Laboratory, mesocosm, and field experiments reveal costs associated with peritoneal fibrosis and cestode infection in threespine stickleback (*Gasterosteus aculeatus*)

By
Kristofer T. Sasser

A dissertation in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
(Integrative Biology)

at the
UNIVERSITY OF WISCONSIN-MADISON
2024

Date of final oral examination: 04/24/2024

The dissertation is approved by the following members of the Final Oral Committee:

Jesse N. Weber, Assistant Professor, Integrative Biology
Olaf P. Jensen, Associate Professor, Integrative Biology
Jessica Hite, Assistant Professor, Pathobiological Sciences
Daniel I. Bolnick, Professor, University of Connecticut
Jonathan Velotta, Assistant Professor, University of Denver
Jonathan A.W. Stecyk, Professor, University of Alaska Anchorage

Acknowledgments

I feel a deep sense of gratitude as I look back on the past five years. It has been an incredible privilege to get to pursue this work and I have many people to thank for their help along the way, especially at the University of Alaska Anchorage, where I began my graduate research, and at the University of Wisconsin – Madison, where I finished it. I could not have completed this work without the assistance of the amazing students, faculty, and staff at both institutions.

I owe my advisor, Jesse Weber, a great deal of gratitude. I've never met anyone with a more energetic and contagious passion for science. You gave me an amazing degree of freedom and support in pursuing my interests and for that I cannot thank you enough. It was a pleasure being your student.

I would also like to extend my deepest appreciation to Jonathan Stecyk, Dan Bolnick, Olaf Jensen, Jessica Hite, and Jon Velotta for serving on my committee. You have all been incredibly generous with your time and your thoughtful insight and feedback improved my work immeasurably. It has been a joy to work with you all.

This experience has been made so much richer by a wonderful group of collaborators including Chelsea Wood, Natalie Steinel, Yoel Stuart, Ken Thompson, Stephen De Lisle, and their associated labs. Thank you all for your kindness and help.

I would also like to thank all of the wonderful people who have passed through the Weber Lab including Vanessa Muhlenbruch, Bryce Solin, Emily Kerns, Max Reynolds, Jeremy Abels, Changyue Yu, Cole Wolf, Javi Benavente, Matt Chotlos, and Lily Wagner. Thank you for being fantastic friends and collaborators. May your fieldwork always be fueled by delicious breakfast burritos and discount ground beef.

Most importantly, I could not have completed this journey without the support of my loved ones. Thank you to the many friends who have been there over the years. Mom and Dad, thank you for always encouraging me to follow my passion. Finally, thank you, Allie for believing in me and supporting me throughout the process. I am in awe of the grace with which you have handled this wild ride.

Table of Contents

Abstract

The evolution of immune responses is shaped by the costs imposed by parasites and immune responses themselves. While substantial work has assessed the cost of immunity and parasitism, how costs relate to each other or vary across populations has received less attention. In this dissertation I assess costs associated with infection by the cestode *Schistocephalus solidus* and an innate immune response – peritoneal fibrosis – in several populations of threespine stickleback (*Gasterosteus aculaetus*).

In my first chapter I used laboratory and mesocosm experiments to measure the energetic cost of peritoneal fibrosis. I found that fibrosis was associated with elevated standard metabolic rate, reduced body mass, and reduced ovary mass. Further, the magnitude of many of these costs increased with response magnitude. This correlation has important implications for the evolution of the trait, including the possibility that a dampened response may be preferable to a large, costly response in some environments.

In my second chapter I surveyed wild stickleback populations to test whether longevity or growth covaried with cestode prevalence and the ability to fibrose. This revealed notable variation in growth rate, longevity, cestode infection prevalence, and the ability to fibrose. However, I found no association between fibrosis and growth or longevity. Notably, I did not find a signature of mortality associated with cestode prevalence suggesting that stickleback may be able to survive cestode infections for a considerable time.

In my third chapter I investigated how global warming will alter *S. solidus* infection outcomes and assessed the thermal tolerance of benthic and limnetic stickleback ecotypes. Unfortunately, the first axis of this experiment failed as cestode exposures did not yield infections. However, this work revealed that stickleback from benthic populations suffered significantly less mortality than those from limnetic populations at elevated temperatures possibly due to heritable differences in thermal tolerance. Additionally, while thermal tolerance is often assessed using aerobic scope, I found no significant relationship between these traits, which may call into question the ecological relevance of this often measured trait.

My fourth chapter is a perspective piece calling for ecoimmunologists to carefully assess the ecological and evolutionary relevance of their methodological choices.

Introduction

While the field of ecoimmunology only came into existence 35 years ago, the question of how organisms partition limited resources has been pondered for far longer. R.A. Fisher noted: “It would be instructive to know not only by what physiological mechanism a just apportionment is made between the nutriment devoted to the gonads and that devoted to the rest of the parental organism, but also what circumstances in the life-history and environment would render profitable the diversion of a greater or lesser share of the available resources towards reproduction” (1930). George C. Williams echoed this sentiment 36 years later: “Expenditures on reproductive processes must be in functional harmony with each other and worth the costs, in relation to the long-range reproductive interest; and the use of resources for somatic processes is favored to the extent that somatic survival, and perhaps growth, are important for future reproduction” (1966). Though Fisher and Williams do not directly reference immunity, they clearly understood that organisms must allocate limited resources between reproduction and costly somatic traits related to survival to maximize their fitness.

At a broad level, the tradeoff between resources and immunity is rather obvious: organisms have access to limited resources, parasites present a ubiquitous threat to host fitness, and resources dedicated to immune responses can reduce infection costs (Lafferty and Kuris 2009; Hudson et al. 1992). However, only recently have costs been investigated as a force shaping the evolution of immune responses (Brock et al. 2014). Some authors have gone so far as to suggest that the cataloging and quantifying costs should be a central

goal of ecoimmunology (Schoenle 2018). Substantial work has been done quantifying the costs of immune responses and parasites, however, a limited number of potential costs are typically examined due to the constraints of time and resources (Brock et al. 2014). This has revealed that costs imposed by immune responses and parasites can come in myriad forms (e.g., depletion of critical nutrients, energy, or damage to host tissue) but it is often unclear which costs play a role in shaping the evolution of different immune responses (Lochmiller and Deerenberg 2000). While we are able to quantify the cost of certain immune responses, we typically have an opaque understanding of how significant this cost is relative to other potential strategies. Further, we know very little about how costs relate to each other (e.g., directionality) or vary across populations within species. One resolution to these difficulties is to quantify a diverse set of costs related to a focal immune response, and do so across multiple populations that are likely to have evolved under divergent parasite and environmental pressures.

The coevolutionary battle between threespine stickleback and the cestode *S. solidus* provides an ideal system to probe the costs shaping the evolution of vertebrate immune responses. Threespine stickleback are the obligate intermediate host of the cestode and pair have been coevolving for ~15 million years (Nishimura et al. 2011). Because the reproductive output of the cestode is size dependent and growth takes place exclusively in the stickleback, the individual fitness of the cestode is entirely dependent upon successfully infecting stickleback and growing to large size (Schärer and Wedekind 1999). Conversely, stickleback are likely under intense selective pressure to resist infections and stunt the growth of cestodes as infections impose numerous costs that are

proportional to the size of the infection. Cestode infections negatively impact host body condition (Tierney et al. 1996), reduce anti-predatory behavior (Milinski 1985), and can siphon up to 46% of host energy (Claar et al. 2023). Further, the cestode may even castrate hosts by occupying the space in which gonads typically develop (e.g., Heins et al. 2004). The cestode is found throughout the distribution of stickleback with the exception of brackish waters in which the cestodes eggs cannot hatch (Nishimura et al. 2011; Simmonds and Barber 2016). Consequently, marine stickleback are naïve and suffer severe infections when exposed to cestodes (Weber et al. 2017). Because stickleback are ancestrally marine, we can infer the evolutionary trajectory of immune evolution (Bell and Foster 1994). Naïve marine stickleback have repeatedly colonized freshwater and evolved a suite of immune responses used to resist and stunt cestode infections (Weber et al. 2017, Weber et al. 2022).

This repertoire of immune defense is diverse and includes ROS production (Scharsack et al. 2004; Weber et al. 2017), antigen responses (Kurtz et al. 2006), and peritoneal fibrosis (Weber et al. 2017; Weber et al. 2022). The peritoneal fibrosis response is a particularly attractive trait to study for several reasons. First, recent work suggests that one of its primary functions is to encapsulate peritoneal-dwelling cestodes in fibrotic tissue, thereby stunting their growth (Weber et al. 2022). Second, the response can be induced in stickleback, regardless of ancestry, via injections of 1% aluminum phosphate — a stimulant that induces innate immune responses (Hund et al. 2022). Third, the ability to fibrose when exposed to cestodes has arisen and subsequently been lost in some freshwater populations (Weber et al. 2022), suggesting that deploying the response entails

significant costs that may be untenable in some environments. Taken together, fibrosis represents an immune response that is biologically relevant, experimentally inducible, varies substantially across populations, and is likely costly. Further, the population level variation allows investigation of how divergent immune phenotypes are associated with evolved differences in the costs of deployment.

This dissertation attempts to quantify several metrics of cost associated with both peritoneal fibrosis and *S. solidus* infections utilizing laboratory, mesocosm, and field studies of multiple populations of threespine stickleback. This work adds to our understanding of how the costs of immune responses scale with response magnitude, interact with other costs, and vary across interspecific populations. Further, it adds to a growing body of work that has revealed the mechanisms that underpin peritoneal fibrosis (Weber et al. 2022; Lohman et al. 2017; Fuess et al. 2021), its role in cestode growth suppression (Weber et al. 2017; Weber et al. 2022), its costs (Weber et al. 2022; de Lisle and Bolnick 2021), and the evolution of the trait (Weber et al. 2022).

Chapter One of this dissertation quantifies the cost of peritoneal fibrosis utilizing laboratory and mesocosm experiments. I hypothesized that fibrosis would be energetically costly and that this would drive reductions in body mass. Further, I predicted that costs would scale with response magnitude. The presence of fibrosis was associated with elevated metabolic rate, reduced body mass, and reduced ovary mass. Further, costs scaled with response magnitude in both the lab and mesocosm. Intriguingly, mass loss was not driven by energetic cost of the response, suggesting that it may instead be driven

by the need to free up key nutrients rather than energy. As a whole, this demonstrates that immune suppression offers a viable mechanism to optimize the scale of the response.

Chapter Two of this dissertation describes a field survey aimed at linking longevity, growth, the ability to mount a peritoneal fibrosis response, and cestode prevalence. I predicted that cestode prevalence would be negatively correlated with longevity due to the costs associated with cestode infections. In contrast, I predicted that there would be a positive association between the ability to fibrose and longevity, but that it would come at the cost of reduced growth rates. This work revealed remarkable variation in both growth rate and longevity across stickleback populations. I did not find a signature of mortality associated with high cestode prevalence, suggesting that stickleback may be able to survive infections for a substantial amount of time. Further, I did not find a negative correlation between the ability to fibrose and growth rate.

Chapter Three of this dissertation describes a laboratory experiment with two axes. First, an assessment of how climate change will alter *S. solidus* infection outcomes. Second, an assessment of the thermal tolerance of two stickleback ecotypes. I predicted that that the incidence and severity of cestode infections would increase with temperature and that benthic stickleback would maintain greater thermal tolerance. Unfortunately, the first axis of this experiment was unsuccessful. Despite exposing 184 stickleback to cestodes, only a single individual was successfully infected. The second axis revealed that Alaskan stickleback are remarkably hardy in the face of rising temperatures. Stickleback did not suffer significant mortality until exposed to temperatures of 26C with fish from

large, deep waterbodies suffering significantly more mortality. Mortality was not preceded by a decline in body condition.

Chapter Four of this dissertation is a perspective piece calling for more ecologically relevant studies of studies of immune cost. This piece challenges researchers to carefully assess their methodological choices with a focus on the biological relevance of the immune responses elicited. This piece also encourages more studies to leverage population level variation.

References

Bell MA, Foster S (1994) The Evolutionary Biology of the Threespine Stickleback. Oxford University Press

Brock PM, Murdock CC, Martin LB (2014) The History of Ecoimmunology and Its Integration with Disease Ecology. Integrative and Comparative Biology 54:353–362. https://doi.org/10.1093/icb/icu046

Claar DC, Faiad SM, Mastick NC, et al (2023) Estimating the magnitude and sensitivity of energy fluxes for stickleback hosts and *Schistocephalus solidus* parasites using the metabolic theory of ecology. Ecology and Evolution 13:e10755. https://doi.org/10.1002/ece3.10755

De Lisle SP, Bolnick DI (2021) Male and female reproductive fitness costs of an immune response in natural populations *. Evolution 75:2509–2523. https://doi.org/10.1111/evo.14266

Fisher RA (1930) The genetical theory of natural selection. Clarendon Press

Fuess LE, Weber JN, Den Haan S, et al (2021) Between-population differences in constitutive and infection-induced gene expression in threespine stickleback. Molecular Ecology 30:6791–6805. https://doi.org/10.1111/mec.16197

Heins DC, Ulinski B, Johnson J, Baker JA (2004) Effect of the cestode macroparasite *Schistocephalus pungitii* on the reproductive success of ninespine stickleback, *Pungitius pungitius*. Can J Zool 82:1731–1737. https://doi.org/10.1139/z04-171

Hudson PJ, Newborn D, Dobson AP (1992) Regulation and Stability of a Free-Living Host-Parasite System: Trichostrongylus tenuis in Red Grouse. I. Monitoring and Parasite Reduction Experiments. The Journal of Animal Ecology 61:477. https://doi.org/10.2307/5338

Hund AK, Fuess LE, Kenney ML, et al (2022) Population-level variation in parasite resistance due to differences in immune initiation and rate of response. Evolution Letters 6:162–177. https://doi.org/10.1002/evl3.274

Kurtz J, Wegner KM, Kalbe M, et al (2006) MHC genes and oxidative stress in sticklebacks: an immuno-ecological approach. Proc R Soc B 273:1407–1414. https://doi.org/10.1098/rspb.2005.3450

Lafferty KD, Kuris AM (2009) Parasitic castration: the evolution and ecology of body snatchers. Trends in Parasitology 25:564–572. https://doi.org/10.1016/j.pt.2009.09.003

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Lohman BK, Steinel NC, Weber JN, Bolnick DI (2017) Gene Expression Contributes to the Recent Evolution of Host Resistance in a Model Host Parasite System. Front Immunol 8:1071. https://doi.org/10.3389/fimmu.2017.01071

Milinski M (1985) Risk of Predation of Parasitized Sticklebacks (Gasterosteus Aculeatus L.) Under Competition for Food. Behav 93:203–216. https://doi.org/10.1163/156853986X00883

Nishimura N, Heins DC, Andersen RO, et al (2011) Distinct Lineages of Schistocephalus Parasites in Threespine and Ninespine Stickleback Hosts Revealed by DNA Sequence Analysis. PLoS ONE 6:e22505. https://doi.org/10.1371/journal.pone.0022505

Schärer L, Wedekind C (1999) Lifetime reproductive output in a hermaphrodite cestode when reproducing alone or in pairs: a time cost of pairing. Evolutionary Ecology 13:381–394. https://doi.org/10.1023/A:1006789110502

Scharsack J, Kalbe M, Derner R, et al (2004) Modulation of granulocyte responses in three-spined sticklebacks Gasterosteus aculeatus infected with the tapeworm Schistocephalus solidus. Dis Aquat Org 59:141–150. https://doi.org/10.3354/dao059141

Schoenle LA, Downs CJ, Martin LB (2018) An Introduction to Ecoimmunology. In: Cooper EL (ed) Advances in Comparative Immunology. Springer International Publishing, Cham, pp 901–932

Simmonds NE, Barber I (2016) The Effect of Salinity on Egg Development and Viability of *Schistocephalus solidus* (Cestoda: Diphyllobothriidea). Journal of Parasitology 102:42–46. https://doi.org/10.1645/14-701

Tierney JF, Huntingford FA, Crompton DWT (1996) Body condition and reproductive status in sticklebacks exposed to a single wave of *Schistocephalus solidus* infection. Journal of Fish Biology 49:483–493. https://doi.org/10.1111/j.1095-8649.1996.tb00043.x

Weber JN, Steinel NC, Peng F, et al (2022) Evolutionary gain and loss of a pathological immune response to parasitism. Science 377:1206–1211. https://doi.org/10.1126/science.abo3411

Weber JN, Steinel NC, Shim KC, Bolnick DI (2017) Recent evolution of extreme cestode growth suppression by a vertebrate host. Proc Natl Acad Sci USA 114:6575–6580. https://doi.org/10.1073/pnas.1620095114

Williams GC (1966) Natural Selection, the Costs of Reproduction, and a Refinement of Lack's Principle. The American Naturalist 100:687–690. https://doi.org/10.1086/282461

Chapter 1

Metabolic, performance, and fecundity consequences of peritoneal fibrosis in threespine stickleback (*Gasterosteus aculeatus*)

Kristofer T. Sasser[1,2], Stephen P. De Lisle[3,4], Ken A. Thompson[5,6], Jonathan A.W. Stecyk[2], Daniel I. Bolnick (UConn), Jesse N. Weber[1,2]

University of Wisconsin – Madison[1], University of Alaska Anchorage[2], Karlstad University[3], University of Connecticut[4], Stanford University[5], University of British Columbia[6]

In review at Functional Ecology

Abstract

Immune responses that organisms have evolved to avoid or dampen the costs of parasitism are energetically costly to maintain and deploy. Quantifying these costs can inform the evolutionary dynamics of immunity. Peritoneal fibrosis stunts the growth of cestodes in threespine stickleback (*Gasterosteus aculeatus*) but appears to negatively affect host reproductive potential. Because fibrosis can be experimentally stimulated and the magnitude of this response varies across populations, this system offers an opportunity to assess whether multiple metrics of cost scale with response severity. Here, we first quantify the energetic cost of fibrosis—in terms of metabolic rate and body condition— across three populations of lab-reared stickleback that differ starkly in their natural cestode immunity. We also induced fibrosis in semi-natural experimental ponds to test for effects on host survival, body condition, and fecundity. Lab fish with fibrosis exhibited an 11% increase in standard metabolic rate independent of shifts in mass. In experimental ponds, fibrosis led to reduced survival, 6% lower body mass, and 34% lower ovary mass. Fibrosis severity correlated positively with complementary measures of physiological cost in both experiments. Taken together, these results provide strong evidence that fibrosis entails significant costs in terms of energy, mass loss, and female fecundity.

1. Introduction

Immune evolution is shaped not only by negative effects of pathogens, but also by the costs of maintaining and deploying immune defenses (Lochmiller and Deerenberg 2000).

Despite a wealth of knowledge about the presence and magnitude of costs across a range of responses and taxa, the relationship between response magnitude and cost remains unclear as few studies correlate cost with response magnitude. The dearth of information on the relationship is surprising given its importance in theory (Boots and Haraguchi 1999). While it may seem intuitive that costs should increase with response magnitude, this is not always the case. The cost of inflammatory responses did not correspond with challenge severity (Nilsson et al. 2007) or response strength (Cutrera et al. 2014) in lab experiments utilizing birds and mammals, respectively. Intraspecific comparisons of natural variation across multiple populations offer a powerful approach for resolving cost-severity relationships, as populations can differ markedly in heritable immune responses (Sparkman and Palacios 2009; Peuß et al. 2020). This comparative approach also enables tests of whether some populations evolve mechanisms to mitigate costs (i.e., if the relationship between response magnitude and cost is fixed or variable). For example, common garden studies of cane toads (*Rhinella marina*) revealed that some populations mount larger innate immune responses (Brown et al. 2015) but pay a lower energetic cost when challenged with an immune stimulant (Llewellyn et al. 2012), suggesting the evolution of cost mitigation mechanisms. Despite the promise of this approach, very little is known about how immune costs vary across populations curtailing our ability to understand how costs shape the distribution of immune responses across environments with distinct parasite and resource pressures (Sasser and Weber 2022). There is also a dearth of information on the duration and accumulation of immune costs as most laboratory studies comprise a few snapshot measurements taken over short timescales

(days to a few weeks), with some notable exceptions that monitor costs continually (Bryla et al. 2022) or span months, years, or even decades (Zhang et al. 2019; Hasselquist and Nilsson 2012; Hayward et al. 2014).

The energetic cost of deploying immune responses provides a useful target for evolutionary studies because it is a common currency that unites diverse processes (e.g., somatic growth, reproduction, activity, and immune responses) and organisms (McNab 2002). However, energetic costs are highly variable across taxa and classes of immune response, ranging from no discernible change in resting metabolic rate to increases of up to 35% (Lochmiller and Deerenberg 2000; Schoenle et al. 2018). Fish exemplify this variation in cost. Zanuzzo et al. (2015a,b) injected formalin-killed bacteria (*Aeromonas salmonicida*) into the body cavity of steelhead trout (*Oncorhynchus mykiss*) to induce an acute phase immune response. The authors detected no differences in immune or metabolic traits but found increased expression of eight immune genes. Injecting Atlantic salmon (*Salmo salar*) with live piscine orthoreovirus (PRV) also failed to affect a suite of metabolic traits within 18 weeks of exposure, despite using a dose that elicited larger viral loads than documented in wild fish (Zhang et al. 2019). In contrast, injecting mosquitofish (*Gambusia holbrooki*) with lipopolysaccharide (LPS), known to stimulate innate immune defenses (Alexander and Rietschel 2001), significantly elevated resting metabolic rate 24 hours post-injection, but not 48 hours or 7 days post-injection (Bonneaud et al. 2016). These examples highlight the large variation in energetic cost, immune challenges deployed, and timing of costs. However, it is also important to consider that the natural relevance of many immune challenges is unclear (Sasser and Weber 2022) and costs may be visible only under certain

environmental conditions (Sokolova 2021). Further, none of the studies of fish cited here investigated how costs vary with response magnitude or across populations.

Threespine stickleback provide an ideal model for studying how costs shape vertebrate immune evolution. Stickleback are the obligate intermediate host of a highly virulent cestode, *Schistocephalus solidus,* that imposes substantial costs on hosts. Infections negatively impact host body condition (Tierney et al. 1996), reduce anti-predatory behavior (Milinski 1985), and multi-cestode infections can siphon up to 46% of host energy (Claar et al. 2023). Stickleback populations vary markedly in their susceptibility and ability to suppress the growth of the cestode (Weber et al. 2017). Additionally, the ancestral state of stickleback is known due to the repeated colonization of freshwater by marine populations (Bell and Foster 1994). In particular, three populations of stickleback from British Columbia, Canada have been extensively studied: a cestode naïve marine population (Sayward Estuary), a cestode tolerant freshwater population (Gosling Lake), and a cestode resistant freshwater population (Roberts Lake). Stickleback from the ancestral cestode naïve marine population are rarely exposed to *S. solidus* in the wild as the eggs of the cestode do not hatch in brackish water (Simmonds and Barber 2016). Experimental exposure to *S. solidus* revealed that individuals from this population are highly susceptible to cestode infection and that cestodes grow to large size once established (Weber et al. 2017). The cestode tolerant and resistant populations vary significantly in cestode infection prevalence in the wild (>50% and 0%, respectively) (Weber et al. 2017). Experimental exposure to cestodes confirmed that the differences in cestode susceptibility are heritable and that the populations vary in their ability to stunt the

growth of cestodes once infected (Weber et al. 2017; Weber et al. 2022). Differences in cestode resistance and growth suppression are mediated by a suite of heritable immune responses, including the production of inflammatory reactive oxygen species (ROS) and peritoneal fibrosis (Weber et al. 2017; Weber et al. 2022). The peritoneal fibrosis response is present in all ray-finned fishes (Vrtilek and Bolnick 2021) and recent work on stickleback suggests that one function of the response is to encapsulate peritoneal-dwelling cestodes in fibrotic tissue, thereby stunting their growth (Weber et al. 2022). The fibrosis response can be induced in the cestode naïve and tolerant populations using injections of 1% aluminum phosphate — a stimulant that induces innate immune responses (Hund et al. 2022). However, fibrosis is rarely seen in wild caught stickleback from these populations and the response is not induced when experimentally exposed to cestodes or when injected with cestode protein (Weber et al. 2022; Hund et al. 2022). In contrast, individuals from the cestode resistant populations mount intense fibrosis responses when exposed to cestodes (Weber et al. 2022). Intriguingly, the ability to fibrose when exposed to cestodes has arisen and subsequently been lost in the cestode tolerant population (Weber et al. 2022), suggesting that deploying the response entails significant costs that may be untenable in some environments.

There is experimental and observational evidence that the presence of fibrosis reduces male and female reproductive output in this species. Specifically, surveys of wild stickleback found that fibrosis was negatively correlated with ovary development, ovary mass, and male nesting probability (Weber et al. 2022; DeLisle and Bolnick 2021). Lab based studies likewise find a significant association between fibrosis and reduced ovary

development, with fibrosis retarding sexual development more severely than cestode infections in the absence of fibrosis (Weber et al. 2022). The proximate mechanisms underpinning the reduction in reproductive output remain unknown. It is possible that the fibrotic tissue effectively castrates the fish by taking up space in the body cavity where gonads develop, though there is evidence that some stickleback populations may be able to heal/remediate fibrotic tissue (Hund et al. 2022). Another (non-exclusive) explanation is that the amount of nutrients or energy necessary to mount a fibrosis response is sufficiently large that fish are unable to develop or maintain reproductive organs and mount the response simultaneously.

We performed lab and field experiments to address four key questions related to the energetic and reproductive costs of fibrosis in threespine stickleback: 1) does fibrosis entail a significant energetic cost?, 2) do energetic costs translate into other quantifiable costs such as mass loss or reductions in fecundity?, 3) do costs scale with the magnitude of the response?, and 4) do costs and cestode resistance covary across populations? In the laboratory (i.e., the 'aquarium' experiment), we used respirometry to quantify the short-term energetic cost of mounting a fibrosis response in terms of standard metabolic rate (SMR) and mass loss. In large outdoor ponds (the 'pond' experiment), which provide a longer term, semi-natural context for our lab-based assays, we assessed the cost of fibrosis in terms of survival, body condition, and reproductive capacity. We hypothesized that fibrosis would be energetically costly (elevated SMR) and lead to reductions in body and reproductive organ mass. We additionally expected that these costs would scale with response severity. Further, we hypothesized that the evolution of cestode resistance may

be accompanied by evolution to reduce the cost of immune responses. Finally, we expected that energetic costs would drive changes in other physiological metrics of cost. Together, these experiments integrate proximate measurements of costs (i.e., energy and condition) with ultimate selective outcomes (i.e., population differences in disease resistance, reproduction, and survival) to understand how tradeoffs shape trait combinations in different environments.

2. Material and Methods

(a) *Animal populations and experimental design: Aquarium experiment*

We assayed fish from 3 populations in British Columbia, Canada: Sayward Estuary, Gosling Lake, and Roberts Lake (hereafter, cestode naïve, tolerant, and resistant, respectively). Animal stocks were established via *in vitro* fertilization and then housed with siblings in 16 L tanks at the University of Alaska-Anchorage for one year. Aquarium temperature ranged between 15-16.5 °C, and we fed fish to satiation once per day with a combination of frozen blood worms, frozen mysis shrimp, and fish flakes (tropical flakes; Aqueon; Franklin, USA). A 16 hour light: 8 hour dark circadian cycle was maintained for the duration of the experiment.

At the start of the experiment, we anesthetized each fish in a pH-buffered solution of MS-222 and then implanted visible implant elastomer tags (Northwest Marine Technologies; Anacortes, USA) on the dorsal side, following the manufacturer's instructions. After each fish received a unique color tag, they were transferred to 16 L mixed population tanks at a density of 24 fish per tank. At least 21 days after marking, we

measured mass specific standard metabolic rate (see “Respirometry assay: Aquarium experiment” below). We then injected 92 fish with an immune stimulant (naïve = 24, tolerant = 26, resistant = 42) and 20 fish with a sham control (naïve = 6, tolerant = 7, resistant = 7) (see “Immune Challenge: Both experiments” below). We remeasured individual SMR at 24 and 240 hours post-injection. After the last respirometry assay we euthanized animals and scored fibrosis levels (see “Fibrosis score: Both experiments” below). We recorded body mass immediately after each metabolic trial to track how mass changed over the course of the experiment.

(b) *Respirometry assay: Aquarium experiment*

We measured mass specific standard metabolic rate at a temperature of 15 °C using intermittent-flow respirometry and a static respirometer. Fish were fasted for 24 hours, then transferred into individual 330 mL respirometry chambers submerged in a temperature-controlled stock tank and oxygen consumption recorded for 23 hr. Temperature was maintained using a Teco-TR20 cooler/heater system (Senkor Group, Inc., Terrell, TX, USA). We used submersible pumps to maintain a constant flow of water within each chamber and to flush oxygenated water from the stock tank into the chambers intermittently. The cycling of the flush pump (10 minutes open, 12 minutes closed, during which time water O_2 did not fall below 80% of air saturation) was automated using AquaResp3 (Morozov et al. 2019). We used oxygen-sensitive REDFLASH (PyroScience, Aachen, Germany) contactless sensor spots connected to a PyroScience Firesting O_2 oxygen meter (Aachen, Germany) to record the temperature corrected oxygen

concentration (mg/L) in each chamber once per second. Background oxygen consumption was recorded for each chamber after each trial. We used the FishResp package in R (Svendsen et al. 2019) to estimate standard metabolic rate by first subtracting background microbial respiration, then averaging the lowest 20% of points from the entire data set after excluding outliers (i.e., a quantile approach (Chabot et al. 2016)). Observations were subset for each time × population × injection combination and outliers were identified using the IQR method and removed.

(c) *Animal populations and experimental design: Pond experiment*

We assessed the long-term impacts of fibrosis on stickleback survival, male and female reproductive output, and body condition using a large-scale, semi-natural pond experiment. Fish used in this experiment were hybrids of 3 freshwater populations from British Columbia, Canada (Paxton, Priest, and Little Quarry Lakes). Experimental stocks were established via *in vitro* fertilization and reared for one year in laboratory aquaria at the University of British Columbia prior to the experiment.

We introduced 528 adult fish into two fishless 25 × 15 m outdoor experimental ponds on the University of British Columbia campus. Prior to introduction, 264 of the introduced fish were injected with stimulant (1% aluminum phosphate) and the remaining 264 injected with a sham control (0.8x phosphate buffered saline). Equal numbers from each treatment were introduced into each pond. Genetic backgrounds were allocated to treatments in a balanced design to ensure that each genotype and rearing tank in the lab was equally allocated to each treatment group. After 2 months, a time period in which we

observed fish breeding naturally, we exhaustively sampled the ponds and recovered 358 surviving adult fish.

(d) *Immune challenge: Both experiments*

In both experiments we used 20 ul intraperitoneal injections of either a sham control (0.8x phosphate buffered saline) or 1% aluminum phosphate ("alum": AlumVax Phosphate from OZ Biosciences; San Diego, USA) delivered using a 31G 0.3ml syringe (Exel International; Salaberry-de-Valleyfield, Canada). Alum is an immune stimulant that induces an innate immune response at the site of injection (Kooijman et al. 2022) and this dose reliably induces a fibrosis response in stickleback within the timeframe that we measured costs (Hund et al. 2022).

Injections followed the protocol of Hund et al. (Hund et al. 2022). We anesthetized fish in a pH buffered solution of MS-222 until nonresponsive to stimuli. We then placed anesthetized fish on a wet sponge and injected 20 microliters of alum or PBS directly into the body cavity of stickleback using a 31G 0.3ml syringe, taking care to avoid organ damage.

(e) *Fibrosis score: Both experiments*

In the lab, we euthanized fish immediately following the final metabolic trial (10 days post-injection) using an overdose of MS-222, measured body mass, then dissected animals and scored fibrosis. In the pond experiment, we similarly euthanized fish at 60 days post-injection, preserved bodies in formalin, and then recorded body mass, gonad mass, and

scored fibrosis once samples were returned to the lab. In both experiments fibrosis was scored on a 0-3 scale: 0 there was no visible fibrosis; 1 organs were attached to the swim bladder; 2 organs were fused together; and 3 organs were fused to the side of the body cavity.

(f) *Statistical analysis: Both experiments*

Different statistical tests were performed on data before and after experimental treatments, using the R statistical framework for computing (R Core Team 2021). To test for pre-injection differences between populations in body mass and SMR we used generalized linear models (GLM), assuming Gaussian error, and corrected for multiple testing with Tukey's HSD post hoc test. We used the same approach to assess how injection altered body mass and SMR depending on the presence of fibrosis and severity of fibrosis. The best fit model was determined via backward stepwise selection for each combination of metrics of cost (change in SMR 24 hours post-injection, change in SMR 240 hours post-injection, change in mass 24 hours post-injection, change in mass 240 hours post-injection) and potential drivers of cost (presence/absence of fibrosis, fibrosis score). The full model for SMR included the appropriate driver of cost, population, the change in mass over the appropriate time period, starting SMR, and interactions between all variables. The full model for mass included the appropriate driver of cost, population, starting mass, and interactions between all variables. We used the emmeans package in R (Lenth et al. 2023) to conduct post hoc pairwise comparisons. Since the regression involved nested variables, we used path analyses to determine whether changes in mass drove changes in SMR or

vice versa. We used binomial GLMs to assess whether stimulant injections more frequently induced fibrosis responses than sham injections and to detect population level differences in fibrosis presence. We applied non-parametric tests to test whether stimulant injection induced a larger fibrosis response than sham injection (two-tailed Kruskal-Wallis test) and to detect population level differences in fibrosis severity (Wilcoxon test).

For the pond experiment, we used two-tailed t-tests to assess whether the presence of fibrosis was associated with changes in body or gonad mass. We used linear models and treated fibrosis scores as numerical values to assess whether changes in body or gonad mass were associated with the severity of fibrosis. We used a chi-squared test to determine if the proportion of fish with and without fibrosis was equal at the end of the mesocosm experiment.

3. Results

(a) *Aquarium experiment: Effect of injection on fibrosis*

Injections with immune stimulant induced fibrosis and the magnitude of response covaried with cestode immunity. Stimulant injection drove both a higher incidence ($z = 5.166$, $p < .0001$) and more intense fibrosis response (chi-squared = 37.744, $p < .0001$; Fig. 1) than sham injections across populations. Additionally, there were significant interpopulation differences in the incidence ($z = 3.082$, $p = 0.002$) and severity of fibrosis (chi-squared = 12.554, $p = 0.0019$). Fish from the cestode resistant population were more likely to fibrose ($z = 2.478$, $p = 0.0353$) and mounted larger responses than fish from the ancestral cestode

naïve population (W = 1020.5, p = 0.0021; Fig. 1), but there were no differences between the resistant and tolerant populations.

(b) *Aquarium experiment: Pre-perturbation differences (mass, SMR)*

Prior to experimental injections, the mean mass of age-matched fish from the cestode tolerant population (mean ± SEM; 3.8 ± 0.10 g) was significantly larger than individuals from the cestode naïve (mean ± SEM; 1.9 ± 0.07 g) and resistant populations (mean ± SEM; 2.1 ± 0.07 g) (Tolerant – Naïve, t = 13.866, p < .0001; Tolerant – Resistant, t = 14.429, p < .0001). There was no difference in body mass between the cestode naïve and resistant populations. The large, cestode tolerant fish had significantly lower mean SMR (mean ± SEM; 109.7 ± 3.5 mgO_2/kg/hr) than individuals from the cestode naïve (mean ± SEM; 141.2 ± 5.1 mgO2/kg/hr) and resistant populations (mean ± SEM; 125.7 ± 5.2 mgO_2/kg/hr) (Tolerant − Naïve, t = -4.626, p = <.0001; Tolerant – Resistant, t = -2.469, p = 0.0407). The SMR of naïve fish was marginally higher than that of resistant fish (Naïve − Resistant, t = 2.328, p = 0.0571) despite individuals of both populations exhibiting similar mass.

(c) *Aquarium experiment: Effect of fibrosis presence*

We next assessed the cost of fibrosis as a binary trait (present/absent) at 24 and 240 hours post-injection. At 24 hours post-injection fibrosis did not by itself induce a general change in either SMR or mass loss (Fig. 2A, Fig. 3A). However, the combined effects of fibrosis and mass loss was associated with significant increases in SMR at this timepoint (mass loss × fibrosis, t = 2.156, p = 0.0350), which was most pronounced in fish that started with higher

mass (mass loss × fibrosis × starting mass, t = -2.263, p = 0.0272). The metabolic rate of individuals from the cestode naïve population also increased significantly more than individuals from the cestode resistant population, independent of fibrosis status, at this timepoint (Resistant − Naïve, t = -2.515, p = 0.0382; Fig. 2B). There was no effect of fibrosis presence on changes in mass or SMR 24 hours post-injection when assessed via path analysis (Supp. fig. 1). Additionally, there was no association between changes in mass and SMR.

In contrast to the earlier measurements, the impacts of fibrosis became more pronounced at 240 hours post injection. Specifically, fibrosis (considered as a binary trait) was associated with significant increases (~11%) in SMR values (t = 4.018, p = 0.0001; Fig. 2A). Mass loss was also connected with elevated SMR values independent of fibrosis status (t = -2.690, p = 0.0089), but this effect was more pronounced when fibrosis was present (mass loss × fibrosis, t = 3.314, p = 0.0015). The mass loss-SMR connection at this last timepoint was most pronounced when comparing the cestode naïve and tolerant populations (Tolerant − Naïve, t = -3.234, p = 0.0053). The SMR of the cestode naïve population increased significantly more than the cestode tolerant population regardless of fibrosis status (Tolerant − Naïve, t = 2.451, p = 0.0438; Fig. 2B), but did not differ significantly from the resistant population. Despite changes in SMR, there was no additive effect of fibrosis presence on mass loss 240 hours post-injection (Fig. 3A). Larger starting mass was associated with mass loss independent of fibrosis severity in the cestode naïve and resistant populations, but not in the cestode tolerant population (Tolerant − Naïve, t = 2.543, p = 0.0334; Tolerant − Resistant, t = 2.513, p = 0.0360). While this could be a

regression to the mean, this seems unlikely in the case of fish mass. Fish were fasted for 24 h prior to experiments and thoroughly dried before measurements were taken, making it unlikely that initial measures were inflated due to statistical noise.

Given that SMR, mass loss, and fibrosis were all interrelated, we used a path analysis to integrate these effects in a single model. The presence of fibrosis led to significant increases in SMR (z = 1.961, p = 0.05) and had a marginal effect on mass loss (z = -1.940, p = 0.052) 240 hours post-injection (Fig. 4A). While there was no association between changes in mass and SMR, starting mass differed between populations (z = -11.512, p < 0.001, Fig. 4A) and fish with larger starting mass lost more mass (z = -3.834, p < 0.001; Fig. 4A). Despite this, there was not a direct effect of population on mass loss.

(d) *Aquarium experiment: Effect of fibrosis severity*

To determine if the costs of fibrosis scaled with the magnitude of the response, we also assessed fibrosis score (0-3) at 24 and 240 hours post-injection. When treated as an ordinal trait, high fibrosis scores were not associated with elevated SMR or changes in mass 24 hours post-injection (Fig. 5A, Fig. 6A). The only significant result at the middle timepoint was that large fish lost more mass when accounting for fibrosis severity at this timepoint (fibrosis score × starting mass, t = 2.365, p = 0.0200). However, several significant shifts occurred at the 240 h timepoint. High fibrosis scores were associated with increased SMR (t = 2.859, p = 0.0057; Fig. 5A,B), the effect being more pronounced when fish lost more mass (fibrosis score × mass loss, t = 2.000, p = 0.04976). Similarly, high fibrosis scores predicted increased mass loss (t = -3.518, p = 0.0006; Fig. 6A). Individuals

with larger starting mass lost more mass independent of fibrosis status (t = -3.203, p = 0.0018); however, the effect of starting mass was most pronounced when fibrosis was severe (fibrosis score × starting mass, t = 3.190, p = 0.0019). This connection between fibrosis severity and mass loss was most pronounced in the cestode tolerant population (Tolerant − Naïve, t = -3.158, p = 0.0059; Tolerant − Resistant, t = -3.449, p = 0.0024; Fig. 6B).

A path analysis of these nested associations at the 240 h timepoint (Fig. 4B) revealed the following causal connections. Increased fibrosis severity drove mass loss (z = -2.080, p = 0.038), but not increased SMR (z = 1.633, p = 0.103). Population of origin also directly affected both starting mass (z = -11.512, p < 0.001) and mass loss (z = -2.001, p = 0.045), with larger fish losing more mass regardless of population (z = -4.149, p < 0.001).

(e) *Pond experiment: Effect of fibrosis*

At the end of the pond experiment, we found significantly fewer individuals with fibrosis present, compared to the 50% expected based on the numbers of injected fish (~34% reduction, chi-squared = 122.28, p < .0001; Fig. 7A). Individuals with fibrosis weighed significantly less (~6%) than those without (t = 2.0326, p = 0.0431; Fig. 7B). Additionally, fibrosed female fish had significantly reduced ovary mass (~34%) (t = 3.3765, p = 0.0009; Fig. 7C) while the testes mass of males was not affected (Fig. 7D). When considering fibrosis on a 0-3 scale, body mass ($F_{1,356}$ = 6.735, p = 0.0098; Fig. 7B) and ovary mass ($F_{1,198}$ = 9.261, p = 0.0026; Fig. 7C) both decreased as response severity increased.

4. Discussion

Our experiments reveal that the induction of fibrosis in threespine stickleback, a response that is beneficial for stopping the progression of cestode infection yet differs heritably across populations (Weber et al. 2022), evolved in the context of substantial costs. Because we induced fibrosis in both lab and semi-natural field settings, we were able to divide estimates of costs at both proximate (i.e., changes in mass and energy usage) and ultimate (i.e. survival and reproduction) levels. When observed under lab conditions, higher fibrosis levels drove increased energy usage and declines in body mass, but these costs only became apparent 10 days post-induction. While we originally expected fibrosis to act primarily through effects on SMR, our results suggest it directly causes both increased SMR and mass loss. The relationship between response magnitude and cost also varied across populations. Fish from a cestode tolerant population grew faster and had larger initial masses, but lost more mass as a function of fibrosis severity than cestode naïve and resistant populations. Thus, the evolution of cestode tolerance, which includes failing to fibrose when naturally infected, may involve two non-mutually exclusive scenarios: the gain of traits that exacerbate the costs of fibrosis and/or the loss of traits that mitigate the costs of fibrosis. Furthermore, under field conditions we found that the presence of fibrosis led to decreased survival, and that increased fibrosis severity led to decreased ovary and body mass. We now address additional interpretations and caveats of these experiments to clarify how tradeoffs may shape the outcome of immune evolution in this system.

(a) *Multiple costs are associated with induction of fibrosis*

The immune-associated energetic costs documented in this study fit firmly within the range (i.e., 0-35%) observed in previous studies across a wide range of taxa (Lochmiller and Deerenberg 2000; Schoenle et al. 2018). Having established that fibrosis entails energetic cost, we additionally tested whether the magnitude of cost varies over time and whether other traits that are more directly connected to fitness were also impacted by fibrosis. These additional connections are important, as costs can vary temporally (Bonneaud et al. 2016) and be compensated for by increasing consumption or downregulating other energetically costly activities (Sara et al. 2014). Although fibrosis did not directly induce mass loss in the aquarium experiment, we found that fibrosed fish had 6% lower body mass at the conclusion of the pond experiment. Notably, wet mass may not be an ideal proxy for resource reserves, as changes in water content can mask changes in tissue mass in fish (Lambert and Dutil 1997). Consequently, the actual costs in terms of reserve catabolism may have been more severe than our estimates.

Our pond experiment also provided insight into how fibrosis affects two traits that directly impact fitness: survival and reproductive capacity. Because fibrosis was stimulated in exactly half of all pond fish and our method of inducing fibrosis is extremely effective (Vrtilek and Bolnick 2021; Hund et al. 2022), the paucity of fish recovered with fibrosis strongly suggests they incurred reduced survival compared to the controls. While the impact of fibrosis on survival in experimental ponds is not a perfect proxy for natural lakes due to reduced exposure to parasites and predators, this result at least provides strong circumstantial evidence for the fitness connection. Moreover, we found that fibrosis entailed significant reproductive cost in terms of gonad mass, but only for females.

Previous studies have similarly reported that fibrosed female stickleback were less likely to become gravid in both lab and field settings (Weber et al. 2022; DeLisle and Bolnick 2021). Here, it is important to note that trade-offs are imposed only between processes that both entail significant costs (Lochmiller and Deerenberg 2000). Thus, it is important to consider where costs associated with reproduction are imposed for both sexes. Female stickleback make massive investments in the production of eggs, with clutches reaching up to 38% of a female's somatic mass, and thus gonad mass provides a good proxy for reproductive cost (Heins et al. 2008). In contrast, male stickleback invest relatively little in gamete production, but are entirely responsible for constructing, maintaining, and defending nests. These behaviors are energetically costly in other systems and likely impose the largest costs associated with reproduction in male stickleback (Mainwaring and Hartley 2013). Thus, the divergent cost in gonad mass is unsurprising and does not imply that fibrosis associated reductions in fecundity are isolated to females. Indeed, fibrosis is associated with reduced male nesting probability in wild stickleback (DeLisle and Bolnick 2021). Taken together, these results suggest that fibrosis entails significant reproductive cost for both sexes, but this is not reflected when solely examining gonad mass.

(b) *Scaling, directionality, and timing of costs*

The positive relationship between immune response magnitude and severity of mass loss suggests that fibrosis costs can be managed by modulating the scale of the response. Thus, selection can optimize the response to local conditions (e.g., likelihood of parasite exposure or resource availability) via immune suppression (Boots 2011). Notably, this

scenario requires that there be a quantitative benefit to increases in immune response (i.e., any increase in fibrosis severity decreases the impact of pathogen) (Urban et al. 2013). Data from wild stickleback support this assumption. Both infection probability and the mass of successful cestode infections declines with fibrosis severity (DeLisle and Bolnick 2021), reducing the energetic burden of successful infections (Claar et al. 2023) and facilitating the encysting of cestodes (Weber et al. 2022).

Our experiments also shed light on the directionality of costs imposed by fibrosis. Contrary to our hypothesis, we found no evidence for energetic costs driving, or being driven by, mass loss. Instead, we found that both energy and mass were independently impacted by fibrosis. This suggests that, in addition to increased energetic demands, fibrosis requires tissue to be catabolized to liberate nutrients (such as nitrogen or phosphorus) (Sanders and Taylor 2018). Alternately, the reduction in body mass may be a consequence of immune related anorexia which is hypothesized to reduce exposure to additional threats and curtail other energetically costly activities (e.g., finding, digesting, and assimilating food) while mounting immune responses (Hite et al. 2020).

Previous studies have emphasized the importance of measuring costs over periods which capture the full course of an immune response (Bonneaud et al. 2016); our results exemplify the necessity of this experimental criteria. Previous work has shown that the deposition of fibrotic tissue begins within 24 hours of stimulant injection and continues up to 90 days post-injection (Hund et al. 2022). However, we found no evidence of cost 24 hours post-injection regardless of response severity. We did find significant costs associated with severe fibrosis in terms of body mass and SMR 10 days after inducing the

response in the aquarium experiment. Although we did not continue the lab experiments long enough to identify when (or if) the costs of fibrosis subsided, our field experiments suggest that the costs may be lasting. Fibrosed fish continued to display lower body mass at the end of the 2 month long pond experiment and costs were ~6% more severe than in the aquarium experiment. Importantly, the field observations may include the direct effects of fibrosis (i.e., tissue catabolism and increased energy demand) or indirect effects on feeding (i.e., immune related anorexia or lethargy). Additionally, changes in food provisioning can profoundly impact measurements of cost (Boots 2011). Future work in laboratory settings could disentangle these effects. Measuring post-induction immune costs over a longer period would clarify the response duration. Experimentally manipulating food availability and monitoring intake could test whether fish can mask costs via compensatory feeding and assess whether fibrosis is associated with anorexia.

(c) *Tradeoffs affecting heritable differences in immunity*

Our aquarium experiment highlights why observations of evolutionarily divergent lineages is critical for understanding the landscape of immune tradeoffs. While we found a generally positive relationship between response severity and cost, there was significant variation across populations. Stimulant injection induced fibrosis in every population, but individuals from the cestode resistant population mounted significantly larger responses than those from the cestode naïve population, supporting the hypothesis that fibrosis should be upregulated in the higher threat freshwater environment. Despite this, resistant fish did not pay higher costs when mounting strong fibrosis responses. In fact, individuals

from the cestode tolerant population lost more mass as a function of fibrosis severity than individuals from the cestode naïve and resistant populations. Additionally, the cost in terms of SMR appears to be decoupled from fibrosis presence and severity in the cestode naïve population, potentially because several individuals injected with stimulant did not fibrose but still likely mounted an innate immune response (Kooijman et al. 2022).

Individuals from the freshwater cestode resistant population also grew significantly more slowly than individuals from the freshwater cestode tolerant population prior to the experiment, replicating previous observations (Weber et al. 2017). While both populations can mount robust fibrosis responses, the cestode tolerant population rarely fibroses in the wild or when exposed to cestode proteins (Hund et al. 2022). This implies that maintaining responsive innate immunity might itself entail significant costs. Artificial selection experiments consistently demonstrate a negative correlation between growth rate and immune response strength across a wide range of taxa (Boots 2011; van der Most et al. 2011; Kraaijeveld and Godfray 1997; Boots and Begon 1993). Growth rate may be under intense selection in stickleback as they have the potential to outgrow many gape-limited predators (Urban 2007; Reimchen 1994). If immune suppression facilitates rapid growth, then this may also be a mechanism to tolerate cestode infections as several species of piscivorous birds (the terminal host of the cestode) on Vancouver Island are gape limited (Reimchen 1994). This is of particular importance as avian piscivores are one of the primary mechanisms of parasite induced host mortality in the system (Milinski 1985; Svensson et al. 2022). Further, rapid growth may also limit the detrimental effects of

cestode infections on host fecundity as female reproductive output is highly correlated with body size in stickleback (Heins et al. 2008).

5. *Conclusions*

This study adds to our understanding of how immune costs scale with response magnitude, relate to other costs, and vary across populations. Further, it adds to a growing body of work that has revealed the immunological and genetic mechanisms that underpin fibrosis (Weber et al. 2022; Lohman et al. 2017; Fuess et al. 2021), its role in stunting cestode growth (Weber et al. 2017; Weber et al. 2022), its cost in terms of fecundity (Weber et al. 2022; DeLisle and Bolnick 2021), and the evolutionary trajectory of the trait in several populations (Weber et al. 2022).

Permits/Acknowledgments/Funding

All experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of the University of Alaska-Anchorage (Protocol #1508704-1) and the University of Connecticut (Protocol #A18-008). Wild fish collections were approved by the Ministry of Forests, Lands, and Natural Resources of British Columbia (Permit #NA18-287893 and #MRNA18-288653), and the aquarium experiment in Alaska was approved by the Alaska Department of Fish and Game (Permit #P-19-03 and #P-20-002).

B. Solin provided animal husbandry assistance. J. Hite provided invaluable statistical assistance. J. Ferguson was instrumental in constructing the respirometry equipment. All authors contributed to the conception and design of the experiment. KTS and KAT collected the data. KTS analyzed the data and led the writing of the manuscript. All authors contributed substantially to the drafts. KTS was supported by an Institutional Development Award from the NIGMS of the NIH (#2P20GM103395). KTS and JNW were supported by the NIGMS of the NIH (#1R35GM142891-01). JAWS was supported by the Division of Integrative Organismal Systems of the NSF (#1557818) and by an Institutional Development Award from the NIGMS of the NIH (#P20GM103395). KAT was funded by an NSERC Canada Graduate Scholarship – Doctoral, a UBC 4-Year Fellowship, and a BC Graduate Fellowship, and is presently funded by an HFSP Long-Term Fellowship. SPD was funded by the University of Connecticut. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Alexander C, Rietschel ETh (2001) Invited review: Bacterial lipopolysaccharides and innate immunity. Journal of Endotoxin Research 7:167–202. https://doi.org/10.1177/09680519010070030101

Bell MA, Foster S (1994) The Evolutionary Biology of the Threespine Stickleback. Oxford University Press

Bonneaud C, Wilson RS, Seebacher F (2016) Immune-Challenged Fish Up-Regulate Their Metabolic Scope to Support Locomotion. PLoS ONE 11:e0166028. https://doi.org/10.1371/journal.pone.0166028

Boots M (2011) The Evolution of Resistance to a Parasite Is Determined by Resources. The American Naturalist 178:214–220. https://doi.org/10.1086/660833

Boots M, Begon M (1993) Trade-Offs with Resistance to a Granulosis Virus in the Indian Meal Moth, Examined by a Laboratory Evolution Experiment. Functional Ecology 7:528. https://doi.org/10.2307/2390128

Boots M, Haraguchi Y The Evolution of Costly Resistance in Host-Parasite Systems

Brown GP, Phillips BL, Dubey S, Shine R (2015) Invader immunology: invasion history alters immune system function in cane toads (*Rhinella marina*) in tropical Australia. Ecol Lett 18:57–65. https://doi.org/10.1111/ele.12390

Bryla A, Zagkle E, Sadowska ET, et al (2022) Measurements of body temperature and oxidative stress reveal differential costs associated with humoral immune function in a passerine bird. Journal of Experimental Biology 225:jeb244897. https://doi.org/10.1242/jeb.244897

Chabot D, Steffensen JF, Farrell AP (2016) The determination of standard metabolic rate in fishes: measuring smr in fishes. J Fish Biol 88:81–121. https://doi.org/10.1111/jfb.12845

Claar DC, Faiad SM, Mastick NC, et al (2023) Estimating the magnitude and sensitivity of energy fluxes for stickleback hosts and *Schistocephalus solidus* parasites using the metabolic theory of ecology. Ecology and Evolution 13:e10755. https://doi.org/10.1002/ece3.10755

Cutrera AP, Luna F, Merlo JL, et al (2014) Assessing the energetic costs and trade-offs of a PHA-induced inflammation in the subterranean rodent Ctenomys talarum: Immune response in growing tuco-tucos. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 174:23–28. https://doi.org/10.1016/j.cbpa.2014.04.001

De Lisle SP, Bolnick DI (2021) Male and female reproductive fitness costs of an immune response in natural populations *. Evolution 75:2509–2523. https://doi.org/10.1111/evo.14266

Fuess LE, Weber JN, Den Haan S, et al (2021) Between-population differences in constitutive and infection-induced gene expression in threespine stickleback. Molecular Ecology 30:6791–6805. https://doi.org/10.1111/mec.16197

Hasselquist D, Nilsson J-Å (2012) Physiological mechanisms mediating costs of immune responses: what can we learn from studies of birds? Animal Behaviour 83:1303–1312. https://doi.org/10.1016/j.anbehav.2012.03.025

Hayward AD, Garnier R, Watt KA, et al (2014) Heritable, Heterogeneous, and Costly Resistance of Sheep against Nematodes and Potential Feedbacks to Epidemiological Dynamics. The American Naturalist 184:S58–S76. https://doi.org/10.1086/676929

Heins D, King R, Baker J, Foster S (2008) An overview of life-history variation in female threespine stickleback. Behav 145:579–602. https://doi.org/10.1163/156853908792451539

Hite JL, Pfenning AC, Cressler CE (2020) Starving the Enemy? Feeding Behavior Shapes Host-Parasite Interactions. Trends in Ecology & Evolution 35:68–80. https://doi.org/10.1016/j.tree.2019.08.004

Hund AK, Fuess LE, Kenney ML, et al (2022) Population-level variation in parasite resistance due to differences in immune initiation and rate of response. Evolution Letters 6:162–177. https://doi.org/10.1002/evl3.274

Kooijman S, Vrieling H, Verhagen L, et al (2022) Aluminum Hydroxide And Aluminum Phosphate Adjuvants Elicit A Different Innate Immune Response. Journal of Pharmaceutical Sciences 111:982–990. https://doi.org/10.1016/j.xphs.2022.01.014

Kraaijeveld AR, Godfray HCJ (1997) Trade-off between parasitoid resistance and larval competitive ability in Drosophila melanogaster. Nature 389:278–280. https://doi.org/10.1038/38483

Lambert Y, Dutil J-D (1997) Can simple condition indices be used to monitor and quantify seasonal changes in the energy reserves of Atlantic cod (Gadus morhua)? 54:

Lenth RV, Bolker B, Buerkner P, et al (2024) emmeans: Estimated Marginal Means, aka Least-Squares Means

Llewellyn D, Thompson MB, Brown GP, et al (2012) Reduced investment in immune function in invasion-front populations of the cane toad (Rhinella marina) in Australia. Biol Invasions 14:999–1008. https://doi.org/10.1007/s10530-011-0135-3

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98. https://doi.org/10.1034/j.1600-0706.2000.880110.x

Lohman BK, Steinel NC, Weber JN, Bolnick DI (2017) Gene Expression Contributes to the Recent Evolution of Host Resistance in a Model Host Parasite System. Front Immunol 8:1071. https://doi.org/10.3389/fimmu.2017.01071

Mainwaring MC, Hartley IR (2013) The Energetic Costs of Nest Building in Birds. Avian Biology Research 6:12–17. https://doi.org/10.3184/175815512X13528994072997

McNab BK (2002) The Physiological Ecology of Vertebrates. Comstock Publishing Associates, Ithaca, NY

Milinski M (1985) Risk of Predation of Parasitized Sticklebacks (Gasterosteus Aculeatus L.) Under Competition for Food. Behav 93:203–216. https://doi.org/10.1163/156853986X00883

Morozov S, McCairns RJS, Merilä J (2019) FishResp: R package and GUI application for analysis of aquatic respirometry data. Conservation Physiology 7:. https://doi.org/10.1093/conphys/coz003

Nilsson J-Å, Granbom M, Råberg L (2007) Does the strength of an immune response reflect its energetic cost? J Avian Biology 38:488–494. https://doi.org/10.1111/j.0908-8857.2007.03919.x

Peuß R, Box AC, Chen S, et al (2020) Adaptation to low parasite abundance affects immune investment and immunopathological responses of cavefish. Nat Ecol Evol 4:1416–1430. https://doi.org/10.1038/s41559-020-1234-2

R Core Team (2021). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Reimchen TE (1994) Predators and Morphological Evolution in Threespine Stickleback. In: The Evolutionary Biology of the Threespine Stickleback

Sanders AJ, Taylor BW (2018) Using ecological stoichiometry to understand and predict infectious diseases. Oikos 127:1399–1409. https://doi.org/10.1111/oik.05418

Sarà G, Rinaldi A, Montalto V (2014) Thinking beyond organism energy use: a trait-based bioenergetic mechanistic approach for predictions of life history traits in marine organisms. Marine Ecology 35:506–515. https://doi.org/10.1111/maec.12106

Sasser KT, Weber JN (2022) A call for more ecologically and evolutionarily relevant studies of immune costs. Evol Ecol. https://doi.org/10.1007/s10682-022-10213-5

Schoenle LA, Downs CJ, Martin LB (2018) An Introduction to Ecoimmunology. In: Cooper EL (ed) Advances in Comparative Immunology. Springer International Publishing, Cham, pp 901–932

Simmonds NE, Barber I (2016) The Effect of Salinity on Egg Development and Viability of *Schistocephalus solidus* (Cestoda: Diphyllobothriidea). Journal of Parasitology 102:42–46. https://doi.org/10.1645/14-701

Sokolova I (2021) Bioenergetics in environmental adaptation and stress tolerance of aquatic ectotherms: linking physiology and ecology in a multi-stressor landscape. Journal of Experimental Biology 224:jeb236802. https://doi.org/10.1242/jeb.236802

Sparkman AM, Palacios MG (2009) A test of life-history theories of immune defence in two ecotypes of the garter snake, *Thamnophis elegans*. Journal of Animal Ecology 78:1242–1248. https://doi.org/10.1111/j.1365-2656.2009.01587.x

Svendsen MBS, Bushnell P, J. F. Steffensen (2019) AquaResp3

Svensson PA, Eghbal R, Eriksson R, Nilsson E (2022) How cunning is the puppet-master? Cestode-infected fish appear generally fearless. Parasitol Res 121:1305–1315. https://doi.org/10.1007/s00436-022-07470-2

Tierney JF, Huntingford FA, Crompton DWT (1996) Body condition and reproductive status in sticklebacks exposed to a single wave of *Schistocephalus solidus* infection. Journal of Fish Biology 49:483–493. https://doi.org/10.1111/j.1095-8649.1996.tb00043.x

Urban MC (2007) THE GROWTH–PREDATION RISK TRADE-OFF UNDER A GROWING GAPE-LIMITED PREDATION THREAT. Ecology 88:2587–2597. https://doi.org/10.1890/06-1946.1

Urban MC, Bürger R, Bolnick DI (2013) Asymmetric selection and the evolution of extraordinary defences. Nat Commun 4:2085. https://doi.org/10.1038/ncomms3085

van der Most PJ, de Jong B, Parmentier HK, Verhulst S (2011) Trade-off between growth and immune function: a meta-analysis of selection experiments. Functional Ecology 25:74–80. https://doi.org/10.1111/j.1365-2435.2010.01800.x

Vrtílek M, Bolnick DI (2021) Macroevolutionary foundations of a recently evolved innate immune defense. Evolution 75:2600–2612. https://doi.org/10.1111/evo.14316

Weber JN, Steinel NC, Peng F, et al (2022) Evolutionary gain and loss of a pathological immune response to parasitism. Science 377:1206–1211. https://doi.org/10.1126/science.abo3411

Weber JN, Steinel NC, Shim KC, Bolnick DI (2017) Recent evolution of extreme cestode growth suppression by a vertebrate host. Proc Natl Acad Sci USA 114:6575–6580. https://doi.org/10.1073/pnas.1620095114

Zanuzzo FS, Urbinati EC, Nash GW, Gamperl AK (2015a) Steelhead trout *Oncorhynchus mykiss* metabolic rate is affected by dietary *Aloe vera* inclusion but not by mounting an immune response against formalin-killed *Aeromonas salmonicida*: no cost to *oncorhynchus mykiss* immune response. J Fish Biol 87:43–53. https://doi.org/10.1111/jfb.12690

Zanuzzo FS, Urbinati EC, Rise ML, et al (2015b) Aeromonas salmonicida induced immune gene expression in Aloe vera fed steelhead trout, Oncorhynchus mykiss (Walbaum). Aquaculture 435:1–9. https://doi.org/10.1016/j.aquaculture.2014.09.010

Zhang Y, Polinski MP, Morrison PR, et al (2019) High-Load Reovirus Infections Do Not Imply Physiological Impairment in Salmon. Front Physiol 10:114. https://doi.org/10.3389/fphys.2019.00114

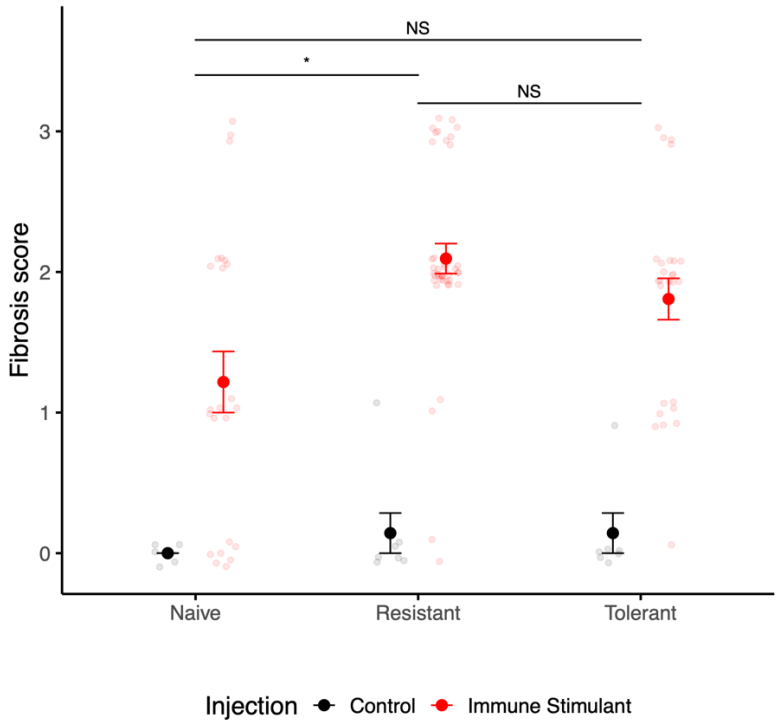


Figure 1. Injection with alum induces fibrosis 240 hours post-injection. Individuals from the cestode resistant population produce significantly more severe fibrosis responses than individuals from the cestode naïve population. Fibrosis scores (mean ± SEM) of individuals injected with PBS and alum 240 hours post-injection. Asterisks denote significant differences between populations; * = $p < 0.05$.

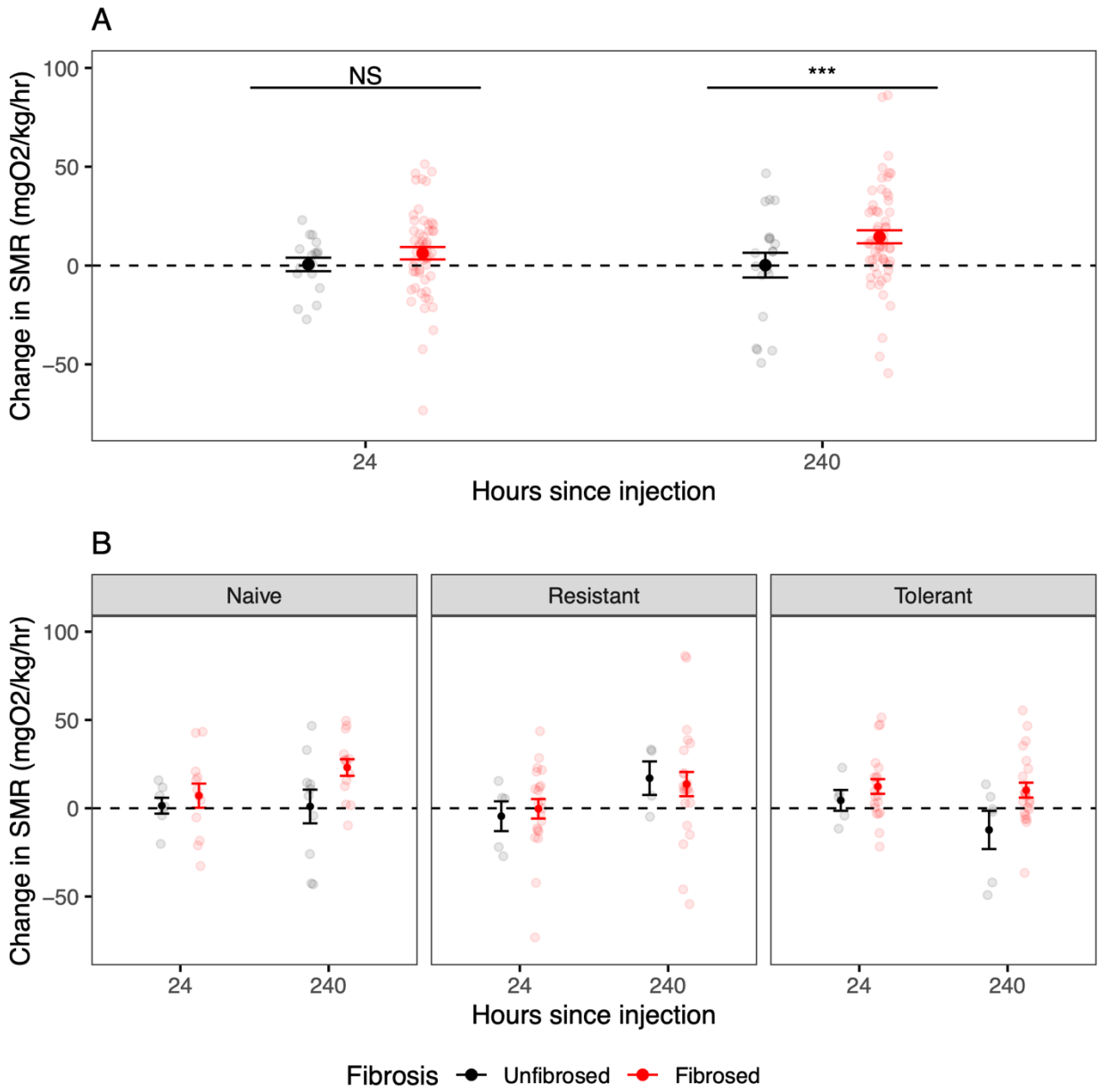


Figure 2. The presence of fibrosis is associated with significantly elevated SMR values 240 h post-injection. Mean (± SEM) change in SMR of unfibrosed and fibrosed individuals 24 and 240 h post-injection (**A**) with all populations pooled and (**B**) shown independently. Asterisks represent a significant effect of fibrosis status when populations are pooled; *** = $p < 0.001$.

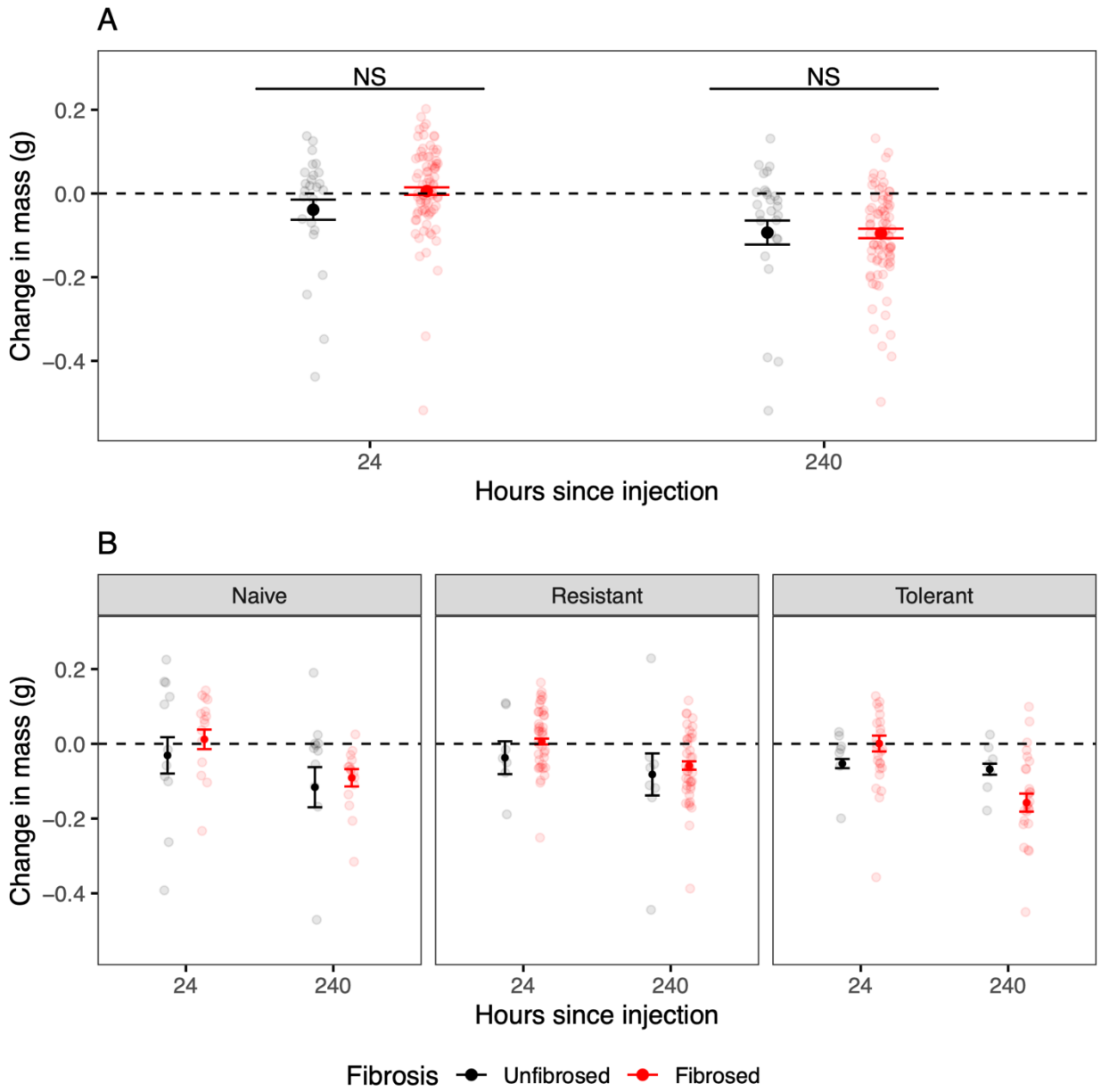


Figure 3. The presence of fibrosis is not associated with significant declines in body mass. Mean (± SEM) change in mass of unfibrosed and fibrosed individuals 24 and 240 h post-injection (**A**) with all populations pooled and (**B**) shown independently.

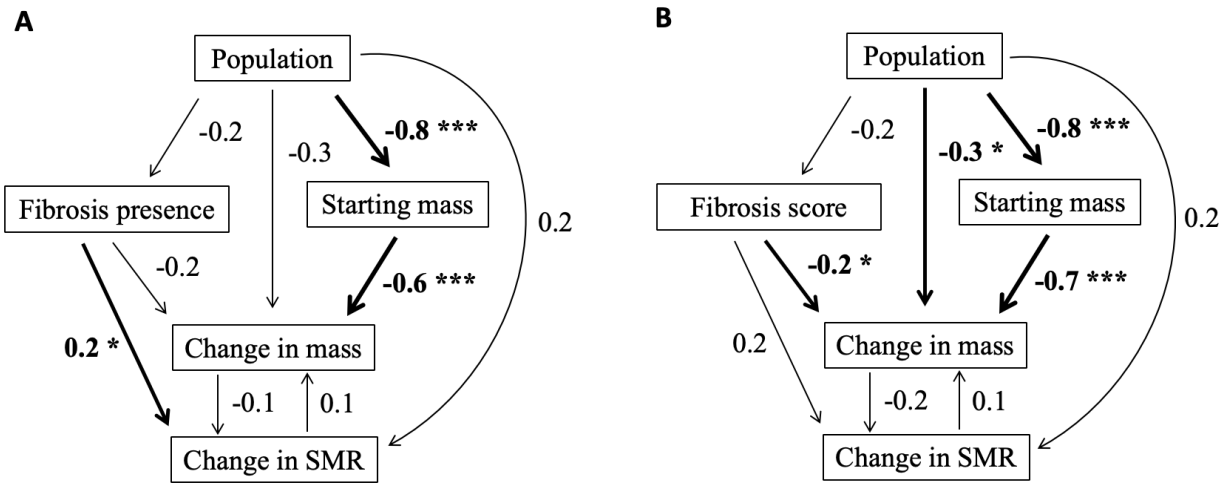


Figure 4. Path analysis detailing the effect of fibrosis (A) presence and (B) severity 240 h post-injection. Asterisks represent a significant effect; * = $p < 0.05$, *** = $p < 0.001$.

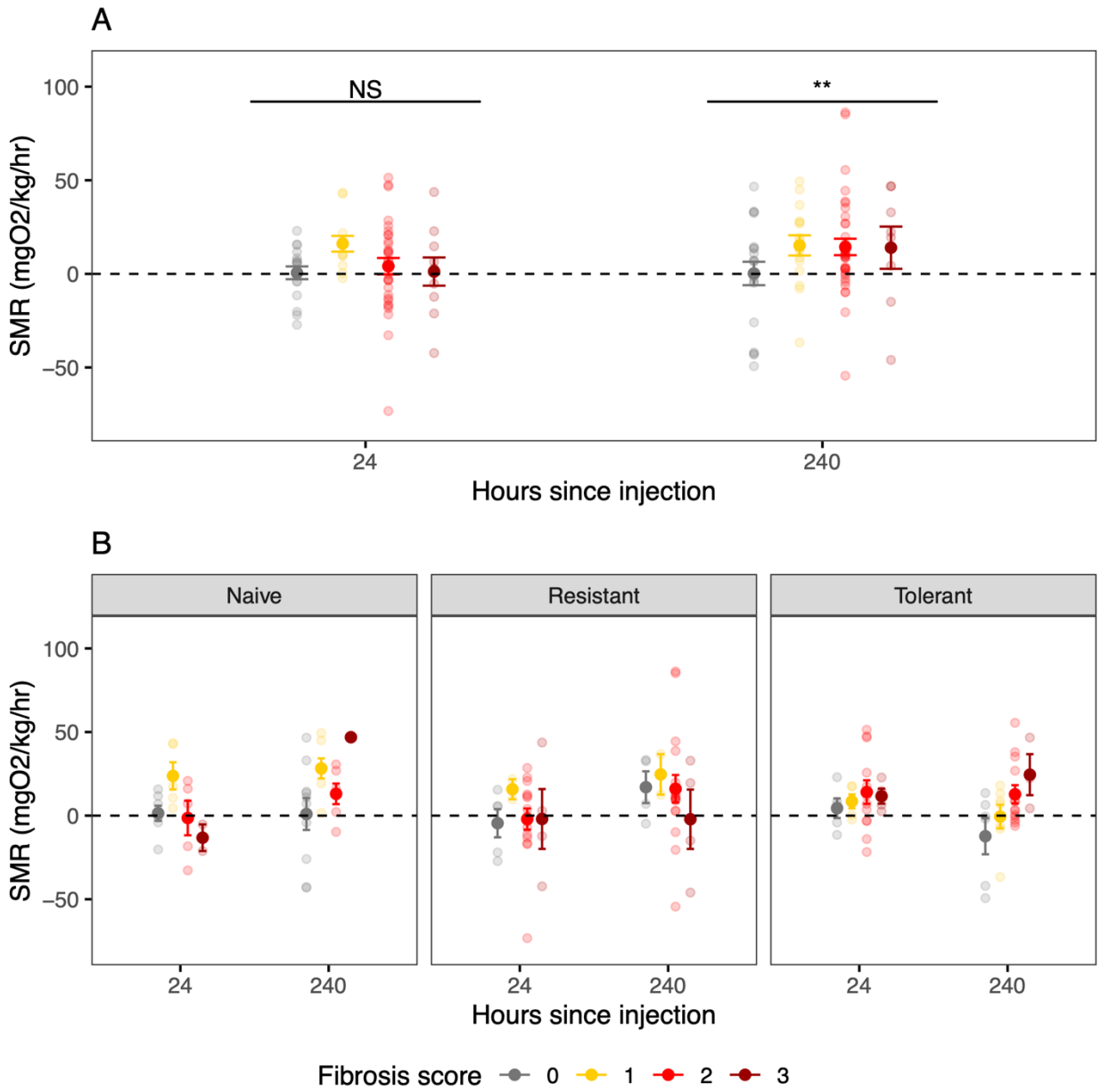


Figure 5. Fibrosis severity is associated with significantly elevated SMR values 240 h post-injection. Mean (± SEM) change in SMR 24 and 240 h post-injection (**A**) with all populations pooled and (**B**) shown independently. Asterisks represent a significant effect of fibrosis status when populations are pooled; ** = $p < 0.01$.

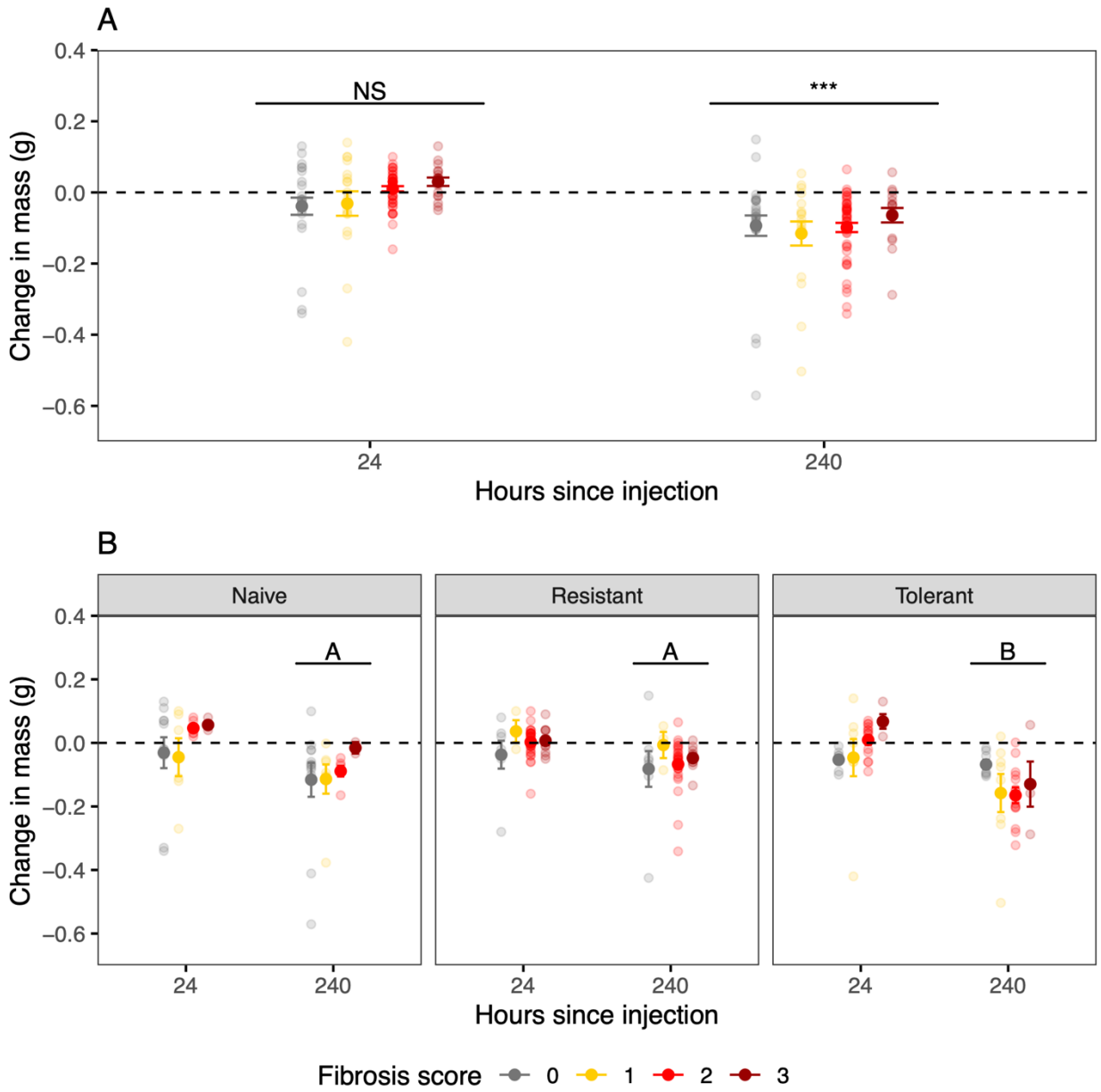


Figure 6. Fibrosis severity is associated with significant declines in body mass 240 h post-injection. Mean (± SEM) change in body mass 24 and 240 h post-injection (**A**) with all populations pooled and (**B**) shown independently. Asterisks represent a significant effect of fibrosis status when populations are pooled; *** = $p < 0.001$. Letters represent significant ($p < 0.05$) population × fibrosis interactions within a time point.

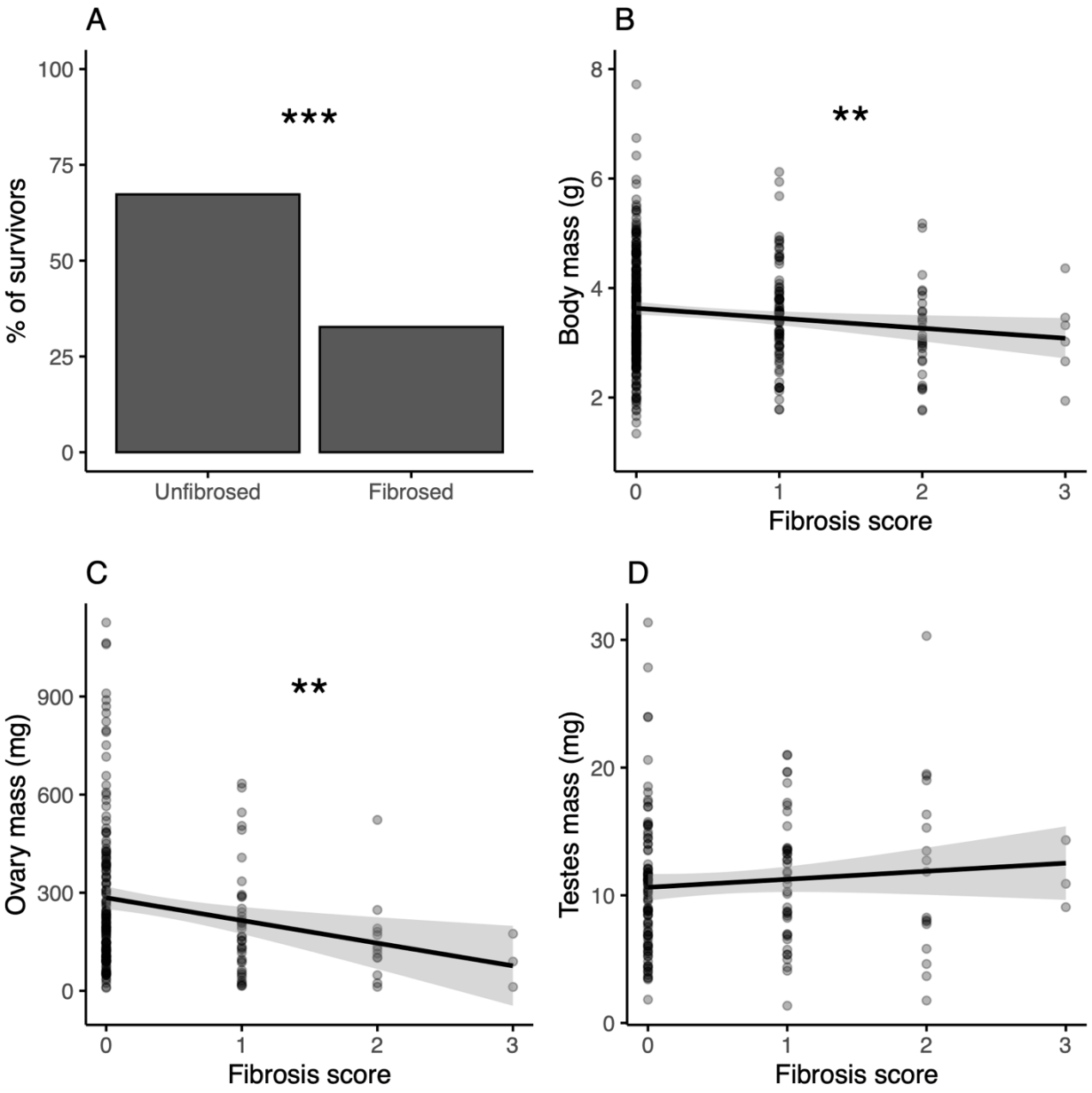
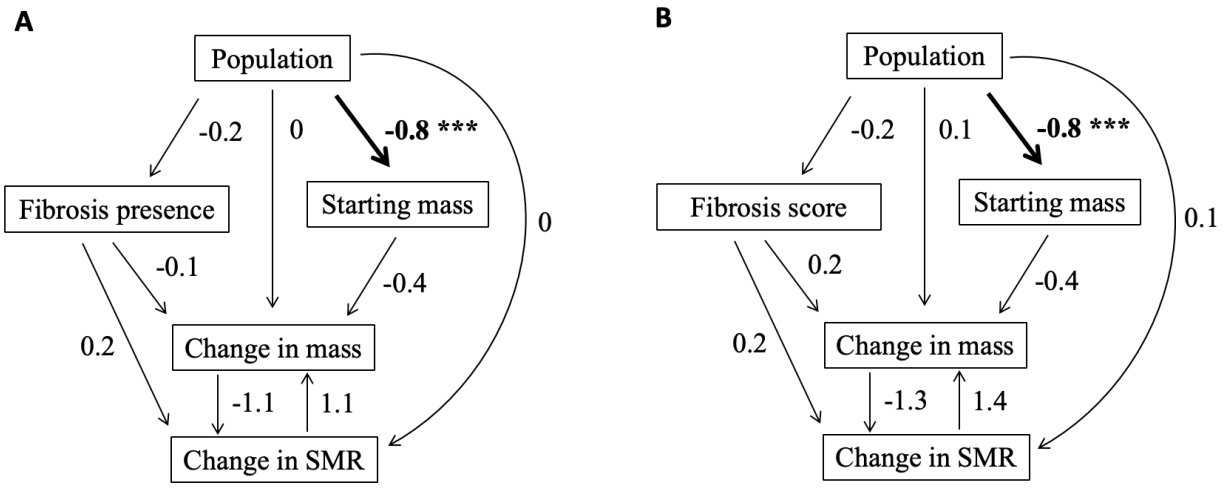


Figure 7. Effect of fibrosis on fitness components in the pond experiment. Fibrosis responses are associated with reduced survival, body mass and ovary mass, but not testes mass. (**A**) Proportion of survivors with and without fibrosis. Mean (± SEM) (**B**) body mass, (**C**) ovary mass, and (**D**) testes mass of individuals at the end of the mesocosm experiment given fibrosis score. Asterisks represent a significant effect; ** = $p < 0.01$, *** = $p < 0.001$.



Supplementary figure 1. Path analysis detailing the effect of fibrosis (A) presence and (B) severity 24 h post-injection. Asterisks represent a significant effect; *** = $p < 0.001$.

Chapter 2

Age structure in Threespine stickleback populations reveals covariation between growth, longevity, and cestode infection prevalence

Kristofer T. Sasser and Jesse N. Weber

University of Wisconsin – Madison

Abstract

Immune responses are costly to maintain and deploy and consequently impose trade-offs on hosts. A trade-off between immune competency and growth rate has been documented in several artificial selection experiments, but there is little evidence from natural systems. We assayed whether growth rate and longevity covaried with cestode infection prevalence and the ability to mount an innate immune response across nine threespine stickleback (*Gasterosteus aculeatus*) populations on Vancouver Island, Canada. We found considerable variation in both life history and immune traits. Despite this, we found no evidence of a trade-off between growth and immune traits. There was not a significant relationship between the ability to mount a fibrosis response and growth rate or longevity. Likewise, we did not find a significant relationship between cestode infection prevalence and growth rate. Further, we found no signature of mortality associated with cestode infection prevalence. In fact, longevity was positively associated with infection prevalence suggesting that cestode-mediated mortality is likely low unless infections are particularly severe and that cestode infections likely accumulate over the life span of stickleback. This study also allowed us to assess whether the widely observed negative relationship between growth and longevity was present in wild stickleback. Interestingly, we found a positive relationship between growth and longevity. One potential explanation for this surprising result is that stickleback may be able outgrow several gape limited predators, thus reducing mortality. This work provides valuable information on stickleback life history variation and lays a foundation for future work investigating links between immune and life history traits in the system.

Introduction

Parasites present a ubiquitous threat to host fitness by reducing host growth (Mavrot et al. 2015), fecundity (Lafferty and Kuris 2009), and survival (Hudson et al. 1992). Hosts reduce the cost of parasites by preventing their establishment, stunting their growth, and clearing them via immune responses. However, organisms must allocate limited resources between processes such as growth, reproduction, and maintenance to maximize fitness (Stearns 1989). Because of this, the evolution of life history traits, such as growth rate or longevity, are shaped by interactions with myriad other traits (Price and Schluter 1991). Because maintaining and deploying immune defenses drains limited resources, it is hypothesized that there is a trade-off between immune defenses and growth (Schmid-Hempel 2003; van der Most et al. 2011). Several experimental and comparative lines of inquiry support this hypothesis. A meta-analysis of poultry selection line experiments found that lines selected for rapid growth showed a significant decline in immune function (van der Most et al. 2011). Conversely, insect lines selected for resistance to parasitoids and viruses grow more slowly than control lines (Boots 2011; Kraaijeveld and Godfray 1997; Boots and Begon 1993). While most evidence comes from laboratory experiments there is some support for this trade-off from natural systems. A survey of 70 tropical bird species found that the most powerful predictor of strong constitutive immune activity was long development time (Lee et al. 2008). Likewise, a common garden experiment involving two populations of diamond-backed watersnake (*Nerodia rhombifer*) found that growth rate was negatively correlated with wound healing and thymus mass (Korfel et al. 2015).

However, more investigations of tractable natural systems that vary in both immune and life history traits are required to provide real world validation of this hypothesized trade-off.

Threespine stickleback may provide just such a system as longevity and growth rate vary markedly across stickleback populations. To our knowledge, at least 16 studies have determined the age of wild-caught stickleback utilizing bony structures from a total of 46 populations (table 1). Stickleback are often assumed to be short lived, but the maximum age of captured stickleback ranges from 2 to 8 years old across populations (table 1). Additionally, these studies demonstrate dramatic differences in growth rate. The mean standard length (SL) of stickleback ranges from 20-50mm at age one, 30-70mm at age two, and 40-100mm at age three (table 1). However, few studies have used standard quantitative population biology techniques to assess growth rate (e.g., von Bertalanffy growth curves; Von Bertalanffy 1938) or mortality (e.g., catch-curve analyses; Smith et al. 2012) (see Yershov and Sukhotin 2014, though).

In addition to a long list of studies on life history variation, there is a growing body of literature documenting substantial heritable variation in stickleback immunity (Weber et al. 2017; Weber et al. 2022; Hund et al. 2022; Barber and Scharsack 2010). Critically, stickleback populations have evolved an assortment of immune responses that facilitate resistance to cestode infection and cestode growth suppression (Weber et al. 2017; Weber et al. 2022; Hund et al. 2022). One such response is peritoneal fibrosis – an innate response that appears to play an essential role in encapsulating cestodes in granulomas (Weber et al. 2022). This stunts the growth of cestodes and can eventually clear infections (Weber et al. 2022). While beneficial for decreasing parasite fitness, fibrosis entails

substantial energetic cost (Sasser et al. *in review*) and leads to reduced host reproductive capacity (De Lisle and Bolnick 2021; Weber et al. 2022; Sasser et al. *in review*). Intriguingly, cestode resistance and growth rate may be negatively linked. Individuals from a highly cestode resistant population grow to significantly smaller adult body size than individuals from a cestode tolerant population in the wild (Bolnick et al. 2020) and this disparity in growth rate persists when reared in a common garden environment and fed *ad libitum* (Weber et al. 2017; Weber et al. 2022; Sasser et al. *in review*). Thus, stickleback provide a tractable natural system with the variation in immune and life history traits required to assess trade-offs.

Here we assess whether demographic parameters vary across 9 stickleback populations and, if so, whether they covary with immunological traits. We used standard quantitative population biology techniques to estimate growth rate, longevity, and mortality. We also examined three proxies of immune competency: cestode prevalence, the incidence and severity of natural fibrosis, and the degree to which fish fibrosed when challenged with an immune stimulant. To control for differences in ecology between lakes we also examined maximum lake depth as this is a primary driver of stickleback diet (Willacker et al. 2010). We hypothesized that there would be a negative relationship between lake depth and growth rate, as deeper lakes are associated with a lower quality planktivorous diet. Further, we predicted that there would be a positive relationship between depth and longevity given that the hunting efficiency of both piscivorous birds and fish can decline with depth (McMahon and Holanov 1995; Vilches et al. 2013). Additionally, we predicted that growth rate and mortality would be negatively associated with strong

immunity (low cestode prevalence, robust fibrosis response) due to the cost of maintaining and deploying costly but beneficial immune responses. In contrast, we hypothesized that longevity would be positively associated with strong immunity due to the negative effect of parasites on host health. This study also allowed us to assess whether growth rate covaried with longevity. We hypothesized that there would be negative relationship between growth and longevity as this is common life history paradigm (Mangel and Stamps 2001; Metcalfe and Monaghan 2003). This study provides a robust test of the trade-off between immunity and growth in a natural system and detailed information on the basic life history of one of the most studied wild vertebrates.

Methods

Sample collection

We captured a total of 917 fish from 9 lakes on Vancouver Island, Canada using unbaited minnow traps during June 2022 (table 2). All of the fish were retained from each trap until a sample size greater than 100 was reached in order to avoid size selection biasing growth and mortality estimates (Goodyear 2019). The maximum depth of each lake was obtained from the Province of British Columbia Freshwater Atlas.

After collection, 40 randomly selected individuals were used to assess the prevalence of *S. solidus* infections and the ability to mount a fibrosis response. Of these fish, a subset of 20 were immediately dissected, screened for *S. solidus* infections, and naturally occurring fibrosis was scored, establishing a baseline of naturally occurring fibrosis in the population. The other 20 individuals were retained in aerated 20 gallon

coolers and injected with 1% aluminum phosphate to assess the ability to mount a fibrosis response when challenged (see immune assay section). These individuals were euthanized 24 hours post-injection, screened for *S. solidus* infections, and fibrosis scored. The remaining individuals were immediately euthanized using an overdose of buffered MS-222. The standard length (SL) and mass of each individual was recorded and both pelvic spines were clipped as close to the base as possible and stored dry in individually labelled tubes.

Immune assay

We used 20ul intraperitoneal injections of 1% aluminum phosphate following the protocol of Hund et al. (2022) to assess the ability of fish to mount a fibrosis response ("alum": AlumVax Phosphate from OZ Biosciences; San Diego, USA). Alum is an immune stimulant that induces a local innate immune response at the injection site (Kooijman et al. 2022). We anesthetized fish in a pH buffered solution of MS-222 until nonresponsive, placed anesthetized fish on a wet sponge, and injected 20ul of alum directly into the body cavity of stickleback using a 31G 0.3ml syringe (Exel International; Salaberry-de-Valleyfield, Canada). Fish were then transferred to aerated 20 gallon coolers where they were held until being euthanized 24 hours post-injection. The severity of fibrosis elicited by this dose of alum varies considerably across populations of stickleback when assessed 24 hours post-injection, allowing us to assess the sensitivity of the innate immune system across populations (Hund et al. 2022).

Fibrosis was scored on a 0-4 scale. A score of 0 was assessed if there was no visible fibrosis, 1 if organs were attached to the swim bladder, 2 if organs were fused together, 3 if

organs were fused to the side of the body cavity, and 4 if the body cavity was entirely filled with fibrotic tissue.

Spine preparation and aging

Counting of growth annuli in pelvic spines is a practical method for determining the age of stickleback due to the ease of collecting and storing spines in the field and because the method's accuracy has been validated utilizing a mark recapture experiment in stickleback (Reimchen 1992). Spines were prepared for bulk sectioning by placing them into a silicone mold lined with air-drying modeling clay (DAS) and casting them in epoxy (105A Epoxy with 206A Slow Epoxy Hardener; West System). The epoxy blocks were then sectioned and polished by the thin sectioning lab at the UW – Madison Geology Department. The epoxy blocks were first cut into billets and flattened using a series of diamond discs. Next, they were bonded to glass slides using epoxy and left to cure for 12 hours. Once cured, the samples were cut off the glass slide to a thickness of approximately 1mm and ground to 200 microns using a 220-grit diamond grinding wheel. The sections were then polished in the following sequence on a 500 rpm lap: 6 micron Buehler polycrystalline diamond suspension with allied gold label pad, 3 micron Buehler polycrystalline diamond suspension with allied gold label pad, 1 micron Buehler polycrystalline diamond suspension with allied white label pad. The final polish was achieved on a vibrapolisher using 0.05 micron colloidal alumina suspension on a Buehler microcloth pad. After polishing we photographed the mounted spine sections using a digital camera attached to an inverted microscope at 10X and 20X magnification. We assigned each individual a

random ID and relabeled the photos with the new ID prior to using the photos for age determination.

Photos of each individual spine at 10X and 20X were viewed side by side and annuli were marked and counted. This process was repeated with a second set of unmarked photos once all individuals had been aged once. We used an Evans-Hoenig test to determine if there was any systematic bias between readings (McBride 2015). After anonymized scoring we identified instances of disagreement, revisited the marked photos, assessed whether the images were clear enough to confidently assign an age, and selected the age we were most confident in. We assessed aging precision by calculating the average coefficient of variation (CV) for the whole dataset and for each population (Campana 2001).

Demographic estimates

Estimation of longevity

To assess differences in longevity across populations, we measured the mean, median, and maximum age of captured individuals. We also recorded the age of the oldest 10% (T_{max10}) of individuals in each population as a more conservative estimate of maximum age (Donovan et al. 2012). We used the Chapman-Robson method to estimate instantaneous mortality (Z) as this is the best performing catch curve estimator of mortality when sample sizes are small (Smith et al. 2012). Age classes prior to the age of peak catch were excluded from catch curve analysis.

Length-Weight

The relationship between standard length (*L*, mm) and weight (*W*, g) for each population was assessed using the length-weight equation: $W = aL^b$ (Ricker 1975). To determine whether either a or b parameter varied across populations we linearized the equation (ln (*W*) = ln (*a*) + *b**ln (*L*)) and performed an ANCOVA test.

VBGF construction and comparison

We used length at age data to generate standard von Bertalanffy (VB) growth models of length at age (L_a):

$$L_a = L_{inf}(1 - e^{-K[a-a0]})$$

where *K* is the rate at which the average maximum size (L_{inf}) is approached and a_0 is the theoretical age that an individual would be zero length for each population. We used Bonferroni corrected likelihood ratio tests to determine if growth varied significantly across populations (Kimura 1980). In addition, we also compared the standard length and mass of 1, 2, and 4 years old individuals from each population. These three ages were selected because the majority of growth in stickleback takes place over the first two years of life and by the age of 4 growth has largely ceased (Fig. 1).

Statistics

We assessed whether there was geographic variation in longevity, mortality, and growth using ANOVAs. We used a chi-square tests to assess whether infection prevalence and the presence of fibrosis varied across geography. We used Kruskal-Wallis tests to assess whether the severity of fibrosis varied across geography. In order to assess if there was a relationship between cestode infection and fibrosis we used linear models. Because post-challenge fibrosis scores were not correlated with cestode infection prevalence and were consistently higher than naturally occurring scores, we chose to use post-challenge fibrosis score as a metric of the ability to fibrose. We used Spearman rank correlation tests to assess if there was a relationships between immune traits, demographic parameters, and limnological traits.

Results

Aging

We were able to assign ages to 76% of individuals captured as part of the study (table 2). Failure to assign an age to individuals was primarily due to some spines being too opaque to confidently visualize growth rings and the loss or damage of spines prior to and during epoxy casting. Aging precision for the complete dataset was moderate (10.87 CV) and varied substantially across populations (6.39-14.59 CV). There was no systematic bias between reading sessions (X_3 = 3.44, p = 0.33).

Geographic variation in demography and immune traits

Longevity and mortality

We uncovered dramatic differences in longevity across populations with median ages ranging from 2 to 4 years and maximum ages ranging from 4 to 9 years (table 2). Mean ages ranged from 1.7 to 4.2 years and varied significantly across populations ($F_{8,684} = 38.31$, $p < 0.0001$). T_{max10}, a more conservative estimate of maximum age, also varied significantly across populations ($F_{8,62} = 19.14$, $p < 0.0001$) with mean values ranging from 3.2 to 6.8 years and median values ranging from 3 to 6. Instantaneous mortality (Z) ranged from 0.57-1.05 across populations, but did not differ significantly between populations.

Size and growth

We also uncovered notable variation in body size and growth rate across the populations studied. Both standard length ($F_{8,908} = 257.6$, $p < 0.0001$) and body mass ($F_{8,908} = 199.4$, $p < 0.0001$) varied significantly across populations. The linearized relationship between length and weight also varied significantly across populations ($F_{8,907} = 70.09$, $p < 0.0001$). Likelihood ratio tests revealed significant differences in growth rate between populations as all but 1 pairwise comparisons were significant (GL-RF; fig. 1). The body size of 1 year old (SL: $F_{6,89} = 10.04$, $p = 0.0002$; mass: $F_{6,89} = 4.879$, $p < 0.0001$), 2 year old (SL: $F_{7,204} = 23.3$, $p < 0.0001$; mass: $F_{7,204} = 12.5$, $p < 0.0001$), and 4 year old individuals (SL: $F_{8,97} = 35.51$, $p < 0.0001$; mass: $F_{8,97} = 25.09$, $p < 0.0001$) confirmed that growth rate and adult body size differed significantly across populations.

Immunity

Cestode infection prevalence varied significantly across populations (X_8 = 153.25, p < 0.0001; fig. 2a), ranging from 0% to 58%. The presence (X_8 = 116.69, p < 0.0001) and severity (X_8 = 118.37, p < 0.0001; fig. 2b) of naturally occurring fibrosis also varied significantly across populations. Likewise, populations varied in their ability to fibrose when challenged with immune stimulant in terms of both the presence (X_8 = 18.448, p = 0.0101) and severity (X_8 = 23.149, p = 0.0016; fig. 2b) of fibrosis 24 hours post-injection. At the individual level, both naturally occurring fibrosis (presence: X_1 = 92.859, p < 0.0001; severity: X_1 = 95.018, p < 0.0001) and post-immune challenge fibrosis (presence: X_1 = 5.3404, p = 0.02084; severity: X_1 = 7.9633, p = 0.0048) were positively associated with cestode infection. At the population level, naturally occurring fibrosis was associated with infection prevalence (presence: $F_{1,7}$ = 19.53, p = 0.0031; severity: $F_{1,7}$ = 15.97, p = 0.0052), but the presence and severity of post-immune challenge fibrosis was not.

Drivers of demographic parameters

Given that lake depth has strong effects on ecological variation, and consequently on local adaptation of stickleback, we first tested whether this parameter correlates with measures of infection and innate immunity. Maximum lake depth was not associated with cestode infection prevalence (R = -0.049, p = 0.91) or mean post-challenge fibrosis score (R = -0.68, p = 0.11). Next we assessed whether growth or longevity were associated with maximum lake depth. We found no evidence that longevity (mean age: R = 0.071, p = 0.88; T_{max10}: R = 0.24, p = 0.58; max age: R = 0.35, p = 0.39; fig. 3a), mortality (Z: R = -0.07, p = 0.88), or

growth rate (mean age 1 SL: R = -0.71, p = 0.14; mean age 2 SL: R = -0.21, p = 0.66; mean age 4 SL: R = -0.12, p = 0.79; fig. 4a) were correlated with depth.

We next assessed whether cestode infection prevalence covaried with growth or longevity. Contrary to our expectation, we found no relationship between cestode infection prevalence and growth rate (mean age 1 SL: R = 0.059, p = 0.9; mean age 2 SL: R = 0.34, p = 0.41; mean age 4 SL: R = 0.56, p = 0.12; fig. 4b). Likewise, we found no association between cestode infection and mortality (Z: R = -0.33, p = 0.38). Indeed, we actually found that cestode infection prevalence was associated with increased longevity, although only the relationship with mean age was significant (mean age: R = 0.68, p = 0.044; T_{max10}: R = 0.63, p = 0.071; max age: R = 0.59, p = 0.091; fig. 3b). We found no relationship between mean post-challenge fibrosis score and longevity (mean age: R = -0.21, p = 0.62; T_{max10}: R = -0.14, p = 0.75; max age: R = -0.049, p = 0.91; fig. 3c), mortality (Z: R = 0.12, p = 0.79), or growth rate (mean age 1 SL: R = 0.54, p = 0.24; mean age 2 SL: R = 0.29, p = 0.56; mean age 4 SL: R = 0.048, p = 0.93; fig. 4c).

Finally, we assessed whether growth covaried with longevity. We found no relationship between body size at age one and longevity (mean age: R = 0.07, p = 0.91; T_{max10}: R = -0.21, p = 0.66; max age: R = 0.07, p = 0.87; fig. 5a). However, we found a positive association between longevity and body size at age two (mean age: R = 0.74, p = 0.046; T_{max10}: R = 0.48, p = 0.24; max age: R = 0.48, p = 0.23; fig. 5b) and four (mean age: R = 0.78, p = 0.017; T_{max10}: R = 0.63, p = 0.076; max age: R = 0.55, p = 0.12; fig. 5c), although only the relationship with mean age was significant.

Discussion

By conducting a widespread survey of life history traits across Vancouver Island stickleback populations, we identified marked variation in growth, longevity, cestode infection prevalence, and peritoneal fibrosis. Further, we uncovered that longevity and growth covaried significantly with both each other and cestode prevalence.

Surprisingly, maximum lake depth did not covary with life history traits. Previous work has demonstrated that benthic stickleback grow significantly larger and faster than limnetic stickleback (Schluter 1995), likely due to differences in diet (Schluter 1993). We found no evidence of this, although maximum depth is a coarse proxy for where fish fall on this spectrum (Haines et al. 2023). We also found no relationship between longevity and maximum depth. This is largely unsurprising as a similar suite of piscivorous fish and birds are present in all of the lakes sampled (BC Reconnaissance Inventories). The lack of relationship between depth and cestode prevalence was unsurprising, as a previous study had likewise failed to find a relationship (Bolnick et al. 2020). No previously published work has linked the ability to mount a peritoneal fibrosis response with abiotic traits. While this study provides a tentative survey of this ability, it should be noted that the short time frame between perturbation and dissection likely influences our findings. Previous work has demonstrated that, while the deposition of fibrotic tissue begins within 24 hours of being challenged, the majority of fibrotic tissue is developed between 24hr and 10 days post-challenge (Hund et al. 2022). Thus, the scores recorded here likely do not represent the full ability to mount a response.

We did not find support for our hypothesis that growth would be positively associated with cestode infection prevalence. This is likely due to a complex interplay between immunity and diet. While laboratory based studies often find a trade-off between growth and parasite resistance, the laboratory environment standardizes both diet and parasite exposure. However, in the case of our study these factors are unaccounted for and likely interact to confound our expected findings. The planktivorous diet of primarily limnetic stickleback is of poorer quality and increases the likelihood of cestode exposure as copepods are the initial host (Schluter 1993; Bolnick et al. 2020). Further, the density of both copepod and stickleback populations likely vary across the lakes studied, both of which could influence stickleback growth and cestode exposure risk (Watson et al. 2022; Buck and Lutterschmidt 2017).

The positive relationship between infection prevalence and longevity was surprising, but several non-mutually exclusive explanations exist. First, older individuals may be more likely to be infected due to declining immune performance or higher cumulative lifetime parasite exposures (Gruver et al. 2007; Franceschi et al. 2006). Second, juveniles may be particularly susceptible to parasite-mediated mortality and thus less likely to be found infected (McElroy and Buron 2014). Indeed, the incidence of infection and richness of parasite communities often increases as a function of host age (Knudsen et al. 2002; Belay et al. 2020; Brandell et al. 2021) even in relatively short lived species (Zelmer and Arai 1998). This pattern of accumulation with age is particularly likely if parasite-mediated mortality is low or only becomes significant once infections become particularly severe. It is well established that *Schistocephalus* infections reduce fecundity (Weber et al. 2022; De

Lisle and Bolnick 2021), inhibit predator avoidance (Giles 1983; Milinski 1985; Svensson et al. 2022), deplete host resource reserves (Tierney et al. 1996), and impose significant energetic cost (Claar et al. 2024). Further many of these costs scale with infection severity including the energetic cost (Claar et al. 2024), reduced anti-predator behavior (Giles 1983), and reduced body condition (Bagamian et al. 2004). Notably, one study has assessed how *Scistocephalus* infection prevalence and severity change with age in wild stickleback (Pennycuick 1971). In this population cestode infection prevalence uniformly exceeded 98% after the first year of life and the mean parasite index (parasite mass/host mass), a measure of infection severity, remained constant at ~0.3 across age classes. Despite the ubiquitous presence of severe cestode infections (averaging 30% of host mass), individuals were able to survive until the age of 3. This suggests that stickleback are able to survive with considerable cestode infections for several years as long as infection severity remains below a critical threshold. Future work assessing how the mean/variance ratio of infection severity changes with age would be particularly informative (Anderson and Gordon 1982). If variation above the mean is curtailed in comparison to variation below the mean, then it would suggest that the mean represents an upper lethal limit of infection severity. This approach was used to great effect by Knudsen et al. (2002) to reveal that the swimbladder nematode *Cystidicola farionis* imposes significant mortality on Arctic charr (*Salvelinus alpinus*), but only when infections become severe. Future work linking age, infection severity, reproductive potential, and body condition in the *Schistocephalus*-stickleback system would provide critical information on how cestode infections of varying severity alter host fitness.

The lack of a relationship between fibrosis and life history traits was unexpected and may be the byproduct of several methodological choices. First, our fibrosis measurements may be a poor representation of overall fibrosis ability. As previously stated, the curtailed timeframe between injection and dissection may substantively alter our findings. Additionally, neither the age nor infection status of individuals was accounted for when individuals were selected for injection, both of which could alter the response elicited (Scharsack et al. 2004; Müller et al. 2013). Second, our hypotheses framed fibrosis as a metric of general immune competency (a very dangerous assumption: Adamo 2004; Martin et al. 2006). However, fibrosis is only one of many immune traits that likely covary with one another. There is robust support for a trade-off between innate and adaptive immunity with examples from mammals (McDade et al. 2015), birds (Minias et al. 2023), and fish (Peuß et al. 2020) including stickleback (Wegner et al. 2007). Thus no single measure is likely to adequately represent the overall immune competency of an individual. Thus, these results should be taken with caution until both innate and adaptive responses are assayed alongside growth in a common garden environment. Notably, work is being done to characterize the role both arms of the immune system play in cestode resistance in stickleback paving the way for this type of work (Steinel and Bolnick, 2017; Lohman et al. 2017).

The positive relationship between growth rate and longevity was surprising as previous work, including studies of threespine stickleback, has established that a negative relationship between growth and longevity is common (Miller et al. 2000; Metcalfe and Monaghan 2003; Mangel and Stamps 2001; Lee et al. 2012; Kraus et al. 2013). One

potential explanation for this disparity is that stickleback are capable of outgrowing several gape limited predators (Reimchen 1994). Further, this advantage is likely particularly acute in cestode infected fish as piscivorous birds (several of which are gape limited) are the definitive host of the cestode and a major source of mortality for cestode infected fish (Reimchen 1994; Svensson et al. 2022). Large body size may also reduce predation by non-gape limited predators as the probability of surviving an encounter with predators increases with body size in stickleback (Reimchen 1991). Indeed, larger bodied stickleback were underrepresented in the stomachs of trout suggesting that trout predation declines with body size (Reimchen 1990).

Going deeper: a case study

A subset of the populations used in this study, Gosling and Roberts (hereafter GL and RL), have been the subject of several field and lab based studies providing context and weight to our findings. These populations vary markedly in growth rate and a number of immune traits which will be described in more detail below. In the laboratory, GL fish put on mass roughly twice as fast as RL fish (Sasser et al. *in review*). Remarkably, we found that this difference in growth rate persists in the wild as GL fish are 20% heavier at age 1, 41% heavier at age 2, and 57% heavier at age 4 (Fig. 1). Fish from GL have historically suffered from high cestode infection rates (ranging from ~20-80% year to year) while cestode infections have not been documented in wild RL fish over the last 20 years (Weber et al. 2017). In line with this, we found no infected fish in RL (table 2). However, the prevalence of cestode infections was historically low in GL this year at 7.5% (table 2). Lab experiments

reveal that these stark differences in infection prevalence are the result of heritable differences in cestode resistance. RL fish are significantly less likely to be infected when experimentally exposed to cestodes and are significantly more effective at stunting the growth of cestodes when infected (Weber et al. 2017; Weber et al. 2022). This is facilitated by a suite of robust innate immune defenses including the production of reactive oxygen species and peritoneal fibrosis (Weber et al. 2017; Weber et al. 2022). Challenging fish with cestode protein or alum reveals that RL fish are more likely to fibrose and mount larger responses at 10 days post-challenge than GL fish (Weber et al. 2022). In contrast to this, we did not find significant differences between the populations in the prevalence or intensity of fibrosis 1 day post-challenge (fig. 2). The most likely explanation for this is that the duration of the challenge was not sufficiently long for differences to develop (Hund et al. 2020). Surprisingly, despite suffering from a higher prevalence and severity of cestode infections we found that GL fish were significantly ($p < 0.0001$) more long lived than RL fish (average age: 3.5 vs 2.1; average T_{max10}: 6.8 vs 4.3; max age: 9 vs 6).

In summary, GL fish mount reduced immune responses (ROS production and fibrosis) and concomitantly suffer more numerous and severe cestode infections than RL fish (Sasser et al. *in review*; Weber et al. 2017; Weber et al. 2022). Despite this, we found no signature of elevated mortality in GL; in fact, they live longer than any other population that we surveyed. This work, along with that of Pennycuick (1971), suggests that cestode infection may not significantly contribute to mortality despite imposing significant costs on hosts (Weber et al. 2022; Claar et al. 2024). The reduction in immune investment may facilitate rapid growth, as GL fish grow significantly faster both in the wild and common

garden settings (Sasser et al. *in review*). Selection on growth rate may be intense as large body size improves survival and fecundity (Reimchen 1994; Heins et al. 2008). The strategy of risking infection to facilitate rapid growth may be a resource dependent strategy facilitated by the nutrient dense diet available to shallow living stickleback (Schluter 1993).

Conclusions

This study provides informative measures of stickleback demographic parameters revealing widespread life history variation across populations and tantalizing links between immunity, longevity, and growth rate. Further, it brings into question the fitness cost imposed by infection with a seemingly daunting parasite. This has important implications for the many fields that frequently use wild stickleback as a natural model system and paves the way for future work investigating immune evolution in this rapidly developing model (Barber and Scharsack 2010; Reid et al. 2021).

Permits/Acknowledgments/Funding

All experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of the University of Wisconsin – Madison (Protocol #L006460). Wild fish collections were approved by the Ministry of Forests, Lands, and Natural Resources of British Columbia (Permit #NA22-679623).

D. Rogers and M. Spicuzza were instrumental in designing the spine sectioning process. D. Bolnick and N. Steinel were of great help during field collections. All authors contributed to the design of the study, data collection, and production of the manuscript. KTS analyzed the data and led the writing of the manuscript. KTS and JNW were supported by the NIGMS of the NIH (#1R35GM142891-01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Adamo SA (2004) How should behavioural ecologists interpret measurements of immunity? Animal Behaviour 68:1443–1449. https://doi.org/10.1016/j.anbehav.2004.05.005

Allen JRM, Wootton RJ (1982) Age, growth and rate of food consumption in an upland population of the three-spined stickleback, Gasterosteus aculeatus L. J Fish Biology 21:95–105. https://doi.org/10.1111/j.1095-8649.1982.tb02827.x

Anderson RM, Gordon DM (1982) Processes influencing the distribution of parasite numbers within host populations with special emphasis on parasite-induced host mortalities. Parasitology 85:373–398. https://doi.org/10.1017/S0031182000055347

Bagamian KH, Heins DC, Baker JA (2004) Body condition and reproductive capacity of three-spined stickleback infected with the cestode *Schistocephalus solidus*. Journal of Fish Biology 64:1568–1576. https://doi.org/10.1111/j.0022-1112.2004.00411.x

Barber I, Scharsack JP (2010) The three-spined stickleback-Schistocephalus solidus system: An experimental model for investigating host-parasite interactions in fish. Parasitology 137:411–424

Bellay S, Oda FH, Almeida-Neto M, et al (2020) Host age predicts parasite occurrence, richness, and nested infracommunities in a pilot whale-helminth network. Parasitol Res 119:2237–2244. https://doi.org/10.1007/s00436-020-06716-1

Bergström U, Olsson J, Casini M, et al (2015) Stickleback increase in the Baltic Sea – A thorny issue for coastal predatory fish. Estuarine, Coastal and Shelf Science 163:134–142. https://doi.org/10.1016/j.ecss.2015.06.017

Bolnick DI, Resetarits EJ, Ballare K, et al (2020) Host patch traits have scale-dependent effects on diversity in a stickleback parasite metacommunity. Ecography 43:990–1002. https://doi.org/10.1111/ecog.04994

Boots M (2011) The Evolution of Resistance to a Parasite Is Determined by Resources. The American Naturalist 178:214–220. https://doi.org/10.1086/660833

Boots M, Begon M (1993) Trade-Offs with Resistance to a Granulosis Virus in the Indian Meal Moth, Examined by a Laboratory Evolution Experiment. Functional Ecology 7:528. https://doi.org/10.2307/2390128

Brandell EE, Cross PC, Craft ME, et al (2021) Patterns and processes of pathogen exposure in gray wolves across North America. Sci Rep 11:3722. https://doi.org/10.1038/s41598-021-81192-w

Buck JC, Lutterschmidt WI (2017) Parasite abundance decreases with host density: evidence of the encounter-dilution effect for a parasite with a complex life cycle. Hydrobiologia 784:201–210. https://doi.org/10.1007/s10750-016-2874-8

Campana SE (2001) Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. Journal of Fish Biology 59:197–242. https://doi.org/10.1111/j.1095-8649.2001.tb00127.x

Claar DC, Faiad SM, Mastick NC, et al (2023) Estimating the magnitude and sensitivity of energy fluxes for stickleback hosts and *Schistocephalus solidus* parasites using the metabolic theory of ecology. Ecology and Evolution 13:e10755. https://doi.org/10.1002/ece3.10755

De Lisle SP, Bolnick DI (2021) Male and female reproductive fitness costs of an immune response in natural populations *. Evolution 75:2509–2523. https://doi.org/10.1111/evo.14266

DeFaveri J, Merilä J (2013) Variation in Age and Size in Fennoscandian Three-Spined Sticklebacks (Gasterosteus aculeatus). PLoS ONE 8:e80866. https://doi.org/10.1371/journal.pone.0080866

Donovan MK, Friedlander AM, DeMartini EE, et al (2013) Demographic patterns in the peacock grouper (Cephalopholis argus), an introduced Hawaiian reef fish. Environ Biol Fish 96:981–994. https://doi.org/10.1007/s10641-012-0095-1

Franceschi C, Bonafè M, Valensin S, et al (2000) Inflamm-aging: An Evolutionary Perspective on Immunosenescence. Annals of the New York Academy of Sciences 908:244–254. https://doi.org/10.1111/j.1749-6632.2000.tb06651.x

Gambling SJ, Reimchen TE (2012) Prolonged life span among endemic *Gasterosteus* populations. Can J Zool 90:284–290. https://doi.org/10.1139/z11-133

Giles N (1983) Behavioural effects of the parasite Schistocephalus solidus (Cestoda) on an intermediate host, the three-spined stickleback, Gasterosteus aculeatus L. Animal Behaviour 31:1192–1194. https://doi.org/10.1016/S0003-3472(83)80025-6

Giles N (1987) Population biology of three-spined sticklebacks, *Gasterosteus aculeatus* , in Scotland. Journal of Zoology 212:255–265. https://doi.org/10.1111/j.1469-7998.1987.tb05988.x

Goodyear CP (2019) Modeling Growth: Consequences from Selecting Samples by Size. Trans Am Fish Soc 148:528–551. https://doi.org/10.1002/tafs.10152

Greenbank J, Nelson PR (1959) Life history of the threespine stickleback in Karluk Lake and Bare Lake, Kodiak Island, Alaska. Fishery Bulletin of the Fish and Wildlife Service 59:

Gruver A, Hudson L, Sempowski G (2007) Immunosenescence of ageing. The Journal of Pathology 211:144–156. https://doi.org/10.1002/path.2104

Haines GE, Moisan L, Derry AM, Hendry AP (2021) Dimensionality and modularity of adaptive variation: Divergence in threespine stickleback from diverse environments. Evolutionary Biology

Haines GE, Moisan L, Derry AM, Hendry AP (2023) Dimensionality and Modularity of Adaptive Variation: Divergence in Threespine Stickleback from Diverse Environments. The American Naturalist 201:175–199. https://doi.org/10.1086/722483

Heins D, King R, Baker J, Foster S (2008) An overview of life-history variation in female threespine stickleback. Behav 145:579–602. https://doi.org/10.1163/156853908792451539

Hudson PJ, Newborn D, Dobson AP (1992) Regulation and Stability of a Free-Living Host-Parasite System: Trichostrongylus tenuis in Red Grouse. I. Monitoring and Parasite Reduction Experiments. The Journal of Animal Ecology 61:477. https://doi.org/10.2307/5338

Hund AK, Fuess LE, Kenney ML, et al (2022) Population-level variation in parasite resistance due to differences in immune initiation and rate of response. Evolution Letters 6:162–177. https://doi.org/10.1002/evl3.274

Jones JW, Hynes HBN (1950) The Age and Growth of Gasterosteus aculeatus, Pygosteus pungitius and Spinachia vulgaris, as Shown by their Otoliths. The Journal of Animal Ecology 19:59. https://doi.org/10.2307/1571

Kimura D (1980) Likelihood methods for the von bertalanffy growth curve. Fishery Bulletin 77:

Knudsen R, Amundsen P-A, Klemetsen A Parasite-induced Host Mortality: Indirect Evidence From a Long-term Study

Kooijman S, Vrieling H, Verhagen L, et al (2022) Aluminum Hydroxide And Aluminum Phosphate Adjuvants Elicit A Different Innate Immune Response. Journal of Pharmaceutical Sciences 111:982–990. https://doi.org/10.1016/j.xphs.2022.01.014

Korfel CA, Chamberlain JD, Gifford ME (2015) A test of energetic trade-offs between growth and immune function in watersnakes. Oecologia 179:343–351. https://doi.org/10.1007/s00442-015-3365-8

Kraaijeveld AR, Godfray HCJ (1997) Trade-off between parasitoid resistance and larval competitive ability in Drosophila melanogaster. Nature 389:278–280. https://doi.org/10.1038/38483

Kraus C, Pavard S, Promislow DEL (2013) The Size–Life Span Trade-Off Decomposed: Why Large Dogs Die Young. The American Naturalist 181:492–505. https://doi.org/10.1086/669665

Lafferty KD, Kuris AM (2009) Parasitic castration: the evolution and ecology of body snatchers. Trends in Parasitology 25:564–572. https://doi.org/10.1016/j.pt.2009.09.003

Lee KA, Wikelski M, Robinson WD, et al (2008) Constitutive immune defences correlate with life-history variables in tropical birds. J Anim Ecology 77:356–363. https://doi.org/10.1111/j.1365-2656.2007.01347.x

Lohman BK, Steinel NC, Weber JN, Bolnick DI (2017) Gene Expression Contributes to the Recent Evolution of Host Resistance in a Model Host Parasite System. Front Immunol 8:1071. https://doi.org/10.3389/fimmu.2017.01071

Mangel M, Stamps J Trade-offs between growth and mortality and the maintenance of individual variation in growth

Martin LB, Han P, Lewittes J, et al (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecology 20:290–299. https://doi.org/10.1111/j.1365-2435.2006.01094.x

Mavrot F, Hertzberg H, Torgerson P (2015) Effect of gastro-intestinal nematode infection on sheep performance: a systematic review and meta-analysis. Parasites Vectors 8:557. https://doi.org/10.1186/s13071-015-1164-z

McBride RS (2015) Diagnosis of paired age agreement: a simulation of accuracy and precision effects. ICES Journal of Marine Science 72:2149–2167. https://doi.org/10.1093/icesjms/fsv047

McDade TW, Georgiev AV, Kuzawa CW (2016) Trade-offs between acquired and innate immune defenses in humans. EMPH 2016:1–16. https://doi.org/10.1093/emph/eov033

McElroy EJ, De Buron I (2014) Host Performance as a Target of Manipulation by Parasites: A Meta-Analysis. Journal of Parasitology 100:399–410. https://doi.org/10.1645/13-488.1

McMahon TE, Holanov SH (1995) Foraging success of largemouth bass at different light intensities: implications for time and depth of feeding. Journal of Fish Biology 46:759–767. https://doi.org/10.1111/j.1095-8649.1995.tb01599.x

Milinski M (1985) Risk of Predation of Parasitized Sticklebacks (Gasterosteus Aculeatus L.) Under Competition for Food. Behav 93:203–216. https://doi.org/10.1163/156853986X00883

Miller RA, Chrisp C, Atchley W (2000) Differential Longevity in Mouse Stocks Selected for Early Life Growth Trajectory. The Journals of Gerontology Series A: Biological Sciences and Medical Sciences 55:B455–B461. https://doi.org/10.1093/gerona/55.9.B455

Minias P, Peng W-XV-H, Matson KD (2023) Evolutionary trade-off between innate and acquired immune defences in birds. Front Zool 20:32. https://doi.org/10.1186/s12983-023-00511-1

Monaghan P, Metcalfe NB (2013) Experimental demonstration of the growth rate–lifespan trade-off. Proc R Soc B 280:20122370. https://doi.org/10.1098/rspb.2012.2370

Müller L, Fülöp T, Pawelec G (2013) Immunosenescence in vertebrates and invertebrates. Immun Ageing 10:12. https://doi.org/10.1186/1742-4933-10-12

Pennycuick L (1971) Differences in the parasite infections in three-spined sticklebacks (*Gasterosteus aculeatus* L.) of different sex, age and size. Parasitology 63:407–418. https://doi.org/10.1017/S0031182000079932

Peuß R, Box AC, Chen S, et al (2020) Adaptation to low parasite abundance affects immune investment and immunopathological responses of cavefish. Nat Ecol Evol 4:1416–1430. https://doi.org/10.1038/s41559-020-1234-2

Pichugin MYu, Pavlov DS, Savvaitova KA (2008) Life cycle and structure of populations of three-spined stickleback Gasterosteus aculeatus (Fam. Gasterosteidae) in rivers of northwestern Kamchatka (with reference to the Utkholok River). J Ichthyol 48:151–161. https://doi.org/10.1134/S0032945208020021

Reid K, Bell MA, Veeramah KR (2021) Threespine Stickleback: A Model System For Evolutionary Genomics. Annu Rev Genom Hum Genet 22:357–383. https://doi.org/10.1146/annurev-genom-111720-081402

Reimchen TE (1990) Size-Structured Morality in a Treespine Stickleback (Gastrosteus aculeatus) – Cutthroat Trout (*Oncorhynchus clarki*) Community. Can J Fish Aquat Sci 47:1194–1205. https://doi.org/10.1139/f90-139

Reimchen TE (1991) Trout Foraging Failures and the Evolution of Body Size in Stickleback. Copeia 1991:1098. https://doi.org/10.2307/1446106

Reimchen TE (1992) Injuries on Stickleback from Attacks by a Toothed Predator (Oncorhynchus) and Implications for the Evolution of Lateral Plates. Evolution 46:1224. https://doi.org/10.2307/2409768

Reimchen TE (1994) Predators and Morphological Evolution in Threespine Stickleback. In: The Evolutionary Biology of the Threespine Stickleback

Ricker W (1975) Computation and interpretation of biological statistics of fish populations. Bulletin of the Fisheries Research Board of Canada

Saito T, Nakano S (1999) Reproductive-timing-dependent alternation of offspring life histories in female threespine sticklebacks. 77:

Sandlund OT, Jónasson PM, Jonsson B, et al (1992) Threespine Stickleback Gasterosteus aculeatus in Thingvallavatn: Habitat and Food in a Lake Dominated by Arctic Charr Salvelinus alpinus. Oikos 64:365. https://doi.org/10.2307/3545058

Scharsack J, Kalbe M, Derner R, et al (2004) Modulation of granulocyte responses in three-spined sticklebacks Gasterosteus aculeatus infected with the tapeworm Schistocephalus solidus. Dis Aquat Org 59:141–150. https://doi.org/10.3354/dao059141

Schluter D (1993) Adaptive Radiation in Sticklebacks: Size, Shape, and Habitat Use Efficiency. Ecology 74:699–709. https://doi.org/10.2307/1940797

Schluter D (1995) Adaptive Radiation in Sticklebacks: Trade-Offs in Feeding Performance and Growth. Ecology 76:82–90. https://doi.org/10.2307/1940633

Schmid-Hempel P (2003) Variation in immune defence as a question of evolutionary ecology. Proc R Soc Lond B 270:357–366. https://doi.org/10.1098/rspb.2002.2265

Singkam AR, MacColl ADC (2018) Otolith development in wild populations of stickleback: Jones & Hynes method does not apply to most populations. Journal of Fish Biology 93:272–281. https://doi.org/10.1111/jfb.13687

Smith MW, Then AY, Wor C, et al (2012) Recommendations for Catch-Curve Analysis. N American J Fish Manag 32:956–967. https://doi.org/10.1080/02755947.2012.711270

Steinel NC, Bolnick DI (2017) Melanomacrophage Centers As a Histological Indicator of Immune Function in Fish and Other Poikilotherms. Front Immunol 8:827. https://doi.org/10.3389/fimmu.2017.00827

Svensson PA, Eghbal R, Eriksson R, Nilsson E (2022) How cunning is the puppet-master? Cestode-infected fish appear generally fearless. Parasitol Res 121:1305–1315. https://doi.org/10.1007/s00436-022-07470-2

Tierney JF, Huntingford FA, Crompton DWT (1996) Body condition and reproductive status in sticklebacks exposed to a single wave of *Schistocephalus solidus* infection. Journal of Fish Biology 49:483–493. https://doi.org/10.1111/j.1095-8649.1996.tb00043.x

van der Most PJ, de Jong B, Parmentier HK, Verhulst S (2011) Trade-off between growth and immune function: a meta-analysis of selection experiments. Functional Ecology 25:74–80. https://doi.org/10.1111/j.1365-2435.2010.01800.x

Vilches A, Arizaga J, Salvo I, Miranda R (2013) An experimental evaluation of the influence of water depth and bottom color on the Common kingfisher's foraging performance. Behavioural Processes 98:25–30. https://doi.org/10.1016/j.beproc.2013.04.012

von Bertalanffy L (1938) A quantitative theory of organic growth. Human Biology 10:181–213

Watson AS, Hickford MJH, Schiel DR (2022) Interacting effects of density and temperature on fish growth rates in freshwater protected populations. Proc R Soc B 289:20211982. https://doi.org/10.1098/rspb.2021.1982

Weber JN, Steinel NC, Peng F, et al (2022) Evolutionary gain and loss of a pathological immune response to parasitism. Science 377:1206–1211. https://doi.org/10.1126/science.abo3411

Weber JN, Steinel NC, Shim KC, Bolnick DI (2017) Recent evolution of extreme cestode growth suppression by a vertebrate host. Proc Natl Acad Sci USA 114:6575–6580. https://doi.org/10.1073/pnas.1620095114

Wegner KM, Kalbe M, Reusch TBH (2007) Innate versus adaptive immunity in sticklebacks: evidence for trade-offs from a selection experiment. Evol Ecol 21:473–483. https://doi.org/10.1007/s10682-006-9129-5

Willacker JJ, Von Hippel FA, Wilton PR, Walton KM (2010) Classification of threespine stickleback along the benthic-limnetic axis: CLASSIFICATION OF THREESPINE STICKLEBACK. Biological Journal of the Linnean Society 101:595–608. https://doi.org/10.1111/j.1095-8312.2010.01531.x

Yershov P, Sukhotin A (2015) Age and growth of marine three-spined stickleback in the White Sea 50 years after a population collapse. Polar Biol 38:1813–1823. https://doi.org/10.1007/s00300-015-1743-7

Yurtseva A, Noreikiene K, Lajus D, et al (2019) Aging three-spined sticklebacks *Gasterosteus aculeatus* : comparison of estimates from three structures. J Fish Biol jfb.14071. https://doi.org/10.1111/jfb.14071

Zeller M, Lucek K, Haesler MP, et al Signals of predation-induced directional and disruptive selection in the threespine stickleback

Zelmer DA, Arai HP (1998) The Contributions of Host Age and Size to the Aggregated Distribution of Parasites in Yellow Perch, Perca flavescens, from Garner Lake,

Alberta, Canada. The Journal of Parasitology 84:24.
https://doi.org/10.2307/3284522

Study	Structure	Location	Waterbody	Sample size	SL at age 1	SL at age 2	SL at age 3	Max age
Jones and Hynes 1950	Otolith	England	Stream	~500	37	41	46	4
Greenbank and Nelson 1958	Otolith	Alaska	Lake	250	35	45		2
Pennycuick 1971	Otolith	England	Lake	774	Unclear	Unclear	Unclear	3
Allen and Wootton 1982	Otolith	England	Lake	480	~33	Unclear	Unclear	3
Giles 1987	Otolith	England	Lake, river, stream	Unclear	25-32	Unclear		2
Reimchen 1992	Otolith, spine	Canada	Lake	23	42	65	~80	8
Sandlund et al. 1992	Otolith	Iceland	Lake	175	21.5	34.1	46.5	5
Saito and Nakano 1999	Otolith	Japan	Stream	234	~50	~70	~70	3
Pichugin et al. 2008	Otolith	Russia	Rivers	559	22	44	53	5 to 7
Gambling and Reimchen 2012	Spine	Canada	Lakes	65 (5 per location)	45-85	45-85	75-100	2 to 6
Zeller et al. 2012	Otolith	Switzerland	Lakes	290 (~140 per location)	~35-39	~40-45	Unclear	2 to 3
DeFaveri and Merilä 2013	Otolith, spine, gillcover	Finland	Marine, lakes, streams	239 (~30 per location)	~37-40	~35-50	~40-57	3 to 6
Yershov and Sukhotin 2014	Gillcover	White Sea	Marine	1437	39	66	72	5
Bergström et al. 2015	Otolith	Baltic Sea	Marine	155 (~50 per location)	~37-60	~60-70	~60	2 to 4
Singkam and MacColl 2017	Otolith	England	Lake, stream	484 (~150 per location)	~33-45	~30-57	Unclear	2 to 3
Yurtseva et al. 2019	Otolith, spine, gillcover	White Sea	Marine	48				4 to 5

Table 1. Studies assessing stickleback age. Data on SL (mm) was drawn from the text or estimated from graphs (indicated with a ~). Length at age estimates represent the mean size of randomly selected adults with the exception of Gambling and Reimchen (2014) and DeFaveri and Merilä (2013) which sampled the largest individuals.

	n			VGBF parameters			Predicted size at age			Longevity			Immune function		
Location	Collected	Aged	Size range	Linf	K	L0	L(1)	L(2)	L(4)	Avg. age	Tmax10	Max age	Avg. natural fibrosis	Avg. induced fibrosis	Infection prevalence
Boot Lake	100	75	36 - 80.9	67.77	2.27	1.41		54.25	69.05	3.93	6	7	2.07		58.3%
Echo Lake	100	57	48.7 - 69							4.246	6.33	7	0.605	0.6	25.4%
Farewell Marsh	112	87	32.7 - 48.5	43.5	0.708	-2.44	39.70	41.62	43.04	2.425	3.78	5	0.55	0.25	0.0%
Gosling Lake	89	62	36.2 - 68.6	74.16	0.186	-3.37	41.18	46.76	55.25	3.548	6.83	9	0.19	0.765	7.5%
Lower Stella Lake	62	45	35 - 48.7							1.711	3.2	4	0.03	0.895	5.7%
McCreight Lake	119	83	31.9 - 60.4	74.79	0.124	-4.31	36.03	40.55	48.06	2.94	5.63	7	0.033	0.3	4.0%
Mary Lake	105	96	36.5 - 59.9	48.18	3.03	0.26	43.03	47.94	48.18	2.5	4.2	6	0.85	1.7	0.0%
Farewell Lake	101	77	33.1 - 64.9	91.87	0.127	-3.16	37.72	44.19	54.89	2.649	5.13	6	0.35	0.35	0.0%
Roberts Lake	129	111	28.9 - 52.9	43.02	0.373	-4.03	36.43	38.48	40.86	2.144	4.36	6	0.368	0.65	0.0%

Table 2. Demographic and immune parameters estimated in this study. n = sample size, size range = minimum and maximum SL (mm), Linf = mean asymptotic SL (mm), K = growth coefficient, L0 = hypothesized size at age 0, predicted size at age = predicted SL (mm) at age (yrs), Tmax10 = average age (yrs) of oldest 10% of fish caught, average natural fibrosis = average unperturbed fibrosis score, average induced fibrosis = average fibrosis score 24hrs post injection with 1% aluminum phosphate, infection prevalence = percent of fish infected with *S. solidus*.

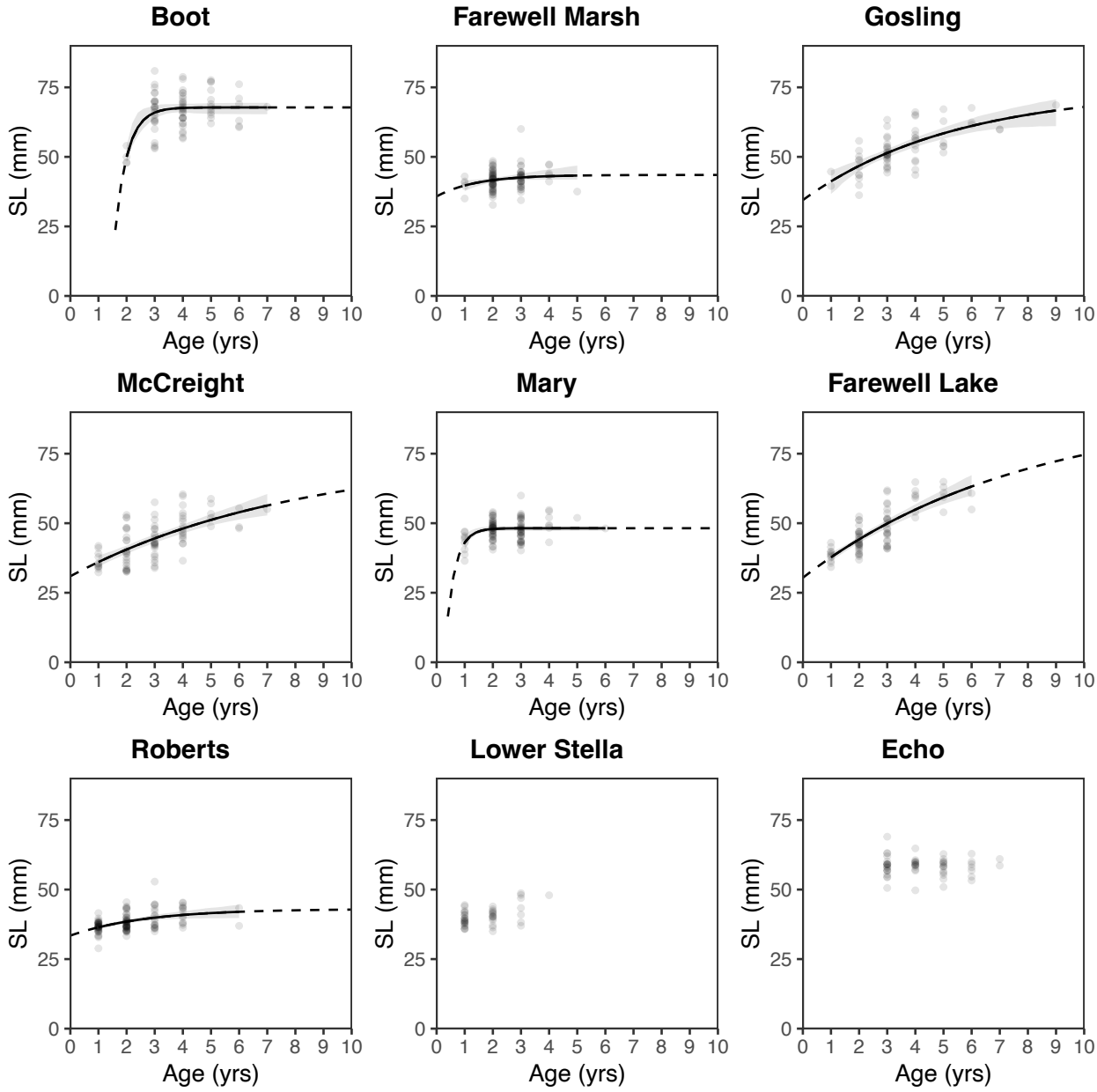


Figure 1. Von Bertalanffy growth curves of threespine stickleback populations from 9 localities on Vancouver Island, Canada. Curves could not be fit for Lower Stella and Echo Lakes due to a lack of data from the ascending portion of the growth curve.

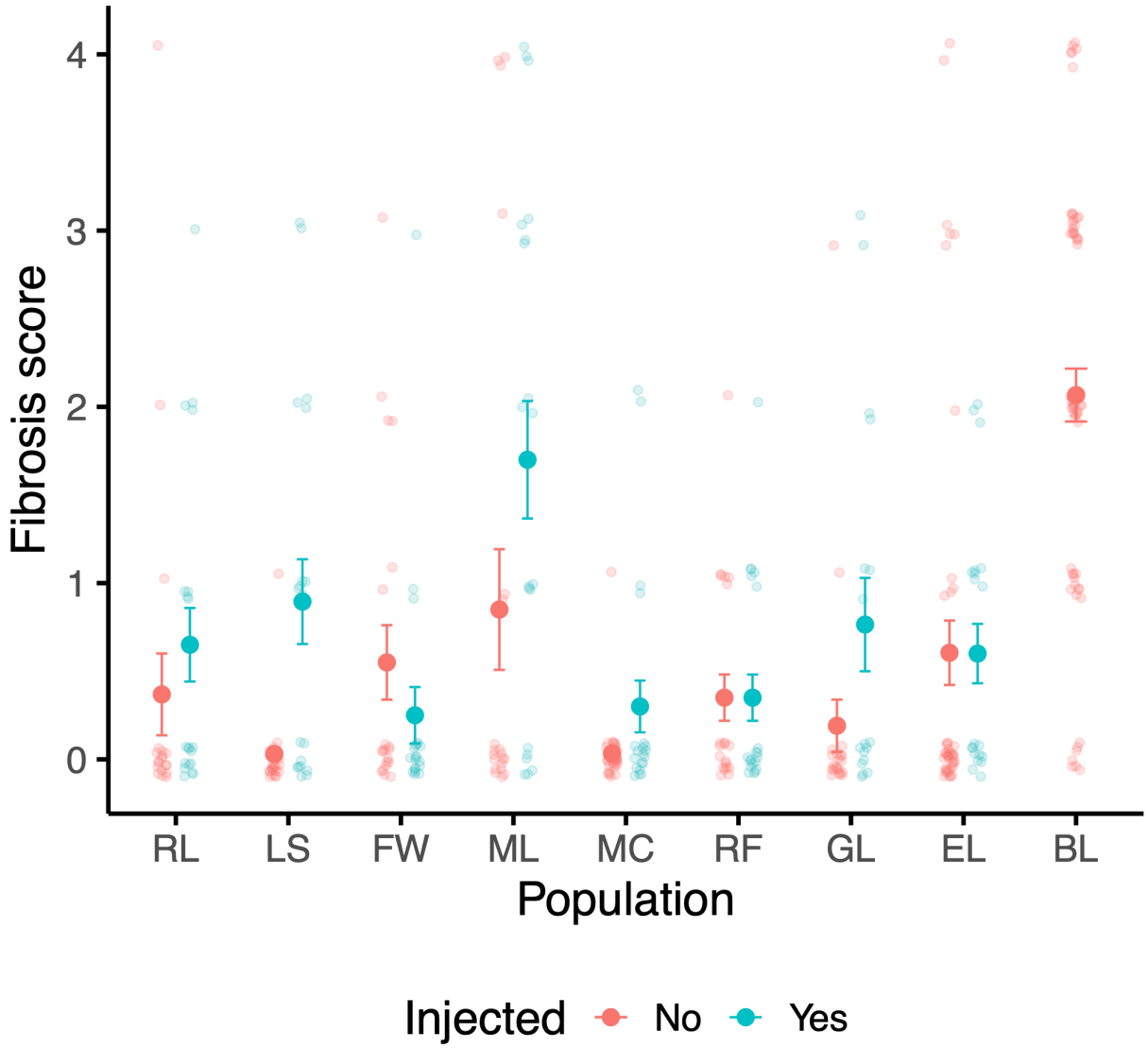


Figure 2. Naturally occurring and post-challenge fibrosis severity varies significantly across populations. Dots represent mean ± SEM.

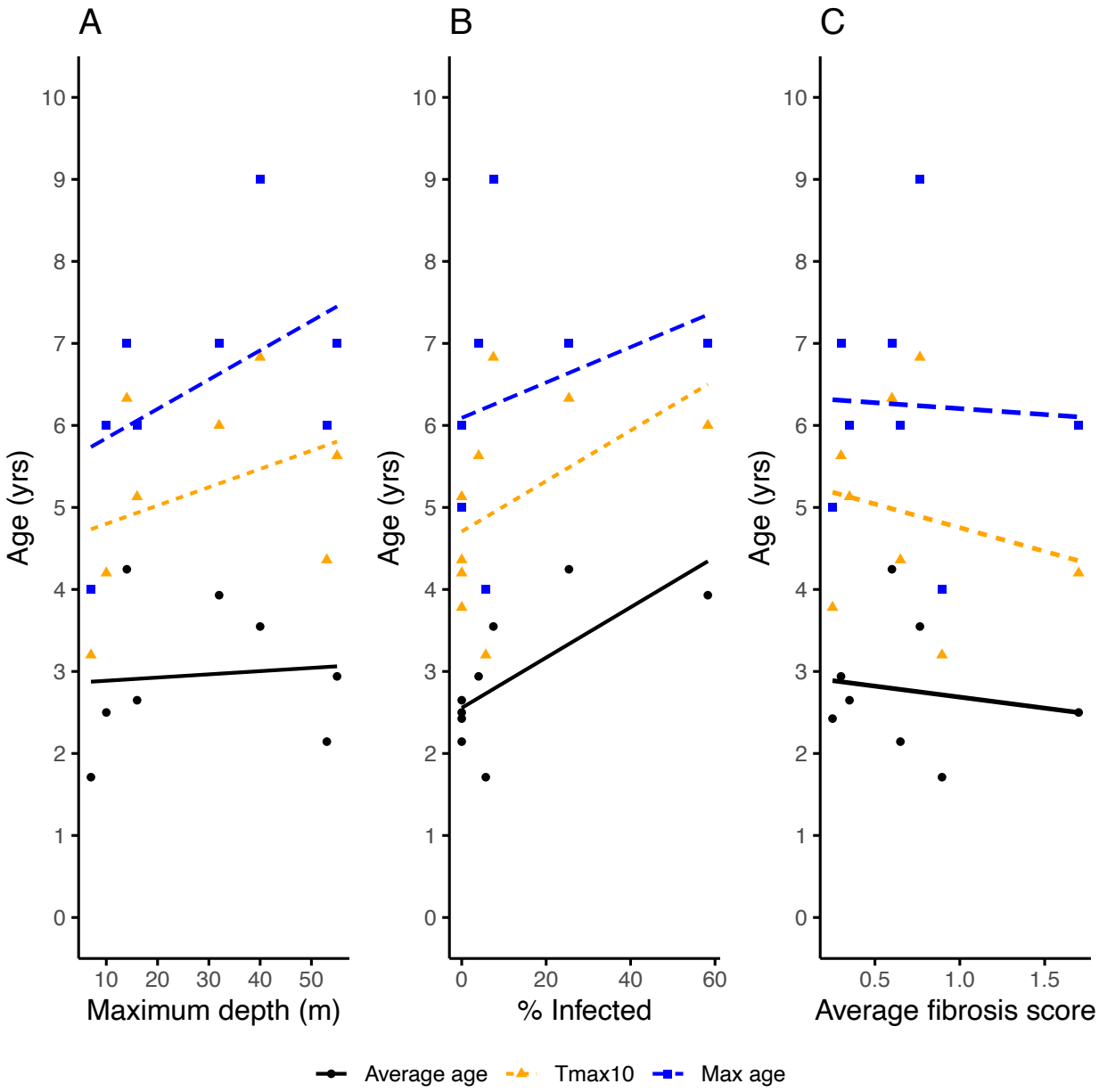


Figure 3. Relationship between longevity and maximum depth (A), *S. solidus* infection prevalence (B), and average post-challenge fibrosis score (C). There was a significant relationship between infection prevalence and average age. No other relationships were significant.

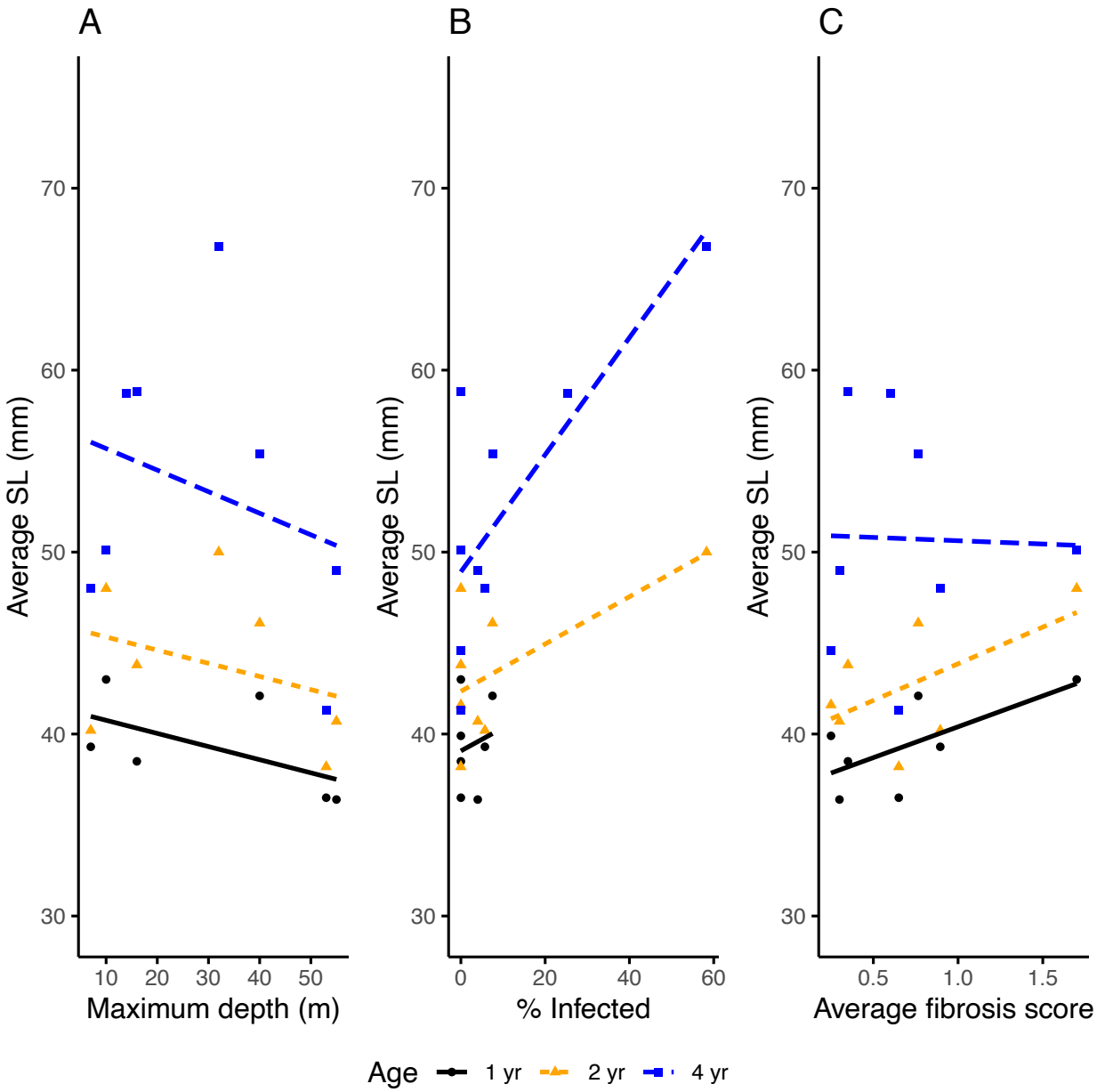


Figure 4. Relationship between size at age and maximum depth (A), *S. solidus* infection prevalence (B), and average post-challenge fibrosis score (C). No relationships were significant.

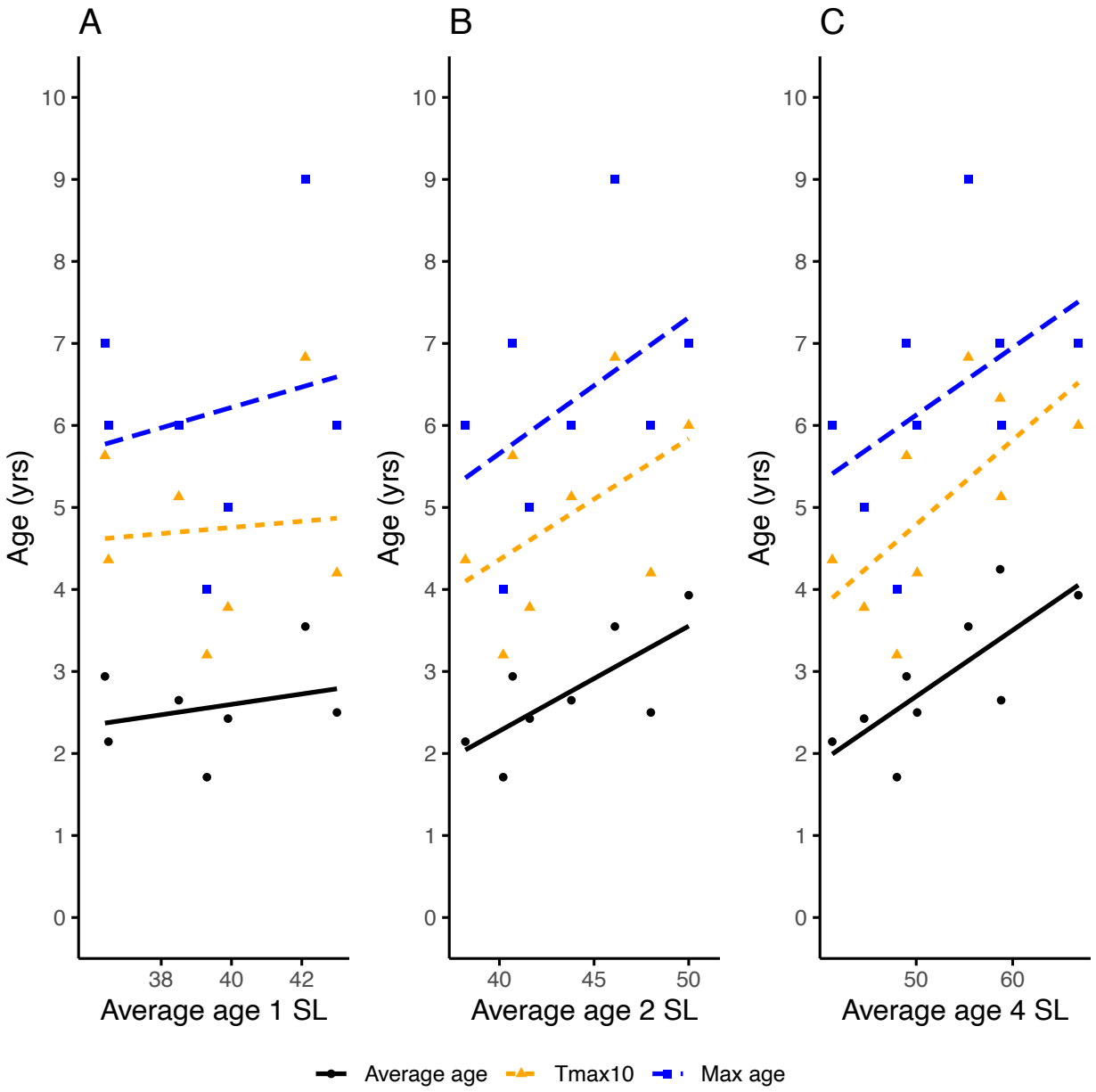


Figure 5. Relationship between size at age and longevity. Size at age 1 (**A**) was unrelated to longevity. Size at age 2 (**B**) and age 4 (**C**) were both significantly associated with average age.

Chapter 3

Rising temperatures impact mortality but not aerobic performance or condition of threespine stickleback (*Gasterosteus aculeatus*)

Kristofer T. Sasser, Emily V. Kerns, and Jesse N. Weber

University of Wisconsin – Madison

Abstract

Global warming is driving population declines and range shifts globally. Aquatic ectotherms, including fish, are particularly vulnerable to elevated temperatures. Assessing the thermal tolerance of species and populations can inform our understanding of which taxa are most at risk. The oxygen and capacity limited thermal tolerance (OCLTT) hypothesis posits that aerobic scope, the difference between resting and maximal metabolic rate, underpins other ecologically relevant metrics of performance. Here we assess the thermal tolerance of benthic and limnetic stickleback ecotypes utilizing multiple metrics of performance including aerobic scope, body condition, and the ability to resist experimental cestode infections. This approach allows us to assess: 1) the thermal tolerance of stickleback ecotypes, 2) the effectiveness of aerobic scope as a metric of thermal tolerance, and 3) how cestode infection dynamics will be impacted by global warming. Although our experiment failed at measuring infection variation, we found that elevated temperatures resulted in significant mortality, with the effect being more severe in limnetic fish. This finding strongly suggests that benthic stickleback are more tolerant of elevated temperatures. However, elevated temperatures were not associated with declines in condition or AS. Further, AS did not decline significantly with temperature nor did it correlate with performance. These results do not support the OCLTT hypothesis and suggest that mortality may be due to acute organ failure rather than deteriorating condition. Together, our findings shed light on the thermal resiliency of a species that forms the foundation of many aquatic ecosystems in the northern hemisphere and adds to a growing body of literature assessing the OCLTT hypothesis.

Introduction

Global warming is altering species distribution ranges (Chen et al. 2011) and driving population declines that can ultimately lead to extinctions (Hof et al. 2011). Aquatic organisms and ectotherms are particularly sensitive to warming temperatures (Pinsky et al. 2020; Ramalho et al. 2023) and fish are consequently at great risk (Comte and Olden 2017). In the case of fish, higher temperatures can reduce growth rates (Huang et al. 2021), increase disease transmission (Karvonen et al. 2010), and ultimately increase mortality (Ern et al. 2023). Maintaining healthy fish populations is critical as fish provide invaluable ecological services (Holmlund and Hammer 1999) and are of great economic and societal value, with fisheries supplying 15% of the protein consumed by humans globally (Boyd et al. 2022). Because of this, assessing the thermal tolerance of fish species and populations has become critically important (Lutterschmidt and Hutchinson 1997; Farrell 2016). These measures of thermal tolerance can be used to project changes in range (Sunday et al. 2012), population declines (Roeder et al. 2021), and identify species and populations at risk of either local or global extinction (Turko et al. 2021).

Many studies of thermal tolerance use a few metrics of performance that provide useful information, but often have unclear ecological relevance. For example, measuring the temperature at which fish can no longer maintain equilibrium (CTMax) is an expedient metric that provides an upper lethal temperature. However, other traits are likely to fail before this point limiting the ecological relevance of this metric (Lutterschmidt and Hutchinson 1997). One trait that has received substantial attention is aerobic scope (AS) – the temperature-specific difference between the minimum and maximum metabolic rate

of an organism (Farrell 2016). Because most routine animal activities are fueled primarily by aerobic metabolism, AS likely constrains activity (Portner et al. 2017). Thus, mechanisms at every step of the oxygen cascade affect the ability of an organism to fuel the actions necessary for survival and reproduction, linking physiology to fitness. Since the metabolic rate of ectotherms is dependent on environmental temperature, maintaining the ability to transport sufficient oxygen to tissue in the face of elevated temperatures may be a determinant of thermal tolerance (Portner et al. 2017). This concept, known as the oxygen and capacity limited thermal tolerance (OCLTT) hypothesis, has garnered widespread attention but remains contentious among ecophysiologists for several reasons (Jutfelt et al. 2018). First, the OCLTT hypothesis predicts that AS will increase with temperature until an optimal temperature is reached, after which AS will decline (Portner et al. 2017). However, a meta-analysis of 73 studies found that almost half failed to find a thermal optima for AS, undercutting a major pillar of the OCLTT hypothesis (Lefevre 2016). Second, the ecological relevance of AS is often poorly defined. AS has been linked to growth (Auer et al. 2015a,b), swimming speed (Fu et al. 2022), and behavior (Metcalfe et al. 2015). However, thermal performance curves can vary dramatically across traits, including somatic growth, activity, digestion, metabolism, and fecundity (Gräns et al. 2014; Sinclair et al. 2016; Ørsted et al. 2022; Kellermann et al. 2019; Mayer et al. 2023). Thus, while AS may provide a tractable and expedient metric of thermal tolerance, its utility for understanding natural organisms depends on forming a more robust body of work demonstrating clear and consistent links to ecologically relevant metrics of performance (Clark et al. 2013).

One area that warrants particular attention is connecting AS with immune competency. Relatively little is known about the effect warming will have on the immune system of ectotherms and downstream parasite infection dynamics. It is hypothesized that global warming will exacerbate the impacts of parasitism by disrupting host immunity and facilitating parasite transmission, infectivity, and growth (Lohmus and Bjorklund 2015; Cable et al. 2016; Byers 2021). Threespine stickleback (*Gasterosteus aculeatus)* fish provide an informative example of immune-temperature connections. When some population are exposed to elevated temperatures their innate and adaptive immunity is suppressed (Dittmar et al. 2014) and the growth rate of cestode parasites increases (Macnab and Barber 2012). Additionally, shorter winters are predicted to increase the seasonal window for cestode transmission (Heins et al. 2016). However, warming might also be detrimental to parasites as it could alter host abundance (Jeppesen et al. 2012) and behavior (Draper and Weissburg 2019; Kua et al. 2020). Additionally, parasites are themselves subject to thermal stress and increasing temperatures may detrimentally impact fitness (Kirk et al. 2022), hindering the ability of parasites to complete their life cycle (Claar and Wood 2020; Barber et al. 2016). Improving our understanding of how immunity might decline in a warming world is critical as parasite infections will have a cascading impacts affecting individuals, populations, species, and communities (Wood et al. 2007).

While thermal tolerance can impact traits differentially, populations too may also differ in tolerance. Significant interpopulation variation in thermal performance has been documented in a number of taxa (ants: Roeder et al. 2021; insects: Sinclair et al. 2012; amphibians and reptiles: Bodensteiner et al. 2020; plants: Angert et al. 2011). Fish also

clearly display this pattern of intraspecific variation. Studies of wild-caught salmon demonstrate that populations differ in the temperature at which performance (maximum swimming speed, MMR, AS) is optimized, and temperature optima closely track the ambient temperature of the streams in which they migrate (Eliason et al. 2011; Lee et al. 2003). However, studies that focus on wild-caught individuals can rarely disentangle whether differences in thermal tolerance are heritable (and thus visible to selection) or plastic. This is consequential, as exposure to elevated or variable temperature regimes can have a significant effect on thermal tolerance, whether it occurs during development (Schaefer and Ryan 2006) or as an adult (Mottola et al. 2022). However, it is notable that the impact of prior exposure appears to be generally weak (Pottier et al. 2022; Weaving et al. 2022).

Multiple studies of threespine stickleback have found heritable differences in thermal tolerance by rearing fish from divergent thermal environments in common garden settings (Barrett et al. 2011; Dittmar et al. 2014). Further, Barrett et al. (2011) established that thermal tolerance is subject to rapid selection by transplanting individuals into a novel thermal environment and retesting thermal tolerance after three years. Dittmar et al. (2014) similarly found evolutionary divergence in individuals that were only recently (17 years prior) established in a novel thermal environment (i.e., a stream to pond transition). Understanding heritable population level variation can inform distribution models (Angert et al. 2011; Diamond 2016) and management decisions (Turko et al. 2021). For example, this method has been used to predict that the range of marine threespine stickleback will

expand in the Bering Sea with global warming as their distribution is currently dictated by lower thermal limits (Smith et al. 2021).

To better understand the impact of global warming on freshwater stickleback we conducted a laboratory study to answer three questions: 1) how will threespine stickleback handle prolonged exposure to projected future temperatures under a moderate emissions scenario?, 2) do benthic and limnetic ecotypes differ heritably in thermal tolerance?, and 3) is AS predictive of other metrics of performance? Understanding the thermal tolerance of this species is important, as stickleback are the base of many aquatic communities across the northern hemisphere (Reimchen 1994). In order to develop a more holistic view of thermal tolerance we used stickleback from both benthic and limnetic ecotypes. Because the benthic-limnetic ecotype spectrum is strongly associated with lake depth (Willacker et al. 2010), benthic fish that inhabit shallow lakes and ponds are also exposed to a warmer and more variable thermal environment. This leads us to predict that benthic-specialized fish may have evolved a greater thermal tolerance than limnetic stickleback. By quantifying thermal tolerance with multiple metrics of performance, this experiment also allowed us to assess whether there is a generalizable relationship between AS and other metrics of performance. Assessing the link between performance and AS is an important step in validating the OCLTT hypothesis (Clark et al. 2013). We predict that AS will decline as temperatures increase and that this will be accompanied by concomitant declines in other metrics of performance. We also predict that the incidence and severity of cestode infections will increase with temperature. This experimental framework provides valuable information on the thermal tolerance of a foundational species in many aquatic

ecosystems, assesses a key assumption of the OCLTT hypothesis, and provides information on how cestode infection dynamics will be altered by global warming.

Methods

Animal husbandry and experimental design

We assayed lab-reared fish descended from 4 lakes in central Alaska: Watson, Wik, Spirit, and Finger. Previous work that characterized the diet and phenotypes of these populations indicated that Watson and Finger are represent benthic ecotypes, Spirit is moderately limnetic, and Wik is highly limnetic (Haines et al. 2023). The lakes also vary considerably in ecology and bathymetry, with Spirit and Wik being considerably deeper (69m and 80m, respectively) than Finger and Watson (44m and 14m, respectively). Animal stocks were established via *in vitro* fertilization and then housed with siblings in 16L tanks at the University of Wisconsin – Madison for one year. Aquaria temperature prior to the experiment ranged between 15-16.5°C. We fed fish to satiation once per day with pelletized fish feed (GP500; Brine Shrimp Direct; Ogden, USA). A 16 hour light: 8 hour dark circadian cycle was maintained for the duration of the experiment.

At the beginning of the experiment, the temperature of the experimental tanks was be raised by 1°C per day until they reached their nominal values. Following this acclimation, we measured SMR and MMR using intermittent flow respirometry (see respirometry section for details). Immediately following respirometry, we exposed 80% of fish from each temperature*ecotype treatment to the cestode *S. solidus* (see infection section). Thirty days post-exposure the SMR and MMR of a subset of fish were remeasured.

At the end of the experiment (32 days) all fish were euthanized using an overdose of pH buffered MS-222, dissected, and infection outcome, peritoneal fibrosis, mass, SL, body condition, spleenosomatic, hepatosomatic, and gonadosomatic indices were recorded. The temperatures used in this experiment were chosen to match the range of projected mean and maximum air temperatures in the Kenai, AK area during the hottest month of the year in 2060 under a moderate emissions scenario (RCP 6.0) (https://snap.uaf.edu/tools/community-charts). While air temperature is a coarse predictor of water temperature, it provides a reasonable estimate of the surface water temperature of lakes (Shuter et al. 1983). Also, based on a previous study (Dittmar et al. 2014), we expected these temperatures to fall below the upper lethal temperature for the species, allowing us to measure phenotypic consequences of thermal increase without inducing mortality in experimental animals.

Respirometry

We measured mass specific SMR and MMR using intermittent-flow respirometry and a static respirometer. Fish were fasted for 24 hours, placed into a holding tank, chased with a net for one minute, then transferred to individual 330mL respirometry chambers submerged in a temperature-controlled stock tank. Water temperature was maintained using a TECO 2000 chiller (Ravena, Italy) and a Hygger tank heater (Shenzhen, China). Submersible pumps maintained a constant flow of water within each chamber and flushed oxygenated water into the chamber intermittently. The flush pump was automated using AquaResp3 (Morozov et al. 2019). We used oxygen-sensitive REDFLASH contactless sensor

spots connected to a PyroScience Firesting O_2 oxygen meter (Aachen, Germany) to record the temperature corrected oxygen concentration (mg/L) in each chamber. We used the FishResp package in R (Svendsen et al. 2019) to estimate SMR, MMR, and AS. SMR was calculated by first subtracting background microbial respiration, then averaging the lowest 20% of points from the entire data set after excluding outliers (i.e., a quantile approach (Chabot et al. 2016). MMR was defined as the maximum value recorded during the trial (Clark et al. 2013). AS was calculated as the difference between SMR and MMR (Clark et al. 2013). Observations were subset for each ecotype*temperature combination and outliers were identified using the IQR method and removed.

Experimental cestode exposure

First, copepods (*Macrocyclops albidus*) were exposed to lab-reared cestode coracidea descended from individuals caught in Walby Lake, Alaska. We then transferred fish into individual temperature-controlled containers and exposed them to infected copepods containing a total of ~8 cestodes overnight. A subset of each batch of copepods were screened prior to exposure to determine the correct number of copepods required to achieve the desired exposure rate. After exposure we seined the water from the containers to ensure that the copepods were eaten and returned fish to their home tanks.

Body condition, infection outcome, and fibrosis

The mass and standard length of fish was measured twice: immediately after respirometry and at the conclusion of the experiment. Spleenosomatic index (spleen mass/overall fish

wet mass), hepatosomatic index (liver mass/overall fish wet mass), and male gonadosomatic index (testes mass/overall fish wet mass) were also recorded at the end of the experiment. Body condition was calculated as the total wet mass of the fish divided by its standard length. Upon dissection, the body cavity was screened for the presence of cestodes and the number of cestode infections was recorded. Peritoneal fibrosis was scored on a 0-4 scale per Hund et al. (2022). Specifically, a score of 0 was assessed if there was no visible fibrosis, 1 if organs were attached to the swim bladder, 2 if organs were fused together, 3 if organs were fused to the side of the body cavity, and 4 if the body cavity was entirely filled with fibrotic tissue.

Statistics

We used ANOVAs and Tukey's HSD post-hoc tests to assess whether performance (SMR, MMR, AS, HSI, SSI, GSI, body condition, fibrosis score) varied between ecotypes, across temperature treatments, or between ecotypes within a temperature treatment. The models included temperature (treated as a factor), ecotype, and the interaction between the factors. We used SEM path analyses to assess whether tank density, fibrosis status, or AS impacted metrics of condition (supp. fig. 1-4). We used a binomial GLM to determine if the incidence of fibrosis varied between ecotypes, across temperature treatments, or between ecotypes as a function of temperature. We used a Cox regression to assess whether mortality varied between ecotypes, across temperature treatments, or between ecotypes within a temperature treatment.

Results

Metabolic traits

As expected, SMR increased significantly with temperature at the start of experiment ($F_{4,177}$ = 77.673, $p < 0.0001$, fig. 1A). Pairwise comparisons revealed that every 2 °C increase in temperature resulted in a significant increase with the exception of the shift from 20-22 °C (fig. 1A). Similarly, MMR increased with temperature ($F_{4,177}$ = 15.704, $p < 0.0001$, fig. 1B). However, only shifts of 4 °C or greater resulted in significant changes in MMR (fig. 1B). AS differed significantly between temperature treatments ($F_{4,177}$ = 3.482, $p = 0.0091$, fig. 1C). Pairwise comparisons reveal that AS peaked at 22 °C, differing significantly from measurements taken at 20°C ($p = 0.0241$) and 26 °C ($p = 0.0135$), but not those taken at 18 °C or 24 °C (fig. 1C). Additionally, we found that AS varied between ecotypes independent of temperature ($F_{1,177}$ = 4.849, $p = 0.0289$), with limnetic individuals displaying ~8% higher values. Despite this, no metabolic trait differed between ecotypes within temperature treatments.

As at the beginning of the experiment, SMR increased significantly with temperature at the end of experiment ($F_{2,64}$ = 22.98, $p < 0.0001$, fig. 1D). Pairwise comparisons revealed that SMR was significantly higher at 26 °C (18-26: $p < 0.0001$; 22-26: $p < 0.0001$), but did not differ significantly between 18-22 °C (fig. 1D). Likewise, temperature had a significant effect on MMR ($F_{2,64}$ = 7.042, $p = 0.0017$) with values at 22 °C being significantly lower than those recorded at 18 °C ($p = 0.0053$) and 26 °C ($p = 0.01$). Ecotype also had a significant effect on MMR independent of temperature ($F_{1,64}$ = 6.411, $p = 0.0134$) with limnetic individuals displaying ~11% higher values. AS differed significantly between temperature treatments

($F_{2,64}$ = 8.181, p = 0.0006, fig. 1C). Pairwise comparisons reveal a significant difference between 18-22 °C (p = 0.0004) but not 18-26 °C or 22-26 °C (fig. 1F). As with MMR, we found a significant effect of ecotype on AS independent of temperature ($F_{1,64}$ = 7.116, p = 0.0096) with limnetic individuals displaying ~21% higher values. No metabolic trait differed between ecotypes within temperature treatments.

SEM path analyses confirmed the effects of temperature and ecotype on AS and revealed that the presence of fibrosis was associated with reduced AS (~14%, z = -2.205, p = 0.027). To determine if reductions in AS were driven by elevated SMR or suppressed MMR measurements we used univariate ANOVAs treating fibrosis as a binary trait. This revealed that the presence of fibrosis significantly reduces both measures (SMR: $F_{1,67}$ = 5.108, p = 0.0271; $MMR_{1,67}$: F = 4.456, p = 0.0385). However, MMR was more severely curtailed by fibrosis than SMR, and this is what led to a reduction in AS.

Condition, immunity, and mortality

We found no evidence suggesting that temperature negatively impacted the condition (HSI, SSI, GSI, or body condition; fig. 2A-D; supp. fig. 1-4) or immune function of stickleback. Only a single individual was successfully infected by a cestode, thus infection outcomes did not differ significantly across ecotypes or temperature. Additionally, the incidence and severity of fibrosis was not associated with temperature, ecotype, or the interaction between the two (fig. 2E). Despite the negligible impact on condition and aerobic scope, we found that temperature had a significant effect on mortality (z = 2.091, p = 0.0365, fig. 3) with the effect of temperature being marginally more severe in limnetic fish

(z = 1.949, p = 0.0512, fig. 3). We found limited support for our prediction that AS would underpin other metrics of performance as it was positively associated with GSI (z = 2.293, p = 0.022, supp. fig. 4), but not any other metric of condition.

Discussion

We found that stickleback from four lakes, including two distinct ecotypes, were remarkably resilient when exposed to temperatures up to 22 °C. Surprisingly, we found no evidence of declining body condition despite a prolonged exposure to elevated temperatures. This is particularly surprising given that 80% of the fish were experimentally exposed to cestodes, which can lead to significant reductions in body condition even in the absence of successful infections (Macnab and Barber 2012). Further, we found no signature of immune dysregulation as there was no association between temperature and the incidence or severity of peritoneal fibrosis. Importantly, the presence of fibrosis suggests that fish were exposed to live tapeworms that managed to invade the host body cavity but did not successfully establish infections. Despite the lack of decline in condition, we began to see substantial mortality around 24 °C (20%) that became pronounced at 26 °C (50%). Under a moderate emissions scenario the average air temperature of south central Alaska is projected rise from 13 °C to 16 °C during the hottest month of the year in 2090 (https://snap.uaf.edu/tools/community-charts). However, the projected mean maximum temperature during the hottest month of the year is projected to reach 26°C by 2060 (https://snap.uaf.edu/tools/community-charts). This suggests that stickleback are unlikely to suffer population declines due to warming in south central

Alaska over the coming decades. Additionally, given that we exposed stickleback to elevated temperatures for a period of 30 days, the levels of mortality recorded here would likely exceed what would be experienced by wild populations. There are also several ways in which our laboratory environment likely limits mortality and decreases the ecological relevancy of our findings, however. For example, elevated temperatures can significantly alter predator-prey interactions (Allan et al. 2015). Ectotherms may be more susceptible to predation by endotherms at elevated temperatures given that the performance of endotherms is less temperature dependent (Dell et al. 2014). Further, while the increased energetic demands associated with elevated temperatures did not impact condition in the lab, this may be because the fish were fed to satiation. This is an important caveat, as costs may be masked when resources are plentiful but apparent when they are scarce, as is likely the case in natural settings (Boots 2011).

While stickleback appear generally tolerant of elevated temperatures, not all populations are uniformly tolerant. We observed that condition and metabolic traits did not vary across ecotypes within temperature treatments; however, mortality differed significantly. We found dramatically higher levels of mortality in limnetic fish at 26 °C (limnetic: 89%; benthic:11%), strongly suggesting that the ecotypes differ in thermal tolerance. These differences should be, at least in part, heritable because the fish used in this study were reared in a common developmental environment. This adds to a growing body of work demonstrating that thermal tolerance is a heritable target of selection that has enabled populations to adapt to local conditions (Angert et al. 2011; Diamond 2016). This has important implications for how stickleback will fare in the face of global warming.

Standing genetic variation has facilitated repeated evolution to freshwater in stickleback (Roberts Kingman et al. 2021) and it is possible that this process could facilitate the rapid evolution of thermal tolerance in currently intolerant populations. Alternately, tolerant populations may recolonize areas where temperature intolerant fish have died or provide source populations for assisted migration (Turko et al. 2021). While our results strongly suggest that thermal tolerance is heritable, it is possible that maternal effects may also play a role in shaping differences in thermal tolerance documented here (Muñoz et al. 2014; Lockwood et al. 2017). Indeed, maternal effects confer advantages in growth rate and mitochondrial function at elevated temperatures in marine stickleback (Shama et al. 2014).

In addition to providing an assessment of stickleback thermal tolerance, this study provides the opportunity to assess a key assumption of the OCLTT hypothesis: changes in AS should underpin other metrics of performance (Clark et al. 2013). Our results largely do not support this hypothesis. First, AS did not decline significantly with temperature. This adds to a growing body of work that have likewise failed to find a significant decline in AS with temperature (Lefevre 2016) including in stickleback (Cominassi et al. 2023). Further, we did not find a relationship between AS and most metrics of body condition. Although we were unable to link AS with survival, mortality generally increased with temperature while AS did not, strongly suggesting that AS did not underpin survival differences. Future work could address this connection between AS and survival more directly by marking and following individuals throughout and experiment. It is worth noting that AS may have a greater impact on condition and survival in wild settings as AS has been linked with

swimming speed, a trait with obvious importance to both feeding and predator avoidance (Fu et al. 2022). Additionally, we found that AS was positively associated with male reproductive potential. Thus, AS may have important implications for the fitness of wild fish, but we found little support for this in our experiment.

Several non-mutually exclusive mechanisms at the molecular, cellular, and organ level may explain the temperature associated mortality documented here (Ern et al. 2023). In particular, brain and heart function are likely points of failure and should be targets of future studies aimed at understanding the mechanisms that underpin thermal tolerance in this system. Both organs are likely to be impacted by temperature mediated declines in neuron function (Ern et al. 2023). Increased temperatures may impair neuron function by altering cell membrane fluidity (Cossins et al. 1977), decreasing electrical excitability within neurons (Vornanen 2016), or denaturing proteins (Basu et al. 2002). Heat related protein denaturation is particularly likely to impact brain function, but may be staved off via heatshock proteins (HSPs) (Basu et al. 2002). Elevated expression of the HSP gene hsp70 is associated with reduced mortality in some populations of Common killifish (*Fundulus heteroclitus*) strongly suggesting that protein denaturation underpins mortality in this system and that HSPs facilitate tolerance (Fangue et al. 2006). The role of HSPs in facilitating thermal tolerance in stickleback remains opaque. The expression of genes that encode HSPs consistently increase when stickleback are exposed to elevated temperatures (26-28°C), but HSP expression has not been associated with differences in thermal tolerance (Dittmar et al. 2014; Metzger et al. 2016; Mottola et al. 2022). Excitingly, the population level variation in thermal tolerance documented here presents the

opportunity to investigate the mechanisms that underpin temperature related mortality and the evolution of traits that facilitate thermal tolerance (Ern et al. 2023).

Conclusions

This work demonstrates that threespine stickleback possess the ability to survive dramatic increases in temperature, but some ecotypes are may fare better than others under global warming. This work also provides a valuable test of the OCLTT hypothesis and suggests that AS does not underpin temperature mediated performance in stickleback. This provides a foundation for future work identifying the mechanistic basis of temperature mediated mortality and thermal tolerance in stickleback.

Permits/Acknowledgments/Funding

All experiments followed the National Research Council's Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committees of the University of Wisconsin – Madison (Protocol #L006460).

B. Solin, M. Chotlos, J. Abels, M. Reynolds, C. Wolf, and J. Benavente assisted with animal care and dissections. All authors contributed to the design of the study and production of the manuscript. KTS and EVK led data collection. KTS analyzed the data and led the writing of the manuscript. KTS, EVK, and JNW were supported by the NIGMS of the NIH (#1R35GM142891-01). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Allan BJM, Domenici P, Munday PL, McCormick MI (2015) Feeling the heat: the effect of acute temperature changes on predator–prey interactions in coral reef fish. Conserv Physiol 3:cov011. https://doi.org/10.1093/conphys/cov011

Angert AL, Sheth SN, Paul JR (2011) Incorporating Population-Level Variation in Thermal Performance into Predictions of Geographic Range Shifts. Integrative and Comparative Biology 51:733–750. https://doi.org/10.1093/icb/icr048

Auer SK, Salin K, Anderson GJ, Metcalfe NB (2015a) Aerobic scope explains individual variation in feeding capacity. Biol Lett 11:20150793. https://doi.org/10.1098/rsbl.2015.0793

Auer SK, Salin K, Rudolf AM, et al (2015b) The optimal combination of standard metabolic rate and aerobic scope for somatic growth depends on food availability. Funct Ecol 29:479–486. https://doi.org/10.1111/1365-2435.12396

Barber I, Berkhout BW, Ismail Z (2016) Thermal Change and the Dynamics of Multi-Host Parasite Life Cycles in Aquatic Ecosystems. Integr Comp Biol 56:561–572. https://doi.org/10.1093/icb/icw025

Barrett RDH, Paccard A, Healy TM, et al (2011) Rapid evolution of cold tolerance in stickleback. Proc R Soc B 278:233–238. https://doi.org/10.1098/rspb.2010.0923

Basu N, Todgham AE, Ackerman PA, et al (2002) Heat shock protein genes and their functional significance in fish. Gene 295:173–183. https://doi.org/10.1016/S0378-1119(02)00687-X

Bodensteiner BL, Agudelo-Cantero GA, Arietta AZA, et al (2021) Thermal adaptation revisited: How conserved are thermal traits of reptiles and amphibians? J Exp Zool Pt A 335:173–194. https://doi.org/10.1002/jez.2414

Boyd CE, McNevin AA, Davis RP (2022) The contribution of fisheries and aquaculture to the global protein supply. Food Sec 14:805–827. https://doi.org/10.1007/s12571-021-01246-9

Byers JE (2021) Marine Parasites and Disease in the Era of Global Climate Change. Annu Rev Mar Sci 13:397–420. https://doi.org/10.1146/annurev-marine-031920-100429

Cable J, Barber I, Boag B, et al (2017) Global change, parasite transmission and disease control: lessons from ecology. Phil Trans R Soc B 372:20160088. https://doi.org/10.1098/rstb.2016.0088

Chabot D, Steffensen JF, Farrell AP (2016) The determination of standard metabolic rate in fishes: measuring smr in fishes. J Fish Biol 88:81–121. https://doi.org/10.1111/jfb.12845

Chen I-C, Hill JK, Ohlemüller R, et al (2011) Rapid Range Shifts of Species Associated with High Levels of Climate Warming. Science 333:1024–1026. https://doi.org/10.1126/science.1206432

Claar DC, Wood CL (2020) Pulse Heat Stress and Parasitism in a Warming World. Trends in Ecology & Evolution 35:704–715. https://doi.org/10.1016/j.tree.2020.04.002

Clark TD, Sandblom E, Jutfelt F (2013) Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. Journal of Experimental Biology 216:2771–2782. https://doi.org/10.1242/jeb.084251

Cominassi L, Ressel KN, Brooking AA, et al (2022) Metabolic rate increases with acclimation temperature and is associated with mitochondrial function in some tissues of threespine stickleback. Journal of Experimental Biology 225:jeb244659. https://doi.org/10.1242/jeb.244659

Comte L, Olden JD (2017) Climatic vulnerability of the world's freshwater and marine fishes. Nature Clim Change 7:718–722. https://doi.org/10.1038/nclimate3382

Cossins AR, Friedlander MJ, Prosser CL (1977) Correlations between behavioral temperature adaptations of goldfish and the viscosity and fatty acid composition of their synaptic membranes. J Comp Physiol 120:109–121. https://doi.org/10.1007/BF00619309

Dell AI, Pawar S, Savage VM (2014) Temperature dependence of trophic interactions are driven by asymmetry of species responses and foraging strategy. Journal of Animal Ecology 83:70–84. https://doi.org/10.1111/1365-2656.12081

Diamond SE (2017) Evolutionary potential of upper thermal tolerance: biogeographic patterns and expectations under climate change. Annals of the New York Academy of Sciences 1389:5–19. https://doi.org/10.1111/nyas.13223

Dittmar J, Janssen H, Kuske A, et al (2014) Heat and immunity: an experimental heat wave alters immune functions in three-spined sticklebacks (*Gasterosteus aculeatus*). J Anim Ecol 83:744–757. https://doi.org/10.1111/1365-2656.12175

Draper AM, Weissburg MJ (2019) Impacts of Global Warming and Elevated CO2 on Sensory Behavior in Predator-Prey Interactions: A Review and Synthesis. Front Ecol Evol 7:72. https://doi.org/10.3389/fevo.2019.00072

Eliason EJ, Clark TD, Hague MJ, et al (2011) Differences in Thermal Tolerance Among Sockeye Salmon Populations. Science 332:109–112. https://doi.org/10.1126/science.1199158

Ern R, Andreassen AH, Jutfelt F (2023) Physiological Mechanisms of Acute Upper Thermal Tolerance in Fish. Physiology 38:141–158. https://doi.org/10.1152/physiol.00027.2022

Fangue NA, Hofmeister M, Schulte PM (2006) Intraspecific variation in thermal tolerance and heat shock protein gene expression in common killifish, *Fundulus heteroclitus*. Journal of Experimental Biology 209:2859–2872. https://doi.org/10.1242/jeb.02260

Farrell AP (2016) Pragmatic perspective on aerobic scope: peaking, plummeting, pejus and apportioning: AEROBIC SCOPE: USES AND LIMITATIONS. J Fish Biol 88:322–343. https://doi.org/10.1111/jfb.12789

Fu S-J, Dong Y-W, Killen SS (2022) Aerobic scope in fishes with different lifestyles and across habitats: Trade-offs among hypoxia tolerance, swimming performance and digestion. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 272:111277. https://doi.org/10.1016/j.cbpa.2022.111277

Gräns A, Jutfelt F, Sandblom E, et al (2014) Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO2 in Atlantic halibut. Journal of Experimental Biology 217:711–717. https://doi.org/10.1242/jeb.096743

Haines GE, Moisan L, Derry AM, Hendry AP (2023) Dimensionality and Modularity of Adaptive Variation: Divergence in Threespine Stickleback from Diverse Environments. The American Naturalist 201:175–199. https://doi.org/10.1086/722483

Heins DC, Eidam DM, Baker JA (2016) Timing of Infections in the Threespine Stickleback (*Gasterosteus aculeatus*) by *Schistocephalus solidus* in Alaska. Journal of Parasitology 102:286–289. https://doi.org/10.1645/14-675

Hof C, Araújo MB, Jetz W, Rahbek C (2011) Additive threats from pathogens, climate and land-use change for global amphibian diversity. Nature 480:516–519. https://doi.org/10.1038/nature10650

Holmlund CM, Hammer M (1999) Ecosystem services generated by fish populations. Ecological Economics 29:253–268. https://doi.org/10.1016/S0921-8009(99)00015-4

Huang M, Ding L, Wang J, et al (2021) The impacts of climate change on fish growth: A summary of conducted studies and current knowledge. Ecological Indicators 121:106976. https://doi.org/10.1016/j.ecolind.2020.106976

Hund AK, Fuess LE, Kenney ML, et al (2022) Population-level variation in parasite resistance due to differences in immune initiation and rate of response. Evolution Letters 6:162–177. https://doi.org/10.1002/evl3.274

Jeppesen E, Mehner T, Winfield IJ, et al (2012) Impacts of climate warming on the long-term dynamics of key fish species in 24 European lakes. Hydrobiologia 694:1–39. https://doi.org/10.1007/s10750-012-1182-1

Jutfelt F, Norin T, Ern R, et al (2018) Oxygen- and capacity-limited thermal tolerance: blurring ecology and physiology. Journal of Experimental Biology 221:jeb169615. https://doi.org/10.1242/jeb.169615

Karvonen A, Rintamäki P, Jokela J, Valtonen ET (2010) Increasing water temperature and disease risks in aquatic systems: Climate change increases the risk of some, but not all, diseases. International Journal for Parasitology 40:1483–1488. https://doi.org/10.1016/j.ijpara.2010.04.015

Kellermann V, Chown SL, Schou MF, et al (2019) Comparing thermal performance curves across traits: how consistent are they? Journal of Experimental Biology jeb.193433. https://doi.org/10.1242/jeb.193433

Kirk D, O'Connor MI, Mordecai EA (2022) Scaling effects of temperature on parasitism from individuals to populations. Journal of Animal Ecology 91:2087–2102. https://doi.org/10.1111/1365-2656.13786

Kua ZX, Hamilton IM, McLaughlin AL, et al (2020) Water warming increases aggression in a tropical fish. Sci Rep 10:20107. https://doi.org/10.1038/s41598-020-76780-1

Lee CG, Farrell AP, Lotto A, et al (2003) The effect of temperature on swimming performance and oxygen consumption in adult sockeye (*Oncorhynchus nerka*) and coho (*O. kisutch*)salmon stocks. Journal of Experimental Biology 206:3239–3251. https://doi.org/10.1242/jeb.00547

Lefevre S (2016) Are global warming and ocean acidification conspiring against marine ectotherms? A meta-analysis of the respiratory effects of elevated temperature, high CO_2 and their interaction. Conserv Physiol 4:cow009. https://doi.org/10.1093/conphys/cow009

Lockwood BL, Julick CR, Montooth KL (2017) Maternal loading of a small heat shock protein increases embryo thermal tolerance in *Drosophila melanogaster*. Journal of Experimental Biology jeb.164848. https://doi.org/10.1242/jeb.164848

Lutterschmidt WI, Hutchison VH (1997) The critical thermal maximum: history and critique. Can J Zool 75:1561–1574. https://doi.org/10.1139/z97-783

Macnab V, Barber I (2012) Some (worms) like it hot: fish parasites grow faster in warmer water, and alter host thermal preferences. Glob Change Biol 18:1540–1548. https://doi.org/10.1111/j.1365-2486.2011.02595.x

Metcalfe NB, Van Leeuwen TE, Killen SS (2016) Does individual variation in metabolic phenotype predict fish behaviour and performance? J Fish Biol 88:298–321. https://doi.org/10.1111/jfb.12699

Metzger DCH, Schulte PM (2017) Persistent and plastic effects of temperature on DNA methylation across the genome of threespine stickleback (*Gasterosteus aculeatus*). Proc R Soc B 284:20171667. https://doi.org/10.1098/rspb.2017.1667

Mottola G, López ME, Vasemägi A, et al (2022) Are you ready for the heat? Phenotypic plasticity versus adaptation of heat tolerance in three-spined stickleback. Ecosphere 13:. https://doi.org/10.1002/ecs2.4015

Muñoz NJ, Anttila K, Chen Z, et al (2014) Indirect genetic effects underlie oxygen-limited thermal tolerance within a coastal population of chinook salmon. Proc R Soc B 281:20141082. https://doi.org/10.1098/rspb.2014.1082

Ørsted M, Jørgensen LB, Overgaard J (2022) Finding the right thermal limit: a framework to reconcile ecological, physiological and methodological aspects of CTmax in ectotherms. Journal of Experimental Biology 225:jeb244514. https://doi.org/10.1242/jeb.244514

Pinsky ML, Selden RL, Kitchel ZJ (2020) Climate-Driven Shifts in Marine Species Ranges: Scaling from Organisms to Communities. Annu Rev Mar Sci 12:153–179. https://doi.org/10.1146/annurev-marine-010419-010916

Pörtner H-O, Bock C, Mark FC (2017) Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. Journal of Experimental Biology 220:2685–2696. https://doi.org/10.1242/jeb.134585

Pottier P, Burke S, Zhang RY, et al (2022) Developmental plasticity in thermal tolerance: Ontogenetic variation, persistence, and future directions. Ecology Letters 25:2245–2268. https://doi.org/10.1111/ele.14083

Ramalho Q, Vale MM, Manes S, et al (2023) Evidence of stronger range shift response to ongoing climate change by ectotherms and high-latitude species. Biological Conservation 279:109911. https://doi.org/10.1016/j.biocon.2023.109911

Reimchen TE (1994) Predators and Morphological Evolution in Threespine Stickleback. In: The Evolutionary Biology of the Threespine Stickleback

Roberts Kingman GA, Lee D, Jones FC, et al (2021) Longer or shorter spines: Reciprocal trait evolution in stickleback via triallelic regulatory changes in *Stanniocalcin2a*. Proc Natl Acad Sci USA 118:e2100694118. https://doi.org/10.1073/pnas.2100694118

Roeder KA, Roeder DV, Bujan J (2021) Ant Thermal Tolerance: A Review of Methods, Hypotheses, and Sources of Variation. Annals of the Entomological Society of America 114:459–469. https://doi.org/10.1093/aesa/saab018

Schaefer J, Ryan A (2006) Developmental plasticity in the thermal tolerance of zebrafish *Danio rerio*. Journal of Fish Biology 69:722–734. https://doi.org/10.1111/j.1095-8649.2006.01145.x

Shama LNS, Strobel A, Mark FC, Wegner KM (2014) Transgenerational plasticity in marine sticklebacks: maternal effects mediate impacts of a warming ocean. Functional Ecology 28:1482–1493. https://doi.org/10.1111/1365-2435.12280

Shuter BJ, Schlesinger DA, Zimmerman AP (1983) Empirical Predictors of Annual Surface Water Temperature Cycles in North American Lakes. Can J Fish Aquat Sci 40:1838–1845. https://doi.org/10.1139/f83-213

Sinclair BJ, Marshall KE, Sewell MA, et al (2016) Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? Ecology Letters 19:1372–1385. https://doi.org/10.1111/ele.12686

Sinclair BJ, Williams CM, Terblanche JS (2012) Variation in Thermal Performance among Insect Populations. Physiological and Biochemical Zoology 85:594–606. https://doi.org/10.1086/665388

Smith SJ, Mogensen S, Barry TN, et al (2022) Evolution of thermal physiology alters the projected range of threespine stickleback under climate change. Molecular Ecology 31:2312–2326. https://doi.org/10.1111/mec.16396

Sunday JM, Bates AE, Dulvy NK (2012) Thermal tolerance and the global redistribution of animals. Nature Clim Change 2:686–690. https://doi.org/10.1038/nclimate1539

Svendsen MBS, Bushnell P, J. F. Steffensen (2019) AquaResp3

Turko AJ, Leclair ATA, Mandrak NE, et al (2021) Choosing source populations for conservation reintroductions: lessons from variation in thermal tolerance among populations of the imperilled redside dace [1]. Can J Fish Aquat Sci 78:1347–1355. https://doi.org/10.1139/cjfas-2020-0377

Vornanen M (2016) The temperature dependence of electrical excitability in fish hearts. Journal of Experimental Biology 219:1941–1952. https://doi.org/10.1242/jeb.128439

Weaving H, Terblanche JS, Pottier P, English S (2022) Meta-analysis reveals weak but pervasive plasticity in insect thermal limits. Nat Commun 13:5292. https://doi.org/10.1038/s41467-022-32953-2

Willacker JJ, Von Hippel FA, Wilton PR, Walton KM (2010) Classification of threespine stickleback along the benthic-limnetic axis: CLASSIFICATION OF THREESPINE STICKLEBACK. Biological Journal of the Linnean Society 101:595–608. https://doi.org/10.1111/j.1095-8312.2010.01531.x

Wood CL, Byers JE, Cottingham KL, et al (2007) Parasites alter community structure. Proc Natl Acad Sci USA 104:9335–9339. https://doi.org/10.1073/pnas.0700062104

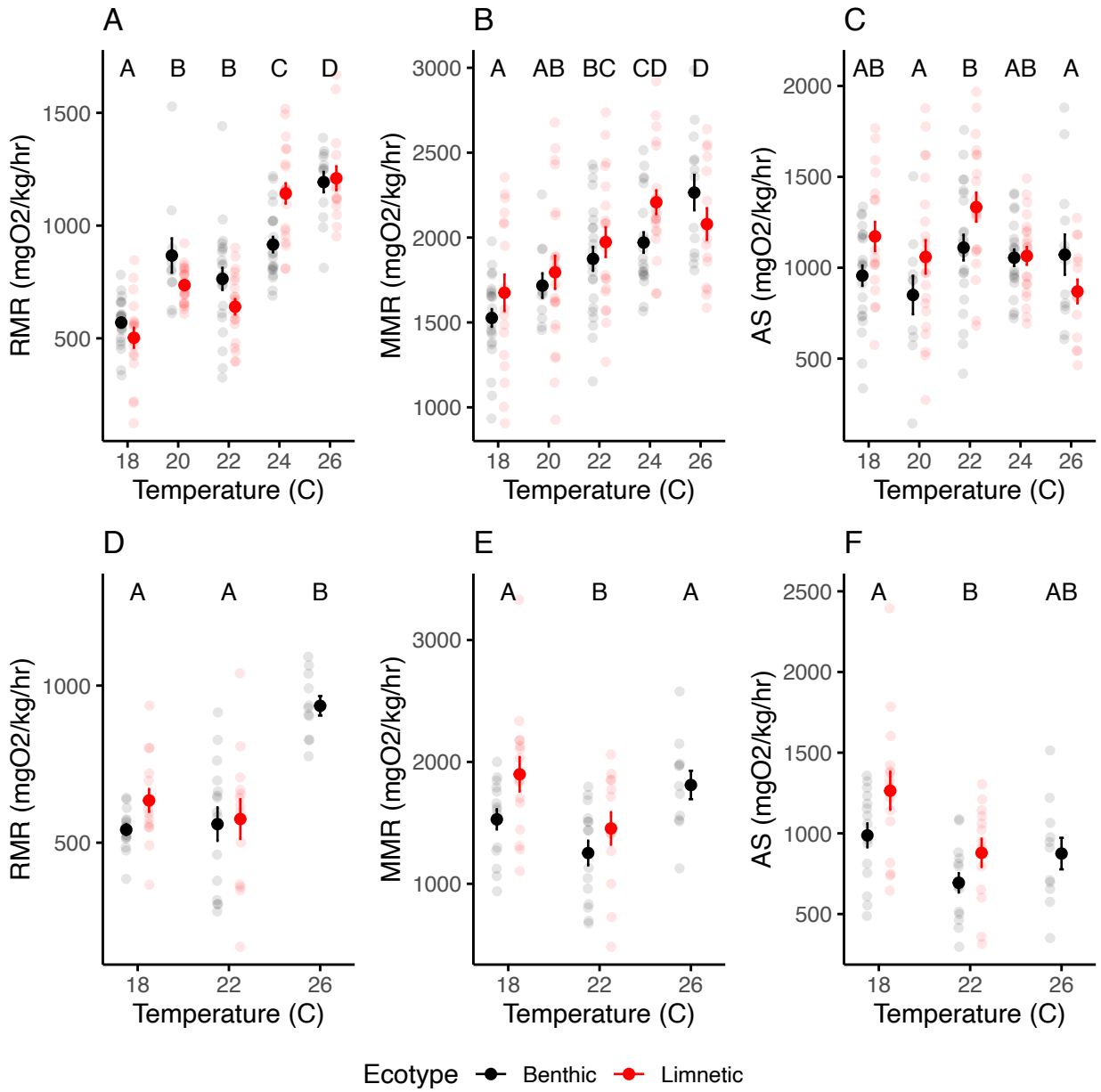


Figure 1. Mean (± SEM) metabolic traits as a function of temperature for each ecotype. At the beginning of the experiment both SMR (A) and MMR (B) increased with temperature. AS peaked at 22C at the start of the experiment (C). SMR increased significantly with temperature at the end of the experiment (D), but MMR did not (E). AS peaked at 18C at the end of the experiment (F).

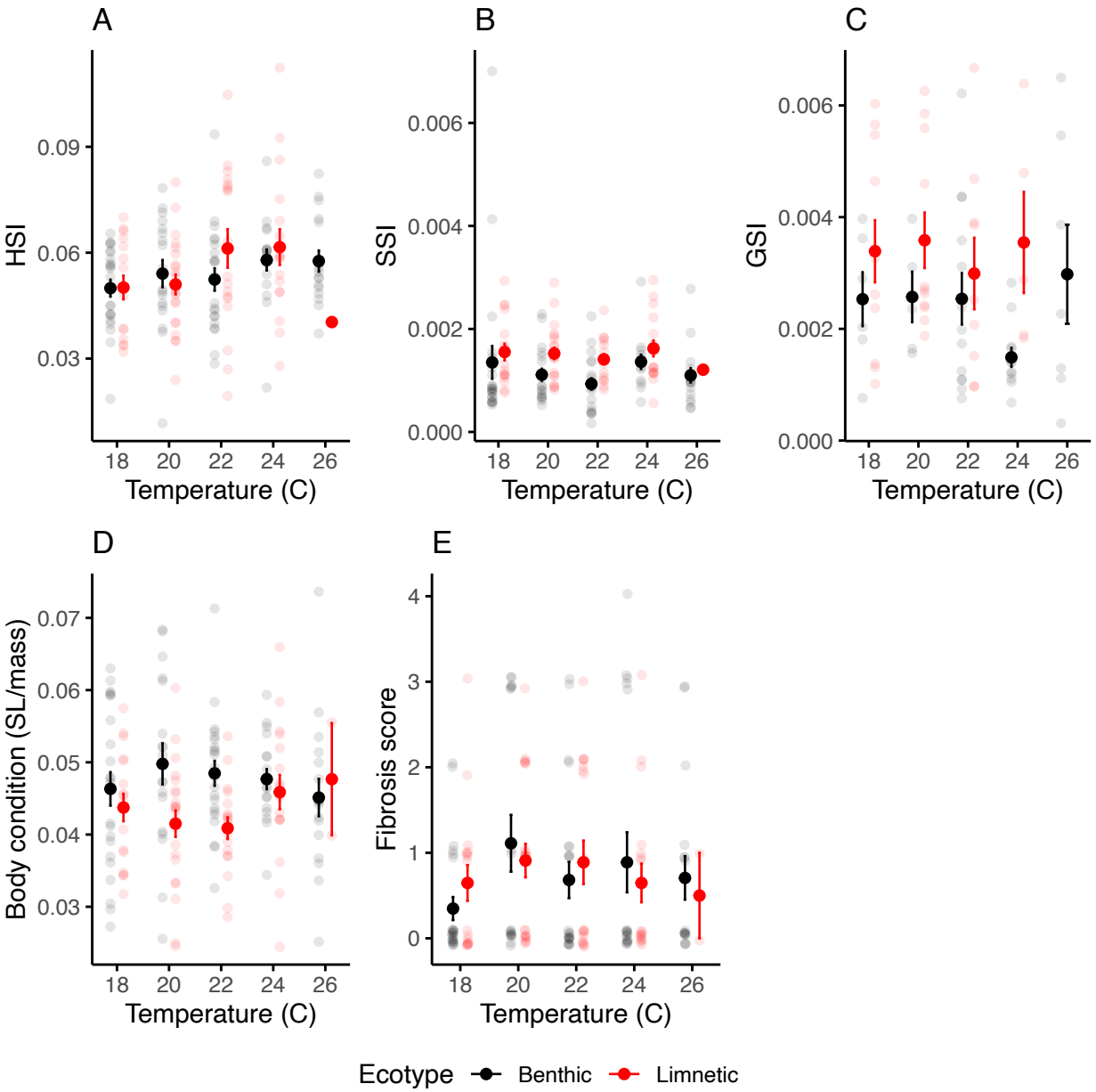


Figure 2. Mean (± SEM) metrics of condition as a function of temperature for each ecotype. Neither HSI (A), SSI (B), GSI (C), body condition (D), or fibrosis severity (E) varied with temperature or between ecotypes within temperature treatments.

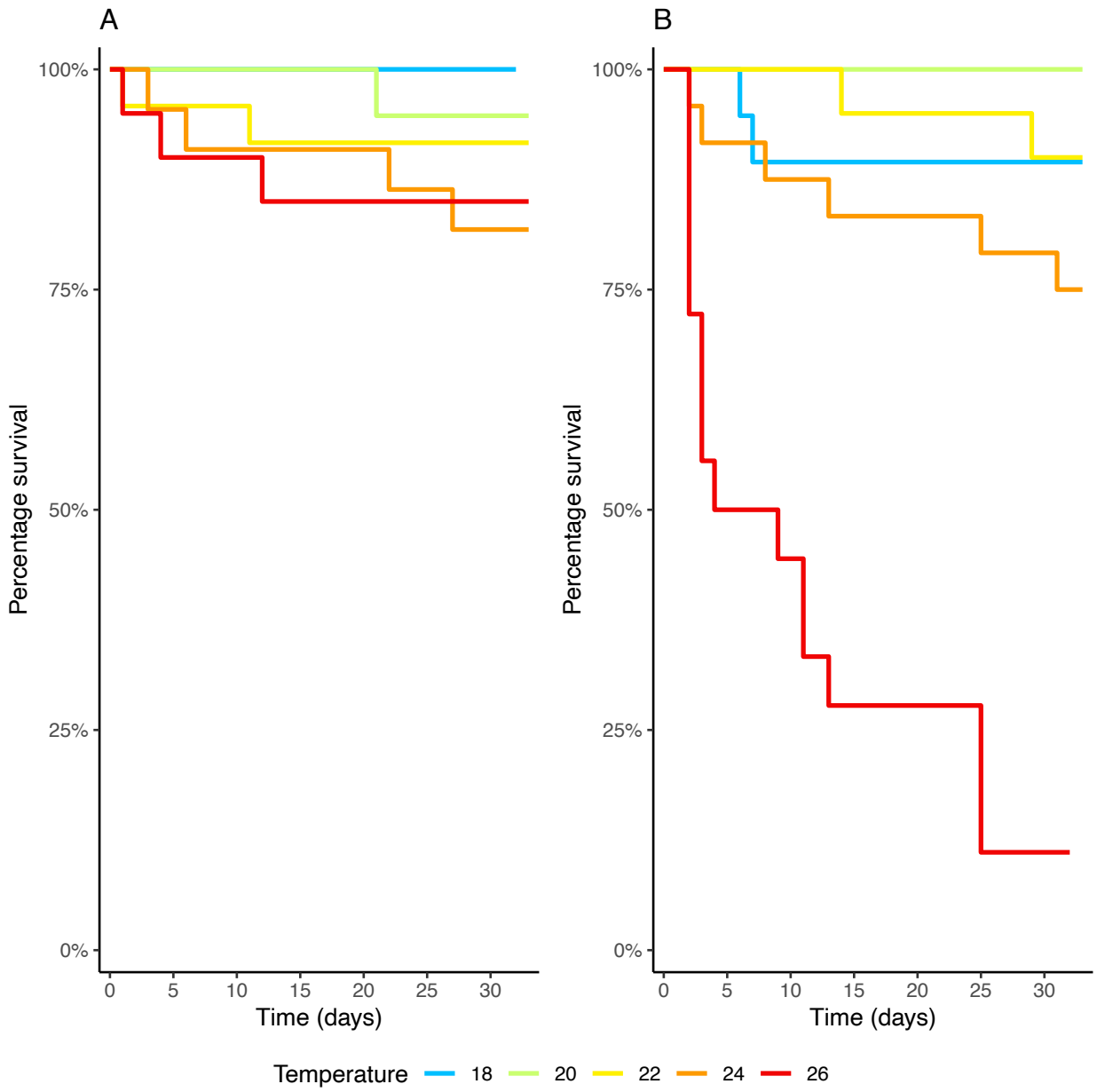
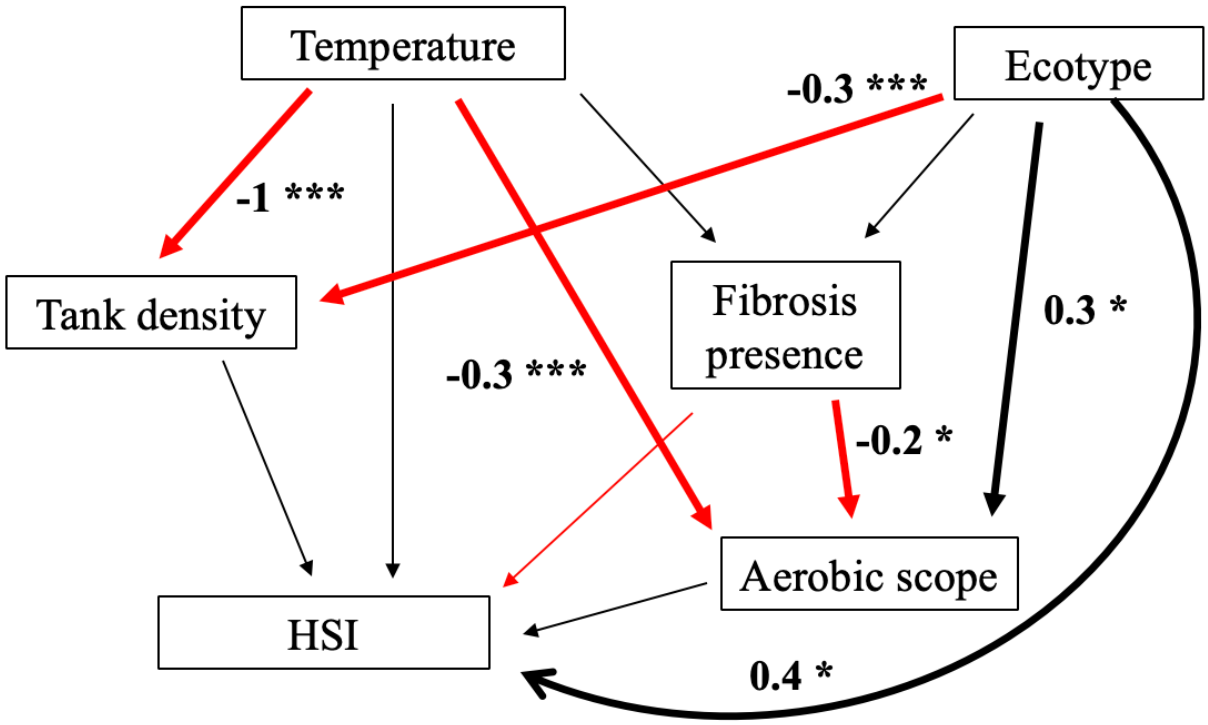
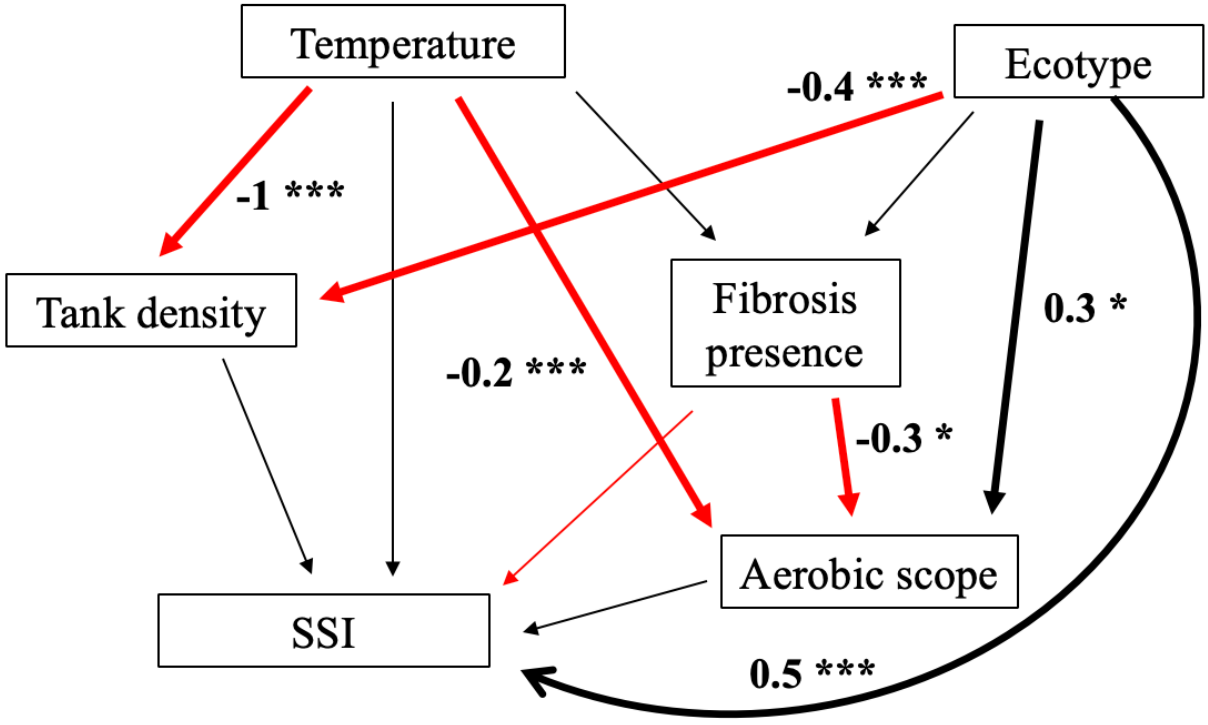


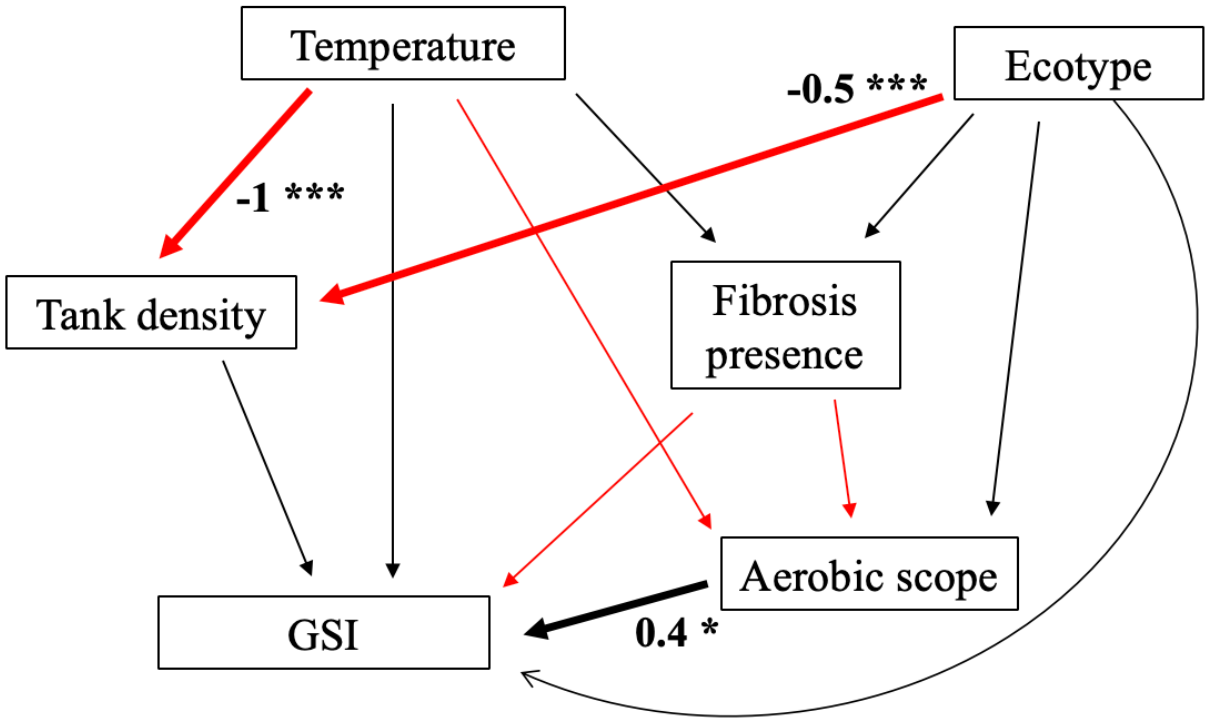
Figure 3. Probability of survival as function of temperature and time for benthic (A) and limnetic (B) stickleback. Temperature had a significant effect on mortality with the effect of temperature being marginally more severe in limnetic fish.



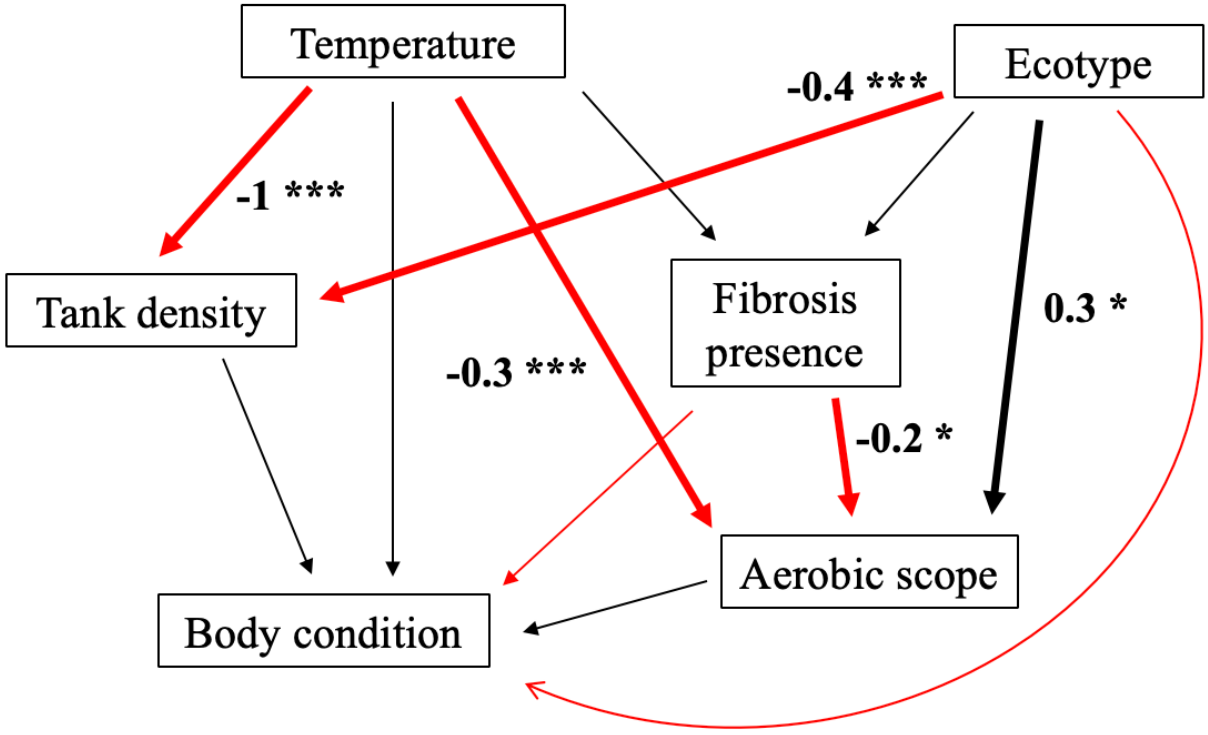
Supplemental figure 1. Path analysis detailing the effect of temperature and ecotype on HSI. Asterisks represent a significant effect; * = p <0.05, *** = p < 0.001.



Supplemental figure 2. Path analysis detailing the effect of temperature and ecotype on SSI. Asterisks represent a significant effect; * = p <0.05, *** = p < 0.001.



Supplemental figure 3. Path analysis detailing the effect of temperature and ecotype on GSI. Asterisks represent a significant effect; * = p <0.05, *** = p < 0.001.



Supplemental figure 4. Path analysis detailing the effect of temperature and ecotype on body condition. Asterisks represent a significant effect; * = p <0.05, *** = p < 0.001.

Chapter 4

A call or more ecologically and evolutionarily relevant studies of immune costs

Kristofer T. Sasser and Jesse N. Weber

University of Wisconsin – Madison

Published February 2023 in Evolutionary Ecology

Volume 37, Issue 1, Pages 203-214; doi: 10.1007/s10682-022-10213-5

Abstract

What are the relative costs and benefits of mounting immune responses? Practitioners of ecoimmunology have grappled with this central question since the field's inception with the main tension being how to make tractable methodological choices that maintain the ecological relevance of induced and measured immune costs. Here, we point out two methodological approaches that we feel are underrepresented in the field, describe risks associated with neglecting these methods, and suggest modern techniques that maximize both the diversity and ecological relevance of collected data. First, it is commonly assumed that frequently used and experimentally convenient immune stimulants will induce ecologically relevant immune responses in study organisms. This can be a dangerous assumption. Even if a stimulant's general immune response properties are well characterized, it is critical to also measure the type and scale of immune responses induced by live pathogens. Second, patterns of immune defenses evolve like other traits, thus a comparative approach is essential to understand what forces shape immune variation. Finally, we describe modern genetic and immunological approaches that will soon become essential tools for ecoimmunologists, and present case studies that exemplify the utility of our recommendations.

Introduction

Biologists have long sought to understand how organisms partition limited resources (Fisher 1930; Williams 1966). Central to the field of ecoimmunology is the idea that maintaining and deploying immune defenses imposes costs on hosts (Sheldon and

Verhulst 1996). Costs are myriad and include depleting nutrient and energy reserves and autoimmune damage incurred when fighting off a pathogen (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003). Further, these costs may impose trade-offs between immune defenses and other resource hungry functions such as growth or reproduction (Schmid-Hempel 2003). Consequently, quantifying the costs of immune defenses and assessing whether costs impose trade-offs has been a major focus of the field over the last three decades (Brock et al. 2014).

Many metrics have been used to demonstrate that mounting immune responses can be costly; however, it is still common to find experiments that do not explicitly state which stimulant options are available, and why the chosen stimulant and dose match the type and scale of responses initiated by live pathogens (Kennedy and Nager 2006; Alexander and Rietschel 2001). These omissions become most severe when the used stimulant is not an ideal fit (i.e., there are limited stimulant options) and/or investigators lack knowledge about natural immune states during infection (i.e., even if the right immune response is elicited, the costs are not accurate). This latter limitation also means that researchers need at least a partial understanding of the spectrum of natural immune responses to a particular pathogen.

To more fully embrace the “eco” and “immunology” sides of our field, we highlight several experimental additions that are both practical and demonstrate that induced immune responses are commensurate with those induced by live pathogens. We also highlight exemplary work from recent literature to serve as models for a new generation of ecoimmunologists. Finally, because the goal of this perspective is to preserve the

ecological context of studies on naturally diverse systems, our case studies primarily focus on “ecological models,” with abundant natural history information, that are now being examined with modern immunological and genetic tools.

Are we measuring ecologically relevant immune responses?

Two types of immune challenges, each with their own strengths and weaknesses, are frequently used to measure biological costs: infections with live pathogens and injection or exposure to immune stimulants. Experiments that use live pathogens clearly have more ecological relevance than those only using stimulants. However, the decision to use stimulants often has great practical import. For example, it is difficult to disentangle the cost directly imposed by the pathogen (e.g., nutrient theft or direct damage to the host) from the cost of mounting an immune response in natural settings (Graham et al. 2011). Furthermore, researchers must consider the possibility that live pathogens may harbor genetic, developmental, or environmental variation that lead to varying levels of virulence or that alter the likelihood or scale of host immune responses (Dupas and Boscaro 1999; Benesh and Kalbe 2016).

Ignoring the difficulties of performing and interpreting experiments with live pathogens, there are also many benefits using immune stimulants. Most stimulants can be delivered in precise doses, providing investigators the ability to elicit and measure a particular class of immune response. Similarly, stimulant-only measurements increase experimental signal in the data that could be diminished by the complex immune suppression or induction mechanisms of pathogens. These approaches are also not

limited to the lab; immune stimulants are easily administered in field settings, and responses can often be detected without controlling for previous exposure history. Despite these advantages, to maintain ecological relevancy researchers must always demonstrate that the immune responses they measure are both qualitatively and quantitatively similar to those induced by live pathogens. These criteria ensure that measured costs are commensurate with the costs paid by organisms in the wild.

Dangerous assumptions

A recent study of three populations of threespine stickleback (*Gastorosteus aculeatus*) demonstrates how immune stimulants can initiate qualitatively and quantitatively dissimilar immune responses. The magnitude of an inflammatory response--peritoneal fibrosis--in these fish depends on an interaction between the method of immune stimulation and the population being studied (Hund et al. 2020). All host populations mount varying levels of fibrotic responses when injected with a commonly used chemical adjuvant (1% aluminum phosphate), even though most populations lack fibrosis in nature. Only one population, which is highly resistant to tapeworm infections, mounts a response to a semi-natural immune challenge (tapeworm homogenate), and this response is more muted than with the chemical adjuvant. This highlights how both the magnitude and overall trend of responses varies with stimulant type and population, and how comparisons with natural pathogen products can inform the results of studies using immune stimulants.

Other commonly used immune stimulants are also often utilized in ways that cloud their ecological relevance. Take for example the commonly used immune stimulant phytohemagglutinin (PHA), which initiates cell mediated immune responses in mammals, reptiles, and birds (Kennedy and Nager 2006; Brock et al. 2014). Although most investigators focus on innate immune responses, PHA also stimulates the adaptive arm of the immune system and the makeup of the response varies across species (Martin et al. 2006; Tylan and Langkilde 2017). Furthermore, several forms of PHA are commercially available, each has a different effect on the type and scale of immune response initiated, and the type of PHA used is frequently not listed (Tylan and Langkilde 2017). To complicate matters further, the cellular makeup of the response can vary temporally on a fine scale (24-48 hours) both locally and systemically (Tylan and Langkilde 2017).

Lipopolysaccharide (LPS) is another commonly used immune stimulant that poses similar risks. The stimulant is a component of bacterial cell walls that induces innate immune responses (Alexander and Rietschel 2001). However, the type and scale of response induced is highly dependent on the strain of the bacteria species used to create the active component of the molecule--lipid A (Alexander and Rietschel 2001; Li et al. 2012). Additionally, the temperature in which certain pathogenic bacteria are grown (e.g., with Yersinia pestis and Francisella) can also influence the structure of lipid A (Li et al. 2012). Thus, the type of bacteria used to produce LPS and the temperature the bacteria are grown in may alter the response initiated, limiting the ability to generalize across studies that utilize LPS.

Taken together, we emphasize that researchers should test a diversity of immune assays to characterize the type and scale of response being initiated, pay close attention to all aspects of time when costs are being assessed (we discuss this more in the section arguing for more population-level analyses), and be willing to change methods on a study-by-study basis. All of these are requisite to determining what type of response is actually inducing costs and how that response compares to responses induced by live pathogens.

Ensuring ecological relevance

Although immune stimulants can initiate ecologically relevant immune responses, the onus of demonstrating their relevance falls upon their users. We offer several suggestions to effectively justify their use. First, it is essential to explain why a stimulant was chosen and how the nominal response relates to natural immune challenges. Second, researchers must demonstrate that the stimulant actually induces the nominal response during the timeframe you are measuring costs. Finally, the field must strive to demonstrate that stimulated response is quantitatively similar to responses induced by natural immune challenges. The first two suggestions are often followed, but to varying degrees of effectiveness. However, the scale of immune responses induced by immune stimulants is rarely compared to responses induced by natural immune challenges. This is a difficult task, but it is critical information that can be gleaned via immune assays of infected and uninfected individuals in the field or by experimentally exposing organisms to a live pathogen (reviewed in Graham et al. 2011).

All hope is not lost when considering immune responses in nature. Both well-developed and newer immunological techniques can be used by creative ecoimmunologists to quantify the type and scale of the immune responses induced by natural and artificial immune challenges (see Demas et al. 2014 for a review of pertinent immunological techniques). We highlight a few of these approaches, along with important caveats to consider while implementing them (Table 1). Enzyme-linked immunosorbent assays (ELISA) allow researchers to quantify the presence of specific antibodies (Garnier and Graham 2014; Garnier et al. 2017). Flow cytometry can characterize the diversity of immune cells, quantify their numbers, and measure the intensity of responses both before and after induction (Blackwell and Garcia 2022). Cell type specific antibodies greatly enhance the utility of flow cytometry approaches, but even basic measures of forward and side scatter provide rough estimates of cell diversity and number. There are also free software packages that can be used to cluster or gate cells of interest, which could be particularly useful for more ecology minded researchers (Hahne et al. 2009). For systems where little is known about the immune cell types or their function, new image based cytometers such as ImageStream (Luminex) can categorize the full extent of cell-type variation while also gathering data on cell function and physiological state (Accorsi et al. 2021). While most of these analyses cannot be done in the field, samples can be frozen with liquid nitrogen and transported back to the lab in some cases (Blackwell and Garcia 2022). By working from the ground up, building the most molecularly and cellularly detailed blueprint of immune variation will put ecologically focused studies in a much stronger position to measure and understand the responses of wild organisms.

Assuming a basic knowledge of immune cell type variation, advances in transcriptomics provide another exciting opportunity to simultaneously detect variation in the type and magnitude of immune responses in specific tissues or even single cells (Guslund et al. 2020; Huang et al. 2021). Although transcriptional data is most powerful when accompanied by protein level data, any of the above techniques will still greatly improve the ability to measure and understand wild immune responses (Liu et al. 2016). A deep toolbox of immunological assays is available to researchers and the right assay will depend upon the immune response of interest and the particulars of the study (e.g., the ability to draw blood from the organism, the ability to save and transport samples from the field, or cost).

An experimental limitation of wild animals is that they are often infected by multiple pathogens, and rarely is it possible to measure the complete prevalence and intensity of all infections via individual dissections. While substantial information can be gleaned from measuring infection intensity of a few focal pathogens, this is yet another case where adoption of modern genetic technology could be of great value to ecological and evolutionary immunologists (Graham et al. 2011). Environmental DNA screens offer insights not only into the diversity and prevalence of pathogens that exist in a specific environment but are also increasingly being used to catalog the infections of individuals (Cabodevilla et al. 2022). Most studies extract DNA directly from natural water or soil samples and develop PCR primers to sequencing genes that exhibit substantial nucleotide variation (e.g., Mitochondrial cytochrome c oxidase subunit I) (Thomas et al. 2022; Sengupta et al. 2018). Although the lab and bioinformatic components of eDNA analyses

are relatively simple, the choice of genes and development of primers requires careful consideration for each system, and only organisms with known sequences (i.e., deposited in GenBank or a similar repository) can be detected (Collins et al. 2019). Alternatively, when a large subset of the pathogens is already known, capture probe based methods offer the opportunity to physically separate pathogen DNA from surrounding host DNA, leading to very precise measures of both pathogen type and quantity at relatively low cost (Ellis et al. 2022; Jones and Good 2016).

The assumption that immune stimulants are effective facsimiles of natural immune challenges is too often left untested and casts doubt on the ecological relevance of much work in ecoimmunology. The work done using immune stimulants in the past was groundbreaking in establishing that immune responses can be costly, but we now must build on that foundation by taking a more nuanced approach. Future work comparing the type, scale, and costs of immune responses induced by natural immune challenges, such as live parasites or parasite protein, to the immune responses induced by stimulants is critical if we want to maximize the potential of these powerful tools. This type of work can be readily accomplished in many systems and will provide essential groundwork to interpret ecologically relevant immune costs in both previous and future studies.

Prospects for population level analyses of immune costs

The ability to mount immune responses varies at every level of biological organization, from individuals to species, and ecoimmunologists must choose which level of organization best suits their line of inquiry (Schoenle et al. 2018). Studies of individuals from a single

population are the foundation of the field and have given researchers an appreciation for the complexity and nuanced costs of immune responses, especially when physically isolated populations have been studied extensively (Lochmiller and Deerenberg 2000; Schmid-Hempel 2003). This approach has generated exquisite fitness data by measuring pedigrees, reproductive success, and mortality for every individual in a population, and illustrated a myriad of costs and benefits associated with deploying immune responses (e.g., the work done on the St. Kilda Island soay sheep over the last 60 years; Hayward et al. 2014). Investigations at the species level have likewise been fruitful, particularly work investigating life history trade-offs (Lee et al. 2008; Johnson et al. 2012).

There is, however, a dearth of work that investigates immune costs across multiple populations. A search of Google Scholar revealed that 17% of 6,810 scientific articles using the phrase “ecoimmunology”, “eco-immunology”, or “ecological immunology” also used any of the terms “cline”, “multiple populations”, “across populations”, or “population level”. Searching Web of Science using the same search parameters yielded a paltry 3%. While this is far from an exhaustive review, it demonstrates the lack of attention given to population level variation by ecoimmunologists. We argue that by studying multiple populations researchers unlock a greater ability to empirically test theories about immune costs and tradeoffs. This approach also allows researchers to leverage natural variation in order to understand which traits are labile and which processes generate and maintain this trait variation.

Studies of multiple populations are particularly powerful when individuals are reared in a common garden. As discussed in our critique of immune stimulants, even slight

environmental differences alter the responses organisms mount. A common rearing environment ensures that environmental effects are minimized, both at the moment of stimulation and during the development of an individual, and remaining phenotypic differences are primarily of genetic origin. Notably, many effects of the environment can be replicated in a common garden setting, allowing researchers to isolate the effects of single variables. For example, the ability to mount immune responses can be influenced by the season, availability of resources, and risk of predation (season: Nelson 2004; resource availability: Boots 2011; predation risk: Navarro et al. 2003). The following case studies illustrate how common garden experiments can shed light on complex ecoimmunological questions.

Using Populations to Empirically Test Theories of Immune Costs

Studies on invasive cane toads (*Rhinella marina*) in Australia exemplify how sampling multiple populations is necessary to test for immunity-dispersal trade-offs. Theory suggests that investment in immune defenses should diminish across an invasion gradient, as range edge populations can maximize dispersal ability by investing less energy or resources in immune defenses (Lee and Klasing 2004; Phillips et al. 2010). Measures of bacterial infections in wild cane toads support this finding. Bacterial infections are significantly more prevalent in range edge populations than range core populations (Brown et al. 2007). However, decreased immune effectiveness may be caused by the stress and demands of sustained movement associated with increased dispersal on range edges, rather than heritable factors. Indeed, the use of radio collared toads revealed that long

distance movements were correlated with diminished immune responses (Brown and Shine 2014).

To test whether infection differences were heritable or environmentally induced, researchers reared toads in a common garden setting prior to initiating immune responses. When researchers reared range edge and range core toads in a common environment and stimulated innate immune response using injections with LPS, range edge toads increased their metabolic rate significantly less than range core toads (Llewellyn et al. 2012). This supports the hypothesis that range edge toads may be investing less in immune responses generally, although it is also possible that range edge toads may be preferentially investing in less expensive humoral immune responses rather than innate responses.

These genetic hypotheses were further tested by using several immunological assays to determine the ability of lab reared toads to mount cell-mediated and innate immune responses (Brown et al. 2015). Contrary to expectations, toads with more range edge ancestry mounted significantly larger innate immune responses (Brown et al. 2015). The blood of toads with more range edge ancestry had significantly higher phagocytotic activity as well as a higher number of circulating neutrophils (Brown et al. 2015). Blood from range edge individuals was also significantly more effective at killing two species of bacteria, including one that commonly infects range edge toads (Brown et al. 2015).

The work using LPS should be approached with caution because it is unclear how the response initiated with LPS compares to a response initiated by a natural immune challenge such as a bacterial infection. However, these findings suggest that range edge toads have evolved a greater ability to mount innate immune responses while

simultaneously paying a lower energetic cost to do so than their range core peers. Although field studies suggested that long range movement came with a cost of increased infection, the common garden data show that natural costs would be even higher if not accompanied by efficient and effective immune evolution. Similar studies on the immune responses of invasive house sparrows (*Passer domesticus*) in Kenya have also revealed complex dynamics (Martin et al. 2009; Martin et al. 2014; Martin et al. 2017). Range edge sparrows pay significantly higher energetic costs when mounting an inflammatory response, yet they also evolved to incur less oxidative damage than range core sparrows (Martin et al. 2017).

Studies of multiple populations are also ideal for studying potential trade-offs between life history and immune traits. Theory predicts that life history and immune traits should evolve in tandem with "fast" living organisms investing primarily in non-specific innate immune responses and "slow'" living organisms investing primarily in the adaptive arm of the immune system (Lee 2006). This prediction has largely been borne out by studies of birds at the species level, but few attempts have been made to test this prediction using populations of the same species (Lee et al. 2008). Studies of western terrestrial garter snakes (*Thamnophis elegans*) provide one of the few examples of researchers leveraging population level variation to test this prediction. Garter snake populations vary in a number of life history traits including growth rate, body size, age at maturity, litter size, and life span making them an ideal system to test this hypothesis (Bronikowski 2000; Bronikowski and Arnold 1999). Consistent with theory, these populations also vary in immune response: individuals from a population that grows rapidly to large size, reproduces at a young age, and has large litters also mount stronger innate

immune responses than individuals from a population that grows slowly, reproduces later in life, and lives longer (Sparkman and Palacios 2009).

A bounty of natural variation begging for deeper immune inquiry

Although we strongly support researchers gathering novel natural history and immunological data, there are numerous species with natural history data and immunological resources that provide outstanding opportunities to study costs and trade-offs. In particular, we recommend looking for species with life history or phenotype-environment associations that strongly suggest that the costs of immune responses shape patterns we see in nature.

Threespine stickleback are a posterchild for how phenotypic variation is produced during an adaptive radiation, including divergence in immune traits. Stickleback populations vary in multiple life history traits hypothesized to trade-off with immune defenses, including lifespan (Gambling and Reimchen 2012; DeFaveri and Merilä 2013), reproductive investment: Heins et al. 2008, Weber et al 2022), and growth rate (Wright et al. 2004; Snyder 1991). Further, wild stickleback show marked variation in the prevalence of tapeworm infection, with some populations never becoming infected while others suffer up to 80% infection prevalence (Weber et al. 2017). This population variation makes stickleback an ideal system to study whether the costs of immune responses impose life history trade-offs, and how parasite variation drives (or is driven by) immune evolution. However, the heritability of many of these traits remained in question until recently. Common garden experiments found that infection outcomes are strongly influenced by

heritable differences. High and no-infection fish differ in a suite of immune traits including reactive oxygen species production, granulocyte abundance, the ability to mount a fibrotic response used to encapsulate tapeworms, and helminth growth suppression (Weber et al. 2017; Weber et al. 2022; Hund et al. 2020).

Mexican tetras (*Astyanax mexicanus*) provide another striking example of a well characterized system that is practically begging for researchers to assess costs. Peuß et al. (2020) compared the immune systems of lab reared offspring of both cave and surface-dwelling Mexican tetras and provided remarkably detailed descriptions of immune system evolution. Using flow cytometry, phagocytosis assays, and transcriptomics, the authors found that cave dwelling populations rely primarily upon the adaptive arm of the immune system while surface fish invest evenly in both innate and adaptive responses. This variation is likely driven by differences in the timing and type of costs imposed by the different arms of the immune system and local adaptation to differing parasite communities (Lee 2006). The deployment of innate immune responses is generalized, short lived, and costly in terms of both resources and the risk of autoimmune damage while the deployment of adaptive immune defenses is pathogen specific, thought to be relatively inexpensive, and provides longer term protection (McDade et al. 2016). Historically, it has been difficult to measure the arms of the immune system independently. Identifying natural populations with heritable differences in immune investment, combined with the ability to measure the type and scale of immune responses, should provide an important avenue for quantifying the cost of particular types of immune responses (Kennedy and Nager 2006; Råberg et al. 2002).

Discussion

Generating empirical measurements to test theories about the costs of mounting immune responses remains a foundational problem for the field of ecoimmunology. We highlight ways to integrate old and new experimental techniques more fully, which need not always entail sophisticated or costly methods. The vast body of work assessing the cost of immune responses has demonstrated that immune defenses can be costly, but the ecological relevance of these costs will remain in doubt until the responses induced using stimulants are compared to those induced by natural pathogens. This extension is readily accomplished using the broad array of immunological assays available. These methods may also be used to identify if and how populations vary in immune responses. Very little is currently known about how the costs of immune responses vary across populations, yet this information is crucial for evaluating theory and explaining the variation in immune responses we see in the wild despite dramatic fitness costs imposed by pathogens and parasites.

Permits/Acknowledgments/Funding

We thank two anonymous reviewers and members of the Weber Lab for valuable feedback on the manuscript. KTS conceived the paper and wrote the initial draft. JNW contributed ideas for additional subject matter, and to writing and editing the final draft. KTS was supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number

2P20GM103395. KTS and JNW were supported by the National Institute of General Medical Sciences of the National Institutes of Health under grant number 1R35GM142891-01.

References

Accorsi A, Box AC, Peuß R, et al (2021) Image3C, a multimodal image-based and label-independent integrative method for single-cell analysis. eLife 10:e65372

Alexander C, Rietschel ETh (2001) Invited review: Bacterial lipopolysaccharides and innate immunity. Journal of Endotoxin Research 7:167–202

Benesh DP, Kalbe M (2016) Experimental parasite community ecology: intraspecific variation in a large tapeworm affects community assembly. J Anim Ecol 85:1004–1013

Blackwell AD, Garcia AR (2022) Ecoimmunology in the field: Measuring multiple dimensions of immune function with minimally invasive, field-adapted techniques. American J Hum Biol

Boots M (2011) The evolution of resistance to a parasite is determined by resources. The American Naturalist 178:214–220

Brock PM, Murdock CC, Martin LB (2014) The history of ecoimmunology and its integration with disease ecology. Integrative and Comparative Biology 54:353–362

Bronikowski AM (2000) Experimental evidence for the adaptive evolution of growth rate in the garter snake Thamnophis elegans. Evolution 54:1760–1767

Bronikowski AM, Arnold SJ (1999) The evolutionary ecology of life history variation in the garter snake Thamnophis elegans. Ecology 80:2314

Brown GP, Phillips BL, Dubey S, Shine R (2015) Invader immunology: invasion history alters immune system function in cane toads (*Rhinella marina*) in tropical Australia. Ecol Lett 18:57–65

Brown GP, Shilton C, Phillips BL, Shine R (2007) Invasion, stress, and spinal arthritis in cane toads. Proceedings of the National Academy of Sciences 104:17698–17700

Brown GP, Shine R (2014) Immune response varies with rate of dispersal in invasive cane toads (Rhinella marina). PLoS ONE 9:e99734

Cabodevilla X, Gómez-Moliner BJ, Abad N, Madeira MJ (2022) Simultaneous analysis of the intestinal parasites and diet through eDNA metabarcoding. Integr Zool 1749-4877.12634

Collins RA, Bakker J, Wangensteen OS, et al (2019) Non-specific amplification compromises environmental DNA metabarcoding with COI. Methods Ecol Evol 10:1985–2001

DeFaveri J, Merilä J (2013) Variation in age and size in fennoscandian three-spined sticklebacks (Gasterosteus aculeatus). PLoS ONE 8:e80866

Downs CJ, Adelman JS, Demas GE (2014) Mechanisms and methods in ecoimmunology: Integrating within-organism and between-organism processes. Integrative and Comparative Biology 54:340–352

Dupas S, Boscaro M (1999) Geographic variation and evolution of immunosuppressive genes in a Drosophila parasitoid. Ecography 22:284–291

Ellis VA, Kalbskopf V, Ciloglu A, et al (2022) Genomic sequence capture of Plasmodium relictum in experimentally infected birds. Parasites Vectors 15:267

Fisher RA (1930) The genetical theory of natural selection. Clarendon Press

Gambling SJ, Reimchen TE (2012) Prolonged life span among endemic Gasterosteus populations. Can J Zool 90:284–290

Garnier R, Graham AL (2014) Insights from parasite-specific serological tools in eco-immunology. Integrative and Comparative Biology 54:363–376

Garnier R, Ramos R, Sanz-Aguilar A, et al (2017) Interpreting ELISA analyses from wild animal samples: Some recurrent issues and solutions. Funct Ecol 31:2255–2262

Graham AL, Shuker DM, Pollitt LC, et al (2011) Fitness consequences of immune responses: strengthening the empirical framework for ecoimmunology. Functional Ecology 25:5–17

Guslund NC, Solbakken MH, Brieuc MSO, et al (2020) Single-cell transcriptome profiling of immune cell repertoire of the Atlantic Cod which naturally lacks the major histocompatibility class II system. Front Immunol 11:559555

Hahne F, LeMeur N, Brinkman RR, et al (2009) flowCore: a Bioconductor package for high throughput flow cytometry. BMC Bioinformatics 10:106

Hayward AD, Garnier R, Watt KA, et al (2014) Heritable, heterogeneous, and costly resistance of sheep against nematodes and potential feedbacks to epidemiological dynamics. The American Naturalist 184:S58–S76

Heins D, King R, Baker J, Foster S (2008) An overview of life-history variation in female threespine stickleback. Behav 145:579–602

Huang Z, Chen B, Liu X, et al (2021) Effects of sex and aging on the immune cell landscape as assessed by single-cell transcriptomic analysis. Proc Natl Acad Sci USA 118:e2023216118

Hund AK, Fuess LE, Kenney ML, et al (2020) Rapid evolution of parasite resistance via improved recognition and accelerated immune activation and deactivation. Evolutionary Biology

Johnson PTJ, Rohr JR, Hoverman JT, et al (2012) Living fast and dying of infection: host life history drives interspecific variation in infection and disease risk: Living fast and dying of infection. Ecology Letters 15:235–242

Jones MR, Good JM (2016) Targeted capture in evolutionary and ecological genomics. Mol Ecol 25:185–202

Kennedy M, Nager R (2006) The perils and prospects of using phytohaemagglutinin in evolutionary ecology. Trends in Ecology & Evolution 21:653–655

Lee KA (2006) Linking immune defenses and life history at the levels of the individual and the species. Integrative and Comparative Biology 46:1000–1015

Lee KA, Klasing KC (2004) A role for immunology in invasion biology. Trends in Ecology & Evolution 19:523–529

Lee KA, Wikelski M, Robinson WD, et al (2008) Constitutive immune defences correlate with life-history variables in tropical birds. J Anim Ecology 77:356–363

Li Y, Powell DA, Shaffer SA, et al (2012) LPS remodeling is an evolved survival strategy for bacteria. Proc Natl Acad Sci USA 109:8716–8721

Liu Y, Beyer A, Aebersold R (2016) On the dependency of cellular protein levels on mRNA abundance. Cell 165:535–550

Llewellyn D, Thompson MB, Brown GP, et al (2012) Reduced investment in immune function in invasion-front populations of the cane toad (Rhinella marina) in Australia. Biol Invasions 14:999–1008

Lochmiller RL, Deerenberg C (2000) Trade-offs in evolutionary immunology: just what is the cost of immunity? Oikos 88:87–98

Martin LB, Alam JL, Imboma T, Liebl AL (2010) Variation in inflammation as a correlate of range expansion in Kenyan house sparrows. Oecologia 164:339–347

Martin LB, Coon CAC, Liebl AL, Schrey AW (2014) Surveillance for microbes and range expansion in house sparrows. Proc R Soc B 281:20132690

Martin LB, Han P, Lewittes J, et al (2006) Phytohemagglutinin-induced skin swelling in birds: histological support for a classic immunoecological technique. Funct Ecology 20:290–299

Martin LB, Kilvitis HJ, Brace AJ, et al (2017) Costs of immunity and their role in the range expansion of the house sparrow in Kenya. Journal of Experimental Biology jeb.154716

McDade TW, Georgiev AV, Kuzawa CW (2016) Trade-offs between acquired and innate immune defenses in humans. EMPH 2016:1–16

Navarro C, de Lope F, Marzal A, et al (2004) Predation risk, host immune response, and parasitism. Behavioral Ecology 15:629–635

Nelson RJ (2004) Seasonal immune function and sickness responses. Trends in Immunology 25:187–192

Peuß R, Box AC, Chen S, et al (2020) Adaptation to low parasite abundance affects immune investment and immunopathological responses of cavefish. Nat Ecol Evol 4:1416–1430

Phillips BL, Brown GP, Shine R (2010) Life-history evolution in range-shifting populations. Ecology 91:1617–1627

Råberg L, Vestberg M, Hasselquist D, et al (2002) Basal metabolic rate and the evolution of the adaptive immune system. Proc R Soc Lond B 269:817–821

Rusch JC, Hansen H, Strand DA, et al (2018) Catching the fish with the worm: a case study on eDNA detection of the monogenean parasite Gyrodactylus salaris and two of its hosts, Atlantic salmon (Salmo salar) and rainbow trout (Oncorhynchus mykiss). Parasites Vectors 11:333

Schmid-Hempel P (2003) Variation in immune defence as a question of evolutionary ecology. Proc R Soc Lond B 270:357–366

Schoenle LA, Downs CJ, Martin LB (2018) An Introduction to Ecoimmunology. In: Cooper EL (ed) Advances in Comparative Immunology. Springer International Publishing, Cham, pp 901–932

Sengupta ME, Hellström M, Kariuki HC, et al (2019) Environmental DNA for improved detection and environmental surveillance of schistosomiasis. Proc Natl Acad Sci USA 116:8931–8940

Snyder RJ (1991) Migration and life histories of the threespine stickleback: evidence for adaptive variation in growth rate between populations. Environ Biol Fish 31:381–388

Thomas LJ, Milotic M, Vaux F, Poulin R (2022) Lurking in the water: testing eDNA metabarcoding as a tool for ecosystem-wide parasite detection. Parasitology 149:261–269

Tylan C, Langkilde T (2017) Local and systemic immune responses to different types of phytohemagglutinin in the green anole: Lessons for field ecoimmunologists. J Exp Zool 327:322–332

Weber JN, Steinel NC, Peng F, et al (2022) Evolutionary gain and loss of a pathological immune response to parasitism. Science 377:1206–1211

Weber JN, Steinel NC, Shim KC, Bolnick DI (2017) Recent evolution of extreme cestode growth suppression by a vertebrate host. Proc Natl Acad Sci USA 114:6575–6580

Williams GC (1966) Natural selection, the costs of reproduction, and a refinement of Lack's principle. The American Naturalist 100:687–690

Wright HA, Wootton RJ, Barber I (2004) Interpopulation variation in early growth of threespine sticklebacks (Gasterosteus aculeatus) under laboratory conditions. 61:7

Table 1: Modern methods to improve wild immune measurements

Category of method	**Measurements**	**Notes**
Cell focused		
1. ELISA	-host antibody levels -abundance of immune cells or secreted molecules	-Requires specific probes, but many have broad utility
2. Flow cytometry		
a. Traditional/ Laser-based	-Forward and side scatter distinguishes general cell categories -Fluorescent antibodies and assays increase cell specificity and physiology	-Requires basic understanding of cell gating, but can be done with opensource software (R gating REFS)
b. Image cytometer	-Catalog cell diversity and abundance without antibodies	-ImageStream machine not accessible at all institutions
3. RNA assays		
a. qPCR	-Expression of genes in key immune pathways	-Multiplexing lowers cost -Must know cell specific expression profiles and signaling cascades
b. RNAseq- Whole tissue	-Transcript abundance of all immune genes	-3'TagSeq methods provide greatest statistical power -Limited ability to infer cell types and abundances
c. RNAseq- Single cell	-Detailed understanding of all cell types and activities	-Good for characterizing new systems, but cost prohibitive for assays in full experiments
Parasite measures		
1. 16sRNA sequencing	-Presence and potentially abundance of parasite communities -Either eDNA or from tissue homogenate	-Strong potential of eDNA for aquatic systems/fecal samples -Need to target particular tissues and optimize DNA extraction
2. DNA capture probes	-Presence and abundance of particular parasite groups	-Starts with expert ID of parasites and DNAseq -High initial cost, but scales for many assays