TEMPERATURE AND HORMONAL DRIVERS OF IMMUNE PERFORMANCE IN VERTEBRATES

by

ASHLEY ANNE LAVERE

(Under the Direction of Vanessa Ezenwa)

ABSTRACT

Immunity is a physiological process crucial to survival. Thus, identifying factors influencing immunity is important for understanding variation in an organism's fitness across contexts. In this thesis, I used microbial killing assays to examine temperature and hormonal drivers of immune performance across vertebrates. First, by testing testosterone-immunity relationships in alligators, I found that interactions with co-circulating hormones and temperature may be important mediators of testosterone-immunity trade-offs. Second, by assessing immune performance of endotherms and ectotherms across temperatures, I found that immune performance across temperatures depended on thermoregulatory strategy and that thermoregulatory strategy determined whether temperature imposed trade-offs on immunity. Third, I showed that variability in immune performance depended on microbial context, with the presence of testosterone-immunity tradeoffs and temperature-dependent shifts in immune performance varying across the different microbes used to quantify immune performance. In aggregate, this work provides insights into immunological trade-offs and intrinsic and extrinsic factors influencing these trade-offs. INDEX WORDS: immunity, ectotherm, endotherm, microbial killing assay, hormones, testosterone, temperature

TEMPERATURE AND HORMONAL DRIVERS OF IMMUNE PERFORMANCE IN VERTEBRATES

by

ASHLEY ANNE LAVERE

BS, University of North Carolina – Greensboro, 2014

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2020

TEMPERATURE AND HORMONAL DRIVERS OF IMMUNE PERFORMANCE IN VERTEBRATES

by

ASHLEY ANNE LAVERE

Major Professor:	Vanessa Ezenwa
Committee:	Kristen Navara
	Benjamin Parrott

Electronic Version Approved:

Ron Walcott
Interim Dean of the Graduate School
The University of Georgia
August 2020

ACKNOWLEDGEMENTS

I would like to first thank my major advisor, Dr. Vanessa Ezenwa, for her support and guidance throughout my graduate research. She provided me with the encouragement to follow new ideas and approach questions from different angles. I would also like to thank my committee members, Dr. Ben Parrott and Dr. Kristen Navara for their guidance and feedback. Further, I want to share my appreciation for my fellow Ezenwa Lab members for providing feedback and lending their support and friendship during my time in the lab.

I would also like to thank the Odum School community for their kindness and endless support. In particular, the mentorship of Dr. Jasmine Crumsey-Forde, who helped me grow as an educator and build professional skills in instructional research and design.

Finally, I am incredibly thankful for my family, partner, and friends who have been there to listen and provide their unconditional support.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Introduction

Immunity is a crucial physiological function comprised of complex interactions among diverse components (Coico and Sunshine, 2015). In literature, the immune system has been depicted as a "micro-ecosystem" or a "finely-tuned orchestra", highlighting its interconnectedness whereby components must work together to generate a coordinated response to pathogen invasions (Coico and Sunshine 2015; Thomas-Vaslin 2017). This complexity has fueled long-standing interest in how the immune system works and the factors that constrain system function. In addition to immunity, organisms are responsible for maintaining several other biological processes, leading to potential interactions among these processes. Thus, factors intrinsic to an organism including body mass, nutritional state (Alonso-alvarez and Tella, 2001; Pérez-Rodríguez et al., 2006), and hormone activity (Hau and Wingfield, 2013), may all influence immune function. Further, organisms operate within variable environments that present a variety of extrinsic factors such as social rank (Ezenwa et al., 2012; Habig et al., 2018), resource availability (Prall and Muehlenbein, 2014) and temperature (Hanson, 1997), that may moderate biological processes. Two factors of particular interest are hormones and temperature. Understanding how intrinsic (i.e. hormones) and extrinsic factors (i.e. temperature) influence immunity can provide important insights into immune performance across ecological contexts.

Hormones and immune function

The effects of hormones on immune function has garnered considerable attention, with much of this interest focused on steroid sex hormones (i.e. testosterone, estrogen, etc.). For example, by comparing males and females, differences in both immune function and rates of parasitism have repeatedly emerged (Klein, 2000; Roved et al., 2017; Vázquez-Martínez et al., 2018; Zuk and McKean, 1996). General trends reveal reduced immune function and greater parasitism in males, especially during the breeding season when males engage in mating displays aided by elaborate secondary sexual traits (e.g. ornamental plumage, weapons). Though these traits are used to enhance reproduction, males may simultaneously experience trade-offs between investment in the development of secondary sexual traits and other physiological needs, such as immune function (Houslay et al., 2017).

One proposed mediator of these trade-offs is testosterone. Folstad and Karter (1992) proposed the Immunocompetence Handicap Hypothesis (ICHH), which postulates that testosterone positively affects development of secondary sexual characteristics while simultaneously suppressing immunity. Thus, this hypothesis implicates testosterone-mediated immunosuppression as a key mechanism facilitating honest signaling in males. However, ongoing evidence suggests that interactions between testosterone and immune function are more complex.

A recent meta-analysis assessed support for the ICHH across multiple taxa for which testosterone was either experimentally manipulated or measured across a natural gradient (Foo et al., 2017). Moreover, the effects of testosterone were examined across a range of immune measures, encompassing both cell-mediated immunity and humoral-mediated immunity. Results showed that across all immune measures, there was general support for the ICHH for

manipulative studies, but not observational studies. These findings suggest that testosterone may indeed have immunosuppressive effects, but that natural variation in testosterone may not be clearly linked to changes in immune function.

Across taxa, other steroid hormones have also been identified as potential regulators of immune function (Guillette et al., 1995). Corticosterone (CORT) and dehydroepiandosterone (DHEA) are two steroid hormones that have been widely linked to immune function. Corticosterone is a steroid hormone that drives stress responses, which are believed to redirect energy away from physiological processes less pertinent to immediate survival, such as immune function (Martin, 2009; Sapolsky et al., 2000). However, CORT has also been linked to both enhancement and suppression of immune components, further demonstrating the complexity of hormone-immunity interactions (Adamo, 2014). Importantly, CORT may interact with testosterone to drive immunosuppressive effects (Roberts et al., 2007), suggesting that interactions among co-circulating hormones may mediate hormone-immunity relationships. Dehydroepiandrosterone (DHEA), which serves as a precursor to testosterone, is known to stimulate certain components of the immune system (e.g. cytokine secretion, lymphocyte function; Regelson et al. 1994; Hazeldine et al. 2010). Several studies on DHEA have suggested that it is used by organisms outside of the breeding season as a "low cost" substitute for testosterone in moderating aggression (Boonstra et al., 2008; Soma and Wingfield, 2001). However, few studies have looked at potential role DHEA may play during the breeding season in counteracting the costs of testosterone, including immune costs.

Temperature and immune function

Temperature is an important driver of many physiological functions, including immune performance (Angilletta, 2009).The most common evidence of temperature effects on immune function comes from studies on fever response. Thermal stress resulting from increases in body temperature by a few degrees above mean temperatures can lead to enhanced immune mechanisms and greater pathogen clearance (Evans et al., 2016; Roberts, 1979). Specifically, febrile temperatures have been associated with increased innate and adaptive immune activity (Evans et al., 2016). Importantly, extreme, uncontrolled fever responses can push organisms outside their thermal limits, potentially resulting in damaging outcomes (Evans et al., 2016) . Conversely, thermal stress induced by temperature declines are often associated with reduced immune performance (Kusumoto, 2014; Mondal and Rai, 2001; Sacchi et al., 2014). Lower metabolic rates that occur under decreased temperatures can lead to reduced immune activity (Bouma et al., 2010). Finally, effects of temperature on host immune defenses and pathogen physiology may interact to drive variable infection outcomes (Cohen et al., 2017).

Organisms often occupy environments that are subject to daily or seasonal fluctuations in temperature. Vertebrates have evolved distinct strategies to deal with thermally fluctuating environments and regulate body temperatures within ranges that optimize physiological performance (Angilletta et al., 2002; Angilletta et al., 2010). Endothermic species regulate their metabolism to produce enough heat to maintain a narrow body temperature range, regardless of fluctuating environmental temperatures (Seebacher, 2009). Ectothermic species conform to surrounding environmental temperatures and use behavioral techniques to alter their body temperature (Seebacher, 2009). Energetic costs and benefits associated with each thermal strategy may play important roles in driving patterns of thermal sensitivity across species.

Though temperature effects on immunity is well recognized, few studies have assessed how thermal strategy may moderate the thermal sensitivity of immune performance and further, how these patterns may inform methodological practices used to quantify immunity across vertebrates.

Study objectives

The objective of this thesis was to provide insights into temperature and hormonal drivers of immune performance. To do this, I first tested the ICHH in a free-ranging population of American alligators (*Alligator mississippiensis*) and examined whether and how both steroid hormones (i.e. DHEA) and temperature mediated testosterone-immunity relationships. These patterns were examined using microbial killing assays to test the innate immune response against three bacteria species: *Escherichia coli, Salmonella typhimurium,* and *Klebsiella pneumonia*. Multiple bacteria were selected to determine if effects were repeatable across bacteria species. Second, I measured microbial killing across temperatures ranging from 15ºC - 45ºC and examined how an organisms' thermal strategy (i.e. endotherm, ectotherm) affected the temperature sensitivity of immune performance. Again, these patterns were assessed across three microbial species, in this case, *Escherichia coli*, *Salmonella typhimurium*, and *Candida albicans*, to identify how the thermal preferences of the microbes may interact with the thermal sensitivity of hosts. Overall, this thesis provides important insights into how factors both intrinsic and extrinsic to an organism may mediate immune trade-offs and also highlights key methodological considerations that should be taken into account when studying immunity in vertebrates.

References

Adamo, S. A. (2014). The effects of stress hormones on immune function may be vital for the adaptive reconfiguration of the immune system during fight-or-flight behavior. *Integr. Comp. Biol.* 54, 419–426.

Albert-Vega, C., Tawfik, D. M., Trouillet-Assant, S., Vachot, L., Mallet, F. and Textoris, J. (2018). Immune functional assays, from custom to standardized tests for precision medicine. *Front. Immunol.* 9, 1–12.

Alonso-alvarez, C. and Tella, J. L. (2001). Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. 79,.

Angilletta, M. J. J. (2009). Thermal sensitivity. In *Thermal Adaptation: A theoretical and empirical synthesis*, pp. 35–87. Oxford University Press, Inc.

Angilletta, M. J., Niewiarowski, P. H. and Navas, C. A. (2002). The evolution of thermal physiology in ectotherms. *J. Therm. Biol.* 27, 249–268.

Angilletta, M. J., Cooper, B. S., Schuler, M. S. and Boyles, J. G. (2010). The evolution of thermal physiology in endotherms. *Front. Biosci.* E2, 861–881.

Assis, V. R. de, Monteiro, S. C., Giorgi, A. M., Assis, V. R. De, Christie, S., Titon, M., Maria, A. and Barsotti, G. (2013). Antimicrobial Capacity of Plasma from Anurans of the Atlantic Forest. *South Am. J. Herpetol.* 8, 155–160.

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. Part A Ecol. Integr. Physiol.* 327, 293–301.

Bergey, D. H. (2005). *Bergey's Manual of Systematic Bacteriology, Volume 2: The Proteobacteria*. (ed. Garrity, G. M.) Springer US.

Boonstra, R., Lane, J. E., Boutin, S., Bradley, A., Desantis, L., Newman, A. E. M. and Soma, K. K. (2008). Plasma DHEA levels in wild, territorial red squirrels: Seasonal variation and effect of ACTH. *Gen. Comp. Endocrinol.* 158, 61–67.

Bouma, H. R., Carey, H. V. and Kroese, F. G. M. (2010). Hibernation: the immune system at rest? *J. Leukoc. Biol.* 88, 619–624.

Calder, P. C. (2007). Immunological Parameters: What Do They Mean? *J. Nutr.* 137, 773S-780S.

Cohen, J. M., Venesky, M. D., Sauer, E. L., Civitello, D. J., McMahon, T. A., Roznik, E. A. and Rohr, J. R. (2017). The thermal mismatch hypothesis explains host susceptibility to an emerging infectious disease. *Ecol. Lett.* 20, 184–193.

Coico, R. and Sunshine, G. (2015). Overview of the Immune System. In *Immunology: A Short Course*, pp. 1–10. John Wiley & Sons, Ltd.

Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. and French, S. S. (2011). Beyond phytohaemagglutinin: Assessing vertebrate immune function across ecological contexts. *J. Anim. Ecol.* 80, 710–730.

Duong, N., Osborne, S., Bustamante, V. H., Tomljenovic, A. M., Puente, J. L. and Coombes, B. K. (2007). Thermosensing coordinates a cis-regulatory module for transcriptional activation of the intracellular virulence system in Salmonella enterica serovar typhimurium. *J. Biol. Chem.* 282, 34077–34084.

Evans, S. S., Repasky, E. A. and Fisher, D. T. (2016). Fever and the thermal regulation of immunity: the immune system feels the heat. *Nat. Rev. Immunol.* 15, 335–349.

Ezenwa, V. O., Stefan Ekernas, L. and Creel, S. (2012). Unravelling complex associations between testosterone and parasite infection in the wild. *Funct. Ecol.* 26, 123–133.

Fallahi-Sichani, M., Honarnejad, S., Heiser, L. M., Gray, J. W. and Sorger, P. K. (2013). Metrics other than potency reveal systematic variation in responses to cancer drugs. *Nat. Chem. Biol.* 9, 708–714.

Folstad, I. and Karter, A. J. (1992). Parasites , Bright Males , and the Immunocompetence Handicap. *Am. Nat.* 139, 603–622.

Foo, Y. Z., Nakagawa, S., Rhodes, G. and Simmons, L. W. (2017). The effects of sex hormones on immune function: a meta-analysis. *Biol. Rev.* 92, 551–571.

French, S. S. and Neuman-lee, L. A. (2012). Improved ex vivo method for microbiocidal activity across vertebrate species across vertebrate species. *Biol. Open*.

Guillette, L. J., Cree, A. and Rooney, A. A. (1995). Biology of stress: interactions with reproduction, immunology and intermediary metabolism. *Heal. Welf. Captiv. Reptil.* 32–81.

Habig, B., Doellman, M. M., Woods, K., Olansen, J. and Archie, E. A. (2018). Social status and parasitism in male and female vertebrates: A meta-analysis. *Sci. Rep.* 8, 1–13.

Hamilton, W. J. (1973). *Life's color code.* McGraw-Hill.

Hanson, D. F. (1997). Fever , Temperature , and the Immune Response. *Annu. New York Acad. Sci.* 813, 453–464.

Hau, M. and Wingfield, J. C. (2013). Hormonally-regulated trade-offs: Evolutionary variability and phenotypic plasticity in testosterone signaling pathways. *Mech. Life Hist. Evol.* 349–361.

Hazeldine, J., Arlt, W. and Lord, J. M. (2010). Dehydroepiandrosterone as a regulator of immune cell function. *J. Steroid Biochem. Mol. Biol.* 120, 127–136.

Hoffmann, A. A., Chown, S. L. and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: How constrained are they? *Funct. Ecol.* 27, 934–949.

Houslay, T. M., Houslay, K. F., Rapkin, J., Hunt, J. and Bussière, L. F. (2017). Mating opportunities and energetic constraints drive variation in age-dependent sexual signalling. *Funct. Ecol.* 31, 728–741.

Huey, R. B. and Hertz, P. E. (1984). Is a Jack-of-All-Temperatures a Master of None? *Evolution (N. Y).* 38, 441–444.

Huey, R. B. and Stevenson, R. D. (1979). Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Integr. Comp. Biol.* 19, 357–366.

Klein, S. L. (2000). The effects of hormones on sex differences in infection: From genes to behavior. *Neurosci. Biobehav. Rev.* 24, 627–638.

Kommanee, J., Preecharram, S., Daduang, S., Temsiripong, Y., Dhiravisit, A., Yamada, Y. and Thammasirirak, S. (2012). Antibacterial activity of plasma from crocodile (Crocodylus siamensis) against pathogenic bacteria. *Ann. Clin. Microbiol. Antimicrob.* 11, 1–9.

Kusumoto, K. (2014). Comparison of Humoral Immune Response Under Low Tempearture in

Non-breeding Gray Red-backed Voles (Myodes rufocanus). *Bull. Fac. Agr.* 99, 43–55.

Liebl, A. L. and Martin, L. B. (2009). Simple quantification of blood and plasma antimicrobial capacity using spectrophotometry. *Funct. Ecol.* 23, 1091–1096.

Martin, L. B. (2009). Stress and immunity in wild vertebrates: Timing is everything. *Gen. Comp. Endocrinol.* 163, 70–76.

Matson, K. D., Tieleman, B. I. and Klasing, K. C. (2006). Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. *Physiol. Biochem. Zool.* 79, 556–564.

Merchant, M., Williams, S., Trosclair, P. L., Elsey, R. M. and Mills, K. (2007). Febrile response to infection in the American alligator (Alligator mississippiensis). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 148, 921–925.

Millet, S., Bennett, J., Lee, K. A., Hau, M. and Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* 31, 188–201.

Mondal, S. and Rai, U. (2001). In vitro effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, Hemidactylus flaviviridis. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 129, 391–398.

Nguyen, H. D. N., Yang, Y. S. and Yuk, H. G. (2014). Biofilm formation of Salmonella Typhimurium on stainless steel and acrylic surfaces as affected by temperature and pH level. *LWT - Food Sci. Technol.* 55, 383–388.

Nicholls, S., Leach, M. D., Priest, C. L. and Brown, A. J. P. (2009). Role of the heat shock

transcription factor, Hsf1, in a major fungal pathogen that is obligately associated with warm-blooded animals. *Mol. Microbiol.* 74, 844–861.

O'Meara, T. R., Robbins, N. and Cowen, L. E. (2017). The Hsp90 Chaperone Network Modulates Candida Virulence Traits. *Trends Microbiol.* 25, 809–819.

Pérez-Rodríguez, L., Blas, J., Viñuela, J., Marchant, T. A. and Bortolotti, G. R. (2006). Condition and androgen levels: are condition-dependent and testosterone-mediated traits two sides of the same coin? *Anim. Behav.* 72, 97–103.

Prall, S. P. and Muehlenbein, M. P. (2014). Testosterone and Immune Function in Primates: A Brief Summary with Methodological Considerations. *Int. J. Primatol.* 35, 805–824.

Pumeesat, P., Muangkaew, W., Ampawong, S. and Luplertlop, N. (2017). Candida albicans biofilm development under increased temperature. *New Microbiol.* 40, 279–283.

Rakus, K., Ronsmans, M. and Vanderplasschen, A. (2017). Behavioral fever in ectothermic vertebrates. *Dev. Comp. Immunol.* 66, 84–91.

Rauw, W. M. (2012). Immune response from a resource allocation perspective. *Front. Genet.* 3, 1–14.

Roberts, N. J. (1979). Temperature and host defense. *Microbiol. Rev.* 43, 241–259.

Roberts, M. L., Buchanan, K. L., Hasselquist, D. and Evans, M. R. (2007). Effects of testosterone and corticosterone on immunocompetence in the zebra finch. *Horm. Behav.* 51, 126–134.

Rome, L. C., Sosnicki, A. and Choi, I. H. (1992). The influence of temperature on muscle

function in the fast swimming scup. II. The mechanics of red muscle. *J. Exp. Biol.* 163, 281–295.

Roved, J., Westerdahl, H. and Hasselquist, D. (2017). Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. *Horm. Behav.* 88, 95–105.

Sacchi, R., Capelli, E., Scali, S., Pellitteri-Rosa, D., Ghitti, M., Acerbi, E. and Pingtore, E. (2014). In vitro temperature dependent activation of T-lymphocytes in Common wall lizards (Podarcis muralis) in response to PHA stimulation. *Acta Herpetol.* 9, 131–138.

Sapolsky, R. M., Romero, M. L. and Munck, A. U. (2000). How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions*. *Endocr. Rev.* 21, 55–89.

Seebacher, F. (2009). Responses to temperature variation: Integration of thermoregulation and metabolism in vertebrates. *J. Exp. Biol.* 212, 2885–2891.

Sherman, E., Baldwin, L., Fernandez, G. and Deurell, E. (1991). Fever and thermal tolerance in the toad Bufo marinus. *J. Therm. Biol.* 16, 297–301.

Siroski, P. A., Pina, C. I., Larriera, A., Merchant, M. E. and Conza, J. Di (2009). Plasma Activity of the Broad-snouted Caiman (Caiman latirostris). *Zool. Stud.* 48, 238–242.

Soma, K. K. and Wingfield, J. C. (2001). Dehydroepiandrosterone in songbird plasma: Seasonal regulation and relationship to territorial aggression. *Gen. Comp. Endocrinol.* 123, 144–155.

Terrell, K. A., Quintero, R. P., Murray, S., Kleopfer, J. D., Murphy, J. B., Evans, M. J., Nissen,

B. D. and Gratwicke, B. (2013). Cryptic impacts of temperature variability on amphibian immune function. *J. exper* 2016, 4204–4211.

Tutar, U., Çelik, C., Ataş, M., Tunç, T. and Gözel, M. G. (2015). Evaluation of biofilm formation activity of standard microorganism strains. *J. Clin. Exp. Investig.* 6, 135–139.

Vázquez-Martínez, E. R., García-Gómez, E., Camacho-Arroyo, I. and González-Pedrajo, B. (2018). Sexual dimorphism in bacterial infections. *Biol. Sex Differ.* 9, 1–20.

Zuk, M. and McKean, K. A. (1996). Sex differences in parasite infections: Patterns and processes. *Int. J. Parasitol.* 26, 1009–1024.

CHAPTER 2

ASSOCIATIONS BETWEEN TESTOSTERONE AND IMMUNE ACTIVITY IN ALLIGATORS DEPEND ON BACTERIA SPECIES AND TEMPERATURE[1]

[1] LaVere, A.A., Hamlin, H.J., Lowers, R.H., Parrott, B.B, and Ezenwa, V.O. Submitted to *Functional Ecology*, 05/08/2020

Abstract

The immunocompetence handicap hypothesis (ICHH) postulates that testosterone supports the development of secondary sexual traits while simultaneously suppressing immune function, creating a trade-off between trait quality and pathogen vulnerability. The nature of interactions between testosterone and immunity are complex. Conflicting patterns from the literature suggest that testosterone-immunity relationships are variable across immune measures and may be modified by factors both intrinsic and extrinsic to the organism. In this study, we tested the ICHH in free-ranging American alligators (*Alligator mississippiensis*) and examined how both intrinsic (steroid hormone levels) and extrinsic (temperature) factors mediate the relationship between testosterone and immunity. Specifically, we quantified the simultaneous effects of testosterone and dehydroepiandrosterone (DHEA) on microbial killing capacity of three bacteria species (*Escherichia coli, Salmonella typhimurium,* and *Klebsiella pneumoniae*) at two challenge temperatures (15ºC and 30ºC). We found that accounting for circulating levels of DHEA was important for predicting testosterone-mediated effects on microbial killing capacity. We also found that testosterone-mediated immunosuppression was dependent on temperature and bacteria species, with negative effects of testosterone present only for *S. typhimurium* at 15ºC.Our results highlight the context dependency of interactions between testosterone and immunity, and illustrate the importance of evaluating the ICHH in natural systems to identify key intrinsic and extrinsic factors mediating testosterone-immunity trade-offs.

Introduction

Males often use elaborate secondary sexual traits (e.g. ornamental plumage, weapons) to enhance reproduction, but simultaneously experience trade-offs between investment in the

development of these traits and other physiological needs (Houslay et al., 2017). One widely hypothesized physiological mediator of the trade-offs associated with secondary sexual traits is testosterone. Testosterone positively affects the development of secondary sexual traits, but can simultaneously suppress immune function, creating a trade-off between sexual signaling and vulnerability to pathogen infection (Folstad and Karter, 1991; Mougeot et al., 2004; Greives et al., 2006). This idea, formalized by the immunocompetence handicap hypothesis (ICHH; Folstad and Karter, 1991), implicates testosterone-mediated immunosuppression as a key mechanism facilitating honest signaling in males. However, ongoing evidence suggests that interactions between testosterone and immunity are complex.

The complexity of testosterone-immunity relationships is evidenced by conflicting (i.e. both positive and negative) patterns reported from studies examining relationships between non-manipulated testosterone levels and components of the immune response (e.g. Ezenwa et al., 2011; Trumble et al., 2016). Indeed, a recent meta-analysis of 52 species spanning from fish to mammals found support for the ICHH for a subset of studies that manipulated testosterone, but there was no significant link between testosterone and immunity for non-manipulative studies (Foo et al., 2017). These findings suggest that while testosterone can indeed have suppressive effects on components of immune function, natural variation in testosterone is not always linked to clear changes in immunity. Therefore, identifying factors that potentially mediate the relationship between testosterone and immunity is central to understanding the relevance of the ICHH in natural systems.

A large number of factors that are both intrinsic and extrinsic to an animal, such as the activity of other hormones or abiotic factors linked to seasonality (e.g. temperature), may exert a strong influence on the relationship between testosterone and immunity. For example,

dehydroepiandrosterone (DHEA) is a steroid hormone that serves as a precursor to testosterone that may play an important role in mitigating the immunosuppressive effects of its derivative. DHEA has been described as a "low-cost" substitute for testosterone because of its ability to maintain a subset of testosterone-associated functions (e.g. aggression; Soma and Wingfield, 2001; Boonstra et al., 2008), without compromising immunity (Wingfield et al., 2001). In fact, DHEA stimulates certain components of immune function such as cytokine secretion (e.g. interleukin 2) and lymphocyte function (Regelson et al., 1994; Hazeldine et al., 2010). Consequently, the immune-stimulating effects of DHEA may compensate for the immune costs of testosterone when both hormones are co-circulating (Owen-Ashley et al., 2004; Hamlin et al. 2011). Alongside the effects of intrinsic factors such as DHEA, extrinsic factors such as temperature may also influence associations between testosterone and immunity. Hormone synthesis, secretion, and metabolism all depend on temperature (Van der Kraak and Pankhurst, 1996). Furthermore, thermal sensitivity in immune performance is well-described in both ectotherms and endotherms (Butler et al., 2013). With temperature potentially acting on both hormone activity and immune performance, testosterone-immunity interactions may be strongly modified by seasonal variation in temperature.

In this study, we tested the ICHH in free-ranging American alligators (*Alligator mississippiensis*) and examined how both intrinsic (DHEA) and extrinsic (temperature) factors mediate the relationship between testosterone and immunity. Alligators are highly sexually dimorphic (Chabreck and Joanen, 1979; Vilet, 1989; Reber et al., 2017). During the breeding season, males compete aggressively for access to females (Garrick and Lang, 1977; Vliet, 1989), and correspondingly, testosterone levels of adult males show defined seasonal cycles, with peaks during the breeding season (Hamlin et al., 2011). Alligators have potent innate immune defenses

against many bacterial species (Merchant et al., 2003; Zimmerman et al., 2013), but whether these defenses are compromised by testosterone is unknown. DHEA concentrations in male alligators have been found to be consistently higher than testosterone concentrations throughout the year, except during the breeding season when levels of both hormones are similar (Hamlin et al., 2011). Thus, given seasonal fluctuations in testosterone, robust innate immune responses to bacteria, and potentially compensatory DHEA levels, alligators represent an excellent model for testing the ICHH and the role DHEA may play in mitigating the immunosuppressive effects of testosterone. Furthermore, the fact that alligators are ectotherms whose physiological functions, including immune function (Merchant et al., 2003; Butler et al., 2013), are strongly dependent on environmental conditions provides a unique opportunity to examine how temperature affects associations between testosterone, DHEA, and immune function. To explore these questions, we quantified the simultaneous effects of testosterone, DHEA and temperature on the microbial killing capacity of alligator blood, focusing on three bacterial pathogens: *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumoniae.* We predicted that: (i) the concentration of DHEA relative to testosterone would be a better predictor of immune performance than testosterone alone; (ii) testosterone would correlate negatively with immune function; and (iii) temperature would affect immunity in ways that modify correlations between testosterone, DHEA and immune function.

Methods

ANIMALS AND SAMPLING

Adult male alligators were captured from Merritt Island, Florida between 2006-2010 with intense monthly sampling occurring in 2008-2009. Individuals were identified using numbered

metal and passive internal transponder (PIT) tags to identify recaptures (Hamlin et al., 2011). Blood samples and morphometric data, including snout-to-vent length (SVL), were collected for each individual at capture (Hamlin et al., 2011). Blood was drawn from the postcranial supra-vertebral sinus into heparinized vacutainer tubes. Plasma was isolated by centrifugation, stored at -20℃ until hormone assays were performed, and then archived at -80℃ prior to immunological assays. Only testosterone and immune data from an individual's first capture were used in this study.

HORMONE ASSAYS

Testosterone and dehydroepiandrosterone (DHEA) concentrations in alligator plasma samples were quantified using solid-phase radioimmunoassays as described in Hamlin et al (2011). Briefly, testosterone and DHEA specific antibodies were used to coat the wells of a 96 well plate and then incubated at room temperature for 2 and 8 hours, respectively. All wells then received 100ul of the sample, standard, or control and 12,000cpm of ^{3}H-labeled steroid, followed by a 3-hour incubation at room temperature. All standards and samples were run in duplicate. Plates were counted using a Microbeta 1450 Trilux counter and concentrations were extrapolated from standard curves as the percentage of bound versus $1og^{10}$ concentration.

IMMUNE ASSAYS

To quantify bacterial killing ability (BKA) of plasma, we followed a spectrophotometer-based protocol described by French and Neuman-Lee (2012) with minor modifications. Assay conditions for alligator samples, including bacterial concentrations, challenge temperatures and incubation times were optimized for three bacteria: *Escherichia coli* (EPower Microorganisms, Microbiologics, St. Cloud, MN, USA, REF# 0483E7, ATCC# 8739), *Salmonella typhimurium* (KwikStik, Microbiologics, St. Cloud, MN, USA, REF# 0363P, ATCC# 14028) and *Klebsiella*

pneumoniae (EPower Microorganisms, Microbiologics, St. Cloud, MN, USA, REF# 0684E7, ATCC # 10031). All optimizations were performed on a subset (n= 20) of the samples used in the larger study. Bacteria were challenged at two different temperatures: 15℃ and 30℃. These two temperatures were selected to reflect the optimal body temperature of alligators during summer (30℃; Lang, 1987) and the minimum body temperature during winter (15℃; Seebacher et al., 2003). A set of four assays were run at each challenge temperature using two challenge times (30 minutes and 60 minutes) and two bacteria concentrations (10^5 and 10^3). A 1:5 plasma dilution, optimized in prior experiments, was used for all assays.

Assay conditions that yielded the highest killing with sufficient among-sample variation were selected for use at each challenge temperature (Table 2.1). For *E. coli*, samples were challenged for 60 and 30 minutes at 15℃ and 30℃, respectively with a 10^5 bacteria concentration. For *S. typhimurium*, samples were challenged for 30 minutes at both 15℃ and 30℃, respectively with a 10^3 bacteria concentration. For *K. pneumoniae*, samples were challenged for 60 minutes at both 15℃ and 30℃, respectively with a 10^3 bacteria concentration. Bacteria were prepared by creating a 10^8 solution from plated colonies using the BD BBL™ Prompt™ Inoculation System, followed by dilution with phosphate buffered saline (PBS) to the appropriate concentration: *E. coli*: 10^5 (~1,500-2,000 CFUs) and *S. typhimurium, K. pneumoniae*: 10^3 (~400-500 CFUs).

Assays were performed by adding 4µl of plasma, 4µl of bacteria, and 16µl of PBS to a single well of a 96 well plate, and each sample was run in triplicate. Positive controls were made by adding 4µl of bacteria to 20µl of PBS and negative controls consisted of 24µl of PBS. Each plate contained 8 positive and 8 negative controls. Samples were mixed by vortexing each plate and plates were then incubated under appropriate challenge conditions (see Table 2.1).

After bacterial challenge, 125ul of tryptic soy broth (TSB) was added to all wells and an initial, background, absorbance reading was obtained for each well prior to bacterial growth. Plates were then incubated for 12 hours at 37℃. Following incubation and homogenization, sample absorbance was re-read. For *E coli* and *S typhimurium* sample wells were homogenized by vortexing. For *K. pneumoniae*, which forms biofilms, sample wells were simultaneously vortexed and stirred to facilitate homogenization. Finally, to calculate sample BKA the following equation was used:

$$BKA = 1 - \frac{(Sample\ Mean\ Absorbance)}{(Positive\ Control\ Mean\ Absorbance)}$$

Background absorbance values were subtracted from all post-incubation absorbance values prior to averaging. Absorbance was read at 300nm on a standard microplate reader.

STATISTICAL ANALYSIS

We examined the relationship between testosterone and immunity and the influence of two different factors on this relationship: DHEA and temperature. Analyses were performed separately for each bacteria. Killing ability scores for each bacteria were checked for normality using a Shapiro-Wilk's test. BKA scores for both *S. typhimurium* and *K. pneumoniae* were approximately normal *(S. typhimurium*: W = 0.980, P = <0.0001; *K. pneumoniae*: W = 0.947, P = <0.0001), while scores for *E. coli* deviated substantially from normality (W = 0.648; P = <0.0001). As a consequence, we used a box cox transformation to normalize *E. coli* BKA scores (W = 0.896; P = <0.0001). We then used linear mixed models (LMMs) for all subsequent analyses and examined model residuals to assess model validity (Zuur et al., 2009).

As a first step, we tested for a possible role of DHEA in mediating relationships between testosterone and immune function by comparing four LMMs with different combinations of

DHEA and testosterone as independent variables to identify which combination of these predictors best accounted for variation in killing ability for each bacteria. The models included either: (i) T only, (ii) the ratio of T to DHEA [T/DHEA] only, (iii) T + DHEA or (iv) T + T/DHEA. In addition to these hormone-related predictors, the following covariates were also included in each model: challenge temperature, testosterone phase and snout-to-vent length (SVL). Interactions between the hormone variables and each covariate were also included in all models. Testosterone phase was used to account for seasonal variation in testosterone secretion. The testosterone phase (primary or secondary) for each sample was classified according to the seasonal window of sample collection. Primary samples were collected during the first half of the year (January-July), which encompassed the breeding season and highest recorded levels of testosterone secretion; while secondary samples were collected during the second half of the year (August-December), which encompassed the non-breeding season (see Table 2.2). Snout-to-vent length (SVL) was used to account for variability in male size since larger males consistently have higher concentrations of testosterone than smaller males (Hamlin et al., 2011; Lance et al., 2015). Finally, sample ID and year were included as random effects in each model. Akaike's Information Criteria (AIC) was used to compare models. The model with the lowest AIC score was considered to be the best supported model, and models with a ΔAIC value ≤ 2 were considered to be of the same rank as the best model (Mazerolle, 2006). The top model was then used to interpret relationships between testosterone, DHEA, challenge temperature and immunity.

Results

Average BKA scores varied across bacteria (*E. coli*, *S. typhimurium* and *K. pneumoniae)* and challenge temperatures (Table 2.3). A comparison of four models including the effects of T alone, T/DHEA, T + DHEA or T + T/DHEA ratio showed that accounting for DHEA (T/DHEA ratio model) best predicted variation in BKA for all three bacteria (Table 2.4).

In addition, we found that the effect of testosterone on immune function depended on bacteria species and was mediated by challenge temperature. Specifically, testosterone only emerged as a significant predictor of BKA for 1 out of 3 bacteria, and when it did, the effect depended on temperature. For *S. typhimurium*, there was no main effect of T/DHEA on killing ability, but killing was significantly higher at 15℃ compared to 30℃ (LMM, n = 625; temperature: estimate = -0.0383, $P < 0.001$; Figure 2.1A; Table 2.5). Importantly, temperature interacted with T/DHEA such that a negative effect of having higher testosterone was apparent only at the 15℃ challenge temperature (T/DHEA × temperature: estimate = 0.0048, P = 0.0056; Figure 2.1B; Table 2.5). Interestingly, in the T + DHEA model including the independent effects of both hormones, the interactions between challenge temperature and T and DHEA were both significant (Table 2.6). In this model, testosterone had a negative effect on killing at 15℃ and no effect at 30℃ (Figure 2.2A), while DHEA had a positive effect on killing at 15℃ and no effect at 30℃ (Figure 2.2B). This result corroborates the pattern seen in the T/DHEA model, while also highlighting the opposing effects of T and DHEA on microbial killing.

For *E. coli*, challenge temperature was the only significant predictor of BKA (Table 2.7), but in contrast to *S. typhimurium*, killing of *E. coli* was significantly higher at 30℃ compared to 15℃ (LMM, n = 622, estimate = 0.0305, P = 0.0019; Figure 2.1C). Neither T/DHEA nor its interaction with temperature had any effect on BKA (Figure 2.1D; Table 2.7). Likewise, there

was no independent effect of either T or DHEA on *E. coli* BKA apparent in the T + DHEA model (Table 2.8).

For *K. pneumoniae,* challenge temperature and testosterone phase emerged as the only significant predictors of BKA (Table 2.9). Killing of *K. pneumoniae* was significantly higher at 15℃ (LMM, n = 622, estimate = -0.0756, P = 0.0011; Figure 2.1E) and during the secondary testosterone phase (phase: estimate = 0.0834, P = 0.0148; Table 2.9). Neither T/DHEA nor its interaction had any effect on BKA (Figure 2.1F; Table 2.9). Similarly, there was no independent effect of either T or DHEA on *K. pneumoniae* BKA in the T + DHEA model (Table 2.10).

Discussion

Testosterone is often described as a "double-edged' sword that facilitates the expression of secondary sexual characteristics while simultaneously suppressing immune function (Folstad and Karter, 1991). However, the magnitude of the trade-off between testosterone and immunity may depend on a range of intrinsic and extrinsic factors that modify the impact of testosterone on immune responsiveness. Here, we found that variation in bacteria killing ability (BKA) across three bacteria (*E. coli, S. typhimurium, and K. pneumoniae*) was best explained when co-circulating levels of the hormone DHEA were simultaneously considered. This result suggests that DHEA may serve to mediate interactions between testosterone and immunity. We also found that testosterone-immunity relationships depended on both bacteria species and challenge temperature. Of the three bacteria we examined, *S. typhimurium* was the only one for which testosterone had a significant negative effect on plasma killing capacity. Moreover, this effect was only apparent at 15℃ and not 30℃, highlighting the role abiotic factors, like temperature, can play in shaping testosterone-immunity trade-offs.

Our results suggest that factors that are intrinsic to an animal, such as circulating levels of other steroid hormones that may interact with immune function or testosterone, should be accounted for when evaluating relationships between natural variation in testosterone levels and immune function. We found that correcting testosterone levels for relative levels of DHEA improved the explanatory power of testosterone as a predictor of plasma killing ability across all bacteria. A low ranked *S. typhimurium* model including the independent effects of T and DHEA supported the presence of opposing effects of these two hormones on immune function (Figure 2.2; Table 2.6). Furthermore, the T/DHEA ratio model was consistently supported across bacteria suggesting that co-circulating levels of DHEA may mediate interactions between testosterone and immunity. For instance, many immune components reported to be suppressed by testosterone have also been reported to be enhanced by DHEA (e.g. T-cell production, immune cell cytotoxicity, and natural killer cell activity; Suzuki et al., 1991; Khorram et al., 1997; Hazeldine et al., 2010). Thus, co-circulating DHEA may play an under-appreciated role in modifying the immunosuppressive effects of testosterone. Another hormone that has been widely proposed as a mediator of testosterone-immunity relationships is corticosterone, a stress-related steroid hormone. Under the stress-linked immunocompetence handicap hypothesis (SL-ICHH), corticosterone is suggested to interact with testosterone to drive immunosuppressive effects (Evans et al., 2000; Poiani et al., 2000; Roberts et al., 2007). Interestingly, DHEA has been shown to counteract immunosuppressive effects of glucocorticoids (Hazeldine et al., 2010), suggesting an additional mechanism by which DHEA may lessen testosterone-immunity trade-offs.

Scrutiny of the best fitting models for all three bacteria further revealed the key role of an extrinsic factor, in this case temperature, in shaping the outcome of testosterone-immunity

relationships. We show that challenge temperature was a significant predictor of killing ability for all three bacteria we examined. Intriguingly, the temperature at which killing was highest differed among bacteria. The highest killing of *S. typhimurium* and *K. pneumoniae* occurred at 15℃, while highest killing of *E. coli* occurred at 30℃. Host immune performance has been linked to temperature in a range of animal taxa (Hanson, 1997; Rios & Zimmerman, 2001; Nickoskelainen et al., 2002; Rollins-Smith and Woodhams, 2012). For instance, in alligators, complement activity, via the alternative pathway, declines at temperatures below 15℃ or above 30℃ (Merchant et al., 2005). Given that our three focal bacteria species elicit unique host immune responses (Lebeis et al., 2006; Broz et al., 2012; Pacoza and Mecsas, 2016), differential effects of temperature on these responses may explain variability in host immune performance at 15℃ versus 30℃ across the different bacteria.

Beyond temperature dependency of the host immune response, pathogens themselves use a variety of mechanisms to evade host immunity, some of which may be thermally sensitive. For instance, *K. pneumoniae* and *S. typhimurium* form protective biofilms when exposed to stressors triggered by host immunity (Tutar et al., 2015), but the process of biofilm production may be inhibited under low temperatures (Nguyen et al., 2014), leaving bacteria more vulnerable to immune attacks. Some pathogens also use temperature cues to regulate expression of virulence genes, often reducing pathogenic activity at temperatures outside of those found in their preferred hosts (Lam et al., 2014). Such temperature-dependent virulence has been described in *S. typhimurium*, which shows reduced virulence at 25℃ compared to 37℃, likely due to its tight association with endothermic hosts (Duong et al., 2007). Both reduced pathogen defense and lower virulence may explain increased killing of *K. pneumoniae* and *S. typhimurium* at 15℃. Temperature can also influence the effectiveness of pathogen immune evasion. For instance, *E.*

coli often use capsule formation to defend against serum bactericidal effects (Miajlovic and Smith, 2014). In alligators, Phospholipase A_2, an enzyme that disrupts microbial membranes (Moreau et al., 2001) such as those composing the capsule, has reduced enzymatic activity at lower temperatures (i.e. 5-10℃; Merchant et al., 2009), which may explain our finding of lower killing of *E. coli* at 15℃.

Finally, temperature effects on host and pathogen physiology may interact to modify testosterone-immunity relationships. We found that the negative relationship between testosterone and immunity observed for *S. typhimurium* was only present at the 15℃ challenge temperature. The overall lower killing of *S. typhimurium* compared to the other two bacteria, and few records of *S. typhimurium* presence in reptiles (Scott & Foster, 1997; Pedersen et al., 2009), suggests that alligators are rarely exposed to this bacteria under natural conditions. Therefore, it is possible that alligators are generally less able to defend themselves against *S. typhimurium.* In addition, *S. typhimurium* might be more impaired in terms of both its defense mechanisms (Nguyen et al., 2014) and virulence activity (Duong et al., 2007) at 15℃ than at 30℃. Taken together, this may explain why individuals were consistently ineffective at killing *S. typhimurium* at 30℃, but not 15℃. Impaired pathogen activity at 15℃, may have allowed for a more effective host response against this pathogen. More generally, this result highlights the importance of accounting for abiotic factors, such as temperature, as well as pathogen species, and potential interactions between the two when assessing testosterone-immunity relationships in nature.

Overall, our integrative approach of evaluating the ICHH provided key insights into the context dependency of relationships between testosterone and immunity. In particular, interactions between co-occurring physiological processes and seasonal drivers may serve as

important mediators of testosterone-immunity trade-offs. Furthermore, these interactions likely vary across different pathogen species to which an individual is exposed, reinforcing the idea that context is key to understanding how the ICHH operates in natural populations.

References

Boonstra, R., Lane, J.E., Boutin, S., Bradley, A., Desantis, L., Newman, A.E.M., & Soma, K.K. (2008). Plasma DHEA levels in wild, territorial red squirrels: seasonal variation and effect of ACTH. *General and Comparative Endocrinology* 158: 61-67.

Broz, P., Ohlson, M.B., & Monack, D.M. (2012). Innate immune response to *Salmonella typhimurium*, a model enteric pathogen. *Gut Microbes*, 3(2): 62-70.

Butler, M.W., Stahlschmidt, Z.R., Ardia, D.R., Davies, S., Davis, J., Guillette Jr, L. J., ... & DeNardo, D.F. (2013). Thermal sensitivity of immune function: evidence against a generalist-specialist trade-off among endothermic and ectothermic vertebrates. *The American Naturalist*, 181(6), 761-774.

Chabreck, R.H. & Joanen, T. (1979). Growth rates of American alligators in Louisiana. *Herpetologica*, 35(1), 51-57.

Duong, N., Osborne, S., Bustamante, V.H., Tomljenovic, A.M., Puente, J.L., & Coombes, B.K. (2007). Thermosensing coordinates a cis-regulatory module for transcriptional activation of the intracellular virulence system in Salmonella enterica serovar Typhimurium. *Journal of Biological Chemistry*, 282(47), 34077-34084.

Evans, M.R., Goldsmith, A.R., & Norris, S.R.A. (2000). The effects of testosterone on antibody production and plumage coloration in male house sparrows (*Passer domesticus*). *Behav Ecol Sociobiol*, 47:156-163.

Ezenwa, V.O., Ekernas, L.S., & Creel S. (2011). Unraveling complex associations between testosterone and parasite infection in the wild. *Functional Ecology*, 26: 123-133.

Folstad, I. & Karter, A.J. (1991). Parasites, bright males, and the immunocompetence handicap. *The American Naturalist*. 139(3): 603-622.

Foo, Y.Z., Nakagawa, S., Rhodes, G., & Simmons, L.W. (2017). The effects of sex hormones on immune function: a meta-analysis. *Biological Reviews*, 92(1), 551-571.

French, S.S. & Neuman-Lee, L.A. (2012). Improved ex vivo method for microbiocidal activity across vertebrate species. *Biology Faculty Publications*, paper 418.

Garrick, L.D. & Lang, J.W. (1977). Social signals and behaviors of adult alligators and crocodiles. *American Zoologist*, 17: 225-239.

Greives, T.J., McGlothlin, J.W., Jawor, J.M., Demas, G.E., & Ketterson, E.D. (2006). Testosterone and innate immune function inversely covary in a wild population of breeding Dark-Eyed Juncos (Junco hyemalis). *Functional Ecology*, 20(5), 812-818.

Hamlin, H.J., Lowers, R.H., & Guillette, L.J. (2011). Seasonal androgen cycles in adult male American alligator (*Alligator mississippiensis*) from a barrier island population. *Biology of Reproduction*. 85: 1108-1113.

Hanson, D.F. (1997). Fever, temperature, and the immune response. *Annals of the New York Academy of Sciences*, 813(1), 453-464.

Hazeldine, J., Arlt, W., & Lord, J.M. (2010) Dehydroepiandrosterone as a regulator of immune cell function. *Journal of Steroid Biochemistry & Molecular Biology*. 120: 127-136.

Houslay, T.M., Houslay, K.F., Rapkin, J., Hunt, J., & Bussiere, L.F. (2017). Mating opportunities and energetic constraints drive variation in age-dependent sexual signaling. *Functional Ecology*, 31: 728-741.

Khorram, O., Vu, L., & Yen, S.S. (1997). Activation of immune function by dehydroepiandrosterone (DHEA) in age-advanced men. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 52(1), M1-M7.

Lam, O., Wheeler, J., & Tang, C.M. (2014) Thermal control of virulence factors in bacteria: A hot topic, Virulence, 5:8, 852-862, DOI: 10.4161/21505594.2014.970949 Lance VA, Elsey RM, Trosclair III PL. 2015. Sexual maturity in male American alligators in southwest Louisiana. *South American Journal of Herpetology*, 10(1): 58-63.

Lang, J.W. (1987). Crocodilian Thermal Selection. *Wildlife management: crocodiles and alligators.* IBSN 0949324094.

Lebeis, S.L., Sherman, M.A., & Kalman, D. (2008). Protective and destructive innate immune responses to enteropathogenic Escherichia coli and related A/E pathogens. *Future Microbiology*, 3(3): 315-328.

Mazerolle, M. (2006). Improving data analysis in herpetology: using Akaike's Information Criterion (AIC) to assess the strength of biological hypotheses. *Amphibia-Reptilia*, 27(2), 169-180.

Merchant, M.E., Roche, C., Elsey, R.M., & Prudhomme, J. (2003). Antibacterial properties of serum from the American alligator (Alligator mississippiensis). *Comparative Biochemistry and Physiology Part B*, 136:505-513.

Merchant, M.E., Roche, C.M., Thibodeaux, D., & Elsey, R.M. (2005). Identification of alternative pathway serum complement activity in the blood of the American alligator (Alligator mississippiensis). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 141(3), 281-288.

Merchant, M., Heard, R., & Monroe, C. (2009). Characterization of phospholipase A2 activity in serum of the American alligator (Alligator mississippiensis). *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 311(9), 662-666.

Miajlovic, H. & Smith, S.G. (2014). Bacterial self-defence: how Escherichia coli evades serum killing. *FEMS microbiology letters*, 354(1), 1-9.

Moreau, J.M., Girgis, D.O., Hume, E.B., Dajcs, J.J., Austin, M.S., & O'Callaghan, R.J. (2001). Phospholipase A2 in rabbit tears: a host defense against Staphylococcus aureus. *Investigative ophthalmology & visual science*, 42(10), 2347-2354.

Mougeot, F., Irvine, J.R., Seivwright, L., Redpath, S.M., & Piertney, S. (2004). Testosterone, immunocompetence, and honest sexual signaling in male red grouse. *Behavioral Ecology*, 15(6), 930-937.

Nikoskelainen, S., Lehtinen, J., & Lilius, E.M. (2002). Bacteriolytic activity of rainbow trout (Oncorhynchus mykiss) complement. *Developmental & Comparative Immunology*, 26(9), 797-804.

Nowak, J., Pawłowski, B., Borkowska, B., Augustyniak, D., & Drulis-Kawa, Z. (2018). No evidence for the immunocompetence handicap hypothesis in male humans. *Scientific reports*, 8(1), 7392.

Nguyen, H.D.N., Yang, Y.S., & Yuk, H.G. (2014). Biofilm formation of Salmonella Typhimurium on stainless steel and acrylic surfaces as affected by temperature and pH level. *LWT-Food Science and Technology*, 55(1), 383-388.

Owen-Ashley, N.T., Hasselquist, D., & Wingfield, J.C. (2004). Androgens and the immunocompetence handicap hypothesis: unraveling direct and indirect pathways of immunosuppression in song sparrows. *The American Naturalist*, 164(4), 490-505.

Paczosa, M.K. & Mecsas, J. (2016). Klebsiella pneumoniae: going on the offense with a strong defense. *Microbiology and Molecular Biology Reviews*, 80(3), 629-661.

Pedersen, K., Lassen-Nielsen, A.M., Nordentoft, S., & Hammer, A.S. (2009). Serovars of Salmonella from captive reptiles. *Zoonoses and public health*, 56(5), 238-242.

Poiani, A., Goldsmith, A.R., & Evans, M.R. (2000). Ectoparasites of house sparrows (Passer domesticus): an experimental test of the immunocompetence handicap hypothesis and a new model. *Behavioral Ecology and Sociobiology*, 47: 230-242.

Reber, S.A., Janisch, J., Torregrosa, K., Darlington, J., Vliet, K.A., & Fitch, W.T. (2017). Formants provide honest acoustic cues to body size in American alligators. *Scientific reports*, 7(1), 1816.

Regelson, W., Loria, R., & Kalimi, M. (1994). Dehydroepiandrosterone (DHEA) – the "mother steroid". *Annuals of the New York Academy of Sciences*, 719: 553-563.

Rios, F.M. & Zimmerman, L.M. (2001). Immunology of reptiles. *eLS*, 1-7.

Roberts, M.L., Buchanan, K.L., & Evans, M.R. (2004). Testing the immunocompetence handicap hypothesis: a review of the evidence. *Animal Behavior*, 68: 227-239.

Roberts, M.L., Buchanan, K.L., Hasselquist, D., & Evans, M.R. (2007). Effects of testosterone and corticosterone on immunocompetence in the zebra finch. *Hormones and Behavior*, 51(1), 126-134.

Rollins-Smith, L.A. & Woodhams, D.C. (2012). Amphibian immunity (pp. 92-143). New York: Oxford University Press.

Scott, T. & Foster, B.G. (1997). Salmonella spp. in free-ranging and farmed alligators (Alligator mississippiensis) from Texas and Louisiana USA. *Aquaculture*, 156(1-2), 179-181.

Seebacher, F., Elsey, R.M., & Trosclair III, P.I. (2003). Body temperature null distributions in reptiles with nonzero heat capacity: seasonal thermoregulation in the American alligator (*Alligator missipssippiensis*). *Physiological and Biochemical Zoology*, 76(3): 348-359.

Soma. K.K. & Wingfield, J.C. (2001). Dehydroepiandrosterone in Songbird Plasma: Seasonal Regulation Relationship to Territorial Aggression. *General and Comparative Endocinology*. 123:144-155.

Suzuki, T., Suzuki, N., Daynes, R.A., & Engleman, E.G. (1991). Dehydroepiandrosterone enhances IL2 production and cytoxic effector function of human T cells. *Clinical Immunology and Immunopathology*, 61(2): 202-211.

Trumble, B.C., Blackwell, A.D., Stieglitz, J., Thompson, M.E., Suarez, I.M., Kaplan, H., & Gurven, M. (2016). Associations between male testosterone and immune function in a pathogenically stressed forager-horticultural population. *American journal of physical anthropology*, 161(3), 494-505.

Tutar, U., Çelik, C., Ataş, M., Tunç, T., & Gözel, M. (2015). Evaluation of biofilm formation activity of standard microorganism strains. *Journal of Clinical and Experimental Investigations*, 6(2), 135-139.

Van der Kraak, G. & Pankhurst. N.W. (1996). Temperature effects on reproductive performance of fish. *Global Warming: Implications for freshwater and marine fish.* Cambridge University Press, pp. 159-176.

Vilet, K.A. (1989). Social displays of the American alligator (*Alligator mississippiensis*). *American Zoology*, 29: 1019-1031.

Wingfield, J.C., Lynn, S.E., & Soma, K.K. (2001). Avoiding the 'Costs' of Testosterone: Ecological Bases of Hormone-Behavior Interactions. *Brain, Behavior, and Evolution*, 57:239-251.

Woodward, H.N., Horner, J.R., & Farlow, J.O. (2011). Osteohistological evidence for determinate growth in American alligator. *Journal of Herpetology*, 45(3): 339-342.

Zimmerman, L.M., Bowden, R.M., & Vogel, L.A. (2013). Red-eared slider turtles lack response to immunization with keyhole limpet hemocyanin by have high levels of natural antibodies. *ISRN Zoology*. 2013: 1-8.

Zuur, A., Ieno, E.N., Walker, N., Saveliev, A.A., & Smith, G.M. (2009). Mixed Effect Models an Extensions in Ecology with R. *Springer Science.*

Tables

Table 2.1 Optimized assay conditions for *E. coli* (ATCC# 8739), *S. typhimurium* (ATCC# 14028), and *K. pneumoniae* (ATCC#10031).

Bacteria	**Challenge Temperature (C)**	**Incubation Time (min)**	**Bacterial Concentration (CFUs)**	**Plasma Dilution**
E. coli (ATCC# 8739)	15 30	60 30	10^5	1:5
S. typhimurium (ATCC# 14028)	15 30	30 30	10^3	1:5
K. pneumoniae (ATCC# 10031)	15 30	60 60	10^3	1:5

Table 2.2 Mean monthly testosterone concentrations for alligators sampled across the entire study period (2006-2010). Samples from the primary testosterone phase appear in white and samples from the secondary testosterone phase appear in in dark gray. Data are from Hamlin et al. 2011.

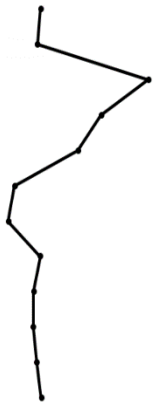
	Month	Mean Testosterone (pg/100ul)	
Primary	January	120.8	
	February	109.7	
	March	472.0	
	April	318.3	
	May	242.8	
	June	35.8	
	July	14.8	
Secondary	August	119.76	
	September	97.4	
	October	96.1	
	November	107.9	
	December	122.4	

Table 2.3 Mean and range of bacteria killing scores for each temperature condition across all bacteria. Killing is represented on a scale of 0-1.

Bacteria	**Range**	**Mean**	**Temperature-Dependent Mean**	
			15	**30**
E. coli (ATCC# 8739)	0 – 1	0.29 ± 0.30	0.24 ± 0.24	0.34 ± 0.35
S. typhimurium (ATCC# 14028)	0 – 0.35	0.13 ± 0.07	0.15 ± 0.07	0.11 ± 0.06
K. pneumoniae (ATCC# 10031)	0 - 1	0.64 ± 0.27	0.67 ± 0.26	0.61 ± 0.28

Table 2.4 Comparison of models explaining variation in *E. coli* (top), *S. typhimurium* (middle), and *K. pneumoniae* (bottom) killing ability. Parameters from the best fitting model for each bacteria are shown in bold.

***E. coli* (n=622)**	K	AIC	Δ AIC	df
T/DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T/DHEA:Phase + T/DHEA:Challenge Temperature + T/DHEA:SVL +[Year] + [ID]	**10**	**-991.57**	**0**	**12**
T+ Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase+ T:Challenge Temperature + T:SVL +[Year] + [ID]	10	-952.52	39.0	12
T + T/DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL + T/DHEA:Phase + T/DHEA:Challenge Temperature + T/DHEA:SVL +[Year] + [ID]	14	-912.90	78.7	16
T + DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL + DHEA:Phase + DHEA:Challenge Temperature + DHEA:SVL +[Year] + [ID]	14	-875.08	116.5	16
***S. typhimurium* (n=625)**	K	AIC	Δ AIC	df
T/DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T/DHEA:Phase + T/DHEA:Challenge Temperature + T/DHEA:SVL +[Year] + [ID]	**10**	**-1672.84**	**0**	**12**
T+ Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase+ T:Challenge Temperature + T:SVL +[Year] + [ID]	10	-1629.19	43.7	12
T + T/DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL + T/DHEA:Phase + T/DHEA:Challenge Temperature + T/DHEA:SVL +[Year] + [ID]	14	-1584.12	88.7	16
T + DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL + DHEA:Phase + DHEA:Challenge Temperature + DHEA:SVL +[Year] + [ID]	14	-1550.92	121.9	16
***K. pneumoniae* (n=622)**	K	AIC	Δ AIC	df
T/DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T/DHEA:Phase + T/DHEA:Challenge Temperature + T/DHEA:SVL +[Year] + [ID]	**10**	**117.70**	**0**	**12**
T+ Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL +[Year] + [ID]	10	154.92	37.2	12
T + T/DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL + T/DHEA:Phase + T/DHEA:Challenge Temperature + T/DHEA:SVL +[Year] + [ID]	14	192.49	74.8	16
T + DHEA + Phase + Challenge Temperature + SVL + Phase:Challenge Temperature + T:Phase + T:Challenge Temperature + T:SVL + DHEA:Phase + DHEA:Challenge Temperature + DHEA:SVL +[Year] + [ID]	14	224.10	106.4	16

Table 2.5 T/DHEA model results for *S. typhimurium* killing ability. Significant predictors (P <0.05) appear in bold.

***S. typhimurium* (n = 625)**				
Fixed Effects	**Estimate**	**Std Error**	**T value**	**P value**
T/DHEA	0.0085	0.0168	0.506	0.6131
Testosterone Phase[Secondary]	-0.0138	0.0081	-1.713	0.0874
Challenge Temperature[30]	**-0.0383**	**0.0052**	**-7.303**	**2.40e-12**
SVL	-0.0002	0.0001	-1.598	0.1110
Phase[Secondary]:Challenge Temperature[30]	-0.0047	0.0072	-0.648	0.5173
T/DHEA:Phase[Secondary]	-0.0083	0.0083	-1.421	0.1564
T/DHEA:Challenge Temperature[30]	**0.0048**	**0.0017**	**2.791**	**0.0056**
T/DHEA:SVL	-0.0001	0.0001	-0.722	0.4709

Table 2.6 T+ DHEA model results for *S. typhimurium* killing ability. Significant predictors (P <0.05) appear in bold.

***S. typhimurium* (n = 625)**				
Fixed Effects	**Estimate**	**Std Error**	**T value**	**P value**
T	0.00005	0.0001	0.344	0.7307
DHEA	-0.00002	0.0002	-0.070	0.9442
Testosterone Phase[Secondary]	-0.0142	0.0154	-0.920	0.3585
Challenge Temperature[30]	**-0.0186**	**0.0085**	**-2.194**	**0.0290**
SVL	-0.0003	0.00003	-1.043	0.2977
Phase[Secondary]:Challenge Temperature[30]	-0.0071	0.0072	-0.980	0.3281
T:Phase[Secondary]	-0.00003	0.00004	-0.778	0.4374
T:Challenge Temperature[30]	**0.00004**	**0.00002**	**2.476**	**0.0138**
T:SVL	-0.00000	0.00000	-0.678	0.4982
DHEA:Phase[Secondary]	-0.00000	0.00008	-0.077	0.9386
DHEA:Challenge Temperature[30]	**-0.0001**	**0.00004**	**-2.886**	**0.0042**
DHEA:SVL	0.00000	0.00000	0.404	0.6864

Table 2.7 T/DHEA model results for *E.coli* killing ability. Significant predictors (p <0.05) appear in bold.

***E.coli* (n = 622)**				
Fixed Effects	**Estimate**	**Std Error**	**T value**	**P value**
T/DHEA	0.0225	0.0271	0.830	0.4071
Testosterone Phase[Secondary]	-0.0149	0.0135	-1.102	0.2711
Challenge Temperature[30]	**0.0305**	**0.0097**	**3.131**	**0.0019**
SVL	0.0000	0.0002	0.102	0.9189
Phase[Secondary]:Challenge Temperature[30]	-0.0056	0.0134	-0.422	0.6734
T/DHEA:Phase[Secondary]	-0.0025	0.0093	-0.270	0.7874
T/DHEA:Challenge Temperature[30]	-0.0032	0.0032	-1.014	0.3114
T/DHEA:SVL	-0.0002	0.0002	-0.915	0.3612

Table 2.8 T + DHEA model results for *E. coli* killing ability.

***E. coli* (n=622)**				
Fixed Effects	**Estimate**	**Std Error**	**T value**	**P value**
T	0.0001	0.0002	0.571	0.569
DHEA	0.0001	0.0004	0.347	0.729
Testosterone Phase[Secondary]	-0.0050	0.0243	-0.208	0.836
Challenge Temperature[30]	0.0178	0.0158	1.130	0.259
SVL	-0.00002	0.0005	-0.033	0.973
Phase[Secondary]:Challenge Temperature[30]	0.0017	0.0135	0.126	0.900
T:Phase[Secondary]	0.00004	0.00006	0.674	0.501
T:Challenge Temperature[30]	0.00003	0.00003	1.040	0.299
T:SVL	-0.00000	0.00000	-0.925	0.356
DHEA:Phase[Secondary]	-0.0001	0.0001	-0.846	0.398
DHEA:Challenge Temperature[30]	0.00000	0.00007	0.072	0.943
DHEA:SVL	0.00000	0.00000	0.046	0.963

Table 2.9 T/DHEA model results for *K. pneumoniae* killing ability. Significant predictors (P <0.05) appear in bold.

***K. pneumoniae* (n = 622)**				
Fixed Effects	**Estimate**	**Std Error**	**T value**	**P value**
T/DHEA	0.0833	0.0706	1.180	0.2391
Testosterone Phase[Secondary]	**0.0834**	**0.0341**	**2.446**	**0.0148**
Challenge Temperature[30]	**-0.0756**	**0.0230**	**-3.284**	**0.0011**
SVL	0.0001	0.0006	0.179	0.8584
Phase[Secondary]:Challenge Temperature[30]	0.0446	0.0316	1.412	0.1590
T/DHEA:Phase[Secondary]	-0.0381	0.0290	-1.525	0.1283
T/DHEA:Challenge Temperature[30]	0.0025	0.0076	0.332	0.7398
T/DHEA:SVL	-0.0004	0.0005	-0.958	0.3390

Table 2.10 T + DHEA model results for *K. pneumoniae* killing ability. Significant predictors (P <0.05) appear in bold.

***K. pneumoniae* (n=622)**				
Fixed Effects	**Estimate**	**Std Error**	**T value**	**P value**
T	0.0008	0.0006	1.435	0.1523
DHEA	-0.0012	0.0010	-1.107	0.2693
Testosterone Phase[Secondary]	0.0559	0.0661	0.845	0.3985
Challenge Temperature[30]	-0.0568	0.0372	-1.525	0.1283
SVL	-0.0004	0.0013	-0.311	0.7563
Phase[Secondary]:Challenge Temperature[30]	0.0350	0.0319	1.097	0.2733
T:Phase[Secondary]	**-0.0003**	**0.0002**	**-2.088**	**0.0376**
T:Challenge Temperature[30]	-0.00006	0.00008	-0.728	0.4673
T:SVL	-0.00000	0.00000	-0952	0.3419
DHEA:Phase[Secondary]	0.0002	0.0003	0.692	0.4897
DHEA:Challenge Temperature[30]	-0.00002	0.0002	-0.112	0.9106
DHEA:SVL	0.00000	0.00000	0.677	0.4991

Figures

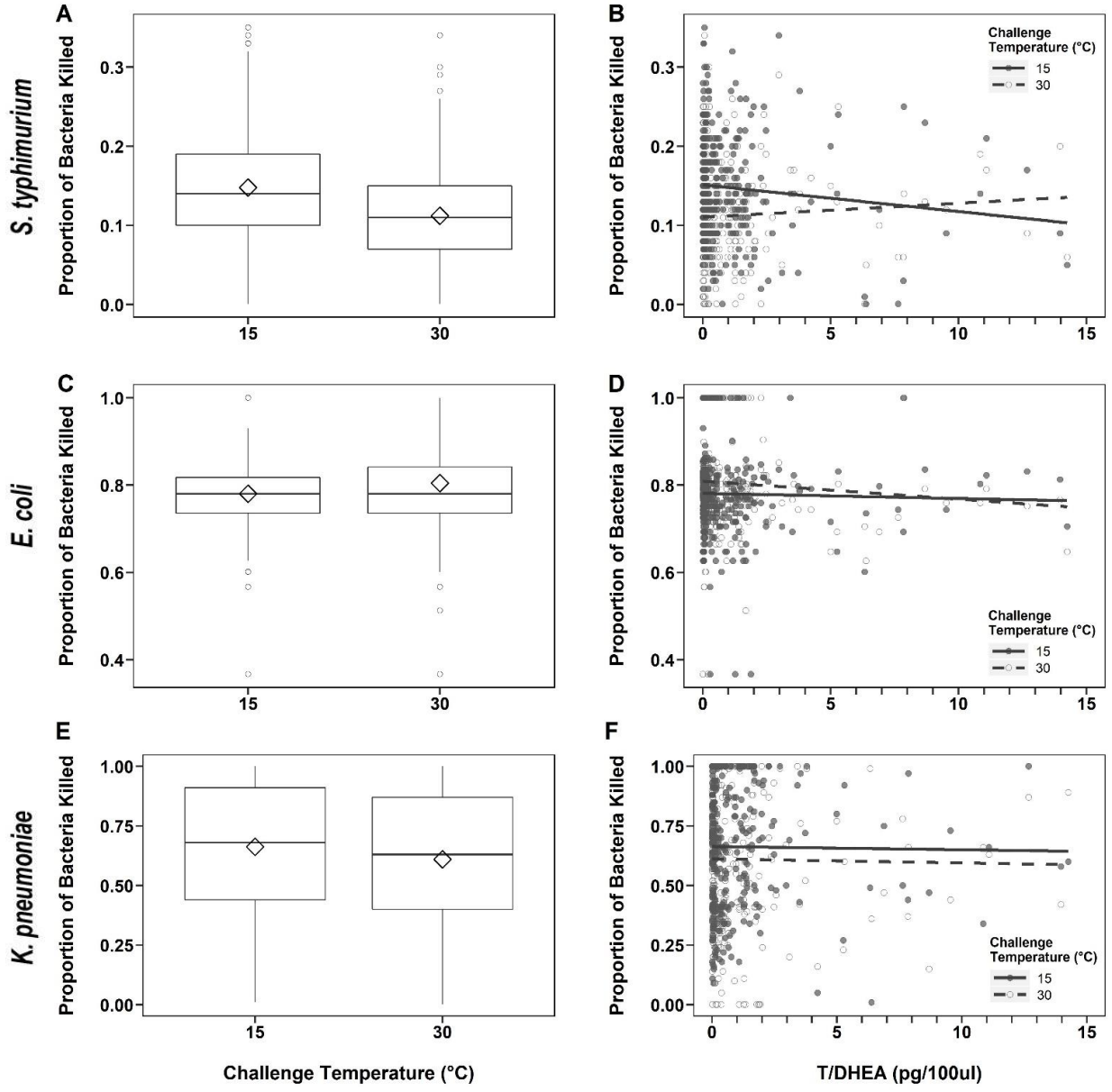


Figure 2.1 (A,C,E) A comparison of killing ability for three bacteria at two challenge temperatures (15℃ and 30℃). (B,D,F) The relationship between bacterial killing and T/DHEA across both challenge temperatures (15℃: solid; 30℃: dashed). For *E. coli*, the transformed BKA scores are shown.

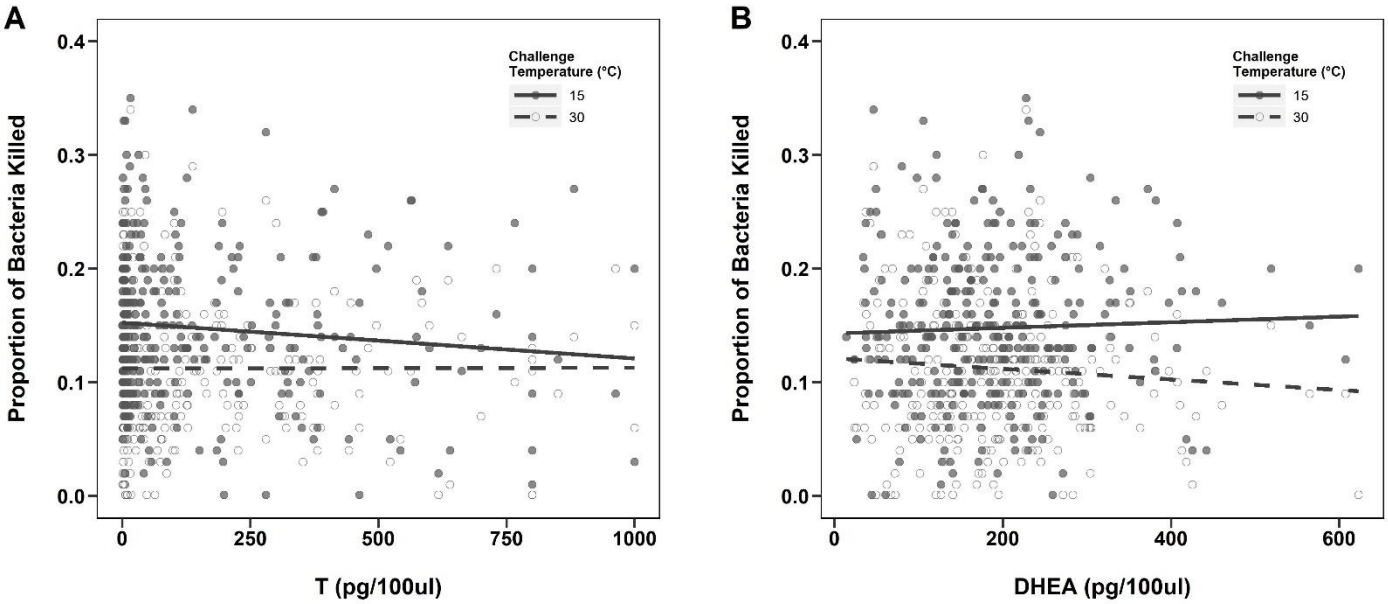


Figure 2.2 The relationship between (A) T and (B) DHEA and *S. typhimurium* killing at two challenge temperatures, 15℃ (solid) and 30℃ (dashed line).

CHAPTER 3

THERMOREGULATORY STRATEGY AND MICROBIAL SPECIES DETERMINE TEMPERATURE EFFECTS ON IMMUNE PERFORMANCE

Introduction

Temperature plays an important role in many biological functions (e.g. metabolism, growth) by placing physical constraints on fundamental biochemical reactions (Rome et al., 1992; Hochochka and Somero, 2002). These constraints scale up to produce thermal sensitivities that may influence an individual's physiology, behavior and fitness (Angilletta, 2009). Organisms are confronted with variable environmental temperatures that fluctuate daily and seasonally. In response, vertebrates use one of two strategies: (1) adjust metabolic function to regulate and stabilize body temperatures within a set thermal range (endotherms) or (2) passively conform to environmental temperatures and use behavioral techniques to increase or reduce body temperatures (ectotherms; Seebacher, 2009). Each thermoregulatory strategy has its costs and benefits that shape thermal sensitivity patterns. Endotherms expend energy to maintain a strict thermal range, allowing them to maximize efficiency of biological processes within that range (Angilletta et al., 2010). In response, they may experience inefficient, or suboptimum performance when temperatures deviate from their set temperature range. Alternatively, ectotherms experience imperfect thermoregulation since they rely on available thermal gradients present within their environment. This likely requires flexibility of biological processes to perform across a broader range of temperatures (Angilletta et al., 2002).

Effects of different temperatures on biological processes are often captured using thermal performance curves that plot performance measures across a range of temperatures. From these curves, the thermal limitations and optimum temperatures for different biological processes can be identified (Huey and Stevenson, 1979). An organisms' optimum temperature is designated as the temperature at which it experiences peak performance (Huey and Stevenson, 1979) and deviation in performance as the organism moves away from the optimum temperature can provide important insights about the nature of an organism's thermal sensitivity. For example, energetic constraints may impose trade-offs between achieving high performance at optimum temperature and maintaining performance across other temperatures (Huey and Slatkin, 1976). Alternatively, high performance at optimum temperature may reflect the ability to consistently perform well across all temperatures, as in the "jack-of-all trades, master of all" hypothesis (Huey and Hertz, 1984).

Immune performance is a biological process that is strongly temperature dependent. For example, immunity has been shown to increase with warmer temperatures, as seen with enhanced pathogen clearance under fever conditions (Evans et al., 2016; Roberts, 1979), driven by increased neutrophil, cytokine, and natural killer cell activity under febrile temperatures (Zanker and Lange, 1982; Kappel et al., 1991; Hasday et al., 2000; Ostberg et al., 2000). Though fever responses are mostly studied in endotherms, many ectotherms show fever responses by seeking out warmer areas in their environments to increase body temperatures at the onset of infection (Merchant et al., 2007; Rakus et al., 2017; Sherman et al., 1991). Moreover, low temperatures have been linked to reduced immune performance, including observations of lower phagocytic capacity, cytokine production, and lymphocyte proliferation in both endotherms (Bouma et al., 2010) and ectotherms (Mondal and Rai, 2001; Sacchi et al., 2014).

Interestingly, assessment of some aspects of vertebrate immune performance relies heavily on temperature. Immunity is typically measured either by quantifying amounts of specific immune components (e.g. white blood cells, antibodies, cytokines), or by assessing the functional response to an immune challenge (e.g. bacteria killing, hemolysis, lymphocyte proliferation; Calder, 2007). Though both types of measures characterize an individual's immune status, the latter more directly quantifies an active immune response against a stimulus (Albert-Vega et al., 2018). Interestingly, functional measures of immunity are often reliant on temperature. For instance, bacteria killing assays measure the capacity of blood (whole blood, serum, or plasma) to kill a fixed concentration of bacteria under a set temperature condition (Matson et al., 2006; Millet et al., 2007). Therefore, the measured response is a reflection of the performance of immune components at a given temperature. In general practice, killing assays are performed under a single temperature, commonly the optimum body temperature of the focal organism (Beck et al., 2017; Liebl and Martin, 2009; Matson et al., 2006; Millet et al., 2007). However, given that most wild organisms function under fluctuating temperatures, measuring immune performance at a single assay temperature may not capture the true range of immune responses that organisms experience under natural conditions.

In this study, we used microbial killing assays to quantify immune performance for vertebrates with different thermal strategies under different temperature conditions. Specifically, we selected 7 species, including 4 ectotherms and 3 endotherms, and quantified their immune performance at four challenge temperatures: 15℃, 25℃, 37℃, and 45℃. These temperatures encompassed each species' optimum temperature and extended across a range both above and below these optimum temperatures. Further, assays were repeated across three microbes (*E. coli, S. typhimurium, and C. albicans*) that also varied in their optimum temperatures. We

characterized thermal sensitivity patterns in immune function by comparing immune performance across challenge temperatures and vertebrate thermal strategies, expecting temperature effects to be greatest at temperature extremes (15℃ & 45℃), and among endotherms given their potentially less flexible thermal sensitivity. We also assessed whether measuring immune performance at a species' optimum temperature was representative of immune performance across temperatures, by examining relationships between immune performance at optimum temperatures and consistency in immune performance across temperatures. We expected a negative relationship to occur in endotherms, since they maximize performance under strict thermal ranges and thus, may experience greater energetic constraints. In ectotherms, we expected a positive relationship reflecting a "jack – of – all – trades" strategy, with greater consistency of immune performance across temperatures, as they are often exposed to broader ranges of body temperatures, and thus, may experience greater flexibility in performance. Finally, we reflected on general methodological practice in light of our findings. Based on our expected outcomes, it would suggest that typical methodological practices may not reliably capture important immune variability.

Methods

ANIMALS AND SAMPLING

To capture temperature-immunity trade-offs across species that employ different thermal strategies and inhabit a broad range of environmental conditions, we focused on seven species, four ectothermic species: axolotl (*Ambysotma mexicanum*), Eastern hellbender (*Cryptobranchus alleganiensis*), timber rattlesnake (*Crotalus horridus*), and American alligator (*Alligator mississippiensis*); and three endothermic species: Japanese quail (*Coturnix japonica*), domestic

chicken (*G. gallus domesticus)* and domestic horse (*Equus caballus*). Using comparable protocols, blood samples were collected from 19 – 30 individuals of each species. Blood was drawn into heparinized syringes, capillary or vacutainer tubes. Following collection, plasma was isolated by centrifugation and stored at -80℃ prior to immunological assays.

Samples were collected across a range of captive, wild, and domestic populations. Axolotl samples were collected from captive individuals housed at the University of Kentucky. Eastern hellbender samples were collected from a wild population at a confidential stream site in western Virginia. Timber rattlesnakes samples were collected from a population residing at the Di-Lane Wildlife Management Area in Georgia. American alligator samples were collected from a wild population at the Tom Yawkey Wildlife Refuge in South Carolina. Japanese quail and domestic chicken samples were collected from captive individuals housed at the University of Georgia (UGA) Poultry Research Center. Finally, domestic horse samples were collected during routine veterinary scans conducted by the UGA Veterinary Teaching Hospital. All samples were collected between 2018-2020, with the exception of the alligator samples, which were collected in 2011.

IMMUNE ASSAYS

To quantify microbial killing ability, we followed a spectrophotometer-based protocol described by French & Neuman-Lee (2012) with minor modifications. Assays were performed with three microbial species: *Escherichia coli* (EPower Microorganisms, Microbiologics, St. Cloud, MN, USA, REF# 0483E7, ATCC# 8739), *Salmonella typhimurium* (KwikStik, Microbiologics, St. Cloud, MN, USA, REF# 0363P, ATCC# 14028) and *Candida albicans* (KwikStik, Microbiologics, St. Cloud, MN, USA, REF# 0443P , ATCC# 10231), at four challenge temperatures: 15℃, 25℃, 37℃, and 45℃. The selected microbes included two

bacteria (*E. coli* and *S. typhimurium*) and one fungus (*C. albicans*) that ranged across different thermal optima. *E. coli* and *S. typhimurium* experience optimum growth at 37°C, while *C. albicans* experiences optimum growth at a, cooler, 30°C (Bergey, 2005). Challenge temperatures were selected to capture appropriate thermal ranges for all focal species (Table 3.1; Nickerson and Mays, 1973; Lance, 1994; McNab, 1996; Beaupre and Duvall, 1998; Björklund and Duhon, 1999; Green et al., 2005). For *E. coli*, samples were challenged for 30 minutes, and for *S. typhimurium* and *C. albicans*, samples were challenged for 60 minutes following standard protocols (French and Neuman-lee, 2012; Liebl and Martin, 2009). Microbial concentrations were prepared by creating a 10^8 solution from plated colonies using the BD BBL™ Prompt™ Inoculation System, followed by dilution with phosphate buffered saline (PBS), to create a 10^5 concentration used in all assays.

To assess microbial killing across a physiological range of plasma concentrations, we serially diluted pooled plasma samples from each species to create nine different dilutions (1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, & 1:256). For all species except quail, samples from 15 individuals were randomly assorted into groups of 3 to produce 5 pools. Due to small plasma volumes obtained from individual quail, samples from 20 individuals from this species were randomly assorted into groups of 5 to produce 4 pools. Assays were performed by first serially diluting pooled plasma samples across a 96 well plate. 18ul of PBS was added to wells in the first 9 columns of the plate, followed by 18ul of pooled plasma. Plasma and PBS were mixed using a multichannel pipette to create a 1:1 dilution and then 18ul of this mixture was removed and transferred to the next column (1:2 dilution) and re-mixed. This procedure was repeated until the 9th column (1:256) for which 18ul of the mixture was removed and discarded. All plasma samples were prepared in duplicate in this manner, then 6ul of microbe solution was added to

each well. All other wells on the plate contained positive (n = 8) and negative (n = 8) controls. Positive controls were made by adding 6ul of microbe solution to 18ul of PBS, and negative controls contained 24ul of PBS. Each plate was vortexed to mix the contents of each well and then incubated at 15℃, 25℃, 37℃, or 45℃ for the appropriate challenge time (30 or 60 minutes). After the allotted challenge time, all plates were removed from the incubator and tryptic soy broth (125ul) was added to all wells, and then the plates were vortexed. Prior to microbial growth, an initial background absorbance reading was recorded for each well at 300nm for *E. coli* and *S. typhimurium* and 340nm for *C. albicans* using a standard microplate reader. Plates were then incubated for 12hrs at 37℃ for *E. coli* and *S. typhimurium* and for 24hrs at 30℃ for *C. albicans*. Following incubation, plates were vortexed to homogenize the contents of each well and re-read to obtain a final, post-incubation, absorbance for each well.

IMMUNE PERFORMANCE METRICS

Three metrics were used to quantify immune performance: microbial killing ability, microbial growth, and minimum killing concentration. To calculate these metrics, first, a dose response curve was fit to microbial killing data for each species-microbe-temperature pairing across plasma dilutions using the *GRmetrics* package in R version 3.6.1 (Clark et al., 2016; Figure 3.1). To do this, plasma dilutions were converted into concentrations (1.0, 0.5, 0.25, 0.125, 0.063, 0.031, 0.016, 0.008, & 0.004), such that a low dilution corresponded to a high concentration, or dose of plasma, while a high dilution corresponded to a low concentration, or dose of plasma. The assay response was then quantified as the relative microbial cell count, using the following equation:

$$\text{Relative Microbial Cell Count} = \frac{\text{Mean (Duplicate Sample Absorbance)}}{\text{Positive Control Mean Absorbance}}$$

Background absorbance values were subtracted from all post-incubation absorbance values prior to averaging across duplicates. The relative cell count reflects the amount of microbial growth that occurred within a sample; a relative cell count of 0 represents no growth, or high killing, and a relative cell count of 1 represents uninhibited growth, or no killing.

From each dose-response curve, we generated a series of metrics to quantify the immune performance of each sample, for all species-temperature-microbe combinations. The three performance metrics were:

(i) Microbial killing ability: a point estimate of microbial inhibition. Microbial killing ability is a traditional metric calculated and reported for microbial killing assays (Demas et al., 2011). We calculated this value by subtracting the relative cell count from 1 to obtain the quantity killed. To select the most appropriate single dilution for calculating microbial killing for each sample, we identified the dilution at which killing was closest to 50% at the temperature closest to the species' optimum temperature (Table 3.1), an approach often used for optimizing microbial killing assay conditions (Beck et al., 2017; French and Neuman-lee, 2012). We then used this single 'optimum dilution' to calculate microbial killing for all challenge temperatures for a given sample. High microbial killing reflects a higher point estimate of microbial inhibition, while low microbial killing indicates a lower point estimate of microbial inhibition.

(ii) Microbial growth: cumulative microbial growth response across the observed range of plasma concentrations. This value was determined by calculating area under the dose-response curve (AUC), a common calculation used for dose-response analysis (Huang, 2012; Fallahi-Sichani et al., 2013). Similar to microbial killing ability, AUC provides an estimate of microbial inhibition, but does so across the full physiological range of plasma concentrations rather than for a single concentration. A high AUC indicates reduced inhibition of microbial growth across

plasma concentrations whereas a low AUC indicates greater inhibition of microbial growth across plasma concentrations.

(iii) Minimum killing concentration: the lowest concentration of plasma that elicits a killing response. This value was determined for each sample by selecting the lowest plasma concentration at which killing, indicated by a relative cell count < 1, occurred. A low minimum killing concentration indicates higher sensitivity of plasma, whereas a high concentration indicates lower sensitivity of plasma.

STATISICAL ANALYSES

First, we examined the effect of temperature on immune performance across thermal strategies and microbes. To do this, we used a series of linear mixed models (LMM), with models for each microbe run separately. LMMs were run using the *lmer* package in R version 3.6.1 (Bates et al., 2015). An immune performance metric served as the response variable in each model, while challenge temperature and thermal strategy were included as fixed effects. An interaction term between challenge temperature and thermal strategy was also included to assess how thermal strategy mediated effects of temperature on immune performance. Finally, pool (i.e. sample) ID was included as a random effect to account for repeated measures across temperature treatments. Pairwise comparisons of least square means were computed from contrasts between temperatures both within and among thermal strategies using the *lsmeans* package in R version 3.6.1 (Lenth, 2016). P-value adjustments to account for multiple comparisons were made using the Tukey HSD method. All three immune performance metrics (microbial killing ability, microbial growth and minimum killing concentration) were checked for normality using Shapiro-Wilk's tests. Cube root transformations were used to normalize distributions for minimum killing concentration for *E. coli*, *S. typhimurium* and *C. albicans*, and microbial killing

ability for *S. typhimurium* and *C. albicans*. In all cases, model residuals were examined to evaluate model validity (Zuur et al., 2009).

Next, we tested for the presence of trade-offs between a species' performance at its optimum body temperature versus its performance across temperatures. For each species, we used the challenge temperature closest to the species optimum body temperature (Table 3.1) as its optimum temperature. We then compared immune performance (microbial killing ability, microbial growth and minimum killing concentration) at this optimum temperature against performance consistency, measured as range (max – min) of performance across all challenge temperatures. A greater range in immune performance indicated a larger difference between the highest and lowest performance value, demonstrating lower overall consistency in immune performance across temperatures. We used a series of LMMs to perform this analysis, with a separate model for each microbe-performance metric combination. In each model, performance range across temperatures served as the response variable and performance at the optimum temperature served the predictor, with thermal strategy as a covariate. An interaction term between performance at the optimum temperature and thermal strategy was also included to examine how thermal strategy may mediate potential trade-offs between performance under optimum temperature conditions vs. consistency across temperatures. Finally, species ID was included as a random effect to account for variation among species. Immune performance metrics were checked for normality using a Shapiro-Wilk's tests. A cube-root transformation was used to normalize the distribution of minimum killing concentration for *E.coli*. Square-root transformations were used to normalize distributions of microbial killing ability for *S. typhimurium* and *C. albicans*. Model residuals were examined for all LMMs to evaluate model validity.

Results

EFFECTS OF TEMPERATURE ON IMMUNE PERFORMANCE

We found that temperature affected immune performance, but that this effect depended on species' thermal strategy and the microbe each species was challenged with. Against *E. coli*, the immune performance of ectotherms uniformly declined across all performance metrics at the highest temperature (45℃), whereas the performance of endotherms was not affected by temperature. This pattern manifested as a significant thermal strategy by temperature interaction for all three performance metrics with ectotherms, but not endotherms, showing sensitivity to temperature (Table 3.2; Figure 3.2 A-C).

Against *S. typhimurium*, microbial killing ability decreased gradually with increasing temperature for both ectotherms and endotherms (Figure 3.2 D). For microbial growth and minimum killing concentration, ectotherms experienced greater reductions in performance at lower temperatures (15℃ – 25℃) that then stabilized across higher temperatures (37℃ – 45℃; Figure 3.2 E – F). Conversely, endotherms showed stable performance across lower temperatures (15℃ – 25℃) with the greatest reductions occurring at higher temperatures (37℃ – 45℃; Figure 3.2 E – F). This pattern manifested as a significant thermal strategy by temperature interaction for microbial growth and minimum killing concentration, with both endotherms and ectotherms showing sensitivity to temperature, but with different trajectories (Table 3.3; Figure 3.2 E –F).

Against *C. albicans*, endotherms showed declines in both microbial killing and microbial growth at the highest temperature (45℃), while the performance of ectotherms was not affected by temperature (Figure 3.2 G – H). This pattern manifested as a significant thermal strategy by temperature interaction for microbial growth, with endotherms but not ectotherms showing

sensitivity to temperature; the exact opposite of the pattern observed against *E. coli* (Table 3.4; Figure 3.2 H). Neither challenge temperature, thermoregulatory strategy or the interaction between the two were significant predictors of minimum killing concentration (Table 3.4; Figure 3.2 I).

TRADE-OFFS BETWEEN PEROFRMANCE AT OPTIMUM TEMPERATURE AND PERFORMANCE CONSISTENCY ACROSS TEMPERATURES

We found evidence of a trade-off between the ability of a species to kill bacteria at its optimum temperature versus its performance range across temperatures, but this trade-off was dependent on thermal strategy. For *E. coli*, immune performance at a species' optimum temperature emerged as a predictor of performance consistency across temperatures (as measured by the performance range) only for a single metric – microbial growth (Table 3.5; Figures 3.3 A – C). Importantly, there was a significant interaction effect in microbial growth where immune performance at the optimum temperature traded off with performance consistency in ectotherms, but showed the opposite pattern in endotherms (Table 3.5; Figure 3.3 B).

For *S. typhimurium*, performance at optimum temperatures emerged as a predictor of performance consistency across temperatures for all metrics (Table 3.6; Figures 3.3 D – F). A significant interaction effect emerged for microbial growth and minimum killing concentration where immune performance at optimum temperature traded off with performance consistency for ectotherms, but showed the opposite pattern in endotherms (Table 3.6; Figures 3.3 E – F). For microbial killing ability, there was a marginal interaction effect where microbial killing at optimum temperature traded off with performance consistency in ectotherms, whereas only a slight trade-off emerged for endotherms (Table 3.6; Figure 3.3 D).

For *C. albicans*, performance at optimum temperatures emerged as a significant predictor of performance consistency for only one metric – microbial killing (Table 3.7; Figures 3.3 G – I). Once again, there was a significant interaction effect for which microbial killing ability at optimum temperature traded off with performance consistency in ectotherms, but not in endotherms (Table 3.7; Figure 3.3 G).

Discussion

Temperature is an important driver of many biological processes, including immune performance (Angilletta, 2009). With organisms residing in thermally fluctuating environments, thermoregulation strategies are important mediators of body temperature variation and may modify thermal sensitivity within vertebrates. Here, we found that challenge temperature affected immune performance, but that this effect was dependent on thermal strategy and microbial species. We also documented a trade-off between performance at optimum temperature and performance consistency across temperatures that was persistent across microbes for ectotherms, but not endotherms. These findings suggest that immune performance demonstrates thermal sensitivity and as expected, greatest sensitivity occurred at temperature extremes. Contrary to expectations, endotherms did not consistently show greater thermal sensitivity than ectotherms. In further contrast to our expectations, ectotherms were unable to maintain high immune performance across temperatures while endotherms could. In consequence, methodological approaches that quantify functional immune responses using a single temperature condition may not accurately reflect the true range of ectotherm immune performance under natural conditions.

Our results show that immune performance, measured using common microbial killing assays, is affected by the temperature at which the immune challenge occurs. Interestingly, these effects are host and microbe specific. For immune challenges using *E. coli* as the focal microbe, ectotherms showed thermal sensitivity while endotherms did not. Specifically, the immune performance of ectotherms declined at 45℃ across all performance metrics. Ectotherms may have experienced greater thermal sensitivity at 45℃ than endotherms due to potential environmental constraints placed on their upper thermal performance limits (Hoffmann et al., 2013). For instance, two of our aquatic ectotherm species, axolotls and hellbenders, are unlikely to be naturally exposed to environmental temperatures of 45℃ and above, suggesting that their physiological processes may not be acclimated to performing under these temperatures. In fact, stream temperatures for hellbenders range on average from 0-30℃, with hellbender critical thermal maximum recorded at just below 40℃ (Terrell et al., 2013). Alternatively, endotherms optimize physiological performance by maintaining warm body temperatures, regardless of environmental temperatures (Seebacher, 2009). Interestingly, endotherm immune performance remained consistent even at low temperatures (15℃). This could be the result of the immune challenge being completed in vitro where potential trade-offs between upregulating metabolism and immune function are removed (Rauw, 2012). Further, *E. coli* exhibits an optimum temperature that more closely matches that of endotherms (~ 37℃; Bergey, 2005). Therefore, temperature may have imposed similar effects on both host and microbe performance as they each moved away from this optimum. This corresponding increase and decrease in performance of both *E. coli* activity and endotherm immune defense may have resulted in more evenly matched host-pathogen interactions across temperatures and thus, greater observed consistency in endotherm immune performance across temperatures.

For immune challenges with *S. typhimurium* as the focal microbe, both ectotherms and endotherms showed thermal sensitivity. Specifically, ectotherms experienced the greatest reduction in immune performance between 15℃ and 25℃, whereas endotherms showed the greatest reduction in immune performance between 37℃ and 45℃. The pattern in endotherms may have been driven by temperature-dependent traits of *S. typhimurium*. For instance, warmer temperatures (~ 37℃) are a cue for the activation of virulence genes in *S. typhimurium* (Duong et al., 2007), likely due to its tight associations with endothermic hosts (Bergey, 2005). Further, *S. typhimurium* can develop biofilms as a defense against host immunity (Tutar et al., 2015), with rates in biofilm formation increasing with temperature (Nguyen et al., 2014). In contrast, the pattern in ectotherms may have arisen if ectotherms were only been able to effectively defend themselves under temperatures at which *S. typhimurium* is likely to be most impaired (15℃).

In immune challenges with *C. albicans* as the focal microbe, endotherms showed thermal sensitivity, while ectotherms did not; directly opposite of what was seen in *E. coli*. Interestingly, optimum temperature for *C. albicans* more closely aligns with those of ectotherms (30℃; French and Neuman-lee, 2012). Thus, similar to *E. coli,* a closer match in optimum temperatures between *C. albicans* and ectotherms, may have allowed for similar effects of temperature on relative performance as they both moved away from this optimum temperature, leading to more consistent immune performance observed in ectotherms across temperatures. Reductions in endotherm immune performance at 45℃ may be due to the ability of *C. albicans* to mount a stress response even at high temperatures that triggers defense mechanisms to protect against host immune defenses (Nicholls et al., 2009; O'Meara et al., 2017; Pumeesat et al., 2017).

To understand if temperature sensitivity can alter interpretations of immune performance measures, we assessed relationships between performance at optimum temperatures and across

temperatures. In contrast to our expectation, we found that ectotherms showed a negative relationship between high immune performance at optimum temperatures and consistency in immune performance across temperatures, a pattern that was consistent across microbes. This negative relationship aligns with expected performance in the presence of energetic constraints (Huey and Slatkin, 1976), whereby ectotherms experience a trade-off between performing well under optimum temperatures and performing consistently well across temperatures. Thus, this finding suggests that for ectotherms, high immune performance measured at a species optimum temperature is not indicative of performance at other, suboptimum, temperatures. Conversely, endotherms consistently showed either a positive relationship, with high performance at optimum temperatures mostly being associated with high consistency in performance across temperatures, or a very weak trade-off. This aligns with the "jack-of-all-trades, master of all" hypothesis, in which processes allowing high performance at optimum temperatures may also allow for high performance across all temperatures (Huey and Hertz, 1984). Thus, for endotherms, immune performance measured at a species optimum temperature should be indicative of how performance at other, suboptimum, temperatures. Endotherms' consistency in performance across temperatures may be explained by the "hotter – is – better" hypothesis, which predicts that organisms with higher optimum temperatures have higher maximum performance (Hamilton, 1973). This hypothesis translates into the general theory that endotherms may experience added physiological benefits from functioning under warmer temperatures that may persist outside of optimum conditions (Hamilton, 1973). Based on our findings, we provide additional support that endothermy serves as a successful thermal strategy, specifically, in terms of immune performance.

Overall, this study reveals patterns of thermal sensitivity of immune performance across vertebrates and identifies methodological considerations that should be taken into account when performing temperature-dependent immune assays. In particular, we found that the temperature sensitivity of immune performance, measured using microbial killing assays, differed in ectotherms and endotherms and depended on the focal microbe. These results indicate that temperature interacts with immunity, and that temperature differences potentially drive variation in immune performance. We also found that while measuring immune performance at a species' optimum temperature may provide an adequate assessment of immune performance across a range of temperatures for endotherms, this is not the case for ectotherms. Thus, characterizing ectotherm immune performance based solely on performance at an optimum temperature does not accurately reflect overall immune functionality across temperatures. This finding is highly relevant to methodological practice since performing functional immune assays to characterize immunity in ectotherms at only a single optimum body temperature is common in the literature (Assis et al., 2013; Kommanee et al., 2012; Siroski et al., 2009), and as a result, important immune variability in ectotherms under natural conditions may frequently be missed. In conclusion, our findings suggest that future studies need to consider temperature, thermoregulatory strategy, microbial species and the interaction among all three when designing experiments involving temperature-dependent immune assays in vertebrates.

References

Adamo, S. A. (2014). The effects of stress hormones on immune function may be vital for the adaptive reconfiguration of the immune system during fight-or-flight behavior. *Integr. Comp. Biol.* 54, 419–426.

Albert-Vega, C., Tawfik, D. M., Trouillet-Assant, S., Vachot, L., Mallet, F. and Textoris, J. (2018). Immune functional assays, from custom to standardized tests for precision medicine. *Front. Immunol.* 9, 1–12.

Alonso-alvarez, C. and Tella, J. L. (2001). Effects of experimental food restriction and body-mass changes on the avian T-cell-mediated immune response. 79,.

Angilletta, M. J. J. (2009). Thermal sensitivity. In *Thermal Adaptation: A theoretical and empirical synthesis*, pp. 35–87. Oxford University Press, Inc.

Angilletta, M. J., Niewiarowski, P. H. and Navas, C. A. (2002). The evolution of thermal physiology in ectotherms. *J. Therm. Biol.* 27, 249–268.

Angilletta, M. J., Cooper, B. S., Schuler, M. S. and Boyles, J. G. (2010). The evolution of thermal physiology in endotherms. *Front. Biosci.* E2, 861–881.

Assis, V. R. de, Monteiro, S. C., Giorgi, A. M., Assis, V. R. De, Christie, S., Titon, M., Maria, A. and Barsotti, G. (2013). Antimicrobial Capacity of Plasma from Anurans of the Atlantic Forest. *South Am. J. Herpetol.* 8, 155–160.

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. Part A Ecol. Integr. Physiol.* 327, 293–301.

Bergey, D. H. (2005). *Bergey's Manual of Systematic Bacteriology, Volume 2: The Proteobacteria*. (ed. Garrity, G. M.) Springer US.

Boonstra, R., Lane, J. E., Boutin, S., Bradley, A., Desantis, L., Newman, A. E. M. and Soma, K.

K. (2008). Plasma DHEA levels in wild, territorial red squirrels: Seasonal variation and effect of ACTH. *Gen. Comp. Endocrinol.* 158, 61–67.

Bouma, H. R., Carey, H. V. and Kroese, F. G. M. (2010). Hibernation: the immune system at rest? *J. Leukoc. Biol.* 88, 619–624.

Calder, P. C. (2007). Immunological Parameters: What Do They Mean? *J. Nutr.* 137, 773S-780S.

Cohen, J. M., Venesky, M. D., Sauer, E. L., Civitello, D. J., McMahon, T. A., Roznik, E. A. and Rohr, J. R. (2017). The thermal mismatch hypothesis explains host susceptibility to an emerging infectious disease. *Ecol. Lett.* 20, 184–193.

Coico, R. and Sunshine, G. (2015). Overview of the Immune System. In *Immunology: A Short Course*, pp. 1–10. John Wiley & Sons, Ltd.

Demas, G. E., Zysling, D. A., Beechler, B. R., Muehlenbein, M. P. and French, S. S. (2011). Beyond phytohaemagglutinin: Assessing vertebrate immune function across ecological contexts. *J. Anim. Ecol.* 80, 710–730.

Duong, N., Osborne, S., Bustamante, V. H., Tomljenovic, A. M., Puente, J. L. and Coombes, B. K. (2007). Thermosensing coordinates a cis-regulatory module for transcriptional activation of the intracellular virulence system in Salmonella enterica serovar typhimurium. *J. Biol. Chem.* 282, 34077–34084.

Evans, S. S., Repasky, E. A. and Fisher, D. T. (2016). Fever and the thermal regulation of immunity: the immune system feels the heat. *Nat. Rev. Immunol.* 15, 335–349.

Ezenwa, V. O., Stefan Ekernas, L. and Creel, S. (2012). Unravelling complex associations between testosterone and parasite infection in the wild. *Funct. Ecol.* 26, 123–133.

Fallahi-Sichani, M., Honarnejad, S., Heiser, L. M., Gray, J. W. and Sorger, P. K. (2013). Metrics other than potency reveal systematic variation in responses to cancer drugs. *Nat. Chem. Biol.* 9, 708–714.

Folstad, I. and Karter, A. J. (1992). Parasites , Bright Males , and the Immunocompetence Handicap. *Am. Nat.* 139, 603–622.

Foo, Y. Z., Nakagawa, S., Rhodes, G. and Simmons, L. W. (2017). The effects of sex hormones on immune function: a meta-analysis. *Biol. Rev.* 92, 551–571.

French, S. S. and Neuman-lee, L. A. (2012). Improved ex vivo method for microbiocidal activity across vertebrate species across vertebrate species. *Biol. Open*.

Guillette, L. J., Cree, A. and Rooney, A. A. (1995). Biology of stress: interactions with reproduction, immunology and intermediary metabolism. *Heal. Welf. Captiv. Reptil.* 32–81.

Habig, B., Doellman, M. M., Woods, K., Olansen, J. and Archie, E. A. (2018). Social status and parasitism in male and female vertebrates: A meta-analysis. *Sci. Rep.* 8, 1–13.

Hamilton, W. J. (1973). *Life's color code.* McGraw-Hill.

Hanson, D. F. (1997). Fever , Temperature , and the Immune Response. *Annu. New York Acad. Sci.* 813, 453–464.

Hau, M. and Wingfield, J. C. (2013). Hormonally-regulated trade-offs: Evolutionary variability and phenotypic plasticity in testosterone signaling pathways. *Mech. Life Hist. Evol.* 349–

361.

Hazeldine, J., Arlt, W. and Lord, J. M. (2010). Dehydroepiandrosterone as a regulator of immune cell function. *J. Steroid Biochem. Mol. Biol.* 120, 127–136.

Hoffmann, A. A., Chown, S. L. and Clusella-Trullas, S. (2013). Upper thermal limits in terrestrial ectotherms: How constrained are they? *Funct. Ecol.* 27, 934–949.

Houslay, T. M., Houslay, K. F., Rapkin, J., Hunt, J. and Bussière, L. F. (2017). Mating opportunities and energetic constraints drive variation in age-dependent sexual signalling. *Funct. Ecol.* 31, 728–741.

Huey, R. B. and Hertz, P. E. (1984). Is a Jack-of-All-Temperatures a Master of None? *Evolution (N. Y).* 38, 441–444.

Huey, R. B. and Stevenson, R. D. (1979). Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. *Integr. Comp. Biol.* 19, 357–366.

Klein, S. L. (2000). The effects of hormones on sex differences in infection: From genes to behavior. *Neurosci. Biobehav. Rev.* 24, 627–638.

Kommanee, J., Preecharram, S., Daduang, S., Temsiripong, Y., Dhiravisit, A., Yamada, Y. and Thammasirirak, S. (2012). Antibacterial activity of plasma from crocodile (Crocodylus siamensis) against pathogenic bacteria. *Ann. Clin. Microbiol. Antimicrob.* 11, 1–9.

Kusumoto, K. (2014). Comparison of Humoral Immune Response Under Low Tempearture in Non-breeding Gray Red-backed Voles (Myodes rufocanus). *Bull. Fac. Agr.* 99, 43–55.

Liebl, A. L. and Martin, L. B. (2009). Simple quantification of blood and plasma antimicrobial

capacity using spectrophotometry. *Funct. Ecol.* 23, 1091–1096.

Martin, L. B. (2009). Stress and immunity in wild vertebrates: Timing is everything. *Gen. Comp. Endocrinol.* 163, 70–76.

Matson, K. D., Tieleman, B. I. and Klasing, K. C. (2006). Capture stress and the bactericidal competence of blood and plasma in five species of tropical birds. *Physiol. Biochem. Zool.* 79, 556–564.

Merchant, M., Williams, S., Trosclair, P. L., Elsey, R. M. and Mills, K. (2007). Febrile response to infection in the American alligator (Alligator mississippiensis). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 148, 921–925.

Millet, S., Bennett, J., Lee, K. A., Hau, M. and Klasing, K. C. (2007). Quantifying and comparing constitutive immunity across avian species. *Dev. Comp. Immunol.* 31, 188–201.

Mondal, S. and Rai, U. (2001). In vitro effect of temperature on phagocytic and cytotoxic activities of splenic phagocytes of the wall lizard, Hemidactylus flaviviridis. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 129, 391–398.

Nguyen, H. D. N., Yang, Y. S. and Yuk, H. G. (2014). Biofilm formation of Salmonella Typhimurium on stainless steel and acrylic surfaces as affected by temperature and pH level. *LWT - Food Sci. Technol.* 55, 383–388.

Nicholls, S., Leach, M. D., Priest, C. L. and Brown, A. J. P. (2009). Role of the heat shock transcription factor, Hsf1, in a major fungal pathogen that is obligately associated with warm-blooded animals. *Mol. Microbiol.* 74, 844–861.

O'Meara, T. R., Robbins, N. and Cowen, L. E. (2017). The Hsp90 Chaperone Network Modulates Candida Virulence Traits. *Trends Microbiol.* 25, 809–819.

Pérez-Rodríguez, L., Blas, J., Viñuela, J., Marchant, T. A. and Bortolotti, G. R. (2006). Condition and androgen levels: are condition-dependent and testosterone-mediated traits two sides of the same coin? *Anim. Behav.* 72, 97–103.

Prall, S. P. and Muehlenbein, M. P. (2014). Testosterone and Immune Function in Primates: A Brief Summary with Methodological Considerations. *Int. J. Primatol.* 35, 805–824.

Pumeesat, P., Muangkaew, W., Ampawong, S. and Luplertlop, N. (2017). Candida albicans biofilm development under increased temperature. *New Microbiol.* 40, 279–283.

Rakus, K., Ronsmans, M. and Vanderplasschen, A. (2017). Behavioral fever in ectothermic vertebrates. *Dev. Comp. Immunol.* 66, 84–91.

Rauw, W. M. (2012). Immune response from a resource allocation perspective. *Front. Genet.* 3, 1–14.

Roberts, N. J. (1979). Temperature and host defense. *Microbiol. Rev.* 43, 241–259.

Roberts, M. L., Buchanan, K. L., Hasselquist, D. and Evans, M. R. (2007). Effects of testosterone and corticosterone on immunocompetence in the zebra finch. *Horm. Behav.* 51, 126–134.

Rome, L. C., Sosnicki, A. and Choi, I. H. (1992). The influence of temperature on muscle function in the fast swimming scup. II. The mechanics of red muscle. *J. Exp. Biol.* 163, 281–295.

Roved, J., Westerdahl, H. and Hasselquist, D. (2017). Sex differences in immune responses: Hormonal effects, antagonistic selection, and evolutionary consequences. *Horm. Behav.* 88, 95–105.

Sacchi, R., Capelli, E., Scali, S., Pellitteri-Rosa, D., Ghitti, M., Acerbi, E. and Pingtore, E. (2014). In vitro temperature dependent activation of T-lymphocytes in Common wall lizards (Podarcis muralis) in response to PHA stimulation. *Acta Herpetol.* 9, 131–138.

Sapolsky, R. M., Romero, M. L. and Munck, A. U. (2000). How Do Glucocorticoids Influence Stress Responses? Integrating Permissive, Suppressive, Stimulatory, and Preparative Actions*. *Endocr. Rev.* 21, 55–89.

Seebacher, F. (2009). Responses to temperature variation: Integration of thermoregulation and metabolism in vertebrates. *J. Exp. Biol.* 212, 2885–2891.

Sherman, E., Baldwin, L., Fernandez, G. and Deurell, E. (1991). Fever and thermal tolerance in the toad Bufo marinus. *J. Therm. Biol.* 16, 297–301.

Siroski, P. A., Pina, C. I., Larriera, A., Merchant, M. E. and Conza, J. Di (2009). Plasma Activity of the Broad-snouted Caiman (Caiman latirostris). *Zool. Stud.* 48, 238–242.

Soma, K. K. and Wingfield, J. C. (2001). Dehydroepiandrosterone in songbird plasma: Seasonal regulation and relationship to territorial aggression. *Gen. Comp. Endocrinol.* 123, 144–155.

Terrell, K. A., Quintero, R. P., Murray, S., Kleopfer, J. D., Murphy, J. B., Evans, M. J., Nissen, B. D. and Gratwicke, B. (2013). Cryptic impacts of temperature variability on amphibian immune function. *J. exper* 2016, 4204–4211.

Tutar, U., Çelik, C., Ataş, M., Tunç, T. and Gözel, M. G. (2015). Evaluation of biofilm formation activity of standard microorganism strains. *J. Clin. Exp. Investig.* 6, 135–139.

Vázquez-Martínez, E. R., García-Gómez, E., Camacho-Arroyo, I. and González-Pedrajo, B. (2018). Sexual dimorphism in bacterial infections. *Biol. Sex Differ.* 9, 1–20.

Zuk, M. and McKean, K. A. (1996). Sex differences in parasite infections: Patterns and processes. *Int. J. Parasitol.* 26, 1009–1024.

Tables

Table 3.1 Preferred thermal ranges for each species matched with its designated optimum challenge temperature.

Species	Preferred Thermal Range	Optimum Challenge Temperature
Axolotl	16 – 18℃	15℃
Hellbender	10 – 22℃	15℃
Alligator	29 – 31℃	25℃
Rattlesnake	21 – 32℃	25℃
Chicken	41 – 42℃	45℃
Quail	41 – 42℃	45℃
Horse	37 – 38℃	37℃

Table 3.2 LMM model results for *E. coli* with immune performance (microbial killing ability, microbial growth, and minimum killing concentration) as the response variable and challenge temperature, thermal strategy, and their interactive term as predictor variables. Significant predictors ($P < 0.05$) appear in bold.

E. coli						
Microbial Killing Ability (n = 132; pool = 33)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F – value**	**P – value**
Challenge Temperature	**0.766**	**0.255**	**3**	**93**	**3.647**	**0.015**
Thermal Strategy	0.056	0.056	1	31	0.796	0.379
Challenge Temperature:Thermal Strategy	**1.016**	**0.339**	**3**	**93**	**4.837**	**0.004**

Microbial Growth (n = 132; pool = 33)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F value**	**Pr(>F)**
Challenge Temperature	**0.125**	**0.042**	**3**	**93**	**12.415**	**6.71E-07**
Thermal Strategy	**0.021**	**0.021**	**1**	**31**	**6.227**	**0.018**
Challenge Temperature:Thermal Strategy	**0.131**	**0.044**	**3**	**93**	**13.022**	**3.58E-07**

Minimum Killing Concentration (n = 132, pool = 33)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	0.155	0.052	3	93	1.978	0.123
Thermal Strategy	0.016	0.016	1	31	0.629	0.434
Challenge Temperature:Thermal Strategy	**0.392**	**0.131**	**3**	**93**	**4.985**	**0.003**

Table 3.3 LMM model results for *S. typhimurium* with immune performance (microbial killing ability, microbial growth, and minimum killing concentration) as the response variable and challenge temperature, thermal strategy, and their interactive term as predictor variables. Significant predictors ($P < 0.05$) appear in bold.

S. typhimurium						
Microbial Killing Ability (n = 136; pool = 34)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	**0.623**	**0.208**	**3**	**96**	**8.919**	**2.85E-05**
Thermal Strategy	**0.182**	**0.182**	**1**	**32**	**7.829**	**0.009**
Challenge Temperature:Thermal Strategy	0.003	0.001	3	96	0.044	0.988

Microbial Growth (n = 136; pool = 34)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	**0.074**	**0.025**	**3**	**96**	**41.546**	**2.68E-17**
Thermal Strategy	0.002	0.002	1	32	2.853	0.101
Challenge Temperature:Thermal Strategy	**0.012**	**0.004**	**3**	**96**	**6.459**	**0.0005**

Minimum Killing Concentration (n = 90; pool = 31)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	**0.768**	**0.256**	**3**	**57**	**7.993**	**0.0002**
Thermal Strategy	0.045	0.045	1	25	1.420	0.245
Challenge Temperature:Thermal Strategy	**0.329**	**0.110**	**3**	**57**	**3.425**	**0.023**

Table 3.4 LMM model results for *C. albicans* with immune performance (microbial killing ability, microbial growth, and minimum killing concentration) as the response variable and challenge temperature, thermal strategy, and their interactive term as predictor variables. Significant predictors ($P < 0.05$) appear in bold.

C. albicans						
Microbial Killing Ability (n = 136; pool = 34)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	**0.595**	**0.198**	**3**	**96**	**7.171**	**0.0002**
Thermal Strategy	**0.180**	**0.180**	**1**	**32**	**6.530**	**0.016**
Challenge Temperature:Thermal Strategy	0.153	0.051	3	96	1.847	0.144

Microbial Growth (n = 136; pool = 34)						
Predictors	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	**0.030**	**0.010**	**3**	**96**	**6.286**	**0.001**
Thermal Strategy	0.003	0.003	1	32	1.695	0.202
Challenge Temperature:Thermal Strategy	**0.021**	**0.007**	**3**	**96**	**4.360**	**0.006**

Minimum Killing Concentration (n = 110; pool = 32)						
Predictor	**Sum Sq**	**Mean Sq**	**NumDF**	**DenDF**	**F - value**	**P - value**
Challenge Temperature	0.194	0.065	3	75	1.029	0.385
Thermal Strategy	0.003	0.003	1	30	0.053	0.820
Challenge Temperature:Thermal Strategy	0.406	0.135	3	75	2.158	0.100

Table 3.5 LMM model results for *E. coli* relating metric performance at optimum temperatures to range in performance across all challenge temperatures. Significant predictors ($P < 0.05$) appear in bold.

E. coli					
Microbial Killing Ability (n = 33)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Microbial Killing	0.095	0.136	25.397	0.704	0.488
Thermal Strategy[Endotherm]	-0.186	0.274	6.569	-0.680	0.520
Optimum Microbial Killing:Thermal Strategy[Endotherm]	0.088	0.216	25.468	0.409	0.686
Microbial Growth (n = 33)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Microbial Growth	**-0.195**	**0.071**	**27.893**	**-2.728**	**0.011**
Thermal Strategy[Endotherm]	**-0.330**	**0.129**	**21.553**	**-2.565**	**0.018**
Optimum Microbial Growth:Thermal Strategy[Endotherm]	**0.357**	**0.159**	**28.998**	**2.253**	**0.032**
Minimum Killing Concentration (n = 33)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Minimum Concentration	0.259	0.179	28.677	1.448	0.158
Thermal Strategy[Endotherm]	-0.166	0.164	7.514	-1.012	0.343
Optimum Min. Concentration:Thermal Strategy[Endotherm]	0.494	0.639	28.772	0.773	0.446

Table 3.6 LMM model results for *S. typhimurium* relating metric performance at optimum temperatures to range in performance across all challenge temperatures. Significant predictors (P < 0.05) appear in bold.

S. typhimurium					
Microbial Killing Ability (n = 33)					
Predictors	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Microbial Killing	**1.207**	**0.544**	**22.218**	**2.216**	**0.037**
Thermal Strategy[Endotherm]	0.226	0.102	6.760	2.208	0.064
Optimum Microbial Killing:Thermal Strategy[Endotherm]	-1.097	0.573	23.474	-1.912	0.068

Microbial Growth (n = 34)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Microbial Growth	**-0.629**	**0.122**	**26.579**	**-5.161**	**2.06E-05**
Thermal Strategy[Endotherm]	**-0.615**	**0.150**	**28.521**	**-4.090**	**3.21E-04**
Optimum Microbial Growth:Thermal Strategy[Endotherm]	**0.651**	**0.146**	**26.732**	**4.463**	**1.31E-04**

Minimum Killing Concentration (n = 20)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Min. Concentration	-0.753	0.395	16	-1.906	0.075
Thermal Strategy[Endotherm]	-0.429	0.232	16	-1.849	0.083
Optimum Min. Concentration:Thermal Strategy[Endotherm]	**1.310**	**0.469**	**16**	**2.796**	**0.013**

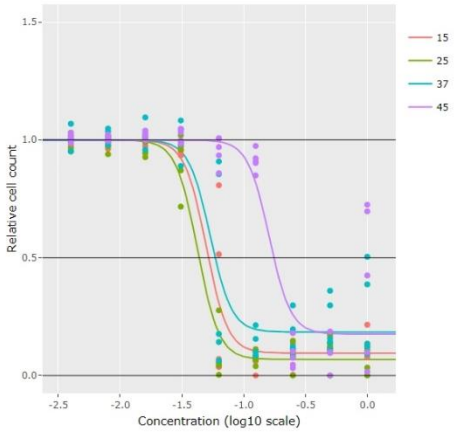
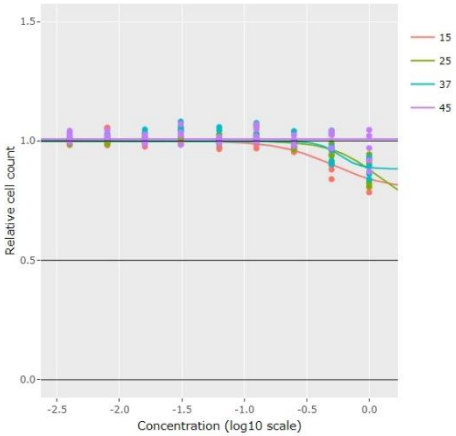
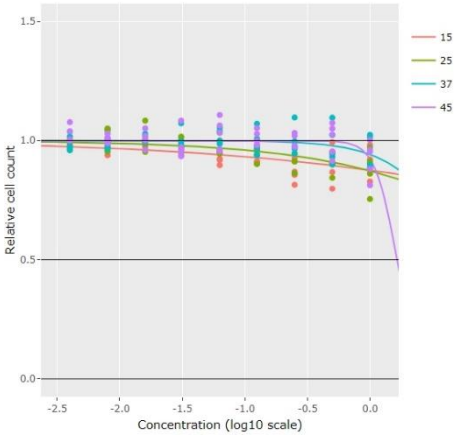
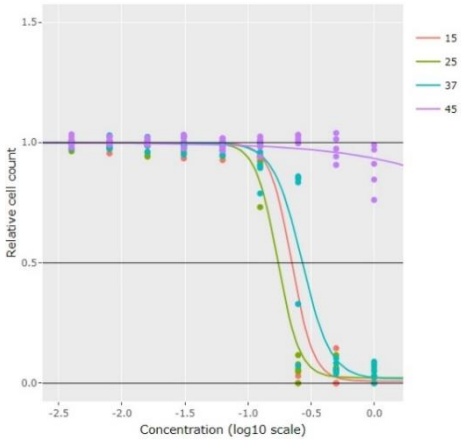
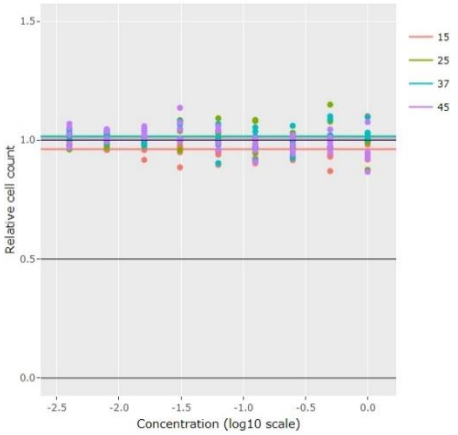
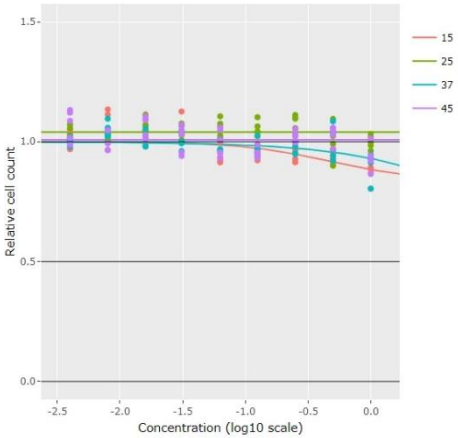
Table 3.7 LMM model results for *C. albicans* relating metric performance at optimum temperatures to range in performance across all challenge temperatures. Significant predictors (P < 0.05) appear in bold.

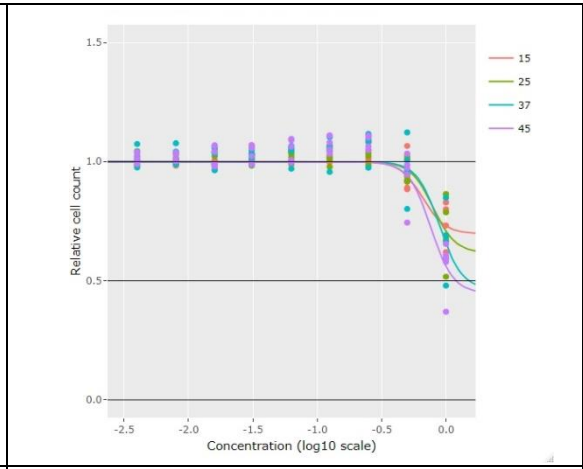
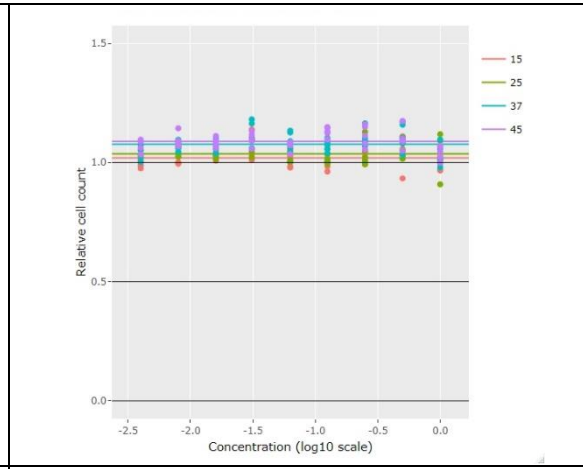
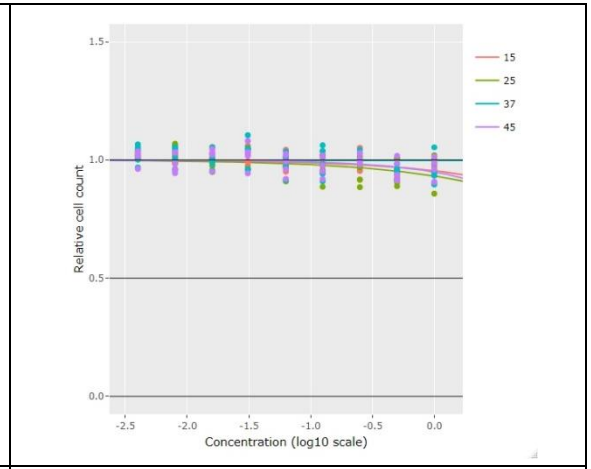
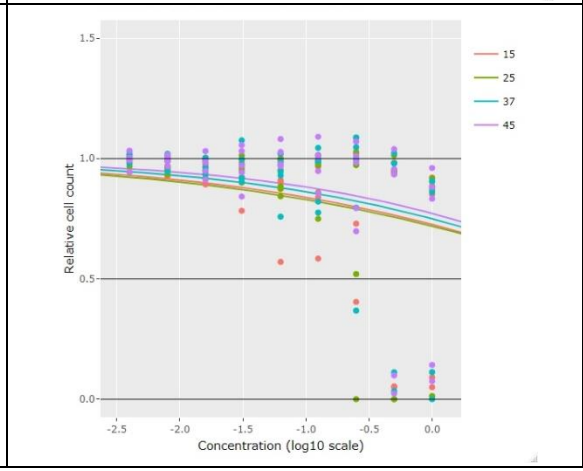
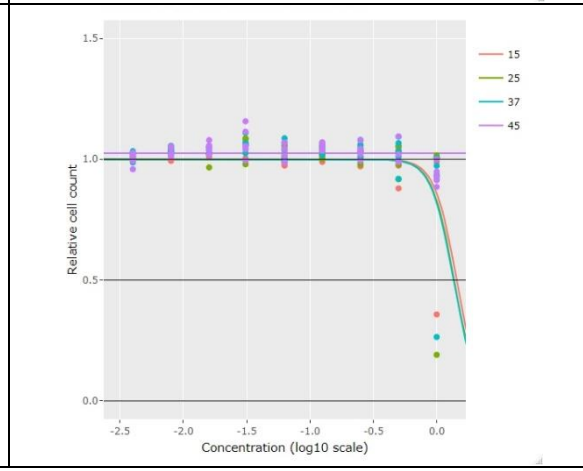
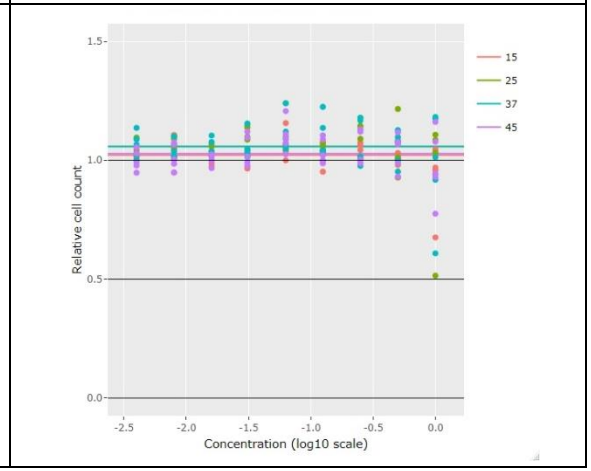
C. albicans					
Microbial Killing Ability (n = 34)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Microbial Killing	**1.201**	**0.176**	**25.253**	**6.827**	**3.53E-07**
Thermal Strategy[Endotherm]	**0.285**	**0.096**	**5.723**	**2.972**	**0.026**
Optimum Microbial Killing:Thermal Strategy[Endotherm]	**-1.125**	**0.215**	**25.957**	**-5.234**	**1.82E-05**

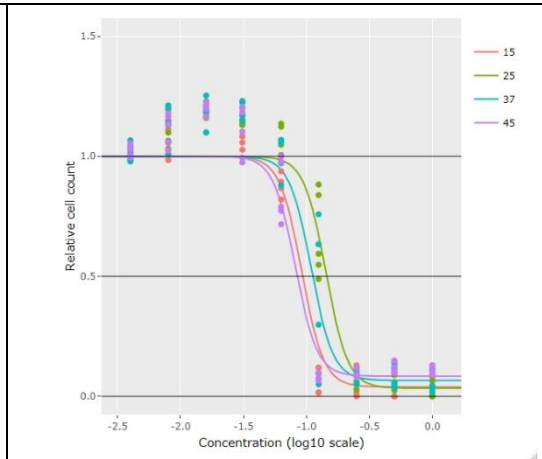
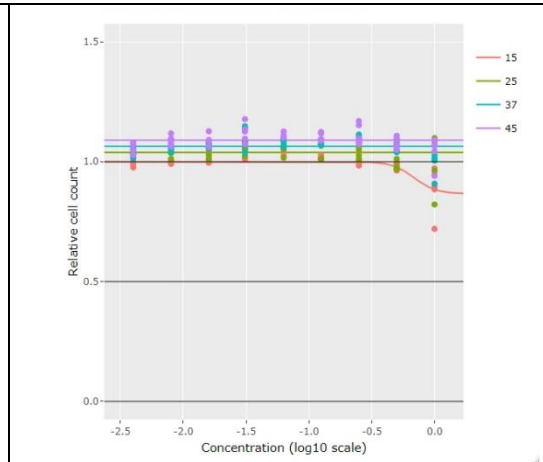
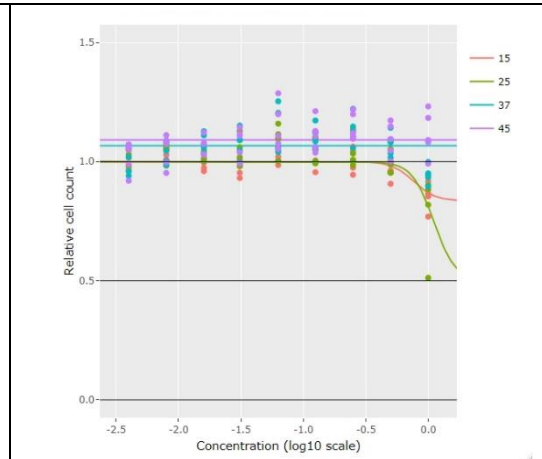
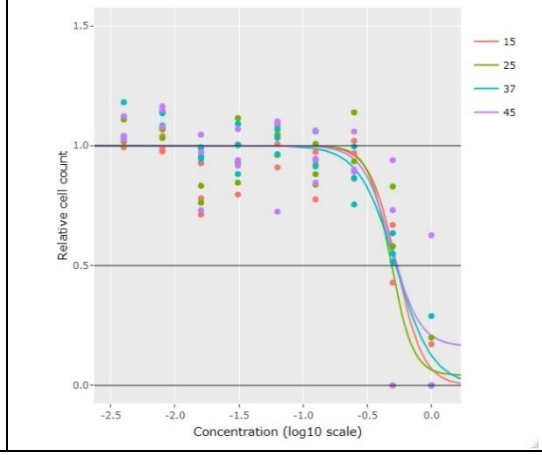
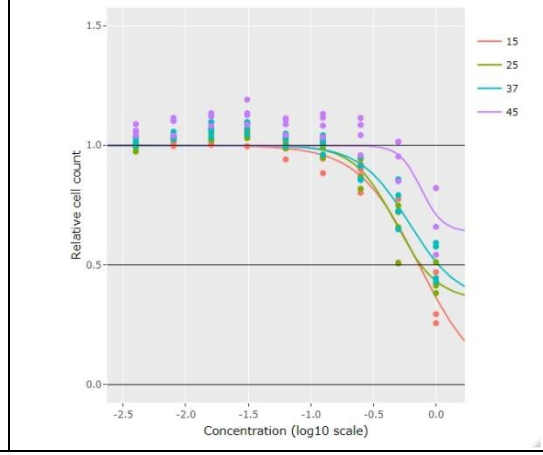
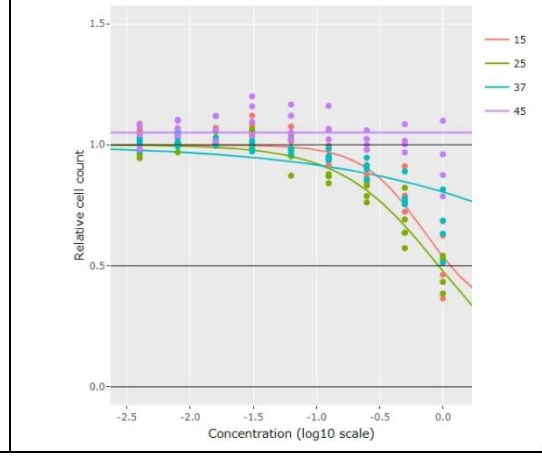
Microbial Growth (n = 34)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Microbial Growth	0.032	0.161	28.04	0.201	0.842
Thermal Strategy[Endotherm]	0.073	0.186	28.102	0.395	0.696
Optimum Microbial Growth:Thermal Strategy[Endotherm]	-0.023	0.185	28.531	-0.122	0.904

Minimum Killing Concentration (n = 25)					
Predictor	**Estimate**	**SE**	**df**	**t - value**	**P - value**
Optimum Min. Concentration	0.070	0.385	11.044	0.181	0.860
Thermal Strategy[Endotherm]	-0.100	0.241	5.763	-0.415	0.693
Optimum Min. Concentration:Thermal Strategy[Endotherm]	0.351	0.462	13.765	0.759	0.461

Figures

Species	*E. coli*	*S. typhimurium*	*C. albicans*
Axolotl			
Hellbender			

Alligator			
Rattlesnake			

Chicken			
Quail			

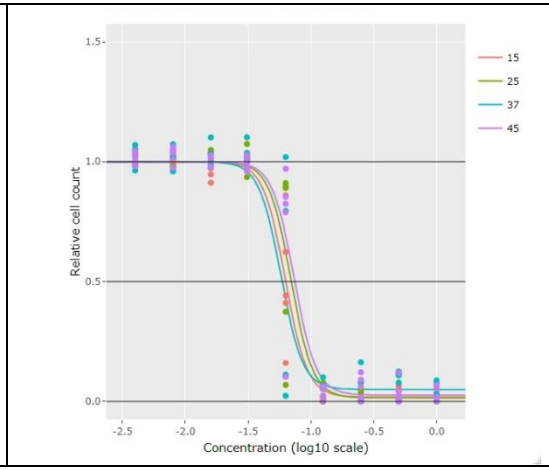
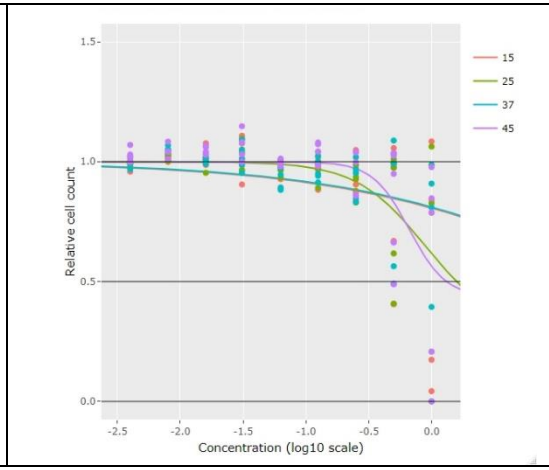
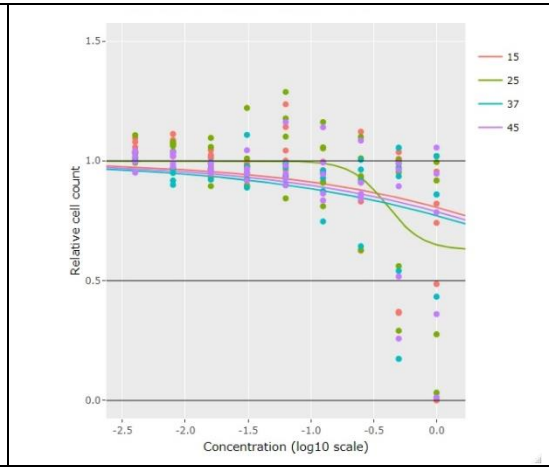
Horse			

Figure 3.1. Dose response curves for each species across microbes.

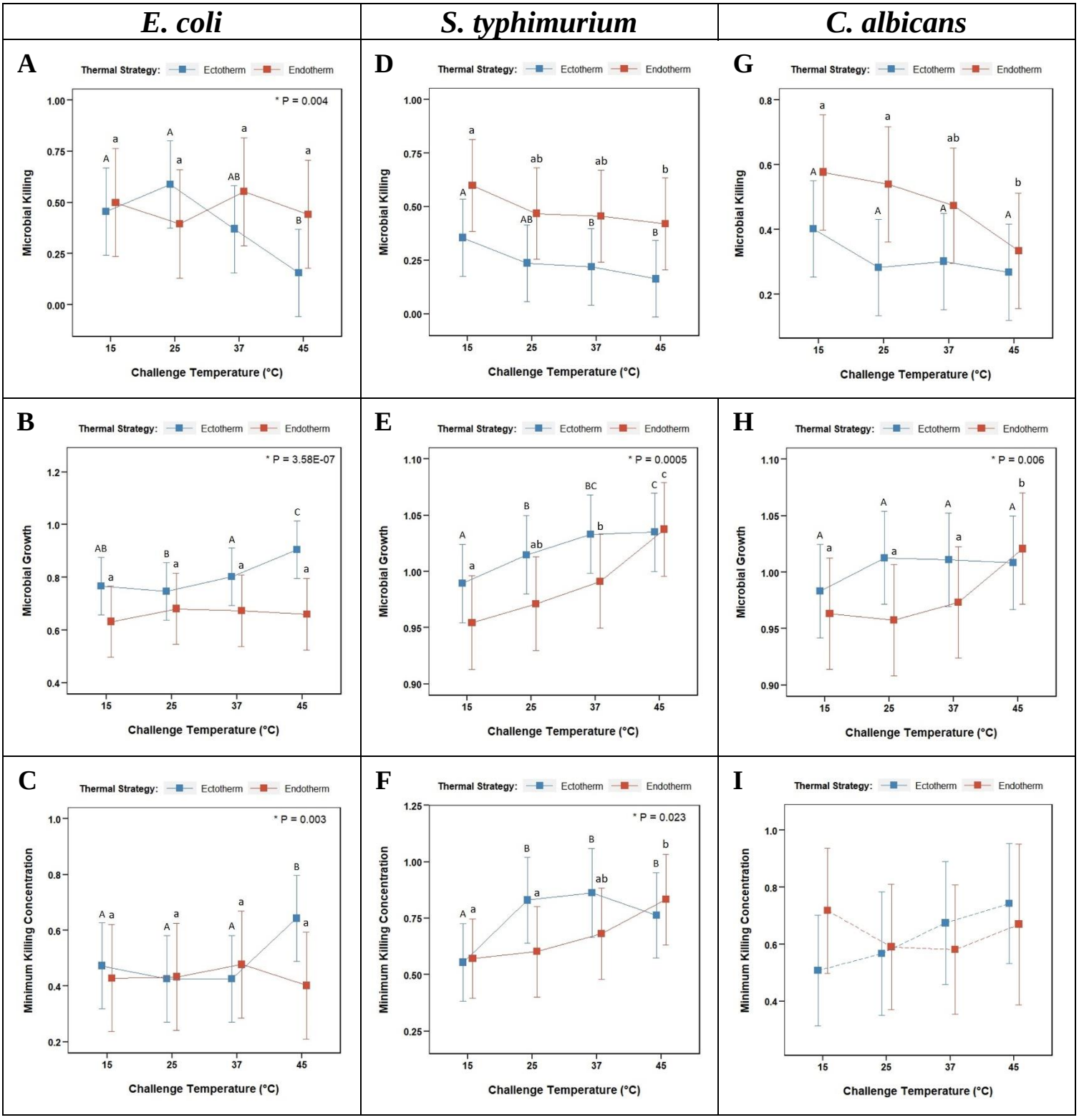


Figure 3.2 Average measure of each performance metric across challenge temperatures for ectotherms (blue) and endotherms (red) across three microbes: *E. coli* (A – C), *S. typhimurium* (D – F) and *C. albicans* (G – I). Error bars indicate 95% confidence intervals. Solid lines indicate a significant main effect of challenge

temperature. Capital letters represent differences between temperatures within ectotherms and lowercase letters represent thermal differences within endotherms. Mean values that share the same letter are not significantly different. The presence of an interaction between challenge temperature and thermal strategy is indicated by an asterisk and the associated p – value.

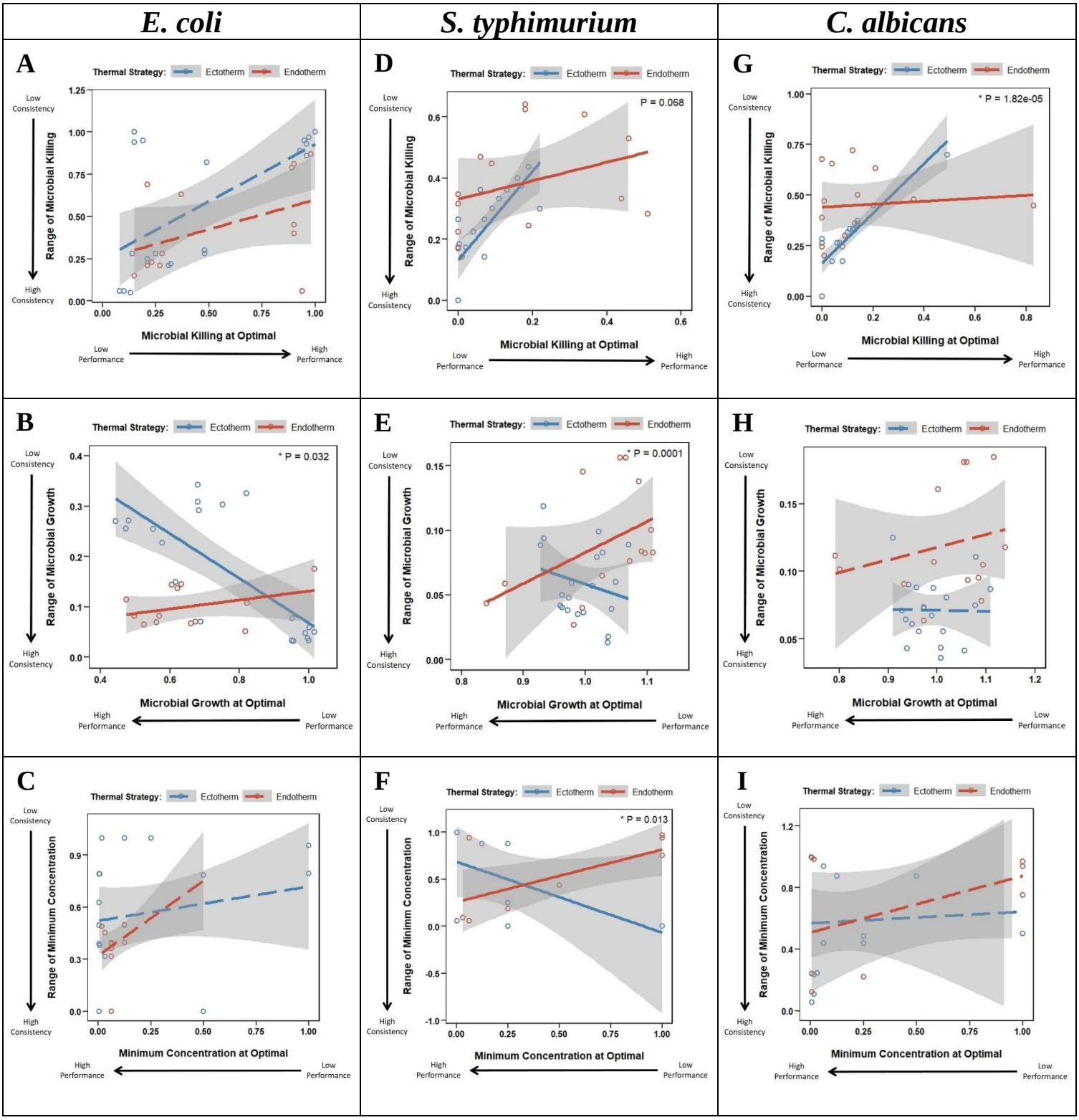


Figure 3.3 Relationship between performance at optimum temperature and range of performance across all temperatures for ectotherms (blue) and endotherms (red) across metrics for *E. coli* (A – C), *S. typhimurium* (D –

F), and *C. albicans* (G – I). Solid lines indicate a significant main effect of optimum temperature. Presence of a significant interaction between performance breadth and thermal strategy is denoted by an asterisk and the associated p - value. For marginally significant ($0.07 < P > 0.05$) interactions the p – value is reported but not denoted by an asterisk.

CHAPTER 4

CONCLUSIONS

The goal of this thesis was to examine temperature and hormone effects on immune performance. Overall, our results demonstrate that temperature affects immune performance and further, serves to mediate testosterone-immunity interactions. Together, these findings illustrate the context dependency of immunity and highlight the importance of accounting for both intrinsic and extrinsic factors when assessing immune patterns in vertebrate populations.

In Chapter 2, we found that trade-offs occurring between testosterone and immune function were modified by both other hormones and temperature. We found that variation in bacteria killing ability across three bacteria (*E. coli, S. typhimurium, and, K. pneumoniae*) was best explained when co-circulating levels of the hormone DHEA were simultaneously considered alongside testosterone. This finding suggests that co-circulating levels of DHEA may serve an under-appreciated role in modifying immunosuppressive effects of testosterone. Further, we found evidence for testosterone-mediated immunosuppression, but only in challenges against S. typhimurium at 15℃. This result suggests that temperature effects on host and pathogen physiology may interact to modify testosterone-immunity relationships. Interestingly, we also found that killing ability varied depending on which bacterial species an animal is challenged with, and the magnitude of killing was dependent on challenge temperature. Specifically, higher killing of E. coli occurred at 30℃, while higher killing of S. typhimurium and K. pneumoniae occurred at 15℃, highlighting the temperature-dependency of host-pathogen interactions.

Together, these findings demonstrate how co-occurring physiological processes and seasonal drivers may serve as important mediators of testosterone-immunity trade-offs and thus, may determine patterns of pathogen vulnerability in natural populations. Therefore, identifying influential contextual factors is key to characterizing how testosterone-immunity trade-offs operate in the wild.

Further investigation into thermal effects on immune function in Chapter 3 found additional support for temperature-dependent effects on immune performance. In this case, the effects of temperature were mediated by vertebrate thermoregulatory strategy and microbial species. Ectotherms and endotherms performed differently across challenge temperatures, ranging from 15℃ – 45℃, for three microbial species (E. coli, S. typhimurium, and C. albicans), with the greatest differences exposed under the most extreme temperatures (15℃ & 45℃). Endotherms performed similarly across all challenge temperatures when challenged with E. coli, but experienced temperature effects on immune performance against S. typhimurium and C. albicans. Ectotherms performed similarly across all challenge temperatures when challenged with C. albicans, but experienced temperature effects on immune performance against E. coli and S. typhimurium. These findings demonstrate that temperature, thermal strategy and microbial species should be considered when assessing immune performance in vertebrates.

We also found significant relationships between immune performance at a species' optimum temperature and consistency in immune performance across temperatures. Specifically, only ectotherms experienced a trade-off between high immune performance at optimum temperatures and maintaining consistent performance across temperatures, as would be expected given energetic constraints. This finding suggests that for ectotherms, measuring immune

performance at optimum temperatures may not be representative of immune performance across a broader range of temperatures.

Our findings from Chapter 3 provide insight into differences in the thermal sensitivities of immune function across vertebrates employing different thermoregulatory strategies. Further, these results highlight the importance of measuring immune performance across temperatures, as measurements at a single optimum temperature may not be representative of performance across broader thermal ranges. Being able to accurately quantify immune responses across thermal profiles, will allow for a better understanding of how daily and seasonal temperature fluctuations may drive important patterns of pathogen vulnerability in natural vertebrate populations.