Quantification of three steroid hormone receptors of the leopard gecko (*Eublepharis macularius*), a lizard with temperature-dependent sex determination: their tissue distributions and the effect of environmental change on their expressions

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**Abstract**

Sex steroid hormones play a central role in the reproduction of all vertebrates. These hormones function through their specific receptors, so the expression levels of the receptors may reflect the responsibility of target organs. However, there was no effective method to quantify the expression levels of these receptors in reptilian species. In this study, we established the competitive-PCR assay systems for the quantification of the mRNA expression levels of three sex steroid hormone receptors in the leopard gecko. These assay systems were successfully able to detect the mRNA expression level of each receptor in various organs of male adult leopard geckoes. The expression levels of mRNA of these receptors were highly various depending on the organs assayed. This is the first report regarding the tissue distributions of sex steroid hormone receptor expressions in reptile. The effects of environmental conditions on these hormone receptor expressions were also examined. After the low temperature and short photoperiod treatment for 6 weeks, only the androgen receptor expression was significantly increased in the testes. The competitive-PCR assay systems established in this report should be applicable for various studies of the molecular mechanism underlying the reproductive activity of the leopard gecko.

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**Keywords:** Reptile; Leopard gecko; Androgen receptor; Estrogen receptor; Progesterone receptor; Competitive PCR; Temperature; Photoperiod

1. Introduction

Squamata are distributed widely around the world, and acquire various characteristics to adapt to their diverse environments. They adapt to and habit various regions from the Frigid Zone to the Torrid Zone, and deserts to marshy areas. For successful adaptation, the reproductive endocrine system will attain to the most efficient reproductive activity. Squamata may be the most successful animal group to adapt to various environments among ground living vertebrates. Thus, squamata are often used to study the field of environmental endocrinology. In the past research, the annual changes in the weight of gonads, and concentration of circulating sex steroid hormones, have been reported in some species (Biurne and Seamark, 1975; Arslan et al., 1978; Courty and Dufaure, 1979; Licht, 1984) and the effects of ambient
environmental factors, such as temperature and light, have also been extensively studied (Marion, 1970; Licht, 1969, 1971; Sexton et al., 1971; Gavaud, 1991; Borrelli et al., 2000). However, the molecular mechanisms underlying such complex traits are still not generally understood in squamata animals.

The leopard gecko, *Eublepharis macularius*, is easily tamed, and is easy to breed in the laboratory, unlike other squamata animals. In this animal, the incubation temperature during embryonic development determines their gonadal sex (Viets et al., 1993), and the effects of embryonic temperature have been studied on within-sex differences in growth, adult morphology, aggressiveness, reproductive behavior (Flores et al., 1994; Tousignant and Crews, 1995; Rhen and Crews, 1999) and brain organization (Coomber et al., 1997). The leopard gecko has also been described as a seasonal breeder (Vosjoli et al., 1998). From such a character, the leopard gecko is expected to be used as an experimental model. However, there are no detailed data regarding its reproductive characteristics during the year or the effect of ambient environmental factors on its reproductive activity.

In this study, the competitive-PCR assay systems to quantify the mRNA expression levels of three sex steroid hormone receptors were established. Sex steroid hormones play a central role in the reproduction of all vertebrates. They directly affect gametogenesis, sex behavior and organization of sex specific phenotypes, and their circulating concentrations have long been used as a popular marker the reproductive activity. These hormones can function through their specific receptor, and the expression levels in an organ may reflect the sensitivity against their counterpart hormones. Therefore, the receptor expression may also provide important information regarding their target organs, such as, in the whiptail lizard, estrogen receptor and progesterone receptor mRNA are increased in the oviduct during vitellogenesis and after estrogen treatment (Young et al., 1995). Therefore, it is very important to know not only the circulating levels of the hormones but also the expression levels of their receptors.

In this report, we developed the competitive-PCR assays to quantify the mRNA expression level of the androgen receptor, estrogen receptor and progesterone receptor of the leopard gecko. The competitive-PCR assay system is simple and sensitive, so this system has been used to quantify various mRNAs (Peten et al., 1992; Fandrey and Bunn, 1993) and well reviewed (Siebert and Larrick, 1992; Zimmermann and Mannhalter, 1996). Two experiments were designed applying these systems. First, to discover the sensitivity and reliability of each assay system, the expression level of each receptor in various organs of male adult leopard geckoes was analyzed. Second, to know the effectiveness of these systems to detect the physiological change of each receptor expression, the effects of a short photoperiod and low temperature were examined. The results in this report demonstrate the utility of the assay systems for the physiological study of sex steroid receptor expression.

2. Material and methods

2.1. Animals

Fertile male leopard geckoes at 15 months of age were used. They were raised in our laboratory and their incubation temperature was 32 °C. The lizards were housed individually in polypolypropylene containers (18×27×13.5 cm³) and supplied with shelter, water and a calcium supplement. Crickets were provided as a main diet three times per week. Animals were maintained on a 14:10 h light:dark cycle, at 29 °C (Fig. 1, the group Cont, n = 3). After this, the short photoperiod and low temperature treatment were carried out on a 8:16 h light:dark cycle, at 18 °C for 6 weeks (Fig. 1, the group A, n = 3). For some of the animals, these environmental conditions were returned to the initial conditions (14:10 h light:dark cycle, 29 °C) and then maintained for 2 weeks (Fig. 1, the group B, n = 3). All environmental changes were done stepwise for a week to minimize stress (Fig. 1). An animal was anesthetized with pentobarbital (50 mg/kg) and killed by complete bleeding. The testes, vas deferens, hemipenes, whole brain, pituitary, livers, adrenal glands, lungs and hearts were quickly taken out, and they were frozen immediately in liquid nitrogen and stored at −70 °C until used.

2.2. RNA extraction and reverse transcription

Total cellular RNA was isolated by Isogen (Nippon Gene, Tokyo, Japan). Reverse transcription of 3 μg total RNA using 100 pmol of oligo dT primer was performed for 1.5 h at 42 °C with
200 units of MMLV1 reverse transcriptase (Takara, Tokyo, Japan). The samples were then heated for 7 min at 70 °C to terminate the reverse transcription reaction, and were diluted to 25 ng/μl with MilliQ water. The pituitary gland was too small to acquire 3 μg total RNA, so 1 μg of total RNA was extracted, reverse transcribed and diluted to the same concentration as the other samples.

2.3. Design of primers

The primers for the amplification of targets and to construct the competitor DNA of each receptor were derived from a sequence of the cDNA for the androgen receptor (Rhen and Crews, 2001; Ikemoto, personal communication), estrogen receptor (Rhen and Crews, 2001; Ikemoto, personal communication) and progesterone receptor (GenBank accession no. AB105375, Ikemoto, 2003) of the leopard gecko.

The target sequences of the androgen receptor, estrogen receptor and progesterone receptor were 323, 291 and 310 bp, respectively, and the competitor sequences of them were 261, 233 and 243 bp, respectively. The sequences of these primers are shown in Table 1, and the positions of the primers are schematically illustrated in Fig. 2.

2.4. Construction of the competitor cDNAs

To construct the competitor DNAs, PCR was performed with 0.25 units of Takara EX taq polymerase (Takara, Shiga, Japan), 25 mM Tris–HCl, 5 mM KCl, 2 mM MgCl₂, 0.25 mM dNTP, 10 pmol of forward (AR-SE or ER-SE, PR-SE) and reverse (AR-COMP or ER-COMP, PR-COMP) primers and 125 ng of testes cDNA (total volume 100 μl). The conditions for the PCR were as follows: after incubation for 5 min at 94 °C, 40 cycles of reactions inducing denaturation for 1 min

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**Table 1**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Primers</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androgen receptor</td>
<td>AR-SE</td>
<td>5’-ATGAAGCAGGGATGACCCCTTGGAGC-3’</td>
</tr>
<tr>
<td></td>
<td>AR-AS</td>
<td>5’-GCAGGGTACGGAATCCTGTAAGGC-3’</td>
</tr>
<tr>
<td></td>
<td>AR-COMP</td>
<td>5’-GCAGGGTACGGAATCCTGTAAGGCAGGAGTGTCAGGCTGTGTGTC-3’</td>
</tr>
<tr>
<td>Estrogen receptor</td>
<td>ER-SE</td>
<td>5’-GATTCGGAAGACCGCAGAGGTGG-3’</td>
</tr>
<tr>
<td></td>
<td>ER-AS</td>
<td>5’-TGCGCAAGCGCATCTAGCAAGGCACCTG-3’</td>
</tr>
<tr>
<td></td>
<td>ER-COMP</td>
<td>5’-TGCGCAAGCGCATCTAGCAAGGCACCTGTCACAGGAAGTTGCAACAGTG-3’</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>PR-SE</td>
<td>5’-CTGCGCATCTTTGGGGGTCG-3’</td>
</tr>
<tr>
<td></td>
<td>PR-AS</td>
<td>5’-GACTACACAAAGGAAGCTGCTTGTGCTGTG-3’</td>
</tr>
<tr>
<td></td>
<td>PR-COMP</td>
<td>5’-GACTACACAAAGGAAGCTGCTTGTGCTGTG-3’</td>
</tr>
</tbody>
</table>
Fig. 2. Schematic illustration of the positions of the primers used to amplify the sex steroid hormone receptors of the leopard gecko.
The sequence of each primer is listed in Table 1.

at 94 °C, annealing for 1 min at 60 °C and extension for 1 min at 72 °C were performed, followed by additional extension for 5 min at 72 °C (all PCR performed in this experiment was done with this program). The PCR products were electrophoresed on a 1% agarose gel and visualized by ethidium bromide staining. The bands of the competitor were cut off and extracted with a QIA Quick gel extraction kit (QIAGEN). The amount of competitor DNA was estimated by the absorbance of 260 nm. The solutions were diluted to various concentrations and stored at −27 °C until used.

To confirm the similarity of the efficiencies of amplification between the cDNA and the competitor, 25 ng of testes cDNA and the 1 arbitrary unit (a.u.) of the competitor of each receptor were amplified independently with 20, 23, 25, 28, 30, 35, 40 and 45 cycles. The products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. A picture of the gel was taken with Digital Image Stocker (TOYOBO, Osaka, Japan), and the intensities of the bands were analyzed densitometrically using the SCION IMAGE program.

2.5. Competitive PCR

Competitive PCR was performed with a fixed amount of the cDNA sample (25 ng) and various concentrations of the competitor DNA. Five microliters of the PCR product was electrophoresed on a 2% agarose gel and analyzed. The sex steroid hormone receptor mRNA expression levels were obtained by comparing the ratio of the band intensity of the target and the competitor. The expression levels were given as an a.u. The averages of the expression levels of the testes cDNA of three geckoes from the group Cont were defined as 1 a.u.

To evaluate the sensitivity and reproducibility of the competitive-PCR assay systems, serially diluted cDNA samples from the testis of one gecko from the group Cont were subjected as samples and the relation between the rate of dilution and the measured value was compared.

2.6. Quantification of sex steroid hormone receptors mRNA expression levels in various organs

For the comparison of the expression levels of sex steroid hormone receptors in various organs, total RNA was extracted, as described above, from various tissues, the testes, vas deferens, hemipenes, whole brain, pituitary, livers, adrenal glands, lungs and hearts from the geckoes of the group Cont. Three micrograms of total RNA from each tissue was reverse transcribed. Then 25 ng of cDNA was subjected to the competitive-PCR assay.

2.7. Effect of a short photoperiod and low temperature on sex steroid hormone receptor mRNA expression levels in the testes and hemipenes

Nine leopard geckoes were divided into three groups (the group Cont, A and B) and treated as described above. The mRNA expression levels of three sex steroid hormone receptors were quantified in the testes and hemipenes of geckoes from three groups.

2.8. Statistical analysis

Analysis of variance followed by Tukey test was used to analyze the data, where $P<0.05$ was
considered as statistically significant. The numbers of samples are expressed in parentheses.

3. Results

3.1. Establishment of the competitive PCR

Each forward and reverse primer for amplification was derived from the regions relatively poorly conserved among other steroid receptors, so the specificity of each PCR amplification would not be compromised by closely related mRNAs. The specificity of each PCR amplification was also confirmed by the sequencing of the PCR product. The primers were designed to include the exon/intron boundary in their target sequences according to the reported genomic structure of human sex steroid receptors (Pongilittmongkol et al., 1988; Brinkmann et al., 1989; Misrahi et al., 1993). However, there was no information regarding the genomic structures of the leopard gecko sex steroid hormone receptor. To examine the effect of genomic contamination in cDNA, the PCR was performed using the primer sets for the competitive PCR with genomic DNA and no band was detected. Therefore, the effect of genomic contamination could be excluded. The bands of the target and competitor were evidently distinguished, and there was no difference in the efficiency of the amplification between the target and competitor (Fig. 3a–c). The lower limit of the quantification of the androgen and estrogen receptors was as little as 10 a.u. mRNA, and this value was 100-fold less than the lowest amount measured in any tissue (Fig. 3d and e). In the case of progesterone receptor, 5×10⁻² a.u. mRNA was the lower limit and it was 10-fold less than the lowest amount of testes. Representative results of the quantitative PCR for the androgen receptor show the typical results of a competitive-PCR assay in Fig. 4.

3.2. Expression levels of various organs

Fig. 5 shows the expression levels of three sex steroid receptors from various tissues. Each dot represents a result from a single animal of the
Fig. 4. Representative results of the quantitative PCR for the androgen receptor. (a) Ethidium bromide staining of the PCR products separated on a 2% agarose gel. Twenty-five nanograms of cDNA of testis (from the group Cont) was mixed with 0, 0.4, 0.5, 0.6, 0.7 and 0.8 a.u. of the competitor DNA (lanes 1–6, respectively), 1 a.u. competitor (lane 7) and 25 ng of total RNA of testis (lane 8) and were amplified with the primer set, AR-SE and AR-AS. The sizes of the amplified bands for the cDNA (313 bp) and the competitor (261 bp) are indicated. (b) Plot of the reaction. Logarithm of the ratio of the fluorescent intensities of the PCR products of the cDNA sample to that of the competitor DNA was calculated for each lane, and plotted on the y-axis against the logarithm of the known input of the androgen receptor competitor DNA (a.u.). The estimated value of the androgen receptor mRNA expression level was 0.4 a.u. in this sample.

Fig. 5. The expression levels of the sex steroid hormone receptor mRNA. The expression levels of the androgen receptor were shown in (AR), estrogen receptor in (ER) and progesterone receptor in (PR), in 25 ng cDNA of various organs of male leopard geckoes. The y-axis was plotted on a log_{10} scale. Each dot represents a result from a single animal of the group Cont. The numbers of each sample are three except the brain and pituitary. N.D. represents non-detected. T, testis; V, vas deferens; H, hemipenis; B, whole brain; P, pituitary gland; A, adrenal gland; H, heart; N, lung; L, liver.
Table 2
The average of the body weight, testis weight and testis weight/body weight

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Body weight (g)</th>
<th>Testis weight (mg)</th>
<th>Testis weight (mg)/body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cont (3)</td>
<td>50.0±7.0</td>
<td>36.2±7.4</td>
<td>0.74±0.10</td>
</tr>
<tr>
<td>A (3)</td>
<td>57.7±5.8</td>
<td>42.0±10.6</td>
<td>0.74±0.24</td>
</tr>
<tr>
<td>B (3)</td>
<td>55.4±6.1</td>
<td>28.0±10.0</td>
<td>0.50±0.13</td>
</tr>
</tbody>
</table>

The numbers of animals in each group are in parentheses. The values are mean (±) S.E.M. There was no significant difference between any two groups.

3.3. The effect of the short photoperiod and low temperature

When the conditions were 18 °C, 8L/16D, leopard geckoes seldom moved or ate. They ate two crickets three times per week when the conditions were 29 °C, 14L/10D, but the number of crickets eaten dropped dramatically, only one or two crickets per month. However, their body weights did not change significantly nor did their testes weights or their testes weights per body weight (Table 2). From the microscopic observations of the testes sections, no significant difference was observed among the control and the experimental groups (data not shown).

In the testes, the expression levels of the androgen receptor significantly increased when the conditions were 18 °C, 8L/16D (P<0.01). This increased expression levels decreased to near the levels of the control 2 weeks after the conditions were returned to 29 °C, 14L/10D. The expression levels of the androgen receptors in the hemipenis, and progesterone receptor in the testis changed like androgen receptor in the testis, but not significantly (Figs. 6–8). In the case of the estrogen receptors, no major change or tendency, like the androgen receptors in the testes, was observed

Fig. 6. The effect of low temperature and a photoperiod on the expression levels of androgen receptor mRNA. The expression levels in 25 ng cDNA of the testes and hemipenes were determined by the competitive PCR. The numbers of animals examined are three in each group. The bar and vertical lines denote mean (±) S.E.M. *P<0.05; **P<0.01.

Fig. 7. The effect of low temperature and a short photoperiod on the expression levels of estrogen receptor mRNA. The expression levels in 25 ng cDNA of the testes and hemipenes were determined by the competitive PCR. The numbers of animals examined are three in each group. The bar and vertical lines denote mean (±) S.E.M. There was no significant difference between any two groups.
Fig. 8. The effect of low temperature and a short photoperiod on the expression levels of progesterone receptor mRNA. The expression levels in 25 ng cDNA of testes were determined by the competitive PCR. The numbers of animals examined are three in each group. The bar and vertical lines denote mean ± S.E.M. There was no significant difference between any two groups.

4. Discussion

Sex steroid hormones play a central role in the reproduction, and the expression levels of receptors in an organ may reflect the sensitivity against their counterpart hormones. Therefore, it is important to know the expression levels of the sex steroid hormone receptors to investigate the reproductive activity of the leopard gecko, as well as to know the circulating levels of the sex steroid hormones. Thus, we developed simple and sensitive assay systems to quantify three sex steroid hormone receptors in the leopard gecko.

In the assay systems of this study, the length of the target and the competitor DNAs were short to make the amplification efficiency high and big enough to be obviously distinguished from primer dimmer. The competitor DNA fragments were designed to be identical to the target sequence, except the short deletion of approximately 20% of the sequence at the 3'-region of the target DNA. This similarity of the inner sequences between the target and the competitor DNA made the difference in amplification small enough to be ignored (Fig. 3). Both the competitor and the target DNA had identical primer binding sites. The competitive-PCR systems could quantify mRNA expression levels as little as approximately 25 ng of the cDNA sample. Therefore, this gives us a strong advantage to quantify the mRNA expression level in a small organ such as a pituitary. As an example, the wet weight of the pituitary gland of the leopard gecko was only approximately 3 mg, and only 1 μg of total RNA could be extracted, which was enough to quantify the expression levels of all the three receptors in this study.

By applying the newly established assay systems for steroid receptors, the expression levels of sex steroid receptors were examined in various organs, some related directly to reproduction and others indirectly. The androgen receptor and the estrogen receptor expressed every organ measured, as was the progesterone receptor except the hemipenis, lung and liver. In the hemipenes, lungs and livers, progesterone receptor could not also be detected by conventional RT-PCR. These wide distributions coincide with the distributions in chicken, rat and human (Pasanen et al., 1997; Pelletier, 2000; Manolagas and Kousteni, 2001). As in these species of amniotes classes, the leopard gecko sex steroid hormone may act in various organs, even indirectly related to reproduction. In non-amniotes classes, such as fish and amphibian, there is no data to compare with the result of this experiment.

Most reptile species are thought to be seasonal breeders and their reproductive phenomena are strongly affected by various ambient environmental factors. Leopard geckoes are also described as seasonal breeders (Vosjoli et al., 1998), however, their reproductive characteristics have still not been elucidated, including the annual cyclicity of gonadal activity. In this study, we tried to analyze the effect of environmental change on the expression of three sex steroid hormone receptors in the testes of the leopard gecko by using the competitive-PCR assay systems established in this study. The hemipenis, also an important reproductive organ as the male copulatory organ of squamata, was examined for the comparison.

During the experiment, leopard geckoes were able to freely access their food, including living crickets, but they seldom moved or ate when the environmental conditions had been adjusted to low temperature and a short photoperiod. However, their body and testes weights were not changed.
This result suggests that there is no significant decrease in the activity of the testes for the 6-week treatment. There were many mature spermatozoa and no major histological changes were observed (data not shown). However, the changes in the expression of each steroid hormone receptor showed an interesting pattern. The expression levels of the estrogen receptor and the progesterone receptor were not changed significantly, whereas that of the androgen receptor was significantly increased after the 6-week treatment of low temperature and short photoperiod (18 °C, 8L/16D). This increase was lost when the environmental conditions were returned to that of the control (29 °C, 14L/10D). This data suggests that the low temperature and short photoperiod up-regulate the androgen receptor mRNA expression in the testes. However, the meaning of the increased androgen receptor expression in the testes of the leopard gecko could not easily be interpreted. In Anolis carolinensis and Sceloporus undulatus, it was suggested that a low temperature induces the restart of gametogenesis after their breeding season (Licht, 1969; Marion, 1970). Therefore, it may reflect the enhanced spermatogenic activity of the leopard gecko. In future, the meaning of this up-regulated androgen receptor expression in the testes should be clarified for the understanding of the reproductive characteristics of the leopard gecko. On the other hand, the expression level of the androgen receptor in the hemipenis was not significantly changed by the same treatment. This organ exclusively expressed the androgen receptor, which suggests that androgen is the only sex steroid hormone to control the activity of the hemipenis.

In this study, we established the competitive-PCR assay systems to quantify the expression levels of the receptors of three sex steroid hormones; androgen, estrogen and progesterone. We demonstrated that the newly established assay systems were able to detect the differences of the tissue diversity of the mRNA expression levels of each hormone receptor. This is the first report regarding the tissue distributions of sex steroid hormone receptor expression in a reptilian species. These assay systems were also able to successfully detect the change in receptor expression level after a change in the environmental factors. These results indicate the utility of these assay systems. The primers and the methodology presented here will promote further molecular study on the mechanisms of the reproductive endocrine system of the leopard gecko.

References


