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Immune stability predicts tuberculosis infection risk in a wild mammal

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Immunity is one of the most variable phenotypic traits in animals; however, some individuals may show less fluctuation in immune traits, resulting in stable patterns of immune variation over time. It is currently unknown whether immune variation has consequences for infectious disease risk. In this study, we identified moderately stable immune traits in wild African buffalo and asked whether the stability of these traits affected bovine tuberculosis (TB) infection risk. We found that adaptive immune traits such as the level of interferon-γ (IFN-γ) released after white blood cell stimulation, the number of circulating lymphocytes and the level of antibodies against bovine adenovirus-3 were moderately repeatable (i.e. stable) over time, whereas parameters related to innate immunity either had low repeatability (circulating eosinophil numbers) or were not repeatable (e.g. neutrophil numbers, plasma bacteria killing capacity). Intriguingly, individuals with more repeatable IFN-γ and lymphocyte levels were at a significantly higher risk of acquiring TB infection. In stark contrast, average IFN-γ and lymphocyte levels were poor predictors of TB risk, indicating that immune variability rather than absolute response level better captured variation in disease susceptibility. This work highlights the important and under-appreciated role of immune variability as a predictor of infection risk.

1. Introduction

Immune function is among the most variable of phenotypic traits in vertebrates, showing high levels of heterogeneity both within and among individuals [1–3]. For example, individual immune responses can shift drastically over time in response to demographic (e.g. age, reproductive status; [4,5]) and environmental (e.g. pathogen exposure; [6,7]) changes. However, recent studies suggest that some individuals maintain fairly stable immune responses over time [2,7–9]. Such longitudinal stability of immune traits could have profound implications for predicting individual responses to a wide range of immune challenges [2,7]. However, whether stable immune traits are predictive of infectious disease risk is completely unknown.

The longitudinal stability of biological traits can be quantified by estimating repeatability, defined as the fraction of phenotypic variation resulting from differences between individuals [10]. Repeatable (or stable) traits are those for which there is relatively low within-individual variance compared to high among-individual variance. In humans and wildlife, high among-individual variance in immune traits such as cytokine, antibody and leucocyte concentrations is common, and the repeatability of such traits can be estimated when the same

individuals are sampled multiple times [2,8]. Interestingly, emerging evidence suggests that there is considerable variability in the degree to which different immune traits are repeatable. For example, across 60 wild voles sampled two to seven times over an average 102 day period, levels of expression of the T-helper (Th) 1 cytokine interferon-γ (IFN-γ) were significantly repeatable, whereas the expression of the Th 2 transcription factor GATA binding protein 3 and the regulatory cytokine interleukin-10 were not [8]. Likewise, in humans, B-cell and CD4+ T-cell subsets consistently showed higher repeatability than did regulatory T cells [2,7,11]. Because most of the immune variation in mammal populations probably arises from environmental exposure to pathogens and commensals [3,7,12], differences in the repeatability of particular immune traits suggest that the environment elicits distinct effects on different components of the immune system. However, the significance of these differences for disease susceptibility is not clear.

Tuberculosis (TB; caused by bacteria in the *Mycobacterium tuberculosis* complex) is one of the most devastating infectious diseases of our time, accounting for nearly 2 million human deaths annually [13]. Domestic animals and wildlife also experience substantial TB-related mortality (mainly owing to *Mycobacterium bovis*, a member of the *M. tuberculosis* complex and causative agent of bovine TB). Bovine TB is responsible for approximately 25% of all disease-related deaths per annum in cattle [14], and spillover of *M. bovis* from animals to humans contributes to the global burden of human TB [15]. Despite the impact of bovine TB on agriculture, public health and conservation, very little is known about the factors contributing to variation in disease susceptibility in animal reservoirs. In sub-Saharan Africa, a region of the world with one of the highest burdens of TB [13,16], African buffalo (*Syncerus caffer*) act as the major reservoir of TB in the wild [16,17]. The prevalence of TB in buffalo is reported to be as high as 40% in some locations [18], and spillover from buffalo to species such as cattle, lions (*Panthera leo*), giraffes (*Giraffa camelopardalis*) and African wild dogs (*Lycaon pictus*) is of great public health and conservation concern [19]. Given the important role buffalo play in the ecology of TB, new insight into the factors that drive variation in infection risk in this species can contribute to disease control and management.

To evaluate the role of immune stability in TB risk, we tested for the presence of repeatable immune traits in wild African buffalo, and asked whether the stability of specific traits was predictive of future risk of bovine TB infection. To do this, first, we tracked multiple immune parameters in the same individuals over time to identify which immune traits were most repeatable. Next, we examined whether individual stability in repeatable immune traits was predictive of TB infection. Our results show that some aspects of immunity are more repeatable than others, and intriguingly, that the stability of certain immune traits is a strong predictor of future risk of infection. These findings suggest that a better understanding of immune stability can facilitate the profiling of individual disease risk.

2. Methods

(a) Animal sampling

One hundred and forty-five female African buffalo, ranging in age from 1.5 to 13 years, were captured in Kruger National Park (KNP), South Africa, between June 2008 and August 2012. These animals represent a subset of a larger group of individuals captured for a study on the effects of anthelmintic treatment on bovine TB outcomes [20]. The animals used for the current study were control individuals that did not receive anthelmintic treatment. Individuals were sampled approximately every 180 days, with an average of six (range: 3–9) captures per animal. For sampling, buffalo were chemically immobilized with a mix of etorphine (M99) and ketamine delivered by dart. Blood samples were collected via jugular venipuncture into EDTA and heparin tubes for use in immunological assays and TB diagnostics. Age was estimated by incisor eruption and tooth wear patterns [21]. Pregnancy status (not pregnant, early, mid or late pregnancy) was assessed by rectal palpation as described for Egyptian buffalo [22]. The presence or absence of milk in the mammary gland (lactation status) was assessed by manually milking all four teats. Body condition was assessed using a manual fat scoring system standardized for African buffalo [23]. All animals were initially captured from two herds in distinct locations: Crocodile Bridge (CB) and Lower Sabie (LS). Upon recapture, herd membership was assigned as CB, LS or Other if an animal dispersed to another location and herd.

(b) Immune parameters

We measured 10 immune parameters that represent both the innate and adaptive branches of the immune response. Bacteria killing ability (BKA) of plasma against *Escherichia coli* and *Staphylococcus aureus* were used as measures of innate humoural immunity. BKAs were performed in a 96-well plate format as described in French & Neuman-Lee [24], using frozen plasma diluted in culture media containing 10^5 colony forming units (CFU) of *E. coli* strain ATCC no. 8739 (BKA *E. coli*) and 10^5 CFUs of *S. aureus* strain ATCC no. 6538 (BKA *S. aureus*) [25]. Plasma samples were processed in bulk at the end of the study to minimize the effects of sample storage time on variation in killing capacity. Killing ability ranged from 0.01 to 100% (mean = 64%) and 0.01 to 60.8% (mean = 25.7%), for *E. coli* and *S. aureus*, respectively. Variation in sample storage time prior to processing had no notable effect on killing ability (*E. coli*: generalized linear mixed model (GLMM): $n = 554$, estimate $= 1.79 \times 10^{-5} \pm 2.1 \times 10^{-6}$, $Z = 0.83$, $p = 0.40$; *S. aureus*: GLMM: $n = 563$, estimate $= -3.48 \times 10^{-5} \pm 2.4 \times 10^{5}$, $Z = -1.4$, $p = 0.162$).

White blood cell and platelet counts were used as measures of cellular innate immunity. Total counts were performed on an automated impedance cell counter (model ABC-VET), and differential counts were done manually from blood smears to estimate the fraction of each white blood cell type [20]. The concentrations (cells μl^{-1}) of neutrophils, lymphocytes, monocytes and eosinophils were calculated by multiplying the proportion of each cell type from blood smears by the total number of white blood cells per microlitre. All cell types, except lymphocytes, were considered as innate immune parameters based on the fact that most lymphocytes in the peripheral blood of mammals (approx. 90%) are B- and T-lymphocytes [26].

In addition to lymphocytes, we measured three other aspects of adaptive immunity: concentrations of the cytokines (i) IFN-γ and (ii) interleukin-4 (IL-4) after stimulation of whole blood with pokeweed mitogen (Sigma Aldrich, St Louis, MO, USA), and (iii) antibodies to bovine adenovirus (BADV-3). IFN-γ concentration in plasma was measured using a bovine IFN-γ ELISA (MCA5638KZZ, Bio-Rad, Hercules, CA, USA) following previously described methods [20]. IL-4 concentration in plasma was measured using a standard sandwich ELISA designed for bovines (CC308 and CC313, Bio-Rad) as previously described [27]. For both IFN-γ and IL-4, *in vitro* stimulation of white blood cells with the B- and T-cell mitogen pokeweed was used to more accurately measure adaptive immune cell reactivity compared to baseline levels. Finally, given that all study animals were positive for BADV-3 at first capture, and the ubiquity of adenoviruses in the bovine respiratory and gastrointestinal mucosae [28], we

used antibody responsiveness to BADV-3 at subsequent captures as a measure of the level of immune reactivity against a specific pathogen. Serum BADV-3 antibodies were measured using an indirect ELISA kit designed against whole BADV-3 viral antigen and following the manufacturer's instructions (BIO K-063, Bio-X Diagnostics, Belgium). Mild to moderate cross-reactivity against other Serogroup-I adenoviruses is expected with this test (BADVs 1–2 and 9), while no cross-reactivity should occur against Serogroup-II adenoviruses (BADVs 4–8) (Bio-X Diagnostics, Belgium). Antibody concentration was semi-quantitatively determined based on the optic absorbance of the sample in relation to a negative control provided by the manufacturer.

(c) Tuberculosis testing

Bovine TB infection status was assessed using a whole-blood IFN-γ assay (BOVIGAM, Prionics, Switzerland) implemented according to the manufacturer's instructions and as described in Ezenwa & Jolles [20]. For each animal, a time series of two to nine test results were used to assign infection status (see [20] for details on the assignment procedure).

(d) Data analyses

GLMMs were used to evaluate the effects of fixed (demographic and environmental) and random (individual identity) factors on the variance of immune traits and to estimate the repeatability of immune traits. Separate models were fitted for each of the 10 focal immune parameters using the R package 'glmmTMB' [29]. Models included five fixed factors: age (in months), body condition, lactation status, pregnancy status, season and one random factor: animal identity (ID). The fixed factors were selected based on their known effects on buffalo immune responses [20,25,27,30,31]. Exploratory data analyses were used to identify the appropriate error distributions for each response variable following recommended statistical methods [32,33]. IFN-γ, BADV-3, platelets and BKAs had an approximately normal distribution, so models with a Gaussian error structure were fitted to these response variables. For leucocytes and IL-4, we evaluated models with both Poisson and negative binomial distributions. For monocytes and lymphocytes, we considered the best model to be the one that minimized the Akaike information criterion (AIC) and overdispersion and that did not have convergence issues. For neutrophils, eosinophils and IL-4, both Poisson and negative binomial models fitted poorly, so we applied logarithmic transformations to these response variables, and then fitted models with Gaussian error distributions to these data. For all models, residuals were checked to ensure normality and homoscedasticity. Because immune traits were sampled longitudinally over time, we also tested for evidence of temporal autocorrelation in the selected model residuals using a Durbin–Watson (DW) test applied to scaled residuals and implemented in the R package 'DHARMa' [34]. There was no evidence of either positive or negative autocorrelation in the residuals of all final models (DW range = 1.960–2.07, $p = 0.489$–0.80).

We estimated the proportion of model variance explained by fixed versus random factors by calculating marginal ($R^2_{\mathrm{GLMM}(m)}$) and conditional ($R^2_{\mathrm{GLMM}(c)}$) pseudo-R^2 values with the r.squared.glmm function in the R package 'MuMIn' [35]. $R^2_{\mathrm{GLMM}(m)}$ represents the percentage of variance explained by fixed factors and $R^2_{\mathrm{GLMM}(c)}$ represents the percentage of variance explained by the full model, including random effects. Therefore, the variance attributed to the random factor (animal ID), or adjusted-repeatability, was calculated as: $R^2_{\mathrm{GLMM}(c)} - R^2_{\mathrm{GLMM}(m)}$ [36]. We also calculated adjusted-repeatability for each model using the R package 'rptR' [37], which provides uncertainty estimates (s.e., 95% confidence interval (CI) and p-value) via parametric bootstrapping. Both methods yielded identical repeatability estimates.

To test whether repeatability in immune traits was associated with the risk of TB infection, we first calculated each animal's pre-TB immune repeatability. For this analysis, we focused only on the three immune traits (BADV-3 antibodies, IFN-γ, lymphocytes) that emerged as moderately repeatable based on our GLMM analyses. We considered repeatability values between 0.3 and 0.5 to be 'moderately repeatable' following the categorical classification of effect sizes proposed by Cohen [38] for correlation coefficients. Using the first three observations (in longitudinal sequence) taken per animal, we calculated the repeatability of immune traits for each individual using the standard formula for raw repeatability:

$$R = \frac{s_A^2}{s^2 + s_A^2}$$

where s_A^2 is the variance among individuals and s^2 is the variance within individuals [10,37]. Because R can be influenced by among-individual variance, we also calculated a measure of immune stability that does not include among-individual variance: the coefficient of variation (CV, s.d./mean). These two stability estimates were highly correlated (Spearman's rank correlation: lymphocytes: $\rho = -0.79$, IFN-γ: $\rho = -0.82$, BADV-3: $\rho = -0.85$), suggesting that variation in R was driven mostly by within-individual variance. Next, we tested if these stability estimates were good predictors of TB infection risk by fitting Cox proportional hazard regression models implemented in the 'survival' package in R [39]. Separate univariate models included the time animals were at risk of TB infection as the response variable, and either lymphocyte, IFN-γ or BADV-3 antibody stability as the predictor variable. Animals that did not acquire TB during the study were right-censored. The R and CV models yielded similar results (see the electronic supplementary material, table S1 for CV models), so we performed all further analyses using R as the measure of immune stability.

Finally, we compared the effect of immune stability versus absolute magnitude on infection risk. To do this, we fitted univariate Cox models using the average values of the three immune traits over the same time period (first three captures) over which immune stability (R) was estimated. We also tested if a combination of repeatability and absolute values was the better predictor of TB infection risk by fitting multivariate Cox regression models with an interaction between repeatability and mean values for each immune trait. We included the age of animals at initial capture and herd membership in these models as covariates to control for the fact that not all animals were captured at the same age and that TB incidence varies with age in buffalo [20], and to account for possible herd effects on TB risk [20]. These models were ranked using AIC corrected for small sample size (AICc). We considered models with a ΔAICc of at least 2.0 to be significantly different from one another. Lastly, to determine if the effect of the repeatability of different immune traits was additive or multiplicative, we compared a series of Cox regression models including different combinations of the predictors emerging as significant in univariate tests (i.e. IFN-γ and lymphocyte repeatability). Age and herd were included as covariates in all candidate models. The best fitting model was selected through a multimodel selection approach implemented in the 'MuMIn' package [35]; ranking of models was based on AICc. For all Cox regression models, the proportional hazard assumption of predictors was checked by plotting the Schoenfeld residuals versus time for continuous predictors and by plotting the log–log-transformed Kaplan–Meier survival curves for categorical predictors [40].

3. Results

(a) Adaptive immune traits are more repeatable than innate immune traits

All four immune traits associated with adaptive immunity were significantly repeatable (table 1). Adjusted-repeatability

Table 1. Coefficients of determination, repeatability estimates and significant fixed factors from generalized linear mixed models with 10 different African buffalo (*S. caffer*) immune traits as response variables. ($R^2_{(c)}$, conditional R^2 (variance explained by the model); $R^2_{(m)}$, marginal R^2 (variance explained by fixed factors); *R*, adjusted repeatability; s.e., standard error; CI, confidence intervals.)

response	model type	*n* animals	*n* observations	R^2		repeatability				significant fixed factors
				$R^2_{(c)}$	$R^2_{(m)}$	*R*	s.e.	CI	*p*-value	
BADV-3 antibodies	Gaussian	144	765	0.43	0.03	0.40	0.074	0.122–0.506	3.21×10^{-5}	pregnancy, lactation
lymphocytes	Poisson	144	769	0.51	0.14	0.38	0.05	0.266–0.512	3.87×10^{-15}	age, season, pregnancy
IFN-γ	Gaussian	144	785	0.36	0.02	0.34	0.06	0.246–0.491	3.87×10^{-15}	season, body condition
eosinophils	Gaussian	144	769	0.30	0.20	0.10	0.077	0.041–0.287	0.0324	season, lactation
IL-4	Gaussian	142	654	0.10	0.03	0.08	0.05	0–0.199	0.0287	pregnancy, season
BKA: *S. aureus*	Gaussian	129	563	0.14	0.06	0.08	0.07	0.02–0.247	0.0501	age, lactation, body condition
platelets	Gaussian	143	763	0.13	0.06	0.07	0.06	0.002–0.29	0.0510	pregnancy
neutrophils	Gaussian	144	769	0.17	0.12	0.05	0.06	0–0.224	0.0514	age, season
monocytes	negative binomial	144	769	0.12	0.07	0.05	0.07	0–0.169	0.0500	season, pregnancy
BKA: *E. coli*	Gaussian	130	554	0.12	0.08	0.04	0.04	0–0.194	0.25	body condition, pregnancy, lactation, season

Figure 1. Adjusted repeatability for 10 different immune traits measured in African buffalo. Traits that measure more specific (i.e. adaptive) arms of immune function (black bars), tended to be more repeatable, whereas traits that measure mostly innate immunity (grey bars) were less repeatable. Error bars represent 95% s.e. BKA, bacterial killing ability.

scores ranged from 34 to 40% for IFN-γ, lymphocytes and BADV-3 antibodies, while for the fourth adaptive trait, IL-4, repeatability was much lower, at 8% (figure 1). By contrast, only one of six traits associated with innate immunity, eosinophils, was significantly repeatable, with a repeatability score of 10% (table 1 and figure 1). All demographic and environmental covariates emerged as significant predictors of at least one immune trait, but these factors typically explained very little variation (table 1). In fact, for all the adaptive immune traits, individual identity explained substantially more variation than did demographic and environmental factors (table 1). By contrast, for all six innate traits, demographic and environmental covariates explained more or similarly low, levels of variation as did individual identity (table 1). Therefore, among-individual differences were generally greater for adaptive immune traits compared to innate traits, explaining the difference in repeatability between the two groups of traits.

(b) Repeatable immune traits predict tuberculosis infection risk

In addition to assaying immune traits, we also tested animals for TB infection at each sampling point. At the beginning of the study, 130 animals were negative for TB infection, but TB prevalence in the study cohort increased over time (electronic supplementary material, figure S1), such that by the end of the study, 37 of 130 animals (28%) had acquired TB. Overall, 89 animals had sufficient pre-TB immune histories to allow individual repeatability estimation, and of these, 27 acquired TB (figure 2*a*). The individual repeatability of IFN-γ and lymphocytes emerged as significant predictors of TB risk, while the repeatability of BADV antibodies did not (electronic supplementary material, table S2).

For IFN-γ, higher repeatability was associated with a higher risk of TB infection. Specifically, for each 1% increase in IFN-γ repeatability, TB risk increased 1.05-fold (univariate Cox regression : hazard ratio (HR) = 1.05, 95% CI = 1.02–1.07, $p = 0.0002$; electronic supplementary material, table S2). This translated into a 5.7-fold greater risk of TB infection for individuals in the highest repeatability quartile compared to

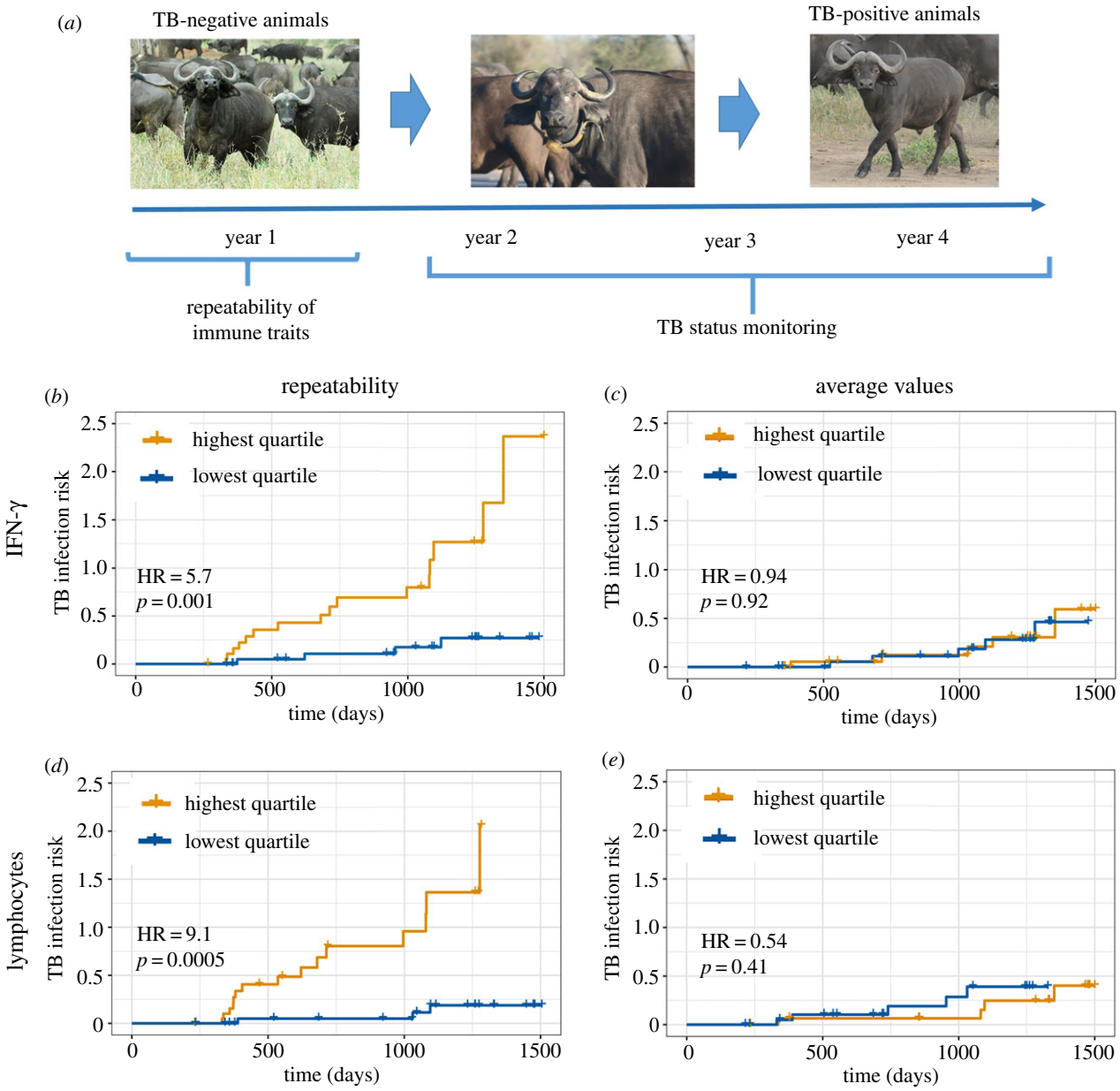

Figure 2. Stable IFN-γ and lymphocyte levels predict TB infection risk. (*a*) For 89 TB-negative animals, individual repeatability for IFN-γ and lymphocytes was estimated based on three samples taken during the first 18 months of study. Individuals were subsequently monitored over 3 years for TB infection, and 27 of 89 had acquired TB by the end of the study. (*b*) IFN-γ repeatability was a significant predictor of TB infection, such that individuals in the highest repeatability quartile ($R > 0.89$) had a 5.7 times higher risk of infection compared to individuals in the lowest quartile ($R < 0.6$); (*c*) by contrast, average IFN-γ level was a poor predictor of TB risk. (*d*) Likewise, lymphocyte repeatability was a significant predictor of TB risk, with individuals in the highest repeatability quartile ($R > 0.9$) being 9.1 times more likely to acquire TB compared to individuals in the lowest quartile ($R < 0.65$); whereas (*e*) average lymphocyte level was a poor predictor of TB risk. Graphs compare the highest and lowest quartiles for each continuous predictor variable. TB infection risk represents the cumulative hazard on a log scale. Reported HRs and *p*-values correspond to the hazard of the highest quartile compared to the lowest quartile. (Online version in colour.)

individuals in the lowest repeatability quartile (figure 2*b*). In stark contrast with the repeatability results, the average IFN-γ level was a poor predictor of TB infection risk (univariate Cox regression: HR = 0.677, CI = 0.25–1.81, $p = 0.44$; figure 2*c*). Using a multivariate model comparison approach, top ranked models for the effect of IFN-γ on infection risk included only a significant effect of IFN-γ repeatability (electronic supplementary material, tables S3 and S4). This suggests that it is the repeatability of this immune trait, not merely its average value, that is a risk factor for TB infection. Surprisingly, the model including an interaction between IFN-γ repeatability and IFN-γ average ranked poorly (electronic supplementary material, table S3), suggesting that stable IFN-γ levels elevate the risk of TB infection irrespective of whether absolute values are low or high.

Similar to IFN-γ, longitudinal stability in the number of peripheral blood lymphocytes was associated with a significant increase in TB infection risk (electronic supplementary material, table S2). Specifically, for each 1% increase in lymphocyte repeatability, TB risk increased 1.06-fold (univariate Cox regression: HR = 1.064, CI = 1.03–1.10, $p = 0.0001$; electronic supplementary material, table S2). This translated into a 9.1-fold greater risk of TB infection for individuals in the highest repeatability quartile compared to individuals in the lowest repeatability quartile (figure 2*d*). The average lymphocyte number was a poor predictor of TB infection risk (univariate

Table 2. Comparison of candidate Cox proportional hazard models of TB infection risk ($n = 88$, number of events = 27). (Models are ranked based on Akaike's information criteria corrected for small sample size (AICc). Detail on the top ranked model (highlighted in italics) is provided in the electronic supplementary material, table S7.)

model	d.f.	logLik	R^2	AICc	ΔAICc	weight
IFN-γ repeatability + lymphocyte repeatability + age + herd	*5*	*−87.6*	*0.76*	*188.71*	*0*	*0.72*
IFN-γ repeatability + lymphocyte repeatability + IFN-γ repeatability × lymphocyte repeatability + age + herd	6	−87.0	0.77	190.22	2.05	0.25
lymphocytes repeatability + age + herd	4	−92.7	0.65	195.33	7.17	0.01
IFN-γ repeatability + age + herd	4	−95.2	0.58	200.25	12.08	0.01

Cox regression: HR = 0.871, CI = 0.61–1.24, $p = 0.45$; figure 2*e*). As observed for IFN-γ, in a multivariate model comparison, only lymphocyte repeatability emerged as a significant predictor of TB infection risk in the top ranked models (electronic supplementary material, tables S5 and S6). Thus, once again, repeatability was more important than the average trait value and high levels of stability in lymphocyte numbers elevated the risk of TB infection irrespective of the magnitude of the absolute values.

(c) Effects of interferon-γ and lymphocyte repeatability on tuberculosis risk are independent and additive

Because the repeatability of both IFN-γ and lymphocytes was associated with TB infection risk, we examined whether there were combinatorial (multiplicative or additive) effects on individuals expressing high repeatability in both traits. We compared a series of models including different combinations of IFN-γ repeatability, lymphocyte repeatability and the interaction between the two, while controlling for individual age and herd membership. The best performing model included the main effects of both IFN-γ and lymphocyte repeatability, but not an interaction between the two (table 2; electronic supplementary material, table S7). This result suggests that the expression of stability in these two different immune traits has non-overlapping and additive effects on the risk of acquiring TB.

4. Discussion

In natural populations, the identification of repeatable immune traits is relatively recent, raising intriguing questions about the consequences of this form of variation at the individual and population level [3,8]. This is especially true if the likelihood of infection or disease outcomes differ for individuals with higher or lower levels of immune stability. In this study, we found that some immune traits are repeatable (i.e. stable) in wild African buffalo, and that individuals with more stable levels of IFN-γ and lymphocytes were at higher risk of acquiring bovine TB. These findings suggest that immune variation over time may be central to understanding infection risk heterogeneity in some populations.

Very few studies have measured how immune traits vary over time in wild animals under natural conditions [41–45]. In humans, though, an emerging body of work is showing that some adaptive and innate cellular immune traits are relatively stable over time, and that this stability is mostly driven by environmental factors [2,3,7,11]. Intriguingly, the studies that exist for other vertebrates (e.g. birds: [5,44]; small mammals: [8]) suggest that stable immune profiles, as described in humans, also apply to wild animals. However, in animals, it is not clear which components of immunity are more likely to exhibit stability. In buffalo, we found that immune stability, quantified as repeatability, was closely associated with the level of specificity of the response. Specifically, for traits closely related to adaptive immunity, we found the responses of individual animals to be significantly correlated over time, with three of four of these traits showing moderate repeatability ($R = 0.34$–0.40), whereas for innate immune traits, there was little correlation in individual responses over time and repeatability tended to be non-significant (with the exception of eosinophils) and uniformly low ($R = 0.04$–0.10). Contrary to our findings, studies on birds have found moderate to high ($R = 0.4$–0.63) repeatabilities for innate traits such as serum bacteria killing capacity against *E. coli* [45,46]. However, repeatability calculations in these studies did not account for variance explained by covariates likely to influence immunity such as body condition, which can inflate repeatability estimates [36,37]. To our knowledge, no studies on wild mammals have simultaneously examined repeatabilities for innate and adaptive immune traits within the same system. However, with respect to adaptive immune traits, a study of wild voles found that the repeatability of IFN-γ was significant at $R = 0.2$ [8], while a study of Soay sheep found the repeatabilities of various antibodies, such as total immunoglobulin M (IgM) and parasite-specific IgM, to be moderate ($R = 0.28$–0.43) [47]. In humans, repeatabilities of adaptive immune traits (B- and T-cell subsets) can be very high (average $R = 0.8$) [2]; however, most human studies monitor study subjects over short time intervals (days–weeks) and measure hundreds of highly specific leucocyte subsets, which probably facilitates the identification of highly repeatable immune traits [2,3,7].

A fundamental difference between innate and adaptive immune traits is that innate effector mechanisms respond to a wide range of stimuli, while adaptive mechanisms respond to a much narrower range of stimuli [48]. Because effector mechanisms of innate immunity are influenced by a wide spectrum of pathogens and environmental exposures [48,49], innate immune experience could accumulate at a similar rate in most individuals in a population, decreasing among-individual differences [3], thereby reducing repeatability. By contrast, the opposite could occur for adaptive traits, as after activation of innate pathways, these immune traits are stimulated by a narrower range of pathogens.

Therefore, the likelihood of differential exposure among individuals in the same population is high, potentially leading to higher among-individual differences in these traits [2,3,7,11], and higher repeatability. In support of this idea, exposure to certain pathogens, such as cytomegalovirus in humans, generates permanent differences in the profiles of innate immune traits between infected and non-infected individuals, increasing between individual variance, and potentially, the repeatability of these traits [7,11,12,50]. Moreover, in our study, all innate immune traits showed 20% lower among- versus within-individual variance (repeatability) when compared with adaptive immune traits (see '*R*' values in table 1). Thus, the contrasting levels of stability we observed in adaptive versus innate immune traits, and across all immune traits, more generally, could relate to how different immune pathways are stimulated and how immunological variation between individuals is created, and maintained, in a population.

TB infection risk is known to be variable across individuals in animals (where infection is caused mainly by *M. bovis*: [51,52]) and humans (where infection is caused mainly by *M. tuberculosis*: [53,54]). However, the factors that underlie these different resistance profiles are not fully understood, although several genetic and immune mechanisms have been explored [51–56]. Our results suggest that the stability of certain immune traits may be an important contributor to infection resistance. Indeed, the two immune traits for which we found strong links between stability and infection risk (IFN-γ and lymphocytes), both play well-described roles in TB defence, providing a strong rationale for why these two traits emerged as predictors of risk. IFN-γ plays a crucial role in the host response to both *M. tuberculosis* and *M. bovis* [57,58]. For example, during *M. bovis* infection in cattle and *M. tuberculosis* infection in humans, IFN-γ activates nitric oxide production within macrophages, enhancing their mycobacterial killing capacity [56–60]. A strong IFN-γ response is also related to high levels of other immune effector molecules, such as nuclear factor kappa-light-chain-enhancer of B cells and tumour necrosis factor alpha (TNF-α), which are associated with early clearance of *M. tuberculosis* infection, and therefore, protection against disease [58]. Similarly, there is extensive evidence that an effective response of lymphocytes, particularly CD4+ T lymphocytes, is critical for protection against *M. bovis* and *M. tuberculosis* infection as well as TB disease progression [59–62]. For instance, the number of CD4+ T lymphocytes was the best predictor of *M. tuberculosis* infection risk among HIV-infected people receiving anti-retroviral therapy [62]. Moreover, during *M. bovis* exposure, most IFN-γ is produced by CD4+ T lymphocytes, and post exposure, a high proportion of these cells change their phenotype to IFN-γ producing cells [58]. IFN-γ producing CD4+ T cells are also crucial for the production of antibodies that have been shown to be protective against *M. tuberculosis* infection in humans and rodent models [55].

Given the role of IFN-γ and lymphocytes in TB defence, the association we found between higher stability of these effectors and higher infection risk may reflect the reduced capacity of some individuals to vary these immune traits as appropriate when confronted with mycobacterial stimuli. For instance, the stability of some arms of the immune response could compromise the activation of effector mechanisms necessary for TB clearance, such as TNF-α-dependent bacterial killing capacity of macrophages [61]. Moreover, some *in vitro* studies suggest that at the cellular level, higher variation in the immune response against *M. tuberculosis* correlates with the presence of genetic factors associated with resistance to TB [63]. These findings reinforce our hypothesis that a sufficient level of variability in the immune response may improve protection against TB in buffalo. Moreover, because IFN-γ and lymphocytes are key players in the immune response against many infectious agents, it is likely that their levels of stability affect the infection risk of other pathogens, and reciprocally, that these pathogens have an effect on immune stability. This reciprocal effect may be particularly important in the context of wildlife systems, like buffalo [64], where individuals are typically infected by many pathogens concurrently.

The level of immune reactivity of an individual has been recognized for some time as an important predictor of protection or time to recovery from infection [65,66]. However, there has been very little work done on developing predictive frameworks based on levels of variation in immune reactivity exhibited by individuals over time [3]. Our finding that average IFN-γ and lymphocyte response levels were poor predictors of infection risk compared to the stability of these responses over time suggests that immune stability may be an important, and under-appreciated, driver of variation in disease outcomes. Indeed, recent work suggests that in humans, responses to vaccination can be predicted by monitoring immune traits that exhibit longitudinal stability [2,7,11]. Here, we provide novel evidence that infection risk is also associated with the stability of some immune traits. Further work is necessary to understand the underlying drivers of the patterns of immune stability we observed, and why higher immune variability confers protection against TB infection. However, our results for TB might translate to other host-pathogen systems where strong CD4+ T lymphocytes and IFN-γ responses are associated with protection and clearance. More generally, our results suggest that quantifying the stability of immune traits may be a powerful new tool for predicting disease risk in natural populations.

Ethics. Animal procedures were approved by the University of Georgia (UGA), Oregon State University (OSU) and South African National Parks Institutional Animal Care and Use Committees (UGA AUP A2010 10-190-Y3-A5; OSU AUP 3822 and 4325).

Data accessibility. Data presented in this paper are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6bv1qn3 [67].

Authors' contributions. M.S. and V.O.E. conceived the research idea; M.S., A.E.J. and V.O.E. designed the study; B.R.B., C.C.C., P.W.S., J.M.S., A.E.J. and V.O.E. collected data; M.S. analysed data; M.S. and V.O.E. wrote the paper; A.E.J. and V.O.E. contributed long-term data. All authors gave final approval for publication and agree to be held accountable for the work performed therein.

Competing interests. We declare no competing interests.

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