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Species Differences in Behavioral and Neural Sensitivity to Estrogen in Whiptail Lizards: Correlation with Hormone Receptor Messenger Ribonucleic Acid Expression

Key Words
Gonadal steroids
Gonadal steroid receptors
Ventromedial nucleus
Sex behavior
Reptiles

Abstract
Cnemidophorus uniparens is a unisexual species of whiptail lizard of hybrid origin whereas *C. inornatus* is a sexual species and the maternal ancestor of *C. uniparens.* Together they represent an excellent model system for investigating the evolution of hormone-brain-behavior relationships. Normal circulating estradiol (E) concentrations in *C. uniparens* are approximately 5-fold lower than those of female *C. inornatus* in a similar reproductive state. Experiments were performed to determine whether (i) *C. uniparens* is more sensitive to E, and (ii) whether the difference in sensitivity is correlated with differences in estrogen receptor (ER)-mRNA expression in the brain. Dose-response curves reveal that ovariectomized *C. uniparens* are more responsive than ovariectomized *C. inornatus* to exogenous estradiol 17β-benzoate (EB). EB is more effective in *C. uniparens* at inducing receptive behavior and progesterone receptor (PR) gene expression in the ventromedial nucleus of the hypothalamus (VMH). In situ hybridization analysis of ER-mRNA expression revealed no species differences in ER-mRNA content in the VMH of ovariectomized animals. Treatment of ovariectomized animals with EB resulted in a greater induction of ER-mRNA expression in the VMH of *C. uniparens* compared to *C. inornatus.* These results indicate that the differences in behavioral sensitivity to E lie in the estrogen target neurons in the brain region controlling receptive behavior, the VMH, and that the difference in sensitivity cannot be explained by species differences in the basal rate of ER gene expression.

The general neuroendocrine mechanisms controlling reproductive behavior in vertebrates are highly conserved [1]. However, a number of genetic, environmental and social factors have resulted in the evolution of species differences in many aspects of the hormonal and neural control of reproductive behaviors [2]. Understanding the nature of species differences at the level of the brain and gene expression is essential for understanding how behaviors evolve under selective pressures. Whiptail lizards (genus *Cnemidophorus*) afford an excellent opportunity to

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investigate the mechanisms by which the brain's response to gonadal hormones may be altered to give rise to novel behaviors important for reproduction [3].

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* [4]. Although the species are genetically very similar, *C. uniparens* and *C. inornatus* differ in at least two aspects of their reproductive physiology: (i) *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 (E) concentrations in reproductively active female *C. uniparens* are approximately fivefold lower than in reproductively active female *C. inornatus* [5, 6]. It is possible that these differences are somehow interrelated.

As in many vertebrates, ovulation in the female lizard is facilitated by the courtship behavior of the male [7, 8]. The absence of males in *C. uniparens* would therefore appear to pose a reproductive dilemma for the species. *C. uniparens* has overcome this obstacle by retaining the reproductive behavior of its sexual ancestors, regularly exhibiting both female-typical receptive behavior and male-like mounting and copulatory courtship behavior [3, 9]. Comparisons of hormone-brain-behavior relationships between *C. uniparens* and female *C. inornatus* provide an opportunity to investigate the evolution of mechanisms involved in species-specific behaviors.

One possible interpretation of the lower circulating concentrations of E in vitellogenic *C. uniparens* compared to female *C. inornatus* is that individual *C. uniparens* are more sensitive to E than are female *C. inornatus*. If true, such a difference in neural sensitivity to estrogen may be relevant to the species differences in expression of male-like pseudosexual behaviors [9]. The following experiments were designed (i) to determine whether *C. uniparens* is in fact more sensitive to E, and (ii) to determine whether that difference is correlated with differences in estrogen receptor mRNA (ER-mRNA) expression in the brain.

Estrogen is effective at inducing receptive behavior in both species, presumably by acting on neurons in the ventromedial nucleus of the hypothalamus (VMH) [10]. Recently, in situ hybridization studies have demonstrated the presence of neurons expressing the messenger RNA for estrogen receptor (ER) and progesterone receptor (PR) in the VHM of both species [11]. Although the role of progesterone (P) in the induction of receptive behavior in whiptail lizards has not been investigated, P is known to act synergistically with E to facilitate receptive behavior in another lizard, the green anole [12]. Estrogen is known to increase PR concentration in the hypothalamus of a number of vertebrate species including rat [13, 14], guinea pig [15], ferret [16], chicken [17], frog [18], and the green anole lizard [19], presumably through increase in PR gene expression.

In order to compare the relative sensitivities to exogenous E for each species, we performed dose-response assays, measuring both the induction of receptive behavior and the expression of PR-mRNA in the VMH. Then, in order to determine whether the differences in sensitivity to E could be related to differences in ER gene expression in the VMH, we compared ER-mRNA concentrations in the VMH of ovariectomized and estrogen-treated lizards of both species.

Materials and Methods

Animals

Adult *C. uniparens* were captured near Portal, Ariz. and *C. inornatus* were captured near Dryden and Sanderson, Tex. After transport to the University of Texas at Austin, animals were housed in environmental chambers as previously described [10]. All animals were housed in groups of four in 29 gal aquaria prior to the onset of the experiment. Female *C. inornatus* were housed with one reproductively active male. Animals were ovariectomized as previously described [10] and housed three per 29 gal aquaria, with each of the three separated by a sheet of opaque Plexiglas. Each animal was supplied with a heat lamp, water dish, a small board for basking and cover, and sand as substrate. Animals were fed crickets or meal worms three times per week.

Ovariectomy was performed as previously described [10] using hypothermia as an anesthetic.

Hormone Treatment

A stock solution of 17β-estradiol 3-benzoate (EB) (Sigma, St. Louis, Mo., USA) was prepared by dissolving it in 95% ethanol to a final concentration of 30 μg/10 μl. This stock solution was diluted with Steroid Suspension Vehicle (SSV; National Cancer Institute, Bethesda, Md., USA) to create suspensions of the desired concentrations and stored at 4°C. One week after ovariectomy, animals were given a single subcutaneous injection above the scapula of 0, 0.002, 0.004, 0.008, 0.016, 0.031, 0.063, 0.125, 0.25 or 0.5 μg EB, each in 10 μl of SSV. Stimulus animals used in behavior tests were ovariectomized *C. uniparens* or castrated male *C. inornatus* given a subcutaneous Silastic capsule packed with crystalline testosterone (T) (Sigma) to stimulate mounting behavior as described in Wade and Crews [10].

In situ Hybridization

Tissue sections were prepared by melting 20-μm thick coronal cryosections on RNase-free polylysine-coated microscope slides and allowing them to dry at room temperature before storing in slide

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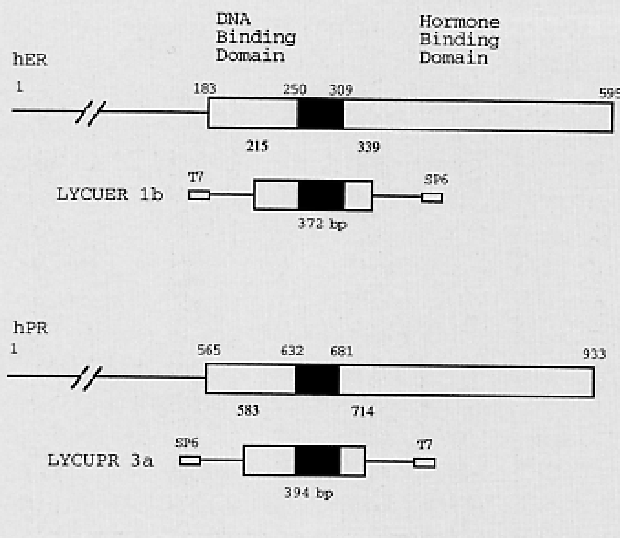

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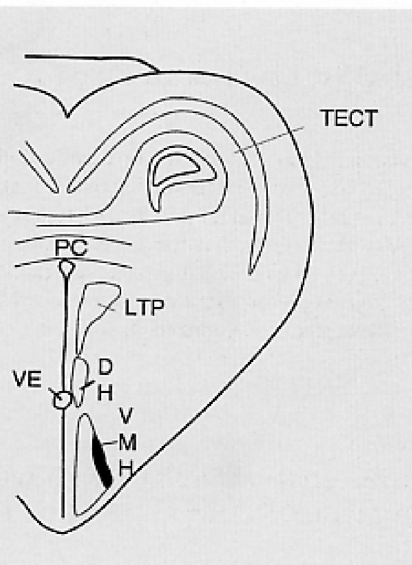


Fig. 1. Schematic diagrams showing the size and position of the *Cnemidophorus* estrogen receptor (LYCUER 1b), and progesterone receptor (LYCUPR 3a) clones relative to the full length human sequences. The structures of the human estrogen receptor (hER), and progesterone receptor (hPR) are illustrated 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 human estrogen receptor [33], and the human progesterone receptor [34].

Fig. 2. Drawing of a coronal section of a *Cnemidophorus* brain illustrating the neuroanatomical position of the ventromedial nucleus of the hypothalamus (VMH). The blackened area within the VMH represents the position of the cells that were analyzed for estrogen receptor and progesterone receptor mRNA content. DH = Dorsal hypothalamus; LTP = lentiformis thalami pars plicta; TECT = optic tectum; VE = ventricular ependymal organ; VMH = ventromedial nucleus of the hypothalamus.

boxes with desiccant at –80°C. In situ hybridization was performed using ^{35}S-radiolabeled antisense ER and PR probes prepared from the whiptail ER and PR clones, LYCUER 1b and LYCUPR 3a, respectively, as described previously [11]. The structures of these clones relative to the human structures are illustrated in figure 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 [20]. The hybridized slides were exposed to Kodak NTB-2 autoradiographic emulsion at 4°C for 2 weeks for PR and 3 weeks for ER, developed in a Kodak D-19 developer and fixed. This developing time was chosen based on optimum silver grain density for quantification. After fixation, the sections were washed in water, stained with cresyl violet, and cover slipped using Permount.

Analysis

Relative abundances of PR- and ER-mRNA in the VMH were quantified using the Grains image analysis program [pers. commun., Donald K. Clifton, University of Washington]. The slides were randomized and coded prior to analysis so that the identity of the slides was unknown to the investigator. The sections to be analyzed were first located using brightfield microscopy. The neuronal population analyzed is located in the lateral edge of the VHM, as illustrated in figure 2. For a more detailed atlas of steroid receptor expression in the *Cnemidophorus* brain, see Young et al. [11]. Analysis of each region began on an anatomically matched section for each brain. Due to the small size of the lizard brain, the area analyzed within the VMH was typically localized to only two sections and could be encompassed in approximately 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. Once the number of silver grains in each cluster in the field of view had been counted, a new field of view was analyzed. This was repeated until a minimum number of 20 cells for each animal was counted. Once the minimum number of cells was counted, the remainder of the clusters in that field of view was counted and the analysis was then complete. In brains where silver grain density was sparse over the cell populations in the VMH which normally express receptor mRNA, silver grain density was calculated over 20 cells within the boundaries of that region. Thus, the same cell population was counted for all treatment groups, regardless of the silver grain density. This analysis produced values which were consistent with the relative silver grain density as judged by visual inspection. Background was calculated by counting silver grains over ten cells immediately adjacent to the VMH 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 labeled cell. For PR the average for the duplicate slides was used in the statistical analysis.

Statistical Analysis

Analysis of receptivity data in experiment 1 was performed using the appropriate nonparametric statistic. Each animal was given a numerical score which equaled the cumulative number of tests out of the 4 posttreatment tests in which that animal was judged to be receptive. The effect of EB on receptivity in both species was examined by comparing behavior scores in each dosage group using a nonparametric analysis of variance, the Kruskal-Wallis test. To test whether the species differed in response to EB treatment, the Mann-Whitney U test was used to compare behavior scores between the species.

In experiment 2, two-way ANOVA was used to compare cellular silver grain density across treatment groups and species. In experiment 3, two-tailed t tests were used to compare silver grain density in the VMH of control and EB-treated *C. uniparens* and female *C. inornatus.*

Experiment 1: Estrogen Induction of Sexual Receptivity

Behavior Testing. Receptive behavior was determined as previously described [10] by placing the test animal into the cage of a conspecific stimulus animal, a testosterone (T)-treated castrated male for *C. inornatus,* or a T-treated ovariectomized parthenoform for *C. uniparens.* The experimental animal was classified as being receptive if it allowed the stimulus animal to mount and attempt copulation without resistance. Under these conditions, nonreceptive animals are typically aggressive to a courting stimulus animal and will roll if the stimulus animal attempts to mount it. The behavior tests were continued until the receptive status of the experimental animal was determined, typically within 3 min of testing.

Pretests began 5 days after ovariectomy and were performed on three successive days until seven days after ovariectomy. Immediately following the third pretest, experimental animals which were not receptive in pretests were given hormone or vehicle treatment (6–7 per dosage). Posttreatment behavior testing began 24 h after injection and continued daily for 4 days. The treatment of the experimental animal was unknown to the observer.

Experiment 2: Estrogen Induction of PR-mRNA in the VMH

Seven days after surgery, ovariectomized female *C. inornatus* and *C. uniparens* were given a single injection of EB solution; 0, 0.004, 0.063 or 0.5 μg as described above (6 per dosage). This design reproduced exactly the conditions used in the receptivity testing experiment. Twenty-four hours after injection, the animals were killed by rapid decapitation, and the brains were removed, frozen on dry ice and stored at –80°C until sectioning.

Experiment 3: Estrogen Receptor-mRNA Expression in the VMH

Animals and tissues were treated exactly as described in experiment 2 except that only two dosages were used, 0 and 0.5 μg EB.

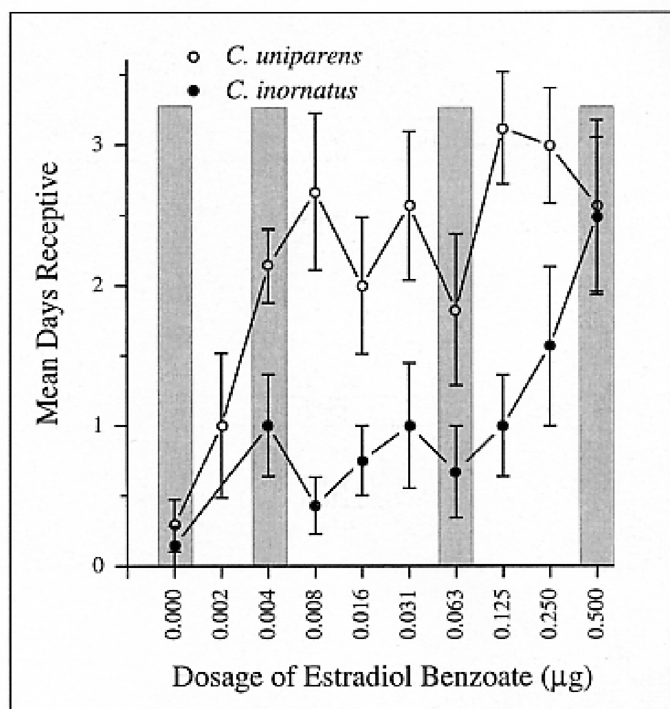


Fig. 3. Species differences in the induction of sexual receptivity by 17β-estradiol-3-benzoate (EB). One week following ovariectomy, animals were injected with various dosages of EB and tested for receptivity on 4 consecutive days. Plotted is the mean number of receptivity tests for each dosage in which the animals were receptive. Vertical bars illustrate SE: The shaded bars indicate dosages at which PR-mRNA expression was analyzed in experiment 2.

Results

Experiment 1

Treatment with EB resulted in a significant induction of receptive behavior in both *C. uniparens* and female *C. inornatus* ($p < 0.005$ and $p < 0.05$, respectively). However, EB treatment was much more effective at inducing sexual receptivity in ovariectomized *C. uniparens* compared to ovariectomized female *C. inornatus* ($p < 0.0001$) (fig. 3). Dosages as low as 0.004 μg facilitated receptive behavior in *C. uniparens* while female *C. inornatus* required up to 0.5 μg to reliably induce receptive behavior.

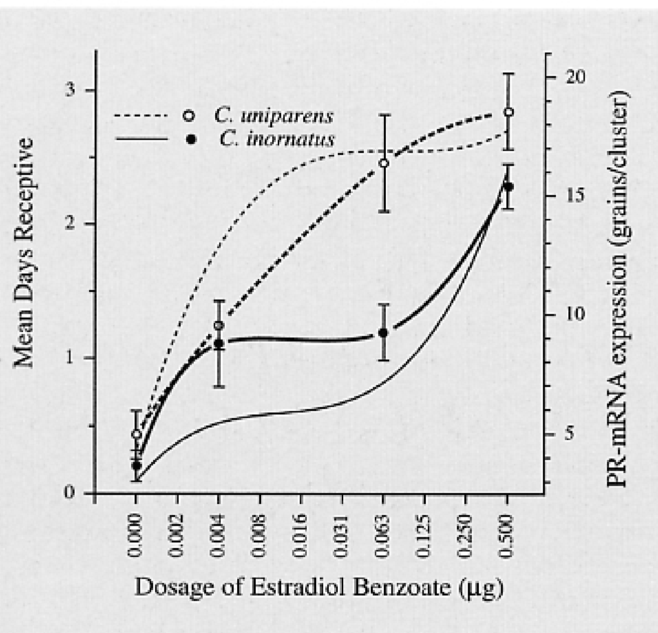
Analysis of the behavioral data indicates that EB is effective at inducing receptive behavior within 24 h after treatment. Of the animals which responded to EB treatment, the majority were receptive 24 h after the EB injection (77% for *C. uniparens* and 64% for *C. inornatus*).

Experiment 2

Treatment with EB significantly increased PR-mRNA expression in the VMH in both species as determined by silver grain density over cell somata in brain slices hybridized to radiolabeled antisense probes ($p < 0.0001$) (fig. 4). Furthermore, EB was more potent at inducing PR-mRNA expression in *C. uniparens* than in female *C. inornatus* ($p < 0.006$). However, at the highest dosage analyzed in this study (0.5 μg), PR-mRNA expression in the VMH of female *C. inornatus* was similar to that of *C. uniparens.*

Experiment 3

No differences in silver grain density were detected between ovariectomized, untreated *C. uniparens* and female *C. inornatus.* EB treatment (0.5 μg) resulted in an increase in ER-mRNA expression in the VMH in both



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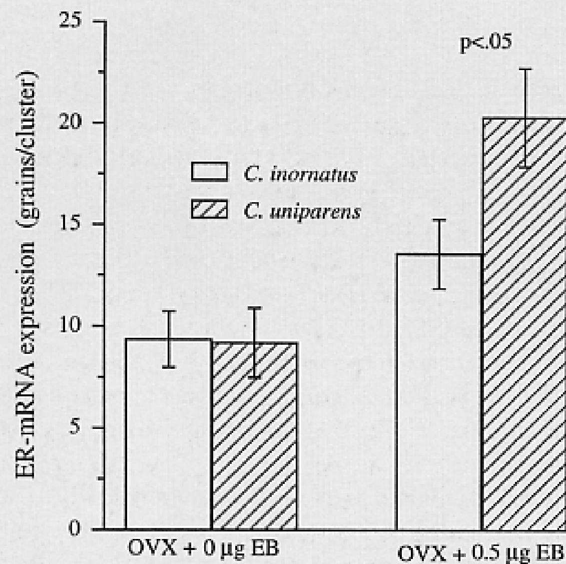

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Fig. 4. Species differences in the effects of 17β-estradiol-3-benzoate on the induction of PR-mRNA expression in the VMH (thick lines). The relative abundance of PR-mRNA was determined by analyzing the density of silver grains over sections hybridized to radiolabeled antisense PR probe. Values represent mean silver grain density clustered over cells plotted with standard error bars. Thin lines represent the receptivity data presented in figure 3.

Fig. 5. Species differences in ER-mRNA expression in the VMH. The relative abundance of ER-mRNA was determined by analyzing the density of silver grains over sections hybridized to radiolabeled antisense ER probe. Values represent mean silver grain density clustered over cells plotted with standard error bars.

species. However, the increase was statistically significant only in *C. uniparens* ($p < 0.01$) (fig. 5). Treatment with EB resulted in a greater increase in ER-mRNA levels in *C. uniparens* compared to *C. inornatus* ($p < 0.05$).

Discussion

The present study demonstrates that *C. uniparens* is more sensitive to E than female *C. inornatus*. This species difference is apparent at both the behavioral and molecular levels. The results indicate that the difference in sensitivity to E in the VMH may not be due to differences in steady state level of ER-mRNA in the absence of E. Estrogen exposure, however, results in elevated concentrations of ER-mRNA in the VMH of *C. uniparens* compared to that of *C. inornatus*. Although the present results only demonstrate a species difference in E-induced ER-mRNA concentration, this difference is likely to translate into a higher concentration of ER protein, resulting in an even greater increased sensitivity to E.

The EB-stimulated increase in ER-mRNA expression in the VMH of whiptail lizards was unexpected since EB treatment results in a decrease in ER-mRNA expression in the VMH of the female rat [21]. This discrepancy in the regulation of ER-mRNA expression between the rat and the lizard suggest that while the neuroanatomical distribution of ER is highly conserved across species of divergent taxa [1, 11], the tissue-specific regulation of ER gene expression may vary according to the physiological requirements of the species.

Although species differences in ER-mRNA concentration were apparent in animals injected with 0.5 µg EB, no differences in PR-mRNA concentration were found in similarly treated animals, although differences were apparent at lower dosages. One possible explanation for this inconsistency is that at 0.5 µg EB, the ER in *C. uniparens* had reached saturation. Supporting this hypothesis is the fact that the PR-mRNA concentration in the VMH of *C. uniparens* did not differ significantly in animals injected with 0.063 vs. 0.5 µg EB (fig. 4).

In the present investigation, the induction of PR-mRNA expression in the VMH was analyzed for the purpose of providing a measure of the neural sensitivity to EB. The behavioral significance of the species differences in EB-induced PR-mRNA expression is difficult to interpret, especially since the species differ in endogenous circulating concentrations of E [5, 6]. Progesterone has been demonstrated to act synergistically with E to facilitate receptive behavior in the female green anole lizard [12],

although E is capable of inducing receptivity in the absence of P in the anole as well as in whiptail lizards. Progesterone has been demonstrated to play a crucial role in the regulation of male-like mounting and copulatory behavior in *C. uniparens* [22].

The nature of the mechanism resulting in the species differences in sensitivity to EB in the VMH cannot be determined from the present study, although several candidates exist. Species differences in posttranscriptional ER-mRNA processing or posttranslational modification could result in species differences in active ER protein, which would result in differences in sensitivity to E. Another possibility is species differences in the ER protein structure, which might result in greater affinity for E, or more efficient activation of gene expression. In mice, strain differences in ER affinity for E have been associated with strain differences in sensitivity to E [23]. Since steroid receptors modulate gene expression by interacting with a number of other proteins, e.g. other transcription factors [24], it is also possible that the species difference in sensitivity to E results from differences in another protein involved in ER action.

Finally, 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 there diploid relatives is that the increased gene dosage may result in higher enzyme and hormone levels [25]. This phenomenon has been well documented for a number of enzymes in several plant species [26]. 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 [27, 28], rather than one chromosome set becoming inactivated or compensating for the increased gene dosage as might be expected. Thus, triploidy is likely to 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 has evolved in a diploid genome.

It should be noted that the above interpretation of the species differences in responses to EB treatment assumes that the two species do not differ in steroid clearance rates, which would result in differences in circulating hormone after similar treatments. To address this possibility, blood samples from both species were taken at 2 and 24 h after animals were injected with various dosages of EB and the samples were analyzed by radioimmunoassay for estradiol immunoreactivity. After 2 h, estradiol immunoreactivity increased in both species in a dose-dependent fashion and no species differences were apparent. Twenty-four hours after treatment, estradiol immunoreactivity was undetectable in all treatment groups [Young and Crews, unpubl. data]. The lack of species differences in plasma estradiol immunoreactivity after EB treatment and the species difference in endogenous circulating E during the ovarian cycle suggest that *C. uniparens* and *C. inornatus* differ in sensitivity to E rather than in steroid clearance rates.

Species Differences in Sensitivity to Steroid Hormones

Species differences in circulating hormone concentrations and receptor characteristics have been reported in rodents and in primates. For example, circulating concentrations of E during proestrus in rats, guinea pigs, and hamsters are 50, 70 and 190 pg/ml, respectively [29]. The species also differ in sensitivity to E. The induction of lordosis by E treatment requires only 1–2 μg/kg BW in rats [30], 2–5 μg/kg BW in guinea pigs, and 90 μg/kg BW in hamsters [31]. These differences in sensitivity to E are paralleled by differences in E-binding capacity in the brain. In a comparative study of neural uptake of tritiated E in the hypothalamus, rat and guinea pig tissue bound 3–5 times the amount of E as hamster tissue [31].

Species differences in steroid hormone dynamics have also been described between old world and new world monkeys [32]. Typical plasma steroid levels are elevated 2- to 5-fold for E and up to 100-fold for P in new world monkeys compared to old world monkeys. Steroid receptor binding assays revealed higher concentrations of ER and PR in the peripheral tissues of old world monkeys compared to new world monkeys [32]. Since circulating concentrations of gonadal steroids are regulated by neural feedback mechanisms located within the brain, perhaps the elevated circulating concentrations of steroids in the new world species are due to decreased steroid receptor concentrations in the brain regions controlling gonadotropin release. The inverse relationship between circulating hormone concentration and sensitivity to steroid in these species parallels those reported here for *Cnemidophorus* spp. It is possible that the lower circulating concentration of E in vitellogenic *C. uniparens* compared to female *C. inornatus* may be the result of a compensatory mechanism for the greater neural sensitivity to E of *C. uniparens.*

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 responses to gonadal steroids.

Acknowledgments

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