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**Interactions between global change and multiple  
infection have complex effects on life-history traits of**

***Daphnia magna* and its parasites**

*by*

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## **Declaration**

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Floriane O'Keeffe



## Summary

Recent outbreaks of disease such as the COVID-19 pandemic have highlighted the need to better understand the drivers of pathogen spread and transmission, especially with disease outbreaks becoming more frequent due to anthropogenic change. Global warming frequently alters the outcome of disease, either increasing or decreasing it, but although studies often focus on constant rising temperatures, climate change is also exacerbating temperature variability and increasing the frequency of extreme weather events such as heatwaves, both of which may have complex impacts on parasites and their hosts. Additionally, rising global temperatures may be accompanied by other ecological changes, such as eutrophication due to increased nutrient concentrations in the environment. The outcome of parasitic infections from combined impacts of temperature and nutrient stress is difficult to predict, especially in cases where the two stressors interact, and seems to be specific to the host-parasite system. Furthermore, although much of the research investigating how diseases respond to changing ecological parameters focuses on single parasites and their hosts, multiple infections with two or more parasites are common in real-world systems, and may add another layer of complexity when trying to make generalisations about the consequences of global change on disease. In this thesis, I use an empirical approach combined with Bayesian modelling to investigate how the parasites of the freshwater invertebrate *Daphnia magna* respond to increasing temperatures and temperature variability, changes in nutrient levels combined with rising constant temperatures, and multiple infection under a scenario of increasing temperatures and heatwave conditions. My results show that different parasites have differing thermal tolerance, but that the breadth of these temperature ranges does not allow for predictions of parasite response to temperature variation and heatwaves. Moreover, I find that increasing temperatures and changing nutrient levels have strong individual effects on infection in a *Daphnia*-microsporidian system, but may also interact leading to varying responses in parasite life-history traits. Finally, I observe that despite the

absence of prior residency effects on co-infection in this system, varying temperatures lead to changes in the outcome of multiple infection at the extremes of the *Daphnia* thermal range. I conclude that these findings add to the growing number of studies which suggest that the outcome of disease under conditions of climate change may be system-specific, and suggest that predictions may be harder to draw than anticipated due to the complexity of the many factors at play.

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## **Chapter 1. Introduction**

The incidence of disease is increasing (Smith *et al.*, 2014), with impacts on human and animal health (Hilgenfeld and Peiris, 2013), economic losses (Sachs and Malaney, 2002), and changes to ecological and evolutionary processes (Lafferty, Dobson and Kuris, 2006). For example, the recent outbreak of COVID-19 caused not only the death of over 7 million people worldwide (WHO, 2024) but was also responsible for major financial damages (Nicola *et al.*, 2020). Among the factors contributing to rising disease incidence are increases in human connectivity (Baker *et al.*, 2022), intensification of plant and animal trade (Smith *et al.*, 2009), and, of course, climate change (Watts *et al.*, 2017; Caminade, McIntyre and Jones, 2019). Changes to species distributions and interactions mediated by climate change (Capinha *et al.*, 2013; Porretta *et al.*, 2013), as well as changes to global weather patterns (Dore, 2005; Trenberth, 2011; Ohmura, 2012; Thornton *et al.*, 2014), are modifying disease outbreak and spread (Karvonen *et al.*, 2010; Rohr and Raffel, 2010; Levinton *et al.*, 2011; Chen *et al.*, 2012). Gaining a better understanding of which factors facilitate or impede disease spread is crucial to better predict and respond to future epidemics, especially given the ongoing anthropogenic changes which may alter disease transmission, virulence and epidemiology.

### **1.1 Increases in constant temperatures**

Rising temperatures mediated by climate change are modifying disease outbreaks, although there is some debate about the direction of their effects. Although increases in temperature associated with global warming may not always lead to higher incidences of disease (Stireman III *et al.*, 2005; Rohr *et al.*, 2011; Gehman, Hall and Byers, 2018; Shocket *et al.*, 2019), they have been reported to lead to lowered host immunity (Cheng, Wang and Chen, 2005), modifications of the life cycle of parasites (Thomas and Blanford, 2003), shifts in the geographical ranges of diseases as previously inhospitable areas become warm enough to sustain disease (Altizer *et al.*,

2013), larger epidemics (Auld and Brand, 2017) and overall higher levels of infection (Poulin, 2006; Stenseth *et al.*, 2006; Burge *et al.*, 2014; Labaude, Rigaud and Cézilly, 2015). Rising temperatures may bring about increased pathogen transmission and within-host growth (Ben-Horin, Lenihan and Lafferty, 2013; Kirk *et al.*, 2018; Kunze *et al.*, 2022), while host fecundity and survival may decrease when hosts are exposed to increasing temperatures (Altizer *et al.*, 2013; Green *et al.*, 2019). However, host and parasite life-history traits may be differentially affected by rising temperatures (Lafferty and Holt, 2003; Dell, Pawar and Savage, 2011; Paull, LaFonte and Johnson, 2012), leading to difficulties in predicting the outcome of infection under scenarios of increasing temperatures. More research is required into the impacts of temperature on disease to be able to better predict the complex outcomes which may arise (Hay *et al.*, 2002; Patz *et al.*, 2005; Rohr *et al.*, 2011), especially given that climate change is not only increasing constant temperatures but also temperature variability.

## **1.2 Increases in temperature variability**

Global warming is predicted to exacerbate temperature variability, as well as increase the number of extreme weather events such as heatwaves (Easterling *et al.*, 2000; Meehl and Tebaldi, 2004; Schär *et al.*, 2004; Rohr and Raffel, 2010). Additionally, temperatures are becoming more autocorrelated due to climate change (Ruel and Ayres, 1999; Wang *et al.*, 2015; Di Cecco and Gouhier, 2018), which may lead to extended stress on organisms exposed to these conditions (García-Carreras and Reuman, 2011) and can change disease dynamics under certain conditions (Chaves *et al.*, 2014). Increases in climate variability must be considered in the context of disease as they may have more of an impact on fitness traits than mean temperature increases alone (Vasseur *et al.*, 2014), and models based solely on constant temperature variation may be misrepresenting the risks of disease (Paaijmans, Read and Thomas, 2009). Although decreases in disease outbreaks have been recorded in response to temperature variability (Stireman III *et al.*, 2005; Dallas and Drake, 2016), many studies have observed

increased disease incidence under conditions of increased temperature variability (Zhou *et al.*, 2004; Raffel *et al.*, 2006; Stenseth *et al.*, 2006; Rohr and Raffel, 2010), and temperature fluctuations may even initiate disease outbreaks, as is the case with malaria (Zhou *et al.*, 2004; Paaijmans *et al.*, 2010). Similarly to temperature variability, extreme weather events such as heatwaves may modify the outcome of disease (Harvell *et al.*, 2019), although specific outcomes may depend on the intensity, duration and starting temperature of the heatwave, as well as the host-parasite system and the life-history traits examined (Landis *et al.*, 2012; Schreven *et al.*, 2017; Abarca, Larsen and Ries, 2019; Zhang *et al.*, 2019a; Kunze *et al.*, 2022). For example, growth of the parasitoid wasp *Diadegma semiclausum* increased when exposed to a 5°C heatwave during their immature phase, but a 10°C heatwave in the same system delayed parasitoid development (Schreven *et al.*, 2017), while a 6°C heatwave in the *Daphnia-Ordospora colligata* system drastically increased spore burden despite having no effect on infection rates (Kunze *et al.*, 2022). The complexity of the impacts of temperature variability and heatwaves makes it difficult to predict the outcome of disease under these conditions, even with previous knowledge of the thermal tolerance of the host-parasite system (Miranda *et al.*, 2019). Further research into these factors is crucial to allow for generalisations regarding organisms' responses to increasing temperature variability driven by climate change.

### **1.3 Interactions between temperature and nutrient regimes**

Global change is not only leading to changes in temperature regimes worldwide, but is also responsible for shifts in nutrient levels in the environment, often due to the pollution associated with industry (Selman and Greenhalgh, 2010) as well as the use of fertilisers in agriculture (Moss, 2008). Increased nutrient concentrations may cause eutrophication (Fink *et al.*, 2018), cyanobacterial blooms (Paerl and Paul, 2012) and artificially enhanced environments (Smith, 2003), which may amplify (Johnson *et al.*, 2007) or reduce (Frost, Ebert and Smith, 2008) disease outbreaks. Similarly, although lower-quality food due to nutrient imbalances may increase

pathogen infection due to increased feeding and thus increased contact between the host and the parasite (Darchambeau and Thys, 2005; He and Wang, 2008; Aalto and Pulkkinen, 2013), nutrient stress may also decrease host immunity and cause higher levels of infection (Field, Johnson and Schley, 2002; Alaux *et al.*, 2010). Additionally, some studies suggest that lower-quality food may lead to decreased in-host parasite growth given a lack of host resources (Darchambeau and Thys, 2005; He and Wang, 2008), or that the relatively higher nutrient requirements of smaller organisms such as pathogens (i.e. the growth rate hypothesis) (Main, Dobberfuhl and Elser, 1997; Acharya, Kyle and Elser, 2004) may alter the outcome of infection. However, although shifting nutrient levels in the environment may be accompanied by changes in temperature, their combined effects on the outcome of disease are somewhat understudied (but see Manzi *et al.* (2020) for their impacts on disease and McKee *et al.* (2003), Moss *et al.* (2003), Lu *et al.* (2018) and Šorf *et al.* (2015) for their impacts on free-living organisms). The combined effects of temperature and nutrient pollution may be dependent on the direction and amplitude of each factor (Moss, 2010; Peter and Sommer, 2013), making it difficult to predict how they will interact to impact the outcome of disease, especially when considered beyond the level of a single parasite and its host.

#### **1.4 The importance of multiple infection**

The impacts of ecological variables such as temperature and nutrient levels on the outcome of disease have frequently been examined in the context of a single parasite and its host, despite multiple infections being common in natural systems (Petney and Andrews, 1998; Lively *et al.*, 2014). Multiply infecting parasites may have synergistic or antagonistic effects (Vaumourin *et al.*, 2015; Cressler *et al.*, 2016; Shen *et al.*, 2019). For example, a study investigating the impacts of co-infection with the protist *Giardia* and helminths such as hookworm and *Ascaris lumbricoides* in humans found that *Giardia* infection was reduced in individuals with helminth infections, and vice versa, helminth infection was dampened for individuals infected with

*Giardia* (Blackwell *et al.*, 2013). In contrast, synergistic effects where co-infection with the helminth *Gyrodactylus derjavini* and the bacterium *Flavobacterium psychrophilum* increased host mortality were observed in rainbow trout (Busch, Dalsgaard and Buchmann, 2003). The outcome of multiple infection may be impacted by a range of factors, including transmission modes of the parasites (Cressler *et al.*, 2016), age of the host at infection (Cote *et al.*, 2000; Ben-Ami, 2019; Davies *et al.*, 2020) and order of exposure of the host to the parasites (i.e. prior residency) (Hood, 2003; de Roode *et al.*, 2005; Ben-Ami, Rigaud and Ebert, 2011; Karvonen, Jokela and Laine, 2019). Regardless of the factors at play, multiple infection may modify parasite virulence (Taylor, Walliker and Read, 1997; Hood, 2003; Schürch and Roy, 2004; Alizon, 2008; Vojvodic *et al.*, 2012), with potential impacts on evolution (Tollenaere, Susi and Laine, 2016), population dynamics (van Baalen and Sabelis, 1995; Heesterbeek *et al.*, 2015) and species coexistence (Escribano *et al.*, 2001), and the effects of co-infecting parasites may be complex and specific to the host-parasite system.

### **1.5 Multiple infection and temperature**

Multiple infection is generally understudied with respect to temperature, despite the impacts of both temperature variation and co-infection being important questions in the field of disease (Rohr *et al.*, 2011; Lively *et al.*, 2014). Few studies have investigated the combined effects of temperature and multiple infection on disease dynamics, though temperature is an important predictor of multiple infection in mathematical models (Brooker and Clements, 2009; Oakgrove *et al.*, 2014; Poterek *et al.*, 2022), suggesting that it may have a strong impact on the outcome of co-infection. Both increases and decreases in infection have been observed in response to interactions between co-infection and temperature variation (Shameena *et al.*, 2021; Herczeg *et al.*, 2023; Terradas *et al.*, 2023), and specific impacts appear to be dependent on the thermal tolerance of the host-parasite system (Fargues and Bon, 2004; Herczeg *et al.*, 2023). Changes to virulence mediated by multiple infection may be further exacerbated by climate change, as

increasing temperatures may be responsible for increased virulence in certain host-parasite systems (Aeby *et al.*, 2010; Maynard *et al.*, 2015; Hutchins *et al.*, 2019). For example, the coral bacteria *Vibrio coralliilyticus* has been shown to have increased virulence at warmer temperatures, leading to elevated coral mortality (Ben-Haim *et al.*, 2003). In addition to changes in virulence, distributions of pathogens are expected to shift with global warming (Harvell *et al.*, 2009), leading to altered co-occurrence of parasites and their hosts, and it will thus become critical to understand how coexistence between co-infecting parasites changes with ongoing global warming.

Rising constant temperatures, increases in temperature variability, interactions between temperature and nutrient regimes as well as interplay between temperature and multiple infection may have a myriad of outcomes with potentially far-reaching effects. Warming-induced outbreaks of disease may cause species declines or even extinctions, with cascading effects across trophic levels (Hughes *et al.*, 2002; Mouritsen, Tompkins and Poulin, 2005; Burge *et al.*, 2014). Similarly, eutrophication mediated by nitrogen and phosphorus enrichment caused an increase in amphibian infections by the flatworm *Ribeiroia ondatrae*, which led to widespread amphibian population declines (Johnson *et al.*, 2007). Moreover, shifts in virulence induced by multiple infection can affect disease dynamics (van Baalen and Sabelis, 1995) or lead to host population extinction (Rafaluk *et al.*, 2015). The combined impacts of these factors may have important repercussions for disease outbreak and spread. Overall, the question remains of how global warming and nutrient pollution impact infection and if predictions based on single infections will be valid in slowly warming natural systems where multiple infections dominate.

## **1.6 Research outline**

In this thesis, I examine how ecological factors, such as temperature and nutrient levels, as well as multiple infection, impact disease in *Daphnia magna*, to decipher whether we can make

predictions regarding the impacts of global change on disease outbreaks. Through its first three chapters, this thesis aims to determine the relationship between temperature (mean and variance) and parasitism in the aquatic invertebrate *Daphnia magna*, investigate the combined impacts of temperature and nutrient levels, and understand how multiple infection affects *D. magna* (both in individuals and in larger populations), before focusing on the combined effects of an ecological stressor (temperature) and multiple infection in the final chapter.

## **Chapter 2: Differing thermal ranges of three *Daphnia* parasites do not allow for predictions of their response to fluctuating temperatures and heatwaves**

Climate change is impacting the spread and outbreak of disease, not only through increases in constant temperatures, but also due to increased temperature variability and extreme weather events such as heatwaves. Despite a growing number of studies investigating the effects of changing temperatures on disease dynamics, it remains unclear whether predictions can be generalised or whether the response to temperature is host-parasite specific. Here, I investigate the impact of increases in constant temperatures, fluctuating temperatures and heatwaves on three of *D. magna*'s parasites (the microsporidians *O. colligata* and *H. tvaerminnensis* and the fungus *M. bicuspidata*). I integrate data on infection rates and spore burden with a Bayesian modelling approach to draw conclusions about the thermal tolerance curves of each of these parasites. Finally, I compare the thermal tolerance of each parasite and their response to fluctuating temperatures and heatwaves to draw conclusions about whether thermal tolerance curves can be used to predict parasites' response to increasing temperature variation.

## **Chapter 3: Low phosphorus levels in combination with moderate temperatures limit infection by a microsporidian parasite in the freshwater invertebrate *Daphnia magna***

Climate change is causing increasing temperatures and modifying nutrient regimes, both of which may alter disease dynamics. However, although these factors are frequently studied

individually, there is a lack of research investigating the combined impacts of these two stressors. Here, I investigate the impacts of rising constant temperatures in combination with increasing levels of phosphorus on *D. magna* and their microsporidian parasite *O. colligata*, in a fully factorial experiment combining seven temperatures and four levels of phosphorus. I integrate infection and spore burden data with a Bayesian modelling approach to predict the thermal tolerance range of *O. colligata* under different phosphorus regimes and investigate how *Daphnia magna* survival is impacted by these two stressors in combination with infection by *O. colligata*. This work allows me to draw conclusions about how changes in temperature and phosphorus regime may impact disease spread in affected organisms.

#### **Chapter 4: Increased virulence due to multiple infection in *Daphnia* leads to limited growth in one of two co-infecting microsporidian parasites**

Recent disease outbreaks highlight the need to better understand the drivers behind the spread and outbreak of disease. However, although much of the experimental work investigating disease dynamics focuses on a single parasite and its host, multiple infections are common in real-world systems and may change the disease dynamics through co-infecting parasite interactions. Furthermore, multiple infection outcomes may be impacted by prior residency, whereby the first parasite to infect may have a competitive advantage over the second. Here, I investigate the outcome of multiple infection in *Daphnia magna* exposed to two microsporidian parasites, *Ordospora colligata* and *Hamiltosporidium tvaerminnensis*, through a fully factorial experiment with simultaneous and sequential infections. This work allows me to infer potential changes in virulence in natural populations where both parasites co-occur, as well as providing a basis for the experimental design of my final chapter (Chapter 5).

#### **Chapter 5: Interactions between microsporidian parasites and temperature modify the outcome of multiple infection at the extremes of the *Daphnia* thermal range**

Climate change is causing increases in temperature which are impacting the outbreak and spread of disease. Much of the research investigating the impacts of rising temperatures has focused on single parasites and their hosts, despite multiple infections being frequent in natural systems. Here, I investigate the impacts of increasing constant temperatures and a heatwave event on multiple infection of *Daphnia magna* by two of its microsporidian parasites (*O. colligata* and *H. tvaerminnensis*). Given that previous work (Chapter 2) has shown that these two parasites have differing thermal tolerance ranges, I anticipate that there may be shifts in the outcome of multiple infection at the extremes of the *Daphnia* thermal tolerance range. This work allows me to add to the few studies which have experimentally investigated the impacts of climate change on multiple infection and shows that disease dynamics in natural populations where these parasites co-occur may shift as a result of increasing temperatures.



## **Chapter 2. Differing thermal ranges of three *Daphnia* parasites do not allow for predictions of their response to fluctuating temperatures and heatwaves**

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**Author contributions:** FEO’K, CVH and PL contributed to the experimental design. FEO’K and PL conducted the experiment with assistance of AB, NMCC and WP. TK wrote the code for Bayesian modelling. FEO’K conducted the final analyses and created the figures. FEO’K wrote the first draft of the manuscript and PL and CVH contributed to revisions. PL and CVH supervised the project.

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### **2.1 Abstract**

Increasing temperatures due to climate change are impacting the spread and outbreak of infectious diseases, with disease outbreaks frequently predicted to increase under warming temperatures. In addition to increases in constant temperatures, global change is also increasing temperature variability and extreme weather events such as heatwaves, which may further modify disease dynamics. Here, we investigate the impacts of rising constant temperatures, fluctuating temperatures, and heatwaves across the thermal range of *Daphnia magna* (between

10.5 and 31°C) and three of their parasites: the microsporidians *Ordospora colligata* and *Hamiltosporidium tvaerminnensis*, and the fungus *Metschnikowia bicuspidata*. We find that these parasites have differing thermal tolerance curves, and that thermal performance does not necessarily allow for predictions of their response to heatwaves and fluctuating temperatures. Indeed, *O. colligata*, while having intermediate thermal tolerance, was most impacted by heatwaves and fluctuating temperatures, as heatwaves decreased the optimal and maximal temperatures for spore growth and fluctuating temperatures decreased the optimal temperature for spore growth and the number of spores produced at this optimum. In contrast, *H. tvaerminnensis* had the widest temperature tolerance and was predicted to produce a higher number of spores when exposed to a heatwave. Finally, *M. bicuspidata*, despite having the smallest temperature tolerance range, had infection rates which were unaffected by exposure to fluctuating temperatures or heatwaves and produced a similar number of spores across the temperature range, regardless of temperature regime. We conclude that parasite responses to increasing temperatures and temperature variability are system-specific, leading to difficulties in predicting the specifics of changing disease dynamics due to climate change.

## 2.2 Introduction

Rising temperatures due to human-induced global change are modifying the effects of infectious diseases (Patz *et al.*, 2005), which may have far-reaching consequences impacting human and animal health (Hilgenfeld and Peiris, 2013), agriculture (Chakraborty and Newton, 2011) and biodiversity (Bates, Hilton and Harley, 2009). Numerous studies have reported that increasing temperatures will lead to higher levels of parasitic infection (Stenseth *et al.*, 2006; Burge *et al.*, 2014; Kunze *et al.*, 2022) and larger epidemics (Auld and Brand, 2017), although some research suggests the opposite may also be true (Stireman III *et al.*, 2005; Karvonen *et al.*, 2010; Gehman, Hall and Byers, 2018). Parasite traits such as transmission and within-host growth can increase under scenarios of rising temperatures (Ben-Horin, Lenihan and Lafferty, 2013; Labaude, Rigaud

and Cézilly, 2015; Kirk *et al.*, 2018), while host traits such as survival and fecundity may decrease with increasing temperatures (Altizer *et al.*, 2013; Green *et al.*, 2019). However, changes in patterns of infection due to rising temperatures are likely to be complex, as temperature may differentially affect individual host and parasite life-history traits (Lafferty and Holt, 2003). For example, in the trematode *Ribeiroia ondatrae* and its amphibian host, increasing temperatures led to higher levels of parasite penetration, but reduced establishment of the parasite within the host, therefore leading to no overall differences in parasite success in warmer temperatures (Paull, LaFonte and Johnson, 2012). The complexity of the outcome of disease under increasing temperatures remains a central issue, requiring more research to better predict how climate change will impact disease (Hay *et al.*, 2002; Patz *et al.*, 2005; Lafferty, 2009; Rohr *et al.*, 2011). Furthermore, despite many studies investigating the impacts of rising constant temperatures on disease outbreaks, climate change is also increasing temperature variability through increased temperature fluctuations (Easterling *et al.*, 2000; Thornton *et al.*, 2014) and extreme weather events such as heatwaves (Meehl and Tebaldi, 2004; Schär *et al.*, 2004; Harris *et al.*, 2018), which may further complicate the outcome of infection.

Increasing temperature variability due to global warming may have more of an impact on fitness traits than rising mean temperatures alone (Vasseur *et al.*, 2014). For example, biodiversity losses in amphibian species attributed to outbreaks of the fungal pathogen *Batrachochytrium dendrobatidis* have been linked to increased temperature variability due to El Niño events (Rohr and Raffel, 2010). Temperature variability may be responsible for shifts in disease patterns (Rohr *et al.*, 2013), either through increasing (Zhou *et al.*, 2004; Raffel *et al.*, 2006; Stenseth *et al.*, 2006) or decreasing (Stireman III *et al.*, 2005; Dallas and Drake, 2016) disease outbreaks, and temperature fluctuations play a role in initiating disease outbreaks (Zhou *et al.*, 2004). For instance, daily fluctuations in temperature drive malaria transmission intensity (Paaijmans *et al.*, 2010), while increased temperature fluctuations led to reduced infection prevalence of the

fungus *Metschnikowia bicuspidata* in its *Daphnia* host (Dallas and Drake, 2016). Furthermore, temperature fluctuations in models examining malaria outbreaks were found to reduce the effect of increases in mean temperature, suggesting that models which ignore temperature fluctuations may be under- or overestimating the risks of disease (Paaijmans, Read and Thomas, 2009).

In addition to temperature fluctuations, it is expected that climate change will increase the number of extreme weather events such as heatwaves (Meehl and Tebaldi, 2004; Schär *et al.*, 2004). Heatwaves may cause increased levels of disease (Harvell *et al.*, 2019), although their specific impacts may depend on the duration, intensity and starting temperature of the heatwave (Landis *et al.*, 2012; Schreven *et al.*, 2017; Zhang *et al.*, 2019a; Abarca, Larsen and Ries, 2019), and the life-history traits investigated (Kunze *et al.*, 2022). For example, a 6°C heatwave increased spore burden almost tenfold in *Daphnia magna* infected with the microsporidian *Ordospora colligata*, but had no impact on infection rates in the same system (Kunze *et al.*, 2022). Given how variable different host-parasite systems' sensitivity to temperature may be and the complexity of a multitude of interacting host and parasite life-history traits, our ability to predict the response of diseases and parasites to altered temperature fluctuations and heatwave events may be limited, particularly if responses are system specific.

Here, we investigate the impacts of changes in constant temperatures, fluctuating temperatures, and heatwaves on the freshwater invertebrate *Daphnia magna* and three of its parasites (the fungus *Metschnikowia bicuspidata* and the microsporidians *Hamiltosporidium tvaerminnensis* and *Ordospora colligata*) over a large thermal range. These three parasites have distinct modes of transmission, with *M. bicuspidata* being horizontally transmitted upon death of the host, *O. colligata* continuously and horizontally transmitted from living hosts, and *H.*

*tvaerminnensis* both vertically transmitted, and horizontally transmitted upon death of the host. Furthermore, all three parasites infect different areas of the host body, as *H. tvaerminnensis* infects the fat and ovarian cells, *O. colligata* the gut epithelium and *M. bicuspidata* the body cavity of the host. The differences between these parasites may cause varying responses to temperature variability. Previous research in this system has found that *O. colligata* infection may increase under conditions of rising temperatures or increased temperature variation ((Kirk *et al.*, 2018; Kunze *et al.*, 2022; Krichel *et al.*, 2023), while both *H. tvaerminnensis* and *M. bicuspidata* infections were negatively impacted by increasing temperatures (Dallas and Drake, 2016; Santos and Ebert, 2022). Here, we combine experimental work with Bayesian modelling to examine the response of these three parasites to constant temperatures, fluctuations in temperature and heatwave events over the full thermal range, allowing us to draw conclusions on the similarities and differences between these parasites and determine if responses are system specific or can be generalised.

## 2.3 Materials and methods

### 2.3.1 Study system

*Daphnia magna* are a widely studied model system often used in ecological and evolutionary studies (Ebert, 2005) due to their ease of use in the laboratory (Ebert, 2022), and are ubiquitous throughout the Northern Hemisphere where they encounter a range of environmental conditions (Lange, Kaufmann and Ebert, 2015). In addition to their ecological importance as a keystone species in freshwater ecosystems (Altshuler *et al.*, 2011; Jansen *et al.*, 2017), they are also a well-established system used to investigate host-parasite interactions as a range of parasites can infect these animals under natural and laboratory conditions (Ebert, 2005). Among these parasites are the fungus *Metschnikowia bicuspidata*, and the microsporidia *Ordospora colligata* and *Hamiltosporidium tvaerminnensis*. *M. bicuspidata* is a fungus which is transmitted

horizontally when spores are released from dead hosts and ingested through filter feeding (Ebert, Lipsitch and Mangin, 2000). Spores are found in the body cavity of infected animals (Ebert, 2005), and infected hosts experience reduced survival and fecundity (Ebert, Lipsitch and Mangin, 2000; Ebert, Zschokke-Rohringer and Carius, 2000). *O. colligata* is a microsporidian parasite that is transmitted horizontally from living hosts (Ebert, Lipsitch and Mangin, 2000) and produces spore clusters which invade the host gut epithelium (Larsson, Ebert and Vavra, 1997). In comparison to other *Daphnia* parasites, *O. colligata* is relatively avirulent, as infection only slightly reduces host fecundity and survival (Ebert, Lipsitch and Mangin, 2000) although recent observations suggest that under some circumstances it may be more virulent (O'Keeffe *et al.*, 2024). Finally, *H. tvaerminnensis* is a microsporidian parasite which infects the fat cells and ovaries of the host (Jirovec, 1936) through vertical transmission, and horizontal transmission upon death of the host (Vizoso, Lass and Ebert, 2005). Similarly to *O. colligata* infections, survival and fecundity of infected hosts are only slightly reduced as *H. tvaerminnensis* is relatively avirulent (Ebert, 2005; Narr and Frost, 2016). The host and parasite isolates used in this study were originally collected from the Tvärminne Archipelago in Finland (*D. magna* genotype Fi-Oer-3-3, *O. colligata* isolate OC3 and *H. tvaerminnensis*) and from the United States of America (*M. bicuspidata*, US-Connecticut Valley Biological Supply).

### **2.3.2 Experimental procedure**

To measure the thermal performance of each of the three parasites (*M. bicuspidata*, *O. colligata* and *H. tvaerminnensis*), we exposed individual juvenile female *D. magna* to each parasite or a placebo, and combined this with three temperature regimes (constant, fluctuating and heatwave) over a range of temperatures between 11°C and 32.5°C (see Figure 2.1 for an illustration of the experimental set up). . Each treatment combination had 18 replicates, which yielded a total of 1980 animals (5 parasite treatments (*H. tvaerminnensis*, *O. colligata*, *M. bicuspidata* and 2 sets of controls (1 set for each experimental block)) x 22 temperature

treatments (8 constant, 7 fluctuating and 7 heatwave) x 18 replicates; see Table 2.1 for temperatures). The study was run in two experimental blocks with identical experimental procedures (with *M. bicuspidata* exposure in the first block, and *O. colligata* and *H. tvaerminnensis* exposures in the second block).

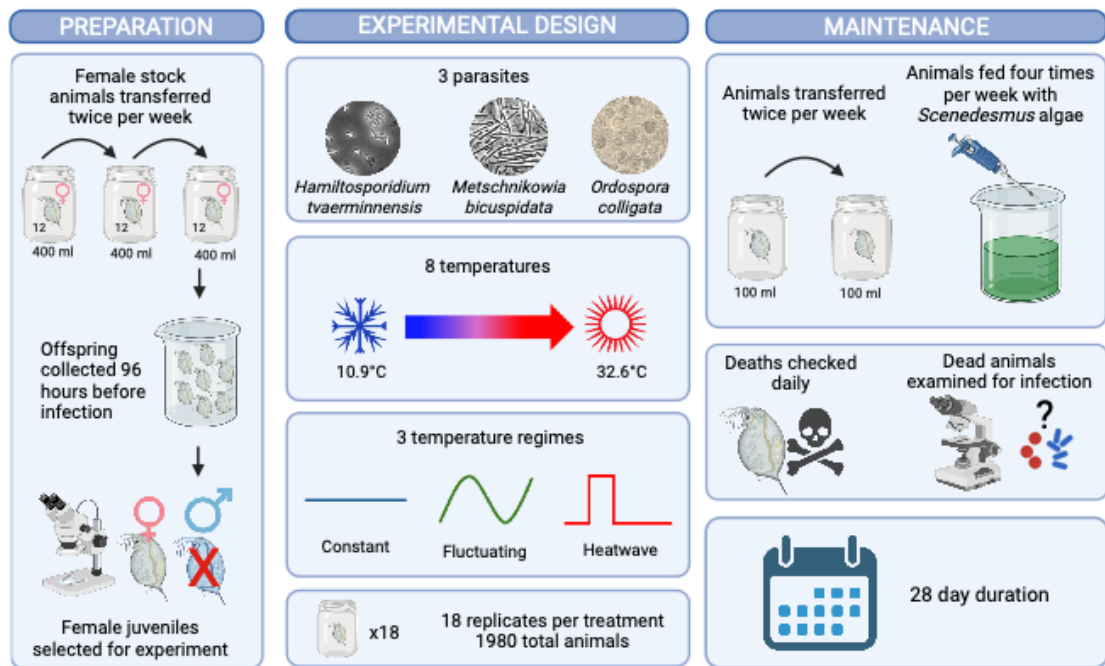


**Figure 2.1.** Illustration of the experimental set up. The left panel shows the preparation and generation of the juveniles used in the experiment, carried out between 28 and 4 days before infection. The central panel shows the experimental design (3 parasites x 8 temperatures x 3 temperature regimes, with 18 replicates per treatment for a total of 1980 animals). The right panel shows the maintenance and measurements taken during the experiment which lasted for 28 days. Figure created on Biorender.com.

(a) Host preparation

To obtain sufficient juvenile females for both experimental blocks and minimise maternal effects, ~600 *D. magna* adult females from clonal line Fi-Oer-3-3 were grown under standardised

conditions for four weeks in advance of each experimental block (10-12 female animals per 400 ml microcosm with 200 ml of artificial *Daphnia* medium ADaM (Ebert, Zschokke-Rohringer and Carius, 1998), transferred to a new microcosm with fresh medium twice per week, kept at 20°C with continuous light and fed *ad libitum* with batch cultured *Scenedesmus* sp. algae). Animals were transferred to new microcosms 5 days prior to each experimental block to ensure that no juveniles were present. Juveniles born within the next 96 hours (4 days) were collected, sexed (using a dissecting microscope at x8 to x12 magnification), randomly assigned to a treatment, placed individually into a 100 ml microcosm containing 60 ml of ADaM and given 24 hours to acclimatise. Microcosms were organised into trays which were placed into the appropriate waterbath and rotated daily to avoid positioning effects.

#### *(b) Temperature*

In the laboratory, we set up 22 water baths ranging from ~11°C to ~33°C. Water temperature was regulated by a controller (Inkbird ITC-308D or ITC-310T-B) interfacing with aquarium chillers (Hailea HC150A & DC300) and heating rods (EHEIM JÄGER 300W). Water was circulated using microjet pumps (NemaMicro MC450) to equalise the temperature. To test for both the effect of changing mean temperatures as well as changing temperature variation, we included three different temperature regimes: one constant temperature regime, one diurnally fluctuating regime and one heatwave regime. Constant temperatures ranged from 10.9°C to 32.6°C in increments of 3.1°C (Table 2.1). Temperatures in the fluctuating regime were centred around the same mean temperatures as the constant temperature regime, but alternated between a lower and higher temperature with a 5.8°C difference every 12 hours (Table 2.1). Temperatures in the heatwave regime were also centred around the same mean temperatures as the constant temperature regime, but included a lower temperature which was used for most of the experiment and a 7.5°C higher temperature which was used under the heatwave scenario for five days between day 9 and day 14 (Table 2.1). To ensure that results from the three regimes

are comparable to each other, temperatures were chosen such that mean temperatures were consistent across equivalent treatments in the three regimes and that variance was consistent across equivalent treatments in the fluctuating and heatwave regimes.

**Table 2.1.** Mean target temperatures used in each of the treatments in °C, and upper and lower target temperatures for the fluctuating and heatwave treatments. Note that the constant regime has 8 treatments whereas the fluctuating and heatwave regimes only have 7 respectively, as the 32.6°C constant temperature was included to provide a comparison point for the highest fluctuating and heatwave temperatures.

| Constant             | 10.9  | 14    | 17.1  | 20.2  | 23.3  | 26.4  | 29.5  | 32.6 |
|----------------------|-------|-------|-------|-------|-------|-------|-------|------|
| Fluctuating - mean   | 10.9  | 14    | 17.1  | 20.2  | 23.3  | 26.4  | 29.5  |      |
| Fluctuating - lower  | 8     | 11.1  | 14.2  | 17.3  | 20.4  | 23.5  | 26.6  |      |
| Fluctuating - higher | 13.8  | 16.9  | 20    | 23.1  | 26.2  | 29.3  | 32.4  |      |
| Heatwave - mean      | 10.99 | 14.09 | 17.19 | 20.29 | 23.39 | 26.49 | 29.59 |      |
| Heatwave - lower     | 9.65  | 12.75 | 15.85 | 18.95 | 22.05 | 25.15 | 28.25 |      |
| Heatwave - higher    | 17.2  | 20.3  | 23.4  | 26.5  | 29.6  | 32.7  | 35.8  |      |

*(c) Parasite spore preparation and exposure*

Animals were exposed to spore doses of either *M. bicuspidata*, *O. colligata* or *H. tvaerminnensis*, or to a placebo dose. Appropriate spore doses were selected based on previous experiments and pilot studies using these host-parasite combinations (see Auld, Hall and Duffy (2012) and Cuco et al. (2020) for *M. bicuspidata*; Kunze et al. (2022) and Kirk et al. (2018) for *O. colligata*; and Visozo, Lass and Ebert (2005) and Krebs, Routtu and Ebert (2017) for *H. tvaerminnensis*). *M. bicuspidata* and *H. tvaerminnensis* spore doses were prepared by crushing infected individuals of each of these three parasites respectively, counting the resulting spore suspension using a haemocytometer (Neubauer improved) and phase contrast microscopy and diluting the

suspension to the required spore dose (90,000 spores per ml and 61,000 spores per ml for *M. bicuspidata* and *H. tvaerminnensis* respectively). The *O. colligata* spore dose was prepared by crushing *O. colligata* infected individuals with known prevalence and burden (determined by phase contrast microscopy on a subsample of the stock population) using a mortar and pestle and diluting the resulting spore suspension to the required dose of 64,000 spores per ml. The placebo dose was prepared by crushing uninfected *D. magna* (of the Fi-Oer-3-3 strain used in this experiment) and diluting the resulting suspension in a similar manner to the spore doses. On infection day (day 0 of the experiment), individual animals were exposed to 1 ml of the appropriate spore dose or placebo, depending on the treatment to which they were assigned. Animals were exposed for 7 days, after which they were transferred to new microcosms with fresh ADaM.

#### *(d) Experimental maintenance*

Animals were kept under a 16:8 light-dark photoperiod and were transferred to new microcosms with fresh ADaM every four days (after the initial exposure period between day 0 and day 7) to avoid build-up of waste products and offspring produced during the experiment. Each animal was fed with 1 ml of batch cultured *Scenedesmus* algae grown in WC medium (Kilham *et al.*, 1998) every two days during the experiment, with the density of algae increasing in increments from five million per ml to 12 million per ml in week one, then remaining constant at 12 million per ml for the remainder of the experiment. Animals were followed until natural death or until the end of the experiment (day 28).

#### **2.3.3 Measurements**

Animals were checked for mortality daily, and any animals that died were subsequently examined for infection. Infection status was checked from day 8 onwards as any earlier infections would not have been detectable. *M. bicuspidata* infections were determined by

crushing animals in 1 ml of water and examining a 50 µl aliquot of each sample on a haemocytometer using phase contrast microscopy at x400 magnification. Similarly, *H. tvaerminnensis* infection status was examined by crushing animals in 100 µl of water on a slide and examining 12.5 µl of this liquid on a haemocytometer using phase contrast microscopy at x400 magnification. *O. colligata* infection status was determined by dissecting animals using a stereo microscope and counting the number of spore clusters present in the gut of the animal (as *O. colligata* is known to form clusters of up to 64 spores) using phase contrast microscopy at x400 magnification.

### 2.3.4 Statistical analyses

Statistical analyses were performed using R (version 4.2.2) interfacing with Stan for Bayesian analyses. Parasite infection rates were calculated using only animals which died after day 14, as infection could not be reliably confirmed prior to this date. Similarly, only animals which died in the last 7 days of the experiment were used to examine spore burden data, to ensure that spore burdens were comparable across treatments. Realised temperatures were used when analysing parasite fitness (infection rates and spore burden).

Bayesian analyses were chosen to examine parasite infection rates and spore burden data, as, in addition to allowing us to fit our data to a model, this approach allowed us to predict values for each of the model parameters with estimates of uncertainty for each prediction. Parasite infection rates and spore burden were analysed by fitting our data to a Beta Function for each temperature and parasite treatment, as

$$res = c_m \left( \frac{t_{max} - t}{t_{max} - t_{opt}} \right) \left( \frac{t - t_{min}}{t_{opt} - t_{min}} \right)^{\frac{t_{opt} - t_{min}}{t_{max} - t_{opt}}}$$

where *res* is proportion infected or spore burden at temperature *t*, *c<sub>m</sub>* is the probability of infection based on temperature, *t<sub>opt</sub>* is temperature at optimal performance, and *t<sub>min</sub>* and *t<sub>max</sub>*

are the critical minimum and maximum temperatures where fitness of the trait becomes unviable, respectively. The Beta function was chosen as it has been shown to accurately represent thermal performance (Niehaus *et al.*, 2012) and parameter estimates from the equation have biological meaning which can be easily interpreted. We used a binomial distribution to evaluate the effect of temperature and food quality treatment on parasite infectivity (infection rates), and a Poisson distribution to assess their effect on spore burden, and fitted the model in a Bayesian framework with Stan using the MCMC fitting algorithm called from R. Priors for temperature were chosen based on previous research (Kirk *et al.*, 2018; Kunze *et al.*, 2022) and satisfied the condition  $t_{min} \leq t_{opt} \leq t_{max}$  (see Table A1 for a list of priors used). The posterior distribution of all parameters was estimated using four chains, and the number of warmup and post-warmup iterations differed depending on which parameter was being fitted (see Table A2 for a list of the number of iterations used). Model fit was checked through MCMC divergence, MCMC convergence (R-hat values) and effective samples.

*M. bicuspidata* spore burden could not be fitted through Bayesian analysis as data collected did not conform to the Beta function used to fit all other response variables. Therefore, *M. bicuspidata* spore burden was assessed by means of a negative binomial generalised linear model (GLM) which was compared to the null model using the  $\chi^2$  distribution, i.e. spore burden  $\sim$  temperature\*temperature regime.

## 2.4 Results

### 2.4.1 *H. tvaerminnensis* response to temperature

The highest rates of *H. tvaerminnensis* infection were observed at intermediate temperatures (between 17.5 and 22.5°C) and were similar across all temperature regimes, regardless of whether animals were exposed to constant temperatures, fluctuating temperatures, or a

heatwave (Figure 2.2, Panels 1A, 1C and 1E; confidence intervals of  $t_{opt}$  and  $c_m$  overlap for all temperature regimes). Infection rates dropped at extremes of the range (between ~6 and 11°C for minimum temperature and ~28 to 37°C for maximum temperature), and minimum and maximum temperatures for infection were unaffected by temperature regime (Figure 2.2, Panels 1A, 1B and 1D; confidence intervals of  $t_{min}$  and  $t_{max}$  overlap for all temperature regimes). In comparison, the highest spore burden observed in *H. tvaerminnensis* infected animals was seen at higher temperatures, with optimum temperatures predicted to be between 24.5 and 27°C (Figure 2.2, Panels 2A and 2C). Furthermore, animals under different temperature regimes had different optimum temperatures and number of spores, with those exposed to a heatwave having the highest optimum temperature and the highest predicted spore burden (Figure 2.2, Panels 2A, 2C and 2E; confidence intervals of  $t_{opt}$  and  $c_m$  do not overlap for all temperature regimes).

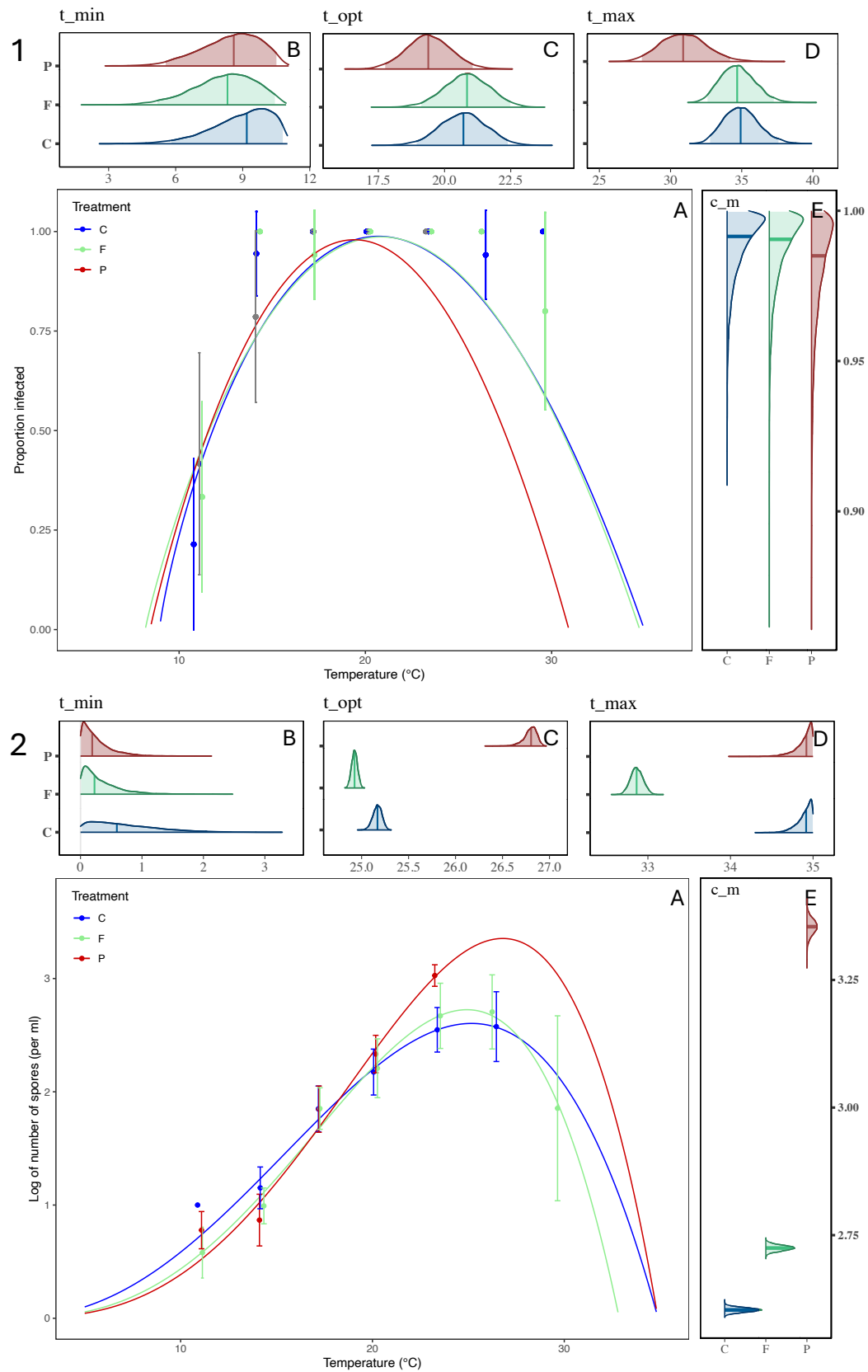


**Figure 2.2.** *H. tvaerminnensis* response to temperature, where panel 1 represents the proportion of animals infected with *H. tvaerminnensis* and panel 2 represents *H. tvaerminnensis*

spore burden. Panels 1A and 2A represent the predicted Beta model fit lines and the datapoints used to fit the model for infection rates and spore burden, respectively. Panels 1B, 1C, 1D and 1E represent the estimations of the parameters of the model for infection rates ( $t_{\min}$ ,  $t_{\text{opt}}$ ,  $t_{\max}$  and  $c_m$  respectively) while panels 2B, 2C, 2D and 2E represent the estimations of the parameters of the model for spore burden ( $t_{\min}$ ,  $t_{\text{opt}}$ ,  $t_{\max}$  and  $c_m$  respectively). For both sets of parameters the shaded area under the curve represents the 95% confidence interval. C, F, and P refer to the constant, fluctuating and heatwave temperature treatments, respectively. Numbers on the x-axis for panels B, C and D refer to temperature for both sets of parameters, while numbers on the y-axis for panels E refer to proportion infected for infection rates (panel 1E) and log of number of spores for spore burden (panel 2E).

#### **2.4.2 *M. bicuspidata* response to temperature**

*M. bicuspidata* infection rates were highest in moderate temperatures (between 17.5 to 22.5°C), and were sensitive to extremely high or low temperatures, with infection rates falling to zero at the extremes of the range (Figure 2.3, Panel 1A). Temperature regime did not affect infection rates, and minimum, maximum and optimum temperatures for infection remained similar regardless of whether animals were exposed to constant temperatures, fluctuating temperatures or heatwaves (Figure 2.3, Panels 1B-D; confidence intervals of  $t_{\min}$ ,  $t_{\text{opt}}$ ,  $t_{\max}$  and  $c_m$  overlap for all temperature regimes). Contrary to infection rates, spore burden of *M. bicuspidata* infected animals did not appear sensitive to temperature, as the number of spores in infected animals remained similar across the temperature range, regardless of which temperature regime animals were exposed to (Figure 2.3, Panel 2; GLM, analysis of deviance,  $df = 55$ ,  $p = 0.784$  and  $df = 53$ ,  $p = 0.564$ , for temperature and temperature regime, respectively).

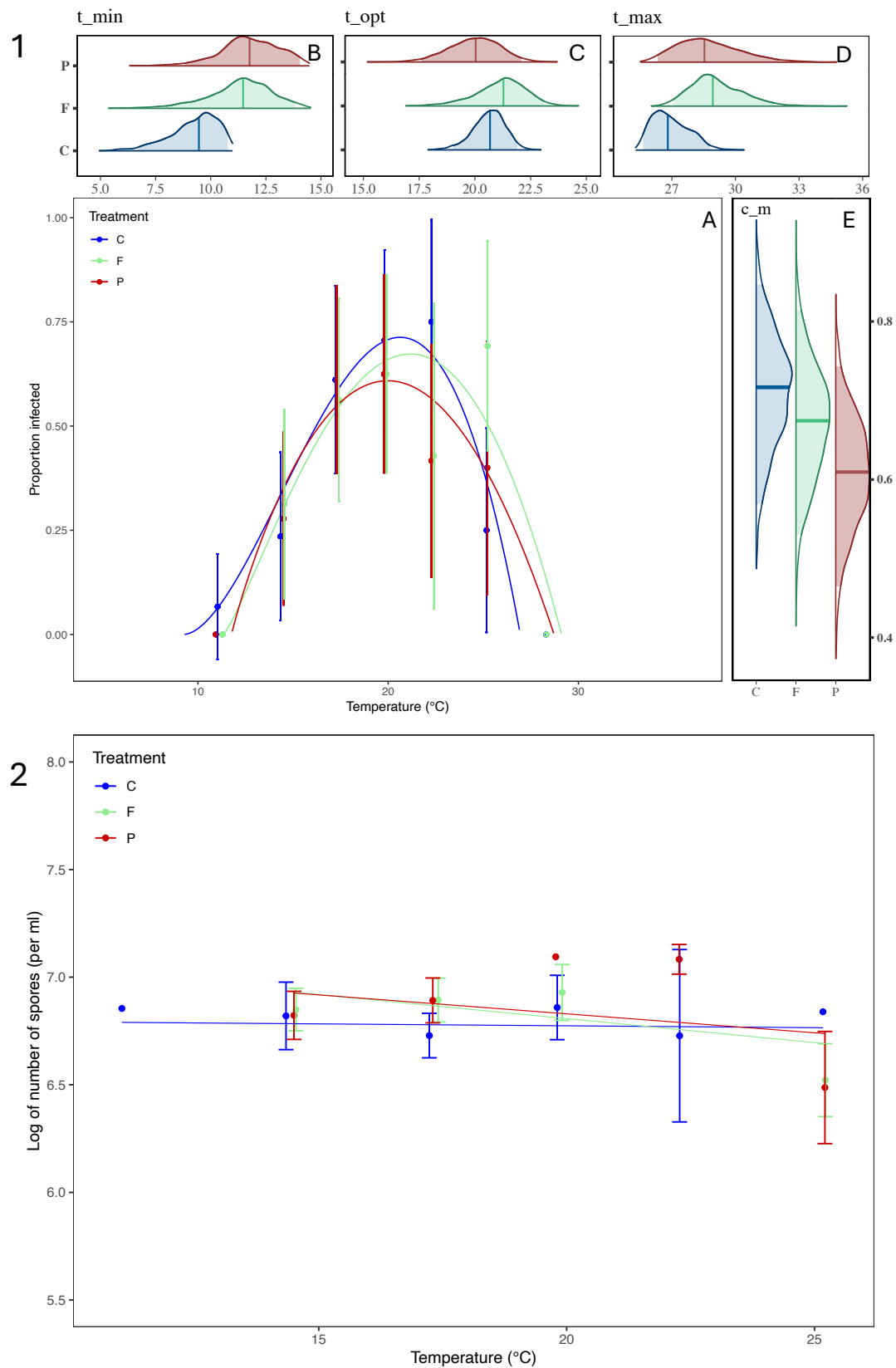


**Figure 2.3.** *M. bicuspidata* response to temperature, where panel 1 represents the proportion of animals infected with *M. bicuspidata* and panel 2 represents *M. bicuspidata* spore burden.

Panel 1A represents the predicted Beta model fit lines and the datapoints used to fit the model for infection rates and spore burden, respectively. Panels 1B, 1C, 1D and 1E represent the estimations of the parameters of the model for infection rates ( $t_{\min}$ ,  $t_{\text{opt}}$ ,  $t_{\max}$  and  $c_m$  respectively) where the shaded area under the curve is the 95% confidence interval. Panel 2 represents spore burden in log of number of spores per ml. Error bars represent standard deviation and lines represent the GLM model fit for panel 2 (fitting the beta function to these data would not be justified). C, F, and P refer to the constant, fluctuating and heatwave temperature treatments, respectively. Numbers on the x-axis for panels B, C and D refer to temperature, while numbers on the y-axis for panels E refer to proportion infected.

#### **2.4.3 *O. colligata* response to temperature**

*O. colligata* infection rates were highest between 18 and 22°C, regardless of whether animals were exposed to constant temperatures, fluctuating temperatures, or a heatwave (Figure 2.4, Panels 1A, 1C and 1E; confidence intervals for  $t_{\text{opt}}$  and  $c_m$  overlap for all temperature regimes). Infection rates dropped to zero at the extremes of the temperature range, but while minimum temperatures were similar for all temperature regimes (Figure 2.4, Panels 1A and 1B; confidence intervals for  $t_{\min}$  overlap for all temperature regimes), animals exposed to a heatwave or to fluctuating temperatures had a lower maximum temperature than those under constant temperatures (Figure 2.4, Panels 1A and 1D; confidence intervals for  $t_{\text{opt}}$  of heatwave and fluctuating temperature regimes do not overlap with those of constant temperature regime). Spore burden of animals infected with *O. colligata* was highly sensitive to changes in temperature (Figure 2.4, Panels 2A-E). Exposure to a heatwave reduced the optimum and maximum temperatures for *O. colligata* spore burden when compared to constant temperatures (Figure 2.4, Panels 2A, 2C and 2D; confidence intervals for  $t_{\text{opt}}$  and  $t_{\max}$  of heatwave temperature regime do not overlap with those of constant temperature regime), although the number of spores produced at the optimum did not differ between heatwave and constant temperature

regimes (Figure 2.4, Panels 2A and 2E; confidence intervals for  $c_m$  of heatwave and constant temperature regimes overlap). Spore burden of animals under fluctuating temperatures had a lower optimum temperature than those in the constant temperature regime, and the number of spores produced at the optimum was lower under fluctuating temperatures (Figure 2.4, Panels 2A and 2C; confidence intervals for  $t_{opt}$  and  $c_m$  of fluctuating temperature regime do not overlap with those of constant temperature regime).

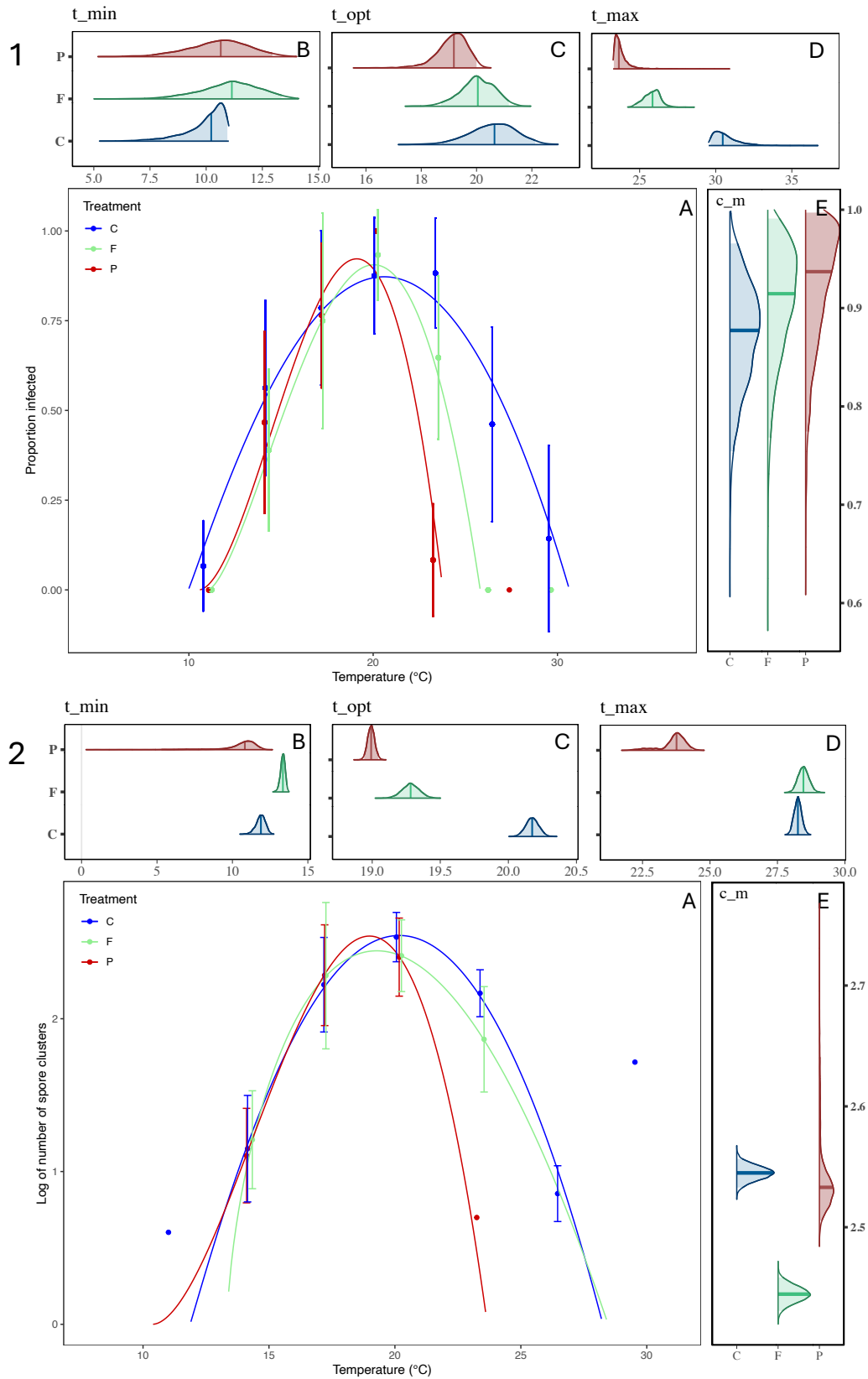


**Figure 2.4.** *O. colligata* response to temperature, where panel 1 represents the proportion of animals infected with *O. colligata* and panel 2 represents *O. colligata* spore burden. Panels 1A and 2A represent the predicted Beta model fit lines and the datapoints used to fit the model for

infection rates and spore burden, respectively. Panels 1B, 1C, 1D and 1E represent the estimations of the parameters of the model for infection rates ( $t_{\min}$ ,  $t_{\text{opt}}$ ,  $t_{\max}$  and  $c_m$  respectively) while panels 2B, 2C, 2D and 2E represent the estimations of the parameters of the model for spore burden ( $t_{\min}$ ,  $t_{\text{opt}}$ ,  $t_{\max}$  and  $c_m$  respectively). For both sets of parameters the shaded area under the curve represents the 95% confidence interval. C, F, and P refer to the constant, fluctuating and heatwave temperature treatments, respectively. Numbers on the x-axis for panels B, C and D refer to temperature for both sets of parameters, while numbers on the y-axis for panels E refer to proportion infected for infection rates (panel 1E) and log of number of spores for spore burden (panel 2E).

#### **2.4.4 Comparison of all parasite responses**

Parasite responses to temperature were not consistent across the three parasites examined (Figure 2.5A and B, Table 2.2). *H. tvaerminnensis* has the largest temperature tolerance range, both in terms of infection rates and spore burden (Figure 2.5A and B), and was mostly unaffected by fluctuating temperatures or heatwaves, although *H. tvaerminnensis* spore burden at optimum temperatures increased when animals were exposed to a heatwave. In contrast to *H. tvaerminnensis*, *M. bicuspidata* had the smallest temperature tolerance range with regard to infection rates (Figure 2.5A), but neither infection rates nor spore burden were impacted by heatwaves or fluctuating temperatures (Figure 2.5A and B). Furthermore, despite the small temperature tolerance range observed for infection rates, *M. bicuspidata* infected animals produced similar numbers of spores across the temperature range and did not appear to have a true optimum (Figure 2.5B). Finally, *O. colligata* appeared to have intermediate temperature tolerance but was the most impacted by exposure to heatwaves and fluctuating temperatures. Exposure to a heatwave decreased the maximum temperature for *O. colligata* infection, and decreased the optimum and maximum temperatures for spore burden, while fluctuating temperatures decreased the maximum temperature for infection, and decreased the optimum

temperature and the number of spores produced at the optimum for spore burden (Figure 2.5A and B).

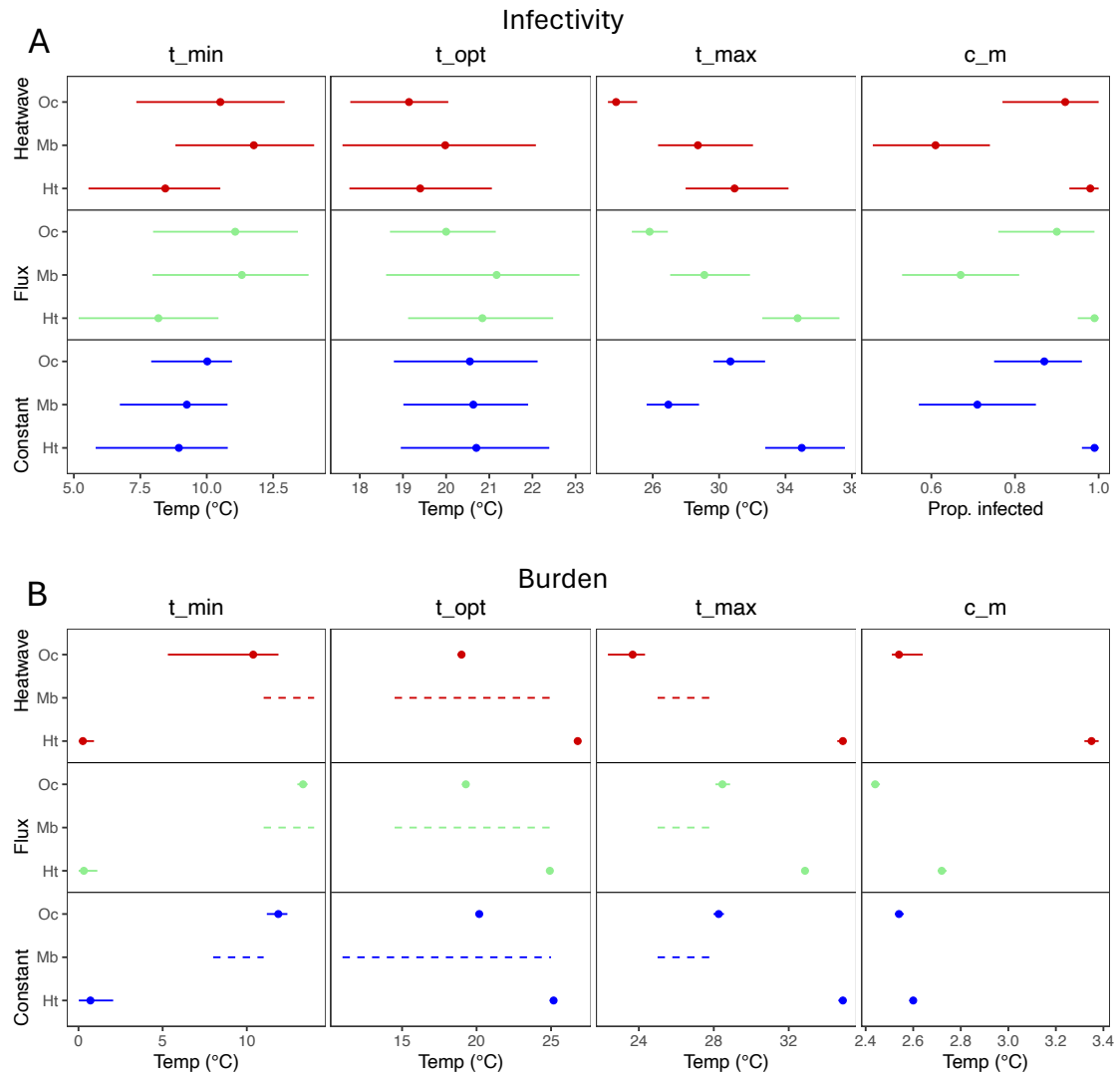


**Figure 2.5.** Comparisons of parameter estimates ( $t_{min}$ ,  $t_{opt}$ ,  $t_{max}$  and  $c_m$ ) for all parasites (*H. tvaerminnensis*, *O. colligata* and *M. bicuspidata*) and temperature regimes (constant, fluctuating and heatwave). Panel A shows comparisons for infection rates and panel B shows comparisons for spore burden. Solid lines represent 95% confidence intervals for parameter estimates, and points represent mean parameter estimates. Dotted lines for *M. bicuspidata* spore burden show uncertainty regarding estimates for this parasite as the data did not fit a Beta function.

**Table 2.2.** Comparisons of the range of each of the three parasites (*H. tvaerminnensis*, *M. bicuspidata* and *O. colligata*) based on the parameter estimates presented in Figure 4.

| Response           | Parasite                 | Result                                                                                                                    |
|--------------------|--------------------------|---------------------------------------------------------------------------------------------------------------------------|
| <b>Infectivity</b> | <i>H. tvaerminnensis</i> | Largest range, unaffected by fluctuating temperatures or heatwave.                                                        |
|                    | <i>M. bicuspidata</i>    | Smallest range, but unaffected by fluctuating temperatures or heatwave.                                                   |
|                    | <i>O. colligata</i>      | Intermediate range, but most affected by fluctuating temperatures and heatwave.                                           |
| <b>Burden</b>      | <i>H. tvaerminnensis</i> | Largest range, relatively unaffected by temperature.                                                                      |
|                    | <i>M. bicuspidata</i>    | Smallest range, but unaffected by temperature across the range.                                                           |
|                    | <i>O. colligata</i>      | Intermediate range, but burden most affected by fluctuating temperatures and heatwave, although less so than infectivity. |

## 2.5 Discussion

Temperature responses were strongly dependent on the identity of the parasite, and temperature variability (i.e. fluctuating temperatures and heatwaves) had differential effects depending on the parasite. *H. tvaerminnensis* infection rates were highest at intermediate temperatures (between 17.5 and 22.5°C) and dropped at temperature extremes regardless of temperature regime, but had the largest temperature tolerance range of the three parasites examined here. *H. tvaerminnensis* has previously been observed to have reduced persistence in populations exposed to a warmer temperature, but this was dependent on the genotype of the host population (Santos and Ebert, 2022). In contrast to *H. tvaerminnensis* infection rates, which were unaffected by fluctuating temperatures and heatwaves, the maximum and optimum temperatures for spore growth were reduced when animals were exposed to fluctuating temperatures, while exposure to a heatwave increased the optimum temperature for spore growth and the number of spores produced. Fluctuating temperatures and heatwaves can alter

parasite performance (Stireman III *et al.*, 2005; Paaijmans *et al.*, 2010; Zhang *et al.*, 2019a), and notably, temperature variation can play a role in initiating outbreaks of malaria in certain parts of Africa (Zhou *et al.*, 2004). Indeed, similarly to *H. tvaerminnensis*, *O. colligata* was affected by temperature fluctuations and heatwaves.

*O. colligata* was impacted by changes in temperature through fluctuating temperatures and heatwaves, but was also more sensitive to changes in constant temperature than *H. tvaerminnensis* given its narrower temperature tolerance. Previous studies have demonstrated the sensitivity of *O. colligata* to both increases in constant temperatures (Kirk *et al.*, 2018) and changes in temperature through fluctuating temperatures and heatwaves (Kunze *et al.*, 2022). Here, we find that animals exposed to fluctuating temperatures had a lower optimum temperature for spore growth and produced fewer spores at the optimum and similarly, that exposure to a heatwave reduced the range over which *O. colligata* could grow. However, contrary to what we observed in the present study, previous studies have suggested that fluctuating temperatures may increase infection prevalence (Krichel *et al.*, 2023), or that spore burden may be unaffected by fluctuating temperatures but may be dramatically increased under heatwave conditions (Kunze *et al.*, 2022). Indeed, results may be dependent on the intensity, duration, and starting temperatures of heatwave events or the type of temperature fluctuations (Dallas and Drake, 2016; Zhang *et al.*, 2019a) and may be specific to the parasite. However, while some parasites are strongly affected by temperature, others may have similar performances regardless of temperature variability.

*M. bicuspidata* spore burden was not affected by temperature. Specifically, despite having a relatively narrow thermal tolerance range for infection rates, *M. bicuspidata* spore burden was unaffected by heatwaves or temperature fluctuations, and infected animals produced a similar number of spores across the thermal range, which mimics results observed in a previous study

(Dallas and Drake, 2016). Similar insensitivity to temperature has been found in the trematode *Gynaecotyla adunca*, where the emergence of infectious stages from its first intermediate host (the mud snail *Ilyanassa obsoleta*) and infection prevalence and intensity in its second intermediate host (the amphipod *Corophium volutator*) were unaffected by increases in temperature (Shim, Koprivnikar and Forbes, 2013). In certain host-parasite systems, the host may be a limiting factor in terms of its response to temperatures, with parasite success being reduced as a consequence of host life-history trait responses to temperature (Studer, Thieltges and Poulin, 2010; Dallas and Drake, 2016). Alternatively, multiple temperature-sensitive life-history traits may act in opposite directions, making the outcome of disease difficult to predict (Lafferty and Holt, 2003). For example, as previously mentioned, increasing temperatures in an amphibian-trematode system increased host penetration of the parasite, but decreases in parasite establishment and survival led to no difference in parasite burden overall (Paull, LaFonte and Johnson, 2012). Overall, each of the three *Daphnia* parasites *H. tvaerminnensis*, *O. colligata* and *M. bicuspidata* studied here showed differential responses to both constant and fluctuating temperatures, suggesting that parasite and disease responses to global change may be system-specific.

Differing parasite thermal ranges may not allow for generalisations regarding the responses of the parasites to fluctuating temperatures and heatwaves. Indeed, despite *M. bicuspidata* having the narrowest thermal range, both infection rates and spore burden of this parasite were unaffected by fluctuating temperatures and heatwaves, while *O. colligata* was the most impacted by fluctuating temperatures and heatwaves of the three parasites despite having an intermediate thermal range. Host thermal ranges may contract under conditions of stress from pathogen infection (Sherman, 2008; Greenspan *et al.*, 2017; Porras *et al.*, 2021), but the degree to which the range may be reduced depends on host and pathogen genotype combinations (Hector, Sgrò and Hall, 2019). Although evaluating thermal performance curves and using

metabolic theory may allow us to draw predictions about organisms' response to climate change (Deutsch *et al.*, 2008; Levy *et al.*, 2015; Kirk *et al.*, 2018), there will likely be limitations given that estimating fitness parameters under variable climates may be complex (Sinclair *et al.*, 2016; Kingsolver and Buckley, 2017) and the application of metabolic theory to fluctuating temperatures is controversial (Marshall and McQuaid, 2011). In the present study, the breadth of the thermal range of a pathogen did not determine how sensitive it was to fluctuating temperatures or heatwaves. Thermal tolerance may not always be indicative of organisms' responses to heatwaves (Miranda *et al.*, 2019). For example, a study investigating whether gastropod responses to heatwaves could be extrapolated from their thermal tolerance found that an interaction between increases in temperature and increased desiccation caused unpredictable outcomes (Miranda *et al.*, 2019). Our findings suggest that assessing species' thermal performances may not be sufficient to predict their responses to increased temperature variability and extreme weather events associated with climate change.

Parasite infection rates and spore burden did not have the same response to changing temperatures. Spore burden of *O. colligata* and *H. tvaerminnensis* were more sensitive to temperature than infection rates of these parasites, while the opposite was true for *M. bicuspidata*. Life-history traits of hosts and their parasites may be differentially impacted by temperature fluctuations, and some traits may be more sensitive to temperature than others, which may complicate predictions of the outcome of rising temperatures (Lafferty and Holt, 2003; Dell, Pawar and Savage, 2011). In *Daphnia*, infection prevalence of *M. bicuspidata* decreased in response to increasing temperature variability but the number of spores produced in infected animals was unaffected (Dallas and Drake, 2016), while exposure to a heatwave drastically increased the number of *O. colligata* spores despite infection rates remaining unchanged (Kunze *et al.*, 2022). Furthermore, infection with the trematode *Ribeiroia ondatrae* in its' amphibian host was unaffected by temperature, as parasite penetration, survival and

establishment had opposing reactions to temperature which cancelled each other out (Paull, LaFonte and Johnson, 2012). Increases in temperature or exposure to a heatwave may be detrimental to host immunity (Cheng, Wang and Chen, 2005; Raffel *et al.*, 2006; Dittmar *et al.*, 2014; Tobin *et al.*, 2024), which may mediate an increase in parasite success, but temperature may interact with other factors such as host age and the nature of the immune challenge to modify the immune response in more complex ways (Murdock *et al.*, 2012). Furthermore, although in certain cases increasing temperatures may have a more negative impact on parasites than on their hosts (Karvonen *et al.*, 2010; Gehman, Hall and Byers, 2018), the acclimatisation theory suggests that parasites may have a competitive advantage over their hosts in scenarios of fluctuating temperatures given their ability to acclimate more quickly due to their faster metabolisms (Gillooly *et al.*, 2001; Raffel *et al.*, 2013). The outcome of infection under scenarios of increasing and fluctuating temperatures remains difficult to predict due to its highly complex nature.

Changes in temperature may have complex effects on host-pathogen interactions. The type of temperature variation (rising mean temperatures, fluctuating temperatures, or heatwave events), as well as the starting temperature of the system, may yield differing results (Kunze *et al.*, 2022). Furthermore, different parasite life-history traits (infection rates and spore burden) may be affected in different ways by changes in temperature (Paull, LaFonte and Johnson, 2012), and the response of pathogens to temperature variation may be specific to the parasite in question. The complexity of the impacts of increasing temperatures and temperature variation on parasites and their hosts may lead to unexpected outcomes on disease outbreaks (Paull, LaFonte and Johnson, 2012; Kunze *et al.*, 2022). For example, in *Daphnia*, exposure to a heatwave increases microsporidian *O. colligata* spore burden tenfold when compared to fluctuating temperatures, but this is dependent on the starting temperature of the system (Kunze *et al.*, 2022). Changes in disease dynamics following increased temperature variability

may have far-reaching consequences such as host population decline (Mouritsen, Tompkins and Poulin, 2005), species extinction (Hughes *et al.*, 2002) and trophic cascades (Bates, Hilton and Harley, 2009). For example, increases in rickettsial disease caused by rising temperatures have led to near extinction in *Haliotis* molluscs (Burge *et al.*, 2014), causing shifts across multiple trophic levels (Burge *et al.*, 2014) and impacting fisheries and aquaculture (Friedman *et al.*, 2003). On a *Daphnia*-specific level, changes to disease dynamics through increasing temperature variability may have widespread impacts due to their role as a keystone species in many freshwater ecosystems (Carpenter *et al.*, 2001; Sarnelle, 2005). Given that the response of pathogens to increasing temperature variability appears to be system-specific, continued research into this response is crucial if we are to be able to draw generalisations and predict impacts on a wider scale.



# **Chapter 3. Low phosphorus levels in combination with moderate temperatures limit infection by a microsporidian parasite in the freshwater invertebrate *Daphnia magna***

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**Author contributions:** KO’N, ID and PL contributed to the experimental design. KO’N and PL conducted the experiment. TK wrote the initial code for Bayesian modelling. FEO’K conducted the final analyses and created the figures. FEO’K wrote the first draft of the manuscript and PL and CVH contributed to revisions. PL and CVH supervised the project.

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## **3.1 Abstract**

Human-induced global change is causing shifts in temperature and nutrient regimes in ecosystems worldwide, which may impact the transmission and outbreak of infectious diseases. Shifting temperatures may cause mismatches between host and parasite temperature tolerance ranges, while changes in nutrient availability may alter host-parasite dynamics. While both temperature and nutrient levels have been examined in the context of disease dynamics, few studies have investigated the impacts of both simultaneously. Here, we investigate the

combined effect of seven temperatures (between 10°C and 31°C) and four levels of phosphorus (4 ml, 2 ml, 1 ml, and 0.5 ml of phosphorus per litre of algae medium) on the aquatic invertebrate *Daphnia magna* and its microsporidian parasite *Ordospora colligata*. The combination of experimental work and Bayesian modelling enables us to predict parasite minimum, optimum and maximum temperatures under different phosphorus regimes. Individual hosts were kept under standard laboratory conditions, exposed to the parasite, and observed for 30 days while recording host mortality, infection rates and spore burden. The highest levels of infection with *O. colligata* were observed at intermediate temperatures (~17°C and 20.5°C) and high phosphorus levels. Spore burden of the parasite followed a similar pattern to infection rate, but Bayesian analysis of spore burden suggests an interaction between temperature and phosphorus, as the highest spore burden was observed at intermediate levels of phosphorus which differs from patterns observed in infection rates. Temperature and phosphorus levels also impacted host survival, as animals exposed to high temperatures and low levels of phosphorus had increased mortality. With both phosphorus and temperature impacting life-history traits of both the host and the parasite, it is clear that ongoing anthropogenic changes may alter disease dynamics. Populations near the temperature optimum for infection may experience a decrease in disease prevalence under conditions of rising temperatures while those below the thermal optimum may see enhanced parasite infection and burden. However, the strength and direction of this effect may depend on the current temperature, the nutrient that is limiting host and parasite growth and the identity of the host and the parasite.

### **3.2 Introduction**

Human-induced anthropogenic changes may alter disease proliferation and outbreaks (Daszak, Cunningham and Hyatt, 2001) with impacts on human and animal health (Hilgenfeld and Peiris, 2013), ecosystem services (Paseka *et al.*, 2020) and biodiversity (Johnson *et al.*, 2007). For example, rising seawater temperatures due to global warming are believed to be responsible for

the densovirus-induced outbreak of sea star wasting disease which has decimated a keystone predator and led to a trophic cascade (Bates, Hilton and Harley, 2009). Similarly, eutrophication caused by nitrogen and phosphorus enrichment has been found to promote amphibian infection by the flatworm *Ribeiroia ondatrae*, leading to reduced amphibian survival and widespread population decline (Johnson *et al.*, 2007). Indeed, it is well established that rising temperatures may lead to latitudinal or altitudinal disease range shifts (Martens *et al.*, 1995; Garamszegi, 2011; Zamora-Vilchis, Williams and Johnson, 2012; Altizer *et al.*, 2013), higher levels of infection (Labaude, Rigaud and Cézilly, 2015) or altered disease dynamics (Thomas and Blanford, 2003). Likewise, increased nutrient concentrations associated with agriculture (Moss, 2008) and industry (Selman and Greenhalgh, 2010) have been shown to cause artificially enriched environments (Smith, 2003), increased eutrophication (Fink *et al.*, 2018) and growth of cyanobacterial blooms (Paerl and Paul, 2012). Moreover, the effects of climate change and nutrient pollution may depend on the direction and the amplitude of each factor (Moss, 2010). For example, in phytoplankton, community cell size decreased under increased temperatures and higher nutrient levels, with a ten-fold difference in the size decrease mediated by nutrient stress in higher temperatures (Peter and Sommer, 2013). Although these interactive effects have been investigated in free-living organisms and ecosystems (McKee *et al.*, 2003; Moss *et al.*, 2003; Šorf *et al.*, 2015), few studies have investigated the simultaneous effects of alterations in food quality and temperature in host-parasite systems (but see Manzi *et al.* (2020)). Overall, the question remains how temperature and nutrient pollution will impact disease outbreak and spread, if their effects interact, and whether their impacts will be consistent across different host-parasite systems.

Numerous factors and mechanisms have been invoked to explain the effects of temperature and nutrient levels on host-parasite systems individually, but their combined effects may lead to unexpected consequences for the outbreak and spread of disease. Indeed, mismatches between

host and parasite thermal curves (Cohen *et al.*, 2017), temperature or nutrient-induced changes in host immunity (Raffel *et al.*, 2006), alterations in host foraging behaviour (Darchambeau and Thys, 2005; Shocket *et al.*, 2018a) or differences in metabolic requirements (Kirk *et al.*, 2019) may provide an advantage to one of the antagonists. Several studies have shown that increasing temperatures are detrimental to host immune function (Cheng, Wang and Chen, 2005; Raffel *et al.*, 2006). For example, in white shrimp infected with the bacterium *Vibrio alginolyticus*, animals which were exposed to warmer temperatures had reduced immune capability and displayed diminished phagocytic activity (Cheng, Wang and Chen, 2005). Furthermore, according to the acclimatisation theory, smaller organisms, such as parasites, may be able to acclimatise to new temperatures more rapidly (Pörtner, 2002; Brown *et al.*, 2004), potentially allowing parasites to gain a competitive advantage over their hosts in conditions of increasing temperatures (Raffel *et al.*, 2013). Despite the complexity of the impact of temperature on parasitic infection, recent work has shown that these effects could be predicted using the Metabolic Theory of Ecology (Kirk *et al.*, 2019). However, the question remains whether these predictions will apply across different host-parasite systems when other factors, such as varying nutrient levels, are introduced. Indeed, nutrient pollution has been shown both to amplify (Johnson *et al.*, 2007) or reduce (Frost, Ebert and Smith, 2008) disease outbreak, with the direction of effects seemingly dependent on the host-parasite system. Lower quality food may encourage animals to increase their feeding or filtering rates and lead to increased contact between the host and the parasite (Darchambeau and Thys, 2005; He and Wang, 2008; Aalto and Pulkkinen, 2013). Nutrient stress can also impair host immunity, thus leading to higher levels of infection, as has been observed in humans (Field, Johnson and Schley, 2002) and honeybees (Alaux *et al.*, 2010). Alternatively, the parasite may experience dampened in-host pathogen growth and multiplication due a lack of host resources as a result of poor quality food (Darchambeau and Thys, 2005; He and Wang, 2008). Moreover, faster-growing organisms have been suggested to require more phosphorus (i.e. the growth rate hypothesis (Main, Dobberfuhl and Elser, 1997; Acharya, Kyle and Elser,

2004)) which could lead to a shift in the balance between hosts and their parasites with changing nutrient levels.

With both nutrients and temperature simultaneously affecting numerous factors and life-history traits of hosts and parasites, it is difficult to predict how host-parasite systems will respond to the interaction of temperature and nutrient levels. Regardless of the mechanisms at play, the interaction of temperature and food quality introduces an added level of complexity to disease spread (Manzi *et al.*, 2020). Thus, to anticipate future disease outbreaks it is crucial to gain an understanding of the relative contribution of rising temperatures, altered nutrient levels and the mechanisms which may shape their interactions. Here, we investigate how the parasite *Ordospora colligata* and its host *Daphnia magna* are affected by seven different temperatures and four levels of phosphorus. This experimental host-parasite model has previously been used in several climate change experiments (Kirk *et al.*, 2019; Kunze *et al.*, 2022) and the effects of nutrient enrichment on *D. magna* life-history traits are well studied (Kilham *et al.*, 1997; Jeyasingh and Weider, 2005; Vale, Stjernman and Little, 2008; Aalto and Pulkkinen, 2013). Here, we focus on how temperature interacts with different levels of phosphorus as this nutrient is often the limiting factor of primary production in aquatic ecosystems (Bracken *et al.*, 2015). Given previous research in this system, we hypothesise that the parasite will be most successful in moderate temperatures, while infection rates and spore burden may be lower at the lower and upper ends of the temperature range (Kirk *et al.*, 2018). Furthermore, we hypothesise that animals in lower phosphorus regimes may experience reduced survival (Aalto and Pulkkinen, 2013), while the parasite may have limited growth under phosphorus-limited conditions given the growth rate hypothesis (Acharya, Kyle and Elser, 2004), although previous research suggests that the host may be more affected by low phosphorus than the parasite (Aalto and Pulkkinen, 2013).

### 3.3 Material and methods

#### 3.3.1 Study system

*Daphnia magna* (Order: *Cladocera*) has been widely studied due to its key role as a primary producer in freshwater ecosystems and is also a well-established system to investigate host-parasite interactions (Ebert, 2005). *D. magna* is ubiquitous throughout the Northern Hemisphere (Lange, Kaufmann and Ebert, 2015), therefore encountering a range of habitats and environmental conditions. The parasite *Ordospora colligata* is present throughout the range of *D. magna* (Angst, Ebert and Fields, 2023). *O. colligata* is a microsporidian parasite of *D. magna* that comes in contact with the host while filter feeding, invades the gut epithelial cells and replicates intracellularly (Larsson, Ebert and Vavra, 1997), and is horizontally transmitted through spores which are shed in the faeces of *D. magna* (Ebert, Lipsitch and Mangin, 2000). Although relatively avirulent, *O. colligata* is easily transmittable from host to host and is known to occur in high prevalences in natural populations (Ebert, 2005). The host and parasite isolates used in this study were originally collected from the Tvärminne Archipelago in Finland (*D. magna* strain Fi-Oer-3-3 and *O. colligata* strain OC3).

#### 3.3.2 Experimental procedure

##### (a) Temperature

We exposed individual juvenile female *D. magna* to spores of *O. colligata* at seven temperatures spanning the natural range of *D. magna* (between 10°C and 31°C, increasing in increments of 3.5°C) and four food treatments grown at varying levels of phosphorus. Each treatment combination had 21 replicates split over two water baths, that yielded a total of 1176 animals. Temperature was manipulated by immersing microcosms in water baths in which temperature was regulated by a controller (Inkbird ITC-308) that interfaced with aquarium chillers (Hailea HC150A & DC300) and/or heating rods (EHEIM JÄGER 300W) depending on the required

temperature. Water in the baths was circulated using microjet pumps (NemaMicro MC450) to equalise the temperature. Each bath held up to 96 microcosms distributed over four trays, that were perforated to facilitate water circulation and rotated daily to avoid positing effects. Realised temperatures in each bath were monitored by a HOBO temperature logger placed into an empty microcosm filled with ADaM to mimic conditions in experimental microcosms. Mean temperatures for each pair of baths targeting the same temperature were within 0.6°C of each other.

#### *(b) Phosphorus levels*

The quality of the food was altered by changing the phosphorus concentration of the WC Medium (Guillard and Lorenzen, 1972) used to grow *Scenedesmus* algae (a genus of green algae), which was used to feed *D. magna* throughout the experiment. Batch cultured *Scenedesmus* were either grown under nutrient saturating conditions (11.4 g/l  $K_2HPO_4$ ), with half (5.7 g/l  $K_2HPO_4$ ), one quarter (2.85 g/l  $K_2HPO_4$ ) or only one eighth (1.43 g/l  $K_2HPO_4$ ) of the recommended phosphorus concentration (therefore amounting to 4 ml, 2 ml, 1 ml, and 0.5 ml of phosphorus per litre of medium and referred to as 4P, 2P, 1P and 0.5P treatments respectively henceforth). As altering phosphorus concentrations can result in changes to both the carbon and phosphorus content simultaneously (Bernot and Poulin, 2018), we determined the carbon content of the algae using a Total Organic Carbon (TOC) analysis. Samples of each food treatment were taken, centrifuged, and diluted with ADaM to mimic feeding conditions. Samples were then counted using a haemocytometer, blended, and acidified using hydrochloric acid, before being run through the TOC analyser. Results showed that there was no change in the carbon content of the algae grown under the different phosphorus concentrations (linear model,  $df = 3$ ,  $p = 0.43$ ). Algae of all treatments were harvested three times a week, concentrations were determined (with a haemocytometer) and the algae were diluted to target concentrations prior to feeding.

### *(c) Host preparation*

To obtain sufficient juvenile females and minimise maternal effects, we grew a large quantity of animals (~425 individuals) of a single *D. magna* clone (Fi-Oer-3-3, originally sampled from Tvärminne, Finland) under standardised conditions for five weeks in advance of the experiment (11-12 female animals per 400 ml microcosm with Artificial *Daphnia* Medium [ADaM] (Ebert, Zschokke-Rohringer and Carius, 1998) that was replaced twice a week, kept at 20 °C with a 16-8 day-light cycle and fed *ad libitum*). Three days prior to the start of the experiment, animals were transferred into new microcosms to ensure that no juveniles were present. Juveniles born during these three days were collected, sexed using a dissecting microscope, placed individually in a 100 ml microcosm filled with 50 ml of medium and randomly assigned to a treatment combination. Female juveniles were allowed to acclimatise for 24 hours to their assigned temperature before infection and food treatments were applied. Any individuals that died during the acclimatisation period were replaced by backups that had been treated identically up to that point.

### *(d) Experimental design*

Animals were exposed to a spore dose of ~32500 spores (chosen based on pilot experiments and previous studies, see Manzi *et al.* (2021)) by adding 1 ml of spore solution prepared by diluting down a suspension of spores that was created by crushing ~8500 infected individuals with known prevalence and burden (determined by phase contrast microscopy on a subsample). Controls were exposed to 1 ml of a placebo (consisting of crushed uninfected animals). Animals were exposed to either the parasite or placebo for seven days (based on previous experiments, see Kunze *et al.* (2022)) and were subsequently transferred to clean microcosms with fresh medium twice a week until the end of the experiment. Animals were fed four times a week with their assigned food treatment, starting at four million batch cultured algae on day 0. Food

amounts were increased to eight million algae on day 2 and 10 million from day 4 onward to accommodate for growth of the animals. Animals were followed up to natural death or to the end of the experiment (day 28). Host mortality was assessed daily through examination of individuals for movement. Any individuals (exposed and controls) that died during the experiment after the initial period of exposure (day 7) were immediately dissected (as reliable detection of *O. colligata* infection is generally not possible before 11 days post exposure (Kirk *et al.*, 2019)). All animals which survived the duration of the experiment were sacrificed and dissected within 4 days of the end of the experiment. All exposed individuals were examined under a 400x phase contrast microscope to determine infection status and parasite load. Half of the control animals were also examined for each treatment to ensure that no contamination had occurred.

### **3.3.3 Statistical analyses**

Statistical analyses were performed using R (version 4.2.2) interfacing with Stan for Bayesian analyses. Parasite infection rates were calculated using only animals which died after day 14, as infection could not be reliably confirmed prior to this date. Similarly, only animals which died in the last 7 days of the experiment were used to examine spore burden data, to ensure that spore burdens were comparable across treatments. Realised temperatures were used when analysing parasite fitness (infection rates and spore burden). Overall host survival was equal between baths, therefore temperature differences between baths were not included in survival analyses.

Bayesian analyses were chosen to examine parasite infection rates and spore burden data, as in addition to allowing us to fit our data to a model, this approach allowed us to predict values for each of the model parameters with estimates of uncertainty for each prediction. Parasite infection rates and spore burden were analysed by fitting our data to a Beta Function for each of the phosphorus treatments, as

$$res = c_m \left( \frac{t_{max} - t}{t_{max} - t_{opt}} \right) \left( \frac{t - t_{min}}{t_{opt} - t_{min}} \right)^{\frac{t_{opt} - t_{min}}{t_{max} - t_{opt}}}$$

where *res* is proportion infected or spore burden at temperature *t*, *c<sub>m</sub>* is the probability of infection based on temperature, *t<sub>opt</sub>* is temperature at optimal performance, and *t<sub>min</sub>* and *t<sub>max</sub>* are the critical minimum and maximum temperatures where fitness of the trait becomes unviable, respectively. The Beta function was chosen as it has been shown to accurately represent thermal performance (Niehaus *et al.*, 2012) and parameter estimates from the equation have biological meaning which can be easily interpreted. We used a binomial distribution to evaluate the effect of temperature and food quality treatment on parasite infectivity (infection rates), and a Poisson distribution to assess their effect on spore burden, and fitted the model in a Bayesian framework with Stan using the MCMC fitting algorithm called from R. Priors (see Table B1) were chosen based on previous research (Kirk *et al.*, 2018; Kunze *et al.*, 2022) and satisfied the condition  $t_{min} \leq t_{opt} \leq t_{max}$ . The posterior distribution of all parameters was estimated using four chains, and number of warmup and post-warmup iterations can be found in Table B2. Model fit was checked through MCMC divergence, MCMC convergence (R-hat values) and effective samples.

Overall survival rates were assessed through a generalised linear model with binomial error distribution and a post-hoc Tukey test, and survival over time was assessed through a Kaplan-Meier survival analysis, log rank test and pairwise comparisons (using the “survminer” package in R), where survival was analysed for phosphorus treatments across the temperature range, and was also compared to placebo exposed control animals.

### 3.4 Results

#### 3.4.1 Infection rates

The highest rates of *O. colligata* infection were observed in *D. magna* at intermediate temperatures (17°C, 20.5° and 24°C), while in treatments approaching the upper and lower boundaries of the natural temperature range of *D. magna* (10° and 28° respectively) *O. colligata* infection rates approached zero (Figure 3.1). The effect of temperature on *O. colligata* infection was consistent across all levels of phosphorus, as lowest and highest levels of infection were achieved at similar minimum, maximum and optimum temperatures (Figure 3.1A, B, C and D; confidence intervals of  $t_{\min}$ ,  $t_{\max}$  and  $t_{\text{opt}}$  overlap for all phosphorus treatments). However, animals in treatments with lower levels of phosphorus (0.5P, 1P) had lower maximum infection rates than those in treatments with the highest levels of phosphorus (4P) (Figure 3.1A and E; confidence intervals do not overlap for  $c_m$  of lower phosphorus treatments (0.5P, 1P), compared to 4P treatment), with *D. magna* in the lowest phosphorus treatment (0.5P) showing no infection across all temperatures except 20.5 and 24°C, where a single animal was infected in each case (Figure 3.1).

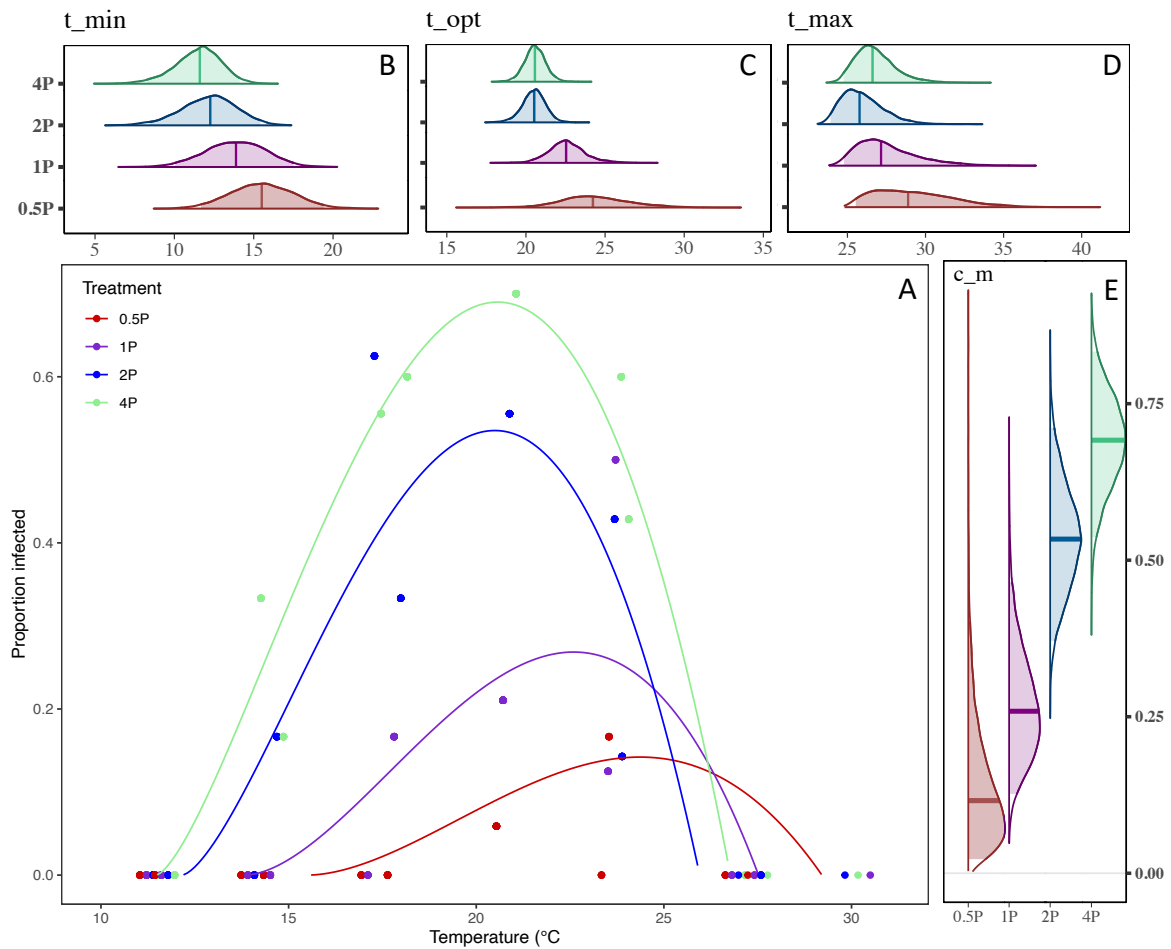


**Figure 3.1.** Proportion of *O. colligata* infected animals across all temperatures and phosphorus treatments. Panel A represents the predicted Beta model fit line and the datapoints used to fit the model. Panels B, C, D and E represent the estimations of the parameters of the model ( $t_{min}$ ,  $t_{opt}$ ,  $t_{max}$  and  $c_m$  respectively) where the shaded area under the curve is the 95% certainty interval.

### 3.4.2 Spore burden

*O. colligata* spore burdens had similar patterns to infection rates across the different phosphorus treatments, as higher spore burdens were seen in intermediate temperatures and were lower at the more extreme temperatures in the range (Figure 3.2A). However, the predicted optimal temperature for spore growth varied depending on phosphorus levels, with the 4P treatment having the lowest predicted optimum and the 2P treatment the highest (Figure 3.2C; confidence

intervals of  $t_{opt}$  do not overlap for 1P and 2P treatments when compared to the 4P treatment). Phosphorus levels also affected the predicted number of spores produced at these optimums, with lower phosphorus levels impeding spore growth when compared to treatments with higher levels of phosphorus (Figure 3.2E; confidence intervals of  $c_m$  do not overlap between 1P, 2P and 4P treatments). However, the highest number of spores were produced in the 2P treatment (Figure 3.2A and E), in contrast to the highest infection rate which was observed in the 4P treatment (Figure 3.1A). This higher peak in the 2P treatment suggests a potential interaction between temperature and phosphorus. Furthermore, although all treatments had similar minimum temperatures required for spore growth, spore growth in the lower 1P phosphorus treatment was more sensitive to higher temperatures, with lower maximum temperatures for spore production predicted in this treatment compared to those with higher levels of phosphorus (Figure 3.2D; confidence interval of  $t_{max}$  for 1P does not overlap with those of 2P and 4P). This suggests that lower phosphorus levels not only prevent *O. colligata* from establishing an initial infection, but may also limit in-host growth of the parasite.

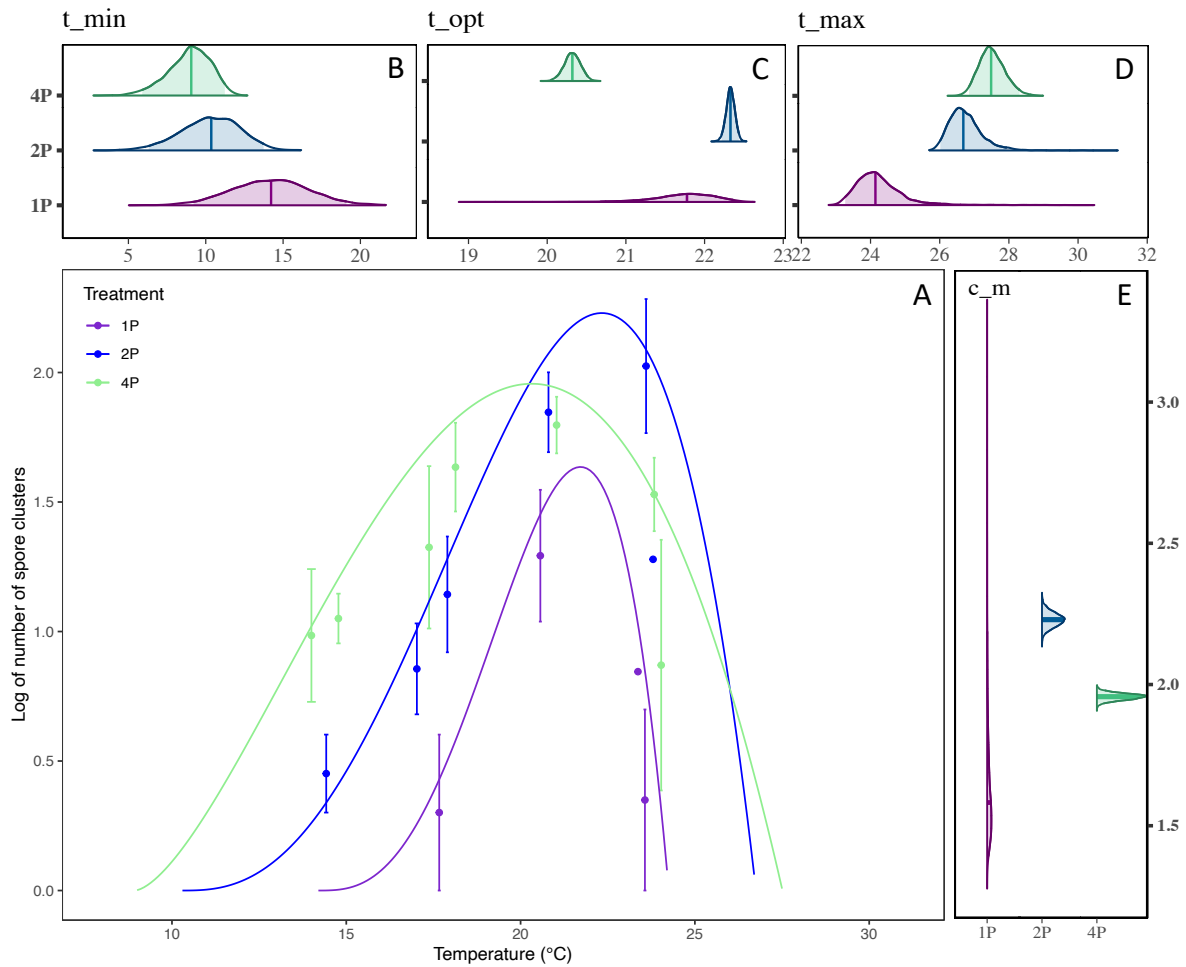


**Figure 3.2.** Mean *O. colligata* spore burden (log of number of spore clusters) across all temperatures and phosphorus treatments. Panel A represents the predicted Beta model fit line and the datapoints used to fit the model. Panels B, C, D and E represent the estimations of the parameters of the model ( $t_{min}$ ,  $t_{opt}$ ,  $t_{max}$  and  $c_m$  respectively) where the shaded area under the curve is the 95% certainty interval. Spore burdens were calculated using animals which died in the final 7 days of the experiment. Error bars represent standard error. The 0.5P treatment was excluded as it had too few replicates due to low infection rates in this treatment to accurately assess model fit.

### 3.4.3 Host mortality

The highest survival rates of *D. magna* were observed in cold temperatures (10°C, 13.5°C, 17°C), with survival decreasing with increasing temperatures (Figure 3.3A; GLM, analysis of deviance,

$p < 2e-16$ ,  $df = 843$ ). At the highest temperature (31°C), no animals survived for the duration of the experiment, regardless of levels of phosphorus (Figure 3.3A). In general, animals in higher phosphorus treatments had higher survival rates than those in lower phosphorus treatments (Figure 3.3A; GLM, analysis of deviance,  $p < 2e-16$ ,  $df = 846$ ), although there was also a two-way interaction between temperature and phosphorus levels (GLM, analysis of deviance,  $p < 0.002$ ,  $df = 833$ ). While survival for exposed and uninfected animals appeared similar, there was a three-way interaction between phosphorus levels, temperature and exposure (GLM, analysis of deviance,  $p = 0.019$ ,  $df = 818$ ), but post-hoc testing did not reveal any meaningful differences between specific treatments. Similarly to overall survival, survival over time was highest in colder temperatures (Figure 3.3B-E). However, differences in survival over time were only significant in the most central temperature regime (20.5°C, Figure 3.3D), where animals exposed to higher levels of phosphorus (2P, 4P) lived longer than animals in lower phosphorus treatments (0.5P, 1P) (Kaplan Meier analysis, all pairwise comparisons with BH adjustment method,  $p < 0.01$  except between 2P and 4P,  $p = 0.174$ ), although animals under the lowest levels of phosphorus (0.5P) lived longer than those in the second lowest phosphorus treatment (1P) (Kaplan Meier analysis, pairwise comparison with BH adjustment method,  $p = 0.0065$ ). However, it is important to note that the duration of our experiment may have been too short for difference in host survival to be detected as hosts infected with *O. colligata* have been observed to live for up to 50 days, with only slight effects to host fitness due to the low virulence of this parasite (Ebert, Lipsitch and Mangin, 2000).

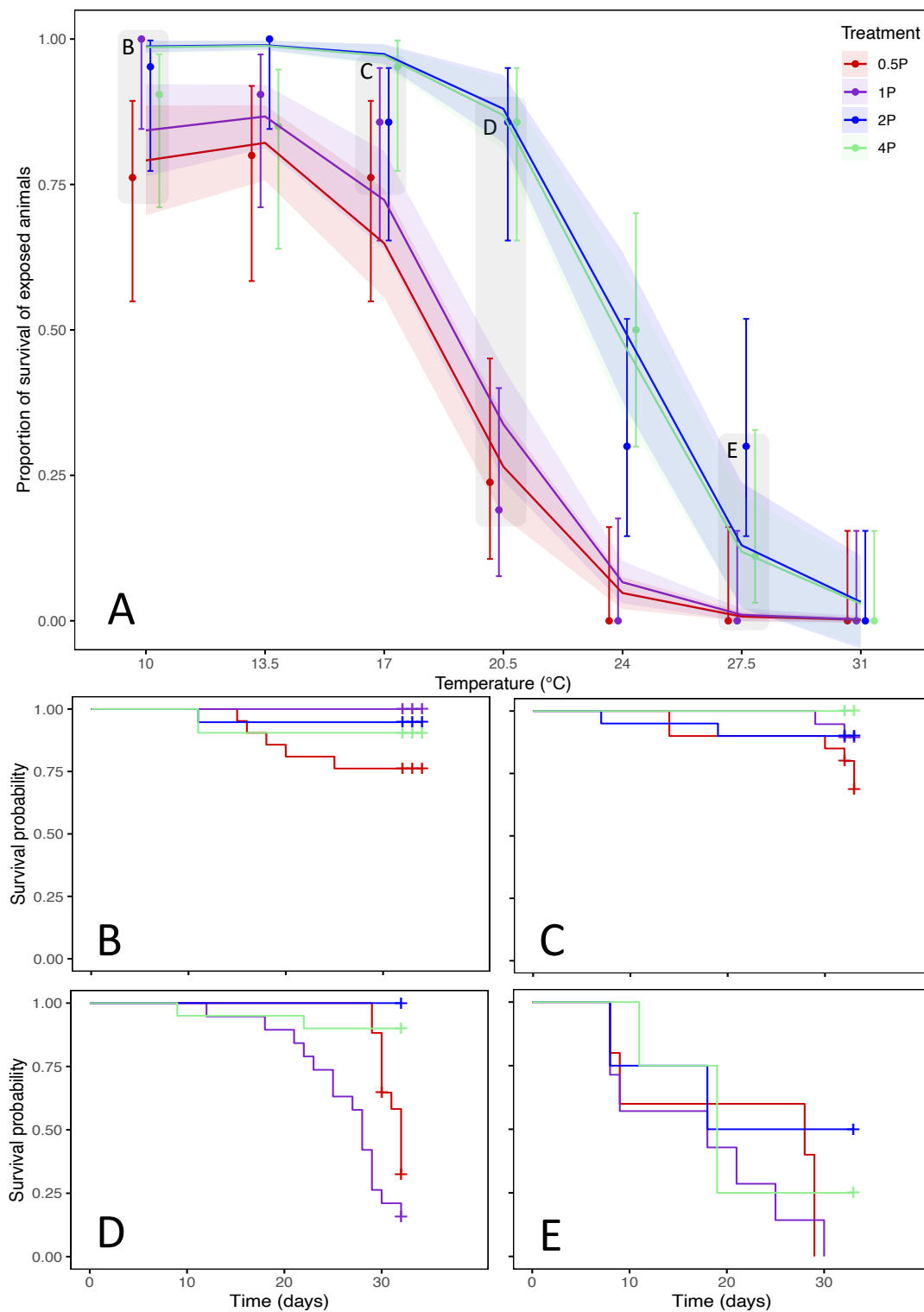


**Figure 3.3.** Survival of exposed animals across all temperature and phosphorus level treatments. Panel A shows overall *D. magna* survival, taken at the end of the experiment. Panels B-E show survival probability over time generated by means of a Kaplan-Meier analysis, at a subset of temperatures as indicated in Panel A: 10°C (B), 17°C (C), 20.5°C (D) and 27.5°C (E). Error bars in

panel A represent binomial 95% confidence interval. Ribbon shading in panel A represents the 95% confidence intervals of the model fit. “+” symbols seen in panels B-E represent animals which were still alive at the end of the experiment. Given that we found no difference between the survival of exposed and unexposed animals, only exposed animals are shown here for clarity.

### 3.5 Discussion

Increased temperature and the amount of phosphorus in the environment both altered infection rates, spore burden and host longevity. Low levels of phosphorus impeded infection of *D. magna* by *O. colligata*, as did extreme temperatures. This resulted in large differences in infection rates and spore burden between different treatment combinations with an up to 13 - fold and 28-fold difference for infection and burden at 20.5°C, respectively. Host survival, another key parameter in epidemiological models (Anderson and May, 1983; May and Anderson, 1983; Kirk *et al.*, 2019), was highest at low temperatures and high levels of phosphorus. The interactions between phosphorus and temperature observed in spore burden and host survival as well as their independent effects simultaneously impacting different host and parasite life-history traits may cause difficulties in predicting their combined effects. It nevertheless is clear that alterations in either temperature or levels of phosphorus may change within-host growth and survival of this microsporidian parasite. Thus, with ongoing global warming and human induced changes to phosphorus concentrations, we may expect altered patterns of disease prevalence and outbreak in the future.

Survival rates of *D. magna* were influenced by both temperature and stoichiometry, with increasing temperatures and poorer quality food yielding lower survival rates, regardless of exposure to *O. colligata*. Temperature is known to be an important factor for *D. magna* survival, with *Daphnia* survival rates, growth rates and reproductive rates generally being highest in more moderate, central temperature regimes (Burns, 1969; Kirk *et al.*, 2018). The high survival rates

observed in the coldest temperatures (10°C, 13.5°C and 17°C) are likely due to the slower host metabolic rates which are generally seen in colder temperatures (Brown *et al.*, 2004), while excessively warm temperatures place increased stress on the host leading to higher rates of mortality. Indeed, warming temperatures are well known to be one of the primary causes of the current decline in biodiversity (Koh *et al.*, 2004), with organisms unable to adapt to rapid changes in the environment. Furthermore, overall survival was higher in better quality food treatments, regardless of exposure to the parasite. This has previously been observed where phosphorus limitation led to lower levels of survival (Urabe and Sterner, 2001; Aalto and Pulkkinen, 2013). However, excessive levels of phosphorus in the environment can lead to eutrophication (Wurtsbaugh, Paerl and Dodds, 2019), which has been linked to increased mortality in organisms such as fish, crustaceans and molluscs (Bauer, 1988; Chislock *et al.*, 2013). Furthermore, some research has suggested that temperature and nutrient levels may interact when both stressors are present simultaneously (Peter and Sommer, 2013), as was the case in the present study with a two-way interaction observed between temperature and phosphorus levels. The effects of temperature and nutrient levels on survival may therefore be difficult to predict, and the current state of an ecosystem may be critical to determining the direction of these effects (Moss, 2010).

Infection rates of *D. magna* with *O. colligata* were highest in intermediate temperatures and at higher levels of phosphorus, but while spore burdens generally followed the same pattern, there was a shift in both the optimal temperature and the number of spores at this temperature for some treatments, complicating infection patterns. Previous studies utilising the *Daphnia-Ordospora* system have observed similar results of increasing temperature on infection rates, with the highest proportion of infected individuals being observed in central temperatures (Kirk *et al.*, 2018; Kunze *et al.*, 2022). The most likely explanation for this pattern of infection relates to feeding rates, with warmer temperatures causing an increase in metabolic rates (Pörtner,

2002; Brown *et al.*, 2004) and therefore facilitating spore uptake through filter feeding (Shocket *et al.*, 2018b). However, past a certain temperature, survival rates of the host drop (Kirk *et al.*, 2018), leading to a corresponding decrease in infection rates, as the parasite does not have sufficient time to establish infection before host death. Furthermore, lowered food quality can affect infection rates by increasing pathogen transmission through altered host behaviour (e.g. increased feeding rates (Darchambeau and Thys, 2005; Aalto and Pulkkinen, 2013)) or lowered host immunity (Alaux *et al.*, 2010). However, although several studies have shown that lower nutrient levels may favour the pathogen (Pulkkinen, Wojewodzic and Hessen, 2014), we found that lower nutrient levels impede pathogen establishment and growth. This may be a result of a lack of host resources, as has been observed in the *Daphnia dentifera*-*Metschnikowia bicuspidata* system where lower levels of phosphorus dampened within-host growth (Hall *et al.*, 2009a; Hall *et al.*, 2009b). This is in accordance with the growth rate hypothesis, which suggests that slower growing species require less phosphorus than faster growing species (Sternler, 1995; Paseka and Grunberg, 2019), and thus that parasites require higher levels of phosphorus for growth than their hosts (Main, Dobberfuhl and Elser, 1997; Acharya, Kyle and Elser, 2004). Overall, several mechanisms may be driving the effects of temperature and nutrient levels observed in this study, and our understanding of these may be further complicated by their potential for interaction.

Pathogen responses to changing temperatures and nutrient levels have been shown to be complex, with several factors influencing the response, and the question remains whether these two factors will interact in a predictable manner in real world systems. Indeed, we did observe a shift in the optimal temperature for parasite growth and in the number of spores produced at this temperature in higher phosphorus regimes (2P and 4P), although our confidence in this pattern is reduced due to our low sample size at higher temperatures. However, several studies have shown that combined changes in temperature and nutrients can lead to unexpected effects

in free-living organisms and whole ecosystems (McKee *et al.*, 2003; McFeeters and Frost, 2011; Šorf *et al.*, 2015). While the combined impacts of both alterations in temperature and nutrient levels have been less studied in pathogens, their individual effects can already be difficult to disentangle. Moreover, temperature and nutrient availability may impact different life-history traits in opposite directions making their combined effect on disease outbreaks difficult to predict (Manzi *et al.*, 2020). For instance, both temperature and nutrient availability can impact immune function (Cheng, Wang and Chen, 2005; Alaux *et al.*, 2010). In addition, the effects of temperature and nutrients may be genotype dependent (Vale, Stjernman and Little, 2008) with different disease outcomes observed for different combinations of host and parasite. Indeed, in *Daphnia longispina*  $\times$  *galeata* hybrids exposed to the fungus *Metschnikowia bicuspidata*, host genotype was found to interact with temperature and food quality to determine spore yield of infected hosts (Manzi *et al.*, 2020). Therefore, it is clear that more insight into how temperature and nutrient levels may impact host-parasite systems is needed to accurately predict the effects of global anthropogenic change.

Overall, our results suggest that global change in the form of increasing temperatures and nutrient pollution may increase parasite success in systems with colder current temperatures, while systems with higher baseline temperatures may see a decrease in disease outbreak. In testing *D. magna*'s entire temperature tolerance range, we observed an optimum for disease outbreak in moderate temperatures with high levels of phosphorus, although this is likely to vary depending on the host-parasite system in question. Models predicting the outbreak of disease rely on all host-parasite traits to make projections (Kirk *et al.*, 2019), and predictions may become increasingly more complex if these traits scale differently with temperature and phosphorus levels. Nevertheless, it may be possible to use a combination of ecological stoichiometry and metabolic theory to predict disease outbreaks (Allen and Gillooly, 2009), despite the complexities of the factors investigated here. Both the individual impacts and the

interactions observed between temperature and phosphorus levels in this study suggest that further investigation into the effects of these factors in free-living organisms and their parasites is crucial to better predict future disease outbreaks.



## **Chapter 4. Increased virulence due to multiple infection in *Daphnia* leads to limited growth in one of two co-infecting microsporidian parasites**

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**Author contributions:** All authors contributed to the experimental design, FEO’K and RCP conducted the experiment with assistance of PL. FEO’K wrote the first draft of the manuscript and all authors contributed to revisions. PL and CVH supervised the project.

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### **4.1 Abstract**

Recent outbreaks of various infectious diseases have highlighted the ever-present need to understand the drivers of the outbreak and spread of disease. Although much of the research investigating diseases focuses on single infections, natural systems are dominated by multiple infections. These infections may occur simultaneously, but are often acquired sequentially, which may alter the outcome of infection. Using waterfleas (*Daphnia magna*) as a model organism, we examined the outcome of sequential and simultaneous multiple infections with two microsporidian parasites (*Ordospora colligata* and *Hamiltosporidium tvaerminnensis*) in a

fully factorial design with nine treatments and 30 replicates. We found no differences between simultaneous and sequential infections. However, *H. tvaerminnensis* fitness was impeded by multiple infection due to increased host mortality, which gave *H. tvaerminnensis* less time to grow. Host fecundity was also reduced across all treatments, but animals infected with *O. colligata* at a younger age produced the fewest offspring. As *H. tvaerminnensis* is both horizontally and vertically transmitted, this reduction in offspring may have further reduced *H. tvaerminnensis* fitness in co-infected treatments. Our findings suggest that in natural populations where both species co-occur, *H. tvaerminnensis* may evolve to higher levels of virulence following frequent co-infection by *O. colligata*.

## 4.2 Introduction

Parasites are ubiquitous across all ecosystems (Karvonen, Jokela and Laine, 2019), with conservative estimates predicting that there must be at least one parasite species for each known free-living species (Poulin, 1999). Pathogens and the diseases they cause can impact human and animal health (Hilgenfeld and Peiris, 2013), as they may influence host fitness (Johnson and Hoverman, 2012), behaviour (Milne, Webster and Walker, 2020) and physiology (Corbin *et al.*, 2017). Furthermore, parasites may affect evolutionary processes (Lafferty, Dobson and Kuris, 2006) and lead to major economic losses (Sachs and Malaney, 2002; Nicola *et al.*, 2020). In addition, despite parasitic organisms representing a relatively small proportion of total animal biomass (Kuris *et al.*, 2008; Paseka, 2017; Preston *et al.*, 2021), parasites may have large ecological effects such as causing trophic cascades (Schultz, Cloutier and Côté, 2016) and impacting nutrient cycling (Mischler *et al.*, 2016). Gaining a better understanding of which factors facilitate or impede disease spread is crucial to prevent the many negative impacts of disease, especially given that the frequency of epidemics is increasing due to anthropogenic change and greater host mobility (Smith *et al.*, 2014). However, although many studies investigating the effects of parasites focus on interactions between a single disease and its

host(s), co-infections are common in real-world systems. Indeed, under natural conditions, parasites rarely occur in isolation (Lively *et al.*, 2014), with multiple infections accounting for up to 80% of parasite infections in some human populations (Petney and Andrews, 1998). Moreover, interactions among multiply infecting parasites can either have synergistic or antagonistic effects (Vaumourin *et al.*, 2015; Cressler *et al.*, 2016) and may cause more variation in the outcome of infection than commonly studied environmental and host factors (e.g., seasonality or host age) (Telfer *et al.*, 2010). This highlights the importance of studying and identifying the principles and mechanisms that drive interactions among parasites.

Numerous mechanisms, traits and factors may affect the outcome of multiple infections. For example, virulence may depend on spatial structure, host longevity, competition for resources, host availability (Godinho *et al.*, 2023) and the immune response, among other factors (see for review Cressler *et al.* (2016)). Studies have shown that co-infection may drive changes in parasite virulence (Alizon, 2008), which may either increase (Taylor, Walliker and Read, 1997; Vojvodic *et al.*, 2012) or decrease (Hood, 2003; Schürch and Roy, 2004). Moreover, virulence of co-infecting parasites may be dependent on the transmission modes of the parasites (Cressler *et al.*, 2016). Indeed, parasites which are vertically transmitted (from mother to offspring) are generally less virulent than those that rely on horizontal transmission (from host to host through the environment), as these maintain host fecundity at a higher level (Ebert, 2013). This may lead to mismatches in virulence levels between co-infecting parasites that have differing transmission modes, which can result in one parasite outcompeting the other. Furthermore, many studies have highlighted the importance of priority effects, suggesting that the order of exposure of the host to multiple parasites may determine the outcome of multiple infection (Hood, 2003; Ben-Ami, Rigaud and Ebert, 2011). Prior residency, where one parasite infects earlier than the second, may lead to a competitive advantage for the first parasite when compared to cases of simultaneous infection where both parasites invade the host at the same time (Hood, 2003;

Karvonen, Jokela and Laine, 2019). For example, a study investigating multiple infection of genetically distinct clones of the protist *Plasmodium chabaudi* infecting mice found that prior residency dictated which clone would succeed at infecting the host, regardless of which strain would typically gain a competitive advantage (De Roode *et al.*, 2005). In addition, the timing of exposure may have implications for the outcome of co-infection, as effects of infection may be different depending on the age of the host at infection (Ben-Ami, 2019). Indeed, many studies have observed differences in host immunity between juvenile and adult animals (Davidar and Morton, 1993; Cote *et al.*, 2000). One notable example of this phenomenon has been observed during the Covid-19 pandemic, with older individuals being more susceptible to the virus (Davies *et al.*, 2020). Overall, the many factors which may influence co-infection make it difficult to predict its outcome which is often specific to the host-parasite system in question.

Here, we investigate the effects of co-infection and prior residency using *Daphnia magna* and two of its microsporidian parasites, *Ordospora colligata* and *Hamiltosporidium tvaerminnensis*, as a model system. *O. colligata* is a horizontally transmitted gut parasite while *H. tvaerminnensis* is found in fat and ovarian cells and is transmitted both vertically (mother to offspring) and horizontally. Animals were exposed to either single, sequential, or simultaneous infections at two timepoints (early and late, Day 0 and 5 respectively) in a fully factorial experimental design. We collected measures of parasite fitness (infection rates and spore burden) and host fitness (survival and fecundity rates) to examine the effects of co-infection and determine whether prior residency plays a role in the outcome of infection in this system. Given previous studies using these parasites, which showed that *O. colligata* is less virulent than *H. tvaerminnensis* (Ebert, 2005), we expected that *H. tvaerminnensis* would outcompete *O. colligata* when competing over host resources. However, these parasites have previously only been studied individually, and given the complexity of the evolution of virulence, other outcomes including additive and synergistic interactions are a possibility.

## 4.3 Materials and methods

### 4.3.1 Study system

*Daphnia magna* (order: *Cladocera*) has been widely utilised as a model organism in ecological and evolutionary studies (Ebert, 2005), due to its role as a keystone species in freshwater ecosystems (Altshuler *et al.*, 2011; Jansen *et al.*, 2017) and ease of use in the laboratory (Ebert, 2022). *D. magna* is ubiquitous throughout the Northern Hemisphere (Lange, Kaufmann and Ebert, 2015) and can be infected by a multitude of parasites in natural systems (Ebert, 2005), often leading to multiple infection (Decaestecker *et al.*, 2005). *D. magna* is known to encounter the microsporidian parasites *Ordospora colligata* and *Hamiltosporidium tvaerminnensis* throughout its range, with the distribution of these two parasites overlapping in Western and Northern Europe (Ebert, 2005; Krebs, Routtu and Ebert, 2017) allowing co-infection to occur in natural populations. Furthermore, both parasites are microsporidians and host resistance loci are known to overlap (Keller, Kirk and Luijckx, 2019), therefore they may trigger a similar immune reaction in the host (Stirnadel and Ebert, 1997). *O. colligata* and *H. tvaerminnensis* spores are ingested by the host *D. magna* during filter feeding (Ebert, 2005), but spores are shed differently for each of these two parasites. *O. colligata* is an obligate gut parasite whose spores are shed continuously into the environment through the faeces of the host, leading to horizontal transmission (Ebert, Lipsitch and Mangin, 2000). In contrast, *H. tvaerminnensis* is transmitted both vertically and horizontally (upon death of the host) and exclusively infects the fat and ovarian cells of *D. magna* (Vizoso and Ebert, 2005b; Urca and Ben-Ami, 2018). Both *O. colligata* and *H. tvaerminnensis* are considered to be relatively avirulent (Ebert, 2005; Narr and Frost, 2016), therefore reducing the risk of competitive exclusion as seen when *Pasteuria ramosa* co-infects with *H. tvaerminnensis* (Regoes *et al.*, 2003; Ben-Ami, Rigaud and Ebert, 2011). The host and parasite isolates used in this study were originally collected from the Tvärminne Archipelago

in Finland (*D. magna* genotype Fi-Oer-3-3, *O. colligata* isolate OC3 and *H. tvaerminnensis*), and both parasites are known to infect this host genotype in natural populations (Haag *et al.*, 2020; Zukowski *et al.*, 2020).

#### **4.3.2 Experimental procedure**

##### *(a) Host preparation*

To obtain sufficient juvenile females for the experiment and minimise maternal effects, ~180 *D. magna* adult females from clonal line Fi-Oer-3-3 were grown under standardised conditions for four weeks in advance of the experiment (10-12 female animals per 400 ml microcosm with 200 ml of medium, transferred to a new microcosm with fresh medium twice a week, kept at 20°C with continuous light and fed *ad libitum* with *Scenedesmus* algae). Four days prior to the beginning of the experiment, animals were transferred into new microcosms to ensure no juveniles were present. Juveniles born within the next four days (96 hours) were collected, sexed (using a dissecting microscope at x8 to x12 magnification), placed individually in a 100 ml microcosm containing 60 ml of medium and were randomly assigned to the different treatments.

##### *(b) Experimental design*

We exposed individual juvenile female *D. magna* to either spores of *O. colligata*, *H. tvaerminnensis*, both parasites, or a placebo comprising of crushed uninfected *D. magna* individuals at two different timepoints (at day 0, “early exposure” and at day 5, “late exposure”) resulting in nine different combinations covering both sequential infection and simultaneous infection. Treatments in this fully factorial design included four single infections (each of the parasites early and late), two simultaneous co-infections (early and late), two sequential co-infections (*O. colligata* early and *H. tvaerminnensis* late; *H. tvaerminnensis* early and *O. colligata*

late), and one control treatment (two placebo doses at early and late timepoints, Figure 4.1). Each treatment included 30 replicates, for a total of 270 animals. Animals were kept under a 16:8 light-dark photoperiod at a constant temperature of 20°C and were transferred to new microcosms with fresh artificial *Daphnia* medium ADaM (Ebert, Zschokke-Rohringer and Carius, 1998) twice per week (after the initial infection period where transfers occurred four days post infection, on day 4 and day 9) to avoid build-up of waste products and offspring produced during the experiment. Each animal was fed with 1ml of batch cultured *Scenedesmus* algae grown in WC medium (Kilham *et al.*, 1998) four times a week during the experiment, with the density of algae increasing in increments from five million per ml to 12 million per ml in week one, then remaining constant at 12 million per ml for the remainder of the experiment. *Daphnia* were monitored until natural death, and host fecundity and survival, and parasite infection and burden (number of spores within the host) were recorded.

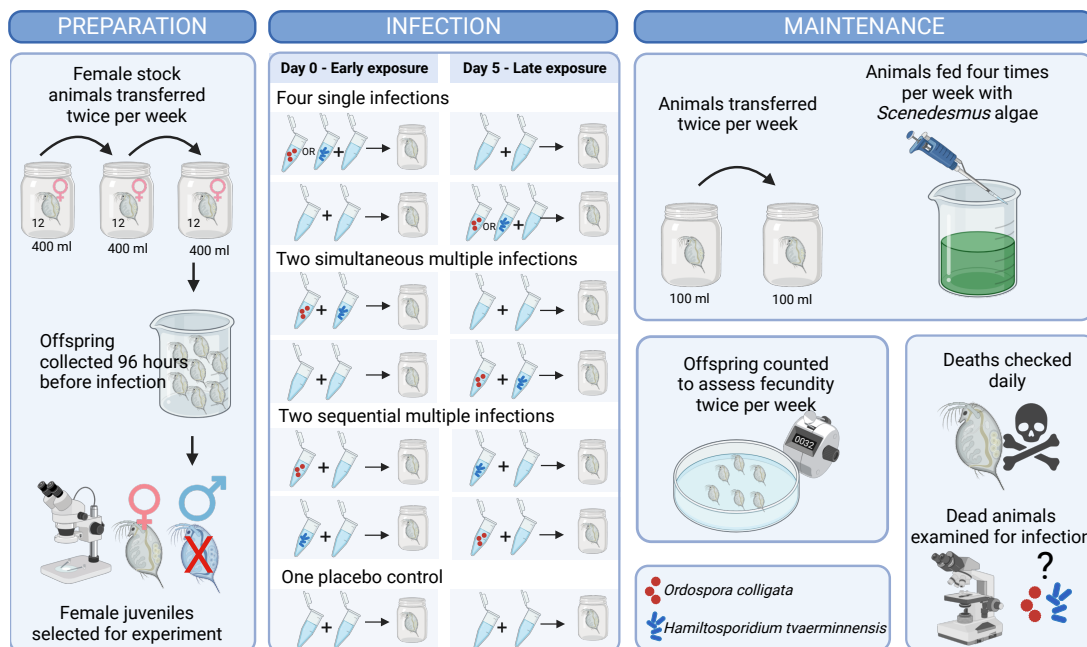


**Figure 4.1.** Illustration of the experimental set up. The left panel shows the preparation and generation of the juveniles used in the experiment, carried out between 28 and 4 days before infection. The central panel shows the infection process carried out between 0 and 5 days after

infection. Nine treatments were included in the experiment: four single infection treatments (“OC Early”: only exposed to *O. colligata* on Day 0; “HT Early”: only exposed to *H. tvaerminnensis* on Day 0; “OC Late”: only exposed to *O. colligata* on Day 5; “HT Late”: only exposed to *H. tvaerminnensis* on Day 5), two simultaneous co-infection treatments (“Both Early”: exposed to both *O. colligata* and *H. tvaerminnensis* on Day 0; “Both Late”: exposed to both *O. colligata* and *H. tvaerminnensis* on Day 5), two sequential co-infection treatments (“OC Early & HT Late”: exposed to *O. colligata* on Day 0 and then exposed to *H. tvaerminnensis* on Day 5; “HT Early & OC Late”: exposed to *H. tvaerminnensis* on Day 0 and then exposed to *O. colligata* on Day 5), and one placebo treatment (Control), which was not exposed to any parasite but given equal volumes of crushed up uninfected *D. magna*. The right panel shows the maintenance and measurements taken during the experiment carried out between 9 and 115 days after infection. Figure created on Biorender.com.

### *(c) Parasite spore preparation and exposure*

On infection days (day 0 and day 5 of the experiment), *Daphnia* were exposed to either 50,000 spores of *O. colligata*, 35,000 spores of *H. tvaerminnensis*, or a placebo dose. Appropriate spore doses were selected based on previous experiments conducted with these parasites. The spore dose of *O. colligata* was prepared by crushing *O. colligata* infected individuals with known prevalence and burden (determined by phase contrast microscopy on a subsample of the stock population) using a mortar and pestle and diluting this suspension of spores to 50,000 spores per ml. Similarly, the *H. tvaerminnensis* dose was generated by crushing *H. tvaerminnensis* infected individuals, counting the resulting spore suspension using a haemocytometer [Neubauer improved] and phase contrast microscopy and diluting down the suspension to 35,000 spores per ml. The placebo dose was prepared by crushing uninfected *D. magna* (of the Fi-Oer-3-3 strain which was also used to grow both parasites). On days 0 and 5, each *Daphnia*

received a total of 2 ml of either placebo, infective doses, or a combination of placebo and infective doses depending on the treatment. For single infections, this included 1 ml of the appropriate infective dose and 1 ml of the placebo dose. For simultaneous co-infection, this meant 1 ml of *O. colligata* infective dose and 1 ml of *H. tvaerminnensis* infective dose. *Daphnia* received 2 ml of placebo doses if no infection was required at the timepoint (Figure 4.1). At both infection timepoints, *Daphnia* were transferred four days after infection (day 4 and day 9 respectively) to allow sufficient exposure time to the spores of the parasites. The same doses were used at both timepoints for *O. colligata*, *H. tvaerminnensis* and the placebo to keep the numbers of spores per ml as consistent as possible over the two infection timepoints, as previous experiments have shown that *O. colligata* and *H. tvaerminnensis* can both persist in the environment for extended periods of time (Vizoso, Lass and Ebert, 2005; Pombert *et al.*, 2015).

#### **4.3.3 Measurements**

Animals were checked for mortality daily, and any animals which died were subsequently examined for infection. Status of infection and spore burden were checked by placing the animals on a slide in 100 µl of water. *O. colligata* infection was determined by dissecting the animals under a stereo microscope and counting the number of spore clusters (as *O. colligata* spores are known to form clusters of up to 64 spores) in the gut of the animal using phase contrast microscopy at x400 magnification. *H. tvaerminnensis* infection was measured by crushing and homogenising the dissected animal on the slide and examining 12.5 µl of this liquid on a haemocytometer using phase contrast microscopy at x400 magnification. Lifetime reproductive success of the host was calculated by summing the offspring counts which were performed twice a week following transfers. Once experimental animals were transferred to fresh microcosms, any remaining offspring were counted in a Petri dish which was placed on a light box to ensure all individuals were visible.

#### 4.3.4 Statistical analyses

All statistical analyses were performed using R (version 4.0.2). All animals were included to determine infection rates as there were no deaths until two weeks after infection, by which point infections could be reliably scored (Larsson, Ebert and Vavra, 1997; Haag *et al.*, 2011). Similarly, all spore counts were included as animals were followed until their natural death and therefore spore burden represent the lifetime reproductive success of *H. tvaerminnensis* (only transmitted upon death of the host) and abundance upon death for *O. colligata* (continuous transmission throughout the lifetime of the host). Analyses on host fitness measures (mortality and fecundity) were done for both animals that were exposed to the parasites and those that became successfully infected to examine whether exposure or establishment of infection was driving the patterns observed in the data. The first analyses using exposure to the parasite(s) as the explanatory variable can be found in the result section, while the second set of analyses where the data were filtered to include only true infections (infections by one parasite in the single exposure treatments and infections by both parasites in the double exposure treatments) can be found in supplemental figure C1. Analyses of infection rates, spore burden, fecundity and average survival used a generalised linear model (GLM) with appropriate error distributions which was compared to the null model using the Chi-squared distribution and Tukey post-hoc tests were used to determine differences between treatments. Response variables measuring parasite success (infection rates and spore burden) were analysed for each of the six treatments (three exposure treatments (single, sequential and simultaneous) at two timepoints (early and late), i.e.,  $y \sim \text{treatment}$ ) for each parasite. Response variables measuring host success (average survival and fecundity) were analysed in the same manner but also included the double-placebo exposed control animals. Survival over time was assessed through a Kaplan-Meier survival analysis and a log rank test, where survival was analysed for all treatments (single *O. colligata*,

single *H. tvaerminnensis*, sequential and simultaneous, at each of the two timepoints) and was also compared to double-placebo exposed control animals.

## 4.4 Results

### 4.4.1 Parasite fitness

Exposure to both parasites and timing of exposure did not influence infection rates across most of the treatments (Figure 4.2A, B and E). *O. colligata* displayed high infection rates across all affected treatments (between 73% and 93%) (Figure 4.2B), regardless of whether animals were single or double exposed and timing of exposure (GLM, analysis of deviance,  $df=5$ ,  $p=0.324$ ). We observed a similar pattern in *H. tvaerminnensis*, where five of the six treatments had comparable infection rates (between 40% and 77%), although the final treatment (late exposed simultaneous infection, 90% infection rate) differed from the early simultaneous treatments and late single *H. tvaerminnensis* treatment (Tukey post-hoc test,  $p=0.049$  and  $p=0.00428$ , respectively). Similarly to *O. colligata* infection rates, there was no effect of treatment on *O. colligata* spore burden, with average spore burden between 301 and 464 spore clusters per animal observed across all treatments (Figure 4.2D) (GLM, analysis of deviance,  $df=5$ ,  $p=0.715$ ). *H. tvaerminnensis* spore burden in single infections (~1.6 million spores per ml), however, was tenfold higher than those observed in double exposed treatments (~0.2 million spores per ml), regardless of whether they were exposed early or late (Figure 4.2C) (GLM, analysis of deviance,  $df=5$ ,  $p<0.001$ ). Furthermore, *H. tvaerminnensis* produced fewer spores in both early co-infected treatments (early sequential and early simultaneous) compared to their late exposed co-infected counterparts (late sequential and late simultaneous) (Figure 4.2C) (GLM, analysis of deviance,  $df=5$ ,  $p<0.001$ ).

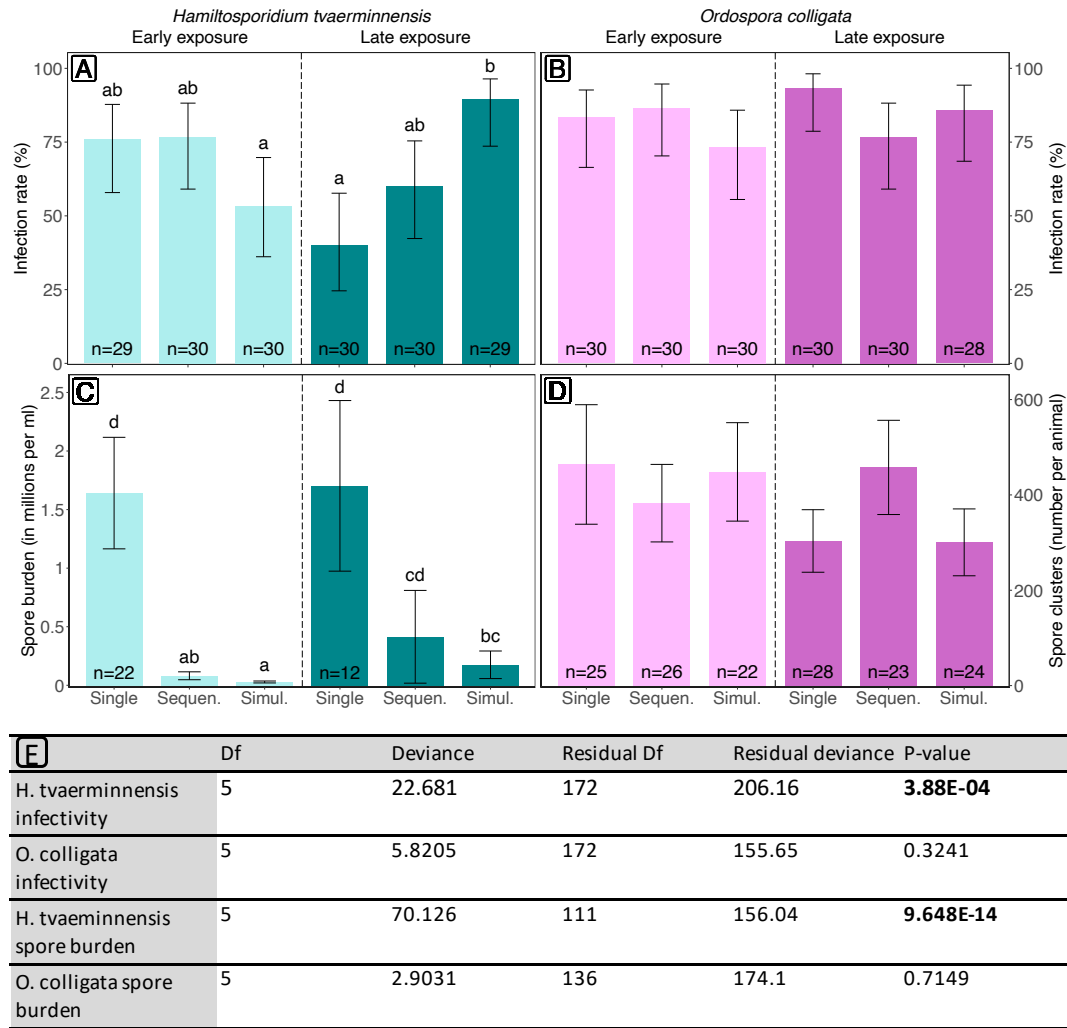


**Figure 4.2.** Impacts of treatment on parasite fitness. Panels A and B show the infection rates of *H. tvaerminnensis* and *O. colligata* respectively. Panels C and D show the number of spores present in infected animals for *H. tvaerminnensis* and *O. colligata* respectively. Treatments may be single infections, sequential co-infections (Sequen.) or simultaneous co-infections (Simul.) and are split into early and late exposures. Error bars represent 95% confidence intervals for infection rates and standard error for spore burdens. Sample sizes for each treatment are indicated on each bar but partially omitted from panel C for legibility. Omitted sample sizes for *H. tvaerminnensis* early exposed sequential, early exposed simultaneous, late exposed sequential and late exposed simultaneous are n=23, n=16, n=18 and n=26 respectively. Statistical significance is indicated through letters visible above each bar which represent the

results of Tukey post-hoc tests. Panel E summarises the GLM statistics for parasite infectivity and spore burden of *H. tvaerminnensis* and *O. colligata* and significance was obtained through a Chi-squared analysis of deviance.

#### **4.4.2 Host fitness**

Animals in single exposed treatments lived longer than those in double exposed treatments regardless of timing of exposure (Figure 4.3B, C and F), and survival analysis by means of the Kaplan-Meier method detected differences in survival over time (Figure 4.3A). Single exposed animals lived on average 18 days longer than their double exposed counterparts (Figure 4.3B and C) (GLM, analysis of deviance,  $df=6$ ,  $p<2.2e^{-16}$ ), except for the early exposed single *O. colligata* treatment which showed no difference in mortality to the early co-infected treatments (Figure 4.3C). In contrast to increased mortality being driven by co-exposure, patterns observed in the fecundity data are driven by age effects caused by early *O. colligata* exposure. Indeed, single and double exposed animals which encountered the *O. colligata* parasite earlier produced fewer offspring than their late exposed counterparts (Figure 4.3E and F) (GLM, analysis of deviance,  $df=6$ ,  $p<2.2e^{-16}$ ), suggesting that early exposure results in an increase in *O. colligata* virulence. Similarly, *H. tvaerminnensis* exposed animals had consistent fecundity across all treatments, except in those where animals were exposed to *O. colligata* at the early timepoint, where offspring production was reduced (Figure 4.3D and F) (GLM, analysis of deviance,  $df=6$ ,  $p<2.2e^{-16}$ ). Results shown here include animals that were infected by at least one parasite at the end of the experiment (although animals with spores of neither parasite were excluded). Analysis using only double infected animals in co-exposed treatments showed similar results although in some cases with reduced significance due to lower replication (Figure C1).

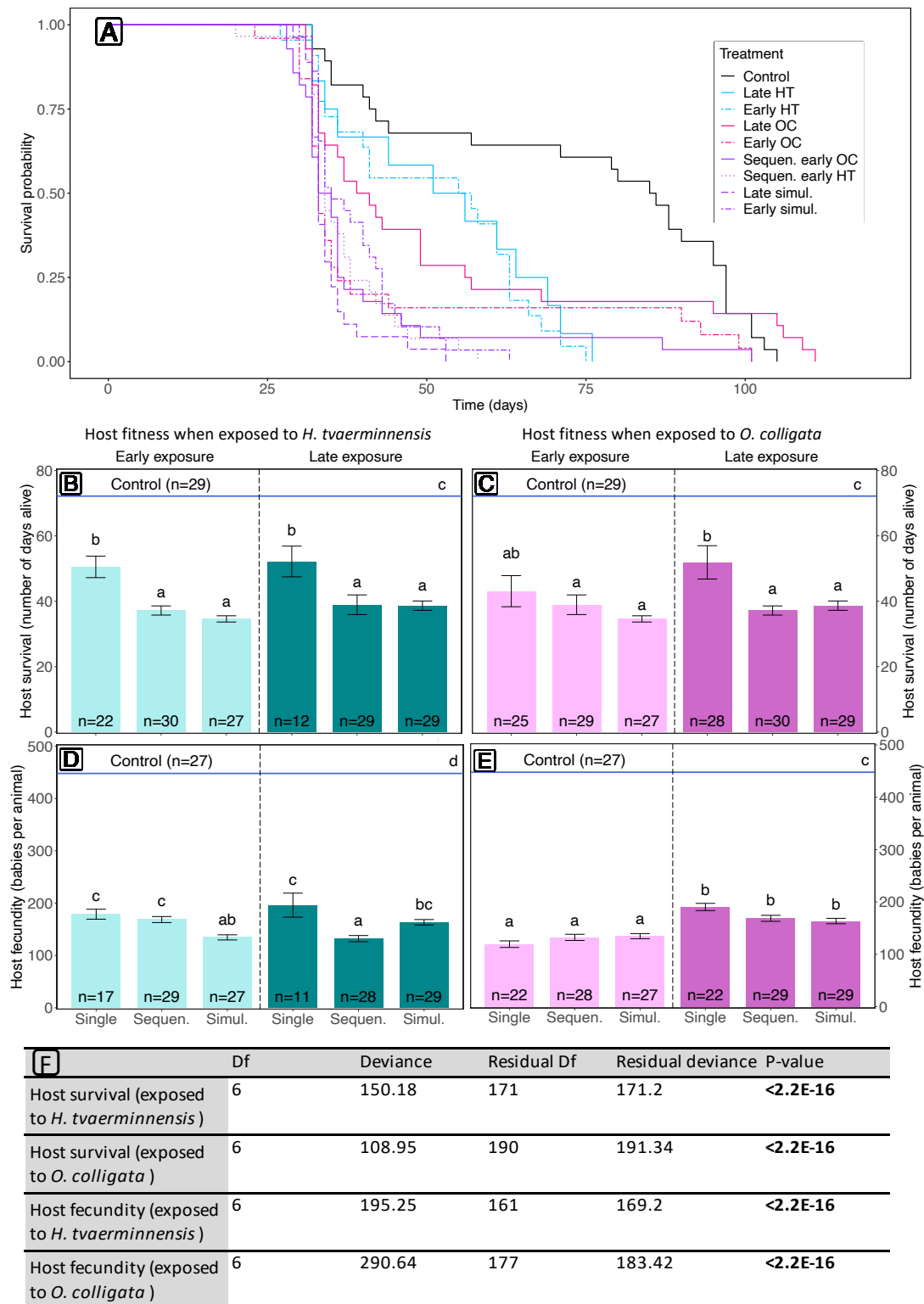


**Figure 4.3.** Impacts of treatment on host fitness. Panel A shows projected survival over time for all treatments using a Kaplan-Meier survival analysis. Panels B and C show host survival for treatments exposed to *H. tvaerminnensis* and *O. colligata* respectively. Panels D and E show

host fecundity for animals exposed to *H. tvaerminnensis* and *O. colligata* respectively. Treatments may be single infections, sequential co-infections (Sequen.) or simultaneous co-infections (Simul.) and are split into early and late exposure timepoints. Error bars represent standard error for panels B-E. Sample sizes for each treatment are indicated on each bar. Blue horizontal lines in panels B-E represent the control treatment. Statistical significance is indicated through letters visible above each bar which represent the results of Tukey post-hoc tests. Panel F summarises the GLM analyses carried out for host mortality and fecundity when exposed to *H. tvaerminnensis* and *O. colligata* and shows the results of a Chi-squared analysis of deviance.

## 4.5 Discussion

We observed age effects on the host with regards to fecundity, where animals which were exposed early to *O. colligata* produced fewer offspring (both from *H. tvaerminnensis* and *O. colligata* perspectives) than animals which were exposed at the later timepoint, regardless of whether exposures were single, sequential, or simultaneous. In addition, we found that multiple exposure to *O. colligata* and *H. tvaerminnensis* led to increased mortality in the *Daphnia* host, with *Daphnia* survival being higher in single exposure treatments (except in hosts exposed early to *O. colligata* where the difference was not significant). These higher rates of mortality in multiple-infected treatments are likely to be responsible for the lower spore burden observed in *H. tvaerminnensis*, as in these treatments this parasite had reduced time to grow.

### 4.5.1 Age effects

We observed age effects in the fecundity of *O. colligata* exposed animals, where early exposed animals experienced higher levels of virulence. Regardless of whether animals were double or single exposed, those that were exposed to *O. colligata* at the early timepoint produced fewer

offspring than those that were exposed later in life, despite similar average survival. The decreased fecundity in earlier infected animals may have been caused by a trade-off between investment in the immune response and reproductive output (Gustafsson *et al.*, 1994; Sheldon and Verhulst, 1996), as immune function requires a high energy expenditure (Demas *et al.*, 1997; Rauw, 2012), or may be mechanistically linked to reproductive function as genes involved in the immune response may also play a role in reproductive function (i.e. pleiotropy) (Fuchs *et al.*, 2014). Additionally, differences in host immunity between juvenile and adult animals, which have been observed in numerous organisms (e.g., in rodents (Cote *et al.*, 2000), purple martin birds (Davidar and Morton, 1993), and honeybees (Roberts and Hughes, 2014)), may have contributed to the observed differences. Changes in disease transmission have also been linked to age in humans, with older individuals being more susceptible to Covid-19 (Davies *et al.*, 2020), exhibiting more symptoms (Davies *et al.*, 2020) and suffering higher fatality rates (Levin *et al.*, 2020). Although immune responses vary depending on the pathogen encountered (Shoham and Levitz, 2005), host susceptibility may be dependent on age of infection (Hamley and Koella, 2021), and previous research in *D. magna* found that younger animals may be less efficient at dealing with parasitic infection (Izhar and Ben-Ami, 2015; Ben-Ami, 2019), potentially explaining the higher costs to fecundity we observe. The existence of age effects in the *D. magna*-*O. colligata* system may have implications for studies using this host-parasite pairing as a model, including studies creating a predictive framework for the effects of climate change on parasitism (Kirk *et al.*, 2018; Kirk *et al.*, 2020; Kunze *et al.*, 2022), as many of these studies often only infect juvenile animals and do not consider age-mediated changes. Indeed, studies have found that transmission of certain diseases is dependent on age structure of the affected population (Cook, McMeniman and O'Neill, 2008; Brooks-Pollock, Cohen and Murray, 2010; Laskowski *et al.*, 2011), and the inclusion of population age structure in some predictive models is critical to their accuracy (Castillo-Chavez *et al.*, 1989; Dowd *et al.*, 2020). In conclusion, while there are clear

effects of age of exposure on reproductive output in the *Daphnia-Ordospora* system, the epidemiological consequences of these effects need be studied in more detail.

#### **4.5.2 Host effects**

Concurrent exposure to both *H. tvaerminnensis* and *O. colligata* resulted in higher virulence, with animals which were exposed to both parasites generally dying earlier than those that were exposed to single parasites. Although host mortality may be exacerbated by multiple infection (Knowles, 2011; Chu *et al.*, 2016; Zilio and Koella, 2020), virulence in co-infection scenarios has frequently been observed to be determined by just one of the co-infecting parasites (De Roode *et al.*, 2005; Ben-Ami, Mouton and Ebert, 2008; Manzi *et al.*, 2021). However, this does not seem to be the case for our study system with single infections of both parasites living longer than multiple infections. Similar results where both parasites contribute to mortality have been found in several other systems (Su *et al.*, 2005; Louhi *et al.*, 2015), although the mechanism behind this increased mortality may depend on the host-parasite system in question. Indeed, within-host competition for resources (Choisy and de Roode, 2010), interactions with the host immune system (Cressler *et al.*, 2016) or unrelatedness of concurrent parasites (Gleichsner, Reinhart and Minchella, 2018) may all lead to increased virulence. However, while both parasites in our system contribute to increased average host mortality, early mortality and fecundity patterns seem more strongly affected by *O. colligata*.

Despite both parasites contributing to overall virulence, *O. colligata* is more virulent in the earlier stages of the infection process, as seen by increased early mortality and decreased offspring production. This may be explained by the difference in life histories of the two parasites. *O. colligata* spores are horizontally transmitted (Ebert, Lipsitch and Mangin, 2000) while *H. tvaerminnensis* spores are transmitted vertically from parent to offspring, as well are horizontally upon death of the host (Vizoso and Ebert, 2005b; Haag *et al.*, 2011). Given that

vertically transmitted parasites increase their fitness by allowing their host to reproduce, they often evolve towards lower virulence (Ebert, 2013; Cressler *et al.*, 2016). Moreover, despite *O. colligata* being a horizontally transmitted parasite, the large reduction in fecundity following exposure to *O. colligata* (-49% when compared to control) is at odds with previously published work (Ebert, Lipsitch and Mangin, 2000) where *O. colligata* has been described as a benign parasite only reducing fecundity by up to 20%. These differences may be attributed to experimental design and/or conditions under which experiments were conducted. Indeed, previous work on microsporidians has shown that they require high levels of phosphorous to grow (Aalto and Pulkkinen, 2013) and that parasite spore loads within a host can be dependent on the genotype of both the host and the parasite (Refardt and Ebert, 2007). However, it is clear that under some circumstances *O. colligata* can have higher virulence than previously reported and that this, when combined with *H. tvaerminnensis* exposure, can reduce survival in multiply exposed treatments which can have consequences for parasite fitness and the evolution of virulence.

#### **4.5.3 Parasite fitness**

While the fitness of *O. colligata* was not affected by multiple infection, the spore burden of *H. tvaerminnensis* decreased in multiply exposed animals, and infection rates of *H. tvaerminnensis* differed when co-exposed with *O. colligata*. Previous studies investigating co-infection have frequently observed that one parasite may impede the establishment or development of the other (Gold, Giraud and Hood, 2009; Ben-Ami, Rigaud and Ebert, 2011; Manzi *et al.*, 2021). In our system, the reduction of *H. tvaerminnensis* spore burden in multiply infected treatments is likely due to the increased mortality in these treatments, giving *H. tvaerminnensis* less time to develop leading to lower spore burdens. In addition, being a gut parasite (Ebert, 2005), *O. colligata* may have monopolised host resources, leading to reduced resource availability for *H. tvaerminnensis*. Indeed, several studies have suggested that parasitic infection may modify host

resource levels (Dobson, 1988; Tocque and Tinsley, 1994; Ebert, 2005), impacting host resource quality, and potentially reducing the fitness of a second parasite (Norton *et al.*, 2008; Randall *et al.*, 2013), especially in cases where the parasites have conflicting interests with respect to resource allocation (Karvonen, Jokela and Laine, 2019). While *O. colligata* may, by its position in the host gut, have easier access to host resources, we found no impact of prior residency, as sequential and simultaneous infections led to consistent infection rates and spore burden across the affected treatments. Absence of prior residency has been reported in several studies (Ben-Ami, Mouton and Ebert, 2008; Lohr, Yin and Wolinska, 2010; Ben-Ami, Rigaud and Ebert, 2011; Hoverman, Hoyer and Johnson, 2013), although it has also been observed numerous times (De Roode *et al.*, 2005; Ezenwa *et al.*, 2010; Karvonen, Jokela and Laine, 2019). Another *Daphnia* study found that prior residency both benefitted and disadvantaged co-infecting parasites, depending on which parasite infected first (Lohr, Yin and Wolinska, 2010). Interactions among parasites combined with prior residency effects can thus lead to complex infection patterns, and despite the lack of prior residency in our study system, there is some evidence that interactions between both parasites could impact parasite fitness.

Interactions between both parasites may explain why late simultaneous infections of *H. tvaerminnensis* have higher spore burden and infection levels than early simultaneous infections, and higher infection rates than late single infections. This pattern could be explained by stronger immunity in younger animals, which would explain the lower *H. tvaerminnensis* infection rate and burden. However, this is at odds with previous research which found that younger *Daphnia* are less efficient at fighting off parasitic infection (Izhar and Ben-Ami, 2015; Ben-Ami, 2019) and with our own finding that young animals experience higher virulence. Alternatively, *H. tvaerminnensis* may elude the immune response when co-infecting with *O. colligata*, therefore causing *H. tvaerminnensis* infection rates to be lower in the late single treatment than in the late simultaneous treatment. However, if co-exposure with *O. colligata* is

indeed driving this pattern, it must be age specific given that the pattern is not visible in the early simultaneous treatment when compared to the early single treatment. While the mechanism underlying these differences remains unclear, an interaction between host age and immunity could potentially be present. Indeed, given that we observed that exposure of the host to the parasite without successful establishment of infection was sufficient to cause the same patterns in host fitness (survival and fecundity) as we observed in infected animals, it seems likely that the immune system did play a role. Immune-mediated competition is known to play a role in within-host interactions of co-infecting parasites, and may facilitate or hinder the existence of co-infection (Read and Taylor, 2001). While immunity may thus modify the outcome of the interaction between *H. tvaerminnensis* and *O. colligata*, the observed reduction in *H. tvaerminnensis* spore burden is due in large part to the increased mortality in multiply exposed treatments, which may in turn influence the evolution of virulence in this system.

#### **4.5.4 Virulence**

Multiple infection may alter the evolutionary trajectories of co-occurring parasites (Tollenaere, Susi and Laine, 2016), which in turn may affect population dynamics (van Baalen and Sabelis, 1995; Heesterbeek *et al.*, 2015) and species coexistence (Escribano *et al.*, 2001). Indeed, both modelling work and empirical data suggest that multiple infection can lead to altered levels of virulence which can change host-parasite interactions (Alizon, 2008; Alizon, de Roode and Michalakis, 2013), whether due to increasing (Taylor, Walliker and Read, 1997; López-Villavicencio *et al.*, 2011; Vojvodic *et al.*, 2012; Susi *et al.*, 2015) or decreasing virulence (Hood, 2003; Massey, Buckling and French-Constant, 2004; Schürch and Roy, 2004). In addition, transmission modes may have strong implications for parasite virulence (Cressler *et al.*, 2016), as horizontally transmitted parasites are generally expected to be more virulent than vertically transmitted parasites due to the latter's need to keep their host alive for longer to effectively reproduce (Ebert, 2013). In our system, given that *O. colligata* seems to be the more virulent of

the two parasites (contrary to expectation), as it inhibits *H. tvaerminnensis* transmission through reduced spore growth and offspring production (vertical transmission), in areas where both parasites co-occur *O. colligata* may reduce the prevalence of *H. tvaerminnensis*. In response, when it is frequently co-infecting with *O. colligata*, *H. tvaerminnensis* may evolve increased levels of growth and horizontal transmission, and therefore higher virulence, to offset the earlier mortality and reduced fecundity caused by *O. colligata*. Indeed, higher virulence is a frequent outcome when parasites compete (Taylor, Walliker and Read, 1997; Vojvodic *et al.*, 2012), although reduced virulence has been observed when parasites cooperate (Hood, 2003; Schürch and Roy, 2004), while in other cases patterns of virulence can be obscured by phenotypic plasticity (Choisy and de Roode, 2010).

Shifts in virulence, in other systems, have been reported to lead to extinction of the host population (Rafaluk *et al.*, 2015) and can affect disease dynamics (van Baalen and Sabelis, 1995). Moreover, co-infecting parasites may alter the outcome of apparent competition (Rovenolt and Tate, 2022), affect host behaviour (Haine, Boucansaud and Rigaud, 2005) and may explain a larger proportion of variation than commonly studied environmental factors (Telfer *et al.*, 2010). Thus, interactions among co-infecting parasites can have far reaching consequences and can impact evolutionary trajectories (Cressler *et al.*, 2016), population dynamics (Laurenson *et al.*, 2003), human health (De Roode *et al.*, 2005) and animal health (Graham, 2008). Indeed, given their key role in aquatic ecosystems, alterations to *Daphnia* population dynamics may have far reaching consequences (Carpenter *et al.*, 2001; Sarnelle, 2005).



## **Chapter 5. Interactions between microsporidian parasites and temperature modify the outcome of multiple infection at extreme temperatures in *Daphnia***

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**Author contributions:** FEO’K and PL contributed to the experimental design. FEO’K conducted the experiment with assistance from VR and PL. FEO’K conducted the statistical analyses and created the figures. FEO’K wrote the first draft of the manuscript and PL and CVH contributed to revisions. PL and CVH supervised the project.

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### **5.1 Abstract**

Human-induced global change is causing rising temperatures as well as increasing the number of extreme weather events such as heatwaves, both of which have been shown to modify the dynamics of disease and may increase parasite fitness and host mortality. However, although co-infections are prevalent in nature, representing up to 80% of infections in certain populations, research investigating the impacts of increasing temperature and temperature variability has focused on single parasites and their hosts. Here, we expose the freshwater

invertebrate *Daphnia magna* to two of its microsporidian parasites *Hamiltosporidium tvaerminnensis* and *Ordospora colligata*, across five constant temperatures ranging from 10.5 to 24.5°C and one heatwave treatment with a mean of 17.5°C. We find that despite their differences in thermal tolerance, *H. tvaerminnensis* is outcompeted by *O. colligata* across the temperature range, as animals in co-exposed treatments produced fewer *H. tvaerminnensis* spores than those in single exposed treatments, although the impact of co-exposure with *O. colligata* on *H. tvaerminnensis* infection rates was exacerbated at warmer temperatures. Additionally, *H. tvaerminnensis* generally mediates host fitness in co-exposed treatments, while *O. colligata* seems to dictate host fecundity in multiply-exposed treatments. Results from this study suggest that *H. tvaerminnensis* may evolve towards higher virulence in scenarios where both parasites are present to avoid being outcompeted by *O. colligata*, although given that *O. colligata* has a lower temperature tolerance than *H. tvaerminnensis*, it is unclear whether this pattern will hold at temperatures above 24.5°C.

## 5.2 Introduction

Widespread global change caused by human activity is leading to changes in climatic patterns such as rising temperatures (Hansen *et al.*, 2006) and increased frequency of heatwave events (Schär *et al.*, 2004; Harris *et al.*, 2018). These modified temperatures are leading to changes in the spread and outbreak of infectious diseases, which in turn may affect human and animal health (Hilgenfeld and Peiris, 2013), food security (Chakraborty and Newton, 2011), and biodiversity (Bates, Hilton and Harley, 2009). Impacts of increasing temperatures and extreme temperature events on host-parasite life-history traits include increased disease transmission (Ben-Horin, Lenihan and Lafferty, 2013; Harvell *et al.*, 2019) and within-host parasite growth (Labaude, Rigaud and Cézilly, 2015; Kirk *et al.*, 2018; Kunze *et al.*, 2022), while traits such as host survival and fecundity may decrease in response to an increase in temperature and extreme weather events (Altizer *et al.*, 2013; Abarca, Larsen and Ries, 2019; Green *et al.*, 2019; Zhang *et*

*al.*, 2019b). However, these effects may be dependent on the host-parasite system examined (Altizer *et al.*, 2013). For example, in a study examining the effects of increasing temperatures on the trematode *Ribeiroia ondatrae* and its amphibian host, despite increasing host penetration and reducing encystment, increases in temperature had no overall effect on tadpole parasite burdens (Paull, LaFonte and Johnson, 2012). Conversely, a study investigating the impacts of increasing temperature and temperature variation on the microsporidian *Ordospora colligata* and its host *Daphnia magna* found the highest levels of infection in moderate temperatures, although exposure to a heatwave decreased the optimum temperature for spore production and increased spore burden almost tenfold (Kunze *et al.*, 2022). Such differences may be explained by temperature-induced changes in host immune function (Cheng, Wang and Chen, 2005; Raffel *et al.*, 2006), differences in metabolic requirements (Kirk *et al.*, 2019), or mismatched host and parasite thermal performance curves. Nevertheless, studies have almost exclusively focused on how changing temperatures associated with global warming impact single parasite systems, while multiple infections are common in nature.

Co-infections are frequent in real-world systems (Lively *et al.*, 2014), and up to 80% of parasitic infections may be multiple infections in certain systems (Petney and Andrews, 1998). Both synergistic and antagonistic effects have been observed in multiply infected systems (Vaumourin *et al.*, 2015; Cressler *et al.*, 2016), as competing parasites may impede one another in establishing or maintaining infection (Norton *et al.*, 2008; Ben-Ami, Rigaud and Ebert, 2011), but may also decrease host fitness (Tocque and Tinsley, 1994; Knowles, 2011; Marchetto and Power, 2018). However, their interactions may be mediated by parasite genotype (Louhi *et al.*, 2015), host availability (Godinho *et al.*, 2023), timing of exposure (Hood, 2003) and host age at infection (O'Keeffe *et al.*, 2024). Furthermore, temperature is an important predictor of where multiple infection outbreaks will be able to occur (Brooker and Clements, 2009; Oakgrove *et al.*, 2014; Poterek *et al.*, 2022), suggesting it may have a strong influence on the outcome of co-infection.

However, the specific effects may be dependent on the thermal tolerance of the host-parasite system (Fargues and Bon, 2004; Herczeg *et al.*, 2023). Increases in temperature may exacerbate infection and cause higher host mortality (Shameena *et al.*, 2021) or facilitate disease transmission (Terradas *et al.*, 2023) in scenarios of co-infection, but the opposite has also been observed, with increased temperatures mediating a decrease in infection intensity of co-infected treatments (Herczeg *et al.*, 2023). Moreover, co-infection may lead to changes in parasite virulence (Alizon, 2008) with repercussions such as changes in disease dynamics (van Baalen and Sabelis, 1995) or host population extinction (Rafaluk *et al.*, 2015), and temperature may mediate changes in competitive interference and within-host dynamics (Sardanyés *et al.*, 2022). Therefore, the complexity of interactions among co-infecting parasites may lead to difficulties in predicting their outcome, especially when considered in the context of increasing temperature variability.

Here, we investigate the impacts of increasing temperatures and a heatwave on *Daphnia magna*, co-exposed to two of its parasites over a large range of temperatures (~10.5-24.5°C). While both *Hamiltosporidium tvaerminnensis* and *Ordospora colligata* are microsporidian microparasites, *H. tvaerminnensis* is found in the fat and ovarian cells of its host and is transmitted both horizontally and vertically (from mother to offspring), while *O. colligata* is an obligate gut parasite which is horizontally transmitted only. Previous research investigating the outcome of co-infection in this system has found that *O. colligata* can outcompete *H. tvaerminnensis*, resulting in decreased *H. tvaerminnensis* infection rates and spore burden in co-infected treatments (O'Keeffe *et al.*, 2024). However, these parasites have different thermal tolerance curves, with *H. tvaerminnensis* seemingly more tolerant to extreme temperatures than *O. colligata* (Chapter 3). Given this difference in the thermal tolerance of the two parasites and their response to co-infection, we expect that competition dynamics may shift at the higher

end of the temperature range, allowing *H. tvaerminnensis* to outcompete *O. colligata* under increased temperatures or in heatwave conditions.

## 5.3 Materials and methods

### 5.3.1 Study system

*Daphnia magna* (order: *Cladocera*) is a widely utilised model organism in ecological and evolutionary studies (Ebert, 2005), given its ease of use in laboratories (Ebert, 2022), available genomic resources (Ebert, 2022) and its role as a keystone species in freshwater ecosystems (Jansen *et al.*, 2017; Altshuler *et al.*, 2011). *D. magna* is ubiquitous throughout the Northern Hemisphere (Lange, Kaufmann and Ebert, 2015), and is the host species for a range of parasites (Ebert, 2005) which have been observed to infect simultaneously in natural systems (Decaestecker *et al.*, 2005; Weigl *et al.*, 2012). Among the parasites infecting *D. magna* are the microsporidians *Ordospora colligata* and *Hamiltosporidium tvaerminnensis*, which are present throughout the range of *D. magna* and are both found in Western and Northern Europe (Ebert, 2005; Krebs, Routtu and Ebert, 2017). In addition to their overlapping ranges, these parasites share host resistance loci (Keller, Kirk and Luijckx, 2019), which may trigger a similar immune reaction in the host (Stirnadel and Ebert, 1997). Both *O. colligata* and *H. tvaerminnensis* are transmitted to the host when they are ingested during filter feeding (Ebert, 2005), but spores are shed differently for each of these two parasites. *O. colligata* is an obligate gut parasite whose spores are horizontally transmitted through continuous shedding into the environment via the faeces of the host. In contrast, *H. tvaerminnensis* is transmitted both vertically (from mother to offspring) and horizontally (upon death of the host) and infects the fat and ovarian cells of *D. magna*. Both of these parasites are considered to be relatively avirulent (Ebert, 2005; Narr and Frost, 2016), therefore reducing the risk of competitive exclusion observed in some other host-parasite pairings (Regoes *et al.*, 2003; Ben-Ami, Rigaud and Ebert, 2011), and have been successfully used in a previous multiple infection study (O'Keeffe *et al.*, 2024). The host and

parasite isolates used in this study were originally collected from the Tvärminne Archipelago in Finland (*D. magna* genotype Fi-Oer-3-3, *O. colligata* isolate OC3 and *H. tvaerminnensis*), and both parasites are known to infect this host genotype in natural populations (Haag *et al.*, 2020; Zukowski *et al.*, 2020).

### **5.3.2 Experimental procedure**

#### *(a) Host preparation*

To obtain sufficient juvenile female animals for the experiment and to minimise maternal effects, ~600 *D. magna* adult females were grown under standardised conditions for four weeks preceding the experiment. Female animals were kept in 400 ml microcosms with 200 ml of artificial *Daphnia* medium (ADaM) (Ebert, Zschokke-Rohringer and Carius, 1998) in densities of 10-12 animals per microcosm under 20°C with continuous light, were fed *ad libitum* with batch-cultured *Scenedesmus sp.* algae grown in WC medium (Kilham *et al.*, 1998) and were transferred to new microcosms with fresh medium twice per week. Four days prior to the beginning of the experiment, animals were transferred into new microcosms to ensure no juveniles were present. Juveniles born within the next 72 hours were collected, sexed (using a dissecting microscope at x8 to x12 magnification), placed individually in a 100 ml microcosm containing 60 ml of ADaM and were randomly assigned to different treatments. These animals were given 24 hours to acclimatise to their new environment before being exposed to infective doses on day 0 of the experiment.

#### *(b) Temperature*

Water temperature was regulated by a controller (Inkbird ITC-308D or ITC-310T-B) interfacing with aquarium chillers (Hailea HC150A or DC300) and heating rods (EHEIM JÄGER 300W), and water was circulated using microjet pumps (NemaMicro MC450) to equalise the temperature.

We included five baths running at constant temperatures (i.e., 10.5, 14, 17.5, 21 and 24.5°C) to test the effects of changing constant temperatures, and one bath with a heatwave at day 10 of the experiment (i.e., 17.1 to 23.5°C with a mean of 17.4°C) to test the effects of a heatwave event as compared to constant temperatures. Each water bath contained 96 microcosms split into four perforated trays which were rotated daily to avoid positioning effects in the baths. Realised temperatures in each bath were monitored by a HOBO temperature logger placed into an empty microcosm containing ADaM.

### *(c) Parasite preparation*

On day 0 of the experiment, animals were exposed to either 50,000 spores of *O. colligata*, 50,000 spores of *H. tvaerminnensis*, or a placebo dose. Appropriate spore doses were selected based on previous experiments conducted using these parasites (see Chapter 1 and O’Keeffe *et al.* (2024)). The *O. colligata* spore dose was prepared by crushing *O. colligata* infected individuals with known prevalence and burden (determined by phase microscopy on a subsample of the stock population) using a mortar and pestle and diluting this suspension of spores to 50,000 spores per ml. Similarly, the *H. tvaerminnensis* dose was generated by crushing *H. tvaerminnensis* infected individuals in a known volume of ADaM, counting the resulting spore suspension using a haemocytometer [Nebauer improved] under phase contrast microscopy and diluting the spore suspension to 50,000 spores per ml. The placebo dose was generated by crushing uninfected *D. magna* female adults of the Fi-Oer-3-3 strain used in this experiment. On day 0 of the experiment, each experimental animal received a total of 2 ml of either infective doses, placebo or a combination of infective doses and placebo depending on the treatment. Single exposed animals received 1 ml of the appropriate spore dose and 1 ml of the placebo, double exposed animals received 1 ml of each of the infective doses (1 ml of *O. colligata* and 1 ml of *H. tvaerminnensis*) and control animals received 2 ml of the placebo. Animals were exposed to infective doses for seven days (based on previous experiments, see Chapter 1 and

Kunze *et al.* (2022)) before being transferred to new microcosms containing fresh medium for the first time.

#### *(d) Experimental design and exposure*

We exposed juvenile female *D. magna* to either constant temperatures between 10.5 to 24.5°C, or to a heatwave event with a mean temperature of 17.4°C, in combination with either single exposures of *O. colligata* or *H. tvaerminnensis*, double exposures to both parasites, or a placebo dose for control animals. Each treatment combination had 24 replicates, for a total of 576 animals (24 replicates x 6 temperature treatments x 4 parasite treatments). Following the first transfer seven days after being exposed to the appropriate parasite doses, animals were transferred every four days for the remainder of the experiment to avoid build-up of waste products and offspring produced during the experiment. Animals were fed with 1 ml of *Scenedesmus* algae every two days, with the density of algae increasing in increments from five million per ml to 12 million per ml in week one, then remaining constant at 12 million per ml for the remainder of the experiment. Animals were monitored until natural death, with the exception of 43 individuals (all in the 10.5°C temperature treatment) that were still alive on day 208 (30 weeks) which were sacrificed and dissected.

#### **5.3.3 Measurements**

Animal survival was monitored daily, and animals which died were subsequently examined for infection. Status of infection and spore burden were checked by placing the animal on a microscope slide in 100 µl of water. *O. colligata* infection was determined by dissecting animals under a stereo microscope and counting the number of spore clusters (as *O. colligata* spores are known to form clusters of up to 64 spores) in the gut of the animals by using phase contrast microscopy at x400 magnification. *H. tvaerminnensis* infection and spore burden were quantified by crushing and homogenising the dissected animal on a microscope slide and

examining a 12.5 µl aliquot of the resulting liquid on a haemocytometer using phase contrast microscopy at x400 magnification. Offspring counts were taken twice per week following transfers of focal animals to new microcosms (leaving the produced offspring in the old microcosm), by counting any offspring in a Petri dish placed on a lightbox to ensure that all individuals were visible. These counts were used to calculate cumulative number of offspring over time and lifetime reproductive success of the host.

#### **5.3.4 Statistical analyses**

All statistical analyses were performed using R (version 4.0.2). All animals which died at least 2 weeks after infection were included in the analyses, by which point infection can be reliably scored (Larsson, Ebert and Vavra, 1997; Haag *et al.*, 2011). All spore burdens were included as animals were followed to their natural death and therefore spore burden represents the lifetime reproductive success of *H. tvaerminnensis* and the abundance upon death for *O. colligata*. All animals which were exposed to the parasite were included in analyses of host fitness, as comparisons using only infected animals and using all exposed animals showed no difference in results. Analyses of infection rates, spore burden, fecundity and average survival used a generalised linear model (GLM) with appropriate error distributions which was compared to the null model using the  $\chi^2$  distribution. Post-hoc comparisons using the *emmeans* package in R were conducted to determine differences between treatments. Response variables measuring parasite fitness (infection rates and spore burden) were analysed for each of the three parasite treatments (*O. colligata* exposed, *H. tvaerminnensis* exposed and double exposed) across the five temperatures examined (10.5, 14, 17.5, 21 and 24.5°C) i.e.  $y \sim \text{parasite} * \text{temperature}$ . Response variables measuring host fitness (fecundity and overall survival) were analysed in a similar manner but also included placebo-exposed control animals. Survival over time was assessed through a Kaplan-Meier survival analysis and a log-rank test using the *survminer* package in R, where survival was assessed for four parasite treatments (*O. colligata* exposed, *H.*

*tvaerminnensis* exposed, double exposed and control) at each temperature. Similarly to constant temperatures, heatwave treatments were assessed by means of a GLM with appropriate error distributions which was compared to the null model using the  $\chi^2$  distribution, and post-hoc comparisons were conducted using the *emmeans* package in R to determine differences between treatments. Analyses for heatwave treatments were identical but only included two treatments for temperature (constant and heatwave), i.e.  $y \sim \text{parasite} * \text{temperature regime}$ . A full table of p-values from emmean custom contrasts and Kaplan Meier pairwise comparisons can be found in the supplemental materials.

## 5.4 Results

### 5.4.1 Parasite fitness

#### (a) *H. tvaerminnensis* fitness

Temperature modifies the outcome of *H. tvaerminnensis* infection in *Daphnia* co-exposed to *H. tvaerminnensis* and *O. colligata*. Generally, higher levels of infection with *H. tvaerminnensis* were observed with increasing temperatures (Figure 5.1A and Table 5.1; GLM, analysis of deviance,  $df = 212$ ,  $p = 4.47e-07$ ), and animals in single exposed *H. tvaerminnensis* treatments had higher infection rates than those in double exposed treatments (Figure 5.1A and Table 5.1; GLM, analysis of deviance,  $df = 211$ ,  $p = 0.0074$ ). However, the difference in infection rates between single *H. tvaerminnensis* exposed and double exposed animals was exacerbated at higher temperatures, with the single exposed infection rates increasing more than their double exposed counterparts (Figure 5.1A and Table 5.1; GLM, analysis of deviance,  $df = 208$ ,  $p = 0.0426$ ). *H. tvaerminnensis* spore burden followed a similar pattern, with higher spore burdens observed in higher temperatures (Figure 5.1C and Table 5.1; GLM, analysis of deviance,  $df = 141$ ,  $p = 1.04e-10$ ) and in single exposed animals (Figure 5.1C and Table 5.1; GLM, analysis of deviance,  $df = 140$ ,  $p = 0.032$ ). However, while *H. tvaerminnensis* spore burden does show an

increasing difference between single and multiple infected individuals with increasing temperature, this difference was not significant (GLM, analysis of deviance,  $df = 138$ ,  $p = 0.734$ ). Furthermore, *H. tvaerminnensis* fitness was not affected by a heatwave event, as animals in constant and heatwave treatments had similar *H. tvaerminnensis* infection rates and spore burden (Figure 5.2A and C, and Table 5.2; GLM, analysis of deviance,  $df = 88$ ,  $p = 0.125$  and  $df = 61$ ,  $p = 0.786$  for infection rates and spore burden, respectively) regardless of whether they were single or double exposed (Figure 5.2A and C, and Table 5.2; GLM, analysis of deviance,  $df = 87$ ,  $p = 0.295$  and  $df = 60$ ,  $p = 0.243$  for infection rates and spore burden, respectively).

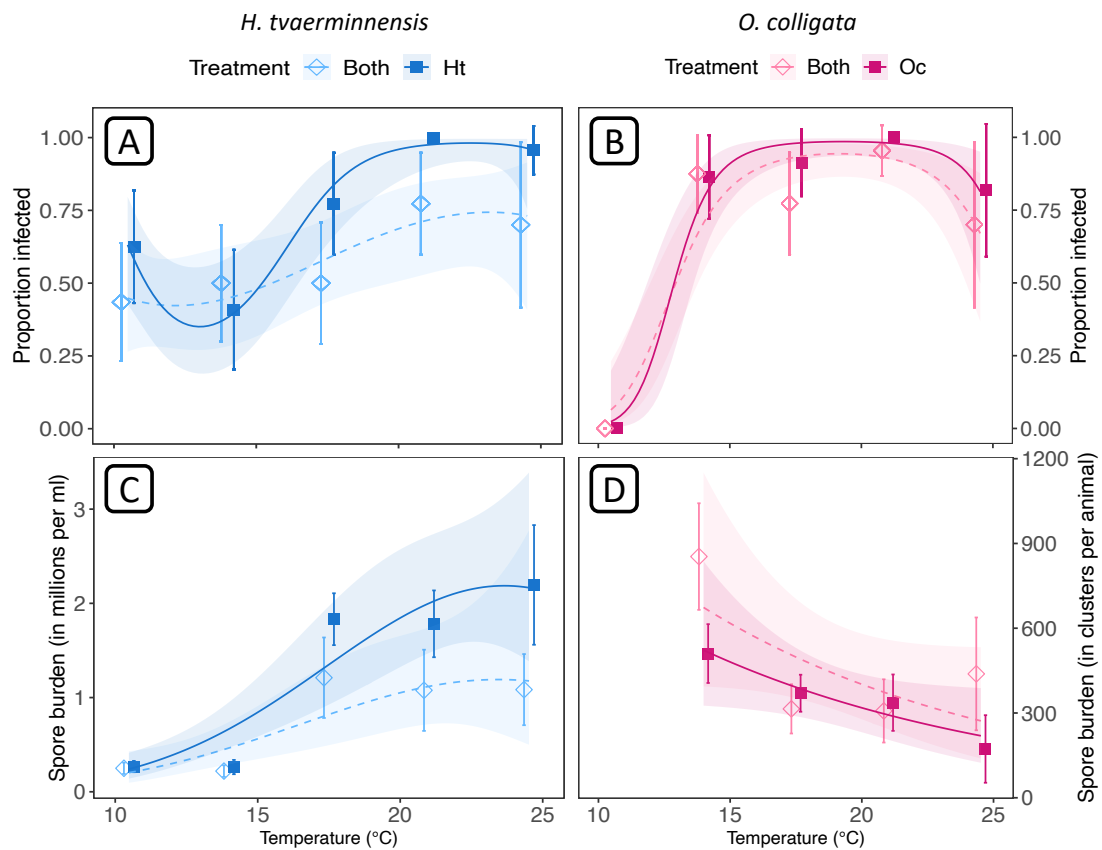


**Figure 5.1.** Impacts of multiple infection on parasite fitness across the range of temperatures tested. Panels A and B show the infection rates of *H. tvaerminnensis* and *O. colligata* respectively. Panels C and D show the spore burden (number of spores present in infected animals) for *H. tvaerminnensis* and *O. colligata* respectively. Treatments may be *H.*

*tvaerminnensis* exposed (Ht), *O. colligata* exposed (Oc) or double exposed (Both). Error bars represent 95% confidence intervals for infection rates and standard error for spore burden. Lines represent the predicted model fit and ribbons represent 95% confidence intervals of the predicted model fit.

**Table 5.1.** Summary of the GLM statistics for constant temperature treatments, including infection rates and spore burden of both parasites, and survival and fecundity of the host. Significance was obtained through a  $\chi^2$  analysis of deviance. Stars represent significance levels, i.e.  $p < 0.1$ ,  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  for •, \*, \*\*, and \*\*\* respectively.

|                                         | Df | Deviance | Residual df | Resid. deviance | p-value  |     |
|-----------------------------------------|----|----------|-------------|-----------------|----------|-----|
| <b><i>H. tvaerminnensis</i> fitness</b> |    |          |             |                 |          |     |
| Infection rates                         |    |          |             |                 |          |     |
| temperature (poly, 3)                   | 3  | 32.324   | 212         | 242.65          | 4.47E-07 | *** |
| parasite                                | 1  | 7.147    | 211         | 235.48          | 0.007397 | **  |
| temperature (poly, 3)*parasite          | 3  | 8.171    | 208         | 227.31          | 0.0426   | *   |
| Spore burden                            |    |          |             |                 |          |     |
| temperature (poly, 2)                   | 2  | 45.976   | 141         | 179.49          | 1.04E-10 | *** |
| parasite                                | 1  | 4.579    | 140         | 174.91          | 0.0324   | *   |
| temperature (poly, 2)*parasite          | 2  | 0.619    | 138         | 174.29          | 0.734    |     |
| <b><i>O. colligata</i> fitness</b>      |    |          |             |                 |          |     |
| Infection rates                         |    |          |             |                 |          |     |
| temperature (poly, 2)                   | 2  | 130.251  | 199         | 125.01          | <2E-16   | *** |
| parasite                                | 1  | 1.202    | 198         | 123.81          | 0.2728   |     |
| temperature (poly, 2)*parasite          | 2  | 1.652    | 196         | 122.16          | 0.4379   |     |
| Spore burden                            |    |          |             |                 |          |     |
| temperature                             | 1  | 7.8443   | 123         | 153.96          | 0.005098 | **  |
| parasite                                | 1  | 1.0862   | 122         | 152.87          | 0.297311 |     |
| temperature*parasite                    | 1  | 0.0036   | 121         | 152.87          | 0.952337 |     |
| <b>Host fitness</b>                     |    |          |             |                 |          |     |
| Overall survival                        |    |          |             |                 |          |     |
| temperature (poly, 3)                   | 3  | 1027.27  | 434         | 533.01          | <2.2E-16 | *** |
| parasite                                | 3  | 65.32    | 431         | 467.69          | 4.28E-14 | *** |
| temperature (poly, 3)*parasite          | 9  | 16.42    | 422         | 451.27          | 0.0586   | .   |
| Overall fecundity (total offspring)     |    |          |             |                 |          |     |
| temperature (poly, 2)                   | 2  | 110.139  | 301         | 405.91          | <2.2e-16 | *** |
| parasite                                | 3  | 48.191   | 298         | 357.72          | 1.94E-10 | *** |
| temperature*parasite (poly, 2)          | 6  | 15.234   | 29          | 342.49          | 0.01851  | *   |
| Fecundity per week (offspring per week) |    |          |             |                 |          |     |
| temperature (poly, 2)                   | 2  | 213.076  | 297         | 441.27          | <2.2E-16 | *** |
| parasite                                | 3  | 56.249   | 294         | 385.02          | 3.72E-12 | *** |
| temperature (poly, 2)*parasite          | 6  | 41.983   | 288         | 343.04          | 1.85E-07 | *** |

**Table 5.2.** Summary of the GLM statistics for heatwave versus constant temperature treatments, including infection rates and spore burden of both parasites, and survival and fecundity of the host. Significance was obtained through a  $\chi^2$  analysis of deviance. Stars represent significance levels, i.e.  $p < 0.1$ ,  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  for •, \*, \*\*, and \*\*\* respectively.

|                                                | Df | Deviance | Residual df | Resid. deviance | p-value    |
|------------------------------------------------|----|----------|-------------|-----------------|------------|
| <b><i>H. tvaerminnensis</i> fitness</b>        |    |          |             |                 |            |
| <b>Infection rates</b>                         |    |          |             |                 |            |
| temperature                                    | 1  | 2.3554   | 88          | 105.85          | 0.1249     |
| parasite                                       | 1  | 1.0954   | 87          | 104.76          | 0.2953     |
| temperature*parasite                           | 1  | 2.822    | 86          | 101.94          | 0.09298    |
| <b>Spore burden</b>                            |    |          |             |                 |            |
| temperature                                    | 1  | 0.07412  | 61          | 74.352          | 0.7854     |
| parasite                                       | 1  | 1.36451  | 60          | 72.988          | 0.2428     |
| temperature*parasite                           | 1  | 0.1406   | 59          | 72.847          | 0.7077     |
| <b><i>O. colligata</i> fitness</b>             |    |          |             |                 |            |
| <b>Infection rates</b>                         |    |          |             |                 |            |
| temperature                                    | 1  | 0.09     | 88          | 74.241          | 0.76419    |
| parasite                                       | 1  | 4.9915   | 87          | 69.25           | 0.02547 *  |
| temperature*parasite                           | 1  | 0.268    | 86          | 68.982          | 0.60466    |
| <b>Spore burden</b>                            |    |          |             |                 |            |
| temperature                                    | 1  | 1.95504  | 65          | 80.149          | 0.162      |
| parasite                                       | 1  | 0.55214  | 64          | 79.597          | 0.4574     |
| temperature*parasite                           | 1  | 0.02011  | 63          | 79.577          | 0.8872     |
| <b>Host fitness</b>                            |    |          |             |                 |            |
| <b>Overall survival</b>                        |    |          |             |                 |            |
| temperature                                    | 1  | 1.303    | 183         | 275.6           | 0.25359    |
| parasite                                       | 3  | 76.327   | 180         | 199.27          | <2e-16 *** |
| temperature*parasite                           | 3  | 8.857    | 177         | 190.41          | 0.03125 *  |
| <b>Overall fecundity (total offspring)</b>     |    |          |             |                 |            |
| temperature                                    | 1  | 2.641    | 125         | 212.39          | 0.1041     |
| parasite                                       | 3  | 76.286   | 122         | 136.1           | <2e-16 *** |
| temperature*parasite                           | 3  | 2.163    | 119         | 133.94          | 0.5393     |
| <b>Fecundity per week (offspring per week)</b> |    |          |             |                 |            |
| temperature                                    | 1  | 0.781    | 125         | 231.6           | 0.3769     |
| parasite                                       | 3  | 86.284   | 122         | 145.31          | <2e-16 *** |
| temperature*parasite                           | 3  | 2.045    | 119         | 143.27          | 0.5631     |

*(b) O. colligata fitness*

Contrary to *H. tvaerminnensis*, *O. colligata* fitness was not affected by multiple infections under constant temperature regimes, as single and double exposed animals had similar levels of infection (Figure 5.1B and Table 5.1; GLM, analysis of deviance,  $df = 198$ ,  $p = 0.273$ ) and spore burden (Figure 5.1D and Table 5.1; GLM, analysis of deviance,  $df = 122$ ,  $p = 0.297$ ). Although temperature affected *O. colligata* fitness through both infection rates (Figure 5.1B and Table 5.1;

GLM, analysis of deviance,  $df = 199$ ,  $p = <2e-16$ ) and spore burden (Figure 5.1D and Table 5.1; GLM, analysis of deviance,  $df = 123$ ,  $p = 0.0051$ ), infection and spore burden patterns observed differed from each other and from those observed in *H. tvaerminnensis*. Indeed, *O. colligata* appeared to be more sensitive to temperature than *H. tvaerminnensis*, with *O. colligata* infection rates falling to 0 at 10.5°C, while *O. colligata* spore burden decreased with increasing temperatures, contrary to what was observed in *H. tvaerminnensis* infected animals. A comparison between constant temperatures and a heatwave at 17.5°C revealed that double exposed treatments had lower *O. colligata* infection rates than animals which were single exposed to *O. colligata* regardless of temperature treatment (Figure 5.2B and Table 5.2; GLM, analysis of deviance,  $df = 87$ ,  $p = 0.0255$ ), although this pattern was not observed in spore burden (Figure 5.2D and Table 5.2; GLM, analysis of deviance,  $df = 64$ ,  $p = 0.457$ ).

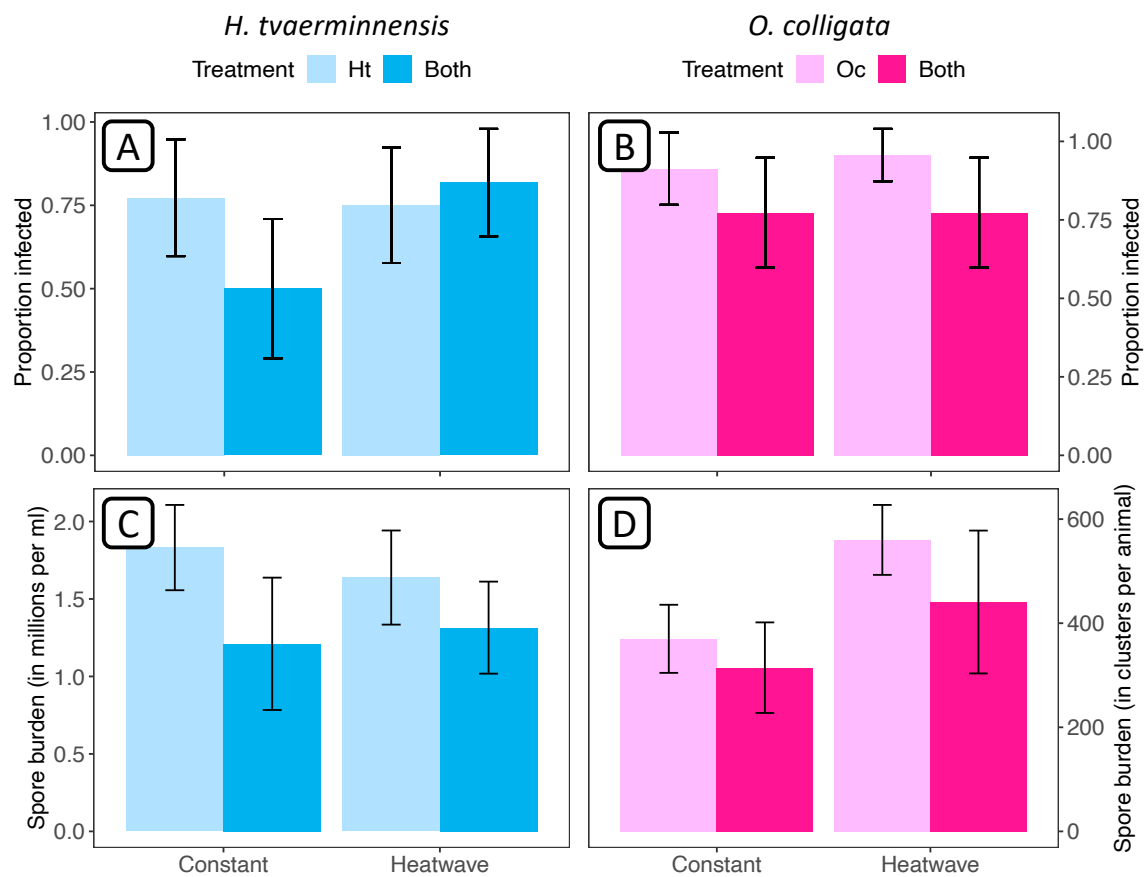


**Figure 5.2.** Impacts of treatment on parasite fitness under constant temperature conditions and heatwave conditions. Panels A and B show infection rates of *H. tvaerminnensis* and *O. colligata* respectively. Panels C and D show spore burden (number of spores in infected animals) of *H. tvaerminnensis* and *O. colligata* respectively. Treatments may be *H. tvaerminnensis* exposed (Ht), *O. colligata* exposed (Oc) or double exposed (Both). Error bars represent 95% confidence intervals for infection rates and standard error for spore burden.

## 5.4.2 Host fitness

### (a) Survival

Survival was dependent on both temperature (Table 5.1; GLM, analysis of deviance,  $df = 434$ ,  $p < 2.2e-16$ ) and parasite treatment (Table 5.1; GLM, analysis of deviance,  $df = 431$ ,  $p = 4.28e-14$ ), with overall host survival decreasing as temperatures increased. Temperature did not alter the outcome of survival under conditions of multiple infection, although the interaction term in the

model was approaching significance (Table 5.1; GLM, analysis of deviance,  $df = 422$ ,  $p = 0.0586$ ). Across all temperatures, animals exposed to either *H. tvaerminnensis* or double exposed animals died earlier than control animals and animals exposed to *O. colligata* (Figure 5.3A and Table D1; emmean, all  $p$ -values  $< 2.14e-5$ ). However, although animals exposed to only *O. colligata* lived longer than their *H. tvaerminnensis* and double exposed counterparts, they still died earlier than unexposed control animals (Figure 5.3A and Table D1; emmean, all  $p$ -values  $< 0.039$ ). Survival over time followed a similar pattern to overall survival (Figure 5.3B-D), with double exposed and *H. tvaerminnensis* animals generally dying earlier than control animals (Table D2; Kaplan-Meier analysis, pairwise comparisons using log-rank test, all  $p$ -values  $< 0.045$ ) and *O. colligata* exposed animals (Table D2; Kaplan-Meier analysis, pairwise comparisons using log-rank test, all  $p$ -values  $< 0.046$ ). Furthermore, although *O. colligata* exposed animals had lower overall survival than control animals, their survival over time was not significantly different from each other except at the highest temperatures (20.5 and 24.5°C) (Kaplan-Meier analysis, pairwise comparisons using log-rank test,  $p = 0.015$  and  $p = 0.00051$ , respectively). Exposure to heatwave conditions did not impact overall survival alone, as animals in both the constant and heatwave regimes had similar longevity (Figure 5.4A, Table 5.2; GLM, analysis of deviance,  $df = 183$ ,  $p = 0.254$ ). However, in addition to survival being affected by parasite treatment (Table 5.2; GLM, analysis of deviance,  $df = 180$ ,  $p < 2e-16$ ), temperature and parasite treatment were found to interact under heatwave conditions to change overall survival (Figure 5.4A and Table 5.2; GLM, analysis of deviance,  $df = 177$ ,  $p = 0.0313$ ). Indeed, animals which were exposed to *O. colligata* under the constant temperature regime lived longer than their counterparts in the heatwave temperature regime (emmean,  $z(\text{inf}) = -2.02$ ,  $p = 0.044$ ) while *H. tvaerminnensis* exposed, double exposed and control animals had similar survival under heatwave and constant temperature conditions (Figure 5.4A and Table D5).

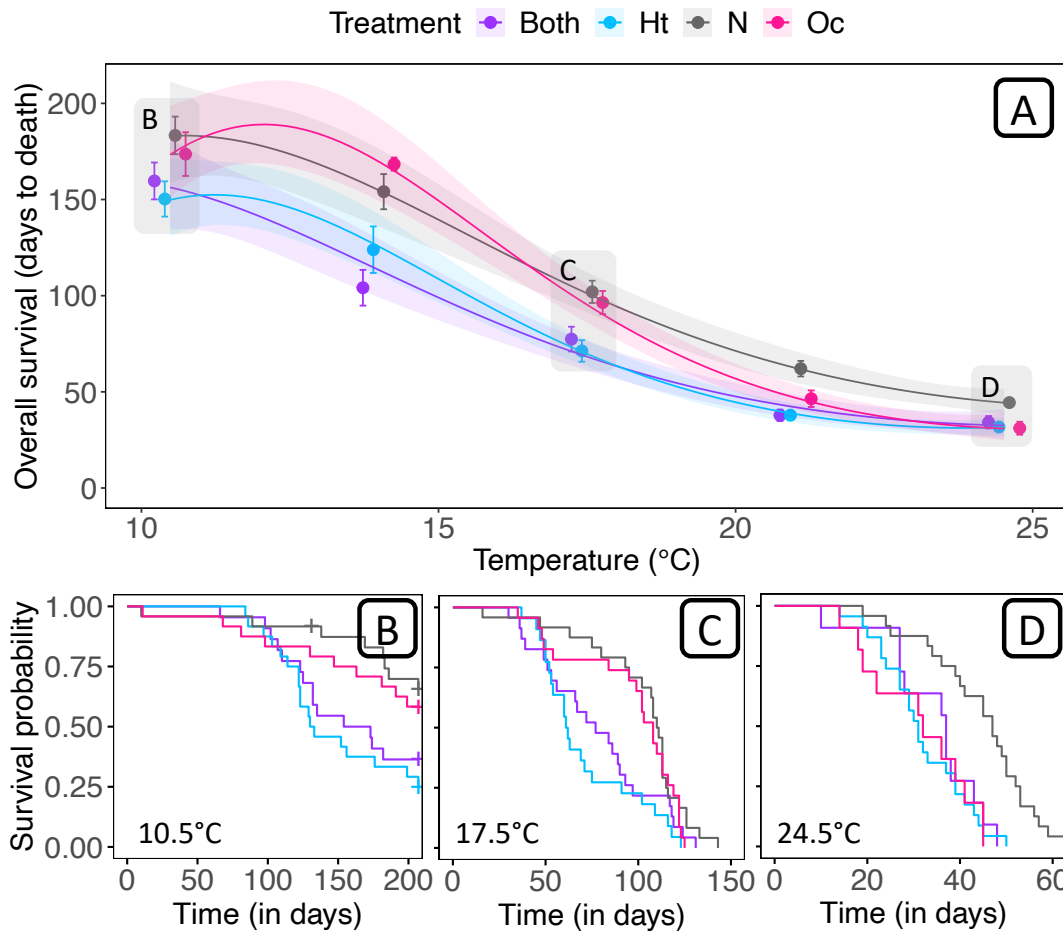


**Figure 5.3.** Impacts of treatment on host survival across the range of temperatures tested. Panels A shows overall survival. Panels B-D show survival over time at a subset of temperatures examined (as shown in Panel A): 10.5, 17.5 and 24.5°C. Treatments may be *H. tvaerminnensis* exposed (Ht), *O. colligata* exposed (Oc), double exposed (Both) or control animals (N). Errors bars represent standard error, lines represent predicted model fit and ribbons represent 95% confidence intervals of the predicted model fit for overall survival. “+” symbols in panel C represent animals which were still alive at the end of the experiment.

*(b) Lifetime reproductive success*

Temperature altered the fecundity reduction experienced by infection. Generally, lifetime reproductive success was negatively affected by increasing temperatures, as animals in warmer temperature treatments produced fewer total offspring (Figure 5.5A and Table 5.1; GLM,

analysis of deviance,  $df = 301$ ,  $p < 2.2e-16$ ) due to decreased survival at these temperatures. However, while the impact of infection on reproductive success was limited at lower temperatures, with all treatments producing similar numbers of offspring at 10.5°C, the effect of parasitism became more pronounced at higher temperatures (Figure 5.5A and Table 5.1, GLM, analysis of deviance,  $df = 292$ ,  $p = 0.019$ ). Overall, multiple exposed and *O. colligata* exposed animals had the lowest fecundity, except at 14 °C where double exposed animals produced fewer offspring (emmean,  $z(\text{inf}) = -2.08$ ,  $p = 0.038$ ). *H. tvaerminnensis* exposed animals produced similar numbers of offspring as control animals at the lowest temperatures (10.5 and 14°C) (Table D3), but the costs of infection became more pronounced with increasing temperatures. Indeed, *H. tvaerminnensis* exposed animals' fecundity dropped below that of control animals from 17.5°C (emmean,  $z(\text{inf}) = -1.96$ ,  $p = 0.0495$ ) and became similar to *O. colligata* exposed and double exposed animals at the higher end of the temperature range (21 and 24.5°C). Fecundity under heatwave conditions resembled that of constant temperatures, but only parasite treatment was found to have an effect on fecundity (Table 5.2; GLM, analysis of deviance,  $df = 122$ ,  $p < 2e-16$ ), as *O. colligata* exposed and double exposed animals produced fewer offspring than *H. tvaerminnensis* exposed animals, which in turn had lower fecundity than control animals (Figure 5.4B and Table D7; emmean, all  $p$ -values  $< 0.0086$ ).

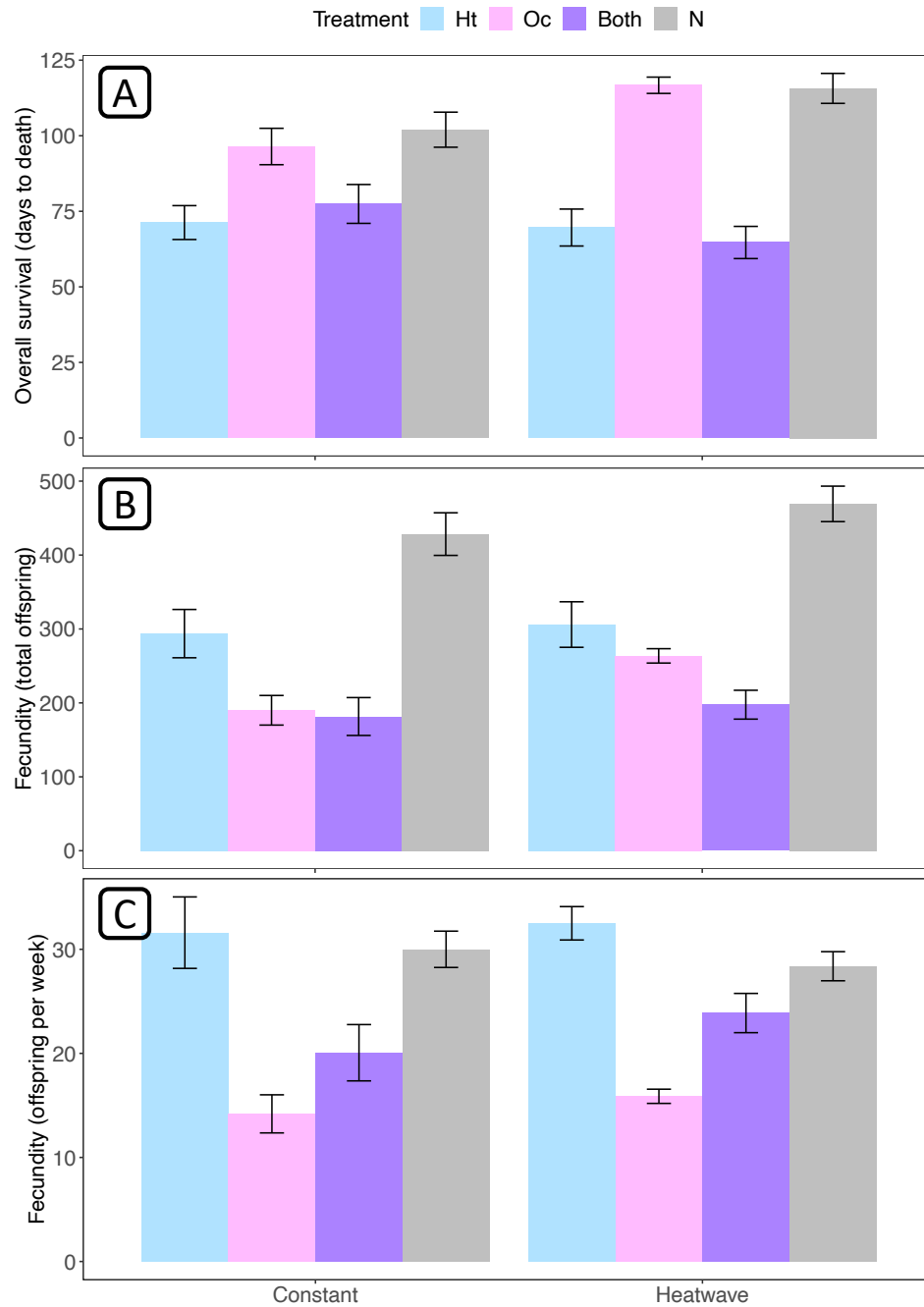


**Figure 5.4.** Impacts of treatment on host fitness under constant temperature conditions and heatwave conditions. Panel A shows overall survival, panel B shows overall fecundity (total offspring produced throughout the experiment), and panel C shows fecundity per week (offspring produced per 7 days). Treatments may be *H. tvaerminnensis* exposed (Ht), *O. colligata* exposed (Oc), double exposed (Both) or control animals (N). Errors bars represent standard error.

Offspring produced per week differed from total fecundity due to differential survival across different treatments (Figure 5.5A and B). In general, animals at higher temperatures had greater offspring production per week, while those in colder temperatures produced fewer offspring per week (Figure 5.5B and Table 5.1; GLM, analysis of deviance,  $df = 297$ ,  $p < 2.2e-16$ ). However, temperature altered weekly offspring production in multiple exposed animals as compared to their single exposed counterparts in central temperatures (14 to 21°C) (Table 5.1; GLM, analysis of deviance,  $df = 288$ ,  $p = 1.85e-7$ ). Overall, *H. tvaerminnensis* exposed animals had the highest fecundity per week compared to *O. colligata* and double exposed animals, and even exceeded the number of offspring produced by control animals at 14°C (Figure 5.5B and Table D4). Both double exposed and *O. colligata* exposed animals had reduced weekly fecundity relative to the control at intermediate temperatures (17.5 and 21°C), and *O. colligata* exposed individuals showed a larger reduction in fecundity per day than double exposed animals at 14°C. (Figure 5.5B and Table D4). The number of offspring produced per week under heatwave conditions was similar to that of constant temperatures (Figure 5.4C and Table 5.2; GLM, analysis of deviance,  $df = 125$ ,  $p = 0.377$ ), with *O. colligata* exposed animals having lower fecundity per week than double exposed treatments (emmean,  $z(\text{inf}) = -2.65$ ,  $p = 0.008$  and  $z(\text{inf}) = -3.22$ ,  $p = 0.0013$  for heatwave and constant treatments, respectively), which in turn had lower weekly fecundity than *H. tvaerminnensis* exposed and control animals (Table D8; emmean, all  $p$ -values  $< 0.0094$ ).

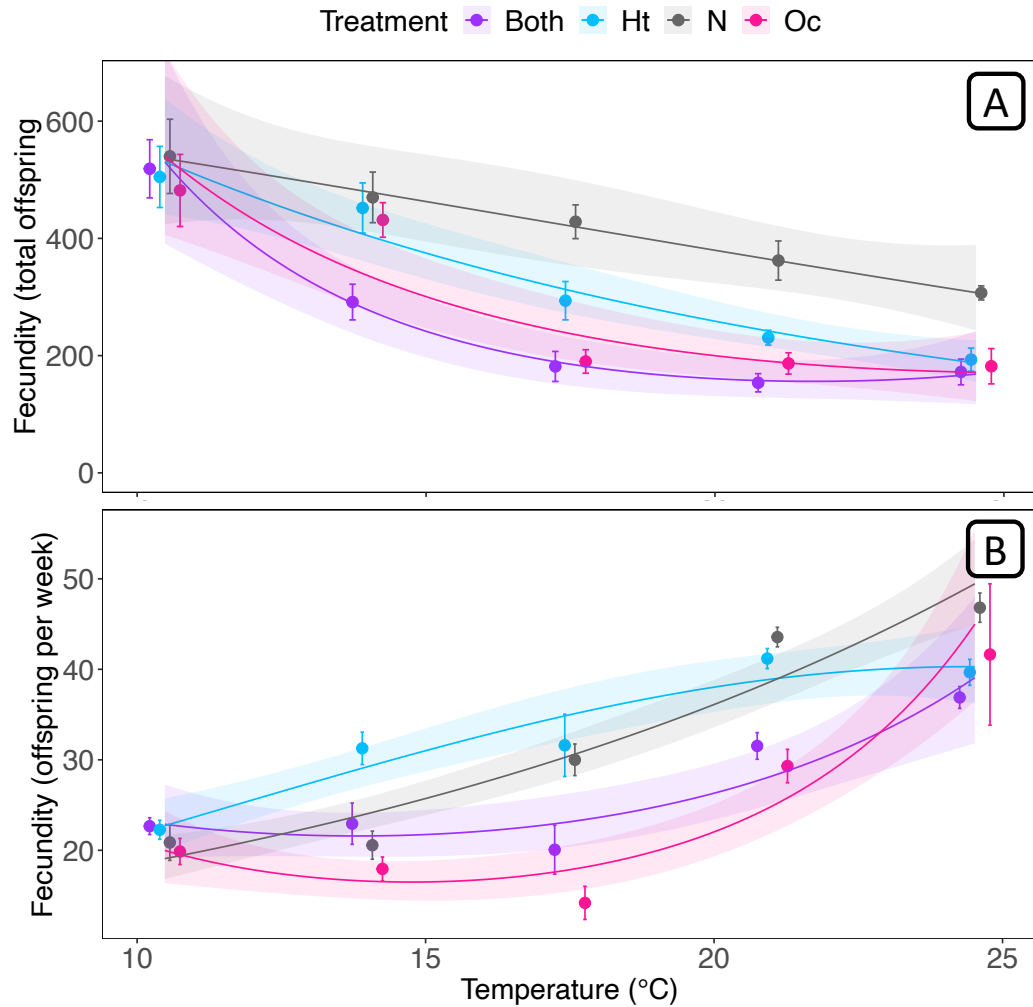


**Figure 5.5.** Impacts of treatment on host fecundity across the range of temperatures tested. Panel A shows total fecundity (total offspring produced throughout the experiment). Panel B shows fecundity per week (offspring produced per 7 days). Treatments may be *H. tvaerminnensis* exposed (Ht), *O. colligata* exposed (Oc), double exposed (Both) or control animals (N). Errors bars represent standard error, lines represent predicted model fit and ribbons represent 95% confidence intervals of the predicted model fit.

## 5.5 Discussion

We observed that co-infection with *O. colligata* lowered *H. tvaerminnensis* fitness across the range of temperatures examined, while *O. colligata* fitness was minimally impacted by co-infection, and that temperature modified the outcome of multiple infection. Indeed, *H.*

*tvaerminnensis* infection rates were higher in single exposed compared to double exposed animals but only at intermediate temperatures. Similarly, both fecundity and survival were altered based on temperature and parasite treatment, and generally appeared to be determined by one of the two parasites. A heatwave did not change the outcome of multiple infection for the most part, as parasite and host fitness responses generally did not differ from those observed in constant temperature treatments, although exposure to a heatwave changed survival outcomes for *O. colligata*.

### **5.5.1 Parasite fitness**

#### *(a) Multiple infection effects*

While *O. colligata* fitness was generally unaffected by co-infection, *H. tvaerminnensis* infection rates and spore burden were lower in animals that were co-infected with *O. colligata*. This suggests that co-infection with *O. colligata* is not only impeding the initial establishment of *H. tvaerminnensis* but is also limiting in-host spore growth. This is consistent with a previous study investigating co-infection in the same system at 20°C (O'Keeffe *et al.*, 2024), and several other studies have also observed antagonistic interactions between co-infecting parasites where one parasite may hinder the establishment or development of the other (Gold, Giraud and Hood, 2009; Ben-Ami, Rigaud and Ebert, 2011; Shen *et al.*, 2019; Manzi *et al.*, 2021). For example, co-infection with *Pasteuria ramosa* competitively excluded *H. tvaerminnensis* in their *Daphnia magna* host, with spore production being determined by *P. ramosa* in co-infected animals (Ben-Ami, Rigaud and Ebert, 2011), while *Wolbachia* bacteria have been observed to reduce vector-driven malaria transmission by inhibiting *Plasmodium* development (Kambris *et al.*, 2010). However, a comparison between animals in the heatwave and constant temperature treatments suggested that *O. colligata* infection rates were also lower in multiple infected animals than in their single infected counterparts, a pattern which was also observed in *O. colligata* infection rates in constant temperatures between 14.5 and 24.5°C (although not significant in this case).

These lower levels of *O. colligata* infection in double exposed animals suggest that *H. tvaerminnensis* may also have a negative impact on *O. colligata* under co-infection scenarios, although to a lesser extent than the inverse. Interactions between co-infecting parasites may be complex, as in addition to competition for physical space or host resources (Dobson and Roberts, 1994; Pedersen and Fenton, 2007; Mideo, 2009), they may be mediated by indirect competition through the hosts' immune system (Read and Taylor, 2001; Lello *et al.*, 2004). Given that *O. colligata* and *H. tvaerminnensis* are known to share resistant genes (Keller, Kirk and Luijckx, 2019), immune interactions may explain the lower level of within-host growth in multiple infections. However, previous studies have also suggested that parasitic infection may change host resource levels (Dobson, 1988; Tocque and Tinsley, 1994), therefore negatively impacting the fitness of a co-infecting parasite (Norton *et al.*, 2008; Randall *et al.*, 2013), although some research suggests this may be more prevalent in cases where co-infecting parasites are competing for similar resources (Karvonen, Jokela and Laine, 2019). While *O. colligata* may have the opportunity to monopolise host resources given its position as a gut parasite (Ebert, 2005), it is unclear whether *H. tvaerminnensis* and *O. colligata* are in direct competition as they infect different parts of the host body (Ebert, 2005). Although the mechanisms behind *O. colligata* outcompeting *H. tvaerminnensis* remain unclear, the presence of *O. colligata* influences the outcome of *H. tvaerminnensis* infection, and this may be further exacerbated by changes in temperature.

#### *(b) Temperature effects*

Temperature moderates the outcome of multiple infection, with the impact of *O. colligata* on *H. tvaerminnensis* fitness being diminished at the lowest temperatures. At 10.5°C, where *O. colligata* was unable to grow, parasite fitness differences between single and multiple exposed *H. tvaerminnensis* individuals were minimal, while *H. tvaerminnensis* infection rates in double exposed treatments were most reduced at 21°C (where *O. colligata* had its highest infection

rates) as compared to their single infected counterparts. Temperature plays a key role in the outcome of in-host competition under scenarios of co-infection, causing changes to within-host dynamics due to increased competitive interference (Sardanyés *et al.*, 2022). In addition, increases in temperature may increase the intensity of co-infection, either through a decrease in host immunity as observed in an ectoparasite (*Argulus* fish louse) and bacterial (*Aeromonas hydrophila*) co-infection in goldfish (Shameena *et al.*, 2021), or due to increased transmission at higher temperatures as found in multiple infections with vector borne arboviruses (Terradas *et al.*, 2023). However, the opposite has also been observed. For example, increased temperatures can reverse the facilitative effects of co-infection by decreasing infection intensity, as seen in fungal and viral co-infections in amphibians (Herczeg *et al.*, 2023). When co-infecting parasites have different temperature tolerance ranges, as is the case with *O. colligata* and *H. tvaerminnensis* in the present study, one may be able to outcompete the other in colder or warmer temperatures, while co-infection may only persist in more moderate temperatures (Fargues and Bon, 2004). This suggests that the combined effects of temperature and co-infection may be dependent on the response of individual parasites to changes in temperature.

*O. colligata* and *H. tvaerminnensis* were individually and differentially impacted by temperature, as *H. tvaerminnensis* infection rates and spore burden increased in higher temperatures, while *O. colligata* infection rates were highest between 14 and 21°C and *O. colligata* spore burden decreased with increasing temperatures. Parasite responses to temperature are known to be variable depending on the host-parasite system (Rohr *et al.*, 2013; Kunze *et al.*, 2022)), with *H. tvaerminnensis* having a wider temperature tolerance range and being less sensitive to temperature than *O. colligata* (Chapter 3). The present study suggests the optimum growth temperature for *O. colligata* to be around 14.5°C (based on observed spore burden), which is lower than expected, as previous studies have found that *O. colligata* infections are most successful between 18 to 21°C (Kirk *et al.*, 2018; Kunze *et al.*, 2022). Furthermore, neither *O.*

*colligata* nor *H. tvaerminnensis* were affected by exposure to a heatwave, despite previous research suggesting that heatwaves may change the outcome of infection (Schreven *et al.*, 2017), including a study examining the *D. magna*-*O. colligata* system (Kunze *et al.*, 2022). However, the outcome of infection after being exposed to a heatwave may vary depending on the host-parasite system, the intensity and the duration of the heatwave, and the initial temperature of the system (Landis *et al.*, 2012; Schreven *et al.*, 2017; Zhang *et al.*, 2019a; Kunze *et al.*, 2022). Overall, individual parasite responses to temperature may influence the outcome of multiple infection, which, when combined with ongoing global change, may lead to shifts in geographic ranges, altered species interactions, and changes in host fitness.

### **5.5.2 Host fitness**

#### *(a) Parasite effects*

Parasite treatment affected host fitness, as both host survival and fecundity were diminished in animals exposed to one or both of the parasites as compared to control animals. Multiple infection with both parasites in this system has been previously reported to lead to higher virulence (O'Keeffe *et al.*, 2024), and while in the present study we did observe higher losses in fecundity in the double exposed treatment at 14°C, at all other temperatures double exposed treatments had similar fecundity to *O. colligata* exposed individuals. Fecundity in co-infected animals may be driven by just one of two co-infecting parasites (Garza-Hernández *et al.*, 2013), including in *D. magna* co-infected with *H. tvaerminnensis* and *P. ramosa* (Ben-Ami, Rigaud and Ebert, 2011). However, while reductions in fecundity appeared to be mediated by *O. colligata*, decreases in survival seemed to be controlled by exposure to *H. tvaerminnensis*. Co-infected host mortality is often controlled by only one of the co-infecting parasites (de Roode *et al.*, 2005; Ben-Ami, Mouton and Ebert, 2008; Manzi *et al.*, 2021), although cases also exist where co-infecting parasites have been shown to cause higher host mortality than either of the single infections (Bandilla *et al.*, 2006; Pylkkö *et al.*, 2006; Knowles, 2011; Chu *et al.*, 2016; Zilio and

Koella, 2020). For instance, co-infection of mosquitoes with the microsporidians *Vavraia culicis* and *Edhazardia aedia* decreased larval survival beyond what was seen in single infections (Zilio and Koella, 2020), and co-infections of the ectoparasite *Argulus coregoni* and the bacterium *Flavobacterium columnare* led to earlier onset of disease and increased mortality in rainbow trout (Bandilla *et al.*, 2006). That fecundity and survival appeared to each be more influenced by a different one of the two parasites suggests that exposure to these parasites in tandem affects different aspects of host fitness. The reductions in survival and fecundity observed in the present study may be explained by the differing life-history strategies of these two parasites. *H. tvaerminnensis* is horizontally transmitted only upon death of the host but is also vertically transmitted to the offspring (Vizoso, Lass and Ebert, 2005), whereas *O. colligata* spores are horizontally transmitted while the animals are alive (Ebert, Lipsitch and Mangin, 2000). This means that higher fecundity benefits *H. tvaerminnensis* reproductive success but not *O. colligata*, while longer survival allows for longer transmission for *O. colligata*. Moreover, discrepancies in the level of virulence of animals double exposed to *H. tvaerminnensis* and *O. colligata* between this study and previous work suggest that the outcome of multiple infection may be context-dependent.

#### *(b) Temperature effects*

The impact of single and multiple infection on host fitness was moderated by temperature. Reductions in fecundity and survival due to parasite exposure were exaggerated at higher temperatures, and host survival and total fecundity decreased with increasing temperatures regardless of parasite treatment. Increasing temperatures have been frequently reported to lead to accelerated or increased mortality (Kirk *et al.*, 2018; Altizer *et al.*, 2013) and reduced fecundity (Walsh *et al.*, 2019; Kunze *et al.*, 2022) across a range of organisms, which may be due to increasing metabolic rates under warmer temperature conditions (Gillooly *et al.*, 2001) or survival constraints dominating over fecundity requirements under extreme environmental

conditions (Buckley, Schoville and Williams, 2021). For example, warmer temperatures led to increased mortality and decreased fecundity in New Zealand mussels (Petes, Menge and Murphy, 2007), as well as increased mortality in migratory birds (Santisteban *et al.*, 2012), while causing decreases in fecundity and extended egg development times in brown planthopper insects (Piyaphongkul, Pritchard and Bale, 2012). Furthermore, delayed maturation in colder temperatures (Angilletta Jr, Steury and Sears, 2004) may lead to decreases in fecundity (Pörtner *et al.*, 2001; Berger, Walters and Gotthard, 2008), as we observed in the present study where fecundity per week increased with rising temperatures. However, in addition to temperature impacting host fitness individually, parasite-mediated fitness effects were also exacerbated at certain temperatures.

At intermediate temperatures, fecundity in *H. tvaerminnensis* exposed animals was higher than *O. colligata* exposed and double exposed animals, while all three treatments produced similar numbers of offspring at the extremes of the temperature range. This may be explained by *O. colligata* having the largest impact on host fecundity at intermediate temperatures where it is most infective, while at more extreme temperatures where it is either absent or has low numbers its impact on the host is decreased. The impacts of parasitism on host fitness have been previously shown to be dependent on ecological conditions such as food quality (Chapter 2), humidity (Moyer, Drown and Clayton, 2002), and temperature (Kunze *et al.*, 2022), and these ecological impacts may have more complex effects when combined with multiple infection. Finally, *O. colligata* exposed animals survived longer under heatwave conditions than *O. colligata* exposed animals in constant temperatures, while there was no difference between constant temperature and heatwave survival for all other parasite treatments. This is somewhat unexpected, as exposure to a heatwave generally increases host mortality (Chen *et al.*, 2019; Green *et al.*, 2019), although this may depend on the starting temperature, duration and intensity of the heatwave (Abarca, Larsen and Ries, 2019; Moore, Hill and Kingsolver, 2022).

Overall, the impact of temperature on patterns of host fitness was condition dependent with temperature able to modify the intensity of these effects.

### **5.5.3 Consequences for parasite virulence**

Rising temperatures and more frequent extreme weather events associated with global change can modify the outcome of multiple infection, which may have consequences for the evolution of virulence (Alizon, de Roode and Michalakis, 2013), species coexistence (Escribano *et al.*, 2001), and population dynamics (van Baalen and Sabelis, 1995). Indeed, temperature is an important predictor of multiple infection in mathematical models (Brooker and Clements, 2009; Oakgrove *et al.*, 2014), and has, in some situations, been shown to be more important than independent population parameters for predicting co-infection incidence (Poterek *et al.*, 2022). Moreover, temperature variation may alter co-infection dynamics if one parasite is less adapted for competition at specific temperatures (Sardanyés *et al.*, 2022; Herczeg *et al.*, 2023; Terradas *et al.*, 2023), and modify the strength of selection by reducing competition. For instance, given that *O. colligata* outcompetes *H. tvaerminnensis* in our system, *H. tvaerminnensis* may evolve to higher levels of virulence in areas where both parasites co-occur, but changes in temperature associated with global change may alter both the chance that parasites co-occur in the same area (given their different thermal tolerance) and may decrease the impacts of *O. colligata* on *H. tvaerminnensis* at more extreme temperatures. Climate-induced shifts in parasite ranges, competitive abilities, and altered evolutionary trajectories could impact *Daphnia* population dynamics, which in turn may have important consequences for ecosystem function given their key role in freshwater ecosystems (Carpenter *et al.*, 2001; Sarnelle, 2005). Given that the outcome of multiple infection may be specific to the host-parasite system (Vaumourin *et al.*, 2015), and that co-infection can have far-reaching consequences including impacting human (de Roode *et al.*, 2005) and animal health (Graham, 2008), and modifying population (Laurenson *et*

*al.*, 2003) and disease dynamics (van Baalen and Sabelis, 1995), more research is required to better predict how temperature may impact the role of co-infections in natural systems.



## Chapter 6. Discussion

Recent disease outbreaks such as the COVID-19 pandemic (Ciotti *et al.*, 2020) and the Ebola virus epidemic (Holmes *et al.*, 2016) have reiterated the need to better understand how anthropogenic change will impact parasites and their hosts. However, parasites may have differing responses to constant temperatures, fluctuating temperatures and extreme weather events, and the combination of temperature variability and other ecological factors such as nutrient pollution increases the complexity of host-parasite responses (Manzi *et al.*, 2020; Kunze *et al.*, 2022). Furthermore, it remains unclear whether predictions from single infections will hold with interactions between co-infecting pathogens (Lively *et al.*, 2014). The aim of this thesis was to examine the impacts of increased temperature variability, changes in nutrient levels and multiple infection in a controlled host-parasite system under laboratory conditions, and draw conclusions about whether predictions from such a system can be scaled up to a range of diseases, or whether the complexities of the many factors at play may impede large-scale predictions.

### 6.1 Rising constant temperatures and the predictability of their effects on disease outbreaks

The parasites examined in this thesis appeared to have varying responses to increases in constant temperatures, adding to the growing body of research showing that thermal tolerance curves are species-specific (Lafferty, 2009; Rohr *et al.*, 2011; Gibbin *et al.*, 2015). However, host-parasite responses to increases in constant temperatures may be predictable through the metabolic theory of ecology (Molnár *et al.*, 2013; Kirk *et al.*, 2019; Kirk *et al.*, 2020), despite gaps in empirical knowledge regarding the temperature tolerance of certain host and parasite traits (Molnár *et al.*, 2017; Kirk *et al.*, 2018). Predictions using the metabolic theory of ecology require empirical data covering the entire thermal performance curve of the host-parasite system

(Molnár *et al.*, 2017), as extreme temperatures are particularly important when considering changes to parasite species ranges (Molnár *et al.*, 2013). Furthermore, various life-history traits may have variable sensitivities to changes in temperature (Kirk *et al.*, 2018) due to differing metabolic enzyme activity (Seebacher *et al.*, 2003). Generally, differing responses of host and parasite life-history traits to temperature, as I observed in this thesis, may increase the complexity of the outcome of disease (Dell, Pawar and Savage, 2011), as if temperature impacts hosts and their pathogens asymmetrically, the severity of infection may be under or overestimated (Lafferty and Holt, 2003; Thomas and Blanford, 2003; Paull, LaFonte and Johnson, 2012; Kirk *et al.*, 2018). Additionally, although organisms' responses to rising constant temperatures may be predictable by means of the metabolic theory of ecology, it is unclear whether this theory extends to responses to temperature variability and extreme weather events, both of which are projected to increase due to global change.

## **6.2 The importance of fluctuating temperatures and heatwaves**

Varying temperatures and extreme weather events can modify the outcome of disease (Rohr *et al.*, 2013), and parasite and host life-history traits may respond differently to predictable fluctuating temperatures as opposed to once-off heatwaves (Kunze *et al.*, 2022). Predictions from the metabolic theory of ecology may not apply in cases where host-parasite systems are exposed to variable temperatures (Kunze *et al.*, 2022), but several other theories regarding host-parasite responses to temperature exist. The thermal stress hypothesis suggests that the outcome of disease is dependent on whether the host or the parasite experiences more stress, therefore proposing that depending on the circumstances, either the host or the parasite can gain an advantage under conditions of increased thermal stress, although this effect may be influenced by the thermal history of the host (Paull *et al.*, 2015). In contrast, the temperature variability hypothesis posits that the faster acclimation rates of parasites relative to their hosts' (Gillooly *et al.*, 2001) experienced under conditions of temperature fluctuation will benefit the

pathogen, therefore leading to higher infection levels in the host (Rohr and Raffel, 2010; Raffel *et al.*, 2013; Krichel *et al.*, 2023). For instance, the fungal pathogen *Batrachochytrium dendrobatidis* exhibited increased growth rates in treefrogs which were not previously acclimated to a 10°C decrease in temperature when compared to growth in hosts which were acclimated to the lower temperature (Raffel *et al.*, 2013). However, the strength of the acclimation response may depend on the direction of the temperature variation (Rohr *et al.*, 2013) or the stage of infection (Altman *et al.*, 2016). Additionally, the thermal mismatch hypothesis suggests that the highest levels of infection will occur where the mismatch between host and parasite thermal range is the greatest, meaning that hosts adapted to colder temperatures will be more susceptible to infection at warmer temperatures, and vice versa (Nowakowski *et al.*, 2016; Cohen *et al.*, 2017). For example, the fungal pathogen *Batrachochytrium dendrobatidis* infecting its amphibian host had the highest growth rates in warm-adapted amphibian species exposed to cold temperatures and cold-adapted hosts exposed to warm temperatures (Cohen *et al.*, 2017). However, as with the temperature variability hypothesis, the impact of thermal mismatches may be dependent on latitude, elevation, life stage and body size of the host population (Cohen *et al.*, 2019), and predictions using this theory require knowledge of the pathogen's thermal performance outside of the host, which may be difficult to measure in certain systems. Studies which have empirically tested these theories have found both supporting and contrasting outcomes (Paull *et al.*, 2015; Cohen *et al.*, 2017; Cohen *et al.*, 2019; Kunze *et al.*, 2022; Krichel *et al.*, 2023), underlining the complexity of applying these hypotheses in real world systems. Krichel *et al.* (2023) suggest that while metabolic theory and mechanistic disease transmission models may be appropriate for representing changes in mean temperatures, rapidly changing conditions such as temperature fluctuations may not be captured by these models. That these theories may only be applicable under certain circumstances demonstrates the difficulties associated with predicting disease outcomes in the context of global change.

### 6.3 Interactions between temperature and nutrient levels

Organisms are likely to be exposed to interactions between temperature and other environmental stressors in the context of global change, which may further complicate the outcome of disease. Research investigating stressor interactions in coral reefs found that temperature frequently had impacts on other stressors, and that pathogen impacts were aggravated by multiple stressors in 78% of cases (Ban, Graham and Connolly, 2014). For example, in fish, mortality in rainbow trout caused by *Myxobolus cerebralis* was exacerbated by concurrent exposure to elevated temperature and gas supersaturation (Schisler, Bergersen and Walker, 2000), while salmon exposed to sublethal doses of malathion pesticide experienced higher levels of mortality caused by the bacterium *Aeromonas salmonicida* when temperatures were elevated (Dietrich *et al.*, 2014). Increases in water temperature have been linked to changes of nutrient levels in the environment by promoting cyanobacterial blooms (Joehnk *et al.*, 2008; Paerl and Paul, 2012). Changes in host diet or nutrient balance may modify host immunity levels (Landolt, 1989; Las Heras *et al.*, 2019) or increase pathogen impacts (Bruno *et al.*, 2003), and low-quality food or lower levels of certain nutrients in the environment have both been linked to decreases in parasite success (Crompton, 1987; Hall *et al.*, 2009b; Hall *et al.*, 2009a; Civitello *et al.*, 2015), meaning that the combination of modified temperature and nutrient levels may have important repercussions for disease. In a previous study examining temperature and host diet effects in a *Daphnia-Metschnikowia bicuspidata* system, although temperature and food quality did not have concurrent effects on the parasite (as low-quality food alone reduced parasite output), animals exposed to a combination of increased temperatures and high densities of cyanobacteria experienced increased mortality, with potential implications for parasite transmission (Manzi *et al.*, 2020). In this thesis, although temperature and nutrient levels were mostly independent, the optimum temperature for *O. colligata* spore production, as well as the number of spores produced, increased unexpectedly,

suggesting an interaction between temperature and nutrient levels. The findings presented here suggest that increases in temperature and nutrient pollution may increase disease outbreaks, but this is dependent on the starting temperature of the system. Overall, the fluctuation of multiple environmental stressors such as temperature and nutrient regimes will further complicate predictions regarding the outcome of disease.

#### **6.4 The complexity of multiple infection**

The inclusion of multiple infection in research investigating the impacts of global change on disease is crucial, given that co-infection is the rule in natural systems and that it may cause unexpected outcomes (Alizon, de Roode and Michalakis, 2013). Co-infections may lead to larger epidemics (Susi *et al.*, 2015) or higher infection success (Karvonen *et al.*, 2012), but the outcome of multiple infection may be dependent on a number of factors such as parasite infectivity and virulence (Mihaljevic, Hoverman and Johnson, 2018), abiotic conditions (Herczeg *et al.*, 2021), and the order of exposure of the hosts to the parasite (Hoverman, Hoyer and Johnson, 2013). For example, multiply-infecting clones of *Plasmodium chabaudi* in mice experienced higher levels of competition when they infected the host simultaneously or sequentially, but sequential co-infection allowed competitively inferior clones to outcompete competitively superior clones if they were first to infect (De Roode *et al.*, 2005). Furthermore, multiple infection may have important repercussions for the evolution of virulence (Alizon, 2008; Alizon and van Baalen, 2008; van Baalen and Sabelis, 1995), although empirical research investigating these impacts has found evidence that both more and less virulent parasites may outcompete the other (Read and Taylor, 2001; Davies, Fairbrother and Webster, 2002; de Roode *et al.*, 2005; Harrison *et al.*, 2006), with host identity (De Roode *et al.*, 2004), prior residency (De Roode *et al.*, 2005) and parasite transmission mode (Vizoso and Ebert, 2005a) all modifying virulence evolution.

The work completed in this thesis suggests that multiple infection in a *Daphnia*-microsporidian system may lead to increases in virulence in the less virulent parasite (*Hamiltosporidium tvaerminnensis*), but extreme temperatures may modify the outcome of co-infection by reducing the success of the more virulent (*Ordospora colligata*) of the two parasites. Considering variations in temperature in the context of multiple infection adds another layer of complexity, but is paramount given that temperature may predict where multiple infection outbreaks will occur (Brooker and Clements, 2009; Oakgrove *et al.*, 2014) and that seasonality in temperature variation has been shown to account for patterns of co-infection more than temperature-independent population parameters (Poterek *et al.*, 2022). Although the outcome of co-infection in the context of temperature may be system-specific, given the range of outcomes observed in empirical studies thus far (Fargues and Bon, 2004; Shameena *et al.*, 2021; Sardanyés *et al.*, 2022; Herczeg *et al.*, 2023; Terradas *et al.*, 2023), it is clear that interactions between variations in temperature and co-infections may modify disease dynamics, and may further complicate predictions of disease outbreaks.

## **6.5 Shortcomings and future directions**

The topics examined in this thesis provide several potential avenues for future research. Although *Daphnia* are a useful system for investigating the impacts of disease, due to their asexual reproduction mode, ease of use in the laboratory and well-studied genome (Ebert, 2022), the focus on a single clonal line in this thesis excludes genetic variation found in natural populations. The outcome of environmental change on disease may depend not only on the host-parasite system examined, but also on the specific host and parasite genotypes involved (Luijckx *et al.*, 2011), with the presence of genetic variation in natural populations potentially altering the outcome of epidemics (King and Lively, 2012). Genetic similarity between hosts increases the risk of infection (Anderson and May, 1986; Keesing, Holt and Ostfeld, 2006) and populations which are more genetically homogeneous may experience larger epidemics than

their more diverse counterparts (Sherman, Seeley and Reeve, 1988; Altizer, Harvell and Friedle, 2003). In *Daphnia* exposed to *M. bicuspidata*, host clone identity interacts with food quality and temperature to modify parasite infectivity and reproduction (Manzi *et al.*, 2020), while host genotype-specific susceptibility to the chytrid parasite *Zygorhizidium planktonicum* in diatoms can be modified by temperature (Gsell *et al.*, 2013). Furthermore, the focus of this thesis has been on experiments involving single hosts, but the outcome of disease may be different in population-level experiments. For example, the concept of density thresholds, whereby a disease may only persist in a population above a certain size, underpins disease control efforts through vaccination or culling programs (Dobson and Meagher, 1996; Donnelly *et al.*, 2003; Lloyd-Smith *et al.*, 2005). Empirical evidence for these thresholds is limited (Packer *et al.*, 1999; Begon *et al.*, 2003; Davis *et al.*, 2004; Lloyd-Smith *et al.*, 2005), and the question of whether changes in temperature may affect disease thresholds remains unanswered. Additionally, at the population level, increases in temperature led to larger epidemics (Auld and Brand, 2017), impacting host populations through parasite-mediated selection (Auld *et al.*, 2013) and population genetic change (Thrall *et al.*, 2012) and potentially modifying the outcome of future epidemics by decreasing genetic diversity (Obbard *et al.*, 2011). Finally, disease dynamics should also be considered on the community-level, bringing with it another layer of complexity due to the number of species interactions present, each with their own response to anthropogenic changes such as variations in temperature, modified nutrient levels, and multiple infection.

## **6.6 Concluding remarks**

This thesis adds to the growing number of studies examining the impacts of global change, but also builds upon existing research by investigating the combined effects of temperature, nutrient levels, and multiple infection. This research examines the effects of temperature variation in greater detail than many previous studies, by studying not only the impacts of rising constant temperatures, but also the effects of fluctuations in temperature and heatwave events. The

inclusion of three distinct parasites in this study allows me to draw conclusions about the specifics and generalities of the impacts of temperature, with data collected here suggesting that parasite responses to climate change are likely to be system-specific. Furthermore, the inclusion of a second stressor in the form of varying nutrient levels further supports the notion that host-parasite interactions under conditions of global change are likely to be complex. The study of multiple infection presented in this thesis is a strong addition to the few existing empirical studies investigating the impacts of climate change on multiple infection, and demonstrates that predictions made in single host-parasite systems regarding their response to climate change may not apply in natural systems where multiple infections are the norm. Given the complexity of host-parasite responses to global change demonstrated in this thesis, there may be implications for predictions of future outbreaks, with ramifications for human and animal health, biodiversity, food security, and the economy. Finally, changes to *Daphnia* population dynamics as a result of the impacts of anthropogenic change may have important consequences in their own right, due to their key ecological role (Carpenter *et al.*, 2001; Sarnelle, 2005), with potentially rippling effects across aquatic ecosystems.

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## Appendix A – Supplementary information for Chapter 2

**Table A1.** Priors for beta function.

| Parasite                 | Parameter        | Infection rate<br>normal (mean, sd) | Spore burden<br>normal (mean, sd) |
|--------------------------|------------------|-------------------------------------|-----------------------------------|
| <i>H. tvaerminnensis</i> | $C_m$            | 0.95, 0.1                           | 4, 4                              |
|                          | $t_{\min}$       | 10, 2                               | 10, 5                             |
|                          | $t_{\text{opt}}$ | 22, 6                               | 19, 3                             |
|                          | $t_{\max}$       | 29, 2                               | 25, 5                             |
| <i>O. colligata</i>      | $C_m$            | 0.8, 0.4                            | 2, 2                              |
|                          | $t_{\min}$       | 11, 2                               | 11, 5                             |
|                          | $t_{\text{opt}}$ | 20, 4                               | 20, 5                             |
|                          | $t_{\max}$       | 25, 4                               | 26, 5                             |
| <i>M. bicuspidata</i>    | $C_m$            | 0.7, 0.2                            |                                   |
|                          | $t_{\min}$       | 11, 2                               |                                   |
|                          | $t_{\text{opt}}$ | 22, 4                               |                                   |
|                          | $t_{\max}$       | 28, 2                               |                                   |

**Table A2.** Number of iterations used for Bayesian modelling.

| Parasite                 | Response<br>variable | Warmup iterations | Post-warmup<br>iterations |
|--------------------------|----------------------|-------------------|---------------------------|
| <i>H. tvaerminnensis</i> | Infection rate       | 4000              | 2000                      |
|                          | Spore burden         | 1500              | 2000                      |
| <i>O. colligata</i>      | Infection rate       | 7000              | 1000                      |
|                          | Spore burden         | 5000              | 2000                      |
| <i>M. bicuspidata</i>    | Infection rate       | 1500              | 500                       |

## Appendix B – Supplementary information for Chapter 3

**Table B1.** Priors for beta function.

| Parasite            | Parameter        | Infection rate<br>normal (mean, sd) | Spore burden<br>normal (mean, sd) |
|---------------------|------------------|-------------------------------------|-----------------------------------|
| <i>O. colligata</i> | $c_m$            | 0.5, 0.5                            | 2, 2                              |
|                     | $t_{\min}$       | 29, 2                               | 12, 2.5                           |
|                     | $t_{\text{opt}}$ | 20, 5                               | 20, 5                             |
|                     | $t_{\max}$       | 25, 3                               | 26, 5                             |

**Table B2.** Number of iterations used for Bayesian modelling.

| Parasite            | Response<br>variable | Warmup iterations | Post-warmup<br>iterations |
|---------------------|----------------------|-------------------|---------------------------|
| <i>O. colligata</i> | Infection rate       | 7000              | 2000                      |
|                     | Spore burden         | 1000              | 2000                      |

## Appendix C – Supplementary information for Chapter 4

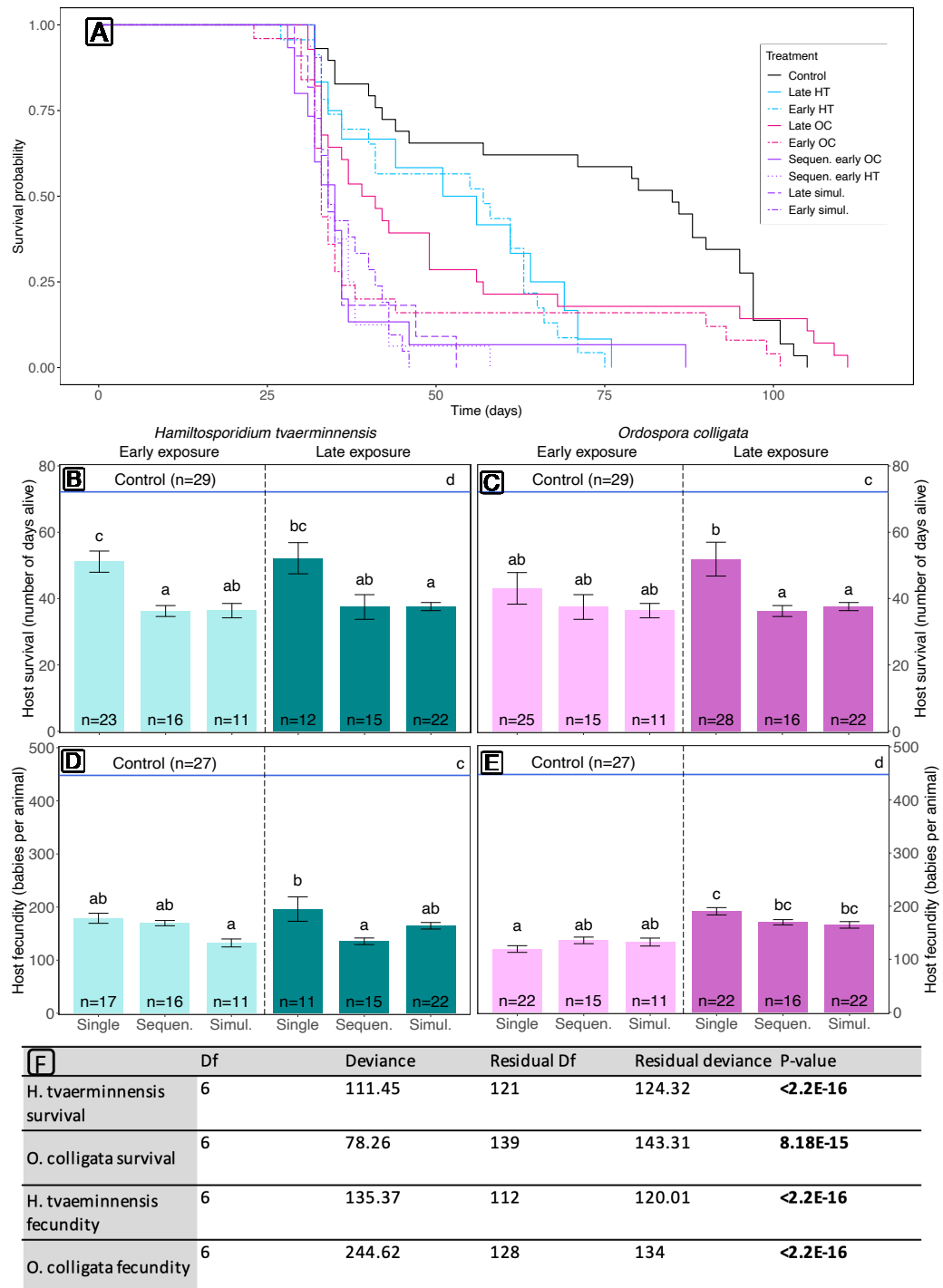


**Figure C1.** Impacts of treatment on parasite fitness where only true infected replicates where included (infections by one parasite in the single exposure treatments and infections by both parasites in the double exposure treatments). Panel A shows projected survival over time for all treatments using a Kaplan-Meier survival analysis.

Panels B and C show host survival for treatments exposed to *H. tvaerminnensis* and *O. colligata* respectively. Panels D and E show host fecundity for animals exposed to *H. tvaerminnensis* and *O. colligata* respectively. Treatments may be single infections, sequential co-infections (Sequen.) or simultaneous co-infections (Simul.) and are split into early and late exposure timepoints. Error bars represent standard error for panels B-E. Sample sizes for each treatment are indicated on each bar. Blue horizontal lines in panels B-E represent the control treatment. Statistical significance is indicated through letters visible above each bar which represent the results of Tukey post-hoc tests. Panel F summarises the GLM analyses carried out for host mortality and fecundity when exposed to *H. tvaerminnensis* and *O. colligata* and shows the results of a Chi-squared analysis of deviance.

## Appendix D – Supplementary information for Chapter 5

**Table D1.** List of custom contrasts for survival under constant temperatures generated through emmeans package in R.

| contrast             | estimate | SE       | df  | z.ratio  | p.value  |
|----------------------|----------|----------|-----|----------|----------|
| 10.5 Both vs 10.5 Ht | -0.00065 | 0.050787 | Inf | -0.01273 | 0.989841 |
| 10.5 Both vs 10.5 Oc | -0.21635 | 0.051792 | Inf | -4.17737 | 2.95E-05 |
| 10.5 Both vs 10.5 N  | -0.31998 | 0.050037 | Inf | -6.39489 | 1.61E-10 |
| 10.5 Ht vs 10.5 Oc   | -0.21571 | 0.050752 | Inf | -4.25018 | 2.14E-05 |
| 10.5 Ht vs 10.5 N    | -0.31933 | 0.048554 | Inf | -6.57688 | 4.8E-11  |
| 10.5 Oc vs 10.5 N    | -0.10363 | 0.050003 | Inf | -2.07245 | 0.038223 |
| 14 Both vs 14 Ht     | -0.00065 | 0.050787 | Inf | -0.01273 | 0.989841 |
| 14 Both vs 14 Oc     | -0.21635 | 0.051792 | Inf | -4.17737 | 2.95E-05 |
| 14 Both vs 14 N      | -0.31998 | 0.050037 | Inf | -6.39489 | 1.61E-10 |
| 14 Ht vs 14 Oc       | -0.21571 | 0.050752 | Inf | -4.25018 | 2.14E-05 |
| 14 Ht vs 14 N        | -0.31933 | 0.048554 | Inf | -6.57688 | 4.8E-11  |
| 14 Oc vs 14 N        | -0.10363 | 0.050003 | Inf | -2.07245 | 0.038223 |
| 17.5 Both vs 17.5 Ht | -0.00065 | 0.050787 | Inf | -0.01273 | 0.989841 |
| 17.5 Both vs 17.5 Oc | -0.21635 | 0.051792 | Inf | -4.17737 | 2.95E-05 |
| 17.5 Both vs 17.5 N  | -0.31998 | 0.050037 | Inf | -6.39489 | 1.61E-10 |
| 17.5 Ht vs 17.5 Oc   | -0.21571 | 0.050752 | Inf | -4.25018 | 2.14E-05 |
| 17.5 Ht vs 17.5 N    | -0.31933 | 0.048554 | Inf | -6.57688 | 4.8E-11  |
| 17.5 Oc vs 17.5 N    | -0.10363 | 0.050003 | Inf | -2.07245 | 0.038223 |
| 21 Both vs 21 Ht     | -0.00065 | 0.050787 | Inf | -0.01273 | 0.989841 |
| 21 Both vs 21 Oc     | -0.21635 | 0.051792 | Inf | -4.17737 | 2.95E-05 |
| 21 Both vs 21 N      | -0.31998 | 0.050037 | Inf | -6.39489 | 1.61E-10 |
| 21 Ht vs 21 Oc       | -0.21571 | 0.050752 | Inf | -4.25018 | 2.14E-05 |
| 21 Ht vs 21 N        | -0.31933 | 0.048554 | Inf | -6.57688 | 4.8E-11  |
| 21 Oc vs 21 N        | -0.10363 | 0.050003 | Inf | -2.07245 | 0.038223 |
| 24.5 Both vs 24.5 Ht | -0.00065 | 0.050787 | Inf | -0.01273 | 0.989841 |
| 24.5 Both vs 24.5 Oc | -0.21635 | 0.051792 | Inf | -4.17737 | 2.95E-05 |
| 24.5 Both vs 24.5 N  | -0.31998 | 0.050037 | Inf | -6.39489 | 1.61E-10 |
| 24.5 Ht vs 24.5 Oc   | -0.21571 | 0.050752 | Inf | -4.25018 | 2.14E-05 |
| 24.5 Ht vs 24.5 N    | -0.31933 | 0.048554 | Inf | -6.57688 | 4.8E-11  |
| 24.5 Oc vs 24.5 N    | -0.10363 | 0.050003 | Inf | -2.07245 | 0.038223 |
| 10.5 Oc vs 14 Oc     | 0.205298 | 0.052938 | Inf | 3.878075 | 0.000105 |
| 10.5 Oc vs 17.5 Oc   | 0.660861 | 0.053221 | Inf | 12.41738 | 2.1E-35  |
| 10.5 Oc vs 21 Oc     | 1.299117 | 0.054445 | Inf | 23.86093 | 7.8E-126 |
| 10.5 Oc vs 24.5 Oc   | 1.53931  | 0.059974 | Inf | 25.66637 | 2.8E-145 |
| 14 Oc vs 17.5 Oc     | 0.455563 | 0.053924 | Inf | 8.448296 | 2.96E-17 |
| 14 Oc vs 21 Oc       | 1.093819 | 0.055139 | Inf | 19.83755 | 1.41E-87 |
| 14 Oc vs 24.5 Oc     | 1.334012 | 0.06064  | Inf | 21.99903 | 2.9E-107 |
| 17.5 Oc vs 21 Oc     | 0.638256 | 0.05541  | Inf | 11.5187  | 1.06E-30 |
| 17.5 Oc vs 24.5 Oc   | 0.878449 | 0.060863 | Inf | 14.43321 | 3.2E-47  |
| 21 Oc vs 24.5 Oc     | 0.240193 | 0.061837 | Inf | 3.884297 | 0.000103 |
| 10.5 Ht vs 14 Ht     | 0.205298 | 0.052938 | Inf | 3.878075 | 0.000105 |
| 10.5 Ht vs 17.5 Ht   | 0.660861 | 0.053221 | Inf | 12.41738 | 2.1E-35  |
| 10.5 Ht vs 21 Ht     | 1.299117 | 0.054445 | Inf | 23.86093 | 7.8E-126 |
| 10.5 Ht vs 24.5 Ht   | 1.53931  | 0.059974 | Inf | 25.66637 | 2.8E-145 |
| 14 Ht vs 17.5 Ht     | 0.455563 | 0.053924 | Inf | 8.448296 | 2.96E-17 |
| 14 Ht vs 21 Ht       | 1.093819 | 0.055139 | Inf | 19.83755 | 1.41E-87 |
| 14 Ht vs 24.5 Ht     | 1.334012 | 0.06064  | Inf | 21.99903 | 2.9E-107 |
| 17.5 Ht vs 21 Ht     | 0.638256 | 0.05541  | Inf | 11.5187  | 1.06E-30 |
| 17.5 Ht vs 24.5 Ht   | 0.878449 | 0.060863 | Inf | 14.43321 | 3.2E-47  |
| 21 Ht vs 24.5 Ht     | 0.240193 | 0.061837 | Inf | 3.884297 | 0.000103 |

**Table D2.** Table of pairwise comparisons for survival over time under constant temperatures generated through Kaplan-Meier analysis in R.

| Pairwise comparisons using Log-Rank test |           |         |         |         |         |         |         |         |           |         |         |         |         |         |         |         |           |         |         |  |  |
|------------------------------------------|-----------|---------|---------|---------|---------|---------|---------|---------|-----------|---------|---------|---------|---------|---------|---------|---------|-----------|---------|---------|--|--|
| data: c01nf_data_survival and combo      |           |         |         |         |         |         |         |         |           |         |         |         |         |         |         |         |           |         |         |  |  |
|                                          | 10.5 Both | 10.5 Ht | 10.5 N  | 10.5 Oc | 14 Both | 14 Ht   | 14 N    | 14 Oc   | 17.5 Both | 17.5 Ht | 17.5 N  | 17.5 Oc | 21 Both | 21 Ht   | 21 N    | 21 Oc   | 24.5 Both | 24.5 Ht | 24.5 N  |  |  |
| 10.5 Ht                                  | 0.42903   | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 10.5 N                                   | 0.02679   | 0.00239 | -       | -       | -       | -       | -       | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 10.5 Oc                                  | 0.14476   | 0.02412 | 0.54521 | -       | -       | -       | -       | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 14 Both                                  | 0.00017   | 0.00079 | 4.0e-09 | 4.1e-07 | -       | -       | -       | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 14 Ht                                    | 0.00961   | 0.03760 | 1.2e-07 | 1.3e-05 | 0.20479 | -       | -       | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 14 N                                     | 0.11349   | 0.36142 | 4.3e-07 | 5.6e-05 | 0.00521 | 0.60005 | -       | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 14 Oc                                    | 0.20479   | 0.56740 | 2.2e-06 | 0.00017 | 0.00098 | 0.29958 | 0.41369 | -       | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 17.5 Both                                | 1.7e-08   | 8.6e-08 | 1.3e-10 | 5.3e-09 | 0.02056 | 0.00057 | 1.5e-09 | 7.6e-12 | -         | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 17.5 Ht                                  | 4.1e-09   | 4.0e-09 | 1.2e-10 | 2.2e-09 | 0.00313 | 0.00018 | 1.6e-09 | 7.9e-12 | 0.30664   | -       | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 17.5 N                                   | 8.7e-06   | 3.2e-05 | 5.1e-10 | 7.1e-08 | 0.43389 | 0.00821 | 3.6e-09 | 1.3e-11 | 0.03063   | 0.00174 | -       | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 17.5 Oc                                  | 8.1e-07   | 3.4e-06 | 4.9e-10 | 5.5e-08 | 0.29678 | 0.00411 | 4.3e-09 | 1.0e-11 | 0.19545   | 0.01778 | 0.33872 | -       | -       | -       | -       | -       | -         | -       | -       |  |  |
| 21 Both                                  | 7.6e-12   | 4.1e-12 | 3.0e-11 | 3.0e-11 | 5.2e-11 | 6.5e-10 | 3.6e-10 | 7.6e-12 | 5.7e-07   | 1.7e-06 | 1.6e-10 | 6.3e-09 | -       | -       | -       | -       | -         | -       | -       |  |  |
| 21 Ht                                    | 7.6e-12   | 4.1e-12 | 2.8e-11 | 2.8e-11 | 1.9e-11 | 6.2e-11 | 2.6e-10 | 7.6e-12 | 7.6e-08   | 3.3e-09 | 5.7e-11 | 4.2e-10 | 0.38760 | -       | -       | -       | -         | -       | -       |  |  |
| 21 N                                     | 5.2e-11   | 7.6e-12 | 3.7e-11 | 2.7e-10 | 5.3e-05 | 8.8e-05 | 7.6e-10 | 7.6e-12 | 0.01500   | 0.24273 | 2.7e-08 | 7.9e-07 | 9.8e-06 | 8.3e-08 | -       | -       | -         | -       | -       |  |  |
| 21 Oc                                    | 3.0e-11   | 7.6e-12 | 5.0e-11 | 1.8e-10 | 1.9e-07 | 6.4e-07 | 8.8e-10 | 1.1e-11 | 0.00022   | 0.00411 | 1.8e-09 | 9.2e-08 | 0.04500 | 0.02436 | 0.01448 | -       | -         | -       | -       |  |  |
| 24.5 Both                                | 9.9e-11   | 2.8e-11 | 1.3e-09 | 1.3e-09 | 5.6e-10 | 5.5e-10 | 9.8e-09 | 9.9e-11 | 4.7e-07   | 6.1e-09 | 2.1e-09 | 3.5e-09 | 0.26215 | 0.33872 | 9.4e-07 | 0.02679 | -         | -       | -       |  |  |
| 24.5 Ht                                  | 7.6e-12   | 4.1e-12 | 2.9e-11 | 2.9e-11 | 8.5e-12 | 2.6e-11 | 1.4e-10 | 7.6e-12 | 2.9e-09   | 1.2e-10 | 3.4e-11 | 3.7e-11 | 0.04942 | 0.03910 | 4.0e-09 | 0.00101 | 0.57307   | -       | -       |  |  |
| 24.5 N                                   | 7.6e-12   | 4.1e-12 | 2.8e-11 | 2.8e-11 | 1.3e-10 | 6.5e-10 | 5.1e-10 | 7.6e-12 | 3.9e-06   | 6.7e-06 | 2.1e-10 | 2.4e-08 | 0.41472 | 0.01502 | 2.3e-05 | 0.21487 | 0.00356   | 4.0e-05 | -       |  |  |
| 24.5 Oc                                  | 1.2e-10   | 2.9e-11 | 1.9e-09 | 1.9e-09 | 3.6e-10 | 6.9e-10 | 1.1e-08 | 1.2e-10 | 3.2e-07   | 2.7e-09 | 1.5e-09 | 7.2e-10 | 0.13335 | 0.07953 | 2.1e-07 | 0.01713 | 0.57936   | 0.85192 | 0.00051 |  |  |

**Table D3.** List of custom contrasts for total fecundity under constant temperatures generated through emmeans package in R.

| contrast             | estimate | SE       | df  | z.ratio  | p.value  |
|----------------------|----------|----------|-----|----------|----------|
| 10.5 Both vs 10.5 Ht | 0.027228 | 0.194887 | Inf | 0.13971  | 0.888889 |
| 10.5 Both vs 10.5 Oc | 0.073835 | 0.191833 | Inf | 0.384892 | 0.700317 |
| 10.5 Both vs 10.5 N  | -0.04006 | 0.191796 | Inf | -0.20889 | 0.834538 |
| 10.5 Ht vs 10.5 Oc   | 0.046607 | 0.191842 | Inf | 0.242946 | 0.808047 |
| 10.5 Ht vs 10.5 N    | -0.06729 | 0.191805 | Inf | -0.35083 | 0.725716 |
| 10.5 Oc vs 10.5 N    | -0.1139  | 0.188701 | Inf | -0.60359 | 0.546116 |
| 14 Both vs 14 Ht     | -0.43878 | 0.192103 | Inf | -2.28407 | 0.022368 |
| 14 Both vs 14 Oc     | -0.39251 | 0.189003 | Inf | -2.07675 | 0.037825 |
| 14 Both vs 14 N      | -0.47799 | 0.188972 | Inf | -2.52942 | 0.011425 |
| 14 Ht vs 14 Oc       | 0.046264 | 0.191922 | Inf | 0.241059 | 0.809509 |
| 14 Ht vs 14 N        | -0.03921 | 0.191891 | Inf | -0.20435 | 0.838081 |
| 14 Oc vs 14 N        | -0.08548 | 0.188787 | Inf | -0.45277 | 0.650715 |
| 17.5 Both vs 17.5 Ht | -0.48107 | 0.192647 | Inf | -2.49718 | 0.012519 |
| 17.5 Both vs 17.5 Oc | -0.04542 | 0.189831 | Inf | -0.23929 | 0.810883 |
| 17.5 Both vs 17.5 N  | -0.8584  | 0.189349 | Inf | -4.53343 | 5.8E-06  |
| 17.5 Ht vs 17.5 Oc   | 0.435648 | 0.192607 | Inf | 2.261852 | 0.023707 |
| 17.5 Ht vs 17.5 N    | -0.37733 | 0.192131 | Inf | -1.9639  | 0.049542 |
| 17.5 Oc vs 17.5 N    | -0.81297 | 0.189308 | Inf | -4.29445 | 1.75E-05 |
| 21 Both vs 21 Ht     | -0.40587 | 0.189843 | Inf | -2.13793 | 0.032522 |
| 21 Both vs 21 Oc     | -0.19451 | 0.190011 | Inf | -1.0237  | 0.305976 |
| 21 Both vs 21 N      | -0.85724 | 0.189584 | Inf | -4.5217  | 6.13E-06 |
| 21 Ht vs 21 Oc       | 0.211357 | 0.189654 | Inf | 1.114436 | 0.265092 |
| 21 Ht vs 21 N        | -0.45137 | 0.189226 | Inf | -2.38535 | 0.017063 |
| 21 Oc vs 21 N        | -0.66273 | 0.189394 | Inf | -3.49919 | 0.000467 |
| 24.5 Both vs 24.5 Ht | -0.11507 | 0.219263 | Inf | -0.5248  | 0.599721 |
| 24.5 Both vs 24.5 Oc | -0.05485 | 0.234711 | Inf | -0.23369 | 0.815222 |
| 24.5 Both vs 24.5 N  | -0.57819 | 0.216231 | Inf | -2.67395 | 0.007496 |
| 24.5 Ht vs 24.5 Oc   | 0.060219 | 0.213127 | Inf | 0.282549 | 0.777523 |
| 24.5 Ht vs 24.5 N    | -0.46312 | 0.192588 | Inf | -2.40473 | 0.016185 |
| 24.5 Oc vs 24.5 N    | -0.52334 | 0.210007 | Inf | -2.49201 | 0.012702 |
| 10.5 Oc vs 14 Oc     | 0.110432 | 0.188779 | Inf | 0.584984 | 0.558559 |
| 10.5 Oc vs 17.5 Oc   | 0.930531 | 0.189265 | Inf | 4.916538 | 8.81E-07 |
| 10.5 Oc vs 21 Oc     | 0.948119 | 0.189281 | Inf | 5.009058 | 5.47E-07 |
| 10.5 Oc vs 24.5 Oc   | 0.974048 | 0.209831 | Inf | 4.642051 | 3.45E-06 |
| 14 Oc vs 17.5 Oc     | 0.820099 | 0.189305 | Inf | 4.332144 | 1.48E-05 |
| 14 Oc vs 21 Oc       | 0.837687 | 0.189321 | Inf | 4.424691 | 9.66E-06 |
| 14 Oc vs 24.5 Oc     | 0.863616 | 0.209867 | Inf | 4.115052 | 3.87E-05 |
| 17.5 Oc vs 21 Oc     | 0.017588 | 0.189806 | Inf | 0.092663 | 0.926172 |
| 17.5 Oc vs 24.5 Oc   | 0.043517 | 0.210306 | Inf | 0.206923 | 0.83607  |
| 21 Oc vs 24.5 Oc     | 0.025929 | 0.210319 | Inf | 0.123284 | 0.901882 |
| 10.5 Ht vs 14 Ht     | 0.110775 | 0.194936 | Inf | 0.568266 | 0.569855 |
| 10.5 Ht vs 17.5 Ht   | 0.54149  | 0.195139 | Inf | 2.774891 | 0.005522 |
| 10.5 Ht vs 21 Ht     | 0.783369 | 0.19221  | Inf | 4.075596 | 4.59E-05 |
| 10.5 Ht vs 24.5 Ht   | 0.960436 | 0.195442 | Inf | 4.914187 | 8.92E-07 |
| 14 Ht vs 17.5 Ht     | 0.430715 | 0.195179 | Inf | 2.20677  | 0.02733  |
| 14 Ht vs 21 Ht       | 0.672594 | 0.19225  | Inf | 3.49854  | 0.000468 |
| 14 Ht vs 24.5 Ht     | 0.849661 | 0.195481 | Inf | 4.346513 | 1.38E-05 |
| 17.5 Ht vs 21 Ht     | 0.241879 | 0.192456 | Inf | 1.2568   | 0.208826 |
| 17.5 Ht vs 24.5 Ht   | 0.418946 | 0.195684 | Inf | 2.140931 | 0.03228  |
| 21 Ht vs 24.5 Ht     | 0.177067 | 0.192763 | Inf | 0.918574 | 0.358318 |

**Table D4.** List of custom contrasts for fecundity per week under constant temperatures generated through emmeans package in R.

| contrast             | estimate | SE       | df  | z.ratio  | p.value  |
|----------------------|----------|----------|-----|----------|----------|
| 10.5 Both vs 10.5 Ht | 0.017805 | 0.112059 | Inf | 0.158887 | 0.873758 |
| 10.5 Both vs 10.5 Oc | 0.131852 | 0.113661 | Inf | 1.160046 | 0.24603  |
| 10.5 Both vs 10.5 N  | 0.082742 | 0.112951 | Inf | 0.732548 | 0.463834 |
| 10.5 Ht vs 10.5 Oc   | 0.114048 | 0.113893 | Inf | 1.001354 | 0.316656 |
| 10.5 Ht vs 10.5 N    | 0.064938 | 0.113185 | Inf | 0.573731 | 0.56615  |
| 10.5 Oc vs 10.5 N    | -0.04911 | 0.114772 | Inf | -0.42789 | 0.668731 |
| 14 Both vs 14 Ht     | -0.30978 | 0.10617  | Inf | -2.91777 | 0.003525 |
| 14 Both vs 14 Oc     | 0.24588  | 0.111433 | Inf | 2.206533 | 0.027347 |
| 14 Both vs 14 N      | 0.109304 | 0.109419 | Inf | 0.998954 | 0.317817 |
| 14 Ht vs 14 Oc       | 0.555659 | 0.109688 | Inf | 5.065794 | 4.07E-07 |
| 14 Ht vs 14 N        | 0.419084 | 0.107642 | Inf | 3.893317 | 9.89E-05 |
| 14 Oc vs 14 N        | -0.13658 | 0.112836 | Inf | -1.21039 | 0.226129 |
| 17.5 Both vs 17.5 Ht | -0.4543  | 0.107889 | Inf | -4.21086 | 2.54E-05 |
| 17.5 Both vs 17.5 Oc | 0.346491 | 0.117169 | Inf | 2.957182 | 0.003105 |
| 17.5 Both vs 17.5 N  | -0.40234 | 0.106802 | Inf | -3.7672  | 0.000165 |
| 17.5 Ht vs 17.5 Oc   | 0.800796 | 0.11371  | Inf | 7.042422 | 1.89E-12 |
| 17.5 Ht vs 17.5 N    | 0.05196  | 0.102996 | Inf | 0.504485 | 0.61392  |
| 17.5 Oc vs 17.5 N    | -0.74884 | 0.11268  | Inf | -6.6457  | 3.02E-11 |
| 21 Both vs 21 Ht     | -0.26709 | 0.100233 | Inf | -2.66469 | 0.007706 |
| 21 Both vs 21 Oc     | 0.073031 | 0.103254 | Inf | 0.707296 | 0.479383 |
| 21 Both vs 21 N      | -0.32315 | 0.099819 | Inf | -3.23736 | 0.001206 |
| 21 Ht vs 21 Oc       | 0.340121 | 0.099286 | Inf | 3.42566  | 0.000613 |
| 21 Ht vs 21 N        | -0.05606 | 0.095709 | Inf | -0.58575 | 0.558043 |
| 21 Oc vs 21 N        | -0.39618 | 0.098869 | Inf | -4.00716 | 6.15E-05 |
| 24.5 Both vs 24.5 Ht | -0.0726  | 0.116284 | Inf | -0.62434 | 0.532405 |
| 24.5 Both vs 24.5 Oc | -0.12106 | 0.123405 | Inf | -0.98103 | 0.326579 |
| 24.5 Both vs 24.5 N  | -0.23824 | 0.113884 | Inf | -2.09196 | 0.036443 |
| 24.5 Ht vs 24.5 Oc   | -0.04846 | 0.108121 | Inf | -0.44823 | 0.653988 |
| 24.5 Ht vs 24.5 N    | -0.16564 | 0.097112 | Inf | -1.70564 | 0.088075 |
| 24.5 Oc vs 24.5 N    | -0.11718 | 0.105535 | Inf | -1.11031 | 0.266865 |
| 10.5 Oc vs 14 Oc     | 0.10215  | 0.115131 | Inf | 0.887251 | 0.374944 |
| 10.5 Oc vs 17.5 Oc   | 0.336682 | 0.119063 | Inf | 2.82776  | 0.004687 |
| 10.5 Oc vs 21 Oc     | -0.38897 | 0.109101 | Inf | -3.56525 | 0.000364 |
| 10.5 Oc vs 24.5 Oc   | -0.73993 | 0.115607 | Inf | -6.40037 | 1.55E-10 |
| 14 Oc vs 17.5 Oc     | 0.234532 | 0.118734 | Inf | 1.975278 | 0.048237 |
| 14 Oc vs 21 Oc       | -0.49112 | 0.108741 | Inf | -4.51642 | 6.29E-06 |
| 14 Oc vs 24.5 Oc     | -0.84208 | 0.115268 | Inf | -7.3054  | 2.76E-13 |
| 17.5 Oc vs 21 Oc     | -0.72565 | 0.112896 | Inf | -6.4276  | 1.3E-10  |
| 17.5 Oc vs 24.5 Oc   | -1.07661 | 0.119196 | Inf | -9.03228 | 1.68E-19 |
| 21 Oc vs 24.5 Oc     | -0.35096 | 0.109246 | Inf | -3.21257 | 0.001316 |
| 10.5 Ht vs 14 Ht     | -0.33946 | 0.108389 | Inf | -3.13188 | 0.001737 |
| 10.5 Ht vs 17.5 Ht   | -0.35007 | 0.108285 | Inf | -3.23282 | 0.001226 |
| 10.5 Ht vs 21 Ht     | -0.61504 | 0.10453  | Inf | -5.8839  | 4.01E-09 |
| 10.5 Ht vs 24.5 Ht   | -0.57742 | 0.106286 | Inf | -5.43272 | 5.55E-08 |
| 14 Ht vs 17.5 Ht     | -0.0106  | 0.10423  | Inf | -0.10174 | 0.918962 |
| 14 Ht vs 21 Ht       | -0.27558 | 0.100323 | Inf | -2.74695 | 0.006015 |
| 14 Ht vs 24.5 Ht     | -0.23796 | 0.102151 | Inf | -2.32948 | 0.019834 |
| 17.5 Ht vs 21 Ht     | -0.26498 | 0.100211 | Inf | -2.6442  | 0.008188 |
| 17.5 Ht vs 24.5 Ht   | -0.22735 | 0.102041 | Inf | -2.22807 | 0.025876 |
| 21 Ht vs 24.5 Ht     | 0.037624 | 0.098047 | Inf | 0.383731 | 0.701178 |

**Table D5.** List of custom contrasts for survival for constant versus heatwaves temperatures generated through emmeans package in R.

| contrast         | estimate | SE       | df  | z.ratio  | p.value  |
|------------------|----------|----------|-----|----------|----------|
| C Oc vs C Ht     | 0.302353 | 0.096892 | Inf | 3.120532 | 0.001805 |
| C Oc vs C Both   | 0.219431 | 0.095537 | Inf | 2.296822 | 0.021629 |
| C Oc vs C N      | -0.05611 | 0.093836 | Inf | -0.59791 | 0.549898 |
| C Ht vs C Both   | -0.08292 | 0.097461 | Inf | -0.85083 | 0.394865 |
| C Ht vs C N      | -0.35846 | 0.095794 | Inf | -3.74197 | 0.000183 |
| C Both vs C N    | -0.27554 | 0.094424 | Inf | -2.91809 | 0.003522 |
| P Oc vs P Ht     | 0.516446 | 0.094429 | Inf | 5.469124 | 4.52E-08 |
| P Oc vs P Both   | 0.590089 | 0.096823 | Inf | 6.094518 | 1.1E-09  |
| P Oc vs P N      | 0.008857 | 0.093159 | Inf | 0.095071 | 0.924258 |
| P Ht vs P Both   | 0.073644 | 0.097116 | Inf | 0.758306 | 0.448268 |
| P Ht vs P N      | -0.50759 | 0.093464 | Inf | -5.43086 | 5.61E-08 |
| P Both vs P N    | -0.58123 | 0.095882 | Inf | -6.06198 | 1.34E-09 |
| C Oc vs P Oc     | -0.1907  | 0.094543 | Inf | -2.0171  | 0.043685 |
| C Ht vs P Ht     | 0.02339  | 0.096781 | Inf | 0.241681 | 0.809027 |
| C Both vs P Both | 0.179956 | 0.097794 | Inf | 1.840161 | 0.065745 |
| C N vs P N       | -0.12574 | 0.092442 | Inf | -1.3602  | 0.173767 |

**Table D6.** Table of pairwise comparisons for survival over time for constant versus heatwave temperatures generated through Kaplan-Meier analysis in R.

|         | 17.5 Both | 17.5 Ht | 17.5 N  | 17.5 Oc | P Both  | P Ht    | P N     |
|---------|-----------|---------|---------|---------|---------|---------|---------|
| 17.5 Ht | 0.34580   | -       | -       | -       | -       | -       | -       |
| 17.5 N  | 0.04486   | 0.00359 | -       | -       | -       | -       | -       |
| 17.5 Oc | 0.23042   | 0.02672 | 0.36967 | -       | -       | -       | -       |
| P Both  | 0.15586   | 0.56984 | 0.00130 | 0.00378 | -       | -       | -       |
| P Ht    | 0.71906   | 0.89356 | 0.02136 | 0.22965 | 0.56542 | -       | -       |
| P N     | 0.00029   | 8.4e-06 | 0.06125 | 0.00417 | 1.0e-06 | 0.00075 | -       |
| P Oc    | 0.00049   | 6.5e-06 | 0.15586 | 0.00624 | 8.6e-07 | 0.00217 | 0.26609 |

**Table D7.** List of custom contrasts for total fecundity for constant versus heatwaves temperatures generated through emmeans package in R.

| contrast         | estimate | SE       | df  | z.ratio  | p.value  |
|------------------|----------|----------|-----|----------|----------|
| C Oc vs C Ht     | -0.28718 | 0.109238 | Inf | -2.62896 | 0.008565 |
| C Oc vs C Both   | 0.171373 | 0.108644 | Inf | 1.577374 | 0.114709 |
| C Oc vs C N      | -0.69032 | 0.108204 | Inf | -6.37977 | 1.77E-10 |
| C Ht vs C Both   | 0.458555 | 0.109357 | Inf | 4.193199 | 2.75E-05 |
| C Ht vs C N      | -0.40314 | 0.10892  | Inf | -3.70121 | 0.000215 |
| C Both vs C N    | -0.86169 | 0.108324 | Inf | -7.95472 | 1.8E-15  |
| P Oc vs P Ht     | -0.28718 | 0.109238 | Inf | -2.62896 | 0.008565 |
| P Oc vs P Both   | 0.171373 | 0.108644 | Inf | 1.577374 | 0.114709 |
| P Oc vs P N      | -0.69032 | 0.108204 | Inf | -6.37977 | 1.77E-10 |
| P Ht vs P Both   | 0.458555 | 0.109357 | Inf | 4.193199 | 2.75E-05 |
| P Ht vs P N      | -0.40314 | 0.10892  | Inf | -3.70121 | 0.000215 |
| P Both vs P N    | -0.86169 | 0.108324 | Inf | -7.95472 | 1.8E-15  |
| C Oc vs P Oc     | -0.13639 | 0.076922 | Inf | -1.77311 | 0.076211 |
| C Ht vs P Ht     | -0.13639 | 0.076922 | Inf | -1.77311 | 0.076211 |
| C Both vs P Both | -0.13639 | 0.076922 | Inf | -1.77311 | 0.076211 |
| C N vs P N       | -0.13639 | 0.076922 | Inf | -1.77311 | 0.076211 |

**Table D8.** List of custom contrasts for fecundity per week for constant versus heatwaves temperatures generated through emmeans package in R.

| contrast         | estimate | SE       | df  | z.ratio  | p.value  |
|------------------|----------|----------|-----|----------|----------|
| C Oc vs C Ht     | -0.8008  | 0.127963 | Inf | -6.25802 | 3.9E-10  |
| C Oc vs C Both   | -0.34649 | 0.130622 | Inf | -2.65263 | 0.007987 |
| C Oc vs C N      | -0.74884 | 0.12661  | Inf | -5.9145  | 3.33E-09 |
| C Ht vs C Both   | 0.454305 | 0.122819 | Inf | 3.698973 | 0.000216 |
| C Ht vs C N      | 0.05196  | 0.118544 | Inf | 0.438317 | 0.661156 |
| C Both vs C N    | -0.40234 | 0.121409 | Inf | -3.31397 | 0.00092  |
| P Oc vs P Ht     | -0.71649 | 0.124103 | Inf | -5.77338 | 7.77E-09 |
| P Oc vs P Both   | -0.40809 | 0.126871 | Inf | -3.21654 | 0.001297 |
| P Oc vs P N      | -0.58076 | 0.125225 | Inf | -4.63777 | 3.52E-06 |
| P Ht vs P Both   | 0.308408 | 0.118669 | Inf | 2.598888 | 0.009353 |
| P Ht vs P N      | 0.135732 | 0.116907 | Inf | 1.161022 | 0.245633 |
| P Both vs P N    | -0.17268 | 0.119841 | Inf | -1.44088 | 0.14962  |
| C Oc vs P Oc     | -0.11238 | 0.13373  | Inf | -0.84038 | 0.400696 |
| C Ht vs P Ht     | -0.02808 | 0.117866 | Inf | -0.23826 | 0.811678 |
| C Both vs P Both | -0.17398 | 0.12359  | Inf | -1.40771 | 0.159217 |
| C N vs P N       | 0.055689 | 0.11759  | Inf | 0.473584 | 0.635797 |