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Antibodies against the *Mycobacterium tuberculosis* complex and *Brucella* spp. in captive and free-living European bison (*Bison bonasus*) in Poland

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Abstract

Background: The European bison (*Bison bonasus*), a symbol of Polish nature, is a protected species that requires active health monitoring. However, conservation efforts are made difficult by the zoonotic diseases such as brucellosis and tuberculosis.

Objective: The aim of this study was to screen the Polish European bison population for exposure to the *Mycobacterium tuberculosis* complex (MTC) and *Brucella* spp.

Methods: A total of 323 free-living and captive European bison from 13 localities were tested serologically for antibodies against the *M. bovis* P22 multi-protein complex (in-house ELISA) and against *Brucella* spp. (commercial ELISA).

Results: Antibodies against the MTC (P22) were detected in 7% (22/323) of the tested European bison. Anti-MTC antibody positivity was not significantly different by sex, age, and captive/free range status. Anti-MTC antibodies were found in six of 13 populations sampled, always in populations with larger sample sizes including the four free-living ones. Antibodies against *Brucella* spp. were detected in 36% (116/323) of the tested bison. While *Brucella* spp. antibody prevalence was not different by sex, it was significantly different by age (lower in adults) and captive/free-living status. *Brucella* spp. seroprevalence decreased with sample size and seropositive bison were found in 12 of 13 sampling populations.

Conclusions: Our findings identify potential emerging threats to the European bison population and confirm the first serological response to P22 in European bison. As Poland is currently officially free of brucellosis and bovine tuberculosis, our results require careful interpretation. Further studies are needed to establish the presence of cross-reactions with atypical mycobacteria in the case of MTC and other bacteria (e.g. *Yersinia enterocolitica* O:9) in the case of *Brucella* spp.

KEYWORDS
Brucella spp, ELISA, European bison, P22, serology, tuberculosis

1 | INTRODUCTION

The European bison (*Bison bonasus*) is an iconic Polish wildlife species, with more than a quarter of the global European bison population living in Poland. The species is currently safe from extinction, having recently changed its conservation status to 'Near Threatened', according to the International Union for Conservation of Nature (IUCN) (Plumb et al., 2020).

The number of European bison in Poland at the end of 2021 was 206 in captivity (most herds are of a low number) and 2223 in free-living populations located in eight different herds (Raczynski & Bołbot, 2022). The European bison in Poland is undergoing dynamic population development (Olech & Perzanowski, 2022) that results in high local population densities; therefore, it is important to monitor infectious diseases and emerging threats (Klich et al., 2020, 2021).

Tuberculosis (TB), caused by members of the *Mycobacterium tuberculosis* complex (MTC), and brucellosis, caused by the genus *Brucella*, not only have a considerable impact on cattle herds worldwide but also are bacterial zoonoses with significant potential impact on public health. Although Poland is officially free of TB and brucellosis in cattle (Regulation [(EU) 2021/620]) both are occasionally noted in Polish wildlife (Szulowski et al., 2013a; Krajewska et al., 2014). It has been suggested that European bison are highly sensitive to *Mycobacterium* spp. infections (Didkowska, Orłowska, et al., 2021); indeed, TB has been confirmed in both captive (Didkowska, Orłowska, et al., 2021) and free-living herds of European bison in Poland (Welz et al., 2005). Although paratuberculosis does not seem to be an emerging problem in Polish European bison herds (Didkowska, Ptak et al., 2021), atypical mycobacteria (*Mycobacterium avium* and *Mycobacterium xenopi*) have been isolated from them (Didkowska, Orłowska, et al., 2021).

TB has also been identified in European bison in a Brazilian zoo (Zimpel et al., 2017) and is endemic in American bison (*Bison bison*) in some North American populations (Himsworth et al., 2010; Nishi et al., 2002; Shury et al., 2015; Tessaro et al., 1990). In recent years, due to the emerging cases of TB in bison, diagnostic procedures have been extended to incorporate new methods, including serological tests (Didkowska, Dziekan, et al., 2021).

One antigen that has recently been studied for use in serological tests in various mammalian species is P22 (Bezos et al., 2018; Casal et al., 2017; Infantes-Lorenzo et al., 2018; Infantes-Lorenzo, Dave et al., 2019; Infantes-Lorenzo, Moreno et al., 2019; Thomas et al 2019; Arrieta-Villegas et al., 2020; Ferreras-Colino et al., 2022). P22 is a multi-protein complex obtained from *Mycobacterium bovis* purified protein derivative (B-PPD) by affinity chromatography (Infantes-Lorenzo et al., 2017). P22 has demonstrated high sensitivity (Se) and specificity (Sp), depending on the animal species and epidemiological situation (Infantes-Lorenzo, Moreno et al., 2019). In the case of cattle Se was 87% (Casal et al., 2017) and Sp was 92.5-99.4 depending on cut-off and epidemiological situation (Infantes-Lorenzo, Moreno et al., 2019). However, no data about the antibody response to P22 in European bison are available.

Another disease of livestock and wildlife that has significant consequences for animal and public health, as well as international trade, is brucellosis (Khurana et al., 2021). *Brucella abortus* is the primary cause of brucellosis in cattle, but it also affects wildlife, including bison (Mackintosh et al., 2002; Rhyan et al., 2001; Tessaro et al., 1990). A recent meta-analysis found that among wild Bovidae, the highest prevalence of brucellosis was noted in American bison (*Bison bison*) (39.5%) (Dadar et al., 2021). Highly similar *B. abortus* variable number tandem repeat patterns have been identified in cattle and American bison, indicating possible transmission between wildlife and livestock (O'Brien et al., 2017; Rhyan et al., 2013). As *B. abortus* was also noted in American bison in northern Canada (Wobeser 2009; Shury et al., 2016), it would be advisable to monitor European bison for brucellosis.

Although only a few studies have been performed on brucellosis in European bison, their findings indicate that antibodies to *B. abortus* are generally absent or only present in small numbers (Kita & Anusz, 1991; Krzysiak et al., 2018). It is important that endangered species are monitored for diseases causing abortions. To this end, recent studies have attempted to evaluate the potential of serological tests against brucellosis (e.g. Sánchez-Sarmiento et al., 2020).

The aim of this article was to describe the serum antibody response of European bison to the P22 protein complex and to *Brucella* spp. and related factors: gender, age and locality.

2 | MATERIALS AND METHODS

2.1 | Materials

Between October 2017 and April 2022, a total of 323 blood samples were collected from captive (n = 152) and free-living (n = 159) European bison. Captive European bison were those living in zoos/breeding centres, and free-living, living in different herds in the wild. The animals were derived from different locations, presented in Figure 1. The age of the sampled bison ranged from three months to 25 years (mean 6.5 years). The age of the bison was assessed by experienced veterinarians based on the appearance of horns and tooth eruption (Krasińska & Krasiński, 2017). Age was categorized into three groups: calves (<1-year old), juveniles (1–3-year old), and adults (>3-year old) as described previously (Krasińska & Krasiński, 2017). The samples originated from males (n = 136) and females (n = 175). The material was collected from living animals following immobilization, or dead ones that had been culled or found dead. Immobilization was performed pharmacologically with the use of a Palmer Cap-Chur tranquilization gun (Palmer Cap-Chur Equipment, Inc.) as described previously (Didkowska et al., 2022). For some animals, it was not possible to acquire data regarding age, locality, or sex; therefore, these animals were not considered when performing statistics.

The blood was collected into 6–9 mL sterile tubes with a clot activator with a needle of a diameter of 1.2 mm from a jugular vein (*vena jugularis externa*), or sometimes from the tail vein (*vena caudalis mediana*). In the case of dead animals, blood was collected from the heart and body cavities. The tubes containing the blood were refrigerated and transported immediately to the laboratory. In the laboratory, the

FIGURE 1 Map of Poland indicating the distribution of European bison (*Bison bonasus*) sampling localities included in the study. The Białowieża Forest, Bieszczady Mountains and Borecka Forest locations include both captive and free-living herds, whereas Knyszyńska Forest only includes a free-living herd. All other localities include only captive herds.

blood was centrifuged (3000 *g*, 10 min) and the serum was separated. To minimize the risk of false positive results, all extensively hemolysed samples were excluded from the study. The samples were stored at −20°C until analysis.

2.2 | Enzyme-linked immunosorbent assays (ELISA)

Serum samples were brought to room temperature (RT) and then subjected to two serological tests: an in-house ELISA for anti-P22 antibodies and a commercial test for detecting antibodies against *Brucella* spp.

To detect anti-P22 antibodies, the sera were tested with an in-house indirect P22-ELISA as described previously (Thomas et al., 2019). Positive controls were derived from TB-positive cervids (compatible lesions, *M. bovis* positive culture, OD > 1 at P22-ELISA). Negative controls were derived from TB-negative cervids (the absence of compatible lesions, negative culture, OD < 0.2 at P22-ELISA). Briefly, the ELISA plate was coated with P22 at 10 μg/mL in phosphate-buffered saline solution (PBS; Panreac Química S.L.U.) and incubated overnight at 4°C. After washing with PBS solution containing 0.05% Tween-20 (PBST) (Tween 20; Sigma-Aldrich Quimica SA), the wells were blocked with 5% skimmed milk powder solution in PBS (SM) for 1 h at RT. Then tested sera and controls were added in 1:10 dilution in SM. After 1-h incubation at 37°C, the plates were washed three times with PBST. Next, protein G horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich Química SA) at a concentration of 0.002 mg/mL in PBS was added. After incubation for 1 h at RT, the plates were washed with PBST, and *o*-phenylenediamine-dihydrochloride substrate (FAST OPD; Sigma-Aldrich Química SA) was added. After incubation for 20 min at RT, the reaction was stopped by adding H_2SO_4·3N. The optical density (OD) was read in a spectrophotometer at a wavelength of 492 nm. The result for each sample was expressed as ELISA percentage (*E*%). *E*% was calculated according to the following formula: [sample *E*% = (sample OD/2 × mean of negative control OD)x 100]. The cut-off value of 110 was chosen based on the plotted results (Figure 2). A conservative cut-off of 110 *E*% was chosen to maximize specificity (Infantes-Lorenzo, Moreno et al., 2019).

To detect antibodies against *Brucella* spp., a commercial ELISA R.10.BRU.K3 INgezim BRUCELLA Compacis (Ingenasa) test based on a blocking enzyme immunoassay was performed according to the manufacturer's instructions. The test allows the detection of specific antibodies to the LPS of *Brucella* spp. in multiple animal species (Muñoz et al. 2010). The cut-off value was 40, as instructed. Absorbance was read with a spectrophotometer at 450 nm. According to manufacturers' instructions, the sensitivity and specificity of the test for cattle are 98% and 99.9%.

2.3 | Statistical analysis

The effects of sex, age, captive/free, year of collecting material and locality were assessed by homogeneity tests (two-tailed Fisher's exact test and chi-square test where needed). In addition, 95% confidence

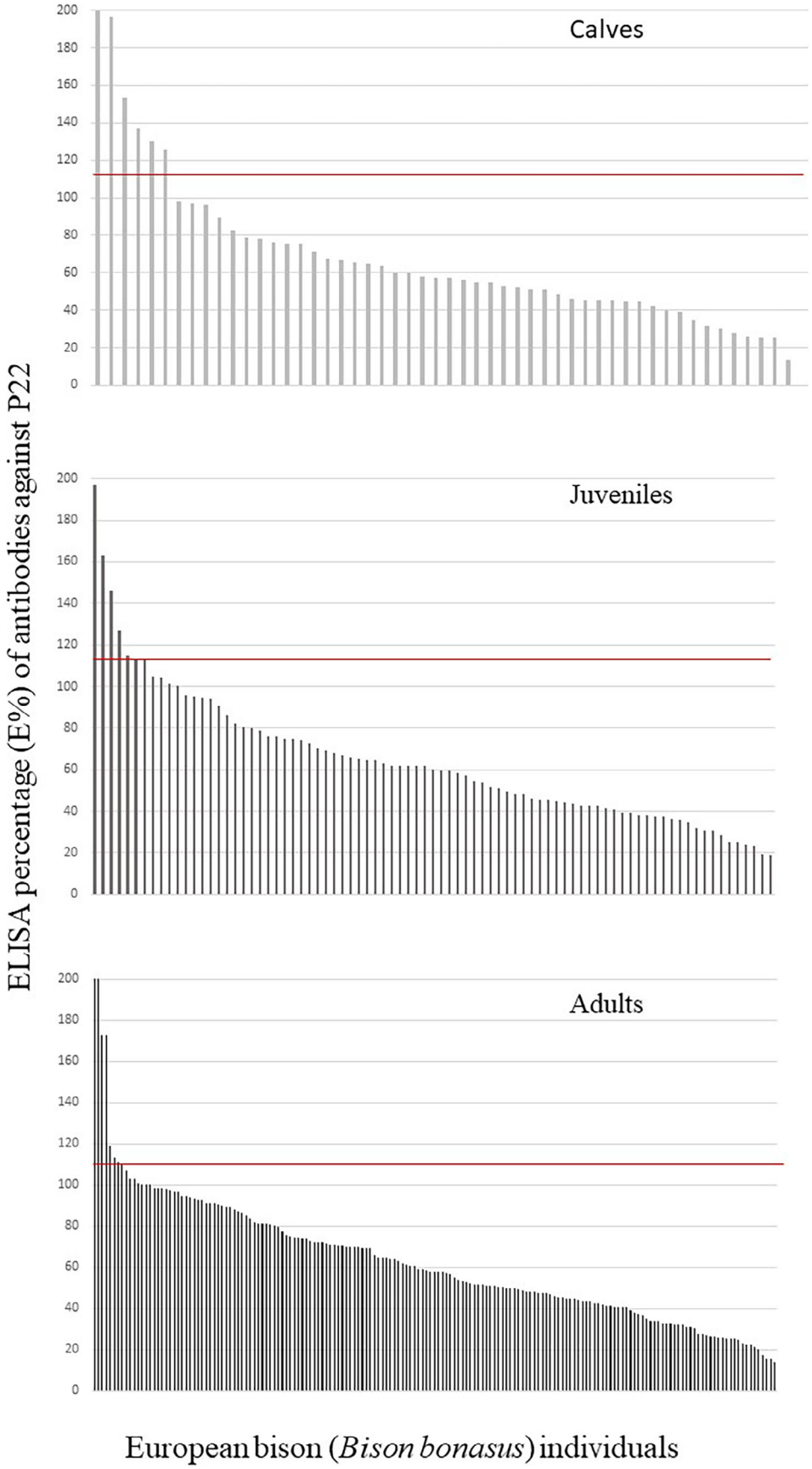

FIGURE 2 ELISA percentage (*E*%) results of Polish European bison (*Bison bonasus*) antibodies against protein complex P22 by age category (calves, juveniles and adults). The solid red line indicates the *E*% value above which the result was considered positive (i.e. cut-off); *E*% = 110.

intervals (95%CI) were calculated for seroprevalence using Sterne's exact method.

2.4 | Ethical statement

No animal was culled or immobilized specifically for this study. *Ante-mortem* sampling (e.g. during translocation, putting the collar) was performed as part of standard veterinary care. The procedures did not require ethical approval according to II Local Ethical Committee for Animal Experiments (Warsaw, Poland).

3 | RESULTS

3.1 | Antibodies against the *M. tuberculosis* complex

Antibodies against P22 were detected in 6.81% (95% CI: 4.44–10.17; 22/323; Table 1) of the tested European bison. The anti-MTC antibody positivity was not significantly different by sex (Fisher's exact test, $p = 0.085$), nor age category (chi-square test, chi-square value = 3.45, degree of freedom = 2, $p = 0.18$). *E*% results had a similar distribution for each age category. For each age category, a few individuals showed high antibody responses (6 of 51 calves, 11.8%; 7 of 83 juveniles, 8.4%; 8 of 170 adults, 4.7%) (Figure 2). The MTC antibody positivity was not significantly different by captive/free-living status (Fisher's exact test, $p = 1$). Data on seroprevalence by year are presented in Table 1.

The seropositive bison were found in 6 of the 13 sampling localities (46.15%), including the four free-living ones (Table 1). In these six localities, at least 19 bison samples were tested (range 19–72); in contrast, in the remaining eight localities, only 1–8 samples were tested. Thus, comparisons among localities were only performed for localities with >19 samples (chi-square test, chi-square value = 3.3, degree of freedom = 5, $p = 0.65$). Seroprevalence slightly increased with sample size (Figure 3).

3.2 | *Brucella* spp. ELISA results

The general seroprevalence for *Brucella* spp. was 35.91% (95% CI: 30.79–41.32; 116/323; Table 2). Anti-*Brucella* spp. antibody prevalence was not significantly different by sex (Fisher's exact test, $p = 0.64$); however, it was significantly different by both captive/free-living status (Fisher's exact test, $p < 0.001$) and age (chi square test, chi-square value = 16.18, degree of freedom = 2, $p = 0.003$). All age categories demonstrated a similar E% distribution, as shown in Figure 4. For each age category, a significant number of individuals were positive: 23 of 51 calves (45.1%), 42 of 83 juveniles (50.6%), and 45 of 170 adults (26.5%). Data on seroprevalence by year are presented in Table 2.

Seropositive bison were found in 12 of the 13 sampling localities (92.31%) (Table 2). The comparisons among localities only included the localities where at least 19 bison samples, that is larger groups, were tested (range 19–72): If the sample size was small, it was impossible to draw reliable conclusions. The anti-*Brucella* spp. seroprevalence was significantly different by locality (chi-square test, chi-square value = 16.383, degree of freedom = 5, $p = 0.006$). The seroprevalence relation to sample size is shown in Figure 3. *Brucella* spp. seroprevalence decreased with sample size.

TABLE 1 The frequencies of seropositive to MTC European bison (*Bison bonasus*) regarding their different characteristics.

Variable	Number seropositive to all samples tested
Population type	**21/311 (6.8%; 95% CI: 4.37–10.08)**
Captive	10/152 (6.6%; 95% CI: 3.52–11.75)
Free-living	11/159 (6.9%, 95% CI: 3.62–12.16)
Sex	**18/311 (5.8%, 95% CI: 3.66–8.96)**
Female	14/175 (8%, 95% CI: 4.76–13.06)
Male	4/136 (2.9%, 95% CI: 1.02–7.25)
Age group	**21/304 (6.9%, 95% CI: 4.47–10.32)**
Calves (<1-year old)	6/51 (11.8%, 95% CI: 5.25–23.36)
Juveniles (1–3-year old)	7/83 (8.4%, 95% CI: 4.03–16.69)
Adults (>3-year old)	8/170 (4.7%, 95% CI: 2.22–9.03)
Sampling year	
2017	0/12 (0%)
2018	10/97 (10.3%, 95% CI: 5.51–17.93)
2019	4/47 (8.5%, 95% CI: 2.96–19.99)
2020	2/45 (4.4%, 95% CI: 0.8–15.20)
2021	2/84 (2.4%, 95% CI: 0.43–8.16)
2022	2/38 (5.3%, 95% CI: 0.95–17.99)
Localities	
Bałtów (captive)	**0/2 (0%)**
Białowiska Forest	**5/72 (6.9%, 95% CI: 2.78–15.13)**
Captive	3/38 (7.9%, 95% CI: 2.19–20.79)
Free-living	2/34 (5.9%, 95% CI: 1.06–20.11)
Bieszczady Mountains	**5/67 (7.5%, 95% CI: 2.99–16.25)**
Captive	2/16 (12.5%, 95% CI: 2.27–37.16)
Free-living	3/51 (5.9%, 95% CI: 1.63–16.36)
Borecka Forest	**2/45 (4.4%, 95% CI: 0.8–15.2)**
Captive	0/8 (0%)
Free-living	2/37 (5.4%, 95% CI: 0.97–18.48)
Gdańsk zoo (captive)	**0/2 (0%)**
Gołuchów (captive)	**0/7 (0%)**
Kiermusy (captive)	**0/8 (0%)**
Międzyzdroje (captive)	**0/1 (0%)**
Niepołomice (captive)	**3/19 (15.8%, 95% CI: 4.45–39.19)**
Pszczyna (captive)	**2/38 (5.3%, 95% CI: 0.95–17.99)**
Knyszyńska Forest (free-living)	**4/37 (10.8%, 95% CI: 3.78–25.38)**
Ustroń (captive)	**0/4 (0%)**
Warszawa zoo (captive)	**0/8 (0%)**

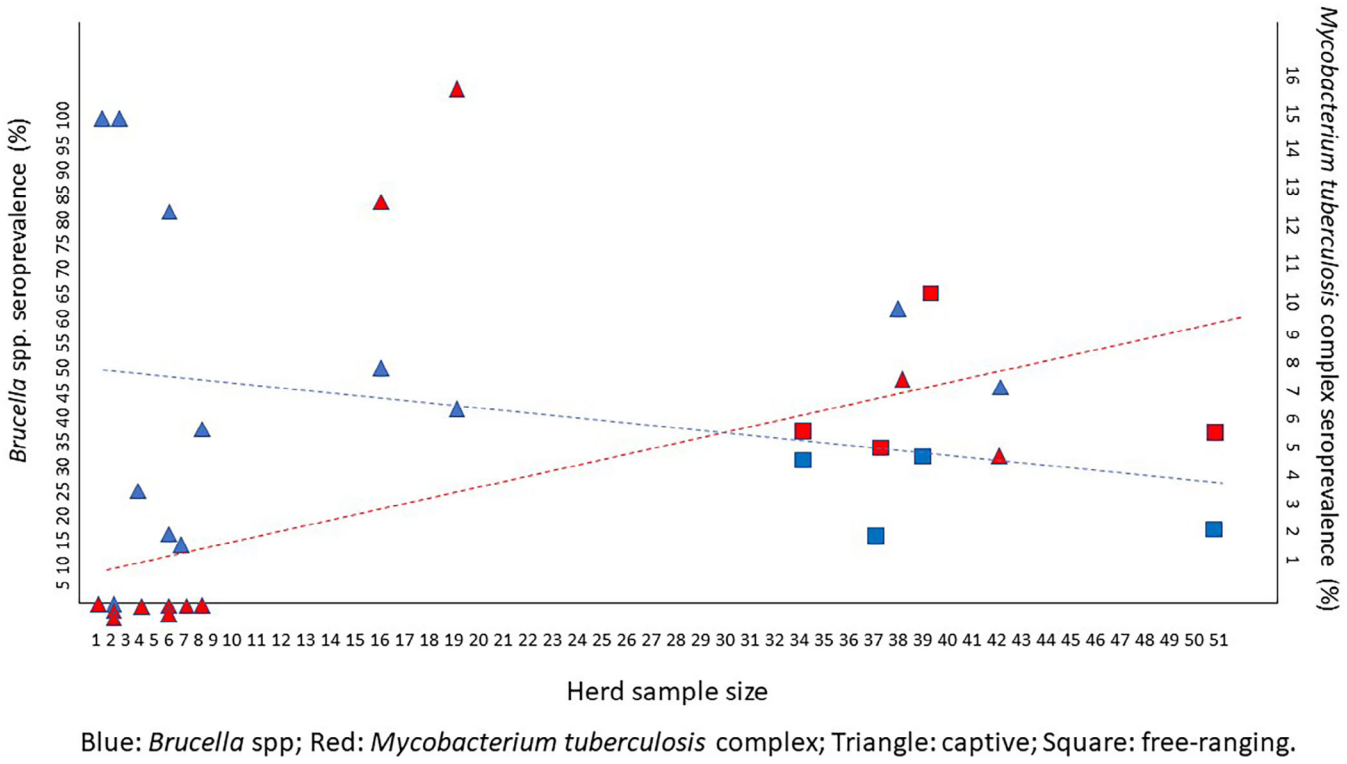

FIGURE 3 The *Mycobacterium tuberculosis* complex (MTC) and *Brucella* spp. seroprevalence in Polish European bison (*Bison bonasus*) in relation to sample sizes. Blue colour: *Brucella* spp. seroprevalence; red colour: MTC (protein complex P22) seroprevalence; triangle: captive herd; square: free-living herd. Dashed regression lines are plotted for MTC and *Brucella* spp. antibody prevalence.

4 | DISCUSSION

Our findings indicate a 6.81% prevalence of anti-MTC antibodies and a 35.91% prevalence of anti-*Brucella spp.* antibodies in European bison from Poland. This is the first confirmation of an antibody response to P22 in bison. The correct diagnosis of TB still presents a challenge in this species, so every new diagnostic tool is desirable (Didkowska, Krajewska-Wędzina et al., 2021). In protected species, there is also a greater need to monitor diseases that can cause reproductive alterations, such as brucellosis, as these can reduce the size of the population and thus its genetic variability, which is already low in European bison (Druet et al., 2020). It should be emphasized that our findings are particularly valuable as they were obtained from a large group of animals over several years; this was necessary as the material was taken from a protected species. This study indicates the current trends regarding the diagnostic value of P22 in European bison and microbiological tests toward *Brucella* spp., highlighting the need for further research.

To confront the problem posed by TB in Polish European bison herds, efforts have been made to find cheap, convenient, and highly sensitive diagnostic tools by improving sample collection (Didkowska et al., 2020) and imaging methods (Didkowska et al., 2021d). Even though culture remains the gold standard for TB diagnosis, it is not a convenient tool for motoring wildlife (Thomas et al. 2021). Microbiological tests are quite expensive, require long wait times and entail difficulties in collecting suitable antemortem material due to periodic shedding (Cousins & Florisson, 2005). Therefore, some studies suggest using serological TB monitoring as an alternative (Thomas et al., 2021). However, few have evaluated the potential of TB serological assays in European bison. Didkowska, Krajewska-Wędzina et al. (2021) found *Mycobacterium caprae*-infected European bison to demonstrate responses to the antibodies ESAT-6, CFP-10, MPB70, MPB83, CFP10/ESAT-6, MPB70/MPB83, DID65 and B-PPD in dual-path platform, multi-antigen print immunoassay (MAPIA) and IDEXX *M. bovis* Ab ELISA (IDEXX Laboratories, Inc.); however, this study (Didkowska, Krajewska-Wędzina, et al., 2021) was performed on a limited number of samples, including only four positives. More recently, serological monitoring based on MPB70 and MPB83 has shown much lower MTC antibody seroprevalence in European bison (1.96%; Krzysiak et al., 2022).

This is the first serological study to use P22 in European bison. Considering Poland that is generally free of brucellosis and TB in cattle, we used caution when interpreting the results. We also considered the lack of bison reference sera and that field samples, as opposed to experimental infection derived ones, can yield lower specificity (Ferreras-Colino et al., 2022). Therefore, we used a conservative cut-off value of 110 instead of the commonly used cut-off value of 100 (Thomas, Infantes-Lorenzo, Moreno, Cano-Terriza, et al., 2019). The cut-off value of 110 was chosen based on the plotted results (Figure 2). With a cut-off of 110, the MTC seroprevalence in European bison

TABLE 2 The frequencies of seropositive to *Brucella spp.* European bison (*Bison bonasus*) regarding their different characteristics.

Variable	Number seropositive to all samples tested
Population type	**113/311 (36.3%; 95% CI: 31.84–40.99)**
Captive	75/152 (49.3%; 95% CI: 41.44–57.52)
Free-living	38/159 (23.9%; 95% CI: 17.87–31.09)
Sex	**112/311 (36%, 95% CI: 30.86–41.63)**
Female	61/175 (34.9%; 95% CI: 27.96–42.27)
Male	51/136 (37.5%; 95% CI: 29.74–45.94)
Age group	**110/304 (36.2%, 95% CI: 30.91–41.76)**
Calves (≤1-year old)	23/51 (45.1%; 95% CI: 31.82–58.87)
Juveniles (2–3-year old)	42/83 (50.6%; 95% CI: 39.72–61.49)
Adults (≥4-year old)	45/170 (26.5%; 95% CI: 20.24–33.78)
Year of colleting blood	
2017	7/12 (58.3%, 95% CI: 29.40–81.89)
2018	45/97 (46.4%, 95% CI: 36.56–56.72)
2019	15/47 (31.9%, 95% CI: 20–46.78)
2020	10/45 (22.2%, 95% CI: 11.84–36.56)
2021	28/84 (33.3%, 95% CI: 26.39–44.02)
2022	11/38 (28.9%, 95% CI: 16.67–45.29)
Localities	
Bałtów (captive)	**2/2 (100%)**
Białowieża Forest	**35/72 (48.6%, 95% CI: 36.95–60.74)**
Captive	24/38 (63.2%, 95% CI: 46.53–77.38)
Free-living	11/34 (32.4%, 95% CI: 18.85–50)
Bieszczady Mountains	**17/67 (25.4%, 95% CI: 16.26–37.24)**
Captive	8/16 (50%, 95% CI: 27.20–72.80)
Free-living	9/51 (17.5%. 95% CI 9.26–30.27)
Borecka Forest	**9/45 (20%, 95% CI: 10.57–34.30)**
Captive	3/8 (37.5%, 95% CI: 11.12–71.07)
Free-living	6/37 (16.2%, 95% CI: 7.31–32.19)
Gdańsk-zoo (captive)	**0/2 (0%)**
Gołuchów (captive)	**0/2 (0%)**
Kiermusy (captive)	**1/8 (12.5%, 95% CI: 0.64–50)**
Międzyzdroje (captive)	**1/1 (100%)**
Niepołomice (captive)	**8/19 (42.1%, 95% CI: 22.19–65.51)**
Pszczyna (captive)	**18/38 (47.4%, 95% CI: 31.35–63.28**
Knyszyńska Forest (free-living)	**12/37 (32.4%, 95% CI: 18.49–48.64)**
Ustroń (captive)	**2/4 (50%, 95% CI: 9.77 to 90.23)**
Warszawa-zoo (captive)	**2/8 (25%, 95% CI: 4.64–63.53)**

was 6.81% (95% CI: 4.44–10.17; 22/323) and varied from 0% to 8.8% depending on the sampling year (Table 1).

A previous study on TB microbiological monitoring carried out from 2017 to 2019 isolated *M. caprae* from 4.44% (4/90) European bison; however, all animals in the study derived from a single captive herd in central Poland (Didkowska, Orłowska, et al., 2021). Animals from this herd have not been tested in the present study. In another region of Poland where TB is being confirmed in wildlife—Bieszczady Mountains (Krajewska-Wędzina et al., 2023), there was no higher seroprevalence than in other free-living sites (Table 2). As such, our findings are higher than expected, and the occurrence of MTC in different localizations is surprising. This can be explained in several ways. The first explanation may be that the P22-ELISA was characterized by high Se; however, it would be necessary to examine the selected animals microbiologically to confirm this. If the test turned out to be very sensitive and specific, it would be a great diagnostic tool for European bison.

Alternatively, a cross-reaction may be present with atypical mycobacteria, a well-known phenomenon in TB diagnostics (Jenkins et al., 2018; Raffo et al., 2020). However, Didkowska, Orłowska et al. (2021) reported that atypical mycobacteria were isolated from only 2.22% (2/90) of tested European bison. It should be highlighted that P22-ELISA offers fair to good specificity (Sp) in detecting antibodies against MTC, depending on the species, and so it also would not necessarily be perfect for European bison. In other ruminants, the tested Sp was 92.5%–99.4% in cattle, 30.9%–78% in goats, 94.4%–100% in sheep and 99.0% in red deer (Thomas, Infantes-Lorenzo, Moreno, Romero, et al, 2019). Another hypothesis is that bison could have contact with other mycobacteria belonging to the MTC, such as *Mycobacterium microti*. *M. microti* infections are increasingly reported in free-living wild animals in Europe (Ghielmetti et al., 2021; Michelet et al., 2021; Pérez de Val et al., 2019; Tagliapietra et al., 2021).

In contrast to most previous studies (Joly & Messier, 2004; Varela-Castro et al., 2020), our findings indicate that the MTC seroprevalence is not significantly different by sex and age. This may be influenced by the behaviour of bison and their gregarious nature (Krasińska & Krasiński, 2017). In addition, the MTC seroprevalence is not significantly different by status and locality. In the case of locality, this is surprising as in previous years, TB was microbiologically confirmed only in two captive herds, namely one herd in Smardzewice, central Poland and another (Wolisko) in Borecka Forest, and in two free-living herds, namely in the Bieszczady Mountains and Borecka Forest (Krajewska et al., 2011, 2016; Welz et al., 2005); therefore, it was anticipated that most positive results would be identified in these locations. In addition, localities with the highest seroprevalence (Białowieża Forest, Bieszczady Mountains, Niepołomice and Pszczyna) are not adjacent to each other (Figure 1, Table 1). In Poland, free-living bison populations are rarely spatially connected with each other (Perzanowski et al., 2019), and new areas are rarely colonised (Ziółkowska et al., 2016); in such cases, therefore, there is probably no bison-driven pathogen transmission between localities. Another important consideration regarding the effect of locality is that, in the case of free-living herds in Poland, the carrying capacity of forest complexes is often exceeded, and so captive herds generally have higher densities (Olech & Perzanowski, 2014). Alternatively, the increase of seroprevalence with sample size (Figure 3) might just point at a higher probability of finding cross-reactions in larger samples.

Previous reports confirmed a sporadic occurrence of *B. abortus* antibodies in European bison (Kita & Anusz, 1991; Krzysiak et al., 2018).

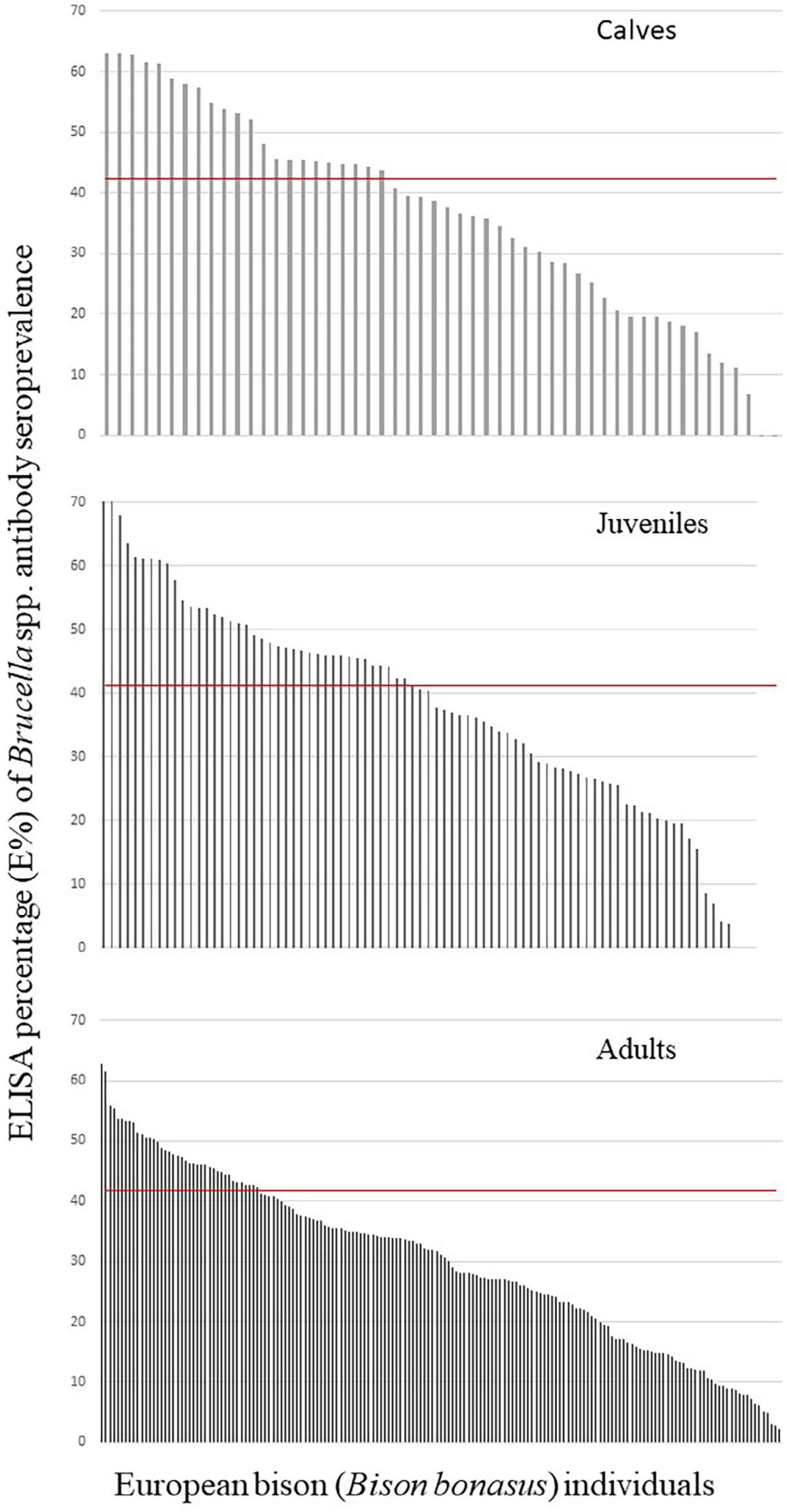

FIGURE 4 ELISA percentage (*E*%) results of *Brucella spp*. antibodies by age category (calves, juveniles and adults) in European bison (*Bison bonasus*) from Poland. The solid red line indicates the *E*% value above which the result was considered positive (i.e. cut-off): *E*% = 40.

The 35.91% (95% CI: 30.79–41.32; 116/323) seroprevalence of *Brucella spp*. identified herein suggests that the Polish European bison population has had contact with these bacteria. The annual seroprevalence has varied significantly over time. The period from 2017 to 2020 saw a downward trend, and this was then followed by a relatively constant level (Table 2). Due to the epizootic situation in Poland, with no case of *B. abortus* being reported since its eradication in 1980, it is likely that any such had been with other species of *Brucella*. Indeed, *Brucella suis* biovar 2 has been isolated from Polish cattle with positive serological results (Szulowski et al., 2013a), and this variant has also been confirmed in Polish wildlife, mainly wild boar (*Sus scrofa*) (Szulowski et al., 2013b). It would be advisable that microbiological monitoring is continued, as *Brucella spp*. can also be a potential threat to European bison, especially in the localities where seroprevalence was high, such as Pszczyna and Białowieża (Table 2).

Our findings indicate that the *Brucella* spp. seroprevalence was significantly different by age; however, unlike most studies, (Alhamada et al., 2017; Asgedom et al., 2016; Chaka et al., 2018), the highest seropositivity was noted in juveniles, not in adults (Table 2). It is possible that in such cases, the animal may have made first contact with the pathogen without the protection offered by colostrum antibodies and these would demonstrate the highest serological response. In American bison, most calves were seronegative by 5 months, and the highest seroconversion was noted at 2 years (Rhyan et al., 2009). In the present study, seropositivity was also more likely in captive than free ranging bison (Table 2). This may be due to the higher animal densities characteristic of captive herds. Localities were also found to have a significant effect, which might be related to a greater presence of bacteria in the areas concerned. Finally, sex was found to have no such effect, and this is in-line with previous studies on American bison, where sex had little influence on the prevalence of *B. abortus* antibodies (Scurlock & Edwards, 2010).

This study has some limitations. First, random sampling was not possible. In addition, the tests were not specially designed for European bison. Furthermore, as the true status of the bison remains unknown, further microbiological testing should be included in later studies. In addition, it should be considered that the controls for MTC seroprevalence testing were taken from a different species (deer). What is more, even though following the recommendations of the OIE-World Organisation for Animal Health, complement fixation tests and serum agglutination tests are being replaced by more sensitive and specific tools for screening brucellosis in mammalian species, including indirect ELISA and fluorescence polarization assay (Greiner et al., 2009; Muñoz et al., 2010; Ragan et al., 2013); most brucellosis serological tests intended for use in wildlife still need validation and standardization (Dadar et al., 2021). One further limitation for *Brucella* sp. results are cross-reactions with *Yersinia enterocolitica* O:9, *Stenotrophomonas maltophilia*, *Salmonella urbana*, *Escherichia coli* O:157, or non-specific reactions associated with the condition of the animal (Ducrotoy et al., 2016; Nielsen et al., 2004).

The obtained results for the MTC and *Brucella* spp. serological surveys raise another important problem. As European bison can be a reservoir of other diseases, care should be taken when introducing them into new areas, as in Spain (Castillo et al., 2019) or Romania (Dănilă et al., 2022).

5 | CONCLUSIONS

This study is the first to report an antibody response to the P22 multiprotein complex in European bison. MTC serological survey showed that 6.81% of the examined European bison are seropositive, which is higher than noted in previous studies using specific *M. bovis* antigens. In addition, more than 1/3 of the tested European bison were seropositive against *Brucella* spp., which shows another direction for future research. Our findings suggest that brucellosis and TB may represent an emerging threat to the largest European bison population. As Poland is TB and brucellosis free, obtaining many positive results might be due to cross-reactions. In the future, it would be worth testing European bison in Poland for pathogens that can cause false positive results.

AUTHOR CONTRIBUTIONS

Conceptualization; data curation; formal analysis; investigation; project administration; resources; writing—original draft: Anna Didkowska. *Data curation; formal analysis; investigation; resources; visualization; writing—review & editing*: Elisa Ferreras. *Data curation; funding acquisition; project administration; resources; writing—review & editing*: Wanda Olech. *Investigation*: Huggette Gloddy. *Supervision; writing—review & editing*: Krzysztof Anusz. *Methodology; supervision; writing—review & editing*: Jose Antonio Infantes-Lorenzo. *K conceptualization; funding acquisition; methodology; supervision; visualization; writing—original draft; writing—review & editing*: Christian Gortazar.

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CONFLICT OF INTEREST STATEMENT

Authors declared no conflicts of interest.

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DATA AVAILABILITY STATEMENT

Data are available in Department of Food Hygiene and Public Health Protection, Institute of Veterinary Medicine, Warsaw University of Life Sciences (SGGW).

ETHICS STATEMENT

No animal was culled or immobilized specifically for this study. *Antemortem* sampling (e.g. during translocation, putting the collar) was performed as part of standard veterinary care. The procedures did not require ethical approval according to II Local Ethical Committee for Animal Experiments (Warsaw, Poland).

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