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Metabolic and endocrine changes during the reproductive cycle of dermatophagic caecilians in captivity

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ABSTRACT

The amphibian order Gymnophiona is poorly known, and studies about their reproduction are mainly comparative and descriptive, focusing on the structure of testes, ovaries and oviducts. However, to understand the reproductive processes, including those of the oviparous dermatophagic species, it is important to know the dynamics of storage and mobilization of energetic substrates to gonads and skin during the reproductive cycle of males and females, as well as the endocrine control associated. For the present study, total lipids and proteins were measured during the annual cycle in the plasma, liver, muscle, testes, ovaries and skin of *Siphonops annulatus* in captivity. Plasma levels of gonadal steroids (estradiol, testosterone and progesterone) were quantified by radioimmunoassay. Histological analyses of ovaries and testes were performed to classify the maturation stages of the animals during the reproductive cycle. Gonadal maturation in males and females of *S. annulatus* was accompanied by metabolic changes in reserve tissues, which supported gonadal development and prepared the females' epidermis for skin feeding by the offspring. Even in confinement conditions, females and males synchronized the reproductive period. However, due to the absence of environmental cues in captivity inadequate levels of the hormones responsible for gamete release were triggered, leading to a lack of reproductive success.

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1. Introduction

The Gymnophiona (or caecilians) are the least studied amphibian order (Wake, 2006; Gomes et al., 2012) and include 200 species divided into 10 families (Wilkinson and Nussbaum, 2006; Kamei et al., 2012), comprising only species with strictly tropical distribution across Southeast Asia, Central and South America, and Africa (Jared et al., 1999). Caecilians are characterized by a reduced visual system, a pair of sensory tentacles and a highly ossified skull that favors the fossorial habit (Jared et al., 1999; Gower and Wilkinson, 2009). All modern caecilians (Teresomata) exhibit oviparity or viviparity (Kupfer et al., 2006a) associated with parental care, and some species present post-hatching parental care that includes food provisioning via dermatotrophy (Jared et al., 1999). The reproductive biology of caecilians remains poorly studied due to their fossorial habits and consequent difficulty of finding individuals in the wild. Most available data are derived from the study of captive animals (Gomes et al., 2012). Altogether, current knowledge about caecilian reproduction comes mainly from descriptive studies that investigated the structure of testes, ovaries and oviducts, often comparing oviparous and viviparous species (Wake, 2006). The sexual cycle of males and females is known to be associated with gonadal endocrine changes perceivable as changes in the morphology of testicular Leydig cells, ovarian follicles, and the corpus luteum of the viviparous females (Wake, 2006).

In caecilians, as in virtually all vertebrates, gonads are known to respond to other endocrine glands such as the pituitary (Wake, 2006), so that their reproductive cycle is assumed to be under metabolic control. However, the metabolism of energetic reserves may be atypical in caecilians because fat metabolism may change in the context of annual cycles related to oogenesis. In general, amphibians regulate fat bodies (Exbrayat and Hraoui-Bloquet, 2006) and caecilians seem no exception. When analyzed in terms of the weight and density of some organs such as the liver, and the extent of fat bodies, the metabolic reserves of the viviparous caecilian *Typhlonectes compressicauda* (males and females) vary during the reproductive cycle (Exbrayat, 1988).

Changes in the metabolism of lipids and proteins, as in many vertebrates, are deeply linked to the synthesis of vitellogenin, the yolk

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precursor molecule (Norris, 2011) that becomes the predominant source of energy and structural components during embryogenesis (Thompson and Speake, 2006; Norris, 2011). Additionally, in some caecilians, lipids may also support skin feeding, a behavior in which the offspring gather the upper layers of the mother's skin as their only nutritional source, using specialized teeth (Kupfer et al., 2006b). In these species, hatchlings are altricial and remain with their mother in underground nest chambers, feeding periodically until becoming able to assume an independent existence. During this period of extended parental care the epidermis of the female becomes hypertrophied and filled with lipids (Wilkinson et al., 2008). In this context, the goal of the present study is to offer new insights on the endocrine and energetic changes that characterize the reproduction of dermatophagic caecilians. Specifically, this paper focuses on the dynamics of storage and mobilization of the energetic substrates, as well as the gonadal steroids, to gonads and skin during the reproductive cycle of female and male caecilians in captivity. The model animal selected for this study is *Siphonops annulatus*, an oviparous caecilian that presents skin feeding during parental care. We hypothesized that *S. annulatus* females exhibit a higher deposition of lipids and proteins in the gonads and in the skin than males during the same period of the reproductive cycle, as a preparation for reproduction and parental care.

2. Materials and methods

2.1. Study animals

Adult male and female *S. annulatus* (Amphibia: Gymnophiona, Siphonopidae) were captured at a cacao farm in the state of Bahia, Brazil, and then transported and maintained in captivity for 7 years in terrariums at the Laboratory of Cellular Biology of the Butantan Institute, according to the authorization of the "Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis" (IBAMA, 2360650). The animals were fed once a week, the diet alternating between newborn mice, beef or chicken, beef heart, and prepared wet food containing meat, chicken or fish. Animals were kept at 23 °C under natural photoperiod and 80–90% relative humidity. High soil humidity was ensured by daily spraying of water inside the terraria.

During the experimental period, a total of 20 males and 21 females were sampled. Samples were grouped as representing four southern-hemisphere seasons (summer: December–March; autumn: March–June; winter: June–September; and spring: September–December), with an average of 5 individuals per sex, per sample.

The sampled animals were anesthetized with 0.5 ml sodium buetal (thionembutal) and morphometric and ponderal data were recorded. Animals were decapitated according to the animal care protocols approved by the Biosciences Institute (University of São Paulo) Ethics Committee (protocol 056/2008), and blood samples were collected from the tail and centrifuged at $655.2 \times g$ for 10 min. After centrifugation, the plasma was separated into aliquots, which were immediately frozen and preserved at −80 °C until processing. After blood collection, fragments of liver, muscle, gonads and skin (with all the layers) were obtained. From these data two indexes relative to organ mass were calculated, the gonadosomatic index (GSI): GSI = (gonad mass/total body mass) × 100 and the hepatosomatic index (HSI): HSI = (liver mass/total body mass) × 100.

Subsequently, tissue samples were transferred to −80 °C until processing. For histological analysis, samples of the middle third of the gonads were collected and fixed in Bouin solution for 24 h and then transferred to 70% ethanol.

2.2. Histological, metabolic and hormonal analysis

To confirm the gonadal maturation stages, gonads were dehydrated in a graded ethanol series, cleared in dimethylbenzene (xylene) and embedded in Paraplast (Erv-Plast; Erviegas Instrumental Cirúrgico, São Paulo, Brazil), following routine histological procedures for the preparation of permanent histological cross-sections (Behmer et al., 1976). Slices of 5 μm were made with a microtome (Leica RM 2255; Leica Microsystems, Wetzlar, Germany), stained with hematoxylin–eosin and examined using a light microscope (Leica DM 1000). The images were analyzed, captured and documented using a computerized image analyzer system (Leica Application Suite Professional).

Total lipids were extracted from tissues according to the method of Folch et al. (1957), by using a solution containing chloroform, methanol and water (2:1:0.5). Then, tissue and plasma total lipid levels were analyzed by the colorimetric method of Frings et al. (1972), with cod liver oil as standard (cod liver oil fatty acid methyl esters; Sigma Diagnostics, St. Louis, MO, USA). Total proteins were extracted from tissues by precipitation and solubilization according to Milligan and Girard (1993). Levels of plasma and tissue proteins were analyzed by the colorimetric method of Lowry et al. (1951), using bovine serum albumin as standard (Sigma Diagnostics, St. Louis, MO, USA).

Plasma levels of 17β estradiol, testosterone and progesterone were determined by radioimmunoassay (RIA) in triplicate (coefficient of variation below 25%) after protein precipitation with methanol. Methanol was evaporated and samples were re-dissolved in RIA buffer (10 mM PBS, 0.1% w/v gelatin, 10 mM EDTA, pH 7.4). Calibration curves were obtained by incubating increasing amounts of the corresponding radio-inert hormones with a constant amount of each labeled hormone (4.54 nCi; NEN Life Science Products, Boston, MA, USA) and antibodies. Polyclonal antibodies were obtained from the Colorado State University, CO, USA. The nonspecific binding was determined in the presence of a 1000× excess of unlabeled hormone. The charcoal–dextran method was used to separate bound and free hormones. Radioactivity in the supernatant (bound) was determined with a liquid scintillation counter (Wallac 1409 DSA; Wallac, Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac, Turku, Finland).

2.3. Statistical analysis

All statistical analyses were carried out using Sigma-Stat 3.1 (Systat Software Inc., San Jose, CA, USA). The concentrations of energetic substrates and plasma steroids in females and males were compared during the annual cycle. Tests of normality and homogeneity of variance were applied and when data fulfilled the requirements for a parametric analysis the results were compared using analysis of variance (one-way ANOVA) followed by the Student-Newman–Keuls test. Otherwise, the non-parametric Kruskal–Wallis test was applied after ANOVA. The differences were considered significant at $P < 0.05$.

3. Results

3.1. Body mass and morphometric parameters

The body mass and length of females and males were similar throughout the year (ANOVA, $F_{3.17}$, $P > 0.001$; Table 1), although the GSI values increased in winter (ANOVA, $F_{3.17}$, $P = 0.023$ for females and $F_{3.16}$, $P = 0.001$ for males; Table 1). HSI values increased in winter relative to spring values (ANOVA, $F_{3.17}$, $P = 0.018$ and $F_{3.16}$, $P = 0.012$, respectively, for females and males; Table 1).

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