



Research Article

ANNUAL RELATION BETWEEN CIRCULATING TESTOSTERONE AND TESTICULAR SIZE IN ADULT MALES OF *PEROMYSCUS MELANOTIS* (RODENTIA: CRICETIDAE) IN A MID-LATITUDE TEMPERATE FOREST

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ABSTRACT

Determination of reproductive season is based on the presence of pregnant females and male testis position and size in several micro-mammals in wild life. However, the endocrine function of the testicles is unknown. Our goal was to evaluate, on a monthly basis during the two reproductive seasons of the year, the relationship between circulating testosterone level and testis recrudescence on adult males of free living Black-eared deer mouse *Peromyscus melanotis* from a mid-latitude temperate forest. Our evidence shows a pattern of raise and fall, though decoupled by a month, between both processes, which sheds light on their physiological relationship in this species, as well as about its plasticity to respond to environmental conditions. They also warn us against customarily inferences about reproductive activity based only in position and size of testes. Finally, our evidence contributes to the scarce knowledge of the endocrine function in some particular events of the reproductive biology of *Peromyscus melanotis*, and on the reproductive biology of *Peromyscus* in temperate mid-latitudes.

Keywords: Rodents, *Peromyscus*, Testicles, Recrudescence, Testosterone, Temperate forest.

INTRODUCTION

Mammalian population maintenance and perpetuation on a certain environment relies on sexually mature individuals. Androgen production, particularly testosterone (T) in adult males with fully functional reproductive physiology, regulates such a complex and contrasting functions like spermatogenesis and mating behavior patterns (Komori *et al.*, 2007; Nelson, 2005). In seasonal species, testes are actively involved in sperm and androgens production only during the reproductive season and can be affected by environmental conditions (Sadleir, 1969; van Tienhoven, 1983). Androgen production and secretion is necessary for other functions besides the reproductive processes, such as territory delimitation and defense, or group and kin recognition (Wyatt, 2009). Endocrine function of testis can be determined in males of various species by evaluating T contents in blood (plasma or serum), and associating its circulating content to particular events in the animal reproductive biology, such as the increased size of testicles

known as recrudescence (Aire, 2007; Bentley, 1998; Norris, 2000).

Testis recrudescence is a phenomenon representing a flurry increase in testicular gamete production (spermatogenesis) associated with a general increase in both volume and cell numbers. In mammals, this phenomenon is associated to the activity of gonadotrophin hormones affecting both germinal and somatic testis cells, giving rise to a volume increase, as well as the descending of the organ to the scrotum (Aire, 2007; Clay & Clay, 1992; Pelletier & Almeida, 1987).

In wild mice of genus *Peromyscus*, population dynamics of reproduction (reproductive pattern) have been customarily reported, based on the abundance of males with scrotal and large testes, together with pregnant/lactating females, along time (Kirkland & Layne, 1989; Kunz *et al.*, 1996), especially on high latitudes. On the other hand, scrotal recrudescence testes have been related

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to production and content of T, only in a few males of Black-eared deermouse (Castro-Campillo *et al.*, 2012; Salame-Méndez *et al.*, 2008; Salame-Méndez *et al.*, 2005; Salame-Méndez *et al.*, 2004), even though relevant information of such processes of gonadal physiology must be documented to really link position, size of testes, and content of T.

In order to learn more about the reproductive biology of *Peromyscus*, inhabiting mid latitude, temperate forests, we have used two species as study models, in periurban forested zones of Mexico City at “Cumbres del Ajusco” and “Desierto de los Leones” National Parks (Castro-Campillo *et al.*, 2012; Castro-Campillo *et al.*, 2008; Salame-Méndez *et al.*, 2008; Salame-Méndez *et al.*, 2005; Salame-Méndez *et al.*, 2004). One of such species, the Black-eared deermouse, *Peromyscus melanotis*, is a quasi-endemic species of Mexico (Álvarez-Castañeda, 2005; Castro-Campillo *et al.*, 2014; Castro-Campillo *et al.*, 2005), whose reproductive activity occurs all year round in both studied areas, but with two distinctive peaks, occurring during the summer and trough autumn-winter, respectively (Castro-Campillo *et al.*, 2014; Castro-Campillo *et al.*, 2008; Salame-Méndez *et al.*, 2008; Salame-Méndez *et al.*, 2004).

From our previous studies, we know that both production and intra-testicular androgen contents are higher during summer and decrease from autumn to winter in adult males of *P. melanotis* in both forested areas (Castro-Campillo *et al.*, 2012; Castro-Campillo *et al.*, 2005; Salame-Méndez *et al.*, 2004). Therefore, to relate physiological evidence to a particular morphological response, we wondered how the profile of T was related to recrudescence processes of testis along a year in this species. To address this question, we documented monthly circulating contents of T, together with changes in testicular volume, in adult males of *Peromyscus melanotis*, along a year.

MATERIALS AND METHODS

Collecting rodents

Adult males of *Peromyscus melanotis* were monthly collected at Cumbres del Ajusco National Park (0.85 Km N, 3.5 Km W Ecuanil, 3180 msnm, CDMEX, 19° 13' 37" N, 99° 15' 37" W), using Sherman traps (8 x 9 x 23 cm, Tallahassee, FL, USA), baited with oat flakes, along two years. Selection of adult individuals was made using conspicuous somatic and diagnostic characters of the species, such as size and pattern of pelage color (Álvarez-Castañeda, 2005; Castro-Campillo *et al.*, 2014; Castro-Campillo *et al.*, 2005). Trapped mice were transferred to laboratory facilities at UAM-Iztapalapa, and killed by cervical dislocation the same day. Each individual was conventionally sexed, measured, and weighted (Kunz *et al.*, 1996; Ramírez-Pulido *et al.*, 1989). Capture of rodents was made according to the Scientific Collector Permit from the National Ministry of Natural Resources (SEMARNAT) and all animal manipulations were made according to

international standards (NIH, 2011) and approved by the UAM-I CBS Ethic Commission.

Samples

Blood samples were taken from each adult mouse from the heart by cardiac puncture and poured into EDTA tubes. Plasma was obtained by centrifugation of each tube to 3000 x g x 5 mn at room temperature, and then transferred into Eppendorf tubes to be stored at -20 °C, until androgen quantification. Testes were removed and were measured (width x length, mm) conventionally to the nearest 0.01 mm. Their volume was calculated using the geometric formula for a prolate spheroid: $V = 4/3 \pi a^2 b$, where a and b are the respective semiaxes, or half axes, of the minor (width) and the major (length) axes, respectively (Castro-Campillo *et al.*, 2012). Corpses of mice were also used in another study, therefore, their remains were prepared as skull and skeleton (Ramírez-Pulido *et al.*, 1989) to be housed as voucher osteological specimens in the mammal scientific collection at Universidad Autónoma Metropolitana-Iztapalapa (UAMI). After skulls were biologically cleaned with dermestid beetles (Salame-Méndez *et al.*, 2008), each individual was assigned to an age category, using wear of the occlusal surface of cheek-teeth *sensu* (Hoffmeister, 1951).

Quantification of testosterone (T)

Methods for valuation of T in plasma by radioimmunoassay (RIA) have been made according to Salame-Méndez *et al.* (2005) with some modifications. Briefly, 50 µL aliquot was taken from each plasma were transferred to an Eppendorf tube and added a phosphate buffer (0.25 M, pH 7, with sodium azide and gelatin to 1%), which contained a diluted solution of specific antiserum and tritiated T as a tracer; the tubes were kept at 4 °C for 18 hrs. After this time, each tube was added 100 µL of a diluted solution of activated charcoal-dextran, separating the steroid bound to the antibody by centrifugation. The supernatant was decanted to vials and these were added Instagel (Packard). Amount of free radioactive steroid was measured in a liquid scintillation spectrophotometer (Beckman, LS-7000), with a maximum efficiency for tritium of 53%. RIA method was validated by means of a standard curve; being the coefficient of variation intra-assay < 4%. Quality control of each RIA was made, according to international specifications of accuracy, precision, and sensitivity (Cekan, 1976; Rodbard, 1974).

Statistical analysis

To determine possible monthly differences in the annual profile of plasma T contents, and testicular volume, we used analysis of variance (ANOVA), followed by Tukey's multiple comparison tests. Both monthly data of T contents and testis size (volume) were plotted, and a polynomial regression model of second grade (parabole) was fitted to their pattern; then the R^2 was calculated assuming that a good empirical fit was achieved when $R^2 \geq 0.7$. All

statistical analyses were carried out at $\alpha \leq 0.05$, using the algorithms of the statistical package GraphPrisma (Motulsky, 1999) and NCSS Data Analysis (version 11, <http://www.ncss.com/software/ncss/demo>). The former and Excel were used to plot results, beginning with December data, for the sake of simplicity.

RESULTS AND DISCUSSION

We analyze 83 adult males of *P. melanotis* (January $n = 12$; February $n = 11$; March $n = 6$; April $n = 5$; May $n = 6$; June $n = 2$; July $n = 6$; August $n = 6$; September $n = 5$; October $n = 4$; November $n = 8$; December $n = 12$). There were no significant monthly differences ($P < 0.05$), between mice of both years when their plasma T contents were compared. Thus, monthly results of both years were pooled as adult male mice.

Androgen plasma profile (Figure 1A) of adult mice followed a pattern of increase from the colder and drier months to the milder and wettest ones in the year; this profile showed significant differences among some monthly means ($F = 22.95$, $df = 11, 71, 82$, $P < 0.0001$, Table 1). Low contents of plasma T lasted from December to February, without significant differences among these months. Up onto March, there was a noticeable difference, since the rising of plasma T was drastic and significant. From then until July, there was a steady increase with plenty of overlap of the standard deviations of T contents, with only low significant differences between March and the other months. Contents of plasma T reached its highest peak in July, from which it started to decrease gently towards August. However, lowering of T was both drastic and statistically significant from August to September and from October to November. Indeed, contents of plasma T reached its lowest amount in the latter month, which is also statistically different from all winter months.

As contents of plasma T, testicular recrudescence (volume) of adult mice (Figure 1B, Table 1), also showed an overall monthly pattern subjected to climate changes, but with some noticeable differences ($F = 5.21$, $df = 11, 71, 82$, $P < 0.001$, Table 1). During the three colder and drier months (December-February), and March, testes volume fluctuated up and down with no major significant differences, except between January and December or February. From March until May, the recrudescence of testes increased sharply with noticeable significant differences between March and April, and between the latter and May, when testes reached its largest size. Then, testes steadily lost volume until August, with no significant differences. From August to September, there was a drastic and statistically significant reduction of volume; but from

then on, reduction of testes became steady again and without significant differences until November.

Both curves of raw means and its standard deviation (Figure 2) were better-fitted ($R \geq 0.7$), using a polynomial regression model of second degree (parabola, $y = ax^2 + bx + c$, Figure 2). Equation for the resulting parabola in monthly contents of plasma T was $y = 2.72 x^2 + 36.63 x - 26.59$ ($R = 0.73$), while that of testicular recrudescence was $y = 5.87 x^2 + 74.29 x - 41.84$ ($R = 0.72$). Parabolas (Figure 2) verified that both the androgen profile and testicular recrudescence are processes of raise and fall during the year. However, there is a decoupling between both parabolas since raising of plasma T, precedes that of testicular recrudescence and keeps on going within a month of difference; *e.g.*, testicular volume is triggered by plasma T to reach its maxima in May, while the latter reaches its own maxima in June.

Finally, it should be noted that it was noteworthy that even during the harsh cold season; all animals had a good morphological profile with no evidences of fasting or other evident alterations in body or coat morphology or texture that could indicate an alteration of the global health. Reproductive activity of free-living wild male mice, is usually assumed from considering only external somatic characters, such as size and location of testes, which in turn implies that individuals with increased testicular size are the ones in which falls reproductive activity (Bronson & Heideman, 1994; Hirschenhauser & Oliveira, 2006; Kunz *et al.*, 1996; Layne, 1968; Lee, 2004; Ramírez-Pulido *et al.*, 1989; Romero-Almaraz *et al.*, 2007).

In the study area, considering testicular recrudescence, together with number of pregnant/lactating females and number of implants, fetus, and newborns, *Peromyscus melanotis* has two reproductive peaks: a main one during the summer and another, but minor, during autumn-winter (Castro-Campillo *et al.*, 2012; Salame-Méndez *et al.*, 2004). Since both contents of circulating T and testicular recrudescence, lowered as months became colder and drier from September to February, while raised as months became milder and more humid from March to August, this behavior suggests that gonadal endocrine function depends on environmental conditions (Figures 1, 2, Table 1). That is, both processes are more efficient during the milder-rainy season and less efficient during the general cold-dry one. This is reinforced by our previous studies in which testicular androgen production is related to the season of the year, being higher during spring-summer and lower during autumn-winter (Salame-Méndez *et al.*, 2004). Therefore, circulating content of T is a reflection of gonadal steroidogenic activity in adult mice of *Peromyscus melanotis*, which in turn, is associated, though decoupled (Figure 2), with gonadal recrudescence.

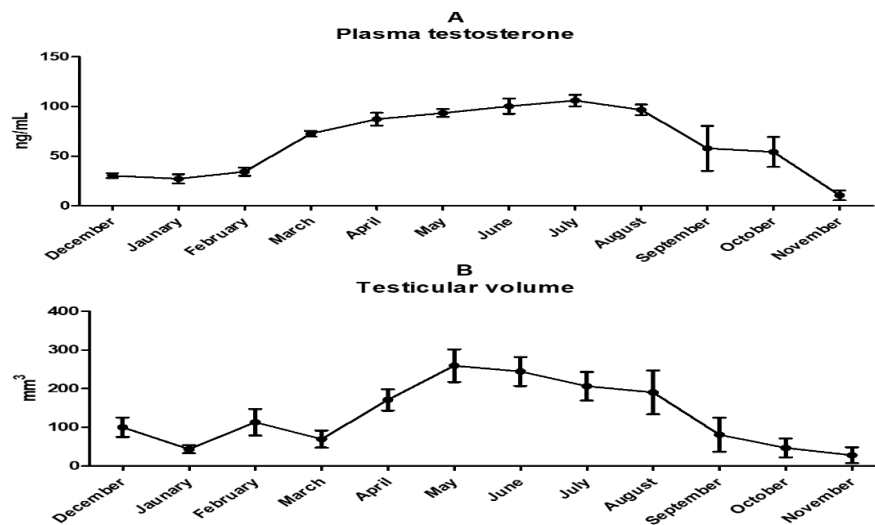


Figure 1. Monthly profile of plasma testosterone contents (A), and testicular recrudescence (B) in adult males of *Peromyscus melanotis* from a mid-latitude temperate forest, along a year. Vertical lines depict a standard deviation at both sides of the mean (points on profile line). Number (n) of adult males for month: January n = 12; February n = 11; March n = 6; April n = 5; May n = 6; June n = 2; July n = 6; August n = 6; September n = 5; October n = 4; November n = 8; December n = 12.

Table 1. Significant differences among monthly mean values of plasma testosterone (above diagonal) and testicular volume (below diagonal) in adult males of *Peromyscus melanotis* from a middle latitude, temperate forest.

	Dic	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov
Dic		ns	ns	***	***	***	***	***	***	ns	ns	ns
Jan	ns		ns	***	***	***	***	***	***	ns	ns	ns
Feb	ns	ns		**	***	***	***	***	***	ns	ns	ns
Mar	ns	ns	ns		ns	ns	ns	ns	ns	ns	ns	***
Apr	ns	ns	ns	ns		ns	ns	ns	ns	ns	ns	***
May	*	***	ns	*	ns		ns	ns	ns	ns	ns	***
Jun	ns	ns	ns	ns	ns	ns		ns	ns	ns	ns	***
Jul	ns	*	ns	ns	ns	ns	ns		ns	**	**	***
Aug	ns	*	ns	ns	ns	ns	ns	ns		*	*	***
Sep	ns	ns	ns	ns	ns	*	ns	ns	ns		ns	**
Oct	ns	ns	ns	ns	ns	*	ns	ns	ns	ns		*
Nov	ns	ns	ns	ns	ns	***	ns	*	*	*	*	

Abbreviations: ns, no significant; > number of asterisks > significant P level.

As a rule, it is claimed that environmental conditions influence reproductive biology during the cold-dry seasons, being lack of food a limitation (Merritt *et al.*, 2001; Sadleir, 1969; Wolff & Sherman, 2008). However, in our study area, circulating levels of T had detectable values in *P. melanotis* during October to February (colder and dryer conditions). Moreover, even when the testicular size decreased significantly in these mice, as compared to testicular size reached during the milder and more humid conditions of May to August ($t = P < 0.0033$; 48.92 ± 12.98

vs. 185.9 ± 64.11 , respectively), these mice also showed spermatogenesis and gametes in the epididymis (Salame-Méndez *et al.*, 2008). Taken together, these facts allow us to confirm that under such conditions in the study area, adult males of *P. melanotis* may be able to reproduce during this unfavorable period (second reproductive peak, (Castro-Campillo *et al.*, 2012; Salame-Méndez *et al.*, 2004) due to its adaptive plasticity, as has been reported for other *Peromyscus* species (Bronson & Heideman 1993; Kaufman & Kaufman, 1989; Munshi-South & Richardson, 2017).

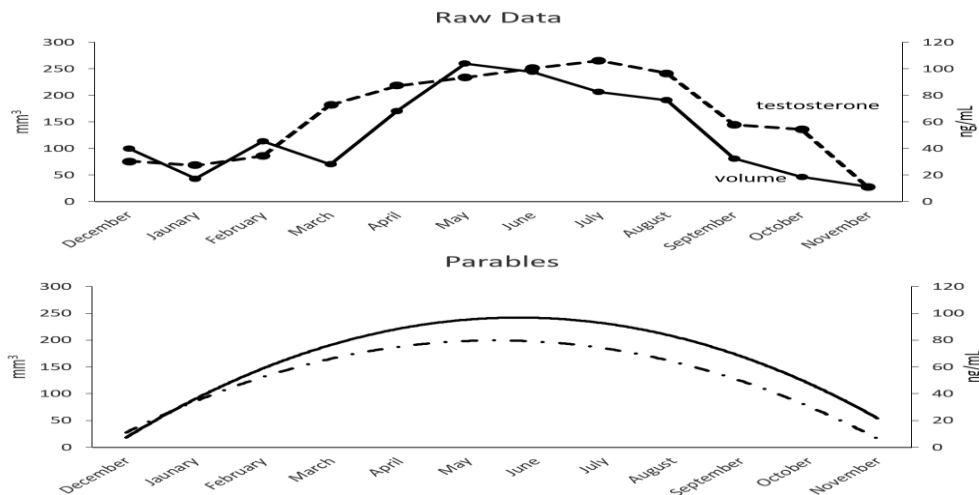


Figure 2. Raw data above and regression curves (Parables) below for plasma testosterone contents (continuous line $R = 0.73$), and testicular recrudescence (discontinuous line, $R = 0.72$) in adult males of *Peromyscus melanotis* from a middle latitude, temperate forest, showing the relationship of these two physiological processes. Triggering of testicular recrudescence (testis volume) by plasma T, occurs in the coldest months (December-February), together with a steady rising until May to a steady fall from then on. Notice decoupling of plasma T that precedes testicular recrudescence and how it remains higher by a month of difference.

The importance of maintaining moderate T content could be reflected on the health of the individual's during colder and dryer conditions. Besides T function on reproduction, it has been shown its activity on different key regulatory cells, such as lymphocytes and macrophages. Macrophages and mononuclear white cells have a positive response to intratesticular and circulating T levels through their respective androgen receptors (Ahmadi & McCruden, 2006; Bebo *et al.*, 1999) and regulate the immunological environment of the testis (Chen *et al.*, 2016). Also, relatively low T levels could explain the contradictory results found by Bronson & Heideman (1993) that even cryptorchidic *Peromyscus* males show normal spermatogenesis and are capable to father normal size litters indicating a full reproductive activity on the natural population. Besides that, low T levels maintain active spermatogenesis (Spaliviero *et al.*, 2004; Walker, 2011; Zhang *et al.*, 2003).

Gonadal recrudescence, involves cell division during the proliferative spermatogenesis phase, testicular angiogenesis and fluid production in the seminiferous tubules, and thus promoting maximum testicular size (Li *et al.*, 2015; Seco-Rovira *et al.*, 2015). On the other side, testicular size regression implies the reverse processes, stopping partial or full cell division, fluid loss, decrease of lumen of seminiferous tubules, drastic vascularization reduction and apoptotic processes of several cells, including germ cells (Alexandre-Pires *et al.*, 2012; Carvalho *et al.*, 2009; Sharpe *et al.*, 1994). Therefore, at the cellular level, T plays an important role on the complex processes involved in the testicular recrudescence-regression cycle (Beguelini *et al.*, 2015; Bueno *et al.*, 2014; Han *et al.*, 2017; Pelletier & Almeida, 1987; Sun *et al.*, 2011).

Therefore, the decoupled pattern of plasma T and testicular recrudescence found in *Peromyscus melanotis*, also warns us about inferring reproductive activity based only in testicular size and/or its location within the scrotum. That is, a captured male on August might have a large scrotal testis but it might also be non-reproductive, since it is undergoing reduction and deactivation of spermatogenesis. In addition to the above, Olivera *et al.* (1986) found reproductively active males in a laboratory colony of *Neotomodon alstoni*, even though they had no scrotal testicles; a fact also reported by Boiani *et al.* (2008) in *Oligoryzomys flavescens* from a temperate boreal habitat.

CONCLUSION

We can conclude that there patterns of circulating T and testis recrudescence are similar, but with a slight delay in testis volume, that actively maintains the reproductive physiology necessary for the two reproductive seasons of *Peromyscus melanotis* along a year. This close association may be used as a first evaluation characteristic of reproductive activity and individual contribution to the maintenance of the community. Therefore, it is important to note that an adult Black-eared mouse, with no conspicuous scrotal testicles, might not be reproductively inactive and no contributing to the mating population, especially during the second breeding season at this middle latitude, temperate forest. Therefore, studies of the population dynamics of wild rodents in temperate forests should be reinforced with physiological information. The above is important to consider, since during autumn-winter, adult males of *Peromyscus melanotis* can produce androgens and spermatozoa, although their testicles are not in the scrotum and/or have a smaller volume with respect to spring-

summer adult males; therefore, such males cannot be considered reproductively inactive. If so, then the reproductive dynamics of this species would be underestimated.

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