Parasites in a warming sea: the performance of trematodes and their ectothermic hosts under thermal stress

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>
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quien me enseñó a apreciar la ciencia,
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Summaries

Summary

Since the start of the industrial revolution, CO_2 emissions have dramatically increased and provoked unusual warming of the Earth (Summerhayes and Zalasiewicz 2018). The Baltic Sea coast, for instance, experiences warming of surface water approximately three times faster than the global average (Reusch et al. 2018). When combined with recurring heatwaves, such temperature regimes may reach unbearable levels for the organisms that inhabit them (Takolander et al. 2017). Ectotherms are particularly vulnerable to global warming because their physiological rates are at the whim of the surrounding water temperature (Somero 2002, Paaijmans et al. 2013). Although the thermal limits and performance of many aquatic ectotherms have been described (Sunday et al. 2012, Wahl et al. 2019), parasites are often neglected in such assessments.

Understanding the fate of parasites in the face of global warming is critical, given their ubiquity and the essential roles they play in nature. These roles include modulating population and community structures, food webs, ecosystem energetics, and ecotoxicological dynamics (Bauer et al. 2000, Dunn and Smith 2001, Wood et al. 2007, Benesh et al. 2008, Hechinger et al. 2008, Kuris et al. 2008, Dunne et al. 2013, Nachev et al. 2013, Sures et al. 2017a, Vivas Muñoz et al. 2021). Trematode parasites are particularly vulnerable to global warming due to their complex life cycle, which includes at least one ectotherm as a host (Galaktionov and Dobrovolskij 2003). These hosts include important grazers in marine ecosystems, such as the gastropod *Littorina littorea* and the amphipod *Gammarus locusta* as first and second intermediate hosts, respectively. The survival and feeding rates of these grazers can be affected by temperature and parasitism (Larsen and Mouritsen 2009). Hence, temperature and parasitism combined could have indirect consequences for competing algae, such as the Baltic native *Fucus vesiculosus* and the invasive alga *Gracilaria vermiculophylla*. Since algae and their associated organisms are essential for the healthy functioning of marine benthic communities, parasitism should be regarded as an important modulator of the effects of global warming on food webs.

Within three chapters, **this thesis aimed** to understand the effect of warming on trematode parasites and the combined effect of warming and parasitism on the survival, feeding, and biochemical condition of their ectothermic hosts (i.e., second intermediate hosts). To fulfill this aim, two species of trematodes, *Himasthla elongata* and *Podocotyle atomon*, and their intermediate hosts from the Baltic Sea were used as study subjects.

In ***Chapter I***, the thermal profile of *H. elongata* parasitizing widespread and ecologically relevant ectotherms (i.e., *L. littorea* and *Mytilus edulis*) was evaluated. The results from this chapter suggest that trematode infection made the gastropod more vulnerable to temperatures corresponding to warm summer events in the area (i.e., 22 °C). The optimal temperature for cercarial emergence (i.e., the emergence of infective free-living larval stages) and infectivity was 22 °C. However, this temperature induced a shorter cercarial survival time and a shorter window for successful mussel infection. After accounting for the temperature-specific gastropod and cercariae survival, we derived that warming negatively impacts trematode transmission to the bivalve host. The results imply that gastropod and cercariae mortality will limit the ability of trematodes to thrive in a warming ocean as a trade-off for the increased emergence and infectivity.

In ***Chapter II***, the role of *H. elongata* on the physiological response of its first intermediate host (i.e., *L. littorea*) to temperature and its potential relevance in modulating the competition between a native and an invasive alga (i.e., *F. vesiculosus* and *G. vermiculophylla*, respectively) to the Baltic Sea was evaluated. In the gastropod, trematode infection increased feces production (i.e., as a proxy for grazing), decreased glycogen concentrations, and increased lipid concentrations. Warming significantly affected glycogen and lipid concentrations, with glycogen peaking at 16 °C and lipids peaking at 22 °C. Infected snails fed more than uninfected snails, while *L. littorea* fed more on the invasive algae than the native one, indicating parasitism as an important indirect modulator of the interaction between these algae. The changes in the gastropod's biochemical condition indicate that thermal stress caused the mobilization of energy reserves, implying that compensatory metabolism may have begun. Finally, the decrease in glycogen in infected snails compared to uninfected snails may make them more susceptible to thermal stress.

Lastly, in ***Chapter III***, the physiological implications of *P. atomon* infection on the thermal response of its second intermediate host *G. locusta* were examined. The survival of gammarids was not significantly affected by trematode infection. Although the difference was statistically insignificant, infected gammarids shred more in colder temperatures than uninfected gammarids. Phenoloxidase activity increased at the lowest and higher temperatures (16 and 18 °C), especially in uninfected females at 18 °C. Catalase activity increased at warmer temperatures for infected males and uninfected females. An increase in the activity of this enzyme at colder temperatures occurred only for infected females. Infection decreased lipid content in gammarids by 14%. Infected males had significantly less glycogen than uninfected, while infected females showed the opposite trend. Results highlight the relevance of parasites and host sex in organismal homeostasis upon thermal stress and provide valuable insights into the stability of the population of a widespread amphipod upon rising temperatures.

The results obtained in these studies clearly highlight the importance of considering the host's performance when attempting to understand the fate of trematodes in a warming sea. The first intermediate host was more sensitive to infection in terms of survival and feeding than the second intermediate host. Accordingly, among trematode larval stages, rediae are expected to have a higher virulence than metacercariae (Galaktionov and Dobrovolskij 2003). Rediae are found in the first intermediate host and actively feed on the host's tissue, while metacercariae, found mainly in the second intermediate hosts, are semi-dormant stages with reduced need for resource consumption (Galaktionov and Dobrovolskij 2003). Moreover, the infection intensity of metacercariae in gammarids (77% of the infections harboring 1-2 cysts per host) is much lower than *H. elongata* redial infections, which generally cover and replace the whole gonadal tissue of the gastropod.

In conclusion, evidence is still insufficient to draw generalizations about the fate of parasites in a warming sea due to the diversity of parasites and hosts. It could be argued that the emergence and infectivity of active-swimming cercariae, such as those of *H. elongata*, are enhanced under warm conditions. However, the increased pathology on the first intermediate host and the heat-induced acceleration of cercarial mortality impede trematodes from thriving in a warming ocean. Moreover, trematodes not only cause the death of their gastropod host under thermal stress, but they also alter the hosts' energy reserves, thereby jeopardizing the ability of ectotherms to compensate metabolically under thermal stress. As trematodes affect the biochemical

state of the host, they also induce the gastropod to consume more food to compensate for the energy the parasite has hijacked. This can imply ecological consequences because the trematode may interfere with the gastropod's ability to modulate the interaction between native and invasive algae species. In the literature, however, the direction of the effect on the feeding behavior of the host is inconsistent, suggesting that results are context-dependent; thus, additional research is required in this area. *Podocotyle atomon* did not significantly affect the thermal performance of the host in terms of survival and feeding behavior, but it did affect the amphipod's biochemical response to temperature. Although some studies suggest that metacercarial infections in amphipods are highly virulent, the published research has focused on a limited number of populations and parasite-host combinations. Hence, it is essential to conduct additional research on the effect of trematodes on the performance of amphipod hosts, considering various ecoregions and host-parasite systems. The chapters of this dissertation contribute to the general understanding of the effects of global change on host-parasite dynamics. As shown in the preceding chapters, parasites can influence the response of individuals (i.e., hosts) to global warming by benefiting some and harming others. To avoid making incorrect assumptions about the magnitude and direction of global warming effects on coastal systems, parasites must be considered when forecasting these effects.

Zusammenfassung

Seit Beginn der industriellen Revolution haben die CO_2-Emissionen dramatisch zugenommen und eine ungewöhnlich schnelle Erwärmung der Erde hervorgerufen (Summerhayes und Zalasiewicz, 2018). An der Ostseeküste zum Beispiel erwärmt sich das Oberflächenwasser etwa dreimal schneller als im globalen Durchschnitt (Reusch et al., 2018). In Kombination mit wiederkehrenden Hitzewellen können solche Temperaturregime für die Organismen, die sie bewohnen, unerträgliche Werte erreichen (Takolander et al., 2017). Ektotherme Organismen sind besonders anfällig für globale Erwärmung, da ihr Stoffwechsel von der Temperatur des umgebenden Wassers abhängt (Somero, 2002; Paaijmans et al., 2013). Obwohl die thermische Leistung vieler aquatischer Ektothermen bereits beschrieben wurde (Sunday et al., 2012; Wahl et al., 2019), werden Parasiten bei solchen Bewertungen oft vernachlässigt.

Angesichts ihres ubiquitären Vorkommens und ihrer wichtigen Rollen und Funktionen in Ökosystemen ist ein Verständnis des Schicksals von Parasiten angesichts der globalen Erwärmung von entscheidender Bedeutung. Zu diesen Funktionen gehört die Regulation von Populations- und Gemeinschaftsstrukturen, Nahrungsnetzen, Energieflüssen in Ökosystemen und ökotoxikologischer Dynamiken (Bauer et al. 2000, Dunn and Smith 2001, Wood et al. 2007, Benesh et al. 2008, Hechinger et al. 2008, Kuris et al. 2008, Dunne et al. 2013, Nachev et al. 2013, Sures et al. 2017, Vivas Muñoz et al. 2021). Trematoden sind aufgrund ihres komplexen Lebenszyklus, der mindestens einen Ektothermen als Wirt umfasst, besonders anfällig für die Folgen der globalen Erwärmung (Galaktionov und Dobrovolskij, 2003). Zu den ektothermen Wirten gehören wichtige Weidegänger in marinen Systemen, wie die Schnecke *Littorina littorea* (als erster Zwischenwirt) und der Amphipode *Gammarus locusta* (als zweiter Zwischenwirt). In Anbetracht der Tatsache, dass sowohl Parasitismus als auch Temperatur die Überlebens- und Fraßraten von Weidegängern beeinflussen können (Larsen und Mouritsen, 2009), könnten Parasitismus und Temperatur in Kombination indirekte Folgen für konkurrierende Algen, wie die in der Ostsee heimische *Fucus vesiculosus* und die invasive Alge *Gracilaria vermiculophylla* haben. Daher ist es wichtig, den Parasitismus als einen bedeutenden Modulator der Auswirkungen der globalen Erwärmung sowohl für die Wirte als auch für andere Arten zu betrachten, die durch Nahrungsnetze miteinander verbunden sind (wie z. B. Algen) und die für das

gesunde Funktionieren mariner benthischer Gemeinschaften von wesentlicher Bedeutung sind.

In den **drei Kapiteln dieser Dissertation** wurden daher die Auswirkungen der Erwärmung auf Trematoden sowie die kombinierten Auswirkungen von Erwärmung und Parasitismus auf das Überleben, die Ernährung und den biochemischen Zustand ihrer ektothermen Wirte (d. h. der zweiten Zwischenwirte) untersucht. Dazu wurden die zwei Trematodenarten *Himasthla elongata* und *Podocotyle atomon* und ihre Zwischenwirte aus der Ostsee als Studienobjekte verwendet.

In **Kapitel I** wurde das thermische Profil des Trematoden *H. elongata*, der weit verbreitete und ökologisch relevante ektotherme Oraganismen (d. h. *L. littorea* und *Mytilus edulis*) parasitiert, bewertet. Die Ergebnisse dieses Kapitels deuten darauf hin, dass eine Trematodeninfektion die Schnecke anfälliger für Temperaturen macht, die den warmen Sommern in dem Gebiet entsprechen (d. h. 22 °C). Die optimale Temperatur für das Ausscheiden der Zerkarien und die Infektiosität lag bei 22 °C, während bei dieser Temperatur eine kürzere Überlebenszeit der Zerkarien und ein kürzeres Zeitfenster für eine erfolgreiche Infektion der Muscheln festgestellt wurde. Unter Berücksichtigung der temperaturspezifischen Überlebensrate der Schnecken sowie der Ausscheidung und Infektiosität der Zerkarien ergaben sich insgesamt negative Auswirkungen der Erwärmung für die Übertragung von Trematoden auf den Muschelwirt. In Anbetracht des Szenarios eines sich erwärmenden Ozeans legen die Ergebnisse nahe, dass die Sterblichkeitsrate der Gastropoden und Zerkarien sowie die Infektiosität der Trematoden, als Gegenleistung für erhöhte Ausscheidungsraten, einschränken wird.

In **Kapitel II** wurde die Rolle des Trematoden *H. elongata* bei der physiologischen Reaktion seines ersten Zwischenwirts (*L. littorea*) auf Temperaturveränderungen und seine potenzielle Bedeutung bei der Regulierung der Konkurrenz zwischen einer einheimischen und einer invasiven Alge (*F. vesiculosus* bzw. *G. vermiculophylla*) in der Ostsee untersucht. Bei den Gastropoden führte die Trematodeninfektion zu einer erhöhten Kotproduktion (stellvertretend für die Fraßrate), zu einem Rückgang der Glykogenkonzentration und zu einer Erhöhung der Lipidkonzentration. Die Erwärmung wirkte sich erheblich auf die Glykogen- und Lipidkonzentrationen aus, wobei die Glykogenkonzentration bei 16 °C und die Lipidkonzentration bei 22 °C ihren Höhepunkt erreichte. Infizierte Schnecken fraßen mehr als nicht infizierte Schnecken,

während *L. littorea* sich mehr von der invasiven als von der einheimischen Alge ernährte, was auf Parasitismus als wichtigen indirekten Modulator der Interaktion zwischen diesen Algen hinweist. Die Veränderungen des biochemischen Zustands der Schnecke deuten darauf hin, dass der thermische Stress die Mobilisierung von Energiereserven verursacht, was möglicherweise auf das Einsetzen des kompensatorischen Stoffwechsels hindeuten könnte. Schlussendlich könnte der Rückgang des Glykogens in infizierten Schnecken im Vergleich zu nicht infizierten Schnecken sie anfälliger für thermischen Stress machen.

In **Kapitel III** wurden die physiologischen Auswirkungen einer Infektion mit *P. atomon* auf die Reaktion seines zweiten Zwischenwirts, *G. locusta*, auf verschiedene Temperaturregime untersucht. Das Überleben der Gammariden wurde durch die Trematodeninfektion nicht signifikant beeinträchtigt. Obwohl der Unterschied statistisch unbedeutend war, fraßen infizierte Gammariden bei kälteren Temperaturen mehr als nicht infizierte Gammariden. Die Phenoloxidase-Aktivität nahm bei den niedrigsten und höchsten Temperaturen (16 und 18 °C) zu, insbesondere bei nicht infizierten Weibchen bei 18 °C. Die Katalaseaktivität nahm bei höheren Temperaturen bei infizierten Männchen und nicht infizierten Weibchen zu, wohingegen ein Anstieg der Aktivität dieses Enzyms bei niedrigen Temperaturen nur bei den infizierten Weibchen auftrat. Die Infektion verringerte den Lipidgehalt der Gammariden um 14%, wobei infizierte Männchen deutlich weniger Glykogen aufwiesen als nicht infizierte, während bei den infizierten Weibchen der umgekehrte Trend zu beobachten war. Die Ergebnisse unterstreichen die Bedeutung von Parasiten und Wirtsgeschlecht für die Homöostase des Organismus bei thermischem Stress und liefern nützliche Erkenntnisse über die Stabilität des Organismus eines weit verbreiteten Amphipoden in einem sich erwärmenden Meer.

Die Ergebnisse dieser Studien machen deutlich, wie wichtig es ist, den Beitrag des Wirts zu berücksichtigen, wenn man versucht, das Schicksal von Trematoden in einem sich erwärmenden Meer zu verstehen. Der erste Zwischenwirt war in Bezug auf Überleben und Nahrungsaufnahme empfindlicher gegenüber einer Infektion als der zweite Zwischenwirt. Dies entspricht den Erwartungen, da man davon ausgeht, dass Redien eine höhere Virulenz aufweisen als Metazerkarien (Galaktionov und Dobrovolskij, 2003). Redien ernähren sich aktiv vom Gewebe des Wirts, wohingegen Metazerkarien halbruhende Stadien sind und entsprechend weniger Ressourcen des Wirts verbrauchen als Redien (Galaktionov und Dobrovolskij, 2003). Darüber hinaus

ist die Infektionsintensität von Metazerkarien bei Gammariden (77% der Infektionen mit 1-2 Zysten pro Wirt) viel geringer als bei Redieninfektionen von *H. elongata*, die normalerweise das gesamte Gonadengewebe der Gastropoden sukzessive ersetzen.

Zusammenfassend lässt sich sagen, dass die Erkenntnisse noch nicht ausreichen, um aufgrund der Vielfältigkeit der Parasiten und Wirte eine allgemeine Schlussfolgerung über das Schicksal der Parasiten in einem sich erwärmenden Meer zu ziehen. Es könnte jedoch argumentiert werden, dass das Auftreten und die Infektiosität von aktiv schwimmenden Zerkarien, wie die von *H. elongata*, unter warmen Bedingungen verstärkt werden. Die erhöhte Pathologie des ersten Zwischenwirts und die hitzebedingte Beschleunigung der Zerkariensterblichkeit verhindern jedoch, dass Trematoden in einem sich erwärmenden Ozean übermäßig gedeihen. Darüber hinaus erhöhen Trematodeninfektionen unter Wärmestress nicht nur die Sterblichkeit ihrer Gastropoden-Wirte, sondern verändern auch die Energiereserven des Wirts und gefährden damit die Fähigkeit der Ektothermen, diesen thermischen Stress metabolisch auszugleichen. Da Trematoden den biochemischen Zustand des Wirts verändern, veranlassen sie die Schnecke ebenfalls dazu, mehr Nahrung zu sich zu nehmen, um die vom Parasiten verwendete Energie zu kompensieren. Dies kann ökologische Folgen haben, da durch die Trematodeninfektion die Fähigkeit der Schnecke beeinträchtigen werden kann, die Interaktion zwischen einheimischen und invasiven Algenarten zu regulieren. In der Literatur ist die Richtung der Auswirkung auf das Fressverhalten des Wirts jedoch uneinheitlich, was darauf hindeutet, dass die Ergebnisse kontextabhängig sind; daher sind weitere Forschungen in diesem Bereich erforderlich. Eine Infektion mit *P. atomon* hatte keinen signifikanten Einfluss auf die thermische Leistung des Wirts in Bezug auf Überleben und Fressverhalten, aber es beeinflusste die biochemische Reaktion des Amphipoden auf Temperaturveränderungen. Obwohl einige Studien darauf hindeuten, dass Metazerkarien bei Amphipoden hochgradig virulent sind, haben sich die veröffentlichten Untersuchungen auf eine begrenzte Anzahl von Populationen und Parasit-Wirt-Kombinationen konzentriert. Daher ist es unerlässlich, die Auswirkungen von Trematoden auf die Performanz von Amphipodenwirten unter Berücksichtigung verschiedener Ökoregionen und Wirt-Parasit-Systeme weiter zu erforschen. Diese Dissertation ist daher ein Beitrag zum allgemeinen Verständnis der Auswirkungen des globalen Wandels auf die Wirts-Parasiten-Dynamik. Wie in den drei Kapiteln dieser Arbeit im Detail gezeigt wird, können Parasiten die Reaktion von Individuen (d. h. von

Wirten) auf den globalen Wandel auf komplexe Art und Weise beeinflussen, indem sie beispielsweise manchen Organismen nützen und anderen schaden. Um falsche Annahmen über das Ausmaß und die Richtung der Auswirkungen der globalen Erwärmung auf marine Küstensysteme zu vermeiden, müssen Parasiten zukünftig bei der Vorhersage dieser Auswirkungen berücksichtigt werden.

General introduction

The Anthropocene and global warming

Humans have altered the natural environment to such an extent that the ramifications and consequences have prompted the proposal of a new geological epoch, the Anthropocene (Steffen et al. 2018). One of the most prominent changes is excessive CO_2 emissions into the atmosphere (Summerhayes and Zalasiewicz 2018). Since the year 2000, the global CO_2 concentration has risen at a rate of ca. 20 ppm/decade, which is up to ten times faster than CO_2 increase rates over the previous 800,000 years (Allen et al. 2018). This CO_2 increase has provoked unusual warming of the Earth (Summerhayes and Zalasiewicz 2018). The Intergovernmental Panel on Climate Change (IPCC) projects that at the end of the century, the global mean surface temperature will increase by 1.5 °C on average relative to preindustrial times (Allen et al. 2018). Although these are mainly projected temperatures, the effects of global warming are already observable in many places around the globe, especially in coastal areas (Torresan et al. 2008).

A warming sea and the effects on the coastal environment

Coastal ecosystems are invaluable both economically and ecologically. Globally, the coastline can extend to more than 2 million kilometers (Liu et al. 2019) and holds a massive amalgam of flora and fauna. Coastal ecosystems provide services (i.e., food, raw materials, climate regulation, erosion prevention, and habitat and cultural services) worth ca. 28,917 \$ ha^{-1} $year^{-1}$ (de Groot et al. 2012). From the ecological point of view, it represents a rich and highly productive system characterized by complex ecological interactions. Being shallow and proximate to land, the coastal environment is characterized by highly fluctuating abiotic conditions, and global warming is expected to and has been observed to highly influence these systems (Harley et al. 2006).

The Baltic Sea coast is an example of a region experiencing unprecedented abiotic changes affecting the organisms that inhabit it. These changes include eutrophication, warming, hypoxia in bottom waters, incoming nonindigenous species, contamination, high shipping intensity, and overfishing (Reusch et al. 2018). The Baltic Sea coast experiences the worst above-average warming (ca. three times faster than the global average) of surface water (Reusch et al. 2018). Combined with recurrent heatwaves,

such temperature regimes might attain unbearable conditions for the organisms that inhabit it (Takolander et al. 2017). Macroalgae assemblages, for instance, have been observed to be sensitive to global warming, which acts either directly by affecting single algal species performance (Verlinden et al. 2014, Poore et al. 2016, McKnight et al. 2021) or indirectly by affecting the efficiency of the grazers' top-down control (Sampaio et al. 2017, Wahl et al. 2019). On top of that, the grazers responsible for keeping marine benthic systems on point are mostly ectotherms and, therefore, sensitive to heat stress.

Ectotherms are sensitive to global warming

Ectotherms are particularly sensitive to global warming since their physiological rates depend entirely on ambient water temperature (Somero 2002, Paaijmans et al. 2013). When stressful conditions arise (e.g., temperatures above the optimal range), such organisms can experience energy limitations and might turn to adaptation or acclimatization pathways that ensure or prolong survival (Somero 2002). Ectotherms can handle thermal stress by upregulating protective enzymes and proteins that help the organism to repair heat-associated tissue and molecular damage (Somero 2002, Dammark et al. 2018). All of these processes are reflected in the energy reserves of the individual. Therefore, organisms often increase feeding rates to suffice the increased energy demand and compensate for thermal stress (Sokolova et al. 2012). Sokolova et al. (2012) described an energetic framework that helps categorize the different stages that organisms experience during heat stress (Figure 1). At their thermal optimum, organisms are fully functional and can spend energy on important long-term processes such as growth, feeding, reproduction, development, energy storage, and activity (Sokolova et al. 2012). However, when an organism starts to experience moderate stress (i.e., the pejus range), metabolic compensation will occur, resulting in a redirection of energy from processes such as reproduction and growth to basal maintenance needed for survival (Sokolova et al. 2012; Figure 1). This is also reflected in facultative anaerobes by reducing the scope of aerobic metabolism (Pörtner 2001). When stress increases, such as during peaks of temperature, and the individual enters the "pessimmum" range (i.e., extreme stress), energy conservation occurs, all activities are halted, and partial anaerobic metabolism begins (Sokolova et al. 2012; Figure 1). However, at this stage, such stress is often fatal. This energy homeostasis framework usually applies to aquatic invertebrates, and its ecological relevance relies on the consideration of traits such as feeding, which are directly linked to ecosystem functioning.

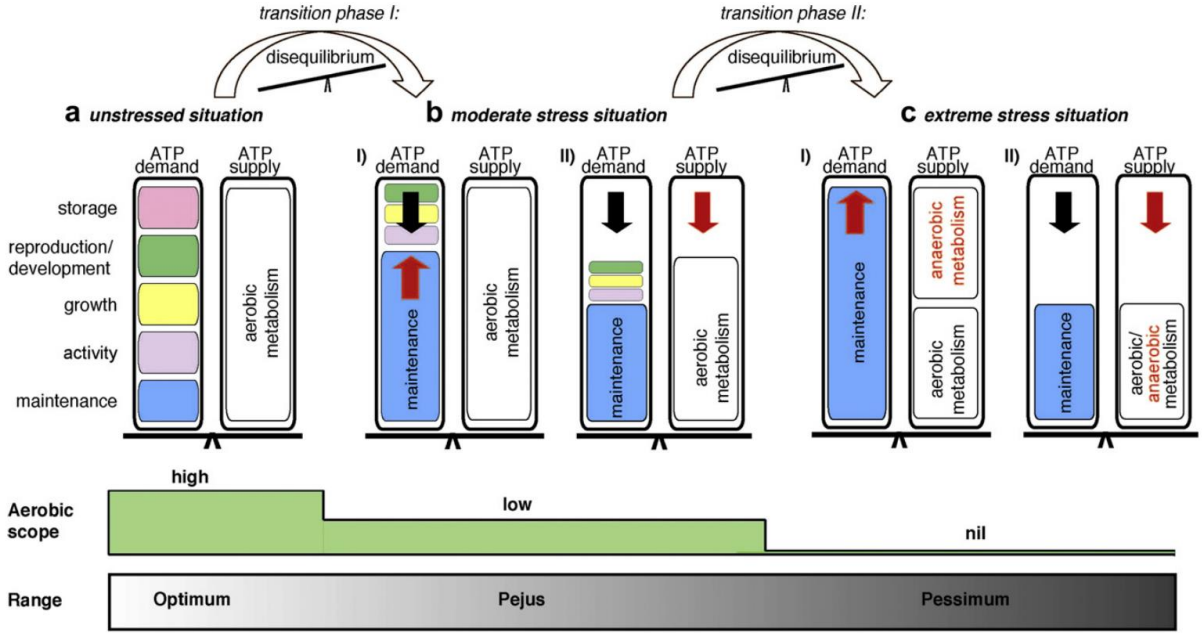

Figure 1 Bioenergetic framework for ectothermic aquatic organisms under different stress scenarios and their physiological implications as described and depicted by Sokolova et al. (2012). Changes in ATP-demand or ATP-supply are represented with red arrows, while the direction of trade-offs is represented with black arrows. It should be noted that the sizes of the boxes corresponding to different energy-demanding operations have been made equal by Sokolova et al. (2012) for clarity's sake and do not reflect the actual energy distribution. Please refer to the original reference for more details (Sokolova et al. 2012).

The fate of parasites upon global warming

Although the thermal limits and performance of many aquatic ectotherms have been described (Sunday et al. 2012, Wahl et al. 2019), parasites are often disregarded in such assessments. Given the plethora and diversity of parasite groups and species and the scarcity of information on their thermal tolerance, it is often difficult to predict the fate of parasites in the face of global warming. Parasites are affected by global warming directly and indirectly. First, several parasites are at the whim of water temperatures, such as ectoparasites or complex life cycle endoparasites with free-living larval stages. Second, parasites are innately dependent on their host. Therefore, anything the host will endure during heat stress will ultimately affect the parasite. Some studies have explored disease ecology under the umbrella of global warming (Lafferty et al. 2004, Mas-Coma et al. 2009, Rohr et al. 2011, Mouritsen et al. 2018). However, the results of the research conducted to date are insufficient to derive general

conclusions regarding the fate of parasites upon global warming and to understand the possible consequences of global change for host-parasite interactions.

The role of parasites in the ecosystem

Understanding the fate of parasites upon global warming is important, given the essential roles that parasites play in nature. Parasitism is the most common species interaction in nature, all organisms are known to serve as hosts for at least one parasite species, and almost half of the species described are parasitic (Goater et al. 2014). Parasites can massively affect their host population, for example, through feminization or castration (Dunn and Smith 2001). Parasites also play an essential role in food webs by modifying their host's diet (Vivas Muñoz et al. 2021), feeding rates (Wood et al. 2007), or by making the host more susceptible to predation (Bauer et al. 2000, Benesh et al. 2008), thus increasing food web density, connectivity, and robustness (Lafferty et al. 2008, Dunne et al. 2013). Some parasites contribute significantly to the biomass of aquatic ecosystems, sometimes surpassing the biomass of top predators (Kuris et al. 2008). Moreover, parasites can serve as pollutant indicators and have been observed to alter metal bioaccumulation patterns by acting as a sink for pollutants reducing the burden their hosts experience upon metal exposure (Nachev et al. 2013, Sures et al. 2017b). Additionally, although most parasites do not pose a zoonotic risk, some are of human relevance since they cause severe diseases (see Carlson et al., 2020). Given parasites' ubiquity and ecological significance, it is crucial to include them in conservation plans (Carlson et al. 2020) and to comprehend their fate under global warming.

Trematodes are sensitive to global warming

Among parasites, trematodes are particularly sensitive to global warming due to their complex life cycle with at least one ectotherm as host (Figure 2). Most trematodes use mollusks (usually a gastropod) as the first intermediate host (Galaktionov and Dobrovolskij 2003). Inside the mollusk, parthenitae (i.e., rediae and sporocysts) produce hundreds of cercariae that emerge from the snail to infect the second intermediate host, which is always an ectotherm, such as amphipods, bivalves, and fish, to name a few (Galaktionov and Dobrovolskij 2003). After reaching the second intermediate host, the cercariae will penetrate the tissue and encyst as metacercariae, a semi-dormant stage. In some trematode species, these metacercariae might grow until becoming infective to the next host, while others do not experience any further

development and thus wait to be transmitted to the next host. Finally, metacercariae are transmitted trophically to the final or definitive host (Figure 2), which is, in most cases, a vertebrate such as shorebirds or fish (Goater et al. 2014). In the final host, the parasite matures into an adult and reproduces sexually, producing eggs which are then shed with the feces. From the egg, a miracidium will hatch upon ingestion by a suitable gastropod host or in the water column. In the case of hatching in the water column, miracidia will actively or passively search and infect the first intermediate host (Galaktionov and Dobrovolskij 2003). Finally, miracidia will metamorphose into redia or sporocysts in the tissue of the first intermediate host, allowing the life cycle to continue. Such a complex life cycle can be affected by global warming in several ways, either indirectly through temperature effects on the host or directly by affecting the performance of free-living infective larval stages (Lõhmus and Björklund 2015).

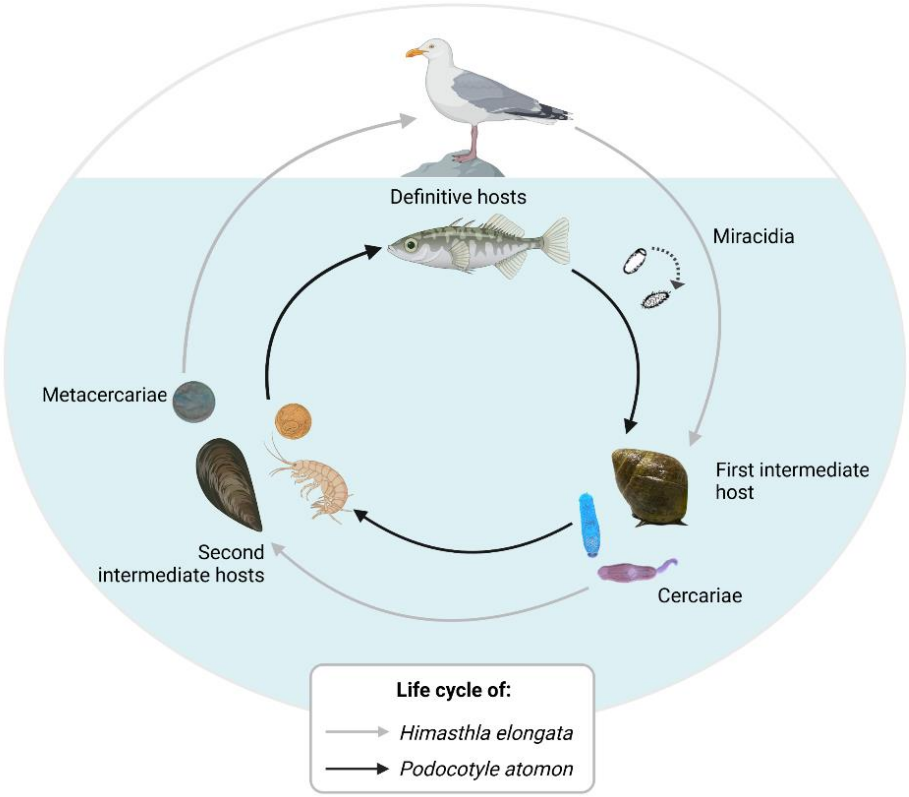

Figure 2 Life cycle of *Himasthla elongata* (gray lines) and *Podocotyle atomon* (black lines) as examples of a typical trematode life cycle involving three hosts. In the first intermediate host (e.g., gastropod), asexual reproduction occurs, and cercariae are produced by parthenitae (i.e., rediae for *H. elongata* and sporocysts for *P. atomon*). Cercariae emerge from the snail and infect the next intermediate host (e.g., bivalve for *H. elongata* or amphipod for *P. atomon*), encysting as metacercariae. The metacercariae are transmitted trophically to the final host (e.g., bird for *H. elongata* or fish for *P. atomon*) where sexual reproduction takes place. After sexual reproduction, eggs are shed with feces, and miracidia hatch to infect the first intermediate host and continue the life cycle.

Different parasite larval stages have different thermal sensitivity and virulence

All life stages described above are affected by temperature (Vanoverschelde 1981, 1982, Galaktionov and Dobrovolskij 2003, Morley 2011a, Morley and Lewis 2013, 2015; Figure 3). Miracidia and cercariae are especially vulnerable because they are the trematode's free-living stages. They are exposed to external environmental conditions and, unlike metacercariae, are highly metabolically active (Galaktionov and Dobrovolskij 2003). Miracidia are known to increase their emergence, hatching, and infection rates in response to rising temperature, while their half-life is reduced (Vanoverschelde 1981, 1982). Rediae and sporocysts are also temperature sensitive, and cercariae development within these larvae is accelerated when temperatures are relatively warm (Ataev 1991). The following free larval stage, the cercaria, is known to be affected by temperature in terms of their emergence, survival, and infectivity (Thieltges and Rick 2006, Studer et al. 2010, Morley 2011a, Morley and Lewis 2013, 2015, Selbach and Poulin 2020; Figure 3). Emergence is always triggered by temperature, and most cercariae species have nil emergence during cold winter months (Galaktionov and Dobrovolskij 2003). However, in the optimal temperature range, cercarial emergence is relatively stable (Morley and Lewis 2013), while there is a decrease in emergence when temperatures rise above the optimum (Thieltges and Rick 2006, Studer et al. 2010). The time of survival and activity is limited since their lecithotrophic character does not allow cercariae to incorporate new energy by feeding. Since higher temperature accelerates metabolic rates, the energy reservoir in cercariae is rapidly depleted, limiting their activity and survival to a few hours (for most species) (Studer et al. 2010, Morley 2011a). Increased temperature benefits infectivity, but as with emergence, once a certain level of warming is reached, infectivity begins to decline (Studer et al. 2010, Morley and Lewis 2015). Metacercariae development within the host is known to be enhanced at warm temperatures up to a threshold, after which metacercariae development is halted (Studer et al. 2010). When it comes to the virulence of trematode developmental stages, it is expected that rediae have the highest virulence since they actively feed on the host’s tissue (Galaktionov and Dobrovolskij 2003) and perform passive sequestration of host resources such as glycogen and lipids (Chengt 1963, Tunholi et al. 2013, Bonfim et al. 2020). Although temperature affects each of these life cycle components separately, they will collectively determine how trematodes will perform in a warming sea and the extent of their ecological impact on coastal ecosystems.

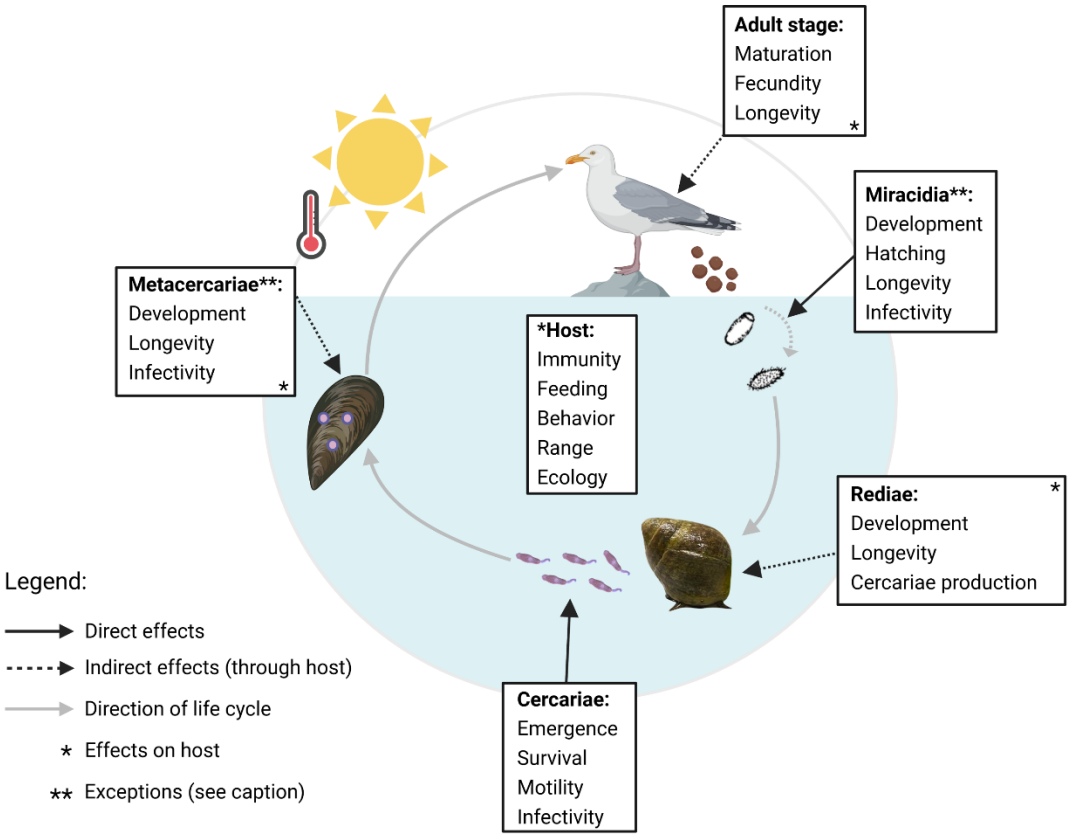

Figure 3 Temperature effects on trematode life cycles using *Himasthla elongata* as an example. Global warming can affect parasite performance either directly (solid black arrows) by exerting effects on free-living larval stages or indirectly (dashed black arrows) by affecting the larval stages inside the host and the host itself through changes in distribution, behavior, physiology, and mortality (Lõhmus and Björklund 2015). Life cycles can vary between trematode species where metacercariae encyst in the environment and are affected directly by temperature or in cases where miracidia hatch inside the gastropod host. This figure was adapted based on Marcogliese (2001).

Cascading ecological effects of temperature and parasitism combined

Parasites are known to mediate complex shifts in ecological interactions (Mouritsen and Poulin 2005, Bernot and Lamberti 2008), particularly in the face of global change (Mouritsen et al. 2018). The combination between temperature and parasitism often results in drastic changes in ecological dynamics, especially when looking at grazers and benthic communities. These effects are mediated through impacts of temperature on the production of infective larval stages (Morley and Lewis 2013) and through the combined effects of temperature and parasitism on the survival of the host (Fredensborg et al. 2005) or its behavior by affecting traits such as feeding rates (Wood et al. 2007) (Figure 3). Temperature, for example, can counteract and nullify the effect of infection on the grazing rates of *Littorina littorea,* probably due to excessive stress

on the host resulting in organismal collapse (Larsen and Mouritsen 2009). On a larger scale, these effects may manifest themselves at the community level. Temperature induces the emergence of cercariae, which increases transmission in benthic invertebrates, leading to increased mortality and altered behavior (e.g., surface swimming) in infected hosts (Mouritsen and Jensen 1997). It has been documented that this increased mortality of infected amphipods under thermal stress creates unstable ecological conditions in coastal ecosystems. Mouritsen et al. (2018), for instance, reported severe parasite-induced extermination of epibenthic amphipods after a prolonged heatwave scenario, favoring infaunal species. This parasite- and temperature-mediated species composition shift is crucial because it can influence interspecific competition by giving a competitive advantage to amphipods that are less susceptible to trematode infections (Larsen et al. 2011).

The role of parasitism in algal invasion ecology

Considering that both parasitism and temperature affect the survival and feeding rates of important grazers (Larsen and Mouritsen 2009), this could have indirect consequences for competing algae such as the Baltic native *Fucus vesiculosus* and the invasive alga *Gracilaria vermiculophylla*. The former is a widespread foundation species in the Baltic Sea, and a decline in its distribution has been recorded in recent decades (Torn et al. 2006, Graiff et al. 2015). Its decline has been attributed to several abiotic factors, such as eutrophication, sedimentation, grazing pressure, and competition with other algae such as *G. vermiculophylla* (Graiff et al. 2015). The latter alga, *G. vermiculophylla,* is an invasive red alga originating from the northwestern Pacific (Thomsen et al. 2007, Hu and Juan 2014). The spread of this invasive species can have several negative consequences, including the suffocation of bivalve communities (Thomsen and McGlathery 2006) and competition for space, light, and nutrients with native algal species such as *F. vesiculosus* (Weinberger et al. 2008, Hammann et al. 2013). Since the invasion range of *G. vermiculophylla* is anticipated to expand (Thomsen et al. 2007, Weinberger et al. 2008, Krueger-Hadfield et al. 2018), it is necessary to gain a deeper understanding of the biotic and abiotic factors that modulate the interaction between this alga and *F. vesiculosus*. As mentioned above, trematodes can drive changes in macroalgae assemblages by affecting their major grazer species (Wood et al. 2007). Since algae and their associated organisms are critical to the health and functioning of marine benthic communities, parasitism should be considered an important modulator of global warming effects within food webs.

Thermal performance curves as a tool to understand and predict global warming effects

Thermal performance curves (TPC) help to understand and predict the performance of marine ectotherms under global warming (Schoolfield et al. 1981, Sinclair et al. 2016, Kontopoulos et al. 2020). TPCs are often derived to describe the thermal profile of biological rates and the performance of several organismal traits in response to temperature (Sinclair et al. 2016). Although TPCs are more often established for adult aquatic invertebrates, they can also help describe the performance and heat tolerance of trematode larval stages. Using TPCs, optimal temperatures can be derived, and critical thermal limits can be identified. Although TPCs do not represent the absolute truth of how organisms will perform in a warming sea (Sinclair et al. 2016), they provide some guidelines regarding which temperatures might be benign or stressful for marine ectotherms. Therefore, TPCs can be considered an initial step-stone guiding future work elucidating marine organisms' thermal responses.

Aims of this thesis

As such, this thesis aims to contribute to the mounting evidence describing the potential fate of trematodes in a warming sea and the role parasites play in the thermal tolerance of their marine ectothermic hosts. Specifically, **Chapter I** addresses the thermal profile of a common trematode (*i.e., Himasthla elongata*) parasitizing widespread and ecologically relevant ectotherms (i.e., *L. littorea* and *Mytilus edulis*). **Chapter II** evaluates the role of this trematode on the physiological response of its first intermediate host (i.e., *L. littorea*) to temperature and its potential relevance in modulating the competition between a native and an invasive alga (i.e., *F. vesiculosus* and *G. vermiculophylla*, respectively) to the Baltic Sea. Finally, **Chapter III** examines the physiological implications of a common trematode (i.e., *Podocotyle atomon*) on the thermal response of its second intermediate host and a fundamental grazer (i.e., *Gammarus locusta*) of the Baltic Sea.

Chapter I: Heat sensitivity of first host and cercariae may restrict parasite transmission in a warming sea

Introduction

Climate change-related temperature shifts are recognized as one of the main drivers of marine benthic community changes (Harley et al. 2006, Poloczanska et al. 2013, Hoegh-Guldberg et al. 2018). However, in realistic natural scenarios, climate change impacts should be addressed as a concert of multiple abiotic and biotic factors (Marcogliese 2008, Ban et al. 2014, Gunderson et al. 2016). Biotic factors such as species interactions can have the capacity of buffering or amplifying climate change effects on individual species as well as on communities (e.g., Urban et al. 2012). Thus, to more realistically predict climate change impacts on species or communities, we need to describe the respective thermal tolerance of closely interacting species systems. Host-parasite systems are one example of such interacting systems that, in response to temperature, can result in complex ecological changes (Mouritsen and Poulin 2002a, Poulin and Mouritsen 2006, James et al. 2018, Mouritsen et al. 2018, Friesen et al. 2021). Moreover, as many free-living species are infected with at least one specific parasite species (Kuris et al. 2008, Poulin 2014), host-parasite interactions are among the most intimately interspecific interactions in ecology. Thus, parasites have to be considered in studies of climate change effects as an important group of biotic drivers. In other words, understanding the fate of host-parasite systems in the context of global warming is crucial and demands consideration of the thermal sensitivity of various life-cycle stages of the involved species.

Trematode parasites are of particular interest in the context of global warming due to their complex life cycle, which often includes three hosts: a first intermediate host (usually a gastropod), a second intermediate host (e.g., crustaceans, fish, bivalves, amphibians), and a final host (often a vertebrate such as shorebirds; Galaktionov and Dobrovolskij 2003). Such a complex life cycle poses a severe constraint to trematode populations faced with global warming due to two main reasons. First, the absence of a single required host groups will directly result in the excision of the parasite from the community in question. Second, the life cycle includes free-living larval stages (i.e., miracidia and cercariae) directly, and potentially differentially, influenced by multiple

abiotic and biotic factors from the external non-host environment (Pietrock and Marcogliese 2003, Thieltges et al. 2008a).

Cercariae have been long known to be sensitive to temperature and one of the most fragile components of the trematode life cycle (Pietrock and Marcogliese 2003, Morley 2011a). Their survival and self-propelling capacity, for example, are highly constrained by warm temperatures due to the lack of feeding ability and, thus, rapid depletion of energy reserves at warmer temperatures (Morley 2011a). However, an increase in temperature can benefit other traits such as cercarial emergence and infectivity (Poulin 2006, Thieltges and Rick 2006). The observations on this thermally-induced tradeoff in favor of higher emergence and infectivity previously suggested that trematode infections will increase in a warming environment (Poulin 2006, Poulin and Mouritsen 2006, Thieltges and Rick 2006). However, growing evidence has shown that the matter is more complicated than previously envisioned, mainly due to important factors such as the hosts' sensitivity to abiotic stressors, the phenology timeline of both hosts and parasites, and the parasites' species-specific idiosyncrasy (Mouritsen et al. 2005, 2018, Marcogliese 2008, Studer et al. 2010, 2013, Morley 2011b, Paull et al. 2012, Paull and Johnson 2014, Selbach and Poulin 2020, Selbach et al. 2020).

Considering the first intermediate host's performance in response to temperature is essential to understand the fate of trematodes under global warming. Trematodes show a high specificity for their first intermediate host (Werding 1969, Galaktionov and Dobrovolskij 2003). This high specificity results in reduced flexibility when searching for appropriate hosts. Even though first intermediate hosts (e.g., gastropods) inhabiting intertidal zones are evolutionarily adapted to stressful environments, even these eurytherms may already live close to their limits of thermal tolerance, and prolonged exposure to heat and recurrent heatwaves can push them over the edge (Somero 2010). The heat sensitivity of first intermediate hosts can be amplified when they are infected by trematode parasites, especially by castrating parasite species, which can take a considerable toll on its host's thermal sensitivity (Fredensborg et al. 2005). Hence, the parasites' greater production of transmission stages could be offset by increased mortality of the first host in warming oceans.

Therefore, to understand the net effect of global warming on host-parasite interactions, it is necessary to consider the most fragile components of the life cycle of trematodes, such as the cercariae and the first-intermediate host. Using *Himasthla elongata*

(Mehlis, 1831) and its intermediate hosts from the Western Baltic Sea as a model system, this study experimentally assessed the impacts of temperature on (*i*) the survival of infected versus uninfected first intermediate host (i.e., the snail *Littorina littorea* (Linnaeus, 1758)) and (*ii*) the emergence of *H. elongata* cercariae along with their self-propelling capacity, survival, encystment, and infectivity to the second intermediate host (i.e., the Baltic *Mytilus edulis* sensu lato (Linnaeus, 1758; Figure 4). The temperatures tested (4, 10, 16, 22, and 28 °C) represent the range of temperatures expected to be relevant to the performance of the parasite (Thieltges and Rick 2006), and include projected end-of-century summer thermal averages and current summer heatwave events in the summer in the Baltic Sea (22 °C) (Franz et al. 2019, Morón Lugo et al. 2020, Wolf et al. 2020), as well as an extreme temperature scenario (28 °C). Specifically, we tested whether the vulnerability of the cercariae and (infected) gastropod host to high temperatures can set a limit on the heat-induced proliferation of the trematode infection to mussels.

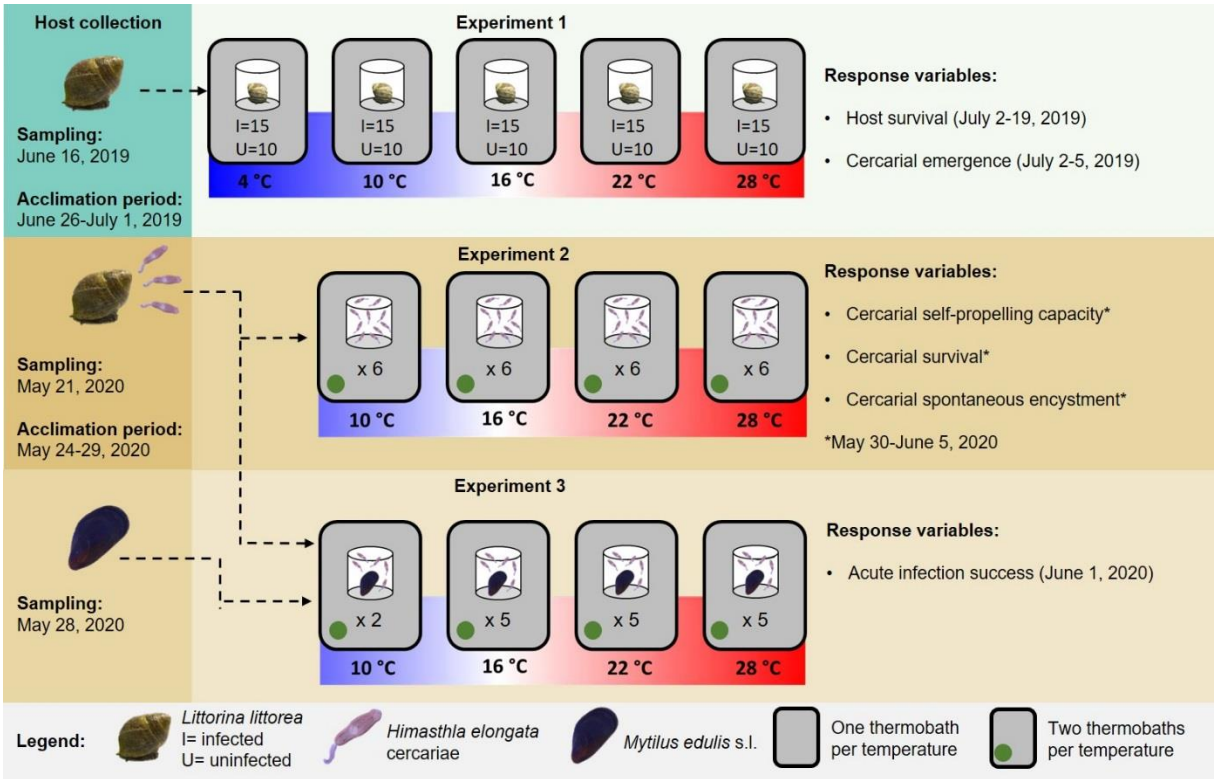

Figure 4 Schematic representation of the experimental design. In Experiment 1, the survival of infected and uninfected *Littorina littorea* was evaluated along with cercarial emergence at 4, 10, 16, 22, and 28 °C. In Experiment 2, the performance of cercariae at 10, 16, 22, and 28 °C was assessed by evaluating their activity, survival, and encystment rate. In Experiment 3, acute infection success of *Himasthla elongata* to *Mytilus edulis* s.l. was evaluated at 10, 16, 22, and 28 °C.

Methods

Collection of hosts

The first intermediate host, the gastropod *L. littorea,* was collected haphazardly by hand from the intertidal at Årøsund, Denmark (55°15'49.0"N 9°42'24.5"E) on June 16, 2019. Snails were transported immediately in portable coolers to the laboratory and kept in a climate chamber at 16 °C under a flow-through system of filtered seawater pumped from the Kiel Fjord. Snails were screened for trematode infections by placing one individual per well in 6-well plates filled with 8 mL of filtered seawater. All wells were covered with transparent lids and placed under lamps for 4 h to induce emergence of the parasite. Infected and potentially uninfected snails were kept in separate tanks at 16 °C until the start of the experiment. Snails were fed *ad libitum* with the seaweed *Fucus vesiculosus* collected from the Kiel Fjord. The second intermediate host, the blue mussel *M. edulis* s.l., was collected from the 'Kieler Meeresfarm' aquaculture facility in Kiel, Germany (54°22'59.1"N 10°09'41.8"E) on May 28, 2020, where no trematode infections have been found after numerous assessments. Mussels were measured, and individuals measuring 30-40 mm were kept in an 8 L plastic tank filled with filtered seawater at 16 °C. Before starting the experiment, mussels were fed once with 125 ng/L of chlorella powder (*Chlorella vulgaris*, Algomed®). To ensure that mussels were uninfected, a sub-sample of 50 mussels was dissected and inspected for metacercarial cysts under a stereo-microscope (Nikon, SMZ1000 body, C-PS160 stand). For a detailed timeline on the collection of hosts and execution of experiments, see Figure 4.

Experiment 1a: Host survival

Snails were acclimated between June 26 and July 1, 2019, to 4, 10, 16, 22, and 28 °C, respectively, in 5 different thermo-baths by increasing or decreasing temperature by increments/reductions of 2 °C every 24 h. This range of temperatures was chosen to cover the range of daily variation in temperatures during summer in shallow waters from the Baltic Sea and the expected critical minimum, maximum and optimal temperatures for the parasite (Thieltges and Rick 2006, Franz et al. 2019). Thieltges and Rick (2006) identified 10-25 °C as the relevant temperature range for the Baltic trematode species *Renicola roscovita*. However, since the present study used another trematode species from the Baltic, this range was expanded down to 4 °C and up to 28 °C to ensure coverage of the *H. elongata* tolerance range, should significant

differences in the thermal tolerance between these trematode species exist. During the acclimation phase, 10 uninfected and 15 infected adult snails per temperature level of similar shell length (Infected: 2.03-2.56 cm; Uninfected: 2.19-2.80 cm) were distributed in two 1-L tanks per infection group and fed *ad libitum* with *F. vesiculosus* (Figure 4). More infected snails were used due to the expected trematode-induced mortality under stressful temperatures. Water was constantly aerated and changed three times a week with temperature equilibrated and previously filtered aerated seawater pumped from the Kiel Fjord. After the acclimation period, snails were transferred individually to 50 mL PLEXIGLAS® beakers with 40 mL of previously aerated and acclimatized filtered seawater pumped from the Kiel Fjord. The photoperiod was set to start of sunrise at 4:00, reaching the maximum experimental intensity of light at 7:00, and sunset starting at 19:00, reaching total darkness at 22:00. Since cercariae are photo-sensitive (Soldánová et al. 2016), we decided to mimic the photoperiod of the season with the highest light intensity in order to avoid underestimations of behavioural responses (i.e., emergence, activity, and infectivity success). Each beaker was covered with a transparent plastic mesh to prevent the snails from escaping. Survival was recorded daily for a total of 17 days. In the case of death, the snail was dissected in order to confirm infection status.

Experiment 1b: Cercarial emergence

In order to evaluate the dependency of cercarial emergence to temperature, water from the Experiment 1a-snails was changed with clean, temperature equilibrated, and previously aerated filtered seawater pumped from the Kiel Fjord. Snails were incubated in recurrent periods of 12 h during July 2-5, 2019, under artificial light as described for Experiment 1a. After incubation, each beaker (including the ones with uninfected snails) was screened for parasites under the stereo-microscope. Water from the snails that did not shed cercariae was directly replaced with new water. When parasites were spotted, the snail was taken out, and the water was poured into a falcon tube. Afterwards, the beaker was rinsed with 10 mL of 70% ethanol and poured again into the falcon tube to fix the cercariae for later counting. The beaker was rinsed once with tap water and once with filtered seawater, filled with 40 mL of new water and placed back with the snails in the thermo-bath for a new incubation period of 12 h. Cercariae were counted under a stereo-microscope (Nikon, SMZ1000 body, C-PS160 stand) by pouring the content of the falcon tube into a petri dish. Metacercariae were also quantified upon appearance. Afterwards, the falcon tube was rinsed with tap water and

poured into the petri dish to ensure all cercariae were counted. In total, cercariae were collected every 12 h over 3 consecutive days. Oxygen levels, salinity, temperature, and pH were monitored before and after water exchanges. During the incubations, snails were fed with 1 cm^2 of *F. vesiculosus.*

Experiment 2: Cercarial activity

Snails collected on May 21, 2020, from Årøsund, Denmark, were acclimated during May 24-29, 2020, by increasing or decreasing 2 °C every 24 h. Once the acclimation to the experimental temperatures finished, cercariae were collected on May 30, 2020, by incubating infected snails in 6-well plates filled with 8 mL of clean temperature equilibrated and aerated seawater at the experimental temperatures (10, 16, 22, and 28 °C). Since almost no cercarial emergence occurred at 4 °C, this temperature was not included in the Experiment 2 and 3. After 1 h of incubation of snails under full light stimulus at the acclimation temperatures, fully active cercariae released by 8-12 snails were pooled together to include potential variation in thermal sensitivity among clones (Solovyeva et al. 2020). After collection, cercariae were immediately distributed in 12 PLEXIGLAS® beakers (10 cercariae in each beaker) filled with 40 mL of aerated, temperature equilibrated and filtered seawater (Figure 4). The beakers were distributed between two thermo-baths per temperature level (6 beakers per thermo-bath). The photoperiod resembled the one described for Experiment 1a. During the first 24 h, the activity of cercariae was recorded every 2 h. After the first 24 h, cercariae were evaluated every 24 h until all cercariae were dead or encysted. Activity traits included self-propelling capacity, premortem encystment, and mortality. “Self-propelling capacity” or functional lifespan was defined as cercariae which were swirling and displacing themselves in the water. “Premortem encystment” is the encystment of cercariae without the presence of the second intermediate host, which was identified by the formation of a defined opalescent cyst inside which the larvae could move (Galaktionov and Dobrovolskij 2003). Cercariae were categorized as “dead” when no movement was detected for 15 s after mechanical stimulus with a thin needle. In order to monitor water parameters (salinity, temperature, pH, and dissolved oxygen) without disturbing the cercariae with the multimeter probe (WTW 3630 IDS, Kaiserslautern, Germany), an additional seawater filled beaker per thermo-bath was added.

Experiment 3: Cercarial infectivity

Infectivity of *H. elongata* was performed as described in Bommarito et al.(Bommarito et al. 2020). In brief, on June 1, 2020, 10 mussels (*M. edulis* s.l.) per temperature measuring 30-40 mm of length were distributed among 50 mL PLEXIGLAS® beakers, each filled with 40 mL of aerated, temperature equilibrated, and filtered seawater (Figure 4). Each set of ten beakers (with mussels), were distributed between two thermo-baths (5 beakers per thermo-bath) previously set to the experimental temperatures (10, 16, 22 or 28 °C). The same photoperiod described for Experiment 1a was used. Before offering the cercariae to the mussels, 2 mL of a chlorella powder solution (2.50 µg/L; *C. vulgaris*, Algomed®) was added to each beaker (final concentration: 100 ng/L) to induce mussel filtering. From the same set of snails acclimatized in Experiment 2, cercariae were collected applying the same approach as described for Experiment 2. Nevertheless, since a significant number of snails died in the 28 °C treatment, the minimal number of snails possible (n=4) was used for cercariae collection in all treatments. After cercariae collection, 20 fully active cercariae were pipetted in each beaker as close as possible to the mussel inhalant siphon. They were then incubated for 24 h to ensure complete cercarial encystment (de Montaudouin et al. 2016). Mussels were removed from the experimental containers and kept at -80 °C until infection intensity evaluation. The intensity of infection was evaluated by counting metacercariae in whole soft body squash preparations under a stereo-microscope (Nikon, SMZ1000 body, C-PS160 stand).

Statistical analyses

All analyses were performed in R (version 4.0.2), RStudio© 1.3.1073 (2009-2020 RStudio, PBC). For Experiment 1a, the significance of differences between the means of infected and uninfected hosts' survival in each temperature was tested with a Mann-Whitney-U test with Holm-corrected p-values. The variance of gross cercarial emergence, encystment proportion, and net cercarial emergence was modeled in response to temperature as a continuous predictor. For cercarial emergence (Experiment 1b), a generalized linear model was applied with Poisson distribution and a second-degree polynomial term (considering the complex nonlinear effect of temperature) using the "glm" function from the "stats" package. The assumption of residual independence was tested by inspecting response (ordinary residuals, $y_i - \mu_i$), deviance, Pearson, and scaled-Pearson residuals against predicted values and

temperature (Zuur et al. 2009). However, since over-dispersion was detected, a correction to the standard errors was performed using a quasi-GLM model (Zuur et al. 2009). For the proportion of encysted cercariae per snail, over-dispersion was also detected. In this case, a negative binomial GLM was chosen over a quasi-GLM with Poisson distribution based on the log-likelihood test and the dispersion parameter (Zuur et al. 2009). Net cercarial emergence was calculated based on a hypothetical population of 10 snails using Equation 1.

$$N_{E,x} = n * E_{i,x} * p_{S,x} \qquad (1)$$

Where $N_{E,x}$ is the net cercarial emergence (# cercariae emerged from survived snails at temperature *x*); n is the number of snails in a population (here, assumed to be 10); $E_{i,x}$ is the # cercariae emerged per snail (replicate *i*) at temperature *x*; $p_{S,x}$ is the snails survival probability at temperature *x*. Although it is known that cercarial emergence rate can change over time (Soldánová et al. 2016, Bommarito et al. 2020), we assume that the emergence per individual in this case represent the natural variability in emergence patterns since snails were naturally infected and are, therefore, not synchronized.

The variation in net cercarial emergence was modeled using a generalized linear model with zero-inflated negative binomial distribution with linear parametrization and a third-degree polynomial term with the function “glmmTMB” from the “glmmTMB” package (Brooks et al. 2017). Model suitability was evaluated using the residual diagnostics tool from the “DHARMa” package (Hartig 2018), which includes quantile-quantile plots with KS test, outlier and dispersion as added tests, and a residual plot against predicted values with a built-in quantile regression to detect deviations from normality (Hartig 2018).

Variations in the proportion of active, dead and encysted cercariae (evaluated in Experiment 2) were modeled as functions of time and temperature as continuous variables using general and generalized additive mixed models (GAMM) with restricted maximum likelihood (REML) as fitting method using the “gam” function from the “mgcv” package (Wood et al. 2016, Wood 2017). GAMMs were selected over GLMMs to allow for the needed flexibility in modeling the variance in the response; the response variables varied differently and non-linearly along time in different temperature treatments, specially encystment rates. A binomial distribution with weights on the number of cercariae per beaker was used for modeling cercarial self-propelling and

mortality, and a gaussian distribution was used for cercarial encystment. Temperature and time were included as smooth terms and their interaction as a tensor product (i.e., non-isotropic smooth), which allows the modeling of an interaction between variables in different units such as time and temperature (Wood 2017). The attributes "thermobath" and "sample id" were included as random (intercept) effects in the global models and time as AR-1 autocorrelation structure to correct for potential dependency in the residuals along time. Model validation was performed by the "gam.check" function from the "mgcv" package and evaluating the residuals histogram plots and boxplots of residuals against each term. All global GAMM models were reduced to having only "sample id" as random factor since the random effects from "thermobath" were not significant. Since no among-residual dependency along time was detected for cercarial self-propelling and mortality, no autocorrelation structure was included in the models. In the case of cercarial encystment, temporal autocorrelation was detected and was therefore corrected for in the model. For cercariae self-propelling capacity the effective time when 50% of the response was reached (ET_{50}) was calculated with imageJ based on the plotted model estimates against time. A similar approach was taken to calculate the half-life or lethal time when 50% of the cercariae were dead (LT_{50}).

For experiment 3, the variation in infection success as a function of temperature was modelled as a continuous variable using GAMM with REML as fitting method. Temperature was included as a smooth term and thermobath as a random (intercept) effect. GAMM was selected over GLMM because it offered the best compromise between model performance and biological plausibility. Model suitability was evaluated using the residual diagnostics tool from the "DHARMa package". Net cercarial infectivity was calculated using the Equation 2.

$$N_{I,x} = \overline{N_{E,x}} * I_{i,x} \qquad (2)$$

Where $N_{I,x}$ is the net infectivity (# infective cercariae adjusted to the proportion of survived snails per temperature at temperature *x*); $\overline{N_{E,x}}$ is the mean net cercarial emergence at temperature *x* (estimated from the corresponding GLM model); $I_{i,x}$ is the infection success (replicate *i*) at temperature *x*.

The variation in net infectivity as a response to temperature was modeled using a generalized linear model with a third-degree polynomial term, thermobath as random factor and zero-inflated negative binomial distribution with linear parametrization using

the “glmmTMB” function. Model suitability was evaluated using the residual diagnostics tool from the “DHARMa” package.

For all models (with the exception of zero-inflated models) marginal and conditional R-squares were extracted using the “r.squaredGLMM” function of the “MuMIn” package (Nakagawa and Schielzeth 2013). For models with a log-link function the trigamma method was used to calculate pseudo-R^2 and for binomial distribution theoretical pseudo-R^2 was used (Nakagawa et al. 2017). For zero-inflated models, R^2 were extracted with the function “r2_zeroinflated” from the “performance” package (Lüdecke et al. 2020). Optimal temperatures for gross and net cercarial emergence, and gross and net infectivity of cercariae were estimated from the respective models using the function “predict” from the “car” package (Fox and Weisberg 2019). The normality of distributions was tested through a Shapiro-Wilk test and further evaluated with histograms and boxplots.

In order to be able to compare among the measured response variables or trematode performance traits at different temperatures, the logarithmic response ratio was calculated in relation to the mean response from the control temperature of 16 °C. The means of the control temperature for each trait were estimated from the above-described models. This was performed according to Lajeunesse (2015), who developed an adjustment to the widely used response ratio described by Hedges et al. (1999). The adjustment procures the avoidance of biases from small sample sizes (n < 15). To this matter, Equation 3 was applied.

$$RR^{\Delta} = \ln\left(\frac{X_T}{\bar{X}_C}\right) + \frac{1}{2}\left[\frac{(SD_T)^2}{N_T\bar{X}_T^2} - \frac{(SD_C)^2}{N_C\bar{X}_C^2}\right] \qquad (3)$$

Where $N_{T/C}$ is the sample size in treatment/control; RR^{Δ} is the adjusted log response ratio; $SD_{T/C}$ is the standard deviation of treatment/control sample; $\bar{X}_{C/T}$ is the estimated mean response of control/treatment; X_T is the response of a sample from a treatment.

Next to the log ratio, Lajeunesse (2015) recommends the employment of a small-sample size adjusted Geary index for both the control and treatment groups as diagnostic tool to validate log response ratios. The adjusted Geary index can be checked using the Equation 4.

$$\frac{\bar{X}}{SD}\sqrt{N} \geq 3 \qquad (4)$$

Results

Experiment 1a: General host infection status and survival

From the initial 125 snails, 82% survived the acclimation period. Specifically, 94% of the uninfected snails survived as compared to 76% of the infected snails. This left us with a total of 102 snails for Experiment 1, from which 54% were infected by trematodes. Among the infected snails, 11% were infected with *Renicola roscovita* and 89% with *H. elongata,* and no co-infections were detected. Statistical analyses were performed only for snails infected with *H. elongata* since the number of specimens infected by *R. roscovita* was too low to conduct meaningful comparisons. At the beginning of the experiment, 96% of the infections with *H. elongata* were patent (i.e., emergence of cercariae detected), which changed to 74% at the end of the experiment. At the end of the experiment, the percentage of patent infections was lowest at 4 °C (18% of the snails) followed by 10 °C, where 80% of the infected snails presented patent infections. Above 16 °C, patency was higher than 88%. Once acclimatized, *L. littorea* generally died faster at 22 and 28 °C than at colder temperatures (Figure 5). Snails infected by *H. elongata* survived less than uninfected ones, mainly at 22 °C, where significant differences were detected (Mann-Whitney-U-Test, $p < 0.01$). At 28 °C, mortality was high in both infected and non-infected snails. However, survival of uninfected individuals was slightly higher (survival range: 3-17 days; Figure 5), although not significant due to high variability.

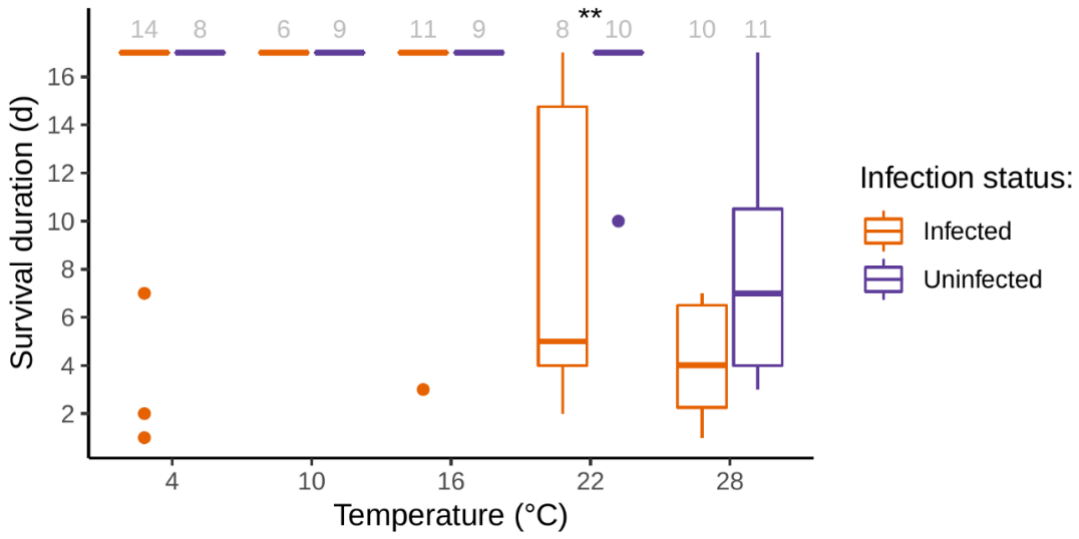

Figure 5 Survival duration of *Littorina littorea* uninfected and infected by *Himasthla elongata* after a 17-day post-acclimation exposure to different temperatures. Asterisks represent significant differences between infected and uninfected individuals (Mann-Whitney-U-Test, Holm-corrected $p < 0.01$). Gray numbers represent sample sizes.

Experiment 1b: Emergence of *Himasthla elongata* cercariae

The emergence of cercariae was significantly affected by temperature (2nd-degree polynomial: $p < 0.001$, $t = -5.34$; $df=38$, $R^2 = 0.85$; Figure 6A, Table S1.1). The optimal temperature for cercarial emergence was 22.7 °C with an estimated mean of 938 (702-1257) cercariae per snail or 313 (234-419) cercariae per snail per day, which decreased on average by 30% at 28 °C in comparison to 22 °C (Figure 6A). Almost no emergence was detected at 4 °C. A significant positive linear relationship in response was detected between emerged cercarial encystment and temperature with the highest encystment rate detected at at 28 °C (38%) and zero values below 16 °C ($p < 0.001$; $Z = 6.78$, $R^2 = 0.78$, $DF= 38$; see Table S1.1 and Figure S1.1). When correcting cercarial emergence for the survival of snails in each temperature, a negative shift in the optimal temperature by almost 4 °C was observed (Figure 6B).

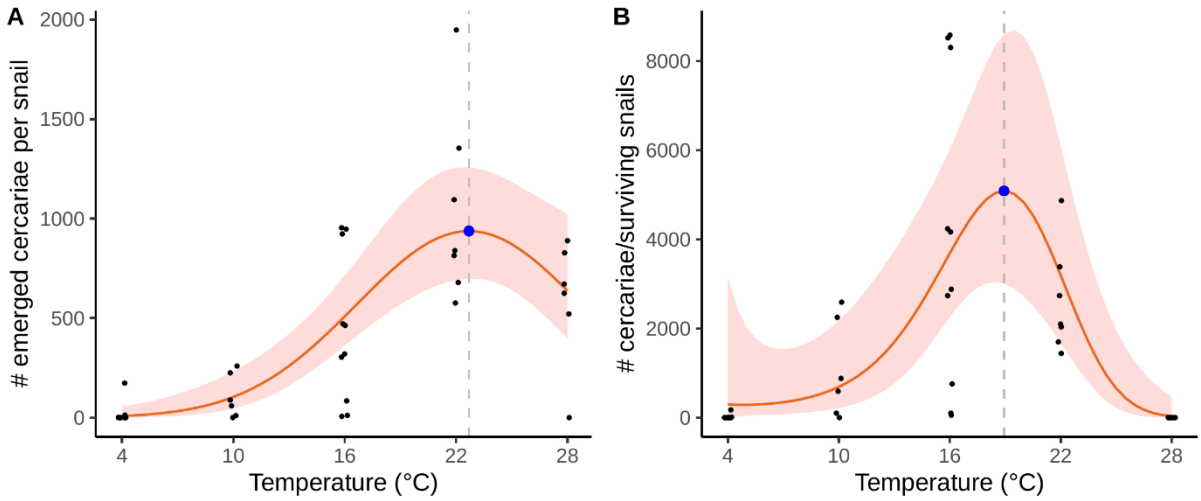

Figure 6 Cercarial emergence per snail (A) and net cercarial emergence per snails that survived from an initial population of 10 snails (B) over a 3-day incubation experiment in different temperatures. Regressions are based on generalized linear models with distributions of overdispersion-corrected Poisson (A) and zero-inflated negative binomial (B). The blue dot represents the optimal temperature [22.7 °C (A) and 18.9 °C (B)] for cercarial emergence [938 cercariae per snail (A) and 5088 cercariae per survived snails (B)].

Experiment 2: Self-propelling capacity, survival, and encystment of *Himasthla elongata* cercariae

For all the response variables, the nonlinear smooth terms for temperature and time, as well as the tensor product interaction of time and temperature, were significant ($p < 0.0001$, GAMM *t*-statistic; Table S1.2). For mortality the time and temperature tensor

product interaction was significant to a lesser degree ($p < 0.01$, GAMM *t*-statistic; Figure 7B; Table S1.2). Regarding cercarial self-propelling capacity, a decrease in a sigmoidal manner was observed across time, while increasing temperatures accelerated this decrease (Figure 7A). The whole model explained 87% of the variance in activity ($R^2 = 0.872$; Table S1.2). For 28 °C the calculated ET_{50} was of 2.96 h (2.56-3.35 h), for 22 °C it was 4.46 h (3.98-4.77 h), for 16 °C 6.31 h (5.95-6.70 h), and for 10 °C 10.65 h (9.98-11.28 h). Moreover, at both 28 °C and 22 °C, the self-propelling capacity ceased completely after 8 h, while at 16 °C and 10 °C cercariae ceased to be active after 10 h and 14 h, respectively. In terms of mortality, an increasing trend was observed across time, and temperature with 85% of the variance explained by the GAMM (Figure 7B).

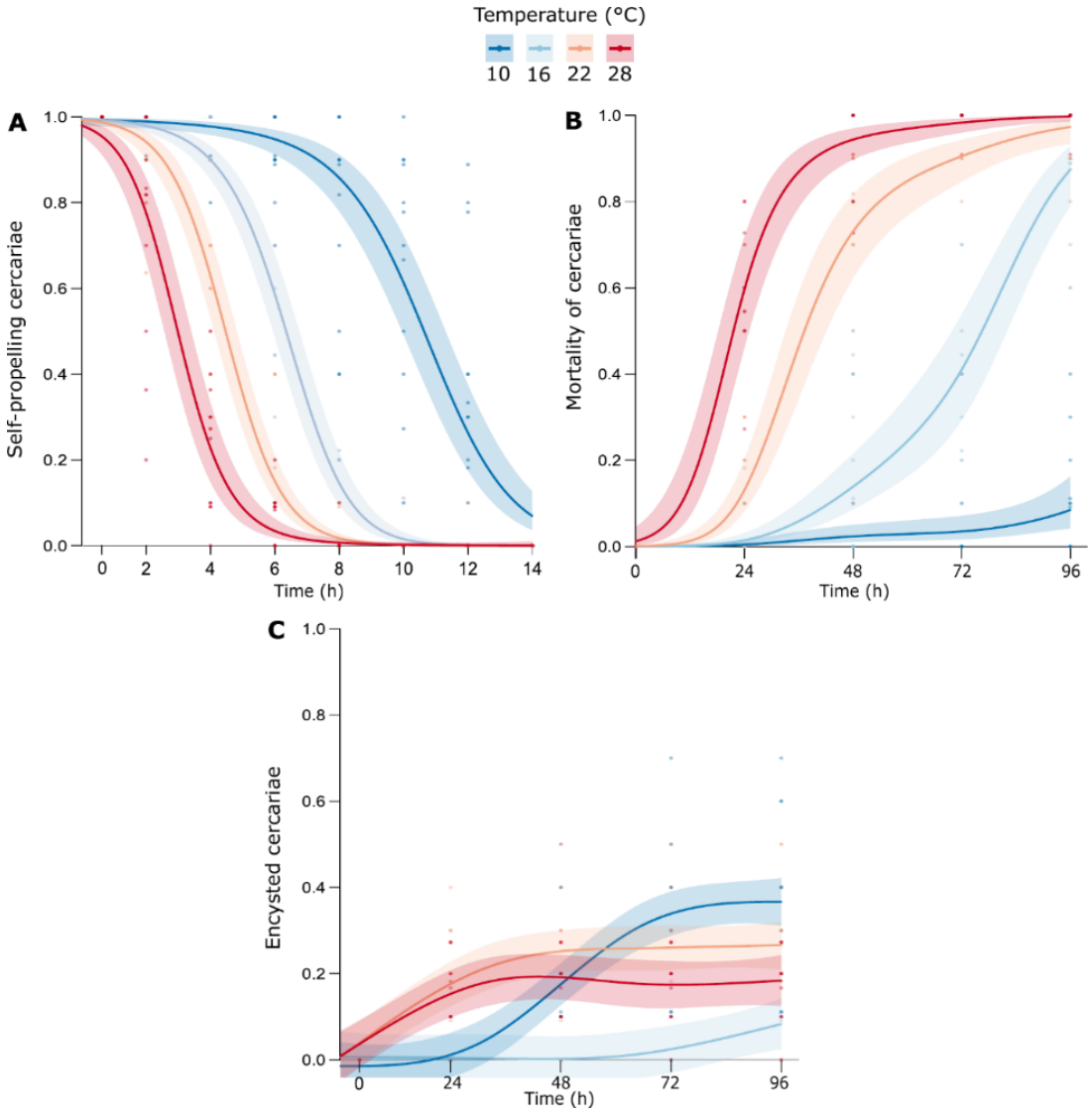

Figure 7 Generalized additive mixed models of *Himasthla elongata* cercariae activity (A), mortality (B), and encystment (C) with temperature (°C) and time (h) as smooth terms. Models explain 87%, 85%, and 78% of the response variance, respectively.

Similar to cercarial self-propelling capacity, the increase in mortality along time was enhanced by temperature. Specifically, the estimated half-life of cercariae (LT_{50}) was 21.88 h (18.28-25.33 h) at 28 °C, 37.57 h (33.82-42.03 h) at 22 °C, and 76.71 h (70.67-81.90 h) at 16 °C. At 10 °C, maximum mortality of 9% was observed at 96 h with cercariae at this temperature surviving up to 120 h. Finally, pre-mortem encystment of cercariae followed a more complicated pattern. At the warmest temperatures (22 and 28 °C), cercariae started encysting earlier but reached an estimated mean encystment rate of 27% (22-31%) and 18% (13-23%), respectively (Figure 7C). Meanwhile, cercariae at 16 °C started to encyst later on but reached a higher encystment proportion of 37% (32-42%). At 10 °C, almost no encystment was observed with an estimated mean of 8% (3-14%). Moreover, the optimal temperature for cercariae pre-mortem encystment decreased over time (Figure S1.2). In the case of pre-mortem encystment, the model explained 78% of the variance.

Experiment 3: Infection success

Both gross and net infectivity significantly correlated with temperature in a bell-shaped curve (Figure 8A,B). For acute infection success, the nonlinear smooth term of temperature was significant ($p < 0.0001$, Table S1.3). The whole model explained 47% of the variance ($R^2 = 0.469$; Table S1.3). The estimated optimal temperature for infection success was 21.5 °C, with an estimated mean of 45 (30-57)% (Figure 8A). In terms of organ partitioning, cercariae encysted mostly in the mussel's foot at 10, 16, and 22 °C. Encystment in the mantle was recorded in all temperatures, but it was highest at 22 °C. Encystment in other organs (e.g., adductor and retractor muscles, and intestine) was highest at 22 °C and minimal in the other temperatures. Regarding the gills, no change in encystment was observed among temperatures except for 28 °C where encystment in the gills was absent. At this temperature (28 °C), infection success was the lowest, and only the foot, the mantle, and muscles were infected without any clear difference among these organs. When considering cercarial emergence and the effect that temperature-specific gastropod survival has on cercarial emergence, the optimal temperature for net infective cercariae was reduced by 2.4 °C, with an estimated number of infective cercariae from surviving snails of 1933 (Figure 8B). For this model, all terms (i.e., first-, second- and third-degree terms) were significant ($p < 0.0001$, GLMM *z*-statistic; Table S1.3).

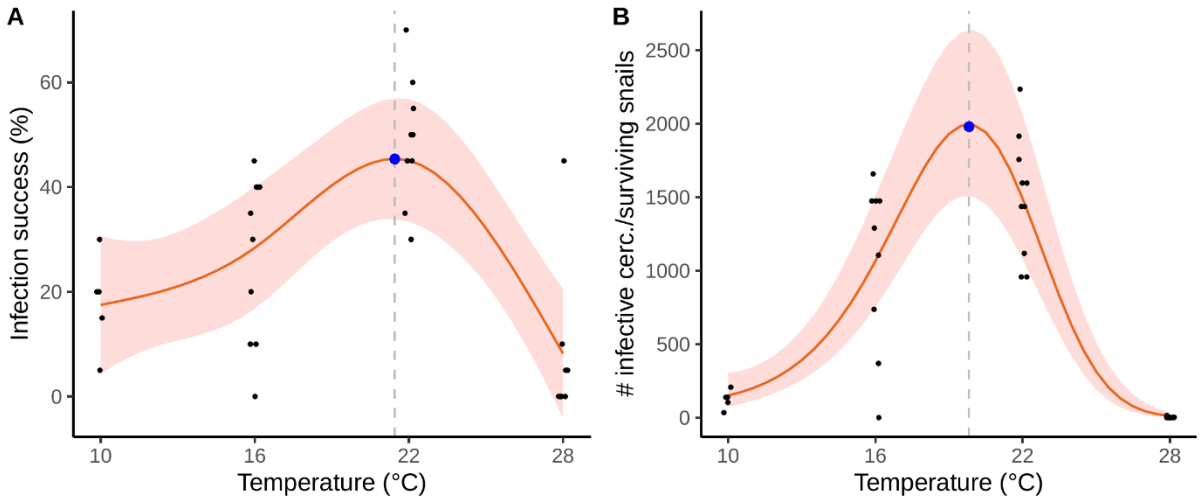

Figure 8 General additive mixed model of *Himasthla elongata* cercarial acute infection success (A) and generalized linear mixed model of cercarial net infectivity (B) after 24 h of exposure to *Mytilus edulis* s.l. under different temperatures with gaussian (A) and zero-inflated negative binomial (B) distributions. The blue dot represents the optimal temperature [21.5 °C (A) and 19.8 °C (B)] for cercariae infectivity [45% infectivity (A) and 1933 infective cercariae from surviving snails (B)].

Summary: log response ratios

When comparing the treatments to the baseline temperature of 16 °C we can see a differential response of *H. elongata* life cycle stages to temperature (Figure 9). Specifically, the treatment of 22 °C was beneficial to cercarial gross emergence, infection success, and net infectivity. In contrast, this treatment decreased survival of infected gastropods, net cercarial emergence, and cercarial self-propelling capacity from 4 h onwards. The 28 °C-treatment was detrimental for all traits except for cercarial emergence and activity at 2 h, where the effect was almost neutral (Figure 9). At 10 °C most traits were unaffected except for net infectivity, which was reduced, and cercarial self-propelling at 8 h, which was greatly enhanced. At 10 °C, no log-response ratio for cercarial emergence was reported since the Geary index suggested by Lajeunesse (2015) was below three. For the self-propelling capacity of cercariae, only the first eight hours are shown since, after this time, the larvae usually lose their self-propelling capacity.

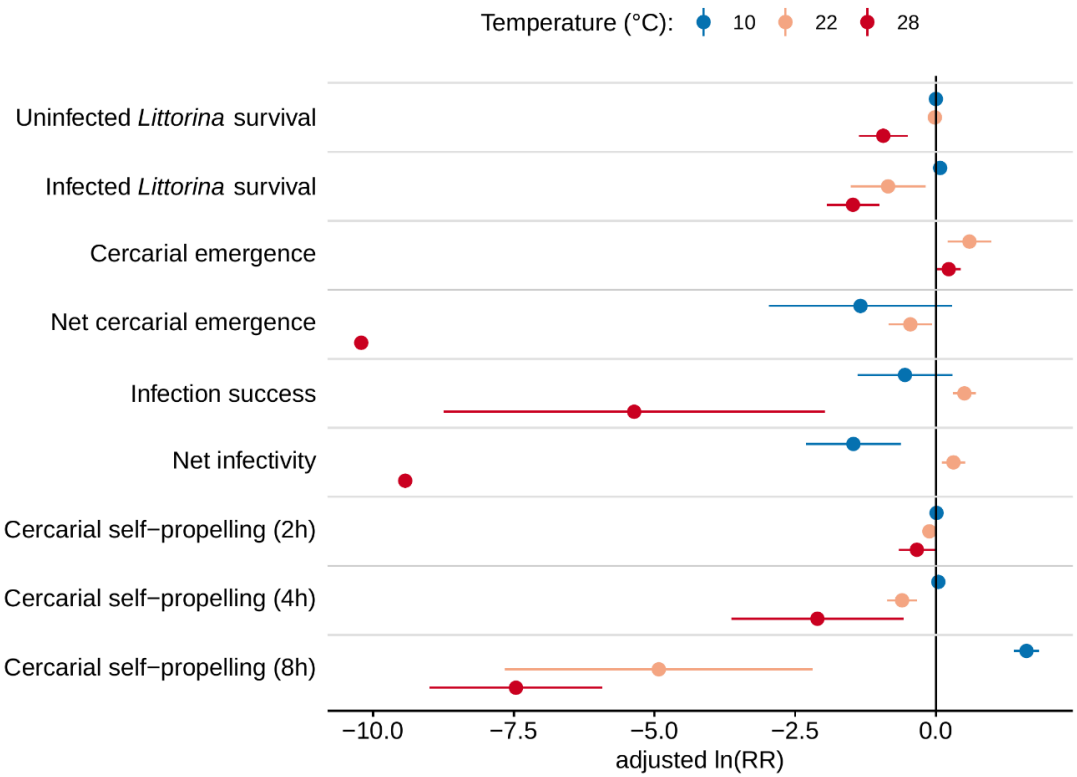

Figure 9 Logarithmic response ratios for crucial traits of the *Himasthla elongata* life cycle in response to temperature deviations. Ratios were calculated and adjusted to small sample sizes according to Lajeunesse (2015) in relation to the baseline temperature of 16 °C. The means of the control temperature for each trait were estimated from the models described in the methods section. Values are given as means and confidence intervals (α = 0.05).

Discussion

The present study illustrates that the first intermediate host (i.e., gastropod) and cercariae represent a fragile link in the life cycle of trematodes under current extremely warm events and projected end-of-century mean temperatures for temperate systems during summer (Gräwe et al. 2013). The ubiquity of trematodes in the environment and their capacity of modulating complex ecological systems makes their consideration in global warming effects predictions an urgent task. The studied *H. elongata* host-parasite system is a good example of the complex dynamics between closely interacting species such as the trematodes and ecologically relevant benthic species (e.g., the common periwinkle, *L. littorea*, and the blue mussel, *M. edulis* s.l.) under the influence of thermal stress. Our results conclusively showed that the optimal temperature range of parasite performance might be overestimated when looking at individual life cycle components. The infected gastropod's thermal sensitivity and reduced functional lifespan and survival of the cercariae resulted in a decreased overall

performance of the parasite with temperatures above the thermal optimum of the host snail.

The trematode-induced gastropod thermal sensitivity is of significant ecological relevance under current and projected thermal regimes for temperate ecosystems as such gastropods play a major ecological role as grazers in their ecosystem. The infected gastropods show substantial mortality already after a few days (i.e., 1-7 days) of exposure to temperatures of 22 °C, projected as mean summer values for the region for the end-of-century (Morón Lugo et al. 2020), as opposed to uninfected individuals which had no mortality at this temperature, and only started to die after three days exposure to 28 °C. Current summer heatwaves in shallow water Baltic Sea habitats can reach up to 22 °C or even higher for several days (Franz et al. 2019, Morón Lugo et al. 2020, Wolf et al. 2020). This means that even the projected average summer temperatures will be stressful for infected gastropods. Although littorinids are assumed to be resistant to harsh conditions due to their evolutionary history in extreme environments (Clarke et al. 2000, Sokolova et al. 2012, Pansch et al. 2018), exposure to other stressors (e.g., pathogens) could be fatal to them.

Trematodes pose significant stress to their gastropod host in many ways. First, they are known to castrate their host by chemical interference of the host's endocrine system and physical destruction in mature infections (Fredensborg et al. 2005). Also, cercarial emergence can severely damage the host tissue since they migrate through the skin of the gastropod (Fredensborg et al. 2005). Enhanced gastropod mortality by parasites has already been observed for other trematodes. McDaniel (1969) showed that *L. littorea* infected with the trematode *Cryptocotyle lingua* have a reduced heat tolerance. In warm temperatures, infected snails show more mature and heavier infections, while at cold temperatures, infection intensity is reduced, and relatively small rediae can be observed (personal observation). Since higher temperatures can accelerate the development of infections (Ataev 1991), snails might benefit from colder temperatures where infection development is arrested and poses less stress to the host.

The parasite-enhanced mortality and decreased overall fitness have been observed both in the marine environment and freshwater ecosystems (Kuris 1980, Paull and Johnson 2011, Paull et al. 2015). Paull and Johnson (2011) showed that the freshwater trematode *Ribeiroia ondatrae* increased its pathology with warmer temperatures by

reducing fecundity in the gastropod *Planorbella trivolvis* (previously *Helisoma trivolvis*). In the same host-parasite system, Paull et al. (2015) showed that infection by *R. ondatrae* increased snail mortality both before and after temperature shifts. These effects of trematodes on their gastropod host can go beyond the individual level and can result in a cascade of effects that compromises the structure and functioning of communities (Mouritsen and Haun 2008). In the case of *H. elongata*, the prevalence of infection has been reported up to 40% in the southwestern Baltic Sea (Bommarito et al. 2021). A high prevalence of infection combined with decreased thermal tolerance could translate into two possible (mutually non-exclusive) scenarios. First, in the near future, infection plus high temperatures could provoke a significant decrease in gastropod populations and, therefore, the scarcity of a functionally important organism on rocky shores. In the long run—bearing in mind the castrating behavior of *H. elongata*—it could provoke an evolutionary advantage for gastropod populations since thermal stress would select for uninfected (i.e., non-castrated) thermal tolerant individuals which can contribute to the reproduction and persistence of the species.

Acute infection success was also optimal at 22 °C. Higher infectivity with higher temperature has been reported for many cases in the literature for both laboratory and field experiments (Mouritsen and Jensen 1997, McCarthy 1999, Thieltges and Rick 2006, Morley and Lewis 2015). Increasing temperatures accelerate the metabolism of cercariae, providing more ATP for penetrating the host tissue and establishing as metacercariae (Galaktionov and Dobrovolskij 2003, Morley and Lewis 2015). The infectivity of trematode larvae can also be compromised by a reduction in the filtering activity of the mussel (de Montaudouin et al. 1998). *Himasthla elongata* targets primarily the foot and the mantle of the mussel, which are usually exposed when the mussel is open. In our case, after offering the cercariae to the mussel, most of them were open with their mantle and foot exposed, except for one mussel at 16 °C and one at 28 °C. Mussel filtration rate has been demonstrated to remain stable up to 24 °C, and beyond this temperature, substantial metabolic depression has been observed (Vajedsamiei et al. 2021a). Our data sustain this fact since the infection of the gills remained relatively constant between 10-22 °C. Therefore, in our experiment, infectivity might be affected by reduced mussel filtration only at temperature 28 °C.

Reduced activity and survival with increased temperature and time were not a surprise. Previous literature has shown that activity and survival of continuously swimming cercariae are limited to a few hours due to their lecithotrophic character (Koehler et al.

2012). The inability to feed makes glycogen (the primary energy reserve for cercariae) a limiting factor. Therefore, higher metabolic rates enhanced by warmer temperatures will deplete the energy reserve faster, which manifests into faster loss of activity and, ultimately, death (Morley 2011a). Accelerated death can be observed when comparing the half-life (ET_{50}) of cercariae, which at 28 °C was almost four times lower than at 16 °C. In terms of self-propelling capacity, the ET_{50} values were three times lower at 28 °C than at 16 °C. Self-propelling capacity resulting in an efficient displacement in the water column is an important trait for trematode overall performance. Since himasthlids do not use chemotaxis and do not search actively for a host, they rely on other important factors aiding transmission (de Montaudouin et al. 1998, Stunkard 2014). Such factors include displacement in the water column via self-propelling, phototactic behavior, positive geotropism, which naturally attracts them to benthic invertebrates (i.e., bivalves), and the siphon current from the host itself (Werding 1969, de Montaudouin et al. 1998). In this experiment, the capacity for self-propelling generally lasted longer than expected, especially at lower temperatures (10 °C), where cercariae were self-propelling for up to 14 h, although they were displacing themselves very slowly (personal observation). Therefore, conducting behavioral studies that characterize cercariae movement patterns (e.g., swimming velocity and distance moved) in response to temperature might be complementary to self-propelling capacity as indicators of infectivity (Selbach and Poulin 2018).

In Experiment 1b, we initially observed increases in the proportion (maximum 40%) of cercariae spontaneously encysted with increasing temperatures. For the marine trematode species *H. elongata,* this type of encystment occurs typically in less than 1% of the cercariae (Gorbushin and Shaposhnikova 2002, Gorbushin and Levakin 2005), and the factors inducing this type of encystment have not been elucidated yet. Only two studies reported higher *in vitro* spontaneous encystment in the presence of hemolymph and plasma extracted from mussels (Gorbushin and Levakin 2005, Levakin et al. 2013). However, these factors are only relevant when the cercariae are inside the mussel. In our case, after closely evaluating encystment and mortality rates as a response to time and temperature (i.e., Experiment 2), we can see that cercariae were not only encysting faster but were also dying faster. Therefore, encystment without the presence of the second intermediate host (i.e., spontaneous encystment) seems to be a before-death response. We thus suggest the term *pre-mortem encystment* as a more accurate term to describe this aspect of the life cycle.

Pre-mortem encystment could be useful for the parasite when the second intermediate host is not present. Instead of merely dying, the cercariae transforms into metacercariae, extending the larvae's lifetime for up to 48 h (personal observation). However, reaching the final host in this way is very unlikely. This reduced probability is attributed to the fact that shorebirds get infected more likely when feeding on infected bivalves than when accidentally ingesting metacercariae directly from seawater (Choisy et al. 2003). Moreover, this external cyst has a thicker impermeable layer that protects against external stressors (Galaktionov and Dobrovolskij 2003). A thicker layer does not offer the same advantages as the cyst formed in the bivalve tissue, which has a thinner permeable layer that allows for obtaining nutrients from the host (Galaktionov and Dobrovolskij 2003). Another interesting observation was that, even though the cercariae were encysting faster at higher temperatures, the maximum encystment proportion was observed at 16 °C. At higher temperatures, pre-mortem encystment might be hindered by enhanced metabolism leading to a faster loss of energy reserves(Pechenik and Fried 1995). The formation of the multiple layers of the cyst requires energy-costly metabolic processes (Galaktionov and Dobrovolskij 2003). Similar results were found by Fried and Ponder (2003), who evaluated *in vitro* encystment of the freshwater echinostome *Echinostoma caproni* at 12, 23, 28, and 37.5 °C. The authors of this study found that maximum encystment in artificial media (Locke's medium mixed with artificial pond water in a 1:1 ratio) was reached at 23 °C with 78.2% of the cercariae encysted, while at 28 °C it decreased to 43.8% and to 0% at the maximum temperature.

When looking at life cycle components individually, we could hypothesize that trematode transmission might be facilitated in current summer heatwave events and end-of-century temperature scenarios (22 °C for the Baltic during the summer). Cercarial emergence and infectivity were optimal near this temperature (22.7 °C for the emergence and 21.5 °C for infectivity). Nevertheless, this assumption might not hold true for three reasons. First, even though we see a peak of emergence and infectivity, the optimal temperature range is approximately 3-6 °C wide (i.e., optimum between ca. 19-25 °C). The stability of cercariae over a wide thermal range at its optimum has been recently explored and challenges the previous assumption that temperature is the most determinant abiotic factor in the transmission of parasites (Morley and Lewis 2013, 2015, Bommarito et al. 2020). Nevertheless, since the amount of data gathered in this experiment does not allow for an accurate estimation of the

optimal temperature range via means of bootstrapping or posterior inference, we highlight the importance of testing the same range of temperatures with higher resolution (i.e., more temperatures). Second, after adjusting cercarial emergence and infectivity to the parasite- and temperature-induced mortality of the gastropod host, we can see that the optimal temperature for emergence and infectivity is shifted to lower temperatures resulting in a costly tradeoff. Specifically, trading first intermediate host survival for higher cercarial emergence as a response to global warming (i.e., prolonged exposure to 22 °C) translates into approximately 41% of loss in net cercarial emergence and 25% of loss in net infectivity in comparison to optimal conditions (i.e., 19 °C). Therefore, this disparity between the thermal performance of the host and the parasite is unstable ground and could translate into a collapse of the host-parasite system overall, as evidenced in other host-parasite systems (Mouritsen et al. 2018). The third reason why trematodes will not necessarily benefit from warmer temperatures is the energetic cost that warming implies for the cercariae. Increasing temperature accelerates the loss of self-propelling capacity and death of cercariae, thus tightening the time window at which cercariae are infective. At 22 °C—temperature representing current summer heatwave events and end-of-century projected averages for the Baltic—the half-functional lifespan of cercariae is only 4 h. With mussel beds declining in biomass due to climate change and displacement by invasive species (Büttger et al. 2008, Jaatinen et al. 2021), a narrow infective time window represents an obstacle for transmission since declining blue mussel populations reduces the probability of cercariae reaching the bivalve host in time before losing infection potential and their chance to continue the life cycle.

Although the overall prognosis for trematode infections in a warming sea does not seem to be auspicious, other scenarios might hold probable. For example, since temperatures are expected to increase gradually until the projected end-of-century scenario is reached, summer thermal averages might initially benefit the parasite in upcoming years. In other words, before we reach a summer thermal average of 22 °C, colder thermal averages (i.e., 18-21 °C) might benefit the parasite initially, and only after, net adverse effects should be expected. Moreover, global warming might create appropriate conditions in seasons that (currently) might be too cold for parasites to proliferate (i.e., in winter). Therefore, we might expect a shift in the season of high infection development and activity instead of an apocalyptic scenario overall. In addition to this, parasites and hosts might be subject to gene adaption and phenotypic

plasticity: temperature resistant genotypes and phenotypes of hosts and parasites might be selected in synchrony through adaptive evolution and thus aid in the sustainability of the host-parasite system (Studer and Poulin 2014). Following this line of thought, future research should consider the possibility and the differences in the adaptation capacity of hosts and parasites considering the difference in generation times and the intraspecific genetic variation (Paull and Johnson 2011, Berkhout et al. 2014).

Even though we did not evaluate the final and second intermediate host's performance in this study, trematodes show low specificity towards them and thus may find alternative ways to prosper. A low specificity gives an advantage to the trematode by having different options to complete the life cycle. Taking *H. elongata* (Mehlis, 1831) as an example, it has several birds as final hosts, such as *Larus* spp., *Haematopus ostralegus*, and *Somateria mollisima* (Werding 1969, de Montaudouin et al. 1998). Regarding the infective larval stage released from the final host (i.e., miracidia), no studies are available specifically on *H. elongata*. However, studies conducted using *H. militaris* showed that, although the half-life of miracidia was significantly reduced at 25 and 30 °C compared to 14 °C, the infectivity of the larvae increased and remained constant at the warmer temperatures (Vanoverschelde 1981). In the case of miracidial eclosion, its proportion increased, and the process was accelerated with increasing temperature (20 and 30 °C) while it was nil at 12 °C (Vanoverschelde 1982), altogether suggesting a high tolerance of miracidia to high temperatures. Moreover, regarding the thermal tolerance of the final host, endotherms have the advantage of modulating their body temperature and are highly mobile, thus being capable of seeking shelter in extreme temperature conditions.
In the second intermediate host's case, cercariae usually parasitize *M. edulis* s.l., but can also infect other bivalves such as the edible cockle *Cerastoderma edule* (Werding 1969, de Montaudouin et al. 2009, Richard et al. 2021). In addition, trematodes encyst as metacercariae—semi-dormant stages of the parasite—in the second-intermediate host (Galaktionov and Dobrovolskij 2003). Although metacercariae are known to negatively affect the host's metabolism, condition index, and biochemistry (de Montaudouin et al. 2012, Stier et al. 2015, Bakhmet et al. 2017, Magalhães et al. 2017, 2018a, 2020), these larval stages are assumed to pose less stress to the host than rediae, which actively feed on the tissue of the gastropod host (Galaktionov and Dobrovolskij 2003). In terms of thermal tolerance, mussels are highly sensitive to

thermal exposure and show significant mortality after recurrent heatwave events (Seuront et al. 2019). Furthermore, since mussels are semi-sessile, they are usually constrained to their habitat and cannot seek shelter as easily as birds or gastropods. However, for infected specimens, recent findings suggest that *H. elongata* metacercarial infections can even protect its host from heat (35 °C compared to 15 °C) at high infection intensities (> 250 metacercariae mussel^{-1}) (Selbach et al. 2020). Although the mechanisms behind this heat-protection are still to be resolved, Selbach et al. (2020) speculate that trematodes might protect the host by pre-equipping it with heat shock proteins.

In summary, by combining multiple traits, we show that the optimal temperature range of parasite performance might be overestimated when looking at individual life cycle components. The thermal sensitivity of the infected gastropod, along with reduced functional lifespan and survival of the cercariae, resulted in a decreased overall performance of the parasite at high temperatures. In addition to this, this study also evaluated the capacity of *H. elongata* cercariae to encyst in the non-host environment as a function of time and temperature for the first time to our knowledge. We determined that increasing temperatures induced faster spontaneous encystment as a consequence of increased cercariae mortality. We show that, as time progresses, the optimal temperature for spontaneous encystment shifts towards colder temperatures down to 16 °C, highlighting the importance of time scale in the life cycle of trematodes.

As with every laboratory study, controlled experiments unavoidably neglect other factors important for the thermal tolerance of marine ectotherms and parasite transmission. Predicting the future of trematodes in a warming sea is difficult due to the numerous factors that are related to warming, which can significantly alter host-parasite dynamics. For example, in warming shallow waters, biotic productivity might be greatly increased (Österblom et al. 2007). Increased biotic productivity can create opportune conditions that invite birds to aggregate, forage, and increase their infection chances (Zander and Reimer 2002, Johnson et al. 2007, Budria and Candolin 2014, Aalto et al. 2015). This increase in chances of infection will benefit allogenic parasites such as *H. elongata*, which use birds as final hosts (Zander and Reimer 2002, Johnson et al. 2007). On top of this, the thermal sensitivity of infected snails can be buffered by daily thermal fluctuations, which can provide relief to intertidal organisms, especially at extreme temperatures (Vajedsamiei et al. 2021b). Moreover, given that temperature can vary within small spatial scales, snails can mobilize to near macro- and micro-

habitats with benign temperatures (Moisez et al. 2020). On the contrary, our experiments restricted snails to constant temperature conditions. Therefore, there is a possibility of an overestimation of the thermal sensitivity of the gastropod. Additionally, parasites can also influence microhabitat selection, especially by trematodes that influence their host's behavior (Shinagawa et al. 1999, Bates et al. 2011, Friesen et al. 2018). Some trematode species (e.g., *Maritrema* spp.) can modify the host's behavior so that the host settles in temperatures matching the thermal optimum of the parasite in natural thermal gradients (Bates et al. 2011). Therefore, we encourage and highlight the importance of conducting more near-natural experiments on larger scales such as mesocosms and field studies, considering other important factors affecting parasite-host interactions such as salinity, predation, and dilution of free-larval stages (de Montaudouin et al. 1998, Thieltges et al. 2008a, Welsh et al. 2014, Bommarito et al. 2020).

Chapter II: Parasitism and temperature affect the feeding, energy metabolism, and stress response of *Littorina littorea*, a prime consumer of a native and an invasive alga in the Baltic Sea

Introduction

As a result of globalization and climate change, marine bioinvasions have increased in the last decades and often are of ecological and economic concern (Ruiz et al. 1997, Schaffelke et al. 2006, Williams and Smith 2007, Sorte et al. 2010, Cuthbert et al. 2021). Consequently, efforts to understand the mechanisms responsible for the success of such invasions have intensified (Hu and Juan 2014, Papacostas et al. 2017). The red algae *Gracilaria vermiculophylla*—originating from the northwestern Pacific—is no exception (Thomsen et al. 2007, Hu and Juan 2014). Its introduction to the western and eastern Atlantic coast was first recorded in 1998 and to the southwestern Baltic coast in 2005 (Mollet et al. 1998, Rueness 2005, Freshwater et al. 2006, Schories and Selig 2006). The invasion success of this algae has been attributed to the avoidance of feeding on it by some native grazers (Wolfe 2002, Nejrup et al. 2012), recruitment versatility (Thomsen et al. 2007), antifouling defenses (Wang et al. 2017b, 2017a), and to its high tolerance to a wide range of environmental factors including temperature, nutrients, and salinity (Thomsen et al. 2007, Weinberger et al. 2008). The spread of *G. vermiculophylla* can result in various negative effects, including the smothering of bivalve communities (Thomsen and McGlathery 2006), and can lead to competition with native algal species, such as the rockweed *Fucus vesiculosus,* for space, light, and nutrients (Weinberger et al. 2008, Hammann et al. 2013). Since the invasion range of *G. vermiculophylla* is expected to expand (Thomsen et al. 2007, Weinberger et al. 2008, Krueger-Hadfield et al. 2018), the biotic and abiotic factors that modulate the interaction between this alga and *F. vesiculosus* require further understanding.

One of the main biotic modulators of algae competition is grazing pressure (Alestra and Schiel 2014). The Enemy Release Hypothesis assumes that invasive species that have lost their enemies during the process of invasion have greater invasion success (Wolfe 2002). Although this might explain part of the invasion success of

G. vermiculophylla (Nejrup et al. 2012), it does not imply that this alga is not consumed at all in its invasion range. In the presence of fast-growing algae like *Ulva* spp., *G. vermiculophylla* is avoided by native grazers such as *Littorina littorea, Gammarus* spp., and *Idotea balthica* (Nejrup et al. 2012). However, when offered together with slower-growing algae (e.g., *F. vesiculosus*), *G. vermiculophylla* is preferred by certain grazers, such as the isopod *I. balthica* (Nejrup et al. 2012). Moreover, by combining field observations and laboratory studies, Weinberger et al. (2008) showed that grazers can efficiently control the growth of *G. vermiculophylla* during the winter season. Therefore, grazers are a potential modulator of the interaction between these native and invasive algal species.

Another biotic factor that can act as an indirect modulator of the interaction among algae is parasitism in grazers. Parasites are considered an essential part of biodiversity and community-level ecological dynamics (Poulin et al. 2016, Sures et al. 2017a). They are known to influence their host's behavior and fitness (Poulin 1994, Magalhães et al. 2020) and induce ecologically-relevant shifts in species-species interactions (Wood et al. 2007, Mouritsen and Haun 2008, Hatcher et al. 2012). Parasites might also indirectly affect the outcomes of biological invasions by mediating a range of competitive interactions among native and invasive species (Prenter et al. 2004). For example, infected and uninfected grazers could differentially alter the interaction between invasive and native algal species. Among parasites, trematodes are particularly common in gastropods and other herbivores such as amphipods and isopods (Mouritsen and Poulin 2002b, Thieltges et al. 2006). Their capacity to modulate grazing behavior has been well documented (Wood et al. 2007, Bernot and Lamberti 2008, Clausen et al. 2008, Larsen and Mouritsen 2009, Morton 2018, Vivas Muñoz et al. 2018, Ayala-Díaz et al. 2019). Therefore, given the ubiquity of trematodes and their potential impact on the dynamics of benthic communities, their consideration in algal invasion ecology is crucial.

Competition between algae can also be modulated by abiotic factors such as temperature. Temperature can directly influence the interaction between invasive and native macroalgae by differentially altering the physiology and performance of the species and, thus, their capacity to compete (Verlinden et al. 2014, Poore et al. 2016, McKnight et al. 2021). Temperature can also act indirectly by 1) altering the palatability of the algae to grazers via changes in their nutritional quality or changes in the production of defense-related secondary metabolites (Connan et al. 2007, Poore et al.

2013, Raddatz et al. 2017); 2) affecting the physiology of grazers and thus their grazing rates (Sampaio et al. 2017, Wahl et al. 2019). Temperature and parasites combined can have further complex effects on grazing behavior. First, warmer temperatures have been shown to neutralize the effects of *Cryptocotyle lingua* infections on *L. littorea* consumption rates of the native alga *Ulva lactuca* (Larsen and Mouritsen 2009). Second, infection and temperature combined can also affect the hosts' fitness and enhance the mortality of grazers (Fredensborg et al. 2004, Paull et al. 2015, Díaz-Morales et al. 2022), compromising the balance in marine benthic assemblages, including the algal components (Mouritsen et al. 2005, 2018, Larsen and Mouritsen 2014).

For this study, we chose *L. littorea* as the grazer of focus because it is widespread in the Baltic Sea and it serves as the first-intermediate host for several trematode species (Werding 1969). Moreover, the asexual reproduction of the trematode in its first-intermediate host is expected to have a more deleterious impact compared to the semi-dormant metacercarial stage in the second intermediate host (e.g., the grazers *Idotea* spp. and *Gammarus* spp.) (Galaktionov and Dobrovolskij 2003). The parasite we focused on, *Himasthla elongata,* is a common trematode infecting *L. littorea* and can reach a prevalence of infection up to 40% in the Baltic Sea (Bommarito et al. 2021). Its second intermediate host is a bivalve (*Mytilus spp.* or *Cerastoderma* spp.; Werding 1969, de Montaudouin et al. 2009), and it uses shorebirds as the definitive host (Werding 1969, Galaktionov and Dobrovolskij 2003). Moreover, *H. elongata* is known to reduce the thermal tolerance of the gastropod host (Díaz-Morales et al. 2022), and it is therefore expected to have a significant effect on the gastropod's physiological response to temperature.

Accordingly, this study aimed to test the effects of *H. elongata* infection and temperature on the defecation rate (as proxy for grazing) of *L. littorea* and thus, its potential role as a modulator of interspecific competition among macroalgae, by: (1) evaluating the difference in *L. littorea* grazing rates on *F. vesiculosus* and *G. vermiculophylla,* and (2) assessing the single and combined effects of parasitism and temperature on the feeding rate of *L. littorea.* Furthermore, since the feeding behavior of *L. littorea* is intrinsically linked to the general condition of the snails, we also evaluated (3) the effect of parasitism, temperature, and algae type on the physiology of *L. littorea* in terms of lipid and glycogen concentrations as markers of energy reserves, and heat shock protein expression as a marker of stress.

Methods

Collection of hosts

Littorina littorea snails were collected haphazardly on October 23, 2019, from the rocky shore (water depth < 1 m) of Årøsund, Denmark (55°15'49.0"N, 9°42'24.5"E). The water temperature at the time of collection was 13.2 °C, the salinity 21 PSU, and oxygen saturation of 138% was measured with a multimeter probe (WTW 3630 IDS, Kaiserslautern, Germany). Snails were transported to the laboratory at GEOMAR, Kiel, Germany, in portable coolers and placed in a climate chamber at 16 °C under a flow-through system consisting of sand-filtered seawater directly pumped from the Kiel Fjord. On October 24, 2019, a subsample of snails (ca. 200) was screened under the stimulus of light for 4 h to induce the emergence of cercariae (i.e., the infective larval stage of trematodes) (Soldánová et al. 2016). However, almost no patent infections were detected. Therefore, a subsample of 40 snails was dissected to confirm the presence of trematode infections, from which 10 (25%) were infected with *Himasthla elongata.*

Grazing experiments

Faeces production was used as proxy for grazing since it is not affected by 'sloppy feeding' behavior (Wahl et al. 2019), and it is linearly positively correlated to ingestion rates (Gaudy 1974, Ayukai and Nishizawa 1986, Tsuda and Nemoto 1990, Båmstedt et al. 1999, Besiktepe and Dam 2002). Moreover, since *L. littorea,* in general, consumed a relatively low amount of algae during the experimental period, the measurement of algal loss as wet weight was not allowing to capture changes in consumption rate and, therefore, faeces production provided a more sensitive measure of feeding rate. The acclimation to experimental temperatures (10, 16, 22, and 28 °C) began on October 25, 2019, one week prior to the onset of the experiment. These temperatures were chosen to cover the range of temperatures occurring in shallow waters (< 1.8 m depth; Wolf et al. 2020) in the southwestern Baltic Sea during the period of the highest abundance of both *G. vermiculophylla* and *F. vesiculosus* (i.e., April-November; F. Weinberger, personal communication). Two thermobaths were randomly assigned to each temperature. The photoperiod was timed to begin with dawn at 4:00, achieving maximum experimental light intensity at 7:00, and end with sunset at 19:00, reaching absolute darkness at 22:00. Two 1.5 L plastic tanks were

filled with 1 µm-filtered fjord seawater (salinity 17.1 ± 0.3 PSU) and placed in each thermobath. Fifteen adult snails (20.6 ± 1.4 mm shell length) were placed randomly in each plastic tank for a total of 30 snails per thermobath and 60 snails per temperature. Snails were acclimated to the target temperatures, by increasing or decreasing 2 °C per day in a way that all tanks reached the target temperature on the same day at the same slope. During the acclimation, water was constantly aerated and exchanged every other day. Snails were fed *ad libitum* with *F. vesiculosus* and *G. vermiculophylla* in similar amounts collected from the beach Falckenstein in Kiel, Germany (54°23'36.8"N, 10°11'21.9"E). At the end of the acclimation (October 31, 2019), snails were transferred individually to 50 mL PLEXIGLAS® beakers filled with 40 mL of aerated temperature-equilibrated 1 µm-filtered seawater. The beakers were covered with a net to prevent the snails from escaping. Before starting the feeding assay, snails were starved for 48 h to standardize hunger levels. After this starvation period, water was replaced with aerated and temperature-equilibrated 1µm-filtered seawater. Water parameters (temperature, salinity, and pH) were monitored before and after water change with a multimeter (WTW 3630 IDS, Kaiserslautern, Germany). After exchanging the water, one half of the snails per thermobath was randomly selected to be fed with two 2 cm long pieces of *G. vermiculophylla*, and the other half was fed with pieces of *F. vesiculosus* of the same size. The pieces of *F. vesiculosus* were cut excluding air-filled vesicles and midribs, and in a similar biomass as *G. vermiculophylla*. Vesicles were excluded since their abundance and thickness are highly variable depending on the age and reproductive status of the algae (Rothäusler et al. 2020) which could potentially affect the palatability of the algae.

The duration of the experiment was set to 48 h to avoid excessive mortality at 22 and 28 °C, which would compromise the number of snails available to conduct biochemical analyses—particularly infected ones, which are known to have a lower thermal tolerance already at 22 °C (Díaz-Morales et al. 2022). At the end of the experiment, snails were removed from the beakers, and the foot was cut off and frozen in liquid nitrogen for further analyses of biomarkers (glycogen, lipids, and HSP). The remaining bodies were inspected under a stereo microscope (Nikon, SMZ1000 body, C-PS160 stand) to confirm infection status. To document the proportion of prepatent and patent infections of the snails, the presence of cercariae in the beakers was recorded. To quantify the faeces produced after 48 h, algae left-overs and cercariae were removed from the faeces under a stereo microscope with tweezers or a micropipette. To ensure

that all faeces were collected, beakers were rinsed with deionized water. The cleaned faeces were collected and filtered through pre-weighed pre-combusted glass microfiber filters (diameter: 25 mm; GF/F Whatman™). Afterward, the filters were washed with 25 mL of deionized water to remove seawater remainders, dried at 80 °C for 24 h, and reweighed.

Biochemical analyses

Tissue homogenization and total protein content analysis

In order to analyze protein, lipid, and glycogen concentrations and relative HSP70 expression, the preserved snail foot was homogenized with a micro pestle in 500 µL of IP lysis buffer with 1% of protease inhibitor cocktail (Sigma-Aldrich© P8340). After vortexing, the samples were centrifuged at 14,000 x *g* for 15 min at 4 °C. From the supernatant, an aliquot of 50 µL was taken for total protein content analysis using a Pierce™ BCA Protein Assay Kit (Thermo Scientific).

Lipid and glycogen quantification

For lipid and glycogen quantification, 20 µL of Na_2SO_4 (2%) and 540 µL of a chloroform:methanol (1:2) solution were added to 40 µL of sample homogenate. Samples were then incubated for 1 h in ice and centrifuged at 3,000 x *g* for 10 min at 4 °C. Lipid concentration was analyzed in the resulting supernatant and glycogen in the remaining pellet using the method by Gismondi et al. (2012), as already described in Chen et al. (2015). In brief, for lipid analysis, 100 µL of the supernatant was evaporated together with cholesterol standards (0, 25, 50, 100, 200, 400, 800, and 1600 µg/mL). Afterward, 200 µL of 95% sulfuric acid was added, and the samples and standards were incubated for 10 min at 90 °C. The samples were then cooled on ice for 5 min and mixed with 4.8 mL of a vanillin-phosphoric acid reagent (300 mg vanillin in 250 ml 68% phosphoric acid). Samples were vortexed for about 20 s each, and 300 µL of each standard and sample solution was loaded in a 96-well plate. The plate was incubated at room temperature for 5 min, after which it was measured in the plate reader at a wavelength of 535 nm (Tecan infinite M200). For the glycogen assay, the pellet was dried and solved in 400 µL of deionized water. Afterward, 100 µL from each sample and glucose standard solutions (0, 25, 50, 100, 150, 200, and 400 µg/mL) was mixed with 4.9 mL of Anthrone reagent (Sigma-Aldrich©; 0.2% Anthrone solved in 95% sulfuric acid). The samples were then heated in a water bath at 95 °C for 17 min

and vortexed for 20 s. After cooling on ice, 300 µL of each sample and standards was pipetted into a 96-well plate in triplicates. The samples were measured at a wavelength of 625 nm in a plate reader (Tecan infinite M200).

Heat Shock Protein analysis

The expression of HSP70 was analyzed following the procedure described in Frank et al. (2013). Briefly, 20 µL of homogenate were mixed with 20 µL of Laemmli buffer, boiled at 95 °C for 5 min, and subsequently cooled on ice. Proteins (10 µg) were separated using discontinuous SDS-PAGE and blotted on a nitrocellulose membrane by semi-dry Western Blot. After blotting, membranes were blocked in a horse serum:TBS 1:1 solution for 30 min on a rocking platform. After blocking, membranes were first incubated overnight in 5 µL of monoclonal HSP70 (Sigma-Aldrich© H5147) and 5 µL of monoclonal β-actin (Sigma-Aldrich© A1978) antibodies diluted in a horse serum:TBS 1:9 solution, followed by a 1 h incubation in 10 µL of HRP-conjugated antibody (Sigma-Aldrich© A4416) diluted in a horse serum:TBS 1:9 solution. To visualize the proteins, membranes were immersed in a staining solution consisting of 40 µL of 30% H_2O_2 and 2 mL of choloronaphtol (3 mg/ml in ethanol) in 20 mL TBS buffer. Stained membranes were scanned with a Canon LiDE 700F flatbed scanner, and bands were analyzed densitometrically using ImageJ (Schneider et al. 2012). HSP70 values are given relative to β-actin, which was used as a reference protein.

Statistical analyses

Statistical analyses were performed in R (version 4.1.0). With respect to the occurring parasites, only snails infected with *H. elongata* were considered for the following analyses since the prevalence of other parasites was too low to conduct meaningful analyses. The variance in faeces production (as a proxy for grazing), glycogen and lipid concentration, and relative HSP70 was modeled using LMM with the function “lmer” from the “lme4” package (Bates et al. 2015). The full model included temperature, infection status, and algae fed (diet) as fixed categorical factors and their interaction. Due to the experimental block design, thermobath (i.e., water bath or experimental block) was set as a random intercept. For infection status, uninfected snails were set as the reference level, and for algae fed and temperature, *F. vesiculosus,* and 16 °C, respectively. Model assumptions were evaluated using the diagnostics tool from the “DHARMa” package (Hartig 2018). This diagnostics tool includes quantile-quantile plots with KS test to detect deviations from normality and

dispersion test via standard deviation of fitted vs. simulated residuals (Hartig 2018). In addition, this package tests for homogeneity of variance using Levene's test (Hartig 2018). The temperature of 28 °C was not included since there were not enough replicates to run a linear mixed model (LMM) due to high snail mortality at this temperature. In the case of faeces production and relative HSP70, the models did not meet the assumptions of normality and homogeneity of variance, and therefore a log-transformation was applied on the response variable. After transformation, the models met all validity criteria. In the case of glycogen and lipid concentrations, models met all validity criteria and no transformation was necessary. The optimal fixed structure of the model was identified using stepwise backward selection following the protocol described in Zuur et al. (2009). Terms were sequentially dropped starting from the interactions. After dropping each term, the reduced models were compared based on the second-order Akaike Information Criterion (AICc) for small sample sizes and the Likelihood Ratio Test (LRT; Zuur et al. 2009). The results of the stepwise backward selection were confirmed using the function "dredge" from the "MuMIn" package using "AICc" as the ranking method. For comparison of reduced models during the backward selection process, models were fitted using Maximum Likelihood estimation (Zuur et al. 2009). Once the optimal fixed structure was identified, the models were refitted with restricted maximum likelihood estimation (REML), and model assumptions were again diagnosed. Finally, in order to compare among response variables, log response ratios (RR) were calculated based on Lajeunesse (2015). For the RR, the following equation was used:

$$RR^{\Delta} = \ln\left(\frac{X_T}{\bar{X}_C}\right) + \frac{1}{2}\left[\frac{(SD_T)^2}{N_T\bar{X}_T^2} - \frac{(SD_C)^2}{N_C\bar{X}_C^2}\right] \qquad (1)$$

Where $N_{T/C}$ is the sample size in treatment/control; RR^{Δ} is the small sample size-adjusted log response ratio; $SD_{T/C}$ is the standard deviation of treatment/control sample; $\bar{X}_{C/T}$ is the mean response of control/treatment; X_T is the response of a sample from a treatment. The variance was calculated as follows:

$$var(RR^{\Delta}) = \frac{(SD_T)^2}{N_T\bar{X}_T^2} + \frac{(SD_C)^2}{N_C\bar{X}_C^2} + \frac{1}{2}\left[\frac{(SD_T)^4}{N_T^2\bar{X}_T^4} + \frac{(SD_C)^4}{N_C^2\bar{X}_C^4}\right] \qquad (2)$$

Next to the log ratio, the Geary index was calculated for both the control and treatment groups to validate RRs (Lajeunesse 2015) using the following formula:

$$\frac{\bar{X}}{SD}\sqrt{N} \geq 3 \qquad (3)$$

To compare the direction and magnitude of temperature effects among the response variables, RRs were calculated using 16 °C as the reference temperature (summer thermal average in the Fjord (< 1.8 m water depth) over the last 5 years; Wolf et al. 2020) for each experimental group. The same was done to compare the effect of infection status among response variables but using uninfected as the control group.

Results

Infection patency and host survival

After four days of exposure to 10, 16, 22, and 28 °C, the proportion of infected snails presenting cercariae emergence (i.e., patent infections) increased with increasing temperature (Figure 10). All snails remained prepatent at 10 °C, while at 16 °C patent infections (7.7% of the snails) started to be detected. At 22 °C a sharp increase in patency was observed, with 65.4% of the infected snails showing patent infections. At 28 °C the highest percentage of patency was observed, with 93.3% of the infections being patent (Figure 10). Indeed, after dissection, rediae appeared mature and much bigger at 22 and 28 °C than the rediae in snails from the 10 and 16 °C treatments (personal observation). The prevalence of infection of other parasite species (*e.g., Renicola roscovita* and *C. lingua*) was always below 3%, including a single co-infection with *H. elongata* and *R. roscovita* (Figure S2.1). In terms of survival, 15% of the snails died at 28 °C, while only 3.2% died at 22 °C. At 10 and 16 °C no snail mortality was recorded.

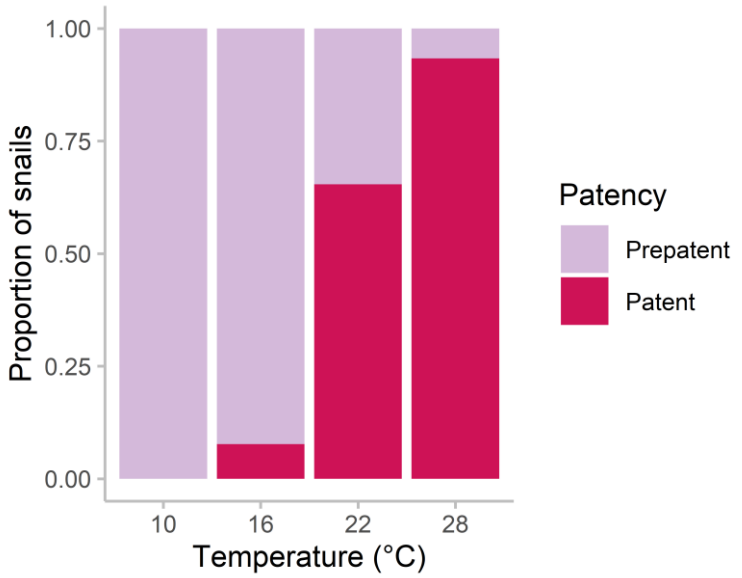

Figure 10 Change in the proportion of infections without cercarial emergence (i.e., prepatent; lilac) and infections presenting cercarial emergence (i.e., patent; pink) in snails naturally infected with *Himasthla elongata* after four days of exposure to acclimation temperatures (10, 16, 22, and 28 °C).

Grazing experiments

During the feeding experiment, snails showed the highest feeding rate at 16 °C for both algae with considerable feeding depression at 28 °C (Figure 11a). According to the full LMM, algae type had a significant effect on faeces production (estimate = 0.38, CI = 0.06 – 0.71, p = 0.022; Table S2.1); specifically, snails feeding on *G. vermiculophylla* produced significantly more faeces (37%) than snails feeding on *F. vesiculosus*. The effect of infection status on faeces production was marginally significant (estimate = 0.34, CI = -0.02 – 0.69, p = 0.062; Table S2.1), with infected snails producing up to 59% more faeces than uninfected snails (Figure 12). Although the interactive terms were not significant, the effect of infection on faeces production was larger at 16 °C (Figure 12). According to the log-RR analysis, at 10 °C and 22 °C the effect of infection was significant only for snails feeding on *G. vermiculophylla.* All results from the log-RR analysis were valid based on the Geary index (i.e., Geary index ≥ 3), including those for the biomarkers. Based on the full model, temperatures 10 and 22 °C had an overall negative effect on faeces production compared to 16 °C (Table S2.1), although not significant. Based on the log-RR analysis, snails decreased defecation rates down to 44% at 10 °C and down to 39% at 22 °C compared to 16 °C (Figure 13). After model selection, the reduced LMM showed that infection status and algae fed were the variables that best explained the variation on faeces production (Figure S2.2a).

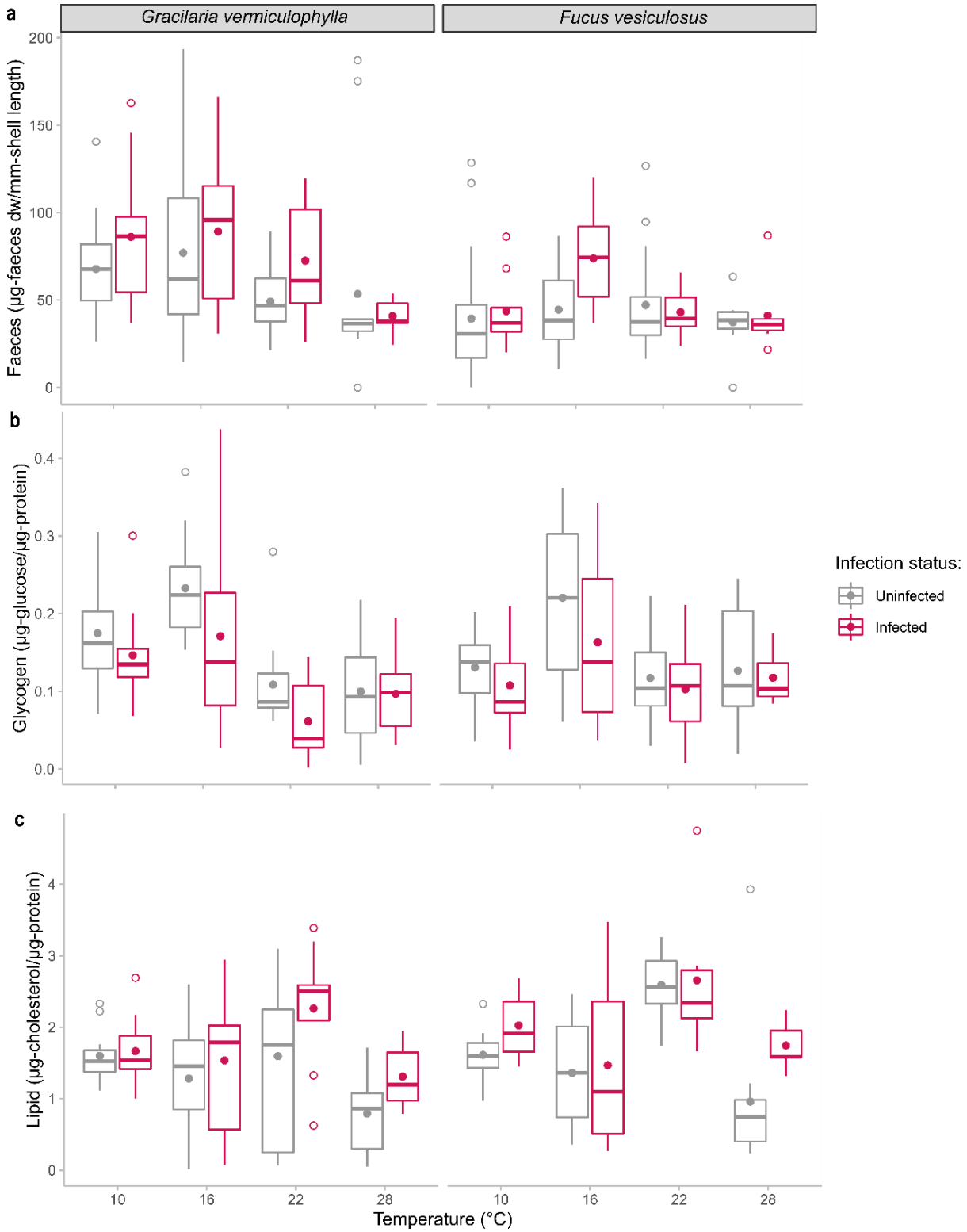

Figure 11 Boxplot of faeces production (a), and glycogen (b) and lipid (c) concentrations in snails fed with *Gracilaria vermiculophylla* (left panel) or *Fucus vesiculosus* (right panel), at 10, 16, 22, and 28 °C. Uninfected snails are represented in gray and infected snails in pink. Open dots represent outliers, solid dots arithmetic means, and horizontal lines the median within the interquartile range.

Biomarker analyses

Snails showed the highest glucose concentration at 16 °C, while lower values were observed at 10, 22, and 28 °C (Figure 11b). Based on the full LMM, snails at 10 and 22 °C had significantly lower glycogen concentrations (34% and 45%, respectively) than at 16 °C (Figure 13). According to the full model, in infected snails the glycogen concentrations were lower than uninfected snails, although not significant (estimate = -0.05, CI = -0.03 – 0.06, p = 0.079; Table S2.2). Overall, infected snails had 20% lower glucose concentration than uninfected snails (Figure 12). Diet or algae type did not influence glycogen concentration significantly. After model selection, the most important variables explaining the variance in glucose concentration were infection status, and temperature (Figure S2.2b). According to the reduced LMM, temperature (10 and 22 °C) and infection status had a significant negative effect on glycogen concentration (Figure S2.2b).

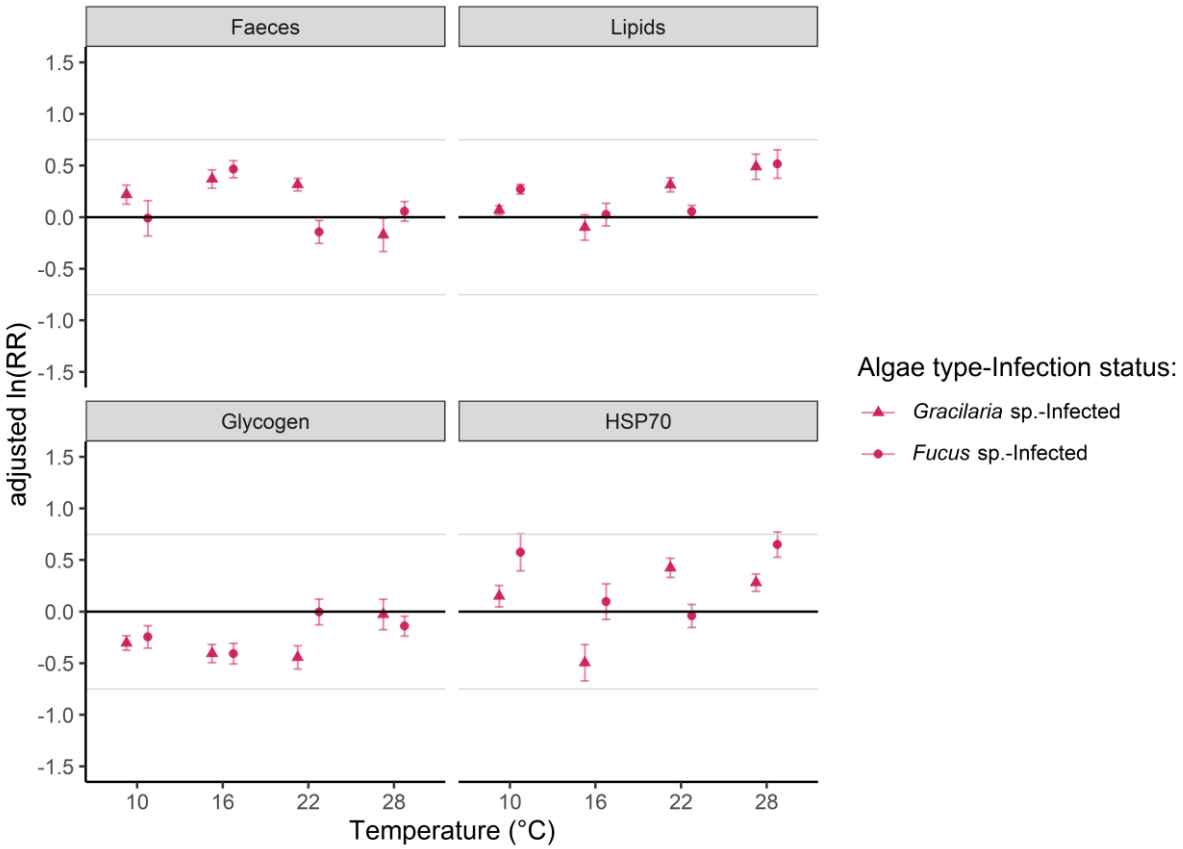

Figure 12 Effect size (adjusted log response ratio) of infection status on faeces production, glycogen, lipids, and HSP70 in *Littorina littorea* fed with *Gracilaria vermiculophylla* or *Fucus vesiculosus*, and uninfected or naturally infected with *Himasthla elongata*. Snails were exposed to acclimation temperatures (10, 16, 22, and 28 °C) for four days. Values are relative to uninfected snails at the respective temperature. Error bars represent 95% confidence intervals. Response ratios were calculated according to Lajeunesse (2015).

Regarding lipids, the highest concentration was observed at 22 °C, particularly for snails feeding on *F. vesiculosus* (Figure 11c). The difference in lipid content between infected and uninfected snails was highest at 28 °C. According to the full model, temperature (22 °C) significantly and positively influenced lipid concentration in comparison to 16 °C (estimate = 1.25, CI = 0.19 – 2.31, p = 0.021; Table S2.3). Based on the reduced LMM, the most important variables for predicting lipid concentration were infection status, temperature, and the interaction between temperature and algae (Figure S2.2c). Snails exposed to 10 °C and 22 °C showed 20% and 67% higher lipid contents, respectively, compared to 16 °C (Figure 13). Temperature (22 °C), infection, and the interaction between algae and temperature significantly affected lipid concentration. Infected snails had 26% higher lipid contents than uninfected snails, while snails fed with *G. vermiculophylla* had lower lipid concentrations at 22 °C than snails fed with *F. vesiculosus* (Figure 12c).

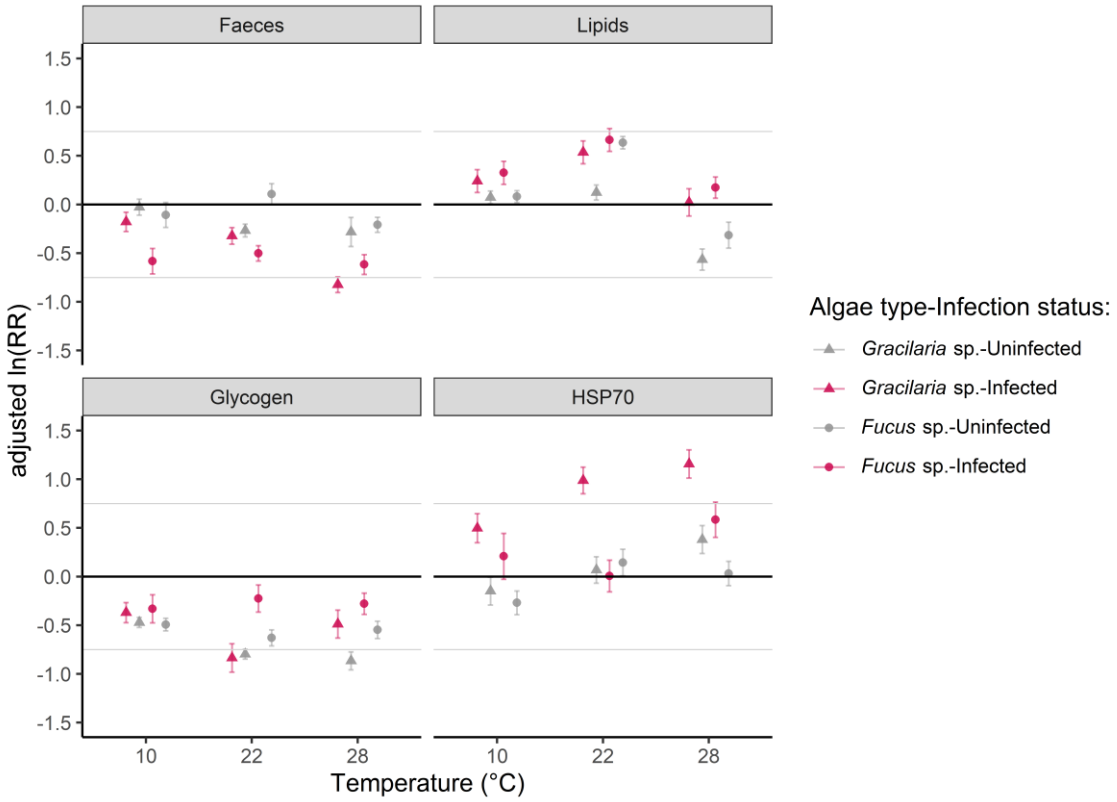

Figure 13 Effect size (adjusted log response ratio) of temperature on faeces production, glycogen, lipids and HSP70 in *Littorina littorea* fed with *Gracilaria vermiculophylla* or *Fucus vesiculosus*, and uninfected or naturally infected with *Himasthla elongata*. Snails were exposed to acclimation temperatures (10, 16, 22, and 28 °C) for four days. Values are relative to snails at 16 °C. Error bars represent 95% confidence intervals. Response ratios were calculated according to Lajeunesse (2015).

Relative to the reference protein β-actin, the expression of HSP70 increased with increasing temperature to a maximum of 35% at 28 °C (Figure 14). In addition, infected snails tended to have higher relative HSP70 values (30% higher on average; Figure 12) than their uninfected counterparts, particularly at 22 and 28 °C. In contrast, diet (i.e., algae type) did not have any influence on the expression of HSP70. Nevertheless, neither the full or reduced LMM showed a significant influence of temperature (10, 16, and 22 °C), infection status, or algae type (Table S2.4).

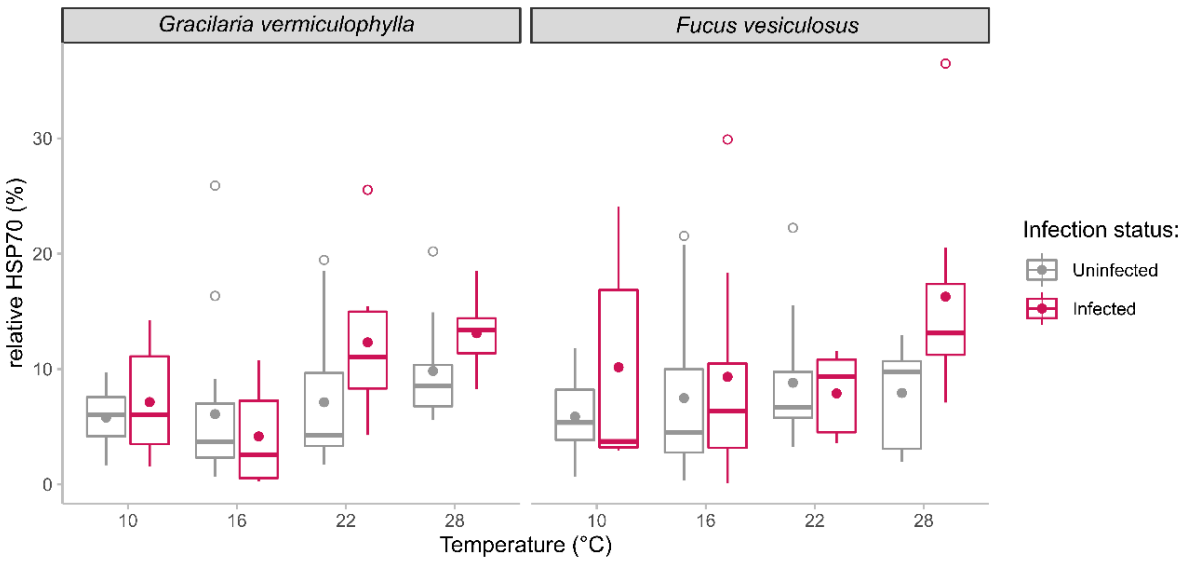

Figure 14 Boxplot for HSP70 (%) relative to β-actin after feeding snails with *Gracilaria vermiculophylla* (left panel) or *Fucus vesiculosus* (right panel) at acclimation temperatures (10, 16, 22, and 28 °C). Snails were uninfected (gray) or naturally infected with *Himasthla elongata* (pink). Open dots represent outliers, solid dots arithmetic means, and horizontal lines the median within the interquartile range.

Effect of patency on faeces production and biomarkers

Since at 22 °C there was a significant proportion of patent (i.e., presenting cercarial emergence) and prepatent (i.e., not presenting cercarial emergence) infections, the data from the two algae types were pooled to evaluate the effect of patency on faeces production, glycogen and lipid concentrations, and expression of HSP70 (Figure S2.3). Snails with prepatent infections had 14% lower feeding rates than snails with patent infections, while lipid concentrations remained similar (Figure S2.3a and S2.3d). However, the most substantial effect was observed for glycogen and HSP70. Prepatent infections showed 38% lower expression of HSP70 than patent infections (Figure S2.3b). The opposite was observed in the case of glycogen concentrations,

with prepatent infections showing 64% higher concentrations than patent infections (Figure S2.3c).

Discussion

To understand the interaction between invasive and native algae, and thus the outcome of invasions, we must contemplate all potential factors involved, including abiotic factors such as temperature and biotic factors such as parasites of important grazer species. In this study, we demonstrate that (1) *L. littorea* grazed 37% more on the invasive alga *G. vermiculophylla* than on the native *F. vesiculosus,* especially so in the cooler temperatures, and that (2) trematode infection modulated the feeding behavior of the gastropod *L. littorea* at temperatures (i.e., seasonal mean thermal averages) relevant to the occurrence of an invasive and a native alga in the field. As a result, trematodes could be an indirect driver in controlling invasive algal species.

In our study, the higher grazing of *L. littorea* on *G. vermiculophylla* compared to *F. vesiculosus* was not expected. Previous studies indicated that *G. vermiculophylla* is less attractive to gastropods than other algae (Weinberger et al. 2008, Nejrup et al. 2012), suggesting that this alga might suffer less from grazing pressure than other species. Nevertheless, in no-choice trials, *L. littorea*, *I. balthica*, and *Gammarus locusta* tended to consume more *G. vermiculophylla* than *F. vesiculosus,* although this was significant only for *I. balthica* (Nejrup et al. 2012). According to Nejrup et al. (2012), the grazer *I. balthica* fed more on *G. vermiculophylla* than *F. vesiculosus* in two-choice feeding assays, suggesting that the invasive algae may be grazed in the invaded range and potentially preferred in the presence of the native *F. vesiculosus*. This finding suggests two possible scenarios: (1) *L. littorea* can decrease the abundance of *G. vermiculophylla* and, thus, benefit *F. vesiculosus*, or (2) *L. littorea* can facilitate the dispersal of *G. vermiculophylla* since it promotes algal fragmentation while feeding (Thomsen et al. 2007). Regarding the latter, an increase of up to 200% in the number of fragments has been observed under grazing pressure by *L. littorea* (Thomsen et al. 2007). Since, in the present study, it was observed that infected snails consumed more algae (i.e., produced more faeces) than uninfected snails, this feeding-enhanced fragmentation can be amplified in the presence of *H. elongata* infections. These fragments can get easily entangled into byssal threads of mussels, feet or feathers of migrating birds, and anthropogenic structures during transportation across regions (Freshwater et al. 2006, Nyberg and Wallentinus 2009,

Hu and Juan 2014). Also, fragments as small as 0.9 mm have been observed to perennate and survive and continue growing even in harsh conditions (Nyberg and Wallentinus 2009, Abreu et al. 2011). Therefore, an indirect enhancement of fragmentation rates by *H. elongata* infections could facilitate the dispersal of the algae and its further colonization. Yet, as mentioned for the first scenario, the enhanced grazing of *G. vermiculophylla* by infected *L. littorea* could possibly result in an overall larger negative effect on this alga, indirectly benefiting *F. vesiculosus.*

Our experimental results suggest that infection status rather than temperature significantly modulated the feeding rate of *L. littorea* at temperatures between 10-22 °C. The temperatures statistically tested herein have been previously reported to have nonsignificant effects on the consumption rate of *L. littorea* (Wahl et al. 2019). Wahl et al. (2019) found that the optimal temperature for *L. littorea* feeding rate is around 18 °C, and it is only significantly suppressed at temperatures below 4 °C and higher than 23 °C. In line with these results, the feeding rate at 28 °C of both infected and uninfected snails showed a considerable decrease, although its significance could not be tested due to low replication resulting from higher snail mortality at this temperature.

To our knowledge, seven peer-reviewed studies previously assessed the impact of trematode infections on the grazing behavior of gastropod hosts: two in freshwater systems (Bernot and Lamberti 2008, Vivas Muñoz et al. 2018) and five in marine systems (Wood et al. 2007, Clausen et al. 2008, Larsen and Mouritsen 2009, Morton 2018, Ayala-Díaz et al. 2019). In general, the direction of infection effects appears to be dependent on the host-parasite system used and their respective populations. The snails *Planorbius corneus* and *Lymnaea stagnalis*, naturally infected with the trematodes *Cotylurus* sp. and *Diplostomum presudopathaceum,* respectively, showed increased specific grazing rates compared to their uninfected counterparts (Vivas Muñoz et al. 2018). In another freshwater study, Bernot and Lamberti (2008) found that *Physa acuta* infected with *Posthodiplostomum minimum* had significantly higher grazing rates than uninfected snails. In contrast, laboratory-infected *Radix lagotis* and *L. stagnalis* with *Trichobilharzia szidatii* and *T. regenti,* respectively, showed a decreased feeding rate (Vivas Muñoz et al. 2018).

In the case of marine systems, it has been shown that trematode infections affect the grazing rate of littorinids (Wood et al. 2007, Clausen et al. 2008, Morton 2018), while

the direction of these effects depends on the size, sex, and population used (Larsen and Mouritsen 2009, Ayala-Díaz et al. 2017). On the one hand, Larsen and Mouritsen (2009) found that large infected snails (shell height > 25 mm) ate less *Ulva lactuca* at 17.9 °C than uninfected snails, but this effect disappeared at 21.2 °C. On the other hand, for small snails (shell height < 25 mm), male infected snails ate more than uninfected ones at 21.2 °C, while infected females' feeding rate was lower than uninfected ones. In another study, Ayala-Díaz et al. (2019) found that three of the four populations studied showed up to 21% reduced grazing rates in trematode infected *Littorina sitkana* compared to uninfected snails, while one population showed 35% increased grazing rates in infected snails. In the present study, we found that infection with *H. elongata* significantly enhanced the consumption rate of *L. littorea* by 18% on average.

Although the mechanisms underlying a higher feeding rate by infected snails compared to uninfected ones remain to be understood, there are a few plausible explanations in this regard. First, trematode infections in gastropods are expected to alter consumption rates since they mechanically and chemically deteriorate the gastropod's digestive gland (Galaktionov and Dobrovolskij 2003, Bonfim et al. 2020). Furthermore, trematodes alter the physiology of their host by altering oxygen consumption (Arakelova et al. 2004, MacLeod and Poulin 2016), carbohydrates metabolism (Arakelova et al. 2004, Faro et al. 2013, MacLeod and Poulin 2016, Bonfim et al. 2020), enzymatic activity (Bonfim et al. 2020), and lipid profiles (Arakelova et al. 2004, Fokina et al. 2018), and by causing castration (Faro et al. 2013) and inducing the production of reactive oxygen species (Hahn et al. 2001). Although the direction and strength of these effects are species-specific and dependent on other abiotic and biotic factors, parasitic infections ultimately can modulate the metabolic requirements of the host (Harland et al. 2016, Fokina et al. 2018). Thus, parasitic infections might enhance feeding rates to suffice the energy lost due to poor gut assimilation efficiency and direct hijack of energy reserves (e.g., glycogen) by the trematode parthenitae (i.e., rediae or sporocyst) (Robson and Williams 1971). Additionally, although castration reduces the energy demand for reproduction of the gastropod, the host still needs additional energy to accommodate acute and chronic immunological responses to the infection (Gorbushin 2019).

The increased need for energy, and thus feeding, is reflected in the glycogen concentrations of infected snails, which showed significantly lower concentrations than

uninfected snails. Since glycogen represents a vital energy reserve for the gastropod's homeostasis (Sokolova et al. 2012), infected snails exhibit enhanced grazing rates to compensate for the glycogen loss. Lower glycogen concentrations in infected snails have been observed in other host-trematode systems (Arakelova et al. 2004, Pinheiro et al. 2009, Faro et al. 2013, Tunholi et al. 2013, Bonfim et al. 2020). Rediae and sporocysts can passively and actively sequestrate glycogen from the snail (McDaniel and Dixon 1967, Pinheiro and Amato 1994). The mechanisms underlying this glycogen sequestration are not yet fully elucidated. However, evidence suggests that the increased energy demand as a response to infection drives the gastropod to upregulate the activity of lactate dehydrogenase (LDH) and accelerates the anaerobic degradation of carbohydrates (Tunholi et al. 2013, Bonfim et al. 2020). Moreover, the combination of reduced glycogen, upregulation of LDH, decreased concentration of pyruvic acid, and increased levels of lactic acids suggest that infected gastropods are prompted to mobilize resources and enter partial anaerobic metabolism (Tunholi et al. 2013). Considering that lipids are solely metabolized aerobically, they will be accumulated when the gastropods enter partial anaerobic metabolism, as occurred in the present study, contrary to glycogen which can be catabolized both aerobically and anaerobically (Sokolova et al. 2012). This transition between full aerobic and partial anaerobic metabolism is usually more drastic in the digestive gland-gonadal (DGG) complex than in the cephalopedal mass (Tunholi et al. 2013). In our case, the glycogen and lipid content was only analyzed in the snail's foot since, in *H. elongata* infections, most of the DGG complex tissue is infested with trematode larvae (Werding 1969). Therefore, using the DGG complex could produce biased results due to glycogen and lipid detections from parasitic tissue.

Temperature had a larger effect on the physiological status of the snails than the trematode infection. Specifically, we can see that glycogen concentration was highest at 16 °C, while the highest concentration of lipids was observed at 22 °C. Glycogen and lipids are considered the pool of energy surplus, and shifts in their metabolism are characteristic of organismal energy homeostasis upon stress in aquatic invertebrates (Sokolova et al. 2012). The mobilization of energy reserves can be driven by high maintenance costs such as protein turnover and protective mechanisms. This could be observed in the HSP70 levels, which, although not significantly, increased at 22 and 28 °C, especially in infected snails. However, based on the higher mortality of snails at

28 °C, the expression of HSP70 did not successfully protect the snails at this temperature.

Moreover, since warm temperatures accelerate the development of trematode infections (Ataev 1991), partial anaerobiosis might also be enhanced by the onset of patent infections. In the present study, snails with patent infections at 22 °C showed a tendency for higher feeding, higher expression of HSP70, lower glycogen concentration, and higher lipid concentrations compared to prepatent infections. These trends are aligned with the expectations of invertebrates undergoing metabolic compensation under thermal stress (Sokolova et al. 2012). In developed trematode infections or patent infections, rediae are active and constantly producing cercariae (Galaktionov and Dobrovolskij 2003). Active rediae might either, but not exclusively, hijack more glycogen from the snail and drive the transition into partial anaerobic metabolism. However, this has not been experimentally tested but deserves attention in future studies.

In addition to trematode infections, stressful temperatures can also drive littorinids to mobilize energy reserves and enter partial anaerobic metabolism (Sokolova and Pörtner 2003). Although the water was constantly aerated in this experiment, organisms can still experience anaerobic metabolism in such conditions (Pörtner 2001, cited in Sokolova and Pörtner 2003). As aforementioned, glycogen can be catabolized aerobically and anaerobically, while lipids can only be catabolized aerobically (Sokolova et al. 2012). Therefore, partial anaerobiosis can result in the usage of glycogen and the accumulation of lipids as observed at 22 °C. The lower glycogen content in infected snails could represent a metabolic disadvantage in the sense that infected snails might be less equipped to sustain thermal stress. Infected gastropods have been reported to have a lower thermal tolerance, and warming often results in fatalities (McDaniel 1969, Kuris 1980, Paull and Johnson 2011, Paull et al. 2015, Díaz-Morales et al. 2022). Nevertheless, determining whether these snails are experiencing anaerobic or aerobic metabolism requires the analysis of metabolic end-products accumulation (e.g., succinate, alanine, and lactate; Sokolova and Pörtner 2001). Therefore, it would also be interesting to increase the temperature resolution between 22 and 28 °C in order to estimate the differences in critical metabolic temperatures between infected and uninfected gastropods. Moreover, it is important to address whether parasites might have the potential to shift or change the widths of metabolic performance ranges (i.e., optimum, pejus, pessimum, or lethal ranges) under exposure

to environmental stressors with the help of dynamic energy budget (DEB) and oxygen- and capacity- limited thermal tolerance (OCLTT) modeling (Sokolova et al. 2012).

Conclusion

This study conclusively shows that *G. vermiculophylla* is consumed by grazers in its recipient environment and might experience more grazing pressure by *L. littorea* than its native competitor *F. vesiculosus.* The grazing pressure experienced by *G. vermiculophylla* might be enhanced in the presence of trematode infections since, in this study, *H. elongata* was shown to increase the grazing rate of *L. littorea* by 18% on average, in particular at intermediate temperature. This parasite infection-enhanced feeding could potentially have repercussions in the invasion course of *G. vermiculophylla* by facilitating the reproduction of the alga via grazing-enhanced fragmentation or else benefit the native alga *F. vesiculosus* by putting more grazing pressure on *G. vermiculophylla*. However, whether parasite infections and grazing pressure will significantly change the course and degree of invasion by *G. vermiculophylla* remains unclear. In order to elucidate this, laboratory experiments should be coupled with field observations and more complex experimental settings that consider relevant factors such as the temperature-dependent palatability of the algae, the season from which the snails were collected, and the complexity of multiple algal choices when the snails are in the field (Nejrup et al. 2012, Poore et al. 2016, Wahl et al. 2019). In addition, secondary metabolites might affect algal palatability that can function as deterrents against consumers (Paul and Fenical 1986). It is, therefore, presumable that grazer feeding preference for one algal species over another may be partially due to species-specific differences in defense mechanisms. So far, this study is the first to address *H. elongata* in the context of grazing behavior, considering invasive and native algal species. Since the studies published so far portray a complex picture regarding the effects of trematodes on grazing behavior, we highlight the importance of conducting laboratory studies that consider multiple variables such as different diets, populations, host's size and sex, trematode species, and complementary field studies.

The trematode infection and temperature effect on the host's metabolism call for more studies that tackle the influence of these stressors combined on the tolerance of gastropod hosts to a changing climate. If trematodes reduce the host's carbohydrate reserve, infected gastropods might be metabolically at a disadvantage, and prolonged

exposure to abiotic stressors might fatally affect them. Given that temperature and heatwave events are expected to increase with climate change (Rahmstorf and Coumou 2011), it is expected that infected grazers will not be able to subsist under such conditions. Moreover, it is important to address the effect of other climate change-related abiotic stressors such as salinity. With expected desalinization upon climate change (Gräwe et al. 2013), parasites might experience a decrease in performance (i.e., emergence, activity, survival, and infectivity; Bommarito et al. 2020) and distribution (Bommarito et al. 2021), thus compromising their success to prevail. Under desalinization, grazers as *L. littorea* are also expected to decrease their activity (Rosenberg and Rosenberg 1972, Berger and Kharazova 1997), while *G. vermiculophylla* can survive at low salinities (Weinberger et al. 2008). Therefore, all in all, with threatened grazer populations by climate change, imbalances in macroalgal assemblages are expected, with potential benefits to invasive species such as *G. vermiculophylla.*

Chapter III: The trematode *Podocotyle atomon* modulates the biochemical response of *Gammarus locusta* to thermal stress but not its feeding rate or survival

Introduction

Climate change-related temperature regimes in marine habitats are unprecedented and challenging for the organisms that inhabit them (Harley et al. 2006). Ectothermic organisms are especially vulnerable since they cannot regulate their body temperature and are hence metabolically at the whim of external temperatures (Somero 2002, Paaijmans et al. 2013). To comprehend and forecast how global warming may affect ectotherms and marine ecosystems in general, it is necessary to develop thermal performance curves (TPCs) to understand their thermal limits of performance (Sinclair et al. 2016, Kontopoulos et al. 2020). Furthermore, TPC evaluation should not be based solely on single responses but rather on a thorough analysis of numerous performance traits (Sinclair et al. 2016). These traits include, but are not limited to, survival rates, feeding rates, and indicators of energetic homeostasis (i.e., lipid and glycogen) (Sokolova et al. 2012).

Although TPCs have been developed for many common and ecologically important ectotherms (Sunday et al. 2012, Wahl et al. 2019), analogue biological factors capable of modulating such TPCs are frequently overlooked. Parasites are one example of such biological factors that have been shown to alter the response of organisms to thermal stress (Magalhães et al. 2018a, Selbach et al. 2020). The inclusion of parasites in such analyses is critical because parasitism is one of the most common species interactions in nature. Trematodes are of particular interest because they have a complex life cycle and use at least one ectothermic organism as an intermediate host (Galaktionov and Dobrovolskij 2003). Furthermore, their ability to influence how organisms deal with thermal stress has been shown to result in complex organismal and community-level responses (Studer et al. 2010, Mouritsen et al. 2018). For instance, trematodes specifically have been observed to modulate the biochemical condition of their host (i.e., oxidative stress, energy metabolism) under warming (Magalhães et al. 2018a) to increase host mortality upon thermal stress (Mouritsen and

Jensen 1997, Studer et al. 2010), and, as a result, drive changes in the community composition by favoring parasite-resistant ectotherms (Larsen et al. 2011) or by crippling coastal benthic communities overall (Mouritsen et al. 2018). Therefore, the inclusion of trematodes in TPCs is of overall importance.

In this study, we focused on the thermal performance of the amphipod *Gammarus locusta*. This gammarid species is abundant in northern temperate waters with the ability to shape marine benthic ecosystems and food webs (Costa and Costa 2000). It is native to the Baltic Sea (Herkül et al. 2016), which is already experiencing many global warming consequences projected elsewhere in the future (Reusch et al. 2018). The thermal tolerance of *G. locusta* has been explored, and temperatures representing projected end-of-century summer thermal averages for the region (ca. 22 °C) are known to decrease the survival (Neuparth et al. 2002, Wahl et al. 2021), the condition index and relative consumption rates (Cardoso et al. 2018), and the density of this amphipod species (Eklöf et al. 2012). The thermal performance of this amphipod can be affected by several factors, such as sex and parasitism. Former studies investigating the response stressors in gammarids detected sex-based differences, with females often displaying higher to abiotic stressors than males (McCahon and Pascoe 1988, Hoback and Barnhart 1996). In terms of parasitism, *G. locusta* serves as a host for several trematode parasites such as *Podocotyle atomon* (Zander 1998, Zander et al. 2002). Although metacercariae are expected to have less virulence than other life stages of trematodes due to their “semi-dormant” state (Galaktionov and Dobrovolskij 2003), *P. atomon* has been observed to reduce the survival and the fitness of its intermediate crustacean host (i.e., *G. zaddachi*; Arundell et al. 2019). However, it is unknown whether *P. atomon* can also affect the thermal performance of *G. locusta* also.

Podocotyle atomon is a common trematode species found in the Baltic Sea (Zander et al. 2002). It uses *Littorina* spp. as the first intermediate host (Kesting et al. 1996), *Gammarus* spp. as the second intermediate host, and fish (e.g., eels or sticklebacks) as the final host (Hunninen and Cable 1943). After sexual reproduction in the final host, eggs are expelled, followed by miracidia hatching, which then actively swim to infect the first intermediate host. Miracidia in *Littorina* spp. develop into sporocysts, which multiply asexually and produce cercariae as the next larval stage (Hunninen and Cable 1943). These cercariae belong to the Cotylocercous group, which normally have a truncated tail and use ambuscade behavior to infect the second intermediate host.

Ambuscade behavior is characterized by an initial still phase of the cercariae while attached vertically to the bottom with the truncated tail. Once the cercariae senses the crustacean approaching (through vibrations produced by the gammarid's pleopods), the cercariae start moving in circular motions in an attempt to perforate the host's cuticle with a stylet (sharp sword-like structure) (Galaktionov and Dobrovolskij 2003). After penetration of the gammarid's cuticle, the cercariae then encyst as metacercariae in the gammarid's hemocoel. During this stage, the gammarid can produce a melanin-rich coat that surrounds the cyst in an attempt to isolate and destroy it (Kostadinova and Mavrodieva 2005). The metacercariae then develop until they reach the progenetic metacercarial stage, after which they are trophically transmitted to the final host.

In many amphipod-trematode systems, the combination of thermal stress and trematodes has been catastrophic (Studer et al. 2010, Mouritsen et al. 2018). The amphipod-trematode system studied herein is common in the Baltic Sea (Zander et al. 2002), which represents a unique habitat in the context of climate change (Reusch et al. 2018). However, this system remains largely unexplored in terms of whether trematodes can exacerbate thermal stress in a key coastal herbivore species. Following this knowledge gap, this study aimed to test the following hypotheses: (1) Warming and metacercarial infection combined will have a synergistic negative effect on the survival of *G. locusta*; (2) Infection will increase compensatory feeding on the gammarid upon warming due to the additional energy drain that the infection represents to the host; (3) Infection status will affect the biochemical condition of the host at different temperatures. The temperatures tested represent temperatures ranging from seasonal averages to temperature extremes (2–30 °C). The response variables included survival, shredding activity, catalase and phenoloxidase activity, and lipid and glycogen concentrations. Due to gammarids' "sloppy" feeding behavior (Wahl et al. 2019), faeces production was also evaluated as a complementary response to shredding activity. Lipid and glycogen contents were evaluated as measures of energy storage (Sokolova et al. 2012). Catalase activity was determined because parasites and temperature are known to alter the expression of this enzyme in ectothermic hosts (Axenov-Gribanov et al. 2016, Magalhães et al. 2018a, 2020). Finally, phenoloxidase activity was assessed as an indicator of immunocompetence as well as an important enzyme for dealing with thermal stress (Matozzo et al. 2011, Labaude et al. 2017a).

Methods

Collection of *Gammarus locusta*

The amphipod *G. locusta* was collected at Bülk, Strande, Germany (54°26'51.9"N 10°11'09.8"E), on July 20, 2020, by shaking the seaweed algae *Fucus vesiculosus* in a bucket filled with site water. Gammarids were transported to the laboratory and distributed in two holding tanks filled with 100 L of filtered seawater (salinity = 13.2 ± 0.53 PSU) and kept in a climate chamber set to 16 °C. The water was aerated and a green plastic mesh and small cylinders were provided as refuge since *G. locusta* can be cannibalistic. The gammarids were fed with *F. vesiculosus* and soft tissue of *Mytilus edulis*. During the following week, adult gammarids (> 6 mm of length) were identified to the species level morphologically under a stereo-microscope (Nikon, SMZ1000 body, C-PS160 stand) according to Köhn and Gosselck (1989) and categorized according to their sex and infection status. The sex was identified by either carefully separating pre-copula pairs or sexual dimorphism (enlarged gnathopods in males and presence of oostegites or eggs/juveniles in brood pouch of females (Costa and Costa 1999)). The identification of parasites in live gammarids under the stereo-microscope was possible owing to their translucent body (e.g. Figure 15a). The gammarids were immobilized with a transparent plastic mesh, and the cysts were detected using the highest possible magnification (480x). This evaluation was performed twice by two distinct observers. Once sorted, gammarids were kept in groups of 100 individuals (separated by sex and infection status) in a climate chamber set to 16 °C in 8 L tanks filled with filtered seawater. Gammarids were provided with refuge and food as described above.

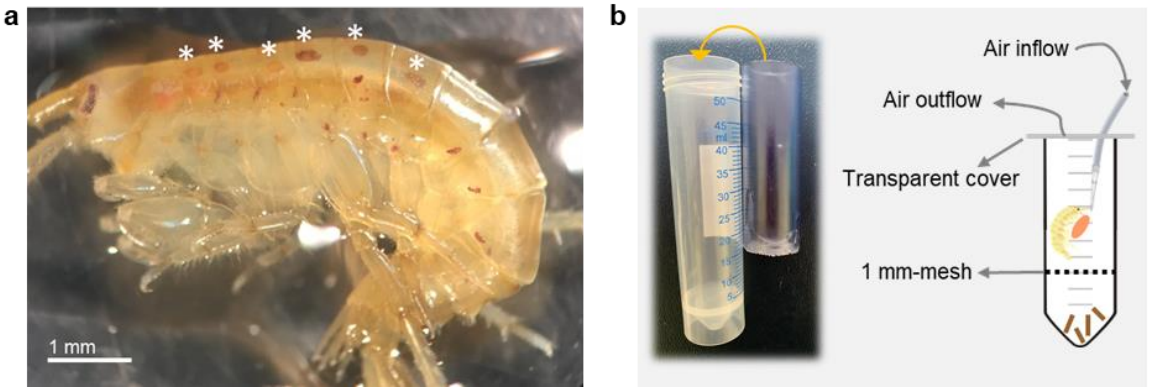

Figure 15 Male *Gammarus locusta* harbouring six melanized cysts (asterisks) of *Podocotyle atomon* (a) and experimental vessel where gammarids were kept during the feeding experiment (b) (left = actual depiction, right = schematic).

Parasite identification

Trematode metacercariae were identified according to their morphological characteristics (Hunninen and Cable 1943). For the identification, additional infected gammarids were dissected, the cysts excysted and morphologically identified under the microscope. Since amphipods are known to serve as hosts for microsporidians (Grabner et al. 2015), which are known to alter the physiology of gammarids (Grabner et al. 2014, Chen et al. 2015), 10 individuals of trematode-uninfected and trematode-infected females and males (for a total of 40) were randomly collected for molecular identification of this parasite. This subsample allowed us to assess the prevalence in the tested amphipod population and the potential confounding effects of microsporidians for the experiment. The intestines were carefully removed to avoid contamination from external ingested DNA. Each degutted gammarid was preserved in molecular grade ethanol (99%) until further processing. DNA extraction was performed by salt-precipitation after the procedure described in Grabner et al. (2015). After DNA extraction, a PCR was performed using the universal primers V1 (Zhu et al. 1993) and Mic-Uni 3R (Weigand et al. 2016). For the PCR, a solution that consisted of 7 µL of Buffer AccuStart PCR Toughmix 2X (Quanta Biosciences), 0.7 µL of each primer, 0.28 µL of loading dye (Quanta Biosciences), 4.62 µL of Milli-Q water and 0.7 µL of DNA was used. The program for the PCR reaction was set to 94 °C for 3 min for the initial denaturation, followed by 35 cycles at 94 °C for 30 s and annealing/elongation of 68 °C for 35 s, and a final elongation step of 68 °C for 3 min. If single, clear bands were visible, samples were sequenced using primer V1 (Microsynth Seqlab), and the species was identified by matching each sequence against the NCBI nucleotide database using BLASTn.

General experimental framework

The performance of infected and uninfected males and females of *G. locusta* was evaluated under eight different temperatures (2, 6, 10, 14, 17, 22, 26, and 30 °C). The range of temperature chosen reflects the range experienced by *G. locusta* in the southwestern Baltic coast (2–26 °C) plus an extreme scenario (30 °C). The response variables included host survival, shredding rate, faeces production as complementary metric to shredding rate, catalase as a marker of stress, phenoloxidase as an immunological marker, and lipid and glycogen concentrations as energy resource. For each experiment, 10 infected and 10 uninfected individuals (male or female) were

measured (i.e., body length from the tip of the rostrum until the end of the pleon) using ImageJ (Schneider et al. 2012). The range of body length was restricted as much as possible (i.e., 6.3-10.0 mm body length for females and 6.4-11.8 mm for males) to avoid size-dependent effects on the response variables. After measuring, each gammarid was individually placed in 50 mL falcon tubes filled with 50 mL of aerated and temperature pre-equilibrated seawater. Falcon tubes were covered with a transparent Plexiglas plate. Above each falcon tube, two holes were drilled, one equipped with a tube that provided constant individual aeration and another one that allowed air exchange. The falcon tubes containing infected and uninfected individuals were placed in thermobaths, 40 falcon tubes in each bath and one bath for each temperature level. The photoperiod began at 4:00, reached maximum experimental light intensity at 7:00, and ended at 19:00, reaching total darkness at 22:00. Temperature acclimation started on July 22, 2020, and finished on July 29, 2020, by increments or decreases of 1 °C every 12 h from a baseline temperature of 16 °C. During the acclimation period, gammarids were fed with freshly cut *Mytilus edulis* mussel feet (half a foot per gammarid from mussels measuring 4 cm in length). Water was exchanged every other day with aerated and temperature pre-equilibrated seawater.

Feeding experiment

On July 30, 2020 (Day 0, 24 h after all target temperatures were reached), the food was removed, and the gammarids were placed inside a Plexiglas chamber suspended inside the falcon tube (Figure 15b). The bottom of this chamber was covered with a transparent plastic mesh (mesh size: 1 mm) that separated the gammarid from the faeces dropped to prevent them from feeding on their own excrements. Gammarids were starved for 24 h to standardize hunger levels and to clean their guts. After starvation, water in the vessels was exchanged, and gammarids were fed with half a foot from *Mytilus edulis* for 30 h. Each mussel foot was freshly prepared and weighed (Sartorius quintix 65-1s, 0.0001g) before providing it to the gammarids. On August 2, 2020 (Day 3 post-acclimation), the food was removed, weighed, and the gammarids were allowed to empty their guts for 24 h. The faeces were collected and filtered using pre-incinerated glass microfiber filters (Whatman® GF/C). After filtering, the falcon tubes were rinsed with deionized water, and the filter was washed with 50 mL of deionized water to remove the remaining salt. The filters were immediately inspected under a stereo-microscope to remove the rests of the gammarid's exoskeleton (i.e.,

after occasional moulting) and other non-faecal matter. After filtering, the filters were dried for 24 h at 80 °C and re-weighed. Three falcon tubes with food but without gammarid were added to account for potential disintegration of the mussels, especially at the highest temperatures. Survival was evaluated daily. On August 5, 2020 (Day 6 post-acclimation), the experiment was ended, and all living organisms were preserved in liquid nitrogen for future biochemical analysis as described below.

Biochemical analyses

Tissue homogenization and total protein content analysis

Since there was high mortality at some temperatures by the time of sample collection, biochemical analyses were conducted only for gammarids exposed to 6, 10, 14, and 18 °C. Each gammarid was crushed with a micro pestle in 200 µL of lysis buffer (25 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA630) with 1% protease inhibitor cocktail (Sigma-Aldrich P8340) to quantifiy protein, lipid and glycogen, and catalase and phenoloxidase activity. Following homogenization, the samples were centrifuged at 14,000 x *g* for 10 min at 4 °C. Aliquots of the supernatant were stored at -80 °C for total protein content and biomarker analyses. Using a serial dilution of bovine serum albumin (BSA) as standard, the total protein content of the lysate was determined using a PierceTM BCA Protein Assay Kit (Thermo Scientific). The protein concentration of the sample was used to standardize all biomarker analyses.

Phenoloxidase

The activity of phenoloxidase was analyzed following the method described by Laughton and Siva-Jothy (2011). Briefly, 20 µL of sample supernatant were added together with 140 µL Milli-Q water and 20 µL of PBS buffer (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM) to a 96-well microplate. Right after, 20 µL of a 3,4-dihydroxy-L-phenylalanine (L-DOPA) solution was added to each well using a multichannel pipette. For the control, 70 µL of PBS and 130 µL of L-DOPA were used. Samples were immediately measured in a plate reader (Tecan infinite M200) every 5 min for 45 min at a wavelength of 490 nm at 30 °C. Results are provided as U-enzyme/µg-protein.

Catalase

Enzymatic activity of catalase was determined following Woermann et al. (2020). A calibration curve of H_2O_2, including the concentrations 0, 5, 10, 15, 20, 25, and 30 mM was performed by diluting H_2O_2 in Tris-Buffer (25 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1 mM EDTA). Samples were diluted to achieve a total protein concentration of around 5-10 µg/mL. After that, 300 µL of the standards and 210 µL of sample were pipetted in triplicates in a UV 96-well plate. In order to induce the activity of catalase, 90 µL of a 100 mM H_2O_2 solution were added to each sample. The plate was immediately measured in a plate reader at a wavelength of 240 nm and 25 °C every 15 s for a total of 4 min. Results are reported as U-enzyme/µg-protein.

Lipid and glycogen quantification

To quantify lipids and glycogen, 40 µL of sample homogenate were mixed with 20 µL of Na_2SO_4 (2%) and 540 µL of a chloroform:methanol (1:2) solution. After that, the samples were kept on ice for 1 h before being centrifuged at 3,000 xg for 10 min at 4 °C. The lipid content in the supernatant and glycogen in the residual pellet were determined using the technique outlined by (Gismondi et al. 2012), as previously described by (Chen et al. 2015). Briefly, 100 µL of the supernatant was evaporated with cholesterol standards (0, 25, 50, 100, 200, 400, 800, and 1600 µg/mL) for lipid analysis. Following that, 200 µL of 95% sulfuric acid was added, and the samples and standards were incubated at 90 °C for 10 min. After cooling for 5 min on ice, the samples were combined with 4.8 mL of a vanillin-phosphoric acid reagent (300 mg vanillin in 250 ml 68% phosphoric acid). Each sample was vortexed for roughly 20 s before being put into a 96-well plate with 300 µL of each standard and sample solution. The plate was incubated for 5 min at room temperature before being measured in a plate reader at a wavelength of 535 nm (Tecan infinite M200). The pellet was dried and dissolved in 400 µL of Milli-Q water for the glycogen assay. Following that, 100 mL of each sample and glucose standard solution (0, 25, 50, 100, 150, 200, and 400 µg/mL) were combined with 4.9 mL of Anthrone reagent (Sigma-Aldrich; 0.2% Anthrone in 95% sulfuric acid). The samples were then vortexed for 20 s after being heated in a water bath at 95 °C for 17 min. After chilling on ice, 300 µL of each sample and standard were pipetted in triplicate into a 96-well plate. The samples were measured in a plate reader (Tecan infinite M200) at a wavelength of 625 nm.

Statistical analyses

All analyses were performed with R in RStudio (Version 1.3.1073). Survival of the gammarids was analyzed as binary data (0 = dead, 1 = alive) after 6 days of exposure to the experimental temperatures. Therefore, a generalized linear model (GLM) with binomial distribution was employed with temperature as continuous explanatory variable and a second-degree polynomial term. Infection status and sex were considered as categorical factors with “uninfected” and “female” as the reference level. Gammarid body length was considered as a covariate. Only gammarids that survived the acclimation period were considered in this analysis. For shredding activity (mussel consumption) the variance in the response variable was modeled with a general linear model (LM) using temperature (with a third-degree polynomial term), sex, and infection status as explanatory variables. The optimal temperature for survival, and shredding was calculated using the “predict()” function of the “car” package (Fox and Weisberg 2019). For faeces production, the variance was modeled using a LM with temperature (as continuous independent variable), sex, and infection status as predictors. The correlation between shredding activity and faeces production was tested using a LM with defecation rate as the response variable and shredding as the predictor. The phenoloxidase and catalase activity, and lipid and glycogen concentration in response to temperature was modeled with a LM with temperature as continuous explanatory variable and a second-degree polynomial term. Infection status and sex were included as categorical factors with “uninfected” and “female” as the reference level and body length as a continuous variable. All main effects and interactions among temperature, sex, and infection status were considered in the models. Models were built using the “glm()” (for survival) and “lm()” (for the other response variables) functions from the “stats” package. Model suitability was evaluated using the DHARMa package, which has built-in analyses of normality, deviance, and homogeneity of variance of the residuals (Hartig 2018). In the case of catalase concentration and phenoloxidase activity transformations were applied to adjust the data's skewness. In the case of phenoloxidase, the LM on square root-transformed data met all model assumptions as per diagnosed with the DHARMa tool, which was not the case when applying a log-transformation. For catalase, a log-transformation (log+1) was applied to the response variable given that using untransformed and square root-transformed data resulted in significant deviation of the residuals from the model-based expectation. Coefficients of determination (R^2) were extracted using the “r.squaredGLMM()” function of the

"MuMIn" package (Nakagawa and Schielzeth 2013). All resulting models were depicted using the "ggpredict()" function of the "ggeffects" package (Lüdecke 2018). Finally, log response ratios (RR) based on Lajeunesse (2015) were calculated to compare the magnitude of the temperature effect among response variables at the different temperatures. RRs were calculated using the following equation:

$$RR^{\Delta} = \ln\left(\frac{X_T}{\bar{X}_C}\right) + \frac{1}{2}\left[\frac{(SD_T)^2}{N_T\bar{X}_T^2} - \frac{(SD_C)^2}{N_C\bar{X}_C^2}\right] \qquad (1)$$

Where $N_{T/C}$ is the sample size in treatment/control; RR^{Δ} is the small sample size-adjusted log response ratio; $SD_{T/C}$ is the standard deviation of treatment/control sample; $\bar{X}_{C/T}$ is the mean response of control/treatment; X_T is the response of a sample from a treatment. The variance was calculated as follows:

$$var(RR^{\Delta}) = \frac{(SD_T)^2}{N_T\bar{X}_T^2} + \frac{(SD_C)^2}{N_C\bar{X}_C^2} + \frac{1}{2}\left[\frac{(SD_T)^4}{N_T^2\bar{X}_T^4} + \frac{(SD_C)^4}{N_C^2\bar{X}_C^4}\right] \qquad (2)$$

Additionally, the Geary index was calculated for both the control and treatment groups to validate RRs (Lajeunesse 2015) using the following formula:

$$\frac{\bar{X}}{SD}\sqrt{N} \geq 3 \qquad (3)$$

To compare the direction and magnitude of temperature effects among the response variables, RRs were calculated using uninfected as the reference category.

Results

General information on gammarids and infection status

The body length of females ranged between 6.32-10.0 mm for infected and 6.78-9.90 for uninfected individuals (Figure S3.1). For males, body length ranged between 7.28-11.8 mm and between 6.45-11.8 mm for infected and uninfected, respectively (Figure S3.1). Therefore, infected gammarids were 4% (among females) and 7% (among males) significantly larger than uninfected ones (estimate: 0.34, CI: 0.02-0.67, p = 0.037; Table S3.1). On the other hand, males were 16% and 13% (infected and uninfected, respectively) significantly larger than females (estimate: 1.03, CI: 0.70-1.35, p < 0.001; Table S3.1). From all infected gammarids, 56% had an infection intensity of 1 cyst gammarid^{-1}, followed by an infection intensity of 2 cysts gammarid^{-1}

representing 21% of the infected gammarids. Less than 1% of the infected gammarids had an infection intensity higher or equal than 7 cysts gammarid^{-1} with a maximum of 12 cysts gammarid^{-1}. Infection intensities higher than 7 cysts gammarid^{-1} were found only in males. In terms of microsporidian infection, 10% of the 40 gammarids evaluated were infected with *Dictyocoela* sp. (similarity between 99.46-100% based on 4 sequences). According to Bacela-Spychalska et al. (2018), three of the sequences correspond to the clone *Dictyocoela* sp. BFAS1 FAS3, and one to *D. berillonum*.

Survival of the host

All gammarids from the 30 °C treatment died already during the acclimation and were not considered for further analyses. The survival of the gammarids from the remaining temperature treatments was not significantly affected by sex, body length, or infection status. Only temperature significantly explained the variance in survival with a bell-shaped curve ($p < 0.001$; Figure 16; Table S3.2). The optimal temperature for survival was approximately 10.1 °C and considerable mortality (ca. 50%) was observed around 2 °C and 18 °C. At 18 °C, some of the gammarids that survived showed a whiteish appearance (personal observation). At 22 °C, most of the gammarids died after 6 days of exposure (Figure 16). The binomial model explained 59% of the variance (Table S3.2).

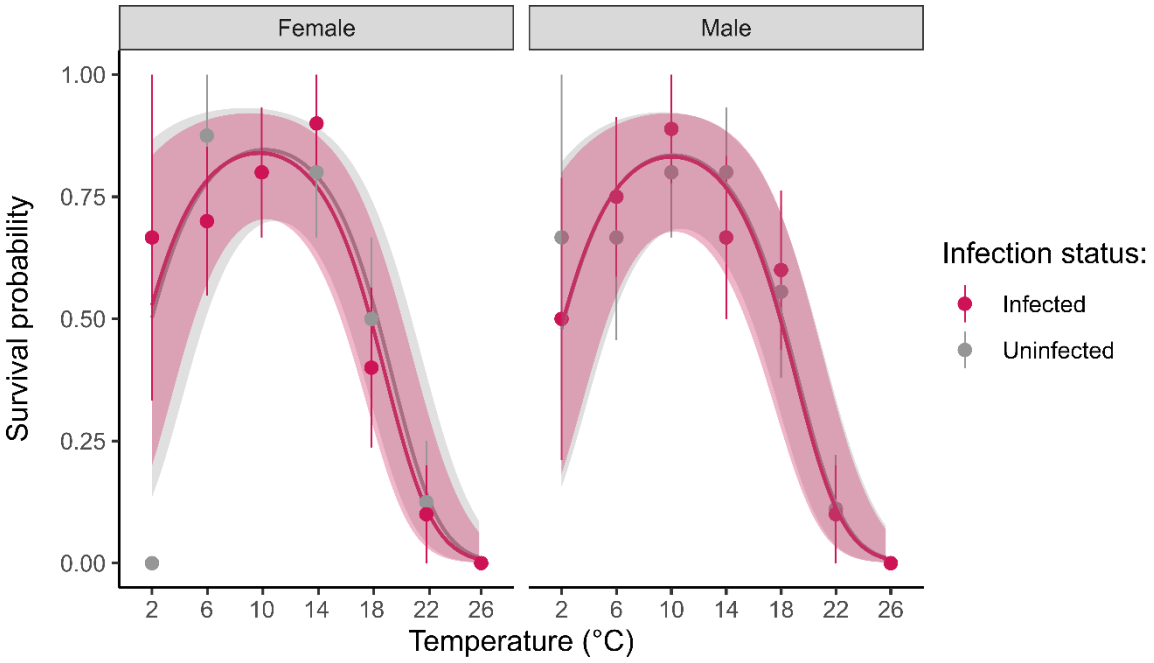

Figure 16 Survival probability of females (left panel) and males (right panel) of *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Dots represent arithmetic means with standard errors, and models represent estimates from a generalized linear model with binomial distribution ($R^2 = 0.586$). Shaded areas represent confidence intervals.

Shredding and feeding behavior

Shredding activity was significantly affected by temperature in a bell-shaped form (estimate = -4.24, CI = -5.08 – -3.39, $p < 0.001$; Figure 17; Table S3.3). Temperature interacted with sex, with males shredding more than females, especially at warmer temperatures (estimate = 0.04, CI: 0.00 – 0.07, $p = 0.035$; Table S3.3). The optimal temperature for shredding for infected females was 15.4 °C and for uninfected 15.9 °C, while for males the optimum for shredding was found at 16.5 °C for infected and 16.9 °C for uninfected (Figure 17). However, the effect of infection was not significant, although a trend was observed for infected gammarids to eat more than their uninfected counterparts at colder temperatures (6–14 °C). The linear model explained 49% of the variance (Table S3.3). The trend for faeces production resembled the one observed for shredding activity. Males defecated more than females, especially at warmer temperatures, although only marginally significant (estimate = 2.05, CI = -0.02 – 4.12, $p = 0.052$; Table S4; Figure S3.2). Although infection status did not have a significant effect on feeding (Table S3.4), the same patterns as for shredding activity were observed. Infected female gammarids consumed more than uninfected. For males, infected gammarids ate more than uninfected at lower temperatures, while uninfected gammarids ate more than infected ones at warmer temperatures. The model explained 19% of the variance. These two response variables (shredding and defecation) were significantly linearly correlated to each other (slope = 15.89 µg-faeces/mg-mussel-shredded, $p < 0.001$, $R^2 = 0.19$; Table S3.5), with increasing defecation rates along increasing shredding activity (Figure S3.3).

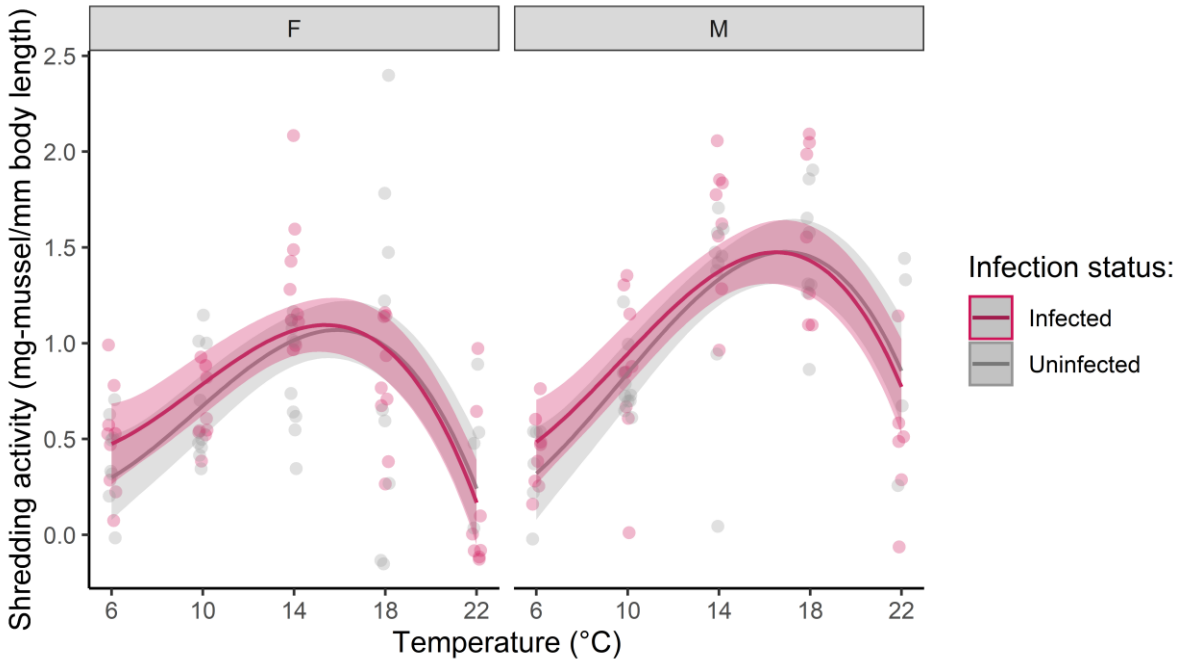

Figure 17 Shredding activity of females (F) and males (M) of *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Jittered dots represent raw data, and models represent estimates from a linear model (R^2 = 0.552). Shaded areas represent confidence intervals.

Biochemical analyses

Phenoloxidase and catalase

The activity of phenoloxidase responded to temperature in an inversed bell shape curve (estimate = 55.13, CI = 43.65 – 66.61, $p < 0.001$; Figure 18a; Table S3.6) with maximum activity of the enzyme at 18 °C. Overall, infection had a significant dampening effect on the activity of phenoloxidase (estimate = 8.51, CI = 1.03 – 15.99, $p = 0.026$; Table S3.6). Nevertheless, this effect was mostly seen for females and was stronger at warmer temperatures (Figure 18a), as supported by the significant interaction term between temperature and infection status (estimate = -0.90, CI = -1.51 – -0.28, $p < 0.01$; Table S6) and the marginally significant three-way interaction term among temperature, sex and infection (estimate = 0.88, CI = -0.00 – 1.77, $p = 0.051$; Table S3.6). In other words, infected females showed lower phenoloxidase activity at 18 °C than uninfected females. The model explained 58% of the variance in phenoloxidase activity (Table S3.6). According to the log-RR analysis, the effect of infection on the activity of phenoloxidase was larger for female gammarids, with a reduction in 59% of the enzyme activity compared to uninfected individuals (Figure 19).

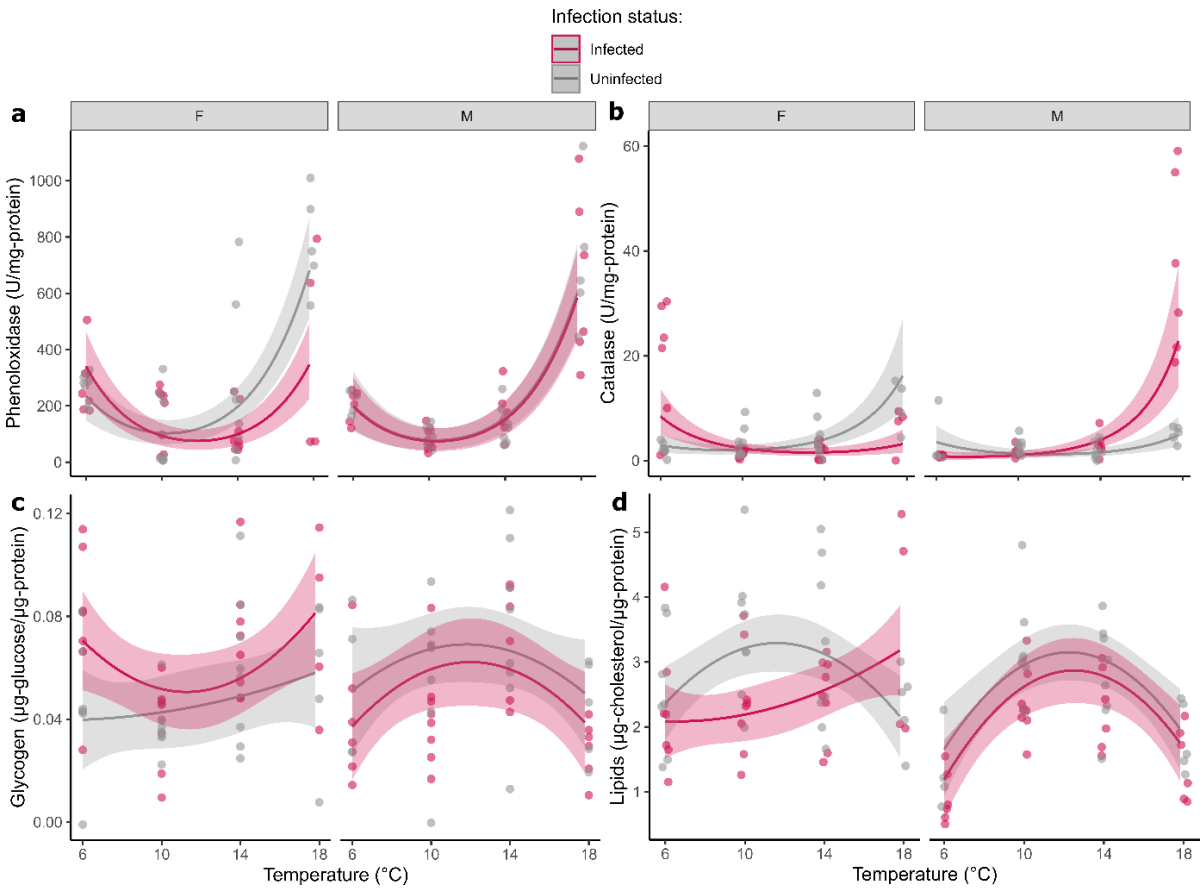

Figure 18 Phenoloxidase activity (a), catalase activity (b), glycogen concentration (c) and lipid concentration (d) in females (F) and males (M) of *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Jittered dots represent raw data, and models represent estimates from general linear models (R^2 = 0.588 (a), R^2 = 0.539 (b), R^2 = 0.195 (c), R^2 = 0.488 (d)). Shaded areas represent confidence intervals.

The activity of catalase was significantly explained by the main effects of temperature and infection status (temperature: estimate = 4.78, CI = 3.15 – 6.41, p < 0.001; infection: estimate = 2.06, CI = 0.99 – 3.12, p < 0.001; Table S3.7), and the interaction between and among temperature, sex, and infection status (Table S3.7). Temperature increased the activity of the enzyme at the extreme temperatures (Figure 19b). However, the direction of the main effect of infection status was dependent on sex and temperature (Figure 19b). For uninfected females the highest activity of catalase was observed at the highest temperature (18 °C; Figure 19b), while almost no activity was detected at 6 °C. In the case of infected females, the opposite was found, with infected females expressing the highest catalase activity at the coldest temperature while at the warmest temperature (18 °C; Figure 18b) activity was reduced compared to uninfected

individuals. A higher expression of catalase was observed for males compared to females, although this was mostly limited to infected males, which showed a steep increase in catalase activity at 18 °C and very low expression at the other temperatures. The model explained 53% of the variance. According to the log-RR analysis, the largest effect of infection was found for males at 18 °C and for females at 6 °C (Figure 19). Moreover, compared to the other physiological parameters, the largest effect of infection was observed for catalase, with infected gammarids showing an increase in catalase activity by three orders of magnitude than uninfected gammarids (Figure 19).

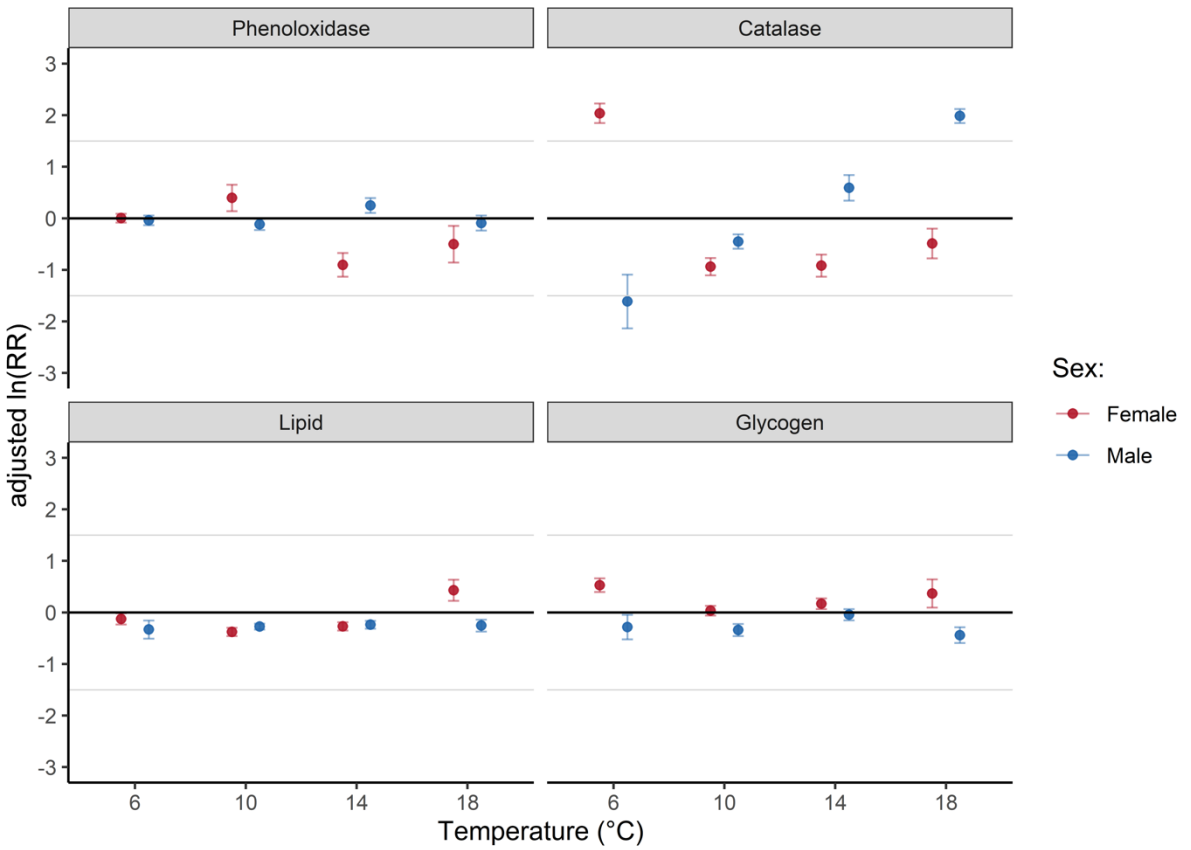

Figure 19 Effect size (adjusted log response ratio) of infection status on phenoloxidase, catalase, glycogen, and lipids in female (red) and male (blue) *Gammarus locusta* uninfected or naturally infected with *Podocotyle atomon*. Gammarids were exposed to acclimation temperatures (6, 10, 14, and 18 °C) for six days. Values are relative to uninfected gammarids at the respective temperature. Error bars represent 95% confidence intervals. Response ratios were calculated according to Lajeunesse (2015).

Lipid and glycogen

The concentration of glycogen in gammarids was significantly explained by infection (estimate = 0.02, CI= 0.00 – 0.03, *p* = 0.021; Table S3.8) and the interaction between sex and infection status (estimate = -0.03, CI= -0.05 – -0.01, *p* = 0.011; Table S3.8). Infected females had higher glycogen content than uninfected females, while uninfected males showed slightly higher glycogen concentration than infected males (Figure 18c). The effect of temperature was not significant, but a bell shaped trend was observed for both infected and uninfected males, meaning that at the coldest and warmest temperatures (6 and 18 °C) there was a slight decrease in glycogen concentrations. In the case of infected females, individuals tended to have higher concentration of glycogen at the extreme temperatures, while an insignificant positive linear relationship was observed for uninfected females (Figure 18c). This model explained 20% of the variance. Lipid concentration followed a significant dome-shaped response to temperature (estimate = -5.74, CI: -9.34 – -2.15, *p* = 0.002; Table S3.9), reaching lowest values at the extreme temperatures (6 and 18 °C) and the highest at 12.0 °C for infected males and 11.8 °C for uninfected males and at 11.2 °C for uninfected females (Figure 18d). The only exception was for infected females for which lipid concentration increased exponentially with temperature, with the lowest concentration at 6 °C and the highest at 18 °C (Figure 18d). There was a significant effect of body length on lipid concentration, with larger gammarids having less lipids (estimate = -0.33, CI= -0.49 – -0.17, p < 0.001; Figure S3.4; Table S3.9). The LM explained 48% of the variance in lipid concentration.

Discussion

The development of thermal performance curves (TPCs) for marine ectotherms is an important tool for understanding and predicting the effects of global warming on the biosphere. Nevertheless, parasites have been largely neglected in such assessments. This article discusses how parasites affect a common marine ectotherm's homeostasis and its response to thermal stress. The infection did not significantly affect survival and shredding thermal profiles, but it did affect physiological responses to temperature. We discuss the potential chronic effects of parasite-driven physiological modulation on host homeostasis.

Gammarus locusta was sensitive to temperature in terms of survival, with an optimum survial rate at 10.1 °C. After 6 days at 18 °C, significantly enhanced mortality was observed, while at 22 °C most gammarids were dead. More than 85% gammarid mortality at 22 °C is alarming, as this temperature has been recorded in shallow subtidal habitats of the Baltic Sea for several days (Wolf et al. 2020). Even higher summer temperatures (up to 26 °C, unpublished data) have been measured in areas closer to river mouths (e.g., Dassower See in Lübeck, Germany), where *G. locusta* has been found occasionally (personal observation). Significantly enhanced mortality at 22 °C was also observed in other studies (Neuparth et al. 2002, Wahl et al. 2021). Although gammarids can move to deeper, cooler waters to avoid fatalities (Axenov-Gribanov et al. 2016), this negative effect of temperature can shift amphipod populations away from the coast, extirpating an important grazer and causing cascading effects in macroalgae assemblages.

Amphipod survival was not affected by trematode infection. Therefore, the first hypothesis is rejected, infection does not exacerbate the mortality of *G. locusta* upon heat stress. Arundell et al. (2019) found that *Podocotyle atomon* significantly increased the mortality of *G. zaddachi.* However, this was detected only after the second week of observations, while no mortality was recorded during the first week. In our case, we limited the exposure to 6 days to avoid exaggerated mortality at temperatures below and above the survival thermal optimum. Furthermore, because larger and thus older gammarids have higher parasite burdens (Arundell et al. 2019b) and a higher probability of dying due to age, it is difficult to distinguish between the effects of age and infection on the survival of naturally infected populations. Although the available evidence does not provide a clear picture of *P. atomon*'s effect on *G. locusta* survival, it makes sense in terms of the parasite's biology that *P. atomon* would have low virulence. *Podocotyle atomon* metacercariae must grow in the host for 30 days before they can infect the next host (Hunninen and Cable 1943). Since one gammarid can host several cysts at different stages of development, the parasite's virulence must be low to keep the host alive and ensure metacercarial maturation and transmission.

The effect of temperature on shredding activity was more pronounced than parasite infection. Gammarids can increase feeding upon stress as a compensatory mechanism (Foucreau et al. 2016, Labaude et al. 2017b), explaining why the optimal temperature for shredding was 7 °C higher than for survival. Compensatory feeding is important in disease ecology because many parasites are transmitted trophically,

increasing the chances of gammarids becoming infected and could therefore represent higher possibilities of amphipods to contract infections in a future warmer sea. However, since the infection effect remained statistically insignificant, the second hypothesis is rejected: infection does not affect compensatory feeding upon thermal stress. Nevertheless, interestingly, there was a significant effect of sex on feeding behavior. Males shredded significantly more than females near the optimum, implying that females could not increase compensatory feeding as efficiently as males due to females' higher sensitivity to thermal stress (Sornom et al. 2010, Labaude et al. 2017b). The difference in stress tolerance between sexes can be explained by the higher energetic burden that females endure during egg production and incubation, which are more energetically costly than sperm production (Sornom et al. 2010). This reproduction-related energy burden is plausible since gammarids were collected during summer, a season when fecundity is high (Steele and Steele 1972). Despite the fact that some food is lost during the shredding process, defecation rate was positively correlated with shredding, indicating that shredding rate is an efficient proxy for feeding rate in *G. locusta*. Moreover, both models explaining the variance in shredding and defecation represent the same trend regarding sex, temperature, and infection effects. Notwithstanding, the variation in defecation rate was higher than that of shredding rate, explaining the low coefficient of determination for the correlation between shredding and faeces production (19%).

Infection significantly altered the biochemical condition of the host at different temperatures and, as such, the third hypothesis can be accepted. The largest effect of infection was observed for catalase activity. However, the direction of the effect was dependent on temperature and sex. The almost constant catalase activity in uninfected males along temperature (6-18 °C) suggests that males are not experiencing unusual oxidative stress in this temperature range. In contrast, uninfected females showed increased catalase activity with increasing temperature. Several studies have also found that female gammarids are more sensitive to stressors than males (McCahon and Pascoe 1988, Hoback and Barnhart 1996, Sornom et al. 2010, Neuparth et al. 2014), probably due to the already compromised metabolism due to oogenesis as discussed above. In terms of infection, infected males had significantly higher catalase activity at 18 °C than uninfected individuals. Infected males at 18 °C actually had the highest catalase activity compared to the other groups in general. A similar trend was also observed in *G. fossarum* infected with the parasite *Polymorphus minutus,* with

infected individuals showing higher catalase activity than uninfected (when exposed to conventionally-treated wastewater, see Rothe et al. 2022). The interactive effect of temperature and infection on catalase activity in males suggests infection may exacerbate oxidative stress at higher temperatures. The trend was more complicated for females, with infected individuals showing increased oxidative stress in colder temperatures but decreased catalase activity at 18 °C. Although the differences in catalase activity between males and females under temperature and infection stress are difficult to explain, they highlight the importance of considering infection and sex when assessing temperature-induced oxidative damage. We do not expect a strong influence of the microsporidian infection (i.e., *Dictyocoela* sp.) due to the low prevalence of infection (10%), although these parasites are known to affect the biochemical condition of the host (Grabner et al. 2014, Chen et al. 2015).

Phenoloxidase activity was enhanced at the lowest and highest temperature. Immunological parameters in crustaceans have previously been observed to respond to temperature in an inverse bell-shaped fashion (Matozzo et al. 2011, Labaude et al. 2017a). As discussed by Labaude et al (2017a), whether phenoloxidase increased activity suggests that gammarids are better equipped to fight infections at warmer temperatures or that the immune system is dysregulated and impaired requires further investigation. In terms of sex, no significant differences were found between females and males, except for infected females at 18 °C. At this temperature, infection by *P. atomon* interfered with the activity of phenoloxidase, suggesting an immunosuppressive effect. Parasite-induced immunosuppression has been observed for other helminths as a result of a long coevolutionary history for the parasite to evade the host's defenses and settle (Rigaud and Moret 2003, Cornet et al. 2009). This immunosuppression at the warmest temperature suggests that gammarids' ability to fight infections may be less effective, making them more susceptible to bacteria and viruses (Cornet and Sorci 2010). Nevertheless, there was high variability in the activity of phenoloxidase in infected females at 18 °C, and whether this pattern is representative of the whole (infected) population must be further evaluated. Since phenoloxidase is involved in melanization—which is a normal amphipod response to metacercarial infections (Kostadinova and Mavrodieva 2005)—the lack of increased phenoloxidase activity in infected gammarids was surprising. Since gammarids were naturally infected and the infections were weeks-old when biochemical samples were collected, melanization may have already occurred, and it is therefore not reflected in

the phenoloxidase analysis. Nevertheless, the lower phenoloxidase activity in infected gammarids compared to uninfected is interesting since it is indicative that already established infections can still have an immunosuppressive effect on the host at warm temperatures.

Energy reserves (i.e., total lipid and glycogen concentrations) were affected by infection, temperature, and sex. The highest and lowest temperatures (6 and 18 °C) induced lipid mobilization. This is consistent with the patterns observed for phenoloxidase, implying that the gammarids may have relied on lipid utilization to maintain organismal homeostasis. This is not a surprise since arthropods mobilize lipids to the hemolymph as a response to immunological stimulation (Arresse and Soulages 2010). The size of the gammarids significantly affected lipid content, with decreasing lipid concentration with increasing size. However, because males are larger than females, females (i.e., smaller gammarids) are likely to have higher lipid concentrations than males (i.e., bigger gammarids) because eggs and oocytes are made up of vitellogenin, a lipoprotein that makes up yolk eggs (Sroda and Cossu-Leguille 2011). Infection status tended to reduce lipid concentration, especially at intermediate temperatures. This resembles patterns found in the literature, and it is explained by the fact that parasites cannot synthesize lipids *de novo* and sequester exogenous lipids from the host to mature and reach the infective metacercarial stage (Marsit et al. 2000, Mondal and Dey 2015, Fokina et al. 2018). This is especially true for *P. atomon* metacercariae because, unlike other species, it grows in the gammarid's body cavity (Hunninen and Cable 1943) and is therefore metabolically active (Siddiqui and Nizami 1981, Galaktionov and Dobrovolskij 2003). Females at 18 °C were the only exception in this regard, with infected females having higher lipid concentrations (as well as glycogen) than uninfected females. Since metacercarial infections can reduce the fertility of female gammarids (Thomas et al. 1995), storage of energy at warmer temperatures can occur in infected individuals. Benign warm temperatures can speed up physiological processes like egg development and enhance lipid mobilization. Should uninfected females have a higher capacity to reproduce than infected ones (Thomas et al. 1995), the energy investment in reproduction might increase in warmer temperatures resulting in lipid usage. The lower lipid concentration in infected amphipods is mirrored in the tendency for infected gammarids to eat more than uninfected gammarids, though this difference was not statistically significant. The lack of significance could be due to the low sample size and high variability in the data.

Conclusion

This study highlighted the importance of thermal performance curves (TPCs) to better predict possible shifts in fundamental grazers as gammarids under global warming and parasite pressure. Metacercarial infection disrupted the biochemical response of *G. locusta* to temperature. Based on these results, we could expect parasites to jeopardize the gammarids' ability to handle thermal stress in the long run. The effect that temperature stress and parasitism might have on chronic metabolic responses and traits such as growth and reproduction remains to be explored. Particularly for female gammarids, it would be necessary to conduct additional biochemical analyses to see whether infected gammarids will remain at a disadvantage and end up in an energetically unstable condition. In this regard, long-term studies in more near-natural scenarios are necessary. Furthermore, the lack of significance for the infection effect on survival and feeding reflects the biochemical parameters' suitability to detect stressful conditions in a more sensitive manner. Internal homeostasis tradeoff could explain the lack of translation of the infection effect on the physiological response to survival and feeding behavior.

General discussion

Given the ubiquity and ecological relevance of both parasites and their hosts, understanding the fate of parasites in the face of global warming and the effect parasitic infections can have on their ectothermic hosts is critical. Therefore, within three chapters, this thesis aims to provide information on the performance of trematodes and their ectothermic hosts under different temperature regimes. More specifically, in **Chapter I**, information on the thermal profile of a common trematode of the Baltic Sea, *Himasthla elongata,* and its effect on the survival of its first intermediate host, *Littorina littorea,* is provided. Temperatures representing extreme summer temperatures and projected end-of-century average temperatures for shallow subtidal habitats of the Baltic Sea promoted cercarial emergence and infectivity while decreasing infected gastropod host and cercariae survival. This parasite- and temperature-induced gastropod mortality resulted in a decrease in the optimal temperature for emergence and infectivity, having a net negative effect on the parasite's performance. Possible mechanisms behind the increased mortality of infected *L. littorea* are suggested in **Chapter II**. Rediae and cercarial development of *H. elongata* in the gastropod reduced glycogen concentrations, possibly jeopardizing the gastropod's ability to perform compensatory metabolism to withstand heat stress. Additionally, *L. littorea* grazed more on the invasive alga *Gracilaria vermiculophylla* than on the native *Fucus vesiculosus*, whereas infection increased grazing by *L. littorea,* indicating that trematodes may act as an indirect modulator of the competition between invasive and native algae. Finally, in **Chapter III**, the effect of *Podocotyle atomon* metacercariae on the response to temperature of its second intermediate host was characterized. The findings in this last chapter suggest that the trematode had a significant impact on the host *Gammarus locusta*'s biochemical response to temperature (particularly catalase activity), while feeding and survival were unaffected. All three chapters are discussed on a broader scale in the following section, providing an overall picture of how trematodes and their hosts may perform in a warming ocean, as well as the anticipated ecological implications.

The future of parasites in a warming sea

Given the variety of parasite groups and the context dependencies that all of these species, and even populations, experience at regional scales, it is challenging to predict whether parasite infections will increase in a warming sea. Nonetheless, a few stepping stones can be addressed to gain a basic understanding of how temperature influences parasite performance. One of these stepping stones is considering the various stages of the parasite's life cycle under different temperatures.

This thesis focuses on trematode parasites. Trematodes have at least one ectothermic host in their life cycle, and the life cycle includes free-living larval stages sensitive to abiotic stressors. Trematode abundance was expected to benefit from a warming sea due to the increasing emergence of infective larval stages (i.e., cercariae) and enhanced infectivity driven by rising temperature (Poulin 2006, Poulin and Mouritsen 2006, Thieltges and Rick 2006). However, recent evidence suggests that the matter is more complicated than previously envisioned due to the numerous variables at play, including the host's thermal performance (Marcogliese 2008, Studer et al. 2013, Paull and Johnson 2014, Mouritsen et al. 2018). Moreover, although cercariae are sensitive to temperature, once the optimal temperature is reached, traits such as emergence and infectivity are quite stable at a wide range of (optimal) temperatures (Morley and Lewis 2013, 2015). In our case, we evaluated different life stages of *H. elongata,* focusing on the transmission between the intermediate hosts. We assessed cercarial emergence, survival, activity and infectivity, encystment capacity, and the survival of the first intermediate host *L. littorea* (Díaz-Morales et al. 2022). The results mirror those from the literature, where the emergence and infectivity increase with increasing temperature while the thermal optimal range is relatively wide (ca. 19–25 °C in the case of *H. elongata*). However, the half-life of cercariae decreased with temperature and higher parasite-induced mortality was observed in the first intermediate host already at 22 °C (Díaz-Morales et al. 2022). In this case a tradeoff of host and cercariae survival for cercarial emergence and infectivity shifted the thermal optima for emergence and infectivity to lower temperatures resulting in an overall net negative effect of warming on trematode performance.

The shift of thermal optima suggests that parasites will have to establish new host-parasite interactions, levels of pathogenicity, or seasonality patterns to assure persistence in the environment. Since surface water temperatures will rise in a

staggered manner (Allen et al. 2018, Hoegh-Guldberg et al. 2018), we might expect that before reaching the critical maximum temperature for trematode performance, temperatures will initially benefit trematode transmission. This benefit will be mainly driven by temperature-enhanced cercarial emergence and infectivity. Therefore, as global warming progresses, trematodes may benefit by slowly increasing their reproduction and thriving in seasons where they could not prosper before (i.e., future warmer winters). This shift in season and initial trigger on the emergence and infectivity of cercariae will have implications for ecosystem energetics (Thieltges et al. 2008b). The increased cercarial emergence and infectivity triggered by rising temperatures in the earliest stages of global warming might result in a zooplankton bloom that can serve as prey for non-host consumers (Thieltges et al. 2008b, Welsh et al. 2014). During this period of enhanced transmission via increased cercarial emergence and infectivity, genes that are more tolerant or efficient under warm temperatures might be selected (Studer and Poulin 2014). However, the low capacity of acclimation (and likely as well low adaption) of intertidal organisms (i.e., hosts) living in fluctuating environments to accelerated warming and more extreme conditions (Somero 2010, Vajedsamiei et al. 2021c) could result in a mismatch between the trematode's and the host's thermal tolerance (Paull and Johnson 2014). As global warming continues, this chain of host-parasite interactions will intensify until the system collapses (at least in the summer), and neither the parasite nor the host can sustain it. As Tom Ray said, "all successful systems attract parasites" (Kelly 1994). Thus, it is safe to say that unsuccessful systems are unattractive to parasites. A future warmer sea does not paint the picture of a stable, successful system that trematodes could exploit to their benefit. Some will argue that this would be ideal since trematodes (especially those of human relevance) would be extirpated from ecosystems. However, given the ecological importance of parasites (Hudson et al. 2006, Hatcher et al. 2012), this would be rather detrimental to a broader scale. This somewhat bleak prognosis for the future of *H. elongata* may be applicable to other trematode species with comparable life cycles and transmission strategies. But are these expectations applicable to all trematode species?

To date, more than 25,000 species of trematodes have been described with a wide range of transmission strategies and metabolic requirements (Combes et al. 1994, Esch et al. 2002). Different transmission strategies and their energy demands imply that trematode thermal sensitivity is rather species-specific (Morley 2011a). Like other

ectotherms, cercariae are at the mercy of external water temperatures. Since water temperature drives metabolic rate in ectotherms, the impact that temperature might have on the performance of a parasite might depend on the costs of transmission to the next host. Therefore, it is expected that with higher temperatures, there will be a selection for trematodes with more passive and low-cost transmission strategies (Figure 20; Morley, 2011). Cercariae with low energy transmission costs can survive weeks or even months without reaching the next host. For instance, cercariae from the family Hemiuridae use a passive transmission strategy that involves floating and waiting for the next host to contract the infection (Galaktionov and Dobrovolskij 2003). In the case of *Halipegus occidualis* (Family: Hemiuridae), a cercaria with a passive mode of transmission (floating instead of active swimming) can maintain a half-life as high as 30 weeks at 4 °C and as low as two weeks at 30 °C (Shostak and Esch 1990). On the contrary, the half-life of cercariae exhibiting active or intermittent modes of swimming range between 4 h to 10 days at temperatures between 0-10 °C and between 2 h to 5 days at temperatures between 20-40 °C (Figure 20; Morley, 2011). Nevertheless, not enough information has been derived on the thermal performance of non-swimming cercariae, and most studies are focused on temperate freshwater species neglecting marine and tropical species. Regarding *P. atomon,* no studies are available on the thermal performance of cercariae except for one record in Russian (Prokofiev 2001). However, given the peculiar transmission strategy of *P. atomon* of ambuscade behavior, where cercariae stay still, waiting for an amphipod to approach before launching towards it and infecting it (Galaktionov and Dobrovolskij 2003), it is plausible that such a transmission strategy has a low energetic cost and might not be as affected by warming as other species.

Host sweet host: an important component of an integrated parasite thermal tolerance assessment

Parasites are generally dependent on their hosts. Thus their survival is dictated by that of their hosts. This thesis analyzed the thermal performance of a first and a second intermediate host of trematodes. As mentioned above, this is particularly important for trematodes because every trematode has at least one ectothermic host. Since all metabolic rates of ectotherms depend on external water temperatures, the host will be highly affected in a warming sea. The processes the host undergoes under warming will reflect on the performance of the internal larval stages of trematodes.

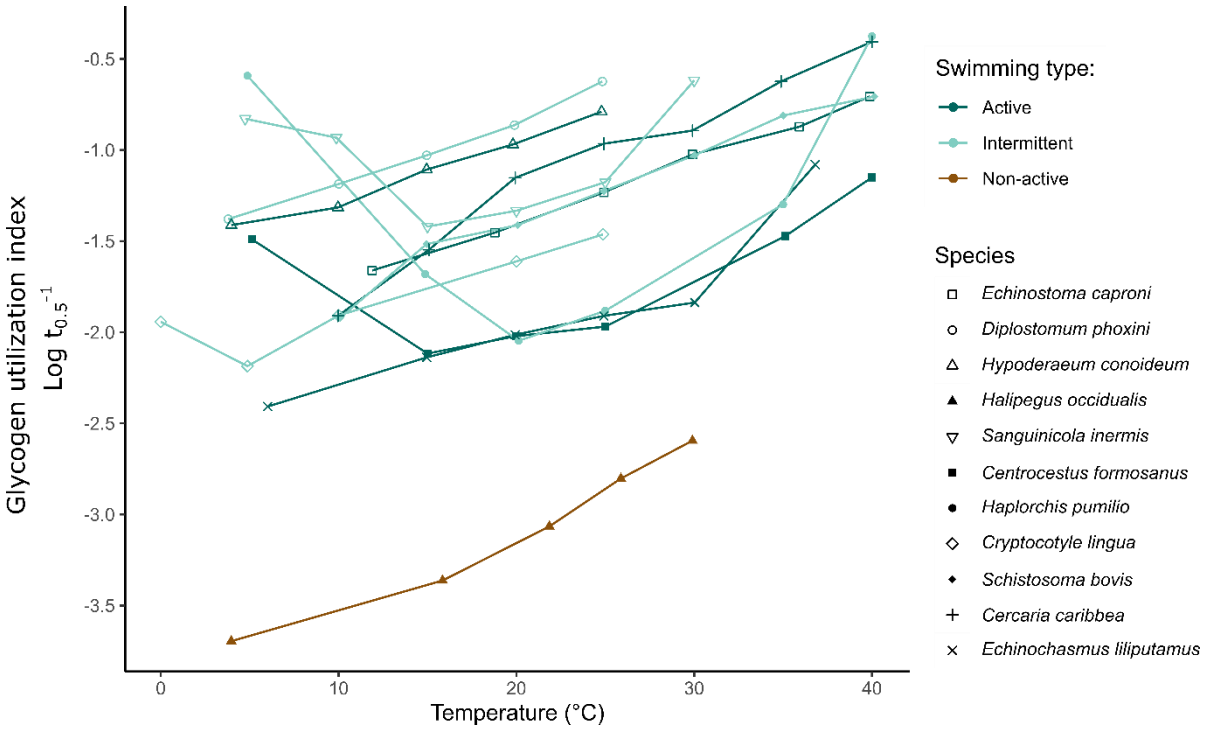

Figure 20 Glycogen utilization index of cercariae species with different swimming behaviors along temperature. The glycogen utilization index is based on the inverse of the logarithm of the cercarial half-life ($Log(t_{0.5})^{-1}$). The lower the index, the lower the glycogen depletion rate. This figure was adapted from Morley (2011).

Among the ectotherms evaluated, the first intermediate host was more sensitive to infection in terms of survival and feeding than the second intermediate host (Chapter I and III). Infected *L. littorea* showed increased mortality already at 22 °C (Díaz-Morales et al. 2022), while no significant difference between infected and uninfected *G. locusta* was detected (Chapter III). This makes sense since rediae are expected to have a higher virulence than metacercariae (Galaktionov and Dobrovolskij 2003). Rediae are actively feeding on the host's tissue, castrate the gastropod and replace the complete gonadal tissue with hundreds of rediae that continuously produce cercariae. In contrast, metacercariae are semi-dormant stages and, although metabolically active, they are not consuming as many resources from the host as the rediae (Galaktionov and Dobrovolskij 2003). Moreover, the infection intensity of *P. atomon* in gammarids is between 1-2 cysts per host (77% of infections), while high infection intensities (> 6 cysts per host) represent less than 1% of the infections (Chapter III). Therefore, the parasite burden is less in gammarids than in gastropods. This difference in the

virulence of redial vs. metacercarial infections is reflected in the feeding behavior. Metacercarial infections did not affect the feeding of *G. locusta,* while redial infections enhanced feeding in *L. littorea* (Chapter II and III). This is biologically senseful since infections with higher virulence should have higher energy drain on the host and therefore prompt higher feeding rates in the host to compensate (Chapter II). Although the trematodes evaluated belong to different species and are not directly comparable, this pattern is likely to apply to most host-trematode systems.

Although the effect of *H. elongata* metacercarial infections on their bivalve host was not assessed in this thesis, several literature records are available. It was identified that *H. elongata* metacercarial infections in the bivalve host could decrease the survival probability of *Mytilus edulis* at 15 °C, while high infection intensity nullifies this effect at 35 °C (Selbach et al. 2020). *Cerastoderma edule* survival has also been compromised by trematode infections under hypoxic conditions (Wegeberg and Jensen 1999). Furthermore, *H. elongata* infection decreased growth rates of *M. edulis* (Bakhmet et al. 2017, 2019), reduced respiration rates in *C. edule* (Magalhães et al. 2020), and affected the biochemical condition of bivalve hosts (Fokina et al. 2018, Magalhães et al. 2018a, 2018b, 2020). In the case of amphipod-trematode systems, a positive effect of *P. atomon* infection on the fluctuating asymmetry index (i.e., random deviations from perfect bilateral symmetry) of *G. duebeni* was found (Arundell et al. 2019a). In laboratory settings, *Podocotyle atomon* also decreased the survival of *G. zaddacchi* (Arundell et al. 2019b), but did not affect the survival of *G. locusta* (Chapter III). Nevertheless, in the latter study, *P. atomon* affected the biochemical response of *G. locusta* to temperature (Chapter III). Under natural field conditions, the trematode *Maritrema novaezealandensis* has been linked to amphipod mass mortalities (Studer et al. 2010, 2013, Mouritsen et al. 2018). Based on this limited number of studies with sometimes contradictory or context-dependent results, it is safe to say that metacercariae can, at the very least, alter the biochemical response of the host to temperature. Because proper metabolic functions ensure that the organism can overcome stress and maintain its ecological function in ecosystems, effects at the molecular level are important.

In terms of the final host, predictions of the effect of global warming on host-trematode interactions become even more complicated. First, most final hosts are vertebrates that are difficult to work with in experimental settings due to ethical and technical reasons. Second, final hosts include a variety of organisms, including both endotherms

(e.g., birds for *H. elongata*) and ectotherms (e.g., fish for *P. atomon*), which by definition are expected to react differently to temperature. Last but not least, many birds are migratory and can cross regional and continental scales increasing the breadth of context dependencies. Despite its complexity, some studies have attempted to predict the future of final host and trematode interactions under climate change (Marcogliese 2001, Lõhmus and Björklund 2015, Galaktionov 2016). In the case of birds, as proposed by Grémillet and Boulinier (2009), they can respond to climate change by (1) modifying their diet and reproduction strategies while keeping their distribution zone, (2) changing their habitat, or (3) extinction. The diet is a particularly important aspect since, on the one hand, a decline in populations of migratory birds has already been observed due to a mismatch between breeding seasons and food availability (Both et al. 2006). On the other hand, due to global warming, the duration of ice cover in lentic systems like lakes is decreasing, allowing birds to feed on fish and organisms that are temporarily covered by ice (Marcogliese 2001). This creates a broader time frame for birds to feed on infected intermediate hosts and increases the birds' chances of getting infected (Marcogliese 2001). Finally, global warming might increase the quantity but decrease the quality of transmission stages (i.e., cercariae and metacercariae) (Mas-Coma et al. 2009), potentially influencing the integrated lifetime reproductive success of adult trematodes (Mouritsen and Elkjær 2020). Decreased fitness in the adult stages can have downstream implications on the parasite's life cycle, particularly in the transmission step between the final host and the first intermediate host (Lõhmus and Björklund 2015). Moving to other final hosts, most fish depend metabolically on temperature. It has been observed that several fish traits are expected to change with global warming such as a suppressed immunocompetence which has direct repercussions on how fish can handle parasitic infections (Dittmar et al. 2014). Although this is just a pinch of the potential effects of global warming on trematodes in terms of their final host, we can conclude that the fate of trematodes in a warming sea is complicated due to the variety of hosts involved in their life cycles and the myriad ways in which temperature can affect both hosts and parasites.

On a broader scale: ecological significance and generalization of results

The three chapters of this thesis addressed mainly the effect of temperature on trematodes (i.e., *H. elongata* and *P. atomon*) and the combined effects of parasitism and temperature on the performance of ectothermic hosts (i.e., *L. littorea* and

G. locusta). However, the results provide insights into the potential indirect ecological effects of climate change and parasitism on coastal marine ecosystems. First, as discussed above, increased cercarial emergence can increase standing crop (Preston et al. 2013, Soldánová et al. 2016) and alter planktonic communities by increasing the numbers of cercariae as zooplankton and, therefore, as prey for non-host organisms (Thieltges et al. 2008b). Second, increased gastropod mortality due to thermal stress combined with infection might affect macroalgae assemblages by extirpating an essential top-down controller of macroalgae epiphytes. Third, even when lethal temperatures are not reached, the energetic drain that trematode infections represent for littorinids impulse these gastropods to increase their feeding, which can have repercussions on the competition between macroalgal species. This could have ramifications on invasion ecology by modulating the success of invasive macroalgae such as *G. vermiculophylla.* Finally, the changes that infection has on the physiological condition of both gastropods and gammarids might dictate the long-term organismal stability of these species compelling them towards tradeoffs between internal homeostasis and ecologically important traits such as feeding, reproduction, and growth. Therefore, the inclusion of trematode infections in understanding the ecological implications of climate change on coastal marine systems is highly relevant.

Trematodes represent only a small fraction of what aquatic parasite diversity looks like. Therefore, we cannot extrapolate the outcomes of this thesis on the fate of parasites, in general, in a changing world. The general fate of parasites will depend on life cycles and the intrinsic biological differences among parasite groups. Parasites are generally grouped into ectoparasites and endoparasites, with simple or complex life cycles (Goater et al. 2014). Ectoparasites—parasites living on their host (e.g., monogeneans)—are expected to increase in intensity and prevalence with global warming, as their oviposition, hatching, and transmission are enhanced at higher temperatures (Jackson and Tinsley 1998, Brazenor and Hutson 2015). In the case of endoparasites—parasites living inside their host (e.g., trematodes)—their performance under warming will depend on how the host reacts to temperature. Therefore, no general pattern can be drawn. Parasites with direct life cycles might be more suited to sustain global warming since they only need one host to proliferate and are not as restricted as complex life cycle parasites (Cizauskas et al. 2017). Parasites with complex life cycles depend on multiple hosts, and all of these hosts must be present in their environment for the parasites to complete their life cycle successfully. This gets

even more complicated for parasites with highly complex life cycles involving more than three hosts. In addition, complex life cycle parasites have free-living transmission stages that are exposed to the external environment, making their life cycle susceptible to disruptions such as those described for trematodes (Pietrock and Marcogliese 2003). However, when a parasite is highly specialized, it makes no difference whether it lives inside or on its host or whether it has a simple or complex life cycle. Specialized parasites, compared to generalist parasites, are less versatile since the breadth of host species they can infect is narrower, restricting them to one genus or even to specific species (Cizauskas et al. 2017). Therefore, with ongoing global warming, we might expect a shift towards more generalist parasites with simpler life cycles. Finally, parasites could also adjust their life history dynamics to find a “happy medium” among transmission, genetic diversity, life cycle complexity, and host virulence (Lõhmus and Björklund 2015). However, accelerated global warming might outpace the rate at which parasites can make these adjustments making it a challenging, if not impossible, road to take.

Future directions of research

An integrated approach towards the prediction of parasitism in a warming sea

To obtain a holistic picture of the fate of parasites in a warming sea, an integrated assessment of parasite thermal tolerance is required. First, we must establish standardized protocols to evaluate the performance of parasites in a warming sea that consider all transmission stages in a life cycle. Although standardization is difficult due to the differences in parasite biology and the variety of contexts in which stressors can be assessed, using Agent-based models or Individual-based models could be a viable option. Agent-based models are microscale models used to simulate a multitude of functions and interactions among the entities of a complex system (agents) to predict their behavior (Gustafsson and Sternad 2010). Such modeling can be used as a starting point to develop experimental designs by identifying the essential components of an organism's life history and to visualize and comprehend the significance and interconnectedness of results derived from thermal tolerance assessments. Moreover, parasites are understudied, and many species lack proper molecular and morphological characterization, leading to an underestimation of parasite diversity (Schwelm 2021). With ongoing global change and a potential sixth-mass extinction approaching (Barnosky et al. 2011, Ceballos et al. 2017), parasites will be extinct along

with their hosts long before they are described, and their performance under stressors is characterized (Carlson et al. 2017). Therefore, incorporating taxonomy into such integrated assessments is crucial for obtaining a clearer picture of what we have and how it might be affected by global warming. Summed to the underestimated parasite diversity is the lack of long-term datasets on the abundance and prevalence of parasites (Wood and Vanhove 2022). Wood and Vanhove (2022) proposed the use of natural history collections dating back hundreds of years to fill this research gap. Together with environmental data, this represents a unique opportunity to look into the past for information that can guide future research and conservation directions (Wood and Vanhove 2022). Lastly, it is crucial to establish an impartial assessment of ecoregions. Most studies are focused on mid-latitudes and temperate systems, leaving polar and tropical systems largely unexplored, although these ecoregions are anticipated to be highly impacted by global change (Allen et al. 2018). Incorporating diverse ecoregions is essential because, as a result of global warming, the length of the seasons will change, which can lead to host range expansion or shifts (Hoegh-Guldberg et al. 2018). These changes in host range and shifts can facilitate bioinvasions and events of parasite spillover or spillback (Power and Mitchell 2004, Prenter et al. 2004, Kelly et al. 2009, Sorte et al. 2010, Goedknegt et al. 2017).

Baltic Sea: a pandora box of abiotic modulators of parasite and ecosystems dynamics

All parasites and hosts addressed in this thesis are native to the Baltic Sea. The Baltic Sea is one of the areas where changes such as warming, hypoxia, eutrophication, contamination, and incoming nonindigenous species have been measured (Reusch et al. 2018). On top of this, biodiversity in the Baltic Sea is powered by abrupt changes in salinity ranging from 2 to 30 PSU (Reusch et al. 2018). Therefore, organisms in the Baltic Sea already experience a heterogeneous abiotic environment that frequently creates stressful conditions. These stressors are important for parasites since they are known to affect their performance. For example, the transmission of trematodes is negatively affected by desalinization through decreased cercarial activity, infection success, and second-intermediate host susceptibility (Bommarito et al. 2020). By affecting their life cycle and the immunocompetence of their host (Birrer et al. 2012), salinity is an important driver of the distribution of parasites and their infection intensities in the Baltic Sea (Gollasch and Zander 1995, Køie 1999, Mellergaard and Lang 1999, Jakob et al. 2009, Thieltges et al. 2010, Lunneryd et al. 2015, Bommarito et al. 2021). However, salinity is not the only driver of changes in parasitic communities'

dynamics, but other stressors, such as eutrophication, can have a significant influence. For instance, infection of reproductive three-spined stickleback *Gasterosteus aculeatus* by the cestode *Schistocephalus solidus* has been observed to increase as an indirect response to eutrophication (Budria and Candolin 2015). A similar pattern was observed in *G. aculeatus* infected with the trematode *Diplostomum* sp., the copepod *Thersitina* sp., and the microsporidium *Glugea anomala* (Heuschele and Candolin 2010). Given the semi-enclosed structure of the Baltic Sea and the myriad of abiotic pressures acting on it, the Baltic Sea represents an ideal study area to address the single effects of these stressors and their interactions on host-parasite systems.

Conclusion

To conclude, we face increasing studies tackling disease ecology in the context of climate change. However, given the diversity of parasites and hosts, research is not yet sufficient to draw a general conclusion regarding the fate of parasites in a warming sea. Nevertheless, there is compelling evidence sustaining general trends that guide us to better understand how trematodes and their hosts might react to a warming sea. Based on this thesis, we could stipulate that active-swimming cercariae (e.g., *H. elongata*) are sensitive to temperature, and their emergence and infectivity are enhanced in warm conditions. However, the increased pathology on the first intermediate host and the heat-induced accelerated cercarial mortality creates a bottleneck for trematodes to flourish in a warming ocean. Trematodes not only induce the mortality of their gastropod host under thermal stress, but they also alter the energy reserves of the host, jeopardizing how ectotherms might metabolically compensate upon thermal stress. As trematodes affect the biochemical condition of the host, they also prompt the gastropod to feed more to compensate for the energy hijacked by the parasite. This can imply ecological repercussions since the trematode might interfere with the capacity of the gastropod to modulate the interaction between native and invasive species of algae. However, the direction of the effect on host feeding behavior is inconsistent in the literature, suggesting that the results are context-dependent. Regarding the second intermediate host, metacercarial infections were less virulent than redial infections. *Podocotyle atomon* did not significantly affect the thermal performance of the host in terms of survival and feeding behavior but affected the biochemical response of the amphipod to temperature. Although some studies suggest that metacercarial infections are highly virulent to amphipods, the studies published so far focus on a limited selection of populations and parasite-host pairs. As such, it is

important to perform more studies addressing the effect of trematodes on the performance of amphipod hosts, including different ecoregions and host-parasite systems.

The chapters of this thesis are grains of sand contributing to the general knowledge of global change effects on host-parasite dynamics. However, it is clear that parasites can influence the response of individuals (i.e., hosts) to global change by benefiting some and harming others. Therefore, when predicting the effects of global change on coastal marine systems, parasites must be considered to avoid inaccurate predictions of the magnitude and direction of these effects. Finally, research must become more accessible in different regions of the world, and taxonomic expertise must be expanded to fill the gaps in less-studied areas worldwide. Until then, we could conclude that global warming will definitely affect parasites, which will take a toll on ecological dynamics in different biomes across the biosphere.

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List of figures

Figure 1 Bioenergetic framework for ectothermic aquatic organisms under different stress scenarios and their physiological implications as described and depicted by Sokolova et al. (2012). Changes in ATP-demand or ATP-supply are represented with red arrows, while the direction of trade-offs is represented with black arrows. It should be noted that the sizes of the boxes corresponding to different energy-demanding operations have been made equal by Sokolova et al. (2012) for clarity's sake and do not reflect the actual energy distribution. Please refer to the original reference for more details (Sokolova et al. 2012). 17

Figure 2 Life cycle of *Himasthla elongata* (gray lines) and *Podocotyle atomon* (black lines) as examples of a typical trematode life cycle involving three hosts. In the first intermediate host (e.g., gastropod), asexual reproduction occurs, and cercariae are produced by parthenitae (i.e., rediae for *H. elongata* and sporocysts for *P. atomon*). Cercariae emerge from the snail and infect the next intermediate host (e.g., bivalve for *H. elongata* or amphipod for *P. atomon*), encysting as metacercariae. The metacercariae are transmitted trophically to the final host (e.g., bird for *H. elongata* or fish for *P. atomon*) where sexual reproduction takes place. After sexual reproduction, eggs are shed with feces, and miracidia hatch to infect the first intermediate host and continue the life cycle. 19

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Abbreviations

AICc	Akaike Information Criterion for small sample sizes
BCA	bicinchoninic acid
BLASTn	Nucleotide Basic Local Alignment Search Tool
BMBF	Bundesministerium für Bildung und Forschung (Federal Ministry of Education and Research)
BSA	bovine serum albumin
d. h.	das heisst (that is)
DANN	deoxyribonucleic acid
DEB	dynamic energy budget
DGG	digestive gland-gonadal
DHARMa	Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models
e.g.	*exempli gratia* (for example)
EDTA	Ethylenediaminetetraacetic acid
ET_{50}	effective time (h) when 50% of cercariae lost their self-propelling capacity
GAMM	generalized additive mixed models
GLM	generalized linear model
HRP	horseradish peroxidase
HSP70	heat shock protein 70 kDa
i.e.	*id est* (that is)
ICC	intraclass correlation coefficient
IGEPAL CA-630	octylphenoxypolyethoxyethanol
IP	immunoprecipitation
IPCC	Intergovernmental Panel on Climate Change
LDH	lactate dehydrogenase
L-DOPA	3,4-dihydroxy-L-phenylalanine
LM	linear model
LMM	linear mixed model
ln(RR)	natural logarithm of response ratio
LRT	Likelihood Ratio Test
LT_{50}	estimated half-life of cercariae (h)

MOST	Ministry of Science Technology
MuMIn	Multi-Model Inference
n	sample size
NCBI	National Center for Biotechnology Information
OCLTT	oxygen- and capacity- limited thermal tolerance
p	p-value
PBS	Phosphate-buffered saline
PCR	polymerase chain reaction
PTJ	Projektträger Jülich
R^2	coefficient of determination
REML	restricted maximum likelihood
RR	response ratio
s.l.	*sensu lato*
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
sp.	species
spp.	species pluralis
TBS	tris-buffered saline
TPC	thermal performance curves
Tris-HCl	Tris hydrochloride
z. B.	zum Beispiel (for example)
σ^2	variance

Appendices

This appendix consists of the following supplementary material to the chapters.

Chapter I

Heat sensitivity of first host and cercariae may restrict parasite transmission in a warming sea

Díaz-Morales, Dakeishla M.; Bommarito, Claudia; Vajedsamiei, Jahangir; Grabner, Daniel S.; Rilov, Gil; Wahl, Martin; Sures, Bernd. *Scientific Reports,* 12:1174, doi: 10.1038/s41598-022-05139-5.

Figure S1.1. Generalized linear model of cercariae encystment after emergence from snail with negative binomial distribution after a 3-day incubation period to different temperatures.
Figure S1.2. Generalized additive mixed model of cercariae pre-mortem encystment with time (h) and temperature (°C) as smooth terms.

Table S1.1: Regression parameters estimated from Generalized Linear Models applied on the variance of cercarial emergence, cercariae encystment, and net cercarial emergence.
Table S1.2: Approximate significance level of smoothing functions from General(ized) Additive Mixed Models applied on the variance of cercariae activity, mortality, and encystment.
Table S1.3: Regression coefficients estimated from Generalized Additive Mixed Model (GAMM) applied on the variance of acute infection success and Generalized Linear Mixed Model (GLMM) on net acute infection success.

Chapter II

Parasitism and temperature affect the feeding, energy metabolism, and stress response of *Littorina littorea*, a prime consumer of a native and an invasive alga in the Baltic Sea

Díaz-Morales, Dakeishla M.; Bommarito, Claudia; Knol, Jeffrey; Grabner, Daniel S.; Noè, Simona; Rilov, Gil; Wahl, Martin; Guy-Haim, Tamar; Sures, Bernd (2022). Submitted to *Science of the Total Environment* on June 09, 2022.

Figure S2.1. Infection status of snails among treatments including infections by *Himasthla elongata, Cryptocotyle lingua, Renicola roscovita* and a co-infection by *H. elongata* and *R. roscovita*.
Figure S2.2. Estimates from reduced linear mixed models explaining the variance in faeces production (log-transformed) (a), glycogen (b), and lipid concentrations (c) in *Littorina littorea* fed with *Gracilaria vermiculophylla* or *Fucus vesiculosus*, and uninfected or naturally infected with *Himasthla elongata* using temperature, infection status, algae type, and their interaction as categorical predictors. Snails were exposed to acclimation temperatures (10, 16, and 22 °C) for four days. Values are relative to uninfected snails fed with *F. vesiculosus* and exposed to 16 °C. Error bars are 95% confidence intervals.
Figure S2.3. Faeces production (a), relative HSP70 (b), glycogen concentration (c), and lipid concentration (d) of *Littorina littorea* infected with prepatent (n=7, lilac) and patent (n=15, pink) infections of *Himasthla elongata* after exposure to 22 °C for 4 days. Treatments of diet were pooled together. Values represent arithmetic means and confidence intervals.

Table S2.1: Full linear model on the variance of faeces production using temperature, algae type, infection status and their interaction as predictors.
Table S2.2: Full linear model on the variance of glycogen concentration using temperature, algae type, infection status and their interaction as predictors.
Table S2.3: Full linear model on the variance of lipid concentration using temperature, algae type, infection status and their interaction as predictors.
Table S2.4: Full linear model on the variance of relative heat shock protein using temperature, algae type, infection status and their interaction as predictors.

Chapter III

The trematode *Podocotyle atomon* modulates the biochemical response of *Gammarus locusta* to thermal stress but not its feeding rate or survival

Díaz-Morales, Dakeishla M.[†]; Khosravi, Maral[†]; Grabner, Daniel S.; Nahar, Nazmun; Bommarito, Claudia; Wahl, Martin; Sures, Bernd (2022). Submitted to *Science of the Total Environment* on July 13, 2022.

Figure S3.1. Boxplot of the size of female and male *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon*.
Figure S3.2. Defecation rate of females (F) and males (M) of *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Jittered dots represent raw data, and models represent estimates from a linear model (R^2 = 0.193). Shaded areas represent confidence intervals.
Figure S3.3. Defecation rate as a response to shredding activity of *Gammarus locusta*. Dots represent raw data, and model represents estimates from a general linear model (R^2 = 0.192). Shaded areas represent confidence intervals.
Figure S3.4. Lipid concentration in *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Dots represent raw data, and models represent estimates from a general linear model (R^2 = 0.488). Shaded areas represent confidence intervals.

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Table S3.2: Effects of infection and temperature on survival of *Gammarus locusta*
Table S3.3: Effects of trematode infection and temperature on shredding activity of *Gammarus locusta*
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Table S3.8: Effects of infection and temperature on glycogen concentration in *Gammarus locusta*
Table S3.9: Effects of infection and temperature on lipid concentration in *Gammarus locusta*

Supplementary material-Chapter I

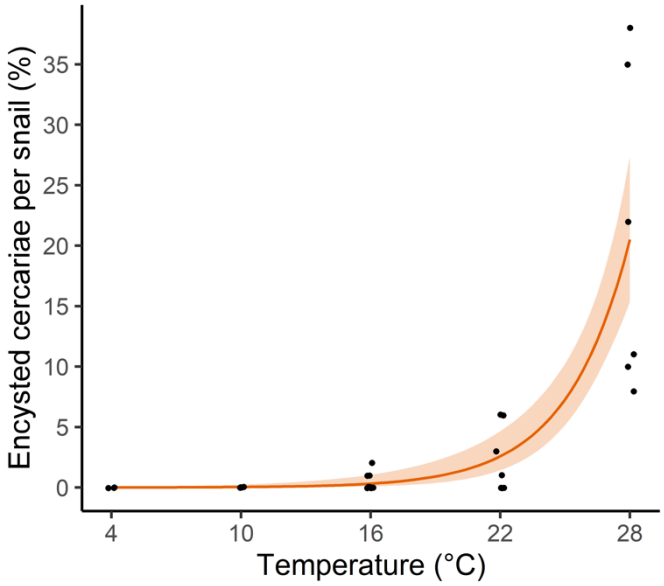

Figure S1.1. Generalized linear model of cercariae encystment after emergence from snail with negative binomial distribution after a 3-day incubation period to different temperatures.

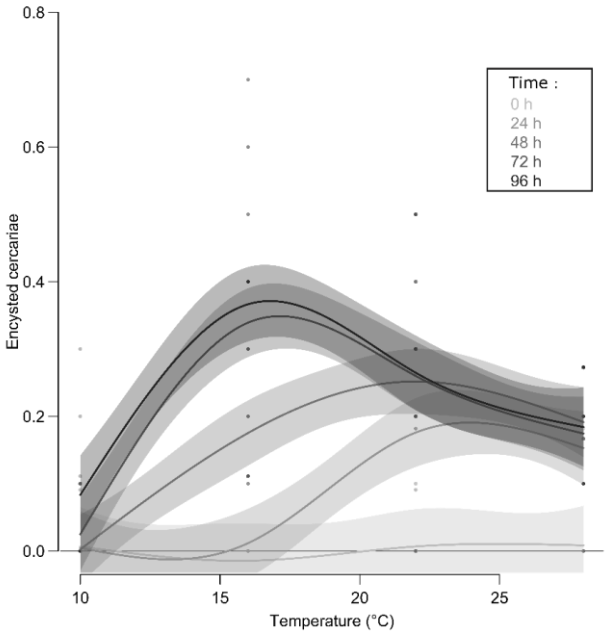

Figure S1.2. Generalized additive mixed model of cercariae pre-mortem encystment with time (h) and temperature (°C) as smooth terms.

Table S1.1: Regression parameters estimated from Generalized Linear Models applied on the variance of cercarial emergence, cercariae encystment, and net cercarial emergence.

Response variable	Model/Distribution	DF	Predictor	Estimate	SE	t	z	p-value	R^2
Cercarial emergence	Poisson*	38	Intercept	4.92	0.36	13.77		<0.001	0.852
			Temperature	11.26	2.45	4.59		<0.001	
			$Temperature^2$	-5.34	1.45	-3.69		<0.001	
Cercariae encystment	Negative binomial	27	Intercept	-6.59	1.24		-5.31	<0.001	0.776
			Temperature	0.34	0.05		6.78	<0.001	
Net cercarial emergence	Negative binomial	34	Intercept	6.51	0.48		13.65	<0.001	0.775
			Temperature	-0.7	3.58		-0.20	0.845	
			$Temperature^2$	-9.53	2.45		-3.90	<0.001	
			$Temperature^3$	-4.35	2.02		-2.15	<0.05	
	Zero-inflation		Intercept	5.04	171.49		0.03	>0.05	
			Temperature	-1.22	45.97		-0.03	>0.05	

*corrected for overdispersion; DF= degrees of freedom; SE=standard error

Table S1.2: Approximate significance level of smoothing functions from General(ized) Additive Mixed Models applied on the variance of cercariae activity, mortality, and encystment.

Response variable	Model/Distribution	Predictor	EDF	Chisq	P-value	R^2
Cercariae activity	Binomial	s(Time)	2.92	607.03	<0.001	0.872
		s(Temperature)	2.02	165.90	<0.001	
		ti(Time, Temperature)	2.95	70.74	<0.001	
		s(Sample_id)*	33.63	122.28	<0.001	
Cercariae mortality	Binomial	s(Time)	1.94	192.96	<0.001	0.811
		s(Temperature)	1.68	113.30	<0.001	
		ti(Time, Temperature)	3.40	15.97	<0.01	
		s(Sample_id)*	30.71	100.96	<0.001	
Response variable	**Model/Distribution**	**Predictor**	**EDF**	**F**	**P-value**	**R^2**
Cercariae encystment	Gaussian	s(Time)	2.44	108.15	<0.001	0.778
		s(Temperature)	2.54	12.65	<0.001	
		ti(Time, Temperature)	8.37	19.37	<0.001	
		s(Sample_id)*	37.9	5.52	<0.001	

*random effect; EDF= effective degrees of freedom

Table S1.3: Regression coefficients estimated from Generalized Additive Mixed Model (GAMM) applied on the variance of acute infection success and Generalized Linear Mixed Model (GLMM) on net acute infection success.

Response variable	**Model/Dist**	**Predictor**	**Estimate**	**SE**	**t**	**p-value**	**mR^2**	**cR^2**
Acute infection success	GAMM/ Gaussian	Intercept	26.35	2.69	9.80	< 0.0001	0.469	0.469
		Predictor	**EDF**	**F-value**	**P-value**			
		s(Temperature)	2.81	10.85	0.0001			
		s(Thermobath)	0.95	0.20	0.2625			
Response variable	**Model/Dist**	**Predictor**	**Estimate**	**SE**	**z**	**p-value**	**mR^2**	**cR^2**
Net infection success	GLMM/ Negative binomial	Intercept	5.58	0.18	30.18	< 0.001	0.786	0.793
		Temp	-6.14	1.23	-5.01	< 0.001		
		$Temp^2$	-9.80	1.06	-9.25	< 0.001		
		$Temp^3$	-2.47	0.67	-3.70	< 0.001		
	GLMM/ Zero-inflation	Intercept	-2.08	3.61	-0.58	0.565		
		Temp	-0.07	0.22	-0.31	0.756		

Dist= distribution family; Temp= temperature; DF= degrees of freedom; SE= standard error; mgR^2= marginal r-square (variance explained by fixed effects); cR^2= conditional r-square (variance explained by fixed effects and random effects)

Supplementary material-Chapter II

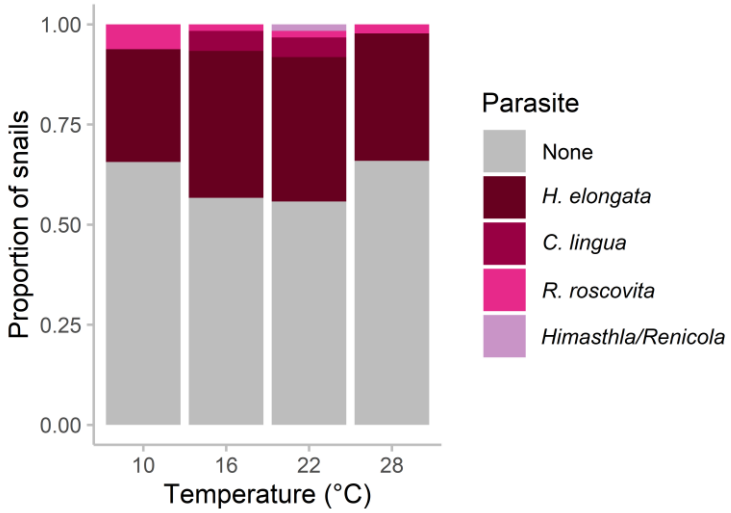

Figure S2.1. Infection status of snails among treatments including infections by *Himasthla elongata, Cryptocotyle lingua, Renicola roscovita* and a co-infection by *H. elongata* and *R. roscovita*.

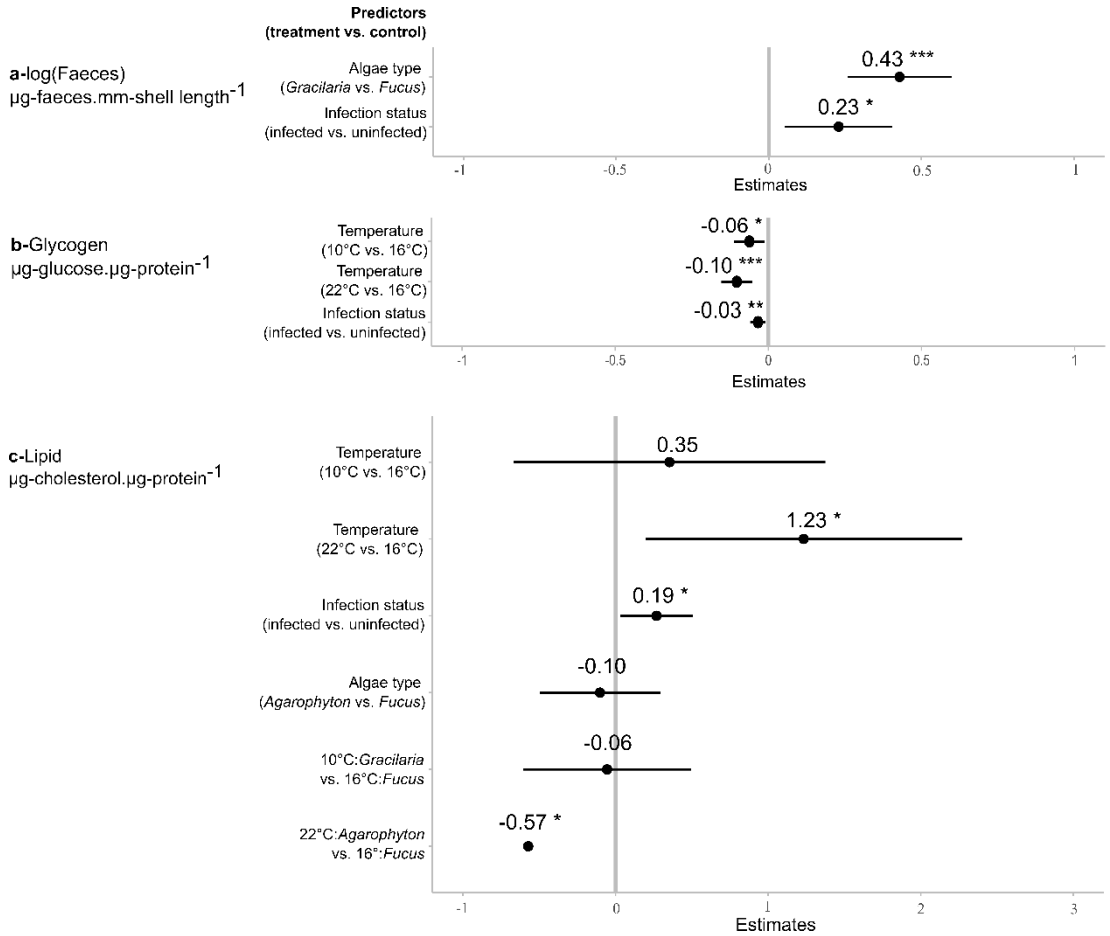

Figure S2.2. Estimates from reduced linear mixed models explaining the variance in faeces production (log-transformed) (a), glycogen (b), and lipid concentrations (c) in *Littorina littorea* fed with *Gracilaria vermiculophylla* or *Fucus vesiculosus*, and uninfected or naturally infected with *Himasthla elongata* using temperature, infection status, algae type, and their interaction as categorical predictors. Snails were exposed to acclimation temperatures (10, 16, and 22 °C) for four days. Values are relative to uninfected snails fed with *F. vesiculosus* and exposed to 16 °C. Error bars are 95% confidence intervals.

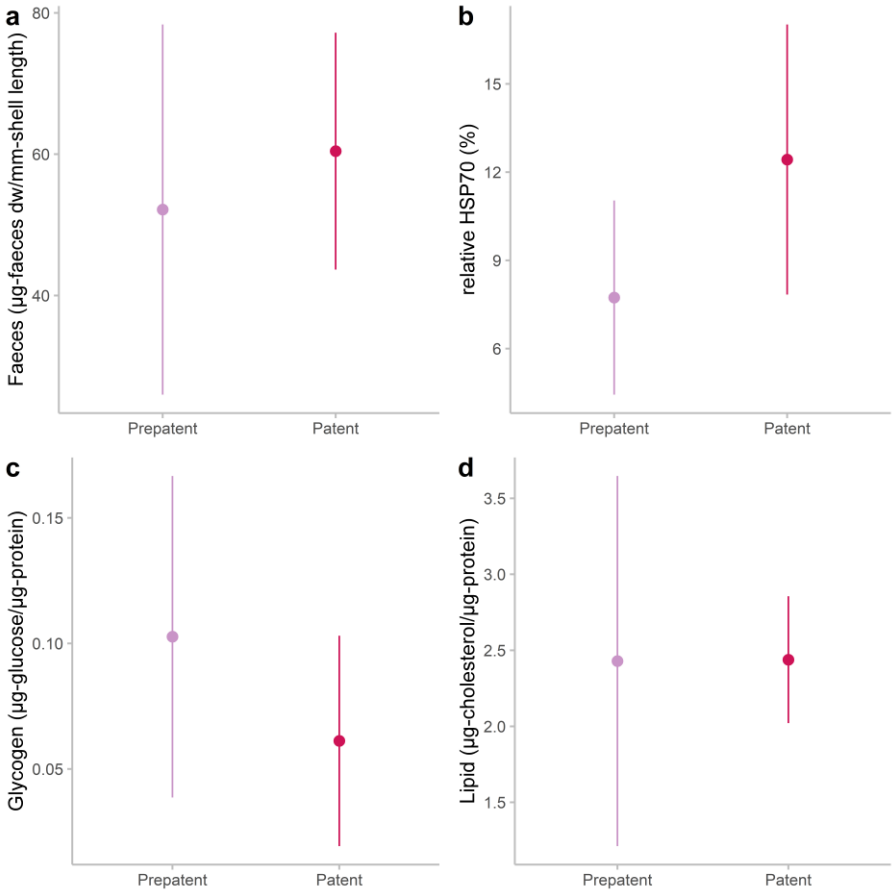

Figure S2.3. Faeces production (a), relative HSP70 (b), glycogen concentration (c), and lipid concentration (d) of *Littorina littorea* infected with prepatent (n=7, lilac) and patent (n=15, pink) infections of *Himasthla elongata* after exposure to 22 °C for 4 days. Treatments of diet were pooled together. Values represent arithmetic means and confidence intervals.

Table S2.1: Full linear model on the variance of faeces production using temperature, algae type, infection status and their interaction as predictors.

	Effects of temperature, infection, and diet on log(faeces production)		
Predictors	*Estimates*	*CI*	*p*
(Intercept: 16 °C,U,Fv)	3.73	3.39 – 4.07	**<0.001**
Temperature (10 °C)	-0.19	-0.65 – 0.27	0.420
Temperature (22 °C)	-0.08	-0.54 – 0.38	0.735
Infection status (Infected)	0.34	-0.02 – 0.69	0.062
Algae type (*Gracilaria*)	0.38	0.06 – 0.71	**0.022**
10 °C:Infected snails	-0.17	-0.61 – 0.26	0.431
22 °C:Infected snails	-0.20	-0.63 – 0.23	0.365
10 °C:*Gracilaria*	0.22	-0.19 – 0.64	0.290
22 °C:*Gracilaria*	-0.12	-0.54 – 0.30	0.581
Infected snails:*Gracilaria*	0.03	-0.32 – 0.38	0.885
Random Effects			
σ^2	0.30		
$\tau_{00\ Thermobath}$	0.03		
ICC	0.08		
$N_{\ Thermobath}$	6		
Observations	166		
Marginal R^2 / Conditional R^2	0.184 / 0.247		
AIC	313.961		

Table S2.2: Full linear model on the variance of glycogen concentration using temperature, algae type, infection status and their interaction as predictors.

	Effects of temperature, infection, and diet on glycogen concentration		
Predictors	*Estimates*	*CI*	*p*
(Intercept: 16 °C,U,Fv)	0.22	0.17 – 0.26	**<0.001**
Temperature (10 °C)	-0.09	-0.15 – -0.03	**0.005**
Temperature (22 °C)	-0.10	-0.16 – -0.03	**0.004**
Infection status (Infected)	-0.05	-0.10 – 0.01	0.079
Algae type (*Gracilaria*)	0.01	-0.03 – 0.06	0.561
10 °C:Infected snails	0.03	-0.03 – 0.09	0.365
22 °C:Infected snails	0.02	-0.04 – 0.09	0.440
10 °C:*Gracilaria*	0.03	-0.03 – 0.09	0.269
22 °C:*Gracilaria*	-0.03	-0.09 – 0.03	0.318
Infected snails:*Gracilaria*	-0.01	-0.07 – 0.04	0.570
Random Effects			
σ^2	0.01		
$\tau_{00\ Thermobath}$	0.00		
ICC	0.07		
$N_{\ Thermobath}$	6		
Observations	141		
Marginal R^2 / Conditional R^2	0.285 / 0.333		
AIC	-257.343		

Table S2.3: Full linear model on the variance of lipid concentration using temperature, algae type, infection status and their interaction as predictors.

	Effects of temperature, infection, and diet on lipid concentration		
Predictors	*Estimates*	*CI*	*p*
(Intercept: 16 °C,U,Fv)	1.27	0.52 – 2.02	**0.001**
Temperature (10 °C)	0.37	-0.67 – 1.40	0.487
Temperature (22 °C)	1.25	0.19 – 2.31	**0.021**
Infection status (Infected)	0.30	-0.19 – 0.78	0.231
Algae type (*Gracilaria*)	-0.10	-0.53 – 0.33	0.650
10 °C:Infected snails	-0.04	-0.61 – 0.53	0.893
22 °C:Infected snails	-0.04	-0.63 – 0.55	0.893
10 °C:*Gracilaria*	-0.05	-0.61 – 0.50	0.851
22 °C:*Gracilaria*	-0.57	-1.15 – 0.00	0.050
Infected snails:*Gracilaria*	-0.00	-0.48 – 0.47	0.986
Random Effects			
σ^2	0.47		
$T_{00\ Thermobath}$	0.23		
ICC	0.33		
$N_{\ Thermobath}$	6		
Observations	140		
Marginal R^2 / Conditional R^2	0.210 / 0.470		
AIC	329.427		

Table S2.4: Full linear model on the variance of relative heat shock protein using temperature, algae type, infection status and their interaction as predictors.

	Effects of temperature, infection, and diet on log(relative HPS70)		
Predictors	*Estimates*	*CI*	*p*
(Intercept: 16 °C,U,Fv)	1.51	0.71 – 2.32	**<0.001**
Temperature (10 °C)	0.10	-1.02 – 1.23	0.856
Temperature (22 °C)	0.40	-0.74 – 1.53	0.488
Infection status (Infected)	-0.14	-0.79 – 0.50	0.662
Algae fed (*Gracilaria*)	-0.35	-0.92 – 0.23	0.235
10 °C:Infected snails	0.31	-0.51 – 1.13	0.461
22 °C:Infected snails	0.38	-0.40 – 1.15	0.341
10 °C:*Gracilaria*	0.39	-0.41 – 1.18	0.338
22 °C:*Gracilaria*	0.43	-0.32 – 1.19	0.253
Infected snails:*Gracilaria*	-0.06	-0.72 – 0.60	0.855
Random Effects			
σ^2	0.77		
$\tau_{00\ Thermobath}$	0.23		
ICC	0.23		
$N_{\ Thermobath}$	6		
Observations	125		
Marginal R^2 / Conditional R^2	0.114 / 0.314		
AIC	352.904		

Supplementary material-Chapter III

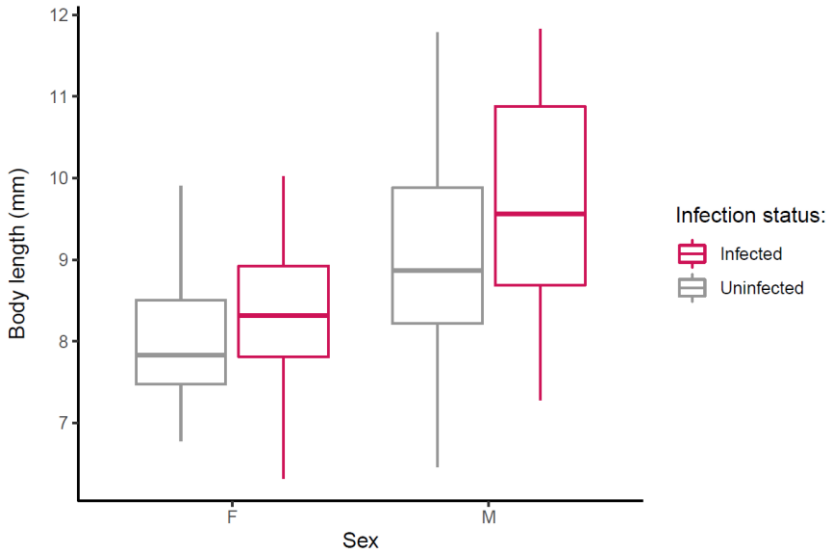

Figure S3.1. Boxplot of the size of female and male *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon*.

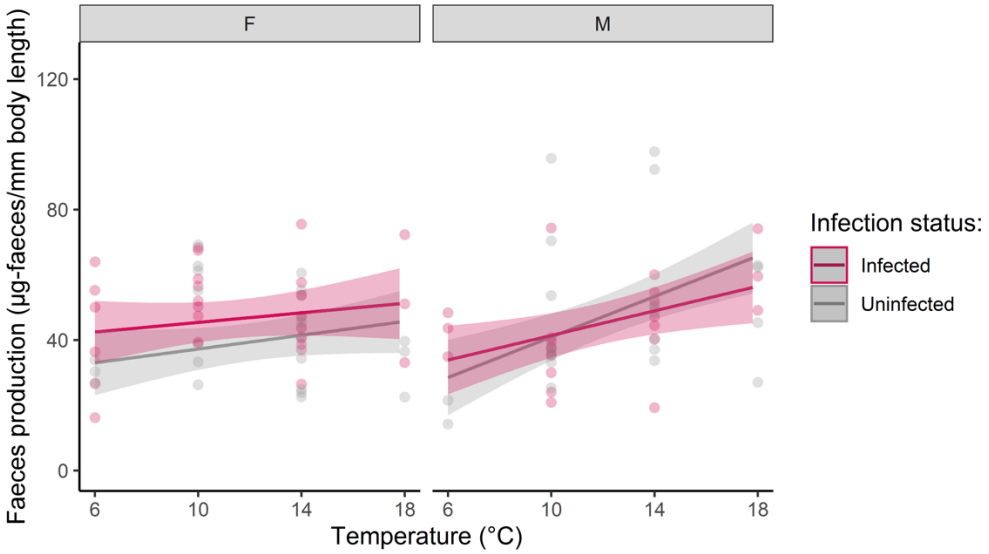

Figure S3.2. Defecation rate of females (F) and males (M) of *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Jittered dots represent raw data, and models represent estimates from a linear model (R^2 = 0.193). Shaded areas represent confidence intervals.

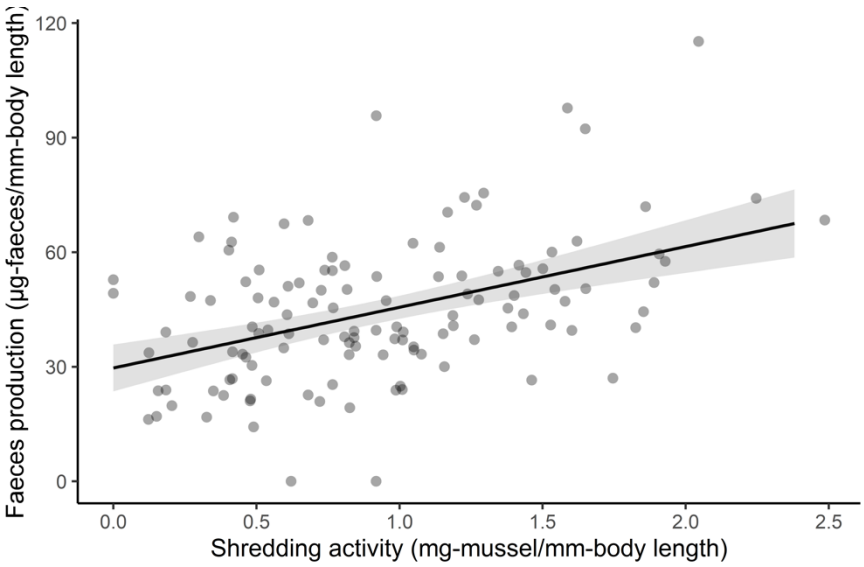

Figure S3.3. Defecation rate as a response to shredding activity of *Gammarus locusta*. Dots represent raw data, and model represents estimates from a general linear model ($R^2 = 0.192$). Shaded areas represent confidence intervals.

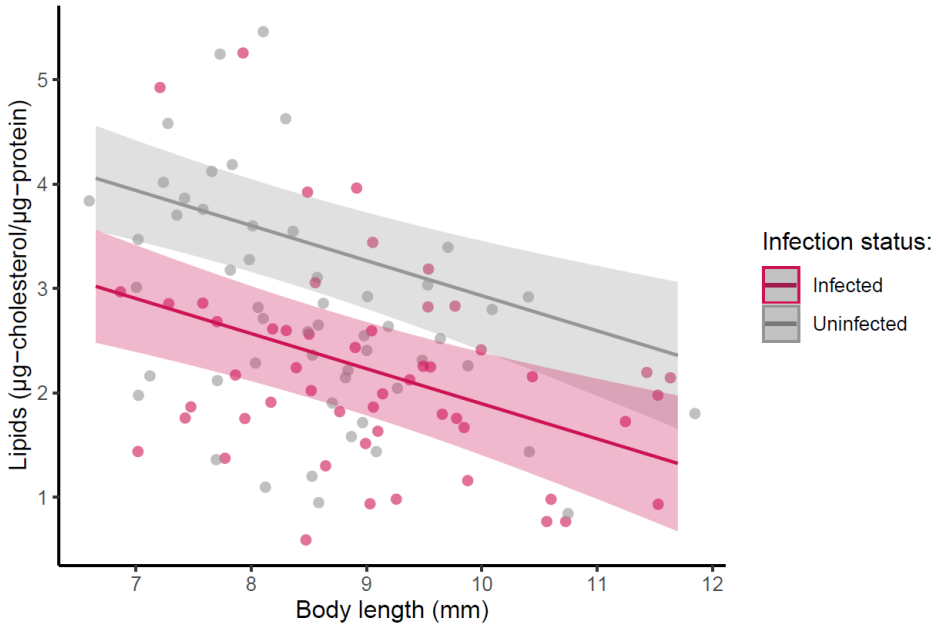

Figure S3.4. Lipid concentration in *Gammarus locusta* uninfected (gray) and naturally infected (pink) with *Podocotyle atomon* after a 6 days post-acclimation exposure to temperature. Dots represent raw data, and models represent estimates from a general linear model ($R^2 = 0.488$). Shaded areas represent confidence intervals.

Table S3.1: Effects of infection and sex on body length of *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	7.97	7.75 – 8.20	**<0.001**
Sex (M)	1.03	0.70 – 1.35	**<0.001**
Infection status (Infected)	0.34	0.02 – 0.67	**0.037**
Sex:Infection	0.33	-0.13 – 0.78	0.157
Observations	320		
R^2	0.284		
AIC	937.212		

Table S3.2: Effects of infection and temperature on survival of *Gammarus locusta*

Predictors	*Odds Ratios*	*CI*	*p*
(Intercept: Uninfected, F)	0.44	0.03 – 7.05	0.565
Temperature[1]	0.00	0.00 – 0.00	**<0.001**
Temperature[2]	0.00	0.00 – 0.00	**<0.001**
Sex (M)	0.91	0.07 – 11.21	0.942
Infection status (Infected)	1.15	0.10 – 12.81	0.908
Body length	1.07	0.76 – 1.51	0.682
Temperature:Sex	1.00	0.85 – 1.19	0.990
Temperature:Infection	0.98	0.83 – 1.16	0.820
Sex:Infection	0.90	0.03 – 25.23	0.949
Temperature:Sex:Infection	1.01	0.81 – 1.27	0.906
Observations	225		
R^2 Tjur	0.586		
AIC	227.324		

Table S3.3: Effects of trematode infection and temperature on shredding activity of *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	0.66	0.54 – 0.78	**<0.001**
Temperature[1]	0.22	-1.57 – 2.02	0.806
Temperature[2]	-4.24	-5.08 – -3.39	**<0.001**
Temperature[3]	-1.33	-2.15 – -0.51	**0.002**
Sex (M)	-0.20	-0.69 – 0.29	0.415
Infection status (Infected)	0.27	-0.18 – 0.72	0.242
Temperature:Sex	0.04	0.00 – 0.07	**0.035**
Temperature:Infection	-0.02	-0.05 – 0.02	0.329
Sex:Infection	-0.01	-0.67 – 0.65	0.973
Temperature:Sex:Infection	0.00	-0.05 – 0.05	0.999
Observations	160		
R^2	0.494		
AIC	158.322		

Table S3.4: Effects of infection and temperature on faeces production of *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	26.70	9.49 – 43.91	**0.003**
Temperature	1.06	-0.28 – 2.39	0.120
Sex (M)	-16.79	-43.36 – 9.77	0.213
Infection status (Infected)	11.38	-13.00 – 35.75	0.357
Temperature:Sex	2.05	-0.02 – 4.12	0.052
Temperature:Infection	-0.32	-2.28 – 1.64	0.745
Sex:Infection	1.29	-35.41 – 37.99	0.945
Temperature:Sex:Infection	-0.90	-3.82 – 2.02	0.544
Observations	123		
R^2	0.193		
AIC	1058.211		

Table S3.5: Correlation between shredding activity and faeces production of *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept)	29.70	23.50 – 35.90	**<0.001**
Shredding activity	15.89	10.05 – 21.73	**<0.001**
Observations	122		
R^2 / R^2 adjusted	0.194		
AIC	1039.715		

Table S3.6: Effects of infection and temperature on sqrt(phenoloxidase) activity in *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	15.70	7.78 – 23.62	**<0.001**
Temperature[1]	55.53	32.72 – 78.33	**<0.001**
Temperature[2]	55.13	43.65 – 66.61	**<0.001**
Sex (M)	-0.89	-9.07 – 7.29	0.830
Infection status (Infected)	8.51	1.03 – 15.99	**0.026**
Body length	0.11	-0.84 – 1.07	0.817
Temperature:Sex	-0.04	-0.68 – 0.60	0.902
Temperature:Infection	-0.90	-1.51 – -0.28	**0.005**
Sex:Infection	-8.57	-19.65 – 2.51	0.128
Temperature:Sex:Infection	0.88	-0.00 – 1.77	0.051
Observations	106		
R^2	0.584		
AIC	642.198		

Table S3.7: Effects of infection and temperature on log(catalase+1) activity in *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	1.03	-0.09 – 2.16	0.072
Temperature[1]	7.30	4.06 – 10.54	**<0.001**
Temperature[2]	4.78	3.15 – 6.41	**<0.001**
Sex (M)	0.77	-0.39 – 1.94	0.190
Infection status (Infected)	2.06	0.99 – 3.12	**<0.001**
Body length (mm)	0.08	-0.05 – 0.22	0.225
Temperature:Sex	-0.10	-0.19 – -0.01	**0.026**
Temperature:Infection	-0.20	-0.28 – -0.11	**<0.001**
Sex:Infection	-4.14	-5.72 – -2.57	**<0.001**
Temperature:Sex:Infection	0.39	0.26 – 0.52	**<0.001**
Observations	106		
R^2	0.528		
AIC	228.703		

Table S3.8: Effects of infection and temperature on glycogen concentration in *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	0.08	0.04 – 0.13	**<0.001**
Temperature[1]	0.08	-0.04 – 0.21	0.190
Temperature[2]	0.02	-0.10 – 0.14	0.738
Sex (M)	0.01	-0.00 – 0.03	0.168
Infection status (Infected)	0.02	0.00 – 0.03	**0.021**
Body length	-0.00	-0.01 – 0.00	0.131
Temperature[1]:Sex	-0.10	-0.29 – 0.09	0.291
Temperature[2]:Sex	-0.12	-0.30 – 0.06	0.175
Temperature[1]:Infection	-0.02	-0.21 – 0.17	0.841
Temperature[2]:Infection	0.12	-0.06 – 0.30	0.181
Sex:Infection	-0.03	-0.05 – -0.01	**0.011**
Temperature[1]:Sex:Infection	0.03	-0.24 – 0.29	0.845
Temperature[2]:Sex:Infection	-0.15	-0.40 – 0.10	0.238
Observations	106		
R^2	0.195		
AIC	-457.175		

Table S3.9: Effects of infection and temperature on lipid concentration in *Gammarus locusta*

Predictors	*Estimates*	*CI*	*p*
(Intercept: Uninfected, F)	5.61	4.30 – 6.92	**<0.001**
Temperature^1	-1.60	-5.37 – 2.16	0.400
Temperature^2	-5.74	-9.34 – -2.15	**0.002**
Sex (M)	-0.30	-0.76 – 0.16	0.202
Infection status (Infected)	-0.18	-0.61 – 0.25	0.413
Body length	-0.33	-0.49 – -0.17	**<0.001**
Temperature^1:Sex	2.13	-3.49 – 7.76	0.453
Temperature^2:Sex	-1.71	-7.03 – 3.61	0.525
Temperature^1:Infection	6.76	1.26 – 12.27	**0.017**
Temperature^2:Infection	7.41	2.23 – 12.60	**0.006**
Sex:Infection	-0.13	-0.75 – 0.48	0.671
Temperature^1:Sex:Infection	-5.67	-13.51 – 2.18	0.155
Temperature^2:Sex:Infection	-7.70	-15.17 – -0.22	**0.044**
Observations	106		
R^2	0.481		
AIC	260.342		

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