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RESEARCH ARTICLE

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The effects of annual cycle, source population, and body condition on leukocyte profile and immune challenge in a basal reptile, the tuatara (*Sphenodon punctatus*)

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Abstract

Leukocyte profiles are broadly used to assess the health status of many species. Reference intervals, and an understanding of the factors that may influence these intervals, are necessary for adequate interpretation of leukograms. Using a data set that spans over three decades, we investigated variation in leukocyte profile in several populations of the evolutionarily unique reptile, the tuatara (*Sphenodon punctatus*). To do this, we first established reference intervals for each leukocyte type according to best practices. Next, we determined that source population and sampling date were the two most important predictors of leukocyte makeup. We found significant differences in the ratio of heterophils: lymphocytes (H:L) between populations, with tuatara on the more resource-stressed sampling island having a significantly higher ratio of H:L. Finally, we found that sampling location, sex, and life stage did not explain variation in the responses of tuatara to stimulation with Concanavalin A and lipopolysaccharide in both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and Griess assay experiments. Our results offer important insight into the function of leukocytes in reptiles.

KEYWORDS

blood smears, immunity, leukocytes, lymphocytes, reptiles, stress

1 | INTRODUCTION

Leukocytes, also called white blood cells (WBCs), are one of the three formed elements that, along with plasma and red blood cells, comprise blood (Ashton, 2007). WBCs are less abundant than red blood cells and function to defend the body against illness and injury and to remove damaged cells and waste products (Ashton, 2007). The five types of leukocytes, ordered from most to least common across species, are: neutrophils (or heterophils in rabbits, birds, and many herpetofauna; Claver & Quaglia, 2009; Sykes & Klaphake, 2008), lymphocytes, monocytes, eosinophils, and basophils (Ashton, 2007). All five types play important roles in the body's immune system. For example, eosinophils respond to helminth infections and function to reduce inflammation (Ashton, 2007; Martin et al., 1996). Comparatively, basophils are extremely rare and primarily function to release histamine and heparin at injury sites (Ashton, 2007; Borriello

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et al., 2017). All leukocytes can respond to chemical stimuli, are capable of ameboid movement, and can be visually distinguished via staining characteristics (Ashton, 2007).

Leukocyte profiles, assessed by the relative proportions of different types of WBCs, are widely used as a proxy for physiological stress in vertebrates (Davis & Maney, 2018; Davis et al., 2008). Lymphocytes are part of the adaptive immune system and respond to antigen-specific threats, whereas heterophils are part of the innate immune system and display high levels of phagocytic activity toward many different stressors (Minias, 2019; Zimmerman, Paitz, et al., 2010). An environment of chronic stress will depress the number of circulating lymphocytes and increase the number of circulating heterophils to maintain a heightened state of preparation against injury and infection. Thus, these two WBC types can be used to broadly assess adaptive and innate immune activity, with high numbers of heterophils relative to lymphocytes indicating high levels of chronic stress (Davis et al., 2008).

The total number of WBCs is another important metric for diagnosing disease and organismal disfunction. Although the total number of WBCs is not a reliable indicator of chronic stress (Davis et al., 2008), leukocytosis, or an increase in the number of WBCs, can be used to diagnose leukemia and other diseases of the blood (Chabot-Richards & George, 2014). Importantly, reference intervals for the expected proportion of each type of WBC are necessary to identify the underlying cause of the leukocytosis. For example, lymphocytosis can be an indicator of an autoimmune disorder, while basophilia is rare and indicative of iron deficiency, chronic inflammation, or hypersensitivity disorders (Arnalich et al., 1987; Chabot-Richards & George, 2014).

Our ability to use leukocyte profiles to assess health is limited by the lack of reference data and experimental research carried out on immune function in many wild species, particularly reptiles. Despite facing an ongoing extinction crisis and serious threats to their conservation in the wild (Böhm et al., 2013; Gibbon et al., 2000), the reptile immune system remains understudied compared to many other taxa. Tuatara (*Sphenodon punctatus*) are the sole surviving members of the once-widespread order Rhynchocephalia, which diverged from other reptiles approximately 250 million years ago (Cree, 2014; Fraser, 1988). Endemic to Aotearoa New Zealand, tuatara have undergone severe range restrictions since the arrival of humans and are now limited to approximately 45 offshore islands and several mainland populations in ecosanctuaries, captive holding centres, and zoos (Cree, 2014; Gaze, 2001).

Despite significant interest and investment in tuatara conservation, our understanding of their immune system remains limited (Burnham et al., 2005; Middleton et al., 2015). Studies on captive tuatara found variation in numbers and proportions of leukocytes between individuals but no seasonal pattern (Burnham et al., 2006). Additionally, captive tuatara had higher numbers of red blood cells than wild tuatara, which displayed an increase in total WBCs with increasing tick burden (Burnham et al., 2006). To date, no standard for leukocyte profiles has been generated for this unique reptile, despite the prevalence of conservation actions and captive holding, including individuals at several zoos outside of Aotearoa New Zealand (Cree, 2014).

Three offshore islands have been longstanding locations for tuatara research in particular: Takapourewa (Stephens Island), the northern island of Ngāwhatu-kai-ponu (henceforth North Brother Island), and Matiu (Matiu/Somes Island). These islands lie on approximately the same latitude but have different terrain and biological communities. At 150 hectares (ha), Takapourewa (40.670°S, 173.997°E) is the largest of these islands and is home to the largest and most dense remaining population of tuatara, with approximately 2700 tuatara/ha in forest areas (Moore, Daugherty & Godfrey, & Nelson, 2009; Moore, Daugherty, & Nelson, 2009). Takapourewa is also host to a rich community of seabirds, invertebrates, and lizards (Cree, 2014). The much smaller (4 ha) North Brother Island (41.115°S, 174.432°E) has scarce resources (Cree, 2014) and an approximate density of 118.25 tuatara/ha (Hoare et al., 2006). In 1998, 54 tuatara from North Brother Island were translocated to Matiu as part of efforts to restore the island and reintroduce tuatara to historical locations within their native range (Cree, 2014). Finally, Matiu (41.258°S, 174.866°E) is a 25 ha island located in Wellington Harbour which plays host to a rich community of lizards, invertebrates, and birds (Crisp et al., 2020; Watts & Gibbs, 2000).

Using blood smears collected from tuatara between 1988 and 2022, we set out to achieve two major aims. First, we created population-specific reference intervals for tuatara leukocyte profiles. These intervals may be used for both routine monitoring of captive populations and to assess the health of wild individuals being considered for inclusion in conservation activity. Second, we investigated the factors influencing immune function, as measured by leukocyte profile composition, T cell proliferation, and cellular activity assays, in wild tuatara.

2 | METHODS

2.1 | Study sites and sample collection

We collected blood samples as part of various projects being undertaken on tuatara between 1988 and 2022 (Table 1). We did not collect blood samples from all three islands during each year or season. During each sampling event, we cleaned the skin over the ventral coccygeal vein of the tuatara using 70% ethanol and drew blood with a sterile syringe. In some cases, the anti-coagulants heparin or ethylenediaminetetraacetic acid (EDTA) were used. To make blood smears, we deposited a drop of blood onto a glass microscope slide and smeared the blood to achieve mono-layering. In November 2010, trips were taken to North Brother Island and Matiu specifically to draw blood for lymphocyte proliferation and cellular activity assays. On these trips, we collected 500–1000 μL of blood from each individual in a heparinized syringe and kept the sample either on ice or between 0°C and 4°C until culture (see below).

In addition to blood samples, we also collected snout-vent length (SVL) (mm), mass (g), and sex for each individual. Using SVL and mass

TABLE 1 Sampling summary for tuatara (*Sphenodon punctatus*) included in this study.

Location	Year	Sampling dates	*n* (Males: Females)	Mean BCI	SE BCI
Takapourewa					
	2005	331–332	15 (11:4)	1.11	0.01
	2006	329	15 (11:4)	1.11	0.01
	2021	49–65	13 (13:0)	1.17	0.01
	2022	50–70	21 (21:0)	1.16	0.01
Total			64 (56:8)	1.14	0.01
North Brother Island					
	1988	21–23	41 (22:19)	1.06	0.01
	2008	331–332	48 (39:9)	1.06	0.01
	2010	306–309	41 (29:12)	1.04	0.01
Total			130 (90:40)	1.05	0.00
Matiu					
	2010	NA	9 (5:4)	1.15	0.02

Note: Body condition index (BCI) is calculated as the ratio of log-transformed mass to log-transformed snout-vent length. Sampling dates are provided as Gregorian day of the year for populations assessed for leukocyte rhythmicity. SE = standard error.

we calculated body condition index (BCI) as the ratio of log-transformed mass to log-transformed SVL. BCI helps to standardize for the effects of sexual dimorphism and tail loss in tuatara and is used as a proxy for overall fitness (Hoare et al., 2006).

2.2 | Blood smears and leukocyte profile generation

Blood smears from 1988, 2008, and 2010 were stained with Wright's stain dissolved in 100% methanol, smears from 2006 were stained with a modified Wright-Giemsa stain (Godfrey et al., 2011), and smears from 2021 and 2022 were stained with a commercial Romanowsky stain. We allowed slides to dry before cover-slipping and sealing with DPX mountant (Sigma-Aldrich) for preservation. We examined all blood smears under light microscope at ×400 magnification. On each slide, we counted the first 100 leukocytes encountered and identified them to type using morphology of the nucleus, cytoplasmic granules, and staining characteristics (Claver & Quaglia, 2009; Desser, 1978) (Supporting Information: Figure S1). Finally, we estimated the number of leukocytes per mL of blood by counting the number of leukocytes in 10 fields at ×400 magnification, calculating the average, and multiplying it by two to get the estimated number of WBCs in $\times 10^9$/L of blood (Sykes & Klaphake, 2008). We present leukocyte cell type data as both % and absolute counts, which were obtained by multiplying the % by the estimated total leukocyte number.

2.3 | Peripheral blood mononuclear cell (PBMCs) assays

We isolated PBMCs from the November 2010 blood samples (Matiu and North Brother Island) following an adapted version of the La Flamme et al. (2010) protocol. Briefly, we diluted between 100 and 300 µL of blood in wash media before layering the sample onto 3 mL of Histopaque-1077 (Sigma-Aldrich) and centrifuging it for 20 min at 400× g at room temperature. We collected PBMCs from the top layer, washed them, and resuspended PBMCs in DMEM containing 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin plus 100 µg/mL streptomycin, 10 mM Hepes, 55 µM β-mercaptoethanol, and 100 µM MEM non-essential amino acids solution (all from Invitrogen). We then cultured PBMCs in 96-well plates at a concentration of 2.5×10^5 cells/well. Some blood samples did not yield a sufficient quantity of PBMCs to plate at this concentration; we plated these more dilute samples at concentrations between 0.34 and 2.1×10^5 cells/well. We stimulated PBMCs with 200 ng/mL bacterial lipopolysaccharide (LPS) (Sigma-Aldrich) and 5 µg/mL concanavalin A (ConA) (Sigma-Aldrich). Finally, we covered and incubated sample plates at 25°C for 72 h in 5% CO_2 (Sanyo CO_2 incubator, model MCO-2OAIC) (La Flamme et al., 2010). To determine the relative proportion of different types of cells in our cultures, we spun cells onto slides using a cytospin (Thermo Shandon), stained samples with Wright's stain, and identified cells as described above.

2.4 | Cell proliferation and nitric oxide assays

Because live cells convert 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) into blue formazan, MTT can be used to assess the proliferation, or activation, of cultured cells (Mosmann, 1983). To measure this shift, we removed 100 µL of supernatant from the cultured cells and added 20 µL of MTT (5 mg/mL in 1× phosphate-buffered saline, Sigma-Aldrich). We incubated the sample for 2 h at 25°C, then added 100 µL of 10% SDS: 45% DMI (pH 4.5) to solubilize the MTT formazan. Finally, we incubated samples overnight (≥9 h). We measured light absorbance at 570 nm using a VERSAmax microplate reader (Molecular Devices). We also measured the nitric oxide (NO) production in the culture supernatants using a Griess reaction (Green et al., 1982; La Flamme et al., 2010).

2.5 | Statistical analyses

We performed the statistical analyses for leukograms using R version 4.2.0 (R Core Team, 2022). We set the alpha level of all analyses to 0.05.

First, we tested the relationship between independent grouping variables and BCI. We ran a Shapiro–Wilk test for normality on BCI and found that data were abnormally distributed and were not able to be brought into normality with transformation ($w = 0.972$, $p < 0.001$). Thus,

we used a series of Kruskal-Wallis tests to investigate differences in BCI among islands and sampling years and a Mann–Whitney *U* test to investigate BCI differences between sexes. To determine which islands differed from each other in BCI, we conducted a post hoc Dunn's test. Finally, we calculated summary statistics (mean and standard error) for BCI, grouped by island and year (Table 1).

As chemical anticoagulants can alter the appearance of blood cells and thus change the observed values, we tested for the effects of anticoagulant presence on blood cell values using the 2010 North Brother Island trip leukocyte percentages. To do this, we first ran Shapiro-Wilk tests on all blood cell parameters and found three WBC types violated the assumption of a normal distribution. We were able to align eosinophils with a normal distribution after log transformation ($w = 0.98$, $p = 0.585$) and heterophils with normal distribution after a transformation of $x^{0.175}$ ($w = 0.98$, $p = 0.753$). However, basophils ($w = 0.90$, $p = 0.002$) could not be transformed into a normal distribution. We ran Welch's *t*-tests on all normally distributed blood parameters and a Mann–Whitney *U* test on basophils; we found no effect of anticoagulant on blood cell differentiation (all *p*-values ≥ 0.093; Supporting Information: Table S1). Moving forward, we removed anticoagulant as a variable from all analyses.

We calculated population-specific reference intervals (RIs) for each leukocyte type absolute count and the estimated total leukocyte count following the best practices of the American Society for Veterinary Clinical Pathology (Friedrichs et al., 2012). Briefly, we used the *rcompanion* package to calculate the λ value that brought each population's leukocyte profiles most strongly into a normal distribution (Mangiafico, 2020). Because leukocyte profiles are inherently compositional data, we removed outliers using a multivariate approach and the R package *MVN* (Korkmaz et al., 2014). Next, we calculated RIs as the central 95% of the data and report 90% confidence intervals for the lower and upper reference limits, in accordance with best practices (Friedrichs et al., 2012). Because reporting RIs calculated for populations of less than 20 individuals is not advised (Friedrichs et al., 2012), we did not calculate RIs for tuatara living on Matiu. However, unfiltered summary data (mean and standard error) is available for all relevant blood parameters, grouped by island, in Supporting Information: Table S2.

We calculated the mean ratio of heterophils:lymphocytes (H:L) and mean total leukocyte count for tuatara blood smears collected from our three study islands and compared values between populations using Kruskal–Wallis tests and post hoc Dunn's tests. We also compared BCI scores between the unfiltered and filtered (individuals with outlying leukocyte profiles removed) datasets using a *t*-test.

Next, we tested for the presence of variation in leukocyte profile parameters by sampling date. Using our filtered data set with multivariate outliers removed ($n = 151$), we created single component cosinor models for each blood parameter using the *circacompare* package in R (Parsons et al., 2020). These models use linear combinations of cosine functions to model physiological processes that are influenced by annual or seasonal variation (Halberg et al., 1967; Refinetti et al., 2007). The equation for a single component cosinor model is written as $Y = M + A^*\cos(r - \varphi)$ where *M* is the midline estimating statistic of rhythm (MESOR, a rhythm adjusted mean), *A* is the amplitude of the physiological rhythm around the MESOR, *r* is the time unit in radian-hours, *and* φ is the acrophase (phase shift in radian-hours). Gregorian dates were available for all samples except the November 2006 trip to Takapourewa, where trip dates, which spanned six calendar days, are known but not individual sampling dates. In this case, we used the middle date of the trip (24 November) for all samples ($n = 15$). For each blood parameter we report the p-value of rhythmicity, *M*, and *A*.

Next, we created a series ($n = 6$) of generalized linear mixed effects models (GLMMs) using the *lme4* package in R (Bates et al., 2015). We created a model for each leukocyte type and the total number of leukocytes, with blood parameter as our response variable, year as a random effect, and location, BCI, and sampling date as fixed effects. Including sex as a random effect led to overdispersion in our models, likely due to sampling imbalances, and was thus excluded from our global model. We centered both Gregorian date and BCI to scale predictors and aid in interpretation of coefficient effect sizes by reducing variance inflation. We then model averaged predictors from the top-ranked models to evaluate each predictor's importance in explaining leukocyte composition in tuatara. Next, we used the *MuMIn* package to assess fit of all possible models and report models with a delta AICc of <2 (Barton, 2022). Finally, we report the sum of model weights (of models with AICc < 2) for each explanatory variable.

For individuals included in our MTT and Griess assay experiments, we assessed normality of BCI values using a Shapiro-Wilk test and tested for differences in BCI between the sample populations using a Welch's *t*-test. We calculated summary statistics for our cytospin cell counts as percentages, as no total leukocyte counts were available, and used a series of *t*-tests to assess for differences in cytospin results. We tested for normality of the responses to stimulation with both LPS and ConA in both assays using Shapiro–Wilk tests. For groups with normal distributions, we compared stimuli response strength using Welch's *t*-tests, and for those with abnormal distributions we used Mann–Whitney *U* tests. We tested for differences between males, females, and juveniles (only included in the PMBC and assay datasets) using grouped analyses of variance (ANOVAs). Finally, we tested for correlation between cytospin results and assay stimulation results using a series of Pearson's correlations.

3 | RESULTS

Kruskal–Wallis tests revealed significant differences in BCI among islands ($H = 90.29$, $df = 2$, $p < 0.001$) and sampling years ($H = 105.26$, $df = 7$, $p < 0.001$). Using a Dunn's test, we found that BCI was significantly different between tuatara living on Matiu and North Brother Island ($z = 4.53$, $p < 0.001$) and Takapourewa and North Brother Island ($z = -8.92$, $p < 0.001$), but not between tuatara on Matiu and Takapourewa ($z = 0.56$, $p = 1.000$). A Mann–Whitney *U*

test showed that BCI differed between sexes ($W = 1249.50$, $p < 0.001$); the mean (±standard error) BCI for female tuatara in this study was 1.03 (±0.01), while the mean BCI for male tuatara was 1.10 (±0.01).

In total, we analyzed blood smears from 203 tuatara. We did not analyze any samples from individuals that appeared sick or wounded, were juvenile, or whose sex could not be determined. We identified 11 outliers in our data from Takapourewa (17% of samples) and 32 in our data from North Brother Island (23% of samples) using strict multivariate normality testing as part of the current best practices for establishing veterinary RIs; subsequently, we created RIs using the 53 remaining values for Takapourewa and 98 values for North Brother Island. After removal of outliers, the data set contained 39 females and 112 males ($n = 151$). We report RIs for each blood parameter and 90% confidence intervals for upper and lower reference limits in Table 2. BCI values for the filtered data set were not significantly different from the unfiltered data set (Table 1) for either Takapourewa ($t = 0.13$, $df = 111.09$, $p = 0.897$) or North Brother Island ($t = -0.37$, $df = 214.86$, $p = 0.715$).

The mean H:L ratios calculated using blood smears from our three sampled islands varied significantly ($H = 87.94$, $df = 2$, $p < 0.001$) (Figure 1). Mean total leukocyte counts ($\times 10^9$/L) among the islands were also significantly different ($H = 25.028$, $df = 2$, $p < 0.001$) (Figure 1).

We found that all blood parameters varied by sampling date (Supporting Information: Table S3, Figure 2). GLMMs created for each blood parameter indicated that location and sample collection date were the two most consistent predictors of leukocyte profile parameters (Table 3). For heterophils, location was the only significant predictor. For lymphocytes and monocytes, both sampling date and location were significant, and for the total leukocyte count, all three predictors appeared in models with Δ AICc < 2 (Tables 3 and 4). For two white blood cell types, eosinophils and basophils, there was no detectable relationship between BCI, location, or sampling date (Tables 3 and 4).

We successfully cultured PBMCs (i.e., lymphocytes and monocytes) for 29 individuals; however, many of these had significant red blood cell contamination. After removal of contaminated cultures, we had 10 individuals from the resource restricted source population of North Brother Island and 6 from the translocated population on Matiu that responded to at least one stimulus (Supporting Information: Table S4). BCI values for these individuals were normally distributed ($w = 0.93$, $p = 0.267$) and differed significantly between islands ($t = 2.96$, $df = 8.31$, $p = 0.017$) (Figure 3a). We found that tuatara from neither island had eosinophils or basophils present in their cytospin samples and the number of heterophils was greatly reduced relative to their presence in blood smears as expected with histopaque purification. No cell count percentage found in our cytospin samples differed significantly between islands, as measured using t-tests (heterophils ($t = -1.75$, $df = 6.66$, $p = 0.126$), lymphocytes ($t = -1.50$, $df = 8.53$, $p = 0.170$), monocytes ($t = 1.96$, $df = 10.93$, $p = 0.076$); Figure 3b). The ratio of lymphocytes to monocytes differed between blood smears and cytospin samples for both North Brother Island (0.36 and 4.61) and Matiu (0.17 and 1.93) suggesting that the histopaque purification favored lymphocytes but affected samples from both tuatara cohorts similarly.

TABLE 2 Reference intervals (RIs) for tuatara sampled on Takapourewa ($n = 53$) and North Brother Island ($n = 98$) were generated following the best practices of the American Society for Veterinary Clinical Pathology (Friedrichs et al., 2012).

Location	n	Blood parameter ($\times 10^9$/L)	RIs	90% confidence interval (CI) for lower limit	90% CI for upper limit
Takapourewa	53				
		Heterophil	0.08–2.67	0.00–0.23	2.52–2.82
		Lymphocyte	0.00–3.08	-	2.88–3.28
		Monocyte	0.15–4.33	0.00–0.39	4.09–4.57
		Eosinophil	0.00–1.54	-	1.44–1.64
		Basophil	0.00–0.29	-	0.27–0.31
		Total white cell count	1.31–10.16	0.80–1.82	9.65–10.67
North Brother Island	98				
		Heterophil	0.34–3.91	0.19–0.49	3.76–4.06
		Lymphocyte	0.00–0.69	-	0.66–0.72
		Monocyte	0.00–1.64	-	1.57–1.71
		Eosinophil	0.00–1.18	-	1.13–1.24
		Basophil	0.00–0.29	-	0.28–0.31
		Total white cell count	0.88–6.88	0.63–1.14	6.63–7.14

Note: Outlier data has been removed using a multivariate, compositional approach.

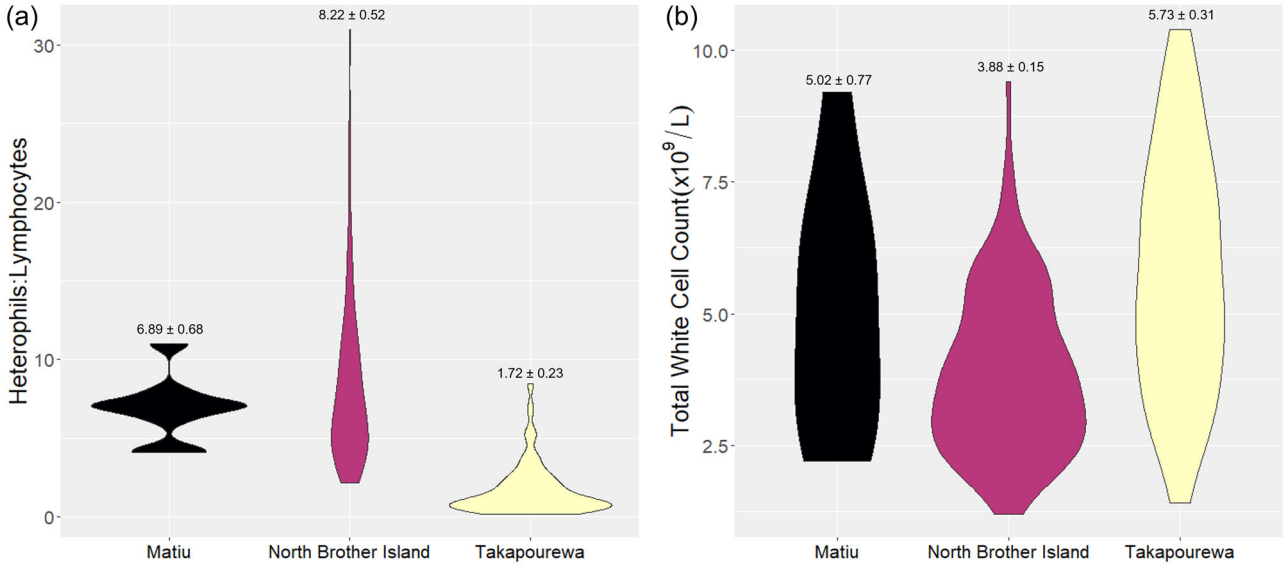

FIGURE 1 Mean values ± standard errors (SE) for (a) heterophil:lymphocyte ratios and (b) total white cell counts, calculated using blood smears from tuatara living on our three sampled islands. Data from Takapourewa (n = 53) and North Brother Island (n = 98) have been filtered for multivariate outliers (see text for more details), while Matiu has not (n = 9).

Among our 16 successful cell cultures, the production of NO, as measured in our Griess assay, followed a normal distribution in response to ConA (W = 0.86, p = 0.182) and LPS stimulation (W = 0.91, p = 0.339). There was no difference between the magnitude of response between islands to either ConA (t = 0.41, df = 3.51, p = 0.707) or LPS stimulus (t = 0.66, df = 4.78, p = 0.537) (Figure 3c). There were no differences between males, females, and juveniles for either measure ($p \geq 0.114$).

Metabolic response, as measured by MTT assay, to both ConA (W = 0.69, $p < 0.001$) and LPS (W = 0.66, $p < 0.001$) stimulation did not meet the assumption of normality as assessed via Shapiro–Wilk. However, there was no difference between islands in the magnitude of response to either stimulus (ConA: W = 19 and p = 0.194; LPS: W = 6 and p = 0.548) (Figure 3d). We did not find any significant differences between sexes or life stages in response to either stimulus ($p \geq 0.439$). Finally, there was only one significant correlation between cytospin result and response to any assay or stimulus: the relationship between monocytes and metabolic response to ConA stimulation (t = 2.48, df = 14, p = 0.027).

4 | DISCUSSION

Using blood samples collected from several island populations and spanning multiple decades, we investigated the leukocyte profile of an evolutionarily distinct reptile, the tuatara. To start, we calculated the first leukocyte RIs for tuatara living outside of captivity. Our sample size for Matiu (n = 9) was not sufficient to generate clinically useful RIs (Friedrichs et al., 2012); thus, we present RIs for tuatara on Takapourewa and North Brother Island. Leukocyte profiles can be influenced by a range of genetic and environmental factors, particularly in ectotherms (Greenspan et al., 2017; Zimmerman, Vogel, et al., 2010). Accordingly, appropriate thought should be given to the genetic background and resource access of populations before application of these RIs. Nonetheless, the availability of leukocyte profiles generated from robust sample sizes for multiple relict populations of tuatara provides both a useful data set for evolutionary comparisons of reptile immune function and clinically sound RIs for the improvement of captive tuatara husbandry worldwide. Further, these RIs allow for more accurate field screenings of tuatara health via leukogram, which significantly improves our ability to confidently select healthy individuals in the field for inclusion in translocations, head-starting programs, and other conservation activities. We note that a significant number of samples were identified as "outliers" according to the strict protocol for establishing RIs for veterinary use. These outliers may reflect natural variation, and thus we present the unfiltered summary data in Supporting Information: Table S2. However, the mean of each blood parameter in the unfiltered data set fell roughly in the middle of all generated RIs, highlighting that while these individuals were removed as outliers, it was through stringent, multivariate normality testing and does not represent any large variations in a single blood parameter. Their outlier status may instead reflect smaller variations in several categories.

There is only one other study that has attempted to created RIs for tuatara blood parameters (Alexander, 2017). This study pooled repeated, multi-season sampling of a captive population of 12 adult tuatara of the northern variant (*S. punctatus*) that were housed in outdoor enclosures at Auckland Zoo in Auckland, New Zealand (36.864°S, 174.720°E). While this study reports that these animals were healthy at the time of sampling, this population suffered from a widespread and persistent period of mycotic dermatitis caused by the

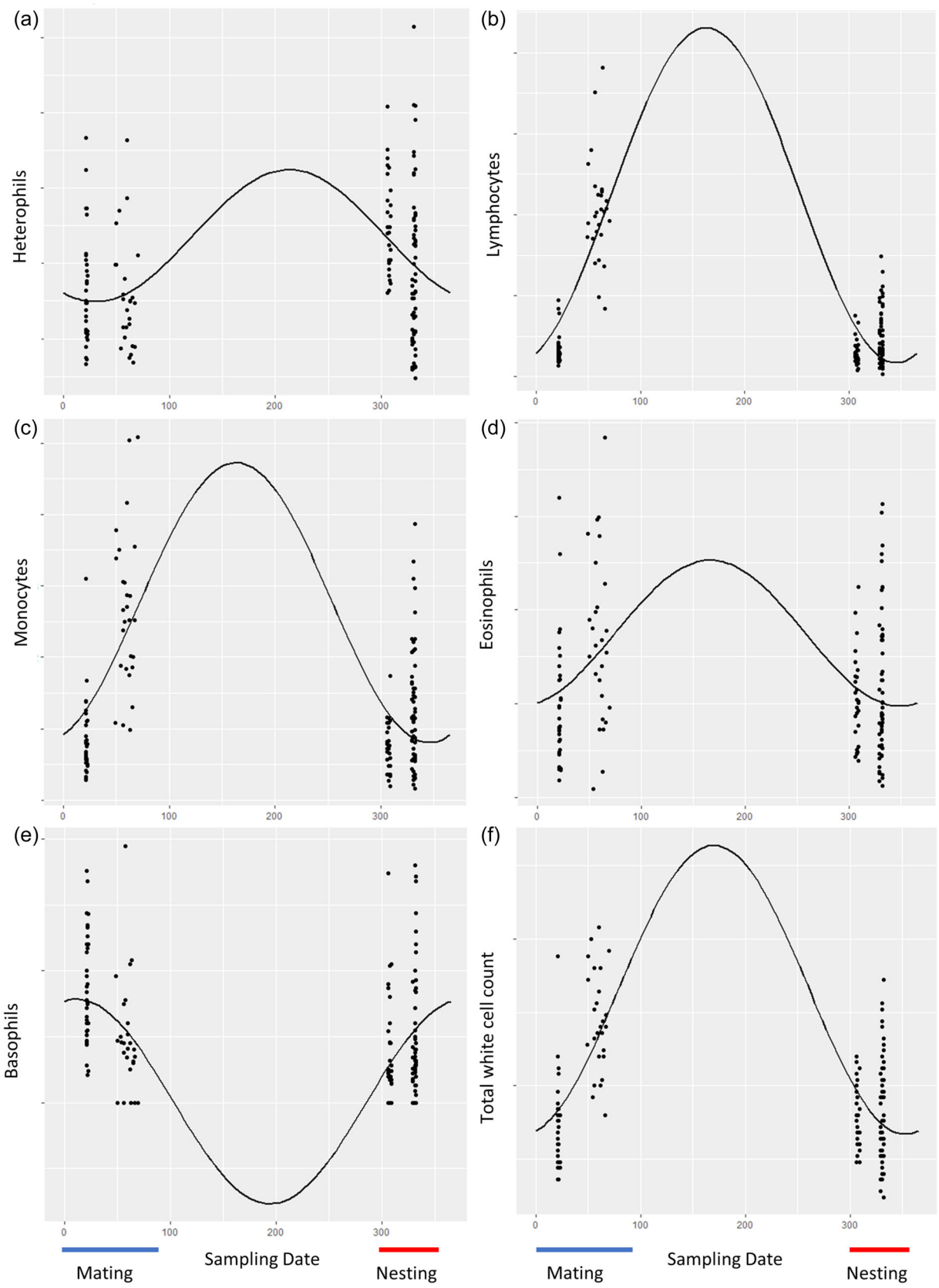

FIGURE 2 Graphs showing the circannual rhythm of different blood cell types found to be rhythmic: (a) heterophils, (b) lymphocytes, (c) monocytes, (d) eosinophils, (e) basophils, and (f) total white cell count. Samples are pooled from both islands, North Brother Island and Takapourewa, and span from 1988 to 2022. The sample date is defined as Gregorian Date. Descriptive bars below the X axis indicate the approximate times of tuatara mating and nesting seasons.

TABLE 3 Model results for each blood parameter, using centered values for body condition index (BCI) and sample date (Gregorian Day).

Blood parameter (×10^9/L)	Model formula	Intercept	AICc	Delta
Heterophils				
	Location + (1 year)	2.15	368.4	0.00
Lymphocytes				
	Gregorian Day + location + (1 year)	0.36	124.7	0.00
Monocytes				
	Location + (1 year)	0.78	315.1	0.00
	Gregorian Day + Location + (1 year)	0.80	316.1	0.98
Eosinophils				
	-	-	-	-
Basophils				
	-	-	-	-
Total white cell count				
	Gregorian Day + location + (1 year)	3.97	577.4	0.00
	Location + (1 year)	3.90	579.0	1.57
	BCI + Gregorian Day + location + (1 year)	4.02	579.4	1.94

Note: We report all models with a delta AICc < 2.

TABLE 4 Summary weights for each fixed effect in our models with delta AICc values < 2.

Response variable	Fixed effect	Sum of weights
Heterophils		
	Location	-
Lymphocytes		
	Gregorian Day	-
	Location	-
Monocytes		
	Gregorian Day	0.38
	Location	1.00
Total white cell count		
	BCI	0.17
	Gregorian Day	0.79
	Location	0.83

Note: There was only one model for both heterophils and lymphocytes with a delta AICc < 2. See text for further details on eosinophil and basophil models.

fungus *Paranannizziopsis australasiensis*. Over the course of 20 years, 57% of tuatara at Auckland Zoo were diagnosed with mycotic dermatitis (Jakob-Hoff, 2014). The RIs reported in the Auckland Zoo study are presented as a mean and min–max of each blood value, making direct comparisons with those generated in our study difficult. Generally, min–max blood values from tuatara at Auckland Zoo fell within the RIs generated in this study. For example, the RI for heterophils (×10^9/L) from tuatara sampled on Takapourewa was 0.08–2.67, from tuatara sampled on North Brother Island was 0.34–3.91, while the min–max values from tuatara living at Auckland Zoo was 0.1–1.3 (Alexander, 2017). It is not surprising that our RIs generated from significantly larger, wild populations experiencing a greater degree of environmental variation encompassed the small, repeatedly sampled population held in captivity.

We found support for annual variation in leukocyte cycle in tuatara living on North Brother Island and Takapourewa. Glucocorticoids are known to suppress the immune system in many taxa (Berger et al., 2005; French et al., 2008), though the impact of steroids on immune parameters varies across species, time scales, and metrics and is not always suppressive (Gangloff & Greenberg, 2023). Corticosterone varies seasonally in tuatara on Takapourewa (Tyrrell & Cree, 1998); thus, it is likely that the variation in leukocyte profile observed in tuatara is at least in part influenced by annual hormonal fluctuations. Plasma corticosterone levels in male tuatara on Takapourewa vary significantly across seasons, peaking in May and reaching their lowest in August. Plasma corticosterone levels in females are also lowest in August but experience roughly equivalent peaks in May and November (Tyrrell & Cree, 1998). Reproductive hormones, such as testosterone and progesterone also have immunomodulatory effects in reptiles, including on peripheral blood leukocytes. The effects of reproductive cycles on immune function may be contributing to the leukocyte patterns observed here, given that tuatara were most frequently sampled during reproductive seasons (Neuman-Lee & French, 2017; Veiga et al., 1998). Immune response can be costly and, in many species, a high immune response

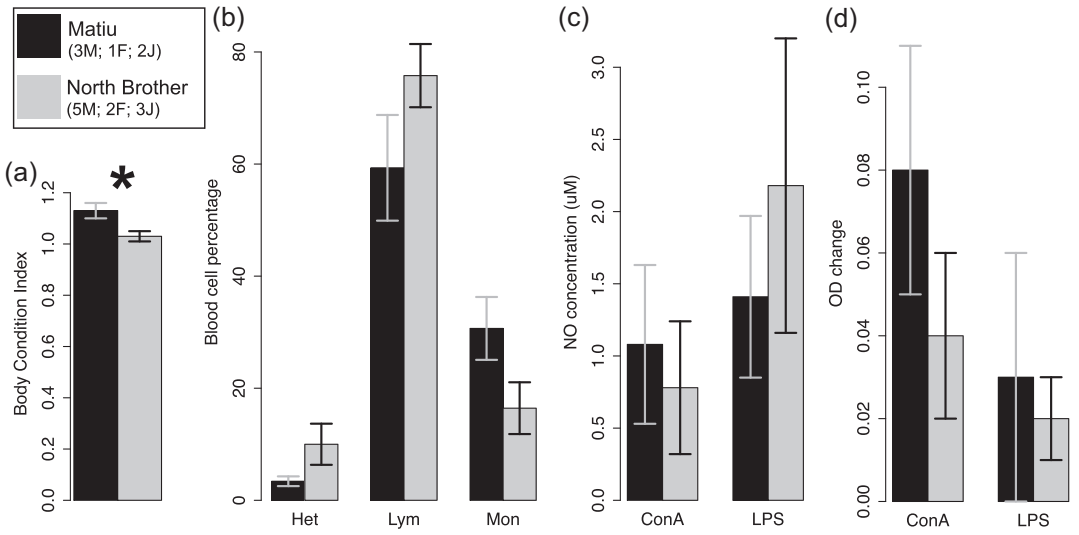

FIGURE 3 Summary information and assay results for cell cultures conducted during the 2010 sampling season. The height of the bars indicate the mean values with error bars reflecting standard errors. Black bars indicate data from Matiu; gray reflects values from North Brother Island. The number and sexes of individuals sampled are indicated in the legend; M, male; F, female; J, juvenile. (a) Body condition of sampled tuatara. (b) Cytospin leukocyte cultures, reported as percentage of the sample. Het, heterophil; Lym, lymphocyte; Mon, monocyte; (c) nitric oxide (NO) production and (d) cell metabolism in response to agonists. ConA, concanavalin A; LPS, lipopolysaccharide. *Indicates significant difference ($p < 0.05$).

is not energetically possible throughout all stages of life history or annual activity cycle (Buehler et al., 2008; Versteegh et al., 2014). Thus, the seasonal variation present in our blood models is not surprising and likely reflects a suite of complex, interactive environmental and physiological factors.

For all blood parameters, excluding eosinophils and basophils, location (ie island population) explained a significant portion of variation in our models. There are two primary possibilities for the large influence of island location on tuatara leukograms. First, the tuatara living on Takapourewa and North Brother Island may have different innate immunity. Tuatara on the two islands are genetically distinct from each other (Gemmell et al., 2020), and innate immunity varies with genetic background in many species (Jones, 2001; Quéméré et al., 2015; Versteegh et al., 2014). The second possible reason for this variation is differences in the strength of stress and resource competition being experienced by tuatara living on the islands. While BCI did vary between the two populations, with Takapourewa tuatara having a significantly higher BCI than those living on North Brother Island, host island was more important than variation in BCI in predicting trends in leukocyte variables. This suggests that while food restriction may play a role in modulating tuatara innate immunity, as seen in many species (Carbillet et al., 2019; Christopher et al., 1999), other stressors are likely more important. These could be population-specific bacterial and viral infections, temperature and climatic stress, or social and territorial competition. Broadscale screening for bacterial and viral infections in tuatara has not been carried out on North Brother Island, but previous work has identified the blood parasite *Hepatozoon tuatarae* (Godfrey et al., 2011) and many diet-related viruses in tuatara sampled on Takapourewa (Waller et al., 2022). The prevalence of these infections in other populations, and the extent of their impact on tuatara physiology, are both unknown. However, neither are thought to impact tuatara BCI significantly (Godfrey et al., 2011; Waller et al., 2022). There is also likely density-dependent competition restricting growth of tuatara living on North Brother Island, exacerbated by a growing male-bias in the population (Grayson et al., 2014; Nelson et al., 2002). Tuatara are solitary reptiles, excluding mate-guarding and antagonistic male-male interactions during mating season (Cree, 2014; Moore, 2008). Males hold and defend consistent territories across years (Moore, Daugherty, & Nelson, 2009), and increases in tuatara density and associated resource competition could impact basal stress levels in tuatara, which would be particularly exacerbated in the North Brother Island population (Grayson et al., 2014). We suspect that eosinophils and basophils, the two least common white blood cell types encountered in our blood smears, did not have large enough sample sizes for our models to detect any significant pattern of prediction.

An increased level of basal stress for tuatara on North Brother Island is supported by differences in the mean ratios of H:L on the islands. North Brother Island tuatara had a H:L ratio of approximately 4.8x higher than Takapourewa tuatara. An increased ratio of H:L in reptiles, birds, and some mammals is characteristic of a hematopoietic stress response or an immune system that has been activated by illness or injury (Carbillet et al., 2019; Davis et al., 2008; Greenspan et al., 2017). Functionally, this occurs because heterophils are the most abundant cell type making up the innate immune system, which serves as the body's first line of defense, while lymphocytes are part of the adaptive immune system (Minias, 2019; Zimmerman, Paitz, et al., 2010). Because no tuatara included in this study had visible signs of injury, and the increased ratio of H:L was robust throughout

the North Brother Island samples, we suspect the increased H:L to reflect a hematopoietic stress response to the resource-poor and highly competitive living conditions experienced by the population. It is worth noting that the ratio of H:L of a small sample of tuatara on Matiu is similar to that of North Brother Island, and significantly different than what we calculated for Takapourewa. This indicates that genetic factors are likely responsible for at least some of the observed differences in leukocyte profile between islands. We suggest further work look at the expression of immune-related genes on tuatara living on Matiu, North Brother Island, and Takapourewa to better understand the impacts of environmental productivity and genetic makeup on immune function in this basal reptile.

Takapourewa tuatara had a higher level of total white blood cells than North Brother Island tuatara, despite having a lower H:L ratio. The difference between the two populations' total WBC counts was not as significant as the difference in H:L ratio, and population-based differences in reptile total white blood cells counts can be marked (Sacchi et al., 2020). The differences observed in our data set appear to be primarily driven by an increase in monocytes in tuatara living on Takapourewa relative to those sampled on North Brother Island.

Finally, we found that tuatara living on North Brother Island and tuatara living on Matiu, itself a translocated population originally from North Brother Island, varied in body condition and the percentage of lymphocytes and monocytes in their cytospin samples. Tuatara living on Matiu were in significantly better body condition than tuatara living on North Brother Island. North Brother Island tuatara had a higher proportion of lymphocytes, while Matiu tuatara had a significantly higher proportion of monocytes. Interestingly, these differences in leukocyte makeup did not translate into functional differences in response to stimulation with ConA and LPS at a population level; there was no difference between islands in response strength or number of individuals responding in either assay.

ConA is a known T cell mitogen, or stimulator of cell proliferation, in tuatara (Burnham et al., 2005; La Flamme et al., 2010). Our results support the almost ubiquitous proliferative response of tuatara T cells to ConA; however, cell viability and the production of NO in response to ConA showed mixed results. NO suppresses cellular proliferation and, in some mammal species, is produced in response to activation of certain toll-like receptors (Abdul-Cader et al., 2016; La Flamme et al., 2010; Sato et al., 2007). Additionally, NO acts as a vasodilator, neurotransmitter, and modulator of inflammatory responses in birds (Sild & Hõrak, 2009) and is important for cardiovascular function and thermoregulation in reptiles (Seebacher & Franklin, 2005). Thus, clarifying differences in NO production in response to differential levels of baseline stress and body condition is important in tuatara and warrants further investigation. Regardless, these preliminary results suggest that source population, sex, and life stage are not determining factors in the ability of individual tuatara to produce NO. This may indicate the presence of a variety of immune responses to challenge within each population, suggesting healthy immunogenetic variability within tuatara on Matiu and North Brother Island.

5 | CONCLUSION

We present the first robust leukocyte reference intervals for an evolutionarily unique reptile of conservation importance, the tuatara, and identified factors that may influence immune function in this species. We found that the primary predictor of variation in leukocyte profile for tuatara in our study was population (location), followed by sampling date, then body condition. We also found that population, sex, life stage, and body condition may not be the most important factors in the ability of tuatara to produce nitric oxide, a significant, multifunctional signaling molecule. We do not yet know if the primary driver of the importance of location to leukocyte profile is the genetic makeup of these inbred, isolated populations or localized environmental and demographic stressors, and suggest further work considering the genetic components of immunity in tuatara be carried out. We also suggest further work be carried out to understand the complex underpinning of the regulation of nitric oxide in this species. Regardless, our results suggest that even tuatara from inbred populations with poor body condition exhibit a variety of immunogenetic responses to immune challenge. This research has important implications for the improved husbandry and welfare of captive tuatara worldwide and provides a useful guide for the interpretation of leukograms generated from wild tuatara being considered for conservation action.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at 10.6084/m9.figshare.24137559.

ETHICS STATEMENT

All samples were undertaken with appropriate Department of Conservation (Wildlife Act) and Victoria University of Wellington (Animal Ethics) permitting.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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