ZOOLOGIA 41: e23032
ISSN 1984-4689 (online)
scielo.br/zool

RESEARCH ARTICLE

Intragonadal evaluation of sexual steroid hormones during three reproductive events in two species of *Peromyscus* (Rodentia: Cricetidae)

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https://zoobank.org/DFBB6A64-5837-4C7F-B331-E2563B45EEAB

ABSTRACT. The ovary of sexually mature females involves oogenetic process for follicular development, ovulation, and luteinization (gametogenic function), which in turn relate to stages of the estrous cycle (EC), as well as the production and biotransformation (steroidogenic function) of sexual steroid hormones (SSH) during EC, pregnancy, and lactation. Depending on their concentrations, SSH play different functions to elicit such reproductive events, but little is known about this in free-living wild species. Here we used ELISA to assess intraovarian concentrations of progesterone, androstenedione, testosterone, and estradiol, during EC, pregnancy, and lactation in wild adult females of *Peromyscus melanotis* J.A. Allen & Chapman, 1897 and *Peromyscus difficilis* (J.A. Allen, 1891), immediately after their capture. Results of intraspecific ANOVA showed statistical differences between concentrations of different SSH in the same reproductive stage or event and between the stages or events for each SSH, according to the steroidogenic Δ_4 pathway. Although ANOVA analyses showed no interspecific differences of the same SSH in the same event, except for more testosterone in *P. melanotis* during heat, profiles of production curves suggest intraspecific peculiarities and interspecific differences that need to be further investigated. Our results contribute to physiological-endocrine evidence during the four stages of EC and first and second part of pregnancy in species of *Peromyscus*, and are the first documentation for overall lactation in wild rodents.

KEY WORDS. Estrous cycle, female mice, lactation, ovarian function, pregnancy, sexual steroid hormones, rodents.

INTRODUCTION

Reproduction in rodents and other mammals is related to the gametogenic and steroidogenic functions of gonads. In the latter, the produced steroid hormones regulate different biological processes in both sexes (van Tienhoven 1983, Ruiz-Cortés 2012, Katsu and Iguchi 2016) and are classified by their physical-chemical characteristics and by their function as corticosteroids and sexual steroid hormones (SSH). The former, including mineralocorticoids and glucocorticoids, participate in stress response, carbohydrate metabolism, protein catabolism, sodium retention in the kidney, regulation of inflammation, bone development, blood electrolyte levels, and behavior (Katsu and Iguchi 2016). SSH, the focus of this study, are mainly produced by gonadal tissues in both sexes (Holst et al. 2004, Pawluski et al. 2009) and participate in several processes of sexual reproduction (Henricks 1991, Ruiz-Cortés 2012). They include progestogens (e.g., progesterone, P_4), androgens (e.g., testosterone, T; androstenedione, A), and estrogens (e.g., estradiol, E_2). In adult reproductive females, these SSH have different functions depending on their respective concentrations and the actual reproductive process. For

example, P_4 contributes to the implantation of embryos in the endometrium and participates in the maintenance of pregnancy (Short 1961, Porter 1970); E_2 stimulates follicle rupture during ovulation and promotes endometrial cell proliferation for future implantation (Austin and Short 1978). Consistent with the Δ_4 pathway, the predominating metabolic route for steroidogenesis in small species of rodents (Henricks, 1991, Salame-Méndez et al. 2004), progestogens are precursors of androgens, and the latter are precursors of estrogens: P_4 can be biotransformed into A, which is biotransformed into T, being the latter the main precursor of E_2 through aromatization. Besides their role as precursors of E_2 during steroidogenesis, androgens such as A and especially T, may also trigger aggressive behavior during mating or maternal care (see Chapman et al. 1998 in *Mus musculus* Linnaeus, 1758; Beehner et al. 2005 in baboons, *Papio* sp.; French et al. 2013 in several mammals, including *P. californicus*).

In females, the gametogenic function of ovaries involves the oogenetic process for follicular development (folliculogenesis), ovulation, and development of corpora lutea (luteogenesis), thus conforming the follicular and luteal phases of the ovarian cycle (OC). Steroidogenic function includes the production and secretion of both SSH and glycoprotein hormones (inhibins and activins; Suzuki et al. 1987, Findlay et al. 2001, Welt and Schneyer 2001), which in turn are related to stages of the estrous cycle (EC). Early characterization of EC in 1922 was accomplished through a very precise correlation between the histological changes of the reproductive tract and the ovaries, using exfoliative vaginal smears (EVS; Long and Evans 1922) in the albino rat *Rattus norvegicus* (Berkenhout, 1769) and the albino mouse *M. musculus* (Allen 1922). More recent studies confirmed the above in these laboratory rodents (Gorbman et al. 1983, Hebel and Stromberg 1986, Freeman 1994, Bentley 1998, Ajayi and Akhigbe 2020), allowing further characterization of the EC into the stages of diestrus, proestrus, estrus, and metestrus (Table 1); drawings and pictures of vagina and vaginal smears in laboratory rodents are available elsewhere (e.g., Gorbman et al. 1983, Heykants and Mahabir 2016, Ajayi and Akhigbe 2020 – the latter also includes histological features in vagina, uterus, and ovary, table 4: 9).

Also, Smith et al. (1975) correlated circulating (plasma) concentrations of progesterone (P_4) and estradiol (E_2), prolactin (PRL), and gonadotropins (FSH, follicle-stimulating hormone; LH, luteinizing hormone) with the stages of the EC and events of the OC, through EVS in *R. norvegicus*, and proposed four stages (I–IV, Table 1) as follows, though it should be noted that between stages III and IV, a short luteal phase occurs with twice more P_4 than E_2 ($P_4 >> E_2$) and twice less FSH than LH (FSH << LH): Stage I, luteolysis, with a proximate duration of 12h where follicular maturation begins at diestrus II, encompassing such stage and early proestrus; $E_2 >> P_4$ and FSH > LH. Stage II, mature follicles, ca. 15 hours, spans from late proestrus to early estrus; $P_4 <<< E_2$ and FSH << LH. Stage III, ovulation, ca. 6–48 hours, occurs from about the second half and towards the end of the estrus and in the metestrus; $P_4 < E_2$ and FSH < LH. Stage IV, folliculogenesis, ca. 60 hours, begins at the end of metestrus and continues throughout diestrus; $P_4 \geq E_2$ or $P_4 < E_2$ and FSH >>> LH. More recently, depending on the functional effect of gonadotropins, E_2, and P_4 on the production and degeneration of tissues, as well as accumulation and discharge of fluids, among others, Pritchett and Taft (2007) considered three phases in the EC (Table 1): i) anabolic phase, starting and ending with the liberation of oocytes, it encompasses proestrus to estrus, and is characterized by growth of reproductive tract and fluid accumulation in tissues; ii) catabolic phase during metestrus, involves depletion of fluid accumulation together with characteristic tissue growth and tissue degeneration by reabsorption or shedding; and iii) quiescent phase during diestrus with an almost static reproductive tract.

Without fertilization, the EC continues (e.g., unsuccessful cycle) in cycling females. On the other hand (Table 1) and depending on the species (González-Flores et al. 2017), when fertilization of ovules occurs, the cyclicity of EC can be interrupted and substituted by pregnancy and subsequent lactation (e.g., temporal anestrus due to a successful EC); also, a definitive anestrus can occur in aged females due to the interruption of the EC. During pregnancy, the corpora lutea of the ovaries mainly produce and secrete P_4 to maintain early pregnancy (Niswender et al. 1994). Likewise, both progestogens and estrogens regulate milk production during pregnancy and lactation (Griffin and Ojeda 1992, Hill et al. 2008). Finally, in species that cease their estrous cycle with aging, the anestrus old females have lower levels of LH, FSH, E_2 and P_4, compared to cycling younger females (González-Flores et al. 2017).

In wild species of rodents, such endocrine events of the EC have been studied mainly under experimental conditions – e.g., *Saccostomus campestris* Peters, 1846 by Westlin-van Aarde (1989), *Microtus pennsylvanicus* (Ord, 1815) by Galea et al. (1995); *Peromyscus* sp. by Eleftheriou (1968) and by Cushing (2016); *Peromyscus polinotus* A.H.Howell, 1920 by Good et al. (2003); *Peromyscus maniculatus* (Wagner, 1845) by Reed (2018), and *Proechymys guyannensis* (É. Geoffroy Saint-Hilaire, 1803) by Sanabria et al. (2019). In other studies, under

Table 1. Summary characteristics for vagina and ovarian cycle during estrous cycle in small rodents. Data gathered mostly from laboratory rat and mouse (Heape 1900, Allen 1922, Mandl 1951, Champlin et al. 1973, Smith et al. 1975, Pritchett and Taft 2007, Cora et al. 2015, González-Flores et al. 2017, Sharma et al. 2020) with some observations of in vitro follicular development in *Peromyscus* (He and Toth 2017). Parentheses include alternate names of stages.

Vaginal appearance and cytology	Ovarian cycle
Proestrus, Pro (late diestrus, diestrus II, early proestrus)	
Wide, moisten vaginal opening; reddish pink. Low to moderate cell density (cellularity): mostly round, small, nucleated epithelial cells (SNEC); 0 to rare (rare in early Pro): distinctive polymorphonucleated leukocytes, neutrophils (NEUT), large nucleated epithelial cells (LNEC), and anucleated cornified epithelial cells (ACEC).	Period of ovarian follicular growth, preceding estrus. Start of anabolic phase with [LH] surge for ovulation, [FSH] concentration peak that triggers next follicles cohort; increase of [E_2] that triggers development of antral follicles, cell divisions in vagina and uterus, and fluid accumulation. Early follicular phase, after regression of corpora lutea of luteal phase; ends with estrus incoming. Includes Stage I luteolysis and follicular maturation in early proestrus and mature follicles in Stage II during late proestrus.
Estrous, Est (heat, rut)	
Open vaginal tissues and vulva; similar to proestrus, but less humid and lighter pink; tissues less moisten and swollen. Moderate to high cellularity: Mostly ACEC; almost no or rare NEUT (late); occasional or no LNEC (early Pro); SNEC rare that increase (late Pro); nucleated epithelial cells can be round, oval or spindle shaped (late Pro).	Period of mating behavior in sexual season where ovulation occurs. Peak and end of anabolic and follicular phases. Elevated [E_2] prompts ovulation; uterus and oviduct grow, fluid accumulation continues (e.g., ampulla); maximum thickness of vaginal epithelium; cornified cells start slough off. Female sexual receptivity during dark phase of circadian cycle. Late follicular phase with mature Graafian follicles. Includes Stage III ovulation, from second half to the end of heat. Also encompasses a short luteal phase.
Metestrus, Met (metestrus I, late estrus, diestrus I, early diestrus)	
Vagina not wide open, unswollen, pale, and dry. Moderate to high cellularity: ACEC and NEUT predominate: NEUT < ACEC (early Met), NEUT = ACEC, NEUT > ACEC (late Met); 0 to rare SNEC and LNEC; NEUT interspersed, clumped to other NEUT or to NEC.	Recovery period after ovulation. Catabolic phase. [E_2] declines with a surge of [LH] and ovulation; corpora lutea and follicular atresia; uterus growth stops; epithelium degeneration; cells slough off. Initial development of corpora lutea (early to middle luteal phase). Includes stage IV with start of folliculogenesis (follicular phase) at the end of metestrus. Only occurs in absence of fecundation.
Diestrus, Die (metestrus II, diestrus II, late diestrus)	
Vaginal opening small or closed; not obvious swollen tissue; dry and bluish-purple tissues; closed mucous membranes; low vaginal mucous, excretions may occur (late Die). Low to moderate cellularity: NEUT predominate; 0 to rare ACEC (conspicuous decrease, but with clumping and shedding of these cells (early Die); some to several LNEC and SNEC, occasional vacuolated cells.	Period of ovarian secretions from corpora lutea that prepare uteri for implantation. Quiescent phase. Maturation of large corpora lutea after ovulation (late luteal phase); without mating, low [E_2] and corpora lutea become inactive; low [P_4], thus, thin uterus mucosa. Includes stage IV with continued folliculogenesis throughout diestrus (follicular phase). It also includes Stage I with the beginning of follicular development; at the end, rapid follicle growth starts (follicular phase) with vagina and uterus regrowth towards proestrus.
Anestrus ([reproductively] "inactive" as for the estrous cycle)	
No changes related to the estrous cycle. Parabasal cells of deep layer of vaginal mucosa predominate; some round or oval cornified cells with well-defined nuclei and uniform size; presence of NEUT and monocytes; little amount of debris, some bacteria at the bottom.	Period of no mating behavior. Interruption of estrus cycle, associated with pregnancy, lactation and/or aging. In general, gonadotropins (LH, FSH), [E_2], and [P_4] decrease; prolactin (PRL) is very high in lactation. No changes in ovary nor endometrium; no mating behavior. In aged females, atrophic ovaries, few primary follicles, infantile uteri, and depleted steroid biosynthesis.

controlled conditions, the OC has also been described for some wild species – e.g., *P. maniculatus bairdi* by Bradley and Terman (1979), *Neotomodon alstoni* Merriam, 1898 by Olivera et al. (1986), *Meriones unguiculatus* (Milne-Edwards, 1867) by Almeida et al. (2001), as well as the activity of females in particular stages of the EC in relation to the circadian rhythm – *P. maniculatus bairdii* by Cushing (1985) – but without considering any endocrine evaluation. Undoubtedly, the results of these studies are important, but it is necessary to document physiological or neuroendocrine conditions in free-living wild rodents through in situ ecophysiological studies that include endocrine assessments during reproductive events in females. Studies that describe the production and content of SSH within the EC and pregnancy in free-living wild species are still scarce – e.g., Nubbemeyer 1999 for *Microtus arvalis* (Pallas, 1779); Salame-Méndez et al. (2003, 2005) for *Peromyscus melanotis* J.A. Allen & Chapman, 1897 and *Peromyscus difficilis* (J.A. Allen, 1891), respectively; Sanabria et al. (2019) for *P. guyannensis*, whereas there is no documentation of such aspects in free-living lactating females. Therefore, here we assessed the intraovarian contents of P_4, A, T, and E_2 during the estrous cycle, two parts of pregnancy, and overall lactation in free-living adult females of *P. melanotis* and *P. difficilis*, which were never held under laboratory conditions.

MATERIAL AND METHODS

Capture and processing of *Peromyscus* females

Females of both species were caught alive in 2008, 2009, 2013 and 2014 in two protected areas near Mexico City: Parque Nacional Cumbres del Ajusco (PNCA: 19°13'49"N, 99°15'19"W; 2800–3500 m.a.s.l.) and Parque Nacional Desierto de los Leones (PNDL: 19°18'17"N, 99°19'14"W; 2180–3200 m.a.s.l.). Environmental characteristics (climate and vegetation) at both sites have been described by Castro-Campillo et al. (2012) and Salame-Méndez et al. (2019, 2020). Capture was selective, using Sherman traps (Tallahassee, FL, USA), baited with oat flakes, and females were transferred to the Mammal Laboratory at Universidad Autónoma Metropolitana, Unidad Iztapalapa (UAM-I) on the same day of capture, to be euthanized by cervical dislocation. Protocols for handling and death of females were carried out in accordance with international standards for animal welfare (Rudran and Kunz 1996) and the guidelines of the Ethics Commission of the Divisional Council of Biological and Health Sciences (CDBS 2010). Conventional somatic measurements were taken postmortem, and voucher specimens were prepared as complete skeletons (Ramírez-Pulido et al. 1989) to be deposited at the scientific mammal collection of UAM-I. Only adult females were selected using the characteristic adult coat pattern of each species (Álvarez-Castañeda 2005 for *P. melanotis*, Fernández et al. 2010 for *P. difficilis*) and postmortem examination of wear in the occlusal surface of cheekteeth sensu Hoffmeister (1951).

EC's stages (proestrus, estrus, metestrus, diestrus, and anestrus) were determined on each female by postmortem exfoliative vaginal smears (EVS) following recognition of cell types sensu Olivera et al. (1986) in unstained preparations at 10x and 40x objectives. During dissection, positive pregnancy was confirmed by the presence of implantations in each uterine horn (right, left). The type and number of implantations were recorded as follows: a) early implant: a recognizable clear point, 1 mm long; b) embryos of 2–5 mm in early stages of development, covered by embryonic membranes; c) uncovered embryo of 6–14 mm; and d) uncovered fetus (with sexual differentiation) of 15–25 mm. Length of the product was recorded as maximum diameter in a and b, and as distance from crown to rump, after removing the embryonic membranes in c and d. Females were then separated, according to the length of their products, into two pregnancy part stages, according to Layne (1968): a) first part of gestation (G1), or early gestation, if products were sized 1–10 mm (implants, buttons, and smaller embryos); b) second part of gestation (G2), or late gestation, when products measured ≥ 11 mm (larger embryos and fetuses). Seemingly, whenever females showed subcutaneous milk tissue, well developed nipples, and surrounding alopecia, they were considered as lactating (Kunz et al. 1996). The corpses were ovariectomized, and the left ovary of each one was frozen for this study (right ovary was used in another analysis).

Intraovarian content of sexual steroid hormones

Left ovaries were homogenized by sonication, and protein concentration was determined following Groves et al. (1968). The extraction of the total contents of sexual steroid hormones (SSH) in each homogenate was carried out in duplicate with diethyl ether. Evaluation of the contents of progesterone (P_4), androstenedione (A), testosterone (T), and estradiol (E_2) was performed in duplicate by enzyme-linked immunosorbent assay (ELISA), using commercial kits (DRG Instruments Inc® Frauenberg, Marburg, Germany) and following instructions in the manufacturer's manual. The concentration of each HES was determined using a spectrophotometer (Microplate Reader, MR 600, Dynatech Product®, Chantilly, VA, USA). The specificity of the hormone antibody and linearity described by the manufacturer of the kit were validated by testing the solutions of the hormones provided therein, as well as the previously purified solutions of P_4, A, T, and E_2 (Sigma Labs®, Santa Fe, NM, USA). Intraovarian concentrations (pg/mg of ovarian protein or pg/mg) of all or particular SSH are referred to in square brackets (e.g., [SSH], [P_4], [A], [T], [E_2]).

Statistical analyses

Data for each [SSH] ([P_4], [A], [T], [E_2]) in females of each studied species were grouped according to: a) four stages of EC (proestrus, Pro; estrus, Est; metestrus, Met; diestrus, Die); b) the first (G1) and second (G2) parts of pregnancy; and c) overall lactation (Lac). Data behavior for each hormone within the groups was explored by computing descriptive statistics along with Shapiro-Wilk's normality tests, and are summarized in the results as the mean ± SE, minimum and maximum values, W and p-values, respectively. Because most data behaved normal, single-paired ANOVA were performed in each species to find statistically significant, differences that allowed us to assess: a) the respective intraspecific production and biotransformation profile of SSH in the gonad during the same reproductive stage/event, according to steroidogenic Δ_4 pathway (e.g., progestogens → androgens → estrogens); i.e., we compared average concentration between pairs of different hormones (e.g., [P_4] vs.

each one of [A], [T], [E_2], during estrus); b) the physiological fluctuations of each [SSH] throughout the reproductive stages/events, according to its role; i.e., we compared the concentration of the same hormone between stages/events (e.g., [P_4] between stages of EC, parts of gestation, and overall lactation). Finally, c) we compared the concentration of the same SSH during the same reproductive stage or event between the two species.

All calculations were performed in PAST (version 4.05, Hammer et al. 2001) with a significance of $\alpha \leq 0.05$, and results are reported using a qualitative scale for significance levels of p-values: * = 0.05–0.01, significant; ** = 0.009–0.001, very significant; *** ≥ than three zeroes before a digit, highly or extremely significant (Note: whenever a p-value is preceded by ≥ 4 zeroes, we use E notation; e.g., 0.0000074 = 7.4E–06). Linear plots of results were constructed in Excel (ver. 13, Microsoft) and represent cyclicity from the estrous cycle through pregnancy and overall lactation in two parts: a) a complete estrous cycle (CEC), from proestrus to diestrus, which was unsuccessful in terms of fecundation and subsequent pregnancy; and b) a successful EC (SEC), from proestrus to lactation, where copulation during estrus heat leads to fecundation and is followed by pregnancy and lactation. Note that the intraovarian contents of proestrus and estrus are repeated in the SEC. The results of the ANOVA were plotted as bars (means) with vertical whiskers (SE) and were also constructed in PAST. Detailed ANOVA tables of results for the two comparative, aforementioned, intraspecific analyses (a, b) are available in Tables S1 and S2.

RESULTS

Descriptive statistics and Shapiro-Wilk's normality tests for the four analyzed [SSH] in the total of the 82 *Peromyscus* females (39 *P. melanotis*; 43 *P. difficilis*) are shown in Table 2. Data were analyzed, according to the stages of estrous cycle, parts of pregnancy, and overall lactation. As shown in Table 1, most intraovarian concentration data for SSH were normally distributed in each reproductive stage or event in the two species, except for [A] during the first part of pregnancy (G1) and for [E_2] during diestrus (Die) in *P. difficilis*.

Qualitative description of the estrous cycle

Peromyscus melanotis (Fig. 1A). In the complete estrous cycle (CEC), [P_4] increased from proestrus to its maximum at estrus, then decreased steadily in metestrus and diestrus. When pregnancy occurred in a successful cycle (SEC), [P_4] increased steadily from diestrus through the next proestrus and estrus, until it reached its maximum in early gestation (G1), but then collapsed into late gestation (G2) and barely increased in overall lactation. The [P_4] peak during G1 was 9.9 pg/mg higher than the [P_4] peak in the estrus, whereas the two lowest concentrations in diestrus and G2 only differed from each other by 0.24 pg/mg. Similarly, during the CEC, [E_2] increased from the proestrus to its maximum peak in the estrus, but then collapsed in the metestrus to increase again in the diestrus. After fecundation in a SEC, [E_2] continued to increase into the next proestrus and estrus, but then collapsed until its minimum in G1 to increase again in G2 and slightly more during lactation. The estrogen was 12.9 pg/mg higher in estrus than in overall lactation and 1.39 pg/mg lower in metestrus than in G1. As for the intermediate androgens, [A] increased steadily in the CEC from proestrus to diestrus, whereas in the SEC, [A] decreased towards the next proestrus, estrus, and G1, but then increased towards G2 and decreased again in lactation. This androgen only differed 2.3 pg/mg between the higher concentrations of diestrus and G2, and 0.85 pg/mg between the lower concentrations at G1 and proestrus, respectively. Opposite to the former, [T] decreased steadily in the CEC through proestrus, estrus, and metestrus, then increased in diestrus and next proestrus after fecundation occurred in the SEC, but then it fell down from estrus to G1, increased again in G2, and decreased slightly in lactation. The highest [T] peak in proestrus differed by 7.25 pg/mg from the peak in lactation, whereas its minimum concentration in G1 was 1.52 pg/mg lower than in metestrus.

Peromyscus difficilis (Fig. 1B). In the CEC, [P_4] increased from its minimum in proestrus to its maximum in estrus; then it decreased steadily in metestrus and diestrus. After fecundation, [P_4] still decreased into a new proestrus, but then increased through the estrus to its maximum peak in early gestation G1 to collapse into late gestation G2, decreasing to its lowest value in lactation. The highest maximum of [P_4] in G1 was 11.45 pg/mg more than in estrous, and the minimum in lactation was 0.21 pg/mg less than in proestrus. [E_2] showed its maximum during estrus in either EC: in the CEC, [E_2] increased from proestrus to estrus, then collapsed into its minimum in metestrus to increase again steadily through diestrus to a new proestrus and estrus after fecundation in a SEC; however, [E_2] collapsed towards G1, increasing again in G2 and slightly more in lactation. The maximum peak of [E_2] in estrus was 14.25 pg/mg higher than in lactation, and the minimum in G1 was 1.63 pg/mg higher than in metestrus. In the CEC, [A] decreased from

Table 2. Intraovarian concentration data for four sexual steroid hormones in two free-living *Peromyscus* species. Concentration data (pg/mg) are arranged into three reproductive events: Estrous cycle (stages: Pro, proestrus; Est, estrus; Met, metestrus; Die, diestrus), pregnancy (two parts: early, G1, and late, G2, gestations), and overall lactation (Lac). Descriptive statistics (with the respective abbreviations) include sample size (n), Mean, standard error (SE), minimum and maximum values (Min-Max). In Shapiro-Wilk's normality tests (W), italicized bold and p-values violate normality ($p \leq 0.05$).

Stage	Estrous cycle				Pregnancy		Nursing
	Pro	Est	Met	Die	G1	G2	Lac
Peromyscus melanotis (n = 39)							
n	5	4	6	5	6	5	8
Progesterone (P_4)							
Mean±SD	11.8±5.1	18.9±2.2	13.4±0.99	8.8±1.7	28.8±2.2	8.6±0.89	8.6±0.75
Min-Max	4.5–17.9	15.0–23.6	10.2–16.7	3.6–13.7	21.2–35.2	5.5–11.5	5.4–11.4
W	0.97	0.86	0.98	0.97	0.89	0.99	0.99
p	0.87	0.27	0.96	0.85	0.26	0.99	0.99
Androstenedione (A)							
Mean±SD	5.0±1.3	6.6±1.1	8.9±0.8	9.9±1.9	4.2±0.9	7.7±0.9	6.7±0.6
Min-Max	2.2–9.0	3.6–8.6	6.4–11.4	4.0–15.5	1.1–7.4	4.8–10.8	4.5–8.5
W	0.92	0.92	0.98	0.96	0.95	0.99	0.94
p	0.54	0.54	0.96	0.83	0.74	0.99	0.63
Testosterone (T)							
Mean±SD	17.2±2.5	11.8±1.1	6.6±0.97	12.2±2.7	5.1±0.7	10.6±0.9	9.9±0.8
Min, Max	10.8–25.2	9.2–14.1	3.6–9.6	6.1–21.3	2.2–7.2	7.3–13.3	6.4–12.4
W	0.96	0.96	0.95	0.91	0.92	0.98	0.95
p	0.84	0.77	0.75	0.46	0.5	0.96	0.73
Estradiol (E_2)							
MeanX±SD	15.1±3.4	24.8±1.7	6.9±0.8	11.2±2.5	8.3±0.6	11.7±0.9	11.9±1.0
Min, Max	3.9–23.8	20–28.2	4.2–9.6	4.5–19.2	6.4–10.4	8.8–14.8	7.3–15.3
W	0.98	0.94	0.99	0.96	0.92	0.99	0.97
p	0.91	0.65	0.98	0.81	0.43	1.00	0.93
Peromyscus difficilis (n = 43)							
n	6	6	7	5	6	5	8
Progesterone (P_4)							
Mean±SD	8.3±1.8	14.7±1.0	14.2±1.3	11.3±0.4	26.1±1.6	9.0±0.7	8.1±0.5
Min-Max	3.6–14.0	11.0–18.0	10.1–19.1	10.5–12.7	22.9–32.0	7.1–11.6	6.4–10.4
W	0.88	0.96	0.96	0.86	0.85	0.91	0.94
p	0.27	0.78	0.82	0.21	0.16	0.44	0.61
Androstenedione (A)							
Mean±SD	7.3±1.3	6.8±1.6	9.2±1.4	7.0±0.9	3.9±0.7	8.7±1.1	7.9±0.5
Min-Max	4.3–12.2	3.0–12.6	4.0–16.0	4.4–8.9	2.6–7.4	6.6–11.7	6.0–10.3
W	0.89	0.87	0.92	0.83	0.72	0.83	0.95
p	0.33	0.23	0.50	0.14	***0.01***	0.13	0.66
Testosterone (T)							
Mean±SD	15.8±2.7	8.1±1.1	9.5±0.9	8.8±0.9	5.5±0.8	9.4±1.8	8.7±0.9
Min, Max	9.4–26.3	5.7–12.7	5.6–12.6	6.8–12.0	2.7–7.8	3.3–13.1	5.2–11.3
W	0.86	0.87	0.97	0.91	0.96	0.9	0.87
p	0.2	0.23	0.89	0.46	0.83	0.44	0.15
Estradiol (E_2)							
Mean±SD	19.9±1.6	27.1±2.7	7.9±0.9	13.3±1.8	9.5±1.9	12.6±1.0	12.8±1.1
Min, Max	16.4–27.2	19.3–37.3	4.3–10.6	6.3–17.0	2.5–15.0	9.3–15.1	8.0–18.0
W	0.84	0.93	0.94	0.77	0.96	0.96	0.92
p	0.12	0.56	0.63	***0.05***	0.79	0.83	0.44

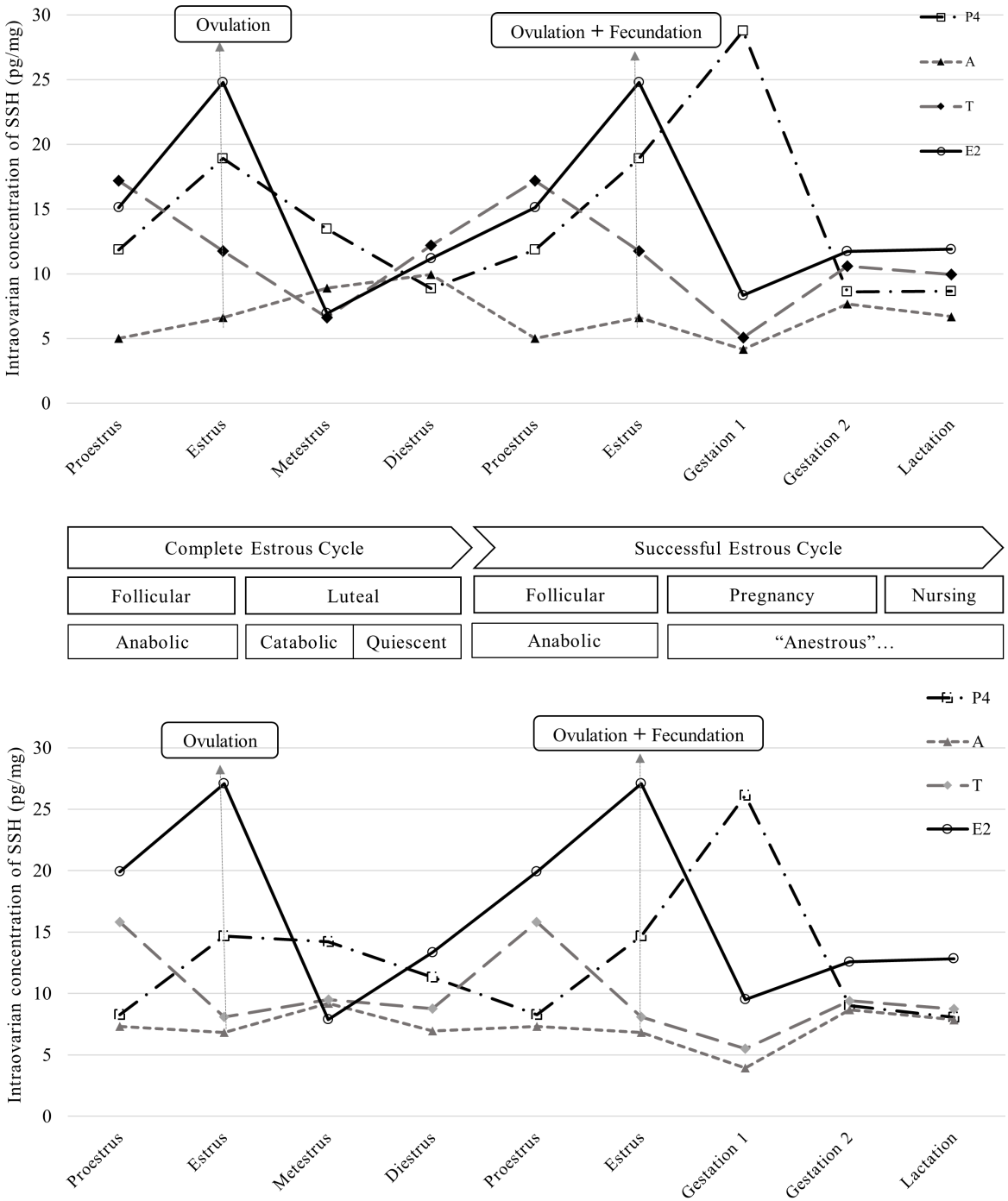

Figure 1. Intraovarian contents of selected Δ_4 pathway's SSH in two *Peromyscus* species. Sexual steroid hormones (SSH: progesterone, P_4; androstenedione, A; testosterone, T; estradiol, E_2) were obtained from free-living, adult females of *P. melanotis* (A) and *P. difficilis* (B), during a complete estrous cycle (CEC: proestrus to diestrus), and after ovulation (vertical arrows) followed by fecundation in a successful estrous cycle (SEC: proestrus, estrus + early gestation 1 and late gestation 2 + overall lactation); note that proestrus and estrus data from CEC are duplicated in SEC). The oogenetic and anabolic/catabolic phases of the ovarian cycle are also depicted (see Table 1).

proestrus to its minimum in estrus, then increased towards its maximum at metestrus, but decreased again in diestrus and only increased a little towards a new proestrus in a SEC; after fecundation, [A] decreased more to its lowest minimum in G1, then increased towards its highest maximum in G2 to decrease a little in lactation. The maximum [A] reached in metestrus was 0.52 pg/mg higher than that in G2, but the minimum in estrus was 2.9 pg/mg higher than in G1. Finally, [T] decreased from proestrus to estrus in the CEC, then increased in metestrus and decreased again in diestrus; after fecundation, [T] increased from diestrus to a new proestrus in a SEC, decreased again from estrus to G1, increased towards G2, and decreased a little more in lactation. The difference between the maximum [T] was 14.25 pg/mg higher in estrus than in lactation, while the difference between the minimum [T] in G1 was 1.63 pg/mg higher than in metestrus.

Comparative biotransformation profiles of [SSH] within each reproductive stage or event

All ANOVA comparisons resulted in statistical differences between the concentrations of one or more pairs of SSH (Fig. 2, Table S1), according to the stages involved in the estrous cycle, the two parts of pregnancy, and overall lactation in adult females of both *P. melanotis* (Fig. 2A) and *P. difficilis*, (Fig. 2B). Such statistical differences and its level of p-value allowed the understanding of the biotransformation of SSH within the ovary, according to the Δ_4 pathway (e.g., progestogens biotransform into androgens and androgens into estrogens). In these analyses, the p-values are reported in three intervals to emphasize the involved biotransformations: significant changes (from 0.01 to 0.04), very significant changes (from 0.001 to 0.008), and extremely significant changes (from 1.1E–04 to 3.5E–07).

Peromyscus melanotis (Fig. 2A, Table S1). Proestrus: [T] was the highest, followed by [E_2], [P_4], and [A], but only the latter was statistically different for being the lowest; in the Δ_4 pathway, the reduction of 6.8 pg/mg from [P_4] to [A] was statistically significant (p = 0.03), the increase of 12.2 pg/mg in [T] from [A] was very significant (p = 0.003), whereas the reduction of 2.1 pg/mg from [T] to [E_2] was not significant; [P_4] was 3.3 pg/mg lower than [E_2] but no significantly different. Estrus heat: [E_2] scored the highest, followed by [P_4], [T] and [A]; in the biotransformation pathway, the decrease of 12.3 pg/mg in [A] from [P_4] was very significant (p = 0.002), the increase of 5.2 pg/mg in [T] from [A] was significant (p = 0.02), and the increase of 13 pg/mg in the highest [E_2] from [T] was extremely significant (p = 7.3E–04); [E_2] was 5.9 pg/mg not significantly higher than [P_4], but 18.2 pg/mg very significantly higher (p = 1.1E–04) than [A]. Metestrus: [P_4] was the highest and the only statistically different, with [A], [E_2], [T] descending in concentration; in the Δ_4 pathway, decrease of 4.5 pg/mg in [A] from [P_4] was very significant (p = 0.004), whereas both the reduction of 2.3 pg/mg from [A] to [T] and the slight increase of 0.3 pg/mg in [E_2] from the latter, were not significant; [P_4] was extremely significantly higher than both [E_2] (6.5 pg/mg more, p = 4.7E–04] and [T] (6.8 pg/mg more, p = 5.8E–04). Diestrus: the descending order was [T], [E_2], [A], and [P_4] without any significant differences; in the biotransformation pathway, [A] increased 1.1 pg/mg from [P_4] and [T] 2.3 pg/mg from the former, whereas [E_2] decreased 1.0 pg/mg from [T]; [P_4] was only 2.4 pg/mg, not significantly lower than [E_2]. Early gestation G1: [P_4] scored the highest, differing statistically from other [SSH] that descended as [E_2], [T], and [A]; in the biotransformation pathway, [P_4] collapsed abruptly into [A] by a 24.6 pg/mg of difference (ca. seven times lower), then there was a slight not significant increase of 0.9 pg/mg in [T] from [A], followed by a very significant increase (p = 0.005) of 3.2 pg/mg in [E_2] from the former; [P_4] was extremely significantly higher than [E_2] by 3.5 times (20.5 pg/mg more, p = 1.0E–06), and by 5.7 times higher (23.7 pg/mg more, p = 2.2E–07) than [T]]; also, [E_2] was very significantly higher than [A] (4.1 pg/mg, p = 0.002) and [T] (3.2 pg/mg, p = 0.005). Late gestation G2: [E_2] was the highest, followed by [T], [P_4], and [A]; as for the Δ_4 pathway, there was a slight not significant decrease of 0.9 pg/mg from [P_4] to [A], then a significant increase (2.9 pg/mg, p = 0.04) in [T] from [A], finishing with a not significant increase of 1.1 pg/mg in [E_2] from [T]; [P_4] was 3.1 pg/mg significantly lower (p = 0.03) than [E_2], whereas the estrogen was 4 pg/mg very significantly higher (p = 0.008) than [A]. Lactation: the overall pattern of [SSH] was somehow similar to G2; in the biotransformation pathway, [A] was 1.9 pg/mg lower and not significantly different from [P_4], whereas the increase of 3.2 pg/mg in [T] from [A] was very significant (p = 0.007), followed by a not significant increase of 2 pg/mg in [E_2] from [T]; [P_4] was also 3.3 pg/mg significantly lower (p = 0.024) than [E_2] and [A] was extremely significantly lower (5.2 pg/mg, p = 7.2E–04) than [E_2].

Peromyscus difficilis (Fig. 2B, Table S1). Proestrus: the descending order was [E_2], [T], [P_4], and [A]; in the Δ_4 pathway, [A] decreased not significantly 1 pg/mg, from [P_4], but the 8.5 pg/mg increase in [T] from [A] was significant (p = 0.02), and the following increase of 4.1 pg/mg in [E_2] from [T] was not significant; [P_4] was 11.6 pg/mg extremely significantly lower (p = 7.3E–04) than [E_2] and 7.5 pg/mg

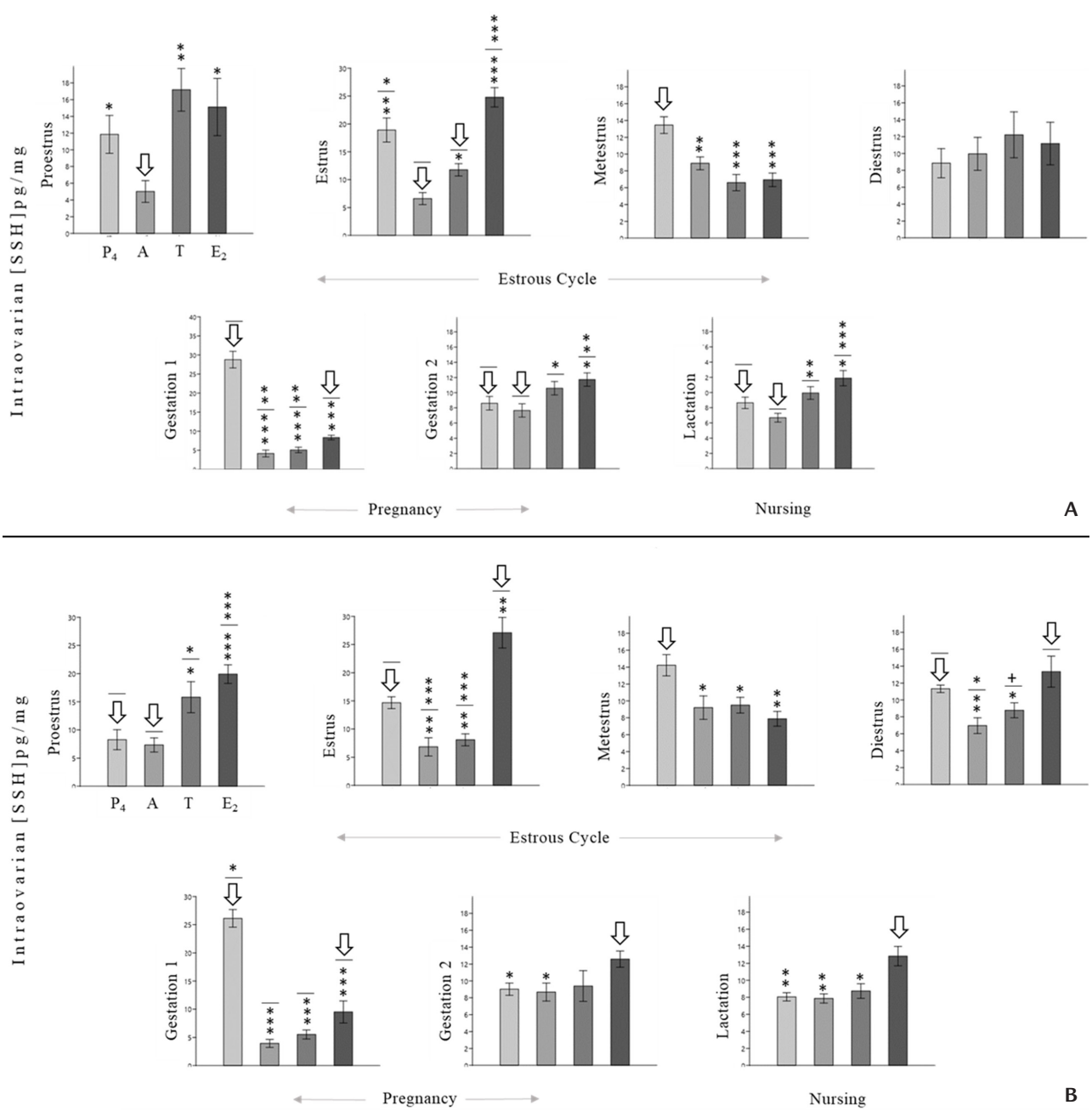

Figure 2. Production and biotransformation profiles in four selected SSH of the Δ_4 pathway by reproductive event in two species of *Peromyscus*. Mean intraovarian concentrations of sexual steroid hormones ([SSH]: progesterone, P_4; androstenedione, A, testosterone, T; estradiol, E_2) were assessed in free-living, adult females of *P. melanotis* (A) and *P. difficilis* (B), during estrous cycle (PRO, proestrus; EST, estrus; MET, metestrus; DIE, diestrus), pregnancy (early gestation, G1; late gestation, G2), and overall lactation. Bars depict mean [SSH] and vertical whiskers the respective standard errors; arrows signal which [SSH] had significant differences with others; when more than one [SSH] was statistically different in ANOVA analyzes, horizontal lines were added and arrows below and above them signal the respective [SSH]; number of asterisks indicate degree of significance in p-values (see Methods, Results, and the ANOVA in Table S1): * = significant; ** = very significant; *** = extremely significant; the plus sign (+) shows an almost statistically significant instance. Note that scales of plots differ.

significantly lower (p = 0.04) than [T]; [A] was 12.6 pg/mg extremely significantly lower (p = 1.2E–04) than [E_2]. Estrus heat: [E_2] was the highest followed by [P_4], [T], and [A]; in the biotransformation pathway, the reduction of 7.9 pg/mg in [A] from [P_4] was very significant (p = 0.002), the slight increase of 1.3 pg/mg from [A] into [T] was not significant, but there was conspicuous increase of 19 pg/mg in [E_2] from [T] extremely significant (p = 6.6E–05); [P_4] was 12.4 pg/mg lower than [E_2] and very significantly different (p = 0.002), 6.6 pg/mg higher than [T] and also very significantly different (p = 0.001); [E_2] was also 20.3 pg/mg and extremely significantly higher than [A] (p = 7.6E–05). Metestrus: [P_4] scored the highest and was statistically different from other [SSH]; in the Δ_4 biotransformation, a decrease of 5 pg/mg from [P_4] into [A] was significant (p = 0.02), but the slight increase of 0.3 in [T] from [A] and the decrease of 1.6 pg/mg in [E_2] from [T] were not significant, respectively; [P_4] almost doubled (6.3 pg/mg) and was very significantly higher (p = 0.001) than [E_2]. Diestrus: [E_2] was the highest, followed by [P_4], [T], and [A]; in the biotransformation pathway, there was a very significant (p = 0.003) decrease of 4.3 pg/mg from [P_4] into [A], a slight, not significant increase of 1.8 pg/mg in [T] from [A], and an almost significant (p = 0.055) increase of 4.5 pg/mg in [E_2] from [T]; [P_4] was 2 pg/mg lower than [E_2] but not significantly different, but it was 2.5 pg/mg significantly higher (p = 0.03) than [T], and [E_2] was 6.3 pg/mg and significantly higher (p = 0.01) than [A]. Early gestation G1: [P_4] was the highest, followed by [E_2], [T], and [A]; in the biotransformation pathway, the collapse of almost seven times with a reduction of 22.2 pg/mg from [P_4] into [A] was extremely significant (p = 1.4E–07), followed by not significant slight increases of 1.2 pg/mg between the two androgens and of 4.4 ng/m in [E_2] from [T], respectively; [P_4] almost tripled [E_2] with an extremely significant difference (p = 5.7E–05) of 16.6 pg/mg and was also about five times more concentrated than [T] with an extremely significant (p = 3.5E–07) difference of 21 pg/mg; [E_2] was also 5.6 pg/mg significantly (p = 0.02) higher than [A]. Late gestation G2: [E_2] was the highest, followed by [T], [P_4], and [A]; in the Δ_4 pathway, there was reduction of 0.3 pg/mg in [A] from [P_4], an increase of 0.7 pg/mg in [T] from the latter, and an increase of 3.2 pg/mg in [E_2], none of which were significant; [P_4] was 3.6 pg/mg significantly lower (p = 0.02) than [E_2] and the estrogen was 3.9 pg/mg significantly higher (p = 0.03) than [A]. Lactation: the concentration order was the same as in G2; there were only slight and no significant changes from the progestogens into androgens because [A] was only 0.2 pg/mg lower than [P_4] and [T] was only 0.8 higher than the former, but the increase of 4.1 pg/mg in [E_2] from [T] was significant (p = 0.01); the estrogen was 4.7 pg/mg very significantly higher (p = 0.002) than [P_4] and 4.9 pg/mg very significantly higher (p = 0.001) than [A].

Fluctuations in the concentration of each SSH through estrous cycle, pregnancy, and overall lactation

The ANOVA (Fig. 3, Table S2) for each [SSH] also resulted in several significant differences in its concentrations between the stages of EC, the two parts of pregnancy, and overall lactation in adult females of *P. melanotis* (Fig. 3A) and *P. difficilis* (Fig. 3B). In these analyses, the significant differences in the concentration of the same SSH were also arranged, according to three intervals for the respective p-values, in order to emphasize its physiological increase or decrease in a particular reproductive stage or event: significant differences fluctuated from 0.01 to 0.05; very significant differences fluctuated from 0.001 to 0.009; extremely significant differences fluctuated from four to eight ceros after a period and before any digit as 1.1E–04 to 3.2E–08.

Peromyscus melanotis (Fig. 3A, Table S2). Overall arrangement from most to least [P_4] was G1 > Est > Met > Pro > Die > Lac > G2. The maximum peak of [P_4] after fecundation and initial development of products in early gestation (G1) was extremely significantly higher than most reproductive stages of a complete estrous cycle (CEC: Pro p = 3.7E–04; Met p = 8.4E–05; Die p = 5.5E–05), as well as than late gestation (G2, p = 6.1E–06) and overall lactation (Lac p = 1.5E–06), being also significantly higher (p = 0.02) than its other conspicuous peak in estrus. Except for proestrus, this second maximum [P_4] at estrus was also statistically significant and conspicuously higher than everything else (Met p = 0.03; Die p = 0.008; G1, p = 0.02; G2 p = 0.001; Lac p = 3.8E–04). In metestrus, [P_4] was also statistically higher than in diestrus (p = 0.04), late gestation G2 (p = 0.005), and lactation (p = 0.002).

Androstenedione [A] was arranged as Die > Met > G2 > Lac > Est > Pro > G1. The lowest [A] in G1 was statistically different from metestrus (p = 0.002), diestrus (p = 0.014), late gestation G2 (p = 0.02), and lactation (p = 0.03); [A] in metestrus was statistically higher than in lactation (p = 0.04) and proestrus (p = 0.02). Thus, the maximum [A] reached at diestrus in the CEC was not statistically different from the maximum during G2 in a successful EC (SEC); however, after fecundation, the minimum [A] in early gestation G1 was significantly lower than in G2 and lactation. In the CEC, the increase of [A] from proestrus to diestrus only had significant differences between proestrus and metestrus,

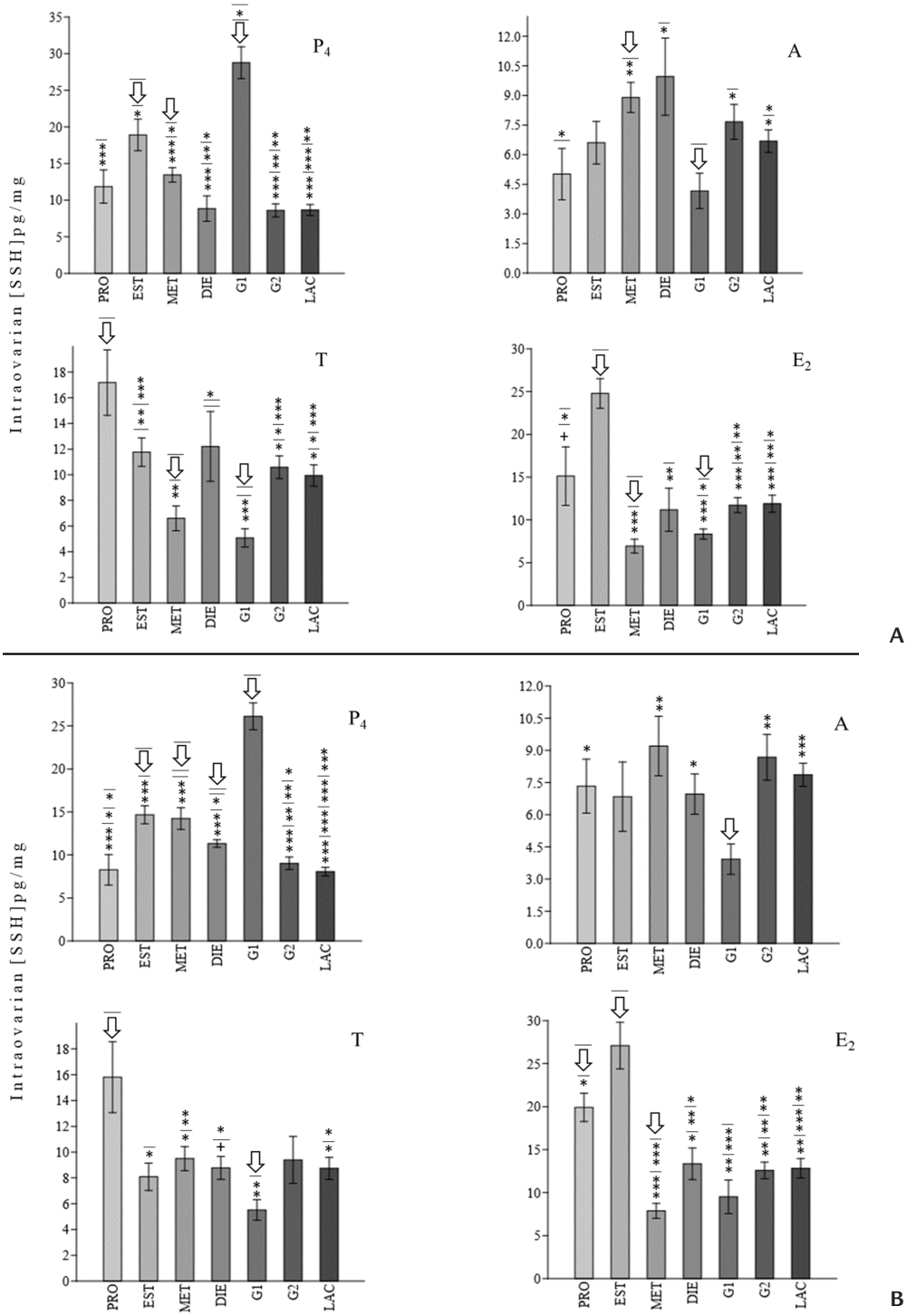

Figure 3. Fluctuations of each intraovarian [SSH] in the Δ_4 pathway throughout three reproductive events in two species of *Peromyscus*. Mean concentrations of sexual steroid hormones, [SSH], were obtained from estrous cycle, pregnancy and lactation in free-living, adult females of *P. melanotis* (A) and *P. difficilis* (B). Symbology as in Fig. 2. Note that scales differ; complete ANOVA information is available in Table S2.

whereas in the SEC, the collapse of [A] from estrus to early gestation G1 was statistically significant, as was the [A] increase from G1 to G2.

The order of [T] was Pro > Die > Est > G2 > Lac > Met > G1. The lowest [T] in early gestation G1 was statistically different from everything else (Pro p = 3.5E–04, Est p = 4.9E–04, Die p = 0.01, G2 p = 4.9E–04, Lac p = 8.2E–04), except for the second lowest [T] in metestrus; the latter also was very significantly segregated from the maximum [T] in proestrus (p =0 0.002) and estrus (p = 0.008), whereas it was significantly different from late gestation G2 (p = 0.01), and overall lactation (p = 0.02). The two collapses of [T] in metestrus and G1 were not significantly different from each other. The maximum [T] in proestrus was significantly higher in either a CEC or a SEC; indeed, the only other [T] peak in G2 was statistically lower. In the CEC, the fall of [T] is interrupted by its statistically significant increase in diestrus, whereas during the SEC, both the collapse from estrus to G1, and its increase towards G2 were statistically significant.

Estradiol [E_2] was arranged as Est > Pro > Lac > G2 > Die > G1 > Met. The maximum [E_2] peak in estrus differed extremely significantly from most [SSH] (Met p = 6.0E–06, G1 p = 1.6E–06, G2 p = 7.4E–05, Lac p = 6.5E–05) and very significantly from diestrus (p = 0.004), being almost significantly different from proestrus (p = 0.054). The minimum [E_2] in metestrus was significantly different from that in proestrus (p = 0.03) and very significantly different from that in both late gestation G2 (p = 0.002) and lactation (p = 0.003), whereas the next minimum in early gestation G1 was significantly different from that in proestrus (p = 0.04) and lactation (p = 0.01) and very significantly different from that in G2 (p = 0.007); [E_2] in metestrus and G1 were not statistically different from each other nor from that in diestrus. The maximum [E_2] in the estrus of either a CEC or a SEC was statistically the highest. On the other hand, both the [E_2] in late gestation G2 and lactation were significantly the lowest. In a CEC, both the sudden increase of [E_2] from proestrus to estrus and the abrupt collapse in metestrus were statistically significant. In the SEC followed by pregnancy and lactation, the collapse of [E_2] into G1 was statistically significant, as was its increase in G2 and lactation.

Peromyscus difficilis (Fig. 3B, Table S2). The order for [P_4] was G1 > Est > Met > Die > G2 > Pro > Lac. Clearly, the maximum [P_4] in early gestation G1 was statistically the highest and extremely significantly different from anything else (Pro p = 2.0E–05, Est p = 1.1E–04, Met p = 8.9E–05, Die p = 1.6E–05, G2 p = 6.8E–06, Lac p = 3.2E–08); the other but lower peak of [P_4] in estrus was also statistically higher than everything else (Die p = 0.02, G2 p = 0.002, Lac p = 4.2E–05), except for metestrus; the three minimum concentrations of [P_4] were also statistically different from the higher concentration in metestrus (Lac p = 3.4E–04, Pro p = 0.02, G2 p = 0.009), whereas the higher concentration in diestrus also differed from lactation (p = 8.0E–04) and G2 (p = 0.03). Therefore, the maximum [P_4] in G1 during a SEC was statistically higher than the maximum [P_4] in the estrus-metestrus of the CEC, while the lowest [P_4] in the proestrus was not different from the minimum [P_4] in G2 and lactation. In the CEC, the increase of [P_4] from proestrus to estrus is statistically significant, whereas in the SEC interrupted by pregnancy, both the sharp increase of [P_4] from estrus to G1 and its abrupt collapse from G2 to G1 were statistically significant.

In [A], the order was Met > G2 > Lac > Pro > Die > Est > G1. Only the lowest [A] in early gestation G1 differed statistically from all other reproductive stages or events (Pro p = 0.04, Met p = 0.008, Die p = 0.003, G2 p = 0.004, Lac p = 7.5E–04), except for estrus. Therefore, the concentration of this anabolic androgen remained almost constant around 6–9 pg/mg; the two slight peaks during metestrus in the CEC and late gestation G2 of the SEC, respectively, were similar to each other. In the CEC, due to the dispersion of data, there were no significant gaps between lower (e.g., Pro, Est, Die) and higher (e.g., Met) [A]. However, in the SEC, both the decrease of [A] from estrus to G1 and the increase from G1 to G2 were statistically significant.

The arrangement for [T] was Pro > Met > G2 > Die > Lac > Est > G1. The maximum [T] in proestrus differed from all others (Pro p = 0.03, Met p = 0.04, G1 p = 0.005, Lac p = 0.02), except for [T] in late gestation G2, and was almost significant for [T] in diestrus (p = 0.052), whereas the minimum [T] in early gestation G1 also differed from [T] in metestrus (p = 0.009), diestrus, and lactation (p = 0.02 both). The fluctuating [T] showed three peaks: the highest [T] in proestrus was statistically significant in the CEC and the SEC; the second peak in metestrus and the third one in G2 were similar to each other. In a CEC, only the fall of [T] from proestrus to estrus was statistically significant. The collapse of [T] at G1 in the SEC was statistically significant, whereas both its decrease from estrus to G1 and its increase from G1 to G2 were statistically not significant.

Estradiol [E_2] was arranged as Est > Pro > Die > Lac > G2 > G1 > Met. The maximum [E_2] peak in estrus was significantly higher than everything else (Pro p = 0.05, Met p = 1.7E–05, Die p = 0.003, G1 p = 3.6E–04, G2 p = 0.001, Lac p = 1.7E–004), and the next highest [E_2] peak in proestrus was also statistically different from the others (Met p = 3.2E–05,

Die p = 0.03, G1 p = 0.002, G2 p = 0.005, Lac p = 0.003); the minimum [E_2] peak in metestrus was statistically lower than everything else (Die p = 0.01, G2 and Lac p = 0.005), except for early gestation G1. Once again, the maximum [E_2] reached at estrus, was clearly statistically defined and higher than the peak in G2-Lac after fecundation. In the CEC, the two increases of [E_2] in Pro-Est and Met-Die were statistically significant. Also, the two abrupt collapses of [E_2] from estrus to metestrus in the CEC and from estrus to G1 in the SEC were statistically significant, respectively.

Interspecific comparisons

Due to the dispersion of data, in general, there were no significant interspecific differences between the concentration of the same SSH in the same stage of the EC, in the same parts of pregnancy or in overall lactation. The only exception was the statistically higher [T] in *P. melanotis* during estrus (df = 1, 8, 9; F = 5.35; p = 0.04). Although [SSH] appears to be practically similar in these species, there are noteworthy qualitative differences in the production curves of SSH along both CEC and SEC (Figs 1, 4).

Notwithstanding the practically similar interspecific [SSH], production curves of SSH along both a CEC and SEC showed noticeable interspecific differences (Figs 1, 4). *Peromyscus melanotis* showed more defined patterns of increase/decrease without any smoothing of slope as in *P. difficilis* (e.g., Fig. 4A-C for P_4, A, and T, respectively). Even in the most similar curves of E_2 (Fig. 4D), there was a slight change of slope in *P. melanotis* towards the second peak in estrus, that was absent in *P. difficilis*. The [A], the least concentrated SSH along the three reproductive events (Fig. 4B), had the most different curves between the species, behaving oppositely in proestrus and diestrus (e.g., minimum vs. maximum), and the same happened for [T] in metestrus (Fig. 4C).

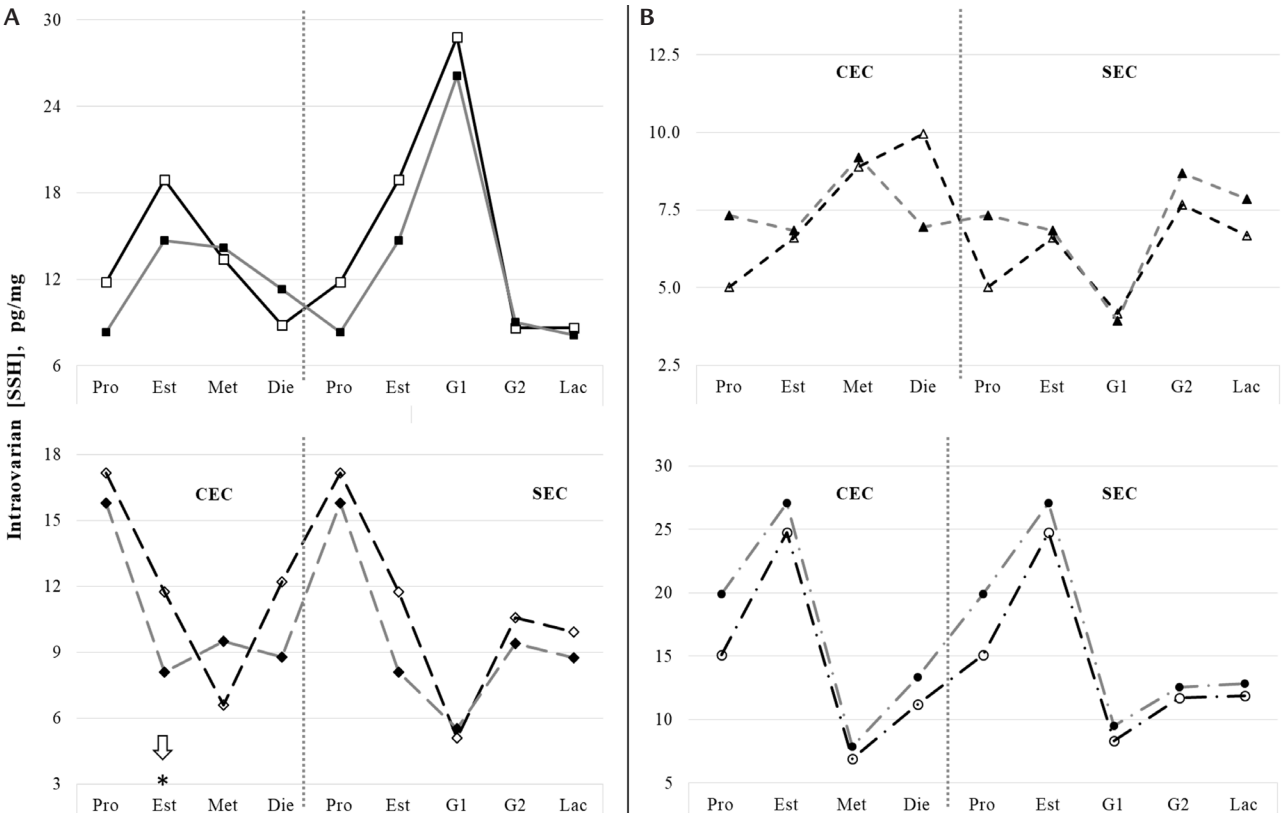

Figure 4. Comparison of intraovarian contents of each steroid hormone in two *Peromyscus* species: (A) progesterone [P_4], (B) Androstenedione [A], (C) Testosterone [T_1], (D) Estradiol [E_2]. Concentrations curves include a complete estrous cycle (CEC) and a successful estrous cycle (SEC; i.e., followed by pregnancy and lactation), in free-living, adult females of *P. melanotis* (open markers) and *P. difficilis* (closed markers). The arrow and asterisk signal the only interspecific significant difference. Symbology after Fig. 1.

DISCUSSION

Our results of intraovarian contents of the four SSH in the steroidogenic Δ_4 pathway during the estrous cycle (EC), pregnancy, and lactation contribute to endocrine evidence on the reproductive physiology in free-living adult females of *P. melanotis* and *P. difficilis*, dwelling in a mid-latitude temperate forest, and provide primary evidence for free-living rodents. These cohabiting *Peromyscus* exert a particular use of the spatial microhabitat (De la Cruz et al. 2019, 2020, 2021), according to their seasonal, species-specific reproductive optima, even though reproductively active individuals can be captured throughout the year in both species (Castro-Campillo et al. 2008). According to [SSH] levels in males (Salame-Méndez et al. 2019), the reproductive optimum for *P. melanotis* occurs in summer, while *P. difficilis* shows a higher peak during late spring and a lower one in winter. Such reproductive endocrine data in males are consistent in each species, with a greater number of captured pregnant and lactating females in the aforementioned mating seasons (Castro-Campillo et al. 2008).

Estrous cycle

The EC is controlled by the central nervous system (CNS), through the Hypothalamus-Pituitary-Gonadal (HPG) axis (Ruiz-Cortés 2012). Sharma et al. (2020) summarize the glandular and hormonal events of the HPG axis. The hypothalamus secretes gonadotropin hormone-releasing hormone (GnRH) into the adenohypophysis, which in turn releases luteinizing hormone (LH] and follicle-stimulating hormone (FSH) into the bloodstream; the highest [GnRH] triggers a pre-ovulatory surge of gonadotropins on proestrus afternoon. Such gonadotropins induce ovarian folliculogenesis, steroidogenesis, ovulation, and the formation of corpora lutea (CL) through the production, biotransformation, and action of sexual steroid hormones (SSH) in the ovary (see fig. 2: 45 in Sharma et al. 2020). According to Sharma et al. (2020), in the rodent estrous cycle (EC), LH and FSH are maintained at low basal levels on the estrus, metestrus, diestrus, and on the early proestrus due to negative feedback by E_2 and P_4. When the circulating level of [E_2] becomes high on the late proestrus, FSH and LH start to rise rapidly, generating the pre-ovulatory increase of [E_2]; increasing [LH] plus surge of [E_2] prompts ovulation at the night proestrus, followed by a decrease in both circulating gonadotropins. In early estrus, LH is at the basal level, but FSH undergoes a secondary surge that declines to the baseline level at late estrus; such an increase in FSH recruits a new cohort of ovarian follicles to another EC. On the other hand, ruptured Graafian follicles by ovulation, are induced by prolactin (PRL) to become functional CL, which secretes [P_4], thus inhibiting LH secretion; a circulating peak of [E_2] prompts a small surge of FSH, followed by a marked increase in [P_4]. In summary, LH induces ovulation, the formation of corpora lutea, and stimulation of ovarian steroidogenesis; whereas FSH stimulates secretion of [E_2] (Sanabria et al. 2019).

Follicles are the functional unit of the ovary (Mlynarcikova et al. 2005, Xu et al. 2022). The follicular phase in the proestrus and estrus involves recruitment, growth, development, and preovulatory maturation of follicles. Folliculogenesis, which is regulated by pituitary gonadotropins and ovarian SSH, involves recruitment and initiation of primordial follicles (with surrounding pre-granulosa cells) into developing preantral follicles (including primary follicles with granulosa cells; secondary follicles with proliferation of granulosa cells' layers and theca cells), preovulatory antral follicles, and mature or Graafian follicles before ovulation (Walters et al. 2019, Xu et al. 2022). Graafian follicles include different layers from outside to inside: surface epithelium, connective tissue, theca interna cells (vascularized, steroidogenically active), granulosa cells (enclosing the fluid-filled antrum), and eccentric oocyte-cumulus-complex (Mlynarcikova et al. 2005, Xu et al. 2022). Mlynarcikova et al. (2005) summarize the ovarian events involved in the ovarian cycle (OC) for gametogenesis and steroidogenesis, according to communication between adenohypophysis and uteri. Gonadotropin surge together with feedback of SSH (E_2, P_4) releases the ovum during ovulation. Intra-follicular processes of the steroidogenesis, expansion of oocyte-cumulus complex, and meiotic maturation of the oocyte have an important role in successful fertilization, and all are mediated by appropriately-timed signals by gonadotropins during EC and OC, thus being dependent on ovarian communication between the oocyte and granulosa cells, as well as between the latter and theca cells. The OC involves the follicular maturation, ovulation, and resorption of corpora lutea in a complete estrous cycle (CEC). Throughout rodent EC and OC, androgens are produced in theca cells through biotransformation of P_4 into A; then this pro-androgen turns into bioactive T; both androgens are aromatized into E_2 in granulosa cells (Walters et al. 2019, Xu et al. 2022). Androgen actions are mediated (Walters et al. 2019) directly via the evolutionarily conserved androgen receptor (AR), or indirectly by their conversion into estrogens, which can interact with the estrogen receptor (ER). In laboratory mice, the availability of androgens may increase follicular recruitment, growth,

and development; bioactive T stimulates primordial follicle initiation whereas A also promotes preantral to antral follicle growth (Walters et al. 2019). Steroidogenesis in follicles involves P_4 and E_2 mainly secreted by granulosa cells, the theca cells-synthesized androgens, and the P_4 synthesized in luteal cells; the progestogen can also be synthesized by the placenta in many species (Walters et al. 2019). After ovulation, SSH contribute to the maturation of the oocytes (from prophase I to metaphase II, plus cytoplasmic changes related to fertilization), its implantation, placentation, maturation of products, and parturition (Mlynarcikova et al. 2005, Ruiz-Cortés 2012).

Proestrus, the stage of follicular development occurring after regression of corpora lutea (Klein 2013), is considered the first part of the anabolic phase of the estrous cycle (McLean et al. 2012) because the increase in $[E_2]$ promotes maturation and development of the antral follicles in the ovary, and developed follicles are the main tissue producing this hormone (Gorbman et al. 1983, Findlay et al. 2009). Estradiol is also an indicator in proestrus for the beginning of "heat" (estrus), thus being a prelude for mating. The lack of significant differences between [T] and $[E_2]$ points to an active aromatization process for the biotransformation of the former into the latter (Norman and Litwack 1997, Simpson et al. 2005). The hormonal pattern found during proestrus in both *Peromyscus* species also agrees with evidence gathered from free-living *P. difficilis* (Salame-Méndez et al. 2005), as well as from *Rattus* sp. (Smith et al. 1975, Freeman 1994) and *M. musculus* (McLean et al. 2012) under controlled conditions. Although in *P. melanotis*, the least [A] was statistically significant with respect to the other [SSH] during proestrus, the ANOVA by hormone showed that this androgen tended to reach its highest concentration at proestrus. In the two *Peromyscus* species, the fluctuating [A] during the estrous cycle indicated that this androgen mainly maintained its function as an anabolic precursor for the biosynthesis of T, rather than behaving as a hormone per se; such an anabolic role of A has been reported in laboratory rats (Sprando et al. 2005). The production and biotransformation profiles of four selected SSH of the Δ_4 pathway, showed some differences between species, especially in the biotransformation from P_4 into the pro-androgen A and from this into the bioactive androgen T; e.g., *P. difficilis* showed a higher rate of aromatization than *P. melanotis*. Nevertheless, in both species, the higher $[E_2]$ and $[P_4]$ coincide with the early follicular phase (Ruiz-Cortés 2012, Sharma et al. 2020).

During the period of sexual receptivity or heat (Klein 2013) and continued follicular phase (anabolic phase), $[P_4]$ and $[E_2]$ reached their optimum in both *Peromyscus* species. In other species of rodents and lagomorphs (Tennent et al. 1980, Bonjour 2019), high levels of these two SSH stimulate receptivity (lordosis) and proceptivity (mating behavior). The ANOVAs by hormone showed the highest $[P_4]$ and $[E_2]$ in wild *P. melanotis* at estrus, and the same occurred for the estrogen in *P. difficilis*, whereas its $[P_4]$ peak in estrus extended to metestrus. Likewise, the lowest [T] occurred during heat in *P. difficilis*, whereas it had the second lowest [SSH] in *P. melanotis*. It is feasible that such decreases of T, due to its aromatization into E_2, also diminish the aggressive behavior of females, making them more tolerant of males and receptive to copulation. In *P. difficilis*, females seem to be more territorial than males, staying closer to their burrows, whereas males exhibit greater dispersion in their microhabitat (De la Cruz et al. 2019, 2020, 2021). In a series of controlled intrusion studies (Davis and Merler 2003) in the territorial species *P. californicus*, using different concentration levels of P_4, E_2, T, and corticosterone alone, as well as different proportions (E_2/P_4, E_2/T, P_4/T), only $[P_4]$ and the $[P_4]/[T]$ ratio decreased, while everything else remained constant. Davis and Merler (2003) proposed the existence of a sex-specific mechanism for aggression towards intruders: if $[P_4]$ decreased, aggression against an invading female was inhibited, whereas a decreased $[P_4]/[T]$ could modify the female aggressive response in future encounters. Increased [T] is usually associated with increased aggression in both sexes (Davis and Marler 2003, French et al. 2013), but treatment with T and E_2 increased aggression in females, whereas P_4 inhibited both non-maternal aggression and male aggression (though all this also depends on what is the dominant sex in the species). Overall, the interspecific production-biotransformation profile of Δ_4 SSH was similar during estrus, except for the significantly higher amount of T in the smaller *P. melanotis*, which may explain its more aggressive and restless behavior sensu González-Jatuff et al. (2012); also, the highest $[E_2]$ indicates ovulation with a surge in $[P_4]$ from CL (e.g., beginning of luteal phase; Sharma et al. 2020).

If ovulation occurs, during metestrus (early metestrus, metestrus, or diestrus I), the stage following the end of estrus (late estrus), then the luteal phase ensues with the formation and endocrine activity of corpora lutea (luteal phase). The two *Peromyscus* species shared an evidently high intraovarian $[P_4]$ during metestrus, statistically different from other [SSH], because the main producer tissue of this hormone, the corpora lutea, begins to develop (Niswender and Nett 1994, Braden et al. 1994, Reynolds and Redmer 1999). Starting after ovulation, the metestrus is considered the final

stage of the estrous cycle, where the resulting [P_4] promotes growth and vascularization of the endometrium for possible implantation (Salame-Méndez et al. 2003) and also promotes the development of the alveolar system in mammary glands for milk production and secretion (Austin and Short 1978, Imagawa et al. 1994, Lamote et al. 2004). Another shared feature of the metestrus in both species was the lowest [E_2], which is also consistent with evidence from the laboratory mouse (*M. musculus*, Walmer et al. 1992, Pritchett and Taft 2007). Both species shared a similar production and biotransformation profile of Δ_4 SSH in estrus with a very high aromatization rate of [E_2] in granulosa cells that make up the mature or Graff follicles, endometrial thickening, and preparation for ovulation (Mlynarcikova et al. 2005, Xu et al. 2022). In both species, [P_4] was significantly higher than the androgens and especially than the decreased [E_2] (Sharma et al. 2020). The surge of [P_4], as compared to the other three [SSH], together with the low production of both androgens ([T] was lower in *P. melanotis*) and the estrogen during metestrus, suggests an active synthesis of the progestogen by the corpora lutea and can be related to the first part of luteal phase (Mlynarcikova et al. 2005, Sharma et al. 2020). Also, the resulting [E_2] in both *Peromyscus* species at metestrus, may be due to the aromatization of [T] by follicular cells that remained inside the ovary after ovulation (Walters and Handelsman 2018, Walters et al. 2019).

Diestrus (diestrus II), the second EC stage in the luteal and catabolic phases, is characterized by lowered levels of [P_4] in the absence of fecundation. Likewise, [E_2] has low intraovarian levels at the beginning of diestrus (McLean et al. 2012), but when the corpora lutea begin to mature, the estrogen increases towards proestrus (Klein 2013), which causes a rapid growth of follicles and a thickening of the uterine lining (Pritchett and Taft 2007). In our study, both *Peromyscus* showed an increase of [E_2] from metestrus to diestrus that continued towards the next estrus, whereas in *P. difficilis* the increase was sustained, the sharper slope from proestrus to estrus in *P. melanotis* suggests a higher rate. Production and biotransformation profiles of SSH during diestrus showed conspicuous differences in the intraovarian contents of sex steroids between the two *Peromyscus*, nevertheless, the intraovarian [P_4], [A], [T], and [E_2] in both *Peromyscus* species suggested the beginning of follicular development, especially due to the amount of estrogen (Ruiz-Cortés 2012). The aforementioned is very clear in *P. difficilis*, because the [T] is significantly lower than the [E_2], which indicates the aromatization process from the androgen into the estrogen by developing follicles (Ruiz-Cortés 2012). It must be noted that differences in proestrus and diestrus during a CEC may be due to difficulties in the differentiation of cell types from vaginal smears, as has been pointed out elsewhere (Cora et al. 2015, Sharma et al. 2020).

Pregnancy and lactation

After ovulation, SSH contribute to the maturation of the oocytes (from prophase I to metaphase II, plus cytoplasmic changes related to fertilization), its implantation, placentation, maturation of products, and parturition (Mlynarcikova et al. 2005). Ruiz-Cortés (2012) summarizes findings in laboratory rodents for the action of SSH and other hormones in pregnancy, post-partum, and lactation, with emphasis on progestogens and estrogens. During fertilization, P_4, androgens and E_2 inhibit abnormalities in oocytes (health maintenance), stimulating its maturation and enhancing fertilization success. Steroidogenesis of P_4 pregnant mice occurs at different times and places, including changes in the uterine-wall induced by implantation and in the extraembryonic giant cells during mid-pregnancy, thus playing an important role for successful implantation and maintenance of pregnancy, respectively; moreover, P_4 could prevent immune rejection of fetuses. P_4 also decreases myometrium contractions and promotes maternal secretions of the endometrium. During placentation, P_4 acts as an immunosuppressant of the placenta, androgens produce a cross-talk between the ovary and the placenta, whereas E_2 contributes to the formation of the latter organ. In laboratory rats, ovarian E_2 together with placental androgen synthesis, suggest interaction between these organs, for estrogens regulate enzymatic expression in the ovary as well as placental mass. In the rats, circulating levels of E_2 and oxytocin (OT) increase throughout pregnancy, whereas P_4 declines after day 19, and prostaglandin E_2 (PGE_2) increases steadily to its maximum peak until parturition. The stimulating action of E_2 in the synthesis of estrogen receptors (ER) and of hypothalamic OT with its receptors (OTR) in the uteri, is a key fact for parturition, whereas reduction and withdrawal of P_4 may participate in the increases of OTR and PGE_2. During pregnancy, P_4 promotes myometrial relaxation, whereas at parturition, E_2, combined with OT and PGE_2, induces the myometrial distensions and contractions necessary for parturition. During parturition, P_4 is actively converted, through androgens, into E_2, and the estrogen increases endometrium lubrication. Post-partum or puerperium starts just after parturition and continues until CEC is restored. During post-partum, E_2 also induces maternal behavior (nursing), androgens participate in cross-talk between ovary and E_2 and in production/control of hypothalamic GnRH. High

[E_2] pre and post-partum regulates OTR and OT, affecting myometrium so that placental and endometrial tissues are discharged in post-partum. Laboratory rodents and many other species, including the two studied *Peromyscus* here, are poliestric with spontaneous ovulation, which means that the SEC is readily turned into a CEC. Finally, Ruiz-Cortés (2012) states that both E_2 and P_4, together with growth factors, play an important role in normal mammogenesis and involution of mammary gland. SSH lipid-soluble, diffuse passively from maternal blood into milk, reflecting, for instance, cyclic ovarian steroidogenesis of E_2 and P_4. During lactation, all SSH participate in development of mammary tissue, mammogenesis, modulation of lactation and in the involution of lactogenic tissues (autophagy).

In our results, compared to the other [SSH], there was a very significant high [P_4], that agrees with reports for laboratory species (*Mus* sp., Pedersen and Peters 1971; *Rattus* sp., Meyer and Bruce 1979, Gibori et al. 1982, Miyauchi and Midgley 1990) and for wild species (*S. campestris*, Westlin-van Aarde 1989, *M. arvalis*, Nubbemeyer 1999, *P. melanotis*, Salame-Méndez et al. 2003). As stated before, after fecundation, the endocrine function of the corpora lutea (CL) becomes very active during the first part of pregnancy (early gestation, G1) in a SEC, especially for production of P_4 (Westlin-van Aarde 1989, Ruiz-Cortés 2012). Such surge of P_4 in the two studied *Peromyscus* species, likely participates in priming reproductive tract for implantation (Westlin-van Aarde 1989) and in maintenance of pregnancy through the development of the first embryonic stages (Niswender et al. 1994, Senger 2006), as well as in the relaxation of the myometrium, decidual quiescence and cervical closure (Csapo 1956). Interspecific similarity of production-biotransformation profile of Δ_4 pathway of SSH highlights the main role of P_4 during G1, and both profiles of [P_4] and [E_2] agree with implantation of zygote and maintenance of early embryo (Ruiz-Cortés 2012). In both *Peromyscus* species the very high [P_4] in G1, may be due to both CL and blood supply (Sharma et al. 2020), whereas the significant [E_2], resulting from lowered androgen concentrations, is likely to promote anabolic processes of reproductive tissues (e.g., endometrial thickening) and fluids (e.g., vascularization) during G1 in the two *Peromyscus* species.

In the second part of pregnancy (late gestation, G2), [P_4] decreased, while [E_2] increased in both *Peromyscus* species, but as compared to G1, hormone levels were noticeably lower. Although [E_2] was not statistically different, its increase indicates that fetal development was nearing completion, as this estrogen is a potent uterotrophic agent that promotes growth and blood flow in the myometrium, the deciduous membrane, and the cervix (Albrecht et al. 2000). Likewise, the estrogen progressively stimulates secretion of prolactin in the hypothalamus during this stage of pregnancy (van Tienhoven 1983, Griffin and Ojeda 1992, Hill et al. 2008). Compared to G1, there is a conspicuous drop in production of P_4 during late gestation (G2) in both *Peromyscus* species, but with an increased concentration of both androgens, which increase and lead to the highest concentrated estrogen (this being significant in *P. melanotis*). Such profile indicates aromatization process to produce the E_2 that will induce follicular development, as well as the changes in reproductive tract, related to proximity of parturition (Ruiz-Cortés 2012, Xu et al. 2022); indeed, in G2 the fetuses had reached the necessary body growth to induce parturition.

During lactation, [E_2] increased slightly more than in G2 and was higher than the other [SSH] in both species. This hormone, together with P_4 and prolactin, stimulates mammary tissue and the growth of lactogenic ducts, together with the synthesis of carbohydrates (e.g., lactose) and proteins (e.g., casein) that make up milk (Griffin and Ojeda 1992, Lamote et al. 2004, Hill et al. 2008). While [P_4] remained as in G2 in *P. melanotis*, it tended to decrease slightly in *P. difficilis*. In the two species of *Peromyscus*, the levels of intraovarian [P_4] and [E_2] during lactation, corroborated that production of both SSH is carried out and that these hormones are involved in milk production, probably through development and growth of milk tissues (Ruiz-Cortés 2012). The profile of [SSH] during lactation is somewhat similar to G2 in both *Peromyscus* species, but with some differences in their respective pattern; e.g., in *P. melanotis*, [A] becomes into higher [T] very significantly. However, in both species [T] becomes [E_2] very or extremely significantly, producing higher contents of the estrogen. The similar profile in G2 and overall lactation is consistent with the relevant role of P_4 and, especially of E_2 in the development of mammary tissues and milk production for nursing of pups (Ruiz-Cortés 2012).

Results of [SSH] during first and second parts of pregnancy, as well as in lactation were obtained from free-living females of *Peromyscus*; therefore, we could not assert evidence of changes in [SSH] reported for laboratory mice and rats (Ruiz-Cortés 2012) or in wild species under controlled conditions (Westlin-van Arde 1989). Studies under controlled conditions are required to obtain evidences of such [SSH] fluctuations throughout pregnancy in both *Peromyscus* species and this is especially true for monitoring them through lactation.

FINAL REMARKS

Information for *P. melanotis* and *P. difficilis* gathered here, constitutes one of the first endocrine evidences for free-living, wild adult females of the genus *Peromyscus*, referring to their intraovarian reproductive physiology in the estrous cycle, the two parts of pregnancy, and especially in overall lactation. The steroidogenic function of the ovary, allowed us to discuss the role of each SSH on the gametogenic function of this gonad, as well as their relationship in the biotransformation of progestogens into androgens and of these into estrogens, according to the steroidogenic Δ_4 pathway in these two *Peromyscus* species. In so doing, our results contribute to the literature on the important role of P_4 and E_2 in EC, pregnancy, and lactation, but also constitute novel documentation on the production and biotransformation roles of androgens into estrogens, through this pathway.

There were no overall differences in SSH contents between the two peromyscine species, except for a higher [T] in *P. melanotis* during heat, which agrees with the more restless and aggressive behavior of this small-sized species, during capture and handling. On the other hand, the shape of production curves suggests interspecific differences in production and biotransformation rates that could be related to their species-specific physiological and genetic adaptations. Such differences are more evident in the intermediate androstenedione, and the timing of maximum peaks along a complete estrous cycle (EC) and in an interrupted EC by pregnancy and lactation. More detailed analyses are needed (e.g., statistical, physiological, and molecular) in order to test whether these are statistically significant differences.

It seems that intraovarian contents of SSH (P_4, A, T, and E_2) during their estrous cycle, two parts of pregnancy and overall lactation in free-living, adult females of *P. melanotis* and *P. difficilis*, have an overall similarity with what has been described for circulating contents of these SSH in females of laboratory rodents. However, further analyses under similar conditions are needed to determine whether there are specific differences in production and biotransformation rates between all these laboratory species and the analyzed *Peromyscus* species; e.g., considering the same source of SSH (circulatory: blood, serum, plasma; intraovaric) and the maintenance of colonies under controlled conditions (e.g., for pregnancy and especially for lactation).

ACKNOWLEDGMENTS

We thank the enthusiastic participation of graduate students in fieldwork, as well as the valuable comments of an anonymous reviewer, the associated editor, and especially of the Editor in Chief, Ricardo Moratelli Mendoça da Rocha, and the managing editor, Sionei Ricardo Bonatto. This study was funded by DCBS, UAM-I through institutional research grants to ASM (DCBS-144.03.07), ACC (DCBS-143.02.46), and JRP (DCBS-143.02.40). GEMJ was granted a scholarship for graduate studies by the Mexican government (CONACyT-10800028).

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Submitted: June 23, 2023
Accepted: November 27, 2023
Editorial responsibility: Carolina Arruda Freire

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Competing Interests

The authors declare no competing interests.

How to cite this article

Salame-Méndez A, Mancera-Jaime G, Castro-Campillo A, Ávila-Valle Z, Ramírez-Pulido J (2024) Intragonadal evaluation of sexual steroid hormones during three reproductive events in two species of *Peromyscus* (Rodentia: Cricetidae). Zoologia 41: e23032. https://doi.org/10.1590/S1984-4689.v41.e23032

Published by

Sociedade Brasileira de Zoologia at Scientific Electronic Library Online (https://www.scielo.br/zool)

Supplementary material 1

Table S1. Intraspecific ANOVA comparisons between different intraovarian sexual steroid hormones (SSH) in the same reproductive event. Mean concentrations (pg/mg) of SSH (P_4, progesterone; A, androstenedione; T, testosterone; E_2, estradiol) in adult females of *P. melanotis* or *P. difficilis* are arranged by estrous cycle (Proestrus, Estrus, Metestrus, Diestrus), pregnancy (Gestation 1–2), and overall lactation (Lactation). Sample sizes (n) and degrees of freedom (DF) are within parenthesis; means of intraovarian [SSH] are bold numbers on diagonal; F statistics are below diagonal and p-values are above it in italics (bolded ones are significant).

Authors: A. Salame-Méndez, G. Mancera-Jaime, A. Castro-Campillo, Z. Ávila-Valle, J. Ramírez-Pulido.

Data type: statistical data.

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Link: https://doi.org/10.1590/S1984-4689.v41.e23032

Supplementary material 2

Table S2. Intraspecific ANOVA comparisons for the concentration of the same sexual steroid hormone between different reproductive events. Compared mean concentrations (pg/mg) of each sexual steroid hormone include estrous cycle (Pro, proestrus; Est, estrous; Met, metestrus; Die, diestrus), pregnancy (Gestation 1–2), and overall lactation (Lac) in free-living adult females of *P. melanotis* or *P. difficilis*. Sample sizes (n) and degrees of freedom (DF) are not shown; means of intraovarian [SSH] are bold numbers on diagonal; F statistics are below diagonal and p-values are above it in italics (bolded ones are significant; underlined ones were almost significant).

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Data type: statistical data.

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Link: https://doi.org/10.1590/S1984-4689.v41.e23032