OXFORD

Immune stress and diet influence reproductive fitness in male tuatara (*Sphenodon punctatus*)

Sarah K. Lamar[a,b,*], [ID] **, Diane K. Ormsby**[a], **and Nicola J. Nelson**[a,b]

[a]School of Biological Sciences, Level 2, Te Toki a Rata Building, Victoria University of Wellington, Wellington 6012, New Zealand
[b]Centre for Biodiversity and Restoration Ecology, Level 2, Te Toki a Rata Building, Victoria University of Wellington, Wellington 6012, New Zealand

*Address correspondence to Sarah K. Lamar. E-mail: sarah.lamar@vuw.ac.nz
Handling editor: Sean C. Lema

Abstract

The theoretical trade-off between immune and endocrine investment in mating animals has received mixed empirical support, particularly in reptiles. We investigated the relationship between male sexual characteristics, diet, and immune response to stress in an island population of tuatara (*Sphenodon punctatus*) across two mating seasons. Tuatara are promiscuous, with a highly skewed mating system where males face significant competition for access to mates and postcopulatory competition for fertilization success. We found that tuatara sperm viability and swim speed were negatively associated with male body condition and the ratio of heterophils to lymphocytes. Additionally, sperm swim speed was negatively associated with spine area, mite load, and the total number of circulating white blood cells, but was positively associated with tick number. This is likely a function of social dynamics in this system where larger male size predicts greater spatial overlap with potential rivals and increased tick load. Because the production of sexual characteristics may be costly, we also investigated the effect of diet on sperm quality. We did not identify an association between diet and sperm viability. However, sperm swim speed was negatively associated with carbon-13 and positively associated with nitrogen-15. We suspect that these results reflect the influence of seabird-based nutrients in this island ecosystem, particularly polyunsaturated fatty acid, and antioxidant damage on tuatara sperm. In total, these results provide evidence of a trade-off between pre- and post-copulatory sexual characteristics and the immune and endocrine systems in male tuatara.

Key words: reproductive fitness, reptiles, sexual selection, sperm characteristics, tuatara.

Sexual selection can lead to the production of expensive characteristics in males, which typically bear the lower cost of gamete production (Darwin 1871; Johnstone et al. 1996). The fitness burden imposed by producing dramatic phenotypes and high-quality sperm in response to the pressures of female mate choice and competition with potential rivals is often the result of complex interactions between the immune and endocrine systems (Guillette et al. 1995). The immune–endocrine trade-off occurring in reproductive males is regulated by increases in testosterone and other androgenic steroids linked to sperm production, surges in mating behavior and aggression, and the development of physical traits under selection through female choice (Zeller 1971; Stokkan 1979; Wingfield et al. 1990; Oppliger et al. 2004). However, increases in androgenic steroids can have negative effects on male fitness. First, testosterone is a known immunosuppressant and can increase an individual's susceptibility to pathogens, parasites, and disease (Hamilton and Zuk 1982; Folstad and Karter 1992). Second, testosterone can increase metabolic rate, which leads to a reduction in body condition via mass loss (Marler and Moore 1988; Marler et al. 1995; Oppliger et al. 2004). These factors, combined with the energetic demands of performing complex mating behaviors, competing with potential rivals, expressing dramatic phenotypes, and producing high-quality sperm, have led to the development of many

theories surrounding the functional trade-offs experienced by reproducing males of anisogamous species.

Although theoretical support for trade-offs between immune function and the quality of reproductive characteristics is strong (Hamilton and Zuk 1982; Folstad and Karter 1992; Rowe and Houle 1996), empirical support in the literature is mixed (e.g. Roberts et al. 2004; Simons et al. 2012). Many studies looking at immune–endocrine trade-offs focus only on secondary sexual characteristics. However, primary sexual characteristics, such as sperm quality, are equally important for fertilization success in males (Friesen et al. 2017; Lüpold and Pitnick 2018). Because pre- and post-copulatory sexual selection often both act within a given mating system, there is a necessary energetic trade-off between the two (Lüpold et al. 2014; Kahrl et al. 2016). Thus, male body condition and the ability to dedicate energetic resources to the production of sexual characteristics may modulate the trade-off between immune and endocrine systems (e.g., Simmons 2012; Dines et al. 2015). Further, dietary intake has been found to directly influence ejaculate traits in some reptile species (Kahrl and Cox 2015). For this reason, energetic factors like diet and nutrient intake should be considered as part of a whole-system approach to investigating reproductive and immune dynamics.

Reptiles, the first truly terrestrial vertebrate ectotherms, have diverse life histories and mating systems (Moore 2008). Although there is great diversity among reptiles, promiscuity of both sexes and pre- and post-copulatory competition between males is common throughout the taxa (Olsson and Madsen 1998). These strong, sexually selective pressures make reptiles an ideal study system for investigating the relationship between primary and secondary sexual characteristics and measures of male reproductive fitness. Experimental studies on the effects of testosterone on immune function, body condition, and reproductive traits have found mixed support for the presence of an immuno-endocrine trade-off in reptiles (Oppliger et al. 2004; LaVere et al. 2021), though the condition-dependence of reproductive characteristics and the modulating role of diet on reproduction in the taxa is broadly supported (e.g., Oppliger et al. 2004; Kahrl and Cox 2015). Still, taxa-specific research, particularly from reptile groups outside of Lacertilia, is needed to fill in the large gaps in our understanding of this foundational relationship.

Tuatara (*Sphenodon punctatus*) are endemic to Aotearoa New Zealand and are the only surviving species of the once widespread and diverse reptile order Rhynchocephalia (Figure 1) (Cree 2014). Relict populations of tuatara are now found only on offshore islands surrounding mainland Aotearoa New Zealand, where tuatara forage on diverse communities of insects, seabirds, and herpetofauna; additionally, restoration efforts have led to the establishment of translocated populations in fenced, mainland ecosanctuaries and reintroductions to islands which would have had historical populations of tuatara. Rhynchocephalians diverged from their closest relatives, squamate reptiles, approximately 250 mya and represent an important evolutionary link to basal amniotes.

Tuatara are sexually dimorphic and reach reproductive maturity at approximately 12–15 years of age (Dawbin 1982; Castanet et al. 1988). Adult male tuatara are larger with wider jaws and larger dorsal and nuchal spines than females of the species (Cree 2014). Tuatara have mating systems with documented polygyny, polyandry, and within-clutch multiple paternity (Moore et al. 2009). Further, access to mating is highly skewed, with only approximately the largest and most aggressive 25% of males having success within a mating season, which occurs at the end of the summer (February–March)

(Moore et al. 2009). The percentage of males that successfully fertilize eggs after mating is not known. Females are only receptive to mating approximately once every four years, though this likely varies among island populations (data collected on Takapourewa, Cree et al. 1992); tuatara display extremely limited maternal care in the form of occasional nest guarding after egg deposition (Guillete et al. 1990). Males do not display any known paternal care. Female tuatara exhibit delayed fertilization but do not have a known organ for prolonged sperm storage (Gabe and Saint Girons 1964; Cree et al. 1991, 1992). The decoupling of mating and fertilization in tuatara allows for the possibility of sperm competition and cryptic female choice. Further, male tuatara are not known to produce a postcopulatory plug. The factors influencing female mate choice are not fully understood in tuatara, though current theory suggests that male body size and certain aspects of coloration, like reflectance, are the best predictors of male mating success (Gillingham et al. 2009; Miller et al. 2009).

To better understand the relationship between male reproductive fitness, immune response to stress, and diet in reptiles, we undertook an in situ study on mating tuatara. Over the course of two mating seasons, we collected a suite of phenotypic characteristics potentially related to male mating success and compared them against hematological markers of immune stress, dietary profiles created using stable isotopes, and measures of sperm quality.

Materials and Methods

Field methods

We sampled mating male tuatara on Takapourewa (Stephens Island), Cook Strait, New Zealand (40.670°S, 173.997°E) during two consecutive seasons (2021 and 2022). Takapourewa is a 150-ha island reserve which hosts the largest and most dense population of tuatara, as well as a rich community of seabirds, invertebrates, and other herpetofauna (Walls 1978; East et al. 1995; Tennyson 1998; Cree 2014; Anderson 2018).

During both sampling seasons (approx. four weeks each), we conducted nightly visual surveys from 16:00 to when activity quieted for the night, typically around 02:00. In each survey we looked for tuatara mating in the heavily populated portion of the island known as Keeper's Bush (~2,732 tuatara/

Figure 1. A male tuatara basks on Takapourewa. Many large, white spines are visible on his dorsal and nuchal crests. Mites are visible as bright orange patches on the skin.

ha; Moore et al. 2009). When we encountered a mating pair, we hand-captured the individuals and used a sterile syringe to collect any sperm visible on the outside of either tuatara's cloaca. We then separated sperm samples into two aliquots to assess sperm viability and swim speed.

To assess sperm viability, we combined 10 µL of sperm with 10 µL Sperm VitalStain™ (Nidacon), which contains both eosin and nigrosine. We pipetted the 20 µL combined sample onto a microscope slide and created duplicate pull-apart slides for each mating within 15 minutes of sperm collection. We allowed slides to dry at room temperature and stored them in a light-proof box until return to the mainland.

To assess sperm swim speed, we pipetted 6 µL of fresh sperm into a 20 µm, two-chambered Leja® slide (LSC-20-01-02-B; Gytex Pty Ltd.) and recorded movement at ambient temperature within 15 minutes of sample collection using a Gigabit Ethernet camera (Basler Scout ACA780-75GC), Eclipse E200 microscope with phase contrast, and a Sperm Class Analyzer program (SCA; Microptic). When sperm samples were too concentrated for sperm to swim in a monolayer in the chamber, samples were diluted by either a 1:2 or 1:3 semen:PBS dilution, which does not affect tuatara sperm motility (Lamar et al. 2021).

For each male tuatara, we recorded snout-vent length (SVL), body mass (g), jaw width (mm), jaw length (mm), number of attached ticks (the tuatara tick, *Archaeocroton sphenodontii*), and mite (*Neotrombicula* spp.) load (scored as an ordinal value from 0 to 4). A mite load score of 0 indicated no mites present, a score of 1 indicated less than 20 mites, a score of 2 indicated 20-50 mites, a score of 3 indicated 50–100 mites, and a score of 4 indicated greater than 100 mites present. We also took a photograph of the nuchal and dorsal spines next to a size standard, collected 1–2 nail trims (approximately ≥ 0.001 grams of nail material) from the distal tip of a rear foot for dietary stable isotope analysis, and drew up to 0.5 mL of whole blood from the ventral coccygeal vein to assess leukocyte profile. Blood draws were carried out within 15 minutes of capture. Using this untreated blood, we made a smear, which was allowed to dry at room temperature until returned to the mainland. Any mating male tuatara that did not have existing permanent identification was given a passive integrated transponder in the left flank. Finally, we marked all tuatara with a temporary number using a non-toxic, xylene-free marker and released individuals at their capture locations.

Lab methods

To assess tuatara sperm viability, we counted a minimum of 100 sperm cells per mating using a Nikon Eclipse E200 microscope at x400 bright field magnification. For three mating events, we were only able to count 59–79 sperm cells due to the small overall sample volume. We report sperm viability values as % viable cells. To measure sperm swim speed (curvilinear velocity; VCL (µm/s)), we analyzed the videos recorded for each sample in the field using SCA software (Lamar et al. 2023).

To assess average dietary makeup over an extended period, we used inert nail tissue from the rear digits (Lamar et al. 2022). We sonicated nail trims in a 1:1 dichloromethane:methanol solution to remove debris and submitted the cleaned samples to GNS Science (Institute of Geological and Nuclear Sciences Limited), Te Pū Ao (Lower Hutt, New Zealand) for carbon-13 and nitrogen-15 analyses. Higher levels of carbon-13 indicate

a relative increase in the amount of marine input into a diet, while nitrogen-15 levels are enriched as the dietary trophic level increases. We present stable isotope values as parts per thousand (‰).

To generate leukocyte profiles, we stained dried blood smears with a commercial Romanowsky stain (Gribbles Veterinary Supply) and fixed slides with a DPX mountant (Sigma–Aldrich) and coverslip. Once dry, we counted the first 100 leukocytes encountered on each slide and identified them to type (Desser 1978; Claver and Quaglia 2009). We estimated the total number of circulating leukocytes (cells × 10^9/L) by counting all leukocytes in 10 fields at ×400 magnification, calculating the per-field average, and multiplying by 2 (Sykes IV and Klaphake 2008). Finally, we calculated the ratio of heterophils:lymphocytes (H:L), a commonly used metric for assessing immune response to stress (Davis et al. 2008).

To calculate the dorsal and nuchal spine area (mm^2) for each individual, we analyzed spine photos using Gimp v2.10.32's "measure" tool and our photographs taken in the field, which included a size standard. We also recorded the total number of dorsal and nuchal spines for each male.

Statistical analysis

We conducted all statistical analyses in R version 4.3.0 (R Core Team 2023) with an alpha level of 0.05. First, we calculated the gape index according to King (2002):

$$\pi \,(\text{jaw length} \times \text{jaw width}) \div 4 \qquad (1)$$

To standardize for the effects of tail loss and regrowth in this species with caudal autotomy, we calculated the body condition index (BCI) as (Hoare et al. 2006):

$$\frac{\log\,(\text{weight})}{\log\,(\text{SVL})} \qquad (2)$$

To assess the swim speed (VCL) of tuatara sperm, we z-transformed velocity data and calculated the between- and within-male variance and intraclass correlation (ICC) for all sperm samples with at least 100 analyzed sperm. Briefly, we calculated ICC as the proportion of between-male variance to the total variance (both between- and within-male variance) of different numbers of fastest sperm per sample (1–5, 1–10, 1–20, 1–50, and 1–100). We aimed to identify the subpopulation that maximized between-male variance while minimizing within-male variance, to allow for comparison of the sperm most likely to enter the female reproductive tract (Nakagawa and Schielzeth 2010; Lara et al. 2020). Based on the results of these analyses, we present all sperm swim speed data as the mean of the 5 fastest sperm per male. We tested for differences in sperm viability and swim speed between sample years using Welch's *t*-tests.

We tested for normality of all quantitative variables (Table 1) using a series of Shapiro–Wilk tests. All variables had a normal distribution except male spine number, H:L ratio, and number of ticks. However, using the *rcompanion* package (Mangiafico 2022), we were able to bring all abnormally distributed values into a normal distribution. Next, we tested for multicollinearity among all quantitative variables. Only one pair of variables had correlation values greater than |0.55|: SVL and gape index (0.73) (Supplementary Table S1). Next, we calculated the coefficients of variance for SVL (4.98) and gape index (11.65). Because tuatara body size, particularly in relation to potential male mating success, is

Table 1. Explanations for variables included in this study

Response variables	Explanation
Sperm viability	% live sperm cells in sample
Sperm VCL	Mean curvilinear velocity (µm/s) of the fastest 5 sperm cells in sample
Fixed effects	
Gape index	Metric quantifying how large tuatara can open their mouths (see Equation 1)
BCI	Body condition index (see Equation 2)
Carbon-13	Unadjusted carbon-13 value, expressed as parts per thousand (‰)
Nitrogen-15	Unadjusted nitrogen-15 value, expressed as parts per thousand (‰)
Spine area	mm² area of the large, white dorsal and nuchal spines
Spine number	Number of dorsal and nuchal spines
Total WBC	Estimated number of total circulating leukocytes
H:L	Ratio of heterophils to lymphocytes, as counted on blood smear
Tick number	Number of *Archaeocroton sphenodonti* of all life stages
Mite score	Ordinal value indicating the level of *Neotrombicula* spp. infestation
Random effect	
Year	2021 or 2022

BCI: body condition index; H:L: heterophil to lymphocyte ratio; VCL: curvilinear velocity (swim speed); WBC: white blood cells

primarily considered using BCI (Hoare et al. 2006; Moore 2008), and because SVL varied relatively little across our data set and was correlated with a much more variable morphological trait potential under female selection (gape index), we removed SVL from further analyses.

To quantify the amount of variation in phenotypic characteristics potentially the object of female mate choice, we calculated the coefficients of variance for BCI, gape index, spine number, and spine area. Because BCI may change throughout the course of the energetically demanding mating season (Jessop et al. 2004), we visualized the relationship between sample date and BCI and calculated the adjusted R^2 and P-value for this relationship using a simple linear model. We also investigated the relationship between male spine area and date captured mating, as spines may be lost in this species through antagonistic interactions with other males as the season progresses; we calculated the adjusted R^2 and P-value for this relationship using another linear model.

Finally, we analyzed the relationship between immune stress, diet, and sexual characteristics using two separate generalized linear mixed effects models (GLMMs) constructed in R's *lme4* package (Bates et al. 2015). An explanation of model parameters can be found in Table 1; we generated two sets of models—one with sperm swim speed as the response variable and another with sperm viability as the response variable. GLMMS were constructed with all possible combinations of noncorrelated predictor variables; the year was included as a random effect to account for stochastic processes beyond the scope of this study. We rescaled all predictor variables to reduce variance inflation and averaged predictors from the top-ranked models using AICc. We report the formulas and sum of predictor model weights for averaged models with a delta AICc of < 2 (Bartoń 2022).

Results

We collected sperm samples from 14 males in 2021 and 24 males in 2022; no males were found mating in both years.

One viability sample was not able to be assessed due to slide quality, thus, for viability $n = 37$ (Table 2).

Six sperm samples had less than 100 sperm with analyzed swim speeds (24–84 cells/sample). Using the remaining 32 sperm samples, we analyzed the between- and within-male variance for different subpopulations of sperm and found that between-male variance was highest (0.887) and within-male variance was lowest (0.140) when analyzing the 5 fastest sperm per sample (Supplementary Table S2). Because all samples had at least 5 analyzed sperm, no samples were excluded from swim speed analysis ($n = 38$) (Table 2).

For both metrics of sperm quality, there was a significant difference between years. Viability scores were significantly higher for sperm samples collected in 2021 than in 2022 ($t = 3.09$, df = 28.27, $P = 0.005$), while the mean sperm swim speed for each sample was higher in 2022 than in 2021 ($t = -3.52$, df = 34.99, $P = 0.001$). However, the range of values for both metrics in 2022 encompassed the values found in the smaller 2021 sample size.

We were able to bring the three non-normally distributed model variables into a normal distribution by transforming with the following lambda values: spine number ($\lambda = 4.075$), H:L ratio ($\lambda = -0.075$), and tick number ($\lambda = -0.25$). The coefficients of variation for male phenotypic characteristics were as follows: BCI = 2.01, gape index = 11.65, spine number = 41.74, and spine area = 49.46. We found that the BCI of males captured during mating decreased slightly as the mating season progressed (adjusted $R^2 = 0.093$, $P = 0.035$). There was no significant relationship between spine area and date captured mating for male tuatara in this study (adjusted $R^2 = -0.01$, $P = 0.578$).

Three GLMMs for each sperm quality metric had Δ AICc scores < 2 (Table 3). For both measures of sperm quality, BCI was the most significant or equal first predictor (Table 3) and was negatively related to sperm quality (Supplementary Table S1). For sperm viability, the H:L ratio was the only other significant predictor and was negatively correlated to % viability. For sperm swim speed, all measures except spine number

and gape index were found in the top model (Table 3). Tick numbers and nitrogen-15 values had a positive relationship with sperm swim speed, although all other variables had a negative predictive relationship in our velocity models: H:L ratio, total WBC count, mite score, carbon-13 value, and spine area. Full model weights for all predictors in significant models are provided in Table 4.

Discussion

We investigated the relationship between male reproductive traits and factors that may influence postcopulatory competition success, particularly sperm viability and swim speed, in a wild, robust population of tuatara. Because mating is highly skewed in this species, with only approximately 1 in 4 males gaining the opportunity to mate within a season (Moore et al. 2009), we did not expect to see significant variation in the phenotypic characteristics of mating males. We suspected that strong directional selection on body condition index (BCI), the likely object of female choice in this species, would act as an evolutionary spandrel driving collinear exaggeration of other characteristics possibly under selection (Gillingham et al. 2009; Moore et al. 2009). Although there was no relationship between sperm quality and two phenotypic measures potentially under female choice (spine number and gape index), there was support for a negative relationship between

BCI and each metric of sperm quality. Further, there was support for a negative relationship between spine area and sperm swim speed.

The identified negative relationship between sperm quality, quantified in this study as highly viable and fast-swimming sperm samples, and body condition in mating male tuatara was significant, despite BCI being the phenotypic characteristic that varied the least in our dataset. This supports theoretical work that suggests increased investment in secondary sexual characteristics should result in a decrease in sperm quality due to testosterone-mediated increases in metabolic rate (Marler and Moore 1988; Marler et al. 1995; Oppliger et al. 2004). Testosterone can also increase the frequency of aggression and other mating-associated behaviors, further increasing energy costs for males. Importantly, although we identified a negative relationship between body condition and sperm quality, the males found mating in this study likely still comprise the top 25% of males on the island in relation to overall body size. Additionally, we identified a weak pattern where males with lower BCI were caught mating for the first time later in the season than larger males. It is important to note that we do not assume to observe every mating event from every male in this study, and thus it is unclear if the males in poorer body condition captured mating for the first time later in the season have undergone losses in mass over the course of the season or have always been in poorer

Table 2. Summary statistics for all quantitative variables in this study. Units are given when applicable

	Mean (±SE)	Range	Units	*n*
Sperm viability	58.44 (±0.04)	3.38–94.53	% live	37
Sperm VCL	78.94 (±6.73)	4.45–172.20	µm/s	38
Gape index	2324.84 (±43.95)	1734–2873	–	38
BCI	1.17 (±0.00)	1.10–1.21	–	38
Carbon-13	-21.89 (±0.12)	–23.60 to –20.50	‰	38
Nitrogen-15	16.86 (±0.18)	15.10–20.00	‰	38
Spine area	455 (±37)	100–939	mm^2	38
Spine number	29.95 (±0.63)	16–36	–	38
Total WBC	7.87 (±0.35)	4.00–13.40	cells $\times 10^9$/L	36
H:L	0.8 (±0.08)	0.21–2.92	–	36
Tick number	20.03 (±3.74)	3–101	–	38

BCI: body condition index; H:L: heterophil:lymphocyte ratio; VCL: curvilinear velocity; WBC: white blood cells.

Table 3. Results for sperm quality metric GLMMs. We report only models with ΔAICc scores < 2

Model formula + (1lyear)	Intercept	AICc	Delta
Sperm viability			
~BCI + H:L	1.69	4.9	0.00
~BCI	3.03	5.6	0.64
~H:L	-0.69	6.5	1.57
Sperm velocity			
~BCI + spine area + H:L + mite score + tick number + total WBCs + c − 13 + n − 15	− 704.00	321.7	0.00
~BCI + H:L + mite score + tick number + total WBCs + c − 13 + n − 15	− 738.10	322.6	0.83
~BCI + spine area + H:L + tick number + total WBCs + c − 13 + n − 15	− 681.60	323.5	1.78

BCI: body condition index; c-13: carbon-13 (‰); H:L: heterophil:lymphocyte ratio; n-15: nitrogen-15 (‰).

Table 4. The sum of weights for predictor variables in models with ΔAICc scores < 2. *n* indicates the number of models containing that fixed effect

Response variable	Fixed effect	*n*	Sum of weights
Sperm viability			
BCI		2	0.79
H:L ratio		2	0.67
Sperm velocity			
BCI		3	1.00
H:L ratio		3	1.00
Tick number		3	1.00
Total WBCs		3	1.00
Carbon-13		3	1.00
Nitrogen-15		3	1.00
Mite score		2	0.80
Spine area		2	0.68

condition; if so, these males may be relegated to late-season mating opportunities due to large males dominating early-season opportunities. Regardless, our results may suggest a decrease in BCI in male tuatara throughout the mating season. We suggest that future work investigate changes in body condition, sperm quality, and mating success of individuals tracked across the duration of a season to better parse the effects of the energetic burden of mating and sperm production in male tuatara.

We identified a negative relationship between sperm swim speed and spine area. The spines of male tuatara able to successfully secure matings on Takapourewa have significantly more white, reflective area than the spines of unsuccessful males. This suggests that female tuatara may be using spine reflectance to choose a mate (Gillingham et al. 2009). The negative relationship found in our study between males with larger, showier spines and slower swimming sperm may therefore reflect a trade-off in investment between primary and secondary sexual characteristics. Importantly, male–male antagonistic interaction is frequent during the mating season, particularly in the densely populated Keeper's Bush area where our sampling occurred (Moore et al. 2009), and we often observed males grabbing onto each other's nuchal crests during aggressive interactions. Thus, although this relationship may be the result of an energetic trade-off, it may also reflect that more aggressive males are likely to be engaged in antagonistic interactions (which result in damage to the dorsal and nuchal spines) and have faster sperm.

Due to the presence of polyandry, polygyny, and the potential for sperm storage in this species, we suspected male tuatara would have significant investment in primary sexual characteristics. This investment could result in immune stress via a testosterone-induced immunocompetence handicap, a theoretical trade-off that has received mixed empirical support. Thus, only the most immunologically fit males would be able to bear the cost of reproduction while staying healthy. This hypothesis is supported by our model results. Besides decreased BCI, the only other significant predictor of high sperm viability in our models was a low ratio of H:L, a measure used to indicate a hematopoietic stress response (Davis et al. 2008; Greenspan et al. 2017; Carbillet et al. 2019). Functionally, this occurs because heterophils are part of the body's first line of defence, the innate immune system,

while lymphocytes are part of the adaptive immune system (Zimmerman et al. 2010; Minias 2019). In addition to a lower ratio of H:L, lower numbers of total WBCs were associated with faster swimming sperm. Leucocytosis, or an increase in WBCs, can be used to indicate stress, though with less confidence than the H:L ratio (Gross and Siegel 1983; Huff et al. 2005; Davis et al. 2008; Han et al. 2020). Thus, tuatara exhibiting reduced hematological signs of stress, illness, or injury, had faster swimming sperm.

In addition to hematological signs of immune challenge, we collected two measures of ectoparasite load: mite score (an ordinal value ranging from 0, no mites, to 4, lots of mites) and the number of attached ticks. Both measures of ectoparasite load were valuable predictors of sperm swim speed. Tuatara with fewer mites had faster-swimming sperm, which supports the presence of an immune trade-off with primary sexual characteristics in this species, as mites and their associated wounds likely trigger an immune response in tuatara. Tuatara play host to two species of trombiculid mites (*Neotrombicula sphenodonti* and *N. naultini*) which are not host-specific and are found on other reptiles living on Takapourewa (Goff et al. 1987; Godfrey et al. 2008). These mites can occur in extremely large densities on tuatara (previous work estimated max of 6,000 mites/tuatara; Godfrey et al. 2008), particularly around the neck and leg folds (Supplementary Figure S1). Tuatara mite loads vary significantly seasonally and annually, peaking in the summer and in years after cooler temperatures (Godfrey et al. 2008). During our sample collection, male tuatara was regularly observed with bloody, damaged skin around large mounds of mites (Supplementary Figure S1). In fact, these were the only cases of external injury observed in males included in this study. Although previous work found no link between body condition and peak mite load (Godfrey et al. 2010b), the presence of a hematopoietic immune response is not always associated with changes in BCI in tuatara (Lamar et al. 2024). Thus, it is possible that the immune challenge presented by extreme mite loads and their associated wounds resulted in immune activation and decreased sperm swim speed in tuatara.

The positive relationship between the numbers of attached tuatara ticks (*Archaeocroton sphenodonti*) and increased sperm swim speed was unexpected. Tuatara body condition and peak seasonal tick load have been found to be negatively correlated in previous years (Godfrey et al. 2010b), a trend also observed in our dataset. Previous work found a strong social factor to tuatara tick load—males with greater network connection, measured via in-strength of individual connectedness, were associated with higher levels of ticks than less connected individuals (Godfrey et al. 2010a). Thus, there may be strong spatial and behavioral influences on tick load, particularly in relation to mating season dynamics, which explain the unexpected patterns found in this study. We suggest further work be carried out looking at the effects of spatial dynamics and social interaction on the fitness of mating male tuatara.

Our indicators of dietary composition, carbon-13 and nitrogen-15, varied with sperm swim speed but not viability. Higher (less negative) values of carbon-13 were positively associated with better body condition and negatively associated with sperm swim speed. The relationship between higher carbon-13 values and an increase in BCI in tuatara is likely a reflection of the dietary increase in seabirds that occurs as tuatara grow larger (Lamar et al. 2022). These seabirds offer a bounty of nutrients not otherwise available in this harsh,

island ecosystem—particularly polyunsaturated fatty acids (PUFAs) (Cartland-Shaw et al. 1998; Cree et al. 1999; Lamar et al. 2022). PUFAs are important for many aspects of male reproduction, including the signaling of sperm towards their target cells (Kubagawa et al. 2006). Additionally, PUFAs increase calcium ion uptake, and calcium levels are associated with increased sperm motility and fertility in many species (Kelly et al. 2003; Rahman et al. 2014; Harchegani et al. 2019). Thus, PUFAs may be a primary reason for the increased sperm swim speed seen in tuatara with diets richer in marine nutrients.

Tuatara with faster swimming sperm had higher nitrogen-15 values, which are useful in calculating the trophic level of the consumer. With each increase in trophic level, there is a subsequent 2 – 4% enrichment in collagen nitrogen-15 (Cabana and Rasmussen 1994). Thus, we assume that tuatara with lower nitrogen-15 values are foraging at lower trophic levels than their conspecifics with higher nitrogen-15 values. There is evidence that vegetarian diets are rich in antioxidants (Benzie 2003), and research on sex-based dietary differences in Magellanic penguins (*Spheniscus magellanicus*) found that significantly lower levels of the antioxidant α-tocopherol were associated with higher trophic level foraging in males (Colominas-Ciuró et al. 2022). The nitrogen-15 signatures of these tuatara with faster swimming sperm, which indicate a diet of prey at lower trophic levels, could reflect a diet richer in antioxidants.

Although PUFAs are beneficial for many cellular processes, they are also oxidizable and highly susceptible to damage by reactive oxygen species (ROS) (Dowling and Simmons 2009; Nassan et al. 2018). Endogenous ROS produced during immune response have been identified as a key factor in male infertility (Dowling and Simmons 2009; Nassan et al. 2018), and dietary supplementation of antioxidants has been suggested as a suitable way to increase sperm quality (Zini et al. 2009; Nassan et al. 2018). However, information about the specific types of antioxidants found in the Takapourewa trophic web and their usefulness against ROS, which cause cellular damage in tuatara, requires further research.

Research looking at ROS and antioxidants in reptile species is extremely limited, but evidence exists for a significant increase in reactive oxygen metabolites during digestion in corn snakes (*Pantherophis guttatus*) (Butler et al. 2016), suggesting that there may be implications for the timing of mating and when tuatara in this study had last eaten. We suggest further work investigating the role of antioxidants in reptile sperm quality be carried out, as our results suggest that diet may play a modulating role in tuatara sperm quality. However, whether this effect is direct, occurs via changes in male body condition, or reflects variation in prey availability in different quality territories among males, is unclear.

In summary, we found evidence for trade-offs between body condition, leukocyte markers of immune stress, and sperm quality in the only living member of the reptile order Rhynchocephalia. We found that two traits previously suspected to be targets of female mate choice, spine area and body condition, were negatively correlated with sperm quality metrics. We suggest that this provides evidence for energetic trade-offs between primary and secondary sexual characteristics in tuatara. We also found evidence for the suppressive effect of immune stress on sperm quality in male tuatara and the modulating effects of diet, particularly trophic level and marine input, on this relationship. It currently remains unclear

whether these effects are the result of an energetic trade-off in investment between the immune and endocrine systems, or whether they represent a more hostile, direct immune challenge within the male reproductive tract. Regardless, these results provide empirical support for the immunocompetence handicap hypothesis in an evolutionarily unique study system and highlight the energetic burden imposed by reproduction on male reptiles.

Authors' Contributions

All authors contributed to funding, project design, and editing. S.K.L. carried out fieldwork, data analysis, and initial manuscript writing. D.K.O. and N.J.N. provided supervision.

Conflict of Interest statement

The authors declare no conflicts of interest.

Ethics statement

The research was carried out under Wildlife Act Authority permit #50568-FAU and Victoria University of Wellington animal ethics committee permits #27041 and #30011.

Supplementary Material

Supplementary material can be found at https://academic.oup.com/cz.

References

Anderson M, 2018. Population of the ngaio weevil (*Anagotus stephenensis*) on Stephens Island/Takapourewa. *The Wētā* **52**:40–54.

Bartoń K, 2022. MuMIn: Multi-model inference. *R package version 1.47.1.*

Bates D, Mächler M, Bolker BM, Walker SC, 2015. Fitting linear mixed-effects models using lme4. *J Stat Softw* **67**(1):1–48.

Benzie IFF, 2003. Evolution of dietary antioxidants. *Comp Biochem Physiol A Mol Integr Physiol* **136**(1):113–126.

Butler MW, Lutz TJ, Fokidis HB, Stahlschmidt ZR, 2016. Eating increases oxidative damage in a reptile. *J Exp Biol* **219**(13):1969–1973.

Cabana G, Rasmussen JB, 1994. Modelling food chain structure and contaminant bioaccumulation using stable nitrogen isotopes. *Nature* **372**(6503):255–257.

Carbillet J, Rey B, Lavabre T, Chaval Y, Merlet J et al., 2019. The neutrophil to lymphocyte ratio indexes individual variation in the

behavioural stress response of wild roe deer across fluctuating environmental conditions. *Behav Ecol Sociobiol* **73**(11):1–13.

Cartland-Shaw LK, Cree A, Skea CM, Grimmond NM, 1998. Differences in dietary and plasma fatty acids between wild and captive populations of a rare reptile, the tuatara (*Sphenodon punctatus*). *J Comp Physiol B* **168**:569–580.

Castanet J, Newman DG, Saint Girons J, 1988. Skeletochronological data on the growth, age, and population structure of the tuatara, *Sphenodon punctatus*, on Stephens and Lady Alice Islands, New Zealand. *Herpetologica* **44**(1):25–37.

Claver JA, Quaglia AIE, 2009. Comparative morphology, development, and function of blood cells in nonmammalian vertebrates. *J Exot Pet Med* **18**(2):87–97.

Colominas-Ciuró R, Bertellotti M, D'Amico VL, Carabajal E, Benzal J et al., 2022. Sex matters? Association between foraging behaviour, diet, and physiology in Magellanic penguins. *Mar Biol* **169**(2):1–12.

Cree A, 2014. *Tuatara: Biology and Conservation of a Venerable Survivor*. Christchurch, New Zealand: Canterbury University Press.

Cree A, Cockrem JF, Brown MA, Watson PR, GuilletteLJ et al., 1991. Laparoscopy, radiography, and blood analyses as techniques for identifying the reproductive condition of female tuatara. *Herpetologica* **47**(2):238–249.

Cree A, Cockrem JF, Guillette LJ, 1992. Reproductive cycles of male and female tuatara (*Sphenodon punctatus*) on Stephens Island, New Zealand. *J Zool* **226**(2):199–217.

Cree A, Lyon GL, Cartland-Shaw L, Tyrrell C, 1999. Stable carbon isotope ratios as indicators of marine versus terrestrial inputs to the diets of wild and captive tuatara (*Sphenodon punctatus*). *NZ J Zool* **26**(3):243–253.

Darwin C, 1871. The Descent of Man, *and Selection in Relation to Sex*. *In The Descent of Man, and Selection in Relation to Sex* (Vols. **1–2**). London, England: John Murray. doi:10.1038/011305a0

Davis AK, Maney DL, Maerz JC, 2008. The use of leukocyte profiles to measure stress in vertebrates: A review for ecologists. *Funct Ecol* **22**(5):760–772.

Dawbin WH, 1982. The Tuatara, *Sphenodon Punctatus: Aspects of Life History, Growth, and Longevity*. Wellington, New Zealand: New Zealand Wildlife Service, Department of Internal Affairs.

Desser SS, 1978. Morphological, cytochemical, and biochemical observations on the blood of the tuatara, *Sphenodon punctatus*. *NZ J Zool* **5**(3):503–508.

Dines JP, Mesnick SL, Ralls K, May-Collado L, Agnarsson I et al. 2015. A trade-off between precopulatory and postcopulatory trait investment in male cetaceans. *Evolution* **69**(6):1560–1572.

Dowling DK, Simmons LW, 2009. Reactive oxygen species as universal constraints in life- history evolution. *Proc R Soc B: Biol Sci* **276**(1663):1737–1745.

East TK, East MR, Daugherty CH, 1995. Ecological restoration and habitat relationships of reptiles on Stephens Island, New Zealand. *NZ J Zool* **22**(3):249–261.

Folstad I, Karter AJ, 1992. Parasites, bright males, and the immunocompetence handicap. *Am Nat* **139**(3):603–622.

Friesen CR, Uhrig EJ, Bentz EJ, Blakemore LA, Mason RT, 2017. Correlated evolution of sexually selected traits: Interspecific variation in ejaculates, sperm morphology, copulatory mate guarding, and body size in two sympatric species of garter snakes. *Behav Ecol Sociobiol* **71**(12):1–17.

Gabe M, Saint Girons H, 1964. Contribution a l'Histologie de *Sphenodon punctatus* Gray (Éditions d).

Gillingham JC, Clark DL, Moore JA, 2009. Studs and duds – Mating success and dorsal spine reflectance in the tuatara, *Sphenodon punctatus*. Joint Meeting of Ichthyologists and Herpetologists.

Godfrey SS, Bull CM, Nelson NJ, 2008. Seasonal and spatial dynamics of ectoparasite infestation of a threatened reptile, the tuatara (*Sphenodon punctatus*). *Med Vet Entomol* **22**(4):374–385.

Godfrey SS, Moore JA, Nelson NJ, Bull C, 2010a. Social network structure and parasite infection patterns in a territorial reptile, the tuatara (*Sphenodon punctatus*). *Int J Parasitol* **40**(13):1575–1585.

Godfrey SS, Moore JA, Nelson NJ, Bull C, 2010b. Unravelling causality from correlations: Revealing the impacts of endemic ectoparasites on a protected species (tuatara). *Parasitology* **137**(2):275–286.

Goff ML, Loomis RB, Ainsworth R, 1987. Redescription of *Neotrombicula naultini* (Dumbleton, 1947) and descriptions of two new species of chiggers from New Zealand (acari: Trombiculidae). *NZ J Zool* **14**(3):385–390.

Greenspan SE, Bower DS, Webb RJ, Berger L, Rudd D et al., 2017. White blood cell profiles in amphibians help to explain disease susceptibility following temperature shifts. *Dev Comp Immunol* **77**:280–286.

Gross WB, Siegel HS, 1983. Evaluation of the heterophil/lymphocyte ratio as a measure of stress in chickens. *Avian Dis* **27**(4):972–979.

Guillete LJ, Cree A, Gross TS, 1990. Endocrinology of oviposition in the tuatara (*Sphenodon punctatus*): I. Plasma steroids and prostaglandins during natural nesting. *Biol Reprod* **43**(2):285–289.

Guillette LJ, Cree A, Rooney AA, 1995. Biology of stress: Interactions with reproduction, immunology and intermediary metabolism. In: *Health and Welfare of Captive Reptiles*. Dordrecht, Netherlands: Springer, 32–81.

Hamilton WD, Zuk M, 1982. Heritable true fitness and bright birds: A role for parasites? *Science* **218**(4570):384–387.

Han X, Hao X, Wang Y, Wang X, Teng L et al., 2020. Experimental warming induces oxidative stress and immunosuppression in a viviparous lizard, *Eremias multiocellata*. *J Therm Biol* **90**:102595.

Harchegani AB, Irandoost A, Mirnamniha M, Rahmani H, Tahmasbpour E et al., 2019. Possible mechanisms for the effects of calcium deficiency on male infertility. *Int J Fert* **12**(4):267–272.

Hoare JM, Pledger S, Keall SN, Nelson NJ, Mitchell NJ et al., 2006. Conservation implications of a long-term decline in body condition of the Brothers Island tuatara (*Sphenodon guntheri*). *Anim Conserv* **9**(4):456–462.

Huff GR, Huff WE, Balog JM, Rath NC, Anthony NB et al., 2005. Stress response differences and disease susceptibility reflected by heterophil to lymphocyte ratio in turkeys selected for increased body weight. *Poult Sci* **84**(5):709–717.

Jessop TS, Hamann M, Limpus CJ, 2004. Body condition and physiological changes in male green turtles during breeding. *Mar Ecol Prog Ser* **276**(1):281–288.

Johnstone RA, Reynolds JD, Deutsch JC, 1996. Mutual mate choice and sex differences in choosiness. *Evolution* **50**(4):1382–1391.

Kahrl AF, Cox RM, 2015. Diet affects ejaculate traits in a lizard with condition-dependent fertilization success. *Behav Ecol* **26**(6):1502–1511.

Kahrl AF, Cox CL, Cox RM, 2016. Correlated evolution between targets of pre- and postcopulatory sexual selection across squamate reptiles. *Ecol Evol* **6**(18):6452–6459.

Kelly O, Cusack S, Jewell C, Cashman KD, 2003. The effect of polyunsaturated fatty acids, including conjugated linoleic acid, on calcium absorption and bone metabolism and composition in young growing rats. *Br J Nutr* **90**(4):743–750.

King RB, 2002. Predicted and observed maximum prey size – Snake size allometry. *Funct Ecol* **16**(6):766–772.

Kubagawa HM, Watts JL, Corrigan C, Edmonds JW, Sztul E et al., 2006. Oocyte signals derived from polyunsaturated fatty acids control sperm recruitment in vivo. *Nat Cell Biol* **8**(10):1143–1148.

Lamar SK, Altobelli JT, Nelson NJ, Ormsby DK, 2022. Investigating the link between morphological characteristics and diet in an island population of omnivorous reptiles (*Sphenodon punctatus*). *Biol Open* **11**(10):bio059393.

Lamar SK, Frank HK, La Flamme A, Gartrell B, Ormsby DK et al., 2024. The effects of annual cycle, source population, and body condition on leukocyte profile and immune challenge in a basal reptile, the tuatara (*Sphenodon punctatus*). *J Exp Zool A Ecol Integr Physiol* **341**(1):60–72.

Lamar SK, Nelson NJ, Moore JA, Taylor HR, Keall SN et al., 2021. Initial collection, characterization, and storage of tuatara

(Sphenodon punctatus) sperm offers insight into their unique reproductive system. *PLoS One* **16**(7):e0253628.

Lamar SK, Nelson NJ, Ormsby DK, 2023. Characterization of sperm and implications for male fertility in the last of the Rhynchocephalians. *Conserv Physiol* **11**(1):coad071. doi:10.1093/conphys/coad071

Lara CE, Taylor HR, Holtmann B, Johnson SL, Santos ESA et al., 2020. Dunnock social status correlates with sperm speed, but fast sperm does not always equal high fitness. *J Evol Biol* **33**(8):1139–1148.

LaVere AA, Hamlin HJ, Lowers RH, Parrott BB, Ezenwa VO, 2021. Associations between testosterone and immune activity in alligators depend on bacteria species and temperature. *Funct Ecol* **35**(5):1018–1027.

Lüpold S, Pitnick S, 2018. Sperm form and function: What do we know about the role of sexual selection? *Reproduction* **155**(5):R229–R243.

Lüpold S, Tomkins JL, Simmons LW, Fitzpatrick JL, 2014. Female monopolization mediates the relationship between pre- and post-copulatory sexual traits. *Nat Commun* **5**:3184.

Mangiafico S, 2022. _rcompanion: Functions to support extension education program evaluation_. *R package version 2.4.18*.

Marler CA, Moore MC, 1988. Evolutionary costs of aggression revealed by testosterone manipulations in free-living male lizards. *Behav Ecol Sociobiol* **23**(1):21–26.

Marler CA, Walsberg G, White ML, Moore M, Marler CA, 1995. Increased energy expenditure due to increased territorial defense in male lizards after phenotypic manipulation. *Behav Ecol Sociobiol* **37**(4):225–231.

Miller HC, Moore JA, Nelson NJ, Daugherty CH, 2009. Influence of major histocompatibility complex genotype on mating success in a free-ranging reptile population. *Proc Biol Sci* **276**(1662):1695–1704.

Minias P, 2019. Evolution of heterophil/lymphocyte ratios in response to ecological and life-history traits: A comparative analysis across the avian tree of life. *J Anim Ecol* **88**(4):554–565.

Moore JA, 2008. *Fitness Implications of the Mating System and Reproductive Ecology of Tuatara*. Wellington, New Zealand: Victoria University of Wellington.

Moore JA, Daugherty CH, Godfrey SS, Nelson NJ, 2009. Seasonal monogamy and multiple paternity in a wild population of a territorial reptile (tuatara). *Biol J Linn Soc* **98**(1):161–170.

Moore JA, Daugherty CH, Nelson NJ, 2009. Large male advantage: Phenotypic and genetic correlates of territoriality in tuatara. *J Herpetol* **43**(4):570–578.

Nakagawa S, Schielzeth H, 2010. Repeatability for Gaussian and non-Gaussian data: A practical guide for biologists. *Biol Rev Camb Philos Soc* **85**(4):935–956.

Nassan FL, Chavarro JE, Tanrikut C, 2018. Diet and men's fertility: Does diet affect sperm quality? *Fertil Steril* **110**(4):570–577.

Olsson M, Madsen T, 1998. Sexual selection and sperm competition in reptiles. In: *Sperm Competition and Sexual Selection*. Cambridge, Massachusetts: Academic Press, 503–577.

Oppliger A, Giorgi MS, Conelli A, Nembrini M, John-Alder HB, 2004. Effect of testosterone on immunocompetence, parasite load, and metabolism in the common wall lizard (*Podarcis muralis*). *Can J Zool* **82**(11):1713–1719.

R Core Team, 2023. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing. https://www.r-project.org/

Rahman MS, Kwon WS, Pang MG, 2014. Calcium influx and male fertility in the context of the sperm proteome: An update. *BioMed Res Int*. doi:10.1155/2014/841615

Roberts ML, Buchanan KL, Evans MR, 2004. Testing the immunocompetence handicap hypothesis: A review of the evidence. *Anim Behav* **68**(2):227–239.

Rowe L, Houle D, 1996. The lek paradox and the capture of genetic variance by condition dependent traits. *Proc R Soc B: Biol Sci* **263**(1375):1415–1421.

Simmons LW, 2012. Resource allocation trade-off between sperm quality and immunity in the field cricket, *Teleogryllus oceanicus*. *Behav Ecol* **23**(1):168–173.

Simons MJP, Cohen AA, Verhulst S, 2012. What does carotenoid-dependent coloration tell? Plasma carotenoid level signals immunocompetence and oxidative stress state in birds-a meta-analysis. *PLoS One* **7**(8):43088.

Stokkan K-A, 1979. Testosterone and daylength-dependent development of comb size and breeding plumage of male willow ptarmigan (*Lagopus lagopus lagopus*). *Auk* **96**(1):106–115.

Sykes IV JM, Klaphake E, 2008. Reptile hematology. *Vet Clin North Am Exot Anim Pract* **11**(3):481–500.

Tennyson A, 1998. Large carabid beetles Stephens Island 30 April–3 May 1996.

Walls G, 1978. The influence of the tuatara on fairy prion breeding on Stephens Island, Cook Strait. *N Z J Ecol* **1**:91–98.

Wingfield JC, Hegner RE, Dufty AM, Ball GF, 1990. The "Challenge Hypothesis": Theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. *Am Nat* **136**(6):829–846.

Zeller FJ, 1971. The effects of testosterone and dihydrotestosterone on the comb, testis, and pituitary gland of the male fowl. *J Reprod Fertil* **25**(1):125–127.

Zimmerman LM, Paitz RT, Vogel LA, Bowden RM, 2010. Variation in the seasonal patterns of innate and adaptive immunity in the red-eared slider (*Trachemys scripta*). *J Exp Biol* **213**(9):1477–1483.

Zini A, San Gabriel M, Baazeem A, 2009. Antioxidants and sperm DNA damage: A clinical perspective. *J Assist Reprod Genet* **26**(8):427–432.