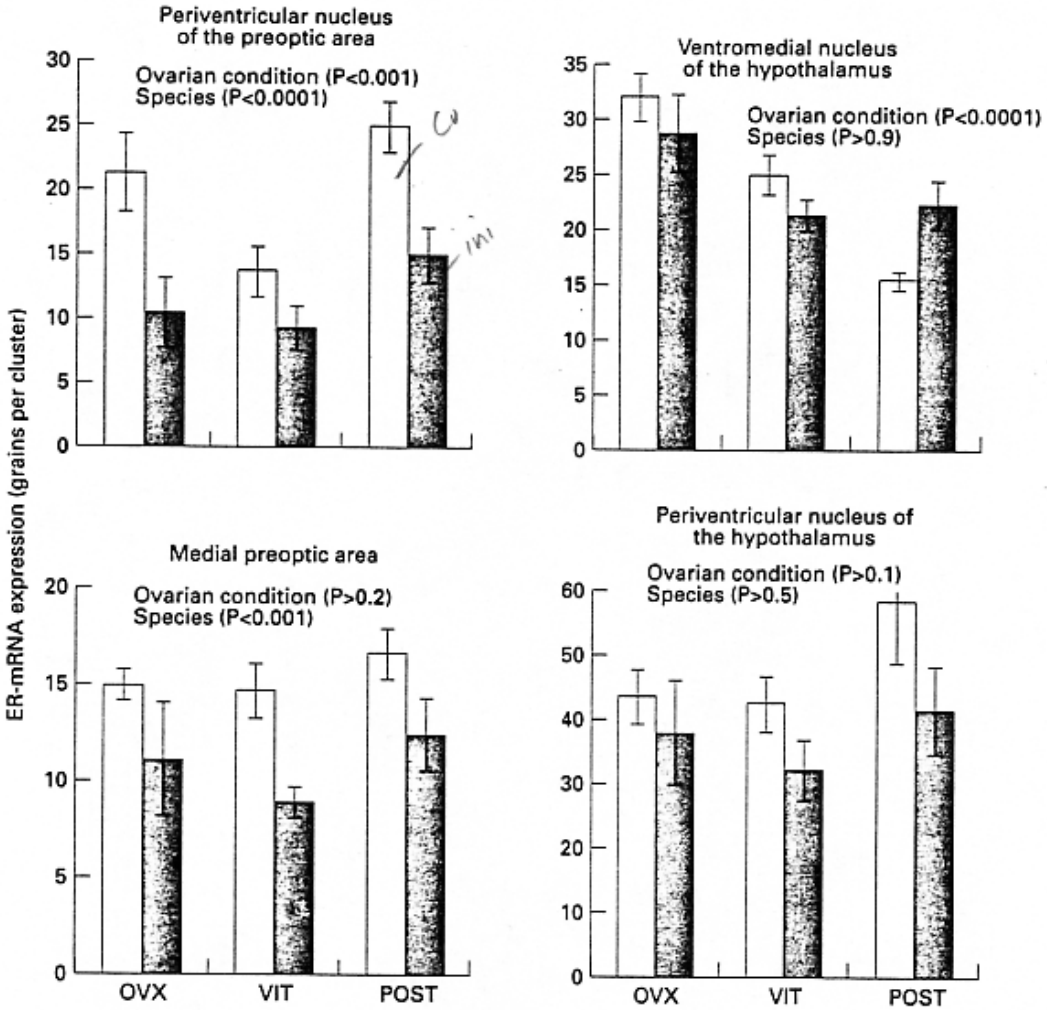

FIG. 4. Estrogen receptor-messenger RNA expression in the brains of ovariectomized (OVX), vitellogenic (VIT), and postovulatory (POST) *Cnemidophorus uniparens* (open bars) and *C. inornatus* (stippled bars). Presented are the mean number of silver grains per cell after hybridization with the radiolabeled antisense ER probe. Vertical bars represent standard errors. The probability values for effects of ovarian state and species differences were generated from a two-way ANOVA.

expression was highest in postovulatory animals, suggesting that PR gene expression in that area may be regulated by luteal progesterone rather than estradiol. ER-mRNA was not found in the lentiformis thalami pars plicta.

Functional significance of steroid sensitive regions of the reptilian brain

Detailed discussions of the functional significance of steroid sensitive regions of the reptilian brain have been presented elsewhere (12, 26). Information relevant to the present data is discussed here. The expression of reproductive behavior in both species varies with the ovarian cycle and circulating concentrations of sex steroid hormones. The ventromedial hypothalamus, anterior hypothalamus, and preoptic area play central roles in the hormonal control of reproductive behavior in reptiles (27). Receptive behavior in both species occurs exclusively during vitellogenesis when estradiol concentrations are elevated (7, 14) and can be elicited by intracranial implantation of estrogen in the ventromedial hypothalamus (9) and abolished by lesions in the ventromedial hypothalamus (28). In the ventromedial hypothalamus, both ER-mRNA and PR-mRNA increase during vitellogenisis as compared to postovulatory animals. Although the role of progesterone in the induction of receptive behavior in whiptail lizards has not been determined, progesterone is known to synergize with estradiol to facilitate receptive behavior in the female green anole lizard. Male-like mounting and pseudocopulat-

FIG. 3. Drawings of coronal sections of a *Cnemidophorus* brain illustrating the neuroanatomical positions of the brain nuclei in which estrogen receptor-mRNA (solid circles) and progesterone receptor-mRNA (open triangles) were quantified. Drawings are arranged in an anterior (A) to posterior (F) order. For a more detailed atlas of the *Cnemidophorus* brain see (12). Abbreviations: PP = periventricular nucleus of the preoptic area, MPOA = medial preoptic area, NSL = nucleus septalis lateralis, LFB = lateral forebrain bundle, VE = ventricular ependymal organ, LTP = lentiformis thalami pars plicta, DH = dorsal hypothalamus, VMH = ventromedial nucleus of the hypothalamus, PH = periventricular nucleus of the hypothalamus, and TS = torus semicircularis.

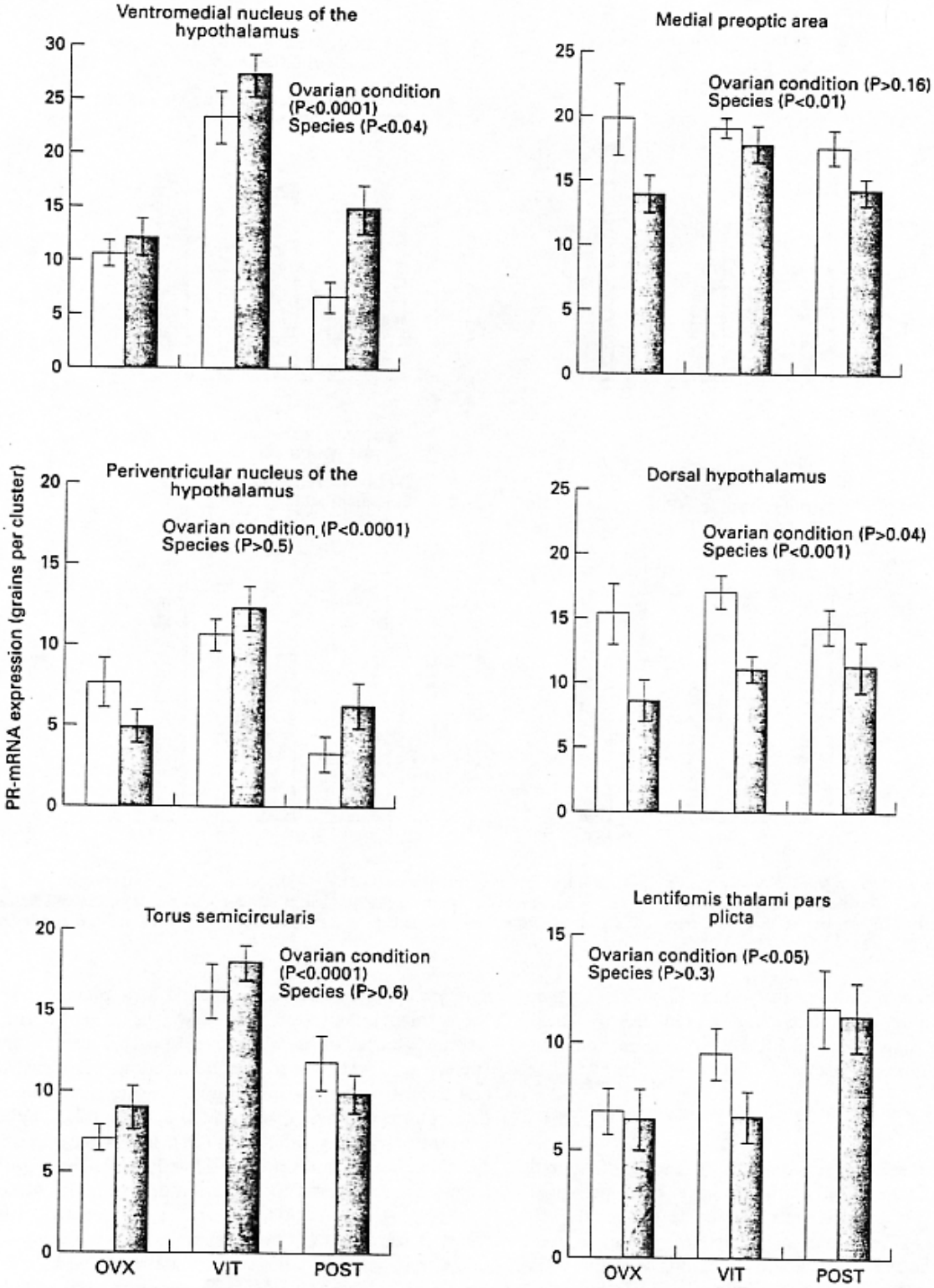

FIG. 5. Progesterone receptor-messenger RNA expression in the brains of ovariectomized (OVX), vitellogenic (VIT), and postovulatory (POST) *Cnemidophorus uniparens* (open bars) and *C. inornatus* (stippled bars). Presented are the mean number of silver grains per cell after hybridization with the radiolabeled antisense PR probe. Vertical bars represent standard errors. The probability values for effects of ovarian state and species differences were generated from a two-way ANOVA.

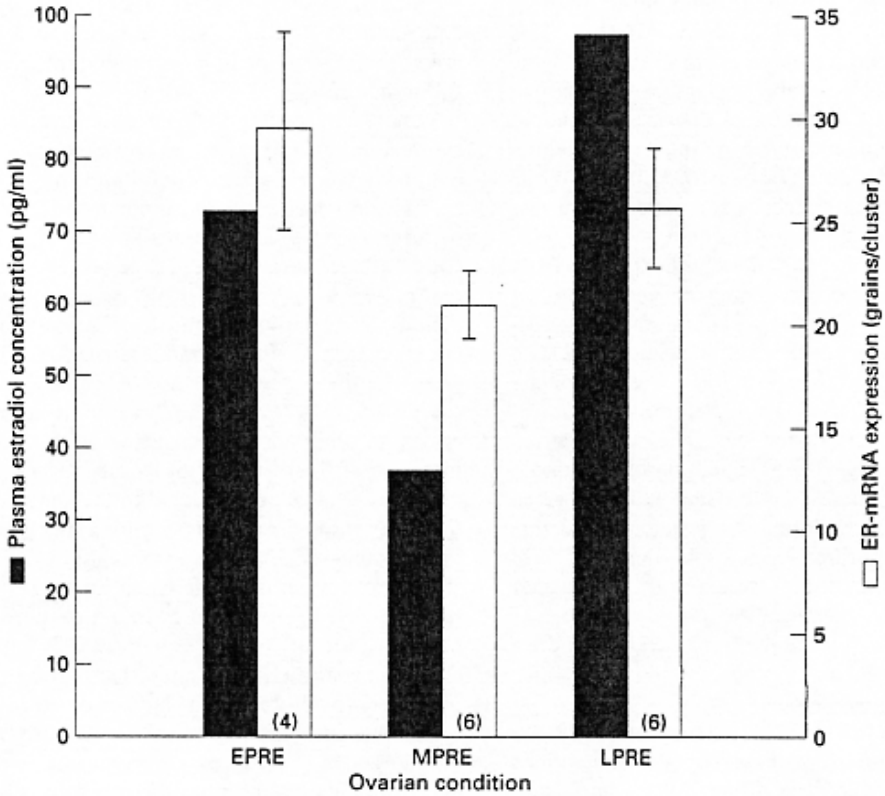

FIG. 6. Circulating estradiol concentration and estrogen receptor-mRNA expression in the ventromedial nucleus of the hypothalamus of *Cnemidophorus uniparens* during early (EPRE), middle (MPRE), and late (LPRE) stages of vitellogenesis. Vertical bars represent standard errors.

ory behavior in *C. uniparens* occurs most frequently following ovulation and is associated with elevated progesterone concentrations (7, 14). This behavior is absent in female *C. inornatus*. Exogenous administration of progesterone (29) and, to some extent, estrogen (30) reinstates male-like pseudosexual behavior in ovariectomized *C. uniparens*. Intracranial implantation of steroid hormone (11) and brain lesion studies (31) indicate that the regions near the anterior hypothalamus and preoptic area are involved in the hormonal control of male-like copulatory behavior in *C. uniparens*. The ventromedial nucleus of the hypothalamus, the medial preoptic area, and the periventricular nucleus of preoptic area in the reptilian brain appear to be homologous and functionally equivalent to the mammalian structures with the same name.

The functional significance of the sex steroid receptors in the other regions analyzed is unknown. The periventricular nucleus of the hypothalamus, however, may be the reptilian equivalent to the arcuate nucleus in the rat. The torus semicircularis is involved in auditory processing in lizards (32) and it has been suggested that this area is homologous to the torus semicircularis of fish and amphibians, the nucleus intercollicularis of birds, and the inferior colliculus of mammals (26). There is no data suggesting that whiptail lizards exchange auditory information in any social context, thus the significance of the presence and regulation of ER-mRNA and PR-mRNA in this region is a mystery.

Nothing is known regarding the functional significance of lentiformis thalami pars plicta or the dorsal hypothalamus in reptiles.

Evolution of hormone-brain-behavior relationships

Although *C. uniparens* and female *C. inornatus* are very similar genetically, two aspects of their reproductive biology differ considerably. First, in addition to female typical receptive behavior, the unisexual *C. uniparens* exhibit female-female mounting, or male-like pseudosexual behavior, a behavior rarely seen in female *C. inornatus*. This pseudocopulatory behavior serves to facilitate the neuroendocrine processes controlling ovarian development and ovulation in the parthenoforms (33). The results of the present study cannot provide an explanation of biochemical basis of this species difference, but are suggestive. Species differences in both ER-mRNA and PR-mRNA were found in the preoptic area, the region involved in the hormonal control of male-like behavior in *C. uniparens*. If the species differences in steroid receptor gene expression in this region results in species differences in sensitivity to steroid hormone, these differences could provide an explanation for the species differences in the expression of male-like mounting and copulatory behavior. Furthermore, since steroid hormones exert powerful organizational effects on the brain during development, it is possible that species differences in sensitivity to steroid hormones during development could influence the sexual organization of brain.

The second difference between the reproductive biology of *C. uniparens* and female *C. inornatus* is that circulating estradiol

concentrations in *C. uniparens* are several-fold lower than in *C. inornatus* of a similar reproductive condition (14, 15). This species difference in circulating hormone concentration and the species differences in ER-mRNA concentrations in the preoptic area may be interrelated. The secretion of ovarian sex steroid hormones in vertebrates is under control of the hypothalamic-pituitary-gonadal axis. Briefly, gonadotropin releasing hormone (GnRH) released at the median eminence stimulates the release of gonadotropins from the pituitary (34). These gonadotropins in turn stimulate an increase in ovarian steroid secretion from the ovary. Neurons sensitive to circulating concentrations of steroid hormones located in the preoptic area monitor the circulating levels of estradiol. In many species, the release of GnRH to the pituitary is inhibited when concentrations of estradiol are elevated above some threshold level. Thus, estradiol secretion is under the control of a negative feedback loop. The exact location of the neurons involved in the feedback regulation of sex steroid hormones is not known in reptiles. Lesion studies in the green anole lizard suggest that the anterior preoptic area and the medial basal hypothalamus are important elements in the control of GnRH release since lesions in these areas result in testicular collapse (35, 36). Immunocytochemical studies in a number of vertebrate species, including reptiles, have demonstrated the presence of GnRH producing neurons in the preoptic area which terminate in the median eminence (37, 38).

The species differences in ER-mRNA in the periventricular nucleus of the preoptic area and the medial preoptic area described in this study suggest a hypothesis regarding the mechanism of species differences in hormone levels. If the periventricular nucleus of the preoptic area or the medial preoptic area are in fact responsible for monitoring and controlling estradiol levels, the higher concentration of ER-mRNA in those areas may result in an increase in sensitivity to estradiol in *C. uniparens* and result in lower circulating estradiol concentration. A similar sensitivity compensation has been reported among old world and new world monkeys (39). Typical plasma steroid levels are elevated 2-5 fold for estradiol and up to 100-fold for progesterone in new world monkeys compared to old world monkeys. Although brain tissues were not analyzed, sex steroid receptor binding assays on peripheral tissues revealed higher concentrations of ER and PR in old world monkeys compared to new world monkeys.

Previously we have demonstrated that ovariectomized *C. uniparens* is both behaviorally and biochemically more sensitive to estrogen (40). Dose-response studies using subcutaneous injection of estradiol benzoate indicate that smaller dosages of estrogen are required to induce receptive behavior, or to upregulate PR-mRNA expression in the ventromedial hypothalamus of *C. uniparens* compared to female *C. inornatus*. This suggests that the lower circulating concentration of estradiol in *C. uniparens* is sufficient to activate estrogen-sensitive sexual behaviors required for normal reproduction.

Comparison of PR-mRNA expression between the two species reveals an interesting pattern. In brain regions where PR-mRNA expression varies with circulating estradiol concentrations (i.e. ventromedial hypothalamus, torus semicircularis, and periventricular nucleus of the hypothalamus), PR-mRNA expression was either similar between the species or in the case of the ventromedial hypothalamus, elevated in *C. inornatus*. In contrast, in those regions where PR-mRNA expression did not vary with estradiol concentrations (i.e. medial preoptic area, amygdala, and dorsal hypothalamus), PR-mRNA was elevated in *C. uniparens*. These data indicate that at basal transcription rates, steroid receptor-mRNA in the brain is more abundant in *C. uniparens* than in *C. inornatus*.

Why such species differences in sex steroid receptor gene expression between ancestral and descendant species have arisen is not known. However, one interesting possibility exists. While *C. inornatus* has a typical diploid genome, with two copies of each target gene, *C. uniparens* hosts a triploid genome with three copies of each target gene. It has been suggested that one reason that polyploid species differ physiologically and ecologically from their diploid relatives is that the increased gene dosage may result in higher enzyme and hormone levels (41). This phenomenon has been well documented for a number of enzymes in several plant species (42). Allozyme analysis of a number of diploid and polyploid whiptail lizards, including *C. uniparens*, demonstrate that each of the three sets of chromosomes are actively transcribing genes at rates proportional to the gene dosage (43, 44), rather than one chromosome set becoming inactivated or compensating for the increased gene dosage as might be expected. Thus, triploidy could possibly result in increased hormone-induced transcription rates for ER, PR, or other hormone-responsive genes involved in the activation of sexual behaviors. It is easy to see how such a change in gene number might disrupt the balance of the regulatory properties for proteins that have evolved in a diploid genome.

Changes in sensitivity to steroid hormones are likely to result in changes in the expression of hormone-dependent behaviors. For example, interstrain differences in ER concentration and affinity for estrogen in the hypothalamus and preoptic area of mice are thought to be responsible for strain differences in estrogen-induced behaviors (45). Perhaps the differences in sex steroid receptor expression in the periventricular nucleus of the preoptic area and/or the medial preoptic area between *C. uniparens* and *C. inornatus* are somehow related to species differences in the frequency of expression of female-female mounting. Changes in neural sensitivity to endogenous hormones via modulation of receptor characteristic may be an important mechanism for the evolution of hormonally mediated behaviors in vertebrates. Since sex steroid hormones have both short term activational and long term organizational effects on the brain and behavior, differences in sensitivity to hormones during development could have a profound effect on the behavioral and neuroendocrine responses to gonadal steroids.

Materials and methods

Animals

Adult *C. uniparens* were captured near Portal, Arizona and, after transport to University of Texas, were housed in environmental chambers as previously described (9). The animals were transported to environmental chambers at UT and housed four per 29 gal aquaria (30 × 75 cm) for *C. uniparens* and three females and one male per aquaria for *C. inornatus*. The stage follicular development was monitored by abdominal palpitation. Prior to dissection, animals were singly removed from there cage, weighed, measured and decapitated with two min. Trunk blood was taken and spun to recover plasma for radioimmunoassay. The brain was immediately removed and frozen on dry ice and stored at −80 °C. The number of follicles or eggs was recorded as well as the diameter of the largest follicle. Animals in the ovariectomized group were gonadectomized as previously described (9) and allowed at least two weeks before tissue was taken. Sample sizes were n = 5 for ovariectomized *C. uniparens* and *C. inornatus* each; n = 16 and 14 for vitellogenic *C. uniparens* and *C. inornatus*,

respectively, and n = 7 and 6 for postovulatory *C. uniparens* and *C. inornatus*, respectively.

In situ *hybridization*

Probe preparation: Probes for *in situ* hybridization were prepared from the whiptail ER clone LYCUER1b (372 bp) and the whiptail PR clone LYCUPR3a (394 bp) as previously described (12). The structures of these clones relative to the human the human structures are illustrated in Fig. 1. It should be noted that the probe for ER spans from exon 3 into exon 4 and therefore should detect both the normal ER as well as the isoform lacking exon 4 (46). Antisense and sense cRNA probes were synthesized using SP6 or T7 RNA polymerase incorporating ^{35}S-UTP (NEN) at a specific activity of 9×10^8 cpm/mg probe. Purified cRNA probes were heat-denatured and added to the hybridization solution (50% formamide, 10% dextran sulfate, 0.3 M NaCl, 10 mM Tris HCl (pH 8.0), 1 mM EDTA, 1× Denhardt's solution, 10 mM dithiothreitol and 0.5 mg/ml tRNA) at a final concentration of 0.3 mg probe × length (kb)/ml.

In situ *hybridization:* Twenty-micron thick coronal cryosections were melted on RNase free polylysine coated microscope slides and allowed to dry at room temperature before storing in slide boxes with desiccant at −80 °C. Sections were placed on a series of six slides so that adjacent sections could be hybridized to different probes. At the time of the assay, the tissue was fixed in 4% paraformaldehyde (pH 7.2) at room temperature for 20 min, rinsed five min in 3× phosphate buffered saline (PBS), followed by two five min washes in 1× PBS. The sections were then dipped in 0.1 M triethanolamine (TEA) (pH 8.0) followed by ten min in freshly prepared TEA/0.25% acetic anhydride, rinsed in 2× SSC, dehydrated in ascending ethanols, delipidated in chloroform, rinsed in 95% ethanol, air-dried and prehybridized overnight (50 °C) in hybridization solution (see above) in which the probe was replaced with tRNA (2.5 mg/ml final concentration). Prehybridization and hybridization took place in air-tight chambers containing moistened paper towels. Prehybridization solution was rinsed off in 2× SSC, and sections were dehydrated in ascending ethanols and air-dried prior to hybridization.

Hybridization solution was applied to the sections and hybridization proceeded overnight at 50°C. The sections were then washed twice for 15 min in 1× SSC, 50% formamide, 0.1% 2-mercaptoethanol (2-ME) at 50 °C, then RNase A digested (20 µg/ml) for 30 min at 37 °C, and washed 30 min in 1× SSC, 0.1% 2-ME (50 °C). Final high stringency washes consisted of two 30 min washes in 0.1× SSC, 1% 2-ME (50 °C). The sections were then dehydrated in alcohols and air-dried.

The slides were then dipped in Kodak NTB-2 autoradiographic emulsion, dried and exposed at 4 °C for two weeks for PR and three weeks for ER, developed in Kodak D-19 developer and fixed. These developing times were chosen based on optimum silver grain density for quantification. After fixation, the sections were washed in water, stained with cresyl violet and coverslipped using Permount.

Analysis: ER- and PR-mRNA in the brain of each animal were quantified using the Grains image analysis program (Personal communication, Donald K. Clifton, University of Washington) as previously described (13). The slides were randomized and coded prior to analysis so that the identity of the slides were unknown to the investigator. The sections to be analyzed were first located using brightfield microscopy. Care was taken to insure that the same cell populations for a given region were analyzed for all brains. Analysis of each region began on an anatomically matched section for each brain and proceeded rostrally. Due to the small size of the lizard brain, the area analyzed for most brain regions was localized to only one or two sections and could be encompassed in two to four fields of view. Silver grains clustered over cell somata were systematically counted using the computerized image analysis system utilizing darkfield microscopy with a 40× objective. Each cluster appearing in the field of view was counted before changing the field of view. This was repeated until a minimum number of labeled cells for each area was counted; 20 cells for the ventromedial hypothalamus and 10 cells for the periventricular nucleus of the preoptic area, medial preoptic area, amygdala, anterior hypothalamus, dorsal hypothalamus, periventricular nucleus of the hypothalamus, lentiformis thalami pars plicta, and torus semicircularis. Once the minimum number of cells were counted, the remainder of the clusters on that field of view was counted and the analysis was then complete. Background was calculated by counting silver grains over ten cells in adjacent areas of the same sections. After decoding, the mean number of silver grains per cluster for each animal was determined by subtracting the mean background silver grains per cell from the mean grains per cell in the area of interest.

Radioimmunoassay

Radioimmunoassay was performed on plasma samples from *C. uniparens* to determine the circulating concentration of estradiol in different stages of vitellogenesis. Plasma samples from animals at similar stages of ovarian development were pooled because the circulating concentration of estradiol in *C. uniparens* is too low to be reliably measured in the small volume of plasma collected from individual animals in this experiment (between 30 and 100 µl per animal). Three stages comprising the preovulatory cycle of follicular development were assessed. Animals with vitellogenic follicles smaller than 4 mm (n = 4) were combined to represent the early preovulatory (EPRE) stage, those with follicles between 4 mm and 7 mm (n = 6) were combined to represent the middle preovulatory (MPRE) stage, and those with follicles greater than 7 mm (n = 6) were combined to represent the late preovulatory (LPRE) stage. The pooled samples were assayed using standard radioimmunoassay techniques according to an established assay protocol (14). Briefly, each pooled plasma sample was spiked with approximately 1000 cpm of tritiated estradiol for extraction efficiency calculation. Samples were then extracted twice with diethyl ether. The ether extract, containing the estradiol, was dried under a stream of nitrogen and reconstituted in 300 µl of assay buffer. Fifty microliters of each sample were counted to determine extraction efficiency; 100 µl samples were assayed in the radioimmunoassay. Each sample was assayed in duplicate in a single assay. Intra-assay covariation was 96% for the assay.

Statistical analysis

The data obtained from the analysis of the *in situ* hybridization were analyzed in a two-way ANOVA to determine the effects of ovarian condition and species. Probability values less than 0.05 were considered statistically significant.

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