Experimental and Applied Acarology (2023) 89:131–140
https://doi.org/10.1007/s10493-022-00770-3

Natural levels of *Rhipicephalus microplus* infestation and *Anaplasma marginale* infection in Angus and Ultrablack calves

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Received: 18 August 2022 / Accepted: 10 December 2022 / Published online: 5 January 2023

Abstract
Infections by *Anaplasma marginale* and infestations by *Rhipicephalus microplus* occur endemically in Brazil, representing an obstacle to expanding the use of taurine breeds, which are more susceptible. In this study, the levels of infection by *A. marginale* and infestation by *R. microplus* were monitored in 31 calves that were either purebred or had a high degree of taurine blood: 17 Angus (100% taurine) and 14 Ultrablack (ca. 82% taurine and 18% Zebu). The animals were evaluated on 13 occasions at 12-day intervals. The levels of *A. marginale* infection were determined by quantification of DNA copy number (CN) by qPCR, and ticks were monitored by two methods: counting adult females (≥4.5 mm) and scoring the level of tick infestation considering all visible instars in the animals' bodies. No significant effects were observed between the means of CN of *A. marginale*, tick counts and scores among Angus and Ultrablack animals. The repeatability estimates for CN of *A. marginale*, tick counts and tick scores were 0.53, 0.12 and 0.16, respectively. The correlations between CN and tick counts and scores were close to zero, whereas the correlations between tick assessment methods were 0.57. The absence of differences between the two genetic groups indicates, under the conditions of the present study, that the low degree of Zebu blood did not influence the levels of infection by *A. marginale* or infestation by *R. microplus*. The results also suggest that the evaluation of the levels of infestation by ticks using scores can provide information closer to the real infestation rate considering that it uses all the visible instars of the parasites.

Keywords qPCR · Resistance · Breed · Correlations · Repeatability

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Introduction

Rhipicephalus microplus is a vector of cattle tick fever that comprises the anaplasmosis produced by the Rickettsiales *Anaplasma marginale*. These tick-borne pathogens cause considerable losses to Brazilian livestock breeders and represent an obstacle to the expanded use of taurine breeds due to their higher sensitivity to ticks and hemoparasites than Zebu breeds (Giglioti et al. 2018). The occurrence of these hemoparasites in bovines is largely dependent on the population dynamics of the tick, which needs favorable climatic conditions for its development. In Brazil, most areas have a tropical climate that favors the tick's occurrence, and consequently, cattle are constantly infected by tick fever agents. The direct damage caused by *R. microplus* infestations is a decrease in milk production and weight gain, mortality, leather damage, morbidity, cost of control and hemoparasites transmitted by this tick (Rodriguez-Vivas et al. 2018). Regarding bovine anaplasmosis, the main symptoms in its acute form are progressive hemolytic anemia associated with increased body temperature, weight loss, decreased milk production, abortion and, in severe cases, the death of the infected animal (Kocan et al. 2003).

The use of quantitative PCR (qPCR) has made it possible to quantify the level of infection by the main hemoparasites that cause tick-borne diseases by quantifying the DNA copy number (Carelli et al. 2007; Bilhassi et al. 2014; Giglioti et al. 2016; Giglioti et al. 2018). In a study using the Canchim breed of cattle (5/8 Charolais + 3/8 Zebu), Giglioti et al. (2018) found repeatabilities moderate–high for *A. marginale* DNA copy number and low–moderate for tick infestations, suggesting that it is possible to identify animals presenting the most resistant phenotype against these parasites.

Currently, in Brazil more than 80% of cattle raised for beef are of the Nellore breed (*Bos taurus indicus*; Zebu), which are adapted to tropical conditions (Ferraz and Felício, 2010). However, these breeds are known to be less productive when compared to taurine breeds (Rodrigues et al. 2017). Therefore, the cross is an alternative way to improve carcass characteristics and meat quality while maintaining the Zebu's adaptive characteristics (Piccoli et al. 2020). Ultrablack is a synthetic breed and has been used mainly in regions with warmer climates. The genetic composition of Ultrablack ranges from 12.5 to 87.5% Brangus, and the remainder is composed of Angus lineage (Waldrip, 2017). According to the International Brangus Breeders Association, the inclusion of a small percentage of *Bos indicus* in Ultrablack produces an animal with hair-slicking ability, increased environmental adaptability and improved reproductive performance in the resulting offspring. However, tick infestation and its associated diseases are significant drawbacks to improving beef cattle productivity in the tropics, mainly when purebred and crossbred taurine animals are used (Cavani et al. 2020). In view of the aspects discussed above, the present study aimed to evaluate the levels of infection by *A. marginale* and infestations by *R. microplus* in Ultrablack calves raised in tropical conditions in Brazil and compare them to Angus animals to verify whether the introduction of a small amount of Zebu blood can effectively improve resistance to these parasites.

Materials and methods

Experimental animals

In this study were used 31 calves from the farm located in José Bonifácio city, São Paulo, Brazil (21° 2′ 23″ S, 49° 41′ 28″ W) that belonged to two genetic groups: 17 Angus (100% taurine; 12 females and 5 males) and 14 Ultrablack (approximately 82% taurine and 18% Zebu; 6 females and 8 males), with an average age of 3 months at the start of the evaluations. Thirteen assessments were performed, with average intervals of 12 days (November 2021–April 2022). This region has already been monitored and was considered endemic for the occurrence of babesiosis (Bilhassi et al. 2014). During the experimental period, the calves remained in rotated paddocks containing coast-cross grass [*Cynodon dactylon* (L.) Pers]. The control of *R. microplus* was carried out every 21 days using fipronil pour-on (Topline), whereas the control of hemoparasites was carried out through clinical symptoms. This study was approved by the Ethics Committee on Animal Experimentation of the Instituto de Zootecnia (Protocol Nr. 328–2021).

Tick counts and blood sample collection

From each animal, all adult female ticks larger than 4.5 mm in diameter were counted on the left side of the body (Utech et al. 1978). In addition, tick infestation scores (IS) were assigned for different levels of tick infestation, based on all stages of parasite development, beyond the adult females. This analyzes followed the methodology proposed by Fraga et al. (2003) with modifications. Thus, animals with no visible instars were considered as IS = 0, whereas IS = 1 for animals with a low infestation (up to 20 ticks), IS = 2 for medium infestations (between 20 and 60 ticks), IS = 3 high infestation (60–100 ticks) and IS = 4 for very high infestation (>100 ticks). Simultaneously, blood samples were taken from the jugular vein using a vacuum system (Vacutainer, Becton Dickinson, Franklin Lakes, NJ, USA) containing EDTA. Approximately 403 samples (blood and tick counts) were evaluated.

DNA extraction

Blood samples from each animal were subjected to genomic DNA extraction using the Wizard Genomic DNA Purification Kit (cat. nr. A1620; Promega, Madison, WI, USA) using the protocol of Isolating Genomic DNA from 300 uL whole blood. The concentration and purity of the DNA samples were determined using a BioDrop spectrophotometer (BioDrop uLITE, Integrated Scientific Solutions, Walnut Creek, CA, USA). All DNA samples were diluted in TE buffer (Tris-EDTA pH 7.8) at a ratio of 1:4 (DNA:TE) and stored in a freezer at −20 °C until analysis.

qPCR assay

The absolute quantification of *A. marginale* DNA copy number (CN) was performed as described by Giglioti et al. (2019) with primers and probes that flank a 119-nucleotide fragment located in the gene encoding the major surface protein 1b (*msp1b*): forward: 5'-TGGATGAAAGCCTGGAGATG-3'; reverse: 5'-TGTTTCCAGACCTTCCCTA-

ACT-3'; probe FAM-5'-AAGGCCAGGCACAGATATCACAGG-3'-BHQ1. The qPCR reactions were carried out with a CFX Real-Time PCR Detection System from BioRad (BioRad, Hercules, CA, USA). The qPCR was conducted in a volume of 10 µL with 2µL 5x HOT FIREPol Probe qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 0.5 µL of each primer (10 µM), 1.0 µL of probe (2.5 µM), 4.0 µL of ultrapure water (Invitrogen, Waltham, MA, USA) and 2.0 µL of DNA sample. The thermocycler conditions were one step of 10 min at 95 °C followed by 40 cycles at 95 °C (denaturation) for 15 s and 60 °C (annealing/extension) for 1 min. The samples were analysed in duplicate, as were the positive and negative controls. The calibration curve for quantification of *A. marginale* DNA was constructed using synthetic DNA, gBlocks Gene Fragments (IDT, Coralville, IA, USA). The gBlocks fragment containing the target sequence of *A. marginale msp1b* was submitted to 10-fold serial dilutions (10^{-1} to 10^{-10}). The method used for serial dilutions of gBlocks, construction of the calibration curve and absolute quantification of the CN of *A. marginale* were performed as described by Giglioti et al. (2019).

Statistical analysis

The quantitative values from CN, counts and tick scores were transformed into $\log_{10}(n+1)$ to approximate normal distributions. The data analyses were described by Giglioti et al. (2018) and used two different models: (*i*) to estimate the repeatability and comparison of means and (*ii*) to estimate associations between variables (VA) (*A. marginale*, counts and tick score). Model (*i*) was applied to each variable separately and included repeated measurements of the same animal and the fixed effects evaluation (EV), genetic group (GG), sex and the interaction EV x GG, and the age effect was included as a co-variable. The first-order autoregressive structure (*AR(1)*) was assumed as the structure for the (co)variance matrix. Model (*ii*) was multivariate with repeated measurements in the same animal, including the fixed effects EV, GG, VA, sex and the interactions EV x GG and EV x GG x VA, and the age effect was included as a co-variable. This model used a structure of the (co) variance matrix of direct product structures (*UN@CS*) designed for multivariate repeated measures. The MIXED procedure of the SAS statistical package was used for the analyses. A p-value ≤ 0.05 was considered statistically significant for the two models.

Results

There was no significant difference in *A. marginale* CN ($p=0.42$) between the two genetic groups. The means and standard errors of the CN_{log10} for Angus and Ultrablack were 4.08 ± 0.15 and 4.27 ± 0.16, respectively. However, there was a significant effect ($p=0.0047$) of the interaction between genetic group and evaluation, which occurred in evaluations 1 and 4, with the CN mean higher for Angus and Ultrablack, respectively (Fig. 1). In the first evaluation, the positive frequencies of *A. marginale* for the Angus and Ultrablack groups were 88.2 and 84.6%, respectively. For the other evaluations (2–13), all animals of both genetic groups were positive for *A. marginale* (Fig. 1).

The tick counts and score means did not differ significantly ($p=0.1824$ for tick counts and $p=0.7585$ for score) between Angus and Ultrablack genetic groups. However, it was verified that the interaction between the genetic group and evaluation was significant

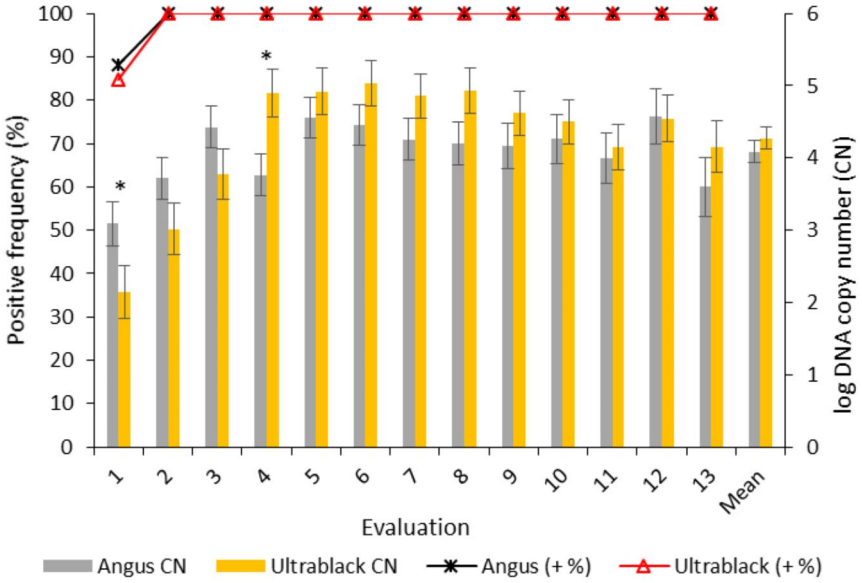

Fig. 1 Distribution of the average DNA copy numbers (CN) and positive frequencies of *Anaplasma marginale* in Angus and Ultrablack calves as determined by qPCR during 13 evaluations (November 2021–April 2022); *significant difference ($p < 0.05$) between two genetic groups

($p < 0.001$) for both counts and tick scores (Fig. 2a and b). Although these differences were found, they varied between the two genetic groups, with higher infestation rates for Angus in some evaluations and lower in others, or vice versa. These differences were more evident in the score measures where in three evaluations the infestations were higher for Ultrablack (3, 4 and 5), and the other three were higher for Angus (7, 9 and 12) (Fig. 2b). The estimated correlation between *A. marginale* with counts and tick scores was close to zero, –0.004 and –0.04, respectively, whereas the correlation between tick count and tick score was 0.57. The repeatabilities estimates for *A. marginale* CN, tick counts and tick score were 0.53, 0.12 and 0.16, respectively.

The dispersion distribution between tick counts and tick scores is shown in Fig. 3. It was observed that in all scores > 0, except 4, there were tick counts equal to zero (Fig. 3). The means and standard deviations of tick counts for scores 1, 2, 3 and 4 were 2.3 ± 2.2, 9.9 ± 6.6, 27.2 ± 20.5 and 88.0 ± 44.5, respectively.

Discussion

Using taurine breeds and their crossbreed is an alternative method to improve the meat quality of beef cattle herds in Brazil. However, the higher degree of taurine blood in the herds may increase the sensitivity to tick infestations and diseases transmitted by this vector. Hemoparasite infections and tick infestations limit growth in productivity when taurine breeds and their crosses are used in beef cattle breeding systems (Oliveira et al. 2013). Thus,

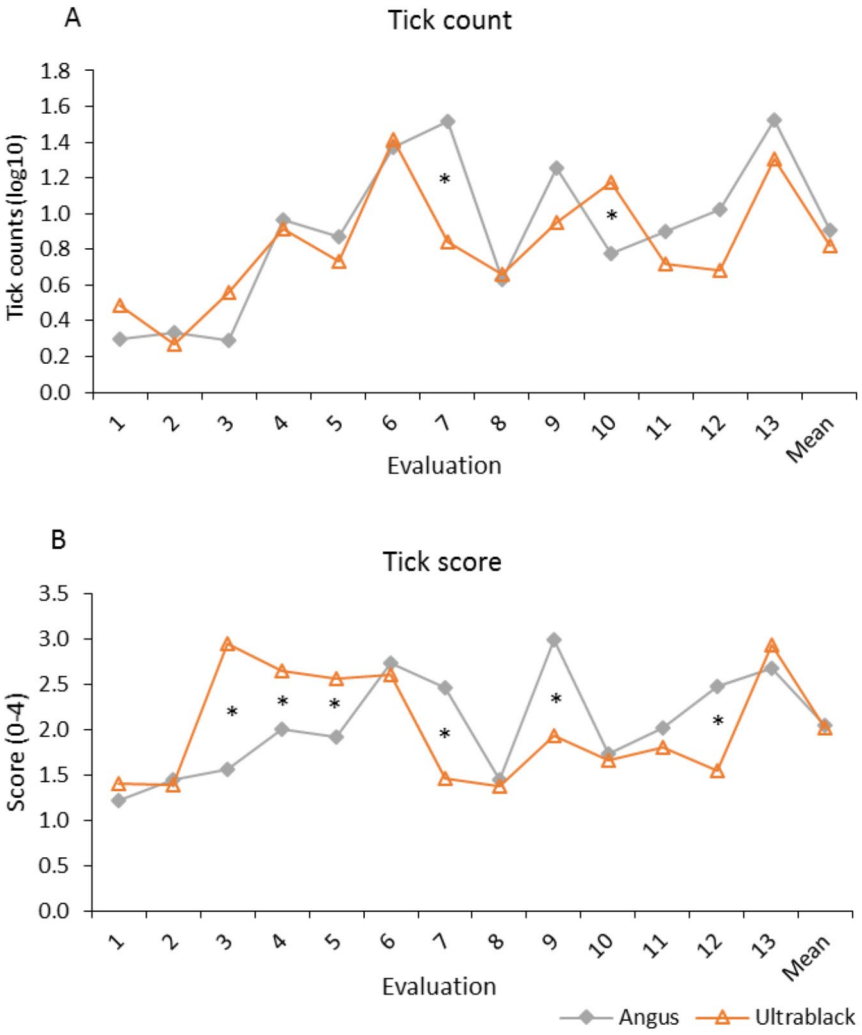

Fig. 2 Distribution of the average log tick counts (A) and tick scores (B) in Angus and Ultrablack calves during 13 evaluations (November 2021–April 2022); *significant difference ($p < 0.05$) between two genetic groups

the present study aimed to verify whether there are differences in susceptibility/resistance to *A. marginale* infections and *R. microplus* infestations between Angus and Ultrablack calves. The hypothesis is that a small proportion of Zebu blood in Ultrablack cattle could effectively reduce tick infestations and *A. marginale* infections in animals. Statistical analyses, however, did not show significant differences between the groups. Nevertheless, as expected, significant effects were found in the collection of CN of *A. marginale*, tick counts and scores. It is known that climatic conditions can influence the development of these parasites in pastures and therefore in the hosts (Jongejan and Uilenberg, 1994). Although no sig-

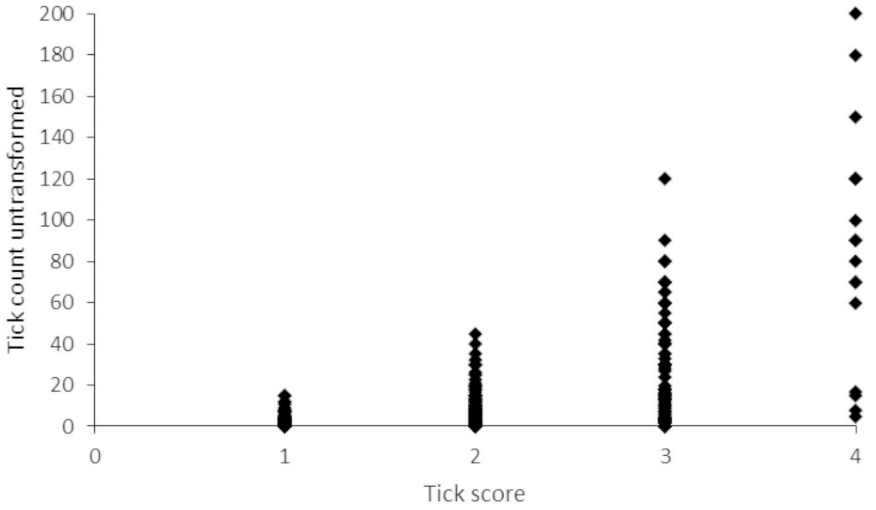

Fig. 3 Scatter plot correlation between tick counts (untransformed) and tick score (0–4)

nificant differences were found between the two genetic groups for the two tick measures, a significant effect of the interaction between the genetic group and evaluation was verified. These differences were more evident for the tick score measure, observed in six evaluations, whereas that for *A. marginale* CN and tick counts were verified only in two evaluations. Silva et al. (2010) studied natural infestations of *R. microplus* in four genetic groups (Nellore, Canchim x Nellore, Angus x Nellore and Simmental x Nellore) in different seasons throughout the year and found that the Nellore group had the lowest mean of tick counts, followed by its crossbreeds with Canchim, Angus and Senepol, respectively. However, the authors emphasized that the differences observed between the different genetic groups also depended on the year's effect and counting periods. The higher *B. indicus* resistance to *R. microplus* compared to *B. taurus* animals is already well established (Oliveira and Alencar, 1990; Wambura et al. 1998; Santos-Júnior et al. 2000; Silva et al. 2007, 2010; Oliveira et al. 2013; Andreotti et al. 2018; Martins et al. 2020).

Although there were no significant differences between the two genetic groups evaluated using two tick measures, we suggest that both measures are substantial. On the farm where the calves were evaluated, acaricide treatments were performed every 21 days. Consequently, in some evaluations and many animals, high tick scores were found (3 and 4) and low tick counts (even equal to zero). The advantage of using the tick score is the possibility of evaluating other life stages of the tick in addition to adult females (tick counts) such as the presence of semi-engorged females (less than 4.5 mm) and nymphs. Due to the greater sensitivity of taurine animals, acaricidal treatments tend to be more constant. In this case, using scores to assess tick burden may be an alternative to counting standard females. In addition, the correlation found between the two tick assessment measures is considered moderate (0.57). The absence of a strong correlation reinforces the use of both measures in tick assessments, contributing to better analysis accuracy.

The quantification of *A. marginale* CN showed no significant difference between the two genetic groups studied. In a similar study, Martins et al. (2020) compared the levels of *A. marginale* infection between Nellore and Brangus cattle and found that the Brangus group had six times more copies of *A. marginale* DNA than the Nellore group. Bilhassi et al. (2014) evaluated *B. bovis* infection in cattle from three different genetic groups (Angus, ½ Angus x ½ Nellore, and Nellore). They found similarities between Nellore and Angus x Nellore crosses concerning infection levels, indicating that the inheritance pattern includes heterotic effects. The absence of a difference in *A. marginale* CN between Angus and Ultrablack groups may be due to the small proportion of Zebu blood in Ultrablack cattle (about 18%), which may be insufficient to generate heterotic patterns as produced in Zebu-taurine half-blood cattle. As verified for two methods of tick evaluations, there was also a significant effect of the interaction between the genetic group and *A. marginale* CN evaluation. However, there were only significant differences in evaluations one and four, the first being greater for CN in Angus and the second greater in Ultrablack. In a similar study, Giglioti et al. (2018) evaluated *A. marginale* CN in Canchim females and found variation, corroborating the present study's findings. In addition, from the second evaluation, all animals of the two genetic groups were positive for *A. marginale*. Similar results to those obtained in the present study, which used qPCR to detect and quantify *A. marginale* infection, were found by Giglioti et al. (2018) and Martins et al. (2020), who found high positive frequencies. This high frequency of positives must be related to the high detection sensitivity of the qPCR assays and the high level of infection by *A. marginale* present in the animals because it is an endemic region for the occurrence of ticks and consequently for infections by *A. marginale*. The absence of a correlation between *A. marginale* CN and tick count found in the present study further reinforces what has already been established in other studies, which is that the variation of parasitemia by the hemoparasites does not depend on tick infestation levels (Giglioti et al. 2016, 2018). Thus, this study indicates it is not possible to use information on *R. microplus* infestations to predict the *A. marginale* infections levels, or vice-versa.

The estimated repeatability for the two tick evaluation measures was low, whereas the repeatability of *A. marginale* CN was high. Although both were low, the repeatability found for the tick score (0.16) was higher than that for the tick count (0.12). Our findings contrast with Fraga et al. (2003) who found repeatabilities for tick counts and scores of 0.29 and 0.21, respectively. Our results corroborate with studies that found low repeatability when studying natural infestations by *R. microplus* in Angus (Giglioti et al. 2016) and Canchim cattle (Giglioti et al. 2018). For *A. marginale* infections, the study conducted by Giglioti et al. (2018) was the first and so far the only study to estimate the repeatability of the *A. marginale* CN. The results found by these authors partially corroborate those found in the present study in which moderate and high repeatability were found, and they are related to the age of the animal. According to those authors, when repeatability is high, the genetic and permanent environmental factors intrinsic to each animal significantly influence the expression of the trait studied. In contrast, low repeatabilities indicate that the most important factor is the environment to which the animal is subjected at the time of measurement.

Conclusion

The evaluations of *R. microplus* infestations and *A. marginale* infections carried out in this study showed no differences in the level of these parasites between Angus and Ultrablack calves. These results suggest that the low proportion of Zebu blood in Ultrablack calves was insufficient to reduce the parasites' load. Furthermore, the absence of a correlation showed that higher tick infestations are not associated with higher *A. marginale* infection or vice versa. In addition, we also suggest using a tick score measurement to complement the counts, especially in taurine breeds, which are constantly treated with acaricide products.

Author contributions A.F.F. and R.G. conceptualization, analysis, investigation, methodology, project administration, software, supervision, validation, visualization, writing – review and editing. L.M.K., A.E.V.F., H.P., and C.J.V. conceptualization, investigation, project administration, supervision. L.C., M.B.S., and T.M.F. methodology, and project administration. H.N.O., and M.C.S.O. conceptualization, investigation, methodology, project administration, supervision, writing – review and editing.

Funding This research project was supported by the São Paulo State Research Support Foundation (FAPESP) (Grant #2019/22675-6, 2018/19452-2) and the Institute of Animal Science (APTA-SAA).

Competing interests The authors declare no competing interests.

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