RESEARCH

Testis and brown adipose tissue xenografts from yellowish myotis (*Myotis levis*)

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Abstract

Yellowish myotis present a seasonal reproduction, influenced by rainfall distribution, in which the testis mass, germ cell composition, and brown adipose tissue (B.A.T.) mass change along the reproductive stages. In the present study, tissue xenografts were performed in immunodeficient mice to investigate spermatogenesis development in a stable endocrine milieu and the possible androgenic role of B.A.T. In this study, 41 adult male bats were captured in the Santuário do Caraça, Minas Gerais, Brazil. The gonads and B.A.T. were collected, weighed, and grafted under the mice's back skin. Mice biometric and hormonal data were evaluated after grafting, and the testis grafts and mice gonads were fixed for histological and immunohistochemical analyses. As a result, testis grafts from adult bats presented a continuous germ cell development in all reproductive stages, showing round spermatids in all testis tissues. Furthermore, testis fragments in the Rest stage presented elongating spermatids as the most advanced germ cell type in the seminiferous epithelium after 7 months of grafting. These data indicated that yellowish myotis spermatogenesis could be continued (presenting a constant spermatogonial differentiation) in a stable endocrine milieu, as found in mice. In addition, the best spermatogenic development was achieved when testis fragments were transplanted at their lowest activity (Rest stage). Regarding the B.A.T. grafts, the adipose tissue consumption by mice increased seminal vesicle mass and testosterone serum levels. This data proves that B.A.T. is related to testosterone synthesis, which may be critical in stimulating the differentiation of spermatogonia in yellowish myotis.

Lay summary

Bats are essential seed dispersers, pollinators, and agricultural pest regulators. Despite their ecological importance, bats face different threats due to environmental destruction and usually have few offspring per year. This study aimed to understand better how bats reproduce, but studying them in captivity is complicated and may not replicate what happens in the natural environment. To overcome this obstacle, we transplanted tissues from bats into mice which allowed in-depth research in lab conditions into bat reproduction. We looked at the tissues of adult bats after they had been transplanted into mice, and this allowed us to see which types of tissue played a critical role in reproduction.

Keywords: ▸ Chiroptera ▸ seasonal reproduction ▸ testosterone ▸ spermatogenesis ▸ gamete

Reproduction and Fertility (2022) **3** 287–300

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https://doi.org/10.1530/RAF-22-0056

Introduction

Bats present a high diversity with more than 1400 species worldwide (Wilson & Mittermeier 2019) and provide essential ecosystem services (Jones *et al.* 2009, Kunz *et al.* 2011, Maine & Boyles 2015, Puig-Montserrat *et al.* 2015, Russo & Jones 2015, Russo *et al.* 2018, Rodríguez-San Pedro *et al.* 2020). Bats adapt well to different reproductive strategies (Racey & Entwistle 2000) but present a low reproductive rate (Barclay *et al.* 2004, Jones *et al.* 2009). This feature makes them more vulnerable to different threats, such as urbanization, climate change (e.g. severe weather), changes in water quality, loss of roost sites, deforestation, hunting, diseases, and exposure to environmental contaminants (pesticides and heavy metals) (Jones *et al.* 2009, Zukal *et al.* 2015, Voigt & Kingston 2016, Frick *et al.* 2020).

Tissue xenograft, the biotechnology used in this study, has been used as a functional and powerful technique to investigate reproductive organ physiology in an *ex situ* manner (Paris *et al.* 2004, Rodriguez-Sosa & Dobrinski 2009, Santos *et al.* 2010, Arregui & Dobrinski 2014). This technique was studied in wild mammals aiming to preserve the genetic material of endangered mammal species (Honaramooz *et al.* 2004, Arregui *et al.* 2008a, Abbasi & Honaramooz 2011, 2012, Gourdon & Travis 2011, Arregui *et al.* 2014, Campos-Junior *et al.* 2014, Pothana *et al.* 2015). Testis xenograft was successful in some wild species, including seasonal species (Abbasi & Honaramooz 2012), generating valuable reproductive biology information, viable sperm and offspring (Honaramooz *et al.* 2004, Campos-Junior *et al.* 2014, Liu *et al.* 2016). Until now, there have been no studies using tissues from bats.

The Neotropical vespertilionid yellowish myotis bat (*Myotis levis*), the investigated species of this study, present a seasonal reproduction in which the development of spermatogenesis is linked to rainfall distribution (Farias *et al.* 2020). Our research group demonstrated that the testis parenchyma of yellowish myotis shows a remarkable variation in the germ cell population, allowing the identification of four reproductive stages known as Rest, Maturing, Mature, and Regressed (Farias *et al.* 2020). The Rest stage is characterized by the presence of the spermatogonial phase only. Primary spermatocytes are observed in the Maturing and Mature stages, coinciding with the peak of rainfall distribution. Sperm formation occurs in the Mature and Regressed stages only, during which the spermiogenic phase is observed. After the conclusion of spermiation, long-term sperm storage in epididymis cauda (~8 months) begins in the Regressed stage (Farias *et al.* 2020). An exciting aspect is related to the cyclic and synchronic fluctuation of the testis and epididymis mass along the reproductive stages. During the reproductive cycle, the maximum testis size and activity (Mature stage) is followed by the highest epididymis volume in the next reproductive phase (Regressed stage). Investigating the impacts of abiotic factors in testis physiology is needed to avoid valuable bat species loss. Testis tissue xenograft is a promising tool for scientific studies of seasonal species because the gonads are transferred to a more stable endocrine milieu.

Another interesting observation is that the brown adipose tissue (B.A.T.) mass varies in the same pattern as the accessory sex gland mass throughout the reproductive stages, indicating a possible role of this organ in the yellowish myotis male reproductive cycle (Farias *et al.* 2020). Furthermore, comparing the Rest stage to the Mature stage, the B.A.T. mass negatively correlates with serum testosterone levels (Farias *et al.* 2020). These findings suggest that the B.A.T. was possibly used for androgenic purposes in the yellowish myotis (Krutzsch & Wells 1960). Current data demonstrate that adipose tissue may contain the steroidogenic machinery necessary to initiate steroid biosynthesis *de novo* from cholesterol (Li *et al.* 2015). In this case, xenografting may offer a novel approach for evaluating the possible involvement of B.A.T. in steroidogenesis.

Therefore, the present study aimed to use testis tissue xenograft from adult yellowish myotis to investigate the resumption of spermatogenesis in tissue derived from different reproductive stages without the influence of environmental factors and to use the B.A.T. xenograft from adult yellowish myotis to evaluate its possible androgenic role in bats for the first time.

Materials and methods

Study area and capture of bats

The yellowish myotis colony lives in Santuário do Caraça, a preserved area in Serra do Caraça, southeastern Brazil (20°04'30"S, 43°24'28"W), which belongs to the Iron Quadrangle geomorphological domain (Moreira & Pereira 2004, Abreu & Palú 2008). The reserve has a great diversity of fauna and flora because it is located in a transition region of Atlantic Forest and Cerrado biomes (Giulietti *et al.* 1997, Moreira & Pereira 2004, Abreu & Palú 2008). A seasonal climate characterizes this region with a rainy summer (rainy season – October to March)

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and a dry winter (dry season – April to September), in which the precipitation occurs mainly during the rainy season (81.5% of the annual average of 1.373 mm) and the remaining percentage of precipitation occurs in the dry season (de Sá Júnior *et al.* 2012).

Bats were captured from August 2017 to November 2020 using mist-nets installed in the attic of Santuário do Caraça church, from 18:00 to 00:00 h. Forty-one male bats were collected representing the reproductive stages Rest, Maturing, Mature and Regressed (Farias *et al.* 2020) for xenograft experiments (Table 1). The forearm length, body mass, age class, and sex of each individual were recorded. Only adult males were used, differentiated from subadults by ossified finger epiphyseal cartilages in the metacarpus (Anthony 1988) and complete testicular descent (Duarte & Talamoni 2010). Bats were sacrificed through intraperitoneal injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight), and the gonads, epididymis, and B.A.T. from the interscapular region were collected.

All specimens were deposited in the Pontifical Catholic University of Minas Gerais reference collection. Captures were performed under license (#28120-4) granted by the Brazilian Chico Mendes Institute for Biodiversity Conservation, and access to animal genetic legacy was granted by license nº A8CA63C of the Genetic Legacy Management Council by the Brazilian Ministry of Environment (SISGen). The Ethics Committee on Animal Use from the Federal University of Minas Gerais (CEUA document 386/2017) approved the study procedures.

Testis and brown adipose tissue xenograft

The xenograft procedure was performed immediately after testis and B.A.T. harvesting. Each testis was sectioned into four fragments (3 × 3 × 5 mm), and half of the B.A.T. was used in each xenograft. After that, the tissues were maintained on Dulbecco's modified Eagle's medium (#12500-062; DMEM/F12, Gibco) at 35°C for 15 min and then the xenograft procedure was performed in the sexually mature immunodeficient recipient mice.

Testis fragments (from each reproductive stage) of yellowish myotis were grafted under the back skin of 15 castrated NSG mice (6 grafts per mouse, 90 grafts in total), resulting in 4 experimental groups (Rest = 6 mice and 36 grafts, Maturing = 3 mice and 18 grafts, Mature = 3 mice and 18 grafts, Regressed = 3 mice and 18 grafts). Mice were anesthetized through intraperitoneal injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight). Then, the animals were castrated and subsequently positioned in the ventral decubitus position, and three incisions of approximately 0.5 cm were performed bilaterally to the dorsal line. The skin was dissected, forming pockets in which the fragments were placed. After receiving the fragment, the incisions were sutured with 5.0 silk threads (Biosut, Brazil).

The B.A.T. of the Maturing stage (0.0499 ± 0.0043 g) (Farias *et al.* 2020) was grafted under the back skin of 8 non-castrated 8-week-old NUDE mice (2 random grafts per mouse, 16 grafts in total). The NUDE mice (without fur) were used to easily observe the B.A.T. mass reduction along the grafting time. The Maturing stage was chosen because it is the phase in which the B.A.T. is consumed by yellowish myotis. Additionally, five NUDE mice (16-week-old) were used as a control group. Anesthesia and surgery procedures were the same as above, but only one incision was performed bilaterally to the dorsal line. The animals were kept on a heated surface (37°C) during surgery to prevent hypothermia and facilitate recovery.

Biometric data and histological evaluation

The body and graft masses were evaluated at 5 and 2 months after grafting for testis and B.A.T., respectively. We waited for 5 months for testis xenografting based on previous reports (Arregui & Dobrinski 2014). B.A.T. xenografting was discontinued (after 2 months) due to the severe reduction of this tissue under the back skin of NUDE mice. The seminal vesicle mass was used to indicate bioactive testosterone for the testis tissue and B.A.T. xenograft experiments (Arregui *et al.* 2008a, 2014). The mice were sacrificed through intraperitoneal

Table 1 Yellowish myotis sampling from August 2017 to November 2020.

Reproductive stages	Months	Number of animals	Analyses
Rest	May to June	6	Testis xenograft
	July to August	6	Testis xenograft
Maturing	November to December	14	Testis and brown adipose tissue xenograft
	January to February	3	Brown adipose tissue xenograft
Mature	March	6	Testis xenograft
Regressed	April	6	Testis xenograft

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injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight) to recover the tissue xenografts.

The epididymis and one testis tissue fragment per bat were not used for grafting, allowing the observation of the original physiologic status of the organs. These organs and the B.A.T. were fixed in Bouin solution, routinely prepared, and embedded in Paraplast® for histological analysis (Fig. 1A). Moreover, these histological images were used as controls for animal age, demonstrating that they were adult animals (spermatozoa identification) (Fig. 1B to E).

After 5 months of grafting, the testis tissue fragments were fixed in Bouin solution and 4% glutaraldehyde for histological analysis. Each germ cell type (undifferentiated spermatogonia, differentiated spermatogonia, spermatocytes, and round spermatids) was counted in 20 seminiferous tubules per reproductive stage. This quantification was performed to determine the spermatogenesis progression after testis tissue xenograft. Concerning the B.A.T. experiments, the NUDE mice gonads were fixed in Bouin solution, routinely processed, and embedded in Paraplast® for histological analysis. As the best outcome was in the Rest stage after 5 months, we qualitatively analyzed the testis graft histologies in this reproductive stage after 7 months to determine if spermatogenesis would advance further than round spermatid steps.

Hormonal analyses

Blood samples of mice were collected by cardiac puncture after anesthesia induction. Plasma was separated through centrifugation (720 ***g***, for 10 min, at 4°C) and stored at −20°C for subsequent hormone evaluation. The samples were analyzed in the automated Cobas e411 (Roche Diagnostics Inc.) platform to assess testosterone directly. Serum testosterone levels were measured using commercial kits (Roche Diagnostics Inc.) through the electrochemiluminescence method (sensitivity of 2.5 ng/dL). Testosterone intra- and inter-assay coefficients of variation (CV) were 1.1 and 1.5%, respectively. The procedures were performed by a Licensed Laboratory specialist in Animal Health (TECSA(R) Laboratory, Belo Horizonte, Brazil).

Immunostaining analyses

For immunohistochemical analysis, deparaffinized sections were dehydrated, and the endogenous peroxidase activity was blocked by incubating the sections in a 3% hydrogen peroxide solution (Sigma). After that, the antigens were exposed to heating in buffered sodium citrate (pH 6.0) at 96°C for 10 min, and the protein was blocked using 10% normal rabbit serum (Sigma #R9133) in PBS for 30 min. The slides were incubated overnight (4°C) with a specific primary antibody against the

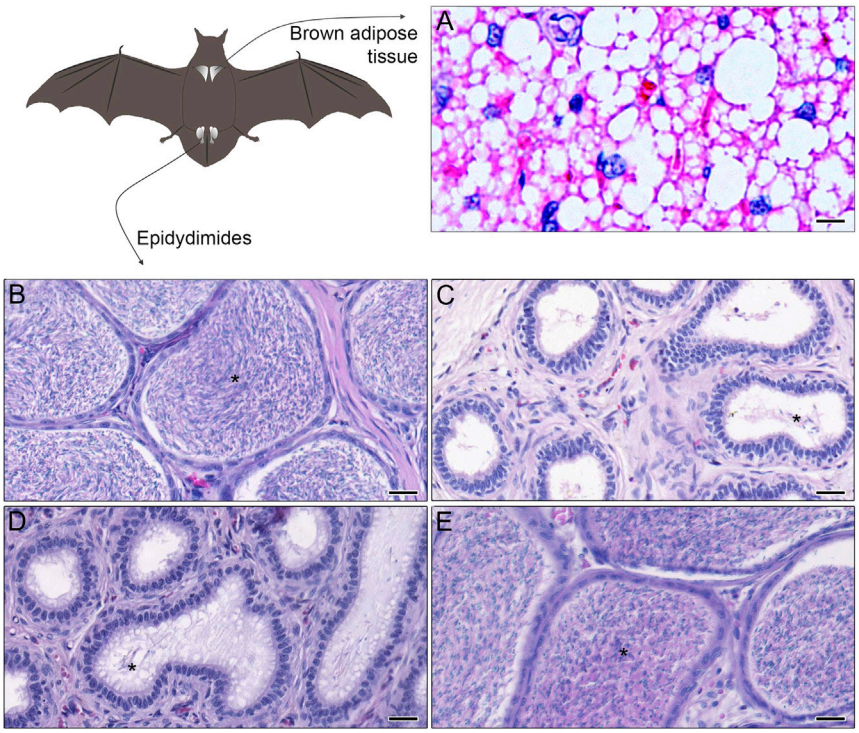

Figure 1 Brown adipose tissue and epididymides from yellowish myotis donors. Yellowish myotis brown adipose tissue is located in the interscapular region and presented several lipid droplets (A). The sperm (*) found in yellowish myotis epididymides (in all reproductive stages), even in different amounts, confirmed that all animals were adults. Sperm cells were found in all animals captured in the Rest (B), Maturing (C), Mature (D), and Regressed (E) stages. Bars: A to D = 20 µm.

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steroidogenic enzyme 3-Beta-HSD (1:100, Santa Cruz Biotechnology, goat polyclonal antibody, sc-30820). Considering that the antibody was raised against the human protein, the protein homology between human and *Myotis* species was tested through *in silico* analysis (Basic Local Alignment Search Tool), showing 76.7% homology. The reaction was developed using rabbit pAb to goat secondary IgG antibody (ab6740; Abcam Inc.). Diaminobenzidine (DAB) was used as chromogen, and the negative control had the primary antibody omitted. For the steroidogenic enzyme 3-Beta-HSD expression, protein labeling was quantified. In this analysis, three random images (30 cells) were captured from the testicular parenchyma of mice using an Olympus BX60 microscope with a coupled camera. The images were treated to convert into gray scale in Photoshop CS6 v13.0, and pixel intensity was measured from the labeled cells, normalized by the pixel intensity obtained from the image's background (lumen of seminiferous tubules or blood vessels).

Statistical analysis

All quantitative data were tested for normality and homoscedasticity of variances by the D'Agostino and Pearson tests. The data obtained were expressed as the mean ±S.E.M., and the statistical analyses were performed using the program GraphPad Prism 6 (GraphPad Software, Inc). The level of significance considered was $P < 0.05$.

For testis tissue xenograft, mice body mass, seminal vesicle mass, and serum testosterone levels presented a normal distribution. These parameters were submitted for one-way ANOVA , and the Newman–Keuls test compared the means of the reproductive stages. The testis tissue graft masses in the Mature and Maturing stages presented normal distribution and were submitted to Student's *t*-test. Testis tissue graft masses in Rest and Regressed stages presented a non-parametric distribution and were submitted to the Kolmogorov–Smirnov test. The percentages of seminiferous tubules with germ cells also showed a non-parametric distribution but were submitted to Kruskal–Wallis and Dunn's test to compare the means of the reproductive stages.

For B.A.T. xenograft, mice body mass, seminal vesicle mass, serum levels of testosterone, and 3-Beta-HSD pixel intensity presented normal distribution and were submitted to Student's *t*-test. The B.A.T. graft mass presented a non-parametric distribution and was submitted to the Kolmogorov–Smirnov test.

Results

Mice seminal vesicle mass and serum testosterone levels did not change among the groups of testis tissue xenograft

The body mass and seminal vesicle masses of mice that received testis tissue xenografts showed no significant differences among the reproductive stages (Fig. 2A and B, Table 2). The absolute values of serum testosterone levels increased from the Rest to the Mature stage and decreased in the Regressed stage. However, it is crucial to mention that no significant variation was observed among the reproductive stages (Fig. 2D, Table 2).

Testis tissue fragments from the Rest stage presented the highest growth index

The testis fragments from the Rest stage demonstrated an expressive and significant volume increase after grafting (prior grafting = 0.0012 ± 0.0002 g and after grafting = 0.0088 ± 0.0013 g) (*Kolmogorov–Smirnov test*, $KS = 0.9667$, $P = 0.0002$) (Fig. 2C). Although in a lower index, the volume of testis fragments from the Maturing stage also significantly increased after grafting (prior grafting = 0.0028 ± 0.0003 g and after grafting = 0.0054 ± 0.0006 g) (*t*-test, $t = 2.618$, $df = 22$, $P = 0.0157$) (Fig. 2C). On the other hand, the testis fragments from the Mature stage presented a significant volume decrease (prior grafting = 0.0150 ± 0.0015 g and after grafting = 0.0091 ± 0.0014 g) (*t*-test, $t = 2.549$, $df = 16$, $P = 0.0215$) (Fig. 2C), and testis fragment size did not differ in the Regressed stage after grafting (prior grafting = 0.0064 ± 0.0007 g and after grafting =0.0161 ± 0.0089 g) (*Kolmogorov–Smirnov test*, $KS = 0.2778$, $P = 0.8782$) (Fig. 2C).

Xenografting in the Rest stage promoted the best development of the spermiogenic phase

The yellowish myotis testis parenchyma presented only Sertoli and undifferentiated spermatogonial cells in the Rest stage (*in situ*) (Fig. 3A). Surprisingly, the testis fragments (xenografting) in this phase resulted in an expressive development of the seminiferous epithelium, displaying germ cells from the three phases of spermatogenesis after 5 (Fig. 3A' and A", 4A) and 7 (Fig. 5) months of grafting.

Undifferentiated and differentiated spermatogonial cells were evident (>95%) in the basal compartment (Fig. 3A" and 4A) of the seminiferous tubule cross-sections (Fig.

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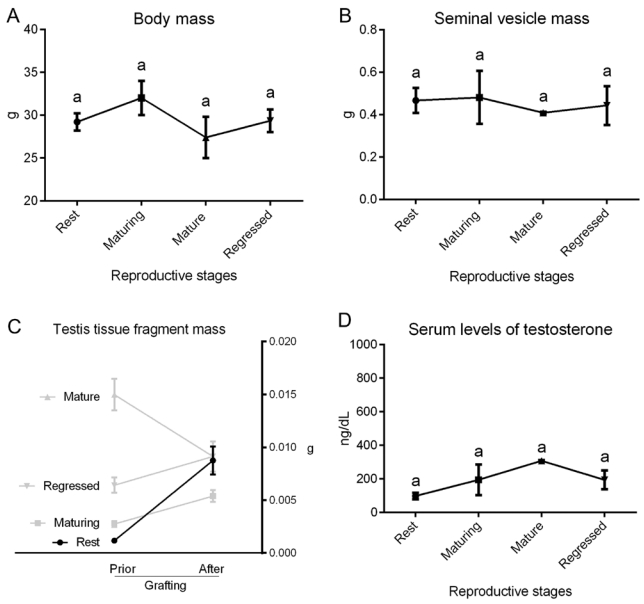

Figure 2 Mean (±S.E.M.) values of biometric and hormonal data of mice grafted with testis tissue fragments in yellowish myotis reproductive stages after 5 months of grafting. The body mass (A) and seminal vesicle mass (B) do not differ among the reproductive stages, while the testis graft mass increased significantly after grafting in the Rest stage (C). No significant differences were observed for the serum testosterone levels (D). Different letters indicate statistically significant differences, $P < 0.05$. Three recipient mice and 18 grafts were analyzed per reproductive stage.

4E and F, Table 2). Primary spermatocytes in pre-leptotene, zygotene, and pachytene cells were readily observed (Fig. 3A" and 4A) in 75–85% of the seminiferous tubule cross-sections (Fig. 4C and D, Table 2).

Regarding the third phase of spermatogenesis, round spermatids were the most advanced germ cell type identified (Fig. 3A" and 4A), showing the highest percentage among the reproductive stages (Fig. 4B, Table 2). Interestingly, testis fragments from the Rest stage presented round spermatids (Fig. 5B and D) and elongating spermatids in the seminiferous epithelium (Fig. 5F) after 7 months of grafting.

Spermatogenesis progressed well until the meiotic phase after xenografting in the Maturing stage

The differentiation of spermatogonia into primary spermatocytes characterizes the yellowish myotis Maturing stage (*in situ*) (Fig. 3B). Similarly, the testis grafts presented differentiated spermatogonial cells and primary spermatocytes (Fig. 3B' and B", 4A) in the majority of the seminiferous tubule cross-sections (85% and 50–70%, respectively) (Fig. 4C to F, Table 2). Although in a lower frequency, it should be mentioned that round spermatids were observed in the seminiferous epithelium (Fig. 3B", 4A and B, Table 2).

Reduced spermatogenic activity was observed in testis fragments xenografted in the Mature stage

In the yellowish myotis Mature stage (*in situ*), a natural gap was observed between undifferentiated spermatogonial cells and primary spermatocytes. Furthermore, more advanced germ cells, including round and elongated spermatids, were identified in this phase (Fig. 3C).

After grafting in this phase, a small percentage of seminiferous tubule cross-sections showed germ cells beyond the undifferentiated spermatogonial cells (Fig. 4A to F, Table 2). Differentiated spermatogonia, spermatocytes, and round spermatids were also identified in a reduced number (Fig. 3C' and C", 4A to E, Table 2).

Reduced activity of meiotic and spermiogenic phases was observed after xenografting in the Regressed stage

In the Regressed stage (*in situ*), the yellowish myotis testis presents unique characteristics, such as seminiferous tubules with a wide lumen and a vast gap between undifferentiated spermatogonial cells elongated spermatids (Fig. 3D). After grafting, approximately half of the seminiferous tubule cross-sections (>52.5%) displayed undifferentiated and differentiated spermatogonia

Table 2 Mean (±S.E.M.) values of mouse and testis fragment parameters after grafting.

Parameters	Rest	Maturing	Mature	Regressed	ANOVA (*F*)	*P* value
BM (g)	29.20 ± 1.02	32.00 ± 2.00	27.40 ± 2.40	29.33 ± 1.33	1.253	0.3473
SVM (g)	0.4664 ± 0.0594	0.4807 ± 0.1247	0.4084 ± 0.0045	0.4427 ± 0.0916	0.1040	0.9556
TSL (ng/dL)	97.13 ± 19.50	193.70 ± 92.03	307.00 ± 5.00	193.20 ± 56.13	1.725	0.2484
UND (%)	95.00 ± 3.44^{a}	87.50 ± 6.15^{a}	45.00 ± 8.03^{b}	57.50 ± 8.33^{b}	27.97*	<0.0001
DIFF (%)	97.50 ± 2.50^{a}	85.00 ± 6.40^{ab}	12.50 ± 7.14^{c}	52.50 ± 10.60^{b}	39.89*	<0.0001
Pl-Z (%)	75.00 ± 7.70^{a}	70.00 ± 6.70^{a}	12.50 ± 6.15^{b}	25.00 ± 8.51^{b}	34.10*	<0.0001
P (%)	85.00 ± 5.26^{a}	50.00 ± 8.11^{ab}	10.00 ± 5.85^{c}	37.50 ± 8.80^{bc}	34.12*	<0.0001
R (%)	37.50 ± 7.14^{a}	5.00 ± 3.44^{b}	5.00 ± 5.00^{b}	7.50 ± 4.10^{b}	24.81*	<0.0001

Different line superscript letters show statistically significant differences, $P < 0.05$. *Kruskal-Wallis test (H).
DIFF, differentiated spermatogonia; P, pachytene spermatocytes; Pl-Z, pre-leptotene to zygotene spermatocytes; R, round spermatids;SVM, seminal vesicle mass; TSL, testosterone serum levels; UND, undifferentiated spermatogonia.

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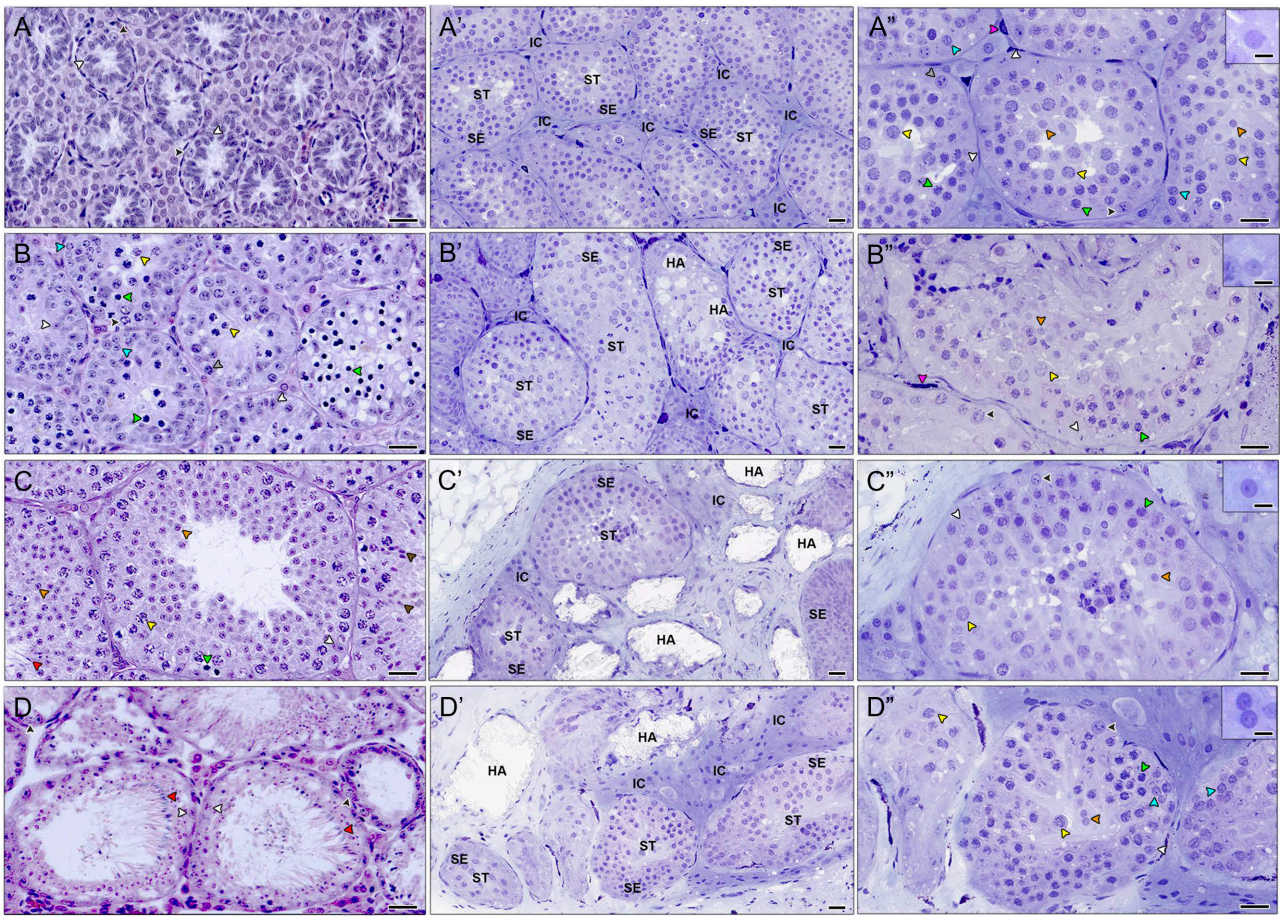

Figure 3 Spermatogenesis development in yellowish myotis testis tissue xenograft after 5 months of grafting. The testis parenchyma of yellowish myotis in the reproductive stages *in situ* showed a huge variation in germ cell composition (A to D). The testis fragments of all the reproductive stages showed the three phases of spermatogenesis after 5 months of grafting. The seminiferous tubules (ST) presented round spermatids as the most advanced germ cell type in the seminiferous epithelium (SE) (A' to D' and A'' to D''). The presence of mast cells in the intertubular compartment (IC) was frequently observed in the Rest and Maturing phases (A'' and B''). Except for the Rest phase fragments, histopathological alterations (HA) were observed in the other phases' fragments (B' to D'). Arrowheads: white (Sertoli cell), black (undifferentiated spermatogonia), gray (differentiated spermatogonia), blue (pre-leptotene spermatocyte), green (zygotene spermatocyte), yellow (pachytene spermatocyte), orange (round spermatid), brown (elongating spermatid), red (elongated spermatid), pink (mast cells). Three recipient mice and 18 grafts were analyzed per reproductive stage. Bars: A to D, A' to D', and A'' to D'' = 20 µm; A'' to D'' inserts = 4 µm.

(Fig. 4E and F). Although in a lower frequency, primary spermatocytes (pre-leptotene, leptotene, zygotene, and pachytene cells) and round spermatids (Fig. 3D'', 4A-D, and Table 2) were also observed.

Mast cells were frequently observed in the most advanced testis tissue fragments

Although the germ cell composition was quite different among the reproductive phases, all testis xenografts led to the formation of round spermatids after 5 months of grafting (Fig. 3 and 4). Interestingly, mast cells were frequently observed in the xenograft interstitial compartment, especially in those fragments that presented the highest development (Rest and Maturing stages) (Fig. 3A'' and B''). Several histopathological alterations were observed in the xenografts of the Maturing, Mature, and Regressed stages (Fig. 3B', C' and D').

The brown adipose tissue graft mass decreased promoting androgenic stimuli

After 2 months of grafting, no significant variation was observed in mice's weight (Table 3). The seminal vesicle mass presented a significant increase (Fig. 6A, Table 3). In an opposite pattern, the B.A.T. graft mass decreased significantly (Kolmogorov–Smirnov test, KS = 0.6875, $P = 0.0010$) (Fig. 6B). Moreover, testosterone serum levels increased more than six-fold in the grafted mice (Fig. 6C, Table 3). The different 3-Beta-HSD immunolabeling

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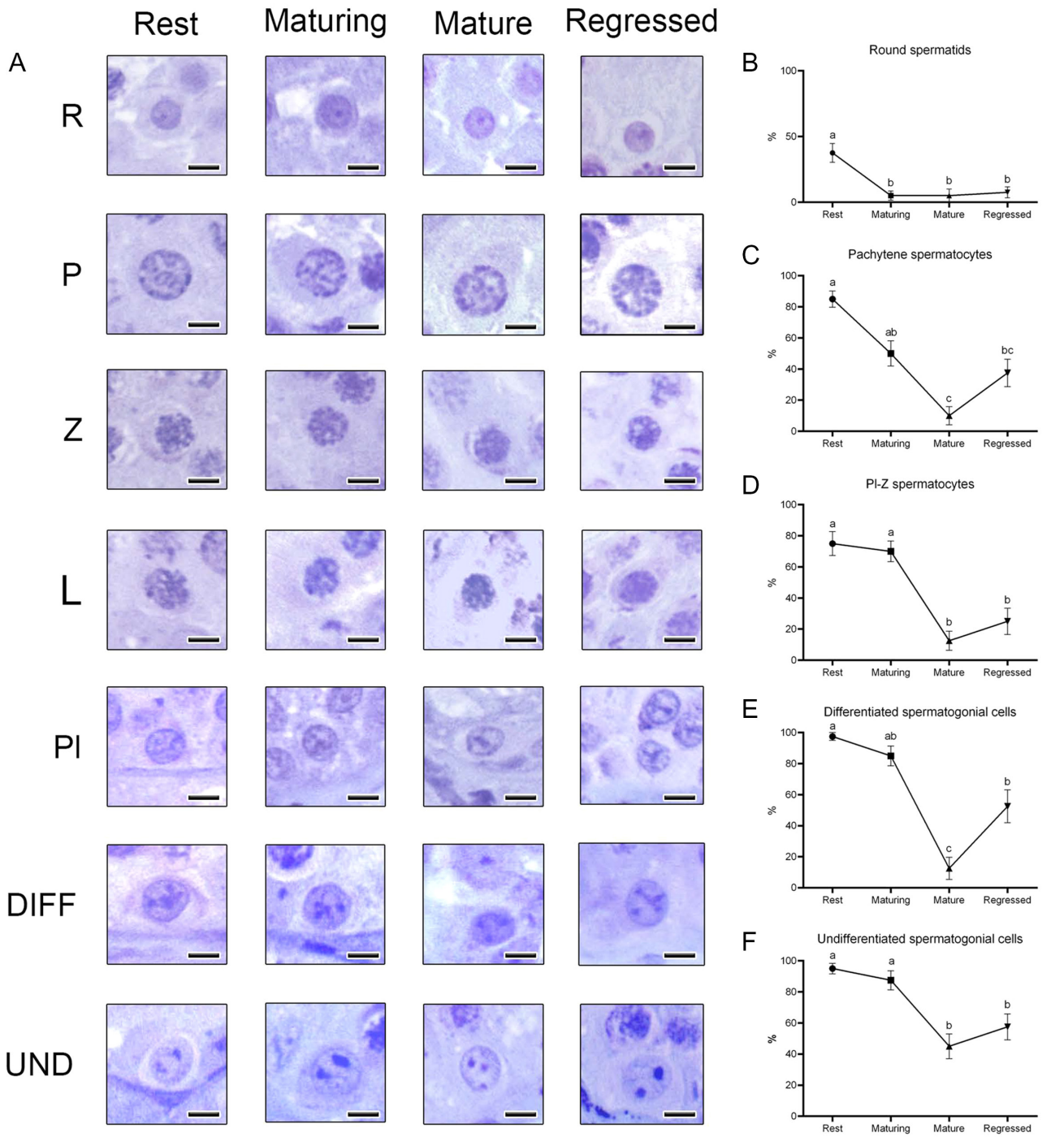

Figure 4 Germ cell composition and quantification in the yellowish myotis testis tissue xenograft after 5 months of grafting. The seminiferous epithelium of the testis grafts presented undifferentiated spermatogonia (UND), differentiated spermatogonia (DIFF), pre-leptotene spermatocyte (PI), leptotene spermatocyte (L), zygotene spermatocyte (Z), pachytene spermatocyte (P), and round spermatid (R) in all the reproductive stages of yellowish myotis (A). However, the testis grafts in the Rest stage presented a significantly higher percentage of round spermatids. In an opposite pattern, the testis grafts in the Mature stage showed fewer germ cells in the seminiferous tubules (B to F). Different letters show statistically significant differences, $P < 0.05$. Three recipient mice and 18 grafts were analyzed per reproductive stage. Bars: 4 µm.

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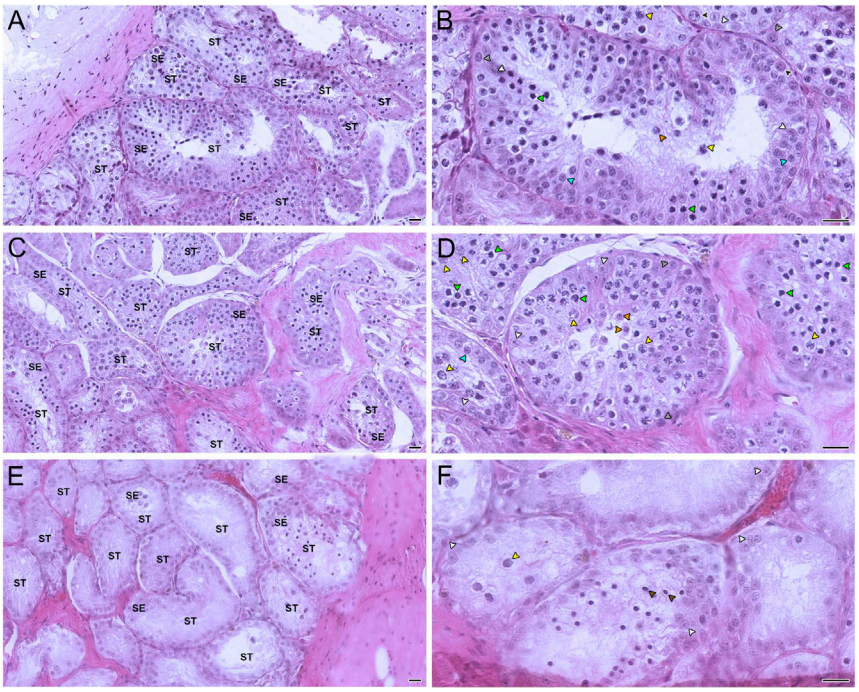

Figure 5 Spermatogenesis progression of yellowish myotis testis fragments in the rest stage after 7 months of grafting. The testis fragments in the rest stage exhibited a significant development of the spermatogenic process (A to F). The seminiferous tubules (ST) were composed of spermatogonial cells, spermatocytes, and spermatids (B, D, and F) in the seminiferous epithelium (SE) (similar pattern of 5 months grafting). Elongating spermatids (F) were the most advanced germ cell type in the testis parenchyma. Arrowheads: white (Sertoli cell), black (undifferentiated spermatogonia), gray (differentiated spermatogonia), blue (pre-leptotene spermatocyte), green (zygotene spermatocyte), yellow (pachytene spermatocyte), orange (round spermatid), brown (elongating spermatid). Three recipient mice and 18 grafts were analyzed in the rest stage. Bars = 20 µm.

patterns in Leydig cell cytoplasm from control mice (Fig. 6E) and grafted mice (Fig. 6F) and the significant increased 3-Beta-HSD pixel intensity evaluation (Fig. 6D) indicated that the B.A.T. consumption is stimulating these cells.

Discussion

Yellowish myotis presents a seasonal reproduction linked to rainfall distribution (Farias *et al.* 2020). To evaluate the gonad and B.A.T. physiology in a stable endocrine milieu, we opted to graft the organs in immunodeficient mice. The recipient mice allowed a constant testosterone production and promoted continuous spermatogenesis in testis tissues of all reproductive stages of this seasonal species. Furthermore, we demonstrated that adult testis tissue xenografts could be well-succeed when testis fragments in low spermatogenic activity are transplanted. For the first time, we also showed that the B.A.T. plays a pivotal androgenic function, as it stimulates the testosterone synthesis of mice (Fig. 7). This data indicated that the cyclic fluctuation of the B.A.T. weight observed in yellowish myotis directly links with the serum testosterone levels oscillation. We suggest that the B.A.T. may stimulate the bat gonad steroidogenic activity, inducing spermatogonia differentiation and spermatogenesis progression (Fig. 8).

In our study, testis tissue xenografts of an adult bat were performed for the first time. The testosterone serum levels of grafted mice did not show significant differences among the reproductive stages, suggesting that a stable LH stimulus in mice regulated the hormonal synthesis. The germ cells progressed until the round spermatid step in testis grafts of all reproductive stages after 5 months of grafting. Furthermore, elongating spermatids were observed in testis fragments of the Rest stage after 7 months of grafting. This finding indicates that testosterone would be vital in promoting undifferentiated spermatogonial differentiation since

Table 3 Mean (±S.E.M.) values (in g, except for TSL: ng/Dl, and PI) of body mass (BM), seminal vesicle mass (SVM), testosterone serum levels (TSL), and 3-Beta-HSD pixel intensity (PI) of control mice and mice that received brown adipose tissue (B.A.T.) xenografts of yellowish myotis.

Experimental groups	BM	SVM	TSL	PI
Control	25.10 ± 1.57	0.1617 ± 0.0326[a]	283.6 ± 101.9[a]	115.3 ± 3.8[a]
B.A.T. grafts	24.05 ± 1.69	0.3158 ± 0.0425[b]	1857.0 ± 274.9[b]	130.2 ± 4.3[b]
Student's *t*-test	0.4237	2.564	4.343	2.523
P value	0.6800	0.0263	0.0012	0.0326

Different column superscript letters show statistically significant differences, $P < 0.05$.

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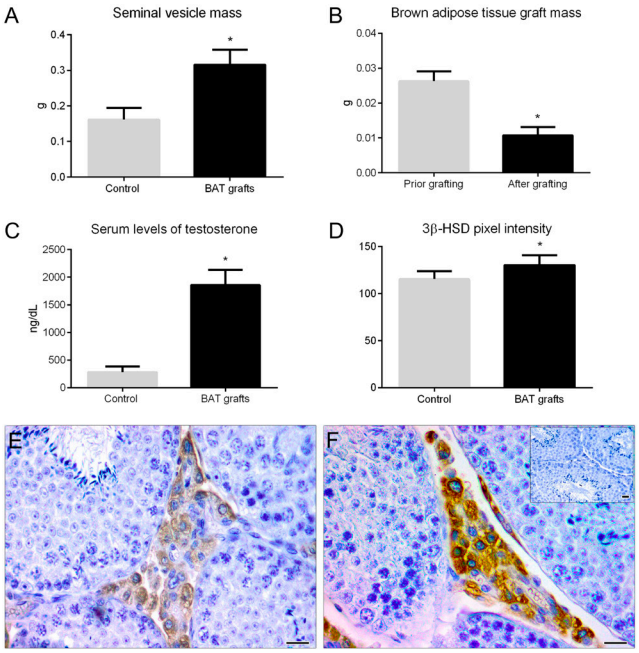

Figure 6 Biometric, hormonal, and immunohistochemical parameters of mice grafted with brown adipose tissue grafts. The seminal vesicle mass increased significantly in the grafted group (A). The brown adipose tissue graft mass decreased significantly after grafting (B), while the serum levels of testosterone significantly increased more than six times in the grafted mice (C). Moreover, there is a difference in the 3-Beta-HSD immunolabeing pattern between the control (E) and grafted group (F), demonstrated by the significant increase in the enzyme expression (D). Insert shows the negative control. Eight recipient mice and 16 grafts were analyzed, while 5 mice served as controls. Bars: 21 μm.*Statistically significant differences, $P < 0.05$.

we do not observe this differentiation in the Rest and Regressed stage of yellowish myotis (Fig. 8A). Testosterone is believed to stimulate spermatogonial differentiation in other wild mammalian species (collared peccary) (Campos-Junior *et al.* 2012). Interestingly, the Rest stage fragments were more successful considering the spermatogenic progression, probably due to a low state of spermatogenesis (Arregui *et al.* 2008b).

In normal conditions (bat reproduction physiology), testis at the Mature and Regressed stage would reach the Rest stage 5 months later. In the Rest stage, the animals present only spermatogonia in the seminiferous epithelium (Farias *et al.* 2020). However, we observed round spermatids when we transplanted testis tissues from the Mature and Regressed stages into mice. The Regressed stage (containing only spermatids in the epithelium) can be expected 5 months after the Maturing stage in nature (Farias *et al.* 2020). However, we found spermatocytes in the seminiferous epithelium of the grafts, indicating continued spermatogenesis. For the Rest stage, we would expect the Maturing stage in natural conditions 5 months after our tissue harvesting time. In the Maturing stage, the animals do not present round spermatids (Farias *et al.* 2020), which is different from the data after the xenograft. All these findings indicate that the steady endocrine environment was more supportive for spermatogenesis development.

The success of testis xenografts is highly variable in adult wild mammal species (Table 4). Using a seasonal and adult animal (Djungarian hamster), Schlatt and colleagues showed that most testis tissues degenerated after grafting (Schlatt *et al.* 2002); however, spermatocytes were found in photoregressed testis tissues 7 weeks post-grafting (Schlatt *et al.* 2002). Unlike the good results achieved in yellowish myotis, testis grafts from adult Lynx (*Lynx pardinus*) and older monkeys (*Macaca mulatta*; 11–12 years old) degenerated after grafting (Arregui *et al.* 2008b, 2014). Grafts from subadult monkeys (6 years old) presented a discrete advance of spermatogenesis, and spermatocytes were observed in 0.3% of the seminiferous tubules cross-sections (Arregui *et al.* 2008b). Grafts from younger subadult monkeys (3 years old) showed higher percentages of seminiferous tubules with spermatocytes (64.1%) after 24 weeks of transplantation. However, it should be mentioned that elongated spermatids were identified in a few seminiferous tubules cross-sections (1.1%) (Arregui *et al.* 2008b).

The testis tissue xenografts from immature donors usually show a better development than tissue grafts from sexually mature donors (Arregui *et al.* 2008b, Arregui & Dobrinski 2014). Several factors could favor juvenile graft development, such as a lower metabolism of spermatogenesis, higher resistance to ischemic conditions, and intense somatic cell proliferation (Schlatt *et al.* 2002, Arregui *et al.* 2008b, Arregui & Dobrinski 2014). As previously mentioned, most grafted tissues from adult donors usually degenerate (Schlatt *et al.* 2002, Arregui *et al.* 2008b, 2014). The first report of complete spermatogenesis resulting in viable and functional sperm occurred in testis tissue xenografts from immature mice, pigs, and goats (Honaramooz *et al.* 2002). This technique was successfully applied to juvenile wild animals, such as bison calves, white-tailed deer, collared peccary, ferret, Djungarian hamster, and rhesus monkey, resulting in sperm production (Schlatt *et al.* 2002, Honaramooz *et al.* 2004, Abbasi & Honaramooz 2011, 2012, Gourdon & Travis 2011, Campos-Junior *et al.* 2014). In the endangered immature Cuvier's gazelle (Arregui *et al.* 2014), a similar spermatocyte percentage was found in testis parenchyma compared to yellowish myotis grafting (Rest stage). Interestingly, the spermatogenic development in testis

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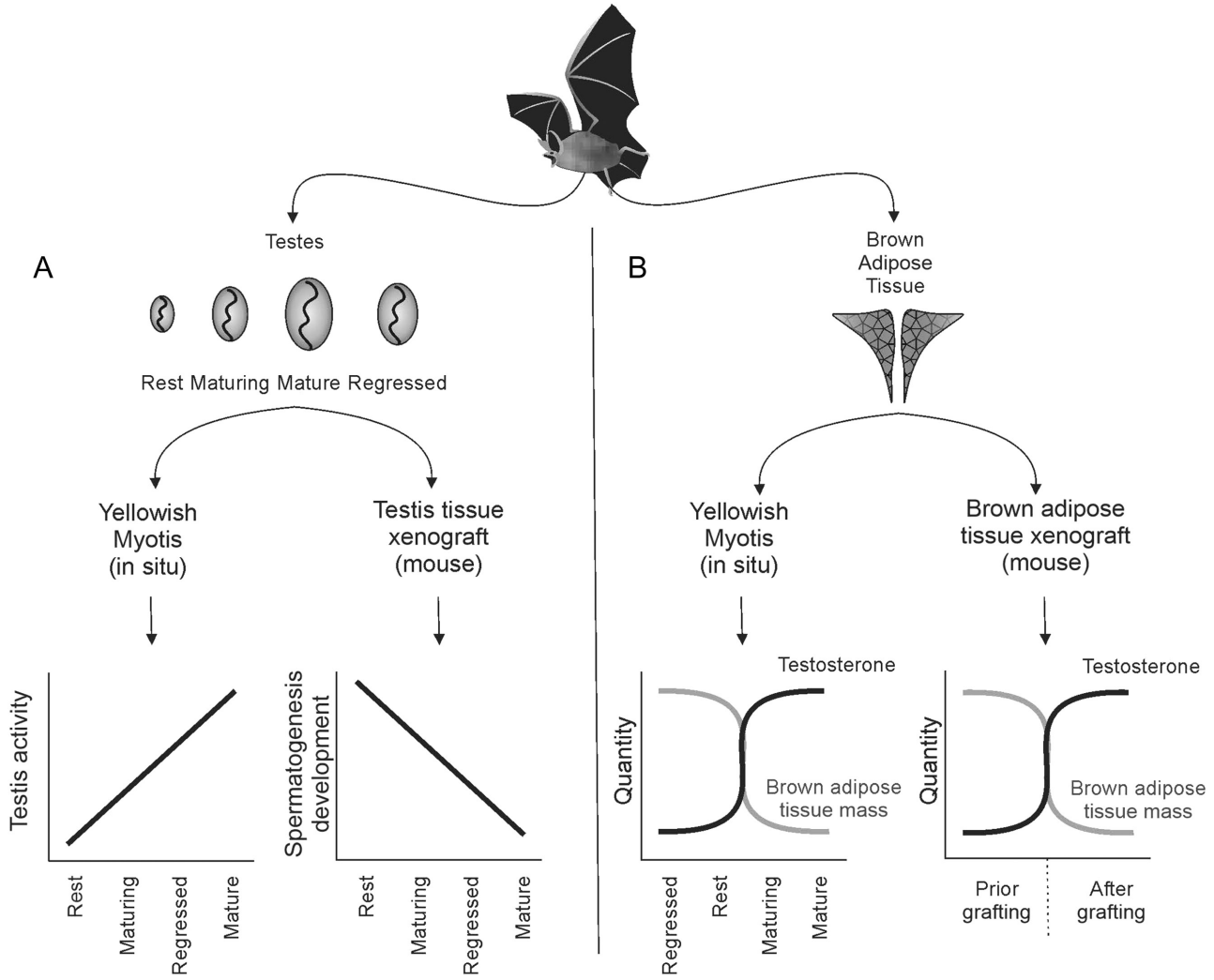

Figure 7 Schematic illustration of the main findings of the present study. (A) Comparison of testicular activity in yellowish myotis and the spermatogenic development in testis fragments after xenografting. (B) Relationship between brown adipose tissue mass and testosterone synthesis in yellowish myotis (in an environmental context) and after xenografting in recipient mice.

grafts from adult yellowish myotis was better than the testis xenografting of bison, white-tailed deer, banteng, and Iberian lynx (Honaramooz *et al.* 2005, Abbasi & Honaramooz 2011, 2012, Arregui *et al.* 2014).

The B.A.T. of yellowish myotis showed a weight fluctuation along the reproductive stages (Farias *et al.* 2020). The consumption of the B.A.T. by yellowish myotis coincided with the better progression of spermatogenesis and production of testosterone. It is thought that testosterone allows the differentiation of undifferentiated spermatogonia (Farias *et al.* 2020). The androgenic activity of B.A.T. was previously explored in hibernating bat *Myotis lucifugus* (Krutzsch & Wells 1960). In this study, non-castrated rats were treated with a fraction (nonsaponifiable) of the interscapular B.A.T. of *M. lucifugus*. This treatment promoted evident seminal vesicle hypertrophy. Furthermore, the authors suggested that 1 g of this fraction corresponded to 676 µg of testosterone, indicating a high androgenic activity of the B.A.T. (Krutzsch & Wells 1960).

To observe if the yellowish myotis B.A.T. influenced testosterone production, we performed a xenograft with this tissue for the first time. The recipient mice consumed the B.A.T. during the 2 months of grafting. Consequently, there was an increase in the mice's seminal vesicle weight (two times higher) and serum testosterone levels (six times higher). These data confirmed the androgenic role of the yellowish myotis B.A.T.. Furthermore, yellowish myotis' consumption of B.A.T. coincided with the spermatogonia differentiation, suggesting that this organ may be linked to germ cell development (Fig. 8). Future molecular studies

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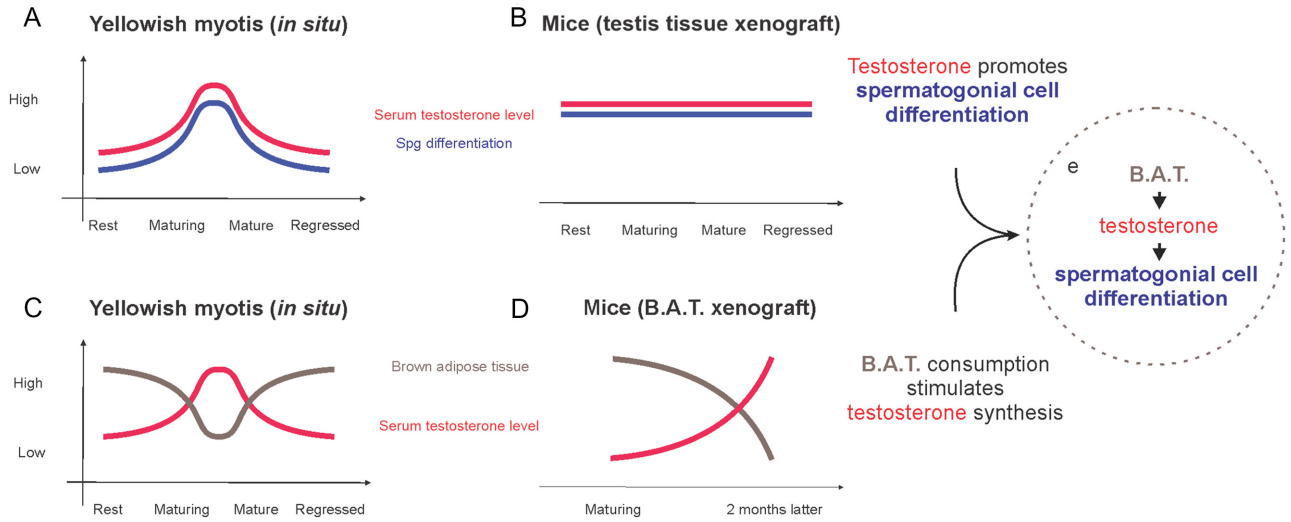

Figure 8 Suggested relationship between brown adipose tissue and spermatogonial differentiation in yellowish myotis. (A) Increased testosterone serum levels promoted spermatogonial differentiation in yellowish myotis (*in situ*). (B) The stability of testosterone serum levels in recipient mice allowed the spermatogonial differentiation in testis tissue xenograft of all reproductive stages. (C) The consumption of the brown adipose tissue (B.A.T.) in yellowish myotis (*in situ*) coincided with the higher testosterone production. (D) The brown adipose tissue xenograft promoted massive testosterone production in immunodeficient mice, confirming its androgenic function. (E) Suggested link between brown adipose tissue and spermatogonial differentiation in yellowish myotis testis.

Table 4 Morphological aspects of testis tissue xenografting in mature wild mammal species.

Xenograft development	Species and age	Reference
Degeneration	Iberian lynx (2 years old), Rhesus monkey (11 and 12 years old)	Arregui *et al.* 2008b, 2014
Sertoli cell only	Rhesus monkey (11 and 12 years old)	Arregui *et al.* 2008b
Spermatocytes	Djungarian hamster, Rhesus monkey (6 years old)	Schlatt *et al.* 2002, Arregui *et al.* 2008b
Round spermatids	Yellowish myotis	Present study
Elongated spermatids	Rhesus monkey (3 years old)	Arregui *et al.* 2008b

must investigate if the Regressed and Rest stages promote undifferentiated spermatogonia expansion before the serum testosterone peak.

According to the 3-Beta-HSD immunostaining and pixel intensity, the B.A.T., directly or indirectly, stimulated the Leydig cell steroidogenic activity. The more robust 3-Beta-HSD immunolabeling pattern was previously demonstrated in yellowish myotis (*in situ*) during B.A.T. consumption (Farias *et al.* 2020). While B.A.T. contributes to heat production during the arousal from hibernation in temperate-zone bats (Smalley & Dryer 1963, Hayward & Ball 1966, Lyman 1970), it is linked to reproduction in Neotropical yellowish myotis, possessing an essential androgenic function. In future studies, we need to investigate the molecular via related to the B.A.T. products to elucidate if they stimulate the hypothalamic–pituitary–gonadal axis (indirect action) or testis parenchyma (direct action).

In conclusion, we observed that the low metabolic status of the testis fragment (Rest stage) is more significant for the success of testis tissue xenografts than the animal's age. Furthermore, the stable endocrine milieu of mice is sufficient for the androgenic support and to generate elongating spermatids of yellowish myotis 7 months after grafting. After the B.A.T. transplantation, we observed a powerful stimulus in the mice's testosterone production. This finding confirmed what was previously speculated for yellowish myotis reproductive physiology, i.e. in the Mature stage of this species, the B.A.T. reaches the smallest size along with the highest serum testosterone levels (Farias *et al.* 2020). These data reinforce that the hypothalamic–pituitary–gonad axis and the B.A.T. precisely regulate the gonad function in yellowish myotis. In general, we can say that the xenograft experiments can elucidate the physiology of wild animals, especially those that are challenging to maintain in captivity.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding
This work was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES), the National Council for Scientific and Technological Development (CNPq), and the Foundation to Support Research of the State of Minas Gerais (FAPEMIG -APQ-01078-21).

Author contribution statement
T.O.F., S.A.T, and G.M.J.C. planned the experiments. T.O.F and G.M.J.C captured the animals, performed the experiments, and wrote the paper. A.F.A.F and N.T.W did the mouse IHC analyzes. A.F.A.F, S.A.T., and N.T.W. did a critical revision of the manuscript. T.O.F. and G.M.J.C. approved the final version of the paper.

Acknowledgements
The support from Image Acquisition and Processing Center (CAPI- ICB/UFMG) were of great importance. The authors thank the Brazilian Chico Mendes Institute for Biodiversity Conservation (ICMBIO) for providing the licence to capture the animals and the staff of the Reserva Particular do Patrimônio Natural Santuário do Caraça for allowing us to collect the bats. The authors also thank Mara Lívia dos Santos, Fr. Lauro Palú, and Dr Aline Abreu for their scientific, technical, and logistical assistance.

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Received 19 May 2022
Received in final form 24 October 2022
Accepted 4 November 2022
Accepted Manuscript published online 4 November 2022