DISEASE DYNAMICS IN FRESHWATER CRAYFISH INFECTED WITH CRAYFISH PLAGUE: LETHAL AND SUBLETHAL EFFECTS OF CUTICLE LESIONS, MARKING METHODS, AND WARMING TEMPERATURE ON CRAYFISH PERFORMANCE

by

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DEDICATION

This thesis is dedicated to crayfish, and to the curious kids and adults who search for them under rocks in creeks, lakes and ditches around the world because it's fun.

For many people, catching a crayfish is the first moment when the hidden world of freshwater ecosystems comes alive. These remarkable animals serve as ambassadors of the waters they inhabit, inviting curiosity, sparking questions, and reminding us that even the smallest creatures can open the door to a lifelong appreciation of nature, as they did for me.

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ABSTRACT

Freshwater ecosystems are increasingly affected by multiple stressors, including emerging diseases and environmental change. Crayfish are important ecosystem engineers that influence sediment structure, nutrient cycling, and aquatic food webs, yet relatively little is known about how disease interacts with environmental stressors and research practices to influence crayfish health and performance. In particular, the effects of crayfish plague and common handling techniques used in ecological studies remain poorly understood. The objective of this thesis was to examine how disease history interacts with research handling and temperature stress to influence survival, growth, molting success, behavior, and lesion development in the crayfish *Faxonius virilis*.

Three laboratory experiments were conducted using crayfish from populations that differed in lesion prevalence history associated with crayfish plague. In the first experiment, crayfish were subjected to two commonly used marking methods, tail punching and visible implant elastomer, to evaluate how handling techniques influenced survival, growth, molting success, and lesion severity. In the second experiment, crayfish were exposed to three temperature treatments to evaluate how thermal stress influenced survival, physiological performance, and lesion dynamics. In the third experiment, an acute thermal ramp assay was used to determine how acclimation temperature influenced thermal tolerance and behavioral responses using a righting response test.

Results showed that marking effects were dependent on disease context. Tail punching reduced survival and molting success and was associated with greater lesion severity, particularly in crayfish from the population with higher lesion prevalence, whereas visible implant elastomer marking had minimal measurable effects. Temperature strongly influenced physiological performance, with warmer conditions increasing mortality and altering behavioral responses. Disease history primarily influenced sublethal responses, including lesion development and body condition.

These findings demonstrate that disease history can modify the effects of both environmental stress and research practices on crayfish performance. Considering disease context when designing experiments and interpreting ecological responses will improve understanding of how freshwater organisms respond to multiple stressors in changing environments.

CHAPTER 1

THESIS INTRODUCTION

Rising temperatures, altered hydrology, species translocations, and increasing human activity are intensifying pathogen impacts on host organisms and producing more variable, and often more severe, disease outcomes for hosts (Rahel and Olden 2008; Rajamanickam et al. 2023; Ramakant et al. 2025). In Montana ,recent wildlife disease events include outbreaks of avian influenza in birds (Shi et al. 2022), white-nose syndrome in bats (Hoyt et al. 2021), chronic wasting disease in cervids (Almberg et al. 2011), and proliferative kidney disease in salmonids (Hutchins et al. 2021). These changes highlight the importance of research aimed at understanding how environmental stressors, pathogens, and host physiology interact.

One group susceptible to disease is freshwater crayfish. Crayfish are dominant benthic consumers, ecosystem engineers, and prey for numerous fish, birds and mammals (Momot et al. 1978; Taylor et al. 1996; Larson and Olden 2011; Albertson and Daniels 2016). As such, they influence sediment dynamics, nutrient cycling, and food web interactions. In the northern Rocky Mountains, crayfish support recreational fisheries and serve as forage for game fishes (Martinez 2012; Kennedy and Adams 2025). However their distribution has expanded through human-mediated translocations, and once established, populations may continue to spread independently, with potential ecological consequences.

Despite their ecological and socioeconomic importance, relatively little research has examined how disease and environmental stressors interact to influence crayfish physiology and population performance (Guiaşu and Labib 2021; Miller et al. 2024; Oficialdegui et al. 2020).

One of the most significant pathogens affecting crayfish worldwide is the oomycete *Aphanomyces astaci* (Schikora 1903), the causative agent of the crayfish plague. Introduced to Europe in the nineteenth century, the pathogen caused widespread collapse of native crayfish populations and continues to threaten European species (Alderman 1996; Holdich et al. 2008; Martín-Torrijos et al. 2021; Jussila et al. 2021). In contrast, North American crayfish have historically been considered tolerant hosts that carry the pathogen with few or no visible symptoms (Söderhäll and Cerenius 1999; Cerenius et al. 2003). However, recent observations in Montana and Europe challenge this assumption (Martín-Torrijos et al. In prep; Adamkowska et al. 2018). Surveys conducted in 2021 documented conspicuous melanized lesions in *Faxonius virilis* and other species, and molecular analyses confirmed the presence of *A. astaci* in several affected individuals from various locations around the state, representing the first documented instance of overt clinical disease consistent with crayfish plague in North American crayfish within North America (Martín-Torrijos et al. in prep.). These findings suggest that environmental conditions, pathogen strains, or host susceptibility may influence disease expression in North American populations.

Montana's crayfish fauna includes five confirmed species, and their distributions remain poorly understood (Sheldon 1988; Adams et al. in prep; Schmidt et al. 2023; Larson et al. 2025; Kennedy and Adams 2025). *Faxonius virilis*, the most widespread species, is believed to be native only to portions of eastern Montana, yet occurs across the western part of the state, likely due to bait bucket transfers and other human-mediated introductions (Cohen and Carlton 1995). Recent phylogenetic work suggests that some species historically regarded as native to western Montana may instead be cryptogenic or introduced (Adams et al. In Prep). Invasive populations

may therefore be encountering novel environmental pressures, pathogen strains, or genotype-host mismatches that could elevate disease expression. Understanding how these environmental factors influence infection outcomes is therefore an important component of crayfish conservation and management in the region.

Genetic studies has also revealed substantial diversity in *A. astaci* strains within North America (Martín-Torrijos et al. 2021; Di Domenico et al. 2021). Modern molecular diagnostic tools, including quantitative PCR assays, now allow rapid identification of pathogen strains (Vrålstad et al. 2009) and have shown that invasive crayfish species often carry different pathogen genotypes across introduced populations within their expanded ranges (Lemmers et al. 2021). Together, these findings suggest that interactions among host origin, pathogen strain, and environmental stressors may contribute to the emerging disease patterns observed in Montana.

Crayfish Marking Methods

Marking crayfish is fundamental for studies of population dynamics, movement, growth, and survival. However, marking techniques vary widely in durability, biological impacts, and logistical feasibility. Most crayfish marking methods fall into three general categories: mechanical tissue marks, internal visual tags, and electronic identification technologies (Ramalho et al. 2010).

Early approaches to crayfish marking often involved physical modification of the exoskeleton, including clipping or hole-punching uropods and telson segments or cauterizing the carapace to create visible marks (Abrahamsson 1965; Ramalho et al. 2010; Guan 1997). These methods are inexpensive and often persistent but involve tissue damage and can influence growth, behavior, or molting in some species (Guan 1997; Buřič et al. 2008; McFarlane et al.

2019). As a result, they are increasingly viewed as less desirable when animal welfare considerations are important. This new perspective may be especially relevant if diseases such as crayfish plague influence external tissues where marks are made.

Internal visual tagging methods, particularly visible implant elastomer (VIE) tags are now widely used alternatives. These tags are injected beneath the ventral cuticle and remain visible through the exoskeleton, allowing individual identification without external attachments. Studies across multiple crayfish species show relatively high tag retention and minimal effects on survival or growth compared to untagged controls (Jerry et al. 2001; Buřič et al. 2008; Isely and Stockett 2001; Mazlum 2007). However, tag loss can occur during molting or in small individuals (Clark and Kershner 2006; Lott 2015).

Electronic tagging technologies such as passive integrated transponder (PIT) tags provide unique electronic identifiers and are widely used in long-term monitoring studies. When used in appropriately sized crayfish, PIT tags show high retention and minimal biological effects (Bubb et al. 2002; Nightingale et al. 2018; Black et al. 2010), although their cost and crayfish size requirements limit their use in some applications (Haddaway et al. 2011; Buřič et al. 2008; Lott 2015; Westhoff and Sievert 2013). Environmental factors such as substrate and burrowing behavior can influence detection probability in field studies (Bubb et al. 2004; Bubb et al. 2006; Bubb et al. 2008).

Although extensive research has evaluated the retention and performance of these marking techniques (Nowicki et al. 2008; Haddaway et al. 2011; Larson and Olden 2016), relatively few studies have examined how marking interacts with underlying host health or disease status. In populations exposed to pathogens such as *A. astaci*, tissue damage associated

with some marking techniques could potentially influence infection dynamics or physiological performance. The experiment presented in Chapter 2 addresses this gap by testing how two commonly used marking techniques influence survival, growth, molting, and lesion expression in crayfish populations with differing levels of disease prevalence.

Temperature in Freshwater Ecosystems

Temperature is one of the most influential environmental variables shaping the structure and function of freshwater ecosystems (Woodward et al. 2010; Bonacina et al. 2022). Because most aquatic organisms are ectothermic, physiological processes such as metabolism, growth, immune function, and behavior are strongly regulated by ambient temperature (Volkoff and Rønnestad 2020; Ren et al. 2021). Even modest temperature changes can alter species interactions, influence nutrient cycling, and shift community composition in streams and lakes (Haase et al. 2019; Verbeek et al. 2018). Therefore, climate warming is expected to have widespread ecological consequences for freshwater systems, including altered species distributions, increased metabolic demand, and changes in the timing and magnitude of biological processes (Heino et al. 2009; Woodward et al. 2010; Woolway et al. 2022).

Temperature also plays an important role in host–pathogen dynamics. In aquatic ectotherms, thermal conditions influence both pathogen growth rates and host immune responses, often determining whether infections remain latent or progress to overt disease (Coates and Söderhäll 2020; Millard et al. 2020; Shields 2019). Elevated temperatures can increase physiological stress, disrupt immune defenses, and accelerate pathogen development (Shields 2019), while cooler temperatures may slow immune processes or prolong infection persistence (Ren et al. 2021). As a result, temperature variation can shape disease outcomes by

modifying both host susceptibility and pathogen performance. Temperature controls when and how efficiently *A. astaci* grows, sporulates, and spreads, and which strains dominate, with ~15-20 °C often optimal and both colder and hotter extremes constraining parts of the life cycle (Unestam 1969; Unestam and Weiss 1970; Diéguez-Uribeondo et al. 1995; Strand et al. 2012; Alvanou et al. 2025).

Crayfish are sensitive to temperature fluctuations because their metabolic rate, molting cycle, and behavioral responses are closely linked to thermal conditions (Westhoff and Rosenberger 2016). Temperature influences growth and activity patterns in many crayfish species and can affect their tolerance to environmental stressors and pathogens (Westhoff and Rosenberger 2016; Yang et al. 2022). In regions such as Montana, where freshwater systems experience substantial seasonal and daily temperature variation, understanding how thermal conditions interact with disease processes is essential for predicting crayfish population dynamics and their effects on ecosystem structure and function.

Thesis Objectives

This thesis examines how interacting stressors influence disease expression and physiological performance in *Faxonius virilis* populations exhibiting different levels of lesion prevalence. Specifically, it evaluates how two common stressors in ecological research (marking methods and temperature) interact with background disease status to influence crayfish health and performance. Infection outcomes depend on intrinsic factors such as host condition and immune function and extrinsic factors such as injury, crowding, temperature, and environmental quality (Sweeny and Albery 2022). In crustaceans, external lesions may facilitate colonization of weakened tissue and may also represent breaches in the cuticle that allow pathogen entry

(Nyhlén and Unestam 1975; Nyhlén and Unestam 1980; Martín-Torrijos et al. 2017). In this thesis, lesion prevalence group (population-level occurrence) and lesion severity (individual-level burden) are used as proxies for disease expression following Reardon et al. (2018).

Chapter 2 investigates how two widely used marking techniques, visible implant elastomer tagging and uropod/telson tail punching, affect survival, growth, molting, and lesion dynamics in crayfish originating from populations with high and low lesion prevalence. Chapter 3 examines how temperature influences survival, growth, molting, behavioral responses to thermal stress, and lesion severity in crayfish from these populations, integrating chronic temperature exposure with acute thermal stress assays. Together, these experiments provide new insight into how disease interacts with both anthropogenic and environmental stressors to shape the physiological performance of freshwater crayfish.

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CHAPTER 2

MARKING METHOD AND LESION PREVALENCE HISTORY INFLUENCE SURVIVAL, GROWTH, AND MOLTING OF CRAYFISH WITH CRAYFISH PLAGUE

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Abstract

The factors that determine disease symptoms in freshwater species are challenging to identify. Recent observations of crayfish with novel external injuries, namely cuticle lesions, resulting from crayfish plague pathogen (*Aphanomyces astaci*) in North America have motivated the need to better understand dynamics of this pathogen and these injuries it causes. Over the course of 7.5 months, we documented lethal and sublethal effects of crayfish plague on *Faxonius virilis* exposed to two factorial experimental treatments in the laboratory: lesion prevalence history (crayfish originating from populations with high- or low- prevalence of plague lesions) and individual marking method (tail punch; visible implant elastomer; unmarked control). Crayfish mortality was affected by marking method, with substantially higher mortality from tail punches (50%) compared to elastomers (5%) or controls (25%). Along with increasing mortality, marking by tail punch reduced the probability of molting among surviving individuals but did not affect growth of those that survived. In contrast, population lesion-prevalence history did not affect survival, molting success, or growth. Lesions were primarily concentrated on posterior body regions, occurring most frequently on the telson and uropods. Marking method affected lesion area over the experiment's duration, with punch-marked crayfish exhibiting larger lesions than elastomer-marked or control animals at the experiment's end. Population lesion-prevalence history also influenced lesion area, but in an unexpected direction. High-prevalence crayfish started with larger lesions, but those lesions declined through time while lesion area remained consistent in low-prevalence history crayfish. Our results suggest that elastomer marking is safe for crayfish with and without plague lesions, but tail punching imposes substantial physiological costs that may exacerbate lesion injuries. To understand the population impacts of symptomatic

crayfish plague in North America, future studies will need to explore how lesions affect crayfish exposed to different temperature and precipitation regimes, under predation pressure or competition, and in natural conditions.

Introduction

Infectious disease can drive population and community dynamics, influencing the survival, growth, reproduction, and species interactions of many taxa (Timi and Poulin 2020; Luca Carraro et al. 2017; Moss et al. 2020; Holt et al. 2021). Host-pathogen dynamics in wildlife populations have been linked to population declines, community restructuring, and compromised ecosystem function (Frick et al. 2017; Silk et al. 2019; Brannelly et al. 2020). However, the ecological effects of disease in freshwater invertebrates remain comparatively understudied, even though freshwater invertebrates are numerically dominant and play critical roles in aquatic food webs (Covich et al. 1999; Johnson et al. 2011; Okamura and Feist 2011). This knowledge gap limits our ability to effectively manage populations experiencing disease in freshwater ecosystems (Dudgeon and Strayer 2025; Reid et al. 2019).

Crayfish are of particular interest for understanding disease dynamics because of their ecological and economic importance (Momot 1995). They are often biomass-dominant omnivores and ecosystem engineers that strongly influence food-web structure, sediment transport, and nutrient cycling (Momot et al. 1978; Taylor et al. 1996; Larson and Olden 2011; Albertson and Daniels 2016). They also as prey for aquatic and terrestrial wildlife and are economically valuable as aquaculture-based food supply in many parts of the world (Dorn and Wojdak 2004; Twardochleb et al. 2013; Haubrock et al. 2021; Geiger et al. 2005). Crayfish function as omnivores that process detritus and plants while strongly benefiting from animal

prey, shifting their diet with size, season, and local conditions (Momot 1995). Some crayfish families are highly susceptible to diseases such as crayfish plague (Svoboda et al. 2015), but relatively few studies have evaluated sublethal effects, phenological events such as molting, or examined disease impacts in North American crayfish populations (e.g. Jussila et al. 2011; Filipová et al. 2013; Basso et al. 2025; Martín-Torrijos et al. 2019; Kozubíková et al. 2009). Additionally, although several crayfish diseases, such as epibiont infections and crayfish plague are recognized globally, the effects of these diseases on crayfish populations in North America remain understudied compared to other regions (Martín-Torrijos et al. 2021; Panteleit et al. 2019; Butler et al. 2020; Petrusek et al. 2025).

Crayfish plague is caused by the oomycete *Aphanomyces astaci*, a pathogenic organism native to North America that infects freshwater crayfish and other decapods (Makkonen et al. 2019; Becking et al. 2021; Casabella-Herrero et al. 2026). In infected crayfish, *A. astaci* hyphae grow withing the cuticle and under nutrient limitation, the pathogen initiates sporangial development, releasing swimming zoospores (i.e., the infective units) that can colonize new hosts (Makkonen et al. 2019; Becking et al. 2021; Martínez-Ríos et al. 2023). North American crayfish can carry the pathogen chronically due to their innate immune defense system (i.e., particularly the prophenol oxidase activating system (proPO-AS), which allows them to encapsulate the growing hyphae through a melanization response. As a result, these North American hosts remain infected without exhibiting severe clinical signs of to the infection. However, this is not the case for many other crayfish species. The introductions of both North American crayfish and *A. astaci* in Europe and Asia have caused widespread and wholesale collapses of native crayfish populations (Söderhäll and Cerenius 1999; Cerenius et al. 2003;

Holdich et al. 2008; Martín-Torrijos et al. 2018, 2019, 2021; Jussila et al. 2021a; Brady et al. 2024). The long-held view that crayfish plague is largely benign in North America but devastating elsewhere has shaped scientific understanding and management decisions for decades, although growing evidence suggests that this pathogen may have sublethal or context-dependent effects on North American crayfish populations.

Recent field observations in Montana, USA, have challenged the historical crayfish plague paradigm. In 2021, during the first statewide crayfish survey, virile crayfish (*Faxonius virilis*) and other crayfish species were documented with conspicuous melanized lesions, with the most severe cases occurring in a few lakes in western Montana. Microscopy and DNA sequencing confirmed that lesions were caused by *A. astaci*, representing one of the first reported instances of clinical disease expression in North American native crayfish (i.e., crayfish plague disease; Martín-Torrijos et al., in prep.). Surveys in the United States have molecularly confirmed *Aphanomyces astaci* infections across multiple crayfish species and regions of North America (Svoboda et al. 2015; Martín-Torrijos et al. 2021; Petrusek et al. 2025), but these surveys did not report visible lesions, and infected individuals were therefore interpreted as chronic carriers. This highlights an urgent need to understand the factors that drive disease progression in native crayfish populations, and to evaluate the field and laboratory methods used to study individual health, growth, and survival under disease risk. Because many crayfish health indicators (such as growth, successful molting, and survival) require repeated individual identification, there is also a need to understand how standard research practices such as tagging and marking methods influence disease outcomes.

Our goal was to determine how disease context and commonly used marking methods influence the health and performance of crayfish in experimental studies. Specifically, we tested whether disease history and marking treatments affect survival, growth, molting success, and lesion dynamics in virile crayfish (*F. virilis*). We conducted a laboratory experiment using crayfish collected from populations with a history of high- or low- prevalence of plague lesions (hereafter high- and low-prevalence history populations) and subjected individuals to three marking treatments: tail punch, visible implant elastomer, or an unmarked control.

We predicted that crayfish from high-prevalence history populations would exhibit lower survival and reduced growth relative to those from low-prevalence history populations. We also predicted that marking methods that breach the cuticle would negatively affect crayfish survival, growth, and molting success by creating potential entry points for infection or increasing energetic costs associated with wound healing. Among marking treatments, we expected the strongest negative effects in tail-punched crayfish. Finally, because molting replaces infected cuticle, we predicted that lesion area and counts would decline following successful molts but would remain elevated or increase over time in individuals with severe infections.

Methods

We conducted a controlled laboratory experiment using wild-collected *F. virilis* to evaluate the effects of marking method and source population lesion prevalence history (high vs. low) on survival, growth, molting success, and lesion dynamics under laboratory conditions. Crayfish were assigned to one of three groups (control, elastomer tag, or tail punch). Individuals were monitored over time to quantify survival, growth, molting and lesion characteristics, including severity and anatomical location. Hereafter, we refer to crayfish primarily by lesion-

prevalence history group throughout the manuscript, with population indicating the source location where we collected each group.

Crayfish Collection, Lesion Prevalence History, and Treatments

We used snorkel and dip-net sampling to collect crayfish from Placid Lake (Missoula County, Montana, USA; 490 ha; 1265 m elevation; 47.11622, -113.52147) on 14 August 2023 and from Lake Mary Ronan (Lake County, Montana, USA; 612 ha; 1130 m elevation; 47.92816, -114.403) on 15 August 2023 (Table 2.1; Fig. A1). Based on previous field monitoring, populations were categorized as high-prevalence (>45% lesion prevalence) history or low-prevalence (<15% lesion prevalence) history. Placid Lake represented a high-prevalence population, with 82% of collected individuals exhibiting lesions ranging from minor to severe (Table A1). In contrast, Lake Mary Ronan represented a low-prevalence population, where lesion prevalence was 8% of collected individual crayfish and all observed lesions were minor. During collection, individual crayfish were qualitatively scored for lesion severity using the rubric in Table A1 for descriptive purposes and to guide selection of individuals representing a range of lesion severities within each population for subsequent pathogen testing.

Table 2.1. Summary of sample sizes by population, lesion prevalence history group, and marking treatment. The experiment took place for 227 days, from 30 September 2023 to 13 May 2024. The one escaped crayfish is not included in final mortality count (Lake Mary Ronan, low-lesion prevalence history) right-censored on 9 January 2024 in survival analyses, contributing survival time until its last observation but not counted as either dead or alive at the end of the experiment.

Crayfish Source Population	Lesion Prevalence History	Marking Method	Number at Start	Number at End	Mortality (%)
Lake Mary Ronan	Low	Control (no mark)	10	9	10
Lake Mary Ronan	Low	Elastomer	10	9	10
Lake Mary Ronan	Low	Punch	10	5	50

Table 2.1. Continued

Placid Lake	High	Control (no mark)	10	6	40
Placid Lake	High	Elastomer	10	10	0
Placid Lake	High	Punch	10	4	60

We used 60 adult *F. virilis* (Fig. 2.1a) in the experiment ranging in post orbital carapace length from 16.5 to 26.0 mm post-orbital carapace length. Crayfish were drawn from two lesion-prevalence history groups (high and low), each represented by a single source population. Field-collected crayfish were transported in coolers where they were isolated in individual Ziploc bags and suspended above ice to encourage minimal movement via cool temperatures, and then gradually acclimated to room temperature in dechlorinated, aerated tap water over 12 hours. If any crayfish died during the laboratory acclimation period (16 August–30 September 2023), it was replaced with a similarly sized crayfish from the same lesion-prevalence history group.

Experimental Setup and Husbandry

The experiment was conducted in a fisheries laboratory in Helena, Montana, USA, between 30 September 2023 and 13 May 2024. Within each lesion-prevalence history group, crayfish were randomly assigned to one of three marking treatments: control (no mark), visible implant elastomer ("elastomer"), or uropod/telson punch ("punch"), yielding 10 individuals per marking treatment per lesion-prevalence history group (n = 30 crayfish per group; Table 2.1). Each crayfish was housed individually in a clear, food-grade, plastic container with a snap tight lid with holes to allow air escape (internal dimension: 14.6 x 14.6 x 7.6 cm). Each container was supplied air via aquarium tubing and small air stone connected to a commercial air pump (Vivosun 1427 GPH, 55 W, 90 L min^{-1}). All crayfish were fed the same diet of a rotating schedule of frozen peas and Hikari® commercial fish food (algae wafers, sinking carnivore

pellets, crab cuisine) 2-3 times per week (Fuertes et al. 2013; Ackefors et al. 1992). Water changes were conducted for all individuals at the same time, usually 24 hours after feeding, to minimize bacterial growth. To prevent cross contamination and mixing of any water, each treatment (described below) was assigned to its own shelf within four shelving units (each ~ 2.2m x 1.2m). The laboratory had natural light from an exterior window plus overhead fluorescent lighting, which was turned on during feeding and water changes (see temperature and light monitoring, below).

All crayfish were photographed dorsally and ventrally at three scheduled time points (start: 30 September 2023; middle: 3 January 2024; end: 13 May 2024), and additionally when individuals molted or died. Post-orbital carapace length (POCL) was measured at the start and end of the experiment using calipers and from dorsal images in Fiji (ImageJ; Schindelin et al. 2012) to quantify growth as a percentage of starting length.

Crayfish were checked four times per week for molts and mortality during routine husbandry visits. These inspections occurred approximately every 2 – 3 days (median interval = 2.5 days). Notable events (e.g., molt, death) were recorded throughout the experiment. At the end of the experiment, all surviving crayfish were euthanized and preserved in 95% ethanol for subsequent genetic analyses.

Marking Treatments

Crayfish from each lesion prevalence history group (high vs low) were randomly assigned to one of three marking treatments: control (unmarked), elastomer, or punch. Red visible implant elastomer (VIE; Northwest Marine Technology, Inc.) was injected into the ventral abdominal musculature at four sites (Figures 2.1d – 2.1e). We injected VIE using 29G, 0.5 mL

insulin syringe needles (½ inch needle length) following the manufacturer's guidelines (Northwest Marine Technology, Inc. 2017).

Tail punch marks were applied through the uropods and telson following the pattern shown in Figure 2.1f – 2.1g adapted from Guan (1997). Stainless steel 20G x ½ inch poly hub needles (Agri-Pro Enterprises) were used to punch through the exoskeleton. All elastomer and punch marks were applied on 1 October 2023. For simplicity, we refer to both tail punching and visible implant elastomer (VIE) tagging collectively as "marking methods" throughout the study, consistent with previous studies (McFarlane er al. 2019; Buřič et al. 2008; Martinez et al. 2013; Mazlum 2007).

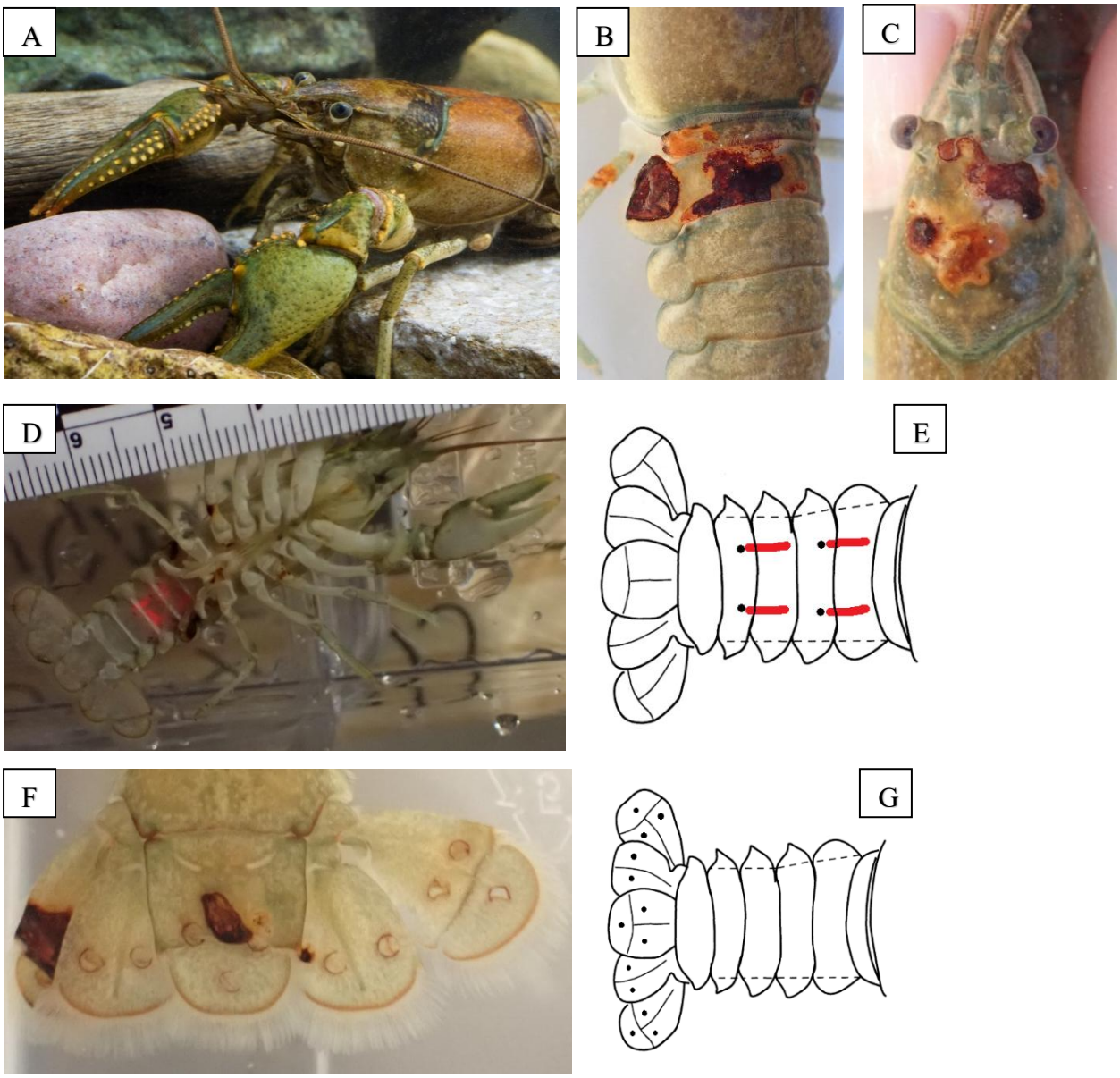


Figure 2.1. (a) *F. virilis* showing no signs of melanized lesions (photograph by Guenter Schuster. (b, c) Variability of lesions in wild crayfish sampled from Montana, 2022 (photo credit: Susan Adams, USFS). (d) Ventral view of experimental crayfish showing visible elastomer implant tags (bright red). (e) Elastomer injection locations (black dots) and elastomer tag sites (red lines; drawing adapted from Guan 1997). Adapted from Guan (1997). (f) dorsal view of a uropod/telson-punch crayfish within hours after marking (small holes), dark areas are crayfish plague lesions that existed prior to marking. (g) Uropod/telson tail punch locations (black dots; adapted from Guan 1997.

Temperature and Light Monitoring

Crayfish treatments were randomly assigned and housed on separate shelves to minimize contamination during water changes. To evaluate shelf-level microclimate differences, we deployed HOBO Pendant temperature and light loggers (UA-002-64, Onset Computer Corporation) on each shelf for seven days after the experiment was finished, with the complete container and aeration setup still running but crayfish removed.

Heteroskedasticity-robust ANOVA and nonparametric tests (Kruskal-Wallis) showed evidence of minor differences in temperature (Fig. A2a) and light (Fig. A2b) among shelves (< 2 °C difference in daily mean temperature. Consequently, shelf was included as a random effect in mixed models where it improved model fit; in models where shelf explained negligible variance or was strongly confounded with lesion prevalence history group and treatment, it was omitted from the statistical model.

Genetic and Molecular Analyses

Because crayfish plague represents a chronic host-pathogen system in North American crayfish (Svoboda et al. 2015; Boštjančić et al. 2022; Martín-Torrijos et al. 2021), and because *A. astaci* virulence can vary among pathogen strains (Diéguez-Uribeondo et al. 1995; Makkonen et al. 2014; Makkonen et al. 2012; Becking et al. 2015), confirming both pathogen presence and strain identity was necessary to inform population-level disease patterns. After the experiment, we used individual lesion severity scores to select subsets of crayfish with mild, moderate, and severe lesions for molecular confirmation of *A. astaci* infection. These subsets of crayfish from the two lesion prevalence history groups were screened at the Real Jardín Botánico (Madrid, Spain) for the presence of *A. astaci* using a combination of molecular detection, microscopic

observation, and photography (Fig. A3) of melanized hyphae (Martín-Torrijos et al. 2018; Appendix A, Genetic and molecular analysis of *F. virilis* and *A. astaci*).

Molecular analyses confirmed the presence of *A. astaci* in crayfish from both lesion-prevalence history populations. Amplification of the ITS region verified pathogen presence, and sequencing of the mitochondrial ribosomal small (rnnS) and large (rnnL) subunits in positive samples identified the A haplotype of the pathogen (Martín-Torrijos et al. 2021; Appendix A, Genetic and molecular analysis of *F. virilis* and *A. astaci*). Population genetic analyses of crayfish mitochondrial DNA, using Cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S rRNA), revealed no detectable genetic differentiation between the two crayfish populations (Appendix A, Genetic and molecular analysis of *F. virilis* and *A. astaci*). These results indicate that differences in lesion prevalence history and experimental outcomes are unlikely to reflect host lineage differences.

Data Collection and Measurements

Lesion Imaging and Metrics Lesion counts and lesion areas were extracted from standardized dorsal and ventral photographs using Fiji. Lesions were manually outlined using drawing tools after scale calibration, and Fiji measured lesion areas (mm^2) and returned lesion counts. Total lesion area per individual was calculated by summing the areas of all lesions across dorsal and ventral views at each sampling point (start, middle, end). Lesion counts represent the total number of distinct lesions recorded per individual at each time point. Lesion analyses involved repeated measurements on the same individuals, so all models included random intercepts for crayfish ID. Shelf was evaluated as a potential random effect for both lesion area and lesion count to account for minor environmental differences among shelves. However, shelf

explained negligible variance in the lesion-area models and was removed from the final model, whereas it accounted for a small portion of variance in lesion counts and was therefore retained in the lesion-count model.

Lesions were classified by body region to evaluate spatial patterns of infection across the crayfish body. Using annotated dorsal and ventral images processed in Fiji, each lesion outline was assigned to one of eight predefined anatomical regions: antennae/antennules, mouthparts/head, claws, carapace, walking legs, abdomen, pleopods/gonopods/annulus ventralis, and uropods/telson. For each individual at each time point, lesion counts and lesion area were then combined across dorsal and ventral views within regions. Regional lesion metrics were summarized descriptively to assess differences in lesion distribution.

Growth was quantified as the natural logarithm of the ratio of POCL at the end versus the start of the experiment, $G = \ln\left(\frac{POCL_e}{POCL_s}\right)$, where G is growth, POCL is post-orbital carapace length, "e" is end and "s" is start, to improve homoscedasticity. Negative POCL changes (apparent "shrinkage") were set to zero prior to analysis to reflect biological realism and remove minor error associated with measurement technique.

Statistical Analyses

All analyses were conducted in R (version 4.5.1; R Core Team 2025). Statistical models and diagnostics were implemented using the packages lmerTest (Kuznetsova et al. 2017), glmmTMB (Brooks et al. 2017), sandwich (Zeileis et al. 2020), lmtest (Zeileis and Hothorn 2002), DHARMa (Hartig 2024), and emmeans (Lenth 2025). Statistical inference focused on effect sizes and associated uncertainty, with $\alpha = 0.05$ used as a threshold for hypothesis tests.

Logarithmic transformations used the natural logarithm. Details of statistical models used to evaluate mortality, growth, molting probability, and lesion metrics are described in the sections below.

Mortality Mortality was monitored throughout the experiment (30 September 2023 through 13 May 2024). For each individual, survival time was calculated as days since 30 September 2023; individuals alive at the end of the experiment were treated as right-censored (i.e., their survival time was recorded up to the last observation but not counted as a mortality event). For survival analyses, we explicitly tested for an interaction between marking treatment and lesion-prevalence history group.

Differences in final mortality (i.e., whether each individual survived to the end of the experiment or died before the end of the experiment) across lesion prevalence history (high vs. low) and marking treatment (control, elastomer, punch) were assessed using Firth's penalized logistic regression to account for small sample sizes and quasi/completely separated data (e.g., no deaths in some treatment combinations). Lesion prevalence history and marking treatment were included as fixed effects. An interaction term was evaluated initially but removed when non-significant to retain a parsimonious model. Model fit and term significance were evaluated using penalized likelihood ratio tests. We report odds ratios (ORs) with 95% profile-likelihood confidence intervals and present model-based mortality probabilities for each lesion-prevalence history × treatment combination using the *logistf* package (Heinze 2025).

One crayfish (LMRP10) escaped the container on 9 January 2024 and was later found dead. Because this death was unrelated to treatment or disease, the individual was treated as

right-censored in the mortality analysis (meaning its survival time was recorded up to the last date it was known to be alive, but it was not counted as a mortality event). For all other analyses (growth, molting, and lesion metrics), data collected prior to the escape were retained and treated normally. Survivorship bias was evaluated separately using a Cox proportional hazards model testing whether lesion area measured prior to death predicted subsequent mortality.

Growth To evaluate crayfish growth, we used a linear mixed effects model with marking treatment and lesion prevalence history as fixed effects. Interaction terms were retained only where biologically motivated and supported by model structure (e.g., time × lesion-prevalence history group for lesion trajectories), in order to maintain model parsimony and avoid overparameterization given sample sizes. Preliminary mixed-effects models with shelf as a random intercept indicated negligible shelf-level variance, so shelf was removed from the final model. Estimated marginal means (EMMs) and 95% confidence intervals were generated using the *emmeans* package (Lenth 2025) and back-transformed to percent change as $(\exp \beta - 1) \times 100$.

Molting We recorded molting as a binary outcome (1 = successful molt observed, 0 = no successful molt) at each monitoring visit. A molt was recorded only when the crayfish had completely shed its exoskeleton with no remaining cuticle. Individuals exhibiting incomplete or failed molts (i.e., portions of the old exoskeleton remained attached) or dying during molting were recorded as no molt for that observation. Because each crayfish contributed multiple observations, we modeled the probability of molting during each monitoring visit using logistic regression with robust standard errors clustered by individual crayfish ID using the *sandwich* (Zeileis et al. 2020) and *lmtest* (Zeileis and Hothorn 2002) packages. Fixed effects included

marking treatment, lesion prevalence history group, and time point (start, middle, end). Mixed-effects models with random intercepts for crayfish and shelf produced nearly identical fixed-effect estimates and near-zero random variance, so we retained the simpler cluster-robust model by removing shelf effects. Estimated marginal probabilities and 95% confidence intervals were computed from *emmeans* (Lenth 2025) on the response scale. We evaluated a marking treatment × lesion-prevalence history interaction for molting probability, but this interaction was not supported and led to unstable parameter estimates due to limited information in some treatment–lesion prevalence history group combinations; therefore, we report additive models for parsimony.

Lesion Area Because total lesion area was right-skewed with many zeros, we analyzed lesion severity using a natural-log–transformation: log(total lesion area + 1). We tested for effects of time point (start, middle, end), lesion-prevalence history group (high vs. low), and marking treatment (control, elastomer, punch) using linear mixed-effects models (*lmerTest*; Kuznetsova et al. 2017). Crayfish identity was included as a random intercept to account for repeated measures. Shelf was initially included as a random effect, but it explained negligible variance and was removed from the final lesion-area model.

We evaluated support for fixed effects and interactions using likelihood ratio tests (LRTs) comparing nested models fit with maximum likelihood (REML = FALSE). Final models were refit with REML for estimation. Predicted marginal means were obtained using emmeans (Lenth 2025) and back-transformed to the original scale for interpretation. Planned contrasts were used to test for changes from start to end within each lesion prevalence history group. To evaluate

whether lesion severity scaled with body size, we tested post-orbital carapace length (POCL) as an additional covariate in the lesion-area model.

Lesion Count To complement the lesion-area analysis, we modeled the total number of lesions per crayfish using a generalized linear mixed model (GLMM) with a Poisson error distribution and log link implemented in the glmmTMB package (Brooks et al. 2017; McGillycuddy 2025). Fixed effects included time point, lesion prevalence, and marking treatment, and total length (mm) was included as a covariate to account for body size. Random intercepts for individual crayfish and shelf were included in this model because shelf variation explained a small but non-negligible portion of the variance in lesion counts, even though shelf was not retained in the final lesion-area model.

Model diagnostics were evaluated with *DHARMa* (Hartig, 2024) residual simulations. Dispersion tests indicated no overdispersion, and tests for zero inflation suggested that a Poisson model provided adequate fit. We therefore retained the simpler Poisson GLMM. Predicted marginal means for lesion count were generated with *emmeans* (Lenth 2025) at the mean body length, with pairwise contrasts among treatments and lesion prevalence history group adjusted for multiple comparisons where appropriate.

Survivorship Bias Analysis To test whether individuals that died during the experiment had larger areas of carapace covered by lesions than those that survived, we constructed a time-to-event dataset with known start and end dates for all crayfish. The dataset included 16 death events; surviving individuals were right-censored at their last observation date.

For each individual, lesion area (mm^2) was extracted as (1) the first recorded value ("start area") and (2) the most recent value prior to death or censoring ("last-prior area"). To evaluate whether lesion burden predicted mortality, we fit Cox proportional hazards models using last-prior lesion area as the predictor, along with lesion prevalence history (high vs. low) and marking treatment (control, elastomer, punch).

Effects of Molting on Lesion Area and Count To evaluate whether molting altered lesion burden, we analyzed paired lesion observations collected opportunistically during "intact molt events." Intact molt events were cases where the exuviae was found intact by an observer before being consumed by the crayfish, which otherwise prevented photography and analysis. For intact molt events, both the shed exoskeleton (hereafter, molt) and the newly molted crayfish (hereafter, post-molt) were photographed. Total lesion area (mm^2) and total lesion count were summed across dorsal and ventral surfaces for each. Analyses were restricted to crayfish with paired molt and post-molt measurements recorded on the same date. This resulted in 17 paired observations. When intact exuviae were recovered before being consumed, both the shed exoskeleton and the newly molted crayfish were photographed and analyzed. Thus the measurements were (1) molt: lesions on the exuviae, and (2) post-molt: lesions on the crayfish immediately after molting (within 24 hours).

Changes in lesion area were analyzed using a linear mixed-effects model with natural-log–transformed total lesion area [log(area + 1)] as the response variable, molting condition (molt vs. post-molt) as a fixed effect, and crayfish identity included as a random intercept to account for repeated measures. Changes in lesion count were analyzed using a negative binomial generalized linear mixed model with the same fixed and random effects structure.

Results

Mortality

Over the 227 days of the experiment, mortality was relatively low overall (16 of 60 crayfish; 26.7%; Fig. 2.2; Table 2.2). Mortality varied among marking treatments overall (penalized likelihood-ratio test: $\chi^2 = 11.73$, df = 3, p = 0.008), although individual pairwise comparisons between treatments were not statistically significant. Predicted mortality probabilities varied among treatments, with elastomer-tagged crayfish showing the lowest estimates (0.05–0.14), controls intermediate (0.14–0.41), and punch-marked crayfish the highest (0.41–0.59; Fig. 2.2). However, none of the pairwise contrasts among treatments were statistically significant (elastomer vs. control: OR = 0.21, 95% CI = 0.02–1.24, p = 0.090; punch vs. control: OR = 2.86, 95% CI = 0.80–11.29, p = 0.110). Lesion-prevalence history group did not significantly affect mortality (OR = 0.46, 95% CI = 0.13–1.56, p = 0.220), and the interaction between marking treatment and lesion-prevalence history group was not significant (elastomer × prevalence: p = 0.150; punch × prevalence: p = 0.590). Differences in time to death were not statistically detectable in Cox models (Table A5).

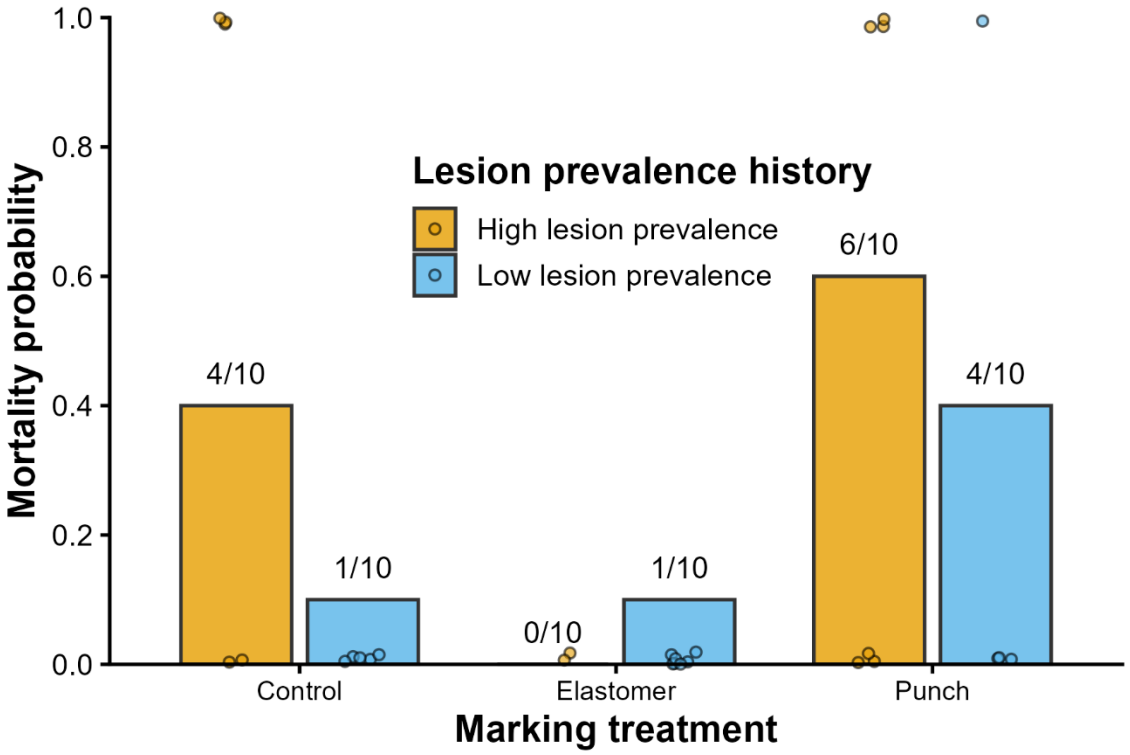


Figure 2.2. Observed mortality probabilities of crayfish exposed to different marking treatments and collected from high- and low-lesion-prevalence history populations. Bars represent observed mortality proportions within each treatment × lesion-prevalence history combination, and labels above bars indicate the number of deaths relative to the total number of crayfish in each group. Jittered points represent individual crayfish outcomes (0 = survived, 1 = died). Mortality patterns were evaluated using Firth's penalized logistic regression. The analysis included 60 crayfish total (n = 10 per treatment × lesion-prevalence history combination), of which 16 died.

Table 2.2. Mortality (dead vs. alive) was analyzed using Firth penalized logistic regression to account for complete separation among marking treatments. The additive model included marking method (control, elastomer, punch) and lesion prevalence history group (high vs. low). Reported values are odds ratios (OR) with 95% confidence intervals. The overall model was statistically significant (likelihood-ratio test: $\chi^2 = 11.73$, df = 3, p = 0.008; n = 60).

Predictor	Odds ratio (95% CI)	p-value
Elastomer vs. Control	0.21 (0.02–1.24)	0.088
Punch vs. Control	2.86 (0.80–11.29)	0.108
Low vs. High-Lesion-Prevalence History Group	0.46 (0.13–1.56)	0.217

Individuals with heavier lesion burdens were more likely to die earlier in the experiment. Crayfish that died had substantially greater lesion coverage than those that survived, averaging 14.8 mm^2 ± 5.0 (SE) compared to 3.0 mm^2 ± 0.9 (SE) in survivors (Wilcoxon p = 0.001). Consistent with this pattern, lesion area measured at the most recent observation ("last-prior area") was positively associated with mortality risk in a Cox proportional hazards model, with the hazard of death increasing by approximately 4% per mm^2 of lesion area (HR = 1.04, 95% CI = 1.01–1.07, p = 0.003). However, lesion area explained only a modest portion of variation in mortality.

Growth

Mean crayfish growth during the experiment was 15% increase in POCL relative to initial size (range = 0 – 35%). Growth did not differ among marking treatments or between lesion-prevalence history groups (linear model: $F_{3,39} = 0.91$, p = 0.440, $R^2 = 0.07$; Fig. 2.3; Table 2.3). Crayfish from the low-lesion-prevalence history group grew an average of 17%, while those from the high-lesion-prevalence history group grew 13%. Growth was also similar among marking treatments: controls averaged 15%, elastomer-tagged crayfish averaged 13%, and punch-marked crayfish averaged 12% (Fig. 2.3).

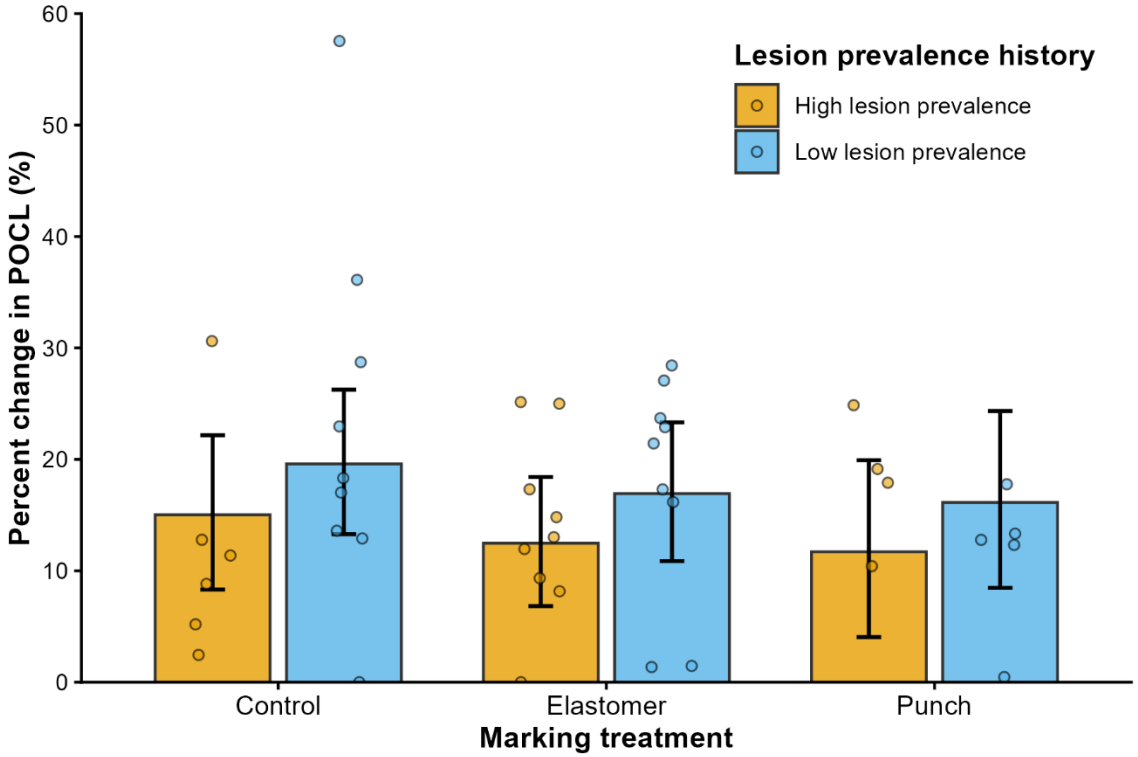


Figure 2.3. Percent growth of crayfish post-orbital carapace length (POCL) over the experiment's duration (September 2023 - May 2024) across marking method and lesion prevalence history groups. Bars show back-transformed means ± 95% confidence intervals from a linear model fit to log-transformed growth ratios. Points represent individual crayfish (jittered for visual clarity). The analysis included 43 crayfish.

Table 2.3. Estimated marginal means (±95% CI) of percent growth in post-orbital carapace length (POCL) for *F. virilis* from high- and low-lesion prevalence history groups across marking treatments. Estimates are back-transformed from the log-ratio linear model.

Lesion Prevalence History	Marking Treatment	Mean % Growth	95% CI
High	Control	15.0	8.3–22.2
High	Elastomer	12.5	6.8–18.4
High	Punch	11.7	4.1–19.9
Low	Control	19.6	13.3–26.3
Low	Elastomer	16.9	10.9–23.3
Low	Punch	16.1	8.5–24.3

Molting

Molting was common during the experiment. Overall, 46 of 52 crayfish (88%) molted at least once during the study. Molting probability differed strongly among marking treatments (Table 2.4; Fig. 2.4). Punch-marked crayfish had substantially reduced molting probabilities compared to those in the control and elastomer treatments, with markedly lower odds of molting than controls (OR = 0.04, 95% CI = 0.01–0.19, $p < 0.001$). In contrast, elastomer marking did not differ significantly from the control treatment (OR = 5.17, 95% CI = 0.58–112.63, $p = 0.180$). The interaction between marking treatment and lesion-prevalence history group was not supported (penalized likelihood-ratio test comparing additive and interaction models: $\chi^2 = 3.35$, $df = 2$, $p = 0.190$), indicating that marking treatment did not differentially affect molting probability across lesion-prevalence history groups. Lesion-prevalence history group alone also did not significantly affect molting probability ($p = 0.260$). In most successful molts, visible lesions were associated with the discarded exuviae rather than the newly exposed cuticle (Fig. A6). One apparent outlier in lesion trajectories occurred in an individual that experienced an incomplete molt, in which portions of the exuviae remained attached and retained visible lesions.

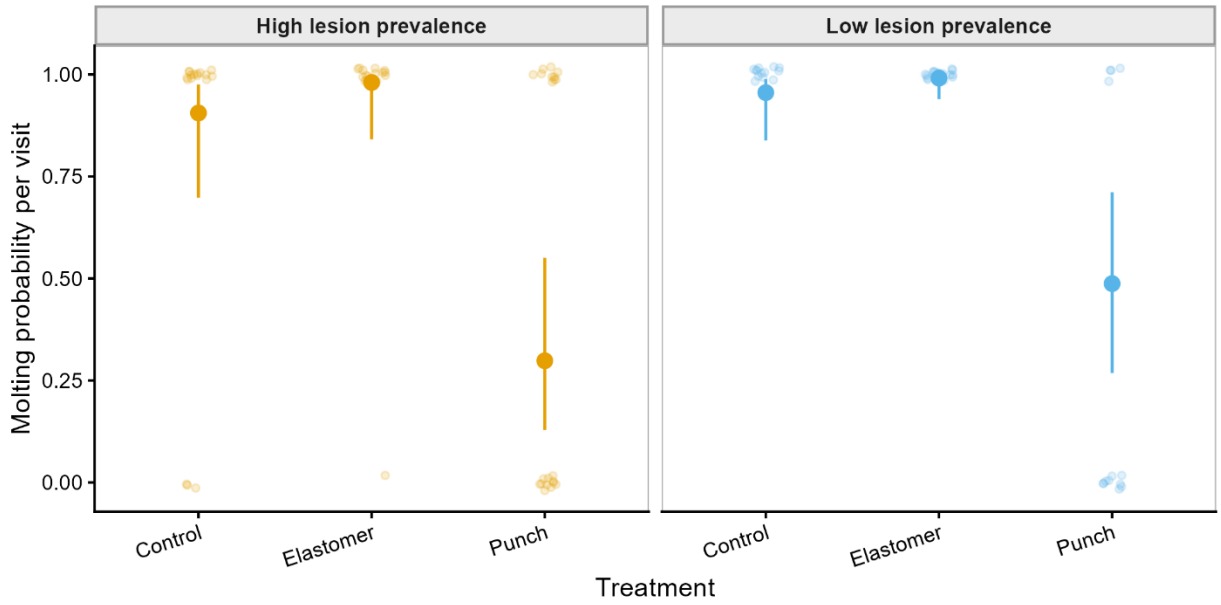


Figure 2.4. Per-visit molting probability for crayfish across marking method and lesion-prevalence history groups. Solid points show model-predicted probabilities and bars show 95% confidence intervals from a cluster-robust logistic regression. Faint orange and blue points represent raw binary observations for high- and low-lesion prevalence history crayfish, respectively (0 = did not molt, 1 = molted). The analysis included 99 observations from 52 crayfish. A "visit" refers to one of the routine husbandry inspections during which crayfish were checked for molting. Model estimates account for repeated observations of individuals and therefore may not align exactly with the distribution of raw binary observations.

Table 2.4. Predicted per-visit molting probabilities from the cluster-robust logistic regression model, shown for each combination of lesion-prevalence history category and marking treatment. These estimates correspond to the values displayed in Figure 4 and illustrate differences in molting likelihood among treatments and lesion-prevalence history groups.

Lesion Prevalence History	Marking Treatment	Predicted prob	95% CI low	95% CI high
High	Control	0.906	0.698	0.975
High	Elastomer	0.980	0.841	0.998
High	Punch	0.298	0.129	0.551
Low	Control	0.955	0.838	0.989

Lesions

Lesion patterns provided a visible indicator of disease severity and progression in infected crayfish. Across the experiment, lesions varied in their spatial distribution, severity, and temporal dynamics.

Location Lesions were observed across all eight crayfish body regions but were unevenly distributed (Fig. 2.5). Lesions were most frequently observed in posterior areas of the body, particularly the abdomen and uropods/telson, where more than half of individuals exhibited at least one lesion. Intermediate prevalence occurred on the walking legs and pleopods/gonopods, while lesions were uncommon on the carapace, claws, and anterior regions such as the antennae and mouthparts. Patterns of mean lesion area mirrored these prevalence patterns, with the largest lesions occurring on the abdomen and posterior appendages, whereas lesions on anterior or heavily sclerotized regions were small and infrequent.

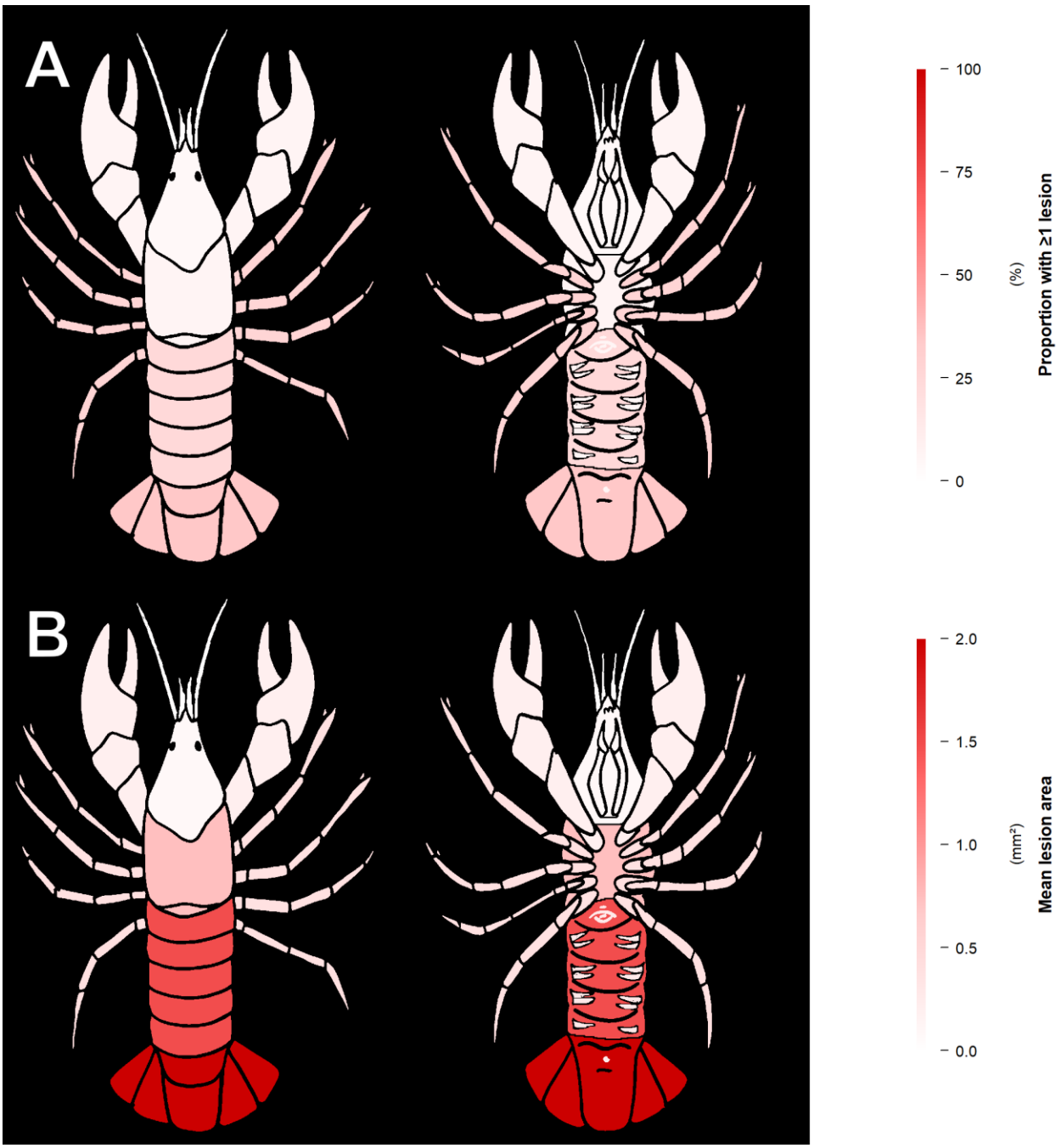


Figure 2.5. Spatial location of lesions across crayfish body locations. Heatmaps show (A) the proportion of individuals with ≥1 lesion per region and (B) the mean lesion area (mm²) for affected regions, combining observations from both dorsal and ventral surfaces within each region. Color intensity corresponds to relative lesion frequency or size, with darker red indicating higher values. Line drawings by Lydia Bushey.

Lesion Severity (Area and Count) Lesion severity differed between lesion-prevalence history groups and changed through time. Across all observations, lesion area ranged from 0 to 59.9 mm² (median = 0.54 mm², mean = 4.18 mm²). Lesion sizes were strongly right-skewed, with more than half of observations <1 mm² (54.7%) and 21.6% exceeding 5 mm² (Fig. A4).

As expected, lesion severity differed markedly between lesion-prevalence history groups. At the start of the experiment, crayfish from the high-prevalence population exhibited substantially greater lesion severity than those from the low-prevalence population, with approximately two- to fourfold greater lesion area and significantly higher lesion counts (area ratio = 2.15–4.15; count IRR = 3.74, 95% CI = 2.43–5.77, $p < 0.001$; Fig. 2.6A; Table 2.5).

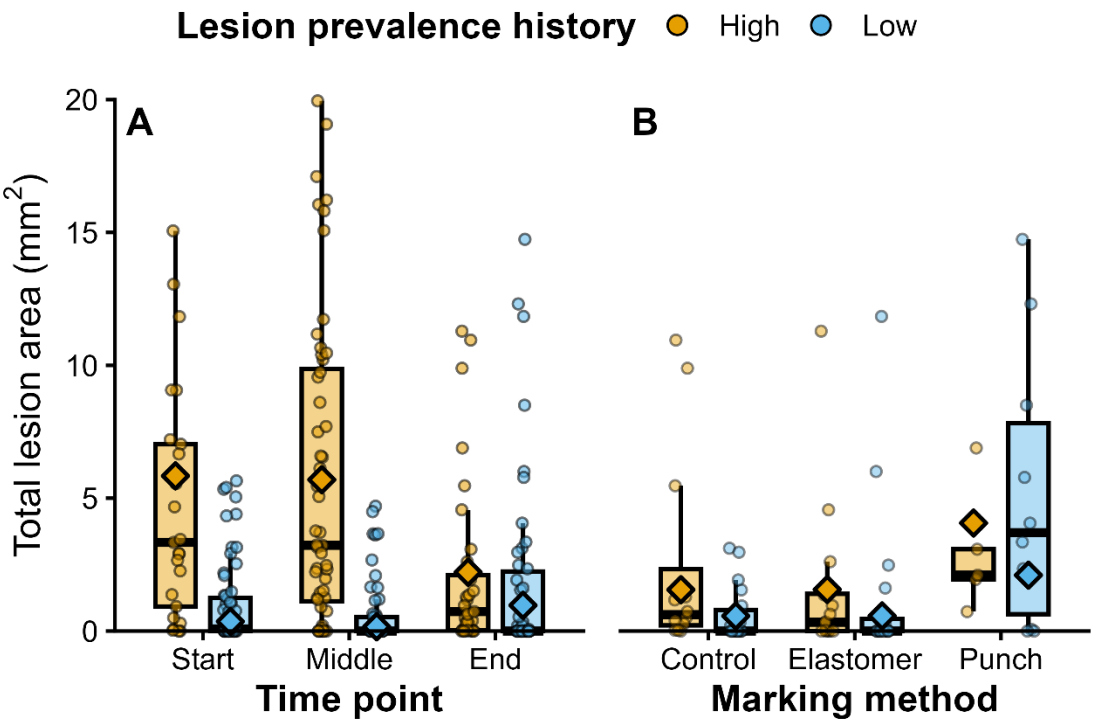


Figure 2.6. Total lesion area (mm²) in crayfish from high- and low-lesion-prevalence history populations across time points pooled across marking treatments (A) and among marking treatments at the end of the experiment (B). Boxplots show the median and interquartile range, jittered points represent individual crayfish observations, and diamonds indicate model-estimated

marginal means from linear mixed-effects models fit to log(total lesion area + 1). Initial treatment groups each contained 10 crayfish per lesion-prevalence history × marking-treatment combination. Sample sizes declined across time points due to mortality.

Table 2.5. Influences on lesion severity in *F. virilis*. Fixed-effects results from a linear mixed-effects model of log(total lesion area + 1) testing the effects of time point (start, middle, end), lesion-prevalence history group (high vs. low), and marking treatment (control, elastomer, punch). Estimates (β) are on the log scale with associated standard errors (SE), degrees of freedom (df), t-values, and p-values. Fold-changes (expβ) are also reported, representing multiplicative changes in lesion area relative to the reference category to facilitate biological interpretation. Significance of fixed effects was assessed using Satterthwaite's approximation, and support for interaction and treatment terms was evaluated using likelihood-ratio tests comparing nested models.

Predictor	Estimate (β)	SE	df	t_value	p-value	Fold change exp(β)
Intercept (Low prevalence, Start, Control)	0.263	0.180	67.881	1.455	0.150	1.300
Middle vs Start (Low prevalence)	-0.178	0.138	216.835	-1.291	0.198	0.837
End vs Start (Low prevalence)	0.176	0.149	224.116	1.180	0.239	1.192
High vs Low (at Start)	1.308	0.226	138.311	5.785	0.000	3.697
Elastomer vs Control	0.041	0.202	46.368	0.203	0.840	1.042
Punch vs Control	0.542	0.206	50.630	2.627	0.011	1.720
High×(Middle vs Start)	0.139	0.227	232.330	0.613	0.541	1.149
High×(End vs Start)	-0.775	0.248	235.324	-3.130	0.002	0.461

Lesion severity also changed differently through time between lesion-prevalence history groups (Fig. 2.6A). Significant time × prevalence interactions were detected for lesion area (χ^2 = 17.88, df = 2, $p < 0.001$) and lesion count ($p < 0.001$). Within-group contrasts indicated that lesion severity declined through time in the high-prevalence group but remained consistently low in the low-prevalence group. Mortality events occurred across treatments and lesion-prevalence history groups but were biased toward individuals with the most severe lesions early in the experiment (see Mortality Results). Consequently, lesion trajectories among surviving individuals likely underestimate the true rate of lesion progression.

Marking treatment influenced lesion severity when measured as lesion area but not lesion count (Fig. 2.6B). Punch-marked crayfish exhibited greater lesion area than control individuals (fold-change = 1.72, 95% CI = 1.15–2.58), whereas elastomer marking had no detectable effect on lesion area relative to controls (estimate = 0.041 ± 0.202 SE on the log scale; $p = 0.840$). In contrast, marking treatment did not influence lesion counts (LRT $\chi^2 = 0.69$, df = 2, $p = 0.710$).

Body size did not explain variation in lesion area after accounting for population, time, and marking treatment ($\chi^2 = 0.22$, df = 1, $p = 0.640$), although larger crayfish exhibited slightly lower lesion counts (IRR = 0.96 per mm, $p < 0.001$). Random effects in the lesion area model indicated moderate variability among individuals (random intercept SD = 0.53 on the log scale) after accounting for fixed effects.

Effects of Molting on Lesion Area and Count Molting reduced both the total area (Fig. 2.7A) and count (Fig. 2.7B) of lesions on the crayfish exoskeleton. Paired within-individual comparisons showed that lesion area and lesion count were lower immediately following molting than on the shed exuviae, indicating that molting temporarily reduced visible lesion severity. Although lesions were largely removed from the exoskeleton during molting, faint pale areas were often visible at previous lesion locations immediately after molting (Fig. A5).

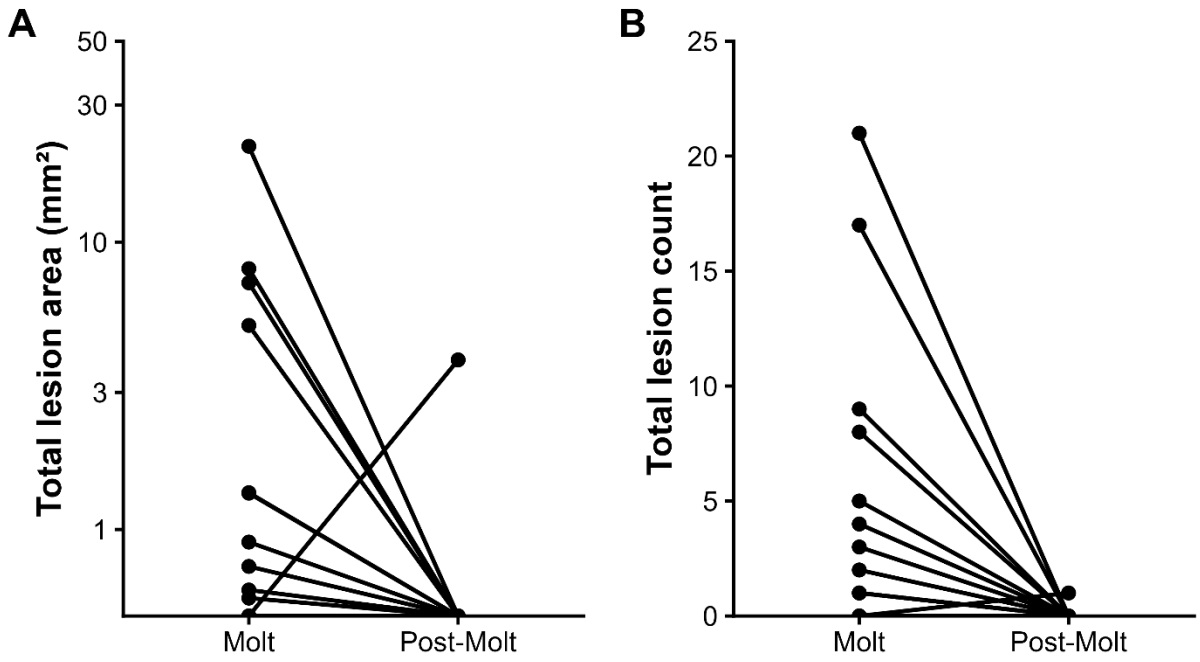


Figure 2.7. Changes in crayfish lesion burden associated with molting (n = 17 crayfish). Paired observations of (A) total lesion area (mm²) and (B) total lesion count are shown for individual crayfish measured from the exuviae immediately after molting ("Molt") and from the corresponding crayfish immediately following ecdysis ("Post-Molt"). Lesion metrics were summed across dorsal and ventral surfaces when available. Points represent individual molt events, and lines connect paired observations for the same crayfish on the same date. Note the logarithmic scale in panel A.

Discussion

Crayfish plague remains the single most significant cause of crayfish population declines and native crayfish extinctions worldwide (Jussila et al. 2021a; Martín-Torrijos et al. 2019; Svoboda et al. 2015). Although North American crayfish often act as tolerant hosts capable of containing infections without severe mortality, visible plague lesions have only rarely been documented in wild North American populations (Jussila et al. 2014, 2021b; Edsman et al. 2015; Petrusek et al. 2025). These observations highlight the need to better understand how visible lesions relate to host performance in tolerant species. In our experiment, marking method had

stronger and more consistent effects on crayfish performance than source-population lesion prevalence history. Tail-punched crayfish experienced higher mortality and reduced molting probability relative to controls, whereas elastomer tagging had no detectable negative effects. In contrast, visible lesion burden had relatively weak effects on growth and molting, and lesion severity often stabilized or declined through time. Together, these results suggest that handling practices that breach the cuticle may impose substantial physiological costs in crayfish already expressing symptoms of crayfish plague.

Across taxa, marking methods that breach tissue or disrupt protective structures often impose physiological costs, whereas minimally invasive approaches tend to have fewer detectable effects. For example, toe clipping in amphibians can reduce growth and survival (Davis and Ovaska 2001), while PIT tagging has been shown to be an effective method of tagging salmonids if appropriate tag sizes are used (Vollset et al. 2020). In fish and crayfish, elastomer-based marking methods generally show high retention and limited impacts on growth or survival (Goldsmith et al. 2003; Dewey and Zigler 1996; Clark and Kershner 2006; Bangs et al. 2013), whereas uropod or telson punching can reduce molt increment (the amount of growth achieved between successive molts) (Guan 1997; Buřič et al. 2008; Eissenhauer et al. 2024; Rácz et al. 2021). Because tail punching disrupts uropod and telson tissue, regions where lesions were most common, the physiological costs of this method may be interacting with underlying disease processes in affected populations.

Sublethal effects of crayfish plague vary widely across host species, pathogen strains, and environmental conditions. Some studies report reduced growth or impaired molting associated with infection (Anderson et al. 2021; Francesconi et al. 2021), whereas others describe chronic

infections with little effect on somatic growth or molt frequency in tolerant hosts (Bouallegui 2021; Martínez-Ríos et al. 2022, 2023). Our results are consistent with this latter pattern: despite clear differences in lesion expression between lesion prevalence history populations, lesion prevalence history had little effect on growth or molting during the experiment. This suggests that infected hosts were able to maintain normal physiological function under laboratory conditions. Mortality outcomes likewise depend strongly on host susceptibility and pathogen virulence. In highly susceptible European crayfish species or under infection by highly virulent *A. astaci* strains, melanization is typically weak or delayed and mortality is rapid (Unestam 1975; Martín-Torrijos et al. 2017; Aydin et al. 2014; Dobrović 2022; Martínez-Ríos et al., 2023). In contrast, more resistant hosts, including many North American species, can maintain long-term controlled infections in which the host immune system is constantly activated and depositing melanin around new growing pathogen hyphae, and mortality primarily occurs when this process is disrupted by additional stressors (Svoboda et al. 2015; Becking et al. 2015; Martínez-Ríos et al. 2022, 2023; Wang et al. 2025).

In our experiment, visible lesion burden alone was a weak predictor of performance outcomes. Although crayfish with larger lesion areas tended to die earlier, lesion severity had minimal effects on growth or molting success. Lesion area frequently stabilized or declined through time, particularly in the high-prevalence population, consistent with previous work showing that visible plague lesions often reflect localized immune responses in which hosts encapsulate pathogen hyphae within melanized tissue. Because lesion area and count varied among individuals and through time, lesion metrics therefore captured dynamic symptom expression and potentially dynamic infection burden rather than a static measure of disease

intensity. External lesions also represent only one dimension of disease expression; other symptoms of crayfish plague, including ataxia, autotomy, and behavioral changes, may occur even when visible lesions are limited (Boštjančić et al. 2023; Alexandrova and Tarasov 2020). In field observations from Montana, most crayfish with visible lesions appeared behaviorally normal, although a small number of individuals with extensive lesions exhibited reduced responsiveness or sluggish movement (Martín-Torrijos et al. In prep.). These observations suggest that visible lesion severity may correspond to functional impairment in some individuals even when population-level patterns are subtle.

One possible explanation for lesion stabilization is the experimental environment itself. Crayfish were maintained in frequently refreshed, dechlorinated tap water rather than lake water, potentially reducing pathogen exposure, organic matter and microbial abundance, or other environmental stressors that may sustain infection in natural systems (Velásquez et al. 2018; Vicente-Santos et al. 2023; Turner et al. 2021; Suzuki et al. 2014). Under these conditions, crayfish may have been able to contain infections rather than experience progressive disease. Strain-specific virulence may also have contributed to these patterns. The a-haplotype of *A. astaci*, which we detected, is less virulent than the b-haplotype even in typically susceptible species such as *Astacus astacus* (Viljamaa-Dirks 2013; Becking et al. 2015; Francesconi et al. 2021). If the a-haplotype is similarly tolerated by *F. virilis*, lesion development may represent a largely sublethal immune response under laboratory conditions.

Molting may have contributed to changes in visible lesion severity through its interaction with host phenology. Infectious agents can alter host phenology across taxa by shifting the timing of key life-history events such as leafing, flowering, breeding, or molting (Altizer et al.

2006; Martinez 2018; O'Brien et al. 2021). These shifts may arise from host responses to infection or from pathogen-driven changes that prolong infection or transmission. Because host physiology, immune investment, and tissue structure vary across life stages, disease impacts are often strongly stage-dependent, with some life stages or organs more susceptible than others. For example, avian blood parasites have been associated with delayed breeding phenology in several songbird species, alongside reduced condition and survival (Grabow et al. 2025). In marine crustaceans, molting phenology interacts with disease dynamics; in American lobsters (*Homarus americanus*), earlier spring molts extend the summer intermolt period and increase exposure to epizootic shell disease (Groner et al. 2018). In our experiment, lesion area and lesion counts declined markedly immediately following molting, consistent with mechanical removal of colonized cuticle during ecdysis. These observations suggest that successful molting may facilitate the removal of lesion-associated cuticle, potentially contributing to reduced lesion severity through time. This pattern suggests that visible plague lesions reflect a dynamic host–pathogen interaction rather than a cumulative measure of infection intensity. Because molting periodically replaces the external cuticle, visible lesion expression may change through time even when underlying infections persist. Immediately after molting, lesions were often absent or appeared as pale areas on the newly exposed cuticle, which sometimes returned to the normal exoskeleton color later in the intermolt period and in some cases were followed by the appearance of new lesions in nearby tissue. Molting may also influence infection dynamics by removing colonized cuticle from the host and, when exuviae are removed from the environment, reducing a potential source of infectious propagules capable of reinfecting the host.

Spatial patterns of lesion occurrence across the crayfish body appear to reflect both morphological vulnerability and exposure. Lesions were observed across all eight crayfish body regions but were most common on the abdomen and uropods/telson. These posterior regions are composed of thinner, more flexible, and less mineralized cuticle than the heavily sclerotized carapace and claws, making them more permeable and mechanically vulnerable to pathogen entry (Kozubíková et al. 2009; Petrusek et al. 2025; Vittori 2024). Similar patterns have been reported in other studies of crayfish plague, where infection prevalence is often highest in the tail fan relative to other body regions (Vrålstad et al. 2011; Makkonen et al. 2018; Hsieh et al. 2016; Akhan et al. 2024). Increased lesion frequency in these regions may also reflect greater surface area and prolonged contact with benthic substrates, where *A. astaci* zoospores tend to concentrate (Vrålstad et al. 2011).

Several limitations of this study should be considered when interpreting these results. Sample size may have limited the ability to detect subtle differences among treatments, particularly given variability in lesion area and the relatively small number of individuals per treatment group. Because mortality events were infrequent and occurred largely toward the end of the experiment, survival analyses likely had limited sensitivity to detect biologically meaningful differences among treatments. Although melanization and visible lesions indicated a localized immune response to infection, lesion area was used as a proxy for disease severity and may not fully capture underlying infection dynamics. External lesions represent only one dimension of disease expression, and additional behavioral or physiological symptoms may occur even when external lesions are limited. Metrics such as pathogen load or hyphal infiltration within the cuticle may better reflect infection intensity (Martín-Torrijos et al. 2018;

Bouallegui 2021; Martín-Torrijos et al. 2019; Martínez-Ríos et al. 2023). In addition, crayfish were categorized based on population-level lesion prevalence history rather than experimentally manipulated infection status, and individual infection intensity likely varied among individuals. The experiment was also conducted under controlled laboratory conditions that excluded ecological factors such as predation, competition, and environmental variability that may influence lesion dynamics in natural systems. Although our 227-day experiment was relatively long compared to many freshwater experimental studies (median = 29 days across 108 studies; Fritz et al. 2026), it still represents only a snapshot of disease dynamics. Many crayfish plague experiments span only a few weeks, whereas resistance and population-level assays typically last 45–60 days and only occasionally extend beyond 120 days (Makkonen et al. 2013; Makkonen et al. 2014; Strand et al. 2012; Svoboda et al. 2019; Becking et al. 2015; Martín-Torrijos et al. 2017; Martínez-Ríos et al. 2022). Because most crayfish species live two to four years in the wild, chronic plague carriers may experience substantial temporal variation in lesion expression when exposed to environmental stressors such as temperature change, eutrophication, other pathogens, or repeated pathogen exposure (Venarsky et al. 2012; Chucholl 2011; Belchier et al. 1998; Wang et al. 2025; Thomas et al. 2020; Aydin et al. 2014). Finally, because tail-punched crayfish experienced higher mortality, growth analyses necessarily included only surviving individuals, which may underestimate treatment effects. Despite these limitations, the controlled experimental design allowed us to isolate the effects of marking method and population disease history on crayfish performance and lesion progression. Improved understanding of invasion history and pathogen distribution in the Rocky Mountains (Kennedy and Adams 2025; Newkirk

et al. 2023; Larson et al. 2018) may further clarify how disease tolerance, environmental stress, and pathogen virulence interact to shape disease dynamics in wild populations.

Taken together, our results show that visible crayfish plague lesions had relatively weak effects on growth, molting, and survival under laboratory conditions, whereas tissue-disruptive marking methods imposed substantial physiological costs and increased lesion severity. These findings highlight that routine research handling practices can interact with underlying disease processes to shape host performance and emphasize the importance of minimizing unnecessary stress in experimental studies involving crayfish. Elastomer tagging emerged as a reliable, low-impact marking method for studies involving crayfish expressing symptoms of crayfish plague, whereas tail punching may amplify stress and lesion expression. More broadly, this study illustrates how common research practices can interact with underlying disease processes, with implications for experimental design, crayfish husbandry, and the interpretation of disease dynamics in freshwater ecosystems. Future work should evaluate how environmental context, pathogen strain, and handling stress jointly influence crayfish plague dynamics in natural populations.

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CHAPTER 3

THERMAL STRESS MODULATES LESION EXPRESSION AND PHYSIOLOGICAL PERFORMANCE IN NORTH AMERICAN CRAYFISH INFECTED BY CRAYFISH PLAGUE

Contribution of Authors and Co-Authors

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Abstract

Rising water temperatures are expected to alter host-pathogen dynamics in freshwater ecosystems, yet consequences for aquatic invertebrates remain poorly understood. Crayfish plague, caused by the oomycete *Aphanomyces astaci*, is a globally important freshwater pathogen, although effects within North American crayfish populations remain less studied than its effects on crayfish on other continents. We experimentally tested how chronic (99 days) and acute (~4 hours) thermal stress influenced performance of virile crayfish (*Faxonius virilis*) from two Montana (USA) populations with contrasting histories of lesion prevalence associated with crayfish plague (high- vs. low- lesion-prevalence). Crayfish were acclimated to 15, 23, or 30 °C and monitored for survival, growth, molting success, lesion severity (count and area), and righting performance. Under chronic temperature exposure, mortality increased with warming (0% at 15 °C; 25% at 30 °C) whereas lesion-prevalence history group did not strongly influence survival. Growth and molting showed little evidence of differing among temperature treatments or lesion-prevalence groups, although crayfish from the high-lesion-prevalence population tended to exhibit lower growth overall. Lesion counts were higher in the high-lesion-prevalence population and generally increased through time, including across molting events, but showed little response to temperature. In contrast, lesion area increased with warming in the low-prevalence population but remained relatively stable in the high-prevalence population. Righting performance during chronic exposure depended on temperature but not lesion prevalence history group, with slower responses observed at 15 °C. Following the chronic temperature exposure experiment, those crayfish were subjected to an acute thermal assay (up to 40 °C) and progressively lost the ability to right themselves as temperature increased. The temperature at

which crayfish lost righting ability depended on prior acclimation temperature but not lesion-prevalence history group. Crayfish acclimated to warmer conditions tolerated higher temperatures before loss of righting response but experienced greater delayed mortality in the 48 hours following the assay. Together, these results suggest that temperature exerted stronger effects on crayfish physiological performance than lesion prevalence history, while population-level disease history was more closely associated with sublethal responses such as lesion severity and growth. Because crayfish function as ecosystem engineers and important components of food webs in freshwater systems, sublethal changes may influence ecological interactions and ecosystem processes even when short-term survival appears unaffected by disease.

Introduction

Abiotic context regulates disease dynamics in aquatic systems by influencing pathogen growth, transmission, and host susceptibility (Poulin et al. 2011; Lipp et al. 2002). Contemporary environmental change, including warming temperatures, altered flow regimes, and species introductions, can modify host-pathogen interactions (Barber et al. 2016; Johnson et al. 2010; Jackson et al. 2016). Increased disease prevalence under warming has been documented in amphibians affected by chytrid fungus (Xie et al. 2016) and in Scleractinian corals affected by temperature-sensitive diseases such as white syndrome (Burke et al. 2023). Yet disease dynamics in freshwater invertebrates remain comparatively understudied in the context of global change (Johnson and Paull 2011; Reid et al. 2018), despite freshwater ecosystems supporting disproportionate biodiversity and ecosystem services relative to their spatial extent (Tickner et al. 2020; Tockner 2021).

Temperature is a particularly influential abiotic driver for ectothermic organisms, shaping metabolic rate, immune function, and physiological performance, while also regulating pathogen development and virulence (Carter et al. 2021; Silva and Elliot 2016; Ellison et al. 2019). For the crayfish plague pathogen *Aphanomyces astaci*, laboratory studies indicate optimal growth and sporulation between approximately 18–23 °C, with impaired growth, reduced sporulation, and decreased survival above ~25 °C (Unestam 1965; 1966; 1969; Alderman et al. 1987; Viljamaa-Dirks et al. 2016). Although these thermal sensitivities suggest that pathogen performance may decline at higher temperatures, host physiological responses to warming may differ from those of the pathogen. Temperature also strongly influences crayfish growth, metabolism, and physiological stress responses. Temperatures near 15 °C correspond to the lower range of active growth and metabolic performance in temperate crayfish, whereas temperatures near 23 °C approximate reported growth optima and preferred temperatures for many North American crayfish species, including *Faxonius* spp. (Westhoff and Rosenberger 2016). In contrast, temperatures approaching 30 °C remain below reported upper thermal tolerance limits but exceed optimal growth conditions and can function as sublethal thermal stressors that elevate metabolic demand and physiological stress (Westhoff and Rosenberger 2016; Tripp et al. 2022). Similar temperature-mediated stress responses have been documented in other aquatic ectotherms under warming conditions (Chadwick and McCormick 2017; Åsheim et al. 2020; Pieper et al. 2025). Consequently, warming may alter disease outcomes through changes in host resilience and resistance rather than pathogen proliferation alone.

Disease impacts are not always expressed through direct mortality (Holt et al. 2021; Morley 2010). Sublethal effects, including altered behaviors, reduced physiological performance,

and diminished stress tolerance, may persist following infection and influence how hosts respond to subsequent environmental challenges (Fontes 2017). In freshwater invertebrates, such cryptic disease effects are rarely quantified, yet they may shape individual fitness and population resilience under changing thermal regimes (Dallas and Ross-Gillespie 2015; Hermann et al. 2024). Behavioral metrics such as righting response provide integrative measures of neuromuscular coordination, physiological condition, and stress tolerance in invertebrates, including crayfish (Newland 1989; Webb 1979; Catteart and Ray 2001) and sea urchins (McClintock 2015), while acute thermal tolerance thresholds reflect limits to short-term physiological resistance. This coordinated behavior reflects nervous system integrity, motor control, and broader locomotor function (Fields 1996; Kivivuori 1980). Righting latency can vary by several seconds across environmental and physiological contexts, including predation-related conditions (Orr et al. 2007) and stress exposure (Tu et al. 2024), with potentially important consequences for wild crayfish survival and escape performance. However, sublethal influences of warming and disease may also manifest beyond locomotor behavior. Measures such as growth, molting success, lesion severity, and survival provide complementary insight into physiological stress, energetic trade-offs, and disease progression (Brosset et al. 2023; Coates and Söderhäll 2020; Rebrina et al. 2015; Sun et al. 2025). Together, these metrics allow for a more comprehensive evaluation of how temperature and pathogen history influence crayfish performance.

Here, we examined how chronic temperature exposure and acute thermal stress interact with disease history to shape physiological performance in freshwater crayfish. We used two laboratory experiments to evaluate (1) mortality, lesion severity, growth, molting success, and

righting performance under chronic warming temperature exposure (15, 23, and 30 °C) over 3 months and (2) loss of righting response during an acute thermal stress assay conducted over several hours. Collectively, these response variables are referred to hereafter as crayfish performance. For the first experiment, we predicted that (1) higher temperatures would impair crayfish performance metrics, (2) crayfish from the high-lesion-prevalence population would exhibit greater reductions in performance under warmer conditions than crayfish from the low-lesion-prevalence population, and (3) survival would remain relatively high despite detectable sublethal impairment. For the second experiment, we predicted that crayfish from the high-lesion-prevalence population would lose righting response more rapidly and at lower temperatures during acute warming, particularly following chronic exposure to warmer water temperatures. Together, these experiments evaluated how crayfish from populations with contrasting lesion-prevalence histories responded to thermal stress and how lethal and sublethal responses contribute to understanding stress in freshwater invertebrates.

Methods

Study System and Source Populations

We conducted a controlled laboratory experiment at Montana State University in Bozeman, MT, USA to evaluate how water temperature and population lesion prevalence (high vs. low) associated with crayfish plague interact to influence physiological performance, lesion severity, and thermal tolerance in Northern crayfish *Faxonius virilis* (Fig 3.1a). Individuals were collected from two wild Montana crayfish populations that differed in prevalence of lesions associated with crayfish plague (Fig. B1): Tally Lake (Flathead County, Montana, USA; high-lesion-prevalence) and Newlan Reservoir (Meagher County, Montana, USA; low-lesion-

prevalence). Previous research confirmed that *A. astaci* was present on crayfish from both populations and that crayfish from the two populations showed no detectable differentiation in mitochondrial COI sequences (Martín-Torrijos et al. in prep.; Schmidt et al. in review; Fig. 3.1b). We used snorkel and dip-net sampling to collect female crayfish from Tally Lake on 26 August 2024 and from Newlan Reservoir on 27 August 2024. Crayfish were collected haphazardly from each population without regard to visible lesion burden. Most of the crayfish with lesions, even those with severe lesions, exhibited what appeared to normal activity, often attempting to evade capture and to escape from the bucket after capture. Only a few crayfish with severe lesions were notably lethargic.

Collections were restricted to females of similar size to reduce variation associated with sex and body size. Study populations were selected based on prior knowledge of population-level lesion prevalence, such that sites represented either high- or low-prevalence conditions. Individuals from both lesion-prevalence groups were transported to the laboratory (in a cooler in individual Ziploc bags in chilled temperatures and placed into the experimental system on 27 August 2024 with a short acclimation period to the assigned temperature treatment (lasting 30 minutes to a few hours for the warmer temperature treatments). Surface water temperatures measured near the date of collection were 21.7 °C in Tally Lake (13 August 2024) and 20.5 °C in Newlan Reservoir (11 August 2024). Hereafter, we refer to experimental treatments by lesion-prevalence group (high vs. low) to represent the source location and underlying population-level lesion prevalence. Temperature treatments were replicated at the bath level, whereas lesion-prevalence history comparisons were based on two source populations and should therefore be

interpreted cautiously as population-level contrasts rather than fully replicated disease-history treatments.

Experiment 1: Chronic Thermal Exposure

Experimental Design and Husbandry The experiment employed a fully crossed design with lesion-prevalence group (high- vs. low-prevalence source populations) and temperature treatment as fixed factors. Crayfish were maintained in insulated 48-qt water baths (coolers; hereafter "baths"), each functioning as an independent thermal unit. Water temperatures were maintained at 15, 23, or 30 °C using aquarium heaters for the 23 and 30 °C treatments and a circulating chiller system for the 15 °C treatment. Continuous aeration and filtration were provided to each bath throughout the experiment.

Within each bath, crayfish were housed individually in perforated plastic containers (14.6 × 14.6 × 7.6 cm), with three crayfish assigned to each bath (Table 3.1). Each container received independent aeration via aquarium tubing connected to a commercial air pump.

Experimental temperatures were selected to represent cool, intermediate, and warm summer thermal conditions relevant to Montana freshwater systems. The 30 °C treatment represented a sublethal thermal stressor expected to elevate physiological demand without causing immediate mortality.

Table 3.1. Experimental components for chronic thermal exposure experiment lasting from August to November 2024. All crayfish were female *F. virilis*.

Factor	Levels	Description
Lesion-prevalence group	Low-lesion-prevalence (Newlan Reservoir) High-lesion-prevalence (Tally Lake)	Source populations differing in historical crayfish plague lesion prevalence
Temperature treatment (°C)	15, 23, 30	Constant-temperature acclimation treatments
Experimental unit	Temperature bath	Each bath contained 3 crayfish housed individually in perforated containers with independent aeration.

Table 3.1. Continued

Crayfish per bath	3	Each bath contained 3 crayfish housed individually in perforated containers with independent aeration.
Baths per treatment	4 per group × temperature	Total of 24 baths
Total crayfish	72	2 lesion-prevalence groups × 3 temperatures × 4 baths × 3 crayfish

Crayfish were randomly assigned to temperature treatments and baths, and bath positions within the laboratory were randomized among treatments. Individuals remained under their assigned temperature treatments for 99 days (27 August–3 December 2024). We assessed crayfish growth and righting response at the beginning (6 September 2024) and end (26 November 2024) of the exposure period.

Temperature was regulated at the bath level; thus, crayfish within a bath experienced a shared thermal environment. Individual responses were analyzed at the level of the crayfish. Bath identity was evaluated as a random effect to account for potential non-independence among crayfish sharing a thermal unit; however, bath-level variance was negligible, and inclusion did not improve model fit, so bath was not retained in final models. Crayfish were maintained under 12-hour photoperiod using artificial lighting. Temperature treatments were replicated at the bath level, whereas lesion-prevalence history comparisons were based on two source populations and should therefore be interpreted as population-level contrasts rather than fully replicated disease-history treatments.

Water temperature and light were continuously monitored in each temperature treatment using HOBO® Pendant temperature/light loggers (Onset Computer Corporation®), with two loggers per temperature treatment and additional loggers recording ambient laboratory

conditions. Daily temperature checks were conducted using calibrated handheld thermometers to verify heater and chiller performance. Early in the experiment, minor equipment-related artifacts were observed, including transient temperature fluctuations associated with the circulation pump clogging, heater displacement within baths, or temporary obstruction or inversion of pendant loggers. These issues were resolved by installing filter socks on circulation pumps, which improved water clarity and stabilized internal bath components. All documented anomalies occurred during the initial phase of the experiment and were corrected within 1-2 days of onset (Appendix B, Temperature control). Periods associated with confirmed mechanical artifacts were excluded from temperature summary calculations.

Crayfish were checked at least once daily Monday through Friday. Feedings occurred twice per week as did water changes. All water used in the experiment consisted of dechlorinated tap water. All crayfish had the same diet consisting of Aquatic Foods Inc. Shrimp Grower (45% Protein Sinking Pellets, 1/16" x 3/8", 4 pellets twice per week). Water changes occurred 24 – 36 hours after feeding. Laboratory space had an artificial 12-hour photoperiod using LED light bars. Molts, deaths, temperature checks, and equipment malfunctions were recorded in a daily laboratory log. Shed exuviae were left in containers following molts, including during water changes, to allow crayfish to consume them.

Response Variables and Measurements All statistical analyses were conducted in R (version 4.5.1; R Core Team 2025). Model structures and assumptions are described within each response-specific subsection below. We interpreted statistical results using an evidence-based framework rather than strict dichotomous significance thresholds. Results with $p \leq 0.05$ were interpreted as strong evidence for an effect, whereas results with $0.05 < p \leq 0.10$ were interpreted

as moderate evidence. Results with $p > 0.10$ were considered to provide weak evidence for an effect. Effect sizes and confidence intervals were also considered when evaluating biological relevance.

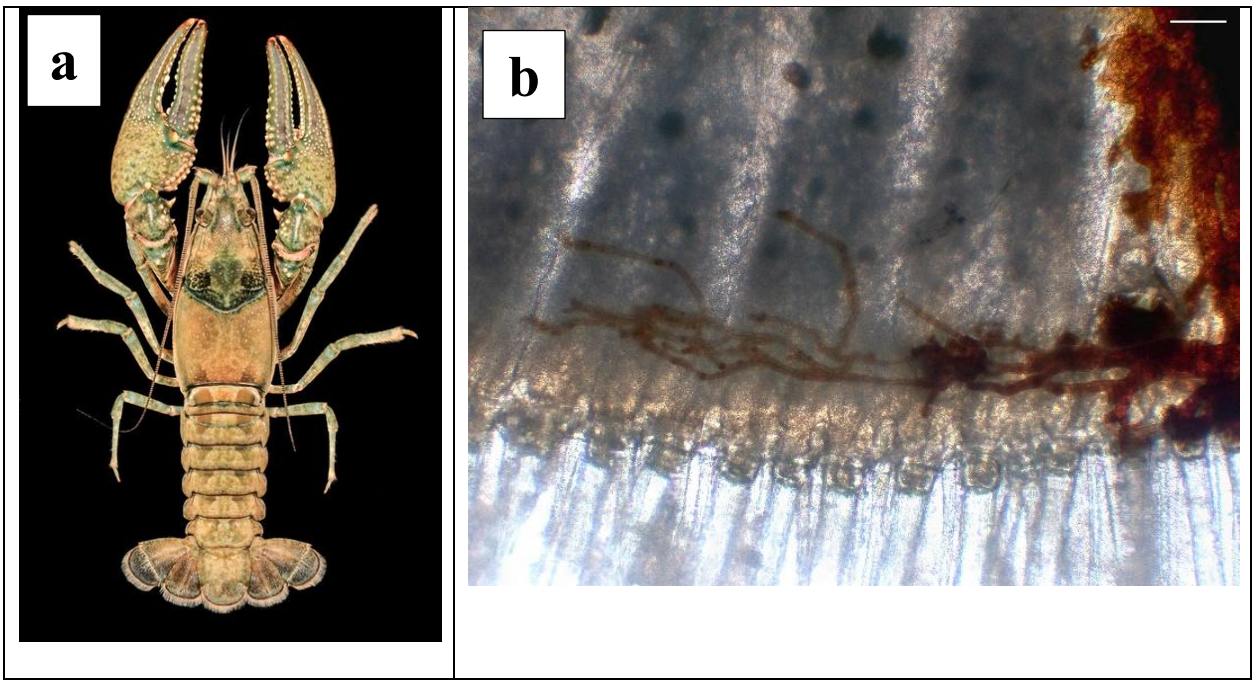
Survival To evaluate effects of temperature treatment and lesion-prevalence group on survival during the chronic exposure period, we fit a Firth-penalized logistic regression model with temperature treatment (15, 23, 30 °C), lesion-prevalence group (high vs. low), and their interaction as fixed effects. Firth logistic regression was used to reduce small-sample bias and provide stable estimates in the presence of low event counts and treatment groups with complete or near-complete survival or mortality outcomes. Model-predicted survival probabilities and 95% confidence intervals were obtained using estimated marginal means on the response scale.

Molting Molting probability was analyzed as a binary response indicating whether an individual molted at least once during the chronic exposure period. We fit a generalized linear model with a binomial error distribution and logit link, including temperature treatment, lesion-prevalence group, and their interaction as fixed effects. Because molting events were rare and individuals experienced a limited number of molt opportunities, the proportion of crayfish that molted at least once was additionally summarized descriptively by treatment group.

Growth Growth was quantified as the change in post-orbital carapace length (POCL; mm) between the start and end of the experiment (final POCL − initial POCL). Negative growth values were assumed to reflect minor measurement error and were set to zero prior to analysis. Growth was analyzed using linear models with temperature treatment, lesion-prevalence history group, and their interaction as fixed effects. Because crayfish were housed in shared temperature

baths, bath identity was initially evaluated as a random effect in mixed-effects models; however, bath explained negligible variance and was not retained in the final growth analysis. Additional analyses incorporating starting POCL as a covariate produced similar conclusions. Supplemental analyses of size-adjusted body condition using wet mass from the end of the experiment are provided in Appendix B (Length-Mass and Body Condition; Table B3).

Lesion Severity Lesion presence, lesion count, and lesion area were quantified from photographs collected at the start and end of the experiment. Lesions were visually identified as melanized or eroded areas consistent with crayfish plague–associated tissue damage and manually delineated in ImageJ/Fiji (Schindelin et al. 2012; Fig. 3.1c). Lesion area (mm^2) was quantified separately for dorsal and ventral surfaces and summed to generate total lesion area per individual at each time point.



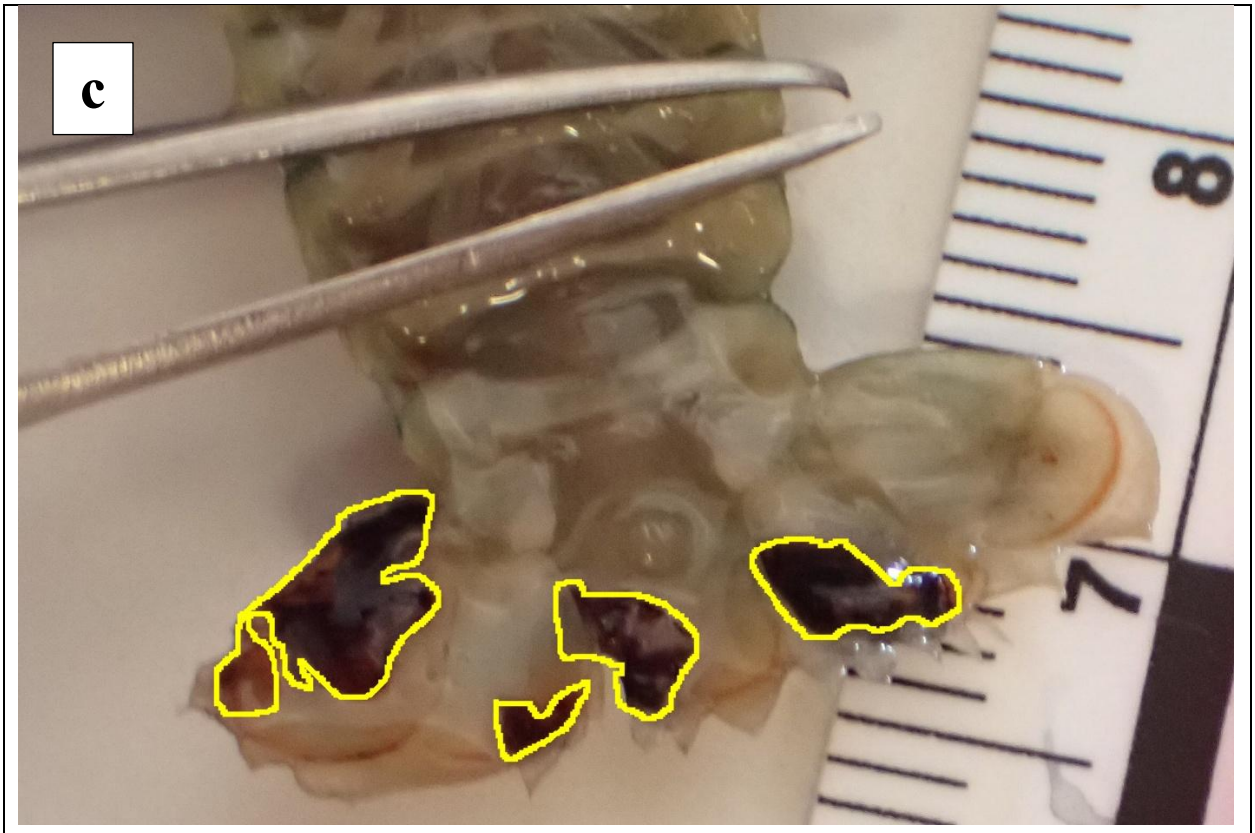


Figure 3.1. (a) Northern crayfish *(Faxonius virilis*) with no lesions (photograph by Guenter Schuster). (b) Microscopic visualization of macroscopic lesions associated with *F. virilis* from Tally Lake (high-lesion-prevalence group). The strong immune response characteristic of the acute infection stage of *A. astaci* was evident as a uniform layer of melanin deposits enveloping the hyphal structures. Micrographs illustrate melanized hyphae in lesions located on telson. Scale bar = 100 µm. (c) Lesions outlined in yellow using Fiji software on a photograph of a crayfish from the experiment.

Lesion presence was summarized descriptively because lesions were present in nearly all individuals from the high-prevalence population, limiting meaningful statistical comparisons of lesion occurrence between populations.

Total lesion count per individual was analyzed using a generalized linear mixed-effects model with a negative binomial error distribution and log link to account for overdispersion. The model included time point (start and end), temperature treatment, lesion-prevalence group, and

their interactions as fixed effects, with crayfish identity included as a random intercept to account for repeated measures. Model structure was simplified using likelihood ratio tests, and inference was based on Type III Wald χ^2 tests.

Because lesion occurrence and lesion severity represent distinct biological processes, lesion-area analyses focused on observations with measurable lesions (>0), thereby evaluating predictors of lesion severity conditional on lesion presence. Total lesion area per individual was analyzed using a generalized linear mixed-effects model with a Gamma error distribution and log link because lesion area was continuous, strictly positive when present, and strongly right-skewed. The initial model included experimental time point (start vs. end), temperature treatment (15, 23, 30 °C), lesion-prevalence group (low vs. high), and all two- and three-way interactions as fixed effects. Repeated measurements from the same individual crayfish across time points were accounted for by including crayfish identity as a random intercept. Model structure was simplified using likelihood ratio tests comparing nested models. The final model retained significant interactions between time and population and between temperature and population. Bath identity was evaluated as an additional random intercept to account for shared thermal environments; however, bath-level variance was negligible and inclusion did not improve model fit, so bath was excluded from final models. Model assumptions were evaluated using residual diagnostics implemented in the DHARMa package, and inference was based on Type III Wald χ^2 tests. Lesion severity models were fit using 102 observations from 60 individuals with measurable lesions.

Lesion Location Lesion location was assessed descriptively using region-specific lesion area measurements derived from laboratory photos at the start and end of the experiment. Lesions were assigned to eight anatomical locations: antennae/antennules, head/mouthparts, claws, carapace, walking legs, abdomen, pleopods/annulus ventralis, and uropods/telson at the time of measurement and count. For each lesion-prevalence group, temperature treatment, time point, and photographic view (dorsal or ventral) lesion area was summed across individuals and expressed as the proportion of total lesion area attributed to each anatomical region, such that the proportions summed to 100% within each group. This descriptive approach was used to evaluate whether temperature-associated changes in lesion severity were accompanied by shifts in anatomical distribution of lesions.

Righting Response During Chronic Thermal Exposure Righting response trials were conducted at the beginning and end of the chronic thermal exposure experiment to assess neuromuscular performance under each temperature treatment. During each trial, crayfish were inverted manually and the time required to return to an upright position was recorded in seconds. Trials were conducted within the assigned temperature treatment for each crayfish. Righting times were analyzed as continuous response variables. Righting time was analyzed using a linear mixed-effects model with time point (start vs. end), temperature treatment (15, 23, 30 °C), lesion-prevalence group (low vs. high), and all interactions as fixed effects. Crayfish identity was included as a random intercept to account for repeated measurements of individuals across time points. Because some individuals died before the final assessment, repeated-measures data were unbalanced across time points. Inclusion of crayfish identity as a random effect allowed the model to accommodate repeated observations while retaining individuals with missing end-point

measurements. Model assumptions were evaluated using residual diagnostics, and inference was based on Type III tests of fixed effects. Post hoc estimated marginal means used to summarize start-to-end contrasts within treatment combinations. Righting response was measured for 71 crayfish at the start of the experiment and for 65 of those individuals at the end (136 total observations; 65 individuals with paired measurements).

Experiment 2: Acute Thermal Stress Assay

Experimental Design To evaluate whether acute thermal stress revealed differences in thermal tolerance associated with disease history and acclimation temperature, we conducted an acute thermal stress assay during the four days following completion of the chronic temperature exposure experiment (3 – 6 December 2024). All surviving crayfish from the chronic experiment (n = 61) were included in the assay. Crayfish had previously been acclimated for 99 days to one of three temperature treatments (15, 23, or 30 °C). Due to equipment limitations, assays were conducted sequentially over multiple days. During each trial, three temperature-controlled water baths were run simultaneously, with one bath representing each acclimation temperature treatment. Each bath contained three crayfish housed individually in separate containers, resulting in nine crayfish tested per trial. Individuals from both lesion-prevalence groups were distributed across trials to minimize potential temporal or procedural bias. Trial identity (Ramp_ID) was included as a fixed effect in statistical analyses to account for potential variation among assay runs.

Thermal Assay Procedure and Temperature Measurement Crayfish remained housed individually within their existing containers, which were transferred into shared temperature-controlled assay baths maintained at each acclimation temperature. Acute warming was initiated by increasing submersible heater settings to 40 °C. Water temperature increased passively under continuous circulation, and heating rate was not manually controlled.

To standardize comparisons among acclimation treatments, heating rate was quantified over the common thermal interval from 30 – 40 °C experienced by all crayfish. Across this interval, warming occurred at a mean rate of 0.041 ± 0.008 °C min^{-1}, with minor variation

among assay runs reflecting differences in heater performance rather than treatment effects. Heating rate did not predict probability of loss of righting response ($p = 0.208$). Trial identity (Ramp_ID) was retained in statistical models to account for potential variation among assay runs.

During the initial warming phase (<30 °C), bath temperature was monitored continuously to track heating progression. Righting response trials began once bath temperature reached 30 °C and continued at approximately 1 °C intervals until temperatures approached 40 °C. At each temperature interval, righting response observations were recorded for individual crayfish and linked to crayfish identity.

Bath temperature was recorded using the water bath controller thermometer (Hygger® temperature controller). Temperatures within individual crayfish containers were also measured during a subset of assay trials when probe thermometers were available. Probe temperatures closely tracked bath temperatures across the assay interval (slope = 1.02, $R^2 = 0.99$), although probe temperatures averaged approximately 0.8 °C lower than bath temperatures. Because probe and bath temperatures remained highly correlated throughout the assay, bath temperature was used as the primary temperature metric in all analyses. Heating rates during the acute thermal stress assay were generally consistent among trials (mean = 0.041 °C min^{-1}, range = 0.028 – 0.067 °C min^{-1}), indicating comparable thermal conditions across assay runs.

Righting Response Trials and Endpoint Definition When bath temperature reached 30 °C, acute righting response trials began. At each subsequent ~1 °C increase in bath temperature (30 – 40 °C), crayfish were manually inverted and assessed for their ability to return to an upright posture within 30 seconds. A 30-second threshold was used to define failure because prolonged

inability to right is widely interpreted as evidence of acute neuromuscular impairment and loss of coordinated motor function under thermal stress.

Crayfish that failed to right themselves within 30 seconds were classified as having experienced loss of righting response (LORR), a commonly used behavioral endpoint indicating acute neuromuscular impairment under thermal stress. LORR was analyzed as a binary response variable (0 = successfully righted within 30 s; 1 = failed to right within 30 s). Because the primary objective of the acute assay was to identify thermal failure thresholds rather than variation in righting latency, analyses focused on the occurrence of LORR as a binary endpoint. Each crayfish was repeatedly assessed across successive temperature increments until LORR occurred or bath temperature reached 40 °C, whichever occurred first.

Loss of righting response was analyzed using a binomial generalized linear mixed-effects model with a logit link. Bath temperature was treated as a continuous predictor. Fixed effects included bath temperature, lesion-prevalence group (high vs. low), acclimation temperature (15, 23, or 30 °C), and interactions between bath temperature and each treatment variable. Trial identity (Ramp_ID) was included as a fixed effect to account for minor variation among assay runs and heating rates. Because individual crayfish were repeatedly assessed across temperature increments, crayfish identity was included as a random intercept to account for non-independence of repeated observations. Model fit and overdispersion were evaluated using simulation-based residual diagnostics implemented in the DHARMa package. All analyses were conducted in R.

Delayed Mortality Monitoring Following completion of the acute thermal stress assay, crayfish were immediately returned to their original acclimation temperatures. Individuals were

monitored for 72 hours for delayed effects associated with the acute stress assay, including post-assay mortality. Because crayfish that tolerated higher temperatures also experienced longer exposure durations and repeated righting trials, the assay represents an integrated measure of acute thermal and handling stress rather than temperature exposure alone.

Post-assay mortality (post_exp_mort) was recorded as a binary response and analyzed using Firth-penalized logistic regression with acclimation temperature and lesion-prevalence group included as fixed effects. Post hoc pairwise comparisons among acclimation temperatures were conducted using Tukey-adjusted estimated marginal means.

Results

Chronic Temperature Exposure Experiment

Survival, Growth, and Molting Responses During the chronic thermal exposure experiment, temperature treatment influenced survival, lesion severity, and righting performance. We analyzed survival for 71 individuals (23 – 24 per temperature treatment; 11 – 12 per temperature × lesion-prevalence group). There was strong evidence that temperature treatment influenced survival ($\chi^2 = 7.28$, df = 2, p = 0.026), whereas there was weak evidence that lesion-prevalence group influenced survival ($\chi^2 = 2.40$, df = 1, p = 0.122). There was weak evidence for an interaction between temperature treatment and lesion-prevalence group ($\chi^2 = 0.63$, df = 2, p = 0.737). Relative to the 15 °C reference treatment, survival was lower at 30 °C (odds ratio = 0.076, p = 0.035), whereas survival at 23 °C showed little evidence of differing from 15 °C (p = 0.180) (Fig. 3.2a; Table 3.2). Mortality occurred only at 23 °C and 30 °C, with no deaths observed at 15 °C; however, overall survival remained relatively high across treatments. Predicted survival probabilities were highest at 15 °C (0.96 in both lesion-prevalence groups),

declined at 23 °C (0.79 low; 0.96 high; mean = 0.88), and were lowest at 30 °C (0.65 low; 0.81 high; mean = 0.73).

Table 3.2. Results of the Firth logistic regression model evaluating effects of temperature treatment, lesion-prevalence group, and their interaction on survival during the chronic thermal exposure experiment. Odds ratios, 95% profile penalized likelihood confidence intervals, likelihood ratio test statistics, and p-values are shown (n = 71).

Term	Odds ratio	95% CI	Chisq	p-value
Intercept	25.000	[3.30, 3203.37]	13.783	0.000
Temp: 23°C (vs 15°C)	0.152	[0.00, 2.16]	1.826	0.177
Temp: 30°C (vs 15°C)	0.076	[0.00, 0.85]	4.447	0.035
Lesion-prevalence: High (vs Low)	1.000	[0.01, 191.81]	0.000	1.000
23°C × High-prevalence	6.579	[0.02, 3452.43]	0.529	0.467
30°C × High-prevalence	2.224	[0.01, 527.06]	0.126	0.722

We found little evidence that temperature treatment, lesion-prevalence group, or their interaction influenced crayfish growth measured as change in POCL (ΔPOCL) over the course of the experiment (all $p > 0.05$; Table 3.3; Fig. 3.2b). Although crayfish from high-prevalence populations were smaller at the start of the experiment, starting POCL showed little evidence of influencing growth, indicating that initial size differences did not confound growth responses during chronic thermal exposure.

Table 3.3. Effects of temperature treatment, lesion-prevalence history group, and their interaction on crayfish growth (change in POCL, mm) during the chronic temperature experiment (n = 64; with both start and end measurements).

Effect	df	F	p
Temperature treatment	2	0.16	0.86
Lesion-prevalence history group	1	1.36	0.25
Temperature × prevalence	2	0.85	0.43

During the 99-day experiment, 47% (33/71) of the crayfish molted at least once. Predicted molting probabilities ranged from 25% to 58% across treatments. There was little evidence that molting probability differed among temperature treatments ($\chi^2 = 1.97$, df = 2, p = 0.370), between lesion-prevalence groups ($\chi^2 = 1.73$, df = 1, p = 0.190), or in their interaction ($\chi^2 = 0.23$, df = 2, p = 0.890; Fig. 3.2c).

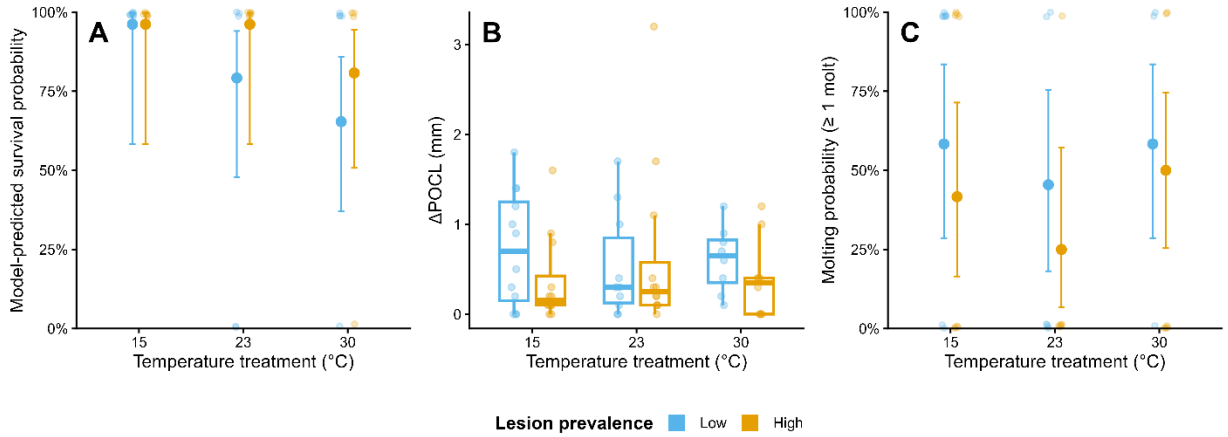


Figure 3.2. Responses of *F. virilis* during the chronic thermal exposure experiment across temperature treatments for low- and high-lesion-prevalence groups. (A) Model-predicted survival probability from a Firth logistic regression, with error bars representing 95% confidence intervals (n = 71 crayfish). (B) Change in post-orbital carapace length (ΔPOCL, mm) over the course of the experiment. Boxes represent interquartile ranges with median values shown as horizontal lines; whiskers extend to 1.5 × the interquartile range (n = 64 crayfish). (C) Probability of at least one observed molt event during the experiment (≥1 molt). Points represent observed proportions with 95% binomial confidence intervals (n = 71 crayfish). In panels A and C, faint points show individual binary outcomes jittered around 0 and 1.

Lesion Dynamics

Lesion dynamics were assessed using two complementary metrics: total lesion count and total lesion area. Lesion count reflects the number of distinct lesions present on an individual, whereas total lesion area reflects the cumulative extent of tissue damage. Although lesion count

and lesion area were generally positively associated, they captured different aspects of lesion progression and responded differently across treatments. Lesion count varied primarily across time and lesion-prevalence groups, whereas temperature-associated patterns were more evident in total lesion area. Because lesion area provided the clearest indication of temperature-related changes in lesion severity, we focus primarily on total lesion area results below.

Lesion Severity Lesion presence differed markedly between lesion-prevalence groups as expected. In the high-prevalence population, lesions were nearly ubiquitous across time points and temperature treatments, with 75 – 100% of individuals exhibiting measurable lesions in all treatment combinations. In contrast, lesion presence in the low-prevalence population ranged from 17 to 50% at the start of the experiment and increased to 58 – 75% by the end of the experiment. Lesion occurrence differed strongly between populations, with nearly all crayfish from the high-prevalence population exhibiting measurable lesions throughout the experiment, whereas lesion-free individuals were more common in the low-prevalence population, particularly early in the experiment. Subsequent lesion-area analyses therefore focused on lesion area among individuals with measurable lesions. Sensitivity analyses including zero-lesion observations produced similar temporal trends in lesion burden (Table B2).

Lesion counts per individual differed between populations and sampling time points. Crayfish from the high-lesion-prevalence population exhibited higher lesion counts overall, and lesion counts generally increased from start to end across most treatment combinations. Temperature treatment did not strongly influence lesion counts (negative binomial GLMM; Fig. B6).

There was strong evidence that total lesion area was influenced by time point, lesion-prevalence group, and interactions between lesion-prevalence group and both time point and temperature treatment (Table 3.4). Lesion area generally increased over time, but temporal patterns differed between lesion-prevalence groups. Similarly, the relationship between temperature treatment and lesion area depended on lesion-prevalence group. The interaction between time point and temperature treatment showed weak evidence ($\chi^2_2 = 0.68$, $p = 0.711$), and the three-way interaction among time point, temperature treatment, and lesion-prevalence group was also not supported ($\chi^2_2 = 1.70$, $p = 0.428$); therefore, the unsupported three-way interaction was removed from the final model.

Table 3.4. Correlations with lesion area. Type III Wald χ^2 tests from the final Gamma GLMM effects model evaluating effects of time point, temperature treatment, lesion-prevalence group, and retained interactions on total lesion area (mm^2) for crayfish with measurable lesions. Individual crayfish identity was included as a random intercept.

Term	χ^2	df	p-value
Time_Point	18.25	1	<0.001
Temperature_Treatment	5.86	2	0.053
Population	32.94	1	<0.001
Time × Population	5.55	1	0.018
Temperature × Population	7.46	2	0.024

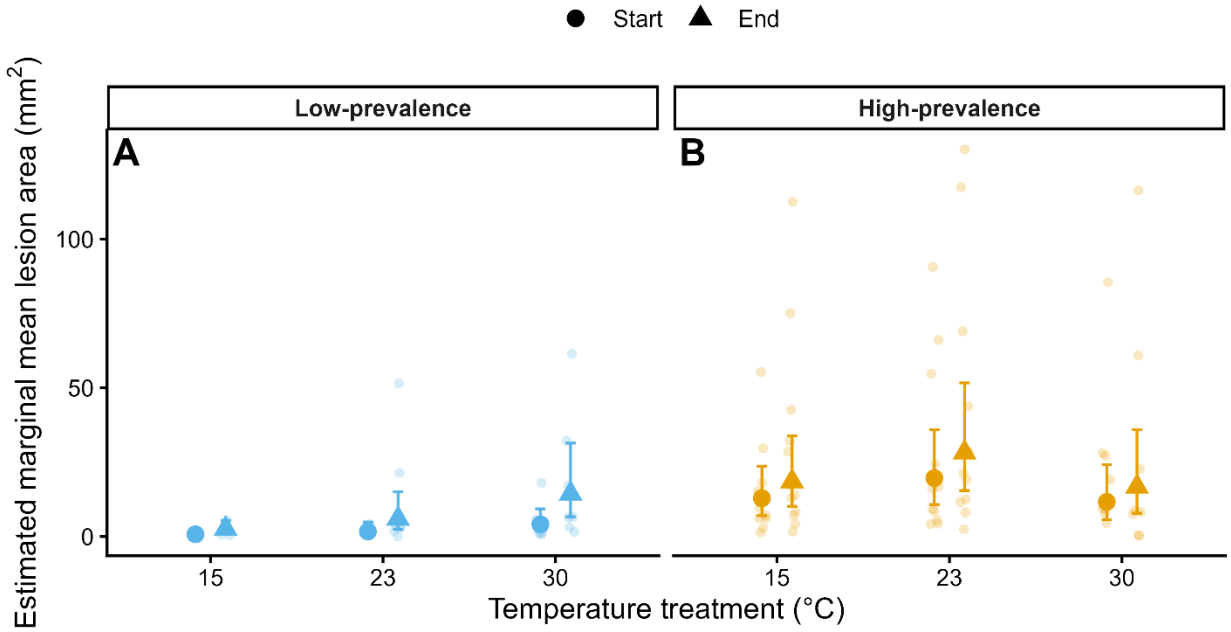


Figure 3.3. Model-estimated mean lesion area (mm²) ± 95% confidence intervals for crayfish with measurable lesions during the chronic thermal exposure experiment (n = 60 crayfish; 102 observations). Panels show crayfish originating from populations with low (A) and high (B) lesion prevalence. Circles represent estimated lesion area at the start of the experiment, and triangles represent estimates at the end for each temperature treatment. Larger points and error bars show model-estimated marginal means and 95% confidence intervals, while faint points represent individual crayfish observations.

Lesion Location Lesions were concentrated primarily in posterior and locomotor-associated regions, particularly the uropods/telson, abdomen, and walking legs (Fig. 3.4). Broad anatomical patterns were generally similar across time points and lesion-prevalence groups, although the relative contribution of specific regions varied among temperature treatments and between dorsal and ventral surfaces. Warmer temperature treatments were associated with greater proportional lesion area in appendage-associated regions in some groups, while posterior regions remained consistently affected across treatments.

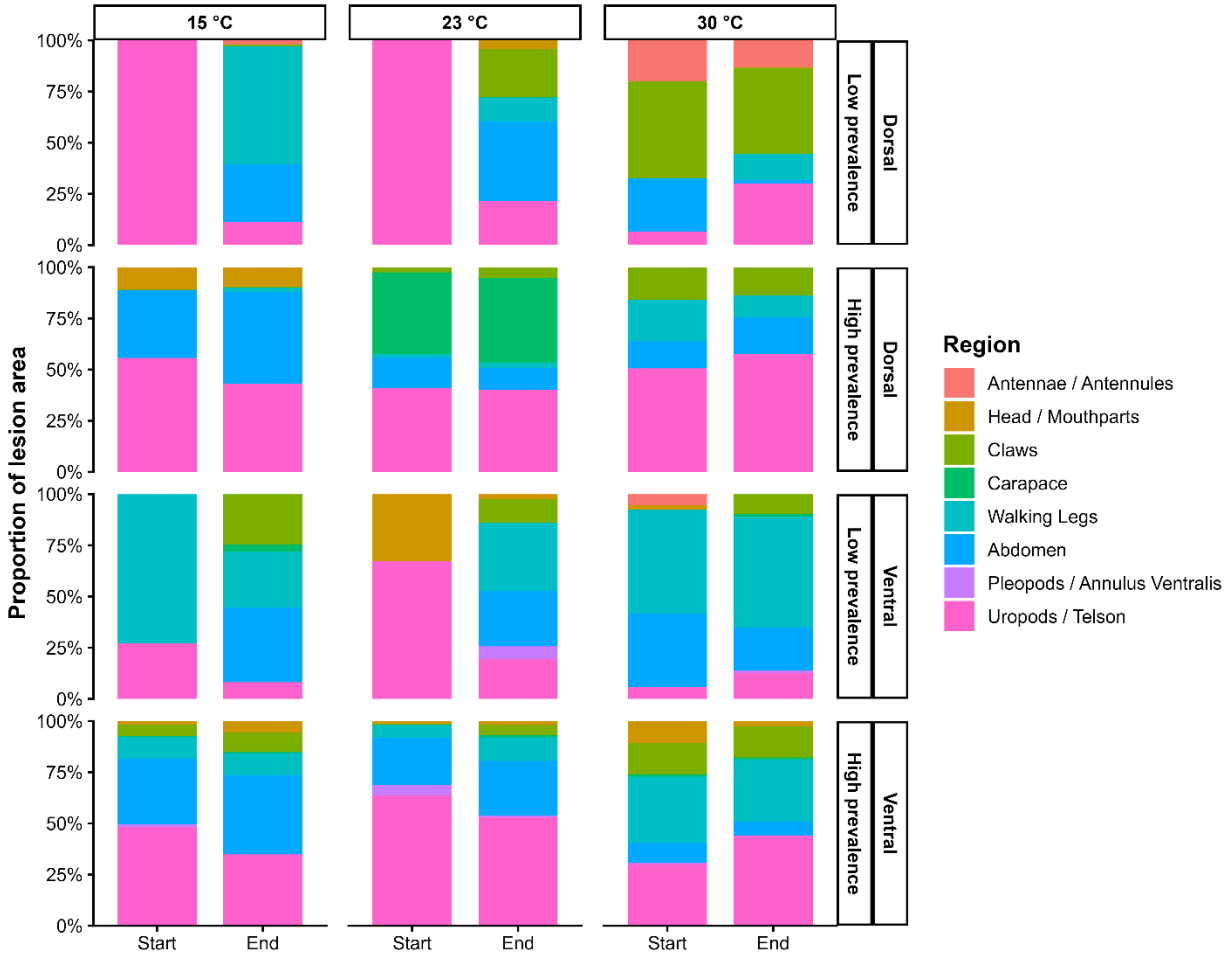


Figure 3.4. Proportional distribution of lesion area across anatomical regions for dorsal and ventral photographs during the chronic thermal exposure experiment. Within each view, lesion-prevalence group, temperature treatment, and time point, lesion area was summed across individuals and expressed as the proportion of total lesion area, such that bars sum to 100%.

Behavioral Performance

Righting response time differed across temperature treatments between the beginning and end of the chronic exposure experiment (Time × Temperature interaction: $\chi^2_2 = 13.38$, $p = 0.001$; Fig. 3.5). Crayfish in the 15 °C treatment exhibited longer righting times at the end of the experiment relative to the start, indicating reduced righting performance under cooler chronic

conditions. In contrast, crayfish maintained similar righting performance over time in the 23 °C and 30 °C treatments.

There was weak evidence for a three-way interaction among time point, temperature treatment, and lesion-prevalence group ($\chi^2_2 = 4.51$, $p = 0.105$), indicating that lesion-prevalence group did not modify temporal responses across temperature treatments There was weak evidence for a main effect of lesion-prevalence group on righting response time ($\chi^2_1 = 1.11$, $p = 0.290$).

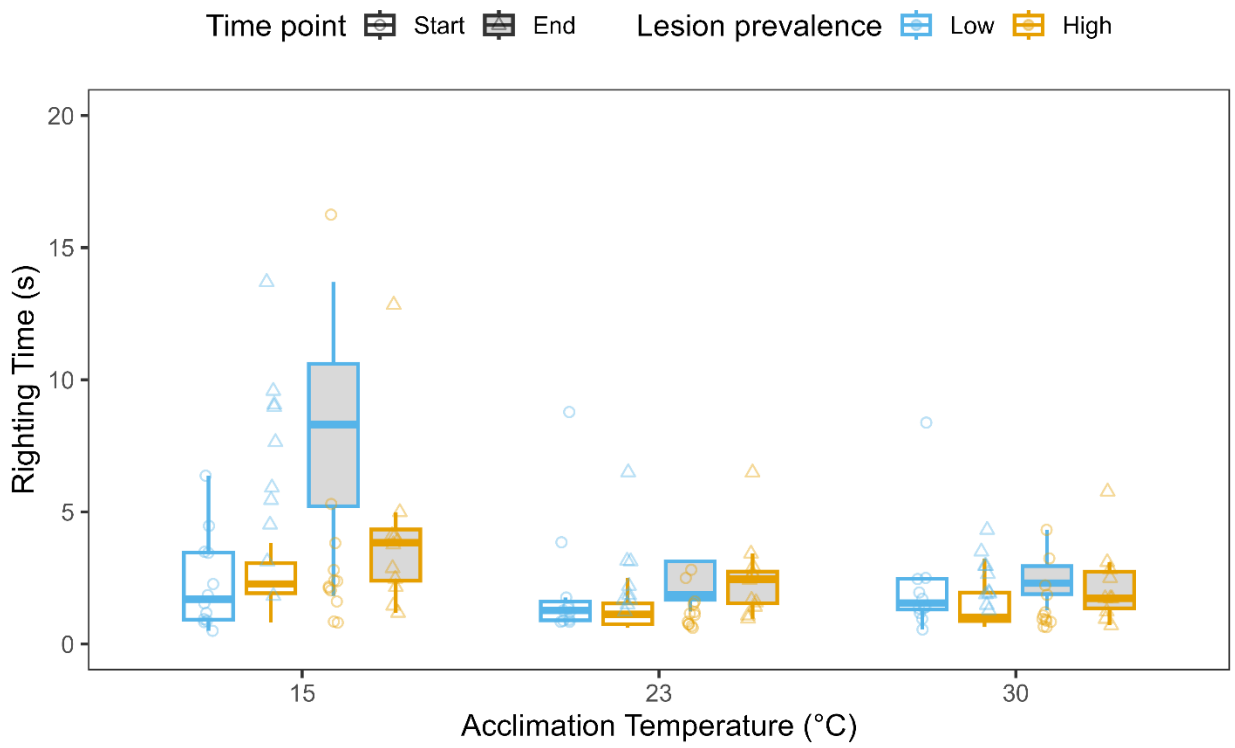


Figure 3.5. Righting response time during the chronic thermal exposure experiment for crayfish from low- and high-lesion-prevalence groups across three acclimation temperature treatments (15, 23, and 30 °C). Righting time (s) was measured at the start and end of the experiment. Boxplots show medians and interquartile ranges (IQR); whiskers extend to 1.5 × IQR and points represent individual observations. Colors indicate lesion-prevalence group and box fill indicates experimental time point (Start vs. End). For visualization purposes, the y-axis is truncated at 20 s, although all observations are shown. Sample sizes included 71 crayfish at the start of the experiment and 65 at the end (136 observations total; 65 paired observations).

Acute Thermal Stress Experiment

Loss of righting response during the acute thermal stress assay increased strongly with water temperature in the assay bath ($\chi^2_1 = 39.36$, $p < 0.001$; n = 61 crayfish, 407 observations; Fig. 3.6; Table 3.5). Prior acclimation temperature also influenced overall probability of loss of righting response ($\chi^2_2 = 6.21$, $p = 0.045$), indicating that chronic thermal history shifted acute heat resistance. However, the bath temperature × acclimation temperature interaction was not supported ($\chi^2_2 = 4.23$, $p = 0.120$), indicating that the rate at which righting failure increased with rising bath temperature did not differ significantly among acclimation treatments.

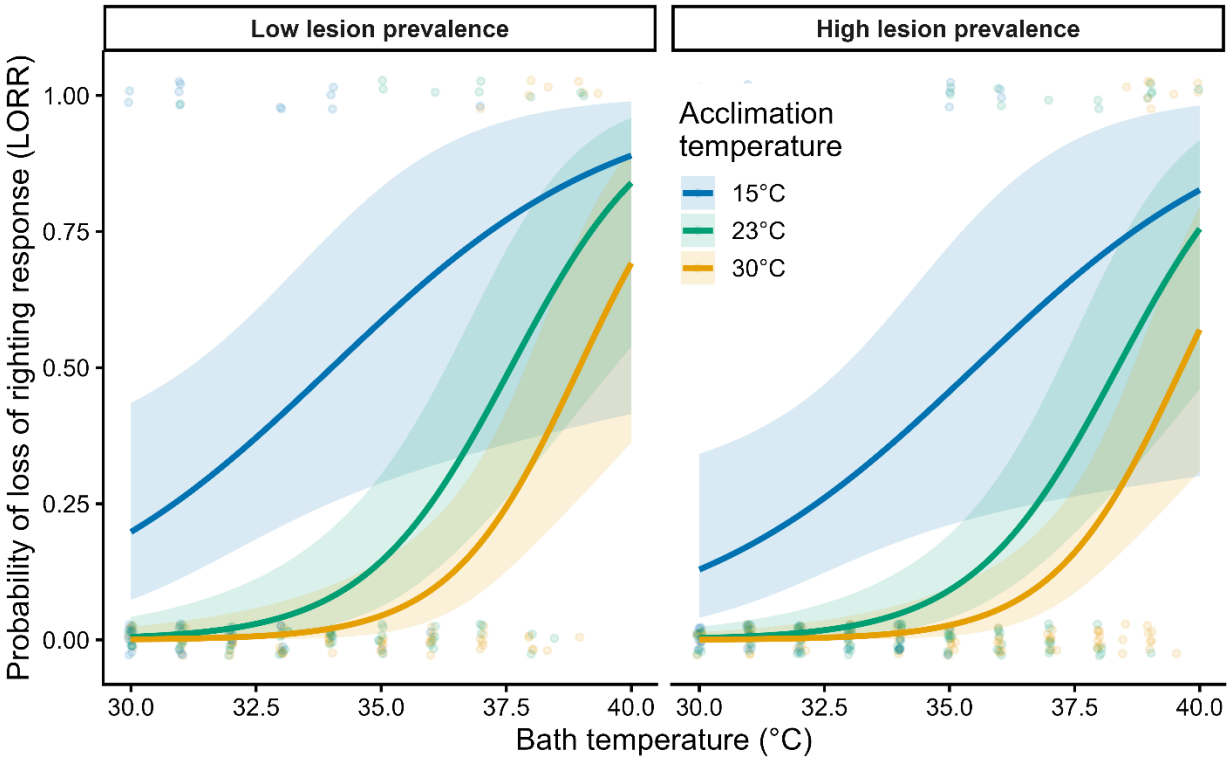


Figure 3.6. Predicted probability of loss of righting response (LORR) during the acute thermal ramp experiment for crayfish from low- and high-lesion-prevalence populations acclimated to 15, 23, or 30 °C prior to thermal exposure. Curves represent model-predicted probabilities from repeated righting-response measurements collected across increasing bath temperatures (30 – 40 °C), with shaded ribbons indicating 95% confidence intervals. Faded points represent individual binary observations (LORR present/absent). A total of 61 crayfish contributed 407 repeated observations.

Table 3.5. Results of a binomial generalized linear mixed-effects model evaluating factors influencing loss of righting response (LORR) during an acute thermal stress assay experiment. Fixed effects include bath temperature, acclimation temperature, lesion prevalence group, and their interactions, as well as assay identity. Estimates are presented as odds ratios with 95% confidence intervals. Bath temperature was mean-centered to facilitate interpretation of interaction terms. The model was fitted with a logit link and included a random intercept for individual crayfish to account for repeated measurements. Terms with p-values < 0.05 are highlighted in bold for reference; p-values reflect Wald tests from the full model.

Term	Odds ratio	95% CI	p-value
Bath temp (per 1°C increase)	2.05	[1.51, 2.80]	**<0.001**
Lesion prevalence: High (vs Low)	0.64	[0.33, 1.28]	0.208
Acclimation: 15°C (vs 23°C)	12.31	[4.02, 37.64]	<0.001
Acclimation: 30°C (vs 23°C)	0.19	[0.03, 1.27]	0.087
Assay: 2 (vs 1)	0.76	[0.34, 1.72]	0.515
Assay: 3 (vs 1)	0.88	[0.39, 2.00]	0.766
Bath temp × High-prevalence	0.95	[0.77, 1.17]	0.622
Table 3.5. Continued			
Bath temp × Acclimation 15°C	0.68	[0.46, 1.00]	**0.050**
Bath temp × Acclimation 30°C	1.17	[0.73, 1.89]	0.506

Crayfish acclimated to cooler temperatures exhibited loss of righting response at lower bath temperatures on average than those acclimated to warmer conditions, but the slope of failure across increasing bath temperatures was similar among treatments. There was weak evidence that lesion-prevalence history influenced probability of loss of righting response either independently ($\chi^2_1 = 0.01$, $p = 0.910$) or in interaction with bath temperature ($\chi^2_1 = 0.0002$, $p = 0.990$). Assay identity was included to account for minor variation in heating rate among blocks but provided weak evidence for additional variation in righting response ($\chi^2_2 = 0.37$, $p = 0.830$).

Delayed mortality following the acute thermal stress assay occurred in 10 of 61 crayfish (16.4%; Fig. 3.7), including individuals regardless of whether they reached loss of righting response (LORR) during the assay. Mortality increased with acclimation temperature (15 °C: 0/23; 23 °C: 2/21; 30 °C: 8/17). Firth-penalized logistic regression provided strong evidence that

acclimation temperature influenced delayed mortality of acclimation temperature on delayed mortality (likelihood ratio test, $p = 0.002$), whereas lesion-prevalence group provided weak evidence that lesion-prevalence group predicted mortality after accounting for acclimation temperature ($p = 0.380$). Estimated marginal means indicated the highest mortality probability in crayfish acclimated to 30 °C (0.46, 95% CI: 0.25–0.69), intermediate mortality at 23 °C (0.11, 95% CI: 0.031–0.32), and lowest mortality at 15 °C (0.021, 95% CI: 0.001–0.26). Pairwise comparisons showed higher delayed mortality in crayfish acclimated to 30 °C relative to 15 °C (Tukey-adjusted $p = 0.040$), whereas the comparison between 23 °C and 30 °C provided moderate evidence for higher delayed mortality ($p = 0.054$).

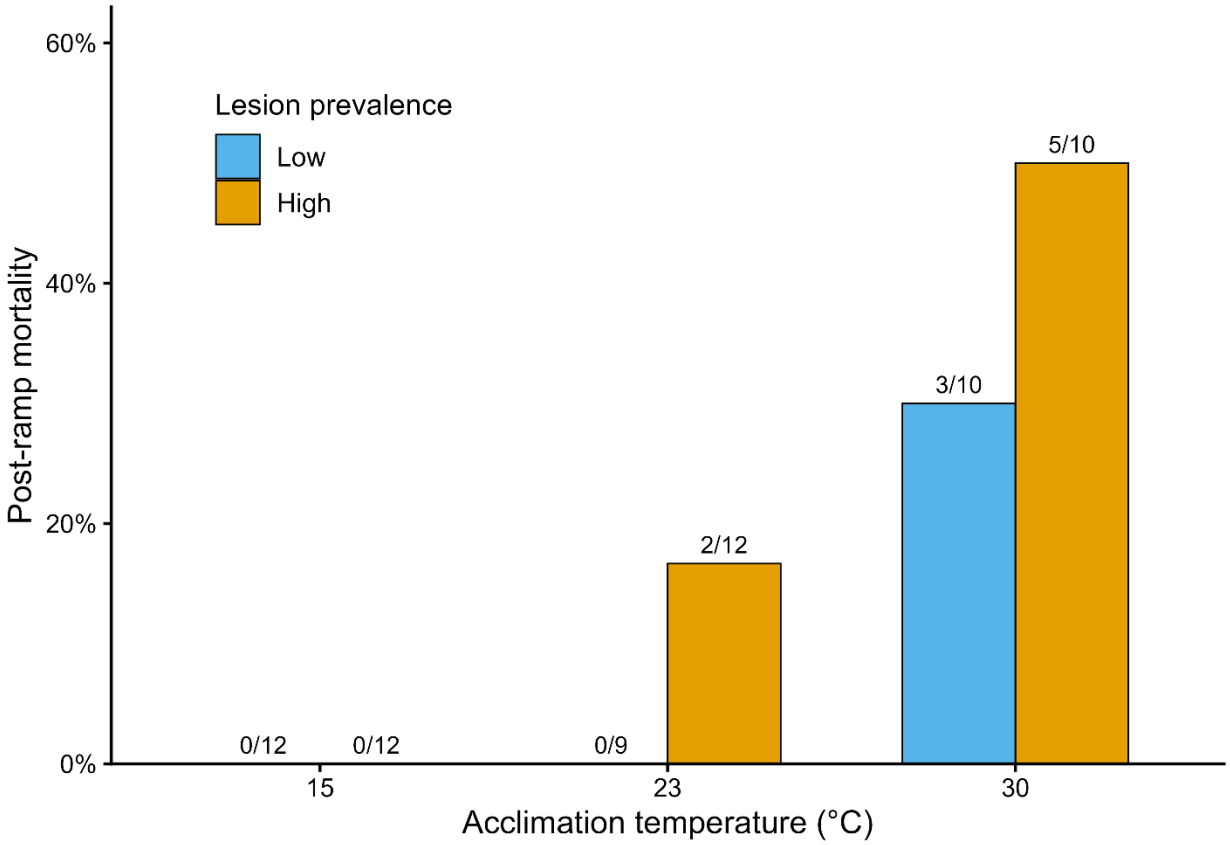


Figure 3.7. Delayed mortality of crayfish following an acute thermal ramp assay by acclimation temperature and lesion-prevalence group (n=65). Bars represent the proportion of individuals that died after the ramp.

Discussion

Understanding how environmental stressors interact with infectious disease is increasingly important in freshwater ecosystems, where warming temperatures and pathogen pressures are intensifying (Jackson et al. 2016; Birk et al. 2020). In this study, temperature emerged as the dominant driver of physiological performance in virile crayfish, while lesion-prevalence history was more strongly associated with sublethal outcomes than thermal limits. Because lesion-prevalence history was represented by only one population per category, population-level differences cannot be attributed solely to crayfish plague history and may also

reflect other unmeasured environmental or genetic differences between source populations. Across both chronic and acute experiments, temperature strongly affected survival, behavioral performance, and physiological tolerance. In contrast, disease history mainly structured lesion severity but had minimal effects on growth, upper thermal tolerance or mortality. Because crayfish grow discontinuously through molting, variation in growth and body condition may partly reflect molt stage and recent molting history rather than continuous somatic growth alone. These results suggest that temperature establishes the physiological boundaries within which crayfish can function, while crayfish plague history may shape how individuals experience stress within those limits. The two righting-response experiments measured different aspects of behavioral performance. The chronic exposure experiment quantified righting time as a sublethal indicator of behavioral impairment under stable temperatures, whereas the acute thermal ramp assay evaluated loss of righting response (LORR) during progressively increasing thermal stress as an indicator of upper thermal tolerance.

Temperature consistently determined the physiological limits of crayfish performance. Mortality increased with chronic warming, and the probability of losing the righting response rose predictably as bath temperature increased during the acute thermal assay. Contrary to our original prediction that crayfish from high-lesion-prevalence populations would exhibit reduced thermal tolerance, lesion-prevalence history did not alter the temperatures at which crayfish lost righting response or experienced delayed mortality. These responses align with fundamental principles of thermal ecology, which predict that ectotherm performance declines as environmental temperatures approach physiological limits (Huey and Kingsolver 2019; Sinclair et al. 2016). The delayed mortality that we observed in crayfish from the warmest acclimation

temperatures following acute heat exposure further indicated that physiological damage accumulated even after organisms lost behavioral function. Similar time-dependent effects of thermal stress have been documented in other ectotherms. For example, in laboratory studies, tobacco hornworm (*Manduca sexta*) larvae growth rates declined with prolonged exposure to heat, demonstrating that physiological consequences of thermal stress can accumulate through time (Kingsolver and Woods 2016). Although immune responses differ substantially between vertebrates and invertebrates, similar patterns of sublethal impairment during infection have been observed across diverse host–pathogen systems.

In contrast to the strong influence of temperature on physiological limits, population groups differing in field-observed lesion prevalence primarily differed in sublethal responses associated with tissue damage and performance. Crayfish originating from populations with historically higher lesion prevalence consistently exhibited greater lesion severity throughout the chronic experiment, although growth responses differed little among lesion-prevalence groups. Despite these differences in lesion severity, survival remained similar between lesion-prevalence groups across experiments. Disease history also did not clearly alter the temperatures at which crayfish lost the righting response during acute thermal stress. However, crayfish from high-lesion-prevalence populations exhibited somewhat greater delayed mortality following acute heat exposure, suggesting that disease-related physiological impairment may become more apparent after thermal limits are exceeded, even when immediate behavioral failure occurs at similar temperatures. Although limited sample sizes may have reduced power to detect subtle population differences. Comparable patterns occur in other host-pathogen systems, where infection increases energetic demand and causes sublethal impairment without necessarily shifting acute

thermal tolerance thresholds (Lafferty and Holt 2003; Rohr et al. 2011). For example, in amphibians infected by the fungal pathogen *Batrachochytrium dendrobatidis,* the infection can reduce host physiological condition and performance even when infected individuals survive environmental stressors (Rohr et al. 2011).

Lesion metrics revealed that warming influenced the expression of infection primarily through increases in lesion area rather than increases in lesion number. Lesion counts were consistently higher in the high-lesion-prevalence population and generally increased through time across most treatment combinations, although temperature treatment had little influence on lesion counts. These patterns differed somewhat from those observed in the marking experiment (Chapter 2), where lesion responses were more strongly associated with handling treatments than with temperature exposure. These differences likely reflect the distinct stressors imposed by the two experimental designs. This pattern suggests that elevated temperatures amplified lesion severity and disease expression rather than increasing infection prevalence itself. Increased metabolic demand under warming may partially explain this response. In ectotherms, rising temperatures elevate metabolic rates and can increase oxidative stress through greater production of reactive oxygen species (Sokolova 2021; Sokolova 2023; Rubalcaba et al. 2020). These physiological pressures can force trade-offs among immune activation, maintenance, and tissue repair (Huey and Kingsolver 2019; DeLong 2018; Messina et al. 2023; Larsen et al. 2018). Under such conditions, crayfish may allocate more energy toward immune responses and physiological maintenance, potentially reducing their ability to contain lesion progression or maintain cuticle integrity under prolonged thermal stress.

Patterns of lesion placement further suggest potential links between lesions and functional performance. Lesions were frequently concentrated on the abdomen, walking legs, and uropod regions, areas associated with locomotion and stability and, in females, reproduction through egg attachment to pleopodal structures. Although the functional consequences of these lesions were not directly assessed, lesion placement on these regions could have ecological implications for movement, reproductive success, or behavioral performance. In other host-pathogen systems, infection can alter locomotor behavior and ultimately contribute to mortality. For example, salmonid fishes infected with the parasite *Myxobolus cerebralis* develop "whirling disease," characterized by abnormal tail-chasing swimming behavior and impaired coordination that can lead to mortality within weeks of infection (Hedrick et al. 1998). Even subclinical infections can affect movement behavior. A tracking study found that birds infected with blood parasites exhibited reduced local movements and smaller foraging ranges despite showing no visible signs of illness (Grabow et al. 2024). Tissue damage in crayfish body regions associated with locomotion and stability may have functional consequences for movement or behavior, although these effects were not strongly reflected in righting-response performance in our study. Expansion of lesions represents cumulative tissue damage that could cause sublethal behavior changes, impair locomotion or increase susceptibility to secondary infections (Bouallegui 2021, Thomas et al. 2020; Boštjančić et al. 2023). More broadly, environmental stressors can reduce swimming speed and exploratory locomotion in aquatic organisms, and physiological stress can directly translate into impaired movement performance (Haney et al. 2020). Future work could investigate whether lesion placement directly affects locomotor performance, escape responses, or other behavioral traits that influence ecological interactions.

Results from the acute thermal ramp experiment showed that acclimation temperature strongly influenced both loss of righting response and delayed mortality following acute thermal stress. Because lesion-prevalence groups were represented by only one population each, differences attributed to lesion-prevalence history may also reflect unmeasured environmental or thermal history differences between populations. Thermal performance curves are not fixed but shift according to prior thermal exposure and acclimation conditions (Sinclair et al. 2016; Kingsolver and Woods 2016). Crayfish acclimated to warmer conditions maintained righting ability at higher temperatures than individuals acclimated to cooler conditions, indicating increased short-term thermal tolerance. Such plasticity suggests that ectotherms may partially buffer gradual warming through acclimation (Gunderson and Stillman 2015; Morley et al. 2019; Seebacher et al. 2014). However, rapid temperature changes such as during heatwaves may outpace acclimatory responses (Rohr et al. 2018; Morley et al. 2019), potentially exposing individuals to thermal stress before physiological adjustment is possible. Once failure began, individuals that were acclimated to the warmest conditions exhibited the most abrupt performance declines and experienced the highest delayed mortality. This pattern suggests a trade-off between increased thermal tolerance and reduced buffering capacity once physiological limits are exceeded. Similar trade-offs occur in other ectotherms, where acclimation to higher temperatures delays the onset of failure but increases vulnerability once thermal thresholds are surpassed (Kingsolver and Woods 2016). Acclimation-driven plasticity in thermal tolerance may contribute to the ecological success of invasive ectotherms like virile crayfish, whose physiological flexibility likely facilitates persistence across novel and rapidly changing environments.

Performance declines were also evident at the cooler end of the thermal spectrum. During the chronic exposure experiment, crayfish maintained at 15 °C exhibited slower righting responses by the end of the experiment than crayfish maintained at 23 °C and 30 °C. Ectotherm performance typically follows unimodal thermal performance curves, with peak performance occurring near an optimal temperature and declining toward both warmer and cooler extremes (Huey and Kingsolver 1989; Huey and Kingsolver 2019; Sinclair et al. 2016; Rezende et al. 2019). Reduced righting performance under cooler conditions likely reflects neuromuscular and metabolic constraints associated with temperatures below recent acclimation conditions. These findings are consistent with the climate variability hypothesis, which predicts that organisms adapted to variable environments may tolerate broad temperature ranges but still experience reduced performance when conditions fall outside recent acclimation windows (Vasseur et al. 2014; Morley et al. 2019). Consequently, both warming and cooling relative to recent thermal history can impair performance through different physiological mechanisms.

Importantly, several of the strongest responses observed in this study were not reflected in survival alone, demonstrating the importance of measuring sublethal responses. Warming increased lesion severity, altered behavioral performance, and produced delayed mortality following acute heat exposure. These findings emphasize that physiological resilience may decline before mortality becomes apparent. Thermal performance curves often differ across levels of biological organization, with behavioral traits frequently exhibiting narrower thermal breadth than survival (Rezende et al. 2019). As a result, sublethal responses such as impaired locomotion, tissue damage, and reduced growth or impaired locomotor performance may provide earlier indicators of environmental stress than survival metrics alone. Similar approaches are

widely used in fish stress physiology, where growth, condition, and behavioral performance are considered integrative indicators of chronic stress and physiological impairment (Sopinka et al. 2016). Similar conclusions have emerged from studies of interacting environmental stressors, where sublethal responses reveal biological consequences that remain undetected when mortality alone is measured (Greenspan et al. 2017; Hector et al. 2021; Campbell et al. 2019; Li et al. 2024).

Because crayfish function as ecosystem engineers and play key roles in freshwater food webs, lethal and sublethal impairments related to global change may have consequences that extend beyond individual physiology. Changes in locomotor performance, tissue integrity, and condition can influence foraging behavior, predator avoidance, and interactions with other species, potentially altering ecosystem processes even when mortality remains low. More broadly, these findings highlight the importance of integrating thermal history, disease context, and sublethal performance metrics when predicting organismal responses to environmental change. In freshwater ecosystems experiencing simultaneous warming and pathogen pressure, temperature may determine physiological limits, but disease can shape how those limits are experienced through changes in lesion severity, growth, and behavioral performance before population-level consequences become visible.

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CHAPTER 4

THESIS CONCLUSION

The findings of this thesis demonstrate that environmental stressors and disease history interact to shape crayfish performance in ways not fully captured by survival metrics alone. In Chapter 2, marking method and lesion-prevalence history influenced survival, molting success, and lesion progression under stable laboratory conditions, suggesting that routine handling methods can impose context-dependent costs. In Chapter 3, temperature emerged as the dominant physiological stressor, while lesion-prevalence history was more strongly associated with sublethal responses such as lesion severity, body condition, and delayed mortality following acute thermal stress. Importantly, many of these effects occurred without widespread immediate mortality, suggesting that virile crayfish may be relatively resilient to chronic pathogen exposure while still experiencing sublethal physiological consequences under environmental stress.

Sublethal responses may provide earlier or more sensitive indicators of environmental stress than mortality alone. In fishes and other aquatic organisms, whole-organism metrics such as growth, body condition, behavior, and disease severity are commonly used to assess chronic stress because they integrate physiological responses across longer time scales and may influence ecological performance before outright mortality occurs. In the present study, crayfish survival remained relatively high across most treatments, yet lesion severity and righting performance revealed measurable effects of thermal stress and disease history. These findings suggest that sublethal responses may better capture early physiological consequences of interacting stressors in chronic host–pathogen systems.

Crayfish are influential ecosystem engineers that modify sediment structure, alter benthic habitats, and shape invertebrate communities through burrowing and foraging. They also serve as prey for many game fishes. Sublethal impairments associated with disease history and warming temperatures may therefore scale beyond individual performance, potentially altering sediment dynamics, habitat structure, and trophic interactions even when survival remains high. Freshwater invertebrates remain comparatively understudied in disease ecology despite their outsized role in ecosystem function. By integrating disease history, physiological performance, and environmental context, this thesis advances a more mechanistic understanding of how endemic pathogens and climate warming may jointly influence freshwater communities. Ultimately, these findings underscore that survival alone is an insufficient metric for assessing ecological resilience; subtle changes in condition and recovery capacity may signal emerging shifts in ecosystem function long before visible collapse occurs.

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APPENDICES

APPENDIX A

CHAPTER 2 SUPPLEMENT

Experimental crayfish source locations

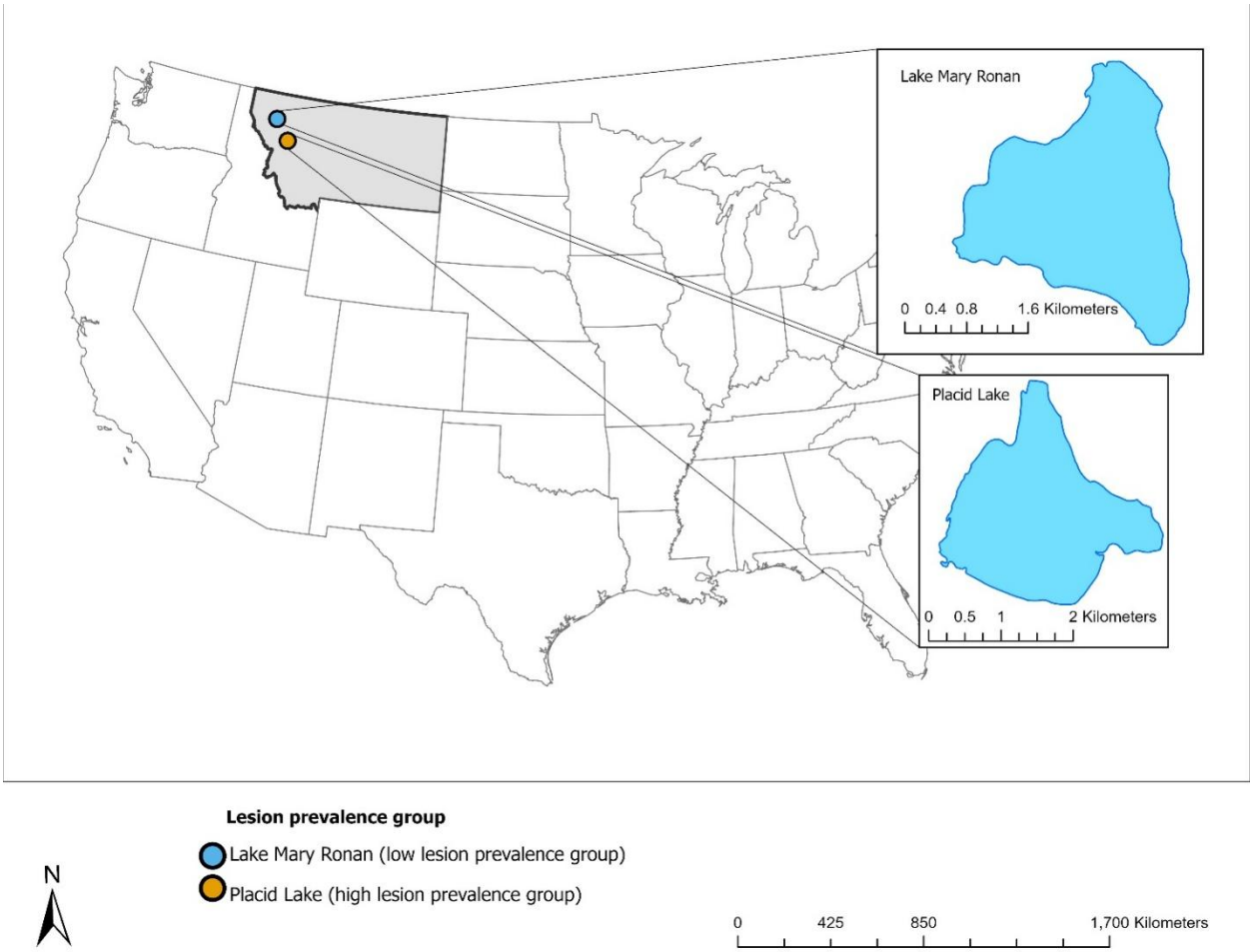


Figure A1. Map of USA with Montana in grey and circles indicating locations of the two *F. virilis* source populations: Lake Mary Ronan (low-lesion-prevalence history group, blue) and Placid Lake (high-lesion-prevalence history group, orange). Lake diagrams shown in insets.

Lesion Categorization

Table A1. Qualitative crayfish plague lesion severity scoring system used during field collection to characterize lesion severity on individual crayfish. Counts represent the number of crayfish observed in each category at the time of collection. This rubric was used to describe lesion severity and to ensure a range of lesion severities were represented in subsamples sent for pathogen confirmation. Population-level lesion prevalence groups were assigned independently based on prior field observations and monitoring rather than on these qualitative severity categories.

Lesion severity	Description	High-lesion-prevalence history (Placid Lake)	Low-lesion-prevalence history (Lake Mary Ronan)
Minor	Lesions few and small, not obviously extending through cuticle or causing deformations in body parts or holes in the cuticle.	31	3
Moderate	Lesions larger OR more abundant OR barely extended through the carapace	9	0
Severe	Many or extensive lesions OR eroded through carapace OR deformed body parts	5	0
No lesions	No lesions or deformities observed	10	32

Experimental Shelf Effects

HOBO® Pendant temperature/light data loggers (Onset Computer Corporation, Bourne, Massachusetts, USA) recorded temperature and light every 15 min on each of the 8 shelves for 7 days after the experiment ended and crayfish were removed from their containers. Data were averaged to daily means and analyzed using one-way ANOVAs to test for shelf differences (Figure S1).

Mean daily temperature and light differed significantly among shelves when analyzed across 64 shelf-by-day observations (temperature: $F_{7,56} = 25.55$, $p < 0.001$; light: $F_{7,56} = 10.12$, $p < 0.001$; Fig. S1). However, shelf-level environmental variation within days was limited. On any given day, individual shelves deviated from daily mean conditions by no more than 2.7 °C in temperature and 20.8 lux in light. Mean daily light levels were low across all shelves (< 8 lux), and even the brightest shelf on the brightest day remained extremely dim relative to typical indoor lighting (>300 lux) or natural daylight (>1,000 lux). Relative to experimental treatments, any shelf-level environmental effects likely had negligible effects on crayfish performance.

Treatments were housed by shelf for logistical reasons and to prevent cross contamination of water that could carry plague; therefore, shelf and treatment were confounded in survival, growth, and molting, and shelf was not included in analyses. For lesion area and count analyses, the experiment had enough replication across shelves to model shelf effects explicitly as a random factor.

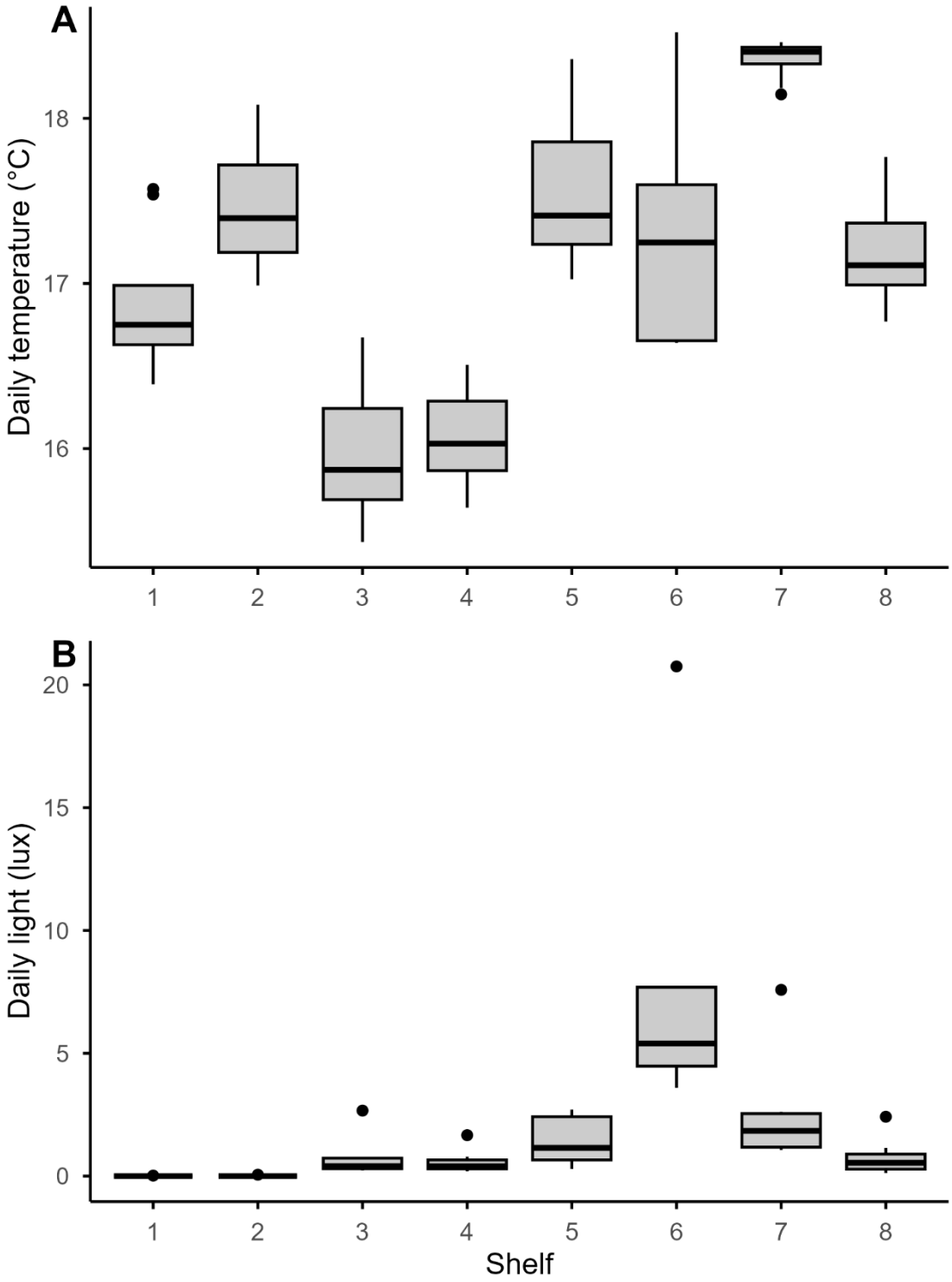


Figure A2. Daily environmental conditions by shelf for 7 days post experiment. (A) Temperature and (B) Light intensity (lux) were calculated from 15-min HOBO logger readings averaged per shelf per day.

Sensitivity Analysis: Treatment × Lesion-Prevalence History Group Interaction

We evaluated whether marking treatment effects on molting probability differed between lesion-prevalence history groups by fitting a binomial GLM with a treatment × lesion-prevalence history group interaction and cluster-robust standard errors (clustered by individual). This interaction was not supported, and the model produced unstable parameter estimates (very wide or non-estimable confidence intervals) due to quasi-complete separation associated with near-100% molting probabilities for control and elastomer crayfish in the low-lesion-prevalence history group. Predicted probabilities indicated that punch marking reduced molting probability in both lesion-prevalence history groups, consistent with the additive model reported in the main text (Table A2).

Table A2. Molting probability per visit: Treatment x lesion prevalence history group interaction model. Binomial GLM with cluster-robust standard errors (clustered by individual crayfish). Odds ratios are reported with 95% confidence intervals.

Term	OR	95% CI (low)	95% CI (high)	p-value
Intercept (End, Control, High-lesion)	1.42	0.32	7.35	0.645
Elastomer vs Control	5.35	0.53	125.13	0.189
Punch vs Control	0.14	0.02	0.75	0.032
Low vs High-lesion-prevalence history	1.12×10^{8}	0.00	NA	0.995
Start vs End	19.84	3.92	144.44	<0.001
Middle vs End	2.64	0.51	16.17	0.259
Elastomer × Low-lesion-prevalence history	0.22	0.00	2.03×10^{44}	1.000
Punch × Low-lesion-prevalence history	0.00	NA	3.02×10^{91}	0.995

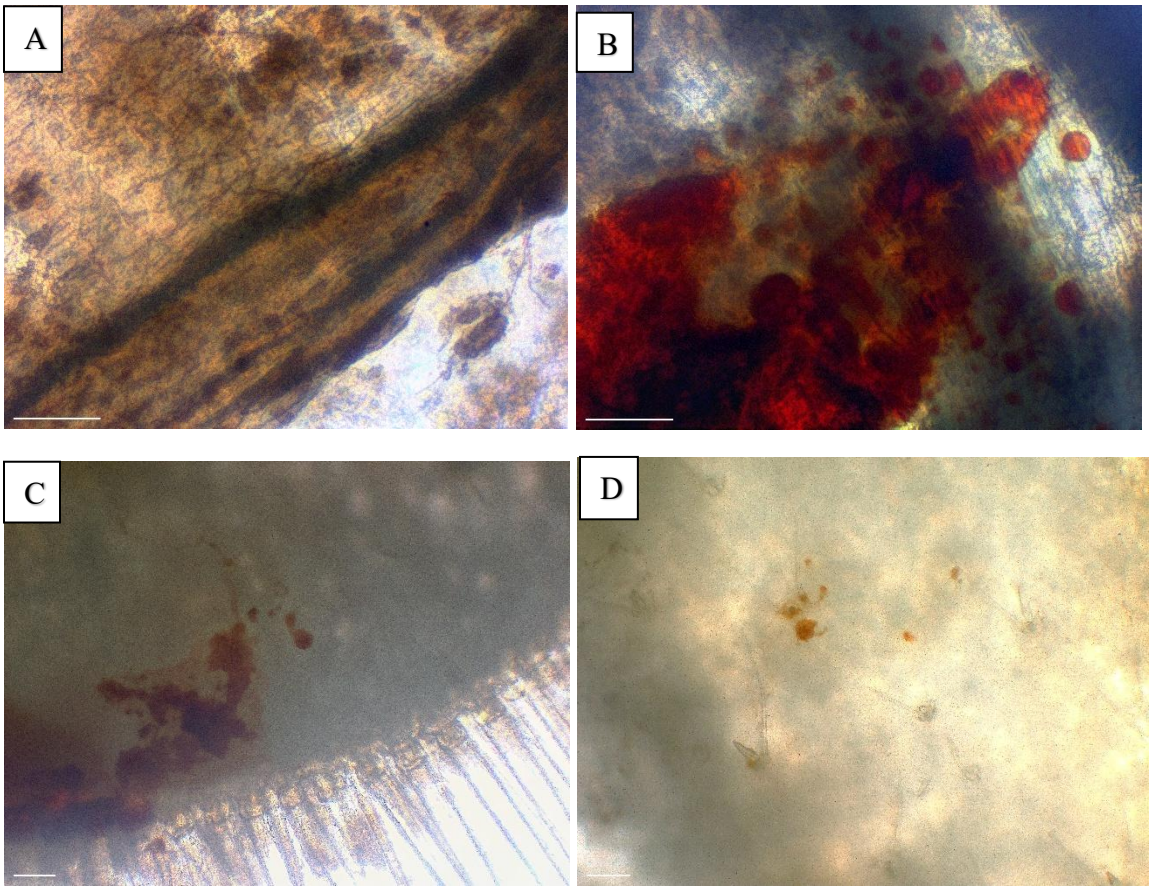
Table A3. Predicted molting probabilities from the interaction model averaged over time points.

Lesion-Prevalence History	Treatment	Predicted probability	95% CI
High	Control	0.84	0.60–0.95
High	Elastomer	0.97	0.77–1.00

Table A3. Continued

High	Punch	0.42	0.21–0.67
Low	Control	1.00	1.00–1.00
Low	Elastomer	1.00	1.00–1.00
Low	Punch	0.34	0.14–0.62

Genetic and molecular analysis F. virilis and A. astaci



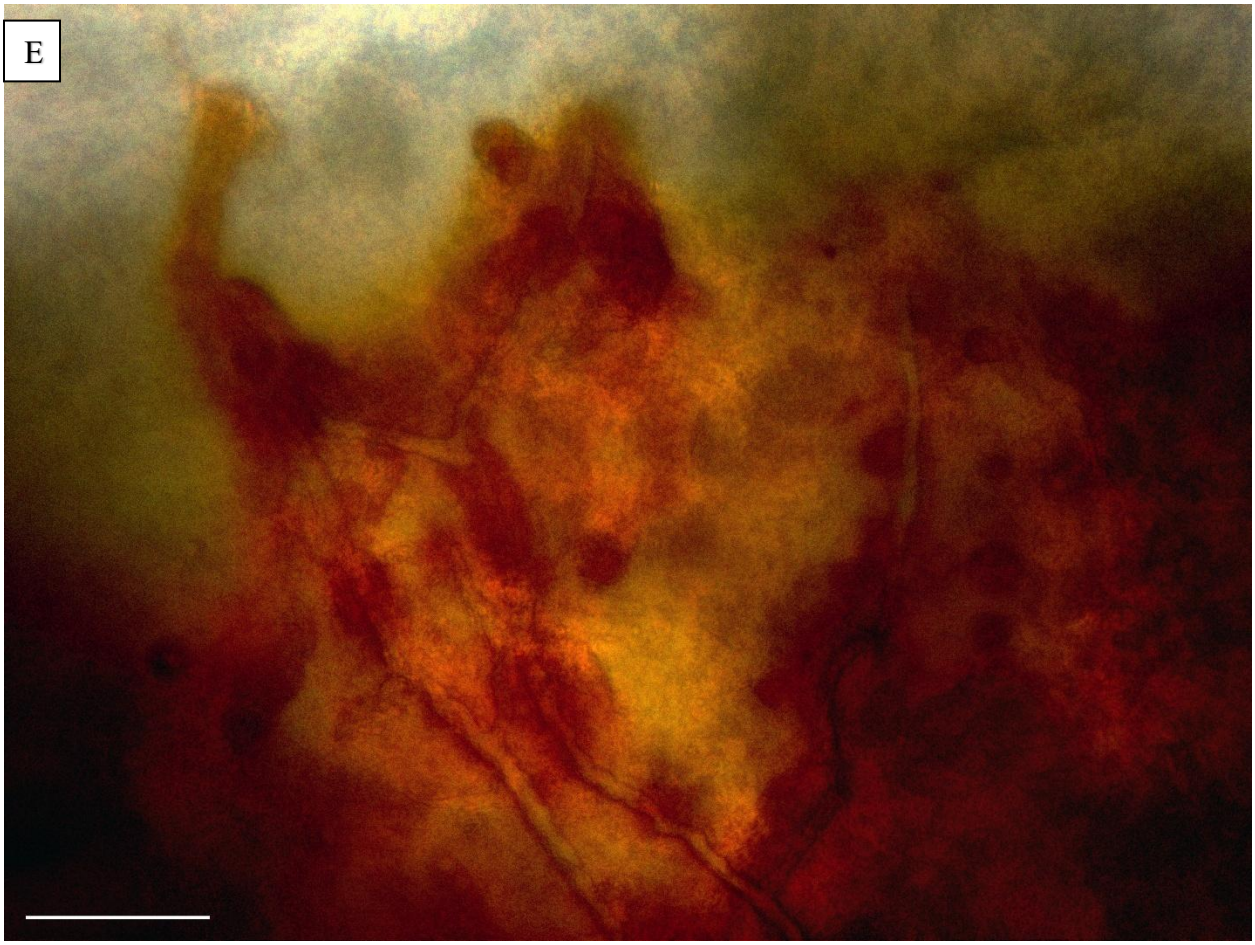


Figure A3. Microscopic visualization of macroscopic lesions associated with *F. virilis* populations in Montana (USA). Panels A and B are from Lake Mary Ronan (low-lesion-prevalence history group), and panels C, D and E are from Placid Lake (high-lesion-prevalence history group). The strong immune response characteristic of the acute infection stage of A. astaci is evident as a uniform layer of melanin deposits enveloping the hyphal structures. Micrographs illustrate melanized hyphae in lesions located on multiple body locations: (telson (C, D, E), carapace, and cephalothorax (A, B). Scale bar = 100 µm.

Faxonius Virilis Genetic Diversity: DNA Extraction and Amplification

To determine that the two source populations used in this experiment were genetically identical, each crayfish was dissected individually, with dissection tools sterilized between specimens to prevent cross-contamination. A piece of the muscle tissue from the tail was preserved in 2-ml tubes with 100% ethanol and labeled with the individual crayfish ID number

until processed at the Laboratory of Molecular Systematics at the Real Jardín Botánico-CSIC, Madrid, Spain.

Samples were rehydrated from ethanol into TE buffer (TRIS 10 mM/ EDTA 1 mM, pH 8) through three 1-hour rinses followed by an overnight wash. Tissue samples were homogenized by bead beating using TissueLyser and genomic DNA was extracted using the E.Z.N.A. Insect DNA Kit (Omega BIO-TEK, Norcross, Georgia, USA) according to the manufacturer's instructions.

Mitochondrial *16S* and *COI* genes were used as markers in this study for detecting intraspecific genetic variation among and within crayfish populations. The primers pair used to amplify the mitochondrial *16S rRNA* gene was 16Sar-L and 16Sbr-H (Palumbi et al., 1991) and the primer pair used to amplify the mitochondrial *Cytochrome c oxidase subunit I (COI)* gene was LCO1490 and HC02198 (Folmer et al., 1994).

Both primers pairs were used in a single round PCR following the protocols described as follows, including negative and positive controls. To generate the 16S sequences, we amplified this region via polymerase chain reaction (PCR) in 25 µl volumes using the MyTaq™ HS DNA Polymerase (Bioline, Meridian), and a touchdown PCR profile as follows: initial denaturation at 96ºC for 5 min followed by denaturation at 96ºC for 30 seconds, annealing at 54ºC /-2ºC per cycle for 30 seconds, and extension at 72ºC for 45 seconds (five cycles), then denaturation at 96ºC for 30 seconds, annealing at 52ºC for 45 seconds, and extension at 72ºC for 45 seconds (30 cycles), with a final extension at 72ºC for 10 min, and hold at 10ºC. To generate the COI data, we amplified this region via polymerase chain reaction (PCR) in 25µl volumes using the MyTaq™ HS DNA Polymerase (Bioline, Meridian), and a PCR profile as follows: initial denaturation at

96ºC for 5 min followed by denaturation at 96ºC for 30 seconds, annealing at 50ºC for 1 minute, and extension at 72ºC for 45 seconds (30 cycles), with a final extension at 72ºC for 10 min, and hold at 10ºC.

Three microliters of aliquots of the amplification products were analyzed to check positive amplicons in 1% agarose TAE gels stained with SBYRSafe (Thermo Fisher Scientific, Waltham, MA, USA). The amplified products were purified using a QIAquick PCR Purification Kit (Qiagen, Germany). Double-stranded PCR products were sequenced using an automated sequencer (Applied Biosystems 3730xl DNA Analyzer, Secugen at Madrid, Spain). Sequences were assembled and edited using the program Geneious 10.0.280, and BLAST search was performed on each sequence to verify its identification with the species *Faxonius virilis*.

Genetic Diversity and Phylogenetic Analyses

To determine the genetic identity of the sampled crayfish populations, we performed phylogenetic analyses with the obtained sequences.We used the Bayesian Inference (BI) phylogenetic approximation, implemented in MrBayes v.3.2.6 and and Maximum Likelihood (ML), implemented in in RAxML v.8 (via raxmlGUI v1.5b). Phylogenetic analyses were run individually for both genes (*i.e.*, the *16S* the *COI* mtDNA genes), and combined (*i.e.*, the concatenated genes). BI analyses were conducted with 10 million MCMC generations, two runs (four chains each), 25% burn-in, and convergence defined by split frequencies <0.01; nodes with posterior probability ≥0.95 were considered supported. ML analyses were performed with 100 independent replicates and 1,000 rapid bootstraps; nodes with bootstrap support ≥75 were considered robust.

Sequences of the mitochondrial *16S* and *COI* genes from previous studies from Montana crayfish were included (*Susie Adams unpulished data*) and mitochondrial *16S* and *COI* sequences from *Cambarus robustus* were used as the outgroup (OM736878 GenBank access number).

Nodes with posterior probabilities (pp) values greater than 0.95 in BI and with nodes with bootstrap support ≥75 in ML were considered supported. Resulting phylogenetic trees were visualized using FigTree v.1.4.2.

Aphanomyces astaci and lesions

Microscopic Examination of Lesions

Each crayfish was dissected individually, with dissection tools sterilized between specimens to prevent cross-contamination. Lesions were labeled with their anatomical location and the individual crayfish ID, and tissue fragments were preserved in 2-ml tubes with 100% ethanol.

Samples were examined under an inverted microscope to detect the presence of growing hyphae. Light micrographs of lesions and melanized hyphae were captured using a QImaging Micropublisher 5.0 digital camera (QImaging, Burnaby, BC, Canada), and image stacking and processing were conducted with Zerene Stacker software (Zerene Systems, www.zerenesystems.com/cms/home).

Each sample was then divided into two subsamples in 2 ml tubes: one for molecular analyses and one retained as backup.

Molecular Analyses

Genomic isolation, PCR amplification, and sequencing

Samples selected for molecular identification were rehydrated from ethanol into TE buffer (TRIS 10 mM/ EDTA 1 mM, pH 8) through three 1-hour rinses followed by an overnight wash. They were homogenized manually, and genomic DNA was extracted using the E.Z.N.A. Insect DNA Kit (Omega BIO-TEK, Norcross, Georgia, USA) according to the manufacturer's instructions.

Presence of *Aphanomyces astaci* was tested by amplifying a fragment of the ITS region using diagnostic primers 42F and 640R (which amplify ITS1, the 5.8S rDNA and ITS2) (Oidtman et al. 2004, 2006). A pure culture of *A. astaci* served as the positive control and sterile Milli-Q water as the negative control. PCR products were checked on 1% agarose gels stained with SYBR Safe, and then purified with ExoSAP-IT, and sequenced bidirectionally on an automated sequencer (Applied Biosystems 3730xl DNA Analyzer, Secugen at Madrid, Spain). Sequences were aligned and edited in Geneious 10.0.280, and their identity was verified via BLAST searches (NCBI database).

Samples tested positive for *A. astaci* were used to determine the genetic diversity of *A. astaci* (i.e., reconstruction of the phylogenetic relationships and haplotype assignment). The mitochondrial ribosomal small (rnnS) and large (rnnL) subunits were amplified using the primer pairs regions described by Makkonen et al. (2018), using the same positive and negative control scheme described above. PCR products were processed and sequenced as described previously.

Phylogenetic relationships were inferred using Bayesian Inference (BI) and Maximum Likelihood (ML). BI analyses were conducted in MrBayes v3.2.6 with 10 million MCMC generations, two runs (four chains each), 25% burn-in, and convergence defined by split frequencies <0.01; nodes with posterior probability ≥0.95 were considered supported. ML analyses were performed in RAxML v.8 (via raxmlGUI v1.5b) with 100 independent replicates and 1,000 rapid bootstraps; nodes with bootstrap support ≥75 were considered robust. Trees from both approaches were visualized in FigTree v1.4.2.

Sequences of rnnS and rnnL from previous studies (Makkonen et al., 2018, Martín-Torrijos et al., 2018, Martín-Torrijos et al., 2019, Panteleit, et al., 2019, Casabella-Herrero et al., 2021, Martín-Torrijos et al. 2021; Martín-Torrijos et al., 2023) were included, and *Aphanomyces fennicus* was used as the outgroup. Analyses were conducted separately for rnnS and rnnL and also for a concatenated dataset using the same parameters described above.

Lesion Area

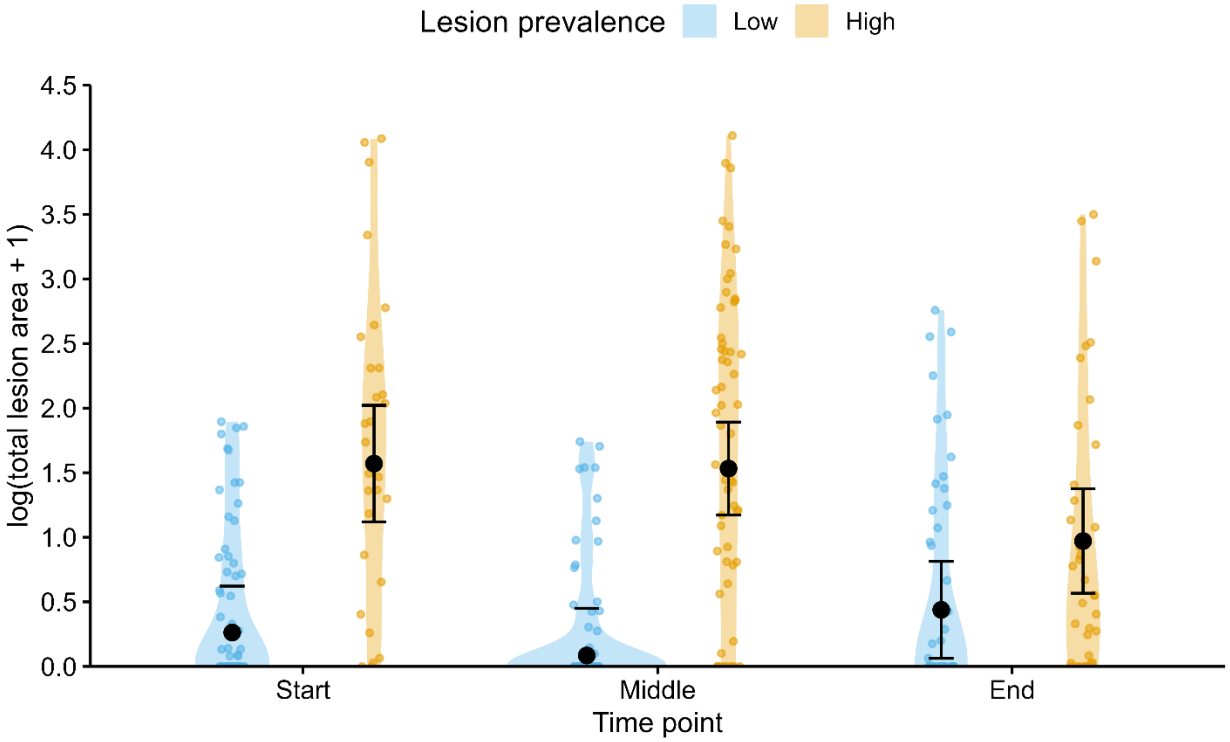


Figure A4. Violin plots showing the distribution of crayfish-level observations of log(total lesion area + 1) across time points and lesion-prevalence history groups. Each point represents one crayfish observation at a given sampling time, and violins summarize the distribution of repeated individual observations within each group. Solid black points indicate model-estimated means and bars show 95% confidence intervals. The analysis included 287 crayfish-by-time-point observations from 60 crayfish.

Lesion Count

Table A4. Fixed effects from the lesion count model (negative binomial GLMM).
Values are expressed as incidence rate ratios (IRRs = exp β) with 95% confidence intervals.
Significant effects are bold. The analysis included 247 observations from 60 crayfish.

Predictor	IRR	95% CI low	95% CI high	p-value
Intercept (Low, Start, Control)	10.64	2.92	38.79	**0.000**
Time (linear) in Low	1.42	0.97	2.09	0.073
Time (quadratic) in Low	2.07	1.37	3.12	**0.001**
High vs Low (Start)	3.79	2.49	5.77	**0.000**
Crayfish length (mm)	0.95	0.93	0.98	**0.000**

Table A4. Continued

Elastomer vs Control	0.99	0.59	1.65	0.971
Punch vs Control	1.24	0.74	2.10	0.415
Time (linear) × High	0.46	0.27	0.77	**0.003**
Time (quadratic) × High	0.45	0.27	0.76	**0.003**

Table A5. Results of a Cox proportional hazards model evaluating whether lesion area, lesion prevalence history group, or marking method predicted time to mortality in crayfish. Although hazard ratios were directionally consistent with observed mortality patterns (higher hazard for punch-marked crayfish and lower hazard for elastomer-marked crayfish), none of the predictors reached statistical significance, likely reflecting limited power due to small sample size and few mortality events. Inference regarding marking effects on survival is therefore based primarily on mortality proportions and Firth penalized logistic regression results (Table 2.2).

Predictor	Hazard Ratio	95% CI	p-value
Lesion area (last observed)	1.03	0.99–1.07	0.12
High-lesion-prevalence history (vs. Low)	1.15	0.67–1.98	0.61
Punch (vs. Control)	2.12	0.78–5.68	0.14
Elastomer (vs. Control)	0.52	0.16–1.69	0.27

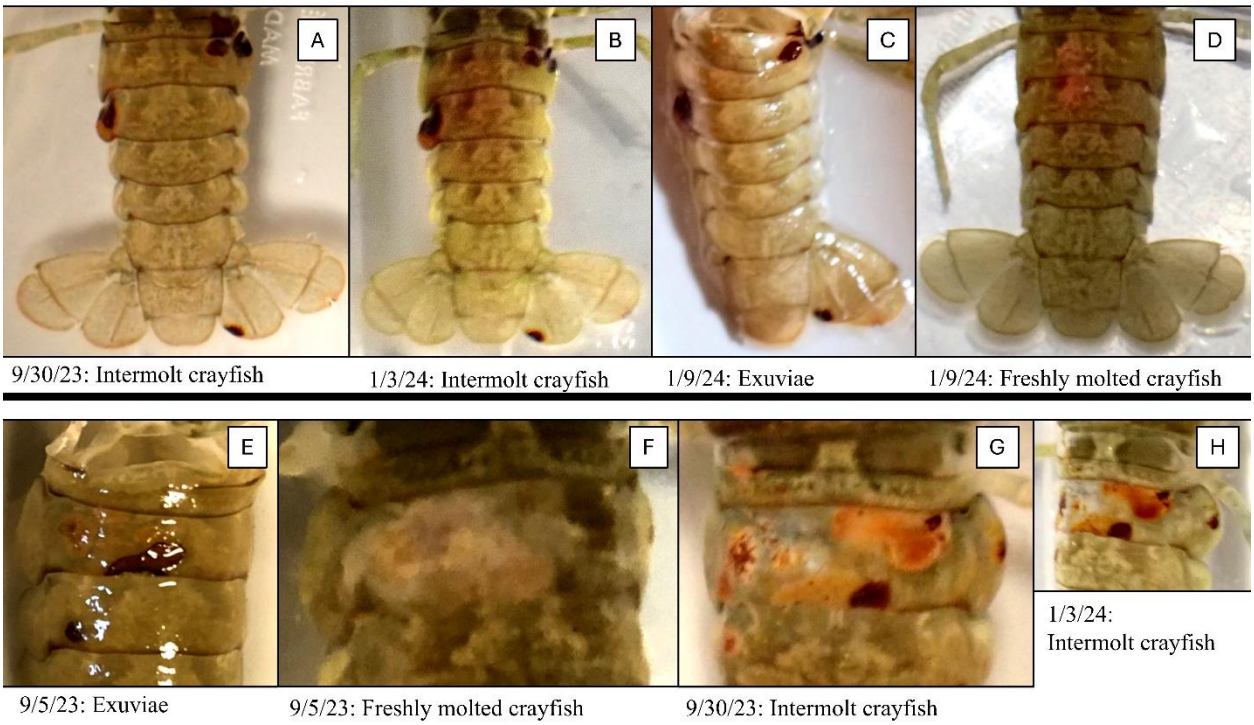


Figure A5. Lesion progression on 2 different crayfish that molted over time (top row is one crayfish and bottom row is a different crayfish). Representative examples of crayfish during

different molting states and lesion expression observed during the experiment. Panels A and B show intermolt crayfish photographed on 30 September 2023 and 3 January 2024, respectively. Panel C shows an exuviae collected on 9 January 2024, and panel D shows a freshly molted crayfish photographed immediately after molting on the same date. Panels E and F show an exuviae and a freshly molted crayfish photographed on 5 September 2023. Panels G and H show examples of lesion expression on intermolt crayfish collected during the experiment (30 September 2023 and 3 January 2024). These images illustrate typical visual characteristics used to distinguish intermolt individuals, recently molted individuals, and shed exuviae during monitoring.

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APPENDIX B

CHAPTER 3 SUPPLEMENT

Experimental crayfish source locations

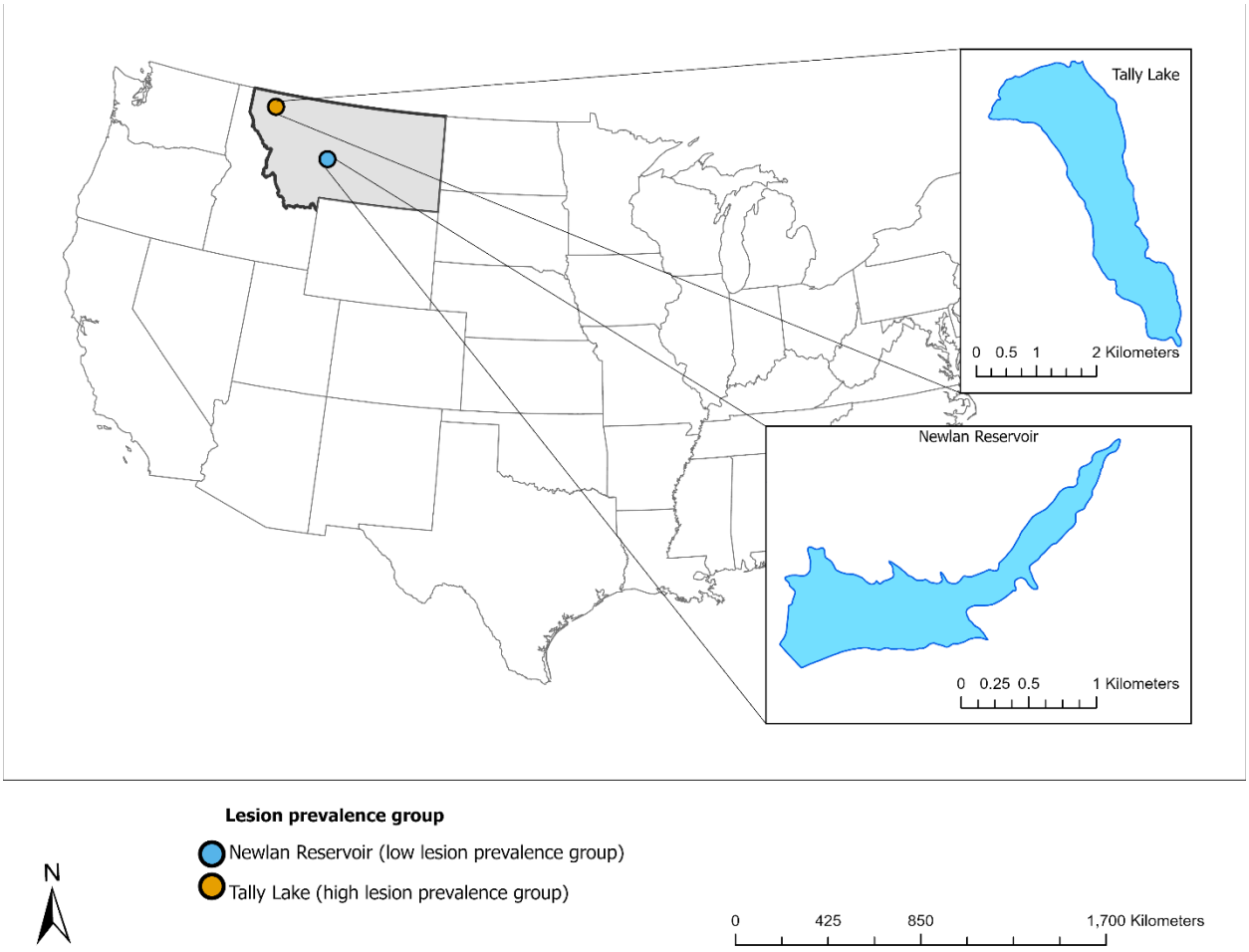


Figure B1. Map of USA with Montana in grey, insets show the two *F. virilis* crayfish population sources at Newlan Reservoir (low-lesion-prevalence group, blue) and Tally Lake (high-lesion-prevalence group, orange).

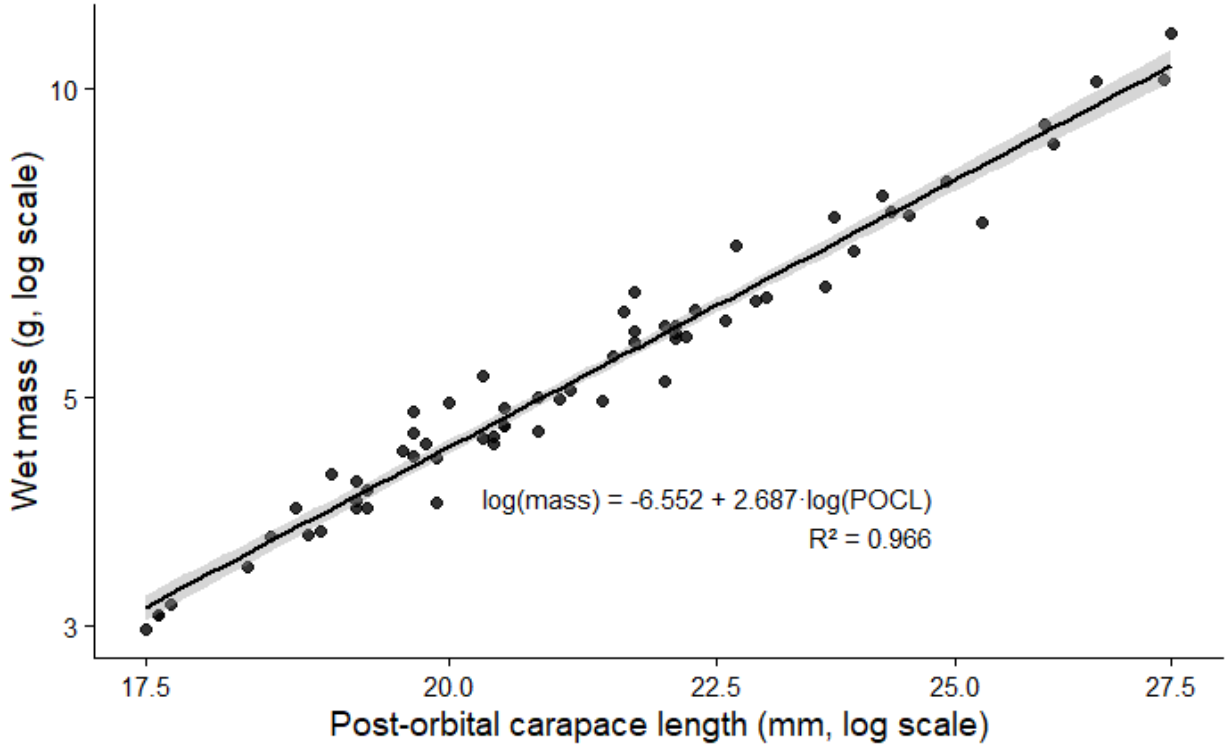


Figure B2. Log-log relationship between post-orbital carapace length (POCL) and wet mass for crayfish in the chronic thermal exposure experiment (n = 64). A linear regression fitted to log-transformed values (solid line; 95% CI shaded) showed strong allometric scaling ($\log(\text{mass}) = -6.552 + 2.687 \cdot \log(\text{POCL})$, $R^2 = 0.966$). This relationship was used to derive a size-independent body condition index from the regression residuals.

Temperature control

In early October, substantial bacterial growth developed in several baths, and filter socks containing activated charcoal were added to all pumps to improve water quality. This

modification temporarily altered water circulation and produced short-term temperature fluctuations detectable in the HOBO logger records.

Heated baths experienced gradual water loss due to evaporation, and room-temperature water was added as needed to maintain water levels. These additions produced brief decreases in bath temperature followed by gradual warming as heaters restored target conditions. Beginning on 16 October, bath temperatures were measured daily using a handheld digital thermometer or the Hygger heater temperature display located on the side opposite the heating element. These measurements were used to track treatment temperatures throughout the experiment.

Daily temperature records indicated that all three temperature treatments remained close to their intended targets during the study (Table B1; Fig. S3). The 15°C baths averaged 15.61°C (range 15.37-15.98°C) and the 23°C baths averaged 22.95°C (range 22.61-23.30°C), both with low day-to-day variation (mean SD ≈ 0.38-0.39°C). The 30°C treatment averaged 29.42°C (range 28.77-29.75°C) but exhibited slightly greater variability (mean SD = 0.73°C). Several brief low-temperature excursions occurred in a subset of baths due to pump or heater issues

documented during the study. Overall, deviations from target temperatures were typically within ±1-2°C, indicating that the thermal environment was well maintained (Fig. S3).

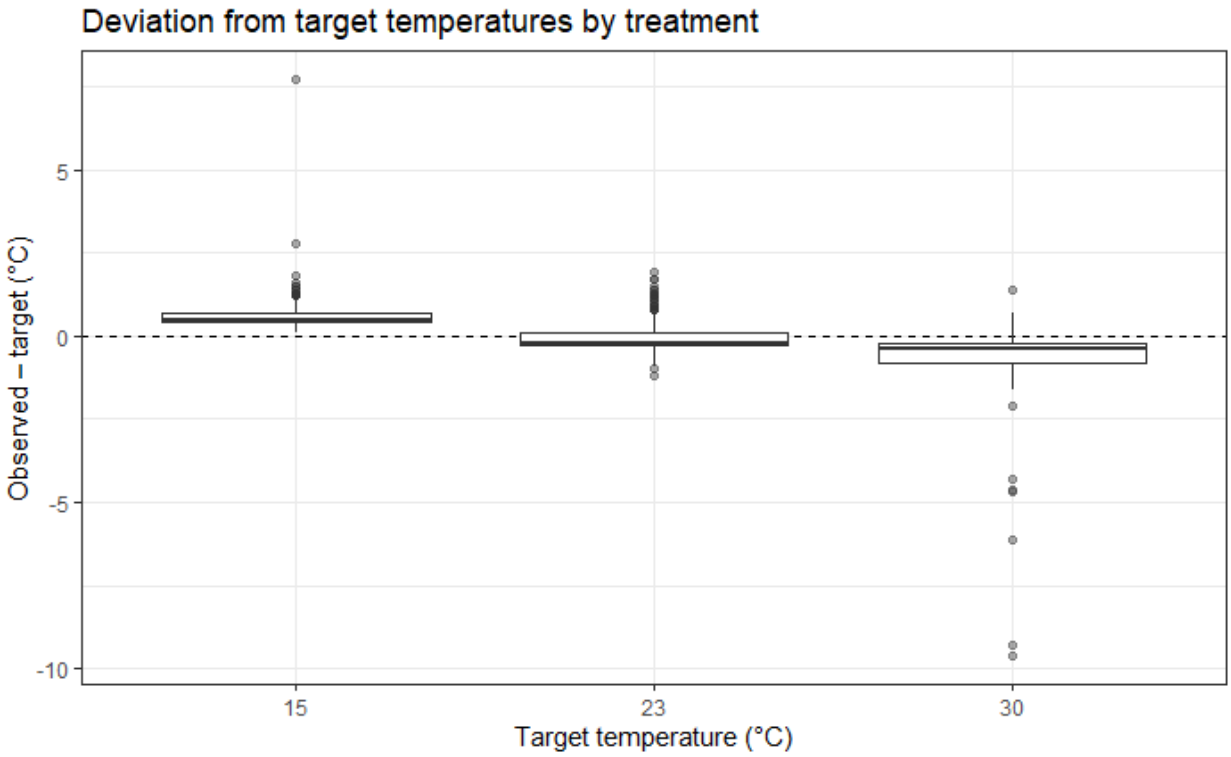


Figure B3. Deviation of observed water temperatures from target setpoints across experimental temperature treatments (15, 23, and 30 °C) for chronic thermal exposure experiment. Values represent the difference between observed and target temperatures (observed − target, °C), with the dashed line indicating perfect agreement (0 °C deviation). Boxes show median and interquartile range, whiskers represent 1.5× the interquartile range, and points indicate individual observations. Temperature control was tight at 15 °C and 23 °C, while greater variability and negative deviations were observed at the 30 °C treatment.

HOBO Data Loggers

Temperature

HOBO temperature loggers were deployed in a subset of coolers (two per temperature treatment) and as ambient room sensors to provide continuous monitoring of thermal conditions during the experiment. Ambient room temperatures recorded by HOBO loggers ranged from 18.3

to 27.9°C (mean ≈ 24°C) during the experimental period. These continuous measurements corroborated daily thermometer records and confirmed that bath temperatures remained close to their intended treatment values.

Light

Light intensity within baths was generally low to moderate and showed clear diel cycling consistent with the programmed 12 h light: 12 h dark photoperiod. Mean light levels ranged from 2.6-15.2 lum/ft² among baths, and distributions overlapped across the 15, 23, and 30°C treatments. Because photoperiod was identical across treatments and light intensity did not vary systematically with temperature treatment, light was not included as a covariate in subsequent analyses.

Summary

Together, daily thermometer records and continuous HOBO logger measurements indicate that temperature treatments were maintained close to their intended setpoints throughout the experiment. Minor deviations were brief and associated with documented equipment malfunctions that were quickly repaired.

Table B1. Summary statistics of observed water temperatures across experimental temperature treatments. Values represent the mean of daily mean temperatures ("Mean of Means"), the minimum and maximum of those daily means, and the mean standard deviation (SD) across sampling periods for each target temperature treatment.

Target Temperature (°C)	Mean of Means	Minimum Mean	Maximum Mean	Mean SD
15	15.61	15.37	15.98	0.38
23	22.95	22.61	23.30	0.39
30	29.42	28.77	29.75	0.73

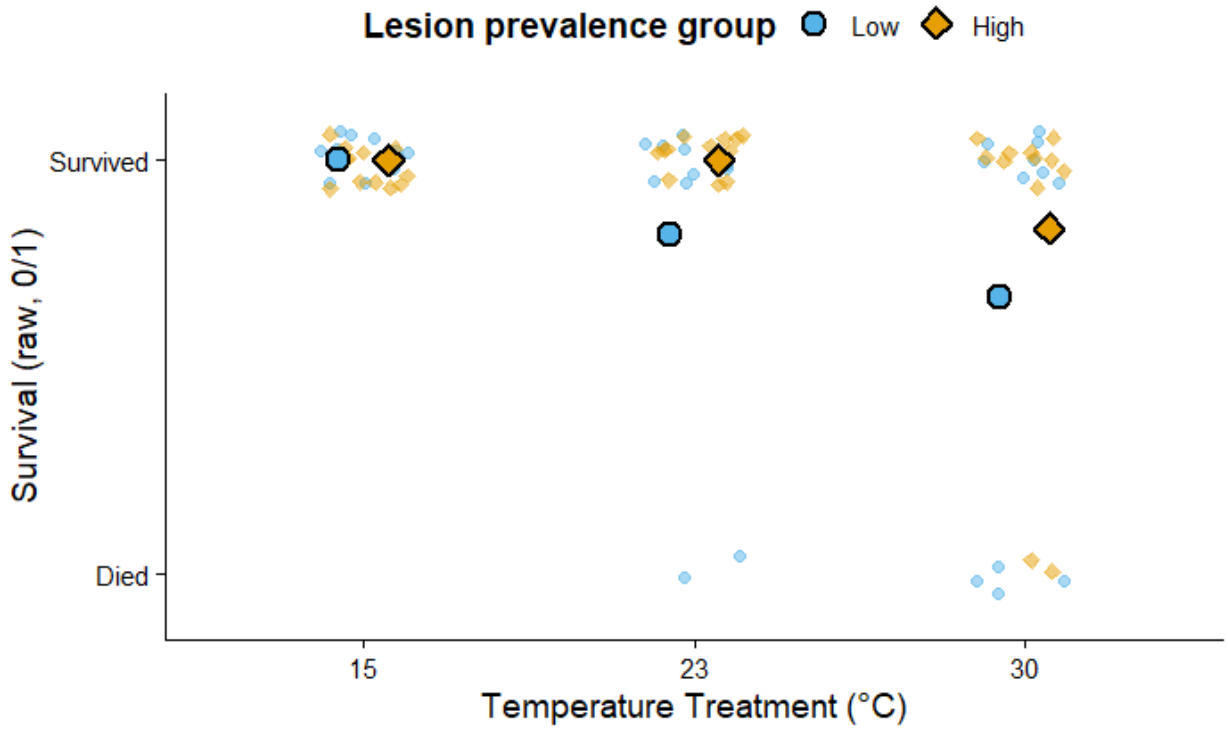


Figure B4. Raw survival outcomes of *F. virilis* across acclimation temperatures and lesion prevalence groups for chronic thermal exposure experiment (n = 65). Points represent individual crayfish (jittered for visibility), with survival coded as 1 (survived) or 0 (died). Filled diamonds indicate lesion-prevalence group mean survival at each temperature, with black outlines added for clarity. Colors denote lesion prevalence group (Low = blue, High = orange).

Table B2. Sensitivity analysis: linear mixed-effects model of log(Total_Lesion_Area_mm² + 0.01) including all individuals (zero and nonzero lesion area). Fixed effects include time point, temperature treatment, and lesion-prevalence population, with a random intercept for individual crayfish ID.

Term	Estimate	SE	df	t value	p-value
Intercept	-2.103	0.559	80.0	-3.765	<0.001
Time: End (vs Start)	1.153	0.333	70.0	3.460	<0.001
Temp: 23°C (vs 15°C)	-0.133	0.654	67.2	-0.203	0.840
Temp: 30°C (vs 15°C)	-0.498	0.658	68.4	-0.756	0.452
Population: High (vs Low)	3.803	0.536	68.0	7.096	<0.001

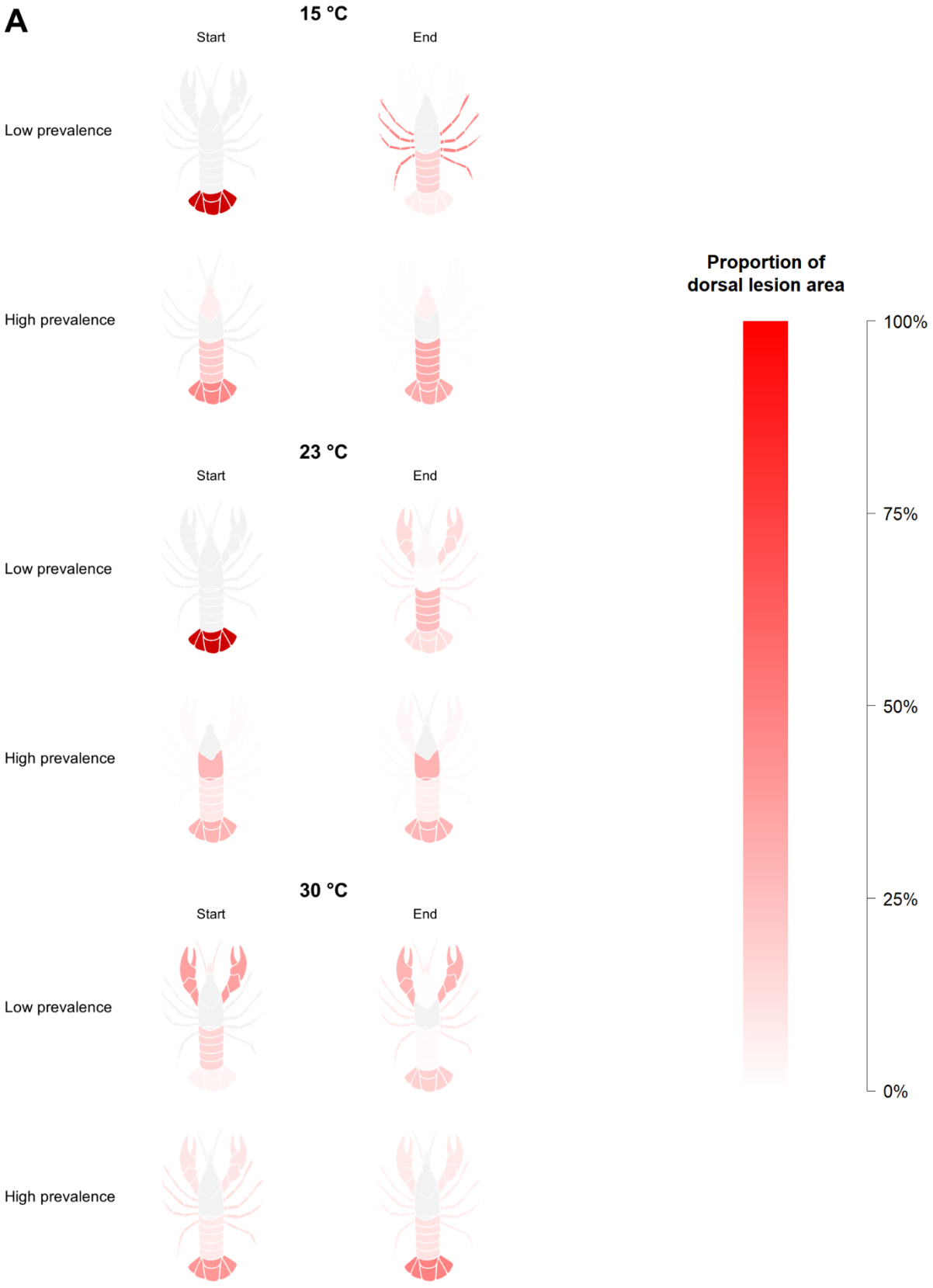

A
15 °C
Start
End
Low prevalence
High prevalence
23 °C
Start
End
Low prevalence
High prevalence
30 °C
Start
End
Low prevalence
High prevalence
Proportion of dorsal lesion area
100%
75%
50%
25%
0%

Figure B5A. Dorsal surface and heatmaps showing the proportion of total lesion area attributed to each body region for crayfish from low- and high-lesion-prevalence populations at the start and end of the experiment across temperature treatments (15°C, 23°C, 30°C). Color intensity reflects the relative contribution of each region to total lesion area within each group, with darker red indicating a greater proportion.

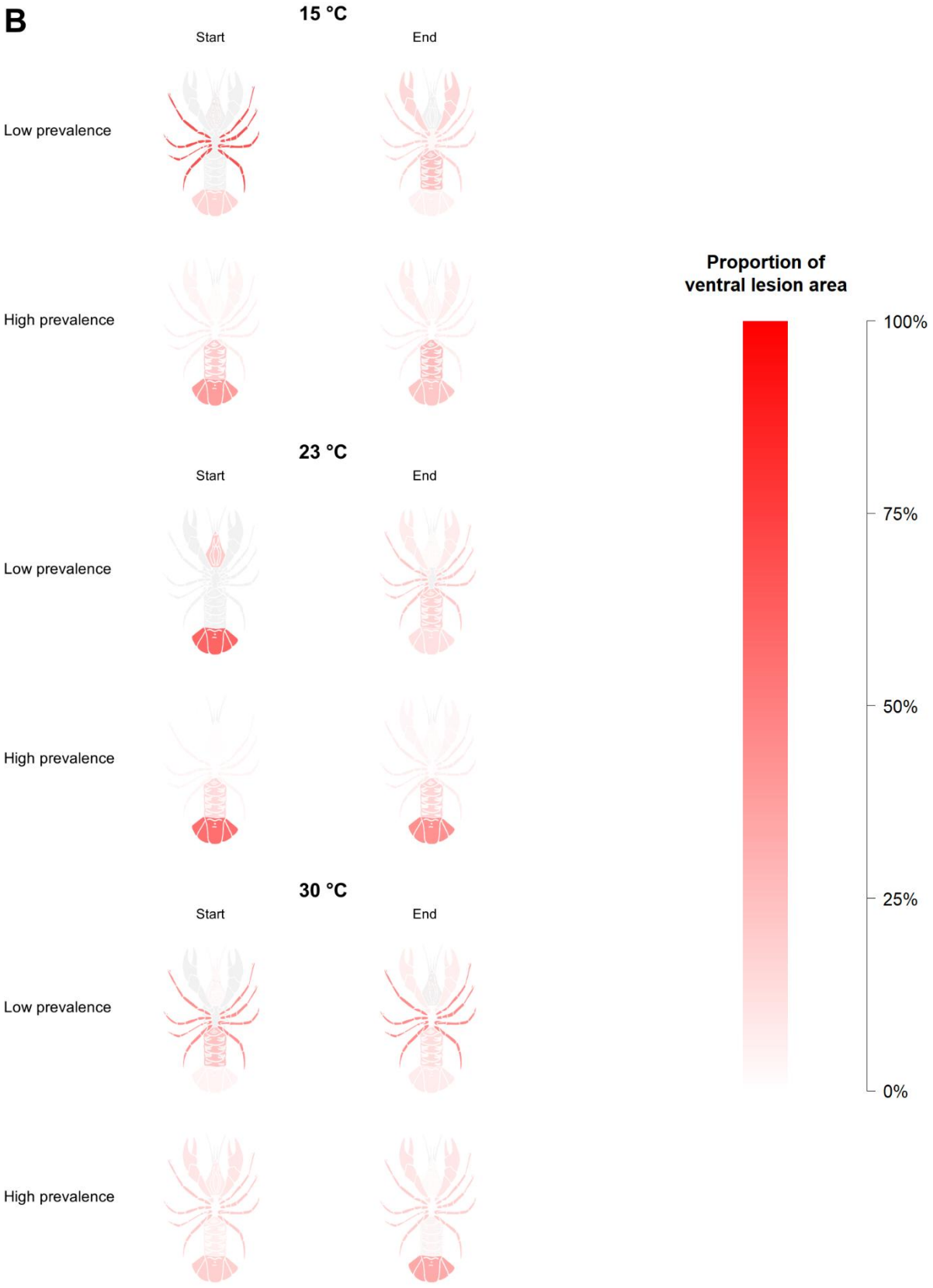

B
15 °C
Start
End
Low prevalence
High prevalence
23 °C
Start
End
Low prevalence
High prevalence
30 °C
Start
End
Low prevalence
High prevalence
Proportion of ventral lesion area
100%
75%
50%
25%
0%

Figure B5B. Ventral surface heatmaps showing the proportion of total lesion area attributed to each body region for crayfish from low- and high-lesion-prevalence populations at the start and end of the experiment across temperature treatments (15°C, 23°C, 30°C). Color intensity reflects the relative contribution of each region to total lesion area within each group, with darker red indicating a greater proportion.

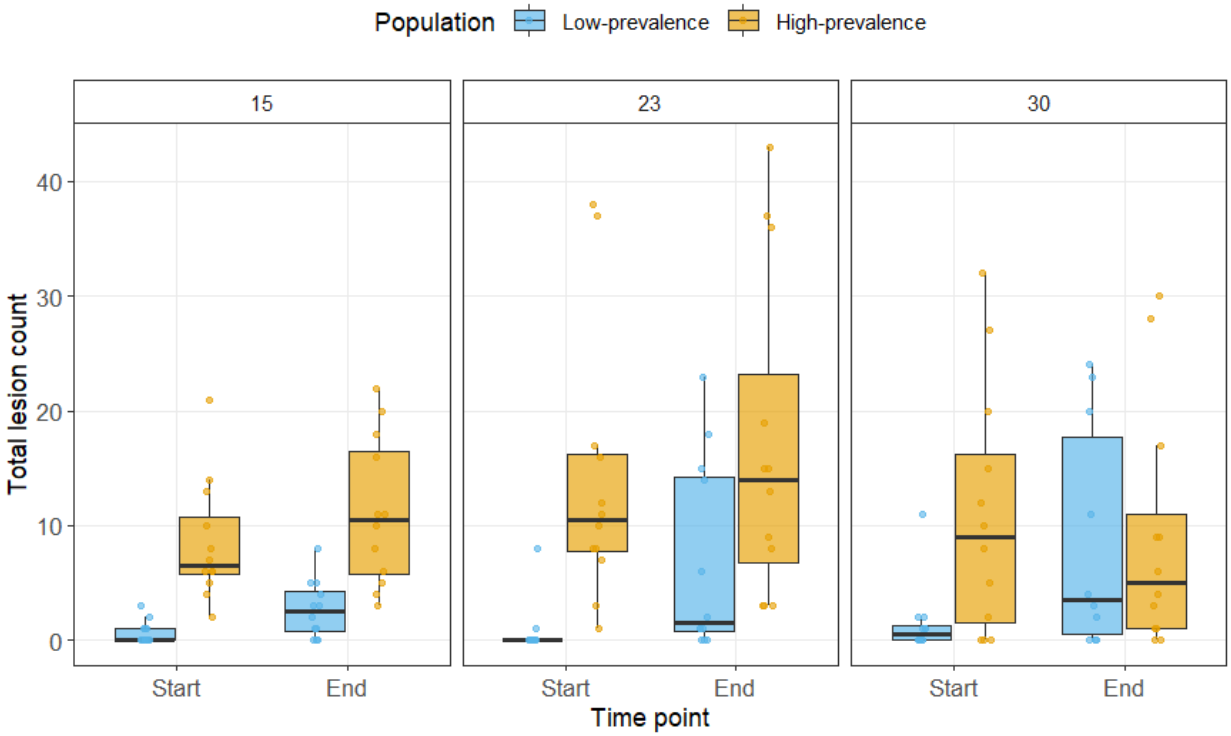


Figure B6. Total lesion counts per individual crayfish at the start and end of the chronic temperature exposure experiment for low-lesion-prevalence (blue) and high-lesion-prevalence (orange) populations across 15, 23, and 30 °C treatments. Points represent individual crayfish and boxplots show medians and interquartile ranges.

Length-Mass and Body Condition

Body condition was used as an integrative indicator of energetic status under the three chronic temperature exposure levels. Body condition was estimated from the relationship between wet mass (g) and post-orbital carapace length (POCL; mm) measured at the end of the experiment. Analyses included 64 individuals with complete final mass and POCL measurements (15°C: n = 24; 23°C: n = 22; 30°C: n = 18).

Both variables were log-transformed, and a linear regression was fit with log(wet mass) as the response and log(POCL) as the predictor. Seven individuals lacked final POCL measurements and were excluded from this regression. Residuals from this regression were used as a size-independent index of body condition, where positive residuals indicated individuals heavier than expected for their length and negative residuals indicate individuals lighter than expected.

To test treatment effects on body condition, body condition index was analyzed using a linear model including temperature treatment, lesion-prevalence group, and the interaction.

Table B3. Linear model results testing the influence of temperature treatment and lesion prevalence group on crayfish. Body condition represents size-adjusted mass derived from residuals of a log–log wet mass–POCL regression. Estimates (relative to 15 °C and low-lesion-prevalence reference levels), 95% confidence intervals, and coefficient-level p-values are reported.

Model	Term	Estimate	95% CI	p-value
Body condition (mass–size residual)	Temp: 23°C (vs 15°C)	-0.011	[-0.058, 0.036]	0.638
Body condition (mass–size residual)	Temp: 30°C (vs 15°C)	-0.024	[-0.074, 0.026]	0.337
Body condition (mass–size residual)	Lesion prevalence: High (vs Low)	0.024	[-0.021, 0.068]	0.295
Body condition (mass–size residual)	23°C × High prevalence	0.022	[-0.042, 0.087]	0.493
Body condition (mass–size residual)	30°C × High prevalence	-0.001	[-0.069, 0.068]	0.984