Differential Testosterone Biosynthesis Relates to Decoupling of Reproductive Pattern in *Peromyscus* Syntopic Species (Rodentia: Muridae)

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Abstract: Syntopic, congeneric species often deploy ecological strategies to avoid interspecific competition for any resource. Here we explore if the monthly and seasonal biosynthesis of sexual steroids involved in the Δ4 pathway, is one of such strategies for syntopic *Peromyscus melanotis* and *Peromyscus difficilis felipensis*, since a differential pattern could affect, in turn, the temporality of their respective annual reproduction patterns. We found, indeed, an ecophysiological relationship between these two *Peromyscus* inhabiting at a midlatitude, temperate forest, since each had a monthly and seasonal species-specific pattern for sexual steroid production. We discuss how such differences are likely to account for the interspecific temporal decoupling of their respective reproductive patterns.

Keywords: Rodents, *Peromyscus*, testes, androgens, testosterone, temperate forest.

1. INTRODUCTION

Syntopic populations of rodents display strategies that allow them to avoid interspecific competition for any resource that could negatively affect their reproduction [1-3]. Among such strategies behavior patterns affected by steroid hormones, such as testosterone, may also be included. Since it regulates stages of spermatogenesis [4, 5], body growth [6, 7, 8], and sexual mating behavior [9, 10], testosterone is considered as one of the main androgens in males. In fact, testosterone can act as a pheromone or it can promote its synthesis [11-13], thus acting as an intra or interspecific signal, which in turn promotes distinctive territorial and breeding behaviors.

Syntopic, congeneric species are ideal models to document the relationship between production of androgens and the shifts on their respective reproductive patterns. Therefore, here we use syntopic populations of *Peromyscus melanotis* and *P. difficilis felipensis*, inhabiting the midlatitude, temperate forests at the western suburbs of Mexico City. The former, the Black-eared deermouse, is a small-sized [14], cursorial, monotypic, and quasi-endemic species from Mexico [15-17]; dwelling in the ground of the alpine zone (> 4300 m) [18], within the mentioned forested areas [19]. The latter belongs to a subspecies of the Mexican Rock deermouse, whose distribution extensively overlaps with the former species at the rural areas of Mexico City (e.g., Ajusco, Contreras) and of those nearby in the State of Mexico [20, 21]; this semiarboREAL deermouse is medium-sized within *Peromyscus* [14].

Both species reproduce throughout the year in the study area but display particular reproductive patterns: *Peromyscus difficilis felipensis* has its greatest peak during spring [22] while *P. melanotis* has it during the summer [23]. Therefore, we wonder whether production of androgens in sexually mature males of these two species, is in accordance or not with the temporal interspecific differences
in their reproductive pattern. In order to address this question, we describe how the testes of adult individuals of each species, carry out monthly as well as seasonal androgen production. Then we discuss both intra and interspecific variations in the biosynthesis of testosterone and its intermediary steroids in the light of possible deployed strategies to coexist in the same habitat.

2. MATERIALS AND METHODS

2.1. Study Area

Parque Nacional Desierto de los Leones (PNDL; Desert of Lions National Park), which is part of the Sierra de las Cruces (The Crosses Mountain Range), a continuation of Sierra del Ajusco (Ajusco Mountain Range), located east of Mexico Valley. Its climate is temperate sub-humid (C (w2) (w') (b') ig; [24]) with highest temperatures recorded in April-July (mean ± standard deviation, 12.6±6°C) and lowest temperatures in December-February (8.1 ± 2°C). The rainy season encompasses May-August (235 ± 30 mm) and the dry season, December-February (12±4 mm). Sampling sites are located at 19°18'17''N, 99° 19'14''W, at 19 km from the park entrance (Alcaldía Álvaro Obregón), within 2180-3200 m of elevation. The arboreal stratum of this temperate forest harbors stocks of pine-oyme (Pinus-Abies) and pine-oak (Pinus-Quercus). The understory includes several shrubs, especially Senecio sp. and Salix sp., together with herbaceous strata and scarce, scattered grasses. The ground stratum holds a variety of fungi, mosses and lichens.

2.2. Rodent Capture and Preparation

We trapped and selected male adult mice, using standard species-specific, pelage patterns [25], every month along a year, using Sherman live-traps (8 x 9 x 23 cm, Tallahassee, Fl.), baited with vanilla-aromatized oat flakes. The same day of capture, we transferred mice to our laboratory facilities and killed by cervical dislocation. Each male was recorded its conventional external measurements and weight and prepared as skull and skeleton [26] to be housed at UAMI mammal collection. All animal manipulations followed international standards [27] and were approved by the Ethic Commission at DCBS, UAM-I.

2.3. Evaluation of Testosterone Biosynthesis

We removed the right testicle from each mouse and detached its tunica albuginea. Then introduced gonadal tissue into an Eppendorf tube, containing a Krebs-Ringer buffer solution with glucose (KRBG, pH 7) without metabolic cofactors. Each tube was added 10,000 cpm of tritiated cholesterol (1, 2-3H specific activity 47.7 Ci/mmol; C273H; New England Nuclear, Boston, MA). Experimental tubes (with tissue) and control tubes (without tissue) were incubated at 36°C for an hour. Metabolic reactions were stopped by freezing, and tubes were stored at -70°C until analyses.

Procedures for homogenization, protein content determination, total steroids extraction, and efficiency determination, as well as those used for separation of each sex steroids followed Salame-Méndez et al. [22], as described below.

2.4. Homogenization of Tissues

Gonadal tissues within experimental tubes were thawed and homogenized by sonication. Two aliquots were taken from each homogenate: one to evaluate biotransformation of precursor (tritiated cholesterol [C273H]) of each sex steroid and the other to determine protein concentration [28].

2.5. Extraction of Total Steroids

Total steroids were extracted from the aliquots of each homogenate by duplicate with diethyl ether. Average of total extraction efficiency was 97.4±0.6%. Results of each of the biotransformation assessment was corrected based on percentage of recovery.

2.6. Separation and Evaluation of Sexual Steroid Production

Each tube containing its corresponding total steroid extract, as well as each control tube, was added a solution of diethyl ether: methanol (2:1, v/v) and then transferred into glass chromatoplates (20 x 20 cm), covered with silica gel and UV radiation indicator (Merck). Fractions in the extracts over the chromatoplates, containing sexual steroids (pregnenolone, P5; progesterone, P4, 17α-hydroxyprogesterone, 17P4; androstenedione, A; and testosterone, T) were separated by TLC, using the
following chromatographic systems as mobile phases: (i) benzene; (ii) benzene:ethyl acetate (7:3, v/v), and (iii) benzene: methanol (95.5, v/v).

The zone corresponding to each reference steroid applied into chromatoplates was visualized with a UV lamp (254 and 366 nm) and Örtel’s reagent (sulfuric acid:ethanol, 2:1, v/v). The area of the steroid to be scored was then scraped; such area coincides with the relative separation distance (Rf) of each reference steroid. The steroid adsorbed to the silica was then separated with diethyl ether:methanol solution (1:1, v/v); the elusion was collected in glass vials.

Instagel (Packard) was added to each vial, and the radioactive metabolite was quantified in a liquid scintillation spectrophotometer (Beckman, LS-7000) with a maximum tritium efficiency of 54%.

Production of 3β-Hydroxy-5-pregnen-20-one (pregnenolone, P5), and 17β-Hydroxy-4-androsten-3-one (T), as well as its intermediaries of the Δ4 metabolic pathway: 4-Pregnene-3,20-dione (P4); 17α-Hydroxy-4-pregnene-3,20-dione (17P4); 4-Androstene-3,17-dione (A), were estimated from assessing biotransformation percentage of precursor (C273H) into each steroid, per 100 mg of gonadal protein per hour of incubation.

Statistical Analyses. After normality and homoscedasticity data tests, we ran analyses of variance (ANOVA), followed by Tukey’s multiple comparison tests, in order to assess the monthly and seasonal concentration patterns for sex steroids, both within and between the syntopic, congeneric species. These quantitative analyses were performed with GraphPrisma routines [29] at a 90% confidence level (α ≤ 0.05).

3. RESULTS

Testes of adult males of both species biotransformed cholesterol into pregnenolone (P5), as well as into each one of the intermediary steroids in the Δ4 pathway (progesterone, P4; 17-hydroxyprogesterone, 17P4, and androstenedione, A) for the synthesis of testosterone (T). However, since the metabolite 17P4 was above the lower limit of detection of radioactivity in all cases, its production was omitted in further descriptions of the synthesis profile of T.

Production of sexual steroids (Fig. 1) mostly followed a statistically significant order in Peromyscus melanotis: P5 > T > A ≈ P4 (F = 63.89, gl 3, 12, 15, P < 0.0001); i.e., there was no significant difference between P4 and A, though the former tended to be higher. From least to most produced steroid hormones in this species, the inter-monthly production profile of P4, A, and T followed a monomodal pattern, whose increase started from February until its maximum in August, to begin its descent from September on (Fig. 2A). In this species, seasonal profile for the biosynthesis of these three steroids (Fig. 2B) increased from the spring (March-May) until reaching its maximum during the summer (June-August); then descended from the autumn (September-November), to attain its minimal production during the winter (December-February).

![Figure 1. Production of pregnenolone (P5), progesterone (P4), androstenedione (A), and testosterone (T) by the testicles of syntopic adult mice of Peromyscus melanotis and P. difficilis felipensis, at the same temperate forest. Synthesis of each steroid corresponds to biotransformation percentage of cholesterol per milligram of gonadal protein for one hour. Bars depict mean values and vertical lines ± standard deviation of the former.](image-url)
most produced, the inter-monthly profile of the biosynthesis of A, P4, and T followed a bimodal pattern (Fig. 2C). One increase starts from October, reaching a first maximum in May that decreases by July; then another increase stops at a second maximum by August to attain its minimum by September, from where the pattern goes on. The seasonal profile for the biosynthesis of A, P4, and T in this species reaches a maximum in the spring that smoothly decreases towards the summer, from where it drastically descends until its lowest values in the autumn, but then towards the winter, it increases again until its spring peak (Fig. 2D).

**Figure 2.** Monthly (left) and seasonal (right) production profiles for progesterone (P4), androstenedione (A), and testosterone (T) in the testicles of adult mice of two Peromyscus species, occurring at the same temperate forest. Synthesis of each steroid corresponds to biotransformation percentage of cholesterol, per milligram of gonadal protein, for one hour; vertical lines depict one standard deviation around the means; n = indicates number of analyzed mice in each month.

In overall, synthesis of T and its Δ4 intermediaries (P4 and A) were not significantly different (P > 0.05) between the two Peromyscus, especially in the production of A, but there was a tendency in P. d. felipensis to surpass P. melanotis in the production of P4 and T (Fig. 1). As noted before, production of T and P4 tended to be higher in P. d. felipensis, while T and A seemed to be higher in P. melanotis on monthly and seasonal basis, respectively (Fig. 2). The monthly synthesis profile of P4, A, and T, highlights the two evident peaks (May and August, respectively; Fig. 2C) in Peromyscus d. felipensis, whereas P. melanotis only presented one peak (August, Fig. 2A). Interspecific differences remain conspicuous during seasons of the year, since for P. difficilis felipensis, the highest biosynthesis of the three steroids occurred during the spring (Fig. 2D), whereas it was highest during the summer for P. melanotis (Fig. 2B).

**4. DISCUSSION**

Androgens such as testosterone (T) are biosynthesized by the interstitial tissue of the testes through two alternative metabolic pathways: the Δ4 pathway and the Δ5 pathway. The name of each pathway depends on the intervening intermediary steroids for the synthesis of T [30], and depending on the species, one path predominates over the other; e.g., in laboratory rodents such as the albino mouse (Mus musculus) and the white rat (Rattus norvegicus), the Δ4 pathway predominates [31, 32]. Results here confirm our previous findings (Peromyscus melanotis, [22]; P. difficilis, [23]) for the production of androgens in adult male mice: (i) in both Peromyscus, T biosynthesis is carried out via the Δ4 pathway, and (ii) T production has a distinctive interspecific profile in either monthly or seasonal basis.
The temporal tendency of a greater production of T and its intermediary steroids in P. d. felipensis with respect to P. melanotis, may have consequences on various processes of its biology, such as its searching behavior for foraging areas or mating territories on suitable habitats, as well as on its sexual behavior. In fact, spatiotemporal marking of species-specific territories for foraging and breeding, can be achieved through the excretion of substances that can act as pheromones in urine and faeces [33]. Pheromones act as stimulating signals for both intra and interspecific distinctive behaviors [34, 35] that can be regulated by T [13, 36]; e.g., they are magnified, becoming thus distinctive, with the concomitant concentration increase of the androgen [37]. Such acting as pheromones by T and some of its metabolites has been reported elsewhere [e.g., 12, 38-40].

Therefore, our results support a species-specific pattern of testicular biosynthesis of testosterone (T) and androstenedione (A), T in adult males of two syntopic species of Peromyscus, sharing habitat and resources in this midlatitude, temperate forest. The fact that both androgens tend to be more produced in P. d. felipensis throughout the year, adds up to a couple of evidences gathered from our previous and current studies. First, the medium-sized P. d. felipensis behaves as a permanent dweller, whereas the small-sized P. melanotis is rather a fluctuating inhabitant in the area [25]. Second, as for the use of space, the former is territorial and while the latter is rather opportunistic, they rarely overlap, especially during mating season [41]. Therefore, together these data support that upon being secreted into the bloodstream and subsequently excreted, such androgens could act as regulatory substances, such as pheromones, in the species-specific marking of temporal territories for foraging and breeding areas, according to shifts in both climate conditions and availability of resources, such as food and shelter.

On the other hand, it is known that sexual behavior is regulated by steroid hormones such as progesterone (P4) and T [10], which stimulate or delay reproduction [36], thus affecting the deployment of diverse mating strategies [42]. As has been documented in other species of the Genus Peromyscus [43, 44], it is highly likely that during their respective breeding season, the testes of P. d. felipensis and P. melanotis, produce these steroid hormones and secrete them into bloodstream from where they reach the hypothalamus and pituitary. Thence, these sexual steroids can act as modulators of agonistic displays towards other males, in order to compete for estrous females.

Our data allow us to propose that differential capacities for testosterone biosynthesis are related to spatiotemporal marking of mating territories, as well as to verify that the production of sexual steroids can explain the seasonal decoupling of the reproductive peaks in P. d. felipensis (spring) and P. melanotis (summer) at PNDL. This pattern of differential production of sexual steroids throughout the year, contributes to document our knowledge of the sexual behavior and reproductive patterns used by syntopic, congeneric Peromyscus dwelling at midlatitude, temperate forests.

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