

Prevalence of bovine trichomonosis and associated risk factors in bulls from Spanish beef herds

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ABSTRACT

Bovine trichomonosis is a sexually transmitted disease considered as an important cause of early reproductive failure in beef cattle. To investigate the occurrence of the infection in different Spanish beef cattle-producing areas, retrospective data from the SALUVET veterinary diagnostic laboratory (Veterinary Faculty, Madrid, Spain) derived from the analysis of samples from beef bulls that were routinely tested for *Tritrichomonas foetus* infection, were compiled from 2011 to 2015. In addition, a number of potential risk factors were assessed. *T. foetus* was detected in 12.7% (385/3016; 95% CI: 11.5%–13.9%) of samples from bulls and in 20.7% (195/941; 95% CI: 18.1%–23.3%) of the herds tested. “Bull age” and “reproductive disorders in the herd” were the risk factors identified in the multivariable analysis. Bulls older than 3 years (19.7%) were more likely to be infected than young bulls (8.2%) and *T. foetus* was more often detected in herds with reproductive problems (27.9%) than in those without reproductive disorders (9.4%). The prevalence in bulls originating in mountain systems (13.9%, 267/1922) was significantly higher than that in “dehesa” (Mediterranean holm-oak pasture) areas (10.8%; 118/1094) ($P < 0.05$), which might be attributable to the use of communal pastures and specific management practices in mountain systems. The results reported here indicate that *T. foetus* infection is substantially spread among beef cattle herds, suggesting that BT could be having a significant negative impact on the reproduction and productivity of Spanish beef herds managed under extensive conditions.

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1. Introduction

Bovine trichomonosis (BT) is one of the OIE Listed diseases of the World Organization for Animal Health [1] and is considered an important cause of early reproductive failure in beef cattle kept under extensive management conditions [2–4]. BT is caused by the flagellated protozoan parasite *Tritrichomonas foetus* [5], which colonizes the preputial cavity of bulls and the urogenital tract of cows and is transmitted during coitus. Bulls act as asymptomatic carriers, whereas the disease in cows causes early embryonic death or abortion [3,4,6]. Economic losses arise from prolonged calving intervals, reduced calf drop caused by failure of or delay in conception, increased culling of infected animals and increased veterinary costs [7–10].

BT is still prevalent in some areas where natural breeding of beef cattle is widely practised, such as in Southern Africa, South America, North America or Australia [2,11,12]. In Europe, the widespread use of artificial insemination (AI) and effective control programmes have greatly reduced the incidence of BT. However, in a previous study, we demonstrated the presence of *T. foetus* in 32% of bulls and 41.5% of herds from one representative beef cattle breed reared in traditional mountain systems in northern Spain [13] which caused a negative impact on the productivity of the infected herds [10]. In another study carried out involving a different Spanish breed, the prevalence of BT was significantly lower than the previously reported prevalence, even though both breeds essentially shared the same areas [14]. Consequently, the prevalence of BT is likely to vary considerably depending on the management of cattle farm. The status of BT in different Spanish beef cattle-producing areas and systems is not well known. Determining the extent and impact of this relevant venereal disease is essential for designing herd-health control programmes focused on improving reproductive efficacy.

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In this study, retrospective data from the SALUVET veterinary diagnostic laboratory (Department of Animal Health, Veterinary Faculty, Madrid, Spain) derived from the analysis of smegma samples from beef bulls that were routinely tested for *T. foetus* infection from 2011 to 2015 were compiled and used to investigate the prevalence of BT in Spanish beef cattle. In addition, a number of potential risk factors potentially involved in the transmission of *T. foetus* were assessed.

2. Materials and methods

2.1. Study design and samples

The study included all bull preputial smegma samples submitted to the SALUVET laboratory for *T. foetus* testing from January 2011 to December 2015. The SALUVET laboratory provides BT diagnostic services to private veterinary practitioners. The study population consisted of bulls used for natural mating (bull age \geq 18 months) originating in farms located in extensive beef cattle-producing areas. Smegma samples were transported at room temperature and the analysis. In order to avoid false negative results, only samples that arrived at the laboratory within 24–48 h post-collection were analysed [13]. Samples were examined for BT using a PCR and cultural exam in parallel, as previously described (see below) [13]. The presence of a cultures trichomonadid parasite that did not yield a PCR-positive result was suggestive of non-*T. foetus* trichomonads (typically of faecal origin) and was considered negative. If *T. foetus* was detected only by PCR, the animal was considered positive. Herds with at least one positive bull were classified as infected. The prevalence was calculated as the ratio of positive bulls/herds to the total number of bulls/herds tested using records of all *T. foetus* diagnostics performed between January 2011 and December 2015. In addition, a questionnaire was sent in order to analyse the association between the status of *T. foetus* infection and specific variables hypothesized to be relevant to the disease, as described below.

The bovine clinical samples used in this study were submitted to the SALUVET laboratory for routine diagnostic testing. The samples were obtained from animals in the field by licensed veterinarians as a part of normal veterinary care and diagnostic investigations, so no formal ethical approval was necessary.

2.2. Sampling technique

Smegma samples were collected by scraping using a plastic cylinder brush scraper with shallow grooves perpendicular to the long axis of the prepuce. The brush was inserted into the prepuce and scraped back and forth. The material was then transferred to a tube containing 5 ml of phosphate buffered saline (PBS, pH 7.0). The PBS suspension was thoroughly mixed to create a homogenous mixture from which aliquots were taken and used as inoculum for culture media or DNA extraction, as described below.

2.3. Culture method for *T. foetus* detection

For culture of *T. foetus*, modified Diamond's medium (MDM) was used according to the previously described protocol [13,15]. Briefly, immediately after sample collection, 1 ml of the PBS mixture was inoculated into 5 ml of MDM medium. After the samples arrived at the laboratory, they were incubated at 37 °C for 7 days in dark conditions and microscopically examined (200 × screening; 400 × confirmations) on days 2, 4, 6 and 7 after sample collection. Parasites were identified based on their typical morphology and motility.

2.4. PCR for *T. foetus* detection

DNA was isolated from an aliquot of 500 μ l of the PBS mixture using a robotic system (Maxwell, Promega) according to the manufacturer's instructions. For *T. foetus* amplification, PCR was performed as previously described [16]. Amplified products were visualized under UV light in a 1.8% agarose/ethidium bromide gel. To avoid false positive reactions, DNA extraction, PCR sample preparation and electrophoresis were performed in separate rooms with different sets of instruments, and aerosol barrier tips and disposable gloves were employed.

2.5. Bull and herd data collection

For all the *T. foetus* diagnostics performed between January 2011 and December 2015, computer records were generated that included the following information: name of the owner or herd affiliated with the sample, sample or bull identification and date the sample was received. In addition, a questionnaire was sent to the veterinarian together with the material for sample collection, in order to analyse the association between the *T. foetus* infection status and variables hypothesized to be relevant to the disease including bull age (\geq or $<$ 3 years old), bull breed (native or imported beef breeds), herd size (small herds: $<$ 100 cows, medium herds: 100–399 cows, and large herds: $>$ 400 cows), bull-to-cow ratio (\geq or $<$ 1:25), the presence of reproductive disorders in the herd (yes or no) and type of management (mountain or “dehesa” systems). In mountain systems, cattle are grazed in communal pastures, whereas in “dehesa” systems (Mediterranean holm-oak pasture), animals are kept in fenced grazing lands. The mountain areas are located in central and northern Spain, and the “dehesa” areas are in western and southern Spain.

2.6. Statistical analyses

The testing apparent prevalence of *T. foetus* infection was calculated from the ratio of positives to the total number of samples examined using exact binomial confidence intervals (95% CIs). In estimating the true prevalence of BT, previously described sensitivity (approximately 80%) and specificity (approximately 98%) results of a combination of PCR and culture were used [17]. Possible associations between prevalence and explanatory variables (farm location province, year, bull age, bull-to-cow ratio, herd size, bull breed, type of management and the presence of reproductive disorders in the herd) were initially screened with a univariable analysis using Pearson's chi-square test or Fisher's exact test when there were fewer than six observations per category. In addition, Student's independent *t*-test was used to evaluate significant differences between means.

Factors with a *P*-value $<$ 0.20 were further scrutinized for associations using Cramer's *V* coefficient between pairs of variables to prevent collinearity. Variables with coefficients greater than 0.60 were considered to be correlated and were not included together in the same model. When collinear variables were detected, only the variable with the a priori stronger biological association with *T. foetus* infection was retained. The third step involved a generalized estimating equation (GEE) model [18]. The number of positive animals was assumed to follow a binomial distribution, and “herd” was included as a random effect. A Poisson distribution and logit link function were used. Biologically plausible confounding factors were assessed using the Mantel-Haenszel method, and confounding was considered to be potentially significant if the odds ratios (ORs) shifted appreciably. Variables that altered coefficients of independent variables of interest by 30% or more were removed from the model and were classified as confounding factors. The model

was re-run until all the remaining variables presented statistically significant values (likelihood ratio and Wald tests, $P < 0.05$). The fit of the model was assessed using the quasi-likelihood under independence model criterion. Statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA).

3. Results

3.1. *T. foetus* prevalence

A total of 3016 preputial samples obtained from mature bulls were analysed from 2011 to 2015, and an increase in the number of sample submissions was detected in 2014 and 2015 (Fig. 1). The affiliated herd was identified for 2867 bulls out of 3016 tested bulls, indicating that these bulls came from 941 herds. In the studied period, *T. foetus* infection was detected in 12.7% (385/3016; 95% CI: 11.5%–13.9%) of the bulls tested, ranging from 17.9% in 2011 (45/252) to 7.3% in 2015 (74/1011). The apparent prevalence of the tested farms was 20.7% (195/941; 95% CI: 18.1%–23.3%), varying from 34.1% (14/41) in 2011 to 12.7% in 2015 (Fig. 1). The bull and herd estimated true prevalence were 13.7% and 24%, respectively.

The tested bulls originated in farms located in 15 provinces out of 50 Spanish provinces; these provinces were representative of the most important beef cow-calf-producing areas in Spain. The distribution of the *T. foetus* bull prevalence in the different Spanish provinces is shown in Fig. 2. The testing prevalence of bulls from mountain systems (13.9%, 267/1922) was significantly higher than that in “dehesa” systems (10.8%; 118/1094) ($P < 0.05$).

In total, 64 out of 3016 (2.1%) samples were PCR-positive and culture-negative, and 2 culture-positive samples (0.06%) were PCR-negative, suggesting infection by non-*T. foetus* trichomonads.

3.2. Risk factors for exposure to *T. foetus* infection

More than 95% of the responses provided information regarding the affiliated farm, location and management system. At least 38.5% of the replies reported herd size, bull breed, bull-to-cow ratio and the presence of reproductive problems. Only 4.6% of the questionnaires were fully completed. Table 1 shows the results of the univariate analysis of the factors associated with *T. foetus* infection. In this analysis, *T. foetus* infection was significantly more frequently detected in bulls older than 3 years; imported breed bulls; bulls from herds <400 cows; bulls from herds with a bull-to-cow ratio $\geq 1:25$; bulls from mountain system herds; and bulls from herds with reproductive disorders (Table 1). Additionally, the mean age of the infected bulls was significantly higher than that of non-infected

ones (5.2 versus 4.3 years old; $P < 0.0001$), and the mean bull-to-cow ratio was significantly higher in *T. foetus* infected herds (38.1 versus 35.4; $P < 0.05$). Comparing mountain versus “dehesa” systems, the herds were significantly smaller (mean herd size 114 versus 212.6 cows; $P < 0.0001$), the bull-to-cow ratio was higher (37.4 versus 33.3; $P < 0.001$) and the bulls were older (4.6 versus 4.3 years old; $P < 0.05$) in herds from mountain farms than from the “dehesa” farms.

In the multivariate GEE analysis, the year was excluded due to collinearity with the variable “region”, while “breed” showed collinearity with “type of management” and “bull-to-cow ratio”. The final model showed that the main risk factors potentially associated with the individual risk of infection by *T. foetus* in breeding bulls were “bull age” and “reproductive disorders in the herd” (Table 2). Bulls older than 3 years (19.7%) were more likely to be infected than young bulls (8.2%). Significantly, a higher rate of *T. foetus* infection was also observed in herds with the presence of reproductive problems than in those without reproductive disorders (27.9% versus 9.4%).

4. Discussion

Bovine trichomonosis is a reproductive problem in beef herds managed under extensive conditions, and is widespread around the world [2,11,12]. In European countries, the BT prevalence has dramatically decreased or even been eradicated (in dairy cattle) due to the implementation of control programmes and the use of AI in the past. A survey conducted in Switzerland involving 1362 preputial samples from bulls and 60 abomasal fluid samples from aborted fetuses from beef and dairy herds revealed the absence of *T. foetus* infection [19]. However, reform of the Common Agricultural Policy (CAP) and increased emphasis on environmentally friendly land management have encouraged the expansion of extensively managed beef herds. For instance, the number of Spanish suckler cattle has doubled over the last 30 years [20]. In these systems, natural breeding and grazing in communal pastures are usually used, which are significant risk factors for BT [2]. In Spain, around 95% of beef cattle use natural mating [12]. In fact, in areas assumed to be largely free of BT, re-establishment of the infection is possible, especially in beef herds farmed under mountain pastoral systems, as we have described previously in northern Spain [13,14]. Similarly, the World Organization for Animal Health reported the presence of BT in Portugal and France in 2016 [12]. Consequently, BT could be emerging in European countries where livestock are extensively managed and natural breed is used.

In the present study, the occurrence of *T. foetus* infection in cow-calf beef herds was investigated at national level using diagnostic retrospective data from laboratory submissions. It is important to mention that there are potential sources of bias of this study, such as the lack of randomization of the analysed bulls; therefore, the studied population is not representative of the entire population of Spanish bulls. The reasons for sample submission include for routine surveillance, regulatory purposes prior to sale, control measures, purchase, breeding, or for the differential diagnosis of early reproductive failure in the herd. In addition, peaks in sample submissions for BT testing in 2014 and 2015 were observed, suggesting that there was growing concern for BT diagnosis in Spanish beef cattle operations due to a better knowledge of the disease and the significant threat it poses to animal health and welfare and due to the establishment of plans for disease control. The results obtained in this study showed a high prevalence for *T. foetus* infection in the tested population (bull prevalence of 12.8% and herd prevalence of 20.8%) and demonstrated that the infection is present in beef cattle herds. Continuous movement of cattle and sharing of bulls can put many herds at risk of venereal infections.

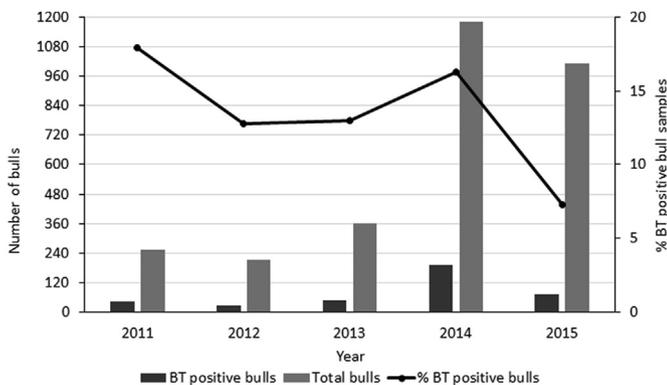


Fig. 1. Bovine trichomonosis prevalence in breeding bulls from Spanish suckler herds derived from samples submitted to the SALUJET diagnostic laboratory between 2011 and 2015.

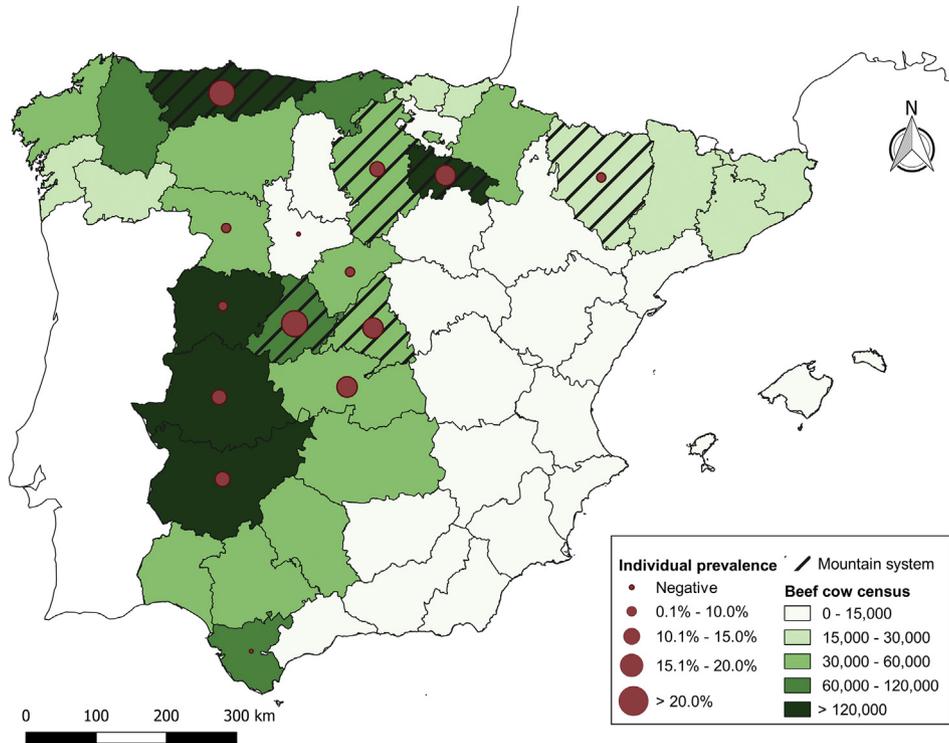


Fig. 2. Choropleth map of Spain showing overlay the beef cattle census and the individual testing prevalence of bovine trichomonosis between 2011 and 2015. Striped and non-striped patterns indicate mountain and “dehesa” areas, respectively.

Table 1
Distribution of *T. foetus* status according to epidemiological data and risk factors analysis.

| Variable | Category | <i>T. foetus</i> status | | | P-value | OR 95% CI | | |
|-------------------------------------|----------|-------------------------|--------------|------------------|---------|-----------|-------|-------|
| | | N | Infected (%) | Non-infected (%) | | OR | Lower | Upper |
| Bull age ^a | <3 years | 512 | 42 (8.2) | 470 (91.8) | <0.0001 | 2.75 | 1.92 | 3.94 |
| | ≥3 years | 815 | 161 (19.7) | 654 (80.2) | | | | |
| Bull breed ^b | Native | 658 | 64 (9.7) | 594 (90.3) | <0.05 | 1.50 | 1.08 | 2.09 |
| | Foreign | 752 | 105 (13.9) | 647 (86) | | | | |
| Bull-to-cow ratio ^c | <1:25 | 284 | 35 (12.3) | 249 (87.7) | <0.05 | 0.65 | 0.44 | 0.97 |
| | ≥1:25 | 876 | 154 (17.6) | 722 (82.4) | | | | |
| Type of management ^d | Mountain | 1922 | 267 (13.9) | 1655 (86.1) | <0.05 | 1.33 | 1.06 | 1.68 |
| | “Dehesa” | 1094 | 118 (10.7) | 976 (89.2) | | | | |
| Herd size (no cows) ^e | <100 | 186 | 39 (21) | 147 (79) | ≥0.05 | 1.13 | 0.68 | 1.90 |
| | 100–399 | 180 | 34 (18.9) | 146 (81.1) | <0.05 | 6.75 | 0.88 | 51.35 |
| | >400 | 30 | 1 (3.3) | 29 (69.6) | <0.05 | 7.69 | 1.04 | 58.29 |
| Reproductive disorders ^f | Yes | 227 | 104 (45.8) | 123 (54.2) | <0.0001 | 4.62 | 2.85 | 7.46 |
| | No | 181 | 28 (15.4) | 153 (84.5) | | | | |

OR, Odds ratio; 95% CI, 95% Confidence interval.

^a Bull age data were recorded for 1327 bulls out of 3016 bulls tested.

^b Bull breed data were recorded for 1410 bulls out of 3016 bulls tested.

^c Bull-to-cow ratio data were recorded for 1160 bulls out of 3016 bulls tested.

^d Type of management data were recorded for all bulls tested.

^e Herd size data were recorded for 396 herds out of 941 herds identified.

^f Reproductive disorders data were recorded for 408 herds out of 941 herds identified.

Consequently, control measures should be routinely implemented in Spanish beef herds managed under extensive conditions. Comparable prevalence rates were recorded in areas with extensive cattle management and natural breeding, such as in California (15.8%) [21], Idaho (40.9%) [22] and Florida (30.4%) [23]. In Argentina, *T. foetus* was found in bulls tested from 24% of herds in Buenos Aires [24], and in Costa Rica, prevalences of 18.4% and 7.2% were reported for farms and bulls, respectively [25]. Currently in

Europe, there are no monitoring programmes to track the incidence and prevalence of BT and there are no mandatory regulations for cattle movement regarding BT. This lack of monitoring in combination with inconsistent testing practices and trade of animals with an unknown health status can lead to underestimation of the prevalence and adverse effects of the disease in the European beef cattle sector where natural mating is used.

In terms of BT control, there are no treatments or commercial

Table 2
Results of the generalized estimating equation model of potential risk factors for *T. foetus* infection in breeding bulls in Spain.

| Variable | Category | β | Sig. | OR | 95% CI | |
|------------------------|--------------------|---------|-------|------|--------|-------|
| Bull age | <3 years old | * | * | * | * | * |
| | ≥ 3 years old | 1.239 | 0.045 | 3.45 | 1.03 | 11.63 |
| Reproductive disorders | No | * | * | * | * | * |
| | Yes | 1.725 | 0.009 | 5.61 | 1.52 | 20.65 |

* Reference category; OR, Odds ratio; 95% CI, 95% Confidence interval.

vaccines available in Europe; therefore, programmes rely on the identification and removal of infected bulls and control of cattle movement [3,10,11]. Culling infected bulls is effective for reducing/interrupting the spread of the disease in areas where mandatory control programmes exist, such as La Pampa (1.1%) [26], Alabama (0.2%) [27], Wyoming (0.2%) [28] and Texas (3.7%) [29]. However, these measures are not mandatory in Spain; this is an important limitation for BT control since without substantial changes in management, BT control will unlikely be achieved because the putative risk factors associated with BT remain present [10].

In terms of diagnosis, the detection of *T. foetus*-infected bulls is complex and may be compromised by many factors, such as sample collection, shipment and testing number, and laboratory techniques [17,30]. A video demonstrating how to perform a preputial scraping to collect smegma and the diagnosis key points was sent to the veterinarian. In addition, some practice seminars were also organized by SALUVET laboratory. The standard testing protocol for controlling BT in an infected herd has been to subject all bulls in the herd to three culture-based tests [31]. PCR is potentially a highly sensitive and specific diagnostic test for BT, and fewer sampling events may be adequate for identifying *T. foetus*-positive bulls [4,30]. In our laboratory, a combination of culture and PCR, previously validated in our laboratory [13] was performed on each sample. Diagnostic testing employing both culture and PCR for *T. foetus* yields a higher sensitivity and improved specificity, therefore this may be a cost-effective and practical approach for assessing bulls [17]. In *T. foetus*-inoculated bulls, both tests combined in parallel on a single sample had a sensitivity and specificity similar to two cultures or two PCR samples tested during consecutive weeks [17]. However, assuming that the diagnostic protocol used had a sensitivity of 80% [17], a number of infected bulls may not have been detected. In addition, the presence of non-*T. foetus* trichomonads were observed in only two young bulls. In previous studies, *Tetratrichomonas* spp. (commensal species of the bovine intestinal tract) were recovered from the bovine preputial cavity [32–34] and have been associated with homosexual behaviour. The data obtained here demonstrate the utility of PCR testing on smegma samples for the diagnosis of BT due to its sensitivity and specificity.

The identification of risk factors is also important to control the spread of *T. foetus* infection. The leading risk factors for trichomonosis are the use of natural mating for breeding, extensive range management and commingling of cattle [2,3]. Moreover, other risk factors have been identified in several studies [9,24,35]. In our study, a number of potential risk factors, whose commonality is their relationship to the transmission of *T. foetus*, were assessed in Spanish beef cattle. “Bull age” and “the presence of reproductive disorders in the herd” were the only risk factors associated with *T. foetus* infection that were identified in the multivariable analysis. According to the data obtained in the present study, the use of bulls older than 3 years seems to be widely practised in herds using natural mating in Spain, which is detrimental for the prevention of *T. foetus* infections. Infection in bulls is reported to persist for more

than 3 years and may persist for life [36,37]. Older bulls tend to retain the infection more permanently than younger bulls. This fact could be due to the increase in number and size of the crypts in the epithelium of the penis, although this hypothesis was questioned recently [30]. Another reason may be that old bulls (chronic carriers) have had a higher number of sexual contacts during their life and consequently have a higher risk of exposure than young bulls [21,38]. These findings have also been observed in numerous studies [13,21,23,25,35,38]. In our study, reproductive disorders were more commonly reported in *T. foetus*-positive herds. BT is a well-documented cause of early reproductive failure in cattle, increasing the interval between calving and successive pregnancies and resulting in reduced fertility rates, higher culling rates and consequent economic losses [8,10]. In epidemiological studies, reporting a “pregnancy rate in cows $\leq 90\%$ ”, “abortion” or an “increased number of repeat breeder cows” were found to be associated with *T. foetus*-positive beef herds [13,24].

In the present study, the testing bull prevalence was higher in mountain systems than in “dehesa” systems. The main reason may be attributable to the use of communal pastures for breeding and grazing in mountain areas. Some cows may be bred at their home farm, but many farmers rely on bulls owned by the community pasture system to breed their cows. Moreover, cows from several herds are usually combined into a breeding group that remains as a unit for the grazing season. The presence of infected bulls and cows in public allotments during the breeding season may play a role as a potential reservoir for the parasite. In a previous study, shared grazing was found to be a significant risk factor [22,24,35,39]. We also observed that particular herd management practices were associated with an increased likelihood of parasite transmission in mountain herds. We found that the bull population was older and the bull-to-cow ratio was higher in mountain herds than in “dehesa” herds; these factors have been identified as *T. foetus* risk factors [9,13,21,23,25,35,38]. The use of a high bull-to-cow ratio may increase the number of potential sexual contacts and thereby increase the probability of being or becoming infected and increase the risk of disease spread. Consequently, mountain herds are at greater risk for the introduction of *T. foetus* based on the relatively high local prevalence of the disease and the use of risk management practices for BT.

5. Conclusions

The results reported here indicate widespread BT in extensive husbandry conditions in Spain, where large numbers of cattle are bred by natural mating. Systematic BT testing of bulls should be part of yearly breeding soundness programmes. The data presented here indicate that management practices are closely related to disease spread. Future control plans for the prevention and control of BT are needed in Spain. In the Europe, there are no global mandatory regulations for cattle movements with regard to trichomonosis in bovine and the disease can be introduced by the trade in animals, both males and females with unknown health status. The increasing importance of natural breeding in European countries in combination with the current low awareness of BT might lead to an unnoticed re-establishment of the infection. More studies should be performed to investigate this disease in other European areas where natural mating is widely practised and where communal grazing areas are used.

Declaration of interest

The authors declare no conflict of interest.

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