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Relationship between ambient temperature at sampling and the interferon gamma test result for bovine tuberculosis in cattle

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ABSTRACT

Bovine tuberculosis (bTB) is a disease of significant economic and zoonotic importance, therefore, optimising tests for the identification of *Mycobacterium bovis* infected cattle is essential. The Interferon Gamma (IFN-γ) Release Assay (IGRA) can diagnose *M. bovis* infected cattle at an early stage, is easy to perform and can be used alongside skin tests for confirmatory purposes or to increase diagnostic sensitivity. It is known that IGRA performance is sensitive to environmental conditions under which samples are taken and transported. In this study, the association between the ambient temperature on the day of bleeding and the subsequent IGRA result for bTB was quantified using field samples from Northern Ireland (NI). Results of 106,434 IGRA results (2013–2018) were associated with temperature data extracted from weather stations near tested cattle herds. Model dependent variables were the levels of IFN-γ triggered by avian purified protein derivative (PPDa), *M. bovis* PPD (PPDb), their difference (PPD(b-a)) as well as the final binary outcome (positive or negative for *M. bovis* infection). IFN-γ levels after both PPDa and PPDb stimulation were lowest at the extremes of the temperature distribution for NI. The highest IGRA positive probability (above 6%) was found on days with moderate maximum temperatures (6–16 °C) or moderate minimum temperatures (4–7 °C). Adjustment for covariates did not lead to major changes in the model estimates. These data suggest that IGRA performance can be affected when samples are taken at high or low temperatures. Whilst it is difficult to exclude physiological factors, the data nonetheless supports the temperature control of samples from bleeding through to laboratory to help mitigate post-collection confounders.

1. Introduction

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis*, is a disease of significant economic and zoonotic importance (Allen et al., 2018; Butler et al., 2010; Humblet et al., 2009; O'Reilly and Daborn, 1995) and therefore several countries where the disease is endemic in the cattle population have developed eradication programmes. Such programmes typically consist of a test and cull strategy.

To detect infected cattle, a diagnostic test with high accuracy and precision is needed to minimise the number of false positive and false negative test results. Three commonly used test to diagnose bTB include the single intradermal tuberculin (SIT) test (Alvarez et al., 2012), the single intradermal comparative cervical tuberculin test (SICCT test) (Amos et al., 2013) and the interferon-gamma (IFN-γ) release assay (IGRA) (Klepp et al., 2019). All three tests are based on cell-mediated immune responses. Animals infected with *M. bovis* may show a delayed-type hypersensitivity response to PPD (De la Rua-Domenech et al., 2006) resulting in clinical signs such as pasty consistency, exudation, necrosis, pain or inflammation of the lymphatic ducts in that region and of the lymph nodes. Next to that, SIT measures the increase in skinfold thickness three days after injection of purified protein derivatives (PPD) from *M. bovis*. The SICCT test compares skinfold thickness differences three days after bovine and avian tuberculin (PPD derivatives) have been injected (Amos et al., 2013). The avian tuberculin is applied to demonstrate infection with cross-reacting organisms (De la Rua-Domenech et al., 2006). The outcome of this test is based on

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the difference in size of the swelling between both injection sites. If a defined threshold in size difference is exceeded, the animal is classified as a reactor (Amos et al., 2013). The IGRA measures the production of a cytokine produced by white blood cells of cattle infected with mycobacteria; infected animals produce elevated levels of IFN-γ compared to non-infected ones (Rhodes et al., 2000). This assay is performed in vitro in two stages. At first, fresh whole-blood samples are collected and transported to the laboratory within eight hours after collection and incubated in the presence of test antigens (PPD's) and a control. Within a day after incubation, the Bovigam™ IGRA test manual states within 16–24 h (Thermofisher Scientific, 2021), supernatants are harvested and the amount of IFN-γ produced is measured by an ELISA (De la Rua-Domenech et al., 2006). The read-outs from all these tests rely on competent immune responses, both *in* and *ex vivo*, so understanding potential confounders of these immunological responses is crucial to ensuring quality and consistency of any diagnostic service. Animal level outcomes from the IGRA are dependent on variance in three broad groups of factors 1. factors influencing exposure to infection (Jennes et al., 2017), 2. physiological factors such as competency of immune response due to variance in host and pathogen genetics (Phelan et al., 2023) and/or environmental impactors (Moriyama and Ichinohe, 2019; Velasco-Arnaiz et al., 2022) and 3. post-sampling factors directly impacting performance of the test.

The test result, and thus the disease status, depends on the cut-off value that is chosen in any diagnostic test but also on factors like the presence of other environmental mycobacteria (Cooney et al., 1997; Hughes et al., 2005) and quality of PPD (Frankena et al., 2018). The diagnostic accuracy of a test is defined by its sensitivity (Se) and specificity (Sp). Estimates for Se and Sp for SIT and SICCT tests have been reported as 50–70% and 99–100%, respectively (Clegg et al., 2011; Lahuerta-Marin et al., 2018; Nuñez-Garcia et al., 2018). Compared to SIT and SICCT, the IFN-γ assay tends to have greater reported Se (83–92%) but poorer Sp (83–97%) (Alvarez et al., 2012; Lahuerta-Marin et al., 2018). However, Se and Sp of both diagnostic tests can vary widely due to the biological activity of PPD (Klepp et al., 2019). Another post-sampling factor that potentially affects the accuracy of the IFN-γ assay is the ambient temperature during the period between blood collection and the start of the actual test in the laboratory (Buddle et al., 2009). Blood samples that were experimentally exposed to temperatures of 37.6 °C before testing showed diminished responses (Waters et al., 2007), potentially leading to false negative test results. Therefore, it might be important to avoid exposing blood samples to extreme ambient temperatures to ensure correct test results (Anonymous, 2005 in: Buddle et al., 2009). When it is not possible to process blood samples immediately, they should be stored and maintained at the same constant temperature until delivered at the laboratory (Waters et al., 2007). Studies have shown that transport of whole blood samples at colder temperatures (below 15 °C) resulted in lower cell recovery and viability compared to transport between 22 and 30 °C (Olson et al., 2011) while storage at 4 °C for 6 or 24 h had marked negative effects on subsequent cell yield compared to storage at room temperature (Jerram et al., 2021). A study, using human blood samples, showed that results of 51 out of 75 diagnostic tests were affected by ambient temperature of the day the sample was drawn; also medical decision-making was affected (Moriyama and Ichinohe, 2019; Obermeyer and Pope, 2021). It is important to note that whilst differences in ambient temperature are related to variance in test outcomes, the causative mechanisms can be difficult to define. Whilst there is a consensus that stable chain transport is important for sample stability *ex vivo*, fundamental physiological parameters such as plasma volume, blood pressure, blood cell counts which can modulate immune responses, both *in* and *ex vivo,* can also be impacted by ambient temperature changes. It has been demonstrated that murine adaptive responses to viral challenge can be impacted by extremes of ambient temperature with autophagy implicated as a potential mechanism (Moriyama and Ichinohe, 2019).

Research on the effect of storage temperature on ELISA optical density (OD) of the IFN-γ assay showed that blood stored at 15.6 and 21.1 °C results in the highest OD, temperatures at and above 37.8 °C gave a severe reduction in OD values. This led to the conclusion that blood samples should be stored and maintained at room temperature (Robbe-Austerman et al., 2006). Moreover, it has been shown that whole blood samples shipped at room temperature yielded the highest and most consistent level of IFN-γ activity in cows sub-clinically infected with *M. paratuberculosis* in comparison to blood samples that were transported at 37 °C (Stabel and Whitlock, 2001). On the basis of these prior findings, we hypothesized that there may be an association between IGRA results and the ambient temperature on the day the herd was sampled, and tested this hypothesis using an unparalleled field derived dataset from Northern Ireland.

1.1. Northern Ireland – local TB conditions

Northern Ireland (NI), situated on the island of Ireland, remains blighted with an ongoing bTB epidemic (Department of Agriculture, 2022). Efforts to control the disease include yearly SICCT testing of all cattle over 42 days old, backed up with ancillary IGRA testing of animals over 180 days old in chronically infected herds. Despite this, state led surveillance via a test and slaughter policy, the annual herd level incidence continues to edge towards 10% (Department of Agriculture, 2022). Therefore, in 2021 it was recommended that the IGRA testing capacity in NI should increase to 45,000 tests annually (Department of Agriculture, 2021a). Eradication of bTB will also require that tests are optimised to their best performance. One aspect of this requires quantifying the association between ambient air temperature and IGRA test results. If ambient air temperature impacts the IGRA outcome then false positive and/or false negative results can be the consequence. Therefore, the objective of this study was to assess the association between the ambient temperature and IGRA test results using field samples collected from cattle in Northern Ireland.

2. Materials and methods

2.1. Data

All data was made available by the Agri-Food and Biosciences Institute (AFBI, location Stormont, Northern Ireland) and covered IGRA test results of samples analysed in the period 2013 until 2018. Also included were animal level data: anonymized cow id, date of birth, date of death, age at the day of testing (in months), breed, sex, production type; herd level variables were: anonymized herd identification, herd type (beef, dairy or mixed), District Veterinary Office and location of the registered farm (x- and y-coordinates in six figure Irish grid reference). From the latter the straight-line (Euclidean) distances of a herd to the nearest weather station and to the laboratory were calculated.

2.2. Interferon-γ testing

In NI, the Bovigam™ IGRA test (Thermofisher Scientific, 2021) is applied as an ancillary test to the SICCT test to those herds with chronic bTB infection. Animals are tested after six months of age, due to the developing immune system and the potential for suboptimal test performance. In NI, the bleed for the IGRA test sample is currently conducted on the day of the SICCT test. Sampling occurs throughout the year and samples are brought to the central laboratory in Stormont, normally within 8 h. The ISO 17025 accredited test procedure in this lab has been described previously (McCallan et al., 2021; Welsh et al., 2002).

The following IGRA test values (optical densities (OD)) were available for this analysis for each sample: PBS (phosphate buffered saline for background, i.e. negative control), PPDa (avian), PPDb (bovine). Test id and test date were also included in this dataset. Net PPDa and net PPDb OD values were calculated by subtracting the background OD. An

animal was classified as IGRA positive if both net PPDb and net PPDb minus net PPDa (i.e. PPD(b-a)) OD values were greater or equal than 0.10. This is the current classification as from February 2016 as recommended by the manufacturer (Thermofisher Scientific, 2021); prior to February 2016, net PPD(b-a) OD value was set to exceed 0.05 (see Lahuerta-Marin et al., (2016) for discussion). The current classification was applied to all samples available for this study.

2.3. Weather data

To estimate the relation between the ambient temperature and IGRA test results, daily weather data was extracted from the archives of Met Office (UK) for the entire study period. The archive holds data of 32 weather stations in Northern Ireland. The maximum and minimum temperature were reported per twelve hours (at 9:00 and 21:00) or per 24 h. For this analysis, temperature data between 9:00 and 21:00 were the most useful as blood sampling took place during daytime. Therefore, fourteen stations were excluded due to incomplete data and/or weather data that was only reported per 24 h so that data of 18 stations remained available for analysis.

2.4. Data analysis

Summary statistics were assessed for animal and herd characteristics in relation to IGRA test results to provide background information. Data were analysed using two types of models. The first type explored the relationship between ambient temperature and the net PPDa, net PPDb and net PPD(b-a) OD values, and the second quantified the association of ambient temperature with IGRA test (binary) results. For the first model type, a multilevel linear regression approach was applied (PROC MIXED, SAS 9.4) where net PPDa, net PPDb or net PPD(b-a) OD values was the independent variable and minimum or maximum ambient temperature the main independent factor, herd was included as random effect (this model is labelled 'univariable model'). To improve model fit, a quadratic term for air temperature was added to the model. Minimum and maximum air temperature were fitted in separate models due to their high correlation (Spearman rank correlation = 0.91). In the multivariable model, age of the animal at bleeding, test year, the distance of the herd to the laboratory, the distance to the nearest weather station, sex and DVO were added as covariates to adjust the estimates of the ambient temperature variables for potential confounding. Season was excluded from the analysis as it is highly related to temperature (see discussion). The relationship between net PPD OD levels and ambient temperature is presented as predicted values and their 95% confidence intervals (CI) over the measured temperature range.

For the second model, the outcome variable was the IGRA test result (negative or positive). For this, a multilevel logistic regression model, based on generalized estimating equations (GEE), was applied (PROC GENMOD, SAS 9.4), with a compound symmetry correlation structure for the random effect of herd. The independent variables were the same as for the first model and model fit was based on the quasi-likelihood under the Independence model Criterion (QIC). The relationship between IGRA test results and ambient temperature is presented as predicted probabilities to test positive over the measured temperature range and their 95% confidence intervals. In addition, odds ratios with their 95% CI are presented for all variables included in the multivariable logistic regression model.

3. Results

3.1. Descriptive analysis

IGRA test results of 106,434 samples were available for analysis; 61 records (0.06%) were excluded due to data quality issues. These samples originated from 1046 herds and 1327 bleeding sessions. Most herds (839) were bled once. The maximum number of bleedings per herd was 5 (4 herds). The mean number of samples per bleeding session was 80 with median 68, mode 30 and an interquartile range (IQR) of 85 (34−119), the minimum being 1 and the maximum 318. The mean number of samples per herd was 102 with median 73, mode 50 and IQR 96 (36−132) with a range of 1–778. The number of bleedings over the temperature range was lower at the more extreme temperatures under NI weather conditions (Fig. 1).

The median net PPDa OD value was 0.074 (IQR: 0.016–0.267), the median net PPDb OD value was 0.038 (IQR: 0.007–0.134) and for net PPD(b-a), the median was 0.026 (IQR:−0.139 to 0.003).

32,240 samples had a PPDb OD value greater or equal than 0.10 and 6220 samples have an OD value greater or equal than 0.10 for PPD(b-a), combined this gives 5896 positive samples. The overall percentage of positive samples therefore is 5896/106,434 = 5.54% (95% CI: 5.40–5.68). The herd adjusted percentage of positive samples, based on an intercept only model, equals 5.96% (95% CI: 5.54–6.42). The average number of positive samples per herd was 5.64, ranging from 0 to 77; 187 herds (17.9%) had no positive samples and 710 (67.9%) had 1–10 positive samples.

The minimum daily air temperature ranged from − 5.1–22.1 °C (average 7.2, median 7.0), 1st and 99th percentiles: − 3.1 and 18.0) while the maximum daily air temperature was between − 0.1 and 29.5 °C (average 12.0, median 11.5, 1st and 99th percentiles: 1.9 and 25.1). Monthly averages (i.e. averaged over all 18 weather stations) of the minimum and maximum daily temperatures are shown in Fig. 2. The crude proportions of positive tests over the ranges of minimum and maximum air temperature are depicted in Fig. S1a and S1b, including second order polynomial trendlines. The distributions of the categorical variables age at test, test year, sex, herd classification and number of animals/DVO are tabulated in Supplementary Table S1.

3.2. Statistical analysis

Both predicted PPDa and PPDb OD values show a declining trend when samples are collected and transported at higher temperatures, where the observed decrease in IFN-γ production as response to PPDa stimulation is larger compared to PPDb stimulation (Fig. 3a and b). This results in increasing OD values for PPD(b-a) at higher temperatures. The curves of predicted values estimated from the multivariable model become more 'spiky' due to the numerous covariate patterns (n = 13,378 and n = 13,367 for minimum and maximum temperature respectively), each having a different predicted value (Supplementary Figs. S2a, Fig. S2b). However, outcomes were not affected by adding potential confounders to the model as magnitudes and significance of the regression coefficients are comparable with the univariable models (Table 1). The quadratic term was significant in all models except for maximum air temperature in the net PPD(b-a) model. Complete results of the multivariable models are presented in Supplementary Tables S2a-S2c for minimum temperature and in Tables S3a-S3c for maximum temperature. In all models less than 10% of the unexplained variation (i.e. variation not explained by the fixed effects) was due to herd, the rest being random error.

Fig. 4a and b show the predicted probability to test positive in the IGRA test estimated by a logistic regression model including the minimum or maximum air temperature and their square (quadratic term) and a random herd effect. This probability is highest (above 6%) at moderate maximum temperatures (6–16 °C). If the maximum temperature is below 3 °C it reduced to below 5% and to below 3% at temperatures above 26 °C. Similarly, for the minimum temperature model, the highest probability to disclose positive results occurred on days with minimum temperatures of 5–7 °C.

At lower and higher temperatures the 95% confidence intervals get wider as these temperatures occur less often under Irish weather conditions and consequently less samples have been collected (Fig. 1). Including the quadratic term reduced the QIC in all four models (i.e. models with and without covariates for both minimum and maximum

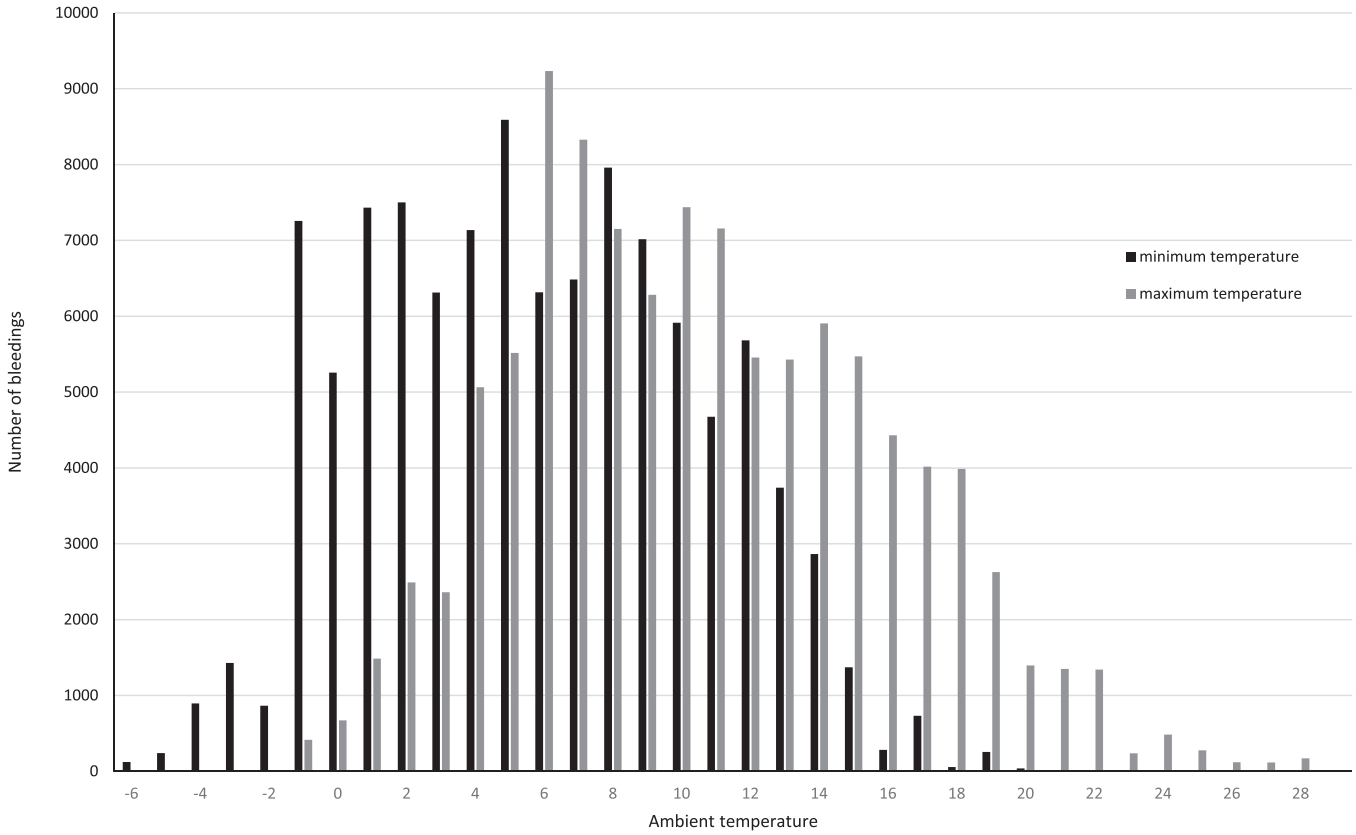

Fig. 1. Distribution of the number of bleedings (n = 106,343) over minimum and maximum ambient temperatures in the period 2013–2018.

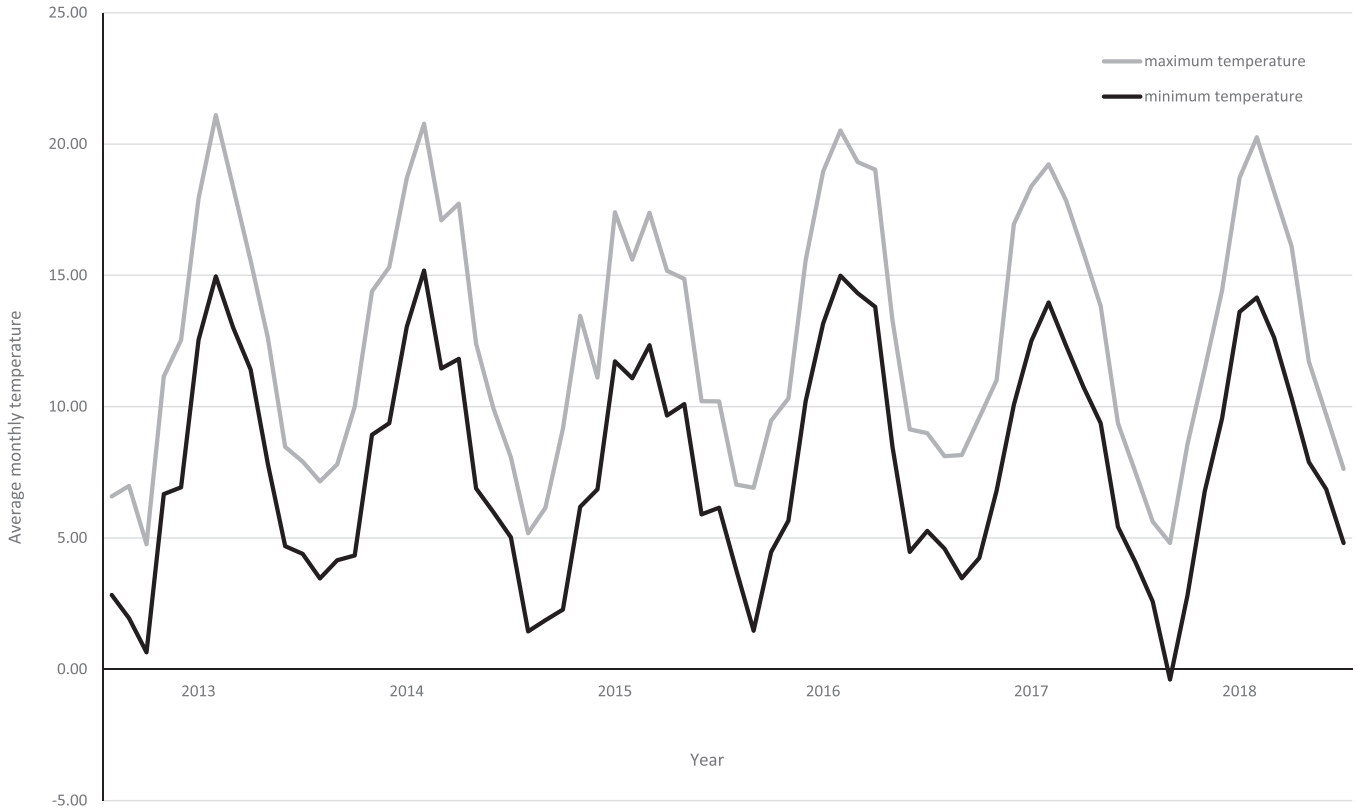

Fig. 2. Average monthly maximum and minimum daily temperature measured at 18 Northern Irish weather stations over the period 2013–2018.

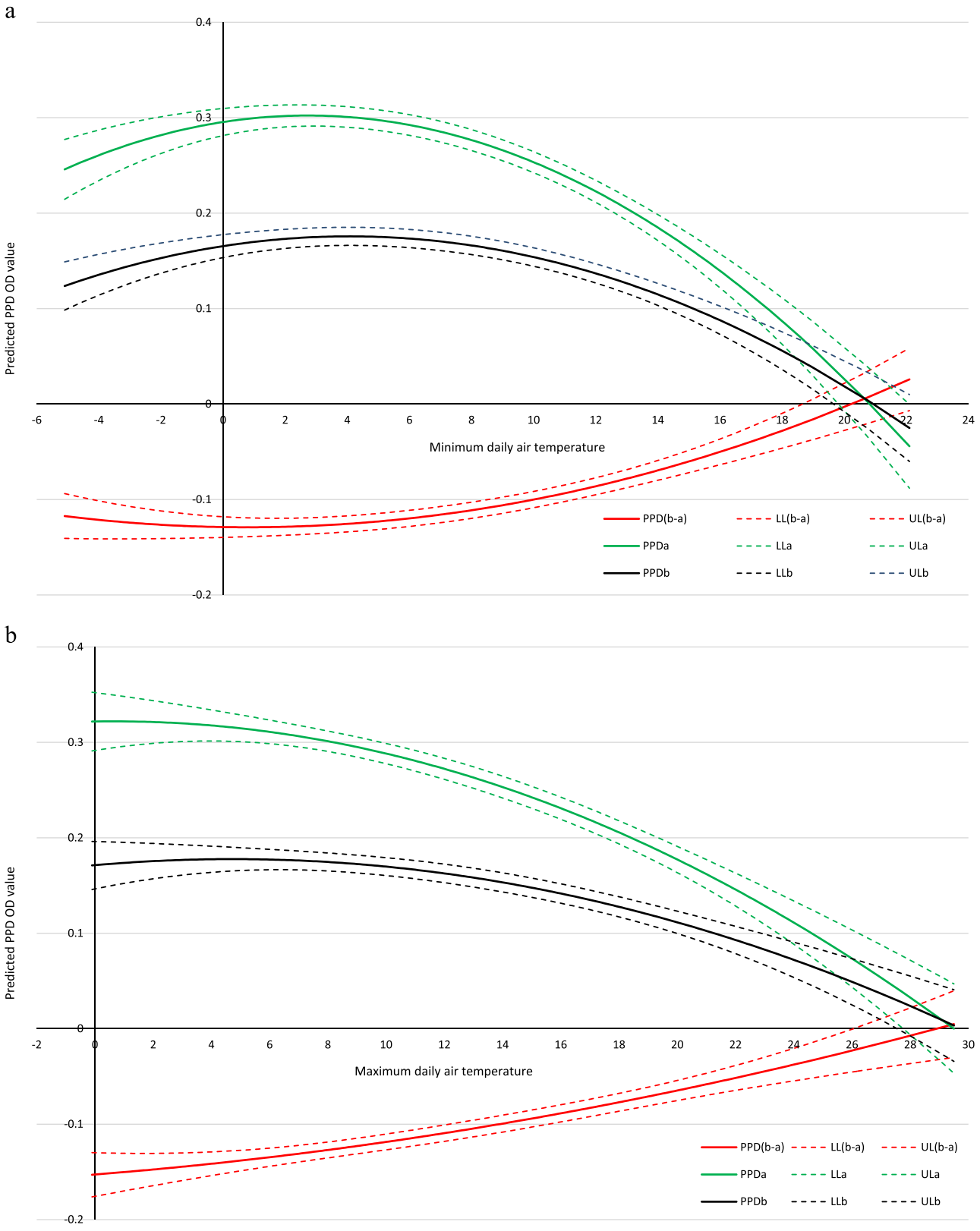

Fig. 3. a. Predicted net PPDa, net PPDb OD values and their difference net PPD(b-a) by a univariable mixed linear regression model including **minimum** daily temperature and its square while adjusting for a random herd effect. Dotted lines represent lower and upper levels of the 95% confidence interval (LL: lower limit, UL: upper limit). b. Predicted net PPDa, net PPDb OD values and their difference net PPD(b-a) by a univariable mixed linear regression model including **maximum** daily temperature and its square while adjusting for a random herd effect. Dotted lines represent lower and upper levels of the 95% confidence interval (LL: lower limit, UL: upper limit).

Table 1
Regression coefficients and their significance of minimum (top) and maximum (bottom) ambient air temperature and its square of the univariable and multivariable (adjusted for covariates) multilevel regression models of IFN- γ response to Purified Protein Derivatives (PPD).

		Univariable model		Multivariable model	
PPD	Variable	Estimate	P-value	Estimate	P-value
PPDa	Intercept	0.295507		0.291353	
	Minimum air temp.	0.005013	0.002	0.005007	0.003
	Minimum air temp. square	-0.000922	< 0.0001	-0.000938	< 0.0001
PPDb	Intercept	0.165487		0.122164	
	Minimum air temp.	0.005030	< 0.0001	0.005705	< 0.0001
	Minimum air temp. square	-0.000618	< 0.0001	-0.000656	< 0.0001
PPD(b-a)	Intercept	-0.128855		-0.169787	
	Minimum air temp.	-0.000526	0.66	-0.000072	0.95
	Minimum air temp. square	0.000341	< 0.0001	0.000331	< 0.0001
PPDa	Intercept	0.321882		0.331209	
	Maximum air temp.	0.000516	0.83	-0.000619	0.81
	Maximum air temp. square	-0.000387	< 0.0001	-0.000358	0.0001
PPDb	Intercept	0.171348		0.138702	
	Maximum air temp.	0.002695	0.17	0.001979	0.33
	Maximum air temp. square	-0.000284	< 0.0001	-0.00026	0.0005
PPD(b-a)	Intercept	-0.152741		-0.195622	
	Maximum air temp.	0.002423	0.19	0.002569	0.18
	Maximum air temp. square	0.000099	0.14	0.000104	0.14

PPDa: IFN- γ response against purified protein derivative from M. avium (PPDa)
PPDb: IFN- γ response against purified protein derivative from *M. bovis*
PPD(b-a): difference in IFN- γ response against PPDb en PPDa

temperature). The quadratic terms, but not the linear, contributed significantly to the univariable and multivariable models (Table 2). Adding potential confounders to the model did neither change the regression coefficients of the temperature variables (Table 2), nor the predicted proportion positives greatly (Supplementary Figs. S3a and S3b). The output of the models with all covariates are presented in Supplementary Table S4a for minimum temperature and in Table S4b for maximum temperature. The exchangeable working correlations were less than 0.05 in both models indicating that the herd effect only explained a small part of the residual variation.

4. Discussion

This study is the first observational epidemiological study to quantify the association between ambient temperature on the day of bleeding, and subsequent IGRA test result based on a large number of field samples. Results show that net PPDa and net PPDb OD values were lower at more extreme maximum and minimum temperatures as recorded in Northern Ireland over the study period. The test binary outcomes were also associated with temperature on bleed day. There were lower probabilities to test positive in the IGRA test at extreme temperature, for example above 26 °C or below − 4 °C. Multiple factors related to animal- and herd characteristics were taken into account as potential confounders but did neither have a major effect on the predicted net PPD OD values nor on the predicted probabilities to test positive.

The analyses of minimum and maximum temperature in relation to net PPD OD values indicate a range of temperatures where OD values are highest for both net PPDa and net PPDb. The effect of air temperature on net PPDa and net PPDb OD values (and thus on net PPD(b-a)) has consequences for the predicted probability of a positive test result (Fig. 4a and b). This probability shows a twofold reduction at ambient temperatures around 25 °C. In several studies the effect of fixed storage and transport temperatures on the test performance of a range of cellular function assays, including the IGRA, have been investigated (Jerram et al., 2021; Olson et al., 2011; Robbe-Austerman et al., 2006; Waters et al., 2007). Though some studies demonstrate little difference in outcomes at temperatures between 4 and 22 °C most point to an optimal temperature range around room temperature (~22 °C) when cellular viability, recovery and overall function (including IFN-γ production) were evaluated. This is consistent with the optimal temperature that the manufacturers of Bovigam™ recommend (22 ± 3 °C). It is however hard to compare the current study with these previous ones as rather than focus on samples held at a few fixed temperatures *ex vivo* we have considered ambient temperature at the day of blood collection which, in addition to sample stability, also introduces the potential for temperature related factors such as infection risk and physiological/immunological changes to impact test outcomes.

Under Northern Irish weather conditions, the effect of low ambient temperatures will be of more importance than high ambient temperatures as ambient temperatures above 20 °C are infrequent in contrast to temperatures below 10 °C (Figs. 1 and 2). The maximum temperature did not exceed 10 °C at 724 (out of 2183) sampling dates during the study period. These relatively cold days especially occurred in November up to March. In these 5 months, 51% of all samples were collected and thus potentially exposed to relatively low ambient temperatures. Therefore, it is of importance to protect samples from the cold during transport to the laboratory at days the ambient temperature is low, transport then should take place under standardized conditions. Moreover, transport in a container with a temperature logger enables more accurate monitoring of temperature fluctuations during the time between bleeding and delivery to the laboratory.

5. Limitations

Despite the study including a large number of samples and herds, the study population might not be representative for all herds in NI as at the time that these historical data were collected when IGRA testing was not compulsory and were especially applied in herds with chronic bTB infections. Although this might affect the absolute level of the proportion positives it is unlikely that the association between temperature and IGRA test result will be affected.

Daily temperatures were collected from weather stations. Distance from the herd to the nearest weather station varied from 0 to 37 km (average 14 km, median 13 km) and was not significantly related to the odds of testing positive in both the minimum and maximum temperature models (Tables S4a and S4b). Measuring the actual temperature at the place and moment of bleeding would be more accurate. Also, with respect to temperature effects *ex vivo,* ambient temperature might not be highly correlated with the temperature in the transporting vehicle and the sample. It cannot be ruled out that extra variability is introduced during blood sample transportation, especially variance related to vehicle temperature during transport. Straight line distance between the herd to the laboratory (average 69 km, median 66 km, minimum 2 km, maximum 149 km) was not significantly related to the test outcome (Tables S4a and S4b). However, recording the actual travel distance, or more importantly the transport time, between bleeding and delivery of the samples to the lab might be more precise than the straight line distance. Whilst not mitigating pre-bleeding, physiological factors that can impact test response transporting the samples to the laboratory under consistent, standardized conditions might rule out some of this potential bias.

The principal finding that ambient daily temperature is associated with responses to PPD and consequently IGRA performance presents an important consideration for the design, operation and expansion of diagnostic schemes and goes beyond previous studies that have focused

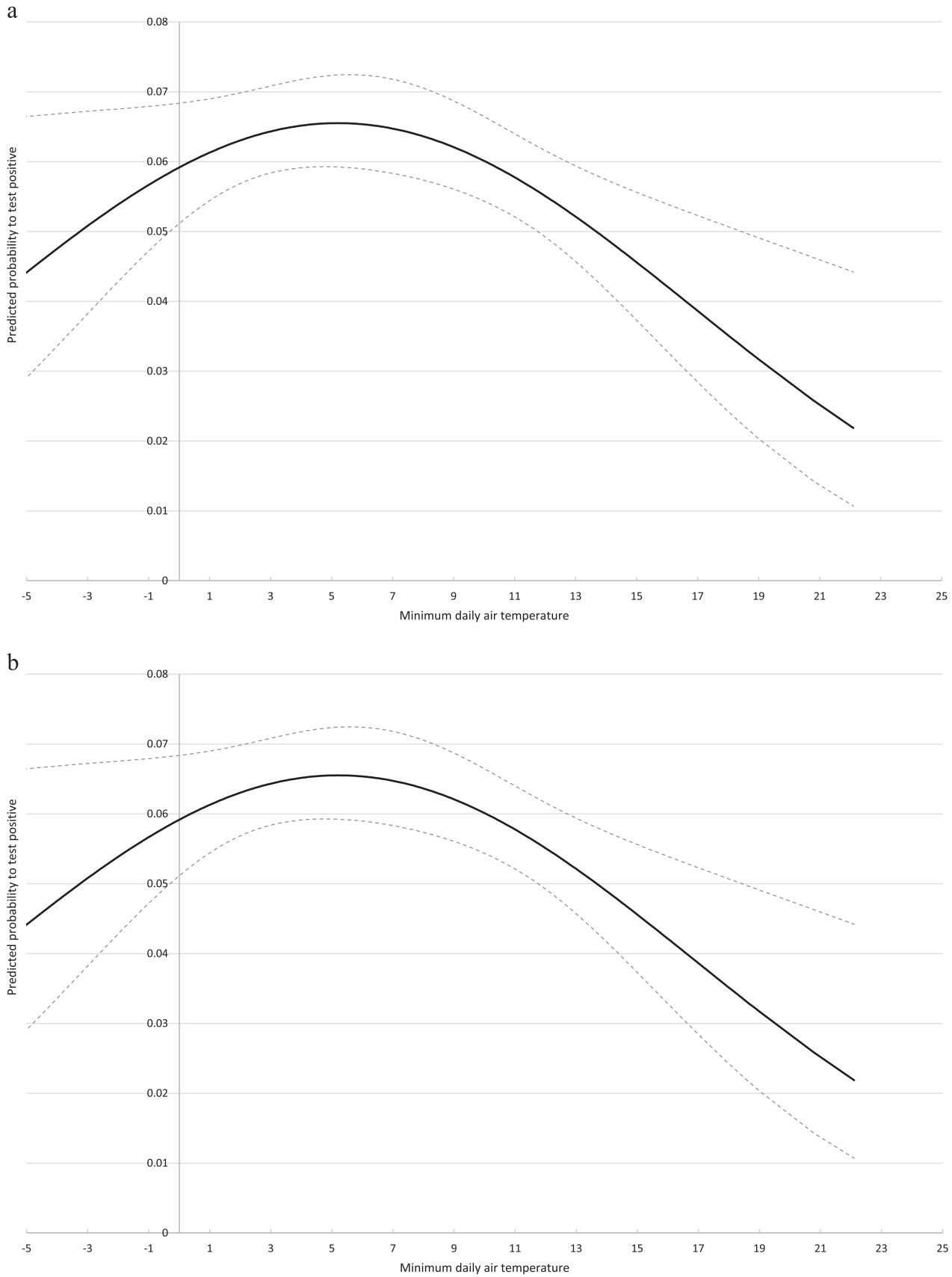

Fig. 4. a. Predicted probability to test positive in the IGRA test by a univariable mixed logistic regression model including **minimum** daily temperature and its square while adjusting for a random herd effect. Dotted lines represent lower and upper limits of the 95% confidence interval. b. Predicted probability to test positive in the IGRA test by a univariable mixed logistic regression model including **maximum** daily temperature and its square while adjusting for a random herd effect. Dotted lines represent lower and upper limits of the 95% confidence interval.

Table 2
Multilevel logistic regression analysis of IFN-γ positive test results for bTB infections during the period 2013 – 2018 based on 106,434 samples from cattle in Northern-Ireland.

	Univariable model		Multivariable model	
Variable	Odds ratio	P-value	Odds ratio	P-value
Minimum air temp.	1.043	0.09	1.041	0.09
Minimum air temp. square	0.996	0.02	0.996	0.02
Maximum air temp.	1.084	0.05	1.076	0.06
Maximum air temp. square	0.996	0.02	0.997	0.03

on the controlled simulation of temperature variance. At low temperatures these findings are consistent with previous work and further underlines the apparent importance of temperature control between the farm and the laboratory. However, to fully understand the mechanisms involved requires additional work that was outside the scope of this study. A similar analysis and comparison with skin test data, an in vivo test, would help to elucidate to what extent post sampling effects rather than physiological effects of temperature extremes have on test outcomes. To further understand the effect of ambient temperature on physiology and baseline immune function focus could also be given to evaluating constituent characteristics of blood samples immediately post blood-draw e.g. full blood counts, immune cell phenotype ratios and plasma volume. Finally another natural extension to this work would focus on comparing sampling time of day with test outcomes as temperature changes associated with circadian rhythms may also have some impact on immune function (Coiffard et al., 2021).

Season was not included in final models, as there is a strong relationship between season and temperature. It is possible that some of the variation in test results associated with temperature could relate to true seasonal effects in terms of cattle exposure, and therefore their likelihood of test positivity. However, cattle can be exposed to *M. bovis* via several transmission pathways, including close contact with infected herd mates (Menzies and Neill, 2000), environmental exposure via fomites (Allen et al., 2021), and through exposure to infected wildlife (Campbell et al., 2020), all of which may have different temporal exposure windows for pasture-based systems. Seasonal effects are further complicated by the lags in animals being exposed, being immunologically primed to give positive tests to tuberculin-based tests, and actually being tested within herds (for example, the mean time from exposure to detection was estimated to be 6–8 months using data from the UK (Brooks-Pollock et al., 2013)). There may be differences in non-tuberculous mycobacteria (NTM) between different soils or weather conditions (Cooney et al. 1997; Jenkins et al. 2018), though it is uncertain how this relates to the variation in non-*M. bovis* mycobacterial exposure during grazing seasons and across farms. Importantly, it is unlikely that NTM exposure would correlate with necessarily bTB exposure, so therefore we feel that it is more likely that the patterns found in the present study relate to forcing by ambient temperature rather than seasonal variation in pathogen exposure.

Finally, we have included in the analysis the avian reactions, which in the test controls for potential non *M. bovis* mycobacterial exposure, and we found temperature related effects, We would not necessarily expect variation in non-*M. bovis* mycobacterial exposure across seasons and for that exposure to correlate with bTB exposure, so therefore we feel that it is more likely that the patterns found in the present study relate to ambient temperature forcing rather than seasonal variation in pathogen exposure.

5.1. Other findings

The crude percentage positive IGRA tests over the study period was 5.54% which increased to 5.96% after adjustment for the multiple sampling per herd. This proportion is in agreement with an earlier study in NI (2004–2010) from which 6.5% of animals tested positive using the IGRA at the more sensitive cut-off of 0.05% and 4.7% of animals tested positive using the IGRA at the cut-off of 0.1 (Lahuerta-Marin et al., 2018). In comparison, data from the Republic of Ireland (ROI) showed a positivity rate of 11.4% (7874/68,993) in 2016–2017, though direct comparisons cannot be made regarding underlying prevalence given the differences in cut-points used and the way the test is used (e.g. ROI use IGRA for quality control of skin testing yielding higher positivity rates (Clegg et al., 2019).

The multivariable models suggested that the odds of test positivity were significantly associated with age-cohort and year. The highest proportion of IGRA positive animals was in the age group of six months to one year olds, and the odds was significantly higher compared to all other age groups. However, risk of test failure increased from 1-year onwards up to 5 years. This finding is somewhat in contrast with previous work where age-dependent accumulation of risk has been described (Byrne et al., 2022; Gormley et al., 2013; Lahuerta-Marin et al., 2016; Male Here et al., 2022). However, it has also been reported that young animals (less than six months old) can produce high levels of IFN-γ due to a high number of natural killer cells that can produce IFN-γ (Olsen et al., 2005). Regarding year, the highest risk year was 2015, while the year with the lowest proportion was 2013, which generally corresponds to national level herd- and animal-level incidence, albeit with a lag period. There were no significant differences in risk between distances to lab, distances to weather station, herd type, DVO region or sex of the animal sampled.

In summary, ambient temperature is associated with the IFN-γ response towards PPD, and thus to the IGRA test result. The probability to test positive was highest at moderate daily ambient temperatures (6–16 °C). This is of particular concern since ambient temperature on the day of sampling is currently not taken into account as a factor that may affect the test outcome. At present, samples are taken under all weather conditions. The results of this study contributes to a better insight into effects of ambient temperature on the IGRA test results, and suggests that, every effort should be made to maintain consistent temperature at each stage of sampling, handling, transporting and storing of samples before testing. With respect to the Northern Ireland programme this is particularly important at lower temperatures as this is the predominant condition with respect to temperature extremes.

Declaration of Competing Interest

The authors declare to have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetmic.2023.109778.

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