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Seasonal changes in plasma steroid levels in relation to ovarian steroidogenic ultrastructural features and progesterone receptors in the house gecko, *Hemidactylus flaviviridis*, in Oman

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ABSTRACT

A comprehensive study of the annual ovarian cycle in the house gecko, *Hemidactylus flaviviridis*, was conducted in Oman. Circulating estradiol (E_2), progesterone (P), and testosterone (T) were measured during the active and inactive phases of the cycle. The steroid levels were related to the steroidogenic ultrastructural features such as the abundance of smooth endoplasmic reticulum (SER), the presence of cisternal whorls, and close association of lipid droplets with swollen vesiculated mitochondria and SER. The steroids were measured using a sensitive detection technique HPLC-MS/MS. E_2 levels began to rise in January at the onset of vitellogenesis and continued to rise between February and May relative to ovulation, postovulation, gravidity and oviposition. Afterwards, E_2 remained low during the inactive phase (June–December). P levels increased significantly in March and peaked in April, which coincided with luteinization. P levels began to decline relative to luteolysis (May–June). Afterwards, it remained low throughout the inactive phase. T levels rose significantly in March–April coinciding with vitellogenesis, but decreased rapidly and significantly in May and remained low during the inactive phase. Progesterone receptors (PR), identified using immunohistochemistry, were strongly expressed during the breeding period, but were absent during the non-breeding period. The appearance of the steroidogenic ultrastructural features in the preovulatory and lutein granulosa cells was correlated with the significant rise in the three steroid levels and the PR. As the steroid levels declined, the granulosa cells underwent a general degeneration and disruption of the associated steroidogenic features.

1. Introduction

Relationships among gonad development, steroidogenesis, and plasma sex steroid levels have been studied in many reptiles [28,30,32,38,13,35,44,54,4,14,2,3,36].

In most reptiles, plasma estradiol (E_2) levels typically rise at the onset of vitellogenesis which is essential for follicular development and maturation [6,25,45,40,20,42,24,4].

Progesterone (P) plays an important role in the maintenance of gravidity in lizards [41,16,13,47,51]. Elevated plasma progesterone levels are related mainly to the secretory activities of corpora lutea. Progesterone levels peak during the gestation period as the eggshell and eggshell membrane are formed [7,8,29,23,40,16,22,50,35,14,51].

Elevated plasma testosterone (T) levels may be involved in the regulation of vitellogenesis and ovulation [6,14] and in the hypertrophy of the oviduct [35,3] and may initiate breeding behavior in females [31].

In squamates, E_2, P, and T concentrations are usually low during the inactive (non-breeding) period but they rise significantly during the active (breeding) period. This indicates that sex hormones play an important role in regulating reproductive phases such as vitellogenesis, courtship, mating, ovulation and uterine secretory activities [9,54,44,14,55,1,53,33].

The ultrastructural changes in the granulosa cells of the ovarian follicles and granulosa lutein cells in reptiles reveal several steroidogenic features correlated with endocrine function [12,35,2,36]. Specifically, steroidogenic ultrastructural features are in the granulosa cells of the ovarian follicle and corpus luteum. These changes in steroidogenic features are associated with the production of

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gonadal steroids mainly E_2 and P which are related to an increase in smooth endoplasmic reticulum (SER), the presence of cisternal whorls, free ribosomes, and close association of lipid droplets with swollen vesiculated mitochondria and SER [5,12,35,2,36].

Al-Kindi et al. [2] reported that when female painted turtles with mature preovulatory and secondary follicles were injected prior to ovulation with synthetic mammalian Gonadotropin Releasing Hormone (GnRH), the granulosa cells acquired more apparent steroidogenic ultrastructural characteristics compared to the controls. Reproductive function is controlled by the hypothalamic release of GnRH in many vertebrates [52,15,48]. GnRH stimulates release of gonadotropins which in turn stimulates steroid secretions which control follicular development and steroidogenesis.

Ultrastructural changes in the granulosa cells under natural condition have been reported in the ovarian follicles of some reptiles [12,35].

Until recently, the relationship between sex hormones and their receptors has not been clearly established in reptiles. Progesterone receptors (PR) have been characterized in lizards [46] and in snakes [26,37,18,11]. There are at least two PR isoforms in reptiles, PRA and PRB with different expression profiles [49,18,11]. It has been reported that PRs are down-regulated by P [18].

The aim of this study was to monitor plasma sex-steroid levels in relation to the development of ultrastructural steroidogenic features and the presence of PR during different phases of the ovarian cycle in the house gecko, *Hemidactylus flaviviridis* which is a widely distributed species in Oman and the rest of the Arabian Peninsula.

Until now, the ovarian cycle of this species has not been studied in this region. Moreover, for the first time this study presents information on the ultrastructural changes related to the hormonal dynamics in this oviparous species.

2. Materials and methods

2.1. Study area

The study area was located in Barka, Al Batina Region, Sultanate of Oman (23°40′33″N, 57°53′13″E). The area is a wooded habitat at sea level and consists mainly of palm trees with some mango and lime trees and scattered farm houses. Annual rainfall averages 30–100 mm, with temperature ranging between 13 and 47 °C. The rainy season occurs between December and April.

2.2. Animals

A total of 129 adult female *H. flaviviridis* [snout–vent length (SVL) < 8 cm] was captured by hand at night from the study area during 2008–2010, in which 28 were sacrificed during the post-breeding (quiescent) phase (June–August), 50 during the prebreeding (recrudescent) phase (September–December) and 51 during the breeding (active) phase (January–May).

The lizards were mostly active year round at temperatures between 18 and 47 °C except briefly during December–January in which they showed limited activity during the cold nights when temperature drops below 15 °C.

2.3. Blood and tissue collection

The lizards were immediately sacrificed after capture to minimize stress which could alter the natural hormonal values [43]. Blood and tissues were collected from all the sacrificed lizards, fixed in appropriate solutions and kept in ice until arrival at the laboratory, which took approximately 12–80 min.

Blood was collected from the carotid vessels in EDTA tubes and then centrifuged, and the plasma samples were stored at −70 °C until analysis.

Tissue samples at different phases of the reproductive cycle were taken from the ovary (follicles, corpus luteum) and the liver for examination under light and transmission electron microscopy and immunohistochemistry.

2.4. Analysis of sex hormones by HPLC-MS/MS

Serum concentrations of E_2, P, and T were measured with the highly specific and sensitive method of high performance liquid chromatography coupled with a tandem quadrupole mass spectrometer HPLC-MS/MS. Initial separation of the steroids from the biological media was performed by the HPLC separation column and individual components were quantified by the mass spectrometer.

The samples were prepared by protein precipitation; a 500 μL aliquot of acetonitrile was added to 500 μL of plasma. The sample was vortex mixed for 5 min and then centrifuged at 3000 rpm for 10 min in an Eppendorf (Hamburg, Germany) microcentrifuge. The precipitated plasma protein was discarded and the top layer containing the extract was analyzed by injecting a sample of 10 μL into the HPLC–MS/MS. A 1 pg/μL standard solution of E_2, P and T (Sigma–Aldrich, UK) was used to tune the mass spectrometer for optimum sensitivity. A tandem quadrupole mass spectrometer (Quattro Ultima Pt, Waters Corp., MA) was used in this analytical procedure and the multiple reaction monitoring (MRM) transitions used for monitoring E_2, P, and T were set to 271.3, 429.2 and 289.2 m/z, respectively. The E_2, P and T were run in negative ion Electrospray and were separated using a high performance liquid chromatography system (Agilent 1100, Palo Alto, CA) with an Xterra C18, 2.1 × 100 mm, 3.5 μm column (Waters Corp., MA). Acetonitrile/water (45/55, v/v) was used as the mobile phase (Sigma–Aldrich, UK), at a flow rate of 0.3 μL/min. Validation of the extraction procedure and HPLC–MS/MS method was achieved by comparing extraction recoveries. The peak area in the chromatogram of a 10 pg/mL standards E_2, P and T solution in water/acetonitrile was compared with standards, at the same concentration, and spiked lizard serum that had been carried through the protein precipitation extraction procedure. A blank plasma sample was run and subtracted from the spiked sample.

E_2, P, and T levels in the blank were below the limit of quantification for the method and this was used to back-calculate the concentrations in the calibration curve standards and subsequent samples. The efficiency of recovery was 98%. The back-calculated concentration from the protein precipitation extraction method was used to evaluate the steroid concentrations in actual lizard serum samples. Calibration curves were constructed in acetonitrile/water, first for validation only and then for the actual samples. The calibration lines were constructed using plasma that was spiked and extracted in the same way as the samples. Back-calculated concentrations were obtained using a calibration curve over the concentration range 0.1–10,000 pg/mL with a correlation coefficient greater than 0.999. This gives an accurate indication of the actual steroid concentration in plasma. The sensitivity of the lowest level concentration was evaluated at 0.1 pg/μL using a signal:noise ratio of 8:1 for all components.

2.5. Light microscopy

Tissue samples from the ovaries were fixed in Bouin's fluid for 48 h. After fixation, the tissues were dehydrated in graded alcohol concentrations (70%, 95% and 100%), cleared in xylene and embedded in paraffin. Tissues were serially sectioned at 3 μm and stained with hematoxylin and eosin. In the ovary sections; follicular