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Assessing the impact of feline immunodeficiency virus and bovine tuberculosis co-infection in African lions

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Bovine tuberculosis (BTB), caused by *Mycobacterium bovis*, is a disease that was introduced relatively recently into the Kruger National Park (KNP) lion population. Feline immunodeficiency virus (FIV_{ple}) is thought to have been endemic in lions for a much longer time. In humans, co-infection between *Mycobacterium tuberculosis* and human immunodeficiency virus increases disease burden. If BTB were to reach high levels of prevalence in lions, and if similar worsening effects would exist between FIV_{ple} and BTB as for their human equivalents, this could pose a lion conservation problem. We collected data on lions in KNP from 1993 to 2008 for spatio-temporal analysis of both FIV_{ple} and BTB, and to assess whether a similar relationship between the two diseases exists in lions. We found that BTB prevalence in the south was higher than in the north (72 versus 19% over the total study period) and increased over time in the northern part of the KNP (0–41%). No significant spatio-temporal differences were seen for FIV_{ple} in the study period, in agreement with the presumed endemic state of the infection. Both infections affected haematology and blood chemistry values, FIV_{ple} in a more pronounced way than BTB. The effect of co-infection on these values, however, was always less than additive. Though a large proportion (31%) of the lions was co-infected with FIV_{ple} and *M. bovis*, there was no evidence for a synergistic relation as in their human counterparts. Whether this results from different immunopathogeneses remains to be determined.

Keywords: feline immunodeficiency virus; bovine tuberculosis; *Mycobacterium bovis*; lion; co-infection; prevalence

1. INTRODUCTION

Both feline immunodeficiency virus (FIV_{ple}) and *Mycobacterium bovis*, causing bovine tuberculosis (BTB), are found in the lion (*Panthera leo*) population in the Kruger National Park (KNP), South Africa. FIV_{ple} is an endemic pathogen in many lion populations in eastern and southern Africa [1–5], and its presence may even date back as far as the species divergence of the genus *Panthera* [6,7]. Differences have been found recently in the CD4^{+}/CD8^{+} T-cell subset [4] and the prevalence of AIDS-defining conditions [8] in FIV_{ple}-infected lions when compared with non-infected lions, contradicting studies that did not find pathological effects associated with FIV_{ple} infection [7,9–11]. This may also depend on differences in pathogenicity between FIV_{ple} subtypes [12]. Common haematological and blood chemistry changes that are found in FIV-infected domestic cats are lymphopoenia, leucopoenia and neutropoenia, anaemia, hyperproteinaemia and hyperglobulinaemia [13–17]. In lions, FIV_{ple} is associated with dehydration and abnormal red blood cell parameters, e.g. anaemia, depressed serum albumin and elevated liver enzymes, total protein, globulin and gamma globulin [8].

Mycobacterium bovis was introduced in the southeast corner of the KNP in the 1960s [18], spreading from infected cattle to buffaloes. The first case of lion BTB was found in 1995, probably resulting from consumption of infected buffalo carcasses [19]. A prevalence of almost 80 per cent of the lion population in the south of the KNP was reported in 2000 [20]. Limited information is available about the effect of (B)TB on haematologic and blood chemistry values, but in humans with minimal active tuberculosis a significant rise in the gamma globulin fraction with a corresponding decrease in albumin was found. In far advanced cases, all globulins were increased, but the mean total protein did not differ from the normal value [21]. In a report of a BTB-infected lion, leukocytosis, monocytosis, anaemia, neutrophilia, hypoalbunemia and hyperglobulinemia were found [22].

In humans, one of the most well-known pathogen–pathogen interactions is that between human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis*. HIV is the strongest known risk factor for TB, affecting on the immunity by T-cell depletion [23]. On the other hand, TB can accelerate the progression of HIV [24]. These synergistic interactions magnify the burden of disease of both infections [23,25,26]. In animals, pathogen–pathogen interactions are also described, for example, between *Babesia* and canine distemper virus in lions [27] or between various infectious agents in voles [28].

Interactions may be synergistic or antagonistic to one or both of the infectious agents [29], which may be explained by a variety of mechanisms influencing host susceptibility, pathogenicity or infectiousness in both positive and negative ways [28–31].

Lions are listed as a vulnerable species by the International Union for Conservation of Nature (http://www.iucnredlist.org/apps/redlist/details/15951/0) and the relatively recent introduction of *M. bovis* in a population where FIV_{ple} is highly prevalent, could be an even more serious threat to lion conservation if the two infections would enhance each other's effects as their counterparts do in humans. Although it has been reported that 53 per cent of the lions in the southern half of KNP are co-infected with FIV_{ple} and *M. bovis* [32], little is known about the effects of their interaction. Previous literature on FIV_{ple} and *M. bovis* infection in lions in the KNP is scarce and has often only been anecdotal, using small groups of animals. We collected data from 1993 to 2008, which resulted in a unique dataset of 669 lions that was used: (i) to assess what variables (area, period, age, sex and body condition) are related with FIV_{ple} and BTB infection in lions, studied by a multivariable logistic regression model, and (ii) to assess the pathogen–pathogen interaction of FIV_{ple} and BTB in lions.

For the latter, we used a sub-group of 205 lions that had been subjected to diagnostic tests for both infections. Body condition and haematological and blood chemistry values, which were deemed relevant based on literature [17,22,33], were used as dependent variables in general linear models to assess this potential interaction.

2. MATERIAL AND METHODS

(a) *The Kruger National Park*
The KNP is a partly fenced, wooded savannah covering about $20\,000\ km^2$. The total KNP lion population is estimated to be about 1600–1700 [34]. For the purpose of this analysis, the KNP was divided into three regions based on the prevalence of *M. bovis* in buffaloes, namely High, Medium and Low Prevalence Zones (HPZ, MPZ, LPZ; roughly corresponding to the southern, central and northern part of the park), separated by the Sabie River (south-central) and the Olifants River (central-north). Prevalences of *M. bovis* in buffaloes in 1998 were, respectively, 38.2 per cent, 16.0 per cent and 1.5 per cent [35]. Data obtained from lions from adjacent game reserves with open access to the KNP were included in the analyses, according their locations.

BTB in the KNP is not controlled. This makes the ecosystem unique, as many other ecosystems with *M. bovis* presence have a test-and-removal [36,37] or culling strategy [37].

(b) *The animals*
Most lions were captured with call-up stations in designated areas in the southern, central and northern part of the park, which were known to have lions, based on ranger information. These stations were randomly distributed as much as logistic considerations allowed (figure 1*a*). About 25 per cent of the study lions were brought to the Veterinary Station as emaciated or problem lions.

Before handling, all lions were immobilized with a combination of tiletamine and zolazepam (Zoletil 100, Virbac). Venous blood samples were obtained from the medial saphenous vein as soon as possible after anaesthesia in heparin,

EDTA and serum Vacutainer tubes, which were kept at ambient temperature and were processed within preferably 8 h, but maximum 24 h. Serum was collected and stored at $-20°C$. All lions in this study were aged by examining dental attrition according to Smuts *et al.* [38]. Body condition score (BCS) was assessed according to criteria that were determined beforehand and ranged from 5 (excellent) to 1 (very poor; table 1). Lions were micro-chipped and were given a unique brand so the animals could be recognized at future captures. Owing to the higher lion density in the south (compared with the north) as well as the location of the veterinary staff headquarters in the south of KNP, almost twice as many study lions originated from the south, when compared with the central and northern areas.

(c) *Sample collection*
(i) *Bovine tuberculosis status*
The BTB status of individual lions was determined by performing the Single Intradermal Cervical Test (SICT/skin test) as described by Keet *et al.* [32]. A lion was considered BTB positive when 3 days after intradermal administration of bovine tuberculin, the skin swelling was 2 mm or larger, irrespective of the response to the avian tuberculin. The SICT has a sensitivity and specificity of, respectively, 86.5 per cent and 81 per cent. The SICT appears not to be influenced by FIV_{ple}, in contrast to the tuberculin skin test in humans, which is affected by HIV infection [32,39].

(ii) *Feline immunodeficiency virus status*
Serum samples were tested for FIV_{ple}–specific antibodies at the Department of the Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, using a protocol described by van Vuuren *et al.* [40]. The sensitivity of the enzyme-linked immunosorbent assay, when using the Western blot as the gold standard, is 78.6 per cent and the specificity 100 per cent [40].

(iii) *Haematology*
Haematology analysis was performed in the KNP with a Coulter AcT diff analyzer (Beckman Coulter).

(iv) *Blood chemistry*
Blood chemistry analysis was conducted with a NExCT/VetEX (Bayer Health) at The Clinical Pathology Laboratory, Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria.

(d) *The dataset*
From 1993 to 2008, a large dataset has been established, consisting of 669 lions from the KNP and adjacent game reserves. Descriptions of the dataset can be found in the electronic supplementary material, tables and figures S1–S5. Small, specific subsets of this extensive dataset have been used in various studies in the past [1,5,20,32,41]. Not all information was available for each animal, and table 2 gives an overview of the cross-sectional data that has been used for the analyses in this study.

(e) *Data analysis*
As age and BCS are subjective characteristics and age is increasingly difficult to determine in the older age classes, these nominal variables were recoded to binary variables to facilitate the modelling, resulting in the following variables: age (binary, less than or equal to 36 months or more than 36 months, reference level: less than or equal to 36 months), BCS (binary, BCS 1,2,3 and BCS 4,5, reference level: BCS 4,5), sex (binary, reference level: female), area

Figure 1. (*a*) Capture locations of lions, covering 93% of the lions from the dataset, of which exact locations were known. Multiple lions may have been captured at one location. Locations outside KNP indicate escaped lions. (*b*) Pie charts representing the numbers of lions in the four co-infection groups ($n = 205$) in the three different areas of the KNP. Size indicates the number of lions captured. Black, $FIV_{ple}^{+}BTB^{+}$; dark grey, $FIV_{ple}^{-}BTB^{+}$; light grey, $FIV_{ple}^{+}BTB^{-}$; off-white, $FIV_{ple}^{-}BTB^{-}$.

Table 1. Definitions of the different BCSs.

BCS	definition
5. excellent	hindquarters well rounded and no ribs showing; general appearance in relation to posture and coat sheen excellent
4. good	hindquarters rounded, but ribs showing slightly
3. fair	hindquarters angular in appearance and ribs well defined
2. poor	pelvic bones and pelvic-femoral joint prominent and ribs protruding. Tail root is sunken in. The dorsal spinae of the vertebrae becomes apparent
1. very poor	skeletal details clearly visible and general appearance, posture and coat condition deteriorated. The dorsal and lateral processes of the vertebrae clearly visible

(nominal, three levels (HPZ, MPZ and LPZ), reference level: MPZ) and period (nominal, three levels (1 : 1993–1998; 2 : 1999–2002 and 3 : 2003–2008), reference level: 1).

To assess which variables potentially influenced FIV_{ple} and BTB infection (both binary, reference level: negative),

the following multivariable logistic regression models were used:

$$FIV = \mu + age + BCS + sex + area + period + e, \qquad (2.1a)$$

Table 2. An overview of the lion dataset ($n = 669$) and the number of lions that were available for the different analyses. (A difference has been made for lions either captured at a call-up station (cal), lions that were brought to the veterinary station (vet) or lions with an unknown capture method (unk). Ht, haematocrit; WBC, white blood cell count; TSP, total serum protein; Alb, albumin; Glob, globulin; A/G ratio, albumin/globulin ratio; gamma glob, gamma globulin.)

	no. lions	
	total (cal.; vet.; unk)	with BTB and FIV$_{ple}$ result (cal.; vet.)
BTB result	240 (191; 49; 0)	205 (165; 40)
FIV$_{ple}$ result	561 (415; 137; 9)	205 (165; 40)
haematology (Ht, WBC)	Ht: 435 (320; 105; 10)	Ht: 164 (124; 40)
	WBC: 375 (260; 105; 10)	WBC: 163 (123; 40)
blood chemistry (TSP, Alb, Glob, A/G ratio, gamma glob)	all: 500 (358; 133; 9)	all: 172 (132; 40)

and

$$BTB = \mu + age + BCS + sex + area + period + e, \qquad (2.1b)$$

where μ represents the intercept, e residual error and where the other variables are coded as mentioned earlier. The e^{β} was used to calculate the odds ratio [42].

A total of 205 lions that had been subjected to both FIV$_{ple}$ and BTB-specific diagnostic tests were used to study the potential synergistic effects of the infectious agents on the body condition and seven blood parameters. To study the effects of FIV$_{ple}$ and *M. bovis* and their interaction on BCS, the following multivariable logistic regression model was used:

$$BCS = \mu + FIV + BTB + FIV \times BTB + e, \qquad (2.2)$$

where μ represents the intercept, e residual error and BCS, FIV and BTB are binary variables coded as defined earlier.

Blood parameters were selected that were deemed relevant in the literature for either or both of the infections: haematocrit (Ht), white blood cell count (WBC), total serum protein (TSP), albumin (ALB), globulin, gamma globulin and albumin : globulin (A : G) ratio [17,22,33,43]. To assess the effects of FIV$_{ple}$ and *M. bovis* and their interaction, as well as other potentially influencing variables on these parameters, the following full general linear regression model was used:

$$\begin{aligned} bloodparameter = &\mu + FIV + BTB + FIV \times BTB \\ &+ age + BCS + sex + area + e, \end{aligned} \qquad (2.3)$$

with variables as defined earlier.

The Akaike Information Criterion (AIC) was used to rank the models [44], following backward stepwise selection. Models with smaller values of the raw AIC values were preferred for each step, unless the difference between the two smallest models was less than 2. In those situations, the principle of Occam's razor was used to select the 'simplest' model with the least parameters, i.e. with the highest information gain [45,46], following standard statistical methods [42]. All models were checked for normality and homoscedasticity. For data analysis, R v. 2.15.0 was used (including packages Hmisc and modeest) [47].

3. RESULTS

The models assessing the dependency of FIV$_{ple}$ and BTB on the variables area, period, age, sex and BCS, showed that FIV$_{ple}$ positivity was significantly related to sex

Table 3. Results of the two final logistic regression models with dependent variables FIV$_{ple}$ and BTB. (Odds ratios are given with their 95% confidence interval between brackets. n.s., these variables were not statistically significant in the model (based on the AIC) and were not included in the final model.)

	FIV$_{ple}$	BTB
sex (M)	1.5 (1.0−2.2)	n.s.
age (more than 36 months)	3.5 (2.4−5.1)	n.s.
BCS (1,2,3)	1.7 (1.1−2.6)	n.s.
area		
LPZ/north	n.s.	0.1 (0.0−0.3)
MPZ/central	n.s.	1
HPZ/south	n.s.	2.2 (1.2−4.3)
period		
period 1 (1993−1998)	n.s.	1
period 2 (1999−2002)	n.s.	0.8 (0.4−1.6)
period 3 (2003−2008)	n.s.	3.4 (1.2−11.1)

(males were more likely to be FIV$_{ple}$ positive), higher age and lower BCS. *Mycobacterium bovis* infection was related to the area (lions were more likely to be infected in the south of the park) and the period (lions were more likely to be infected in the last time period; table 3). Additional information can be found in the electronic supplementary material, tables and figures S7−S10).

The 205 lions with test results for both FIV$_{ple}$ and BTB were divided into four groups: FIV$_{ple}^{+}$BTB^{+}, FIV$_{ple}^{-}$BTB^{+}, FIV$_{ple}^{+}$BTB^{-} and FIV$_{ple}^{-}$BTB^{-}. No significant differences were found between the observed group sizes and the expected group sizes based on the FIV$_{ple}$ and BTB prevalences (see the electronic supplementary material, table S7a); neither for the total KNP nor for the three areas separately (figure 1b, for details, see the electronic supplementary material, table S6). This may indicate that there is no significant relationship between the pathogens, but may also result from a balance between an increased incidence and an increased mortality caused by co-infection of FIV$_{ple}$ and BTB, and should thus be interpreted with caution. When assessing the effects of FIV$_{ple}$ and BTB on the BCS, the co-infected lions had a slightly higher percentage of lions with a low BCS (figure 2) compared with the other groups, but
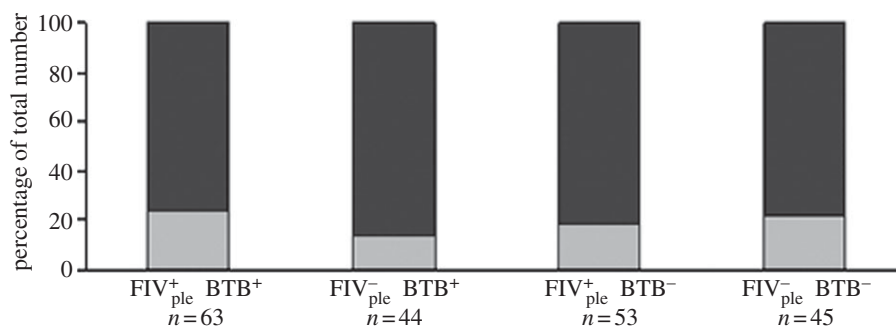
Figure 2. Lions ($n = 205$) grouped according to their FIV$_{ple}$ and BTB status and their BCS. Light grey, lions with a low BCS (BCS 1,2,3). Dark grey, lions with a high BCS (BCS 4,5).

this was not statistically significant (the logistic regression model confirmed this finding) equation (2).

Linear regression models to determine associations with FIV$_{ple}$ and/or *M. bovis* infection showed that in co-infected animals there is a statistically significant antagonistic interaction between FIV$_{ple}$ and *M. bovis* for three of the seven blood parameters, resulting in less deviation than expected from the sum of the individual effects (table 4). Effects of FIV$_{ple}$ were more pronounced, except for the hyperproteinaemia. Neither FIV$_{ple}$ nor BTB, nor their interaction had a significant influence on the WBC or the gamma globulins. Comparisons with reference values from zoo lions can be found in the electronic supplementary material, table S11.

4. DISCUSSION

Infectious diseases are an important issue in conservation, having the power to dramatically influence the dynamics of wildlife species and populations [27,48,49], especially in dwindling populations [50,51]. Because it is difficult to determine their effect, it is common to deal with each infectious disease as a separate entity caused by a single pathogen [31]. However, in nature, multiple pathogens are often encountered simultaneously by individual hosts, which can lead to additive, antagonistic or synergistic effects on hosts and pathogens [52]. It was expected that co-infection with FIV$_{ple}$ and *M. bovis* in lions would have a synergistic effect, similar to the human counterparts [25]. However, although there was an indication that co-infected lions more often had a low BCS, this was not statistically significant (figure 2). FIV$_{ple}$ alone was significantly correlated with a lower BCS. However, although *M. bovis*-infected lions were noted to often have a 'scruffy and unthrifty look' (D. F. Keet 2008, personal observations), BTB was not significantly correlated with BCS. This was surprising as pathologic lesions have been described in *M. bovis*-infected lions with a poor condition [19,20]. In a study of BTB in buffaloes, it was found that 70 per cent of the infected animals examined post mortem had only a mild infection and were unlikely to have shown symptoms while alive [53]. Our dataset contained several lions with necropsy results, but unfortunately their number was too small to allow statistically significant conclusions. Therefore, whether there are patterns in the clinical signs in lions, like in buffaloes, remains to be seen.

Effects of both FIV$_{ple}$ and *M. bovis* infection on the various blood parameters may indicate chronic disease,

such as anaemia [43,54], but could also be exaggerated by the effect of age, for example, the hyperglobulinaemia, as older lions are more likely to be FIV$_{ple}$ or BTB positive, and in general show an increase in globulins [54]. The values might be slightly biased by the delay between sampling and analysis and possible temperature differences during this delay, although the majority of the blood samples was collected at night in the winter season. For all blood parameter values, the interaction of the infections was shown to be less than additive. Explanations for this may be that the immune response already reaches maximum capacity for one infection, or that the body is able to keep the various parameters between homeostatic limits.

Although not specific for either infection, the direction of changes in blood parameter values was comparable to that observed in previous studies on FIV in cats [13–17] and FIV$_{ple}$ in lions [8] and to results from the less abundant literature on (B)TB [21,22]. Anaemia and hypoalbuminaemia are associated with progression to AIDS and death in human pre-AIDS patients [55,56]. In contrast to the decrease of CD4$^+$ and CD8$^+$ T-cell counts observed in HIV-infected humans, previously also reported for FIV$_{ple}$ in lions [4], no decrease of white blood cells was seen in FIV$_{ple}$-positive lions in the present study. White blood cells were not further typed, which would be needed to determine possible changes in the numbers of the different cell types.

Mycobacterium bovis prevalence was significantly different between the three areas in the KNP, coinciding with observations on *M. bovis* prevalence in the buffaloes [57], one of the four preferential prey species of lions in the KNP [58]. Surprisingly, this did not result in an age distribution skewed to the younger ages in the HPZ compared with the LPZ (see the electronic supplementary material, table and figure S5). Also, the prevalence of *M. bovis*-infected animals in the northern part of the KNP increased significantly with time from 0 to 41 per cent (Fisher's exact test, two tailed, $p = 0.014$; electronic supplementary material, table S7c). These findings suggest that *M. bovis* infection in lions is caused by an external source of infection, i.e. that lions are spillover hosts, in agreement with [59,60]. This contradicts suggestions from the past about their role as a maintenance host [19,61]. FIV$_{ple}$, on the other hand, shows no significant relationship with the external factors measured, supporting the intraspecies transmission route.

It was expected that, like HIV and *M. tuberculosis* in humans, a synergy between FIV$_{ple}$ and *M. bovis* was to

Table 4. Final multivariable models for seven haematologic and blood chemistry values. (Beta coefficients are given with their 95% confidence interval between brackets. To determine the effect of interaction, all values of a blood parameter should be added, for example, for globulin: $9.3 + 7.5 − 6.6 = 10.2$. n.s., these variables were not statistically significant (based on the AIC) and were not included in the final model.)

| | dependent variables | | | | | | |
explanatory variables	Ht (%)	WBC ($\times 10^9\,l^{-1}$)	TSP (g l^{-1})	Alb (g l^{-1})	Glob (g l^{-1})	gamma glob (g l^{-1})	A : G ratio
N	164	163	172	172	172	172	172
observed mean values in population	34.3	18.6	84.0	28.3	55.7	24.9	0.5
FIV_ple	−2.0 (−3.5 to −0.6)	n.s.	6.9 (3.1 to 10.6)	−2.4 (−3.5 to −1.2)	9.3 (5.7–12.9)	n.s.	−0.1 (−0.2 to −0.1)
BTB	n.s.	n.s.	7.8 (3.3 to 12.3)	n.s.	7.5 (3.3–11.8)	n.s.	−0.1 (−0.2 to 0.0)
FIV_ple × BTB	n.s.	n.s.	−6.7 (−12.2 to −1.1)	n.s.	−6.6 (−11.9 to −1.3)	n.s.	0.1 (0.0 to 0.2)
age	1.7 (0.2–3.2)	2.6 (0.7 to 4.5)	n.s.	n.s.	n.s.	3.5 (1.9–5.0)	n.s.
BCS	−6.9 (−8.5 to −5.3)	4.8 (2.8 to 6.9)	−4.8 (−8.1 to −1.5)	−6.7 (−8.1 to −5.4)	n.s.	1.9 (0.2–3.7)	−0.1 (−0.2 to −0.1)
sex	1.8 (0.4–3.2)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
TB area							
HPZ/south	−3.3 (−4.9 to −1.8)	n.s.	−3.8 (−7.1 to −0.5)	−0.4 (−1.8 to 0.9)	−3.3 (−6.4 to −0.2)	n.s.	n.s.
MPZ/central (reference)							
LPZ/north	2.0 (0.0 to 3.9)	n.s.	4.1 (0.3–7.9)	2.3 (0.7 to 3.8)	1.4 (−2.2 to 5.0)	n.s.	n.s.
intercept	36.1	15.6	80.0	30.8	49.8	22.2	0.7

be found in lions, but this large dataset shows no proof for detrimental synergy in the tested parameters. There may be various reasons for not finding a similar relationship of FIV_ple and *M. bovis* like in humans.

— The immunopathological characteristics of BTB can vary in different species [62] and macroscopic lesions in *M. bovis* positive lions have been found to be very different from those in ungulates and non-human primates [20]. This may mirror a difference in susceptibility to infection, but the knowledge on immunopathogenesis is still very limited in lions. Differences in immune response in feline and simian species have also been noted for immunodeficiency viruses, related with specific virus–host co-adaptation and viral load [9,12,63]. One reason for a difference in immunopathogenesis may be that lions have co-evolved with the endemic disease FIV_ple, whereas *M. bovis* is a recently introduced pathogen, in contrast to the situation in humans, where *M. tuberculosis* has been in the population for many centuries, and HIV/AIDS was introduced relatively recently [63].

— It remains possible that even our extensive dataset was not suitable to detect a pathogen–pathogen interaction. In literature on the HIV–TB interaction, emphasis has been laid on the changes in $CD4^+/CD8^+$ T-cell counts, pathology and the collection of longitudinal data [25,64,65]. The present dataset were not collected for the purpose of assessing FIV_ple–*M. bovis* interaction, and therefore lacks results on these important parameters. This precludes determining the directionality of any interactions, while order of infection can be crucial in the outcome of the pathogen–pathogen interaction [66]. Also, stage of infection and time of infection for both BTB and FIV_ple were not known, but may affect, for example, blood parameter values such as WBC, and this could thus be a potential source of error. Selection pressures that were not determined in this study, for example, prey availability, could also be confounding the pathogen–pathogen interaction [34].

— Although the use of call-up stations is accepted for the non-lethal capture of wildlife [67], our sampling method is likely not to have been truly random. For example, relatively few young animals have been captured (see the electronic supplementary material, table and figure S3). These are likely to be more cautious approaching a call-up station. This may also count for animals in a bad condition, since fewer animals than expected were captured at call-up stations in poor condition. Lions that were brought to the veterinary station were collected with different effort over the three areas. They had a lower mean BCS and higher mean age compared with lions captured at call-up stations, but the prevalence of FIV_ple and *M. bovis* infection for the lions (respectively, 63% and 47%) brought to the veterinary station were not statistically different (χ^2-tested, *p*-value, respectively, 0.40 and 0.82) from prevalence found for the lions sampled at call-up stations (respectively, 61% and 55%), and either including or excluding the emaciated lions had little influence on the various statistics (results not shown); therefore, they were included to increase the power of the analyses. Although we tried to control for bias as much as possible, field

datasets like these may include unmeasured biases, and results should be interpreted with caution.

Finally, we remark that the pathogen–pathogen interaction may become more important when the lions are under additional stress, for example, owing to high parasite load or bad nutritional status when there is a low prey density [30,49,66,68]. This complexity of disease in general and especially the interaction of pathogens, necessitates an extensive, long-term research programme requiring large sample sizes from the host population [31]. In future studies, besides $CD4^+/CD8^+$ T-cell counts, macro parasite infestation [69,70], and social interaction networks to assess the infectiousness of individual animals [71] could be valuable inclusions.

With the tested parameters, no evidence was found that FIV_{ple} or *M. bovis*, or a co-infection of these, is currently causing a serious conservation threat to KNP lions. However, a significant spatio-temporal increase of BTB was found, which may impact on lion health, as previous studies have related BTB to diverse pathological lesions [72]. In buffaloes, the population growth rate was negatively affected by BTB without altering the population age distribution significantly, thus reducing the resilience of the population to disturbances [53], which may also apply to lions. With the recent creation of the Greater Limpopo Transfrontier Conservation Area and the knowledge that the co-infection of FIV_{ple} and *M. bovis* is probably also present in lions in other African parks [7,73], it should be closely monitored how the KNP and other co-infected lion populations respond to a severe environmental perturbation compared with populations that are infected with only one or neither of these two agents.

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