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Effect of parasite dose and host age on the infection with *Besnoitia besnoiti* tachyzoites in cattle

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Abstract

Bovine besnoitiosis is continuing to spread in Europe. Therefore, the development of ruminant animal models of infection is urgently needed to evaluate therapeutic and prophylactic tools. Herein, we studied the effect of parasite dose and host age on the infection dynamics with *Besnoitia besnoiti* tachyzoites in cattle in two independent experimental infections. In experiment A, twelve 3-month-old male calves were inoculated intravenously with either three different doses of tachyzoites (G1: 10^8; G2: 10^7; G3: 10^6) or with PBS (G4). In experiment B, six 14-month-old bulls were inoculated with 10^6 tachyzoites based on results obtained in experiment A. In both trials, clinical signs compatible with acute and chronic besnoitiosis were monitored daily; blood and skin samples were collected regularly for 70–115 days post-infection (pi). Finally, animals were killed, and tissues were collected for lesion and parasite detections. Infected animals developed mild–moderate signs compatible with acute besnoitiosis. Lymphadenopathy and fever were observed in both calves (from 12 hr until 7 days pi) and bulls (from 6 days until 9 days pi). Seroconversion was detected at 16–19 days pi, and antibody levels remained high. Infected animals did not developed characteristic clinical signs and macroscopic lesions of chronic besnoitiosis. However, successfully, parasite-DNA was detected in a reduced number of target tissues: conjunctiva, ocular sclera, epididymis, skin of the scrotum and carpus in calves (n = 10, 6 of which belonged to G3), and pampiniform plexus and testicular parenchyma in bulls. Remarkably, one tissue cyst and mild microscopic lesions were also detected. In summary, inoculated animals developed the acute besnoitiosis and chronic infection was evidenced by microscopic findings. However, our results suggest that tachyzoite dose and host age are not key variables for inducing clinical signs and macroscopic lesions characteristic of chronic besnoitiosis. Thus, a further refinement of this model should evaluate other parasite- and host-dependent variables.

KEYWORDS
Besnoitia besnoiti, bull, calf, experimental infection, tachyzoites

1 | INTRODUCTION

Besnoitia besnoiti is a cyst-forming apicomplexan parasite responsible for bovine besnoitiosis. At present, the disease is considered to be re-emerging in Europe by the European Food Safety Authority due to an increased number of cases and the geographic expansion of besnoitiosis into cattle herds in several Western and Central European countries (Álvarez-García, 2016; European Food Safety Authority, 2010). The control and prevention of this disease relies only on diagnostic and management measures, as no chemotherapeutics are available and no vaccines are licensed in Europe (Álvarez-García, Frey, Mora, & Schares, 2013). Promising drugs have been tested in vitro (Cortes, Muller, Boykin, Stephens, & Hemphill, 2011; Jiménez-Meléndez et al., 2017). However, an appropriate ruminant experimental model has not been developed yet; this model is a requirement to test the safety and efficacy of these potential prophylactic and therapeutic tools.

In this regard, only *B. besnoiti* tachyzoite and bradyzoite stages have been used so far for the inoculation of animals. *Besnoitia besnoiti*-oocysts are not available as the definitive host remains unknown (Basso, Schares, Gollnick, Rutten, & Deplazes, 2011). Several laboratory rodents (hamster, gerbils and various strains of white mice) have been infected with *B. besnoiti* (Basso et al., 2011; Shkap, Pipano, & Greenblatt, 1987). Although gamma-interferon knockout mice are very susceptible to the acute infection (Schares et al., 2009), they are not recommended as an appropriate laboratory model due to the rapid onset of the acute stage and death. Rabbits showed susceptibility to experimental infection by developing clinical signs of the acute phase of the disease (Lienard et al., 2015), but the results were variable with respect to the chronic stage (Basson, McCully, & Bigalke, 1970; Bigalke, 1968). The disease was successfully replicated in these animals through subcutaneous inoculation of bradyzoites (6×10^6) (Lienard et al., 2015) resulting in mild clinical signs and the formation of cysts in the leg dermis, nasal mucosa, eyelid and penis. However, the authors suggested that the establishment of *B. besnoiti* and its adaptation to a heterologous host was difficult.

Between the 1960s and 1980s, there were a few attempts to establish a reproducible in vivo model for bovine besnoitiosis, but failed due to the difficulty in inducing the characteristic clinical signs (Álvarez-García, García-Lunar, Gutiérrez-Expósito, Shkap, & Ortega-Mora, 2014). Unfortunately, all the inoculations were carried out under different experimental conditions (e.g., different ages of the infected animals, unknown previous health status and use of immunosuppressive therapies). Moreover, the inocula were obtained from different sources, such as blood or tissue cysts from cattle with chronic besnoitiosis (Bigalke, 1967), blood from acutely infected rabbits (Basson et al., 1970) or from a blue wildebeest strain maintained in cell culture (Bigalke, Schoeman, & McCully, 1974). In infected animals, fever and lymphadenopathy have been detected on several occasions (Basson et al., 1970; Bigalke, 1968; Bigalke et al., 1974). However, specific clinical signs of the acute phase, such as oedema and anasarca, and of the chronic phase (tissues cysts) were only reported in infected adult animals (Basson et al., 1970), as well as in immunosuppressed animals that had been infected with bradyzoites by Diesing et al. (1988). This heterogeneity in the experimental assay parameters does not allow for the development of an experimental model of besnoitiosis in cattle. Additionally, most results have been based on clinical inspection and histopathology, as molecular tests were not available and serological tests were rarely employed, which is crucial as immunity to re-infections has been reported (Basson et al., 1970; Bigalke, 1968; Janitschke, De Vos, & Bigalke, 1984; Shkap et al., 1987).

Nevertheless, relevant information concerning pathogenesis has been derived from these trials. It is known that both tachyzoite and bradyzoite stages are infective for cattle (Bigalke, 1967, 1968; Diesing et al., 1988; Pols, 1960). We expect that the parasite dose is important in determining the severity of the infection. Moreover, the incubation period may depend on the route of infection. Information regarding age suggests that the disease is mainly present in older animals due to longer periods of exposure (Álvarez-García et al., 2014). However, the disease was recently confirmed in a 4-month-old calf (Diezma-Díaz et al., 2017), demonstrating that animals younger than 6 months old can also be chronically infected.

The aim of this study was to study the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle and their impact on both the acute and chronic phases of the disease. Accordingly, two independent experimental infections were carried out in calves and bulls. We addressed the limitations of previous trials by: (a) the employment of a well-characterized *B. besnoiti* isolate; (b) checking the viability and quality of the inoculum and (c) checking the animal health status prior to inoculations. Moreover, exhaustive clinical monitoring and regular samplings were carried out, employing complementary serological, histological and molecular tools.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All our experimental procedures were approved by the Animal Welfare Committee of the Community of Madrid, Spain, following proceedings described in Spanish and EU legislations (PROEX 92/14, Law 32/2007, R.D. 53/2013), and Council Directive 2010/63/EU.

Animals were housed in clinical facilities belonging to the Faculty of Veterinary Sciences of Complutense University of Madrid (Number of register: ES280790000101).

2.2 | Animals, experimental design, clinical monitoring and collection of samples

The experimental design followed in both trials is shown in Figure 1. The same clinical and laboratory parameters were evaluated with slight differences in their periodicity.

2.2.1 | Experiment A

The calves used in this study came from a single Holstein Friesian dairy herd located in Madrid province and were free from relevant infectious (tuberculosis and brucellosis) and parasitic diseases of dairy cattle. Twelve 2-month-old male calves were selected after assessing the absence of specific antibodies against *B. besnoiti* and other closely related sarcocystidae parasites (*Neospora caninum*, *Toxoplasma gondii* and *Sarcocystis* spp.) by Western blot. Prior to the inoculation and to guarantee the health status of the animals, the calves were vaccinated against Bovine Respiratory Syndrome (Cattle Master®). The absence of gastrointestinal parasites was checked by means of coprological analyses. Additionally, haematological and biochemical analyses were also carried out.

Calves were randomly allocated into four different groups of three animals each. Group 1 (G1) was inoculated intravenously with 10^8 tachyzoites, group 2 (G2) with 10^7 tachyzoites and group 3 (G3) with 10^6 tachyzoites by the same route of administration. Group 4 (G4), used as a non-infected control group, was inoculated with PBS and MARC-145 cells, where tachyzoites are routinely propagated (see Section 2.3 for the inoculum). Rectal temperatures and other clinical signs characteristic of either acute or chronic phase infections were monitored periodically along the experimental period (Figure 1). Any temperature above 39.5°C was considered as a fever. A clinical scoring system was established to classify the severity of the outcome of the infection (Table 1). The number of tissue cysts in the ocular conjunctiva was estimated and assigned to the following categories: 1: 1–10 cysts; 2: 11–20 cysts; 3: 21 to >30 cysts in both eyes (Gollnick, Scharr, Schares, & Langenmayer, 2015).

A criterion for the humanitarian slaughtering of the animals consisting of 1 day of total anorexia and prostration was established.

Blood samples were collected regularly until the end of the experiment by jugular venipuncture into 5-ml Vacutainer tubes (Becton Dickinson and Company, Plymouth, UK) with and without ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Haematological and biochemical parameters were assessed in blood samples at 7 days prior to infection and at 4, 7, 14 and 21 days post-infection (pi). For biochemical and serological analyses, the blood samples that were collected in vacutainers without anticoagulant were allowed to clot and were centrifuged (1,200× *g* for 10 min) to obtain serum samples (stored at −80°C until use). For haematological analyses and detection of parasitaemia, the blood samples that were collected in vacutainers containing EDTA were immediately processed to obtain peripheral blood mononuclear cells (PBMC).

Once a week, two skin biopsies from each animal were collected from the inner thigh using 6- to 8-mm-diameter biopsy punches to perform *B. besnoiti* ITS-1 PCR and histopathology. At the end of the

TABLE 1 Clinical score employed and based on the severity of the clinical signs compatible with acute and chronic phases of bovine besnoitiosis monitored throughout the experimental infections

Score	Category	Acute besnoitiosis	Chronic besnoitiosis
0	Absence of infection	None	None
1	Mild	Local lymphadenopathy	Systemic lymphadenopathy and/or congestive conjunctiva and/or conjunctival cysts (cat. 1) and/or ocular secretion[a]
2	Moderate	Fever and/or systemic lymphadenopathy and/or cough/nasal secretion and/or congestive conjunctiva[a]	Systemic lymphadenopathy and conjunctival cysts (cat. 2) and/or ocular secretion
3	Severe	Oedema, orchitis, lameness, anorexia	Systemic lymphadenopathy and conjunctival cysts (cat. 3) and/or ocular secretion and/or skin lesions[a]

[a]At least the detection of two clinical signs.

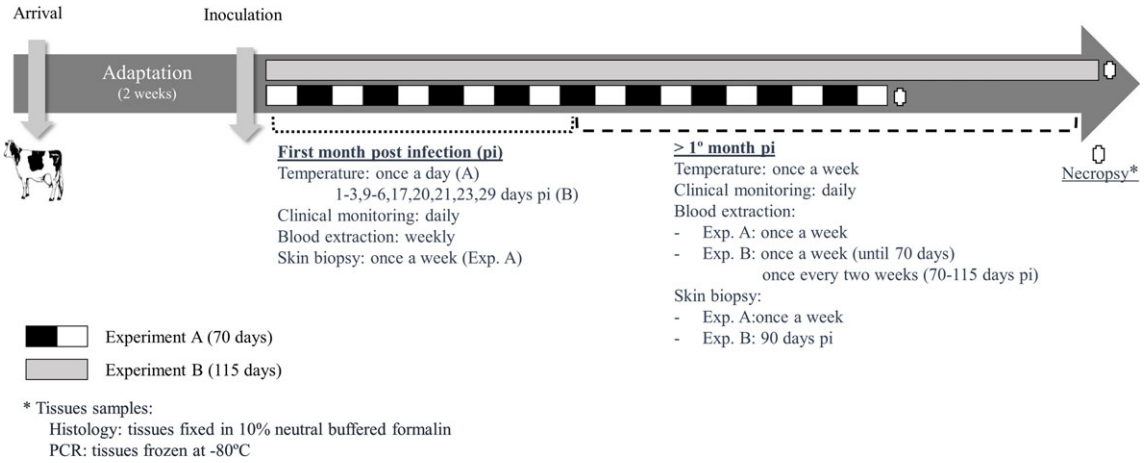

FIGURE 1 Timeline, clinical monitoring and sample collection in experimentally infected calves (a) and bulls (b) [Colour figure can be viewed at wileyonlinelibrary.com]

experiment, the animals were sedated with xylazine hydrochloride (Rompun®; Bayer, Mannheim, Germany) and immediately killed by an intravenously overdose of embutramide and mebezonio iodide (T61®; Intervet, Salamanca, Spain). Post-mortem examination of the calves was carried out immediately after euthanasia and tissue samples were collected for PCR and histopathological analyses. Tissues collected were as follows: reproductive system (testis, epididymis head, body and tail, vasa deferential, bulbourethral gland, prostatic gland, seminal vesicles and penis), respiratory tract (nostrils, nasal turbinates, larynx, pharynx, epiglottis, trachea, bronchi and lungs), digestive tract (tongue), lymphatic system (submandibular, subscapular, inguinal and tracheobronchial lymph node, thymus, tonsils and spleen), skin of different locations (neck, upper eyelid, carpus, tarsus, perineum, pinna, thigh and scrotum) as well as other organs (ventriculum, atrium, ocular conjunctiva, sclerotic, distal fascia-tendon from the rear leg and hoof corium).

2.2.2 | Experiment B

Six 14-month-old young bulls (B1-B6) of the Asturiana de la Montaña breed were included in this trial. Health status monitoring of the animals and laboratory analyses were done as in experiment A. The animals were inoculated with 10^6 tachyzoites through jugular venipuncture (see Section 2.3). The experimental design in experiment B was similar to A. The periodicity of clinical monitoring and skin biopsies and blood samplings are shown in Figure 1.

Bulls were killed at 115 days pi. Tissues from the reproductive tract (testicular parenchyma, epididymis, pampiniform plexus and penis), respiratory tract (nostrils, epiglottis and trachea), skin (scrotum, carpus, tarsus, elbow, perineum and pinna), spleen and ocular conjunctiva were collected for PCR and histopathological analyses.

2.3 | Parasites

Tachyzoites from the Bb-Spain 3 isolate were inoculated in both experiments. This isolate was obtained from a calf with chronic besnoitiosis and showed similar in vitro traits as other Spanish isolates (Diezma-Díaz et al., 2017). Tachyzoites were propagated in a MARC-145 cell monolayer (passage number = 4) according to a previously described protocol (Fernández-García et al., 2009) and were free of bovine viral diarrhoea virus (BVDV) and *Mycoplasma* spp. Tachyzoites were recovered from the cell monolayer when the majority of the parasites were still intracellular. The tachyzoites were then counted using a Neubauer chamber and parasite viability was determined using trypan blue exclusion and a plaque invasion assay by counting lysis plaques and parasitophorous vacuoles (Rojo-Montejo et al., 2009).

To prepare the inocula, tachyzoites were resuspended in phosphate buffer saline (PBS) at the required dose (10^6, 10^7 and 10^8 tachyzoites in a final volume of 2 ml) and were administered to animals within 1 hour of harvesting from cell culture. MARC-145 cells were also counted using a Neubauer chamber to determine the number of cells per dose. These were resuspended in PBS and inoculated in G4.

2.4 | Biochemical and haematological analyses

Biochemical and haematological analyses were performed according to procedures previously described (Langenmayer, Scharr, Sauter-Louis, Schares, & Gollnick, 2015). They were analysed by comparing values from infected animals with bovine reference values, taking into account that values may vary due to age and environmental factors (George, Snipes, & Lane, 2010).

2.5 | IFN-γ innate responses

IFN-γ levels were measured in sera by the Bovine IFN-γ ELISA development kits (Mabtech AB, Stockholm, Sweden), following manufacturer's recommendations. Colour reaction was developed by the addition of 3, 3′, 5, 5′-tetramethylbenzidine substrate (TMB, Sigma–Aldrich, Madrid, Spain) and incubated for 5–10 min in the dark. Reactions were stopped by adding 2N H_2SO_4. Then, plates were read at 450 nm. The cytokine concentrations were calculated by interpolation from a standard curve generated with recombinant cytokines provided with the kits.

2.6 | Humoral immune responses

Besnoitia besnoiti–specific IgG was determined by ELISA using lyophilized *B. besnoiti* tachyzoites as antigen (García-Lunar, Ortega-Mora, Schares, Diezma-Díaz, & Álvarez-García, 2017). Serum samples were analysed and the value of the optical density (OD) was converted into a relative index per cent (RIPC) using the following formula: RIPC = (OD_{405} sample – OD_{405} negative control)/(OD_{405} positive control – OD_{405} negative control) × 100. An RIPC value ≥17.34 indicates a positive result.

Besnoitia besnoiti–specific IgG1 and IgG2 serum isotypes were also determined by ELISA. Briefly, ELISA was performed as described above using a 1:100 dilution of sera samples and anti-bovine IgG1 or IgG2 peroxidase conjugated as secondary antibody (1:1,000; Bio-Rad, Hercules, California, USA). Sera from naturally infected and non-infected cattle were used as positive and negative controls respectively. For each plate, the values of the optical density read at 405-nm wavelength (OD405) were converted into the corresponding RIPC value as described above.

2.7 | DNA extraction and PCR determinations

Whole blood collected in a heparin tube was used to obtain PBMC. An aliquot of 800 μL of blood was gently mixed for 5 min with 960 μl of cold lysing solution (0.83% NH_4Cl + 2.06% Tris) at room temperature. After a centrifugation for 30 s at 12,000× *g*, the supernatant was decanted, and the process was repeated until the liquid was clear red. The pellet was stored at −80°C. Genomic DNA was extracted from PBMC and tissue samples using the Maxwell® 16

MouseTail DNA Purification Kit (Promega, Wisconsin, USA). The DNA content of each sample was measured using a NanoPhotometer® (Implen, Munich, Germany) and adjusted to 40 ng/μl. ITS-1 PCR was performed as described previously (Cortes et al., 2007).

2.8 | Histopathology and immunohistochemistry

Tissue samples and biopsies were fixed in 10% neutral buffered formalin and conventionally processed through a graded alcohol series before being embedded in paraffin wax. Four-μm-thick sections were obtained and stained with haematoxylin and eosin (H/E).

The immunohistochemical labelling was performed on those samples with histological lesions consistent with *B. besnoiti* infection and those where PCR-positive results were obtained. A primary in-house rabbit polyclonal antibody against *B. besnoiti* tachyzoite antigens (Gutiérrez-Expósito et al., 2012) was used for this purpose at 1:4,000 dilution. Briefly, an enzymatic digestion was carried out with trypsin, the primary antibody was incubated overnight at 4°C and, later on, a polymer-based detection system was used (EnVision+ System Labelled Polymer-HRP anti-rabbit; Dako, Glostrup, Denmark). The reaction was developed with 3, 3′-diaminobenzidine tetrahydrochloride (DAB Peroxidase Substrate Kit; Vector Laboratories, California, USA). The sections were counterstained with Mayer's haematoxylin. The specificity of the technique was assessed by omitting the primary antibody and also using pre-immune rabbit serum.

2.9 | Statistical analysis

Rectal temperatures and serology were analysed using a two-way ANOVA repeated measures test followed by a Tukey post-test. Statistical significance for all analyses was assessed with $p < 0.05$. All statistical analyses were carried out using GraphPad Prism 6.01 software (San Diego, CA, USA).

3 | RESULTS

3.1 | Clinical signs and clinical score

From the first day pi onwards, the mean temperatures of the infected calves were significantly higher than the uninfected group ($p < 0.001$) (Figure 2). However, there were no significant differences between infected groups ($p > 0.05$; two-way repeated measures ANOVA, Tukey post-test). All infected animals presented with fever from 1 day pi onwards until the end of the first week. The onset of fever was earlier in the higher dose–infected group (G1). These animals showed temperatures higher than 39.6°C at 12 hr pi. The highest temperature values were observed in one animal from G1 at 1 and 3 days pi (40.8°C and 40.3°C respectively). The remaining calves from G1 also developed high temperatures up to 40.3°C. A febrile response was also observed in all calves from G2 for as long as 7 days pi. The highest values from G2 were observed in one animal at 1 and 3 days pi (40.3°C and 40.7°C respectively). In G3, all animals developed fever. The highest values were observed in two animals at 4 days pi (40.1°C and 40°C). Animals from the uninfected control group (G4) had temperatures below 39.5°C throughout the experimental period.

The only temperatures higher than 39.5°C at 8 days ($n = 3$) and 9 days pi ($n = 1$) were detected in four of six infected bulls (Figure 2).

All calves from G1, G2 and G3 developed lymphadenopathy at 4 days pi in at least two of the three lymph nodes (submandibular, pre-scapular and pre-crural) examined. Lymphadenopathy remained longer in G1, for as long as 34 days pi *versus* 30 days pi in G2 and G3. Respiratory signs were also detected sporadically in calves from infected groups. Most animals coughed and had respiratory distress with an increased expiratory effort, at least once during the first month pi, which is characteristic of diseases that affect the lower respiratory tract.

Other signs characteristic of the acute stage of the disease, such as oedema, orchitis or lameness, did not develop in any of the infected animals. The outcome of the acute infection was classified as a 'mild-moderate infection' according to the clinical score presented in Table 2a, as no severe clinical signs (orchitis, oedemas or lameness) were found. In vivo clinical signs characteristic of the chronic stage, such as skin lesions, were not detected.

All infected bulls developed lymphadenopathy in one or both lymph nodes (only pre-crural or pre-scapular was monitored in bulls). This enlargement was observed at 3 days pi in five of six bulls and after 6 days in all infected animals. Lymphadenopathy was maintained until 55 days pi but was more evident within the first 24 days. Respiratory signs such as cough and nasal discharge were observed mainly between 8 and 20 days pi. Respiratory distress with an increased expiratory effort was observed in all animals between 17 and 20 days pi. Similar to the calves, the bulls did not develop other signs of severe acute stage or chronic phase of the disease, and the outcomes of the infections were classified as mild–moderate based on the clinical score shown in Table 2b.

3.2 | Kinetics of IFN-γ innate responses

Maximum serum IFN-γ levels were observed at 7 days pi in G1 and at 4 days pi in G2 and G3 and were significantly higher compared to the control group ($p < 0.05$; two-way ANOVA). At 4 days pi, IFN-γ levels were significantly higher in G2 and G3 ($p < 0.05$; Tukey post-test) compared to G1 and bulls. IFN-γ levels decreased to basal values in all three infected groups from day 10 pi and remained low onwards (Figure 3a).

In bulls, IFN-γ levels peaked at 8 days pi and were significantly higher compared to calves ($p < 0.05$; Tukey post-test). Levels decreased to basal values at 16 days pi. (Figure 3a).

3.3 | Humoral immune responses

After infection, seroconversion was detected at 16 days in bulls, 17 days in G1 and G2 and at 21 days in G3. The antibody levels were significantly higher in infected calves than in G4 from 17 days

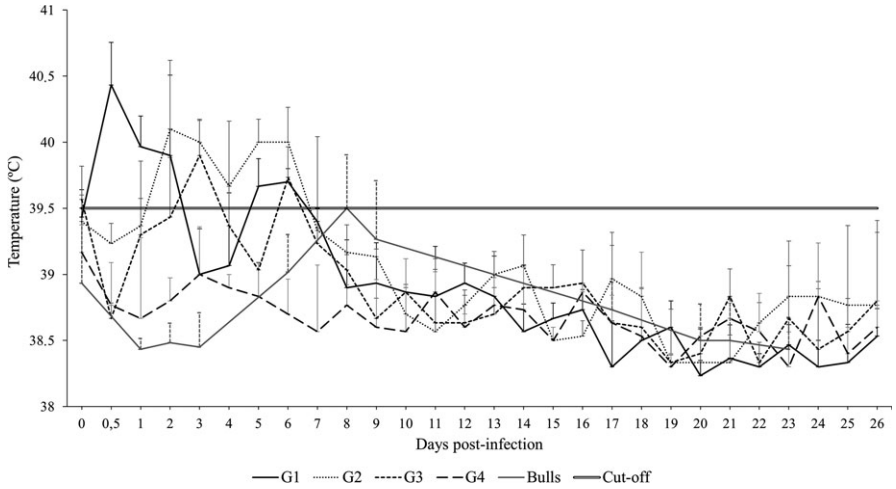

FIGURE 2 Mean rectal temperatures (+*SD*) recorded during the 1st month post-infection. The cut off for fever was ≥39.5°C (see legend)

TABLE 2 Clinical score estimated in calves (A) and bulls (B)

		Days post-infection										
A	Group	0–2	3–6	7–9	10–13	14–16	17–20	21–23	24–27	28–30	31–34	34–70
C1	1	2	2	2	2	2	1	1	1	0	1	0
C2	1	2	2	2	2	2	2	2	1	1	1	0
C3	1	2	2	2	2	2	1	1	1	1	1	0
C4	2	2	2	2	2	2	2	1	1	1	0	0
C5	2	2	2	2	2	1	1	1	1	1	0	0
C6	2	2	2	2	2	1	1	1	0	1	0	0
C7	3	2	2	2	2	2	2	1	0	1	0	0
C8	3	0	2	2	1	2	1	1	1	1	1	0
C9	3	0	2	2	2	2	2	1	0	1	0	0
C10	4	0	0	0	0	0	0	0	0	0	0	0
C11	4	0	0	0	0	0	0	0	0	1	0	0
C12	4	0	1	0	0	0	0	1	1	1	0	0

	Days post-infection																	
B	0	1	2	3	6	8	9	17	20	21	23	29	31	35	42	48	55	>64
B1	0	1	1	2	2	2	2	2	2	2	1	1	1	2	1	1	1	0
B2	0	1	1	1	2	2	2	2	2	1	1	1	1	1	1	1	1	0
B3	1	1	1	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0
B4	0	1	1	2	2	2	1	2	2	1	1	1	1	1	1	1	1	0
B5	0	0	0	0	1	1	1	2	1	1	1	1	1	1	1	1	1	0
B6	1	1	2	2	2	2	2	2	2	1	1	1	1	1	1	1	1	0

C: Calf (1–12); B: Bulls (1–6).

pi onwards ($p < 0.05$; two-way repeated measures ANOVA, Tukey post-test).

In calves, the higher dose–infected group (G1) showed significantly higher antibody levels compared with the lower dose–infected group (G3) starting at 17 days and remained higher from 24 days pi onwards ($p < 0.05$; two-way repeated measures ANOVA, Tukey test). However, there were no significant differences between G1 and G2 ($p > 0.05$; two-way repeated measures ANOVA, Tukey post-test). The antibodies kinetics were similar in calves and bulls with a few significant differences. The antibody levels in bulls were significantly higher than in the G4 from 16 to 70 days pi ($p < 0.05$; two-way repeated measures ANOVA, Tukey post-test) (see Figure 3b).

Significantly higher levels of IgG1 were detected in the infected groups compared to uninfected animals from 17 days pi ($p < 0.01$; two-way repeated measures ANOVA, Tukey post-test) (Figure 3c). Infected calves, G1 had significantly higher levels than the other

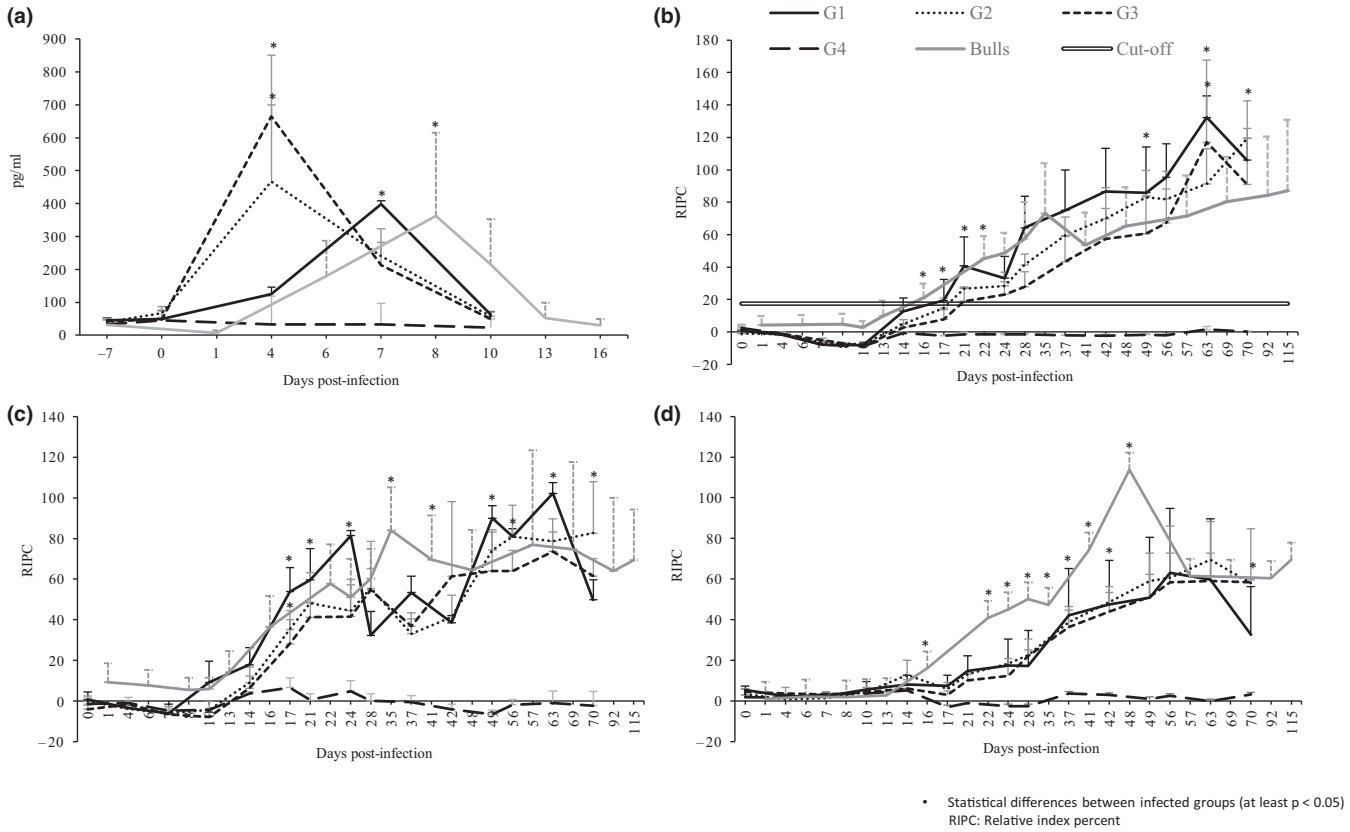

FIGURE 3 Immune responses measured in calves and bulls. (a): Innate IFN-γ responses measured in serum samples; (b): *B. besnoiti*–specific IgG responses; (c): Mean Relative Index (+*SD*) IgG1 antibody levels; (d): Mean Relative Index (+*SD*) IgG2 antibody levels

infected groups between 21 and 24 days pi ($p < 0.01$) and higher than G3 at 56 and 63 days pi ($p < 0.05$). The levels in G2 were significantly higher than G3 at 56 days pi, and higher than the other infected groups at the end of the trial ($p < 0.05$). IgG1 levels in bulls were significantly higher than infected calves between 37 and 41 days pi, ($p < 0.05$).

In calves, IgG1 levels were higher than IgG2 levels throughout the whole trial. Although IgG2 levels were detected approximately 21 days pi, significant differences between infected animals and the negative control group were not detected until 37 days pi (two-way repeated measures ANOVA, Tukey post-test $p < 0.05$). As IgG2 levels decreased in G1, significant differences were only observed between G1 and the other two infected groups in day 70 pi ($p < 0.01$).

An increase in IgG1-IgG2 levels was observed in bulls at 16 days pi and levels remained high until the end of the experiment. IgG2 levels were always lower than IgG1 except for 41 and 48 days pi, when IgG2 levels reached their highest values (Figure 3d). IgG2 levels were higher in bulls than in infected calves between 16 and 48 days pi ($p < 0.001$).

3.4 | Detection of *B. besnoiti* DNA in blood and tissues by PCR

Parasitaemia was detected sporadically in calves from G1 and G2 until 7 days pi. Parasitaemia was observed in two bulls between 10 and 24 days pi. However, the parasite was not detected in skin biopsies in any of the groups (Table 3).

After necropsy, 10 tissue samples from calves were PCR positive with the highest number of PCR-positive tissues (skin, reproductive and eyes samples) in G3. In the experiment B, only two tissue samples (testicular parenchyma and pampiniform plexus) from the same animal were positive by PCR (Table 3).

3.5 | Histopathology

Macroscopic lesions were not detected at necropsy. In contrast, compatible and characteristic microscopic lesions of chronic infection were found. Vasculitis was found in infected calves, particularly, in the skin (neck, tarsus, carpus, pinna and perineum), reproductive tract (testis, epididymis, vasa deferens and bulbourethral gland) and other locations (nostrils, tonsils, pre-scapular lymphnode, lung and hoof corium) (Supporting information Figure S1a). This lesion was characterized by the infiltration of lymphocytes, plasma cells, macrophages and eosinophils within the arteriole and venule walls and hyaline deposits at the arteriolar wall. Granulomatous vasculitis with foreign body giant cells in the testicle and reticular dermis of the perineum was observed in four positive-PCR animals: one from G1 (C3), two from G2 (C5, C6) and one from G3 (C9) (Table 3).

TABLE 3 Detection of *Besnoitia besnoiti* DNA in blood and tissues

Groups		Blood: days post-infection (animals)	Tissues (animals)
Calves	G1	4 (C2, C3); 7 (C1,C2,C3)	Carpal (C2) and scrotum skin (C3)
	G2	7 (C5, C6)	Proximal vas deferens (C5), epididymis body (C5) and scleral (C6)
	G3	Negative	Epididymis tail (C9), ocular conjunctiva (C7, C8), scleral (C8) and carpal skin (C7)
	G4	Negative	Negative
Bulls		10 (B3, B6), 13 (B6), 16 (B6), 24 (B3)	Parenchyma testicular (B2) and pampiniform plexus (B2)

C: Calf (1–12); B: Bulls (1–6).

Focal lymphoplasmacytic inflammatory infiltrates with the presence of eosinophils and macrophages were found in all infected animals. These infiltrates, often perivascular, were more numerous in lamina propria of the respiratory mucosa (larynx and nostrils), in skin dermis (scrotum, tarsus and neck) as well as in the stroma of genital organs (epididymis). Regarding other lesions in the male genital tract, C3 from G1 and C9 from G3 (both positive by PCR) showed testicular degeneration characterized by the formation of multinucleated giant cells in the lumen of seminiferous tubules.

A tissue cyst was only found in one animal (C2) from G1 in carpal skin (Supporting information Figure S1b). This tissue cyst was located in the deep dermis near vessels, surrounded by inflammatory cells (lymphocytes, macrophages and eosinophils) accompanied by fibrinous necrosis of the muscular of the arterioles.

In bulls, mild local inflammation was detected in most of the tissue samples from skin, reproductive and respiratory tract. These foci were formed mainly by lymphocytes and, to a lesser extent, macrophages and plasma cells. They were located at the skin, dermis and lamina propria of the respiratory mucosa. Specifically, these lesions were mainly found at the trachea and scrotum (all animals), epiglottis and skin of the elbow, carpus and thigh (five of six animals) and skin of the tarsus (four of six animals). In addition, the spleen of two bulls showed mild lymphoid hyperplasia. Regarding the genital tract, three animals showed foci on non-purulent inflammatory infiltrate adjacent to vessels at the epididymis and one of them (B2) also at the pampiniform plexus.

All immunohistochemistry labelled samples were negative for *B. besnoitii* antigen but for the carpal skin sample from C2G1.

4 | DISCUSSION

An in vivo ruminant model of besnoitiosis is a major need. Thus, we studied the effect of parasite dose and host age on the infection dynamics with *B. besnoiti* tachyzoites in cattle with the ultimate goal of developing a reproducible experimental model of *B. besnoiti* infection in cattle. We have successfully reproduced the acute phase of the disease and microscopic findings also evidenced the chronic infection. Indeed infected animals without detectable clinical signs and macroscopic lesions characteristic of the chronic phase are more frequently found than clinically affected animals in endemically infected herds (Gollnick et al., 2018). However, to test potential drugs or vaccines, an ideal experimental bovine model should be able to reproduce the acute stage followed by the characteristic clinical signs of the chronic stage of the disease. Fulfilling this requirement is not an easy task based on the variable results obtained by others in the past, where the absence of a well-standardized experimental design hampered the ability to infer conclusions about the key variables and, consequently, to reproduce the results (Álvarez-García et al., 2014).

To address the previous limitations, we carried out two experimental infections where we examined the tachyzoite dose and host age as parasite- and host-dependent variables respectively. Three key elements were carefully controlled to ensure reproducible results: (a) the employment of a well-characterized *B. besnoiti* isolate; (b) the viability and quality of the inocula and (c) the animal health status prior to infections.

We chose the Bb-Spain 3 isolate for several reasons: first, it was obtained from an animal younger than 4 months with marked skin lesions and, second, its recent isolation rules out the possibility of in vitro adaptation that might compromise its ability to form tissue cysts in vivo. Moreover, it has shown similar in vitro behaviour compared to other Spanish isolates (Diezma-Díaz et al., 2017). We also carefully monitored: (a) the inocula to ensure that the tachyzoites were free of BVDV and *Mycoplasma* spp. infections; (b) the animal health status prior to inoculations and (c) the numerous clinical signs and laboratory parameters measured by complementary techniques.

On the other hand, we selected males for these experiments, even though both sexes may be equally infected (Álvarez-García et al., 2014). However, bulls are at higher risk of acquiring and transmitting the infection as they serve a large number of cows and they appear to show more acute signs and a higher mortality rate (Álvarez-García et al., 2014; Gazzonis et al., 2017). Moreover, testicles are target organs for *B. besnoiti* replication and persistence (Kumi-Diaka, Wilson, Sanusi, Njoku, & Osori, 1981; Pols, 1960) and recent studies have provided evidence for the serious consequences of male sterility for herd fertility due to *B. besnoiti* infection (Esteban-Gil et al.,2016; Gazzonis et al., 2017).

Herein, we successfully reproduced the acute stage of the disease. Calves and young bulls developed fever followed by lymphadenopathy and respiratory signs. All infected calves developed fever during the 1st week pi and the incubation period lasted less than 12 hours, which agrees with the less than 2-day incubation period established previously using different intravenous inocula (Bigalke, 1968). However, the incubation period was longer in bulls. Four of six inoculated bulls showed temperatures higher than 39.5°C at 8–9 days pi. Respiratory signs of infection were observed in bulls later and the lymphadenopathy was maintained longer. It is possible

that a longer incubation period leads to a delay in the appearance of acute clinical signs. This delay could also have influenced on parasitaemia, as the parasite was detected in blood until 7 days pi in calves versus 24 days pi in bulls. These findings agree with previous studies that detected proliferative organisms in blood smears from the 3rd to the 12th day after the beginning of the febrile reaction in experimentally infected cattle (Basson et al., 1970). However, this finding was not corroborated in natural infections (Gollnick et al., 2015), where examinations of blood smears from naturally infected cows during the acute phase did not reveal either free or intracellular tachyzoites in peripheral blood. However, the sensitivity of blood smears is lower than PCR, which might explain these negative results. Our analyses of haematological and biochemical parameters showed very little relevant changes, which is in agreement with the mild-to-moderate outcome of the infection in both calves and bulls (unpublished data). We only observed an increase in serum globulin fractions that was correlated with seroconversion in infected animals. There is only one previous study where haematological and biochemical alterations in sera were studied in *B. besnoiti*-seropositive cows during disease progression of naturally acquired bovine besnoitiosis (Langenmayer et al., 2015). Disturbances in Aspartate Aminotransferase or Creatine Kinase activities, and typical alterations of chronic inflammatory diseases such as hyper-(gamma)-globulinemia or reduced erythrocyte were described by these authors.

No relevant differences were observed during the acute stage regarding the parasite dose and host age variables studied. It has been suggested that tachyzoite dose plays an important role in determining a severe versus a mild infection (Álvarez-García et al., 2014). In this study, this variable influenced only the occurrence of fever and the incubation period, but not the clinical score of the disease. Higher temperature values were detected in the group inoculated with the highest tachyzoite dose, similar to previous observations (Basson et al., 1970), but no relevant differences in the severity of the acute phase were observed among groups. It is tempting to hypothesize that doses higher than 10^8 tachyzoites would not significantly exacerbate clinical signs as relevant differences in the outcome of the infection were not observed among the three infected groups. In four experimental infections carried out in the past, tachyzoite doses higher than 10^8 were inoculated in cattle (Álvarez-García et al., 2014; Basson et al., 1970; Bigalke, 1968; Diesing et al., 1988). Acute phase was observed in all inoculated animals with mild–moderate severity, but for one immunosuppressed cow that died during this phase (Basson et al., 1970). However, severe clinical signs of chronic phase were only reported in one bull that had been splenectomiced previously (Basson et al., 1970). We must carefully consider these results as the inoculations were carried out under different experimental conditions. Moreover, a high number of zoites are not likely to be involved in natural infections with *B. besnoiti*, where horizontal transmission seems to be the main route of parasite transmission, either by cyst rupture during direct contact or by blood suckling arthropods bites (Álvarez-García et al., 2013). In fact, in naturally infected herds where animals might be exposed to similar infective doses, only a few animals develop characteristic clinical signs of bovine besnoitiosis, which may be due not only to parasite dose but also to other parasite and host-dependent factors. Regarding host age, according to previous experimental infections (Bigalke, 1970; Bigalke et al., 1974), the incubation period as well as the onset and duration of the febricula were highly variable in adult animals and were probably influenced by the different sources of the inocula. Herein, the inocula employed in both experiments were the same so that the most feasible explanation for the delay in the onset of clinical signs observed in bulls compared to calves might be the maturity of the immune system. The immune system maturation progresses in calves from birth until 6 months, when innate and adaptive immune responses reach values close to the ones developed by adults (Chase, Hurley, & Reber, 2008).

After the acute stage of the disease, the chronic stage of the infection was evidenced by parasite detection and microscopic lesions in target tissues despite no macroscopic lesions characteristic of the chronic stage of the disease, such as hyperqueratosis or alopecia, were detected in vivo. In this study, vasculitis was the most relevant lesion present in skin, genital and respiratory tract in all infected groups. Despite being a non-specific lesion, target locations where vasculitis was found suggested that this might be a consequence of endothelial damage produced by the parasite during the acute phase. Similar lesions have been described under natural infection (Langenmayer et al., 2015). Focal degenerative lesions in testicle of the calves were observed only in relation to vasculitis that probably contributed to mild degenerative changes in the germinal epithelium. However, the lesions observed in the reproductive tract were compatible with normal testicular function. Remarkably, in this study, a tissue cyst was located at the reticular dermis of the carpus region, a less frequent location than the papillary dermis (Langenmayer et al., 2015). As expected, a higher parasite intra-organic distribution was found by PCR compared with histopathological results. PCR-positive tissues were predilection sites for *B. besnoiti* that show tropism for the connective tissue of the superficial skin layers, scleral, conjunctiva and mucous membranes of distal genital tract (Frey et al., 2013; Gentile et al., 2012; Manuali et al., 2011; Nobel, Neumann, Klopper, & Perl, 1981). The DNA detected is expected to belong to the bradyzoite stage according to the infection dynamics as it was detected at 70–110 days pi during the chronic phase of the disease (Álvarez-García et al., 2014). Slight differences were found among infected groups as a higher number of PCR-positive samples (six of the 10) belonged to calves inoculated with 10^6 tachyzoites. It appears that inoculation with a lower dose of the parasite could have caused less stimulus of immune response, facilitating the evasion of the parasite and this could have allowed a higher early intra-organic distribution. This fact could be in agreement with what happens in natural conditions where low parasite doses might be inoculated under natural conditions through either direct contact or via haematophagous vectors as mentioned above (Álvarez-García et al., 2013). Based on the similar results between infected calves, the dose of 10^6 tachyzoites was chosen for the inoculation of bulls, where only two testicular samples were PCR positive. Regarding host age, 3- to 6-month-old Holstein friesian calves were

experimentally infected with *B. besnoiti* bradyzoites in the past and animals only developed tissue cysts and skin lesions under immunosuppressive treatment regardless the route of infection employed (Diesing et al., 1988). When tachyzoites were used as inocula only one of eight infected animals develop a few cysts without skin lesions in agreement with the results obtained in this study.

Herein, efficient immune responses elicited by immune competent animals were likely to have cleared most parasites, which is evidenced by the low parasite load detected by histology and correlated with mild lesions despite the high doses of parasites and the route of inoculation employed and reported by others (Álvarez-García et al., 2014; Bigalke, 1968). Accordingly, in both calves and bulls, the infection was controlled, and the immune response kinetics were similar in all infected groups. After infection, an innate IFN-γ response was developed during the 1st week pi, followed by an acquired immune response evidenced by seroconversion at 2–3 weeks pi and the maintenance of high antibody levels until the end of the experiment. IgG1 levels increased simultaneously with total IgG levels followed by an increase in IgG2 levels a few days later. Similar immune response kinetics have been reported in experimentally infected cattle with closely related apicomplexan parasites such as *Neospora caninum* (Regidor-Cerrillo et al., 2014). The basis of an effective immune response that governs the control of *B. besnoiti* infection remains to be clarified. However, a few remarkable findings were obtained. First, we corroborated that high antibody levels are neither predictive of the outcome of the infection nor protective against the infection, as no relevant differences were observed among groups regardless tachyzoite dose and host age. In contrast, it has been suggested that cell-mediated immune responses may play a key role in the control of the infection (Álvarez-García et al., 2014). Indeed, *B. besnoiti*-induced neutrophil extracellular trap formation was recently demonstrated as an important innate immune response mechanism of PMN acting against *B. besnoiti* (Caro, Hermosilla, Silva, Cortes, & Taubert, 2014) that might be influenced by parasite dose and host age based on the results obtained. It was also reported that *B. besnoiti* infection triggers early innate immune responses in endothelial cells (Maksimov, Hermosilla, Kleinertz, Hirzmann, & Taubert, 2016). Although the information we obtained is limited to IFN-γ and IgG2 responses, the results are in agreement with this hypothesis. The late IFN-γ response displayed by G1 and G2 calves and bulls, together with the higher IgG2 levels observed in bulls might be responsible for a better control of the infection. This mild dose-dependent modulation of the immune response has been previously reported by Rojo-Montejo et al. (2012), who suggested that when a high parasite dose is administered, a large number of tachyzoites may remain extracellular and stimulates the immune response more efficiently, whereas lower parasite doses might facilitate parasite immune evasion. Further works should elucidate the roles played by the immune T-cells repertoire, the Th1/Th2 balance and other cytokines, among others, in the pathogenesis and control of *B. besnoiti* infection.

In summary, infected animals developed clinical signs compatible with the acute stage and microscopic lesions characteristic of the chronic stage of the disease. Neither parasite dose nor host age seem to be relevant parasite- and host-dependent factors when tachyzoites were inoculated, as they did not significantly influenced the outcome of the infection. Moreover, we have set the stage for carrying out further controlled experimental infections in bovines to refine the present experimental model and induce chronic clinical signs. Thus, other variables such as alternative inoculation routes or parasite stages (e.g. bradyzoites) should be investigated in order to be able to reproduce clinical signs and macroscopic lesions characteristic of chronic besnoitiosis.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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