Thermal Sensitivity of Immune Function: Evidence against a Generalist-Specialist Trade-Off among Endothermic and Ectothermic Vertebrates

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abstract: Animal body temperature (T_{body}) varies over daily and annual cycles, affecting multiple aspects of biological performance in both endothermic and ectothermic animals. Yet a comprehensive comparison of thermal performance among animals varying in T_{body} (mean and variance) and heat production is lacking. Thus, we examined the thermal sensitivity of immune function (a crucial fitness determinant) in Vertebrata, a group encompassing species of varying thermal biology. Specifically, we investigated temperature-related variation in two innate immune performance metrics, hemagglutination and hemolysis, for 13 species across all seven major vertebrate clades. Agglutination and lysis were temperature dependent and were more strongly related to the thermal biology of species (e.g., mean T_{body}) than to the phylogenetic relatedness of species, although these relationships were complex and frequently surprising (e.g., heterotherms did not exhibit broader thermal performance curves than homeotherms). Agglutination and lysis performance were positively correlated within species, except in taxa that produce squalamine, a steroidal antibiotic that does not lyse red blood cells. Interestingly, we found the antithesis of a generalist-specialist trade-off: species with broader temperature ranges of immune performance also had higher peak performance levels. In sum, we have uncovered thermal sensitivity of immune performance in both endotherms and ectotherms, highlighting the role that temperature and life history play in immune function across Vertebrata.

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Introduction

Environmental temperature profoundly influences many biological processes (e.g., metabolism, locomotion, and fecundity) in a wide range of taxa (reviewed in Angilletta 2006). Many organismal performance metrics exhibit an inverted-U-shaped relationship with temperature (i.e., a thermal performance curve [TPC]), with peak performance occurring over a limited temperature range (optimal temperature, or T_{opt}; reviewed in Angilletta 2006). Relationships between temperature and performance emerge largely as a result of temperature-dependent enzyme kinetics (Angilletta et al. 2002; Hochachka and Somero 2002; Angilletta 2009). Interspecific variation in TPCs is likely driven by interspecific variation in body temperature profiles (T_{body}; e.g., T_{opt} should approximate operating T_{body}) and by competing selection on other aspects of thermal performance. For example, selection for peak performance can be particularly strong for organisms maintaining a relatively constant T_{body} (homeotherms), whereas selection for performance breadth (e.g., B_{95}, the temperature range at which performance is ≥95% of peak) can be strong for organisms that experience significant variation in T_{body} (heterotherms). Because taxa use different combinations of isoenzymes that exhibit varying properties of thermal sensitivity (Hochachka and Somero 2002), a trade-off between TPC breadth and peak performance is predicted: temperature specialists exhibit TPCs

with small breadths and high peaks, and temperature generalists exhibit TPCs with large breadths and low peaks. Yet empirical support for the breadth-peak trade-off is equivocal—that is, an individual or taxon that is a jack-of-all-temperatures can also be a master of all temperatures (Gilchrist 1995; reviewed in Angilletta 2009). Angilletta (2009) summarizes the proximate mechanisms that can create and maintain a master-of-all-temperatures phenotype, including increased isoenzyme concentrations that can offset enzymatic inefficiency because of relatively poor stability at some temperatures.

Vertebrates offer a compelling system in which to examine selection on aspects of thermal performance because this clade contains species that vary widely with respect to thermal life history, including the magnitude of T_{body} (cool and warm bodied), the stability of T_{body} (heterotherms and homeotherms), and the capacity for endogenous heat production (ectotherms and endotherms). In contrast to other areas of ecology and evolution (e.g., Bonnet et al. 2002; Stahlschmidt 2011), work on thermal performance in vertebrates has been predominately focused on ectothermic animals. However, endotherms also exhibit variation in body temperature (e.g., McKechnie and Lovegrove 2002) and thermal sensitivity of performance (reviewed in Angilletta et al. 2010). Research on TPCs in endotherms is likely constrained by the logistics associated with measuring performance at different body temperatures. Yet TPCs of physiological parameters can be measured in vitro, which allows for the direct comparison of thermal performance between endotherms and ectotherms. Understanding the role that temperature plays in physiological performance is critical for addressing ecological phenomena such as range limits and habitat preferences (Pörtner et al. 2006; Hoffmann 2010) and applied questions such as responses to global climate change (Fields et al. 1993; Tewksbury et al. 2008; Chown et al. 2010).

Immune performance is a physiological trait that is vital to fitness (Graham et al. 2011); is sensitive to temperature in taxa including arthropods (Adamo and Lovett 2011), fish (Hung et al. 1997; Nikoskelainen et al. 2002; Jokinen et al. 2010), reptiles (Mondal and Rai 2001; Merchant et al. 2003, 2005), and mammals (Nikoskelainen et al. 2002); and contains elements that can be measured in vitro. Yet thermal performance of immune function has been relatively understudied (Angilletta 2009), and to our knowledge, detailed comparative examinations of TPCs of immune function are nonexistent.

We compared TPCs of immune function in 13 phylogenetically and biologically diverse vertebrate species to assess how thermal life-history characteristics (e.g., endo- vs. ectothermy, homeo- vs. heterothermy, low vs. high T_{body}, and seasonal temperature variation) influence thermal performance of immune function. Vertebrates possess a highly complex and integrated immune system, with multiple pathways available to combat pathogens and parasites (Manning 1979). While there is significant variation within Vertebrata regarding mechanisms of both innate and acquired immune function (Kimbrell and Beutler 2001; Fujita 2002), the innate immune system is an ideal candidate for testing interspecific differences in immunity because it (1) does not evaluate a single, antigen-specific in vivo response in taxa that have had differential exposures to antigens during their evolutionary histories, (2) is more evolutionarily basal than the acquired component, and (3) is the chief mechanism of host defense for most taxa (Litman et al. 2005). Two aspects of the innate immune system that reflect organismal and life-history variation across vertebrate taxa are natural antibody function and lytic capacity (fish: Magnadóttir 2006; reptiles: Merchant et al. 2005; birds: Matson et al. 2005; mammals: Nikoskelainen et al. 2002). Natural antibodies (NAbs) are cross-reacting immunoglobulins, usually IgM (Matson et al. 2005), that are produced before antigen exposure and play an important role in immune defense via agglutination (Magnadóttir 2006). Natural antibodies also activate complement, which consists of plasma proteins involved in signaling cascades that can cause the lysis of foreign cells (Janeway et al. 2001; Matson et al. 2005). The effectiveness of antibody-complement interactions in lysing cells is heavily dependent on temperature, at least in mammals, with IgM ineffective at a very low temperature (4°C; Frank and Gaither 1970).

Because of the potential links between immune function and thermal life history in both ectotherms and endotherms, we measured TPCs of two metrics of innate immune function of blood plasma—agglutination and lytic capacity—in multiple vertebrate species to test three main hypotheses. First, we tested whether innate immune function of vertebrates adopts a TPC sensu those for other biological processes. On the basis of this hypothesis, we predicted that immune function would exhibit an inverted-U-shaped response to temperature. Second, we tested whether thermal life history influences thermal sensitivity of innate immune function. We predicted that species-specific thermal life-history characteristics would influence thermal performance (e.g., that species with higher T_{body} would perform better at higher temperatures regardless of their level of endogenous heat production). Further, we predicted that heterotherms would tend to be temperature generalists and thus would exhibit both greater performance breadths and lower peak performances relative to homeotherms (i.e., that a generalist-specialist trade-off exists). Related to this, we predicted that TPCs of immune function would be more labile (dependent on species-specific magnitude and variance in T_{body}) than con-

served (dependent on phylogeny) because of the potentially strong selection on the immune system and the length of time since the divergence of most vertebrate species in our analyses (>100 million years ago; fig. 1). Third, we tested whether TPCs for agglutination and lytic capacity show similar temperature sensitivity because of similar selection pressures nested within each species' thermal life history. On the basis of this hypothesis, we predicted that TPC traits for agglutination and lysis would be correlated (e.g., that there would be a significant positive correlation between T_{opt} for agglutination and T_{opt} for lysis).

Methods

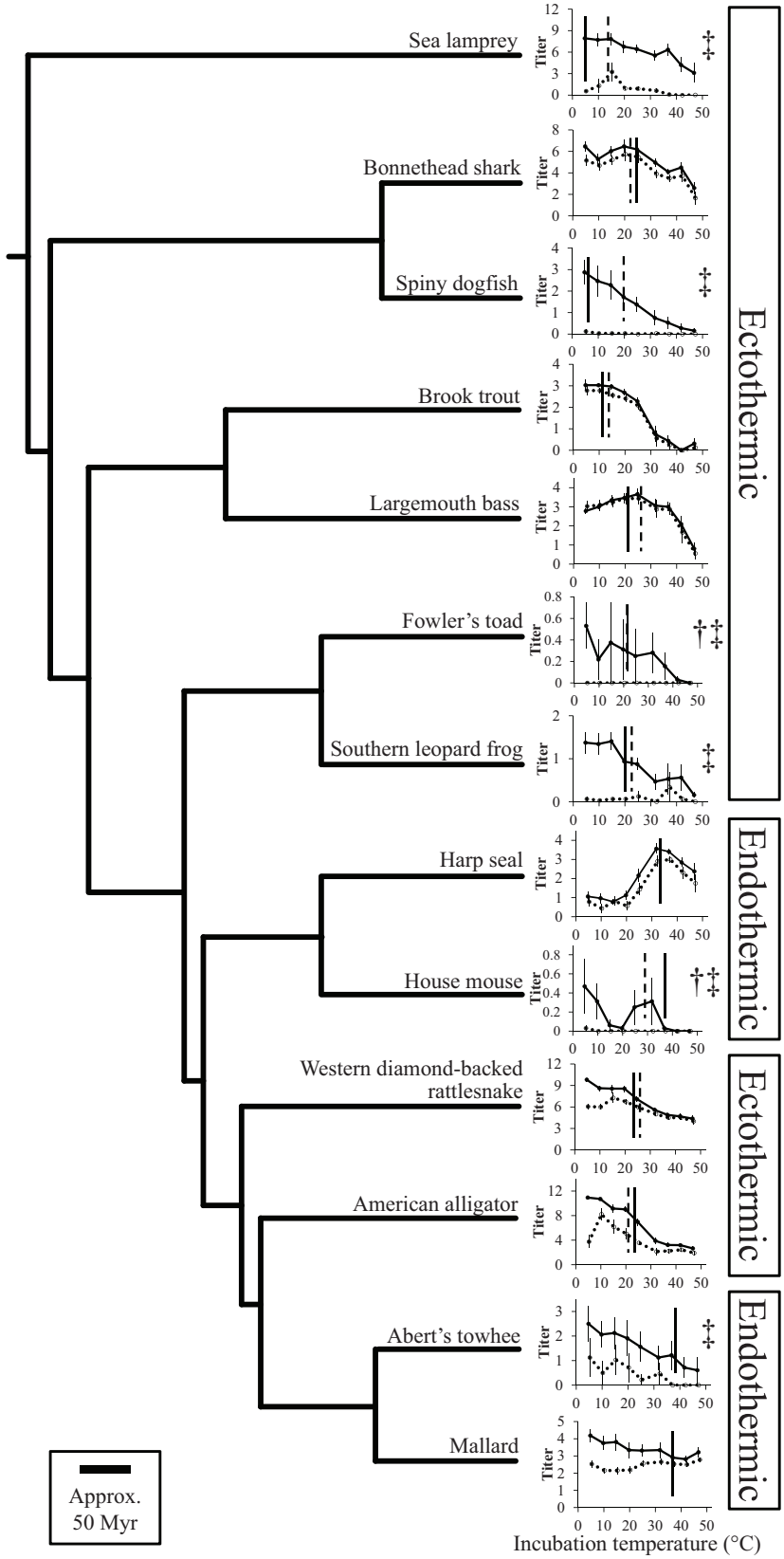
Study Species

We acquired plasma samples from eight individuals of each of 13 species spanning the seven vertebrate classes (fig. 1): (1) sea lamprey (*Petromyzon marinus*; land-locked spawning phase), superclass Agnatha; (2) spiny dogfish (*Squalus acanthias*), class Chondrichthyes; (3) bonnethead shark (*Sphyrna tiburo*), class Chondrichthyes; (4) largemouth bass (*Micropterus salmoides*), class Osteichthyes; (5) brook trout (*Salvelinus fontinalis*), class Osteichthyes; (6) southern leopard frog (*Lithobates sphenocephalus*), class Amphibia: (7) Fowler's toad (*Anaxyrus fowleri*), class Amphibia; (8) western diamond-backed rattlesnake (*Crotalus atrox*), class Reptilia; (9) American alligator (*Alligator mississippiensis*), class Reptilia; (10) Abert's towhee (*Melozone aberti*), class Aves; (11) mallard duck (*Anas platyrhynchos*), class Aves; (12) house mouse (*Mus musculus*), class Mammalia; and (13) harp seal (*Pagophilus groenlandica*), class Mammalia. Because of small plasma volumes, each of the eight samples for both amphibian species was a pooled sample from multiple individuals. All samples were stored at −20°C for a maximum of 2 weeks and at −80°C thereafter. See table A1, available online, for details about individuals, blood collection, plasma isolation and storage, and animal protocol information.

Innate-Immunity Assays

Natural antibodies are primarily involved in antigen recognition and are cross reactive with a variety of antigenic types, leading directly to agglutination (Cotter et al. 2005). By contrast, complement activity reflects a triggered enzymatic cascade that results in target cell lysis (Matson et al. 2005). Because NAbs can bind directly to mammalian red blood cells (RBCs) and complement activation can lyse these same cells, investigators have developed an assay that can assess both agglutination and lytic capacity in vitro. This hemoagglutination-hemolysis assay does not require any species-specific antibodies, and it is recommended for comparing innate immunity among species (Matson et al. 2005). Prior work with teleost fish (Magnadóttir et al. 1999; Nikoskelainen et al. 2002), alligators (Merchant et al. 2005), and humans (Nikoskelainen et al. 2002) has already established that these components of the immune system are temperature sensitive, although TPCs were not explicitly calculated. Understanding the thermal sensitivity of innate immunity is critical and underappreciated, as avian biologists using the hemolysis-hemagglutination assay (nearly 140 species; ISI Web of Science, accessed April 2012) typically incubate plasma at 37°C, which is approximately 4°C lower than the T_{body} of most birds (Clarke and Rothery 2008).

To assess NAb agglutination and lysis in plasma, we modified a previously described protocol (Matson et al. 2005) that quantifies the most dilute concentration of plasma that can still generate an agglutination or lytic response to foreign red blood cells. We serially diluted 20 μL of each plasma sample from 1 : 2 to 1 : 2,048 with phosphate-buffered saline (PBS) along a row of a 96-well plate and added only PBS to the final well in the row as a negative control. To each well, we then added 20 μL of 50% heparinized whole sheep blood diluted 1 : 100 (HemoStat Laboratories, Dixon, CA; SBH050). We gently vortexed each plate, covered the plates with parafilm, and incubated them for 90 min at 5°, 10°, 15°, 20°, 25°, 32°, 37°, 42°, or 47°C (±0.5°C at all temperatures) in a modified 150-L incubator controlled by a datalogger (21X; Campbell Scientific Instruments, Logan, UT), with plasma from each individual represented at each temperature to control for individual immunological history across all temperatures. We chose this range of temperatures because it provided a reasonable breadth of body temperatures experienced by our study species (table A2, available online). We then tilted the plates for 20 min at room temperature and scanned them with a flatbed scanner (Hewlett-Packard ScanJet 3670) at 600 dots per inch to measure agglutination. We then left plates flat at room temperature for 70 min and rescanned them to measure for lysis (see Matson et al. 2005 for scoring procedures). Each plasma sample was present on nine plates (one at each incubation temperature), but we randomly assigned samples to each plate so that species was not nested within plate. Both M. W. Butler and Z. R. Stahlschmidt independently scored the plates blind to sample identity, and their scores were repeatable (Lessells and Boag 1987) for both agglutination ($F_{933,934} = 209.2$, $R = 0.99$, $P < .0001$) and lysis ($F_{933,934} = 185.95$, $R = 0.99$, $P < .0001$). Although NAbs are not specific (Cotter et al. 2005), this assay is predicated on the assumption that sheep RBCs act as a novel antigen when stimulating hemagglutination (Matson et al. 2005). Thus, previous exposure to sheep RBCs might lead to


Sea lamprey
Bonnethead shark
Spiny dogfish
Brook trout
Largemouth bass
Fowler's toad
Southern leopard frog
Harp seal
House mouse
Western diamond-backed rattlesnake
American alligator
Abert's towhee
Mallard
Ectothermic
Endothermic
Ectothermic
Endothermic
Titer
Incubation temperature (°C)
Approx. 50 Myr

production of memory cells or cause an immunoglobulin shift, which could cause either increased or decreased responses. Most often, dietary exposure to RBC antigens results in oral tolerance, or a reduction in immune activity following digestive exposure to antigens (André et al. 1975). However, none of the species used in our study consumed sheep products (table A3, available online), and thus oral tolerance did not affect our results.

Thermal Performance Curves (TPCs)

Optimal methods for estimating and analyzing TPCs have long been a source of discussion among thermal biologists (reviewed in Angilletta 2006). Thus, we analyzed our data in multiple ways to best evaluate their biological and statistical significance. First, we used both ANOVAs and nonlinear function fitting to estimate and characterize TPCs. We used repeated-measures ANOVAs to determine whether a given species' immune performance (agglutination or lytic capacity) varied by incubation temperature, utilizing the Greenhouse-Geisser (G-G) adjusted P values if the G-G epsilon was less than 0.7 (table A4, available online). We omitted species that did not exhibit temperature dependence (e.g., agglutination and lysis in the house mouse; fig. 1) from this first stage of TPC analysis. Separately, we used graphing software (GraphPad Prism, ver. 5; GraphPad Software, La Jolla, CA) to determine the polynomial or Gaussian function that best fitted each individuals' data set (i.e., the function with the lowest Akaike's information criterion; Angilletta 2006). We used these best-fit functions to determine the following metrics for agglutination and lysis from each individual: peak performance (highest titer level), optimal temperature (T_{opt}), and 95% performance breadth (B_{95}). The data from some individuals were not amenable to function fitting (e.g., agglutination in several spiny dogfish; fig. 1). In these cases, we used the peak performance and T_{opt} values from the raw data obtained in our study, with mean values taken in the event of ties (e.g., if titer peaked at both 5° and 10°C, T_{opt} was determined to be 7.5°C), although B_{95} values could not be obtained in these cases. See table A5, available online, for details about each species' TPC.

In addition, we used principal-components analysis to generate principal-component (PC) scores for each individual's TPC for agglutination and lysis. Using the agglutination scores across all nine temperatures for all individuals, we found eigenvalues for PC1 of 7.51 and for PC2 of 0.87, explaining 83%, and 9.7% of the variation, respectively. For lysis, the eigenvalues were 6.96 for PC1 and 0.83 for PC2, explaining 77% and 9.3% of the variation, respectively. We elected to use the first two PCs because all subsequent PCs explained less than 4% of the variation in the data. Generally, PC1 for both agglutination and lysis loaded positively with the overall magnitude of the response, whereas PC2 included information about whether the individual performed better at warmer or cooler temperatures (fig. A1, available online).

Thermal Life History

To determine whether agglutination and lytic capacity were optimized at species' operating T_{body} (magnitude and variance) and/or at T_{opt} for other performance metrics (e.g., locomotor ability), we searched the literature for available information on each species' thermal life history (table A2) or, when feasible, collected T_{body} measurements directly from the individuals used in our study. Nearly all of the species in our study exhibit temporal variation in T_{body}. Thus, we determined the amplitude with which T_{body} varied on daily and annual timescales. We also used this information to estimate the T_{body} for each species at the time it was sampled in our study. For example, the American alligator exhibits a T_{body} annual amplitude of 12°C, and its T_{body} varies depending on time of year (Seebacher et al. 2003). Because the plasma from American alligators in our study was collected in May, we estimated T_{body} more precisely (specifically, 24°C, from Seebacher et al. 2003). For some species, T_{body} annual amplitude was unknown, so we used T_{body} daily amplitude to conservatively estimate T_{body} annual amplitude. We also used available information in the literature to determine most species' mean T_{opt} for other performance metrics, such as growth rate, locomotion, and enzymatic activity (table A2).

Phylogenetically Controlled Comparisons

We used two comparative phylogenetic approaches to test relationships between thermal variables of interest (e.g., body temperature and optimal temperature for agglutination or lytic capacity) while accounting for phylogenetic

Figure 1: Phylogeny and thermal performance of innate immune function in 13 vertebrate species, including four endothermic species and nine ectothermic species. Solid lines in each species' panel denote agglutination capacity, and dotted lines denote lytic capacity. Values are displayed as mean ± SEM. Vertical lines in each panel represent the mean body temperature (solid) and the mean temperature at which another performance metric (e.g., growth rate) is optimized (dashed) for each species. Each panel has the same *X*-axis scale, but the *Y*-axis scale varies among species to best illustrate the temperature dependence of innate immune function of each species. Daggers (†) and double daggers (‡) symbolize species that did not exhibit temperature dependence with regard to agglutination and lytic capacity, respectively.

relationships among species. A comparative phylogenetic approach is necessary when analyzing data compiled from multiple species because species are inherently related to one another and thus their data are typically neither biologically nor statistically independent (Felsenstein 1985; Garland et al. 1992, 2005). First, we used phylogenetically independent contrasts (PICs) to correct for phylogenetic dependence. PIC is a robust method that uses information about phylogenetic relationships among species and the differences in a given trait value (e.g., mean body temperature) between nodes and/or sister species to create standardized contrasts, which are independent and amenable to statistical comparisons (e.g., regression analyses; Felsenstein 1985; Garland et al. 1992).

Second, we used the phylogenetically corrected generalized least squares (PGLS) method, which is a more recently developed method to control for the effects of phylogeny (Grafen 1989; Martins and Hansen 1997; Freckleton et al. 2002). Unlike the PIC method, the PGLS method tests for the relationships between original trait values rather than between contrasts. The PGLS method controls for phylogeny by accounting for any phylogenetic autocorrelation of the data in the error structure (Martins and Hansen 1997; Freckleton et al. 2002). PGLS accomplishes this by employing the maximum likelihood estimation of a parameter of phylogenetic dependence (alpha) to control for the dependence on phylogeny. If alpha is high, data are relatively independent of phylogeny, whereas a low alpha (near 0) indicates that data are strongly dependent on phylogeny. Both PIC and PGLS methods assume a Brownian model of trait evolution to describe the expected interspecific covariance of traits, but the PGLS method is particularly flexible in the model specification (Grafen 1989; Martins and Hansen 1997; Freckleton et al. 2002; Freckleton 2009).

To determine phylogenetic relationships (topology and branch lengths) among the species in our study, we generated a phylogenetic tree (fig. 1), using a web-based program (Interactive Tree of Life; Letunic and Bork 2007, 2011). We generated our tree using interspecific variation in mitochondrial genes (vertebrate mitochondrial code), determined through the National Center for Biotechnology Information database.

Statistical Analyses

To determine relationships between thermal variables of interest (e.g., body temperature and optimal temperature for agglutination or lytic capacity; see fig. 2 for specific variables) across our study taxa, we used COMPARE software (ver. 4.6b; open-access distribution by E. P. Martins, Bloomington, IN) to perform linear regression analyses on both raw data (not corrected for phylogeny) and phylogenetically controlled data using both PIC and PGLS methods, as well as to determine the maximum likelihood estimate of alpha for each pair of variables. Although we performed regression analyses, we inferred correlation between variables, not causation. We also performed parametric statistics without controlling for phylogeny, because of the large degree of phylogenetic separation among our study species (e.g., no two species belonged to the same order, except the Amphibia, which had species from different families). Specifically, we performed a series of mixed models in SAS (PROC MIXED; ver. 9.2, SAS Institute, Cary, NC) to test whether the TPC traits for lysis and agglutination (peak, T_{opt}, B_{95}, PC1, and PC2) differed between endotherms and ectotherms, with species as random factor. Using another suite of mixed models, we tested whether TPC traits were predicted by thermal life-history characteristics (e.g., T_{body} mean and daily and annual amplitude; T_{opt} in other performance metrics), again using species as a random factor.

Results

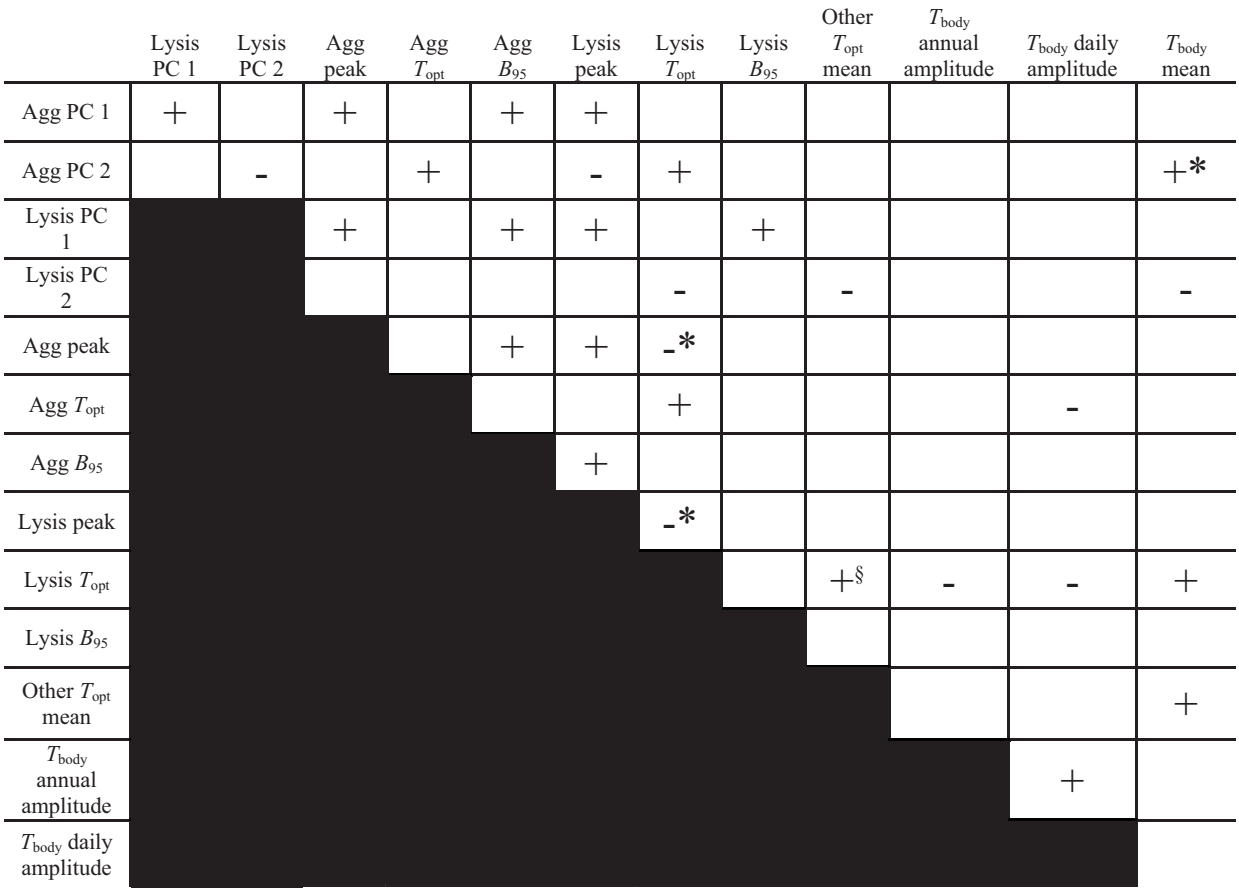
Temperature Dependence of Immune Capacity

Incubation temperature influenced agglutination capacity of blood plasma in 85% of species (11 of 13), whereas it influenced lytic capacity of plasma in only 54% of species (7 of 13; fig. 1; table A5). Although temperature dependent, agglutination capacity in some species was a negative linear function of temperature rather than the inverted-U function that is typical of TPCs (fig. 1).

Relationships between TPC Traits

Within the agglutination metrics for all individuals and species, overall immune performance (PC1) and peak immune performance (maximum titer) were positively correlated. In addition, species with the highest performance had the widest thermal breadth of performance (B_{95}; figs. 2, 3*c*). Within the lysis metrics, we found the same patterns, with PC1 positively correlated with both peak performance and B_{95}, indicating that species with the highest immune performance had the greatest thermal breadth. As expected, species with increased agglutination performance at warmer temperatures (agglutination PC2) had higher agglutination T_{opt} (fig. 2). The same was true for lysis, as species with higher lysis performance at cooler temperatures (lysis PC2) had a lower lysis T_{opt} (fig. 2). There was a link between performance and T_{opt}, but only in lysis; lysis peak performance was higher in species with a lower lysis T_{opt}(PIC method only; fig. 2).

There was a link between performance in agglutination and performance in lysis, as species with high levels of



Agg PC 1: First principal component for agglutination capacity

Agg PC 2: Second principal component for agglutination capacity; greater responses at warm temperatures

Lysis PC 1: First principal component for lytic capacity

Lysis PC 2: Second principal component for lytic capacity; greater responses at cooler temperatures

Agg peak: Peak level of agglutination capacity

Agg T_{opt}: Temperature at which agglutination capacity peaks

Agg B_{95}: Temperature breadth over which agglutination capacity is ≥95% of peak level

Lysis peak: Peak level of lytic capacity

Lysis T_{opt}: Temperature at which lytic capacity peaks

Lysis B_{95}: Temperature breadth over which lytic capacity is ≥95% of peak level

Other T_{opt} mean: Mean temperature at which another performance metric (e.g., growth rate) is optimal

T_{body} annual amplitude: Annual fluctuation in body temperature

T_{body} daily amplitude: Daily fluctuation in body temperature

T_{body} mean: Mean body temperature

Figure 2: Relationships between immune characteristics in 13 vertebrate species. Significant relationships between variables are denoted by a plus sign for a positive relationship and a minus sign for a negative relationship. We performed linear regression analyses on both raw data (not corrected for phylogeny) and phylogenetically controlled data (phylogenetically independent contrasts [PIC] and phylogenetically corrected generalized least squares [PGLS] methods; see text for details). We also ran mixed-model analyses, with species as a random factor, on the subset of variables for which dependent and independent variables made biological sense; that is, the four rightmost variables in the top row served as independent variables, and the 10 metrics of agglutination and lysis served as dependent variables. Relationships were similar for all four methods except those with an asterisk, which denotes significance for the PIC method only, and those with a section sign (§), which denotes significance for mixed-model analyses only. Significance was determined at $P < .05$.

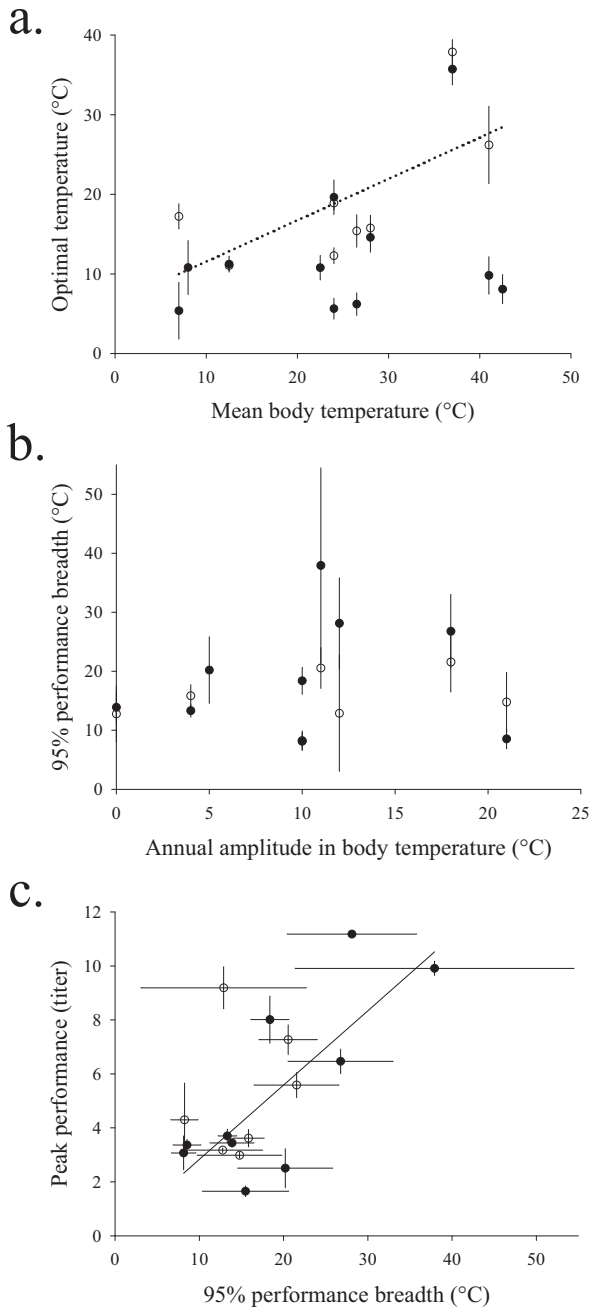


Figure 3: Relationships among thermal life history and innate immune function (agglutination and lysis): *a*, mean body temperature and optimal temperature for immune performance; *b*, annual amplitude in body temperature and 95% performance breadth (B_{95}; temperature range at which performance is ≥95% of maximal) of immune function; and *c*, B_{95} and peak performance of immune function. Filled symbols represent agglutination, and open symbols represent lysis. Significant relationships are represented by regression lines (solid for agglutination, dotted for lysis), and all values are displayed as mean ± SEM. See text for results of phylogenetically controlled analyses. If a species' lysis or agglutination did not vary with temperature or exhibit a particular thermal performance curve trait (see fig. 1), it was excluded.

agglutination activity (agglutination PC1) had high levels of lysis activity (lysis PC1 and lysis peak; figs. 2, 4*a*). This agglutination-lysis link persisted in a temperature-related manner via a positive correlation between agglutination T_{opt} and lysis $T_{opt.}$ (figs. 2, 4*b*). In addition, agglutination PC2 (performance at high temperatures) and lysis PC2 (performance at low temperatures) were negatively correlated, indicating that species with high performance at higher temperatures in one immune metric also had high performance in the other metric at similar temperatures. Similarly, lysis T_{opt} was positively correlated with agglutination PC2, linking lytic capacity at high temperatures to warm-biased agglutination performance. However, the negative correlation between lysis peak and agglutination activity at high temperatures (PC2) indicates that species with the highest lysis performance had weak agglutination activity at warm temperatures. Interestingly, there was no significant relationship between agglutination B_{95} and lysis B_{95} (figs. 2, 4*c*), although lysis peak performance was positively correlated with agglutination B_{95} (fig. 2). Both types of TPC analysis (PC analysis and traditional methods) yielded similar results (e.g., that PC1 was related to peak while PC2 was related to T_{opt}; fig. 2).

Relationships between TPC Traits and Thermal Life History

Mean T_{body} of species was positively correlated with agglutination capacity at warmer temperatures (PC2; PIC method only), with T_{opt} for lysis (fig. 3*a*), and with T_{opt} for other performance metrics (fig. 2). Mean T_{body} was negatively correlated with peak lysis performance at low temperatures (PC2; fig. 2). Species with greater daily fluctuations in body temperature had their peak immune performance at lower temperatures, as daily amplitude in T_{body} was negatively correlated with T_{opt} for both agglutination and lysis and positively correlated with annual amplitude in T_{body} (fig. 2). Optimal temperature for other performance metrics was positively correlated with lysis T_{opt} (mixed-model method only) and negatively correlated with lytic capacity at lower temperatures (PC2; fig. 2). There was less of a relationship between annual amplitude in T_{body} and immune performance metrics, as it was negatively correlated with T_{opt} for lysis, but not with B_{95} (fig. 3*b*) or other variables (fig. 2).

There were few differences between endotherms and ectotherms in thermal performance of innate immunity. Endotherms and ectotherms did not differ in peak response, T_{opt}, or B_{95} for agglutination and did not differ in peak response or B_{95} for lysis (fig. 5). Overall agglutination and lytic capacity (PC1) did not differ between endotherms and ectotherms (all $P > .17$). However, endotherms possessed higher agglutination capacity at warmer tem-

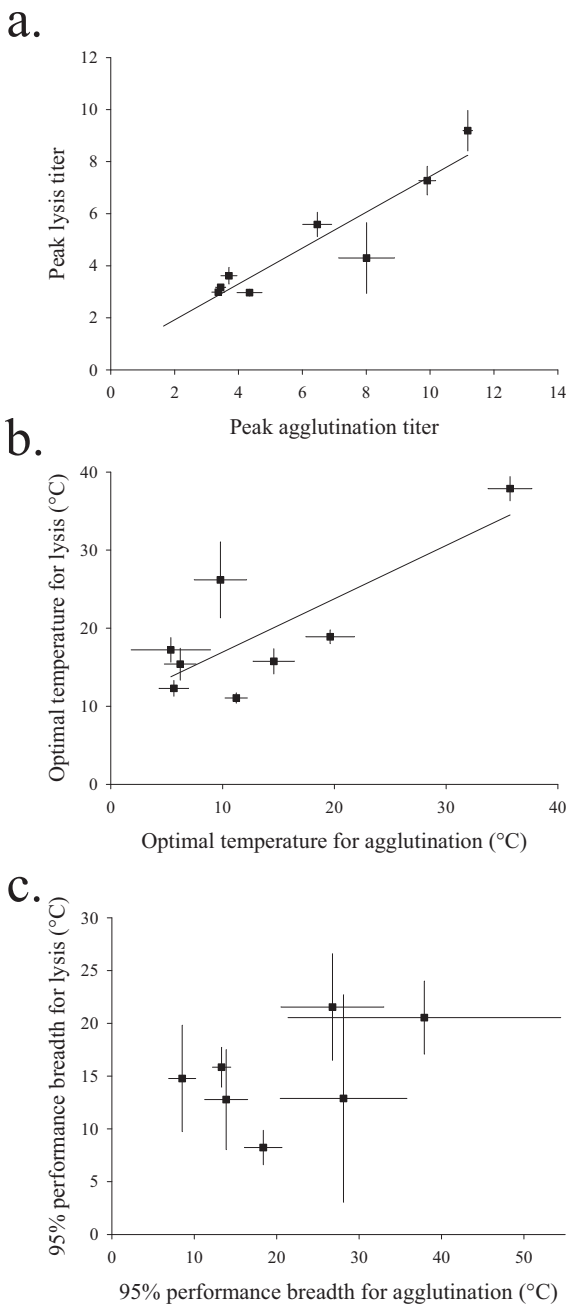


Figure 4: Relationships between innate immune parameters (agglutination and lysis): *a*, peak performance; *b*, optimal temperature; and *c*, 95% performance breadth (B_{95}; temperature range at which performance is ≥95% of maximal) of immune function. Significant relationships are represented by regression lines, and all values are displayed as mean ± SEM. See text for results of phylogenetically controlled analyses. If a species' lysis or agglutination did not vary with temperature or exhibit a particular thermal performance curve trait (see fig. 1), it was excluded.

peratures (PC2; $F_{1,75} = 4.04$, $P = .048$) and higher T_{opt} for lysis ($F_{1,49} = 20.18$, $P < .0001$, fig. 5*b*). In addition, lytic capacity at cooler temperatures (PC2) was lower in endotherms ($F_{1,49} = 12.32$, $P = .0010$).

Aspects of thermal life history and metrics of TPCs for innate immune function were generally independent of phylogeny. Maximum likelihood estimates of alpha from regression analyses were typically quite high, indicating a weak effect of phylogeny. Specifically, alpha was greater than 2 in 86% of significant relationships, and it was greater than 1 in 96% of significant relationships (fig. 2). Further, results from phylogenetically controlled analyses (PIC and PGLS regression) and noncontrolled analyses (raw regression and mixed model) were broadly similar, and discrepancies were not explained by the level of phylogenetic control (e.g., there were no incidences of finding a significant effect with raw and mixed-model methods alone; fig. 2).

Discussion

These data demonstrate that temperature-dependent immune function is related to species-specific variation in thermal biology for species representing all major vertebrate taxa. Interspecific variation was relatively independent of phylogeny, suggesting a lack of phylogenetic constraint on immunological adaptations to diverse thermal environments. Furthermore, the few differences in immune performance that existed between endotherms and ectotherms were linked to broad differences in temperature sensitivity (e.g., PC2 for both agglutination and lysis), and thermal performance of immune function was more clearly explained by differences in species' mean T_{body} and daily amplitude in T_{body}. Thus, the TPCs of endotherms do not appear to be intrinsically different from those of ectotherms. We also demonstrate a master-of-all-temperatures paradigm (in contrast to a generalist-specialist trade-off) with regard to immune performance, as species with broader temperature ranges of immune performance also had higher peak levels of performance.

We first hypothesized that the innate immune response, like many other aspects of animal performance, would exhibit a TPC, reflecting decreased performance below and above an intermediate T_{opt}. Our data partially supported this hypothesis, because all but two of the 13 species showed thermal dependence in agglutination and all but six showed thermal dependence in lysis. However, even in those species that exhibited thermal dependence of agglutination and lysis, only a subset displayed the classical shape of a TPC. Interestingly, in those species for which immune performance was temperature independent, there was a strong tendency to be low responders overall, rarely achieving an agglutination or lysis titer greater than 1 (fig.

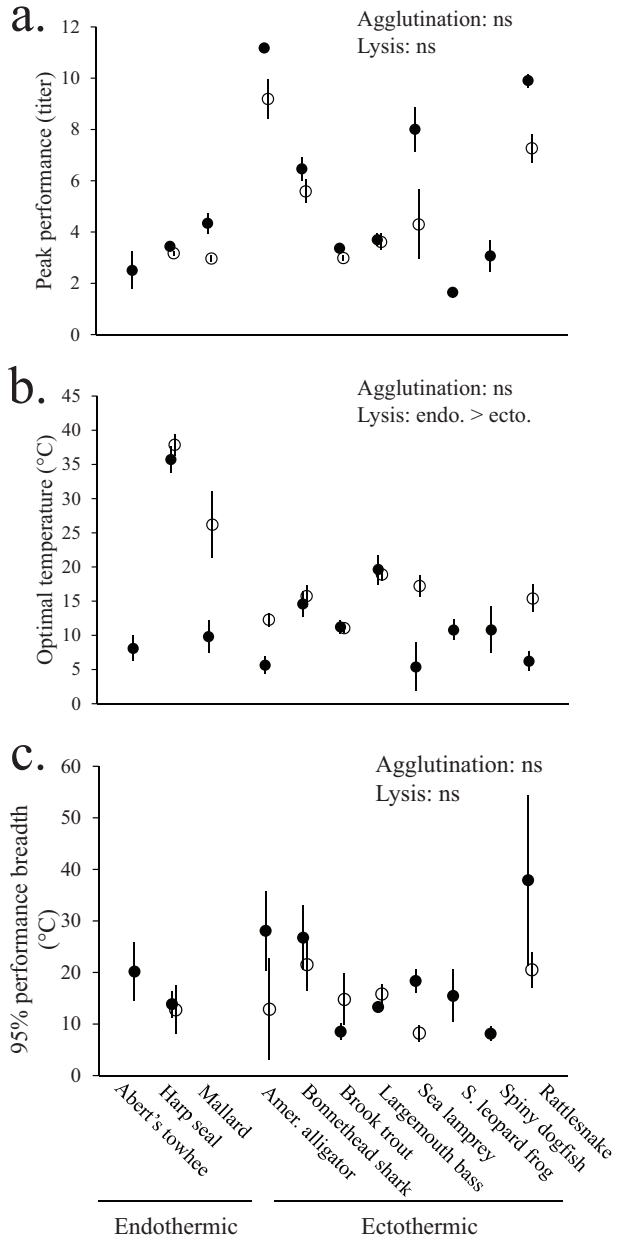


Figure 5: Variation in innate immune function among endothermic and ectothermic species: *a*, peak performance; *b*, optimal temperature; and *c*, 95% performance breadth (B_{95}; temperature range at which performance is ≥95% of maximal) of immune function. Filled symbols represent agglutination, and open symbols represent lysis. All values are displayed as mean ± SEM. If a species' lysis or agglutination did not vary with temperature or exhibit a particular thermal performance curve trait (see fig. 1), it was excluded. endo. = function in endotherms; ecto. = function in ectotherms; ns = not significant.

1). For example, the titers of the Fowler's toad and house mouse never exceeded 1, and these species did not exhibit temperature dependence for either lysis or agglutination. In all cases except lytic ability of plasma in amphibians, the sister taxa of low responders demonstrated thermal sensitivity, indicating that the lack of temperature-sensitive immune performance was not necessarily the ancestral condition. In a similar pattern, variation in performance was likely not due to how closely related taxa were to the species from which the antigen was derived (sheep) in our assays, because performance within several sister-taxa pairs varied considerably (e.g., within sharks or birds; fig. 1), including the sister pair most closely related to sheep (i.e., mammals; fig. 1).

Interestingly, not all thermal responses were in the shape of an inverted U (fig. 1; table A5). Rather, most species, including at least one member of each of the seven major vertebrate taxa, showed general downward trends, with peak immune performance at colder temperatures (e.g., agglutination in sea lamprey, the spiny dogfish, and both nonavian reptiles). Some trends were difficult to interpret, as mallard ducks seemingly displayed a noninverted U, with the trough near the species' mean T_{body} (fig. 1). The overall pattern of decreasing performance at higher temperatures could simply reflect a generally superior ability of these innate immune metrics to function more effectively at colder temperatures. The increased performance of certain enzymes at colder temperatures due to conformational variation could provide an adaptive explanation based essentially on variation in protein tertiary structure (Fields 2001). In addition, low-temperature-biased agglutination and lysis could simply reflect the complexity and integration of the vertebrate immune system. Specifically, incidences of low agglutination or lysis (e.g., at warmer temperatures in most species and at all temperatures in the mouse and toad) could be balanced by the high performance of other aspects of immune function (e.g., acquired immunity or antimicrobial peptides on the skin of amphibians). In accordance with this idea, innate immune function in fish is utilized more at lower temperatures, whereas acquired immunity is relied on at higher temperatures (Magnadóttir 2006). However, specific components of immunity may vary in temperature dependence. For example, fever is part of the innate immune response, and our results generally suggest poorer performance of two innate-immunity metrics at elevated T_{body}, yet cell-mediated innate immunity, which is associated with fever, was not measured in our study and may respond differently to temperature. Alternatively, because the immune system is a tightly coordinated network, poor immune performance at higher temperatures in our assays may reflect the increased activity of inhibiting enzymes, such

as proteases associated with immune regulation, at higher temperatures (e.g., Siroski et al. 2011).

Our second hypothesis stated that the shape of TPCs would be dependent on species' thermal life histories and that heterothermic ectotherms would tend to be temperature generalists relative to homeothermic endotherms. We found only partial support for this hypothesis, as we failed to detect a negative relationship between peak and breadth of immune performance (B_{95}) for either lysis or agglutination, suggesting that the traditional specialist-generalist trade-off does not apply to these two measures of innate immune function. In fact, we found more support for a master-of-all-temperatures paradigm, because sometimes performance and breadth were positively correlated (e.g., a positive relationship between peak performance and B_{95} for agglutination; figs. 2, 3*c*). Thus, species that were high responders for both agglutination and lysis were also more likely to have a broader TPC. Such findings are not uncommon in TPC studies, with several examples of high-performing individuals also having a broader curve (reviewed in Angilleta 2009). Given the complexity of the immune system, it is possible that selection can hone investment in immunity in each species to fit a particular set of environmental and pathogen conditions, rather than through constraints on possible phenotypic combinations (Ardia et al. 2011). Therefore, selection could favor utilization of different immune performance metrics at different body temperatures (Magnadóttir 2006), rather than through specialist or generalist temperature profiles within a single component of immunity.

We also did not find evidence that heterotherms generally exhibited greater performance breadth or that homeotherms exhibited greater peak performance, a prediction of theoretical models that a reduced range of operating temperatures should be linked with higher performance under specialist-generalist models. Specifically, species with broad daily or annual amplitude in T_{body} did not exhibit large thermal breadths (B_{95}) for either agglutination or lysis (figs. 2, 3*b*), and there was no evidence of higher performance in homeotherms (fig. 2). However, we did find multiple threads of evidence linking the temperature of peak performance (T_{opt}) and each species' thermal environment; species with large variation in T_{body} (heterothermic species) tended to have low T_{opt} for both agglutination and lysis (fig. 2), and performance tended to be optimized at the operating temperatures of each species (i.e., lysis T_{opt} was positively correlated with mean T_{body}; fig. 3*a*). Taken together, these data suggest that innate immune performance (particularly lytic capacity) reflects species-specific thermal environments, with ectotherms (variable and generally cooler T_{body}) able to optimally lyse foreign cells at cooler temperatures, especially relative to endotherms. We also found that endotherms and ectotherms differed somewhat in agglutination and lytic capacity, with endotherms possessing warm-biased agglutination and lysis responses (PC2), and that lysis T_{opt} was higher in endotherms. Together, these results support the hypothesis that TPC shape reflects species' thermal life histories, as endotherms generally have an elevated T_{body} relative to ectotherms. Given the independent evolution of endothermy in birds and mammals, this result is evidence of the labile nature of thermal performance of innate immunity.

In support of our second hypothesis, thermal performance of innate immunity in vertebrates appears to be relatively labile, because we did not uncover a strong phylogenetic signal despite the large evolutionary differences among taxa. Our range of statistical approaches yielded the same results 97% of the time, although some tests controlled for phylogeny and others did not. For all analyses using PGLS regressions, we also determined alpha levels to determine the extent to which variation in agglutination and lysis metrics were phylogenetically independent. Because alpha levels were typically very high, our immunological results cannot be explained through conserved evolutionary history of thermal dependence. Therefore, the link between T_{body} (mean and variance) and T_{opt} for lysis and agglutination (figs. 2, 3*a*) suggests that some aspect of the species' life history, including thermal environment or ecological niche, is more important to determining aspects of innate immunity than is evolutionary relatedness, at least on the broad scale that we examined.

Our third hypothesis stated that the thermal performances of agglutination and lysis would be similar. In support of this hypothesis, we uncovered positive correlations between overall agglutination and lysis performance (PC1s) and their peak responses (figs. 2, 4*a*). We also demonstrated positive correlations between their thermal sensitivities (T_{opt}), but not their thermal breadths (B_{95}; figs. 2, 4*b*, 4*c*). This positive relationship between agglutination and lysis was frequently detected, consistent with their having a mechanistic link; that is, lysis and agglutination are biochemically related because complement activation (and resultant lysis of RBCs) through the classical pathway requires antigen-bound IgM (e.g., natural antibodies; Juul-Madsen et al. 2008), although there are other immune factors that may stimulate complement activity. Interestingly, we also uncovered several instances of decoupling of agglutination and lysis performance. For example, we found high agglutination but low lysis responses in some species (sea lamprey, spiny dogfish, and southern leopard frog), and these species may use a different means of complement activation, relative to the other species. For example, the lectin pathway of complement activation differs from the classical pathway by not requiring antigen-antibody binding, and it is ancestral among vertebrates

(Dodds and Matsushita 2007). Thus, while our work shows that there are general correlations between natural antibody and lysis activities, the relative utility of the different complement pathways in response to a variety of infectious agents could vary as a function of both taxon and temperature.

Within this context, we found that sea lamprey plasma contained factors that agglutinated mammalian red blood cells very effectively at cold temperatures (only two other species had higher maximum titers), despite sea lampreys' production of variable lymphocyte receptor antibodies rather than the immunoglobulins produced by jawed vertebrates that are involved in agglutination (Anderson and Rast 2009). Agglutination titers in sea lampreys were uncoupled from lytic capacity, which was generally low and not dependent on temperature (fig. 1). This pattern was shared with spiny dogfish but not with a species from its sister group, the bonnethead shark. Interestingly, sea lampreys and dogfish are unique among vertebrates in that they produce squalamine, a water-soluble steroidal antibiotic that destroys viruses and many foreign cells (e.g., gram-positive and gram-negative bacteria, fungi, and protozoa) but not foreign red blood cells (Moore et al. 1993; Yun and Li 2007; Zasloff et al. 2011). If sea lampreys and spiny dogfish rely heavily on squalamine to serve the same role as complement, they may exhibit very different lytic performance for pathogens other than RBCs.

It is possible that our results may have been influenced by prior dietary exposure to mammalian red blood cells, which could have led to modified responses, thus masking true species-level differences (although TPCs within species would likely be unaffected). We are confident, however, that previous dietary exposure to sheep red blood cells is more likely to tolerize responses than to hyperstimulate (André et al. 1975) and is therefore unlikely to have driven the high agglutination values seen in some carnivores (e.g., alligators). Lymphocytes isolated from tolerized hosts have decreased proliferation, cytokine production, and antibody production, resulting in systemic tolerance (Miller and Hanson 1979; Challacombe and Tomasi 1980; Melamed and Friedman 1994; Yoshida et al. 1997). In addition, while tolerance to dietary antigens may increase secretory immunoglobulin levels, there is no increase in circulating antibody levels (Challacombe and Tomasi 1980). Thus, the patterns that we report here do not represent hyperstimulated responses in those species that may have been previously exposed to mammals through diet, and no species were previously exposed to sheep by-products specifically (table A3).

In summary, we found that two measures of innate immune function in vertebrates, NAbs and complement-mediated lysis, were temperature dependent and varied in accordance with the natural thermal biology of the species. We also found the potential for correlated selection for both lysis and agglutination, although more work is needed to determine the direct regulatory effect of NAbs on complement. We provide new insights into thermal adaptation in broad relationships between widespread abiotic and biotic traits (environmental temperature and immunity, respectively), and this work should encourage many future research directions. Specifically, we encourage determination of TPCs for other immune performance metrics, including oxidative burst capacity (Sild and Hõrak 2010), opsonization ability (sensu Nikoskelainen et al. 2004), phagocytosis, chemotaxis, and the activity of haptoglobin, an acute phase protein. In addition, as ichthyologists have demonstrated (e.g., Jokinen et al. 2010), in vivo work with immunity as a function of temperature is an important next step in ecoimmunology for both ectotherms and endotherms, many of which are heterothermic to some degree (McKechnie and Lovegrove 2002; Geiser 2004). Future research could also examine a greater representation of species or undertake a more thorough investigation of a smaller clade, to more comprehensively assess the relative importance of evolutionary relatedness and species-specific thermal biology (or other life-history traits) on innate immune function. Finally, it will be useful to examine the role of global climate change on vertebrates' abilities to operate within ranges of effective physiological efficiency (Tewksbury et al. 2008), including the ability to effectively fight pathogens. Because peak performance for agglutination and lysis was lower than T_{body} for most species in our study, the immune systems of ectotherms could be particularly sensitive to environmentally induced changes in T_{body}.

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