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Distribution of Androgen and Estrogen Receptor mRNA in the Brain and Reproductive Tissues of the Leopard Gecko, *Eublepharis macularius*

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ABSTRACT

Incubation temperature during embryonic development determines gonadal sex in the leopard gecko, *Eublepharis macularius*. In addition, both incubation temperature and gonadal sex influence behavioral responses to androgen and estrogen treatments in adulthood. Although these findings suggest that temperature and sex steroids act upon a common neural substrate to influence behavior, it is unclear where temperature and hormone effects are integrated. To begin to address this question, we identified areas of the leopard gecko brain that express androgen receptor (AR) and estrogen receptor (ER) mRNA. We gonadectomized adult female and male geckos from an incubation temperature that produces a female-biased sex ratio and another temperature that produces a male-biased sex ratio. Females and males from both temperatures were then treated with equivalent levels of various sex steroids. Region-specific patterns of AR mRNA expression and ER mRNA expression were observed upon hybridization of radiolabeled (^{35}S) cRNA probes to thin sections of reproductive tissues (male hemipenes and female oviduct) and brain. Labeling for AR mRNA was very intense in the epithelium, but not within the body, of the male hemipenes. In contrast, expression of ER mRNA was prominent in most of the oviduct but not in the luminal epithelium. Within the brain, labeling for AR mRNA was conspicuous in the anterior olfactory nucleus, the lateral septum, the medial preoptic area, the periventricular preoptic area, the external nucleus of the amygdala, the anterior hypothalamus, the ventromedial hypothalamus, the premammillary nucleus, and the caudal portion of the periventricular nucleus of the hypothalamus. Expression of ER mRNA was sparse in the septum and was prominent in the ventromedial hypothalamus, the caudal portion of the periventricular nucleus of the hypothalamus, and a group of cells near the torus semicircularis. Many of these brain regions have been implicated in the regulation of hormone-dependent, sex-typical reproductive and agonistic behaviors in other vertebrates. Consequently, these nuclei are likely to control such behaviors in the leopard gecko and also are candidate neural substrates for mediating temperature effects on behavior. J. Comp. Neurol. 437:385–397, 2001.

Indexing terms: androgens; estrogens; hypothalamus; sex behavior; temperature-dependent sex determination

Sex steroid hormones activate various gender-typical reproductive and agonistic behaviors in the adult leopard gecko, *Eublepharis macularius* (Rhen and Crews, 1999, 2000). For example, the display of male-typical scent-marking, courtship, and mounting behaviors depends on elevated levels of androgens in the circulation. Likewise, female-typical receptive behavior is activated by estrogen treatment. However, there are also gender differences in behavioral responses to hormone treatments in adulthood

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that strongly suggest a developmental influence of sex steroids on the brain. In fact, sex differences in circulating levels of testosterone (T), dihydrotestosterone (DHT), and 17β-estradiol (E2) are evident shortly after leopard geckos hatch and persist throughout postnatal life (Sakata et al., 1998). Like what is seen in many mammals, adult male leopard geckos are more responsive than adult females to the activational effects of androgens on copulatory behavior and are relatively insensitive to the activational effects of estrogens on sexual receptivity (Rhen and Crews, 1999, 2000). Hence, sex steroids play a central role in the differentiation of behavior both during development and in adulthood in the leopard gecko. Although these findings indicate that sexual differentiation of brain and behavior, at least in part, is conserved evolutionarily among amniotic vertebrates (reptiles, birds, and mammals), there are aspects of sexual differentiation in the leopard gecko that are not observed in birds and mammals.

Incubation temperature during embryonic development determines gonadal sex in the leopard gecko as it does in some other lizards, many turtles, and all crocodilians (Ewert et al., 1994; Lang and Andrews, 1994; Viets et al., 1994). In the leopard gecko, low temperatures (26°C) and high temperatures (34°C) produce females almost exclusively, a temperature of 30°C produces a female-biased sex ratio (≈1 male:3 females), and a temperature of 32.5°C produces a male-biased sex ratio (≈3 males:1 female). It is interesting to note that embryonic temperature also influences behavioral responses to adult hormone treatments. Mounting and scent-marking behaviors, for instance, are affected by an interaction between gonadal sex and incubation temperature (Rhen and Crews, 1999, 2000). Females from the female-biased (i.e., 30°C) or male-biased (i.e., 32.5°C) incubation temperatures do not mount other females or scent mark even when they are treated with androgens that activate these behaviors in males. In contrast, incubation temperature influences behavior in males, such that males from the male-biased temperature scent mark significantly more than males from the female-biased temperature when they are treated with the same levels of DHT or T. Conversely, and across various hormone treatments, males from the female-biased temperature mount significantly more than males from the male-biased temperature.

Although the mechanism by which embryonic temperature influences sensitivity to sex steroids later in life is unknown, the finding that temperature and gender interact to influence certain hormone-dependent behaviors in adulthood suggests that embryonic temperature and sex steroids act upon a common neural substrate. The anterior hypothalamus-preoptic area (AH-POA), for instance, controls hormone-dependent, male-typical sex behavior in all amniotic vertebrates studied (Crews and Silver, 1985). Moreover, this region of the brain contains distinct populations of temperature-sensitive and/or steroid-sensitive neurons in the rat (Silva and Boulant, 1986) and plays a critical role in thermoregulation in both reptiles and mammals (Cabanac et al., 1967; Heath et al., 1968; Boulant, 1981). In the red-sided garter snake, elevated androgens in the autumn prime the male for courtship behavior, but hibernation at cool temperatures in the winter and increasing temperatures in the spring are required to activate the AH-POA and the display of male courtship behavior (Krohmer and Crews, 1987). Taken together, these observations suggest that the hypothalamus plays a conserved role in the integration of thermal and hormonal signals in vertebrates.

In short, gender differences in levels of sex steroids during postnatal development appear to regulate the differentiation of behavior in the leopard gecko. Sex steroids also have activational effects on behavior in adult leopard geckos. Finally, incubation temperature during embryonic development alters behavioral responsiveness to sex steroids in adulthood. Nevertheless, there have been no studies to date of either androgen or estrogen receptors in the brain of the leopard gecko. Consequently, we have mapped the distribution of androgen receptor (AR) and estrogen receptor (ER) mRNA using in situ hybridization histochemistry. Androgen- and estrogen-responsive peripheral tissues were included in this study as positive controls for AR and ER mRNA expression. By first identifying hormone-responsive regions of the gecko brain, we hope to identify candidate nuclei that may integrate sex steroid and temperature effects on reproductive and agonistic behaviors in the leopard gecko.

MATERIALS AND METHODS

Animals

Animals were treated according to a research protocol approved by the Institutional Animal Care and Use Committee at the University of Texas. The methods used to produce the animals for this experiment have been described in detail previously (Rhen and Crews, 1999, 2000). Briefly, leopard gecko eggs from the captive breeding colony at the University of Texas were collected and candled for fertility. Fertile eggs were placed in individual cups filled with moist vermiculite (1 part water:1 part vermiculite) and split between two constant incubation temperatures (i.e., 30.0°C and 32.5°C ± 0.1°C) that produce female- and male-biased sex ratios, respectively. Geckos hatched from these eggs were raised in isolation for 49–52 weeks as described previously (Flores et al., 1994). Leopard geckos reach sexual maturity at roughly 40–45 weeks of age (Tousignant et al., 1995; our unpublished observations).

A total of 78 adult males and 93 adult females from each incubation temperature were gonadectomized while under cold anesthesia. At the same time, these animals were implanted with Silastic tubing (1.47 mm inner diameter and 1.95 mm outer diameter) containing cholesterol, E2, DHT, or T. A subset of brains from 4 males and 4 females from each incubation temperature and hormone treatment group (a total of 64 geckos) were used for in situ hybridization. Animals were allowed 4 weeks to recover after surgery/implantation. Experimental geckos were then tested with female or male stimulus animals for 5 minutes on each of 6 consecutive days. On days 1–3, behavior tests used sexually receptive females. On days 4–6, behavior tests used sexually active males. Results of these behavior tests have been described in detail previously (see Rhen and Crews, 1999, 2000) and are summarized above and below (see Discussion) in this paper. After behavior testing was completed, a blood sample was taken to confirm hormone delivery. The procedures and data for the radioimmunoassay analyses also have been described in detail previously (see Rhen and Crews, 1999). Briefly, treatment with Silastic capsules containing E2, DHT, and T elevated plasma levels of these hormones above the

levels observed in geckos treated with cholesterol. The steroid levels produced by these implants were also in the normal physiological ranges for intact males and/or females. More importantly, treatment with a given steroid resulted in equivalent levels of hormones in gonadectomized female and male leopard geckos from each embryonic temperature. Consequently, our experimental manipulations separated the normally confounding effects of embryonic temperature and gonadal sex on adult sex steroid physiology and behavior. After blood samples were drawn, the animals were killed by rapid decapitation, their brains were immediately removed, frozen in isopentane, and stored at −80°C until sectioning.

Frozen, 10-μm-thick sections were melted onto RNAse-free, polylysine-coated microscope slides (Sigma, St. Louis, MO). Sections from experimental animals were placed on a series of slides for hybridization to different probes. Sections were allowed to dry at room temperature and then stored in slide boxes at −80°C. The brain and hemipenes from an intact male at the 32.5°C temperature were used as positive control tissues for AR mRNA expression. The brain and oviduct from an intact, vitellogenic female at the 30°C temperature were used as positive control tissues for ER mRNA expression. Three sets of slides from these control animals were used for hybridization to labeled antisense probe. Another three sets were used for hybridization to labeled sense probe. Two sets of slides were hybridized to labeled antisense probe in the presence of 100-fold excess unlabeled antisense probe. A final two sets of slides were used for hybridization to labeled antisense probe after pretreatment with RNAse A (20 μg/ml for 30 minutes).

RNA extraction, cDNA synthesis, and polymerase chain reaction cloning

Reproductively active female leopard geckos were killed, and their oviducts were dissected for the isolation of total RNA using the RNAgents Total RNA Isolation System (Promega, Madison, WI). The poly (A) mRNA fraction was then separated with the PolyATtract mRNA Isolation System (Promega). Purified mRNA was used in reverse transcription reactions with oligo (dT) or gene-specific degenerate primers.

One microliter of the reaction product(s) from the first strand cDNA synthesis was then used as a template for one round of polymerase chain reaction (PCR), which was conducted according to the manufacturer's protocol (SuperScript PCR Kit; Gibco BRL, Gaithersburg, MD). A 2-μl aliquot of the first round PCR reaction was then used as a template for a second round of PCR. In both rounds, the PCR mixture was heated to 94°C, Taq polymerase was added, and the temperature was maintained for 3 minutes. The PCR mixture was then subjected to extension at 72°C for 3 minutes, denaturation at 94°C for 30 seconds, and primer annealing at 55°C for 1 minute for a total of 30 cycles. The specific primers used for PCR have been used previously to clone fragments of the AR and ER in two lizard species and a turtle (Young et al., 1994; Bergeron et al., 1998).

PCR products of the expected size were excised from a 1.5% low-melting-temperature agarose gel for ligation into a cloning vector. Gel slices were melted at 70°C for 10 minutes and cooled to 37°C. Subsequently, 6 μl of the liquid gel were removed and added to a tube containing 1 μl of 10 × ligation buffer, pH 8.5; 2 μl of pCR 2.1 cloning vector (25 ng/ml); and 1 μl of T4 ligase. The solution was mixed, placed on ice, and then incubated at 15°C for 24 hours. After ligation of the PCR product into the cloning vector, *E. coli* cells were transformed and selected using the Original TA Cloning Kit (Invitrogen, La Jolla, CA).

Cells from 10–12 transformed colonies were then grown in 10 ml LB medium. Plasmids were purified for insert sequencing using minipreps (GeniePrep; Ambion). Sequencing verified that the inserts were indeed the target cDNAs (see Results, below). Maxipreps were then used to purify sufficient amounts of plasmid for use as templates in probe synthesis. For production of antisense and sense probes, two plasmids with oppositely oriented cDNA inserts were prepared for both AR and ER.

Probe preparation and hybridization

Radiolabeled (^{35}S-CTP; NEN, Boston, MA) antisense and sense cRNA probes were synthesized by in vitro transcription using T7 RNA polymerase. Unlabeled antisense cRNA probes were synthesized in an identical manner, with the exception that cold CTP replaced ^{35}S-CTP in the transcription reaction. Probes were separated from unincorporated nucleotides by ethanol precipitation in ammonium acetate. Resuspended cRNA probes were heat denatured at 85°C and added to hybridization solution at a final concentration of 0.3 μg probe × length in kb per ml. For the unlabeled competitor control, an additional 100-fold excess of competing unlabeled antisense probe was added to the hybridization solution. The hybridization solution consisted of 50% deionized formamide; 10% dextran sulfate; 0.3 M NaCl; 10 mM Tris HCl, pH 8.0; 1 mM ethylenediamine tetraacetic acid (EDTA); 1 × Denhardt's solution; 10 mM dithiothreitol; and 0.5 mg/ml tRNA.

Prior to prehybridization, tissues were fixed in 4% paraformaldehyde, pH 7.2, for 20 minutes; rinsed in 3 × phosphate-buffered saline (PBS), pH 7.0, for 5 minutes; rinsed twice for 5 minutes (10 minutes total) in 1 × PBS, pH 7.0; dipped in RNAse-free water; and washed in fresh 0.1 M triethanolamine, pH 8.0, and 0.25% acetic anhydride for 10 minutes. Tissues were then rinsed in 2 × standard saline citrate (SSC; 0.3 M sodium chloride and 0.03 M sodium citrate, pH 7.0); dehydrated in 70%, 95%, and 100% ethanol for 3 minutes each; delipidated in chloroform for 5 minutes; rinsed in 100% and 95% ethanol; and air dried.

Tissues were prehybridized overnight at 45°C in hybridization solution in which the cRNA probe was replaced with tRNA at a final concentration of 2.5 mg/ml. Prehybridization took place in plastic containers containing moist paper towels. Tissues were rinsed clean of the prehybridization solution using 2 × SSC for 3 minutes. Tissues were then dehydrated in 70% and 95% ethanol for 3 minutes each and air dried.

Hybridization solution was then applied to the tissues and incubated overnight at 45°C. After hybridization, tissues were washed twice for 15 minutes each at 50°C in 1 × SSC, 50% formamide, and 0.1% β-mercaptoethanol. Tissues were then RNAse A (20 μg/ml) digested in a buffer, pH 7.0, consisting of 10 mM Tris HCl, 100 mM sodium chloride, and 2 mM EDTA for 30 minutes at 37°C. RNAse A was then washed off using the same buffer for 30 minutes at 37°C. Two consecutive washes for 15 minutes each at 50°C were done in 1 × SSC, 50% formamide, and 0.1% β-mercaptoethanol. Two washes for 30 minutes each at 50°C were then done in 0.1 × SSC and 1%

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        10         20         30         40         50         60
ATGACCCTTGGAGCCCGCAAACTGAAGAAACTGGGCAACCTGAAGATGCAGGAGGAAGGA
TACTGGGAACCTCGGGCGTTTGACTTCTTTGACCCGTTGGACTTCTACGTCCTCCTTCCT

        70         80         90        100        110        120
GAAGCGGCCGGGCCTTCAAGCCCCACAGAGGAGCAAGCCCCAAAGATGACCATGACCCGT
CTTCGCCGGCCCGGAAGTTCGGGGGTGTCTCCTCGTTCGGGGGTTTCTACTGGTACTGGGCA

       130        140        150        160        170        180
ATCGAGAGCCTGGAATGCCAGCCCATCTTCCTCAATGTCCTGGAAGCCATTGAGCCCGGT
TAGCTCTCGGACCTTACGGTCGGGTAGAAGGAGTTACAGGACCTTCGGTAACTCGGGCCA

       190        200        210        220        230        240
GTGGTTTGTGCGGGCCACGACAACAACCAGCCTGACTCCTTCGCTGCCCTGCTGACCAGC
CACCAAACACGCCCGGTGCTGTTGTTGGTCGGACTGAGGAAGCGACGGGACGACTGGTCG

       250        260        270        280        290        300
TTGAATGAGCTTGGTGAGAGGCAGCTGGTGCATGTGGTGAAATGGGCCAAAGCCTTACCA
AACTTACTCGAACCACTCTCCGTCGACCACGTACACCACTTTACCCGGTTTCGGAATGGT

       310        320        330        340        350        360
GGATTCCGTAACCTGCATGTGGATGACCAAATGGCAATCATTCAGTACTCCTGGATGGGT
CCTAAGGCATTGGACGTACACCTACTGGTTTACCGTTAGTAAGTCATGAGGACCTACCCA

       370
CTGATGGTCTTTGC
GACTACCAGAAACG
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Fig. 1. Nucleotide sequence for a fragment of the leopard gecko androgen receptor.

β-mercaptoethanol. Final washes were with 50% and then 85% ethanol and ammonium acetate for 3 minutes each and 100% ethanol for 3 minutes. Tissues were dried and dipped in Kodak NTB-2 autoradiographic emulsion (Eastman Kodak, Rochester, NY), dried, and exposed at 4°C for 12 days or for 15 days for ER and AR mRNA, respectively. The slides were developed in Kodak D-19 developer, fixed, rinsed in water, stained in cresyl violet, and coverslipped using Permount (Fisher Scientific, Fair Lawn, NJ). Tissues were examined using darkfield and brightfield microscopy, and images were captured digitally. Brightness and contrast were adjusted using Adobe Photoshop software (version 5.0; Adobe Systems, Mountain View, CA) to improve image quality.

RESULTS

The nucleotide and predicted amino acid sequences for the leopard gecko AR fragments are shown in Figures 1 and 2, respectively. The nucleotide and predicted amino acid sequences for the leopard gecko ER fragments are shown in Figures 3 and 4, respectively. Comparisons of the predicted amino acid sequences with the amino acid sequences of whiptail lizard, chicken, rat, and human AR and ER indicated high sequence homology in both the DNA and the ligand-binding domains (Figs. 2, 4). Conversely, there was more interspecific variation in amino acid sequence within the hinge region.

In situ hybridization validation

The distribution of AR and/or ER mRNA in the brain, hemipenes, and oviduct of the leopard gecko was determined by examining tissues hybridized to radiolabeled antisense probes. Specificity of labeling for AR and ER mRNA was verified by comparing tissues hybridized to radiolabeled antisense probe with tissues hybridized to radiolabeled sense probe. In addition, specific labeling by antisense probe was eliminated in adjoining tissue sections by competitive hybridization with 100-fold excess of unlabeled probe or pretreatment with RNAse A.

AR and ER mRNA distribution in positive control tissues

Expression of AR mRNA was extremely high in the epithelial layer of male hemipenes but was much lower within the body of the hemipenes (Fig. 5). This pattern of tissue-specific expression was evident throughout the hemipenes. AR mRNA expression also was high through the entire length of the female oviduct. Labeling for AR mRNA was especially prominent in the luminal epithelium of the oviduct (Fig. 6). Although expression of ER mRNA was very low or absent from the luminal epithelium, ER mRNA was colocalized with AR mRNA in the myometrium of the oviduct (Fig. 6).

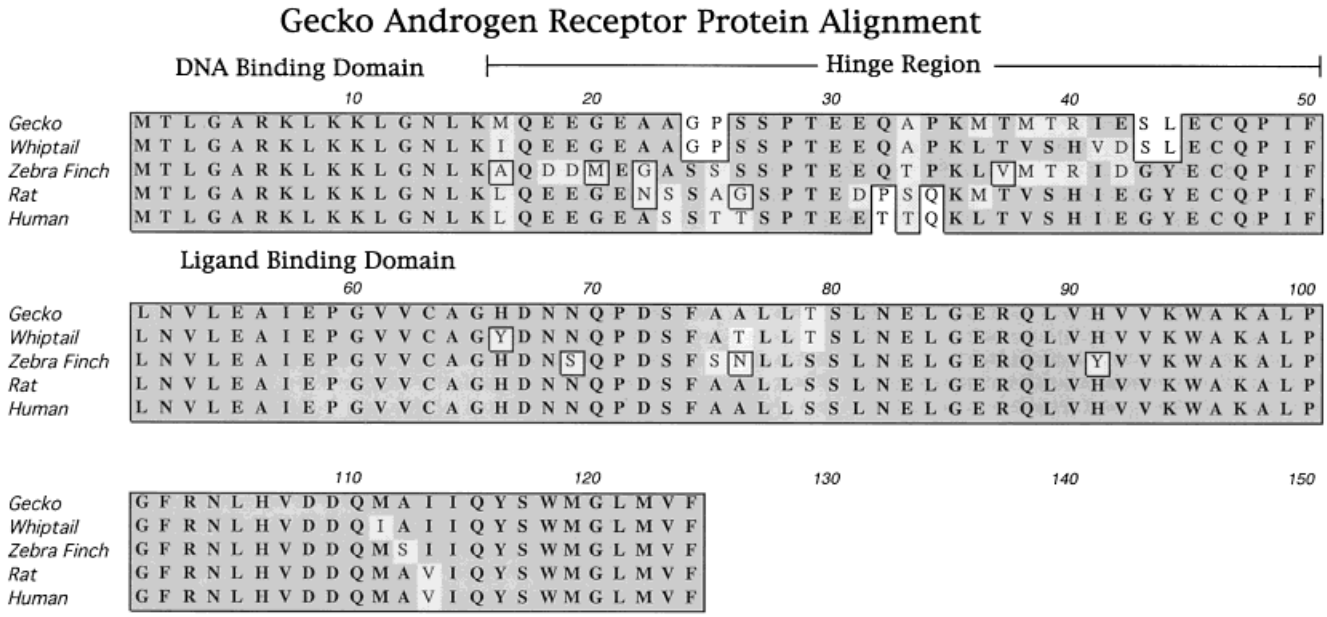


Fig. 2. Comparison between the predicted amino acid sequence for the leopard gecko androgen receptor and the whiptail lizard, zebra finch, rat, and human androgen receptors.

AR mRNA distribution in the brain

The pattern of AR mRNA expression was consistent in all geckos, regardless of their embryonic incubation temperature, gonadal sex, or adult hormone treatment. AR mRNA expression of moderate intensity was distributed fairly evenly in the anterior olfactory nucleus (Fig. 7A). AR mRNA also was expressed at a moderate level within the lateral septal nuclei (Fig. 7B). Labeling was more intense but was concentrated over fewer cells within the medial preoptic area and the periventricular preoptic area (Fig. 7B). Expression of AR mRNA within the lateral septal nuclei extended caudally to the level of the external nucleus of the amygdala, where labeling was especially strong (Fig. 7C). AR mRNA also was expressed in the anterior hypothalamus (Fig. 7D). Much like cells in the medial preoptic area, cells within the ventromedial hypothalamus were labeled intensely (Fig. 7E). Labeling in the ventromedial hypothalamus was concentrated in the dorsolateral aspect and continued more caudally to the level of the premammillary nucleus (Fig. 7F). There was also distinct labeling for AR mRNA in the caudal portion of the periventricular nucleus of the hypothalamus (Fig. 7F). A summary of the nuclei that expressed AR mRNA is shown in a line drawing of a sagittal section from the leopard gecko brain in Figure 9 (shaded regions).

ER mRNA distribution in the brain

The pattern of ER mRNA expression was consistent in all geckos, regardless of their embryonic incubation temperature, gonadal sex, or adult hormone treatment. However, the distribution of ER mRNA was less extensive relative to the distribution of AR mRNA. ER mRNA expression was found in the septum, the ventromedial hypothalamus, the caudal portion of the periventricular nucleus of the hypothalamus, and a group of cells near the tectum. Cells labeled for ER mRNA were scattered throughout the rostral portion of the septum (Fig. 8A) but were not concentrated within any particular septal nucleus (e.g., AR mRNA was localized to the lateral septal nuclei). The ventromedial hypothalamus also expressed ER mRNA at moderate levels (Fig. 8B). In the caudal portion of the diencephalon, labeling for ER mRNA appeared more intense in the periventricular nucleus of the hypothalamus (Fig. 8C). A cluster of cells in the mesencephalon near the rostral portion of the torus semicircularis also expressed ER mRNA at high levels (Fig. 8D). A summary of the nuclei that expressed ER mRNA is shown in a line drawing of a sagittal section from the leopard gecko brain in Figure 9 (cross-hatched regions).

DISCUSSION

Previous studies have demonstrated that androgens and estrogens activate sexual and agonistic behavior in the adult leopard gecko, *Eublepharis macularius* (Rhen and Crews, 1999, 2000). However, developmental differences between the sexes were also evident: Males and females behave differently even when treated with similar levels of the same sex steroids in adulthood. Such gender differences in behavioral responses to hormone treatments are most likely organized by gender differences in circulating levels of T, DHT, and/or E2 prior to reproductive maturity (Sakata et al., 1998). Because steroid hormones regulate gene expression through cognate intracellular receptors, we set out to identify putative AR- and ER-containing regions of the leopard gecko brain. For the most part, the neuroanatomical distribution of AR and ER mRNA-containing cells in this species is similar to that reported in other vertebrates (Simerly et al., 1990; Young et al., 1994). Likewise, the predicted amino acid sequences for fragments of the gecko AR and ER are very similar to their homologs in another lizard species, the zebra finch,

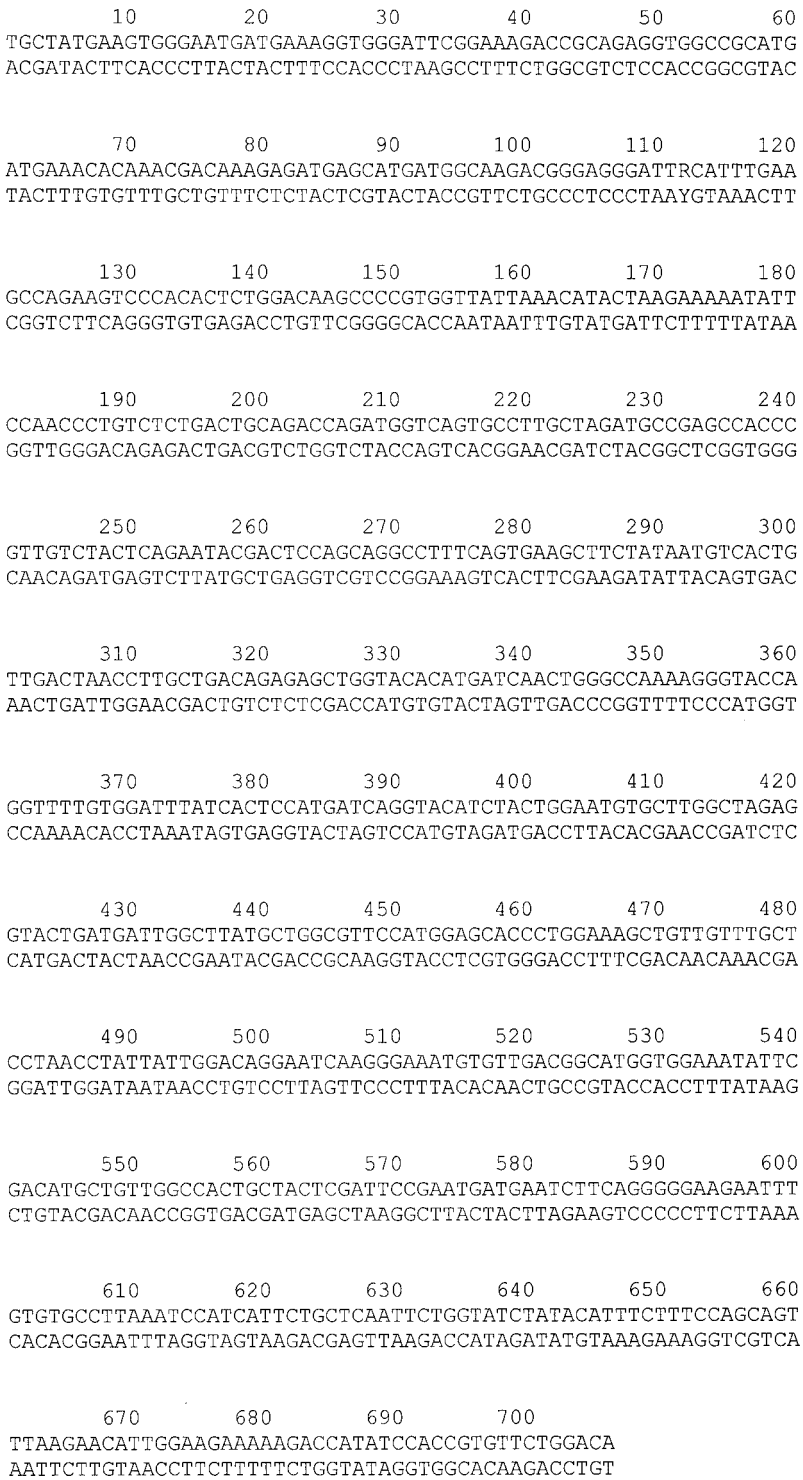


Fig. 3. Nucleotide sequence for a fragment of the leopard gecko estrogen receptor alpha.

the rat, and the human. This conservation of gene structure and tissue-specific gene expression reflects the primary role that sex steroids and their receptors play in the regulation of various reproductive functions, including behavior.

In addition to these evolutionarily conserved effects of sex steroids on gecko comportment, we have shown that embryonic incubation temperature alters behavioral responses to androgen and estrogen treatments in adulthood (Rhen and Crews, 1999, 2000). For example, an

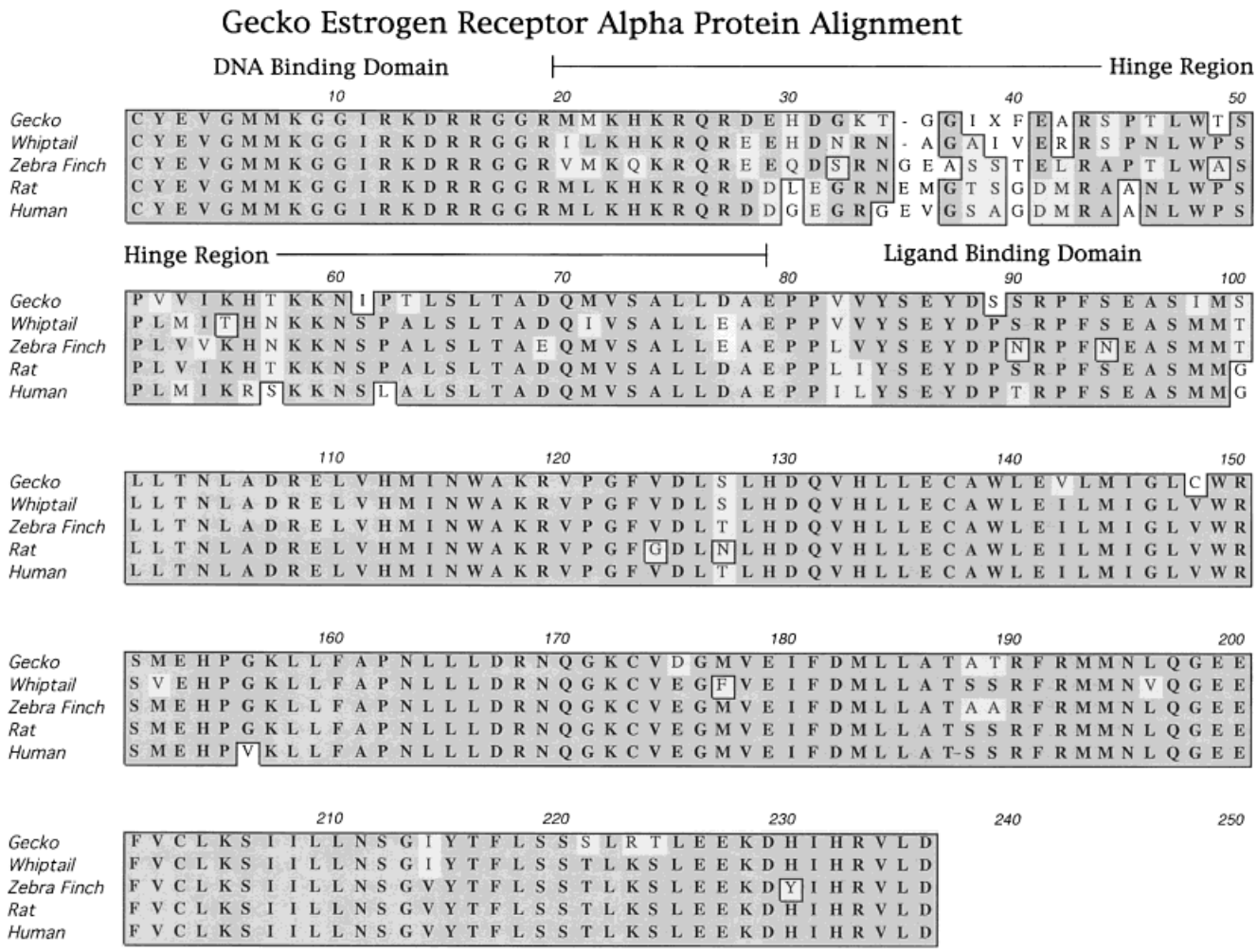


Fig. 4. Comparison between the predicted amino acid sequence for the leopard gecko estrogen receptor alpha and the whiptail lizard, zebra finch, rat, and human estrogen receptor alphas.

interaction between incubation temperature and gonadal sex influences male-typical scent-marking and mounting behaviors. Females do not mount other females or scent mark regardless of their incubation temperature (or their hormone treatment in adulthood). In contrast, incubation temperature strongly influences these behaviors in males. Males from the male-biased temperature scent mark significantly more than males from the female-biased temperature when treated with equivalent levels of androgens. On the other hand, males from the female-biased temperature mount significantly more than males from the male-biased temperature. Such findings suggest that temperature and sex steroids act upon a common neural substrate (or substrates) during development. Therefore, another goal of the current study was to identify candidate, hormone-responsive nuclei where temperature and sex steroid effects on behavior may be integrated. Below, we discuss the potential behavioral relevance of AR and ER expression within the leopard gecko brain.

Expression of AR mRNA in the anterior olfactory nucleus of the leopard gecko suggests that androgens may influence how leopard geckos initially perceive and then behave toward conspecifics (Rhen and Crews, 2000). In fact, pheromones are particularly important cues for sex recognition and act as strong releasers of sexual and agonistic behaviors in the leopard gecko (Mason and Gutzke, 1990; Cooper and Steele, 1997; Steele and Cooper, 1997; Rhen and Crews, 2000). Moreover, the accessory olfactory bulb, which initially processes pheromonal information, has projections to the anterior olfactory nucleus in another lizard species (Martinez-Garcia et al., 1991). These observations are consistent with the finding that the anterior olfactory nucleus contains AR mRNA in the rat (Simerly et al., 1990) and is important for the processing of pheromonal cues from other individuals in the rat and in sheep (Armstrong and Brunjes, 1997; Jansen et al., 1998). In contrast, there was no detectable expression of ER mRNA in the anterior olfactory nucleus of the leopard gecko. This is in accord with the low levels of ER mRNA found in this nucleus in the rat (Simerly et al., 1990).

Both AR mRNA and ER mRNA were expressed in the septum of the leopard gecko, just as they are in the rat (Simerly et al., 1990). However, there were receptor-specific patterns of expression in the leopard gecko that

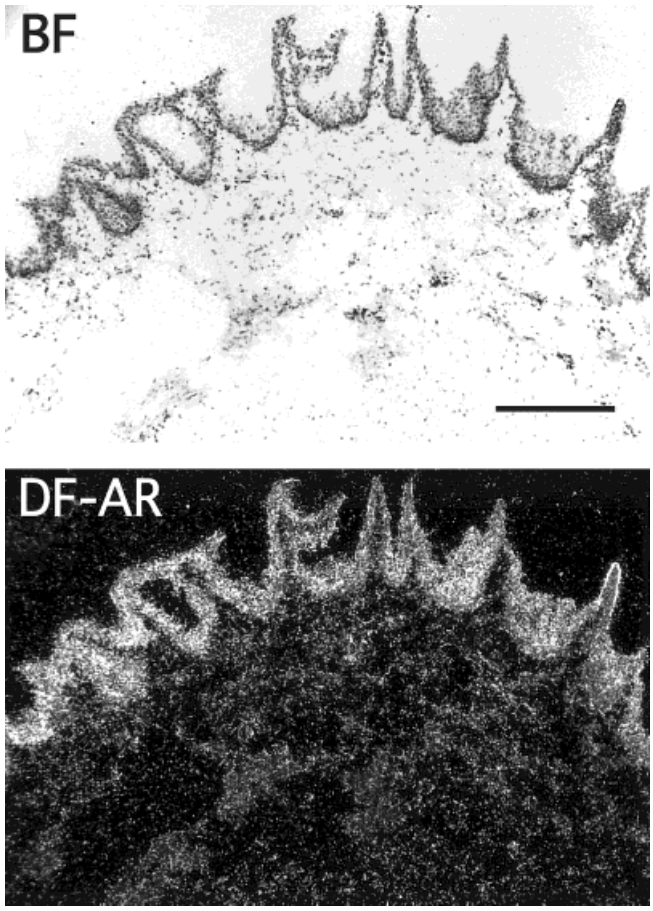


Fig. 5. Brightfield (BF; top) and darkfield (DF; bottom) images of a male leopard gecko hemipenis hybridized to androgen receptor (AR) antisense probe. Scale bar = 250 μm.

were more similar to what is found in the whiptail lizard (Young et al., 1994). Whereas AR mRNA was concentrated and expressed at a high level in the lateral septal nuclei, ER mRNA was expressed in a few cells distributed sparsely throughout the septum. In other words, septal ER mRNA was not localized to any particular cell mass or nuclei. The functional significance of sex steroid receptors in the septum is unknown in the leopard gecko. Nevertheless, data from other species suggest that the lateral septum may modulate agonistic interactions. For example, this brain region is involved in the regulation of aggressive behaviors in both birds and mammals (Goodson et al., 1999; Sheehan and Numan, 2000). There are also sex differences in vasopressinergic innervation of the lateral septum in rats that correlate with the effect of vasopressin on aggressive behavior (Sheehan and Numan, 2000). A comparable sexually dimorphic pattern of vasotocinergic innervation within the lateral septum in the Tokay gecko, *Gecko gecko* (Stoll and Voorn, 1985), raises the possibility that androgens and vasotocin act and possibly interact to produce sex differences in aggression in the leopard gecko. Because the lateral septum, androgens, and vasopressin have been implicated in flank-marking behavior in rodents (Sheehan and Numan, 2000), it will be particularly interesting to determine whether the septum is involved in androgen-dependent scent-marking behavior in the leopard gecko (Rhen and Crews, 1999). In this regard, androgens activate scent marking in a sex-dependent and incubation temperature-dependent manner: Only males from a male-biased incubation temperature scent mark when treated with T or DHT.

Labeling for AR mRNA also was observed in brain regions that have been implicated consistently in the regulation of male-typical copulatory behavior in other vertebrates. AR mRNA was expressed at a high level within the medial preoptic area, the periventricular preoptic area, and the anterior hypothalamus, which is concordant with our previous finding that androgens activate copulatory behavior and tail vibrations (a male-typical courtship behavior) in the leopard gecko (Rhen and Crews, 1999). Male garter snakes that display courtship behavior accumulate more 2-deoxyglucose in the AH-POA than noncourting males, which presumably reflects differences in neural activity associated with mating (Allen and Crews, 1992). The premammillary nucleus also was labeled intensely for AR mRNA in the leopard gecko. The function of the premammillary nucleus has not been defined in the gecko, but it contains AR and is strongly activated during male-typical copulatory behavior in the rat (Yokosuka et al., 1997; Greco et al., 1998). The medial preoptic area, the periventricular preoptic area, the anterior hypothalamus, and the premammillary nucleus did not contain detectable ER mRNA, even though exogenous estrogen activated tail vibrations in male leopard geckos (Rhen and Crews, 1999). Region-specific hormone implants and lesions should help define the exact function of each of these nuclei in various aspects of male sex behavior. It also will be interesting to examine these areas for incubation temperature-induced differences in neurochemistry, because males from the female-biased incubation temperature are more sexually active than males from the male-biased incubation temperature.

Expression of AR mRNA within the external nucleus of the amygdala was especially strong, just as it is in the rat (Simerly et al., 1990). In contrast to the rat, however, ER mRNA expression was absent from the external nucleus of the amygdala in the leopard gecko. This receptor-specific pattern of expression is very much like that previously reported in whiptail lizards (Young et al., 1994). The conserved nature of AR expression in the external nucleus of the amygdala reflects an apparently conserved function in processing pheromonal information and mediating aggressive behavior (Greenberg et al., 1984). Testosterone, for example, activates aggression when it is implanted directly within the amygdala of gonadectomized male mice (Simon et al., 1996).

In addition, the external amygdala receives direct projections from the main olfactory bulb in garter snakes and from the accessory olfactory bulb in the lizard *Podarcis hispanica* (Martinez-Garcia et al., 1991; Lanuza and Halpern, 1997, 1998). The nucleus sphericus, which receives a direct projection from the accessory olfactory bulb in the garter snake, also projects its axons to the external amygdala (Lanuza and Halpern, 1997, 1998; Martinez-Marcos et al., 1999). Various parts of the amygdaloid complex, including the external amygdala, project in turn to the ventromedial hypothalamus in a lizard and in garter snakes (Bruce and Neary, 1995; Lanuza et al., 1997). Although the nucleus sphericus receives projections from the accessory olfactory bulb in squamates in general (Lo-

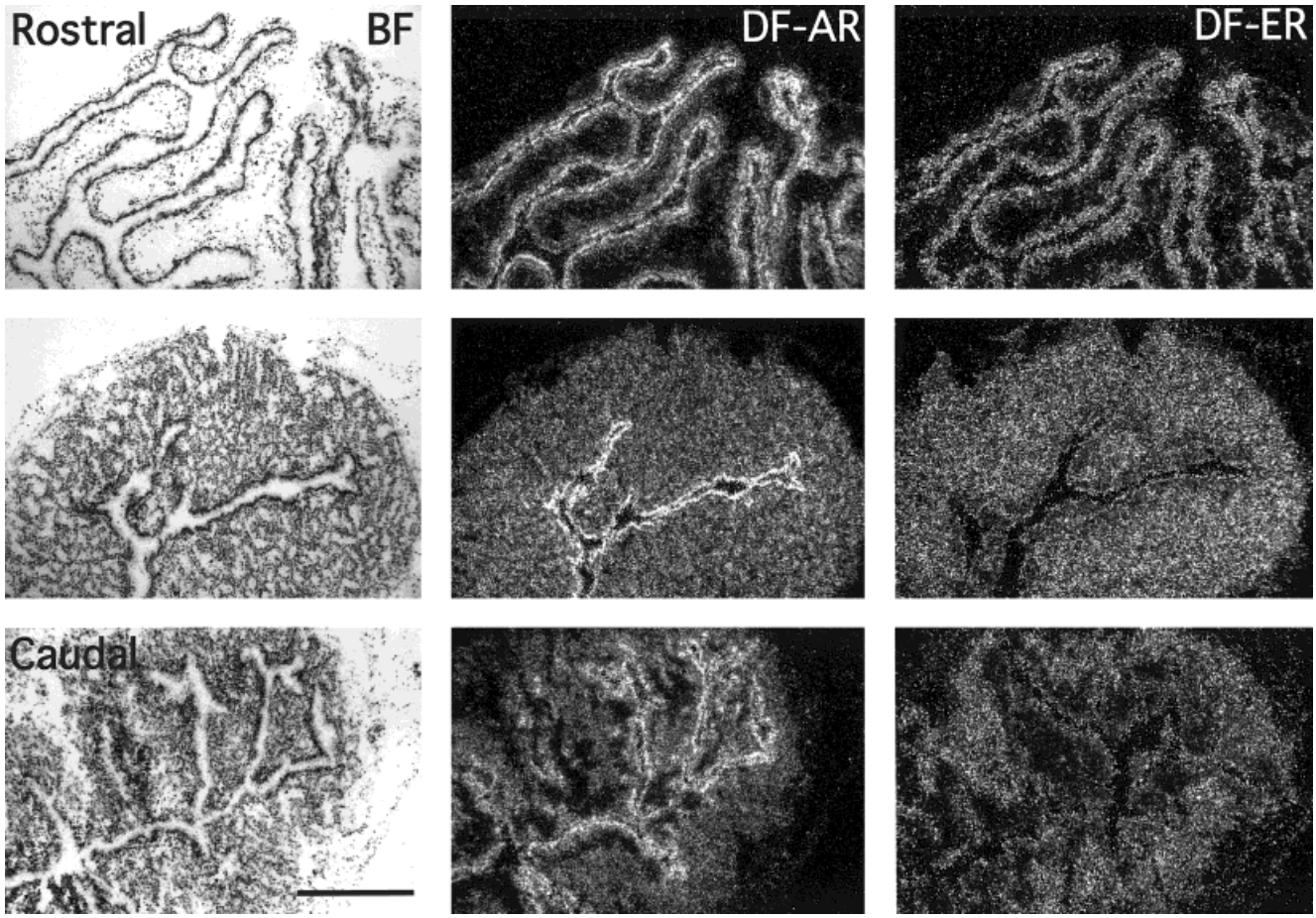


Fig. 6. Brightfield (left column) and darkfield images of a female leopard gecko oviduct hybridized to AR (middle column) and estrogen receptor (ER; right column) antisense probe. Anterior-to-posterior sections of the oviduct are arranged from top to bottom. Scale bar = 250 μm.

hman and Smeets, 1993), it was not labeled for AR or ER mRNA in the leopard gecko or the whiptail lizard (Young et al., 1994; this study). Moreover, the nucleus sphericus only has a small projection to the lateral hypothalamus and no direct connections to the ventromedial hypothalamus in garter snakes (Lanuza and Halpern, 1997). Consequently the amygdaloid complex is in an excellent position to integrate both olfactory and vomeronasal information and then relay that information to the hypothalamus.

Our finding that the ventromedial hypothalamus, a region critical for the display of female-typical sex behavior in other vertebrates, expresses both AR mRNA and ER mRNA in the leopard gecko is consistent with our previous finding that androgens and estrogens activate receptive behavior in female leopard geckos (Rhen et al., 1999; Rhen and Crews, 2000). Although both types of receptors are found in this region of the brain, there are distinct patterns of expression within subregions of the ventromedial hypothalamus. Whereas AR mRNA expression was apparent in cells throughout the dorsolateral aspect of the ventromedial hypothalamus, ER mRNA expression was not concentrated in this part of the ventromedial hypothalamus but was more homogenous throughout the nucleus. In the whiptail lizard, a species with estrogen-dependent receptivity, ER is restricted to the dorsolateral aspect of the ventromedial hypothalamus (Young et al., 1994). In addition, intracranial E2 implants into this subregion stimulate receptivity, whereas lesions of this subregion abolish receptivity (Wade and Crews, 1991; Kendrick et al., 1995). Additional differences in receptor expression were apparent in the caudal portion of the periventricular nucleus of the hypothalamus and farther back in a group of cells near the torus semicircularis. The latter nuclei contained dense concentrations of cells labeled for ER mRNA but were not labeled for AR mRNA.

Nonneural tissues known to be responsive to androgens and estrogens also expressed AR and ER mRNA. Specifically, the hemipenes of an intact male and the oviduct of an intact, vitellogenic female were included as positive controls for sex steroid receptor expression. AR mRNA was expressed at a high level in the epithelial layer of the male hemipenes but was virtually absent from the body of this organ. A comparable tissue-specific pattern of AR expression is observed in sexually mature male rats: There is high AR expression in the skin and urethra, but not the body, of the penis (Takane et al., 1991). In contrast, AR expression is high in all tissues of the immature

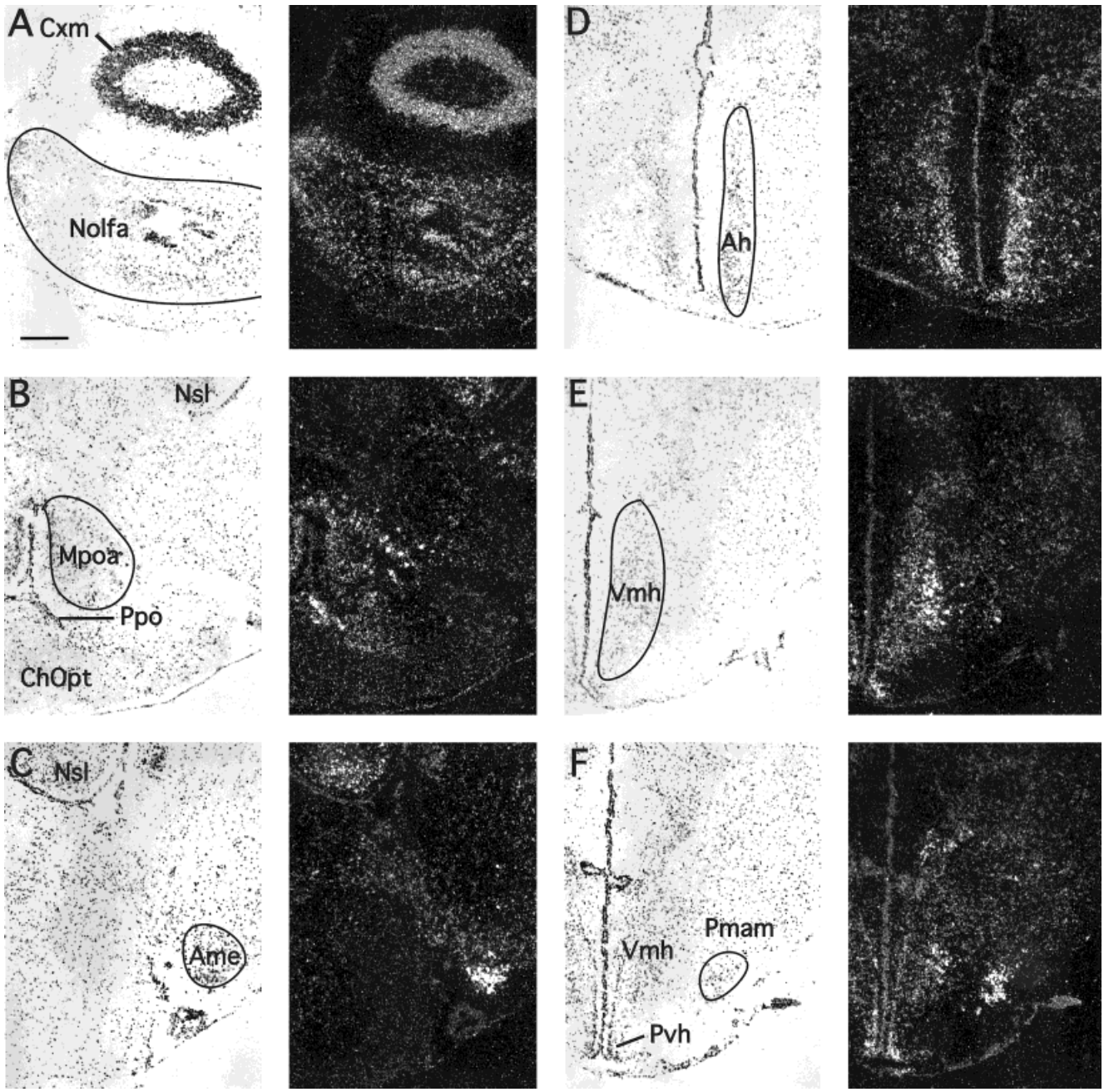
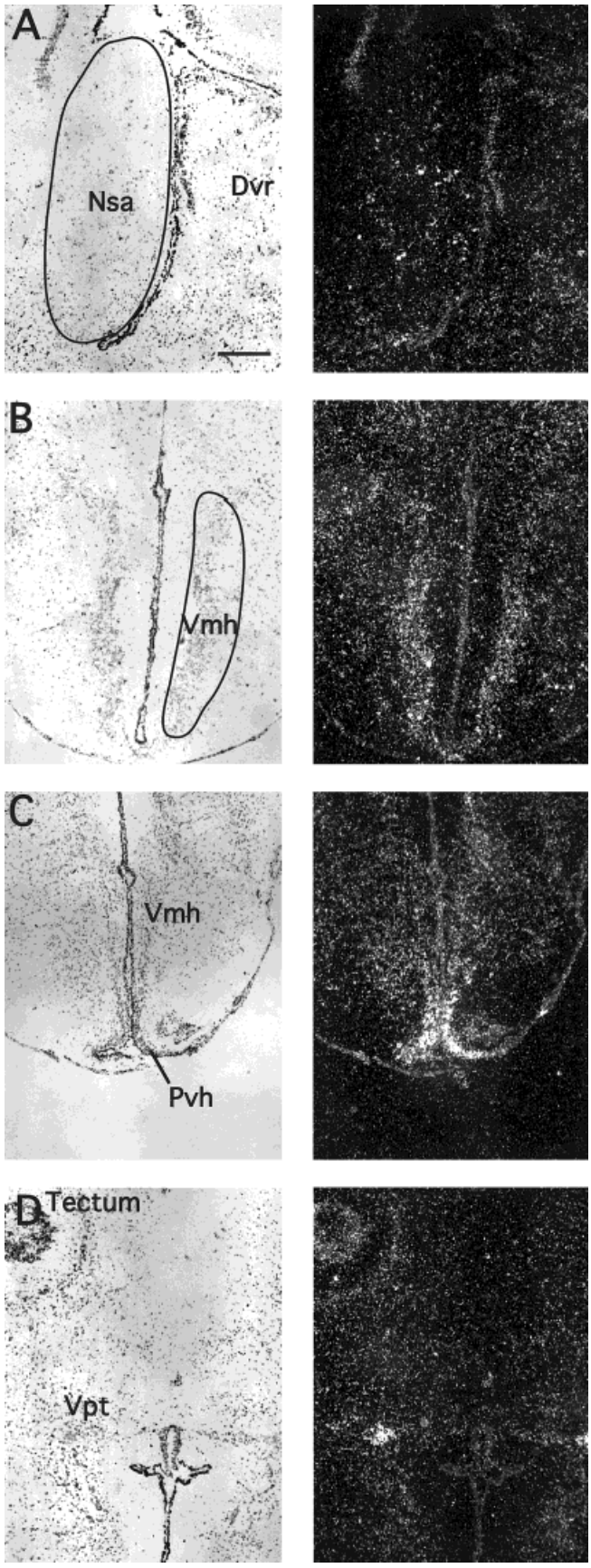


Fig. 7. Brightfield (left columns) and darkfield (right columns) images of coronal sections of the leopard gecko brain hybridized to AR antisense probe. **A:** Medial cortex (Cxm) and anterior olfactory nucleus (Nolfa). **B:** Lateral septal nucleus (Nsl), medial preoptic area (Mpoa), periventricular preoptic area (Ppo), and optic chiasm (ChOpt). **C:** Lateral septal nucleus (Nsl) and external nucleus of the amygdala (Ame). **D:** Anterior hypothalamus (Ah). **E:** Ventromedial hypothalamus (Vmh). **F:** Ventromedial hypothalamus (Vmh), premammillary nucleus (Pmam), and caudal portion of the periventricular nucleus of the hypothalamus (Pvh). Scale bar = 250 μm.

rat penis but decreases within the body of the penis as the male rat attains sexual maturity and circulating levels of androgens rise. It would be interesting to determine whether developmental changes in AR expression parallel changes in androgen levels in the leopard gecko (Sakata et al., 1998). In fact, hemipenile development is entirely under the activational influence of androgens: Long-term castration results in almost complete regression of the hemipenes in males, and androgen treatment induces the development of fully evertable hemipenes in sexually mature females (Rhen et al., 1999).

In contrast to the male hemipenes, high levels of AR mRNA expression were observed in all parts of the female leopard gecko oviduct. Nevertheless, the most intense labeling for AR mRNA was found in the epithelial layer of the oviduct. Androgens probably regulate at least some



aspects of oviduct function, because there are enormous changes in circulating androgen levels during the female reproductive cycle (Rhen et al., 2000). Testosterone levels are low during the previtellogenic stage, increase slightly during early vitellogenesis, increase dramatically during late vitellogenesis, and fall to previtellogenic levels after ovulation. Although circulating levels of DHT are lower than circulating levels of T, DHT shows the same general pattern of changes across the reproductive cycle. This preovulatory surge in androgen levels, along with the finding that AR mRNA expression is high in the oviduct, suggests that androgens may regulate oviduct functions like egg shell formation. In fact, AR is expressed in most tissue types in the chicken oviduct, and androgens appear to play a role in growth and differentiation of the oviduct (Yu and Marquardt, 1973a,b; Joensuu et al., 1992).

Estrogens also are likely to influence oviduct development and function in the gecko, because there is high ER mRNA expression in the female reproductive tract. Moreover, there are cyclic changes in estrogen levels, such that the concentration of E2 is low during the previtellogenic stage, increases gradually during early and late vitellogenic stages, and finally falls to previtellogenic levels after ovulation. Considering the correlated but not perfectly concordant changes in androgen and estrogen levels during the female reproductive cycle, it will be important to examine how both types of hormones act and interact to regulate differentiation of the oviduct. For example, estrogen stimulation of epithelial cell proliferation in the rat uterus is mediated by ER in the stroma, which produces a paracrine signal to the epithelial cells (Cooke et al., 1997). Progesterone, which rises later in the cycle, then regulates decidualization.

Before we conclude, it is important to note that we did not quantify levels of AR and ER mRNA as a function of incubation temperature, gender, or adult hormone treatments. Although some laboratories, including our own, have quantified results from in situ hybridization experiments, there are two problems with trying to quantify results from the present study. First, we used brains from 4 animals in each experimental group (there were 16 groups in all). Consequently, statistical power to detect group differences would be low. There were, however, a very large number of slides from those animals, which precluded running all of the slides in a single in situ hybridization. Thus, two hybridizations were done with an equal number of animals from each group in each hybridization. The fact that in situ hybridizations can be quite variable makes it difficult to compare quantitatively results from the two experiments. In sum, these factors make any statements about potential differences among groups uncertain. Hence, we have chosen to be conservative and simply describe the distribution of AR and ER mRNA within the gecko brain. The pattern of sex steroid

Fig. 8. Brightfield (left column) and darkfield (right column) images of coronal sections of the leopard gecko brain hybridized to ER antisense probe. **A:** Anterior region of the septum (Nsa) and dorsal ventricular ridge (Dvr). **B:** Ventromedial hypothalamus (Vmh). **C:** Ventromedial hypothalamus (Vmh) and the caudal portion of the periventricular nucleus of the hypothalamus (Pvh). **D:** A cell group called the ventral pretectal nucleus (Vpt) near the tectum. Scale bar = 250 μm.

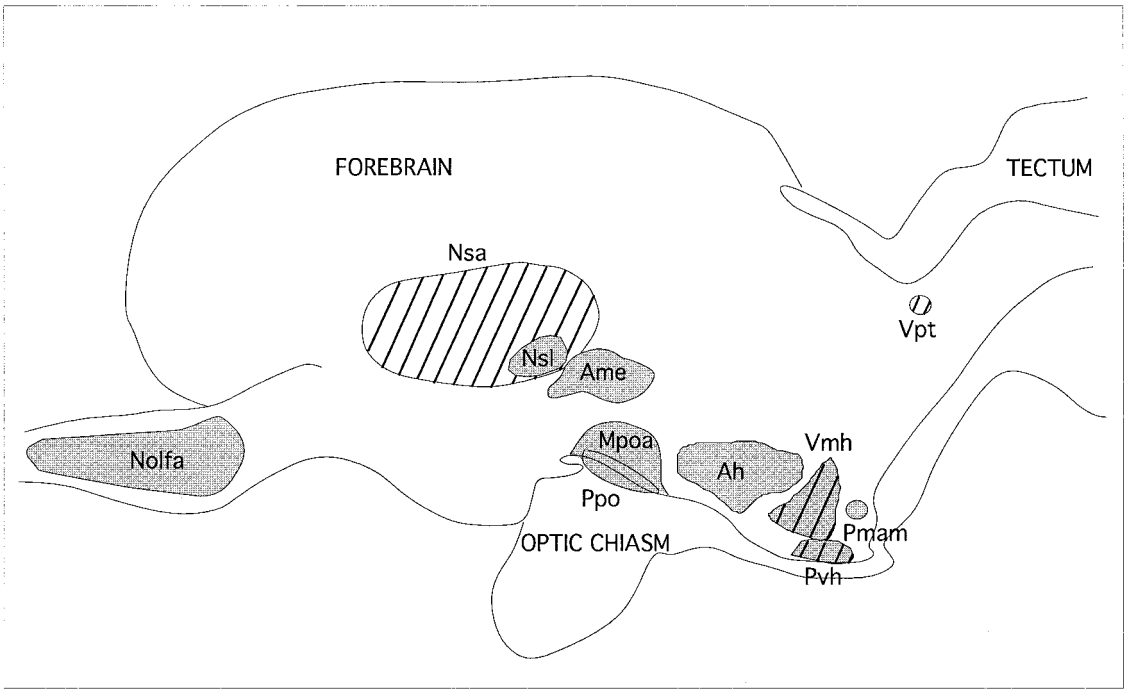


Fig. 9. Line drawing of a sagittal section of the leopard gecko brain. Nuclei expressing AR mRNA are shaded with gray. Nuclei expressing ER mRNA are cross hatched. For abbreviations, see Figures 7 and 8.

receptor expression was generally consistent, with no gross differences among experimental groups. Nevertheless there were clear and consistent regional differences in levels of mRNA expression; i.e., compare the AR mRNA expression in the lateral septum with that in the external nucleus of the amygdala in Figure 7C. Such differences raise the possibility that one may be able to detect incubation temperature, gender, and hormone treatment effects on AR and ER mRNA levels with larger sample sizes. This will be an important hypothesis to test in the future, because temperature-induced and gender differences in behavior potentially may be mediated by differences in receptor levels within hormone-responsive brain nuclei.

In conclusion, the neuroanatomical distribution of AR and ER mRNA in the leopard gecko is in accord with the pattern of sex steroid receptor mRNA distribution found in other vertebrates (Simerly et al., 1990; Young et al., 1994). In the leopard gecko, clear labeling for AR mRNA was observed in the anterior olfactory nucleus, the lateral septal nuclei, the medial preoptic area, the periventricular preoptic area, the external nucleus of the amygdala, the anterior hypothalamus, the ventromedial hypothalamus, and the premammillary nucleus. It is important to note that labeling for AR mRNA in these areas coincides perfectly with AR protein expression as detected by immunohistochemistry in another lizard species (Moga et al., 2000). Expression of ER mRNA was observed in the septum, the ventromedial nucleus of the hypothalamus, the caudal portion of the periventricular nucleus of the hypothalamus, and a cell group near the torus semicircularis. Such fundamental information on the heterogeneous expression of AR and ER mRNA in the brain, in conjunction with the tissue-specific pattern of AR and ER mRNA expression in reproductive organs, provides the foundation for future studies of the functional significance of sex steroid receptors in these tissues. For example, studies might examine whether the AH-POA and the external nucleus of the amygdala, respectively, mediate the activational effects of sex steroids on male-typical reproductive and aggressive behaviors. It will also be critical to determine how androgens and estrogens control the expression of female-typical sex behavior at the level of the ventromedial hypothalamus, because both types of hormones induce receptivity in female geckos. This is a significant question, because AR and ER mRNA display distinct but somewhat overlapping distributions in the ventromedial hypothalamus. Moreover, it should be possible now to ascertain systematically whether any of these brain regions integrate embryonic incubation temperature and sex steroid effects on behavior.

LITERATURE CITED

Allen EE, Crews D. 1992. Sexual behavior and 2-deoxyglucose uptake in male red-sided garter snakes (*Thamnophis sirtalis parietalis*). Brain Behav Evol 40:17–24.

Armstrong AM, Brunjes PC. 1997. Immunohistochemical changes in the anterior olfactory nucleus of the developing rat. Dev Psychobiol 31: 181–192.

Bergeron JM, Gahr M, Horan K, Wibbels T, Crews D. 1998. Cloning and in situ hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. Dev Growth Diff 40:243–254.

Boulant JA. 1981. Hypothalamic mechanisms in thermoregulation. Fed Proc 40:2843–2850.

Bruce LL, Neary TJ. 1995. Afferent projections to the ventromedial hypothalamic nucleus in a lizard, *Gecko gecko*. Brain Behav Evol 46:14–29.

Cabanac M, Hammel HT, Hardy JD. 1967. *Tiliqua scincoides*. Temperature sensitive units in lizard brain. Science 158:1050–1051.

Cooke PS, Buchanan DL, Young P, Setiawan T, Brody J, Korach KS, Taylor J, Lubahn DB, Cunha GR. 1997. Stromal estrogen receptors mediate mitogenic effects of estradiol on uterine epithelium. Proc Natl Acad Sci USA 94:6535–6540.

Cooper WE Jr., Steele LJ. 1997. Pheromonal discrimination of sex by male and female leopard geckos (*Eublepharis macularius*). J Chem Ecol 23:2967–2977.

Crews D, Silver R. 1985. Reproductive physiology and behavior interactions in nonmammalian vertebrates. In: Adler N, Pfaff D, Goy RW, editors. Handbook of behavioral neurobiology, vol 7. New York: Plenum Press. p 101–182.

Ewert MA, Jackson D, Nelson C. 1994. Patterns of temperature-dependent sex determination in turtles. J Exp Zool 270:3–15.

Flores D, Tousignant A, Crews D. 1994. Incubation temperature affects the behavior of adult leopard geckos (*Eublepharis macularius*). Physiol Behav 55:1067–1072.

Goodson JL, Eibach R, Sakata J, Adkins-Regan E. 1999. Effect of septal lesions on male song and aggression in the colonial zebra finch (*Taeniopygia guttata*) and the territorial field sparrow (*Spizella pusilla*). Behav Brain Res 98:167–180.

Greco B, Edwards DA, Zumpe D, Clancy AN. 1998. Androgen receptor and mating-induced fos immunoreactivity are co-localized in limbic and midbrain neurons that project to the male rat medial preoptic area. Brain Res 781:15–24.

Greenberg N, Scott M, Crews D. 1984. Role of the amygdala in the reproductive and aggressive behavior of the lizard, *Anolis carolinensis*. Physiol Behav 32:147–151.

Heath JE, Gasdorf E, Northcutt RG. 1968. The effect of thermal stimulation of anterior hypothalamus on blood pressure in the turtle. Comp Biochem Physiol 26:509–518.

Jansen HT, Iwamoto GA, Jackson GL. 1998. Central connections of the ovine olfactory bulb formation identified using wheat germ agglutinin-conjugated horseradish peroxidase. Brain Res Bull 45:27–39.

Joensuu TK, Blauer MK, Sannisto T, Tuohimaa PJ. 1992. The effect of dihydrotestosterone on the estrogen-induced cytodifferentiation of the chick oviduct. J Steroid Biochem Mol Biol 41:701–709.

Kendrick AM, Rand MS, Crews D. 1995. Electrolytic lesions to the ventromedial hypothalamus abolish receptivity in female whiptail lizards, *Cnemidophorus uniparens*. Brain Res 680:226–228.

Kromer RW, Crews D. 1987. Temperature activation of courtship behavior in the male red-sided garter snake (*Thamnophis sirtalis parietalis*): role of the anterior hypothalamus-preoptic area. Behav Neurosci 101: 228–236.

Lang JW, Andrews HV. 1994. Temperature-dependent sex determination in crocodilians. J Exp Zool 270:28–44.

Lanuza E, Halpern M. 1997. Afferent and efferent connections of the nucleus sphericus in the snake *Thamnophis sirtalis*: convergence of olfactory and vomeronasal information in the lateral cortex and the amygdala. J Comp Neurol 385:627–640.

Lanuza E, Halpern M. 1998. Efferents and centrifugal afferents of the main and accessory olfactory bulbs in the snake *Thamnophis sirtalis*. Brain Behav Evol 51:1–22.

Lanuza E, Font C, Martinez-Marcos A, Martinez-Garcia F. 1997. Amygdalo-hypothalamic projections in the lizard *Podarcis hispanica*: a combined anterograde and retrograde tracing study. J Comp Neurol 384:537–555.

Lohman AHM, Smeets WJAJ. 1993. Overview of the main and accessory olfactory bulb projections in reptiles. Brain Behav Evol 41:147–155.

Martinez-Garcia F, Olucha FE, Teruel V, Lorente MJ, Schwerdtfeger WK. 1991. Afferent and efferent connections of the olfactory bulbs in the lizard *Podarcis hispanica*. J Comp Neurol 305:337–347.

Martinez-Marcos A, Lanuza E, Halpern M. 1999. Organization of the ophidian amygdala: chemosensory pathways to the hypothalamus. J Comp Neurol 412:51–68.

Mason RT, Gutzke WHN. 1990. Sex recognition in the leopard gecko, *Eublepharis macularius* (Sauria: Gekkonidae) possible mediation by skin-derived semiochemicals. J Chem Ecol 16:27–36.

Moga MM, Geib BM, Zhou D, Prins GS. 2000. Androgen receptor-immunoreactivity in the forebrain of the Eastern fence lizard (*Sceloporus undulatus*). Brain Res 879:174–182.

Rhen T, Crews D. 1999. Embryonic temperature and gonadal sex organize male-typical sexual and aggressive behavior in a lizard with temperature-dependent sex determination. Endocrinology 140:4501–4508.

Rhen T, Crews D. 2000. Organization and activation of sexual and agonistic behavior in the leopard gecko, *Eublepharis macularius*. Neuroendocrinology 71:252–261.

Rhen T, Ross J, Crews D. 1999. Effects of testosterone on sexual behavior and morphology in adult female leopard geckos, *Eublepharis macularius*. Hormones Behav 36:119–128.

Rhen T, Sakata JT, Zeller M, Crews D. 2000. Sex steroid levels across the reproductive cycle of female leopard geckos, *Eublepharis macularius*, from different incubation temperatures. Gen Comp Endocrinol 118: 322–331.

Sakata JT, Rhen T, Crews D. 1998. Ontogeny of secondary sex structures and gonadal steroids in the leopard gecko. Annual meeting of the Society for Integrative and Comparative Biology, Denver Colorado [abstract 86A]. Am Zool 38:297.

Sheehan T, Numan M. 2000. The septal region and social behavior. In: Numan R, editor. The behavioral neuroscience of the septal region. New York: Springer-Verlag. p 175–209.

Silva NL, Boulant JA. 1986. Effects of testosterone, estradiol, and temperature on neurons in preoptic tissue slices. Am J Physiol 250:R625–R632.

Simerly RB, Chang C, Muramatsu M, Swanson LW. 1990. Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an in situ hybridization study. J Comp Neurol 294:76–95.

Simon NG, McKenna SE, Lu SF, Cologer-Clifford A. 1996. Development and expression of hormonal systems regulating aggression. Ann NY Acad Sci 794:8–17.

Steele LJ, Cooper WE Jr. 1997. Investigations of pheromonal discrimination between conspecific individuals by male and female leopard geckos (*Eublepharis macularius*). Herpetologica 53:475–484.

Stoll CJ, Voorn P. 1985. The distribution of hypothalamic and extrahypothalamic vasotocinergic cells and fibers in the brain of a lizard, *Gecko gecko*: presence of a sex difference. J Comp Neurol 239:193–204.

Takane KK, Husmann DA, McPhaul MJ, Wilson JD. 1991. Androgen receptor levels in the rat penis are controlled differently in distinctive cell types. Endocrinology 128:2234–2238.

Tousignant A, Viets B, Flores D, Crews D. 1995. Ontogenetic and social factors affect the endocrinology and timing of reproduction in the female leopard gecko (*Eublepharis macularius*). Hormones Behav 29: 141–153.

Viets B, Ewert MA, Talent LG, Nelson CE. 1994. Sex determining mechanisms in squamate reptiles. J Exp Zool 270:45–56.

Wade J, Crews D. 1991. The effects of intracranial implantation of estrogen on receptivity in sexually and asexually reproducing female whiptail lizards, *Cnemidophorus inornatus* and *Cnemidophorus uniparens*. Hormones Behav 25:342–353.

Yokosuka M, Prins GS, Hayashi S. 1997. Co-localization of androgen receptor and nitric oxide synthase in the ventral premammillary nucleus of the newborn rat: an immunohistochemical study. Dev Brain Res 99:226–233.

Young LJ, Lopreato G, Horan K, Crews D. 1994. Cloning and in situ hybridization of estrogen receptor, progesterone receptor, and androgen receptor expression in the brain of whiptail lizards (*Cnemidophorus uniparens* and *C. inornatus*). J Comp Neurol 347:288–300.

Yu JY, Marquardt RR. 1973a. Synergism of testosterone and estradiol in the development and function of the magnum from the immature chicken (*Gallus domesticus*) oviduct. Endocrinology 92:563–572.

Yu JY, Marquardt RR. 1973b. Interaction of estradiol and testosterone in the regulation of growth and development of the chicken (*Gallus domesticus*) oviduct. Comp Biochem Physiol B 44:769–777.