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Research Article

Intraspecific chromosome polymorphisms can lead to reproductive isolation and speciation: an example in red brocket deer (*Mazama americana*)

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Abstract

The red brocket (*Mazama americana*) is a South American deer with a wide geographical distribution that presents different chromosomal variants depending on their location. At least six different cytotypes belonging to two distinct evolutionary lineages have been described. This study aimed to verify the existence of postzygotic reproductive isolation between cytotypes of *M. americana* by comparative evaluation of pure and hybrid males. Seven 18-month-old bucks were submitted to seminal collection and evaluation and testicle histological evaluations. The pure males showed normal parameters for sperm quality and testicular histology. Hybrids from the same evolutionary lineage ($\leq$3 chromosomes different from the progenitors) showed similar results to pure males, except for the reduced ratio of round spermatids to pachytene spermatocytes. Hybrids between cytotypes of different evolutionary lineages ($\geq$10 chromosomes different from progenitors) presented azoospermia and evidence of testicular degeneration. Despite the striking morphological similarities, we can conclude that populations with more distinct karyotypes possess an effective reproductive barrier; moreover, there is evidence that reproductive isolation mechanisms exist between some closer karyotypes, corroborating the hypothesis that *M. americana* is best characterized as a superspecies. Thus, the future description of several new species for this taxon is expected, since the tendency is to establish efficient mechanisms of postzygotic reproductive isolation, preventing the introgression and fusion of genomes from different populations through chromosome variation.

Summary Sentence

Differences equal to or greater than seven chromosomes produce an effective reproductive isolation between red brocket deer cytotypes and a depression in fertility occurs among karyotypes with at least two chromosome diferences.

Key words: cervidae, genetics, chromosomes, evolution, meiosis, fertility.

Introduction

According to Weber and Gonzalez [1], the red brocket (*Mazama americana*) is the deer with the largest geographical distribution in Latin America, since it is found from southern Mexico, throughout Central America and up to northern Argentina in South America. Morphologically, individuals of this species are very similar [2]; however, there are numerous reports of the existence of different chromosome numbers among its populations. The first cytogenetic analysis in *M. americana* was conducted by Jorge and Benirschke [3], who described the karyotype of the subspecies *M. americana temama* from Tamaulipas, Mexico, as 2n = 49/50 and FN (fundamental number) = 72. In a second karyotype analysis, Neitzel [4] identified a different pattern following analysis of a female deer from Paraguay with 2n = 52 and FN = 56, plus four to five supernumerary chromosomes (B chromosomes). Neitzel [4] argued that while the subfamily Cervinae presents karyotype divergence exclusively through Robertsonian translocations, tandem fusions predominate in the genera *Mazama* and *Muntiacus*.

In Brazil, Duarte [5] reported the same conflicts in chromosomal pattern following an analysis of four specimens. To achieve a clearer understanding of these variations, Duarte and Jorge [6] analyzed the karyotypes of 33 *M. americana* from several locations in Brazil, identifying extensive polymorphism, 2n = 42–53 and FN = 48–57, and reporting that some karyotypes were characteristic of certain regions. By 1997, Duarte and Merino [7] were already proposing the existence of multiple species within *M. americana* based on this chromosomal polymorphism. In 2008, Duarte et al. [34] described high levels of molecular and cytogenetic differentiation in this species, reporting it as one of the most surprising cases of convergent morphological evolution and of cryptic species in mammals.

In 2010, Abril et al. [8] proposed a phylogenetic tree based on molecular biology and cytogenetics. They described two lineages of karyotypes that diverge from a common ancestor (2n = 52/53; FN = 54): one lineage composed of deer with low diploid numbers (Juína cytotype: 2n = 44/45, FN = 48; Rondônia cytotype: 2n = 42/43, FN = 46); and another with populations with high diploid numbers (Paraná cytotype: 2n = 52/53, FN = 56; Santarém cytotype: 2n = 50/51, FN = 56; Jari cytotype: 2n = 48/48, FN = 56; and Carajás cytotype: 2n = 50/51, FN = 54). They also affirmed that the degree of genetic differentiation between these lineages is greater than between different species of the genus *Mazama*, corroborating the hypothesis that each lineage is a different species.

It is believed that the cumulative occurrence of centric fusions alone is capable of reproductively isolating two species [9]. Some studies have shown that in cases where chromosomal rearrangements occur, genetic and/or morphological differences are not sufficient to identify distinct species, even though they become reproductively isolated [10–13]. According to the biological concept, species are groups of natural populations that interbreed and are reproductively isolated from other groups [14]; hence, the sterility or subfertility of hybrids is regarded as an important rule in the differentiation and maintenance of distinct species [15]. However, little is known concerning the level of chromosomal differentiation that leads to the reproductive isolation of populations.

The purpose of this study was to verify the existence of postzygotic reproductive isolation between chromosomal variants of *M. americana* by assessing the fertility of pure deer (progenitors with the same chromosome constitution) and hybrids (progenitors with different chromosomal constitutions).

Materials and methods

Ethics committee

This research was conducted following the agreement and approval of the Ethics Committee on Animal Use (CEUA) of the Faculty of Agricultural and Veterinary Sciences of São Paulo State University (FCAV-UNESP), Jaboticabal campus, under protocol no. 000834–09.

Animals

To achieve the mating that produced the male deer studied herein, we used three males and six females obtained from legal breeding centers and zoos that were originally captured in the wild from several Brazilian regions and for whom cytogenetic analysis had been performed. The chromosomal characterization of the progenitors of each deer analyzed in this work is presented in Table 1. The mating between individuals with same cytotypes produced two males (pure, P: P1 and P2) and the mating between individuals with different karyotypes produced five males (hybrids, H: H1 to H5) with differences between the progenitors ranging from one to six chromosomal rearrangements (Table 1).

Restraint and material collection

From 6 to 18 months of age, the male deer were maintained in individual stalls and provided with the same diet and water ad libitum. The individuals were exposed to natural light fluctuations. At 18 months old, the deer were anaesthetized using the protocol described by Pinho et al. [16]. Following chemical restraint, the deer were submitted to semen collection by electroejaculation (eletroejaculator P-T Electronics 11241 SE 362°—Boring, OR 97009, USA) according to the protocol described by Duarte and Garcia [17]. After semen collection, the deer were submitted to unilateral orchiectomy for histological evaluation.

Reproductive evaluation

Semen evaluation

Semen was collected in 15 mL graduated conical tubes and the volume was recorded. Next, a 10-μL aliquot was removed and fixed in saline formaldehyde solution (1:200 v/v) to evaluate its concentration in a Neubauer chamber (spermatozoa/mL), together with the morphology and morphometry of the spermatozoa. The remainder of each semen sample was diluted in a Tris-egg yolk medium [18] to evaluate individual motility (%) and vigor under a light microscope at 400× magnification, in duplicate (two evaluators).

The evaluation of sperm morphology was performed using the wet preparation method under phase contrast microscopy, according to the classification proposed by Blom [19], in which defects resulting from primary testicular problems or secondary problems resulting from inadequate maturation are described. When a sperm cell presented more than one defect, the primary problem was considered most important.

For the morphometric evaluations, one drop of fixed semen was smeared over a microscope slide and allowed to dry in air for 10 min, after which it was stained with Giemsa. The stained slides were examined under a light microscope (Leica DM 5000 B®) and 100 random sperm per individual were measured. The length, largest and smallest width of the sperm head were measured under immer-

sion (1000×), and then the area was calculated using the Folium of Descartes, as proposed by van Duijn [20].

Histology

The testicular sections were processed according to Luna [21]. Briefly, 5 μm thick histological sections were stained with hematoxylin and eosin. Histological analysis was performed using a light microscope (Leica DM 5000 B), and 60 cross sections of seminiferous tubules were measured per testicle, as recommended by Berndtson and Picket [22].

The mean diameter of the seminiferous tubules was obtained from 10 cross sections of tubules that were more circular, regardless of the stage of the seminiferous epithelium cycle and in same tubules cross sections, the height of the epithelium (distance considered was from basal membrane to the luminal border) was measured using the Image J software®. The population (in 10 cross sections) of the sperm cell lineage was quantified, and the ratio between round spermatids and pachytene spermatocytes (spermatid-to-spermatocyte ratio, SSR) was calculated to determine the normality of meiosis II progression, as suggested by Turner et al. [23]. The morphology of spermatic cells was analyzed based in nuclear and cytoplasm shape as proposed by Roosen-Runge [24].

Statistical analysis

The results of the morphometric evaluations of the sperm, histological measurements (tubule diameter and epithelium height) and percentage of intratubular cells were submitted to the Anderson-Darling normality test. The diameter of the seminiferous tubules showed normal distribution, while the height of the germinal epithelium showed lognormal distribution; thus, they were submitted to the Tukey test.

The cell populations of the seminiferous tubules were compared using the nonparametric Bonferroni test, and spermatozoon morphometry was compared using the nonparametric Dunn test. Differences less than or equal to 0.05 ($P \leq 0.05$) were considered significant in all tests and were presented as the mean, standard deviation.

All the statistical processing was performed using GraphPad Prism software Inc. (version 5.01; GraphPad software Inc., San Diego, USA).

Results

Semen evaluation

The semen parameters are presented in Table 2. All individuals in the present study had a low semen volume (100–320 μL). Despite that, the sperm concentration of P1 and P2 was greater than H1, H2, and H3. Notably, hybrids H4 and H5 were azoospermic. In addition, a greater number of sperm defects (Table 3) were observed. In general, most of the defects were identified in the flagellum (P1, P2, H1, and H3); however, H2 showed a greater proportion of midpiece defects.

The sperm cell morphometry (Table 4) showed a negative correlation between smallest head width and the number of chromosomes. Individuals with lower chromosome numbers presented higher values for sperm head width, while those with higher chromosome numbers presented the lowest values, with P2 presenting the lowest value of all.

Testicular histology evaluation

The pure males, P1 and P2, presented greater mean values both for seminiferous tubule diameter (Figure 1A) and for seminiferous

Table 1. Characterization of experimental animals and their respective progenitors.

Deer analyzed	Progenitors	Progenitor chromosomal constitution*						>Difference between progenitors
		2n	FN	Group A*	Group C*	Group D*	Group E*	
P1	T251^{M}	45	48	02	00	08	32	–
	T248^{F}	44	48	02	00	08	32	
P2	T205^{M}	53	56	02	00	06	42	–
	T255^{F}	52	56	02	00	06	42	
H1	T251^{M}	45	48	02	00	08	32	One tandem fusion; Two pericentric
	T236^{F}	42	48	02	02	07	29	inversions in heterozygous
H2	T251^{M}	45	48	02	00	08	32	One tandem fusion; two pericentric inversions
	T211^{F}	42	48	02	02	08	28	(one in homozygosis, the other in heterozygosis)
H3	T260^{M}	51	56	04	00	04	40	Two centric fusions
	T256^{F}	52	56	02	00	06	42	
H4	T251^{M}	45	48	02	00	08	32	Three tandem fusions; one centric
	T257^{F}	52	56	02	00	06	42	fusion; one pericentric inversion
H5	T269^{M}	42	46	02	01	06	26	Four tandem fusions; one centric
	T255^{F}	52	56	02	00	06	42	fusion; one pericentric inversion

P = pure; H = hybrid; M = male; F = female; 2n = diploid number; FN = fundamental number.

*The chromosomes were classified as proposed by Abril and Duarte [37] according to their arms ratio and were organized into groups according to their relative lengths (RL): A (biarmed chromosomes with RL > 2.5%), C (biarmed chromosomes with RL < 2.5%), D (acrocentric chromosomes with RL > 3.0%), E (acrocentric chromosomes with RL < 3.0%).

Table 2. Semen parameters of pure and hybrid red brocket deer (*Mazama americana*) offspring.

Animal	Volume (μL)	Concentration (Sptz $\times 10^9$/mL)	Motility (%)	Vigor (0–5)	Defects (%)		Normal Spermatozoa (%)
					Primary	Secondary	
P1	110	3.59	80	3	20.0	10.0	70.0
P2	160	4.03	75	4	20.0	10.0	70.0
H1	220	2.10	70	3	3.0	30.0	67.0
H2	320	2.33	80	3	54.0	7.5	38.5
H3	200	2.08	55	2	29.0	13.0	58.0
H4	100	0.00	–	–	–	–	–
H5	200	0.00	–	–	–	–	–

Table 3. Percentage of sperm morphological defects in red brocket deer (*Mazama americana*) offspring.

Animal	Sperm Defects (%)					
	Flagellum	Cytoplasmic droplets	Head	Midpiece	Acrosome	Absence of defects
P1	15.0	2.5	2.0	10.5	0.0	70.0
P2	14.5	5.5	5.0	5.0	0.0	70.0
H1	30.0	1.0	1.5	0.5	0.0	67.0
H2	6.0	1.5	6.0	48.0	0.0	38.5
H3	20.0	8.0	7.0	6.0	1.0	58.0
H4	–	–	–	–	–	–
H5	–	–	–	–	–	–

Table 4. Mean (standard deviation) of the sperm micromorphometry of red brocket deer (*Mazama americana*) offspring.

Animal	Length (μm)	Largest width (μm)	Smallest width (μm)	Area* (μm^2)	Diploid number
P1	7.82 ± 0.56	4.22 ± 0.22	2.37 ± 0.28	27.23 ± 2.86	45
P2	7.85 ± 0.29	4.32 ± 0.27	1.67 ± 0.25	27.10 ± 2.26	53
H1	7.87 ± 0.35	4.31 ± 0.19	2.52 ± 0.32	28.03 ± 1.91	44
H2	7.17 ± 0.28	4.12 ± 0.27	2.06 ± 0.30	24.09 ± 1.95	44
H3	8.61 ± 0.52	4.63 ± 0.50	1.91 ± 0.53	32.12 ± 5.27	47
H4	–	–	–	–	49
H5	–	–	–	–	47

*Area calculated using the Folium of Descartes, as proposed by van Duijn [20].

epithelium height (Figure 1B) compared with hybrid males (H1–H5) ($P < 0.05$).

Table 5 presents the mean and standard deviation of the percentage of cell types presented in the seminiferous epithelium of the offspring of *M. americana*. The pure males presented similarities in the proportions of each cell types. However, the hybrids H1, H2, and H3 were similar to each other and differed from H4 and H5, which showed a reduction in later cell types, i.e., the late stages of the second meiotic division producing rounded spermatids (Figure 2).

When calculating the rate of meiotic II progression (SSR), P1, P2, H1, and H2 obtained a ratio of 4.0 or more round spermatids for each pachytene spermatocyte, H3 obtained a ratio of 1.50, and H4 and H5 showed a meiotic arrest in spermatocyte stage (SSR = 0.00).

Histological evaluation of the testes of H4 and H5 showed several germ cell degeneration with condensed pyknotic nuclei, making it impossible to identify the cell stage (Figure 3) and probably affecting the seminiferous epithelium which presented numerous sites of vacuolization.

Discussion

Deer fertility

Semen evaluation verified that the physical (seminal volume, concentration, motility, and vigor) and morphological semen parameters of pure males (P1 and P2) were generally better, followed by hybrid males H1, H2, and H3. Hybrid H4 and H5, the result of matings between progenitors with large cytogenetic differences (seven and ten chromosomes, respectively), were azoospermic.

Very little is known about semen characteristics of brocket deer and their fertilizing potential; however, Favoretto et al. [18] reported similar values for semen volume (365.3 ± 120.5 μL) and sperm concentration (2.7 ± 0.8 × 10^9 sptz/mL) and Rola et al. [25] reported similar values for motility (69.6 ± 8.9%) and vigor (3.5 ± 0.5). There are several reports in the literature of hybrid mammals that possess the capacity to produce sperm, including hybrids between white-tailed deer (*Odocoileus virginianus*) and mule deer (*O. hemionus*) [26] and between cryptic species of common shrew (*Sorex araneus*) [27].

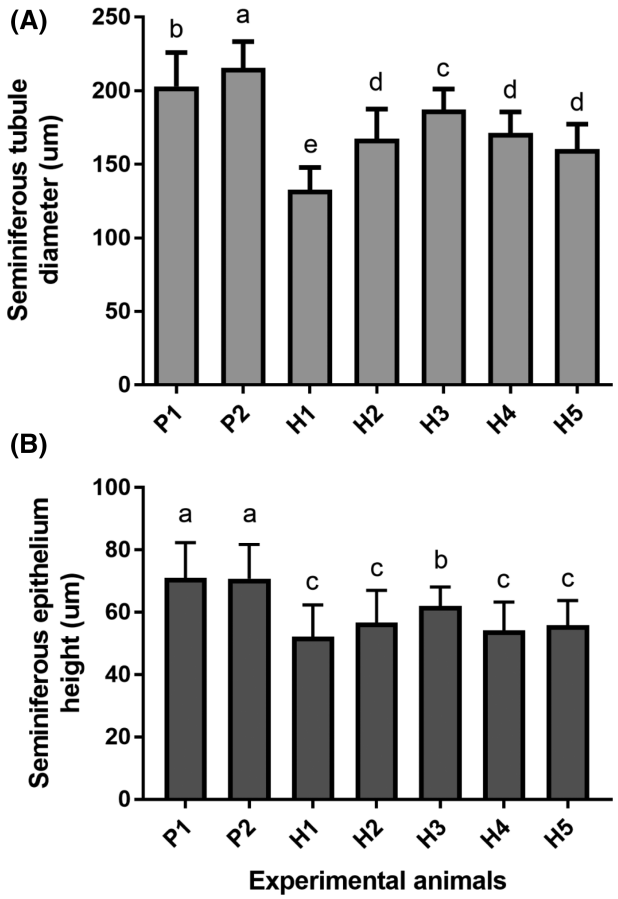

Figure 1. Morphometry of the seminiferous tubules (A, seminiferous tubule diameter; and B, seminiferous epithelium height) of red brocket deer (*Mazama americana*) offspring. Columns with the same letter do not differ statistically by the Tukey test ($P > 0.05$).

According to the classification of sperm defects proposed by Blom [19], the prevalence of sperm defects in our sample might be originated during spermatogenesis (primary defects), except for H1, where epididymal maturation seems to have had greater influence (secondary defects). Evaluation of sperm morphology showed that the most affected region was the flagellum, corroborating results obtained by Rola et al. [25], again except for H1, which presented the largest number of defects in the midpiece.

Analysis of sperm head morphometry showed that the variation in the smallest head width (Table 4) was related to the number of chromosomes. According to Beatty and Fechheimer [28], the shape (morphometry) of the sperm head is fundamentally controlled by genetics. Turner et al. [23] observed changes in the shape of the sperm head as a consequence of the hybridization process when studying the fertility of matings between *Mus musculus muluscus* and *Mus musculus domesticus*, while Wishart et al. [26] made similar observations researching hybrids between white-tailed deer (*Odocoileus virginianus*) and mule deer (*O. hemionus*).

The evaluation of hybrid sterility compared with pure male was based on the results of testicular histology. Several pathologies were verified, particularly in the seminiferous epithelium of the azoospermic males (H4 and H5), which presented vacuolization in Sertoli cell and pyknosis of germ cells suggestive of apoptosis (Figures 2 and 3). The azoospermic hybrids showed blockage of meiosis before spermatocyte II stage, maybe during the pachytene phase, with depletion of spermatids (spermatocyte II) and the consequent atrophy of tubular epithelium. To a lesser extent, Tuner et al. [23] reported similar results in house mouse (*Mus musculus*) hybrids.

These failures, the result of problems in homologous pairings, often occur in the pachytene, which is the prophase I stage, in which chromosomes are completely aligned with their homologs in a process called synapse [29].

The pure males P1 and P2 and hybrids H1 and H2 showed a ratio of four round spermatids to each spermatocyte I. In hybrid male H3, however, this ratio decreased to 1.5 (on average), even though its progenitors only presented a difference of one centric fusion. This finding highlights the crucial role that chromosomes play in reproductive isolation, even with small rearrangements. This was further emphasized when the karyotype differences were larger, as occurred with the progenitors of hybrids H4 and H5, which caused a blockage during the pachytene phase and characterized an effective reproductive barrier between these chromosomal variants.

In general, the reduction in tubule diameter and height of the epithelium appears to be a basic characteristic of hybrids, as evidenced in donkeys [30], mice [10] and in this study. This decrease is probably related to the failure of spermatogenesis, since in the studies cited above, all the hybrid animals showed reductions in the seminiferous tubules and height of the germinal epithelium and were unable to complete the spermatogenesis process, corroborating the results obtained for the azoospermic red brocket deer hybrids H4 and H5.

Table 5. Mean (standard deviation) of the percentages of cell types of the seminiferous epithelium of red brocket deer (*Mazama americana*) offspring.

Animal	Spermatogonia A (%)	Spermatogonia B (%)	Leptotenes/zygotenes (%)	Pachytenes (%)	Rounded Spermatids (%)	Sertoli cells (%)	SSR*
P1	16.99 ± 3.21	17.64 ± 2.08	15.38 ± 2.24	8.54 ± 1.79	34.43 ± 3.86	7.02 ± 1.13	4.03
P2	17.89 ± 2.89	17.09 ± 2.20	15.56 ± 2.09	7.95 ± 1.46	34.47 ± 3.99	7.05 ± 0.86	4.34
H1	9.73 ± 3.03	12.99 ± 3.75	14.233 ± 0.89	10.44 ± 1.43	42.33 ± 4.50	10.28 ± 2.19	4.05
H2	11.36 ± 3.08	11.36 ± 2.82	12.40 ± 2.50	10.70 ± 3.67	46.83 ± 6.31	7.35 ± 1.72	4.38
H3	10.84 ± 1.53	12.82 ± 2.14	19.96 ± 6.35	18.71 ± 5.27	28.04 ± 8.30	9.62 ± 0.93	1.50
H4	26.95 ± 5.94	29.89 ± 7.38	23.08 ± 13.60	0.79 ± 1.36	0.00	19.29 ± 3.19	0.00
H5	28.49 + 4.71	29.89 + 5.55	21.26 + 7.27	1.99 + 2.72	0.00	18.36 ± 2.53	0.00

*SSR, spermatid-to-spermatocyte ratio: number of spermatids for each pachytene spermatocyte.

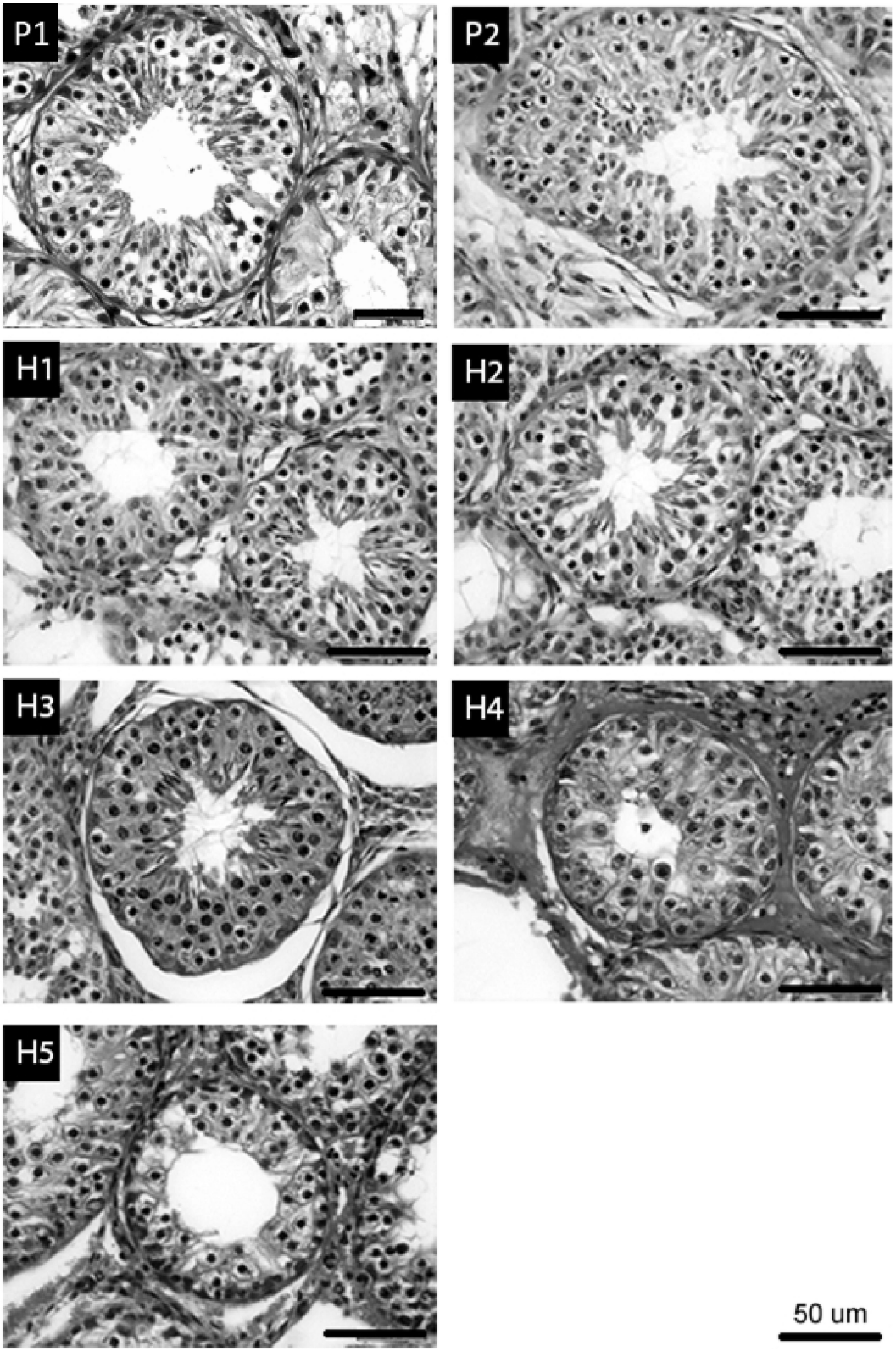

Figure 2. Photomicrographs of the seminiferous tubules of red brocket deer (*Mazama americana*) offspring.

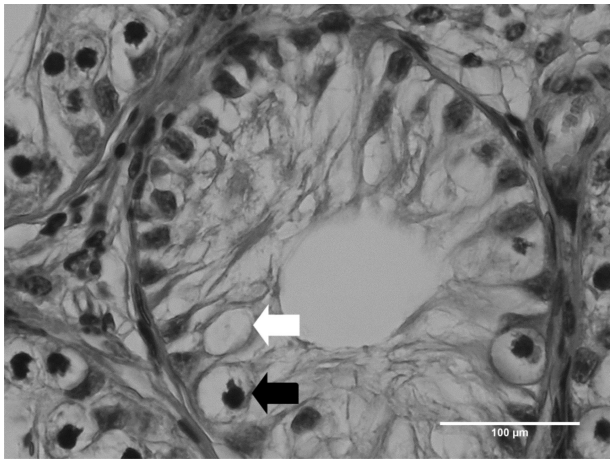

Figure 3. Photomicrograph of a seminiferous tubule of hybrid male H5 showing vacuolation of the epithelium (white arrow) and unidentifiable cells with condensed pyknotic nuclei (black arrow).

In our experiment, the hybrid males H1, H2, and H3 showed tubular morphology similar to the pure males P1 and P2, which confirms that they were able to conclude spermatogenesis, even if only partially.

The role of chromosomes in the fertility of hybrid mammals and speciation

Sterility is commonly observed in hybrids, resulting from the chromosomal or genic incompatibility of the progenitors [29]. However, depending on the chromosomal rearrangements or genic incompatibilities between the progenitors, the sterility of the offspring can be subdued and subfertility may not be detected. The species involved begin to form a hybrid zone, in which this loss of fertility or viability is important for species maintenance, differentiation and/or adaptation (Barton 31]).

For Dobigny et al. [9], only the cumulative occurrence of multiple chromosomal rearrangements, such as centric fusions (Robertsonian), is capable of reproductively isolating two species. In agreement with this statement, red brocket hybrid males only presented sterility when the differences between the progenitors included several chromosomal rearrangements. Regarding the progenitors of H4, the father possessed seven chromosomes less than the mother, because its karyotype had accumulated three tandem fusions, one pericentric inversion, and a centric fusion. As for H5, besides the rearrangements cited above, the father presented one more tandem fusion than the father of H4. All these karyotypic differences between the progenitors led to the generation of sterile (azoospermic) progeny due to pachytene blockage during spermatogenesis.

Progenitors that presented minor differences between them with regard to the number of chromosomes generated males with subfertility (H1, H2, and H3) compared with pure males. This was most evident for hybrid male H3, whose progenitors differed by only one rearrangement (centric fusion) that generated an important reduction in the round spermatid rate per cell in pachytene (SSR = 1.5).

Works in the literature indicate that tandem fusions and inversions can have a more significant effect on fertility [32]; however, this was not verified herein. The hybrids H1 and H2, which presented these rearrangements in heterozygosis resulting from the differences between their progenitors, showed no significant compromise in semen qualities or testicular histology. In a study similar to this one [33], analyzing *M. americana* females from matings between the same progenitors used in this study, postzygotic reproductive isolation was observed between deer with more distant karyotypes. Even female progeny from small chromosomal differences showed potential subfertility, evidenced by ovarian functionality and histology.

The results obtained from males in this study provide support for the claim that the mating between individuals of *M. americana* from different chromosomal constitutions leads to sterile or subfertile offsprings, confirming that an effective barrier of postzygotic reproductive isolation exists.

In the case of red brocket deer, the description of hybrids H4 and H5 as azoospermic supports the statement that there are at least two karyotypically distinct species, one with a high chromosome number (2n = 52–53) and another with a low number (2n = 42–45). However, the results obtained for the spermatogenesis rates of hybrids H1, H2, and H3 provide some evidence of an isolation mechanism that is not fully effective between progenitors with small karyotypic differences.

This work thus corroborates the hypothesis of the existence of several species within the taxon currently grouped as *M. americana* [8,33,34]. Despite this, the marked morphological similarity between individual deer from different populations [2] could suggest the existence of a system of cryptic species for red brocket deer. This leads to errors in the identification of these animals and to ignorance of valid species, which could be threatened with extinction within the vast area that *M. americana* is known to occupy. An example of the problem can be observed in Figure 4, which shows the deer bred in this study and raised under the same conditions at 6 months of age. The morphological similarity between pure males of different cytotypes and among the hybrids is striking, leading to the discussion of the existence of a morphological convergence or a very recent speciation, grounded in chromosomal rearrangements.

Brocket deer are widely used as a food resource for a number of populations in South America [35] and they likely suffer strong predation pressures. This could lead to a decline in numerous populations, since overhunting has been described in several locations [36]. Knowledge of typical karyotypes of the different populations of red brocket deer will assist in developing protective policies for the "chromosomal populations" most threatened, thus preventing their extinction.

Therefore, it is essential to know the effect that chromosomal rearrangements have on reproductive isolation and, by extension, on speciation, in order to determine which chromosomal differences could produce reproductive isolation and, consequently, form new species. This study makes important advances on this theme, since it defined that a tandem or centric fusion alone was unable to produce hybrid infertility, and contrary to what might be expected, centric fusion showed a greater effect on reproductive isolation than tandem fusion for red brocket deer. The limitations of analyzing a small number of deer, due to the difficulty of reproductive management in captivity and the time required to obtain sexually mature F1, mean that multiple similar studies are required to determine the consequences of matings between progenitors with few chromosomal differences.

We conclude that an effective reproductive isolation mechanism exists between *M. americana* cytotypes with differences equal to or greater than seven chromosomes, and that a depression in fertility occurs among karyotypes with at least two chromosome differences. This suggests that chromosomal changes are involved in the underlying process of speciation in this taxon.

Figure 4. Male deer analyzed in this study at 6 months of age.

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