Mammalian Biology 79 (2014) 225–229

Contents lists available at ScienceDirect

Mammalian Biology

journal homepage: www.elsevier.com/locate/mambio

Original Investigation

Seasonal changes in testosterone levels in wild Mexican cottontails *Sylvilagus cunicularius*

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ARTICLE INFO

Article history:
Received 24 September 2013
Accepted 17 February 2014
Handled by Adam John Munn
Available online 26 February 2014

Keywords:
Hormones
La Malinche
Net traps
Reproductive state
Seasonal reproduction

ABSTRACT

We studied serum testosterone levels in the endemic Mexican cottontail, *Sylvilagus cunicularius*, which has been reported to show seasonal breeding. Animals were trapped in the wild and in a field enclosure in the National Park La Malinche in central Mexico over a period of five years. Serum testosterone (T) levels were quantified by ELISA from blood samples. T levels of adult males were lowest around 4 months after the onset of the annual reproductive season and were already high prior to the onset of breeding. As expected, the T levels of adult females were consistently lower than in males, and there were no differences in T level with respect to female reproductive state. There were no detectable sex-specific differences in juveniles and subadults, but there was a marked increase in T levels between juvenile and adult males. Overall, our study clearly reflects and confirms the seasonal breeding strategy of this species, showing high similarities to the much better studied European rabbit.

Introduction

Seasonal breeding in mammals is a strategy for adapting to seasonal environments, and these changes in reproductive activity along the season are typically paralleled by changes in the underlying hormonal status of the animals (Bronson and Heideman, 1984). Testosterone (T) is the major hormone involved in sexual development, function and behavior of mammalian males (Nelson, 2005), and males can show increased testosterone levels when females are in estrous (e.g. rodents: Schradin, 2008; lagomorphs: Blottner et al., 2000; ungulates: Lincoln and Kay, 1979; Sempéré et al., 1992; Blottner et al., 1996; Mooring et al., 2004; primates: Cavigelli and Pereira, 2000). However, T can also be involved in female reproductive behavior in rabbits. For example, T shows an increase at days 10–30 of pregnancy followed by a significant decrease at day 1 postpartum in the domestic rabbit (*Oryctolagus cuniculus*: González-Mariscal et al., 1994). At early gestation T levels contribute to digging behavior, promote hair loosening (playing an important part in the rabbit's nest building behavior) and reduce food intake at the end of this period (González-Mariscal et al., 2003).

Although, it seems that in some seasonal breeders, males become reproductively active before most of females do. For example, male European rabbits (*O. c. cuniculus*) in Great Britain show an increase in weight of sexual organs and in the percentage of individuals with active spermatogenesis before the fecundity peak in females (Boyd and Myhill, 1987). This is confirmed by studies in the subspecies *O. c. algirus* in Tunis and Portugal, where peaks in testosterone level in males were found approximately one month prior to the onset of the main mating season (Ben Saad and Baylé, 1985; Gonçalves et al., 2002). A similar pattern in the changes of T levels can be seen in the European hare (*Lepus europaeus*: Blottner et al., 2000). However, almost no published information is available for the seasonal changes in T levels in wild rabbits of the genus *Sylvilagus*.

Here, we present data on the endemic Mexican cottontail *Sylvilagus cunicularius*. A recent report on this so far poorly studied species has shown a clear pattern of seasonal reproductive activity (Vázquez et al., 2007a). We studied animals from the wild and from field enclosures situated in the National Park "La Malinche" in Mexico over 5 years. Our main goal was to (i) describe the seasonal pattern of T levels in males and females and (ii) to investigate differences in the T levels with respect to the animals' age and reproductive state. In particular, we (iii) intended to test whether

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http://dx.doi.org/10.1016/j.mambio.2014.02.002

males of this lagomorph species also show a distinct peak in T levels prior to the onset of female reproductive activity.

Material and methods

Study species

The Mexican cottontail (*S. cunicularius*) is one of the largest species of this genus, weighing around 1800–2300 g, and it has the widest distribution of all endemic cottontails in Mexico (Cervantes et al., 2005). The diet is mainly based on grasses (Cervantes et al., 1992; Hudson et al., 2005). Mexican cottontails breed all the year around and mainly with an increase in breeding from February to October (Vázquez et al., 2007a). The gestation period is 34 days and females give birth to litters of on average 3 pups (range 1–6; LR, pers. obs.).

Study site

La Malinche National Park is located in the central high plateau of Mexico, in the state of Tlaxcala (19°14′06″ N and 97°59′04″ W). The climate in the area is temperate semi-humid with 56% of the annual rainfall in summer (mean temperature 9.6 °C), and less than 2% in winter (mean temperature 6.5 °C). The vegetation is a mixture of rough pasture, scrub and open woodland dominated by the trees (details in Sánchez et al., 2005). Mexican cottontails were relatively abundant in our study area with about 27 individuals per km^2 (González et al., 2007).

In addition to our field study of wild Mexican cottontails, we studied changes in T levels in animals kept in two field enclosures. These are located in the Malinche Research Station of the Universidad Autónoma de Tlaxcala, at 3100 m above sea level. The enclosures (530 and 108 m^2) were surrounded by a wall (2 m high) with a wire mesh fence on top (2.5 cm mesh diameter). Nylon cord strung at approximately 20-cm intervals across the top of the enclosure prevented entry of aerial predators. The enclosures contained wooden boxes and shelters made from native tussock grass (*Muhlenbergia macroura*) that served as refuges. They also contained a central concrete pond and food and water troughs. Each enclosure contained three to six rabbits of both sexes in different age classes, all individually marked by a color-coded vinyl ear tag and with an identification number tattooed on one ear. In addition to the natural vegetation growing in the enclosure, we regularly provided dried alfalfa as food source. The animals were introduced into the enclosures in June 2006.

Study period and data collection

The study was carried out from January 2005 to April 2009, when animals were caught from the wild twice a month using net traps baited with alfalfa (Vázquez et al., 2007b). Animals from the field enclosure were caught monthly since August 2006. Once an individual was captured, it was taken to the research station. Animals were weighed and blood was taken from the medial ear vein (around 1 ml). Serum samples were then stored at −20 °C until analysis. Before release, wild animals were marked individually in the same way as animals in the enclosures.

Permission to live-trap *S. cunicularius* in the National Park was obtained from the Secretaría del Medio Ambiente y Recursos Naturales, México (registration N° SGPA/dgvs/03502/06).

Hormone analysis

Serum testosterone was quantified at the Centro Tlaxcala de Biología de la Conducta using an enzyme immunoassay (Diagnostic Automation Inc., Calabasas, USA). The lower detection limit was 0.05 ng/ml. The inter- and intra-assay coefficients of variation were 6.3% and 4.5% (n = 6), respectively.

Data analysis and sample sizes

Overall, our data set consisted of 221 measurements (128 samples from males and 93 samples from females) from a total of 82 animals (46 males, 36 females) stemming from 5 different years. Note that animals were frequently re-trapped and thus were measured repeatedly. This was accounted for in our analyses by the use of mixed-effects models (see below), allowing for the inclusion of repeated measurements. 99 of the measurements taken were collected from animals trapped in the wild (68 individuals) and 122 measurements were taken from animals living in a field enclosure (14 individuals).

We assigned three different age classes based on the animals' body mass. Animals weighing less than 1000 g were referred to as "juveniles", those with a body mass of 1001–1400 g were classified as "subadults", and animals with a body mass of more than 1400 g were classified as "adults". In addition, maturity of males was verified by the presence of exterior testes. The reproductive condition of females was diagnosed by manual palpation in order to check for pregnancies and by palpation of the mammary glands in order to check for signs of lactation (Vázquez et al., 2007a).

In rabbits, the annual onset of breeding can vary strongly among years due to variation in environmental conditions (e.g. *O. cuniculus*, Rödel et al., 2005), and such a variation can be also observed in the Mexican cottontail. Thus, we estimated the annual onset of the breeding season based on the first signs of reproductive activity of the trapped females, considering the 34-day gestation and lactation period (Ceballos and Galindo, 1984; LR pers. obs.). This procedure revealed annual onsets of breeding between mid February and late April (on average late March). In order to analyze changes in T-levels along the year, we assigned 45-day time intervals with respect to the estimated annual onset of the breeding season (see Fig. 1).

Analyses were done using the software R version 3.0.0 (R Core Team, 2013). We calculated multivariate linear mixed-effects models using the R package lme4 (Bates and Maechler, 2010). We extracted the P-values using likelihood-ratio tests based on changes in deviance (based on maximum likelihood estimates) when each term was dropped from the full model including all predictor variables (Faraway, 2006). Normality of the model residuals was checked by the Shapiro–Wilk test and visually by normal probability plots. We assured the homogeneity of variances and goodness of fit by plotting residuals versus fitted values (Faraway, 2006).

Our main goal was to test for the effects of different predictor variables on serum testosterone levels. For this, we included the individual identity as a random factor in order to allow repeated measurements of the same animals, and we included the assay identity in order to statistically correct for potential between-assay variation. We included the origin of the animals (wild or field enclosures) as a fixed factor in order to detect possible differences. Since this factor was never significant (P > 0.10), we concluded that pooling the two data sets from animals stemming from the wild and from the field enclosure did not bias the results of our study.

Results

Seasonal changes in T levels of adult males and females

In adult males, serum testosterone levels (averaged over 45-day intervals, see Fig. 1a) varied significantly during the year ($\chi^2_5 = 32.31$, P = 0.001). Post hoc comparisons revealed a maximum peak in T levels around 90–45 days prior to the estimated annual