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RESEARCH ARTICLE

Maternal provisioning and fluctuating thermal regimes enhance immune response in a reptile with temperature-dependent sex determination

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ABSTRACT

The Charnov–Bull model of differential fitness is often used to explain the evolution and maintenance of temperature-dependent sex determination (TSD). Most tests of the model focus on morphological proxies of fitness, such as size traits, whereas early life physiological traits that are closely related to lifetime fitness might provide a framework for generalizing the Charnov–Bull model across taxa. One such trait is the strength of the early-life immune response, which is strongly linked to early-life survival and fitness. Here, we manipulated temperature, variance in temperature, and sex to test the Charnov–Bull model using a physiological trait, immune system strength, in the snapping turtle (*Chelydra serpentina*). We found no evidence of sex-specific differences in bactericidal capacity of hatchling blood, and no evidence that mean temperature influences bactericidal capacity. However, we did find that fluctuating incubation temperature (i.e. a more naturalized incubation regime) is associated with a greater bactericidal capacity compared with constant temperature incubation. We also found that egg mass, a proxy for maternal provisioning, is positively associated with bactericidal capacity. Our findings suggest that the evolution of temperature-dependent sex determination in reptiles is unrelated to our measure of early-life innate immunity. Our study also underlines how immune response is condition dependent in early life, and questions the biological relevance of constant temperature incubation in experimental studies on ectotherm development.

KEY WORDS: Fitness, Bacteria-killing assay, Thermal fluctuations, Maternal provisioning, *Chelydra serpentina*

INTRODUCTION

The environment experienced in early life can have important and irreversible effects on phenotype and fitness (Lindström, 1999). In a variety of taxa, for instance, both stress and parental care in early life can influence adult fitness components (Burton et al., 2020; Cohen et al., 2013; Lee et al., 2013). Among ectotherms, it is well established that temperature experienced during embryonic development exerts pervasive effects on phenotypes (Booth, 2006; Deeming, 2004; Noble et al., 2018), and these effects are particularly well studied in reptiles (Noble et al., 2018). The widespread study of thermal traits in the early lives of reptiles arises, in part, because many reptiles exhibit temperature-dependent sex determination (TSD), whereby incubation temperature experienced by an embryo permanently affects its sex (Charnier, 1966).

Theory suggests the adaptive significance of TSD may be related to a differential effect of incubation temperature on male versus female fitness (Charnov and Bull, 1977). Specifically, females are produced at a temperature that confers a fitness advantage relative to males, and vice versa, indicative of a sex-by-environment interaction for fitness; this is known as the Charnov–Bull model (Charnov and Bull, 1977; Warner and Shine, 2008). Because TSD is phylogenetically widespread in reptiles, the Charnov–Bull model has been explored broadly (Valenzuela and Lance, 2004). A handful of studies have shown that the model is supported for fitness-related traits such as body size (Warner et al., 2020) and locomotor ability (Webb et al., 2001), but it is rare that a sex-by-environment interaction for fitness is observed (Warner and Shine, 2008). Further, despite support for the Charnov–Bull model in a few isolated studies, there is a dearth of hypotheses that outline a general mechanism underpinning a sex-by-environment interaction for fitness in reptile species (Bókony et al., 2019; Schwanz et al., 2016; but see Lawson and Rollinson, 2020 preprint; Warner et al., 2009).

Early-life physiological traits that are closely related to lifetime fitness might provide a framework for generalizing the Charnov–Bull model across reptiles (Hulbert et al., 2017). One such trait is the strength of the early-life immune response, which is strongly linked to early-life survival and lifetime fitness (Cichon and Dubiec, 2005; Møller and Saino, 2004; Schneeberger et al., 2014; Watson et al., 2016). Indeed, recent work underlines that the immune response is context dependent and sex specific, highlighting that it is a plausible candidate for a sex-by-environment interaction under the Charnov–Bull model. For instance, a sex-specific immune response in early life has been documented in non-reptilian taxa (Kraaijeveld et al., 2008; Laughton et al., 2011; Love et al., 2008; Tschirren et al., 2003), and sex has been found to influence hatchling immune function in a reptile with genetic sex determination (GSD), and adult immune function in a reptile with TSD (Judson et al., 2020; Palacios et al., 2020). Yet, despite the promising candidacy of immune strength underpinning a sex-by-environment interaction for fitness, it remains unclear how temperature affects immune strength in reptiles. For instance, immune strength is negatively associated with constant incubation temperature in two turtle species (Dang et al., 2015; Freedberg et al., 2008), but it is not clear whether or how this result is relevant under natural temperature regimes where temperatures fluctuate diurnally and vary across seasons (Les et al., 2009; Paitz et al., 2010). Indeed, the Charnov–Bull model, more broadly, is usually tested by comparing fitness from constant-temperature incubations (Janzen, 1995; Warner and Shine, 2005, 2008), despite recognition that these are evolutionarily novel environments and poorly reflect natural environments where

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temperature fluctuates (Kingsolver et al., 2015; Massey and Hutchings, 2020; Niehaus et al., 2012). Thus, investigating the immune response under incubation regimes that are at least partly naturalized may provide new insight into the origin of a sex-by-temperature interaction for fitness in reptiles.

The immune system may have been largely overlooked in the context of TSD because it is not a phenotype that is readily visible or easily measured. Additionally, many traits affect immune function, making it difficult to directly link immune function, sex and temperature. For instance, because the immune system is energetically costly (East et al., 2015; Leivesley et al., 2019; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996), immune function is condition dependent, where greater energetic reserves (i.e. high condition) are associated with improved immune strength (Garbutt and Little, 2017). If immune function is also condition dependent in early life (Martínez-Padilla, 2006; Rubolini et al., 2006; but see Krist, 2011), then the amount of maternal provisioning that occurs before sex is determined should exert a strong positive effect on early-life immune strength. Maternal provisioning must therefore be carefully controlled in TSD studies in order to directly link immune function, sex and temperature. Fortunately, unlike for birds and mammals, the contents of reptile eggs usually reflect the entirety of offspring provisioning (Congdon and Gibbons, 1985). Egg mass can therefore be used as an unambiguous proxy for offspring provisioning, which can be leveraged to provide a clear test of whether provisioning per se affects immune strength while also controlling any potentially confounding effects of condition on immune function.

In the present study, we used a reptile with TSD to test whether immune strength exhibits a sex-by-environment interaction that is consistent with the Charnov–Bull model. We incubated snapping turtle, *Chelydra serpentina* (Linnaeus 1758), eggs at two mean temperatures, with and without thermal variance, using immune strength as a proxy for fitness. Critically, we also decoupled sex and temperature using a hormonal manipulation, allowing us to assess the independent effects of sex and temperature on immune strength. Applying the Charnov–Bull model to our study system, we predicted a relatively greater immune response (more bacteria killed in a bactericidal assay) for males at 24°C than females, and a greater response of females at 28°C than males. We also controlled for maternal provisioning across all environments, thereby holding constant any effect of hatchling condition while also providing an unambiguous test for a role for maternal provisioning in early-life immune function.

MATERIALS AND METHODS

Study population and egg collection

The present study is part of a long-term study on snapping turtles at the Algonquin Wildlife Research Station (45°35′N, 78°31′W) in Algonquin Provincial Park, Canada. In 2019, we collected and weighed 302 eggs (9 nests) soon after laying. Eggs were kept in containers with moistened vermiculite (1:1 vermiculite and water) and during the experiment, we added water regularly to bring containers back to their initial weight. We randomly assigned clutches to one of four temperature regimes: 24°C constant, 24±4°C, 28°C constant and 28±4°C in a split clutch design. At 24 and 28°C constant temperatures, we would expect the proportion of males produced to be 0.98 and 0.20, respectively (Massey et al., 2019). To produce male hatchlings at female-producing temperatures (28°C/28±4°C), we applied 95 µg of an aromatase inhibitor (fadrozole, Sigma-Aldrich, St Louis, MO, USA) in 100% ethanol to half of the eggs in each experimental group (Table 1) at the start of the thermosensitive period (day 13 and 22 for the 28 and 24°C regimes, respectively). We estimated the timing of the thermosensitive period using a thermal performance curve for embryo development in Algonquin snapping turtle populations (Rollinson et al., 2018). On the same days, we also applied 5 µl of 100% ethanol to the rest of the eggs as a control. Overall, we created eight experimental groups: either oestrogen-inhibited or control treatments in each of four incubation regimes. All animal work was done in accordance with the University of Toronto Animal Care Committee protocols.

Blood collection and sex determination

Within 48 h of hatching, we euthanized hatchlings by decapitation and pithing, in accordance with animal use protocol #20011948 from the University of Toronto. On decapitation, we collected trunk blood in a sterile microcentrifuge tube stored over ice. To limit the effect that stress could have on the bactericidal capacity of blood, we collected blood within 10 min of disturbing the individuals (Beck et al., 2017). We centrifuged blood samples at 5000 rpm for 5 min, removed the plasma fraction and froze this at −80°C until we carried out the bactericidal assay. Plasma samples can be frozen at −80°C for up to 6 weeks without affecting plasma complement (Beck et al., 2017). We determined hatchling sex upon dissection and took photos of the gonads. Three researchers determined sex individually and we assigned sex by at least 2 of 3 researchers reaching consensus.

Bacteria killing assay optimization

We completed a bacteria killing assay (BKA) following previously reported techniques (Beck et al., 2017). Optimization for hatchlings was necessary to determine the dilution of plasma and concentration of bacteria needed to achieve 50% bacteria inhibition, thereby maximizing the variation detectable. We used a pooled plasma sample from eight hatchlings (one from each treatment) to optimize the BKA. We created three plasma dilutions using sterile phosphate-buffered saline (PBS): 1:3, 1:5 and 1:10 (e.g. 1:10=one part plasma to nine parts PBS); and three concentrations of *Escherichia coli* (ATCC 8739, E^{power} microorganisms, Microbiologics, St Cloud, MN, USA); 10^5, 10^6 and 10^7 colony forming units (CFU) per ml. We mixed 72 µl of each plasma dilution with 25 µl of each bacteria concentration in sterile microcentrifuge tubes (i.e. 9 samples – 3 plasma dilutions×3 bacteria concentrations) and included positive controls for each bacteria concentration (25 µl of bacteria mixed with 72 µl of PBS). All samples were incubated at 26°C, the mean incubation temperature in the study, for 30 min to complete the immune challenge. Next, we placed 20 µl of the bacteria mix in 96-well microplates in triplicate and added 140 µl of sterile tryptic soy broth (TSB) to each well; this serves as a food source for *E. coli*. Positive controls were also placed in triplicate on each plate, with 140 µl of sterile TSB. We incubated microplates at 37°C and measured absorbance at 600 nm at 6, 8, 10 and 12 h of incubation with a microplate reader (Synergy HTX, BioTek, Winooski, VT, USA). Absorbance is positively related to bacterial concentration; thus, we averaged the absorbance of each sample and calculated the proportion of bacteria killed as 1−(sample absorbance/positive control absorbance).

Bacteria killing assay

We followed the above method using 1:3 dilution of plasma and 10^6 CFU ml^{-1} of *E. coli* (see Results). Briefly, we incubated a mixture of 72 µl diluted plasma and 25 µl of *E. coli* at 26°C for 30 min. Then, we placed 20 µl of the bacteria mix in 96-well microplates in triplicate and added 140 µl of TSB to each well,

Table 1. Experimental design

Experimental group	No. of males hatched		No. of females hatched		Total no. of eggs allocated (hatched)	
	Treated	Control	Treated	Control	Treated	Control
24°C	16	12	0	3	32 (16)	35 (16)
24±4°C	13	12	6	2	31 (19)	32 (16)
28°C	8	4	12	17	43 (22)	43 (23)
28±4°C	16	13	14	19	42 (32)	44 (36)

Data under the first two column headings are the number of male and female hatchlings in each experimental group; the total number of eggs allocated to each experimental group is shown with the number of successfully hatched eggs in parentheses under the final column heading.

including positive controls. We incubated microplates at 37°C and measured absorbance at 600 nm after 8 h of incubation with a microplate reader (Synergy HTX, BioTek). We averaged the absorbance of each sample and calculated proportion of bacteria killed as 1−(sample absorbance/positive control absorbance), as above. All BKAs were run on the same day.

Statistical analyses

We used a generalized linear mixed effect model with a beta distribution to determine whether temperature, temperature type, sex, initial egg mass and a two-way interaction between sex and temperature influenced the proportion of bacteria killed. The beta distribution was used to accommodate the proportional nature of the response variable. The interaction between sex and temperature serves to test the Charnov–Bull model (i.e. a sex-by-temperature interaction for fitness), where for simplicity we collapsed temperature across fluctuating and constant environments, thereby ensuring that each treatment received a complement of males and females from 'male-producing' and 'female-producing' environments. We included maternal ID as a random effect to account for potential clutch effects. We also re-ran the same model using maternal carapace length in place of egg mass to determine whether maternal quality or age influenced the proportion of bacteria killed by hatchlings. Thirty-two measures of bactericidal capacity that were less than zero were set to equal zero as we assumed these to be zeros with measurement error. For beta regression, we constrained the response to fall between 0 and 1 using the equation:

$$p* = \frac{p(n-1)+(1/2)}{n}, \quad (1)$$

where p is the proportion of bacteria killed and n is the sample size (Douma and Weedon, 2019). The mixed effect model was fitted using the R package glmmTMB (Brooks et al., 2017), and pairwise differences were determined using estimated marginal means calculated within the emmeans package (https://github.com/rvlenth/emmeans).

RESULTS

Overall, 180 out of 302 eggs successfully hatched (Table 1), and 167 had sex determined. Egg mortality was not related to incubation temperature ($t_{4.062}$=−1.639, P=0.175), temperature type ($t_{3.305}$=−2.329, P=0.094) or treatment ($t_{5.534}$=−0.187, P=0.858). Ninety-four males hatched across the four treatments, with a mean±s.d. body mass 10.9±0.94 g and carapace length 28.3±1.75 mm. Seventy-three females hatched and had a mean±s.d. body mass of 10.8±1.1 g and carapace length of 27.9±2.26 mm. The sex manipulation was effective, as the sex ratio was male biased in three of the four hormonally manipulated temperature treatments, and in the fourth, the number of males was doubled relative to the control (Table 1).

BKA optimization

Three combinations of bacteria concentration and plasma dilution produced close to 50% bactericidal capacity. These were 10^5 CFU ml^{-1} and 1:3 dilution at 6 h, 10^5 CFU ml^{-1} and 1:5 dilution at 6 h, and 10^6 CFU ml^{-1} and 1:3 dilution at 8 h, which resulted in a bactericidal capacity of 40%, 41% and 62%, respectively (Fig. 1). The 10^6 CFU ml^{-1} and 1:3 dilution at 8 h was chosen for the BKA as this allowed sufficient time for bacterial growth to take place. Other studies employing similar methods have also allowed 8 h of incubation for *E. coli* to grow after an immune challenge (Beck et al., 2017).

Drivers of variation in bacteria killing ability

We successfully extracted blood from 119 hatchlings for use in the BKA. Intra-assay coefficient of variation (CV%) was 19.2, while inter-assay CV% was 22.7. Hormonal manipulation did not

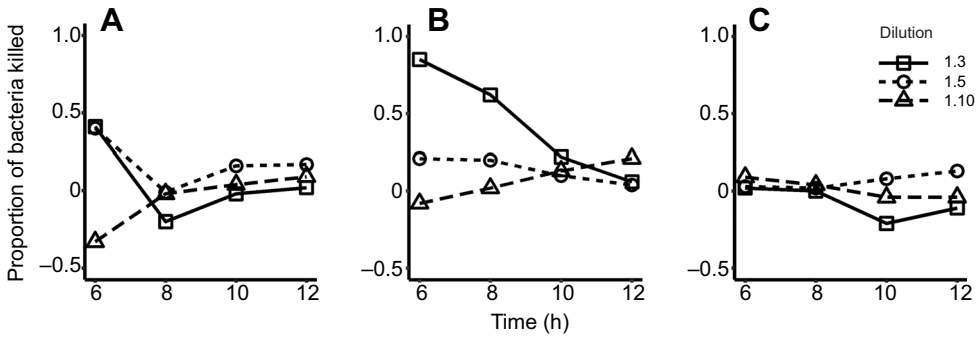

Fig. 1. Results of the bacteria killing assay optimization. Three concentrations of *Escherichia coli* (ATCC 8739) were used: (A) 10^5 CFU ml^{-1}, (B) 10^6 CFU ml^{-1} and (C) 10^7 CFU ml^{-1}. The bacteria were mixed with a pooled sample of plasma from eight snapping turtle hatchlings (one from each experimental group) at three plasma dilutions: 1:3, 1:5 and 1:10. Absorbance was measured after 6, 8, 10 and 12 h of incubation. The proportion of bacteria killed is equal to 1−(sample absorbance/positive control absorbance). Symbols represent the proportion of bacterial killed across three triplicates.

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Table 2. Effects of sex, incubation temperature, incubation temperature type and egg mass on immune system strength of hatchling snapping turtles estimated from a generalized linear mixed effect model.

Variable	Effect type	Estimate	s.e.	*P*
Maternal ID	Random	2.85×10^{-10}	1.69×10^{-5}	–
Intercept	Fixed	−5.21	1.73	0.002*
Sex (male)	Fixed	0.70	0.58	0.225
Temperature (28°C)	Fixed	0.58	0.58	0.314
Temperature type (fluctuating)	Fixed	0.74	0.25	0.003*
Egg mass	Fixed	0.30	0.13	0.020*
Sex×temperature	Fixed	−0.76	0.65	0.243

Parameter estimates for random effects are variance estimates; for fixed effects, the coefficients displayed are log-odds. Standard error estimates for random effects are standard deviation estimates. Asterisks indicate significance.

significantly affect the proportion of bacteria killed ($\beta_{Control}$=−0.049, s.e.=0.224, z=−0.216, P=0.829) and was not included in further analyses. Egg mass was significantly positively associated with the proportion of bacteria killed (Table 2, Fig. 2A); however, in the model replacing egg mass with maternal carapace length, maternal carapace length did not explain variation in the proportion of bacteria killed by hatchling plasma (z=0.812, P=0.417). Neither mean temperature nor sex was significantly associated with the proportion of bacteria killed, but temperature variance was positively associated with the proportion of bacteria killed (Table 2; z=3.00, P=0.003). Hatchlings incubated under fluctuating conditions had an immune response that was nearly 2 times stronger (mean±s.e. 0.453±0.044 bacteria killed) than that of those incubated under constant regimes (0.283±0.048 bacteria killed; Fig. 2B).

DISCUSSION

There are three major findings from the present study. First, we did not find support for the Charnov–Bull model of TSD evolution and maintenance using a single measure of functional immune response. Second, snapping turtles raised in constant temperature environments had a lower functional immune response than those from more naturalized environments, independent of mean temperature, thereby questioning the relevance of studies that use constant-temperature incubation to understand ecologically relevant phenotypes. Finally, the study demonstrates a clear and positive association between egg mass, a measure of maternal provisioning, and a measure of early life functional immune response in an ectothermic vertebrate.

A major goal of the present study was to explore whether the evolution of TSD may be related to early-life functional immune response, a phenotype that is rarely measured in TSD reptiles. Under the Charnov–Bull model, we predicted a relatively greater immune response for males at 24°C than females, and a greater response of females at 28°C than males (Charnov and Bull, 1977). Yet, despite a reasonable sample size (N=11–62 per treatment; Table 1), no such relationship was observed, nor was a main effect of sex observed. It is therefore possible that the immune response is an important aspect of an individual's early-life survival (Cichon and Dubiec, 2005; Møller and Saino, 2004), regardless of the sex of hatchlings, and that any sex specificity in functional immune response emerges later in life.

One version of the Charnov–Bull model, the 'survival to maturity' hypothesis, does not depend on a sex-by-temperature interaction in immune function. Instead, it suggests that temperature affects annual survival (i.e. immune function) equally in the two sexes and that the sex-by-temperature interaction on lifetime fitness and selection for TSD arises if temperature alone impacts immune function and the sexes differ in age at maturity (Schwanz et al., 2016). The logic is that the sex maturing later will experience relatively more mortality prior to maturity and will therefore experience a greater fitness benefit (greater juvenile survival) from developing at temperatures that confer high survival rates. The survival to maturity hypothesis has previously received some empirical support (Bókony et al., 2019), and it is applicable to snapping turtles, where males mature later than females (Steyermark et al., 2008). Further, the hypothesis is indeed in line with our finding of no difference in functional immune response between male and female hatchlings. However, under this hypothesis, we might expect to observe an effect of mean temperature on functional immune response, which we found no evidence for. Interestingly, we did find that temperature fluctuations enhanced the bactericidal capacity of blood compared with constant temperatures. It follows that the survival to maturity hypothesis would be supported if temperature fluctuation is associated with male-producing temperatures in the wild, but whether this is true is unknown. More broadly, we did not find any evidence that functional immune response is related to models of TSD evolution, but we underline that we used only one measure of immune response. It may therefore be premature to discount the possibility that immune response plays a role in the maintenance of TSD, and future studies on TSD may benefit from the use of multiple markers of immune response.

Two previous studies investigated how temperature fluctuations alter immune response compared with constant temperatures (Les et al., 2009; Paitz et al., 2010). Both used delay-type hypersensitivity tests to quantify the swelling response of painted turtle (*Chrysemys picta*) hatchlings, but the studies found mixed results. Les et al. (2009) found that a ±3°C temperature fluctuation did affect the swelling response (which is a measure of immune activity at a site of potential infection). However, the mean temperatures compared by Les et al. (2009) differed by 8°C and appeared to approach the

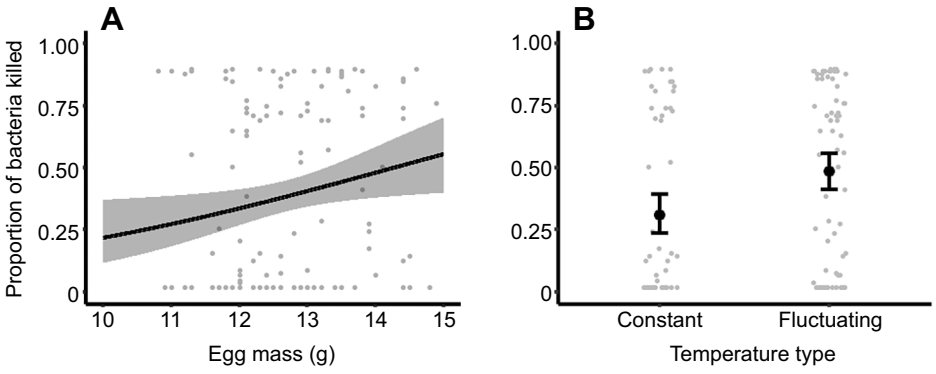

Fig. 2. Effect of egg mass and temperature variation on immune system strength. A generalized linear mixed effects model with a beta distribution was used to determine the effect of (A) egg mass and (B) incubation temperature type on the proportion of bacteria killed by hatchling snapping turtle blood. A total of 119 hatchlings were tested (69 under fluctuating temperature and 50 under constant temperature). Marginal effects and 95% confidence intervals are shown proportionally averaged over experimental group.

thermal limits of their study species. In contrast, Paitz et al. (2010) found no difference in swelling response between constant temperature, and ±4°C and ±8°C fluctuating treatments. Our study may provide insight into these contradictory results. Specifically, while limiting the degree of mean thermal difference between treatments, we found support for the notion that fluctuating temperature enhances the immune response. We therefore suggest that temperature fluctuations may have a transient effect on the immune response: Paitz et al. (2010) measured immune strength at 90 days post-hatching, whereas Les et al. (2009) measured it at 21 days post-hatching and beyond, and the present study measured it immediately after hatching. Thus, our study provides support for Les et al. (2009) and suggests the effect is transient, which may still cause important variation in early-life survival.

Better-provisioned offspring have advantages such as starvation resistance, increased gape size and foraging efficiency, and increased ability to avoid predation (Marshall et al., 2018; Rollinson and Hutchings, 2013a,b), all of which translate into enhanced survival (Rollinson and Rowe, 2015). As the immune system is energetically costly (East et al., 2015; Leivesley et al., 2019; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996), greater energetic reserves should allow better-provisioned offspring to invest more in immune system development and maintenance. Indeed, we found egg mass to be significantly positively associated with bactericidal capacity. As no parental care is provided in snapping turtles, egg provisioning represents total maternal investment in offspring, and thus we have arguably provided a relatively direct test of whether maternal investment in offspring affects the immune response. However, we did not measure maternal antibodies in the eggs or maternal hormones in the eggs, nor did we experimentally manipulate egg size, so we cannot discount the possibility that egg mass is confounded with an unmeasured physiological variable. Nevertheless, there are two reasons we believe it is the energetic reserves in the egg driving the association between egg size and immunity. First, there is no evidence to date that egg mass covaries with maternal antibody provisioning. For instance, female flycatchers that are in greater general health may provision eggs with more antibodies, although these females do not produce relatively larger eggs (Hargitai et al., 2006). Further, across 16 wild populations of flycatchers, egg mass and antibody levels were uncorrelated (Ruuskanen et al., 2011), suggesting antibody provisioning is unrelated to egg size per se. Second, it is possible that variation in yolk hormone concentrations (especially in androgens) could have influenced our results, as yolk hormones have previously been linked to differences in offspring size, growth, behaviour and immunity (Bowden et al., 2004; Schwabl, 1993, 1996; Uller et al., 2007). In particular, recently matured female painted turtles (*Chrysemys picta*) produce relatively small eggs with high testosterone concentration (Bowden et al., 2004), suggesting that hormonal differences rather than provisioning differences can explain the positive association between egg size and immune response. It seems more likely that our result is explained by provisioning, however, as adult body size did not explain immune strength in our study. Our results are therefore in line with theory (Smith and Fretwell, 1974), and suggest that immune strength is an additional axis on which maternal investment in egg size influences neonate quality (Garbutt and Little, 2017; Krist, 2011; Martínez-Padilla, 2006; Pelayo and Clark, 2002; Rollinson and Rowe, 2015; Rubolini et al., 2006).

In sum, the present study does not support a role for early-life immune response in the evolution and maintenance of TSD, although future studies could be strengthened by using multiple markers of immunity to explore the temperature sensitivity of the immune response. We do, however, show that small fluctuations around mean temperatures likely have beneficial effects on hatchling fitness, and we suggest that these effects of temperature fluctuation on immunity are important but transient. This finding is interesting to consider given that studies of reptilian embryos still overwhelmingly use constant temperature incubation regimes to study reptile ecology, or conserve reptiles through artificial egg incubation and hatchling release. Finally, we also show that variation in maternal investment per offspring, through egg provisioning, is positively associated with variation in functional immune response. Importantly, maternal provisioning is not confounded with other sources of information transfer between parent and offspring, and we suggest our result may be generalizable to other groups without parental care.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: J.A.L., N.R.; Methodology: J.A.L., N.R.; Formal analysis: J.A.L.; Data curation: J.A.L.; Writing - original draft: J.A.L.; Writing - review & editing: J.A.L., N.R.; Supervision: N.R.; Funding acquisition: N.R.

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Data availability
The full dataset is available from the Dryad digital repository (Leivesley and Rollinson, 2021): dryad.zs7h44j79.

References
Beck, M. L., Thompson, M. and Hopkins, W. A. (2017). Repeatability and sources of variation of the bacteria-killing assay in the common snapping turtle. *J. Exp. Zool.* **327**, 293-301. doi:10.1002/jez.2089

Bókony, V., Milne, G., Pipoly, I., Székely, T. and Liker, A. (2019). Sex ratios and bimaturism differ between temperature-dependent and genetic sex-determination systems in reptiles. *BMC Evol. Biol.* **19**, 57. doi:10.1186/s12862-019-1386-3

Booth, D. T. (2006). Influence of incubation temperature on hatchling phenotype in reptiles. *Physiol. Biochem. Zool.* **79**, 274-281. doi:10.1086/499988

Bowden, R. M., Harms, H. K., Paitz, R. T. and Janzen, F. J. (2004). Does optimal egg size vary with demographic stage because of a physiological constraint? *Funct. Ecol.* **18**, 522-529. doi:10.1111/j.0269-8463.2004.00861.x

Brooks, M. E., Kristensen, K., Van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Mächler, M. and Bolker, B. M. (2017). glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *R Journal* **9**, 378-400. doi:10.32614/RJ-2017-066

Burton, T., Rollinson, N., McKelvey, S., Stewart, D. C., Armstrong, J. D. and Metcalfe, N. B. (2020). Adaptive maternal investment in the wild? Links between maternal growth trajectory and offspring size, growth, and survival in contrasting environments. *Am. Nat.* **195**, 678-690. doi:10.1086/707518

Charnier, M. (1966). Action of temperature on the sex ratio of the Agama agama (Agamidae, Lacertilia) embryo. *C. R. Seances Soc. Biol. Fil.* **160**, 620-622.

Charnov, E. L. and Bull, J. (1977). When is sex environmentally determined? *Nature* **266**, 828-830. doi:10.1038/266828a0

Cichon, M. and Dubiec, A. (2005). Cell-mediated immunity predicts the probability of local recruitment in nestling blue tits. *J. Evol. Biol.* **18**, 962-966. doi:10.1111/j.1420-9101.2005.00910.x

Cohen, M. M., Jing, D., Yang, R. R., Tottenham, N., Lee, F. S. and Casey, B. J. (2013). Early-life stress has persistent effects on amygdala function and development in mice and humans. *Proc. Natl. Acad. Sci. USA* **110**, 18274-18278. doi:10.1073/pnas.1310163110

Congdon, J. D. and Gibbons, J. W. (1985). Egg components and reproductive characteristics of turtles: relationships to body size. *Herpetologica* **41**, 194-205.

Dang, W., Zhang, W. and Du, W.-G. (2015). Incubation temperature affects the immune function of hatchling soft-shelled turtles, Pelodiscus sinensis. *Sci. Rep.* **5**, 10594. doi:10.1038/srep10594
Deeming, D. C. (2004). *Reptilian Incubation: Environment, Evolution, and Behaviour*. Nottingham, UK: Nottingham University Press.
Douma, J. C. and Weedon, J. T. (2019). Analysing continuous proportions in ecology and evolution: a practical introduction to beta and Dirichlet regression. *Methods Ecol. Evol.* **10**, 1412-1430. doi:10.1111/2041-210X.13234
East, M. L., Otto, E., Helms, J., Thierer, D., Cable, J. and Hofer, H. (2015). Does lactation lead to resource allocation trade-offs in the spotted hyaena? *Behav. Ecol. Sociobiol.* **69**, 805-814. doi:10.1007/s00265-015-1897-x
Freedberg, S., Greives, T. J., Ewert, M. A., Demas, G. E., Beecher, N. and Nelson, C. E. (2008). Incubation environment affects immune system development in a turtle with environmental sex determination. *J. Herpetol.* **42**, 536-541. doi:10.1670/07-133.1
Garbutt, J. S. and Little, T. J. (2017). Bigger is better: changes in body size explain a maternal effect of food on offspring disease resistance. *Ecol. Evol.* **7**, 1403-1409. doi:10.1002/ece3.2709
Hargitai, R., Prechl, J. and Török, J. (2006). Maternal immunoglobulin concentration in collared flycatcher (Ficedula albicollis) eggs in relation to parental quality and laying order. *Funct. Ecol.* **20**, 829-838. doi:10.1111/j.1365-2435.2006.01171.x
Hulbert, A. C., Mitchell, T. S., Hall, J. M., Guiffre, C. M., Douglas, D. C. and Warner, D. A. (2017). The effects of incubation temperature and experimental design on heart rates of lizard embryos. *J. Exp. Zool. Part A Ecol. Integr. Physiol.* **327**, 466-476. doi:10.1002/jez.2135
Janzen, F. J. (1995). Experimental evidence for the evolutionary significance of temperature-dependent sex determination. *Evolution* **49**, 864-873. doi:10.1111/j.1558-5646.1995.tb02322.x
Judson, J. M., Reding, D. M. and Bronikowski, A. M. (2020). Immunosenescence and its influence on reproduction in a long-lived vertebrate. *J. Exp. Biol.* **223**, jeb223057. doi:10.1242/jeb.223057
Kingsolver, J. G., Higgins, J. K. and Augustine, K. E. (2015). Fluctuating temperatures and ectotherm growth: distinguishing non-linear and time-dependent effects. *J. Exp. Biol.* **218**, 2218-2225. doi:10.1242/jeb.120733
Kraaijeveld, A. R., Barker, C. L. and Godfray, H. C. J. (2008). Stage-specific sex differences in *Drosophila* immunity to parasites and pathogens. *Evol. Ecol.* **22**, 217-228. doi:10.1007/s10682-007-9171-y
Krist, M. (2011). Egg size and offspring quality: a meta-analysis in birds. *Biol. Rev.* **86**, 692-716. doi:10.1111/j.1469-185X.2010.00166.x
Laughton, A. M., Boots, M. and Siva-Jothy, M. T. (2011). The ontogeny of immunity in the honey bee, Apis mellifera L. following an immune challenge. *J. Insect Physiol.* **57**, 1023-1032. doi:10.1016/j.jinsphys.2011.04.020
Lawson, L. and Rollinson, N. (2020). A simple model for the evolution of temperature-dependent sex determination explains the temperature sensitivity of embryonic mortality in imperiled reptiles. *bioRxiv*. doi:10.1101/791202
Lee, P. C., Bussière, L. F., Webber, C. E., Poole, J. H. and Moss, C. J. (2013). Enduring consequences of early experiences: 40 year effects on survival and success among African elephants (*Loxodonta africana*). *Biol. Lett.* **9**, 20130011. doi:10.1098/rsbl.2013.0011
Leivesley, J. A. and Rollinson, N. (2021). Maternal provisioning and fluctuating thermal regimes enhance immune response in a reptile with temperature-dependent sex determination. *Dryad, Dataset*. https://doi.org/10.5061/dryad.zs7h44j79
Leivesley, J. A., Bussière, L. F., Pemberton, J. M., Pilkington, J. G., Wilson, K. and Hayward, A. D. (2019). Survival costs of reproduction are mediated by parasite infection in wild Soay sheep. *Ecol. Lett.* **22**, 1203-1213. doi:10.1111/ele.13275
Lenth, R. V. (2017). emmeans: Estimated Marginal Means.
Les, H. L., Paitz, R. T. and Bowden, R. M. (2009). Living at extremes: development at the edges of viable temperature under constant and fluctuating conditions. *Physiol. Biochem. Zool.* **82**, 105-112. doi:10.1086/590263
Lindström, J. (1999). Early development and fitness in birds and mammals. *Trends Ecol. Evol.* **14**, 343-348. doi:10.1016/S0169-5347(99)01639-0
Lochmiller, R. L. and Deerenberg, C. (2000). Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**, 87-98. doi:10.1034/j.1600-0706.2000.880110.x
Love, O. P., Salvante, K. G., Dale, J. and Williams, T. D. (2008). Sex-specific variability in the immune system across life-history stages. *Am. Nat.* **172**, E99-E112. doi:10.1086/589521
Marshall, D. J., Pettersen, A. K. and Cameron, H. (2018). A global synthesis of offspring size variation, its eco-evolutionary causes and consequences. *Funct. Ecol.* **32**, 1436-1446. doi:10.1111/1365-2435.13099
Martínez-Padilla, J. (2006). Prelaying maternal condition modifies the association between egg mass and T cell-mediated immunity in kestrels. *Behav. Ecol. Sociobiol.* **60**, 510-515. doi:10.1007/s00265-006-0193-1
Massey, M. D. and Hutchings, J. A. (2020). Thermal variability during ectotherm egg incubation: a synthesis and framework. *J. Exp. Zool. Part A Ecol. Integr. Physiol.* **335**, 59-71. doi:10.1002/jez.2400
Massey, M. D., Holt, S. M., Brooks, R. J. and Rollinson, N. (2019). Measurement and modelling of primary sex ratios for species with temperature-dependent sex determination. *J. Exp. Biol.* **222**, jeb190215. doi:10.1242/jeb.190215
Møller, A. P. and Saino, N. (2004). Immune response and survival. *Oikos* **104**, 299-304. doi:10.1111/j.0030-1299.2004.12844.x
Niehaus, A. C., Angilletta, M. J., Sears, M. W., Franklin, C. E. and Wilson, R. S. (2012). Predicting the physiological performance of ectotherms in fluctuating thermal environments. *J. Exp. Biol.* **215**, 694-701. doi:10.1242/jeb.058032
Noble, D. W. A., Stenhouse, V. and Schwanz, L. E. (2018). Developmental temperatures and phenotypic plasticity in reptiles: a systematic review and meta-analysis. *Biol. Rev.* **93**, 72-97. doi:10.1111/brv.12333
Paitz, R. T., Clairardin, S. G., Griffin, A. M., Holgersson, M. C. N. and Bowden, R. M. (2010). Temperature fluctuations affect offspring sex but not morphological, behavioral, or immunological traits in the Northern Painted Turtle (Chrysemys picta). *Can. J. Zool.* **88**, 479-486. doi:10.1139/Z10-020
Palacios, M. G., Gangloff, E. J., Reding, D. M. and Bronikowski, A. M. (2020). Genetic background and thermal environment differentially influence the ontogeny of immune components during early life in an ectothermic vertebrate. *J. Anim. Ecol.* **89**, 1883-1894. doi:10.1111/1365-2656.13271
Pelayo, J. T. and Clark, R. G. (2002). Variation in size, composition, and quality of ruddy duck eggs and ducklings. *Condor* **104**, 457-462. doi:10.1093/condor/104.2.457
Rollinson, N. and Hutchings, J. A. (2013a). The relationship between offspring size and fitness: integrating theory and empiricism. *Ecology* **94**, 315-324. doi:10.1890/2-0552.1
Rollinson, N. and Hutchings, J. A. (2013b). Environmental quality predicts optimal egg size in the wild. *Am. Nat.* **182**, 76-90. doi:10.1086/670648
Rollinson, N. and Rowe, L. (2015). Persistent directional selection on body size and a resolution to the paradox of stasis. *Evolution* **69**, 2441-2451. doi:0.1111/evo.12753
Rollinson, N., Holt, S. M., Massey, M. D., Holt, R. C., Nancekivell, E. G. and Brooks, R. J. (2018). A new method of estimating thermal performance of embryonic development rate yields accurate prediction of embryonic age in wild reptile nests. *J. Therm. Biol.* **74**, 187-194. doi:10.1016/j.jtherbio.2018.03.008
Rubolini, D., Romano, M., Bonisoli Alquati, A. and Saino, N. (2006). Early maternal, genetic and environmental components of antioxidant protection, morphology and immunity of yellow-legged gull (Larus michahellis) chicks. *J. Evol. Biol.* **19**, 1571-1584. doi:10.1111/j.1420-9101.2006.01121.x
Ruuskanen, S., Siitari, H., Eeva, T., Belskii, E., Järvinen, A., Kerimov, A., Krams, I., Moreno, J., Morosinotto, C., Mänd, R. et al. (2011). Geographical variation in egg mass and egg content in a passerine bird. *PLoS ONE* **6**, e25360. doi:10.1371/journal.pone.0025360
Schneeberger, K., Courtiol, A., Czirják, G., Voigt, C. C. (2014). Immune profile predicts survival and reflects senescence in a small, long-lived mammal, the greater sac-winged bat (Saccopteryx bilineata). *PLoS ONE* **9**, e108268. doi:10.1371/journal.pone.0108268
Schwabl, H. (1993). Yolk is a source of maternal testosterone for developing birds. *Proc. Natl. Acad. Sci. USA* **90**, 11446-11450. doi:10.1073/pnas.90.24.11446
Schwabl, H. (1996). Maternal testosterone in the avian egg enhances postnatal growth. *Comp. Biochem. Physiol.* **114**, 271-276. doi:10.1016/0300-9629(96)00009-6
Schwanz, L. E., Cordero, G. A., Charnov, E. L. and Janzen, F. J. (2016). Sex-specific survival to maturity and the evolution of environmental sex determination. *Evolution* **70**, 329-341. doi:10.1111/evo.12856
Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**, 317-321. doi:10.1016/0169-5347(96)10039-2
Smith, C. C. and Fretwell, S. D. (1974). The optimal balance between size and number of offspring. *Am. Nat.* **108**, 499-506. doi:10.1086/282929
Steyermark, A. C., Finkler, M. S. and Brooks, R. J. (eds.). (2008). *Biology of the Snapping Turtle (Chelydra Serpentina)*. Baltimore: John Hopkins University Press.
Tschirren, B., Fitze, P. S. and Richner, H. (2003). Sexual dimorphism in susceptibility to parasites and cell-mediated immunity in great tit nestlings. *J. Anim. Ecol.* **72**, 839-845. doi:10.1046/j.1365-2656.2003.00755.x
Uller, T., Astheimer, L. and Olsson, M. (2007). Consequences of maternal yolk testosterone for offspring development and survival: experimental test in a lizard. *Funct. Ecol.* **21**, 544-551. doi:10.1111/j.1365-2435.2007.01264.x
Valenzuela, N. and Lance, V. (eds.). (2004). *Temperature-Dependent Sex Determination in Vertebrates*. Washington, DC: Smithsonian Books.
Warner, D. A. and Shine, R. (2005). The adaptive significance of temperature-dependent sex determination: experimental tests with a short-lived lizard. *Evolution* **59**, 2209-2221. doi:10.1554/05-085.1
Warner, D. A. and Shine, R. (2008). The adaptive significance of temperature-dependent sex determination in a reptile. *Nature* **451**, 566-568. doi:10.1038/nature06519
Warner, D. A., Uller, T. and Shine, R. (2009). Fitness effects of the timing of hatching may drive the evolution of temperature-dependent sex determination in short-lived lizards. *Evol. Ecol.* **23**, 281-294. doi:10.1007/s10682-007-9222-4

Warner, D. A., Mitchell, T. S., Bodensteiner, B. L. and Janzen, F. J. (2020). Sex and incubation temperature independently affect embryonic development and offspring size in a turtle with temperature-dependent sex determination. *Physiol. Biochem. Zool.* **93**, 62-74. doi:10.1086/706786

Watson, R. L., McNeilly, T. N., Watt, K. A., Pemberton, J. M., Pilkington, J. G., Waterfall, M., Hopper, P. R. T., Cooney, D., Zamoyska, R. and Nussey, D. H. (2016). Cellular and humoral immunity in a wild mammal: variation with age and sex and association with overwinter survival. *Ecol. Evol.* **6**, 8695-8705. doi:10.1002/ece3.2584

Webb, J. K., Brown, G. P. and Shine, R. (2001). Body size, locomotor speed and antipredator behaviour in a tropical snake (Tropidonophis mairii, Colubridae): the influence of incubation environments and genetic factors. *Funct. Ecol.* **15**, 561-568. doi:10.1046/j.0269-8463.2001.00570.x