This is the author's accepted manuscript of the following article:

Buzdugan, S. N., Chambers, M. A., Delahay, R. J. and Drewe, J. A. (2017) 'Quantitative interferon-gamma responses predict future disease progression in badgers naturally infected with Mycobacterium bovis', *Epidemiology and Infection*, 10/11, 1-10.

The final publication is available at Cambridge Journals via https://doi.org/10.1017/S0950268817001960.

The full details of the published version of the article are as follows:

TITLE: Quantitative interferon-gamma responses predict future disease progression in badgers naturally infected with Mycobacterium bovis

AUTHORS: Buzdugan, S. N., Chambers, M. A., Delahay, R. J. and Drewe, J. A.

JOURNAL TITLE: Epidemiology and Infection

PUBLICATION DATE: 11 October 2017 (online)

PUBLISHER: Cambridge University Press: STM Journals

DOI: 10.1017/S0950268817001960



# Quantitative interferon-gamma responses predict future disease progression in badgers naturally infected with *Mycobacterium bovis*

# S.N. BUZDUGAN<sup>1</sup>, M.A. CHAMBERS<sup>2</sup>, R.J. DELAHAY<sup>3</sup>, J.A. DREWE<sup>1</sup>

- 1. Veterinary Epidemiology, Economics and Public Health Group, Royal Veterinary College, London, UK
- 2. Animal and Plant Health Agency, Weybridge, UK
- 3. National Wildlife Management Centre, Animal and Plant Health Agency, Woodchester Park, Gloucestershire, UK

\*Corresponding author: J A Drewe, Veterinary Epidemiology, Economics and Public Health Group, Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire AL9 7TA, UK. Email: <u>jdrewe@rvc.ac.uk</u>

# 1 Summary

2 The diagnosis and control of Mycobacterium bovis infection (bovine tuberculosis: TB) continues to 3 present huge challenges to the British cattle industry. A clearer understanding of the magnitude and 4 duration of immune response to M. bovis infection in the European badger (Meles meles) - a wildlife 5 maintenance host – may assist with the future development of diagnostic tests, and vaccination and 6 disease management strategies. Here, we analyse 5,280 diagnostic test results from 550 live wild 7 badgers from a naturally-infected population to investigate whether one diagnostic test (a gamma 8 interferon release [IFNy] assay, n=550 tests) could be used to predict future positive results on two 9 other tests for the same disease (a serological test [n=2,342 tests] and mycobacterial culture 10 [n=2,388 tests]) and hence act as an indicator of likely bacterial excretion or disease progression. Badgers with the highest IFNy optical density (OD) values were most likely to subsequently test 11 12 positive on both serological and culture tests, and this effect was detectable for up to 24 months 13 after the IFNy test. Furthermore, the higher the original IFNy OD value, the greater the chance that a 14 badger would subsequently test positive using serology. Relationships between IFNy titres and 15 mycobacterial culture results from different types of clinical sample suggest that the route of 16 infection may affect the magnitude of immune response in badgers. These findings identify further 17 value in the IFNy test as a useful research tool, as it may help us to target studies at animals and 18 groups that are most likely to succumb to more progressive disease.

#### 19 Introduction

20 The diagnosis and control of Mycobacterium bovis infection (bovine tuberculosis: TB) continues to

21 present huge challenges to the British cattle industry [1]. The problem is compounded by the

22 presence of *M. bovis* infection in European badgers (*Meles meles*) which, in addition to cattle, can

23 act as maintenance hosts. A clearer understanding of the magnitude and duration of immune

response to *M. bovis* infection in badgers may aid in disease control, for example by informing the

25 development of vaccines and diagnostic tests [reviewed in 2]. For example, cytokines such as gamma

26 interferon (IFNγ) released from activated T cells, appear to be useful diagnostic and prognostic tools

27 in humans and other animals [3-5]. Information on the magnitude and duration of immune

responses to *M. bovis* infection in badgers may aid development of management strategies for this
disease.

30

31 A recent study found that the magnitude of early IFNy responses in badgers naturally infected with

32 *M. bovis* was positively correlated with a likelihood of subsequent disease progression [2]. However,

that analysis was based on a small sample size (56 badgers) and so the generality of their conclusions

is uncertain. Here, we use a much larger sample size (>500 badgers) to investigate the

35 representativeness of Tomlinson *et al.*'s [2] results on a wider scale.

36

37 Badgers may become infected with *M. bovis* through a variety of routes including inhalation, bite-38 wounding and, potentially, ingestion [6]. Tomlinson et al. [2] hypothesised that the route of infection 39 may influence the magnitude of the IFNy response and subsequent disease progression, but they had insufficient data to investigate this. Previous studies have suggested that badgers infected 40 41 through biting may subsequently experience particularity aggressive pathology [7, 8] and 42 experimental intradermal injection of *M. bovis* has been linked to progressive systemic infection [9]. 43 Seropositivity is also more likely in situations of progressed disease which suggests that while not 44 directly measuring infectiousness, a positive serological test result may indicate a greater likelihood 45 that this is the case [10]. In the present study, our rich dataset allowed us to examine for 46 relationships between the locations of positive *M. bovis* culture results (from specific lesions or body 47 areas, which may reflect the route of infection or excretion) and IFNy responses. For bite wounds, 48 this provides insight into how the route of infection may affect the magnitude of the immune 49 response to *M. bovis* infection in badgers.

50

51 The duration of immune response is also important. Tomlinson *et al.* [2] showed that the magnitude 52 of IFNy responses to infection in badgers declined over time, but they did not investigate how soon

- 53 an animal is likely to become infectious after the first IFNy test. We were able to do so in the present
- 54 study by focusing on short-term associations (up to 24 months). This revealed insights into the
- 55 differential timing of the immune responses which is likely to be particularly useful from an
- 56 operational perspective, because disease management programmes typically operate over these
- 57 sorts of timeframes [11].
- 58

59 We hypothesise that, in a badger population naturally infected with *M. bovis*, individuals producing 60 the highest IFNy titres will be the ones most likely to subsequently test positive using TB tests that 61 measure other arms of the immune system (the humoral response) or detect the pathogen itself 62 (mycobacterial culture). Should this be the case, then it may be possible to use IFNy test results as an

- 63 indicator of likely future disease progression.
- 64

# 65 Materials and methods

66 Ethics statement

67 Badger capture and sampling was carried out under licences from Natural England and the UK Home

68 Office. The protocols were approved by local ethical review within the Food and Environment

69 Research Agency and the Animal Health and Veterinary Laboratories Agency (now the Animal and

70 Plant Health Agency).

71

# 72 Study site and sample collection

73 Samples and data were collected from between July 2006 and October 2013 from a population of 74 wild badgers living in Woodchester Park, an area of south-west England which is the focus of a long-75 term study into badger ecology and TB epidemiology [12]. Badgers were captured, anaesthetised 76 and sampled using well-established methods [13] with each badger social group being trapped four 77 times per year, resulting in repeated observations of the same individuals throughout the study 78 period. Trapping was suspended between 1st February and 30th April inclusive when most cubs are 79 very young, confined to the sett, and/or totally dependent on their mother [14]. During January 80 (and, weather dependent, during December and May), when some females may be lactating, traps 81 were checked during the night, and females deemed to be lactating or pregnant on the basis of 82 cursory examination, were released immediately without sampling. 83

On first capture each badger was given a unique alpha-numeric tattoo which allowed individuals to
be identified thereafter [15]. The location, sex, body weight and condition, reproductive status and

age class (cub [<1y] or adult [1y+]) of each animal was recorded. The following samples were

collected for mycobacterial culture: faeces, urine, tracheal aspirate, oesophageal aspirate, swabs of
bite wounds (where present) and swabs of suppurating submandibular lymph node lesions (where
present). Bite wounds and suppurating submandibular lymph nodes were sampled separately
because they are likely to represent different routes of infection. Up to 12 ml of jugular blood was
taken for serology and IFNy testing. After recovery from anaesthesia, badgers were released at the
point of capture.

93

94 Three diagnostic tests were conducted: the IFNy test; the Stat-Pak serological test; and 95 mycobacterial culture of clinical samples (for details of all three tests see reference [16]). Briefly, the 96 IFNy test quantified the secretion of the cytokine IFNy by T-cells following stimulation with purified 97 protein derivatives of bovine (PPD-B) and avian (PPD-A) tuberculin [4]. Results from the IFNy test 98 were available on a continuous scale as optical density (OD) readings of IFNy production. The Stat-99 Pak (Chembio Diagnostic Systems, New York) identified antibodies produced in response to specific 100 antigens associated with *M. bovis* [10], giving a binary (positive or negative) test result. The third test 101 was the mycobacterial culture of clinical samples [17] with a positive result recorded for any sample 102 from which *M. bovis* was isolated. All three tests (IFNy, Stat-Pak and culture) were conducted on 103 each badger every time it was trapped, except on 2% of occasions when an insufficient volume of 104 blood was available to allow Stat-Pak or culture to be run. Estimates of the sensitivity and specificity 105 of each of these three tests have been reported separately [18].

106

#### 107 Data description and analysis

108 Data included IFNy, Stat-Pak and culture test results on 550 captured badgers. Animals were 109 enrolled in the study on the date of their first IFNy test (usually the first time they were sampled 110 within the study period) and were followed until the date of their last Stat-Pak or culture test during 111 the period of study. This resulted in a median total observation period per badger of 10 months (range: one day to 86 months (7.2 years) per badger). Badgers with an observation period of one day 112 113 were trapped and tested just once: therefore a true follow-up time period was not recorded for 114 these animals. However, it was considered possible that their infection status might have been 115 different to those that were trapped more than once (e.g. badgers that were lost to follow-up may 116 have been more likely to have advanced infection) and so to reduce exclusion bias resulting from 117 their omission, the test results of these badgers were included in the analysis by artificially 118 increasing the time period between IFNy and subsequent tests by one day. 119

120 The 'risk factor' of interest included as the explanatory variable in the model was the IFNy titre at 121 the first time each badger was tested. The IFNy titre for each badger was calculated as the quantity of IFNy produced following stimulation with bovine tuberculin purified protein derivative (PPD-B) 122 123 minus the amount of IFNy produced by stimulation with avian tuberculin purified protein derivative 124 (PPD-A) [4]. Continuous IFNy optical density (OD) values were used to produce five categories of 125 IFNy results for analysis (Table 1). Because the distribution of data points was highly right-skewed it 126 was not considered appropriate to simply divide the range of values by the number of categories in 127 order to obtain cut-off values. Therefore, negative values (arising from cases where the OD of PPD-A 128 was higher than that for PPD-B) were coded as category 0 (zero), while values higher than zero but 129 less than 0.044 – the current cut-off value for infection in adult badgers [4] – were coded as category 130 1. Categories 2 and 3 were equally spaced (starting from 0.044) using an interval step of 0.33. The 131 category coded as 4 included values higher than 0.70 with the highest IFNy OD value being 1.92 132 (Table 1).

133

134 Relationship between magnitude of IFNy response and other diagnostic test results

135 Associations between the categories of the independent variable (IFNy titre) and the dependent 136 variables (subsequent Stat-Pak and culture results) were initially assessed using Chi-square tests. For 137 this analysis, the results of mycobacterial culture of different clinical samples (e.g., urine, faeces, 138 tracheal aspirate) were pooled into one culture result (positive or negative) per badger per trapping 139 event. A Cox proportional hazards regression analysis was performed to estimate the rates 140 (probabilities) of subsequent positive Stat-Pak or culture results relative to the different categories 141 of initial IFNy titre. Survival analysis was chosen because this method focuses on 'time-to-event' 142 which permits the calculation of rate ratios. For each badger, the time intervals that elapsed 143 between the first IFNy test and subsequent other TB tests (Stat-Pak or culture) were modelled, to 144 determine whether values of IFNy can be used as a measure of the likelihood of progression of 145 infection. Kaplan-Meier and Nelson-Aalen curves were plotted for visual assessment of data 146 distribution and to check if the proportional hazards assumption was upheld. Data were formally 147 assessed using a plot of -log (-log) survival lines and a Schoenfeld residuals test [19] which revealed 148 that the proportional hazards assumption was not met. Therefore, data were corrected by splitting 149 the observation time over the first year into three-month intervals (Table S1 in Supplementary Material). A Schoenfeld test indicated that following this step the data no longer violated the 150 151 proportional hazards assumption. When fitting the Cox regression model to the data, the clustering 152 of multiple observations per badger was specified. This analysis was performed using Stata version 153 11.2 (Statacorp LP, College Station, Texas, USA). Final models were checked for goodness of fit by

using Cox-Snell residuals [20]. The hazard function followed approximately the 45° line and was
exponentially distributed with a hazard ratio that approximated one. Therefore it was concluded
that the data fitted the models adequately.

157

158 Estimates of rate ratios (the relative probabilities of subsequently obtaining a positive Stat-Pak or 159 culture result following a given IFNy result) were produced for inter-test periods of up to three, six, 160 nine and 12 months, and for the period between 12 and 24 months. For time periods greater than 161 12 months, annual time categories were used (1 to 2 years, 2 to 3 years, ...) until the end of the study 162 (up to just over seven years). For follow-up periods in excess of 24 months, the proportional hazards 163 assumption was violated for both Stat-Pak and culture tests. Consequently, only observations made 164 within 24 months of each badger's first IFNy result were included in subsequent analyses. A log-rank 165 test was used to assess equality in survival function between categories of IFNy to determine 166 whether the differences in survival between groups were more than would be expected by chance 167 alone [21].

168

Analyses were conducted to investigate whether age class or sex confounded or modified the
 predictive effect of IFNγ category on subsequent StatPak or culture test results, and whether the
 predictive ability of IFNγ category significantly differed between these categories of age and sex.

173 Relationship between IFNy titres and culture results from different types of clinical sample 174 We looked for relationships between the mycobacterial culture test results from each type of 175 sample (some of which may be considered as potential proxies for the route of infection: for 176 example: a positive culture of a bite wound swab was taken to indicate that infection had occurred 177 through being bitten) and the IFNy response in the same animal. Mixed effects linear regression 178 models were used, with individual animals as a random effect to avoid pseudo-replication, using 179 data from badgers that were tested by both culture and IFNy test on the same trapping occasions 180 (median of three occasions per badger, range: one to 21). Gamma interferon titres (optical density 181 values on a continuous scale) were the response variable, while individual culture sample test results 182 (positive or negative) were the explanatory variables in the model. The IFNy test results were log-183 transformed to meet the assumption of Normal distribution of regression models' residuals. Any 184 IFNy test results below zero (indicating a higher titre with PPD-A stimulation than with PPD-B 185 stimulation) were considered inconclusive and were removed from the analysis. 186

187

### 188 **Results**

189 Summary of data and associations

190 The majority of badgers (403/550, 73%) had multiple serological (Stat-Pak) and culture test results.

191 There were 2,342 Stat-Pak results (median: 3 per badger, range: 1 to 21) and 2,388 'sets' of

192 mycobacterial culture results (median: 3 per badger, range: 1 to 21). A 'set' of culture results related

to the suite of different samples collected from the same badger on the same sampling occasion.

194 The distribution of Stat-Pak and culture test results by category of IFNγ titre can be found in Table

195 S2. Each of the 550 badgers contributed one IFNγ test result because the 'risk factor' of interest was

196 the IFNy titre at first capture. At the time of this IFNy test, 78 badgers tested positive on Stat-Pak

- 197 and 8 badgers tested positive on culture.
- 198 199

examined ( $\chi^2 = 105.7$ , p < 0.001), and between categories of IFNy and culture test results for observation periods up to nine months only ( $\chi^2 = 21$ , p < 0.001). Log-rank tests indicated that the survival function of Stat-Pak test results was not the same for all categories of IFNy in all time periods and for the culture test there was good evidence against equality for a follow-up period of

Associations were detected between categories of IFNy and Stat-Pak test results in all time periods

204 less than a year. This suggests that a difference exists between badgers of different IFNy titres in

205 relation to the probability of subsequently testing positive on Stat-Pak or culture.

206

207 Can IFNy be used as a predictor of future Stat-Pak test results?

The highest rates of Stat-Pak positive test results occurred following the highest IFNy optical density values across all time periods (Table 2). Predictive ability gradually declined, however, and became inconclusive when follow-up time was more than 12 months (Table 2 and Figure 1). An exception was for badgers in the highest IFNy category, where the association was sustained over the longest time period (up to 24 months between IFNy and Stat-Pak tests being conducted on the same badger), albeit with a rate ratio of only 3.14 (95% CI: 1.09 - 9.02) for this longer period (Table 2).

214

215 Can IFNy be used as a predictor of future M. bovis culture test results?

Gamma interferon results were generally of less value in predicting future culture test results than
they were at predicting subsequent Stat-Pak results. Only badgers with the highest IFNy OD values
(category 4) predicted a future positive culture result over every time interval up to two years (Table
3). Low numbers of positive culture test results (Table S1), which are likely to reflect the low
sensitivity of culture for detecting infected animals [18] explain the wide confidence intervals and

221 why reliable estimates could not be produced for badgers with lower IFNγ OD values.

222

223 No significant changes in the predictive effects of IFNy categories on subsequent StatPak or culture 224 test results were detected when data were stratified and adjusted for age and sex using the 225 Maentel-Haenszel stratified analysis of rate ratios method. This was true when data were assessed 226 visually – by inspecting the degree of change in rate ratios to examine for confounding, and formally 227 - by testing for unequal rate ratios to examine for effect modification. No significant associations 228 were detected between age and sex variables on the rates of positive StatPak or culture results 229 when univariate Cox regression was fitted to study the predictive effect of age and sex explanatory 230 variables. Consequently, age and sex were excluded from the final model.

231

232 Comparison with the currently-used IFNy test cut-off level

233 Putting these findings in context, the predictive ability of IFNy over one subsequent year can be seen 234 by using as an example badgers with IFNy OD values equal to or greater than 0.044 ([the current cut-235 off for an adult badger to be considered infected [4]). These badgers had at least a six times higher 236 chance of subsequently testing positive on Stat-Pak and culture within 12 months than did animals 237 testing negative (Tables 2 and 3). Gamma interferon results remained associated with other test 238 results two years later but the association was less pronounced: badgers with the highest IFNy OD 239 values (category 4: IFNy OD > 0.70) had at least a three times higher chance of subsequently testing 240 positive on Stat-Pak, and at least a five times higher chance of subsequently testing positive on culture, than animals with IFNy OD values of zero (Tables 2 and 3). 241

242

243 Relationship between IFNy titres and culture results from different types of clinical sample 244 The mixed effects linear regression provided a better fit to the data than a fixed effect linear 245 regression model for both IFNy and culture test results (likelihood ratio test p<0.001). The 246 distribution of IFNy titres varied by *M. bovis* culture result and type of clinical sample (Figures 2 and 247 S1-S6). The likelihood of obtaining a positive *M. bovis* culture result from three types of clinical 248 sample (urine, faeces and bite wound swabs) was positively associated with an increase in IFNy OD 249 value. For example, a positive urine culture result was associated with an IFNy OD value 3.5 times 250 higher than the IFNy OD value associated with a negative urine culture sample (z-test = 3.68, 251 p<0.001; 95% CI: 1.8-6.9). Similarly, a positive faeces culture result was associated with a 3.5 times increase in IFNy OD value (z-test =3.20, p=0.001; 95% CI: 1.6-7.4), and a positive bite wound swab 252 253 culture result was associated with a 3.3 times increase in IFNy OD value (z-test = 2.85, p=0.004; 95% 254 CI: 1.4-7.7). However, IFNy OD values were not found to be significantly associated with the 255 probability of obtaining any other type of clinical sample (tracheal aspirate, oesophageal aspirate,

- submandibular lymph node, or non-bite-related wounds). It should be noted that the sample sizes
- 257 for the latter two clinical sample types were very small (samples sizes are given in Table S3).
- 258

# 259 **Discussion**

260 Our findings indicate that badgers with the highest IFNy titres were those most likely to 261 subsequently test positive on two other types of diagnostic test (serology and culture) and this effect 262 was detectable for up to 24 months. The Stat-Pak serological test showed a positive trend in its 263 dose-response relationships with IFNy, meaning that the higher the original IFNy OD value, the 264 greater the chance that a badger would subsequently test positive on Stat-Pak. The relationship 265 between IFNy and culture was less clear, which may be a real effect or could have been influenced 266 by the low number of culture-positive badgers in the analysis (69/2388 or 3%). These results concur 267 with and build on those reported by Tomlinson et al. [2] which, due to strict restriction criteria, was 268 based on a substantially smaller dataset (56 badgers, as compared to 550 in the present study). We 269 believe that this positive association between IFNy response and subsequent diagnostic test results 270 indicating disease progression is robust, because two very different analytical approaches - linear 271 modelling [2] and survival analysis (this study) - led to the same conclusion. Further, research in 272 cattle infected with TB has shown the magnitude of the IFNy response (to ESAT-6) to be proportional 273 to disease progression [5]. The present study of badgers adds new evidence supporting the 274 proposition that IFNy appears to be a useful prognostic immunological marker in several species. 275

276 We detected no association between a badger's age class (cub versus adult) or sex and the 277 predictive effect of IFNy category on subsequent StatPak or culture test results. This appears to 278 contrast with previous research [2] where associations were found between the magnitude of IFNy 279 titres and age (lower IFNy responses in cubs) and sex (lower responses in males than females). A key 280 difference in study design may explain this apparent discrepancy: Tomlinson et al. [2] analysed a 281 very small set of data from badgers pre-selected as positive based on their IFNy test (OD values of 282  $\geq$ 0.044 for adults and  $\geq$ 0.023 for cubs) whereas the present study analysed a much larger dataset of 283 IFNy test results regardless of whether they were considered positive for infection (i.e. the present 284 analysis included IFNy test OD values of <0.044 for adults and <0.023 for cubs). This means the 285 present study is likely to have included badgers before they were infected, or at earlier stages of 286 infection.

287

In their previous study, Tomlinson *et al.* [2] showed that the magnitude of IFNγ responses to
 infection in badgers declined over time, but they did not investigate how quickly an animal is likely

290 to become infectious. Analysing the larger dataset in the present study allowed us to tease out 291 differential information on the relative rates of positive diagnostic test results, subsequent to the 292 IFNy test, over a range of fairly short time periods (particularly 6, 9, 12, and 24 months). It is less 293 easy to interpret the results for the 0 to 3 month time period because badgers were rarely caught 294 more frequently than every 3 months (due to trapping occurring four times a year), and hence a high 295 proportion of the positive Stat-Pak and culture test results recorded in this time period occurred at 296 the time of the initial IFNy test. For these badgers, this prevented us from investigating correlations 297 between IFNy titres and disease progression because the available information was limited to the set 298 of diagnostic test results obtained at the time of the IFNy test. This was much less of a problem for 299 time periods longer than 3 months because badgers that contributed data had by then been 300 sampled at least twice. Overall, our findings provide insights into the differential timing of the 301 immune responses, and may enhance the value of the IFNy test as a research tool. Identification of 302 those individuals and groups that may be more likely to experience disease progression may be 303 particularly valuable for field investigations of the behavioural consequences and transmission 304 dynamics of TB in badgers.

305

306 Although IFNy test results are generated on a continuous scale (OD values), the diagnosis of 307 infection status in badgers is currently based on whether the OD value falls above or below a pre-308 determined cut-off (0.044 for adult badgers, 0.023 for cubs: ref. [22]). Hence, as the diversity in the 309 range of OD values is not fully used for diagnosis, some information is lost. Results of our analyses 310 indicate that by using the raw OD values it is possible to go beyond answering whether or not an 311 animal is 'positive', and to potentially infer the stage of infection and the likelihood that it will 312 subsequently test positive on other diagnostic tests, within an up to 24-month time window. Those 313 animals producing the highest values of IFNy (i.e. category 4 in the present analysis) were most likely 314 to go on to also test positive on culture (indicating detection of excretion). On the basis of this 315 evidence it would be tempting to apply a cut-off for IFNy OD values of 0.697 (the lower boundary of 316 our category 4) rather than the currently used cut-off of 0.044 in order to identify animals most 317 likely to go on to become infectious. However, our results suggest that the current cut-off is useful, 318 as badgers with an OD value greater than or equal to this cut-off are likely to go on to test positive 319 on Stat-Pak. This is relevant because previous studies indicate that a positive StatPak result is more 320 likely to occur in badgers at advanced stages of TB [10] and seropositivity identifies badgers with the 321 greatest probability of transmitting infection [22]. Moreover, raising the IFNy cut-off on the basis of 322 culture results would likely result in some future excretors being missed because the culture of 323 clinical samples is known to be an insensitive diagnostic approach in live badgers [16].

324

325 The finding that increased IFNy OD values were associated with positive culture results from some 326 clinical samples (bite wound swabs, urine and faeces) but not others (tracheal aspirates and 327 oesophageal aspirates) suggests that perhaps the routes of infection (bite wounds) or subsequent 328 dissemination of infection (e.g. to the kidneys giving rise to bacteria in urine) may affect the 329 magnitude of immune response in badgers. This is consistent with evidence from studies of TB 330 pathology in badgers which indicate that disease progression in animals with bite wounds may be 331 rapid [6]. No relationship could be established between tracheal aspirate culture results and IFNy 332 titres because of the low proportion of positive culture results from this type of sample (0.1% 333 compared to 15% for bite wound swabs: Table S3). The relationships between IFNy OD values and 334 both the pathogenesis and expression of TB in badgers are worthy of further research.

335

336 There are some limitations inherent in our analysis, one of which concerns the proportional hazards 337 approach, which assumes that the effect of the predictor variable (the IFNy OD value) was constant 338 for the duration of the study. This is unlikely to have been truly the case, as a badger's IFNy titre is 339 expected to vary over time and with the course of infection [2]. Nevertheless, the assumption was 340 not violated for a follow-up period of two years (as indicated by the formal assessment of survival 341 lines and a Schoenfeld residuals test) and hence the analyses and conclusions appear valid. A second 342 limitation was the uneven distribution of observations amongst categories of IFNy response. In order 343 to address the limited number of observations in the highest categories of IFNy (due to few badgers 344 giving very high IFNy OD readings) we focussed on interpreting the trends in outputs rather than 345 individual values. The two highest IFNy categories accounted for only approximately 4% of 346 observations for both Stat-Pak and culture (Table S2), which is likely to have resulted in low 347 statistical power for the parameters estimated. Moreover, the mycobacterial culture test has limited 348 sensitivity in live badgers: as low as 10 per cent in some cases [16] which means that the true 349 predictive ability of IFNy is very likely to be higher than that described here. Only 3% of culture test 350 results were positive in comparison to 21% of Stat-Pak tests, thus any relationship between IFNy and 351 culture may be masked by inaccurate data and/or a low sample size. These limitations could 352 potentially be addressed in future studies by improving the sensitivity of the culture test, possibly by 353 using an extended sampling protocol involving more types of samples or more frequent sampling 354 although this is unlikely to be practical. A more practical alternative would be to repeat the analysis 355 in the future when more data become available.

356

357	In c	onclusion, we have shown that the magnitude of the IFNy response in badgers naturally infected					
358	witł	M. bovis is positively associated with the subsequent likelihood of disease progression,					
359	refle	flected in rates of positive results to two different diagnostic tests over a range of time periods.					
360	Alth	hough this knowledge would be of some value in field operations, for example by helping to					
361	ider	entify individual badgers most likely to become infectious to others, the practical requirements for					
362	perf	rforming the IFNγ test – such as the overnight incubation of blood samples and the relatively large					
363	volu	lumes required – severely limit its potential applications as a management tool. Nevertheless,					
364	mea	easurement of the magnitude of the IFNγ response is a useful research tool as it may help us to					
365	targ	et studies at animals and groups that are most likely to succumb to more progressive disease.					
366							
367							
368	Ack	nowledgements					
369	We	thank the fieldworkers at the Animal and Plant Health Agency (APHA) who contributed to data					
370	coll	ollection in Woodchester Park, and staff of the Bacteriology Department of APHA for generating the					
371	test	est results and for technical support. Glyn Hewinson and Martin Vordermeier provided helpful					
372	com	iments on the manuscript.					
373							
374	Fina	Financial support					
375	This research was funded by the UK Department for Environment, Food and Rural Affairs (project						
376	SE3265). RVC manuscript number: PPH 01437.						
377							
378	Con	flicts of interest					
379	None.						
380							
381	Re	ferences					
382	(1)	Defra. Latest statistics on tuberculosis (TB) in cattle in Great Britain. National Statistics from the					
383		Department for Environment, Food & Rural Affairs and the Animal and Plant Health Agency.					
384		Available online at: <u>https://www.gov.uk/government/statistics/incidence-of-tuberculosis-tb-in-</u>					
385		cattle-in-great-britain (accessed 14 October 2016). 2016.					
386	(2)	Tomlinson AJ, et al. Association of quantitative interferon- $\gamma$ responses with the progression of					
387		naturally acquired Mycobacterium bovis infection in wild European badgers (Meles meles).					
388		Immunology 2015; <b>144</b> : 263-270.					
389	(3)	Lalvani A, Millington KA. T Cells and tuberculosis: Beyond interferon-y. Journal of Infectious					
390		Diseases 2008; <b>197</b> : 941-943.					

Page 12 of 19

- 391 (4) Dalley D, et al. Development and evaluation of a gamma-interferon assay for tuberculosis in
   392 badgers (*Meles meles*). *Tuberculosis (Edinburgh, Scotland*) 2008; 88: 235-243.
- 393 (5) Vordermeier HM, et al. Correlation of ESAT-6-specific gamma interferon production with
- pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine
   tuberculosis. *Infection and Immunity* 2002; **70**: 3026-3032.
- 396 (6) Corner LA, et al. The distribution of *Mycobacterium bovis* infection in naturally infected
  397 badgers. *Veterinary journal (London, England : 1997)* 2012; 194: 166-172.
- (7) Clifton-Hadley RS, Wilesmith JW, Stuart FA. *Mycobacterium bovis* in the European badger
   (*Meles meles*): epidemiological findings in tuberculous badgers from a naturally infected
- 400 population. *Epidemiology and infection* 1993; **111**: 9-19.
- 401 (8) Jenkins HE, et al. The prevalence, distribution and severity of detectable pathological lesions in
  402 badgers naturally infected with *Mycobacterium bovis*. *Epidemiology and infection* 2008; 136:
  403 1350-1361.
- 404 (9) Pritchard DG, et al. Experimental infection of badgers (*Meles meles*) with *Mycobacterium bovis*.
  405 *Epidemiology and infection* 1987; 98: 145-154.
- 406 (10) Chambers MA, et al. Validation of the BrockTB Stat-Pak assay for detection of tuberculosis in
- 407 Eurasian badgers (*Meles meles*) and influence of disease severity on diagnostic accuracy.
- 408 *Journal of Clinical Microbiology* 2008; **46**: 1498-1500.
- 409 (11) **Defra.** Bovine TB: summary of badger control monitoring during 2015. Policy paper from the
- 410 Department for Environment, Food & Rural Affairs. Available online at:
- 411 https://www.gov.uk/government/publications/bovine-tb-summary-of-badger-control-
- 412 <u>monitoring-during-2015</u> (accessed 14 October 2016). 2015.
- 413 (12) Delahay RJ, et al. The spatio-temporal distribution of *Mycobacterium bovis* (bovine
- 414 tuberculosis) infection in a high-density badger population. *Journal of Animal Ecology* 2000; 69:
  415 428-441.
- 416 (13) **Delahay RJ, et al.** Long-term temporal trends and estimated transmission rates for
- 417 *Mycobacterium bovis* infection in an undisturbed high-density badger (*Meles meles*) population.
   418 *Epidemiology and infection* 2013; **141**: 1445-1456.
- 419 (14) Woodroffe R, et al. Welfare of badgers (*Meles meles*) subjected to culling: development and
  420 evaluation of a closed season. *Animal Welfare* 2005; 14: 19-25.
- 421 (15) Cheeseman CL, Harris S. Methods of marking badgers (*Meles meles*). Journal of Zoology 1982;
  422 197: 289-292.
- 423 (16) **Drewe JA, et al.** Diagnostic accuracy and optimal use of three tests for tuberculosis in live
- 424 badgers. *PLoS One* 2010; **5**: e11196.

- 425 (17) Gallagher J, Horwill DM. A selective oleic acid albumin agar medium for the cultivation of
  426 *Mycobacterium bovis. Journal of Hygiene* 1977; **79**: 155-160.
- 427 (18) **Buzdugan SN, et al.** Diagnosis of tuberculosis in groups of badgers: an exploration of the impact
- 428 of trapping efficiency, infection prevalence and the use of multiple tests. *Epidemiology and*429 *infection* 2016; **144**: 1717-1727.
- 430 (19) Grambsch PM, Therneau TM. Proportional hazards tests and diagnostics based on weighted
  431 residuals. *Biometrika* 1994; 81: 515-526.
- 432 (20) Cox DR, Snell EJ. A general definition of residuals. *Journal of the Royal Statistical Society Series B*433 (*Methodological*) 1968; 30: 248-275.
- 434 (21) Allison PD. Event History Analysis: Regression for Longitudinal Event Data. Newbury Park,
- 435 California: Sage Publications, Inc., 1984.
- 436 (22) Chambers MA, et al. Performance of TB immunodiagnostic tests in Eurasian badgers (*Meles*
- 437 *meles*) of different ages and the influence of duration of infection on serological sensitivity.
- 438 *BMC Veterinary Research* 2009; **5**: 1-7.

- 439 **Table 1.** Distribution and categorisation of optical density (OD) values from gamma interferon (IFNγ)
- 440 test results conducted on the first blood sample collected from each of 550 live badgers trapped at
- 441 Woodchester Park from July 2006 to October 2013.

IFNy category	IFNγ OD values (PPD-B minus PPD-A)	Number of observations	Percentage of observations
0	<0	181	33
1	0.000 - 0.043	277	50
2	0.044 - 0.366	66	12
3	0.367 - 0.696	15	3
4	0.697 - 1.920	11	2
Total	0.000 - 1.920	550	100

442 PPD = purified protein derivative; B = bovine; A = avian.

443 Table 2. Relative incidence of positive Stat-Pak test results in badgers in relation to previous IFNy 444 titre results over varying time periods. Data are derived from five Cox regression models, each of 445 which was run for a different time period (defined as the interval between the IFNy test being 446 conducted and a subsequent Stat-Pak test on the same badger). Rate ratios were calculated by 447 comparing the incidence of positive Stat-Pak test results for badgers in each IFNy category to a 448 baseline rate (category zero in Table 1), which was allowed to vary by time period (proportional 449 hazard assumption). Significant differences from baseline are shaded in grey. As an example, to determine the relative chance of a badger with an IFNy titre of 0.50 subsequently testing Stat-Pak 450 451 positive 12 months later, compared to a badger with an initial IFNy titre of zero, first determine the 452 category of IFNy using Table 1: in this example it would be category 3 (because the IFNy OD value 453 falls within the range of 0.367 - 0.696); then look at the rate ratio for this category in time period 0 454 to 12 months. The rate ratio of 9.05 can be interpreted as badgers with an IFNy OD value of 0.50 455 being nine times as likely to test Stat-Pak positive up to a year later than are badgers with an IFNy

456 OD value of zero.

Time period	IFNγ	Rate ratio	SE	Z	P >   z	95% CI
	category*					
0 to 3 months	1	3.32	1.19	3.35	0.001	1.64 - 6.70
	2	12.53	4.54	6.99	0.000	6.17 - 25.48
	3	15.19	6.86	6.03	0.000	6.27 - 36.83
	4	13.90	6.22	5.89	0.000	5.79 - 33.42
0 to 6 months	1	3.00	0.94	3.53	0.000	1.63 - 5.54
	2	12.75	3.97	8.18	0.000	6.93 - 23.46
	3	13.56	5.12	6.91	0.000	6.47 - 28.41
	4	16.33	6.63	6.88	0.000	7.37 - 36.18
0 to 9 months	1	2.27	0.66	2.81	0.005	1.28 - 4.02
	2	9.14	2.72	7.43	0.000	5.10 - 16.38
	3	10.75	3.72	6.87	0.000	5.46 - 21.17
	4	12.13	4.81	6.30	0.000	5.58 - 26.38
0 to 12 months	1	1.91	0.56	2.40	0.016	1.13 - 3.43
	2	6.80	2.00	6.51	0.000	3.82 -12.12
	3	9.05	3.66	5.44	0.000	4.09 - 20.00
	4	13.77	5.05	7.16	0.000	6.72 - 28.24
12 to 24	1	0.96	0.29	-0.14	0.892	0.53 - 1.74
months	2	1.18	0.45	0.43	0.669	0.56 - 2.50
	3	1.56	0.76	0.91	0.364	0.60 - 4.08
	4	3.14	1.69	2.12	0.034	1.09 - 9.02

\*IFNy categories are detailed in Table 1.

- 457 **Table 3.** Relative incidence of positive *M. bovis* culture test results in badgers in relation to previous
- 458 IFNγ titre results over varying time periods. Data derived from five Cox regression models as
- 459 described for Table 2. Significant differences from baseline are shaded in grey. For method of
- 460 interpretation, see legend to Table 2.

Time period	IFNγ	Rate ratio	SE	Z	P >   z	95% CI
	category*					
0 to 3 months	1	1.29	1.57	0.21	0.834	0.12 - 14.04
	2	8.47	9.80	1.85	0.065	0.88 - 81.71
	3	14.60	20.76	1.88	0.060	0.90 - 237.22
	4	15.89	22.61	1.94	0.052	0.98 - 258.61
0 to 6 months	1	1.37	1.69	0.26	0.796	0.12 - 15.26
	2	12.14	13.75	2.21	0.027	1.32 - 111.68
	3	12.69	18.13	1.78	0.075	0.77 - 208.90
	4	16.60	23.66	1.97	0.049	1.02 - 271.18
0 to 9 months	1	2.08	2.41	0.63	0.530	0.21 - 20.23
	2	15.68	17.27	2.50	0.012	1.81 - 135.70
	3	16.26	23.54	1.93	0.054	0.95 - 277.51
	4	19.32	27.87	2.05	0.040	1.14 - 326.78
0 to 12 months	1	2.03	1.35	1.07	0.284	0.55 - 7.47
	2	5.54	4.04	2.34	0.019	1.32 - 23.17
	3	8.15	9.68	1.77	0.077	0.80 - 83.49
	4	12.84	14.94	2.19	0.028	1.31 - 125.82
12 to 24	1	1.03	0.67	0.05	0.959	0.30 - 3.61
months	2	0	0	-63.93	0.000	0 - 0
	3	0	0	-51.75	0.000	0 - 0
	4	5.80	3.99	2.56	0.011	1.508 - 22.33

\*IFNγ categories are detailed in Table 1

#### 461 Figure 1.

462 Rate ratios for subsequently obtaining a positive Stat-Pak test result in badgers after varying follow-

463 up periods, in relation to their initial IFNy titre (category 1 = lowest IFNy titre; category 4 = highest

464 IFNy titre: see Table 1 for details of categories) compared to badgers with a negative IFNy titre

- (category zero). A badger with a rate ratio of 15 can be interpreted as having a 15 times higher 465
- 466 chance of testing positive on Stat-Pak within the indicated follow-up period than a badger with a
- 467 negative IFNy titre. Solid lines indicate significant relationships, and dashed lines indicate
- 468 relationships that were not found to be significant (see Tables 2 and 3 for 95% confidence intervals).
- 469 Data derived from 550 badgers tested with Stat-Pak a total of 2,342 times at Woodchester Park from 470 July 2006 to October 2013.



# 473 Figure 2.

- 474 Distribution of IFNγ OD values stratified by mycobacterial culture result across a range of different
- 475 clinical samples. Data shown includes 2,205 observations from 546 badgers. + = positive culture
- 476 result, = negative culture result. Outliers not shown.



477

Type of sample and Mycobacterium bovis culture result