Impact of environmental stressors on host-parasite interactions in mass-reared *Tenebrio molitor*

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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Rationale for thesis by alternative format

Publishing scientific papers is an internationally acknowledged approach for sharing discoveries with the scientific community. Sharing results of studies through peer-reviewed journals ensures that the new knowledge is available to other researchers. Moreover, in the context of the mass-production of insects, scientific publications are of high relevance to move this field forward by distributing research findings quickly to professionals working in the industry, government, and other organisations. This thesis is therefore presented in the alternative format with the aim to publish the work presented in chapters 2 – 5 in individual publications.

This thesis consists of an introductory chapter, a published literature review, three data chapters, and a discussion chapter. In the introduction (chapter 1), the context and rationale of this thesis are outlined. The work presented in chapter 2 is a literature review on environment-host-parasite interactions, which has been published in the journal Trends in Parasitology. Experimental studies investigating the impact of environmental stressors on host-parasite interactions in *Tenebrio molitor* are presented in chapters 3 – 5. Finally, chapter 6 is a general discussion with recommendations for the insect mass-rearing industry, future research directions, and overall conclusions.

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Abstract

The mass-production of insects is expanding globally and the industry of producing insects as feed and food predicted to grow rapidly in the coming years. Insects kept in closed rearing systems at high densities may be exposed to various abiotic and biotic stressors, often occurring simultaneously. It is crucial to understand the impact of these stressors because they can reduce the productivity of reared insects. In chapter 2 of this thesis, I review the literature on environment-host-parasite interactions in mass-reared insect species and produce a workflow to optimise environmental conditions to reduce the impact of parasite infection in mass-production systems. In my experimental studies, I explore the impact of multiple stressors on host-pathogen interactions, focusing on *Tenebrio molitor*, which is one of the most promising insect species mass-reared for protein production. In chapter 3, I find that a short heat stress of two hours can increase the survival probability of larvae exposed to the fungus *Metarhizium brunneum*, which correlates with the antimicrobial activity measured in the hemolymph. Nevertheless, these beneficial effects wane within five days after the heat stress, accompanied by reduced larval weight gain. Elevated carbon dioxide (CO_2) reduces the viability and persistence of the spores of the bacterium *Bacillus thuringiensis*, but it accelerates the germination of *M. brunneum* conidia. Interestingly, *T. molitor* larvae reared at elevated CO_2 conditions have a reduced susceptibility to both pathogens (chapter 4). However, there is no evidence that elevated CO_2 affects the interactions between the two pathogens during co-exposure of *T. molitor* larvae. Furthermore, in chapter 5, I find mostly antagonistic or additive interactions between the two pathogens when using a mixture toxicity model, indicating that the risk during co-exposure to the two tested pathogen species does not increase in early-stage larval populations of *T. molitor*. In chapter 6, I discuss how this novel understanding of different abiotic stressors interacting with parasites of mass-reared insects, may be used to optimise the environmental conditions to simultaneously enhance productivity, and mitigate the risk of parasite infections.

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List of acronyms and abbreviations

AdDV	*Acheta domesticus* densovirus
AI	Artificial intelligence
AMP	Antimicrobial peptide
ANOVA	Analysis of variances
BAS	Bovine albumin serum
BmNPV	*Bombyx mori* nucleopolyhedrovirus
CCD	Colony collapse disorder
cfu	Colony forming units
CHIKV	Chikungunya virus
CH_4	Methane
CO_2	Carbon dioxide
CrPV	Cricket paralysis virus
Cry	Crystalline
Cyt	Cytolytic
C:N	Carbon-to-nitrogen ratio
DCV	Drosophila C virus
dH_2O	Deionised water
DL	Dose level-dependent
DNA	Deoxyribonucleic acid
DR	Dose ratio-dependent
DWV	Deformed wing virus
d.f.	Degrees of freedom
EC_{50}	Median effective concentration
EU	European Union
HCT	Hydrolysate of casein tryptone
HR	Hazard ratio
HS	Heat stress
HSD	Honestly Significant Difference
HSP	Heat shock protein
IA	Independent action
IIV-6	Invertebrate iridescent virus

Imd	Immunodeficiency
IR	Immune response
JNK	c-Jun-N-terminal kinase
LB	Lysogeny broth
LD_{50}	Median lethal dose
L-DOPA	l-3,4-dihydroxyphenylalanine
MIXTox	Mixture toxicity
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NaClO	Sodium hypochlorite
NaOH	Sodium hydroxide
NH_3	Ammonia
NPK	Nitrogen, phosphorus, and potassium
NPV	Nucleopolyhedrovirus
N_2O	Nitrous oxide
OD	Optical density
PBS	Phosphate-buffered saline
PO	Phenoloxidase
ppm	Parts per million
ProPO	Prophenoloxidase
QQ-plot	Quantile-quantile plot
RNA	Ribonucleic acid
RNAi	RNAinterference
Rpm	Revolutions per minute
SDA	Sabouraud Dextrose Agar
SDAY/4	¼ Sabouraud Dextrose Yeast Agar
SE	Standard error
SEM	Standard error of the mean
SeNPV	*Spodoptera exigua* nucleopolyhedrovirus
siRNA	Small interfering RNA
SIT	Sterile insect technique
S/A	Synergistic/Antagonistic

UK	United Kingdom
US	United States
USA	United States of America
Vmax	Maximal velocity of the reaction
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight
χ^2	Chi-square

1. Chapter: Introduction

1.1 Outline

The class Insecta is seen as the dominant group in the animal kingdom [1] and it consists of approximately 80% of the known animal species [2]. Insects are a very diverse and abundant group, and they are highly adaptable. They therefore inhabit almost all ecological niches in terrestrial and freshwater ecosystems of the planet [1, 2]. Insects have always been in a close relationship with humans. Certain insect species harm human life by, for example, vectoring diseases or feeding on crops and stored products. However, it is estimated that the minority (<1%) of all insect species are harmful to humans [1]. Most insect species pose no threat to humans, and many fulfil various critical functions in ecosystems offering essential 'ecosystem services' (i.e., "Benefits that humans recognise as obtained from ecosystems that support, directly or indirectly, their survival and quality of life" [3]). Insect pollination, for example, is required for 60-90% of all plant species, many of them used as crops for food production [4]. Insects are also a food source for humans, and it is estimated that more than 2,000 insect species are harvested from the wild and eaten predominately in tropical countries [5]. Moreover, insects provide ecosystem services by decomposing organic matter and controlling insect pest populations [6, 7]. Because insects provide all these ecosystem services, they are seen as critical in achieving the sustainable development goals proposed by the United Nation member states (Figure 1.1) [7].

To make improved use of insects, people have started to mass-rear them for numerous reasons such as for food and feed, pollination, or biological control purposes [8]. The sector of insect production for animal feed and human consumption is predicted to grow tremendously in the coming years [9]. Through the utilization of organic side-streams to produce insect-derived protein, this industry also presents a sustainable solution for repurposing waste streams, thereby offering a substantial avenue to contribute to global food security [10, 11].

Mass-reared insects may be exposed to both biotic and abiotic stressors affecting productivity, welfare, and biosafety in these systems. This thesis investigates how environmental stressors affect host-parasite interactions in mass-reared insects. Understanding these interactions will facilitate optimising environmental conditions and assessing risks of stressors in mass-reared insects. I aim to study different stressors in combination with each other and provide specific recommendations for producers of insects.

In this chapter, I introduce this work. I start with giving an overview on mass-rearing insects (section 1.2), focusing on insects produced for food and feed. I then introduce host-parasite interactions in insects (section 1.3) and provide reasons why insect health is important for mass production (section 1.3.1). Thereafter, I present stressors in insect mass-rearing systems (section 1.4). I introduce the study organisms used in my experimental studies (section 1.5) and finally I provide the thesis plan (section 1.6).

1.2 Mass-rearing insects

Insect species such as domestic silk moths (*Bombyx mori* L. Lepidoptera: Bombycidae) and Western honey bees (*Apis mellifera* L. Hymenoptera: Apidae) have been reared for several thousand years for the production of silk and honey, respectively [12]. Today, numerous additional insect species are reared for the biological control of insect pests, the sterile insect technique to control vectors of human and animal diseases, for pollination of crops, management of waste, for research, and the production of food [8]. Moreover, the by-products of insect production are used in other sectors, such as the use of insect frass (i.e., excreta) for the production of fertilisers [13] or the use of insect chitin for the production of cosmetics [14]. Together insects contribute to all of the 17 sustainable development goals defined by the United Nations [7]. Certain insect species act as a threat to the goals but Dangles and Casas (2019) identified many species that act as allies or solutions (Figure 1.1 [7]).

A major recent development is the production of insects for human consumption and as feed for aquaculture, livestock, and pets. Insects reared for these purposes have been identified to contribute to 13 of the 17 sustainable development goals as either allies or solutions (Figure 1.1 [7]). The most common insect species reared for human consumption are yellow mealworms (*Tenebrio molitor* L. Coleoptera: Tenebrionidae), lesser mealworms (*Alphitobius diaperinus* P. Coleoptera: Tenebrionidae), superworms (*Zophobas morio* F. Coleoptera: Tenebrionidae), house crickets (*Acheta domesticus* L. Orthoptera: Gryllidae), banded crickets (*Gryllodes sigillatus* W. Orthoptera: Gryllidae), two-spotted crickets (*Gryllus bimaculatus* D. G. Orthoptera: Gryllidae), migratory locusts (*Locusta migratoria* L. Orthoptera: Acrididae), and desert locusts (*Schistocerca gregaria* F. Orthoptera: Acrididae) [15]. The species frequently produced for animal feed are black soldier flies (*Hermetia illucens* L. Diptera: Stratiomyidae), common houseflies (*Musca domestica* L. Diptera: Muscidae), and *T. molitor* [10, 15]. Insects have a lower environmental footprint than conventional livestock [15]. Moreover, insects reared for food and feed generally have high protein contents and some of them can be reared on low-value organic side streams, which makes them a sustainable alternative to common protein sources such as soy and fishmeal [10, 16].

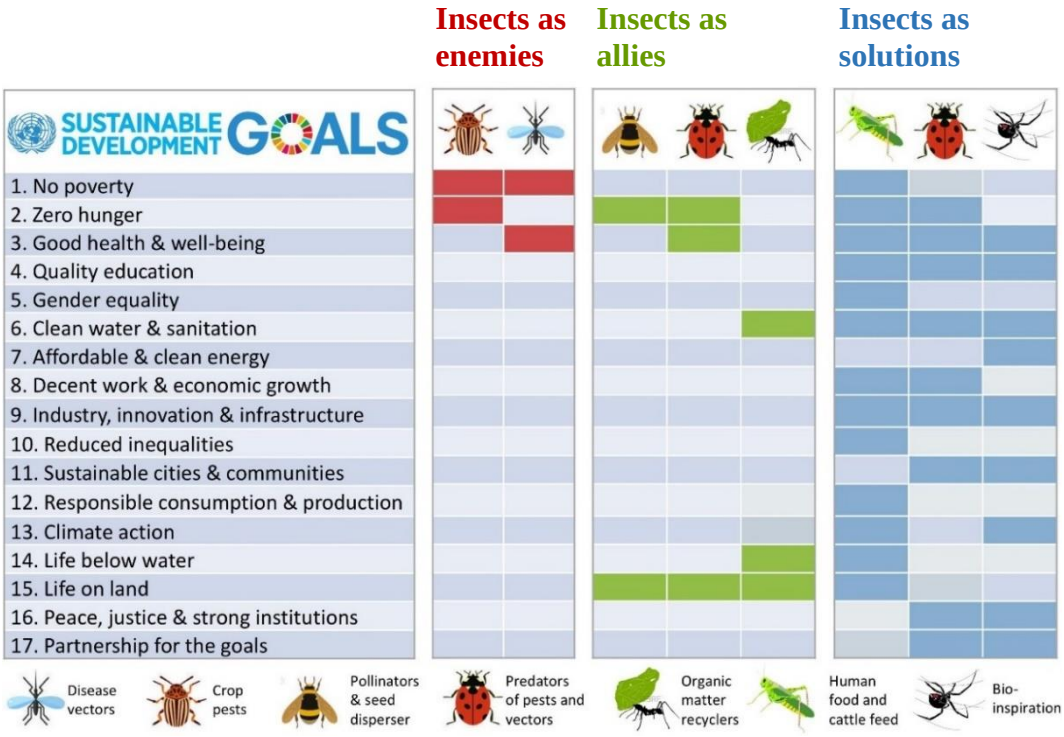

Figure 1.1 Insects and the 17 sustainable development goals.

Insects act as enemies (red), allies (green), and solutions (blue) in relation to the 17 sustainable development goals defined by the United Nations. Figure adapted from Dangles and Casas (2019) [7].

The production of insects as food and feed can take place in open, semi-open, and closed systems [17], ranging from systems with a high proportion of human labour to fully automated systems. All of these systems have in common that the insects are kept at high densities. Such high densities can alter the environmental conditions, for example by increasing temperature due to metabolic heat production (section 1.5.4) or increasing CO_2 because of respiration (section 1.5.5). Moreover, insects kept at high densities are at an increased risk of infections by parasites as the close proximity to other individuals and populations increases transmission rates of infectious agents [18].

1.3 Host-parasite interactions in insects

Insects can defend themselves from parasites with their immune response. In brief, insects can utilise physical defences, behavioural defences, and their innate immune

response to protect themselves. The insect cuticle prevents the entry of many parasites, especially microbial parasites. However, entomopathogenic fungi can penetrate through the cuticle using enzymes and physical pressure [19]. Similarly, entomopathogenic nematodes may enter through the cuticle but they might also enter through natural openings [20]. Other entomopathogens (e.g., bacteria and viruses) need to be ingested to be able to infect insects and as such, these pathogens need to overcome the unfavourable conditions in the gut lumen, such as very low or high pH, and digestive enzymes [21]. Behavioural defences include the increasing or decreasing of the insect's body temperature by actively seeking places with these differential temperatures in response to parasite infection (i.e., behavioural fever or cooling) [22] and avoidance of parasites [23]. Examples of behavioural defences are described in chapter 2.

If entomopathogens can overcome the physical or behavioural defences, they will then face the insect's innate immune system in the hemocoel [21]. Once a pathogen is recognised within the insect host's immune system, several responses are initiated; these responses can be broadly classified as humoral and cellular responses [24]. The humoral response involves the prophenoloxidase cascade and the production of AMPs (antimicrobial peptides), which are typically cationic molecules mainly being produced in the fat body [21, 25]. The production of AMPs is induced by the immunodeficiency (Imd), Toll, and c-Jun-N-terminal kinase (JNK) signal transduction pathways [25]. The cellular response involves hemocytes [24]. In *T. molitor*, three types of hemocytes are distinguished: granulocytes, plasmatocytes and oenocytoids [26]. Granulocytes are involved in phagocytosis and encapsulation, plasmatocytes are involved in encapsulation, phagocytosis and nodulation, whereas oenocytes produce prophenoloxidase [25, 26]. All three hemocyte types are differentiated from stem cells called prohemocytes [21, 26].

The cellular and humoral response are intertwined and they are both involved in a process called melanisation (a pathogen encapsulation process), which can, for example, block the entrance of fungal germ tubes [21]. The melanisation process begins when prophenoloxidase turns into phenoloxidase only minutes after infection

[27]. Thereafter, plasmatocytes are recruited to the pathogen. Through lysis of the plasmatocytes, the produced melanin is released and it encapsulates the pathogen [21].

The impact of the environment on the immunity of mass-reared insects and interactions of the immunity with different parasite groups are reviewed in chapter 2. Furthermore, the innate immune response of *T. molitor* are introduced in chapter 3 together with methods to measure phenoloxidase activity, antimicrobial activity of the hemolymph, and hemocyte concentrations.

1.3.1 Why is insect health important in mass-rearing?

There are three main reasons why we are interested in keeping populations of mass-reared insects healthy. Firstly, infections by parasites often have lethal or sublethal effects on insect populations, leading to economic losses [18]. This reason is the main motivation of this thesis and examples of economic losses caused by parasites in insect mass-rearing systems are given in box 1 in chapter 2. However, healthy insect populations are also desirable for insect welfare reasons [28]. It is unclear if insects are sentient (i.e., "have the capacity to experience negative affective states" [28]) and they should therefore be given the benefit of the doubt [28, 29]. Physical health, which includes the 'freedom of disease', is one of the five domains of animal welfare together with the other four domains (i.e., nutrition, environment, behaviour, and the mental domain) [28]. Finally, insect populations should be kept free from infectious diseases to prevent the spill over of diseases to wild insect populations [18, 30]. Certain mass-reared insect species may be produced in regions of the world where they are not native. This harbours the risk of introducing parasites from production systems into nature, which potentially may harm native insect species in the region [30].

1.4 Stressors in insect mass-rearing systems

Mass-reared insects may be exposed to environmental (e.g., heat stress, elevated CO_2, or vibration) or biotic (e.g., microbial pathogens) stressors (Figure 1.2). In addition, other stressors such as pesticide residues, nutritional stress, or crowding

may occur in production systems. However, in this thesis I focus on the impact of abiotic environmental stressors and parasite infections. The term ‘stressor’ is defined in this thesis as a “variable that causes a quantifiable change, irrespective of its direction (increase or decrease), in a biological response” [31].

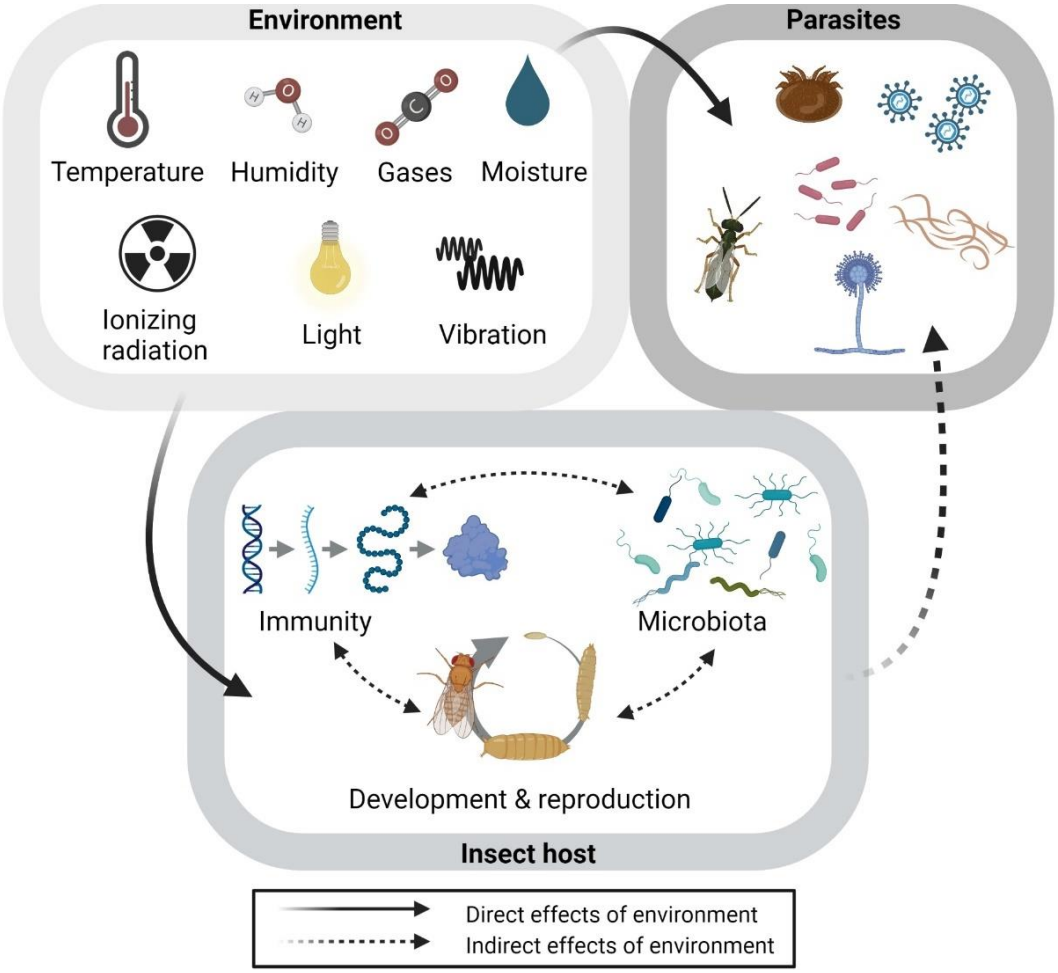

Figure 1.2 Interactions between the environment, parasites, and insect host.
The environmental conditions in mass-rearing systems may have a direct effect on parasites. The insect host’s immunity, microbiota, and development and reproduction may be directly affected by the environment, which has an indirect effect on the interaction between insect host and parasites. Figure created using BioRender.com.

Different abiotic and biotic stressors may occur simultaneously in insect mass-rearing systems. While the effects of multiple stressors on insects are widely studied in natural [e.g., 31, 32, 33] or agricultural [e.g., 34, 35] contexts, studies are also needed on the effects of multiple stressors on mass-reared insects, especially those

produced for food and feed, as this is a new emerging field. It is critical to conduct such studies because the conditions that mass-reared insects are kept in are vastly different to the conditions in nature.

Different stressors may interact with each other in a synergistic way, resulting in a greater effect than predicted from the individual effects. Additivity between stressors describes the case when the resulting effect is equal to that predicted for the individual effects. Finally, stressors can also interact in an antagonistic way, resulting in a smaller effect than predicted. Different stressors can directly interact with each other (e.g., high relative humidity in the environment favours the germination of fungal spores [17]) or they can indirectly interact with each other via the insect host (e.g., high temperature alters the host's microbiota making it more protective against a trypanosomatid parasite [36]), which is shown in Figure 1.2. The term 'cross-protection' describes an indirect interaction in which one stressor prompts a response that is beneficial to the host when encountering another stressor [37]. Cross-protection can be further divided into 'cross-tolerance' (i.e., "stressors share protective mechanisms" [37]) and 'cross-talk' (i.e., "stressors share signalling/regulatory pathways that activate independent protective mechanisms" [37]).

1.5 Study organisms and environmental stressors

Here, I introduce the host species, pathogen species, and environmental stressors used in the experimental studies (chapters 3 – 5). Further introductions are given in the respective chapters.

1.5.1 *Tenebrio molitor*

The yellow mealworm (*Tenebrio molitor* L. Coleoptera: Tenebrionidae) is one of the most common insect species mass-reared for human consumption and animal feed [15, 38]. I therefore chose this insect species as the host organism in the experimental studies (chapters 3 – 5) presented in this thesis. The larval stage of *T. molitor* is produced for its high quality protein, fat, and micronutrients [e.g., 39, 40]. In addition, the frass of *T. molitor* larvae is used as a plant fertiliser, which is

comparable to mineral NPK (nitrogen, phosphorus, and potassium) fertiliser when it comes to uptake of the minerals by agricultural crops such as barley (*Hordeum vulgare* L. Poales: Poaceae) and plant biomass accumulation [41]. Moreover, *T. molitor* larvae have been suggested to be used for the biodegradation of plastics [e.g., 42]. However, before companies started to produce this species for commercial reasons, *T. molitor* had already been used for many years as a model organism to study insect ecology, host-pathogen interactions, and insect physiology [e.g., 43, 44] due to its relatively simple rearing requirements, making it an ideal laboratory organism [45].

Tenebrio molitor is native to central Europe [46] and it naturally occurs in rotting wood [46, 47]. Nowadays, *T. molitor* has become distributed around the world [46] and it is a common pest of stored grains [e.g., 26]. *Tenebrio molitor* larvae hatch from eggs after approximately four days [45]. The larvae go through 9 to 23 instars thereafter depending on environmental conditions and diet availability [45, 48]. In the literature the larval stage is given at between eight weeks at optimal conditions and up to 89 weeks at suboptimal conditions [45], although in my experiments (chapter 3), the larval stage lasted approximately seven weeks. The pupal stage lasts between six and 20 days before the adults emerge [45]. Adult *T. molitor* beetles live up to 24 weeks and the females start to lay eggs approximately three days after emergence [45] (Figure 1.3).

Tenebrio molitor that are produced for food and feed purposes are usually collected in the larval stage when they reach a weight of 100 to 110 mg [49]. The larvae are kept in stackable trays in closed rooms or halls [50]. The frass either falls through a mesh at the bottom of the trays or it needs to be removed on a regular basis using sieves [50]. The larval stages can feed on a variety of diets [40], but the highest growth rates and reproduction are achieved by using carbohydrate-rich (approximately 80%) diets supplemented with sources for protein, lipids, and micronutrients [40]. The larvae and the adults benefit from a water source [50] even though they are able to take up water vapour through their rectum if the relative humidity is high enough [51]. High relative humidity (84%) has been shown to

increase the growth of *T. molitor* larvae [52]. However, with increasing relative humidity the risk of infection by fungal pathogens also increases [17]. In mass-rearing systems, the relative humidity is maintained between 50 and 75% and the temperature is set to 25 to 30°C [50]. *Tenebrio molitor* is generally kept in complete darkness, as light provision has been shown to reduce growth and increase the development time of the larvae [53].

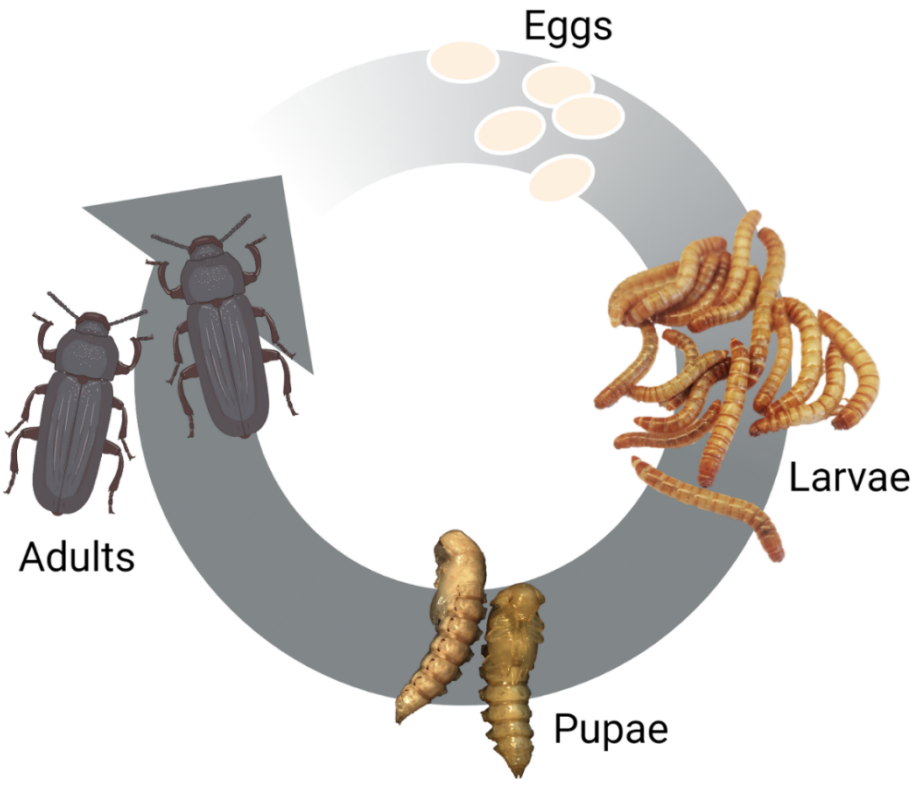

Figure 1.3 Lifecycle of *Tenebrio molitor*.
The eggs are oviposited by the female adults directly inside the diet. After approximately four days, the eggs hatch and the larvae go through 9 to 23 instars before pupation. The adults emerge after six to 20 days. Figure created using BioRender.com.

Tenebrio molitor populations are at risk of parasite infection, ranging from microbial infectious agents (i.e., viruses, bacteria, fungi, microsporidia, and protists) to invertebrate parasites (i.e., acari, cestodes, nematodes, and parasitoids) [17, 54]. Some of the parasites that are able to infect *T. molitor* have this species in their ecological host range (i.e., “The sum of all host species a parasite is capable of

encountering and infecting in the natural environment" [54]), whereas others have *T. molitor* in their physiological host range (i.e., "The sum of all host species a parasite has been found to infect under laboratory conditions" [54]). Both of these parasite groups are relevant for the mass-rearing of *T. molitor*, because these insects are kept under artificial conditions, which may also allow infection of parasites that are not able to infect under natural conditions (i.e., parasites that have *T. molitor* in their physiological host range) [54]. In this thesis, a fungal (*Metarhizium brunneum*) and a bacterial (*Bacillus thuringiensis*) parasite organism of *T. molitor* were chosen to be used in the experimental studies (chapters 3 – 5). Their biology, and the reason they were chosen as study organisms is described in the sections below (1.5.2 & 1.5.3).

1.5.2 *Metarhizium brunneum*

The ascomycete species *Metarhizium brunneum* is an entomopathogenic fungus belonging to the order Hypocreales. Generalists and specialists exist in the genus *Metarhizium*, but *M. brunneum* is considered a generalist, able to infect various insect orders [55]. Fungi from the genus *Metarhizium* have been known since 1880 to infect insects and the disease they cause was called 'green muscardine'[56]. Together with other species of this genus, *M. brunneum* is used worldwide as a biopesticide to control insect pests [55].

The asexual spores of *M. brunneum* are called conidia, they are oval-shaped and 5-8 µm long [55, 57] (Figure 1.4). The infection process starts when conidia land on a susceptible insect host. If the environmental conditions are conducive (especially a high relative humidity is important), the conidia germinate and penetrate through the insect cuticle with the assistance of mechanical pressure and cuticle-degrading enzymes [55, 58]. Inside the hemocoel, the hyphae start to form blastospores, which are then transported to different parts of the insect body via the hemolymph. The mycelium of the fungus uses the nutrients of the insect body and secondary metabolites are produced by the fungus, which can be toxic to insects [58]. Eventually, the insect is killed and thereafter the hyphae grow out of the cadaver and start to produce conidia (Figure 1.4), which can initiate a new infection [58]. Entomopathogenic fungi interact with the prophenoloxidase (ProPO) and the Toll

pathway [59]. Hyphal bodies and blastospores may be encapsulated or phagocytised in the hemocoel by the insect's hemocytes [58]. Furthermore, increased cuticular melanisation can lead to reduced susceptibility when insects are exposed to entomopathogenic fungi [60].

Entomopathogenic fungi have been reported to cause mortality in mass-rearing systems of *T. molitor* [18, 61]. Grains, which are commonly used as a feedstock for mass-reared *T. molitor*, can be contaminated with conidia of the genus *Metarhizium* and other entomopathogenic fungi [62]. It is important to note that *Metarhizium* spp. are facultative entomopathogens, which means that they do not need an insect host for reproduction; they can also live as saprotrophs, rhizosphere colonizers and even endophytes [55, 63]. Therefore, there exists a potential risk that this pathogen can reproduce in the feedstock before or while being fed to *T. molitor*.

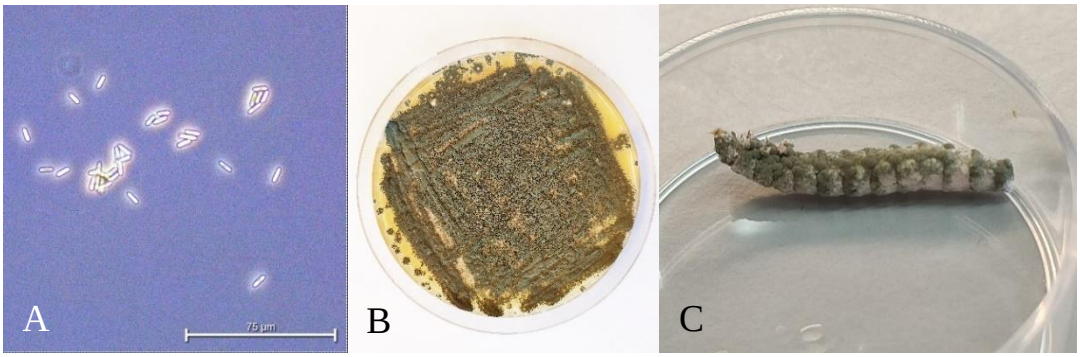

Figure 1.4 *Metarhizium brunneum*
A The conidia (asexual spores) under a light microscope at 400 times magnification. **B** *Metarhizium brunneum* growing on artificial medium producing the typical green conidia. **C** *Tenebrio molitor* cadaver infected with *M. brunneum*.

While the pathogenicity of *Metarhizium* spp. towards *T. molitor* has been shown before, it remains unclear how it interacts with other pathogens in this insect host. In chapter 5, I explore the interaction of *M. brunneum* with *B. thuringiensis* to understand if infection outcomes, and thereby the risk of pathogen infection, changes when *T. molitor* are co-exposed to these two pathogens. Moreover, it is unclear how the infection outcome is affected by different environmental stressors. I therefore study the impact of exposure of *T. molitor* larvae to heat stress before *M. brunneum* exposure in chapter 3. Moreover, I study the effect of elevated CO_2 on the virulence

of this pathogen individually in chapter 4 and in combination with *B. thuringiensis* in chapter 5.

1.5.3 *Bacillus thuringiensis*

Bacillus thuringiensis is a gram-positive entomopathogenic bacterium, which belongs to the *Bacillus cereus sensu lato* group. Other members of this group are *Bacillus mycoides*, *Bacillus weihenstephanensis*, *Bacillus pseudomycoides*, and the vertebrate-pathogenic *Bacillus cereus sensu stricto* and *Bacillus anthracis* [64, 65]. *Bacillus thuringiensis* was first isolated in 1901 in Japan by Shigetane Ishiwata, who discovered it in infected mass-reared *B. mori* [66]. In 1915, Ernst Berliner isolated it from an infected flour moth (*Ephestia kuehniella* Z. Lepidoptera: Pyralidae) in a mill in the region Thuringia (Germany) and he named it *B. thuringiensis* [67]. In the meantime, numerous subspecies have been described and *B. thuringiensis* has become the most used biopesticide in the world [e.g., 68]. Furthermore, the toxins of *B. thuringiensis* are widely used in transgenic crops to protect them from herbivorous insects [69].

The vegetative cells of *B. thuringiensis* are rod-shaped (ca. 5 μm long) and they form spores (Figure 1.5) and proteinaceous δ-endotoxins, which can be crystalline (Cry) and/or cytolytic (Cyt) [64, 70]. The spores and toxins are ingested by the host and thereafter the toxins are solubilized and activated by gut proteases in the insect gut [71]. In a susceptible host, the toxins cross the peritrophic matrix in the insect's midgut in which they then bind to the receptors of the epithelial cells. This leads to host cell death and eventual disruption of the midgut epithelium barrier, which increases the pH of the hemolymph [64]. The disruption allows spores and vegetative cells to colonise the hemocoel, which may lead to septicaemia and ultimately to death [71]. Furthermore, the binding of the toxins to the receptors of the epithelium can lead to gut paralysis, which results in starvation of the insects [64]. Common symptoms of infected insects are paralysis, termination of feeding, and vomiting [64]. The vegetative cells of *B. thuringiensis* multiply in the insect host and after the host's death, they start to produce spores and toxins when the nutrients are depleted. Thereafter, the spores and toxins are released into the environment where they are

potentially ingested by new hosts [71]. As a gram-positive bacterium, *B. thuringiensis* interacts with the Toll pathway of the insect's humoral immune response [59, 72]. Moreover, the ProPO pathway might be involved in the defence [73] and bacterial cells may be phagocytised or encapsulated by hemocytes [73].

Epizootics (i.e., outbreaks of disease with unusual high numbers of cases) in natural environments caused by *B. thuringiensis* are rarely reported but they can occur in relation to crowding [64], which is a common condition in insect mass-rearing systems. In fact, *B. thuringiensis* has been reported to be the causative agent of several disease outbreaks in both laboratory [74, 75] and mass-reared insect cultures [66, 76]. Recently, it has been suggested that *B. thuringiensis tenebrionis* may have been involved in the collapse of a mass-reared *T. molitor* colony [77]. This subspecies produces the Cry3Aa δ-endotoxin and it is able to infect *T. molitor* and other coleopteran species [70], although it is currently not understood how this subspecies kills *T. molitor* [71]. Moreover, there is a gap of knowledge on how this pathogen interacts with other pathogens and environmental stressors of *T. molitor*. In chapter 4, I study the effect of CO_2 on the virulence, viability, and persistence of *B. thuringiensis*. Moreover, I assess the effect of co-exposing *T. molitor* larvae to *B. thuringiensis*, *M. brunneum*, and CO_2 in chapter 5.

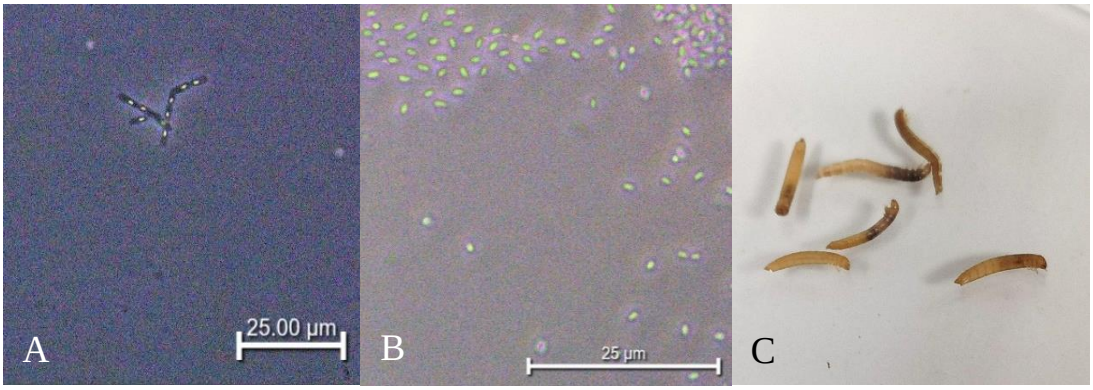

Figure 1.5 *Bacillus thuringiensis*
A The vegetative cells (dark elongated structures) containing spores (bright oval structures) under a light microscope at 1,000 times magnification. **B** The spores after sporulation under a light microscope at 1,000 times magnification. **C** *Tenebrio molitor* cadavers killed by a *B. thuringiensis* infection.

1.5.4 Temperature

The environmental temperature is critical for insects as they are poikilotherms and cannot regulate their internal temperature, being dependent on environmental temperatures for their internal body temperature. It is thereby important to note that different traits (e.g., growth rate or immunity) of the same insect species can differ in thermal optima [78]. The optimal temperature for rearing *T. molitor* in relation to total biomass production, is likely to be between 25 and 31°C [45, 79]. However, immune responses (i.e., encapsulation rate) have been recorded as higher at 18°C than at 28°C [80], which indicates that the determination of thermal optima for rearing *T. molitor* and other insect species is not trivial, and requires a careful assessment of potential trade-offs. At the same time, parasites also have thermal optima, which might differ from host thermal optima [81]. Such mismatches might be useful in preventing disease outbreaks in mass-reared insect populations, which is further discussed in chapter 2.

There are different reasons why the temperature in insect mass-rearing systems can vary. Firstly, temperature is typically higher in upper rearing crates than in lower crates within enclosed systems, especially when ventilation is not optimised [50]. Secondly, the temperature can diverge during the transport of insects [82]. Finally, insects kept at high densities may produce metabolic heat [50, 83, 84]. The temperature created inside crates can be >10°C above the ambient temperature in *T. molitor* production systems [83, 84]. The risk of the formation of such temperature hot spots may be decreased by reducing insect density [50], changing crate materials, or changing the structure of the substrate to increase heat dissipation [85].

The temperature does not only affect development but it also affects reproduction, the immune response, and the insect's microbiota as outlined in chapter 2. There exists a gap of knowledge on how heat stress affects host-parasite interactions in *T. molitor*. Furthermore, it is unknown what the physiological costs of heat is in this insect species. These objectives were studied in chapter 3.

1.5.5 Carbon dioxide

Carbon dioxide (CO_2) affects insects in different ways and different species are adapted to different CO_2 concentrations based on their environmental niche [86]. For example, CO_2 is an important sensory cue for various insect species [87]. Moreover, elevated CO_2 can increase the photosynthesis of plants and thereby increase the C:N ratio (carbon-to-nitrogen ratio), which in turn reduces diet quality for herbivory insects [87, 88]. This indirect effect of CO_2 has been found to result in decreased insect fitness [e.g., 89, 90]. Additionally, elevated CO_2 may lead to water loss because it forces insects to keep spiracles open for a prolonged time to take up enough oxygen [86].

Tenebrio molitor produces CO_2 among other gases (i.e., ammonia: NH_3, methane: CH_4, nitrous oxide: N_2O) [91].The production of gases is likely to be greatest in actively growing larvae [91], and CO_2 and potentially other gases are likely to accumulate when insects are kept in closed systems [50]. CO_2 is known to affect entomopathogens [92, 93] and the immune response of certain insect species [94, 95]. However, there is generally a lack of knowledge on how CO_2 affects host-parasite interactions of mass-reared insects (especially at CO_2 concentrations that are relevant for mass-rearing systems), which is discussed in chapter 2. Because CO_2 is likely to accumulate in production systems of *T. molitor*, it is crucial to understand how industry relevant CO_2 concentrations of this abiotic stressor affect *T. molitor* and potentially interact with pathogens, which is the objective in chapter 4 and 5.

1.6 Thesis plan

In this thesis, I investigate the effects of environmental stress on host-parasite interactions in mass-reared insects. Understanding the interactions between different stressors facilitates risk assessment and can eventually be used to protect mass-reared insects from infectious agents through appropriate risk management. This thesis is structured in a general introduction (chapter 1), four chapters that are individual manuscripts (chapters 2 – 5), and a general discussion with conclusions (chapter 6). I use *T. molitor* in all experimental studies (chapter 3 – 5), but I start broadly in my literature review considering all literature in which mass-reared insect species are

studied (chapter 2). In Figure 1.6, an overview of the interactions investigated in each chapter is given.

In chapter 2, I review the impact of abiotic environmental conditions on different aspects of the insect host performance and how that may alter the outcomes of host-parasite interactions in mass-reared insects. Furthermore, I identify implications and applications for the mass production of insects. This chapter has been published in the journal Trends in Parasitology [96].

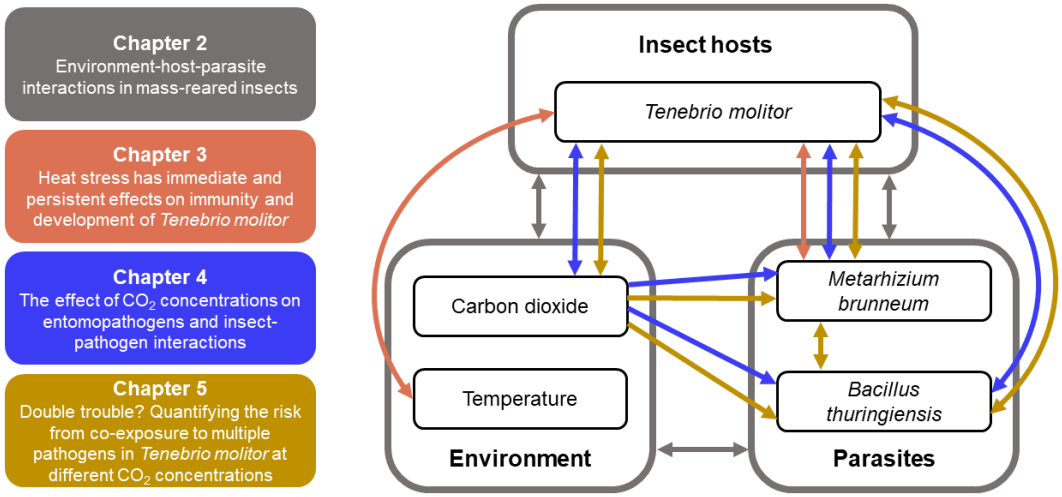

Figure 1.6 Thesis overview.
Chapters 2 – 5 (left side) are colour coded to illustrate the interactions that are tested in each chapter (arrows on the right side).

Mass-reared *T. molitor* larvae may be exposed to elevated temperature because of metabolic heat production [83, 84] and it is currently unknown how heat stress affects host-pathogen interactions in *T. molitor* larvae. In chapter 3, I evaluate the short- and long-term effects of heat stress on immunity, development, and susceptibility to a pathogen in *T. molitor*. I specifically test if heat stress has immediate beneficial effects on immunity and pathogen susceptibility of *T. molitor* larvae, as shown in other insect species [97, 98]. Moreover, I test if increased energy investment in immunity after heat stress will have persistent negative effects on fitness, pathogen susceptibility, and immunity of the larvae. I use either a short (2 h)

or a long (14 h) heat stress on *T. molitor* larvae and assess their susceptibility to exposure to *M. brunneum* immediately or five days after the heat stress. Moreover, I assess the development of heat stressed larvae and I measure phenoloxidase activity, hemocyte concentration, and antibacterial activity of the hemolymph to understand the effect of heat stress on the immune response. This manuscript is currently under revision in the Journal of Insects as Food and Feed.

In chapter 4, I conduct an explorative study on the effects of elevated CO_2 concentrations on *T. molitor* larvae, entomopathogens, and insect-pathogen interactions. CO_2 concentrations can be increased in mass-reared *T. molitor* populations due to respiration. It is currently unknown what the effects of industrially relevant CO_2 concentrations are on *T. molitor*. Moreover, there have been only very few studies investigating host-parasite interactions under elevated CO_2 in insects in general [93, 96]. Therefore, I test if previous exposure of entomopathogens or *T. molitor* larvae to elevated CO_2 affects the host-pathogen interactions *in vivo* (i.e., mortality of exposed larvae). Furthermore, I test if elevated CO_2 affects *M. brunneum* (i.e., germination of conidia and growth of colonies) and *B. thuringiensis* (i.e., viability and persistence of spores) *in vitro*.

Finally, I quantify the risk of co-infections (*M. brunneum* and *B. thuringiensis*) in *T. molitor* larvae under ambient and elevated CO_2 concentrations in chapter 5. The two pathogens may occur simultaneously in mass-rearing systems of *T. molitor*. For risk assessment, it is therefore crucial to study the effect of these pathogens in combination. Studies on mixed infections in insects often describe overall interactions (i.e., overall synergism, antagonism, or additivity), which is imprecise because the interactions might vary depending on the pathogen doses. I therefore aim to use an ecotoxicological mixture toxicity model, which supports detecting more nuanced interactions related to dose-ratio and dose-level dependencies.

1.6.1 Specific objectives with hypotheses:

Chapter 2

Objective 2.1: To synthesise literature on the effects of abiotic environmental conditions on host-parasite interactions in mass-reared insects.

> ***Hypothesis:*** The environment can have positive and negative impacts on insect hosts, which alters their interactions with parasites.

Objective 2.2: To develop a workflow for choosing environmental conditions in insect mass-rearing systems.

Chapter 3

Objective 3.1: To test the short-term and persistent effects (susceptibility to fungal pathogen, immune response, development, and reproduction) of heat stress on *T. molitor* larvae.

> ***Hypothesis:*** Heat stress increases the immune responses of *T. molitor* and decreases their susceptibility to a fungal pathogen.
>
> ***Hypothesis:*** The effects of heat stress on *T. molitor* become detrimental (i.e., increase of susceptibility to pathogen) for the larvae when exposed to the fungal pathogen, five days after the heat treatment.

Chapter 4

Objective 4.1: To assess the effects of industrially relevant elevated CO_2 concentrations on *M. brunneum* and *B. thuringiensis in vitro*.

> ***Hypothesis:*** Elevated CO_2 increases growth and germination of *M. brunneum* as shown for other fungal pathogens.
>
> ***Hypothesis:*** Elevated CO_2 decreases viability and persistence of *B. thuringiensis* spores as shown for other bacteria.

Objective 4.2: To assess the effects of elevated CO_2 on *in vivo* interactions between *T. molitor* larvae when exposed to the two aforementioned pathogens.

Hypothesis: The *in vitro* effects of elevated CO_2 on the pathogens (objective 4.1) are reflected in their relative virulence *in vivo* when subsequently, *T. molitor* larvae are exposed to the two pathogens separately.

Chapter 5

Objective 5.1: To assess the interactions between *M. brunneum* and *B. thuringiensis* in *T. molitor* larvae.

Hypothesis: *M. brunneum* and *B. thuringiensis* interact in a synergistic manner with each other, as shown in other coleopteran species.

Objective 5.2: To use a mixture toxicity (MIXTox) model to study interactions between *M. brunneum* and *B. thuringiensis* using larval biomass, individual weight gain, and survival of *T. molitor* larvae as endpoints.

Objective 5.3: Assess the impact of elevated CO_2 on the interactions between *M. brunneum* and *B. thuringiensis* in *T. molitor*.

Hypothesis: The measured *in vitro* effects of elevated CO_2 on the interaction between the two pathogens, are reflected in the *in vivo* interactions, when *T. molitor* are exposed to both pathogens (i.e., measured as larval biomass, individual weight gain, and survival).

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2. Chapter: Literature review on environment-host-parasite interactions in mass-reared insects

2.1 Abstract

The mass production of insects is rapidly expanding globally, supporting multiple industrial needs. However, parasite infections in insect mass-production systems can lower productivity and can lead to devastating losses. High rearing densities and artificial environmental conditions in mass-rearing facilities affect the insect hosts as well as their parasites. Environmental conditions such as temperature, gases, light, vibration, and ionizing radiation can affect productivity in insect mass-production facilities by altering insect development and susceptibility to parasites. This review explores the recent literature on environment–host–parasite interactions with a specific focus on mass-reared insect species. Understanding these complex interactions offers opportunities to optimise environmental conditions for the prevention of infectious diseases in mass-reared insects.

2.2 Highlights

- Mass-reared insects are kept in artificial environments different from their natural habitats. Additionally, insect populations kept at high densities are generally more susceptible to parasites, which can have devastating impacts on insect mass-rearing systems.

- Environmental conditions affect parasites directly and indirectly by altering insect immunity, microbiota, development, and reproduction, which are all important aspects in combatting parasites. In this way, host–parasite interactions are altered by the environment.

- The environmental conditions in mass-rearing systems can often be precisely controlled. Optimising environmental conditions in insect rearing is therefore a promising tool to reduce the risks caused by parasites in combination with existing hygiene practices.

2.3 Glossary

Antimicrobial peptide (AMP): a heterogeneous group of short-chained amino acids involved in the insect humoral immune response active against a broad range of parasites.

Blastospores: asexual fungal spores formed from hyphae inside the insect host during the infection process.

Diapause: the period in which insect development is delayed due to adverse environmental conditions, such as cold temperatures during winter.

Ectotherm: an organism that depends on the environmental conditions to regulate its body temperature.

Hemocytes: cells involved in the immune response of insects. Hemocyte concentrations can increase in response to infection in order to encapsulate, phagocytose, or lyse parasites.

Hemolymph: fluid in invertebrates analogous to blood in vertebrates. It contains and transports hemocytes, nutrients, and other compounds.

Heat shock proteins (HSPs): a family of proteins expressed after stress to protect denaturation of polypeptides or helping other proteins to refold.

Host microbiota: the community of microorganisms that exists inside or on the host.

Melanisation: a process involved in parasite encapsulation, formation of cytotoxic components, and wound healing, which results in dark pigmentation of melanised areas.

Metabolic heat production: generation of heat due to physiological processes of the insects.

Phenoloxidase: a key enzyme in the cascade leading to melanisation. Phenoloxidases occur in the hemolymph as prophenoloxidases before they are activated.

Phototaxis: movement of an organism towards a light source.

RNAi: a process in eukaryotic cells in which double-stranded RNA molecules suppress mRNA either for host gene regulation or as a defence mechanism against parasites.

Sterile insect technique (SIT): a method in which mass-reared males of an insect species are treated with ionizing radiation and thereby sterilised. The sterile males

are thereafter released in massive numbers to mate with wild females, which then cannot produce offspring.

Symbiont: one partner in an intimate ecological relationship (symbiosis). The relationship can be either mutualistic, commensal, or parasitic.

2.4 Parasites and environmental stress in mass-reared insects

For thousands of years, humans have mass-reared domesticated insects such as honeybees and silkworms. The mass rearing of insects on an industrial scale, however, is a relatively new concept, and this burgeoning industry is vital in producing insects for research, pollination services, and biological control of pests and vectors [1, 2]. The most recent development is the production of insects, such as flies, mealworms, crickets, and locusts, as a protein source for aquaculture, livestock, and human consumption [1, 3, 4].

A key threat to insect rearing is the risk of infection by parasites (we use the word 'parasite' to refer collectively to microbial pathogens, macroparasites, and parasitoids). Parasites might be present as covert infections in mass-reared insect populations [5] or they might be introduced via the feed, addition of insect stocks, the air, or wild insects [6]. High prevalence and transmission of parasites are more likely in mass culture than in natural populations as insects are reared at very high densities [3]. Parasites can cause lethal or sublethal effects leading to substantial economic losses [3, 7-11] (section 2.5).

Mass-reared insects are also exposed to a range of abiotic environmental stressors. High insect densities can lead to elevated temperatures due to metabolic heat production, and this may be exacerbated by low air exchange [12-14]. In addition, high insect densities lead to accumulation of carbon dioxide (CO_2) [15-17] and other gases [17] due to respiration. Moreover, relative humidity and moisture content might be increased when insects are kept at high densities [5]. Certain insects (e.g., dipteran, hymenopteran, or orthopteran species) require a supplementary controlled lighting supply during the day [18-20], which can become stressful if the intensity or duration of light exposure are unsuitable. Rearing processes often include transport,

handling, and sieving of insects, which result in mechanical vibrations [21]. In addition, ionising radiation is used in the sterile insect technique (SIT) which is employed to control insect pests and vectors of human diseases [1, 22, 23].

The susceptibility of insect hosts and the virulence of their parasites depend on, and may be altered by, environmental conditions. Because hosts and parasites often have different environmental conditions at which performance is maximised, the impacts of the environment on host–parasite interactions are not trivial [24]. For example, if a host and a parasite have different thermal optima, there will be a mismatch of thermal performance, making the outcome of infection dependent on temperature [24, 25]. Different host traits (e.g., growth and immune response) often have different environmental performance curves [26]. In mass-reared insect cultures, these trade-offs need to be considered when optimising environmental conditions for the most relevant host traits.

It is critical to understand how the environment affects host–parasite interactions to avoid losses caused by parasites (section 2.5). The effects of abiotic environmental conditions on parasites of mass-reared insects have recently been reviewed [5]. Our aim here, by contrast, is to describe the impact of abiotic environmental conditions on different aspects of the insect host performance and how that may alter the outcomes of host–parasite interactions in mass-reared insects (Figure 2.1). We identify implications for the mass production of insects and knowledge gaps in this area. We focus predominantly on insect species that are commonly mass reared [1, 4], using literature from other insect species in some cases to give a better overview of possible interactions.

2.5 Examples of economic losses caused by parasites in mass-reared insects

- Several parasites have caused losses in silkworm production systems since their establishment more than 6,000 years ago in China [10]. In more recent times, the largest economic impact on the silkworm industry was caused by the microsporidium *Nosema bombycis* causing the highly lethal disease ‘pébrine’ in the domestic silkworm (*Bombyx mori*) [27].

Nosema bombycis was first described in 1857, and in 1865 it caused the annihilation of the French and Italian silkworm industries [9, 10]. To this day, *N. bombycis* is considered to be the main risk in silkworm production systems [27, 28]. The severe impact of this parasite has led to the implementation of hygienic measures and the keeping of eggs and early instars in specialised well-equipped facilities to reduce the risk of infection [10].

- The mite *Varroa destructor* is considered to be the parasite with the biggest economic impact on colonies of the Western honeybee (*Apis mellifera*) [29]. This parasite suppresses its host's immune system, and is a vector of several viruses such as deformed wing virus (DWV) [30]. *Varroa destructor* came to Europe in the 1970s, and in the 1980s it was found in the USA [31]. Together with other interacting factors, such as pesticides, climate change, and other parasites, *V. destructor* is likely one of the major causes of colony collapse disorder (CCD) [30, 32]. A variety of methods are nowadays used to control *V. destructor* ranging from the application of chemical treatments to hygienic practices [10].

- *Acheta domesticus* densovirus (AdDV) can severely affect mass-reared house crickets (*Acheta domesticus*). This virus was first identified in 1977 in a Swiss mass-rearing facility of *A. domesticus* [33] and has frequently led to devastating epidemics in European mass-rearing facilities [7, 11] , forcing many producers to discontinue *A. domesticus* production [11]. Since 2009, severe outbreaks of AdDV have also been recorded in Northern America, with losses of hundreds of millions of dollars in the production of *A. domesticus* as pet feed [7]. It is suggested that the virulence of AdDV is increased when the crickets are exposed to other stressors such as crowding, high relative humidity, or temperatures above 35°C [11].

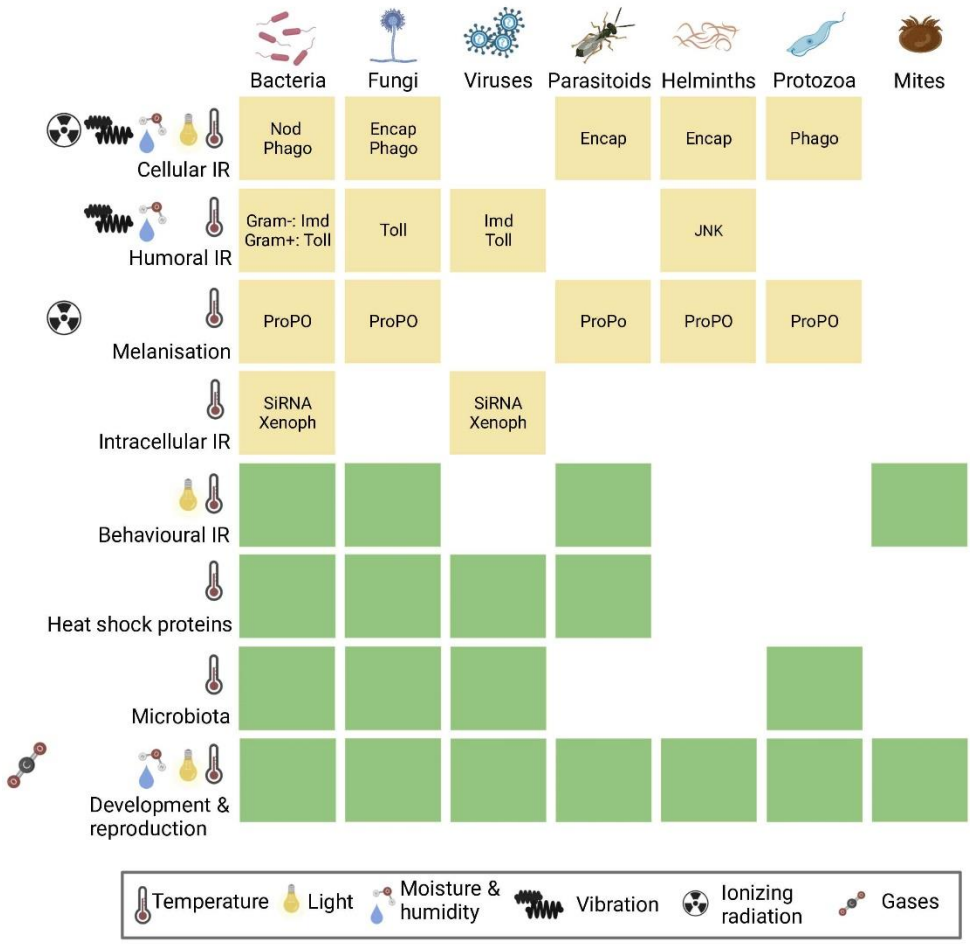

Figure 2.1 Possible effects of the environment on host–parasite interactions.
Different parasites are known to interact with innate immune responses (shown as yellow boxes) and other components (shown as green boxes) of mass-reared insects. Conversely, environmental stressors (left panel, explained in legend) are known to affect the insect hosts, and they can thereby affect the infection outcome. The cellular immune response involves different types of hemocyte. The differentiated hemocytes are formed from stem cells called prohemocytes, which are released into the hemolymph [34]. Smaller targets can be engulfed by single hemocytes through phagocytosis (Phago). For various bacteria, several hemocytes form nodules (Nod) surrounding the targets, and for larger parasites this process is called encapsulation (Encap). The humoral immune response involves the production of antimicrobial peptides. Different parasites trigger different signal transduction pathways – immunodeficiency (Imd), Toll, and c-Jun-N-terminal kinase (JNK) pathways [35, 36]. The cellular and humoral response are both involved in the melanisation process, which is initiated by the activation of the prophenoloxidase (ProPO) pathway to produce phenoloxidase. As a result, the dark pigmented melanin is produced surrounding a parasite in the encapsulation process or around a wound [34, 36]. Intracellular immune responses, such as RNAi and xenophagy (Xenoph), are active against viruses and intracellular bacteria [36]. RNAi is regulated by the siRNA pathway [35] and it silences essential parasite genes by producing small RNA sequences by the host, which interfere with the parasite RNA [36]. Figure created using BioRender.com.

2.6 Environment and immunity

Different parts of the innate immunity and the behavioural immunity in insects act specifically against different parasite groups (Figure 2.1). The relationship between environmental conditions and innate immunity can be a result of crosstalk (parasite infection and environmental stress induce the same signalling pathway) or cross-tolerance (the same mechanism protects from both parasite infection and damage by environmental stress) [37]. Cross-tolerance appears to be important in mass-reared insects in relation to temperature as outlined in the following examples.

2.6.1 Effects of temperature on innate immunity

Temperature is the environmental condition most frequently studied in relation to insect immunity (Table 2.1 and Figure 2.1). A temperature change can increase or decrease the insect's innate immune response, which often correlates with altered susceptibilities to parasites (Table 2.1). Temperature stress may also have transgenerational effects. Greater wax moth (*Galleria mellonella*) larvae in diapause induced by low temperature, for example, had a reduced encapsulation rate in the hemolymph, which coincided with a decreased survival probability when exposed to the fungal parasite *Cordyceps militaris* [38]. By contrast, elevated temperature frequently increases innate immune responses, such as antimicrobial peptide (AMP) production, increasing survival probabilities of *G. mellonella* larvae when exposed to parasites [39, 40]. Interestingly, temperature stress can also induce transgenerational changes in innate immune responses; cold stress applied to the parental generation of red flour beetles (*Tribolium castaneum*) increased phenoloxidase activity in their offspring and decreased mortality of the offspring when exposed to a bacterium (*Bacillus thuringiensis*). Moreover, *T. castaneum* larvae from cold-stressed parents had an increased development time until pupation compared with larvae from parents that did not receive a cold stress, which indicates a trade-off between immune response and development [41]. The effect of temperature on intracellular immunity has been studied in mosquitoes (*Aedes aegypti*). RNAi was hindered in adult mosquitoes reared at 18°C compared with those reared at 28°C, which coincided with elevated infection levels of chikungunya virus (CHIKV) and yellow fever virus at low temperatures [42].

Table 2.1 Overview of recent studies focusing on the effects of environmental stress on the innate immune response and the impact on susceptibility to parasites of mass-reared insect species.[1]

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Ref.
Elevated temperature	*Drosophila melanogaster* (common fruit fly)	Female and male larvae and pupae	*Leptopilina boulardi* (parasitoid wasp) strain ISm	↔ Encapsulation	Susceptibility to becoming parasitized: ↔	[43]
			L. boulardi strain ISy	↓ Encapsulation	Susceptibility to becoming parasitized: ↔	[43]
		Female and male adults	*Pseudomonas aeruginosa* (bacterium)	↓ Cuticular melanisation	↑	[44]
		Female adults	*P. aeruginosa*	Expression of immune response genes (↔ Pgrp-LC, ↔ relish, ↔ diptericin)	↔	[45]
			Lactococcus lactis (bacterium)	Expression of immune response genes (↓ spatzle, ↔ cactus, ↔ metchnikowin)	↔	[45]
	Galleria mellonella (greater wax moth)	Female and male larvae	*Candida albicans* (fungus)	↑ Hemocyte conc., ↑ AMP gene expression (gallerimycin, transferrin, inducible metalloproteinase inhibitor, galiomicin)	↓	[39]
			Malassezia furfur (fungus)	↓ Melanisation	↔	[46]
			Metarhizium robertsii (fungus)	↑ Lysozyme-like activity, ↑ PO, ↔ encapsulation, ↓ AMP gene expression (galiomicin and gallerimycin)	↓	[47]
			Streptococcus agalactiae (bacterium)	↔ Cuticular melanisation	↔	[48]
	Megachile rotundata (alfalfa leafcutting bee)	Female and male larvae	*Ascosphaera aggregata* (fungus)	↑ Overall expression of immune response genes	↓	[19]

Table 2.1 (continued)

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Ref.
Reduced temperature	*D. melanogaster*	Female and male larvae and pupae	*L. boulardi* strain ISm	↔ Encapsulation	Susceptibility to becoming parasitized: ↔	[43]
			L. boulardi strain ISy	↑ Encapsulation	Susceptibility to becoming parasitized: ↓	[43]
		Female adults	*P. aeruginosa*	Expression of immune response genes (↑ Pgrp-LC, ↔ relish, ↔ diptericin)	↔	[45]
			L. lactis	Expression of immune response genes (↔ spatzle, ↔ cactus, ↔ metchnikowin)	↔	[45]
			Metarhizium anisopliae (fungus)	↔ Hemocyte conc., ↔ PO, expression of immune response genes (↔ drosomycin, ↔ defensin,↔ diptericin, ↑ Turandot-A, ↔ cecropin, ↔ metchnikowin, ↔ drosocin, ↔ vir-1), ↔ wound-induced melanisation	↔	[49]
	G. mellonella	Female and male larvae	*Aspergillus fumigatus* (fungus)	24, 48 or 72 h after stress: ↓↔↔ hemocyte conc., expression of immune response genes (↔↔↔ apolipophorin, ↔↔↔ arylophorin, ↔↔↔ prophenoloxidase)	↑ when exposed to parasite 24 h after stress, ↔ when exposed to parasite 72 h after stress	[50]
			Steinernema feltiae (nematode)	↓ PO, ↑ lysozyme activity	↔	[51]
			Steinernema carpocapsae (nematode)	↓ PO, ↑ lysozyme activity	↓	[51]
			Heterorhabditis bacteriophora (nematode)	↓ PO, ↑ lysozyme activity	↓	[51]

Table 2.1 (continued)

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Ref.
			Bacillus thuringiensis (bacterium)	↓ PO, ↑ lysozyme activity	↑	[51]
			C. albicans	↑ Hemocyte conc., ↑ AMP gene expression (gallerimycin, transferrin, inducible metalloproteinase inhibitor, galiomicin)	↓	[39]
			Cordyceps militaris (fungus)	↓ Antifungal peptide gene expression, ↑ antibacterial peptide gene expression	↑	[52]
			C. militaris	↓ Encapsulation, ↔↓ PO (depending on temp.)	↑	[38]
	M. rotundata	Female and male larvae	*A. aggregata*	↑ Overall expression of immune response genes	↓	[19]
Short (<2 h) elevated temperature	*G. mellonella*	Female and male larvae	*B. thuringiensis*	↑ Antimicrobial activity of larval hemolymph, ↑ expression of gallerimycin, cecropin and galiomicin in the fat body, ↔ expression of the metalloproteinase inhibitor-IMPI	↓	[40]
			Beauveria bassiana (fungus)	↓ Expression of gallerimycin and galiomicin, ↑ lysozyme-like activity, ↑ antifungal activity of hemolymph	↓	[53]
	Tribolium castaneum (red flour beetle)	Female and male adults (exposed to stress), adult offspring (effects measured)	*B. thuringiensis*	↓ PO	↔	[41]
Short (<2 h) reduced temperature	*D. melanogaster*	Female adults	*M. anisopliae*	↑ Hemocyte conc., ↔ PO, expression of immune response genes (↔ drosomycin, ↔ defensin,↑ diptericin, ↑ Turandot-A, ↔ cecropin, ↔ metchnikowin, ↔ drosocin, ↔ vir-1), ↔ wound-induced melanisation	↔	[49]

Table 2.1 (continued)

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Ref.
	T. castaneum	Female and male adults (exposed to stress), adult offspring (effects measured)	*B. thuringiensis*	↑ PO	↓	[41]
Fluctuating temperature	*Apis mellifera* (Western honeybee)	Female pupae and adults	*M. anisopliae*	↑ PO	↑	[54]
Vibration	*G. mellonella*	Female and male larvae	*A. fumigatus*	24, 48 or 72 h after stress: ↑↑↔ hemocyte conc., expression of immune response genes (↑↔↔ apolipophorin, ↑↑↔ arylophorin, ↔↔↔ prophenoloxidase)	↓ when exposed to parasite 24 h after stress, ↔ when exposed to parasite 72 h after stress	[50]
			C. albicans	↑ Hemocyte conc., ↑ AMP gene expression (galiomicin, inducible metalloproteinase inhibitor), ↔ AMP gene expression (transferrin, gallerimycin)	↓	[55]
Ionizing radiation	*Ceratitis capitata* (Mediterranean fruit fly)	Female and male larvae	*Diachasmimorpha longicaudata* (parasitoid wasp)	↓ Encapsulation	↑	[18]

[1]Abbreviations: AMPs, antimicrobial peptides; conc., concentration; PO, phenoloxidase activity.

The investment in immune responses requires energy. However, energy for immune responses has been suggested to be limited when insects cope with thermal stress [54, 56], and potentially other environmental stresses. Adult fruit flies (*Drosophila melanogaster*) kept at 18°C, as opposed to 25°C, downregulated the expression of AMP genes. By inducing downregulation in mutant flies independently of temperature, the authors demonstrated that AMP downregulation led to prolonged lifespans and augmented stress resistance, for example, in the case of starvation [57]. Thermal stress can also have the opposite effect, resulting in increased immunity [19, 39, 40, 47, 55]. One possible mechanism for this phenomenon is cross-tolerance. Tang et al. [58] found that the expression of heat shock proteins (HSPs) in housefly (*Musca domestica*) larvae increases after heat stress but also after bacterial challenge (*Escherichia coli* or *Staphylococcus aureus*). Interestingly, a lack of these HSPs (due to silencing of HSP gene expression using RNAi) then led to lower survival after bacterial infection or heat stress, which proves that the same mechanism protects from heat stress and infection [58]. Similarly, HSP gene expression has been shown to increase after exposure of *D. melanogaster* to RNA viruses [Drosophila C virus (DCV), Cricket paralysis virus (CrPV), or Invertebrate iridescent virus (IIV-6)] [59]. HSPs are traditionally not considered as part of innate immunity in insects. Nevertheless, we know from other invertebrates that HSPs can enhance phagocytosis, increase phenoloxidase production, and protect host protein denaturation during parasite infection [60]. An upregulation of HSP gene expression after infection is thought to be beneficial to the host in *B. mori* eggs parasitised by a parasitoid wasp (*Telenomus theophilae*) [61] or in *G. mellonella* infected with the fungus *Conidiobolus coronatus* [62]. In *B. mori* cells, however, HSPs support the proliferation of *B. mori* nucleopolyhedrovirus (BmNPV) [63, 64], demonstrating that the role of HSPs is not always beneficial to the insect host.

Temperature might also affect insect immunity through its effect on the insect cuticle. Yellow mealworm (*Tenebrio molitor*) larvae reared at 28°C had darker cuticles (higher degrees of cuticular melanisation) as adults, compared with adults that had been reared at lower temperatures (18°C or 23°C) during their larval stage [65]. In addition, darker beetles also had an increased survival probability compared

with lighter beetles when exposed to a fungal parasite (*Metarhizium anisopliae*) [66]. However, it is unclear if this was a result of decreased ability of the fungus to penetrate highly melanised cuticles or if it was because increased melanisation correlates with other innate immune responses [67], leading to the lower mortality in beetles exposed to *M. anisopliae* [66]. In adult *D. melanogaster*, darker cuticles correlated with increased survival when exposed to a bacterium (*Pseudomonas aeruginosa*) [44], a parasite that does not infect the host by penetrating the cuticle. Here, on the contrary, higher temperatures led to lighter cuticles [44]. Melanisation as a result of temperature change possibly demonstrates cross-tolerance [44], as cuticular melanisation is known to play a role in thermoregulation [68] but also in immunity.

2.6.2 Effects of ionising radiation, humidity, light, and vibration on innate immunity

In contrast to our knowledge of the effect of temperature, our understanding of the effects of other environmental conditions on innate immunity in mass-produced insects is limited. The effect of ionising radiation on the immune response has been studied in Mediterranean fruit flies (*Ceratitis capitata*), which are commonly reared for SIT. In contrast to control larvae, irradiated larvae (40 gray – the SI unit of ionising radiation dose) did not accumulate phenoloxidase over the course of development [22]. Similarly, encapsulation rates and adult emergence decreased in *C. capitata* with increasing ionising radiation when parasitised by a parasitoid wasp (*Diachasmimorpha longicaudata*) [18]. Ionising radiation is thought to affect the cellular immune response of insect hosts directly; as hemocytes lack pigmentation, they are thought to be sensitive to this type of radiation [23]. This highlights the need to optimise the ionising radiation dose, taking into account sterility, fitness, and other traits.

Humidity affects parasite survival outside the host as well as both parasite virulence [5, 69] and host susceptibility in insects. For example, in larvae of the lepidopteran pest species beet armyworm (*Spodoptera exigua*), increasing relative humidity led to decreased antioxidant activities, which correlated with decreased survival when

exposed to a nucleopolyhedrovirus (SeNPV) [70]. Conversely, the larvae of the Mediterranean flour moth (*Ephestia kuehniella*) showed increased nodulation at an elevated relative humidity (85%) when infected with *B. thuringiensis* compared with larvae kept at a low relative humidity (43%) [71]. However, the effects of humidity on the immune responses of mass-reared insects remain to be investigated more thoroughly.

The effects of artificial light have been studied in the Australian black field cricket (*Teleogryllus commodus*), which is not commonly mass-reared but used as a model organism. Durrant et al. [72] found that dim artificial light during the night decreased hemocyte concentration in the hemolymph, which could have negative effects on infection outcome in case of an exposure to parasites [72].

In contrast to the decreased immune responses observed in response to ionising radiation and light, short periods of vibration have been shown to increase hemocyte concentrations and the expression of several AMP genes in *G. mellonella* larvae, coinciding with lowered mortalities when exposed to the fungal parasites *Candida albicans* [55] and *Aspergillus fumigatus* [50].

2.6.3 Effects of the environment on behavioural immunity

Environmental conditions are also of high relevance in behavioural immunity (host behaviours that lead to the avoidance or mitigation of parasite infection [73, 74]). When given the choice, certain insect species increase their body temperature by seeking places with elevated temperatures to suppress the development of parasites [20, 74-76], a phenomenon called behavioural fever or fevering. Adult migratory locusts (*Locusta migratoria*) infected with *M. anisopliae*, for example, had an 85% higher survival rate when given the opportunity to increase their body temperature for at least 4 h per day compared with adults that were not enabled to fever [74]. The reduced mortality rate due to behavioural fever is potentially a combined effect of parasite inhibition at elevated temperatures and the triggering of the immune response of the host (Figure 2.1). Sangbaramou et al. [20] found that *L. migratoria* nymphs exposed to the fungal parasite *Beauveria bassiana* had an elevated hemocyte

concentration and an absence of circulating fungal blastospores in their hemolymph when given the opportunity to increase their body temperature behaviourally. By contrast, nymphs kept at constant temperatures had fewer hemocytes, and circulating fungal blastospores were observed. This correlated with higher mortality in nymphs kept at constant temperatures compared with fevering nymphs [20].

It is important to note that behavioural fever is not exhibited against all parasite species. Adamo et al. [77] found that *A. domesticus* increased their body temperature when infected with the Gram-negative bacterium *Rickettsiella grylli*, leading to increased survival. However, when the crickets were infected with another Gram-negative bacterium (*Serratia marcescens*), they did not increase their body temperature. Moreover, increasing the temperature artificially did not increase survival when exposed to *S. marcescens*, demonstrating that behavioural fever can be targeted by the insects against parasites that are affected by the temperature change (i.e., *R. grylli*) but not against parasites that remain unaffected by the temperature increase (i.e., *S. marcescens*) [77]. In adults of *M. domestica*, the intensity of the behavioural fever was found to be positively correlated with the dose of *B. bassiana* they received [78], indicating that insects can optimise the costs and advantages of behavioural fever.

In contrast to increasing the body temperature by fevering, buff-tailed bumblebees (*Bombus terrestris*) actively lower their body temperature by remaining in the field overnight when parasitised with parasitoid conopid flies to delay parasite development [79]. Similarly, adult *D. melanogaster* infected with a fungal (*Metarhizium robertsii*) or bacterial (*P. aeruginosa*) parasite exhibited a preference for cooler temperatures compared with uninfected flies; the survival probabilities of the flies when infected was increased under these cooler temperatures [80, 81]. This demonstrates that the ability to raise and to lower temperature behaviourally can be beneficial for infected hosts.

A change in temperature could also act as a cue for parasitism, allowing social insects to react to infested broods. Brood cells of *A. mellifera* parasitised by *V.*

destructor have a higher temperature than nonparasitised cells. Bauer et al. [29] therefore suggested that worker bees might use these elevated temperatures as a cue to remove infested brood cells, a hygienic behaviour that can decrease parasite dispersal inside the beehive. The causal relationship between parasitism, elevated temperature, and brood removal however remains to be demonstrated [29]. A further form of behavioural immunity through phototaxis has been shown recently in adult flies (*Drosophila nigrospiracula*) being infested by mites (*Macrocheles subbadius*) whereby the risk of mite infestation is higher in the dark than in the light. Hence, flies demonstrated avoidance behaviour, spending more time in lightened areas than in dark areas when mites were present [73].

2.6.4 Investigating the effects of environment on immunity is complex

To deal with the complexity of the insect immune system, several immune responses can be studied simultaneously and over time [45, 50] in combination with experimentally establishing the susceptibility of hosts to their parasites, as done in the studies presented in Table 2.1. Several factors will define how the environmental conditions affect immune responses and susceptibility to parasites of insect hosts. Different genetic strains of *C. capitata* show, for example, different parasitoid encapsulation rates [18]. Furthermore, immune responses can depend on species (Table 2.1) and on sex, as shown in *T. molitor* with increased encapsulation rates in females [65] and in *T. commodus* with increased hemocyte concentrations in females [72]. In social insects, the interactions between temperature and immune response are even more complex, as different castes are adapted to different environments. When worker bees, queens, and drones of *A. mellifera* were exposed to a heat stress during the pupal stage, the phenoloxidase activities in the adult stages were either increased, decreased, or remained unchanged, respectively [54]. Last, the effect of the environment on host immunity also depends on the parasite species [51], and the genetic strain of the parasite [43].

2.7 Environment, host microbiota, and parasites

Protection from parasites in insects derives not only from their own immune system, but also from their associated host microbiota [34], for example, by the production of

antimicrobials by bacterial symbionts [82]. Additionally, the host microbiota can increase the ability of the host to cope with environmental stress. Adults of *D. melanogaster* exposed to the nonparasitic fungus *Aspergillus oryzae* had an increased survival probability under heat stress [83]. The parasites in turn can also affect the host microbiota. The toxins of *B. thuringiensis*, for example, can alter the gut bacterial community composition and reduce the total bacterial load in the guts of *L. migratoria* [84]. In a recent review, Savio et al. [85] found that members of the bacterial genus *Lactobacillus* appear to be of great importance in decreasing susceptibility of mass-reared insects to fungal and bacterial parasites, whereas members of the genera *Wolbachia* and *Spiroplasma* reduce susceptibility to viral infection [85].

2.7.1 Temperature affects host microbiota and thereby infection outcome

Thermal stress can act directly on parasites, but it can also affect the host's microbiota and thereby affect the outcome of infection by parasites. Studies of these interactions need to contend with complex systems and understanding of multiple interactions. For example, bacterial symbionts are suggested to have a temperature-dependent effect on the infection by a fungal parasite (*C. militaris*) in *G. mellonella* larvae. Mortality caused by *C. militaris* infection at high temperature (25°C) was reduced in comparison to lower temperature (15°C). This coincided with an increased abundance of enterococci and enterobacteria (both of which have inhibitory effects on *C. militaris in vitro*) in the hemolymph and in the gut at high temperatures in response to infection [52]. In addition, the host responses to different parasites were also temperature dependent. Expressions of lysozyme genes, which play a role in the Toll and prophenoloxidase pathways mainly active against bacteria and fungi, were increased. However, the expressions of cecropin genes, which play a role in the Imd pathway against Gram-negative bacteria (Figure 2.1), were decreased at high temperatures potentially favouring the Gram-negative enterobacteria [52]. Similarly, in common Eastern bumblebees (*Bombus impatiens*), increasing temperature leads to decreased infection intensity of a trypanosomatid parasite (*Crithidia bombi*) [86]. At high temperatures (above 32°C), this can be explained by a direct growth inhibition of *C. bombi* [87]. However, at temperatures below 32°C

the parasite is not directly inhibited by temperature and indirect inhibition might stem from acid-producing bacteria [86] (many bacterial gut symbionts of bees transform carbohydrates into short-chained fatty acids, acidifying the gut [88]), which increase their metabolic rates with increasing temperatures [87]. However, the relationship between temperature, host microbiota, and gut pH remains poorly understood [86]. These examples demonstrate that different infection outcomes in response to temperature may result from multiple interactions.

2.8 Environment, host development, reproduction, and parasites

Two of the most important parameters in the mass production of insects are development and reproduction, which define the productivity of the systems. As ectotherms, the physiology of insects is directly linked to the environmental temperature. Altered development rates due to environmental conditions might lead to a trade-off with immunity [41] as discussed before. However, highest growth rates under conditions optimised for growth, may lead to lowest susceptibility to infection because the parasite dose per host mass decreases with increasing body mass. For example, larger larvae of *G. mellonella* showed decreased mortality when exposed to the bacterium *S. aureus*, which correlated with increased lipid weights in larger larvae [89]. A similar effect might occur when optimising other environmental conditions for higher growth rates. For example, *T. molitor* larvae show increased growth rates when kept in darkness versus alternating light/dark conditions [90] and grow faster with increasing relative air humidity [91]. Larger *T. molitor* larvae show higher survival probabilities than smaller larvae when exposed to *B. thuringiensis* [92], yet the causal relationship between environment, insect body mass, and parasite susceptibility remains to be investigated.

The interaction between reproduction and immunity of the insect host when affected by the environment can lead to different outcomes. First, different energy investments under altered environmental conditions can lead to trade-offs between reproduction and immunity. In brown-banded cockroaches (*Supella longipalpa*) parasitised by an acanthocephalan parasite (*Moniliformis moniliformis*), for example, elevated temperatures led to decreased reproduction compared with healthy

cockroaches, whereas at lower temperatures both parasitised and healthy cockroaches had similar reproductive outputs [93]. One possible explanation might be that the immune response is increased at elevated temperature (e.g., in response to increased parasite performance), which leaves less energy for reproduction. Second, reproduction and immunity may both be increased or decreased under environmental stress. *Bombus impatiens* queens exposed to a short CO_2 narcosis showed increased reproduction and at the same time an increased ability to eliminate bacteria (*Providencia rettgeri*) in their hemolymph [94], which may result in trade-offs with other energy-consuming processes that were not measured. Finally, trade-offs between immunity and reproduction depend on the parasite species. In Texan field crickets (*Gryllus texensis*), differing responses to temperature were shown when infected by different parasites; control crickets kept 7°C above average field temperature showed higher reproduction, as did crickets exposed to a sublethal dose of the Gram-negative bacterium *S. marcescens*. By contrast, when exposed to a sublethal dose of the Gram-positive bacterium *Bacillus cereus*, the elevated temperatures did not lead to higher reproduction [95]. This could not be explained by different thermal optima of the parasites, but the authors suggest that the immune response against *B. cereus* was more energy intensive than that against *S. marcescens*, leading to lower investment in reproduction [95]. These examples demonstrate that the effects of environmental conditions on potential trade-offs between immunity and reproduction and other important energy-consuming processes need to be carefully assessed before choosing environmental rearing conditions (Figure 2.2).

2.9 Implications and applications for mass rearing of insects

Mass-rearing conditions are set to maximise productivity (i.e., output of nondiseased insects). However, parasitic infections can lead to devastating losses in production systems. Environmental conditions play an essential role in the defences of mass-reared insects to parasites (Figure 2.1 and Table 2.1). Adjusting these conditions to increase defences against parasites should therefore be considered when choosing environmental conditions (Figure 2.2), which will in turn lower the risk of lethal and

sublethal effects caused by parasites and maintain productivity of insect mass rearing.

The environmental conditions can be constant or fluctuating throughout the mass-rearing process. Extreme environmental conditions can also appear as ‘shocks’ (i.e., short-term changes of conditions) (Figure 2.2). These different regimes of environmental conditions can either be a result of intentional measures to increase productivity or they can be a result of the rearing process itself. Fluctuating temperatures in an innocuous thermal range are often beneficial for a variety of different response variables of insects (e.g., reproduction, growth, thermal tolerance, and development) [26]. Spring field crickets (*Gryllus veletis*) that were acclimatised to fluctuating temperatures had higher survival probabilities when infected with the fungal parasite *Metarhizium brunneum* compared with crickets kept at constant temperatures [25]. Similarly, *T. molitor* larvae had increased antibacterial activity in their hemolymph when exposed to a fluctuating temperature regime (± 8°C) compared with larvae kept at constant temperatures [96]. Interestingly, fluctuating temperatures have also been found to mediate the course of co-infections. The fungus *M. anisopliae* is highly virulent to desert locusts (*Schistocerca gregaria*) under constant temperatures (30°C). However, under a fluctuating temperature regime (20–42°C) the survival of *S. gregaria* infected with *M. anisopliae* decreased only when they were additionally exposed to another fungus (*B. bassiana*). This is interesting as *B. bassiana* on its own did not decrease survival under fluctuating temperatures compared with uninfected locusts [97]. In certain systems, the outcomes of host–parasite interactions under fluctuating temperatures can be predicted by calculating the averages of the outcomes at constant maximal and minimal temperatures [25], although this is not always possible in other systems [98].

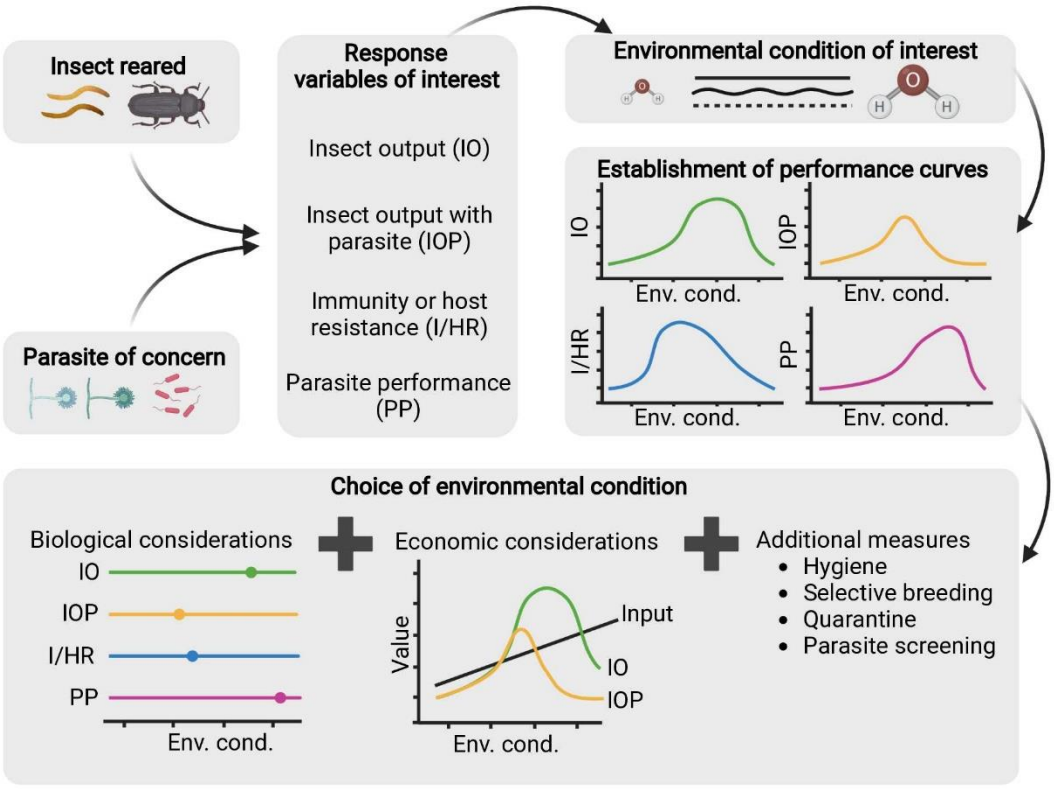

Figure 2.2 Workflow for choosing environmental conditions in mass-rearing systems.
The insect (species, strain, and life stage) reared and the parasite (species and strain) of concern define the response variables to be measured. The response variables should at least consist of a measure of insect output (e.g., insect biomass) when the population is parasite-free and when parasitised, a measure of host resistance or immunity (e.g., encapsulation), and a measure of parasite performance (e.g., germination of fungal spores). Thereafter, the environmental condition of interest is chosen (e.g., humidity is illustrated here). Environmental conditions can remain constant over time (continuous line), occur as fluctuations (wavy line), or as shocks (broken line). The chosen response variables are measured over a range of levels of the environmental condition to establish performance curves (shown as hypothetical curves). Finally, choosing the environmental condition is based on biological and economic considerations, and additional measures that are feasible. Different response variables potentially have different peak performances in response to environmental conditions, which allows choosing a condition that is furthest away from peak parasite performance but closest to maximised insect immunity and output. For the economic considerations, the risk of parasite presence needs to be assessed. In a situation of low parasite infection risk (influenced by additional measures), maximised insect output (green curve) and monetary input value are used for calculating the optimal environmental condition. In a situation of high parasite infection risk (e.g., because of costly additional measures), maximised insect output with parasite infection (yellow curve) and input value are used to identify the optimal environmental condition. Figure created using BioRender.com.

Insects kept at high densities produce metabolic heat leading to temperature gradients in rearing containers, with the highest temperatures in the centre, compared with the lowest temperatures at the edges [12-14]. Such temperature gradients provide the opportunity for mass-reared insects (i.e., insects of the orders Orthoptera, Diptera, and Hymenoptera) to exhibit behavioural fever or cooling that may lead to avoidance or suppression of parasites. To our knowledge, there have been no studies to date that explore temperature selection in response to parasite infection in mass-rearing settings.

Short thermal or physical shocks can occur during handling of mass-reared insects, such as transportation or during sieving processes. These shocks had beneficial effects on the host immunity and survival when exposed to parasites [39, 40, 53, 55]. Browne et al. [50] found that the beneficial effects of thermal and physical shocks peak 24 h and diminish 72 h after the stress [50], which indicates that these short exposures might not be useful to increase immunity over a prolonged period. The regulation of immune response gene expression depends on duration and frequency of thermal shocks [99]. However, the frequent applications of thermal and physical shocks on the immune response and susceptibility of mass-reared insect species have, to our knowledge, not been tested thus far. Such studies would be essential to understand the long-term effects and potential trade-offs in reproduction, growth, or other traits. Furthermore, transgenerational trade-offs between immune response and development are possible [41] and should be considered in the context of using environmental stress to decrease offspring susceptibility to parasites.

In order to make informed decisions regarding choice of environmental conditions, performance curves of relevant response variables are needed. In addition to biological considerations, economic considerations need to be taken into account in any mass-rearing system that is commercially producing insects [26]. Depending on the degree of risk of parasite infection, the chosen environmental conditions may differ to mitigate against infection, whilst maintaining biological and economic optimal outcomes (Figure 2.2).

2.10 Outstanding questions

- Does increased investment into immunity following environmental stress lead to negative impacts in mass-produced insects?

- How do co-infections alter host and parasite performance under different environmental conditions? What are the implications for mass-rearing insects?

- Can mismatches in parasite and host performance under different environmental conditions (specifically relative humidity, moisture content, and gas concentrations) be utilised to alter infection outcomes in mass-reared insects?

2.11 Concluding remarks

Environmental conditions affect host–parasite interactions in mass-reared insects directly or indirectly by changing immunity, microbiota, development, and reproduction of the insect hosts. Optimising environmental conditions merely for increased production (i.e., growth and reproduction) may lead to higher susceptibility to parasites as energy investment into a certain trait (e.g., reproduction) can reduce energy investment into another trait (e.g., immunity) [95]. However, environmental conditions can also be optimised to reduce the risk of parasite infection in mass-rearing systems (Figure 2.2) whilst maintaining an adequate level of insect quality.

Although our knowledge of how the environment affects host–parasite interactions in mass-reared insect species has expanded recently, several key questions remain (see Outstanding questions). Additional efforts are needed to understand sublethal effects (e.g., effects on weight gain or reproductive output) of environment–host–parasite interactions, as they have a tremendous potential to reduce productivity in the mass production of insects in the long term. Moreover, efforts are needed to investigate key mass-reared insect species as well as the parasite species that are challenges in mass-production systems, as our current knowledge stems from a few model organisms (Table 2.1). We also need to acknowledge that the environment in which

insects are reared is a combination of both differing environmental conditions and potentially multiple parasites infecting hosts simultaneously. Future research should therefore consider how different environmental stressors and parasites interact with each other, as the outcomes of such interactions might not be predictable by studying the stressors or parasites individually [97, 100]. There remains a significant dearth of knowledge on how moisture content, gas concentrations, and relative humidity affect host–parasite interactions in mass-reared insect species and how the host microbiota is affected by different environmental conditions. Finally, it should be considered that optimising environmental conditions is one of many options available to maintain insect health in mass-reared systems. The simultaneous use of other interventions and tools (Figure 2.2) will all help to keep parasites under control in insect mass-rearing systems.

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3. Chapter: Heat stress has immediate and persistent effects on immunity and development of *Tenebrio molitor*

3.1 Abstract

The yellow mealworm (*Tenebrio molitor*) is a promising insect species for mass-rearing for the production of feed and food. In mass-production systems, insects may be exposed to abiotic stressors such as heat stress as well as potentially lethal pathogens. To ensure mass-reared *T. molitor* populations are healthy and productive there is a need to understand the risks and potential benefits of heat stress on the fitness of insects and their susceptibility to pathogens. In this study, we investigated the effects of a short (2 h) or a long (14 h) heat stress (38°C) exposure on the susceptibility and the immune responses of *T. molitor* larvae exposed to a fungal pathogen (*Metarhizium brunneum*). Larvae were exposed to the pathogen either immediately or five days after the heat stress treatments. The development of heat stressed larvae and their offspring was also assessed. A short heat stress immediately before exposure to *M. brunneum* increased the survival probability of *T. molitor* larvae, which correlated with increased antibacterial activity in the hemolymph. The exposure of larvae to short or long heat stresses five days before pathogen exposure did not affect their survival despite a temporary lowered body mass gain of heat stressed larvae. However, heat stressed larvae showed decreased hemocyte concentrations when exposed to *M. brunneum*. We also found an increased body weight in offspring of females that had been exposed to a short heat stress as larvae. These findings demonstrate the importance of understanding the effects of heat stress in the long-term. The beneficial effects of heat stress on pathogen susceptibility in *T. molitor* and the negative effects on body mass gain are only transient, whereas negative effects on immune response (hemocyte concentrations) persist over an extended time.

3.2 Introduction

The mass-rearing of insects for human consumption and livestock feed is a growing sector as insects are a promising sustainable source of protein [1]. Insects generally

are more efficient than conventional livestock in converting their feed substrate into body mass and they require less water to produce an equivalent mass of protein [2]. Moreover, insects produce less greenhouse gases than for example pigs and chickens, and they are able to convert organic materials from waste streams into proteins [1, 3].

The yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), is one of the most important species currently mass-produced for food and feed purposes. In addition, it is an important model organism [4], a pest of stored grains [5], and it has been shown to be useful for biodegradation of plastics [6, 7].

A major challenge when mass-rearing *T. molitor* is the risk of infections by entomopathogens [8, 9]. Such infections may be lethal or sublethal for the insects and can lead to significant and severe economic losses when insects are being mass-reared for food and feed [9]. High prevalence and spread of entomopathogens are more likely in cultured insects than in the wild due to high rearing densities which facilitate transmission between infected and non-infected hosts [10, 11].

In addition to biotic stressors (we use the term 'stressor' to describe a "variable that causes a quantifiable change, irrespective of its direction (increase or decrease), in a biological response" [12]), mass-reared insects are also exposed to environmental stressors [13]. Temperature is one of the most important environmental factors determining insect growth [14], metabolic rate, and body composition [15]. Mass-reared insects are kept at high densities and they can therefore be exposed to elevated temperatures due to accumulated metabolic heat [16, 17]. Furthermore, *T. molitor* can absorb water vapour from the environment [18], which is an energy-consuming process that also produces metabolic heat [19]. The highest growth rates in *T. molitor* larvae were recorded at a temperature of 31°C [15]. However, it has been reported that at high densities *T. molitor* larvae elevate the temperature inside rearing containers by 5 – 10°C [16] or even 14°C above the set rearing temperature [17]. Such hotspots of temperature may be present in a rearing crate for a short amount of time (e.g., when detected quickly and the densities in the rearing crates are adjusted)

or the conditions can occur for a prolonged period (e.g., when remaining undetected overnight).

Besides affecting the growth and metabolism, temperature has also been shown to affect both immune response and susceptibility to pathogens in insects [20-23]. Insect immunity can be broadly divided into cellular and humoral immune responses. Hemocytes are the main component of the cellular response to infection in insects. These cells can kill pathogens through encapsulation or phagocytosis [24]. Oenocytes (a type of hemocyte) produce prophenoloxidase [5], which is the zymogen of phenoloxidase, an important enzyme in the melanisation process, which begins when prophenoloxidase turns into phenoloxidase only minutes after infection [25]. Thereafter, plasmatocytes (a type of hemocyte) are recruited to the pathogen. Through lysis of the plasmatocytes, melanin is released and encapsulates the pathogen [26]. Another important part of insect immunity is the humoral immune response, which involves the production of AMPs (antimicrobial peptides) that are active against bacteria and fungi [26]. *Tenebrio molitor* larvae kept at 30°C were found to have increased phenoloxidase activity and antibacterial responses compared to larvae kept at 10 or 20°C [27]. Moreover, increasing temperature (18, 23 and 28°C) led to an increased cuticle darkness (an index of melanisation of the cuticle) of adult *T. molitor* [28], which previously has been linked to increased resistance to the entomopathogenic fungus *Metarhizium anisopliae* [29]. Increased cuticular melanisation also correlated with increased phenoloxidase activity and increased hemocyte concentrations in the hemolymph [30]. The effects of temperatures above 30°C on immune response and host-pathogen interactions in *T. molitor* are currently not known. However, the effect of heat stress has been studied in the lepidopteran species *Galleria mellonella*, which has a similar optimal temperature for larval development (29-33°C) as *T. molitor* [31]. In *G. mellonella*, heat stress generally leads to an increased immune response and decreased susceptibility to different fungal and bacterial pathogens [21, 32, 33].

Because of the potential beneficial impacts of temperature stress on the immune response of insects, some authors have suggested using temperature stress to increase

the immunity of mass-reared insects [4, 34]. However, it remains unclear for how long any beneficial effects of heat stress on immunity persist. Moreover, temperature can affect host-pathogen interactions indirectly by affecting the development of insects and thereby influencing their susceptibility to pathogens. In *G. mellonella*, for example, the susceptibility to pathogens decreases with increasing body mass and length of the larvae [35]. Hence, insects that have a slower body mass gain due to temperature stress might be at risk over a longer period to acquire a lethal infection. Furthermore, temperature can affect the timing, as well as the number of moults in the larval development of *T. molitor* [36]. Moulting may reduce the risk of infection following exposure to fungal pathogens because conidia are removed with the moulted exoskeleton before they can penetrate the cuticle [37-39]. However, after moulting the new cuticle might be more susceptible to fungal penetration until it is completely sclerotized and melanised [40].

In this study, we aimed to answer the question if the exposure of *T. molitor* larvae to heat stress affects their immunity and susceptibility to a fungal pathogen in the short- and long-term. We hypothesised that heat stress will have immediate beneficial effects on immunity and pathogen susceptibility of *T. molitor* larvae, as shown in other insect species. Furthermore, we hypothesised that the increased energy investment in immunity after heat stress will have persistent negative effects on fitness, pathogen susceptibility, and immunity of the larvae. In this study, the generalist fungal entomopathogen *Metarhizium brunneum* was used to test pathogen susceptibility of *T. molitor* larvae. *Tenebrio molitor* larvae can be naturally infected by fungi of the genus *Metarhizium* [41]. Wakil et al. [42] found that these fungi can be present in stored grains [42], which makes them important pathogens in the production process of *T. molitor*, because stored grains are commonly utilised in mass-rearing facilities to feed *T. molitor* [43].

The research reported here documents the susceptibility (i.e., survival) of *T. molitor* larvae when exposed to a fungal pathogen immediately after the exposure to either of two heat stresses (short 2 h or long 14 h) or exposed to the pathogen five days following exposure to the heat stresses. Secondly, the immune responses

(phenoloxidase activity, hemocyte concentration and antibacterial activity of the hemolymph) and moulting of larvae exposed to the same combination of fungal pathogen and heat stresses are measured. Finally, the development (i.e., weight gain, pupal weight, time until pupation, and number of exuviae) of heat stressed larvae and their offspring (i.e., number of offspring and weight of larval offspring) are assessed.

3.3 Materials and methods

3.3.1 Experimental design overview

The larvae of *T. molitor* were exposed to *M. brunneum* immediately or five days after exposure to a short (2 h) or a long (14 h) heat stress (38°C) (Figure 3.1). All insects were constantly kept at 28°C (except during the heat stress treatments) for the remaining duration of the experiment. One group of larvae was exposed to a lethal dose (LD_{50}, previously established in pre-experimental bioassays) of *M. brunneum* immediately after the heat stress treatments. A control group of larvae was not exposed to *M. brunneum*. Similarly, two other groups of larvae were exposed to either *M. brunneum* (LD_{50}) or no *M. brunneum* five days after the heat stress treatments. Subsequently, survival was assessed in all groups daily for 12 days. To compare the immune responses in the different treatment groups, measurements of hemocyte concentration, phenoloxidase activity, and antibacterial activity were made two days after *M. brunneum* or control exposure in all four groups. Similarly, shed exuviae were counted two days after exposure to *M. brunneum* or control treatments.

To compare the development and reproduction of larvae exposed to heat stress (without *M. brunneum* treatment), larval mass (one day before, and five and 10 days after heat stresses), moulting frequency, development duration until pupation, pupal weight, number, and weight of offspring were recorded.

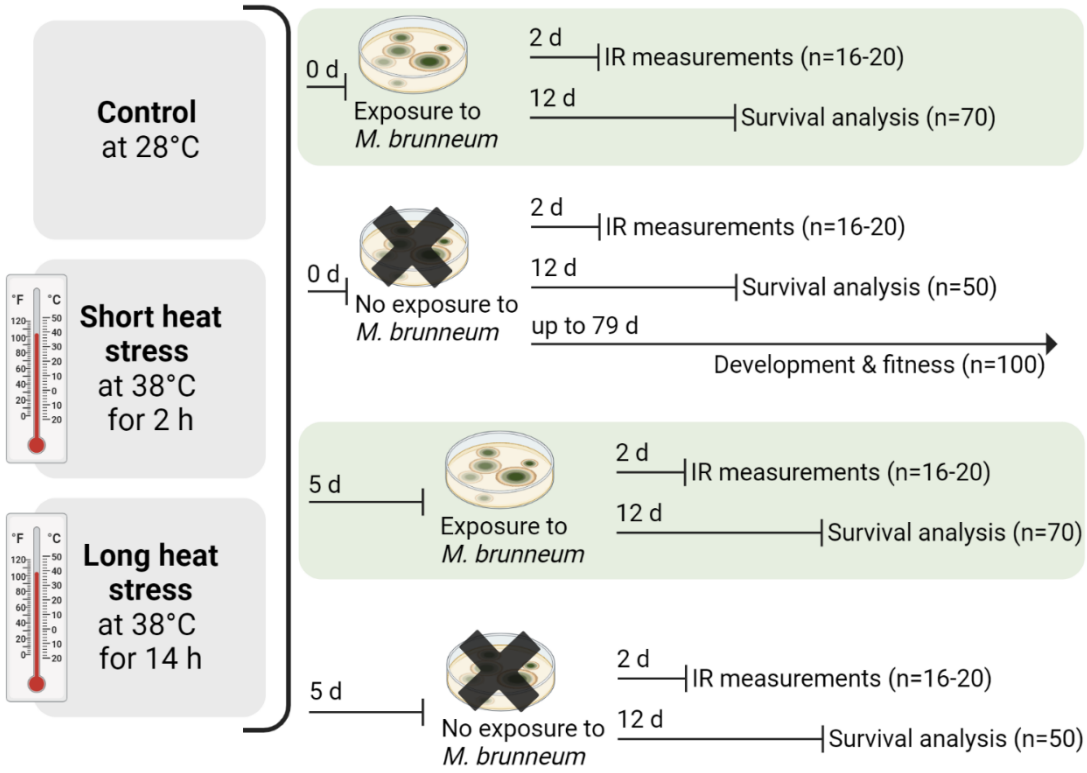

Figure 3.1 Schematic representation of the experimental design.

Tenebrio molitor larvae were either exposed to a control (28°C), a short (2 h), or a long (14 h) heat stress (38°C) and all groups then remained at 28°C. Two groups of larvae were either exposed to a lethal dose of *M. brunneum* or a control treatment immediately after the heat stress treatments. Similarly, two other groups were exposed to the lethal *M. brunneum* dose or control treatment five days after the heat stress treatments. The subsequent survival was assessed daily for 12 days in all groups and immune responses (IR) in the different treatments were measured two days after the *M. brunneum* exposure together with the measurement of moulting. Additionally, development and fitness were measured in a group of larvae exposed to the different heat stress treatments but not exposed to *M. brunneum*. Figure created using BioRender.com.

3.3.2 Insect culture

Tenebrio molitor larvae were initially sourced from the company Ÿnsect (Evry, France). The insects were kept in continual laboratory culture over more than five generations before the start of the experiments. Adult *T. molitor* (40 females and 40 males) were kept in 750 ml plastic containers (15 cm × 9.5 cm = 142.5 cm^2) containing 100 g diet for one week. The diet was provided by Ÿnsect and consisted of wheat bran (35.9%), corn dried distillers grains (30%), wheat (23.7%), and beer

yeast (10.4%). The adults were fed with 5 g bacteriological water agar (1% w/v) twice a week. The containers containing eggs and hatching larvae received 10 g bacteriological water agar (1% w/v) twice a week starting one week after removal of the adults. Three weeks after removal of adults, 50 g of diet was added to each container. All breeding and rearing took place at 28°C (± 0.5°C) in complete darkness. Open containers with water (560 cm^2 surface) were placed in the top of the incubator to maintain a relative humidity of approximately 65% (± 5%) and filled with fresh water once a week. The temperature and relative air humidity in the incubators were monitored every 15 min using EasyLog™ EL-SIE-2 dataloggers throughout the breeding, rearing, and during experiments.

3.3.3 Exposure of larvae to heat stress

The larvae for experiments were chosen based on age and individual weight rather than larval instars because it has been shown that common methods to determine instars are unreliable in *T. molitor* [44]. Larvae (29 days after removal of adults), weighing 40 – 65 mg/larva, were placed in groups of 10 larvae in transparent cups (5.2 cm diameter) each containing 6 g diet and 1 g bacteriological water agar (1% w/v) for one day at 28°C (± 0.5°C). Thereafter, the larvae were exposed to either a short heat stress [38°C (± 0.5°C) for 2 h, 65% RH (± 5%)], a long heat stress [38°C (± 1°C) for 14 h, 65% RH (± 5%)] or no heat stress [constant 28°C (± 0.5°C), 65% (± 5%)]. All groups of larvae were immediately put back to an incubator at 28°C (± 0.5°C) after their treatments.

3.3.4 Exposure to *Metarhizium brunneum*

Metarhizium brunneum isolate KVL12-30 (culture collection of the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark) was grown on Petri dishes (9 cm diameter, triple vented) containing Sabouraud Dextrose Agar (SDA) media (65 g/l) at 23°C (± 0.2°C) in complete darkness for 21 days and sealed with Parafilm™. These were considered the first subcultures (one transfer since stock culture). To harvest conidia, 10 ml of Triton-X (0.05% v/v) was added to each of five Petri dishes, and conidia were harvested using a Drigalski spatula. The subsequent suspension containing the conidia was then poured into a 50 ml Falcon

tube and centrifuged at 3,000 rpm (1,872 *g*, Eppendorf Centrifuge 5810 R) for 3 min. Afterwards, the supernatant was discarded and 20 ml of Triton-X (0.05% v/v) was added to the Falcon tube and the centrifuging step was repeated with the settings as above. The supernatant was again discarded and another 20 ml of Triton-X (0.05% v/v) was added to the tube. The obtained stock suspensions were then diluted ten times by adding 0.1 ml of each stock suspension to 0.9 ml Triton-X (0.05% v/v). This was repeated twice to obtain a 1,000-times diluted suspension. From this suspension, 20 µl was added to a 0.2 mm Fuchs-Rosenthal hemocytometer and the conidia were counted under a light microscope, at 400 times magnification.

Conidial viability was assessed by spreading 100 µl of 10^6 conidia/ml on each of three Petri dishes with SDA, which were incubated at 28°C (± 0.5°C) for 18 h. Thereafter, 300 conidia were counted on each Petri dish and the numbers of germinated and un-germinated conidia were noted. A conidium was considered as germinated when it had a germ tube at least as long as the smallest diameter of the conidium.

The cups containing larvae were checked for shed exuviae before putting the larvae in new cups (5.2 cm diameter) in groups of 10 (same groups as before) containing a filter paper in the bottom (3 × 3 cm) moistened with 0.15 ml sterile water and a soaked filter paper (2 × 5 cm) on the inner side of the cup. The larvae that were exposed to *M. brunneum* immediately after the heat stress received 2 µl of 3.5×10^5 conidia/ml suspension (7×10^2 conidia per larva), directly applied on the metathorax of each larva using a pipette. The larvae in the control treatments were exposed in the same way to 2 µl Triton-X (0.05% v/v). The cups were closed with ventilated lids to ensure high air humidity. After 24 h, the larvae (same groups as before) were moved to new cups containing 6 g diet and 1 g bacteriological water agar (1% w/v) without lids. The cups were checked daily for shed exuviae during the first two days. Twice a week, 1 g bacteriological water agar (1% w/v) was added to each cup.

The larvae that were exposed to *M. brunneum* five days after the heat stress were treated in the same way as the larvae that were exposed to *M. brunneum* immediately

after the heat stress, except that they received 2 µl of 10^6 conidia/ml suspension (2×10^3 conidia per larva) instead of 3.5×10^5 conidia/ml. These different *M. brunneum* doses at different time points were chosen based on data from previous pre-experimental bioassays (see Assessment of EC_{50} values in appendices to Chapter 3) to achieve similar survival rates in the *M. brunneum* groups not exposed to heat stress treatments.

3.3.5 Survival of larvae

The survival of the larvae was checked daily for 12 days and dead larvae were removed from cups. To check the infection status of the larvae, cadavers were surface sterilized by dipping them in a Sodium Hypochlorite (NaClO) solution (2 - 3% v/v) for 20 s. They were then rinsed two times in sterile water and put individually in Petri dishes (3 cm diameter) for 24 h. Next, wet filter papers were added inside the lids of the Petri dishes and they were sealed with Parafilm™. The cadavers were checked visually for fungal outgrowth and sporulation every second day.

3.3.6 Immune response measurements

3.3.6.1 Extraction of hemolymph from larvae

Hemolymph samples were taken from larvae two days after exposure to *M. brunneum*, which is long enough to allow the fungus to germinate, penetrate the cuticle, and cause an immune response, but before any mortality occurs in the larvae. The hemolymph samples were used to measure antibacterial activity, phenoloxidase activity, and hemocyte concentration. Three Eppendorf tubes (0.5 ml) were prepared for each hemolymph sample. In the first Eppendorf tube 22 µl PBS (phosphate-buffered saline) (A), in the second Eppendorf tube 21 µl PBS (B), and in the third Eppendorf tube 16 µl PBS (C) was added (Figure S3.2). All Eppendorf tubes were kept on ice during hemolymph extraction.

The larvae were held using forceps so that they could not move and the tibia of the posterior left leg was severed using forceps so that a droplet of clear hemolymph formed. Immediately afterwards, 2 µl of hemolymph was extracted and added to

Eppendorf tube (A) using a pipette. The Eppendorf tube (A) was vortexed and afterwards 14 µl of this sample was added to Eppendorf tube (B), which was put immediately in liquid nitrogen to analyse phenoloxidase activity later. Another 4 µl from Eppendorf tube (A) was added to Eppendorf tube (C). Eppendorf tube (A) was then put in liquid nitrogen to analyse antibacterial activity and the sample in Eppendorf tube (C) was used for counting hemocytes (Figure S3.2). Once all the samples were collected, the Eppendorf tubes (A) and (B) were transferred from liquid nitrogen to a freezer at -80°C.

A total of 20 hemolymph samples were extracted in each treatment originating from larvae from four different cups (five larvae per cup). If samples contained additional tissue fragments smaller than hemocytes, they were considered most likely not pure and they were therefore excluded from analysis resulting in variable sample sizes (hemolymph from 16 - 20 larvae per treatment was analysed).

3.3.6.2 Antibacterial activity

The antibacterial activity of hemolymph is an indication of the content of AMPs in a sample [45, 46]. The method was adapted from Haine et al. [46]. This method is used to measure antibacterial activity in hemolymph, expressed as the diameter of inhibited growth of the bacterium *Arthrobacter globiformis*. The squared diameter of the inhibited zone is linear to the log of AMP concentration in the hemolymph [45]. However, Haine et al. [46] suggested measuring antibacterial activity as the diameter of the inhibited zone "to avoid compounding marginal measurement errors by multiplying them" [46].

One colony of *A. globiformis* isolate 20124 previously grown on solid P1 media (15 g bacteriological agar, 10 g peptone, 5 g yeast extract, 5 g glucose, and 5 g NaCl in 1 l dH_2O) at 30°C was added to a 250 ml Erlenmeyer flask containing 25 ml liquid P1 media (10 g peptone, 5 g yeast extract, 5 g glucose and 5 g NaCl in 1 l dH_2O). The Erlenmeyer flask was incubated on a platform shaker at 200 rpm at 30°C in complete darkness. After 24 h, the bacterial suspension was poured into a 50 ml Falcon tube and centrifuged for 10 min at 3,000 rpm (1,872 *g*, Eppendorf Centrifuge 5810 R) at

4°C. The supernatant was discarded and 20 ml sterile dH_2O was added. The centrifuging step was repeated with the settings mentioned above. The supernatant was again discarded and another 20 ml sterile dH_2O was added to the Falcon tube. The obtained stock suspension was serially diluted ten times by adding 0.1 ml of the stock suspension to 0.9 ml sterile dH_2O to obtain 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 times dilutions. Subsequently, 10 µl of each of the six latter dilutions were pipetted on solid P1 media (three dilutions per Petri dish). By tilting the Petri dish (9 cm diameter, triple vented) on one side, the suspensions ran down on the media forming straight lines. Three replicates per dilution were prepared. The Petri dishes were incubated at 30°C and the stock suspension was kept in the fridge in the meantime. After 48 h, the colony forming units (cfu) from at least three different dilutions were counted and the concentrations in the different dilutions were calculated using the formula:

$$\frac{cfu}{ml} = \frac{Counted\ cfu}{0.01\ ml} * Dilution\ factor \qquad Equation\ 3.1$$

Thereafter, the average of at least three concentrations was calculated. Solid P1 medium was autoclaved and afterwards put in a water bath at 45°C. A bacterial suspension of *A. globiformis* containing 10^7 cfu/ml was prepared in dH_2O from the stock suspension. Once the P1 medium reached 45°C, 10 µl of the prepared bacterial suspension was added per ml of medium. Thereafter, the medium containing the bacterial suspension was put on a magnetic stirrer and Petri dishes (9 cm diameter, triple vented) with 5 ml medium were prepared. In the meantime, the samples from Eppendorf (A) were thawed on ice. Once the Petri dishes were dry, a pipette with a 2 ml tip was used to form eight holes (2.5 mm diameter) in the medium of each Petri dish. Thereafter, the holes were labelled on the outside and 2 µl of the hemolymph samples was added to each hole (2 µl of PBS was added to each Petri dish as a control; seven hemolymph samples and one PBS control per Petri dish). Two technical replicates (originating from the same biological replicate, in this case the same larva) were prepared on two different Petri dishes and the Petri dishes were sealed with Parafilm™. After incubation at 30°C for 48 h, two perpendicular diameters of the inhibited zones were measured using a digital calliper. The inhibited

zone was calculated as the average of the two diameters minus the average of the two diameters of the well of the control of the same plate. The average of the two technical replicates was used as one data point.

3.3.6.3 Measurement of phenoloxidase activity

The samples in Eppendorf tubes (B) were stored at -80°C for at least 24 h. L-DOPA (l-3,4-dihydroxyphenylalanine; 20 mM; 4 mg/ml) was prepared in dH_2O and put on a magnetic stirrer until it dissolved completely. Thereafter, frozen samples were thawed on ice for 30 min. Each sample was vortexed and afterwards centrifuged for 10 min at 6,500 rpm (3,873 *g*, Beckman Coulter Microfuge 20R) at 4°C. This step disposed of cell debris in the samples, which potentially could inhibit the reaction between phenoloxidase and L-DOPA. Afterwards, two technical replicates per sample were prepared on the same 96-well plate by pipetting 10 µl of the sample per well. Next, 10 µl of PBS was added in each of five control wells per 96-well plate. A volume of 90 µl of L-DOPA was added to each well of the hemolymph sample and PBS controls. The 96-well plate was read using a Synergy™ HT MultiDetection Microplate Reader with Gen5 software. The optical density (OD) was measured every minute for 90 min using a wavelength of 490 nm at 30°C. The maximal velocity of the reaction (Vmax) was measured as the slope during 15 min of the linear phase. Vmax was expressed as ΔOD490 = milliunit/min. The average of the two technical replicates was used as one data point.

3.3.6.4 Counting of hemocytes

The samples from Eppendorf tubes (C) were vortexed and 14 µl of the sample was immediately pipetted on a Fast-read102 counting slide (Sigma Ltd). The hemocytes were left to settle for 1 min and afterwards, the hemocytes from all ten squares (one square containing 16 grids) were counted on the counting slide. The resultant hemocyte concentration was calculated using the formula:

$$\frac{Hemocytes}{\mu l} = \frac{Average\ number\ of\ hemocytes\ per\ square}{0.1\ \mu l} * 60 \qquad Equation\ 3.2$$

The volume above each square is 0.1 µl and the sample was 60 times diluted. The average of the counts from the ten squares was used as one data point. Even though there are multiple types of hemocytes present in the hemolymph of *T. molitor*, we decided to focus on the total number rather than distinguishing between the different types.

3.3.7 Development and fitness measurements

Larvae from each of the heat treatments were weighed per cup (10 larvae together) one day before then five and 10 days after exposure to the heat stress treatments. The weight gain per 10 larvae was calculated as the weight of the larvae five or 10 days after the heat stress minus the weight one day before the heat stress. During the larval development, 1 g bacteriological water agar (1% w/v) was added twice a week. The shed exuviae were counted and removed every second day and larvae were also checked for pupation every second day. Newly emerged pupae were weighed and the sex of each pupa was noted. Males and females were distinguished by examination of the developing genital structures on the ventral side of the eighth abdominal segment. The male has only a small swelling on the seventh visible sternite, whereas the female has a pair of clearly separated papillae on the sternite [47] (Figure S3.3). The first 25 male and female pupae in each heat stress treatment were put individually in separate 50 ml Falcon tubes, each containing 1 g of diet. These pupae were checked every second day for adult emergence after which they remained in their tubes for four to eight days. Thereafter, one male and one female beetle of the same heat treatment (n = 25) were put together in a 50 ml Falcon tube containing 7 g of diet. The pairs were moved to new Falcon tubes with 7 g diet every week for three weeks and they were provided with 0.5 g bacteriological water agar (1% w/v) twice a week. After three weeks, the adult beetles were removed. The larvae in each Falcon tube were counted and weighed in groups 23 days after the adult beetles were removed.

3.3.8 Statistical analysis

The experimental investigation of survival, immune responses, and moulting of larvae after exposure to *M. brunneum* and heat stresses were performed on two

independent occasions (included as random effects in all statistical models), whereas development and fitness measurements of larvae only exposed to heat stresses were performed once. Differences were considered significant at $p < 0.05$. Data was only subjected to one- or two-way ANOVAs (analysis of variances) when normality (QQ-plots) and homogeneity of variances (Levene test, $p > 0.05$) assumptions were satisfied. All statistical analyses were performed with R v. 4.1.0 [48].

3.3.8.1 Survival analysis

Survival analyses of larvae exposed to *M. brunneum* immediately and five days after heat stress were performed separately using the survival [49], and the coxme [50] packages. Only the treatment groups that showed any mortality were analysed using the Cox model, because in the groups with no mortality (treatments without *M. brunneum* exposure) the Cox model resulted in degenerate estimates. A mixed effects Cox model was used to analyse the survival data. The cups, initially containing 10 larvae per cup, as well as the experiments (repetition on two independent occasions) were included as random effects. Pairwise comparisons of the heat stress treatments were carried out using Tukey contrasts with single step adjustment for multiple testing in the multcomp package [51]. To confirm that *M. brunneum* exposure resulted in different survival to no exposure to *M. brunneum*, the survival was additionally analysed using a log-rank test in the survival package [49]. In addition, we used a mixed effects Cox model (with cup and experimental repetition as random effects) to analyse the effect of using different pathogen doses at 0 or five days after heat stress (analysis only done on larvae that were not exposed to heat stress but to *M. brunneum*) to understand if both doses achieved comparable mortality rates.

3.3.8.2 Immune responses and moulting during two days

Immune response and moulting data assessed two or seven days after heat stress were analysed independently. To compare hemocyte concentrations across heat treatments and exposure to *M. brunneum*, a generalized linear mixed model with a negative binomial error distribution (used for overdispersed count data) was implemented using the lme4 package [52]. To compare antibacterial and

phenoloxidase activity across heat treatments and exposure to *M. brunneum*, generalized linear mixed models with gamma error distributions (used for right-skewed data) were implemented using the lme4 package [52]. To compare the number of shed exuviae two days after *M. brunneum* exposure across heat treatments, a generalized linear mixed model with a Poisson error distribution was implemented using the lme4 package [52]. Experiment (repetition on two independent occasions) and cups were included as random effects in all models. Models were selected using the drop1 function and removing non-significant terms and pairwise comparisons were performed using the emmeans package [53]. Statistics for terms that were excluded in the final models were extracted from the stage prior to their elimination.

3.3.8.3 Development analysis

Data on weight gain five and 10 days after the heat stress, number of exuviae until pupation, and number of offspring over three weeks were subjected to one-way ANOVAs using Tukey's HSD (Honestly Significant Difference) tests to separate the means. Pupal weights were analysed using a two-way ANOVA with sex and heat treatments as fixed effects and cup as random effect. Data on duration of development from heat stress until pupation were analysed using a mixed effects Cox model with sex, heat stress treatment, and their interaction as fixed effects. Moreover, the cups were added as a random effect. The analysis was performed using the coxme package [50]. The data on the average larval weight of each mated pair per week were subjected to one-way ANOVA using Tukey's HSD tests to separate the means. Data from one mated pair in the group that received a long heat stress was excluded because the female beetle died in the first week of egg laying.

3.4 Results

3.4.1 Short-term effect of heat stress on pathogen susceptibility

To test the short-term effect of heat stress on pathogen susceptibility of *T. molitor*, larvae were exposed to a lethal dose of *M. brunneum* immediately after a short (2 h), long (14 h) or no heat stress. There was no difference in survival probabilities of control larvae exposed to *M. brunneum* 0 or 5 days after the heat stress, indicating

that the two tested pathogen doses achieved comparable mortality rates (p = 0.670, Table 3.1). The germination rates of *M. brunneum* conidia in all experiments were >99%. *Metarhizium brunneum* exposure had a significant effect on survival (p <0.001). A short and a long heat stress treatment had a significant effect on the survival probabilities of larvae exposed to *M. brunneum* (p <0.001 and p = 0.022, respectively, Table 3.1). After post-hoc tests the survival probability of larvae exposed to *M. brunneum* was still significantly increased when they received a short heat stress compared to larvae that were constantly kept at 28°C (p <0.001, Figure 3.2) but not compared to larvae that received a long heat stress (p = 0.279, Figure 3.2). The survival probability of larvae exposed to a long heat stress was not significantly different compared to larvae that were constantly kept at 28°C (p = 0.057, Figure 3.2). There was no effect of heat stress on mortality in larvae that were not exposed to *M. brunneum* (Figure 3.2), indicating that all the mortality observed in the pathogen treatments can be attributed to exposure to *M. brunneum*. Of all the cadavers, 76, 100, and 84% showed mycosis (visible fungal outgrowth) in no, short, and long heat stress treatments respectively. Subsequently, all of these cadavers sporulated, producing typical green conidia of the *Metarhizium* genus.

To measure the short-term effect of heat and pathogen stress on immune responses, hemolymph samples of larvae were extracted two days after a short, long or no heat stress with either exposure to a lethal dose of *M. brunneum* or no pathogen exposure. Exposure to *M. brunneum* had no significant effect on antibacterial activity (p = 0.063, Figure 3.3A, Table 3.2) and there was no significant pathogen exposure × heat stress interaction (p = 0.388, Figure 3.3A, Table 3.2). The heat stress treatment alone had a significant effect on antibacterial activity (p <0.001, Figure 3.3A, Table 3.2) and post-hoc tests revealed that both a short and a long heat stress increased antibacterial activity in the hemolymph significantly compared to no heat stress (both p <0.001, Figure 3.3A).

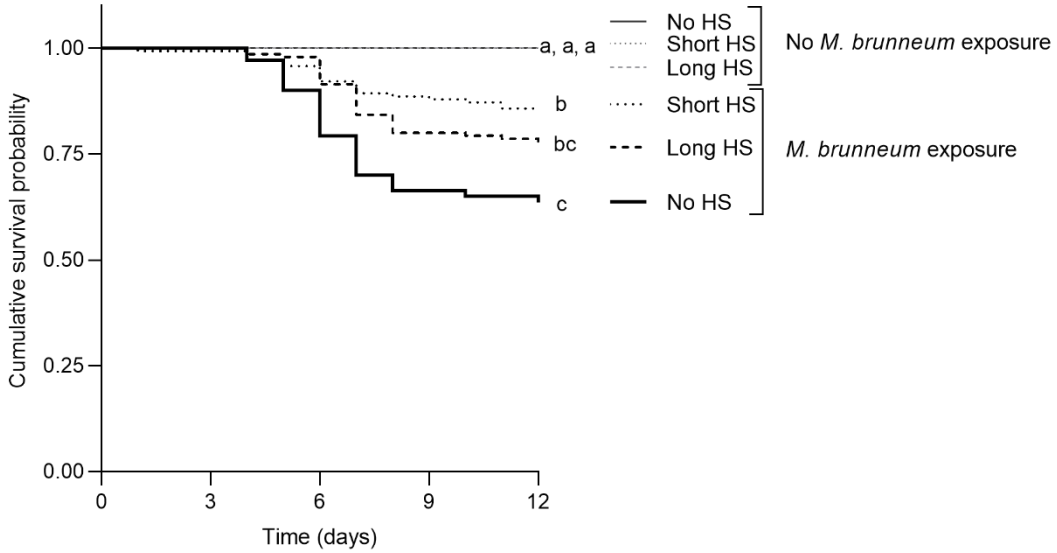

Figure 3.2 Cumulative survival probabilities of *T. molitor* larvae exposed to *M. brunneum* (7×10^2 conidia per larva) immediately after a short (2 h), long (14 h) or no heat stress (HS).

The results for each treatment are reported as the median values of two independent experiments. Each group in each experiment consisted of seven cups with 10 individuals per cup resulting in n = 140. Letters at the end of the curves designate significant differences between the three treatment groups exposed to *M. brunneum* (pairwise comparisons of means, $p < 0.05$). Larvae only exposed to no, short and long heat stresses without exposure to *M. brunneum* were included in both experiments and showed 100% survival in all treatments. Each of these control groups in each experiment consisted of five cups with 10 individuals per cup resulting in total n = 100. Figure created with GraphPad Prism version 9.3.1.

Hemocyte concentration was significantly affected by exposure to *M. brunneum* ($p = 0.012$, Figure 3.3B, Table 3.2), although no significant differences could be identified when treatment combinations were compared with each other individually in the post-hoc tests. Furthermore, heat stress treatments had a significant effect on hemocyte concentration ($p < 0.001$, Figure 3.3B, Table 3.2); a long heat stress increased hemocyte concentration in the hemolymph compared to the short heat stress ($p < 0.001$, Figure 3.3B) and no heat stress ($p = 0.003$, Figure 3.3B). There was no significant pathogen exposure × heat stress interaction on hemocyte concentration ($p = 0.790$, Figure 3.3B, Table 3.2). The phenoloxidase activity was not affected by exposure to *M. brunneum*, heat stress, or pathogen × heat stress interaction ($p = 0.963$, $p = 0.134$, and 0.916, respectively, Figure 3.3C, Table 3.2).

Table 3.1 Results of the statistical analyses using mixed effects cox proportional hazards models to analyse survival and duration until pupation of *T. molitor* larvae (results of pairwise comparisons for survival analyses are mentioned in the text).

Survival of control larvae exposed to *M. brunneum* immediately or five days after heat stress	**Hazard ratio ± standard error**	***p***
M. brunneum dose	1.087 ± 0.196	0.670
Survival of larvae exposed to *M. brunneum* immediately after heat stress		
Short heat stress	0.324 ± 0.308	<0.001
Long heat stress	0.532 ± 0.275	0.022
Survival of larvae exposed to *M. brunneum* five days after heat stress		
Short heat stress	1.126 ± 0.235	0.610
Long heat stress	1.303 ± 0.231	0.250
Duration until pupation after heat stress		
Short heat stress	1.132 ± 0.189	0.510
Long heat stress	1.017 ± 0.188	0.930
Sex	1.402 ± 0.203	0.097
Short heat stress:Sex	0.925 ± 0.287	0.780
Long heat stress:Sex	0.729 ± 0.289	0.270

The number of exuviae shed per 10 larvae over the two days after heat and pathogen exposure was significantly affected by heat stress treatments (p <0.001, Figure 3.3D, Table 3.2) but not by exposure to *M. brunneum* (p = 0.434, Figure 3.3D, Table 3.2) or by the pathogen exposure × heat stress interaction (p = 0.414, Table 3.2). The number of exuviae per replicate was significantly lower in larvae that received a long heat stress compared to larvae that did not receive a heat stress (p = 0.006, Figure 3.3D) or a short heat stress (p <0.001, Figure 3.3D). There was no significant difference in the number of exuviae between short and no heat stress (p = 0.071, Figure 3.3D). The number of exuviae shed during the first day (during which larvae were exposed to the heat treatments and before exposure to *M. brunneum*) were <0.1 exuviae per replicate on average in each treatment. Moulting during the heat treatments was therefore disregarded.

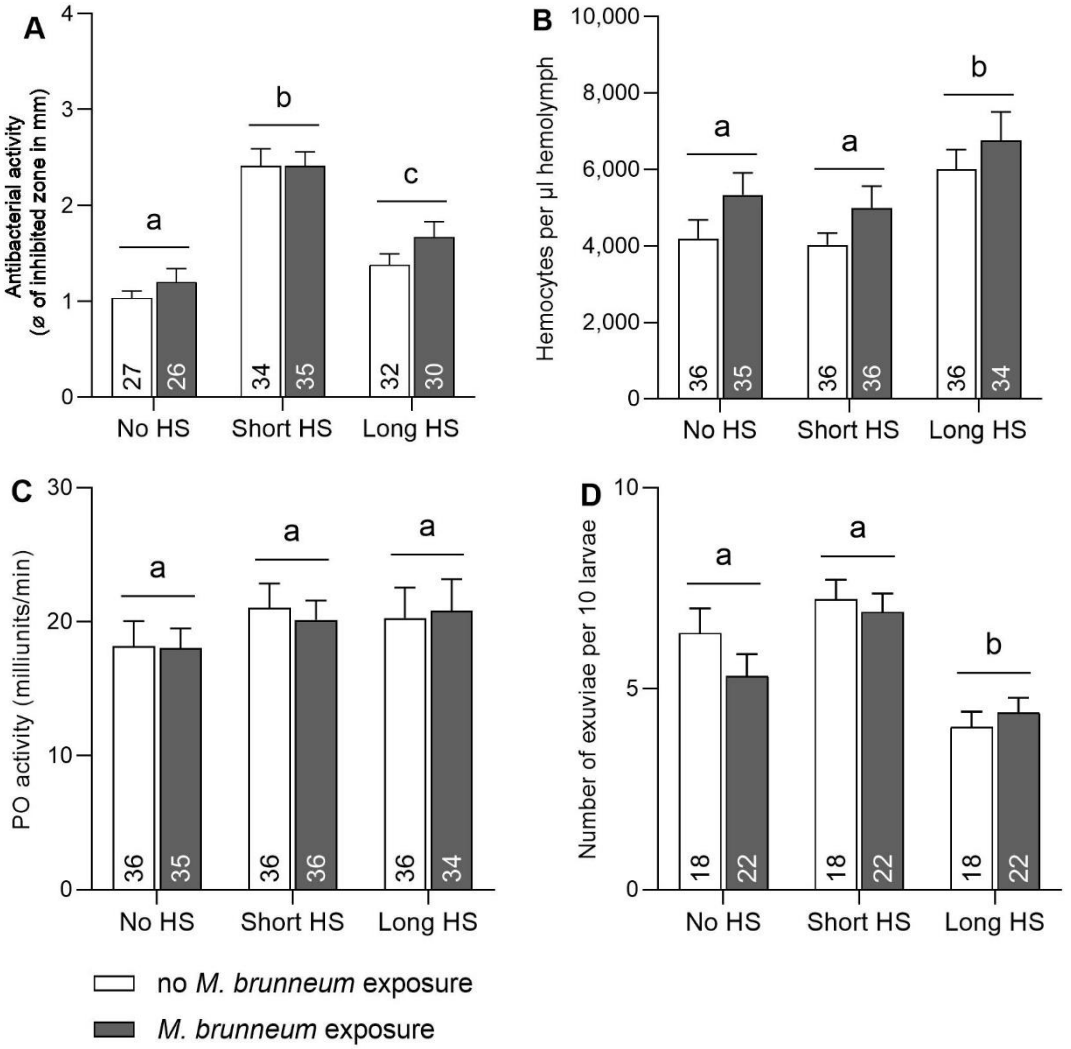

Figure 3.3 Immune responses and moulting of larvae two days after no, short or long heat stress (HS) either control (white) or exposed to *M. brunneum* (grey).

The results are based on two independent experiments. **A** Mean (+ SEM) antibacterial activity (diameter of inhibited zone in mm). **B** Mean (+ SEM) hemocytes per µl hemolymph. **C** Mean (+ SEM) phenoloxidase (PO) activity (milliunits/min). **D** Mean (+ SEM) number of exuviae per replicate during two days after pathogen exposure. Different lowercase letters denote statistically significant differences between heat stress treatments (pairwise comparisons of means, $p < 0.05$). **A - C** number of samples (individuals) per treatment are given in each bar. **D** number of replicates (each containing 10 larvae) are given in each bar. Figure created with GraphPad Prism version 9.3.1.

Table 3.2 Results of statistical analyses using generalized linear mixed models and general linear models (results of pairwise comparisons are mentioned in the text). Bold terms were retained in the final models.

Purpose	Test	Independent variable	χ^2 (chi-square)	d.f.1, d.f.2	*p*
Compare **hemocyte concentrations** between heat treatments and exposure to *M. brunneum* two days after heat stress	Generalized linear mixed model with a negative binomial error distribution	**Heat**	**10.901**	**2, 208**	**<0.001**
		Pathogen	**6.359**	**1, 208**	**0.012**
		Heat × Pathogen	0.472	2, 208	0.790
Compare **hemocyte concentrations** between heat treatments and exposure to *M. brunneum* seven days after heat stress	Generalized linear mixed model with a negative binomial error distribution	**Heat**	**39.793**	**2, 211**	**<0.001**
		Pathogen	2.617	1, 211	0.106
		Heat × Pathogen	**14.044**	**2, 211**	**<0.001**
Compare **antibacterial activity** between heat treatments and exposure to *M. brunneum* two days after heat stress	Generalized linear mixed model with gamma error distribution	**Heat**	**91.647**	**2, 179**	**<0.001**
		Pathogen	3.461	1, 179	0.063
		Heat × Pathogen	1.895	2, 179	0.388
Compare **antibacterial activity** between heat treatments and exposure to *M. brunneum* seven days after heat stress	Generalized linear mixed model with gamma error distribution	Heat	2.985	2, 197	0.084
		Pathogen	0.032	1, 197	0.858
		Heat × Pathogen	1.135	2, 197	0.567
Compare **phenoloxidase activity** between heat treatments and exposure to *M. brunneum* two days after heat stress	Generalized linear mixed model with gamma error distribution	Heat	2.242	2, 208	0.134
		Pathogen	0.002	1, 208	0.963
		Heat × Pathogen	0.175	2, 208	0.916
Compare **phenoloxidase activity** between heat treatments and exposure to *M. brunneum* seven days after heat stress	Generalized linear mixed model with gamma error distribution	**Heat**	**5.123**	**2, 211**	**0.024**
		Pathogen	1.084	1, 211	0.298
		Heat × Pathogen	3.912	2, 211	0.141
Compare the **number of shed exuviae** during two days after *M. brunneum* exposure between heat treatments (immediately after heat stress)	Generalized linear mixed model with a Poisson error distribution	**Heat**	**27.535**	**2, 115**	**<0.001**
		Pathogen	0.613	1, 115	0.434
		Heat × Pathogen	1.764	2, 115	0.414
Compare the **number of shed exuviae** during two days after *M. brunneum* exposure between heat treatments (five days after heat stress)	Generalized linear mixed model with a Poisson error distribution	Heat	0.230	2, 115	0.631
		Pathogen	1.363	1, 115	0.243
		Heat × Pathogen	5.776	2, 115	0.056

3.4.2 Persistent effects of heat stress on pathogen susceptibility

To test the persistent effects of heat stress on pathogen susceptibility of *T. molitor*, larvae were exposed to a lethal dose of *M. brunneum* five days after a short, long or no heat stress. The germination rates of *M. brunneum* conidia in all experiments were

>99%. *Metarhizium brunneum* exposure had a significant effect on survival (p <0.001). Of all the cadavers from the pathogen exposed treatment, 93, 84, and 94% showed mycosis in no, short, and long heat stress treatments respectively, with all these cadavers also showing sporulation. A short or a long heat stress treatment had no significant effect on the survival probability of larvae exposed to *M. brunneum* (p = 0.610 and p = 0.250, respectively, Table 3.1 and Figure 3.4). Larvae that were not exposed to *M. brunneum* but only the short, long or no heat stress treatments did not show mortality.

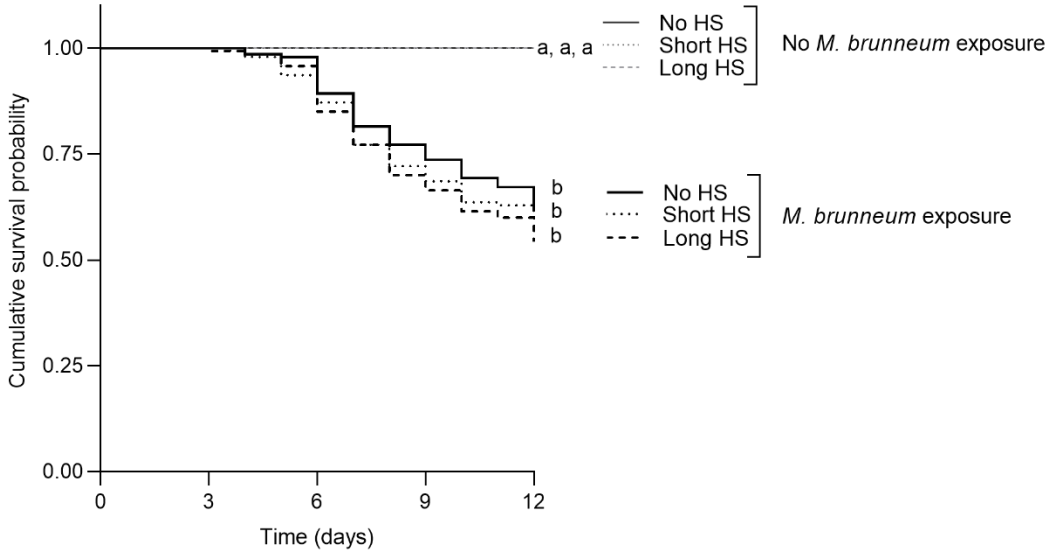

Figure 3.4 Cumulative survival probabilities of *T. molitor* larvae exposed to *M. brunneum* (2×10^3 conidia per larva) five days after a short (2 h), long (14 h) or no heat stress (HS).

The results for each treatment are reported as the median values of two experiments. Each group in each experiment consisted of seven cups with 10 individuals per cup resulting in n = 140. Pairwise comparisons of means showed no significant differences between the treatment groups (p <0.05). Larvae only exposed to no, short and long heat stresses without exposure to *M. brunneum* were included in both experiments and showed 100% survival in all treatments. Each of these control groups in each experiment consisted of five cups with 10 individuals per cup resulting in total n = 100. Figure created with GraphPad Prism version 9.3.1.

To measure the persistent effects of heat and pathogen stress on immune responses, hemolymph samples of larvae were extracted seven days after a short (2h), long (14

h) or no heat stress with either exposure to a lethal dose of *M. brunneum* or no pathogen exposure (two days before). The exposure to *M. brunneum*, heat stress or their interaction did not have a statistically significant effect on antibacterial activity (p = 0.858, p = 0.084, and p = 0.567, respectively, Figure 3.5A, Table 3.2). The interaction between *M. brunneum* exposure and heat stress had a significant effect on hemocyte concentration in the hemolymph (p <0.001, Figure 3.5B, Table 3.2) and was therefore retained in the model (consequently treatments were compared with each other individually). In the larvae exposed to *M. brunneum*, increasing durations of heat stress treatments led to significantly decreasing hemocyte concentrations in the hemolymph (Figure 3.5B).

Phenoloxidase activity was significantly affected by heat stresses (p = 0.024, Figure 3.5C, Table 3.2), although significant differences could not be identified when treatments were compared with each other individually in the post-hoc test. There was no significant effect of exposure to *M. brunneum* on phenoloxidase activity (p = 0.298, Figure 3.5C, Table 3.2) and there was no significant pathogen exposure × heat stress interaction (p = 0.141, Figure 3.5C, Table 3.2). The number of exuviae shed during the two days after *M. brunneum* exposure was generally very low and there was no significant effect of exposure to pathogen, heat or their interaction (p = 0.243, p = 0.631, and p = 0.056, respectively, Figure 3.5D, Table 3.2).

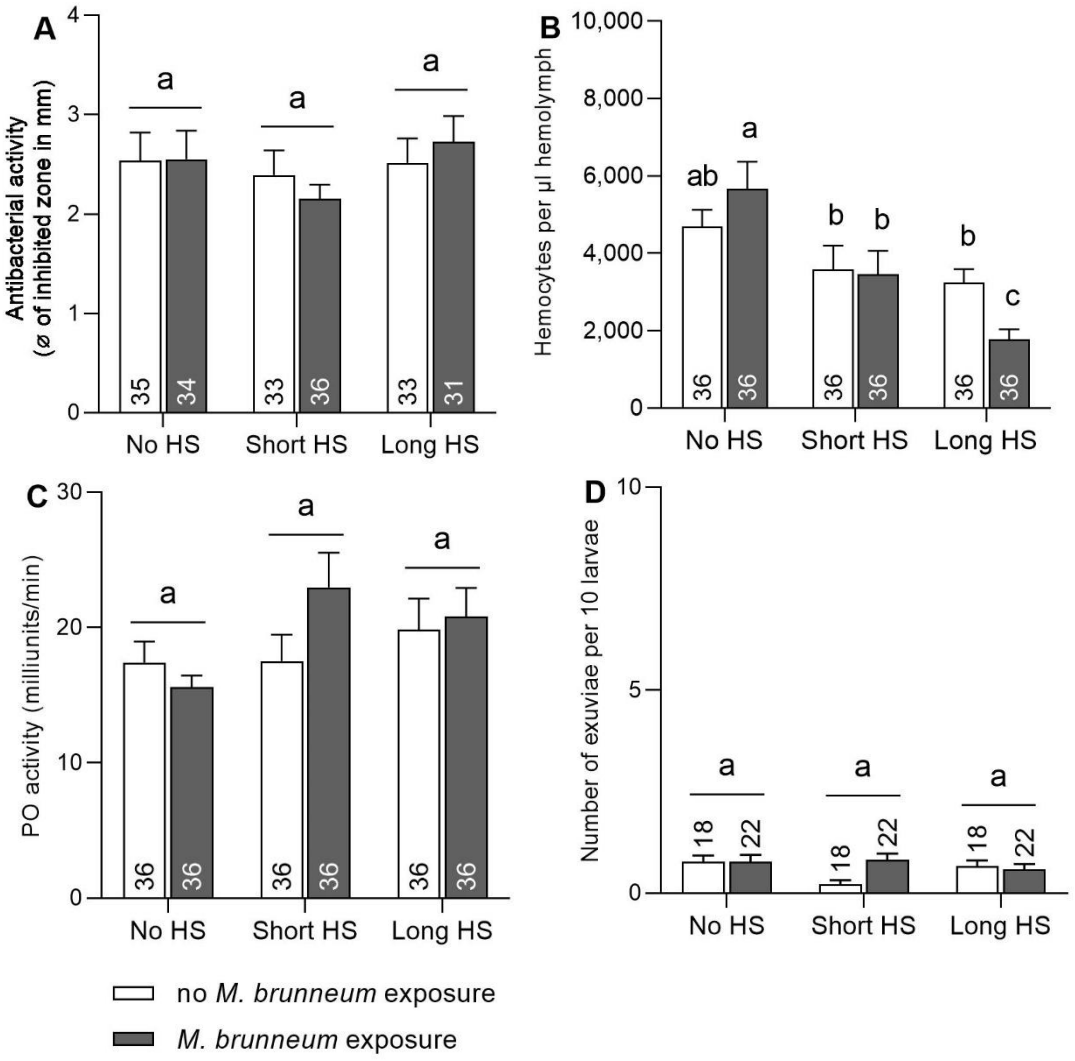

Figure 3.5 Immune responses and moulting of larvae seven days after no, short or long heat stress (HS) either control (white) or exposed to *M. brunneum* (grey).

The results are based on two independent experiments. **A** Mean (+ SEM) antibacterial activity (diameter of inhibited zone in mm). **B** Mean (+ SEM) hemocytes per µl hemolymph. **C** Mean (+ SEM) phenoloxidase (PO) activity (milliunits/min). **D** Mean (+ SEM) number of exuviae per replicate during two days after pathogen exposure. Different lowercase letters denote statistically significant differences between heat stress treatments or individual treatments used for hemocyte per µl hemolymph because interaction between *M. brunneum* and heat stress exposure was significant (pairwise comparisons of means, $p < 0.05$). **A - C** number of samples (individuals) per treatment are given in each bar. **D** number of replicates (each containing 10 larvae) are given above each bar. Figure created with GraphPad Prism version 9.3.1.

3.4.3 Effect of heat stress on development and fitness

To examine the effects of heat stress on development and reproduction, larval weight gain, development duration until pupation, pupal weight, and the number and weight of offspring of females that had been exposed to heat stress as larvae were measured. Heat stress treatments had a significant effect on larval weight gain in the first five days (p <0.001, Tables 3.3 and 3.4). Post-hoc tests revealed that weight gain was highest in the control treatments, and lowest in the long-heat stress treatment (p <0.001, Tables 3.3 and 3.4). After 10 days, the larvae that had been exposed to either a short or long heat stress had both compensated the weight gain and there were no significant differences between treatments anymore (p = 0.115, Tables 3.3 and 3.4).

Heat stress treatments had a significant effect on the total number of exuviae shed until pupation per replicate (p = 0.007, Tables 3.3 and 3.4). Post-hoc tests revealed that a short heat stress increased the total number of exuviae shed in comparison to no heat stress (p = 0.007, Tables 3.3 and 3.4) and long heat stress (p = 0.045, Tables 3.3 and 3.4). The duration of the larval development until pupation was not affected by heat stress treatments or by sex (Tables 3.1 and 3.4). Similarly, the heat stress treatments and sex did not affect the pupal weights (p = 0.158 and p = 0.660, respectively, Tables 3.3 and 3.4). The number of offspring per female over a three week duration was not affected by the heat treatments (p = 0.993, Tables 3.3 and 3.4).

The number and weight of offspring from heat stressed parents were measured to assess the effect of heat stress during the larval stage on reproductive success and offspring. Heat stress treatments experienced as larvae had a significant effect on the weight of their larval offspring (p = 0.036, p <0.001, p <0.001, in weeks 1-3, respectively, Table 3.3). A short heat stress increased the weight of their offspring compared to the offspring of larvae that were not exposed to a heat stress (in weeks 2 and 3) and compared to those exposed to a long heat stress (in weeks 1-3) (significant p-values are shown in Figure 3.6).

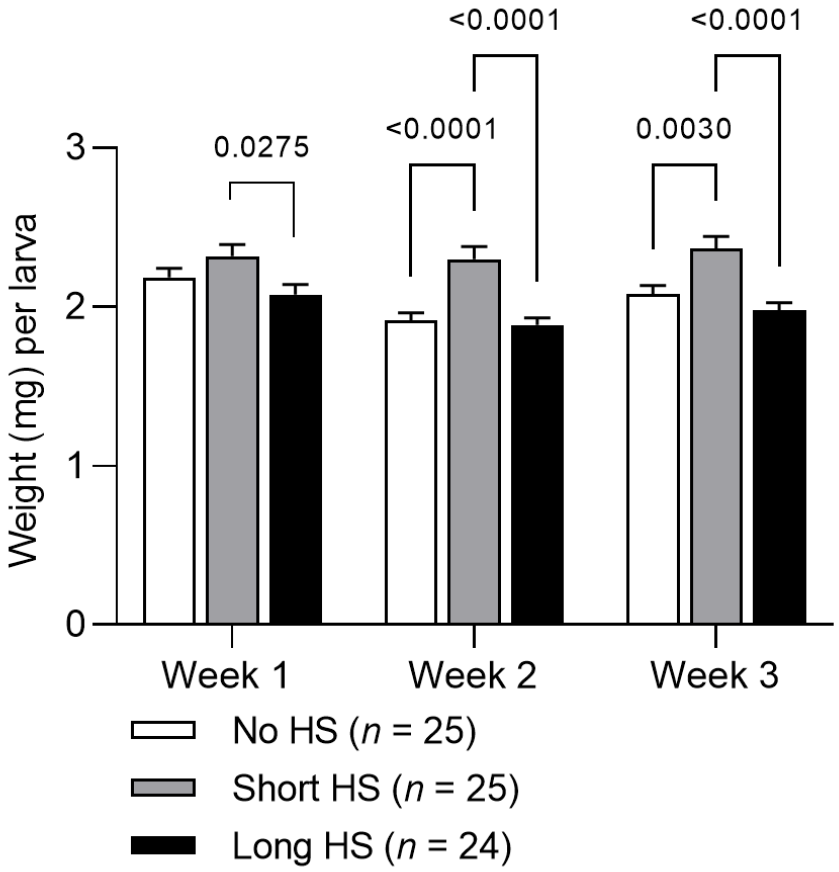

Figure 3.6 Mean (+ SEM) weight (mg) per larva of parents that received no (white), a short (grey) or a long (black) heat stress (HS).

The eggs were laid in three consecutive weeks by the same females. Larvae were weighed 23 days after removal of parents. Significant differences between heat stress treatments per week are denoted by p-values. Figure created with GraphPad Prism version 9.3.1.

Table 3.3 Results of statistical analyses using ANOVAs.

Purpose	Test	*F*	d.f.1, d.f.2	*p*
Compare **weight gain during five days** after heat stress between heat treatments	One-way ANOVAs using Tukey's Honestly Significant Difference (HSD) tests to separate the means	116.300	2, 27	ANOVA: <0.001 Tukey's HSD: long-control: <0.001; short-control: 0.0273; short-long: <0.001
Compare **weight gain during 10 days** after heat stress between heat treatments	One-way ANOVAs using Tukey's Honestly Significant Difference (HSD) tests to separate the means	2.347	2, 27	ANOVA: 0.115
Compare **number of exuviae** until pupation between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	6.075	2, 27	ANOVA: 0.007 Tukey's HSD: long-control: 0.700; short-control: 0.007; short-long: 0.045
Compare **number of offspring** during three weeks between heat treatments	One-way ANOVA	0.007	2, 71	0.993
Compare **pupal weights** between sexes and heat treatments	Two-way ANOVA	Treatment: 1.857 Sex: 0.194 Treatment:Sex: 1.759	Treatment: 2, 293 Sex: 1, 293 Treatment:Sex: 2, 293	Treatment: 0.158 Sex: 0.660 Treatment:Sex: 0.174
Compare **weights of offspring** from heat-stressed parents from first week between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	3.494	2, 71	ANOVA: 0.036 Tukey's HSD: long-control: 0.481; short-control: 0.302; short-long: 0.028
Compare **weights of offspring** from heat-stressed parents from second week between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	15.010	2, 71	ANOVA: <0.001 Tukey's HSD: long-control: 0.939; short-control: <0.001; short-long: <0.001
Compare **weights of offspring** from heat-stressed parents from third week between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	11.150	2, 71	ANOVA: <0.001 Tukey's HSD: long-control: 0.466; short-control: 0.003; short-long: <0.001

Table 3.4 Larval weight gain, number of exuviae shed until pupation, development duration until pupation, pupal weight and number of offspring per female that were exposed to no, short (2 h), or long (14 h) heat stress (HS) as larvae.[1]

	Weight gain 5 d after stress per 10 larvae (mg) (n = 10)	**Weight gain 10 d after stress per 10 larvae (mg)** (n = 10)	**Number of exuviae per 10 larvae** (n = 10)	**Days until pupation**		**Weight per pupa (mg)**		**Number of offspring per female during 3 w** (n = 24-25)
				♀ (n = 54-61)	♂ (n = 39-45)	♀ (n = 54-61)	♂ (n = 39-45)	
No HS	475.9 ± 5.5a	637.6 ± 11.2a	18.6 ± 0.9a	23.3 ± 0.5a	22.4 ± 0.4a	142.4 ± 2.5a	140.9 ± 3.1a	129.6 ± 9.1a
Short HS	443.3 ± 8.5b	675.9 ± 10.5a	21.9 ± 0.5b	23.0 ± 0.4a	22.4 ± 0.4a	148.3 ± 2.4a	145.7 ± 3.3a	130.3 ± 7.4a
Long HS	305.7 ± 9.4c	617.1 ± 28.1a	19.4 ± 0.6a	23.5 ± 0.4a	23.3 ± 0.5a	140.9 ± 2.8a	148.3 ± 3.5a	128.7 ± 10.5a

[1]Means (± SEM) followed by different letters within a column indicate significant differences among the treatments (pairwise comparisons of means, $p < 0.05$).

3.5 Discussion

In this study, we demonstrate that *T. molitor* larvae exposed to a short heat stress have a higher survival probability when exposed to *M. brunneum* as hypothesised. This result correlates well with our finding that short heat stressed larvae had an increased antibacterial activity in their hemolymph, indicating an increased concentration of AMPs in the hemolymph that can fight fungal cells. In contrast, we found that the survival probability of larvae receiving a heat stress treatment five days prior to pathogen exposure was no different to that of non-heat stressed larvae despite the reduced weight gain of the heat stressed larvae.

Our result on increased survival after a pathogen exposure in combination with a short heat stress is in accordance with studies conducted on *G. mellonella* larvae [21, 32, 33]. It has been suggested that temperature stress might induce immune priming in insects [4, 21], a form of innate immune memory [5, 54], which protects insects from pathogens when they have been previously exposed to a sublethal dose of the pathogen [55]. It is however important to note that the term immune priming should not be used to describe the immediate effect of a stress on an infection as this does not involve any form of immune memory. Previous studies have only investigated

larval susceptibility to pathogens immediately after the heat stress [21, 32, 33]. In contrast, we exposed the larvae to the pathogen both immediately after and five days after the heat stress. Although we found an increase of survival probability and antibacterial activity immediately after the short heat stress, we could not detect within-generation immune priming caused by heat stress, as larvae exposed to the pathogen five days after the heat stress were equally susceptible compared to larvae that were kept at constant temperatures. Moreover, heat stressed larvae did not show any increase in the measured immune responses after seven days.

In addition to key immune responses, we also measured the number of shed exuviae after heat and pathogen exposure. This is an important parameter to measure in experiments involving fungal pathogens and *T. molitor* larvae; *T. molitor* can have a variable number of moults [36] and moulting might affect the ability of the fungus to penetrate the insect's cuticle [37]. Larvae exposed to a long heat stress moulted less during the first two days compared to larvae exposed to no or short heat stress. Larvae that received a long heat stress, therefore, may have had a higher probability of becoming infected by the fungus, which could be an additional explanation why their survival probability after pathogen exposure was not as high as the one of the short heat stressed larvae. This is, however, only speculative because the direct relationship between moulting and fungal penetration through the cuticles was not measured in this study.

To understand the potential long-term benefits and risks related to heat stress in *T. molitor* production systems we also tested the effect on reproduction and body mass of the offspring. We found increased body mass in offspring of females that had been exposed to a short but not to a long heat stress as larvae. This indicates that heat stress duration on the parental generation might follow a hormetic response, with short heat stress having a beneficial impact on body mass of offspring. However, in other insect species, heat stress on the parental generation decreases body mass of offspring [56] and increases the developmental time of offspring [57]. To confirm the long-term implications of heat stress on the offspring's body mass, further studies should compare the offspring's pupal mass adjusted for their developmental time.

Mass-reared *T. molitor* larvae can be exposed to elevated temperatures due to metabolic heat production [16, 17]. For the mass-rearing of these insects, it is therefore important to understand the impact of heat stress to determine if short durations of heat stress should be avoided or even facilitated, for example, to increase resistance to diseases as previously suggested [4, 34]. The intensities of the tested heat stresses in this study did not result in a persistent increase of disease resistance. Only immediate exposure to the fungal pathogen after the heat stress led to increased survival probability. To use heat stress as a method to decrease mortality in cultured *T. molitor* populations, the exact timing of pathogen exposure would be needed, which does not seem feasible. It is, however, important to note that in the current study, insects were exposed to a single heat stress and repetitive or pulsed exposures to heat stresses might occur when *T. molitor* larvae are mass-reared, which would be relevant to investigate in future studies.

Future work should also investigate if there are transgenerational effects on immune responses such that *T. molitor* larvae from heat stressed parents express altered immune responses as shown in other insect species [57]. Furthermore, the infection outcome after heat stress might differ when testing other strains of *T. molitor* and different pathogen species. The infection outcome following heat stress could also be affected by symbionts with different thermal requirements. *Gregarina* spp., frequent commensals in the gut of *T. molitor* [58, 59], for example, do not survive temperatures over 36-37°C [60], hence temperature stress might be disadvantageous to these species. Therefore, more research is required to fully understand the effects of heat stress on immunity, insect health, and resistance to disease infections.

In conclusion, we show that heat stress has an impact on host-pathogen interactions in *T. molitor*. Not only the duration of the heat stress but also the timing of the exposure to the pathogen affects the outcome of infection and immune responses. The results in this study show that increased survival after heat stress comes with a cost (i.e., decreased hemocyte concentrations at a later stage of growth and slower body mass gain). The persistent effects are, however, not as severe as hypothesised (i.e., no difference in survival of larvae exposed to the pathogen five days after heat

stress). Moreover, we show that heat stress can affect the body mass of the offspring, which demonstrates the importance of investigating effects of temperature in the long-term and not only in the short-term. Beneficial effects on pathogen susceptibility wane rapidly and the reduction in weight gain after heat stress is only temporary. However, there are negative prolonged effects of heat stress on immune responses, which, together with the impact of repeated exposures to heat stress, should be investigated in further studies. This understanding will help safeguard insect health in mass-reared populations, which is crucial for the rapidly growing industry of rearing insects for feed and food.

3.6 References

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4. Chapter: The effect of CO_2 concentrations on entomopathogens and insect-pathogen interactions

4.1 Abstract

Numerous insect species and their associated pathogens are exposed to elevated CO_2 concentrations in both artificial and natural environments. However, the impacts of elevated CO_2 on the virulence of entomopathogens and the susceptibility of insects to pathogens are not well understood. The yellow mealworm, *Tenebrio molitor*, is commonly produced for food and feed purposes in mass-rearing systems, which increases risk of pathogen infections. Additionally, entomopathogens are used to control *T. molitor*, which is also a pest of stored grains. It is therefore important to understand how elevated CO_2 may affect both the pathogen and impact on host-pathogen interactions. We demonstrate that elevated CO_2 concentrations reduced the viability and persistence of the spores of the bacterial pathogen *Bacillus thuringiensis*. In contrast, conidia of the fungal pathogen *Metarhizium brunneum*, germinate faster under elevated CO_2. Pre-exposure of the two pathogens to elevated CO_2 prior to infection did not affect the survival probability of *T. molitor* larvae. However, *T. molitor* larvae reared at elevated CO_2 concentrations were less susceptible to both pathogens compared to larvae reared at ambient CO_2 concentrations. Our findings indicate that whilst elevated CO_2 concentrations may be beneficial in reducing host susceptibility in mass-rearing systems, they may potentially reduce the efficacy of the tested entomopathogens when used as biological control agents of *T. molitor* larvae. We conclude that CO_2 concentrations should be carefully selected and monitored in laboratory bioassays as an additional environmental factor in experiments that investigate insect-pathogen interactions, as is common practice with other environmental factors.

4.2 Introduction

Carbon dioxide (CO_2) has the potential to affect host-pathogen interactions if either the host, pathogen, or both are affected by changes in CO_2 concentrations. Numerous insect species are constantly exposed to CO_2 concentrations above the atmospheric

level, which is currently recorded as approximately 415 ppm (parts per million) [1]. Elevated CO_2 concentrations can be a result of the respiration of insects [2, 3] or a product of increased microbial activity and subsequent accumulation in enclosed areas [4]. The CO_2 concentration in soil air (inside soil pores), for example, is typically higher than the atmospheric CO_2 concentration due to decreased gas exchange [4], hence soil-dwelling insect species are exposed to elevated CO_2 concentrations in their environment. Furthermore, it is known that CO_2 can accumulate in colonies of social insects reaching up to 60,000 ppm in leaf-cutting ant colonies [5], and 92,000 ppm in termite mounds [6]. Insects that are mass-reared for food and feed purposes can also be exposed to elevated CO_2 concentrations because they are typically kept at high densities in closed systems [7], which facilitates the accumulation of CO_2 [8].

The yellow mealworm, *Tenebrio molitor*, is an insect species that is increasingly being mass-reared for the production of proteins and fats to feed livestock and for use in aquaculture [9, 10]. Respiration of *T. molitor* larvae produces approximately 60 g CO_2 per kg of body mass per day or approximately 1,000 g CO_2 per kg body mass gain [2]. Despite the utilisation of appropriate ventilations, CO_2 is still likely to accumulate in production facilities of *T. molitor* [8, 11]; for example, in a closed experimental *T. molitor* rearing, CO_2 concentrations reached up to 6,000 ppm [12]. The maximum permitted CO_2 concentrations in production facilities are regulated by law in most countries to ensure the health and safety of employees [13]. For example, the long-term (8 h) exposure limit of CO_2 concentration in the workplace is 5,000 ppm in many countries including the UK [14], the US [15], and countries belonging to the EU [16], which is tenfold higher than atmospheric concentrations.

Besides the use of *T. molitor* for the production of feed, the yellow mealworm is also a global pest of stored grains and grain by-products [17]. The CO_2 concentrations inside stored grains can exceed atmospheric CO_2 concentration [18] and when there is microbial or insect activity, CO_2 concentrations may increase even further [19, 20]. Various entomopathogenic (insect-pathogenic) organisms such as bacteria, fungi, protists, nematodes, and viruses can infect *T. molitor* [21, 22]. Such

entomopathogens are used as biological control agents against *T. molitor* in stored grains [23, 24] but at the same time, infectious diseases can be lethal or sublethal for mass-reared insects for food and feed leading to economic losses in production systems [21]. Currently, there is a dearth of knowledge on how CO_2 affects host-pathogen interactions in both mass-reared and wild insects [25]. Gaining a better understanding of the effects of CO_2 on entomopathogens and interactions with their insect hosts will help to decide if CO_2 is a relevant factor to include for insect-pathogen interaction experiments and in the design of insect mass rearing facilities.

CO_2 is known to affect entomopathogenic organisms; for example, *Pseudoxylaria* spp., an entomopathogenic fungus infecting termites (*Odontotermes obesus*), showed reduced growth when exposed to elevated CO_2 concentrations [6]. Furthermore, the number of conidia produced by different strains of the entomopathogenic fungal species *Metarhizium anisopliae*, *Isaria farinosa*, and *Beauveria bassiana* were generally decreased at 1,000 ppm CO_2 compared to 350 ppm CO_2 [26]. CO_2 has also been found to affect the virulence of pathogenic organisms of humans [27]; in the human-pathogenic bacterium *Bacillus cereus*, for example, the expression of virulence genes was higher at elevated CO_2 concentrations [28] and *Candida albicans*, a fungal pathogen of humans, switches from the monocellular to the more virulent filamentous growth at elevated CO_2 concentrations [27]. Nevertheless, the impact of CO_2 on the virulence of entomopathogenic organisms that can infect economically important insects remains unknown.

In this study, we examined the effects of CO_2 on a bacterial pathogen *Bacillus thuringiensis*, and a fungal pathogen *Metarhizium brunneum*, which both naturally infect *T. molitor* [21, 22]. We used *in vitro* experiments and full-factorial bioassays to study interactions between CO_2, insects, and pathogens. The pathogens were selected because both *B. thuringiensis* and *M. brunneum* can be found in stored grains [29-31]; grain products are both an important habitat of *T. molitor* and often used to feed *T. molitor* larvae in production systems [11]. Species of the genus *Metarhizium* are facultative entomopathogens, which can also colonize the rhizosphere of plants or live as saprotrophs [32, 33]. The impact that CO_2 in the

environment externally to the insect host has on the growth of fungi of this genus is therefore highly relevant. *Bacillus thuringiensis* on the other hand is thought to only multiply inside the insect host and the environment (external to the insect host) constitutes a transition compartment for the spores and crystals without reproduction [34]. Therefore, the effects of CO_2 on the viability and virulence of spores and crystals in the environment (e.g., soil or stored grains) are relevant.

The first question we aimed to answer in this study is what the effects of different CO_2 concentrations on *M. brunneum* and *B. thuringiensis* are. The second question is how the *in vivo* interactions between the pathogens with *T. molitor* larvae are affected by the different CO_2 concentrations. We hypothesised that elevated CO_2 leads to increased growth and germination of *M. brunneum*, and decreased viability and persistence of *B. thuringiensis* spores as shown for other fungi and bacteria, respectively. Furthermore, we hypothesised that the *in vitro* effects of elevated CO_2 on the pathogens are reflected in their relative virulence *in vivo* when subsequently, *T. molitor* larvae are exposed to the two pathogens separately. We therefore assessed the effects of elevated CO_2 (4,500 ± 500 ppm) on: (1) the *in vitro* germination of conidia and mycelial growth of *M. brunneum*, (2) the *in vitro* viability and persistence of *B. thuringiensis* spores, and (3) the *in vivo* interactions between *M. brunneum* or *B. thuringiensis* and the larvae of *T. molitor*.

4.3 Materials and methods

All insect rearing and experiments took place in two separate 50-litre LEEC Culture Safe CO_2 incubators adjacent to each other, one used for low [450 ppm (± 50 ppm)] CO_2, and one used for high [4,500 ppm (± 500 ppm)] CO_2 concentrations. The low CO_2 corresponds approximately with ambient CO_2 concentration, whereas the choice of the high CO_2 concentration was based on maximum permitted concentrations for human safe working [13-16] and data from experimental setups [12]. *Metarhizium brunneum* isolate KVL12-30 (culture collection of the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark) and *Bacillus thuringiensis* serovar *morrisoni tenebrionis* 4AA1 *(Bacillus* genetic stock center,

Ohio State University, USA) were used in experiments. The *in vitro* and the *in vivo* experiments were performed on three and two independent occasions, respectively.

4.3.1 Control of environmental conditions

To assure appropriate ventilation, the LEEC Culture Safe CO_2 incubators were fitted with 40 mm ELUTENG USB fans through the access hole at the back of the incubators, which were turned on for 15 min every 45 min. The CO_2 concentrations were monitored every 15 min using Rotronic CL11 loggers. The incubator used for low CO_2 was set to 400 ppm whereas the incubator used for high CO_2 was set to 5,000 ppm. The actual CO_2 concentrations measured were 450 ppm (± 50 ppm) and 4,500 ppm (± 500 ppm) CO_2 in the incubators set to low and high CO_2, respectively. Moreover, the temperature and relative air humidity were monitored every 15 min using two EasyLog EL-SIE-2 loggers per incubator positioned on the top and bottom shelves. The temperature in both incubators was maintained at 28°C (± 0.5°C) and the relative humidity at 75% (± 10%) in complete darkness. To allow for maximum gas exchange in the Petri dishes in which the microorganisms were grown, the lids of all Petri dishes (unless otherwise stated) were elevated by adding 2 cm wide plastic strips between the lids and the lower dish.

4.3.2 Preparation of *Metarhizium brunneum* suspension

Metarhizium brunneum was grown on Petri dishes (9 cm diameter, triple vented) containing 30 ml SDAY/4 (16.25 g Sabouraud dextrose agar, 2.5 g yeast extract, and 11.25 g agar in 1 l dH_2O) media sealed with Parafilm™ in the incubator at low CO_2 for 14 days. Thereafter, conidia were harvested using a Drigalski spatula after adding 5 ml TritonX-100 (0.05% v/v) on each Petri dish. The conidia suspensions were dispensed into 50-ml Falcon tubes and centrifuged at 3,000 rpm (1,872 *g*, Eppendorf Centrifuge 5810 R) for 3 min. After disposing of the supernatant, additional TritonX-100 (0.05% v/v) was added and the centrifuging was repeated using the same settings. The stock suspension was obtained by disposing of the supernatant and adding fresh TritonX-100 (0.05% v/v). The stock suspension was serially diluted to obtain a 1,000 times diluted suspension and the concentration of the conidia was enumerated in a 0.2 mm Fuchs-Rosenthal hemocytometer. The final stock

concentration was diluted to achieve the concentrations used in the respective experiments.

To assess conidial viability, 100 µl of a 10^6 conidia/ml suspension was spread on each of three replicate Petri dishes (9 cm diameter, triple vented) containing 10 ml SDAY/4 media and sealed with Parafilm™. After 18 hours incubation time at 28°C, 300 conidia were counted on each Petri dish using a light microscope at 100 times magnification. If the germ tube of a conidium was equal to, or longer than its smallest diameter, it was considered as germinated during the counting.

4.3.3 Preparation of *Bacillus thuringiensis* suspension

Bacillus thuringiensis was grown overnight on Petri dishes (9 cm diameter, triple vented) containing LB-Agar (lysogeny broth agar; 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g bacteriological agar in 1 l dH_2O) at 30°C in complete darkness. One colony was then picked from the plate using a sterile inoculation loop and added to a 500 ml Erlenmeyer flask containing 50 ml liquid HCT medium (5 g tryptone, 2 g bacto casamino acids, 6.8 g KH_2PO_4, 0.1 g $MgSO_4$, 0.002 g $MnSO_4$, 0.014 g $ZnSO_4$, 0.15 g $CaCl_2$ and 0.022 g Ammonium ferric citrate in 1 l dH_2O) sealed with Parafilm™. The culture was incubated on a platform shaker at 200 rpm at 30°C for four days. Thereafter, the bacterial suspension was poured into a 50 ml Falcon tube and centrifuged for 10 min at 3,900 rpm (3,231 *g*, Eppendorf Centrifuge 5810 R) at 4°C. The supernatant was discarded and 20 ml sterile dH_2O was added. This washing step was repeated twice thereafter with the settings mentioned above. The obtained stock suspension was incubated at 75°C for 10 min to obtain a spore and crystal suspension without viable vegetative cells. Spores and crystals were confirmed to be present through examination under a light microscope at 1,000 times magnification. Thereafter a ten-fold serial dilution was prepared. Subsequently, 10 µl of each of the six diluted suspensions (10^4 - 10^9) were pipetted in triplicate onto 10 ml LB-Agar plates (three dilutions per Petri dish). By tilting the Petri dish on one side, the diluted suspensions ran down the media forming straight lines. All Petri dishes were incubated at 28°C for 16 hours and the stock suspension was kept in the fridge at 4°C in the meantime. The average of the cfu/ml (colony forming units per ml) from at

least three different diluted suspensions was calculated for each diluted suspension using equation 4.1:

$$\frac{cfu}{ml} = \left(\frac{counted\ cfu}{0.01\ ml}\right) * \ dilution\ factor \qquad Equation\ 4.1$$

4.3.4 *In vitro* experiments

4.3.4.1 Germination and growth of *Metarhizium brunneum*

The germination of *M. brunneum* conidia was assessed by adding 100 µl of 10^6 conidia/ml on each of three replicate (per condition and time point) 10 ml SDAY/4 Petri dishes. The suspensions were spread using a Drigalski spatula and the Petri dishes were incubated at either low or high CO_2 for 6, 8, 10, 12, 14, 18, or 24 hours. Thereafter, 100 conidia were counted at three different locations on each Petri dish (300 conidia per Petri dish) and the numbers of germinated and non-germinated conidia were noted. A conidium was considered as germinated when it had a germ tube at least as long as the smallest diameter of the conidium.

The colony growth rates of *M. brunneum* at different CO_2 concentrations were assessed by adding 2 µl of 10^6 conidia/ml on the centre of each of ten replicate 30 ml SDAY/4 Petri dishes and subsequent incubation at either low or high CO_2. The area of each colony was measured using a digital calliper on two perpendicular diameters, every second day for eight days, starting two days after the preparation of the Petri dishes. The average of the two diameters per colony was used as one data point for calculating the growth rate (mm/day) between days two and eight. Petri dishes that dried out before the end of the experiment were excluded from the analysis.

4.3.4.2 Viability and persistence of *Bacillus thuringiensis*

The *in vitro* viability of *B. thuringiensis* spores was assessed by adding 100 µl of 10^3 spores/ml on each of ten replicate 10 ml LB-Agar Petri dishes. The suspensions were spread using a Drigalski spatula and the Petri dishes were incubated at either low or high CO_2. At both CO_2 concentrations, 100 µl of sterile dH_2O was spread on each of three replicate 10 ml LB-Agar Petri dishes as controls (in the case of contamination

this would be apparent on these Petri dishes). The numbers of colonies per Petri dish were counted after 24 h to calculate cfu/ml.

To measure *in vitro* persistence of *B. thuringiensis* spores, the method of Wood et al. [35] was adapted. Nine replicate autoclaved glass coverslips (22 × 22 mm) were placed inside an empty sterile Petri dish (three coverslips per Petri dish). On each coverslip, 100 µl of 6×10^5 spores/ml were added and the Petri dishes containing the coverslips were incubated at either low or high CO_2. Additionally, 100 µl of sterile dH_2O was added on a separate coverslip in each Petri dish as a control (in the case of contamination this would be apparent on these coverslips). After two days the coverslips were transferred individually to 50 ml Falcon tubes containing 15 ml PBS (phosphate buffered saline) with TritonX-100 (0.1% v/v) and the tubes were put on an orbital shaker at 200 rpm at 25°C for 15 min. Thereafter, 10 µl of the resulting suspensions were pipetted onto LB-Agar plates. By tilting the Petri dish on one side, the diluted suspensions ran down the media forming straight lines (three technical replicates on different Petri dishes were prepared). The average of the three technical replicates was used as one data point to calculate cfu/ml.

4.3.5 *In vivo* bioassays

Tenebrio molitor larvae were reared at either low or high CO_2 concentrations for 18 days. *Bacillus thuringiensis* spores and crystals mixed in diet were exposed to either low or high CO_2 concentrations for two days. *Metarhizium brunneum* was grown at either low or high CO_2 concentrations for 14 days. The pathogens were mixed into the larval diet [wheat bran (96% w/w) and dried egg white (4% w/w)]. The larvae were exposed to lethal concentrations (previously determined in pre-experimental bioassays) of each pathogen separately in a full-factorial bioassay (n = 150, five cups per treatment). Furthermore, two groups of unexposed larvae (one at low, and one at high CO_2) were prepared as control treatments (Figure 4.1). The larvae per cup were weighed as a group the day before, and on the day of exposure to the pathogens. Two days after the start of the exposure to the pathogens, the larvae were transferred to fresh cups. The larvae and the remaining diet in each cup were separated from frass by using a sieve (0.5 mm) 2, 4, 6, 8, 10, 12 and 14 days after exposure. Larval

mortality was also assessed on the same days after exposure and dead larvae were removed. The leftover diet and the live larvae were weighed individually and new diet (0.6 × weight of live larvae) and water agar (1% w/v; 0.6 × weight of live larvae) were added on the same days (the value 0.6 was established in a pre-experimental bioassay to ensure that the larvae did not starve in between feeding time points). The larvae from one cup treated with *B. thuringiensis* in the second experimental repetition were excluded from analysis because the cup was tipped over during the experiment.

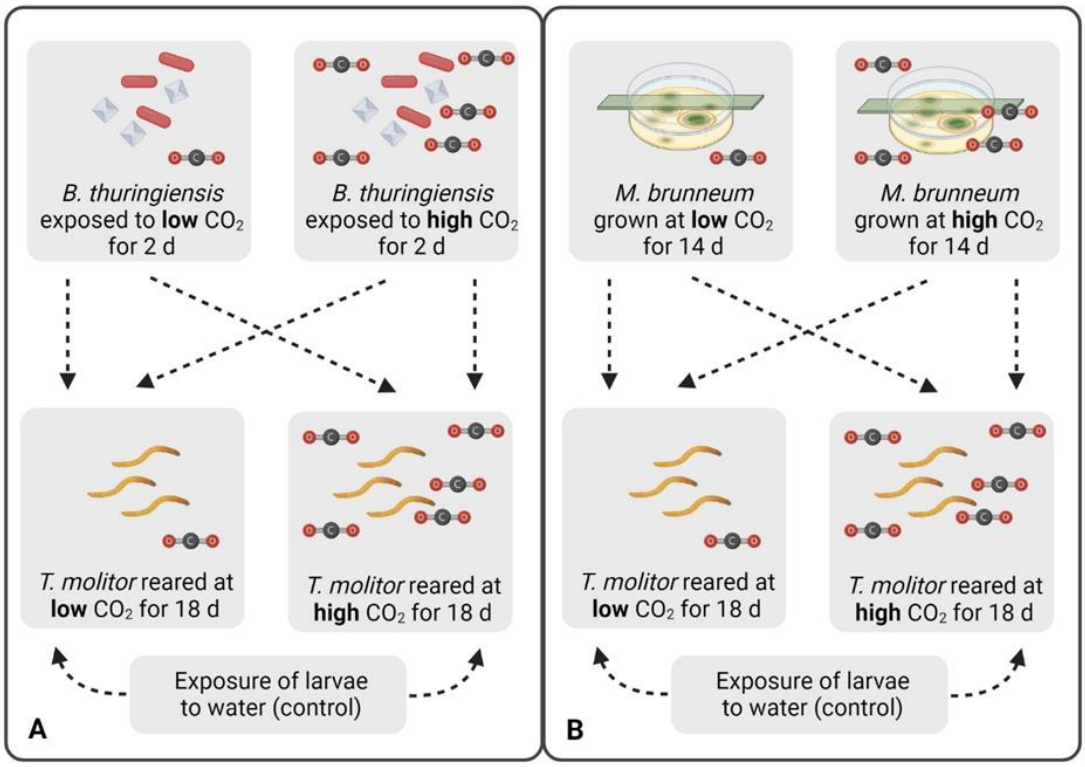

Figure 4.1 Schematic representation of the experimental design.

A Larvae reared at either low or high CO_2 for 18 days were exposed to *B. thuringiensis* previously exposed to either low or high CO_2 for two days or to water as a control. **B** The larvae reared at either low or high CO_2 for 18 days were exposed to *M. brunneum* grown at low or high CO_2 for 14 days. The lids of the Petri dishes were elevated by adding a 2 cm wide plastic strip between the lower dish and the lid. **A, B** Each arrow represents one treatment (n = 150 larvae equally distributed to five cups, two experimental repetitions). The survival, feed intake and weight of the larvae were assessed every second day for a period of 14 days after pathogen exposure. Figure created using BioRender.com.

4.3.5.1 Insect culture

Tenebrio molitor larvae were originally provided by the company Ÿnsect (Evry, France). The insects were kept in a lab culture over more than six generations before the start of the experiments at the UK Centre for Ecology & Hydrology, UK. Adult *T. molitor* (40 females and 40 males) were kept in 750 ml containers (15 cm × 9.5 cm = 142.5 cm^2) containing 50 g diet for four days. The diet consisted of wheat bran (96% w/w) and dried egg white (4% w/w). Additionally, the adults were fed with 5 g agar (1% w/v). Thereafter, the adults were removed and half of the containers containing eggs were transferred to the incubator set to high CO_2, whereas the other half of the containers remained in the initial incubator at low CO_2. The larvae received 10 g agar (1% w/v) twice a week starting one week after the removal of the adults and the number in each container was counted 17 days after the removal of the adults.

4.3.5.2 Exposure of larvae to *Bacillus thuringiensis*

A suspension of *B. thuringiensis* was prepared as described above. In addition to estimating the spore concentration, the crystal concentration in the stock suspension was assessed by using a Bradford assay (see below). The larvae were divided into groups of 30 larvae and placed inside 60-ml cups (3.4 cm diameter) without lids 17 days after removal of the adults. The larvae were starved for 24 h in their respective CO_2 treatments. Sixty ml cups containing the diet and the spore suspensions were prepared by adding 100 µl of 4×10^9 spores/ml (4,999 and 1,678 µg/ml crystals in exp. 1 and 2, respectively) to 100 mg of diet in each of the cups. As a control 100 µl of dH_2O was added to 100 mg of diet. The diet and the conidia suspension (or dH_2O in the control) in each cup were mixed thoroughly with a sterile inoculation loop to make sure that the inoculum was homogeneously distributed. The 60-ml cups containing the diet and the spore suspensions (or dH_2O in the control) were then incubated at either low or high CO_2 for two days. After 2 days, the larvae were exposed; 100 µl of dH_2O was added to each cup and the starved larvae were added to the respective cups. The larvae remained in the cups containing *B. thuringiensis* spores and crystals for two days in total.

Bradford assay

A BAS (bovine albumin serum) solution (1 mg/ml) was prepared in dH_2O. Eight 1-ml-cuvettes were prepared with different volumes of a NaOH solution (0.1 M; 800, 798, 796, 794, 792, 790, 785 and 780 µl). Thereafter, different volumes (0, 2, 4, 6, 8, 10, 15, and 20 µl, respectively) of the BAS solution as well as 200 µl of the Bradford reagent were added to the cuvettes resulting in a total volume of 1,000 µl in each cuvette. A piece of Parafilm^{TM} was put on each cuvette to avoid spillage and then all the cuvettes were vortexed and incubated at room temperature for 5 min. Afterwards, the OD (optical density) in each cuvette was measured at 595 nm using a WPA biowave CO8000 Cell Density Meter. From the obtained data points, the calibration curve was calculated using equation 4.2:

$$y = ax + b \qquad Equation\ 4.2$$

where y = OD, x = BAS volume in µl, a = slope, and b = intercept. Either 2, 5, or 10 µl of the *B. thuringiensis* stock suspension, NaOH solutions (0.1 M; 798, 795 and 790 µl, respectively), as well as 200 µl of the Bradford reagent, were added to 1ml-cuvettes in triplicates. Thereafter, the OD in these cuvettes was measured at 595 nm and the average crystal concentration was calculated using equation 4.3:

$$toxin\ conc.\ in\ \frac{\mu g}{mL} = \frac{\frac{OD - b}{a}}{sample\ volume\ in\ mL} \qquad Equation\ 4.3$$

4.3.5.3 Exposure of larvae to *Metarhizium brunneum*

Metarhizium brunneum (100 µl of 10^6 conidia/ml) was grown on Petri dishes (9 cm diameter) containing 30 ml SDAY/4 media at either low or high CO_2. After 14 days, conidial suspensions were prepared as described above, except that dH_2O was added in the last washing step instead of TritonX-100 (0.05% v/v). Furthermore, conidial viability was assessed (see above). Larvae were starved as described above (section 4.3.5.2). Thereafter, 100 µl of 10^8 conidia/ml was added to 100 mg of diet in new 60-ml cups. As a control 100 µl of dH_2O was added to 100 mg of diet. The diet and the

conidia suspension (or dH_2O in the control) in each cup were mixed thoroughly with a sterile inoculation loop to make sure that the inoculum was homogeneously distributed. The larvae were then put inside the respective cups and they remained in the cups containing *M. brunneum* conidia for two days in total.

4.3.6 Statistical analysis

Differences were considered as significant at $p < 0.05$ and data was only subjected to one-, two- or three-way ANOVAs (analysis of variances) when normality (QQ-plots) and homogeneity of variances (Levene test, $p > 0.05$) assumptions were satisfied. Tukey's HSD (Honestly Significant Difference) tests were used to separate the means. All statistical analyses were performed using R v. 4.1.0 [36].

The effect of CO_2 on *M. brunneum* conidia germination was described using a three-parameter log-logistic model given in equation 4.4:

$$y = \frac{d}{(1 + e^{(b(\ln(x) - \ln(i))})} \qquad Equation\ 4.4$$

where y = germinated conidia (%), i = inflection point (i.e., hours to 50% germination), b = slope, d = upper limit, and x = time in hours using the drc package [37]. The times to 50% germination at different CO_2 concentrations were compared using the compParm function [37]. *Metarhizium brunneum* growth rates at different CO_2 concentrations were analysed using a one-way ANOVA. Experimental repetitions were combined, as no interactive effect of repetition and CO_2 was found in a previous two-way ANOVA. *Bacillus thuringiensis* spore persistence, spore viability, and density of larvae before the start of the *in vivo* assays at different CO_2 concentrations were compared by implementing generalized linear mixed models with a negative binomial error distribution (used for overdispersed count data) using the lme4 package [38] with experimental repetition included as a random effect.

Mixed effects cox proportional hazards models were used to analyse the survival of the larvae in the *in vivo* assays (fixed effects: pathogen exposure, CO_2 exposure of

larvae, CO_2 exposure of pathogens; random effects: experimental repetition, cup) using the coxme package [39]. Only significant fixed effects were retained in the final models and pairwise comparisons of treatments were performed using Tukey contrasts with single-step adjustment for multiple testing using the multcomp package [40]. The effect of CO_2 on larval weight at the start of the experiment was analysed using a generalized linear mixed model with a gamma error distribution using the lme4 package [38] with experimental repetition included as a random effect. Weight gain per larva for the duration of the experiments and feed intake during exposure data were analysed separately for both experimental repetitions (Exp. 1 and 2) using two-way ANOVAs, because interactive effects of experimental repetition and exposure of larvae or pathogens to CO_2 were found in previous three-way ANOVAs.

4.4 Results

First, the effects of CO_2 on different pathogen traits outside of the host were tested. The time to 50% germination of *M. brunneum* conidia was significantly lower at high CO_2 (e = 7.77 (confidence limits = 7.66 and 7.87); b = -9.83; d = 99.90) compared to low CO_2 (e = 9.58 (confidence limits = 9.49 and 9.66); b = -12.74; d = 99.83; comparison of times to 50% germination at low and high CO_2: t = 26.07; p <0.001). At both CO_2 levels, the germination of conidia was >99% after 24 hours (Figure 4.2).

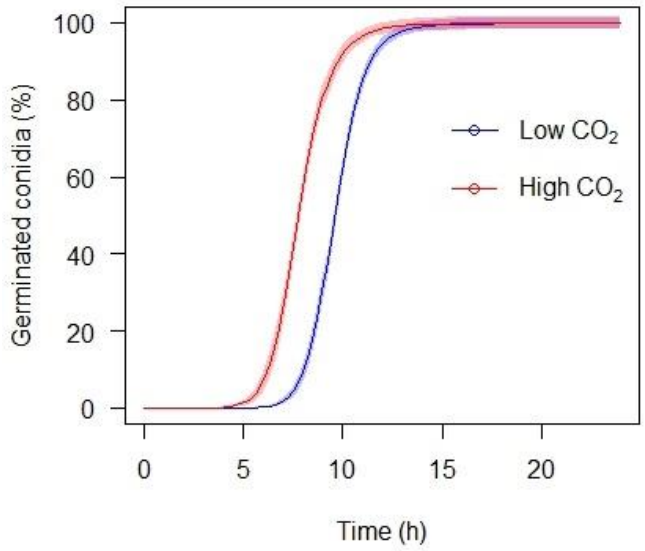

Figure 4.2 Three-parameter log-logistic models for germination of *M. brunneum* conidia over time (hours) at either low or high CO_2 concentrations.
The shaded areas represent the 95% confidence intervals.

Metarhizium brunneum colony growth rate was not affected by CO_2 (Table 4.1). In contrast, *Bacillus thuringiensis* spores incubated at high CO_2 showed significantly lower viability than spores incubated at low CO_2 (Table 4.1). Similarly, *B. thuringiensis* spore persistence was significantly decreased at high compared to low CO_2 concentration (Table 4.1).

Table 4.1 *Metarhizium brunneum* colony growth rate, *Bacillus thuringiensis* spore viability and persistence at either low or high CO_2 concentrations.[1]

	M. brunneum colony growth rate (mm/d ± SEM)[2]	*B. thuringiensis* spore viability (cfu/ml ± SEM)[3]	*B. thuringiensis* spore persistence (cfu/ml ± SEM)[4]
Low CO_2	3.67 ± 0.04[a]	762.33 ± 61.11[a]	650.62 ± 98.07[a]
High CO_2	3.73 ± 0.81[a]	682.00 ± 58.76[b]	213.58 ± 43.11[b]

[1]Means (± SEM) followed by different letters within a column indicate significant differences among the treatments. SEM, standard error of the mean; cfu, colony forming units
[2]$p = 0.305$, F = 1.077, d.f. = 1,43
[3]$p < 0.001$, $\chi 2 = 16.971$, d.f. = 1
[4]$p < 0.001$, $\chi 2 = 13.419$, d.f. = 1

To investigate host-pathogen interactions, full-factorial bioassays were performed in which the pathogens and the host were exposed to either low or high CO_2 (Figure 4.1). We tested the larval density in the two CO_2 conditions before the start of the experiments to ensure that it did not affect our results. The larval density in the rearing containers at low and high CO_2 was indeed not affected by CO_2 ($p = 0.311$, $\chi2 = 1.026$, d.f. = 1). Likewise, CO_2 did not affect the weight of the larvae at the start of the experiment ($p = 0.387$, $\chi2 = 0.748$, d.f. = 1). The germination rates of *M. brunneum* conidia were >99% in all treatments and experiments. Larvae reared at high CO_2 were significantly less susceptible (i.e., less likely to die) to *B. thuringiensis* ($p <0.001$, Figure 4.3A, Table 4.2) and *M. brunneum* ($p = 0.047$, Figure 4.3B, Table 4.2) than larvae reared at low CO_2 resulting in approximately 12 and 8% higher survival after 14 days, respectively. There was no effect of CO_2 on survival of control larvae ($p = 0.771$, Figure 4.3A, B). Moreover, exposure of the pathogens to different CO_2 concentrations before exposure of the larvae did not affect the virulence of *B. thuringiensis* ($p = 0.930$, Table 4.2) or *M. brunneum* ($p = 0.620$, Table 4.2).

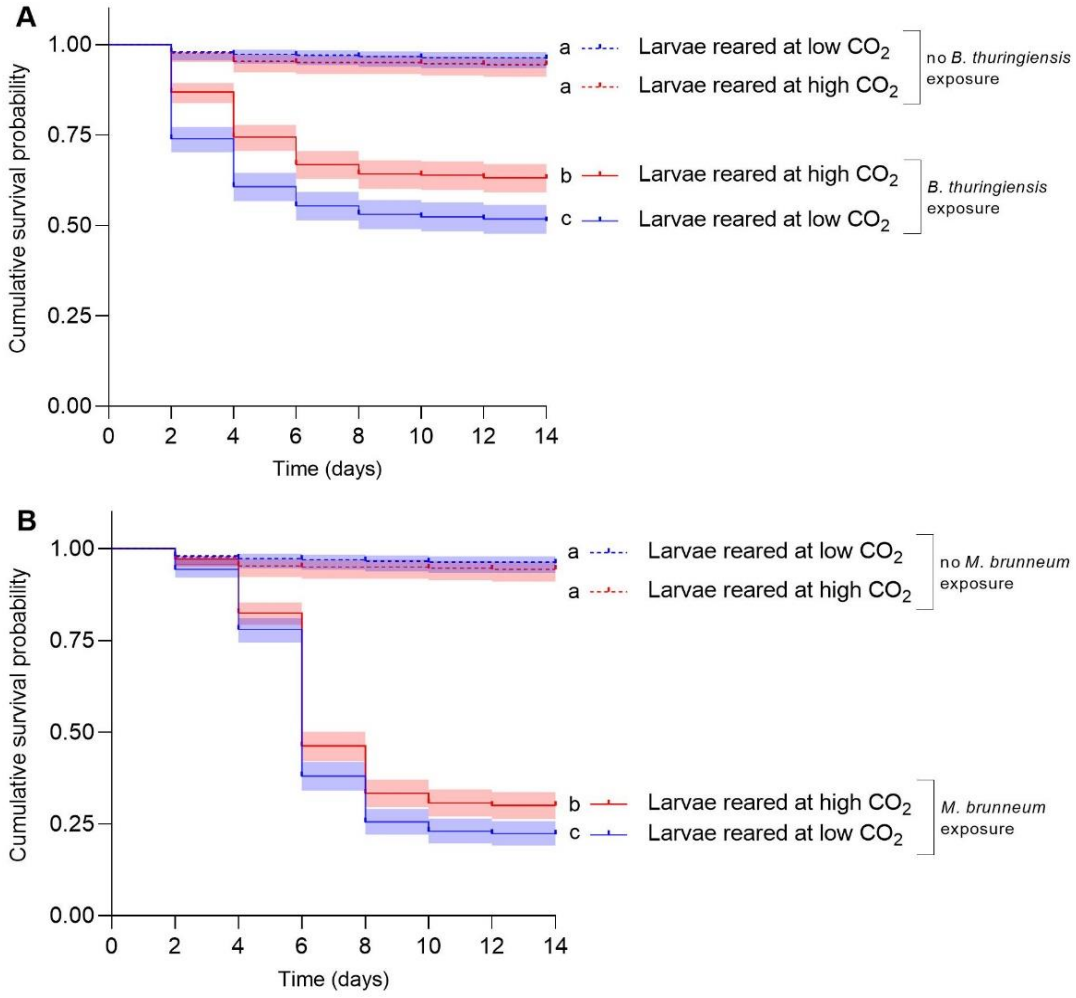

Figure 4.3 Survival of *T. molitor* larvae reared at either low (blue) or high (red) CO_2 concentrations after exposure to pathogens for a period of 14 days.

A Cumulative survival probability of larvae exposed to either low or high CO_2 without (dotted survival curves) and with *B. thuringiensis* exposure (continuous survival curves). **B** Cumulative survival probabilities of larvae exposed to either low or high CO_2 without (dotted survival curves) and with *M. brunneum* exposure (continuous survival curves). **A, B** Different letters to the right of the survival curves indicate statistical differences among treatments at $p < 0.05$. The shaded areas represent the 95% confidence intervals. Hazard ratios and *p*-values of fixed and random effects of the mixed effects cox proportional hazards models are displayed in Table 4.2. Figure created with GraphPad Prism version 9.3.1.

Table 4.2 Results of mixed effects cox proportional hazards models to analyse survival of *T. molitor* larvae.[1,2]

Bacillus thuringiensis (*Bt*)	HR ± SE	*p*
Exposure of larvae to *Bt*	11.505 ± 0.233	**<0.001**
Exposure of larvae to CO_2	0.693 ± 0.088	**<0.001**
Exposure of *Bt* to CO_2	0.992 ± 0.089	0.930
Cup	-	0.963
Experimental repetition	-	0.969
Metarhizium brunneum (*Mb*)		
Exposure of larvae to *Mb*	31.352 ± 0.242	**<0.001**
Exposure of larvae to CO_2	0.816 ± 0.091	**0.025**
Exposure of *Mb* to CO_2	0.954 ± 0.093	0.620
Cup	-	**0.011**
Experimental repetition	-	**<0.001**

[1]HR ± SE (hazard ratio ± standard error) and *p-values* of fixed effects: Exposure of larvae to *Bt*, Exposure of larvae to CO_2, Exposure of *Bt* to CO_2, Exposure of larvae to *Mb*, Exposure of *Mb* to CO_2; and *p*-values of random effects: Cup and Experimental repetition.
[2]Bold *p*-values denote statistical significance at $p < 0.05$.

The effect of CO_2 concentration and pathogen exposure on feed intake was measured during pathogen exposure. The feed intake per larva was reduced by *B. thuringiensis* exposure (Figure 4.4A), but CO_2 did not affect feed intake in either the control or *B. thuringiensis* exposed larvae (Figure 4.4A, Table 4.3). Similarly, feed intake was reduced by *M. brunneum* exposure in the second iteration of the experiments, and in certain treatments of the first iteration (Figure 4.4B). CO_2 did not affect the feed intake during *M. brunneum* or control exposure (Figure 4.4B, Table 4.3).

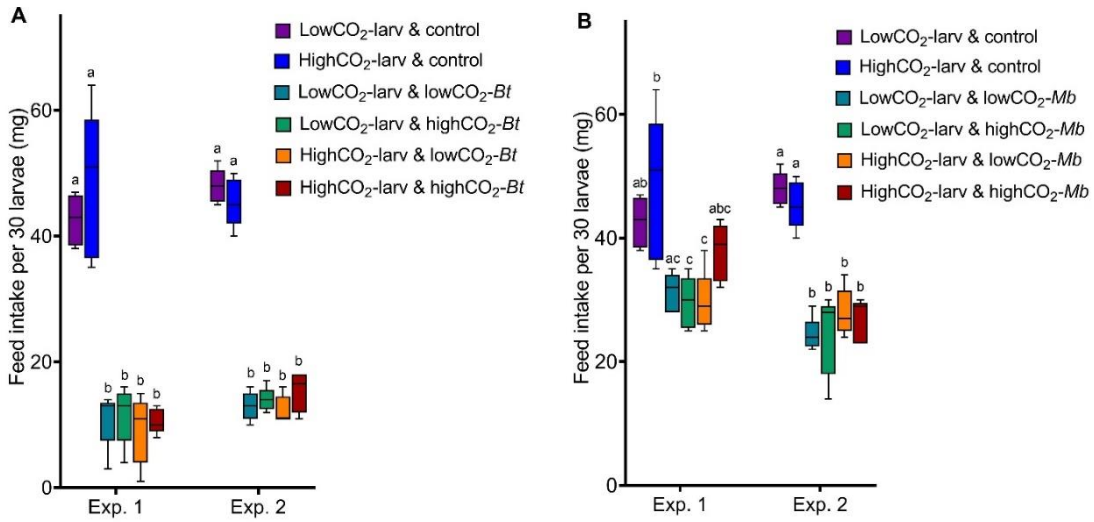

Figure 4.4 Feed intake per 30 larvae during exposure (two days) to the pathogens.

A Feed intake during exposure to *B. thuringiensis* in experiment (Exp.) 1 and 2: no exposure to *B. thuringiensis* (control), larvae exposed to low CO_2 (lowCO_2-larv), larvae exposed to high CO_2 (highCO_2-larv), *B. thuringiensis* exposed to low CO_2 (lowCO_2-*Bt*), *B. thuringiensis* exposed to high CO_2 (highCO_2-*Bt*). **B** Feed intake during exposure to *M. brunneum* in experiment (Exp.) 1 and 2: no exposure to *M. brunneum* (control), larvae exposed to low CO_2 (lowCO_2-larv), larvae exposed to high CO_2 (highCO_2-larv), *M. brunneum* grown at low CO_2 (lowCO_2-*Mb*), *M. brunneum grown at* high CO_2 (highCO_2-*Mb*). **A, B** Boxplots show median, interquartile range, and minimum and maximum. Different letters above boxplots indicate statistical differences among treatments at $p < 0.05$ for each experiment separately. Degrees of freedom, F-values and *p*-values of the two-way ANOVAs are displayed in Table 4.3. Figure created with GraphPad Prism version 9.3.1.

Exposure of larvae to *B. thuringiensis* significantly reduced weight gain of the larvae over the course (14 days) of the experiments (Figure 4.5A). However, weight gain was not affected by exposure of either the larvae or *B. thuringiensis* to different CO_2 concentrations (Figure 4.5A, Table 4.3). Exposure of larvae to *M. brunneum* did not affect the weight gain over the course of the experiment except for one treatment in the second iteration of the experiment (Figure 4.5B). Furthermore, weight gain was not affected by exposure of either the larvae or *M. brunneum* to different CO_2 concentrations (Figure 4.5B, Table 4.3).

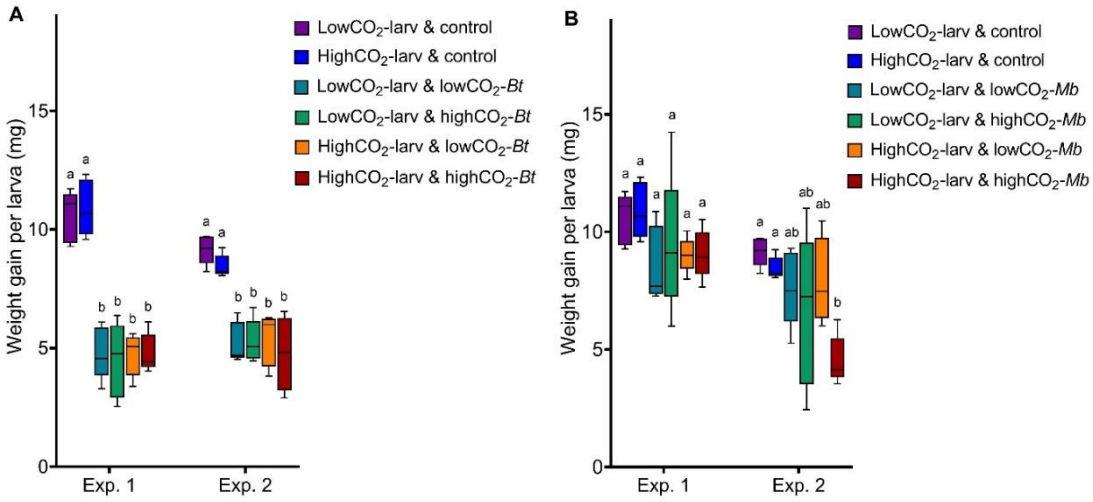

Figure 4.5 Weight gain per larva (mg) during 14 days after exposure to the pathogens.

A Weight gain after exposure to *B. thuringiensis* in experiment (Exp.) 1 and 2: no exposure to *B. thuringiensis* (control), larvae exposed to low CO_2 (lowCO_2-larv), larvae exposed to high CO_2 (highCO_2-larv), *B. thuringiensis* exposed to low CO_2 (lowCO_2-*Bt*), *B. thuringiensis* exposed to high CO_2 (highCO_2-*Bt*). **B** Weight gain after exposure to *M. brunneum* in experiment (Exp.) 1 and 2: no exposure to *M. brunneum* (control), larvae exposed to low CO_2 (lowCO_2-larv), larvae exposed to high CO_2 (highCO_2-larv), *M. brunneum* grown at low CO_2 (lowCO_2-*Mb*), *M. brunneum grown at* high CO_2 (highCO_2-*Mb*). **A, B** Boxplots show median, interquartile range, and minimum and maximum. Different letters above boxplots indicate statistical differences among treatments at p <0.05 for each experiment separately. Degrees of freedom, F-values and p-values of the two-way ANOVAs are displayed in Table 4.3. Figure created with GraphPad Prism version 9.3.1.

Table 4.3 Results of two-way ANOVAs to analyse feed intake of *T. molitor* larvae during the two days of pathogen exposure and individual weight gain of larvae during 14 days of individual experimental repetitions.[1,2]

	Feed intake during exposure			**Individual weight gain**		
B. thuringiensis, Experiment 1	d.f.1, d.f.2	*F*	*p*	d.f.1, d.f.2	*F*	*p*
Bt	2,24	105.01	**<0.001**	2,24	94.34	**<0.001**
Larv-CO_2	1,24	0.17	0.684	1,24	0.19	0.668
Bt × Larv-CO_2	2,24	1.07	0.358	2,24	0.09	0.917
Experiment 2						
Bt	2,23	471.13	**<0.001**	2,23	48.22	**<0.001**
Larv-CO_2	1,23	0.01	0.945	1,23	0.50	0.488
Bt × Larv-CO_2	2,23	1.30	0.29	2,23	0.58	0.570
M. brunneum, Experiment 1						
Mb	2,24	16.24	**<0.001**	2,24	3.96	**0.033**
Larv-CO_2	1,24	3.24	0.084	1,24	0.04	0.837
Mb × Larv-CO_2	2,24	1.69	0.207	2,24	0.18	0.838
Experiment 2						
Mb	2,24	87.37	**<0.001**	2,23	8.54	**0.002**
Larv-CO_2	1,24	0.58	0.452	1,23	2.03	0.167
Mb × Larv-CO_2	2,24	1.64	0.216	2,23	1.10	0.350

[1]Abbreviations: *Bt*: *B. thuringiensis* treatment, including exposure of *B. thuringiensis* to CO_2; *Mb*: *M. brunneum* treatment, including exposure of *M. brunneum* to CO_2; Larv-CO_2: exposure of larvae to CO_2

[2]Bold *p*-values denote statistical significance at $p < 0.05$

4.5 Discussion

In this study, elevated CO_2 concentrations were found to decrease the viability and persistence of *B. thuringiensis* spores *in vitro,* whilst decreasing the duration to germination of *M. brunneum* conidia. Interestingly, exposure of the pathogens to different CO_2 concentrations before infection did not affect the virulence of these entomopathogens toward *T. molitor* larvae, but larvae reared at elevated CO_2 were less susceptible (i.e., less likely to die) to the pathogens than larvae reared at ambient CO_2. These findings are important because *T. molitor* larvae are often exposed to CO_2 concentrations above ambient conditions [8, 11, 18]. Here we show that CO_2 levels affect the susceptibility of *T. molitor* to entomopathogens, which has implications for mass-rearing and for biocontrol of this insect species. In addition to our main findings, we also found that CO_2 did not affect the feed intake of the larvae during exposure to the pathogens and overall, did not affect the individual weight gain of the larvae. Investigating sub-lethal effects such as these is crucial, especially

for the production of insects because a reduction in weight gain leads to economic losses as the overall mass of insects produced is reduced.

It is challenging to put our study in context with other studies on CO_2 because the few other studies that have been published investigating the effects of CO_2 on insect-pathogen interactions either use lower (<1,000 ppm) or significantly higher (>50,000 ppm) CO_2 concentrations than in this present study. To our knowledge, this is the first study to measure the effect of industrially relevant CO_2 concentrations for the mass-rearing of *T. molitor* and other reared insect species. Elevated CO_2 concentrations have been suggested to act as a cue promoting the germination of an entomophthoralean fungus (*Entomophaga maimaiga*) as CO_2 concentrations might be elevated near the insect cuticle [41]. This increased germination of fungal conidia is in accordance with our study. However, decreased germination and mycelial growth of a hypocrealean fungus (*B. bassiana*) were reported as a result of a very high CO_2 concentration (400,000 ppm) [42]. Similarly, 50,000 ppm CO_2 decreased the mycelial growth and sporulation of *M. brunneum, Aspergillus* sp., and *B. bassiana in vitro* [43]. Moreover, it was proposed (without statistical analyses) that the growth rates of different *M. anisopliae* strains are either positively or negatively affected by elevated CO_2 (650 and 1,000 ppm) [26]. We, in contrast, did not find an effect of CO_2 at industrially relevant concentrations on the growth rate of *M. brunneum in vitro*.

This is, to our knowledge, the first study that measures the direct effects of elevated environmental CO_2 on the persistence and viability of a bacterial entomopathogen. However, it is known from other species that CO_2 can reduce bacterial growth [44]. We found that the persistence of *B. thuringiensis* spores was almost three times lower at elevated CO_2. Surprisingly, there was no effect of exposure of *B. thuringiensis* to elevated CO_2 on the subsequent virulence in the insect host. This could be because the crystals of *B. thuringiensis* that are essential for the infection process might not be affected by CO_2. Moreover, we speculate that the spores kept at elevated CO_2 could have been only temporarily inactivated (dormant) and might be reactivated in the host. It has been shown for other species of the *Bacillus* genus that

suboptimal thermal and pH conditions during incubation can increase the time to germination of spores [45].

Interestingly, we could not detect any sublethal effects of elevated CO_2 on the larvae. In contrast, in a study by Li et al. [12], *T. molitor* larvae reared in a closed system had a lower weight gain compared to larvae reared in an open system, which was argued to be due to higher CO_2 concentrations in the closed system [12]. However, these differences could also have been due to other factors such as different relative humidity or different concentrations of other gases in the two systems. It is important to note that elevated CO_2 concentrations may be more detrimental to insects when the relative humidity is low, because elevated CO_2 forces the insects to keep their spiracles open, which can result in water loss [46].

Our study supports prior findings by Borisade & Magan, 2015 who exposed desert locusts (*Schistocerca gregaria*) and house crickets (*Acheta domesticus*) to elevated CO_2 concentrations (1,000 ppm). The authors suggested that *S. gregaria* and *A. domesticus* kept at elevated CO_2 showed increased survival and lethal times, respectively, when exposed to *B. bassiana*, although this was not statistically validated [26]. In contrast to these findings, the survival of red flour beetles (*Tribolium castaneum*) exposed to *B. bassiana* was significantly decreased at very high CO_2 concentrations (440,000 ppm) [42]. Due to our experimental design, we are able to disentangle the effects of CO_2 on the interactions between the pathogens and *T. molitor*, demonstrating that previous exposure of the pathogens to elevated CO_2 did not affect the virulence of the pathogens, but that rearing the larvae at elevated CO_2 decreases the susceptibility of the larvae to the pathogens. One possible explanation is that CO_2 may affect the insect immune response. For example, in *Drosophila melanogaster* the production of antimicrobial peptides was inhibited by CO_2 (130,000 ppm) correlating with increased susceptibility to bacterial infections [47]. Moreover, in *T. castaneum* CO_2 increased the production of benzoquinones [48] (a Quinone that is also produced by *T. molitor* [49]), which inhibit *B. bassiana* [50]. The mechanism underlying the decreased susceptibility of *T. molitor* to pathogens at elevated CO_2 concentrations remains to be investigated. Moreover, it

would be beneficial for the production of *T. molitor* and other mass-reared insect species to investigate the CO_2 concentrations *T. molitor* is evolutionarily adapted to in order to optimise rearing conditions. *Tenebrio molitor* might be adapted to elevated CO_2 concentrations whereas other species may be adapted to different CO_2 concentrations.

Here, we demonstrate that CO_2 directly affects a bacterial and a fungal entomopathogen *in vitro* and their *in vivo* interactions with an insect host. Based on these results, we conclude that the tested elevated CO_2 concentration (4,500 ± 500 ppm) in *T. molitor* mass-rearing systems is beneficial for larvae exposed to the tested pathogens by increasing larval survival. Furthermore, we did not find any sublethal effects of CO_2 on *T. molitor* larvae that would affect the overall productivity of the mass-rearing system. For biocontrol of *T. molitor*, our results indicate that the efficacies of the two tested entomopathogens may be lowered at elevated CO_2 concentrations, which has implications for understanding the reliability of biocontrol of storage pests. To ensure meaningful conclusions, we suggest it is crucial to consider CO_2 effects (i.e., through monitoring and using pertinent CO_2 concentrations) when studying any insect pathogen systems that are likely to be exposed to elevated CO_2 in their natural or artificially maintained environments.

4.6 References

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5. Chapter: Double trouble? Quantifying the risk from co-exposure to multiple pathogens in *Tenebrio molitor* at different CO_2 concentrations

5.1 Abstract

Insects in mass-reared production and in the wild frequently encounter multiple pathogens and environmental stressors simultaneously. Pathogen infection and exposure to abiotic stressors in insect production can lead to significant economic losses and reduce insect welfare. Our understanding of the interactions between different stressors remains limited, and existing methods primarily focus on determining overall patterns of additivity, synergism, or antagonism. However, the interactions between different stressors may exhibit more intricate response patterns, such as time or dose dependency. With the expanding industry of insect production, it becomes vital to conduct comprehensive risk assessment of insect health, using approaches that are able to detect both lethal and sublethal effects of multiple stressors. Here, we assess the risk of co-exposure to a fungal (*Metarhizium brunneum*) and a bacterial pathogen (*Bacillus thuringiensis*) in the yellow mealworm (*Tenebrio molitor*). We performed the experiments at ambient and elevated carbon dioxide (CO_2) concentrations because the accumulation of CO_2 in closed production system is a common issue and the effects on the outcomes of co-infections are unknown. As endpoints, we assessed total larval biomass per group, survival, and individual weight gain 14 and 20 days after pathogen exposure. To analyse the data, we used a mixture toxicity (MIXTox) model, which identifies dose ratio or dose level dependency in addition to overall antagonism or synergism. The interactions between the two pathogens were mostly antagonistic or additive at both CO_2 concentrations and time points, indicating that the observed effects during co-exposure did not exceed the expected combined effects of the individual exposure. Furthermore, we did not find evidence that the interactions between the pathogens substantially change at elevated CO_2. The antagonistic interactions measured in the bioassays are likely to be indirect via the insect host, as we did not detect direct interaction between the two pathogens in *in vitro* experiments. Here we show that

using the MIXTox model is a powerful tool to assess the effects of co-exposure to pathogens and quantify risk of disease in mass-reared insects.

5.2 Introduction

The production of insects as feed and food is an alternative to traditional sources of protein such as livestock, soy, or fishmeal [1]. This new industry is predicted to reach an annual production of 500,000 tons of insect protein by 2030 [2]. Mass-reared insects are at an increased risk of entomopathogenic (insect pathogenic) infections due to the high densities they are cultured in [3, 4], which can severely affect production [4]. Different pathogens may occur simultaneously in insect mass-rearing systems [3]. Furthermore, mass-reared insects may be exposed to environmental stress such as elevated temperature, humidity, or changes in gas concentrations [3, 5]. The interactions between stressors may be synergistic (i.e., larger effect than predicted), antagonistic (i.e., smaller effect than predicted), or additive (i.e., as predicted). For the risk assessment of the impact of individual stressors in insect mass-rearing systems, it is therefore critical to understand how they interact with other common stressors in the system.

Among the insect species reared for feed and food, the yellow mealworm, *Tenebrio molitor*, is considered one of the most promising species for industrial mass-rearing [6]. *Tenebrio molitor* is susceptible to infection by various microbial entomopathogens [7]. Two important pathogens are the gram-positive bacterium *Bacillus thuringiensis* and the ascomycete fungus *Metarhizium brunneum* [7]. Both of these pathogens are a threat to the production of *T. molitor* as they can be introduced into the mass-rearing system via the feed [8, 9] and *B. thuringiensis* has recently been reported from a *T. molitor* mass-rearing system [10]. In addition, mass-reared *T. molitor* may be exposed to elevated carbon dioxide (CO_2) concentrations in closed rearing systems [11]. The legal long-term (8 hours) human exposure limit of CO_2 is set to 5,000 ppm (parts per million) in many countries [12, 13], a concentration that has been reached in experimental setups with larval populations of *T. molitor* [14]. Such levels are approximately 12 times higher than the ambient CO_2 concentration (415 ppm) [15]. In a previous study, we showed that these industrially

relevant CO_2 concentrations affect host-pathogen interactions in *T. molitor* larvae (unpublished data, chapter 4). However, it remains unknown if, and how, the interactions change under co-exposure to the two tested pathogens.

Environmental conditions can have a direct effect on pathogens and insects outside of any host-pathogen interaction, but the environment can also affect pathogens indirectly via the host [5]. Similarly, the interactions between co-infecting pathogens can either be direct (e.g., one pathogen produces a toxin that inhibits the other pathogen) or indirect (e.g., the host immune response is affected by one pathogen, resulting in an altered response to the other pathogen) [16]. In the Colorado potato beetle (*Leptinotarsa decemlineata*) exposure to mixtures of *B. thuringiensis* and either *Beauveria bassiana*, *Metarhizium robertsii*, or *Metarhizium anisopliae* led to increased mortality of the larvae (i.e., synergistic interaction) [17-19] or no significant change in susceptibility (i.e., additive interaction) [20]. *Bacillus thuringiensis* inhibits feeding in infected hosts through disruption of gut membranes, which may in turn aid the fungal pathogen in the infection process of such weakened hosts [19, 21]. In addition, *B. thuringiensis* infection inhibited the cellular immune response of *L. decemlineata* and it increased the germination of fungal conidia on the insect cuticle, which correlated with the synergistic interaction of higher observed mortality in co-infected larvae [17]. In the larvae of the spotted asparagus beetle (*Crioceris quatuordecimpunctata*), a sublethal dose of *B. thuringiensis* toxin led to increased mortality when additionally exposed to *B. bassiana* [22]. The authors showed that the larvae develop more slowly when exposed to the *B. thuringiensis* toxin, which increases the time between moults, possibly leading to increased penetration by *B. bassiana* [22]. Moreover, *B. thuringiensis* in combination with destruxins (fungal secondary metabolites) of *M. anisopliae* led to synergistic or additive effects in spruce budworm (*Choristoneura fumiferana*) larvae depending on the dose applied [23].

Studies on the impact of co-infections in *T. molitor* are rare and the simultaneous exposure to a bacterial and a fungal pathogen has to our knowledge, not been tested so far. Moreover, there exists a gap in knowledge on how CO_2 affects host-pathogen

interactions in mass-reared insects in general [5]. The outcomes of co-exposure of insects to different stressors cannot be predicted without studying them together (i.e., simultaneously or sequentially) and interactions between entomopathogens are often investigated by using methods that only determine overall additivity, synergism, or antagonism. However, interactions may follow more complex response patterns such as dose and time dependency.

In this study, we aimed to answer the question of how *M. brunneum* and *B. thuringiensis* interact with each other in *T. molitor* larvae under ambient and elevated CO_2 concentrations. We hypothesised that *M. brunneum* and *B. thuringiensis* interact in a synergistic way in *T. molitor* as it has been shown for other insect species [17-19, 23]. Furthermore, we measured the *in vitro* effects of elevated CO_2 on the interaction between the two pathogens, to test the hypothesis that the *in vitro* effects are reflected in the *in vivo* interactions when *T. molitor* are exposed to both pathogens (i.e., measured as larval biomass, individual weight gain, and survival). Testing these different stressors in combination is crucial for the risk assessment of each stressor in *T. molitor* production systems for insect health. We use a mixture toxicity (MIXTox) model [24] at different time points after exposure and *in vitro* assays to assess the interactions and thereby quantify the risks caused by co-exposures to different stressors. The MIXTox model was originally developed to study interactions between chemicals. However, it has been suggested before by our lab to be used for studying interactions between entomopathogens [25]. Using the MIXTox model has the advantage that dose-level dependent or dose-ratio dependent effects of mixtures can be detected [24]. Here, we demonstrate the use of the MIXTox model to study *in vivo* interactions between the two entomopathogens, *M. brunneum* and *B. thuringiensis*.

5.3 Materials and Methods

All experiments were conducted on two independent occasions (except for the initial establishment of EC_{50} values, which was run once). The experiments were conducted in two 50-litre LEEC Culture Safe CO_2 incubators adjacent to each other, set at 28°C (± 0.5°C), 75% (± 5%) relative humidity, and ambient (450 ± 50 ppm) or elevated

(4,500 ± 500 ppm) CO_2 concentrations in complete darkness. Each of the incubators had 40 mm ELUTENG USB fans installed at the rear opening. These fans were activated for 15 minutes at 45-minute intervals to ensure that the CO_2 concentrations did not increase due to insect respiration. The environmental conditions were monitored every 15 min using EasyLog EL-SIE-2 loggers for temperature and relative humidity and Rotronic CL11 loggers for CO_2 concentrations.

5.3.1 *Bacillus thuringiensis* culture

Bacillus thuringiensis serovar *morrisoni tenebrionis* 4AA1 (*Bacillus* genetic stock center, Ohio State University, USA) was grown on LB-agar (lysogeny broth agar; 10 g tryptone, 5 g yeast extract, 10 g NaCl, and 15 g bacteriological agar in 1 l dH_2O) in Petri dishes (9 cm diameter, triple vented) overnight at 30°C. Subsequently, one bacterial colony was introduced into each of eight 500 ml Erlenmeyer flask that held 50 ml HCT medium (5 g tryptone, 2 g bacto casamino acids, 6.8 g KH_2PO_4, 0.1 g $MgSO_4$, 0.002 g $MnSO_4$, 0.014 g $ZnSO_4$, 0.15 g $CaCl_2$ and 0.022 g Ammonium ferric citrate in 1 l dH_2O). The flasks were sealed with Parafilm™ and incubated for 96 hours on a platform shaker (200 rpm at 30°C). This duration is long enough for the bacterial cells to sporulate and release crystals into the medium. Afterwards, the suspension was centrifuged at 3,900 rpm (3,231 *g*, Eppendorf Centrifuge 5810 R) at 4°C for 10 minutes. The resulting supernatant was disposed of and 20 ml of sterile dH_2O was added and the pellet re-suspended. After repeating this washing process twice using the aforementioned settings, the resulting stock suspension was incubated at 75°C for 10 minutes to ensure that the suspension was purely containing spores and crystals without any viable vegetative cells, which do not survive these high temperatures. Confirmation of the presence of bacterial spores and crystals was performed by examining the suspension under a light microscope (Leica DM2000 LED; 1,000 times magnified). The number of colony forming units (cfu) per ml were assessed by plating 10 µl of ten-fold serial dilutions (10^4-10^9) of the stock suspension on LB-agar in triplicate and counting after 28°C incubation for 16 hours.

5.3.2 *Metarhizium brunneum* culture

Metarhizium brunneum isolate KVL12-30 (culture collection of the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark) was cultured in Petri dishes (9 cm diameter, triple vented) containing SDAY/4 medium (16.25 g Sabouraud dextrose agar, 2.5 g yeast extract, and 11.25 g agar in 1 l dH_2O) for 14 days at 28°C. Thereafter, the conidia were suspended in TritonX-100 (0.05% v/v) after agitating from the plate using a Drigalski spatula. The resultant suspension was centrifuged at 3,000 rpm (1,872 *g*, Eppendorf Centrifuge 5810 R) at 18°C for 3 min before disposing of the supernatant and adding fresh TritonX-100 (0.05% v/v). This washing procedure was repeated once using the aforementioned settings and afterwards sterile dH_2O was added to produce the stock suspension. The conidia concentration was estimated by counting using a 0.2 mm Fuchs-Rosenthal hemocytometer under a light microscope (400 times magnified). Fresh conidia suspensions were produced for each experiment on the day of use. Conidia germination was assessed alongside each experiment by spreading 100 µl of 10^6 conidia/ml on Petri dishes (9 cm diameter, triple vented) containing SDAY/4 medium in triplicate and incubated for 18 hours at 28°C. Thereafter, 300 conidia per Petri dish were examined under a light microscope and germination was assessed; a conidium was counted as germinated if its germ tube was at least the length of the smallest diameter of the conidium.

5.3.3 Insect culture

Tenebrio molitor larvae were initially supplied by the company Ÿnsect (Evry, France) and reared in the laboratory for more than eight generations. For regular culturing, adult *T. molitor* (40 females and 40 males) were kept in 50 g diet (96% w/w wheat bran and 4% w/w dried egg white) and supplied with 5 g water agar (1% w/v) in 750-ml containers (n = 6) for four days at ambient CO_2. Thereafter, the adults were removed and for the mixture experiment, the containers were distributed to incubators set at either ambient or elevated CO_2 concentrations. The initial assessment of EC_{50} values was only done with larvae reared at ambient CO_2 concentrations (see Assessment of EC_{50} values in appendices to Chapter 5). The

larvae received 10 g water agar (1% w/v) as a water source twice a week starting one week after removal of the adults.

5.3.4 *In vivo* mixture experiment

The larvae in each 750-ml container were distributed in groups of 30 larvae to empty 60-ml cups 17 days after removal of the adults. Larvae were starved for 24 hours to make sure that all of them ingested the diet mixed with the pathogens thereafter. Spore suspensions of *B. thuringiensis* (4×10^7, 6×10^8, and 10^{10} spores/ml) and conidia suspensions of *M. brunneum* (2×10^7, 6×10^7, and 1.2×10^8 conidia/ml) were prepared. The concentrations for each pathogen in the medium was based on the EC_{50} values that were previously established (see Assessment of EC_{50} values in appendices to Chapter 5) for the endpoint larval biomass (mg/cm^2) 14 days after exposure (Table S5.1). To prepare the individual suspensions, each of the aforementioned suspensions was mixed with dH_2O in a ratio 1:1. This was done in order to use the same stock suspensions for the individual and mixture suspensions. Mixture suspensions were prepared by mixing the aforementioned suspensions of *B. thuringiensis* spores and *M. brunneum* conidia in a ratio 1:1 (Figure 5.1). Thereafter, 100 µl of the individual and mixture suspensions was added to 100 mg diet in 60-ml cups (n = 4). The diet and suspension were mixed thoroughly with a sterile inoculation loop. Control cups were also prepared in the same way with 100 µl dH_2O in 100 mg diet (n = 5). Afterwards, larvae were added to these cups containing the suspensions and the diet. This experiment was conducted at either ambient or elevated CO_2 concentrations at the same time.

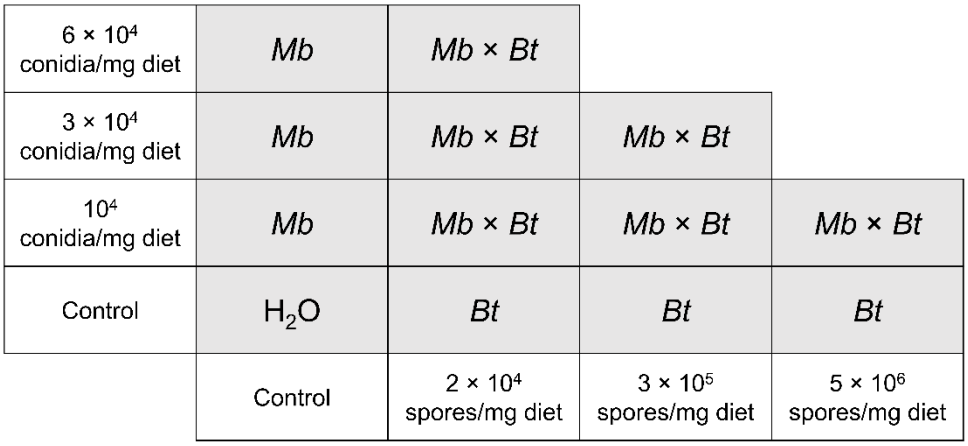

Figure 5.1 Experimental design of the *in vivo* mixture experiment conducted in the same way at ambient (450 ± 50 ppm) and elevated (4,500 ± 500 ppm) CO_2 concentrations.

The treatments consisted of three individual *M. brunneum* (*Mb*) conidia concentrations (green), three individual *B. thuringiensis* (*Bt*) spore concentrations (yellow), six mixture treatments (blue), and one control (grey) in both CO_2 concentrations. The preparation of the suspensions were prepared per ml, but they are given here per mg diet. Two experimental repetitions.

After two days of exposure to the pathogens, the larvae were moved to new cups containing clean diet (0.6 × fresh weight of larvae) and water agar (1% w/v; 0.6 × fresh weight of larvae). The weight of the larvae was multiplied by 0.6 to ensure that the larval biomass to diet ratio was similar in each cup throughout the experiment. Larvae were weighed in groups every second day for 20 days. The total larval mass of each group was divided by the number of alive larvae in that group to obtain the individual weight of larvae. The individual weight at each time point was subtracted by the individual weight at day 0 of the experiment to obtain the individual weight gain. Any dead larvae were removed and fresh diet and water agar were added as detailed before.

5.3.5 *In vitro* experiments

5.3.5.1 Effect of *M. brunneum* on *in vitro* spore viability of *B. thuringiensis*

Seven mixture suspensions of *B. thuringiensis* spore/crystal and *M. brunneum* conidia were prepared. The *B. thuringiensis* : *M. brunneum* ratios were 1:0; 1:3; 1:1.5; 1:0.5; 1:0.1; 1:0.033; 1:0.002. These ratios were chosen based on the ratios used in the *in vivo* experiments. For each suspension, 10 µl was added to Petri dishes (9 cm diameter, a 2 cm wide plastic strip was put between each lid and dish to allow for air circulation) containing 10 ml LB-agar (n = 9). By tilting the Petri dishes, the suspensions formed straight lines. After 16 hours of incubation at either ambient or elevated CO_2 concentrations, the cfu were counted and used to calculate cfu/ml. This time point was chosen to prevent the colonies becoming too big, which would make counting impossible.

5.3.5.2 Effect of *B. thuringiensis* on *in vitro* germ tube length of *M. brunneum*

Seven mixture suspensions of *M. brunneum* conidia and *B. thuringiensis* spore/crystal were prepared. The *M. brunneum* : *B. thuringiensis* ratios were 1:0; 1:500; 1:30; 1:10; 1:2; 1:0.66; 1:0.33. These ratios were chosen based on the ratios used in the *in vivo* experiments. For each suspension, 100 µl was spread on Petri dishes (9 cm diameter, a 2 cm wide plastic strip was put between each lid and dish base to allow for air circulation, n = 3) containing 10 ml bacteriological agar (15 g in 1 l dH_2O). After 15 hours of incubation at either ambient or elevated CO_2 concentrations, three cover slips (22 × 22 mm) were added on each Petri dish. Six pictures were taken for each Petri dish (two per cover slip, location randomly chosen) using a Flexacam C3 mounted on a light microscope (Leica DM2000 LED) at 400 times magnification. The lengths of all germ tubes were measured using the segmented line tool in ImageJ 1.54d. Data from the ratio 1:500 was excluded from analysis because the germ tubes could not be identified between the *B. thuringiensis* spores/crystal suspension.

5.3.6 Statistical analysis

5.3.6.1 The effect of CO_2 on individual pathogen concentration–response

The single pathogen concentration-response relationships of the mixture experiment were modelled by fitting the data to three-parameter log-logistic models using the 'LL.3' function in the 'drc' package [26] using R v. 4.1.0 [27] and equation 5.1:

$$y = \frac{\mu_{max}}{1 + (\frac{c}{EC_{50}})^{\beta}} \qquad Equation\ 5.1$$

where *y* is the response (either larval biomass, survival, or weight gain) 14 days after exposure, μ_{max} is the upper limit (i.e., response when the pathogen concentration is 0), *c* is the pathogen concentration, EC_{50} is the pathogen concentration that results in 50% reduction of μ_{max}, and β is the slope parameter. The effect of exposure to different CO_2 concentrations was performed comparing EC_{50} values using the 'compParm' function.

5.3.6.2 *In vivo* mixture experiment

The data of the mixture experiment was analysed using the 'MIXTox' model [24] assuming independent action (IA) as the reference model for each endpoint given in equation 5.2:

$$y = \mu_{max}(\frac{1}{1 + \left(\frac{c_{Mb}}{EC_{50_{Mb}}}\right)^{\beta_{Mb}}})(\frac{1}{1 + \left(\frac{c_{Bt}}{EC_{50_{Bt}}}\right)^{\beta_{Bt}}}) \qquad Equation\ 5.2$$

using the same parameters as noted above for *M. brunneum* (*Mb*) and *B. thuringiensis* (*Bt*). An F-test was performed to confirm that the reference model of IA provided a statistically significant fit with the null hypothesis of no relationship between observed mixture effects and exposure concentrations. To check for significant interaction deviation patterns from the IA model it was then extended by adding a parameter (*a*) to test if there was overall synergism or antagonism (S/A) interaction. Thereafter, an additional parameter (*b*) was included to allow the degree of synergism or antagonism to vary depending on the dose ratio in which the two stressors are present (DR; parameter b_{DR}) or to allow the degree of synergism or

antagonism to vary depending on the overall dose level (DL; parameter b_{DL}) [24]. The 'Solver' add-in function in Microsoft® Excel 2016 was used to fit the models to the data by minimising the sum of squared residuals. During all model fitting, care was taken to test a range of starting parameter values to ensure a global minimum was identified and the model iterations not having got caught in a local minimum. The degree and significance of the improvement in fit achieved with the extra parameters were tested using Chi-square tests (X^2) for all nested model pairs. To identify which of the nested models provided the best fit description of the observed dose-response surface, first IA vs S/A (one d.f. different) were compared. Where S/A fitted significantly better, then the S/A model fit was compared to the DR and DL fits, respectively (one d.f. different). Where S/A did not provide a significant improvement over the IA model, any improvement of fit achieved by DR or DL was tested against the IA model (two d.f. difference). Depending on the parameters of a, b_{DR}, and b_{DL}, different interaction patterns of antagonistic or synergistic effects are described as defined in Jonker et al. [24]. Where DR was identified as the best fit model, the switch ratio for DR was calculated using equation 5.3:

$$c_{Mb} = \left(\frac{-b_{DR}}{a} - 1\right)\frac{ECx_{Mb}}{ECx_{Bt}}c_{Bt} \qquad Equation\ 5.3$$

We tested the interactions between *M. brunneum* and *B. thuringiensis* at both 14 and 20 days after exposure to understand if the interactions might change temporally.

5.3.6.3 *In vitro* experiments

The data on germ tube length of *M. brunneum* and number of viable spores of *B. thuringiensis* were analysed using two-way analysis of variance (ANOVA) in R v. 4.1.0 [27]. The two experimental repetitions were combined as there was no interactive effect of experimental repetitions with treatment or CO_2 exposure found in initial three-way ANOVAs. The normality (QQ-plots) and homogeneity of variances (Levene test, $p > 0.05$) were tested to check if assumptions were satisfied. The data on germ tube length was log-transformed before analysis to satisfy assumptions. Tukey's HSD (Honestly Significant Difference) tests were used to

separate means. For all statistical analysis, differences were considered significant when the *p*-value was <0.05.

5.4 Results

5.4.1 The effect of CO_2 on individual pathogen concentration–responses

We compared the responses in *T. molitor* (larval biomass, survival, and individual weight gain) to exposure to the two individual pathogens at the two CO_2 concentrations (Figure 5.2, S5.2). The comparison of the EC_{50} values revealed that for larval biomass and survival the EC_{50} values were lower for larvae exposed to *M. brunneum* under elevated CO_2 in the experimental repetition 1, i.e., the larvae were more susceptible to the fungus under these conditions (Figure 5.2, Table 5.1). However, there were no differences between the EC_{50} values of larvae exposed to *M. brunneum* reared under different CO_2 concentrations in the experimental repetition 2 (Figure S5.2, Table S5.2). Moreover, there were no differences between EC_{50} values for larvae exposed to *B. thuringiensis* under the different CO_2 concentrations in either of the two experimental repetitions (Figure 5.2, S5.2, Tables 5.1 and S5.2). The *p* and *t* values of the comparisons between the EC_{50} values are shown in Table S5.3.

Table 5.1 Experimental repetition 1. EC_{50} values, slopes (β) and upper limits (μ_{max}) of three-parameter log-logistic models of larval biomass (mg/cm^2), survival, and individual weight gain (mg/larva) of larvae exposed to individual pathogen (*M. brunneum* or *B. thuringiensis*) concentrations at either ambient (Amb.) or elevated (Elev.) CO_2 concentrations 14 days after exposure.
EC_{50} values followed by different letters for the same endpoint and pathogen indicate significant differences among the treatments. SE, standard error.

	Larval biomass (mg/cm^2)		Survival		Individual weight gain (mg/larva)	
	Amb. CO_2	Elev. CO_2	Amb. CO_2	Elev. CO_2	Amb. CO_2	Elev. CO_2
M. brunneum						
[1]EC_{50} ± SE	3.07±0.43a	1.86±0.17b	4.24±0.45a	2.61±0.20b	9.45±2.99a	4.60±0.90a
β ± SE	1.70±0.38	1.86±0.24	1.60±0.32	1.60±0.17	1.07±0.38	1.24±0.36
μ_{max} ± SE	48.61±2.60	48.17±1.73	0.97±0.04	0.98±0.03	11.17±0.53	10.98±0.64
B. thuringiensis						
[2]EC_{50} ± SE	3.52±1.09a	1.86±0.17a	51.26±17.20a	74.93±15.35a	6.99±2.54a	9.43±6.25a
β ± SE	0.54±0.08	1.86±0.24	0.50±0.09	0.66±0.12	0.40±0.06	0.27±0.06
μ_{max} ± SE	48.78±1.88	48.17±1.73	0.99±0.03	0.99±0.02	11.10±0.41	10.90±0.51

[1]× 10^4 conidia/mg diet
[2]× 10^5 spores/mg diet

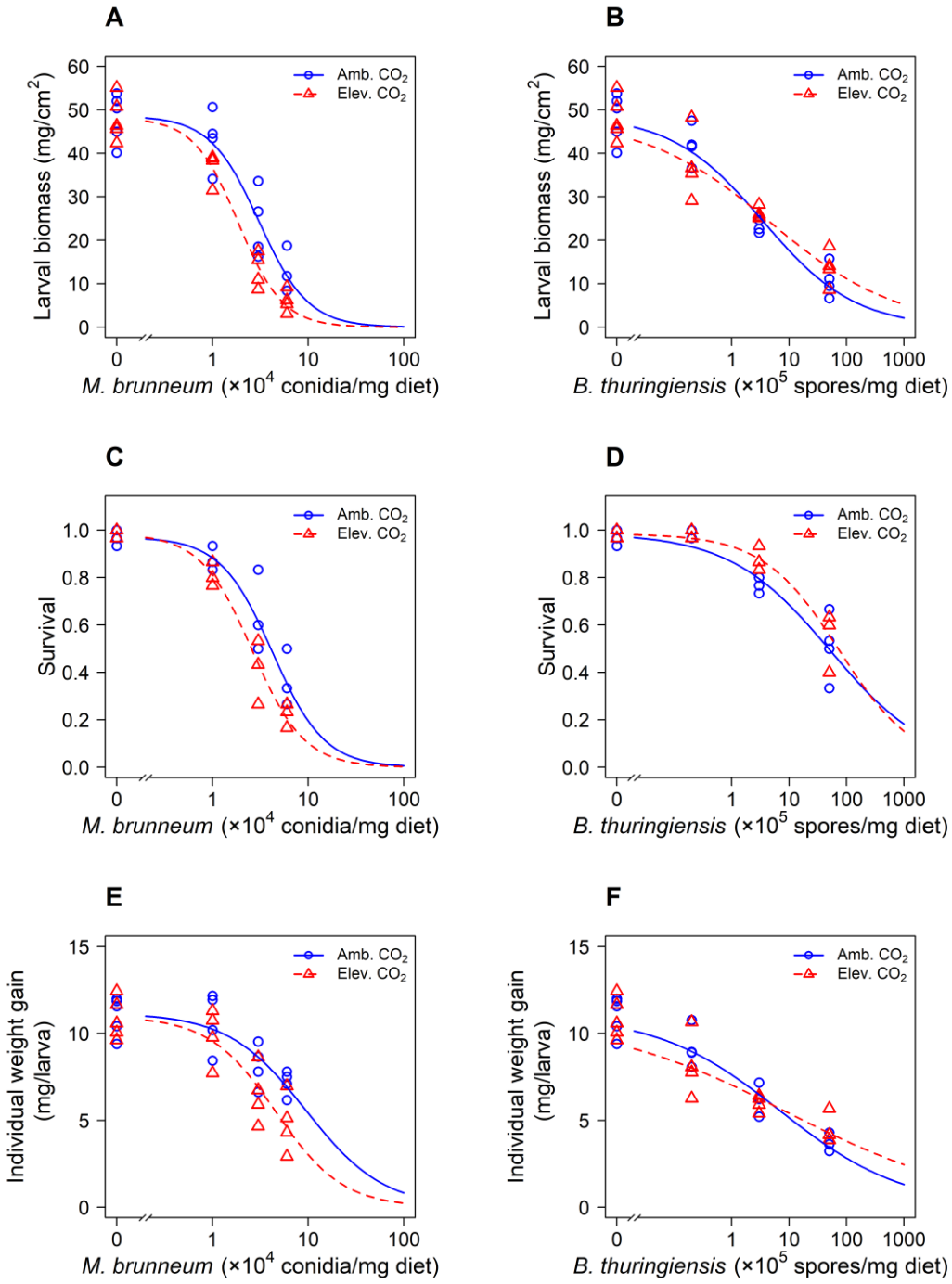

Figure 5.2 Experimental repetition 1. Three-parameter log-logistic models of larval biomass, survival, and individual weight gain of larvae exposed to individual pathogen concentrations at either ambient (Amb.; blue, circles) or elevated (Elev.; red, triangles) CO_2 concentrations 14 days after exposure.

A Larval biomass (mg/cm^2) after *M. brunneum* exposure. **B** Larval biomass (mg/cm^2) after *B. thuringiensis* exposure. **C** Survival after *M. brunneum* exposure. **D** Survival after *B. thuringiensis* exposure. **E** Individual weight gain (mg/larva) after *M. brunneum* exposure. **F** Individual weight gain (mg/larva) after *B. thuringiensis* exposure.

5.4.2 *In vivo* mixture analysis

The results of the mixture toxicity analyses for larval biomass, survival, and individual weight gain for the experimental repetition 1 are visualised in Figures 5.3, 5.4, and 5.5, respectively. An antagonistic interaction in this study is found when survival, larval biomass, or individual weight gain are higher than predicted from the IA reference model, whereas a synergistic interaction is found when the mentioned endpoints are lower than predicted from the IA reference model. The conidial viability of *M. brunneum* was ≥99% in all experiments.

In the experimental repetition 1, the parameter *a* was >0 in all cases, which indicates antagonism between the pathogens. Adding parameter *a* by using S/A models for larval biomass, survival, and weight gain of larvae (Table 5.2) significantly decreased the residuals at both time points and CO_2 concentrations, and provided a significantly improved fit (as indicated by the Chi-square tests values [$p(X^2)$] all being below 0.05). The subsequent addition to some of the data sets of the additional parameter *b* further improved the fit to the data. Adding parameter *b* using dose ratio-dependent deviation (DR) further decreased the residuals for larval biomass at ambient CO_2 20 days post exposure (R^2 S/A = 0.86; R^2 DR = 0.87, Table 5.2) and at elevated CO_2 14 and 20 days post exposure (R^2 S/A = 0.89; R^2 DR = 0.91 and R^2 S/A = 0.89; R^2 DR = 0.90, respectively, Table 5.2). Survival was also better described by DR than S/A at ambient CO_2 14 days post exposure (R^2 S/A = 0.81; R^2 DR = 0.82, Table 5.2) and at elevated CO_2 14 and 20 days post exposure (R^2 S/A = 0.87; R^2 DR = 0.89 and R^2 S/A = 0.87; R^2 DR = 0.90, respectively, Table 5.2). For all endpoints that were better described using the DR model, parameter *a* was >0 and parameter *b* was <0 indicating antagonism when the mixture was dominated by *M. brunneum* switching to synergism when the mixture was dominated by *B. thuringiensis*. The shifts between antagonism (above dotted lines) and synergism (below dotted lines) are shown in Figures 5.3 and 5.4 where applicable.

The results of the mixture toxicity analyses for larval biomass, survival, and individual weight gain for the experimental repetition 2 are visualised in Figures S5.3, S5.4, and S5.5, respectively. As shown in Table S5.4 for the experimental

repetition 2, adding parameter *a* to the IA model for larval biomass 20 days post exposure at ambient CO_2 significantly decreased the residuals (R^2 IA = 0.78; R^2 S/A = 0.80, Table S5.4). Similarly, residuals were significantly decreased by adding parameter *a* to the IA models for survival 14 and 20 days post exposure at ambient CO_2 (R^2 IA = 0.76; R^2 S/A = 0.78 and R^2 IA = 0.76; R^2 S/A = 0.78, respectively, Table S5.4). Similar to the experimental repetition 1, parameter *a* was >0 indicating antagonism. Moreover, residuals were significantly reduced when using DR to describe individual weight gain 14 days after pathogen exposure at elevated CO_2 with *a* >0 and *b* <0 (R^2 S/A = 0.72; R^2 DR = 0.75, Table S5.4). All the data from other endpoints in the experimental repetition 2 were best described using IA (Table S5.4).

Table 5.2 Experimental repetition 1. Results of MIXTox models for larval biomass (L. biom. in mg/cm^2), survival (Surv.), and individual weight gain (Ind. wg. in mg/larva) at ambient and elevated CO_2 either 14 or 20 days after pathogen exposure. Models that described the data best are highlighted in green. IA, independent action; S/A, synergistic/antagonistic; DR, dose ratio-dependent; DL, dose level-dependent.

	14 days								**20 days**							
	Ambient CO_2				**Elevated CO_2**				**Ambient CO_2**				**Elevated CO_2**			
	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL
L. biom.																
μ_{max}	47.32	49.28	49.11	49.56	44.82	47.69	48.05	47.60	103.62	107.09	106.71	107.69	99.07	105.09	105.92	104.87
β_{Bt}	8.56	7.50	7.20	7.69	8.07	6.87	6.16	6.40	9.18	8.24	7.84	8.68	8.84	7.68	6.85	7.01
β_{Mb}	1.42	1.30	1.52	1.33	1.82	1.56	1.93	1.41	1.40	1.31	1.56	1.38	1.91	1.64	2.03	1.44
$^1EC50_{Bt}$	5.68	5.41	5.49	5.40	5.88	5.49	5.57	5.49	5.67	5.43	5.51	5.42	5.88	5.51	5.59	5.52
$^2EC50_{Mb}$	4.16	3.23	3.13	3.13	2.81	2.08	1.93	2.07	3.94	3.10	3.01	3.01	2.74	2.01	1.86	1.99
a	NA	1.63	3.74	1.07	NA	1.98	5.51	4.23	NA	1.57	3.99	0.45	NA	2.10	5.84	4.88
b	NA	NA	-4.09	-0.88	NA	NA	-6.84	0.76	NA	NA	-4.76	-4.04	NA	NA	-7.28	0.81
Residuals	1504.78	1178.07	1102.12	1176.14	1499.38	1077.15	915.00	1040.86	8274.43	6787.17	6310.01	6723.82	8261.33	5976.44	5173.39	5715.22
R^2	0.84	0.88	0.88	0.88	0.85	0.89	0.91	0.90	0.83	0.86	0.87	0.86	0.84	0.89	0.90	0.89
$^3p(X^2)$	<0.001	<0.001	0.060	0.768	<0.001	<0.001	0.003	0.178	<0.001	0.001	0.049	0.481	<0.001	<0.001	0.006	0.124
Surv.																
μ_{max}	0.97	0.97	0.98	0.97	0.98	0.98	0.98	0.98	0.96	0.97	0.98	0.97	0.98	0.98	0.98	0.98
β_{Bt}	9.50	8.76	8.48	8.46	10.84	10.57	9.96	9.80	9.56	8.74	8.32	8.43	10.83	10.59	9.98	9.82
β_{Mb}	1.43	1.34	1.58	1.24	1.50	1.41	1.84	1.09	1.43	1.32	1.53	1.22	1.55	1.46	1.86	1.13
$^1EC50_{Bt}$	6.59	6.44	6.54	6.45	6.65	6.54	6.70	6.56	6.60	6.44	6.53	6.42	6.65	6.55	6.69	6.56
$^2EC50_{Mb}$	6.18	4.79	4.28	4.87	3.67	3.00	2.71	2.71	6.11	4.71	4.18	4.18	3.59	2.97	2.69	2.69
a	NA	1.88	4.84	2.87	NA	1.49	5.67	4.70	NA	1.87	4.67	3.43	NA	1.43	5.45	4.64
b	NA	NA	-5.60	0.71	NA	NA	-8.32	1.11	NA	NA	-5.26	0.69	NA	NA	-7.98	1.13
Residuals	100.68	77.26	72.42	76.87	89.35	71.56	56.66	69.72	98.72	75.96	73.97	77.21	85.74	69.50	55.97	67.33
R^2	0.75	0.81	0.82	0.81	0.83	0.87	0.89	0.87	0.75	0.81	0.81	0.80	0.84	0.87	0.90	0.88
$^3p(X^2)$	<0.001	<0.001	0.028	0.532	<0.001	<0.001	<0.001	0.175	<0.001	<0.001	0.159	NA	<0.001	<0.001	<0.001	0.141

Table 5.2 (continued)

	14 days								20 days							
	Ambient CO_2				Elevated CO_2				Ambient CO_2				Elevated CO_2			
	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL
Ind. wg.																
μ_{max}	10.84	11.10	11.21	11.22	10.20	11.05	11.01	11.15	26.49	27.42	27.17	27.36	24.93	27.06	26.89	27.28
β_{Bt}	5.60	4.96	4.85	5.09	5.20	3.85	3.82	3.47	6.11	5.22	5.27	5.50	6.08	4.33	4.28	3.91
β_{Mb}	1.08	0.89	1.06	1.06	1.52	1.21	1.26	1.06	0.99	0.95	1.02	0.98	1.48	1.17	1.27	1.01
[1]$EC50_{Bt}$	6.00	5.86	5.81	5.78	6.36	5.79	5.84	5.76	6.13	5.93	6.02	5.92	6.52	5.99	6.07	5.98
[2]$EC50_{Mb}$	12.74	12.74	9.45	9.45	6.76	4.70	4.70	4.70	12.92	9.48	9.48	9.48	6.81	4.51	4.51	4.51
a	NA	1.12	1.86	0.12	NA	2.15	2.60	5.38	NA	1.35	3.02	0.02	NA	2.37	3.20	5.68
b	NA	NA	-0.95	-19.03	NA	NA	-0.86	0.93	NA	NA	-2.78	-121.13	NA	NA	-1.64	0.93
Residuals	50.23	45.67	44.48	44.19	89.61	66.68	66.51	64.63	335.58	292.79	287.64	287.44	605.53	437.14	433.44	423.46
R^2	0.83	0.85	0.85	0.85	0.68	0.76	0.76	0.77	0.81	0.83	0.83	0.83	0.64	0.74	0.75	0.75
[3]$p(X^2)$	<0.001	0.025	0.237	0.186	<0.001	<0.001	0.714	0.198	<0.001	0.007	0.332	0.323	<0.001	<0.001	0.502	0.194

[1]log(spores/mg diet)

[2]$\times 10^4$ conidia/mg diet

[3]resulting from F-test for IA (IA vs Null model), or from Chi-square tests (X^2) for S/A (IA vs S/A), DR (DR vs S/A), and DL (DL vs S/A).

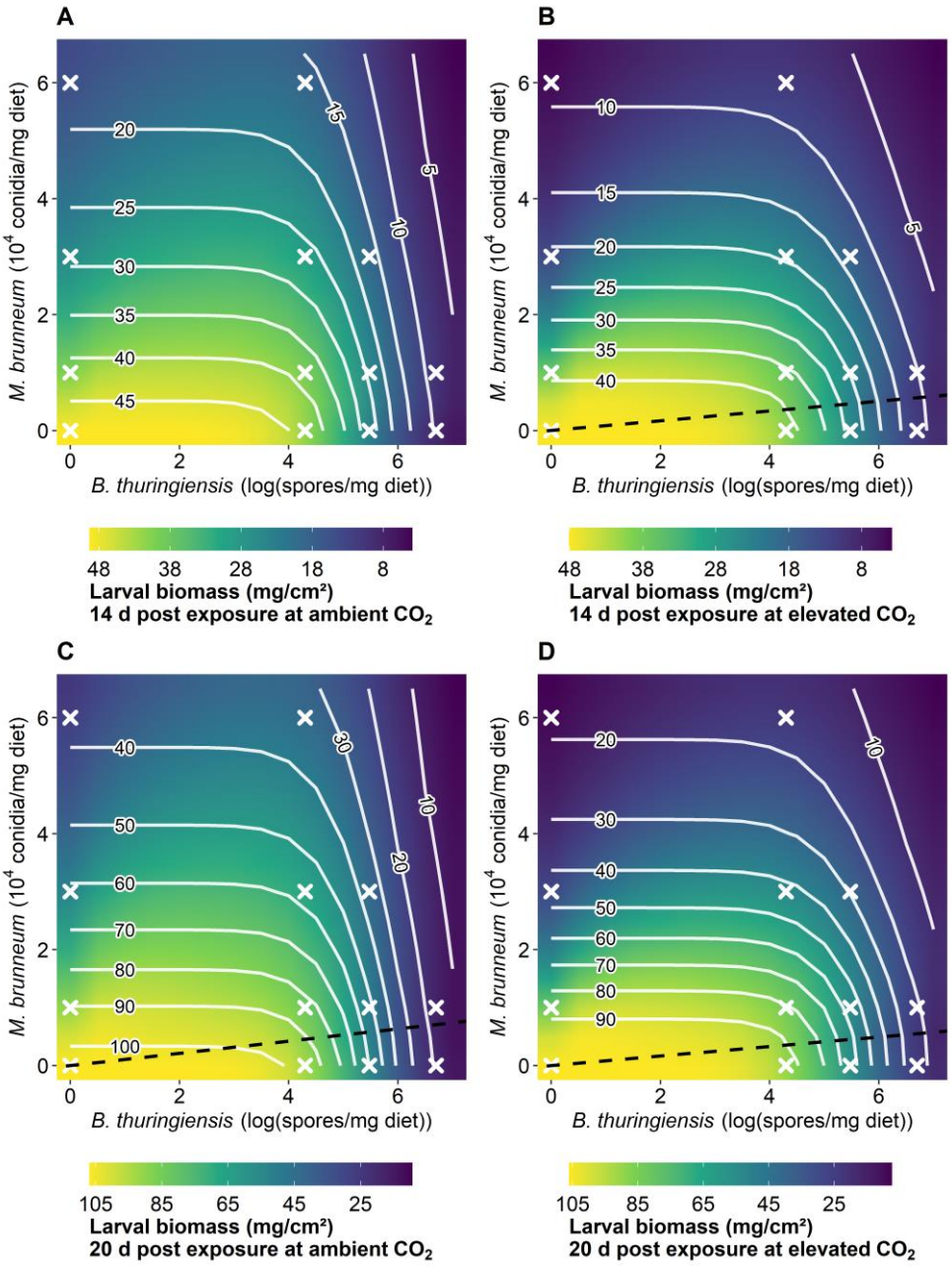

Figure 5.3 Experimental repetition 1. Contour plots of the endpoint larval biomass (mg/cm^2).
The *B. thuringiensis* concentrations are plotted on the x-axes, the *M. brunneum* concentrations are plotted on the y-axes. The white isobole lines show independent action (IA) prediction of the joint effects, whereas the colour gradients show the outcome of the models that describe the data best (Table 5.2). The white crosses show the tested individual (along x- and y-axis) and mixture (inside the plots) concentrations. The black dotted lines mark the shifts from antagonism (above the dotted lines) to synergism (below the dotted lines) for data that was best described using dose ratio-dependent deviation (DR). **A** 14 days post exposure to pathogens at ambient CO_2. **B** 14 days post exposure to pathogens at elevated CO_2. **C** 20 days post exposure to pathogens at ambient CO_2. **D** 20 days post exposure to pathogens at elevated CO_2.

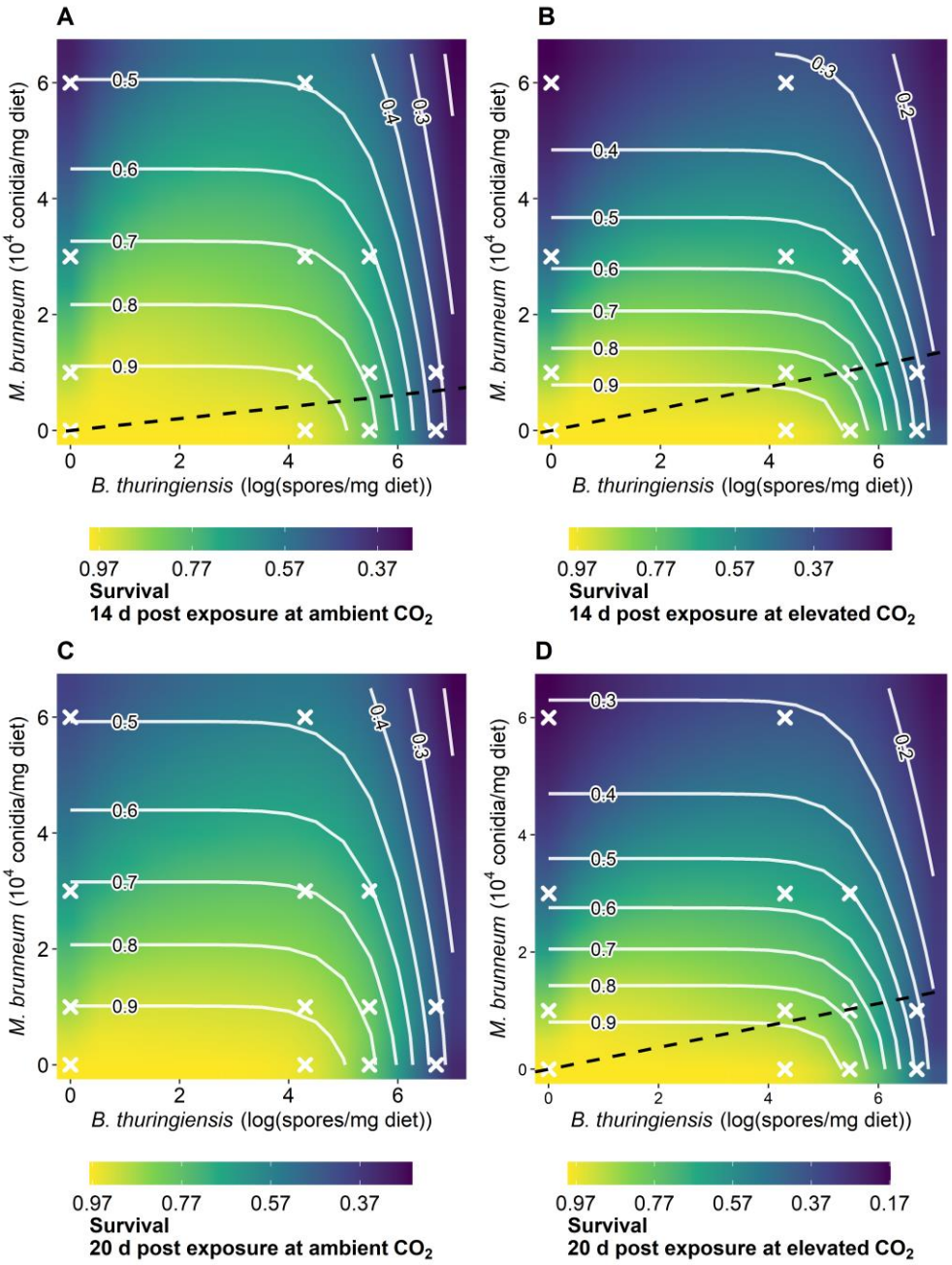

Figure 5.4 Experimental repetition 1. Contour plots of the endpoint survival of larvae.

The *B. thuringiensis* concentrations are plotted on the x-axes, the *M. brunneum* concentrations are plotted on the y-axes. The white isobole lines show independent action (IA) prediction of joint effects, whereas the colour gradients show the outcome of the models that describe the data best (Table 5.2). The white crosses show the tested individual (along x- and y-axis) and mixture (inside the plots) concentrations. The black dotted lines mark the shifts from antagonism (above the dotted lines) to synergism (below the dotted lines) for data that was best described using dose ratio-dependent deviation (DR). **A** 14 days post exposure to pathogens at ambient CO_2. **B** 14 days post exposure to pathogens at elevated CO_2. **C** 20 days post exposure to pathogens at ambient CO_2. **D** 20 days post exposure to pathogens at elevated CO_2.

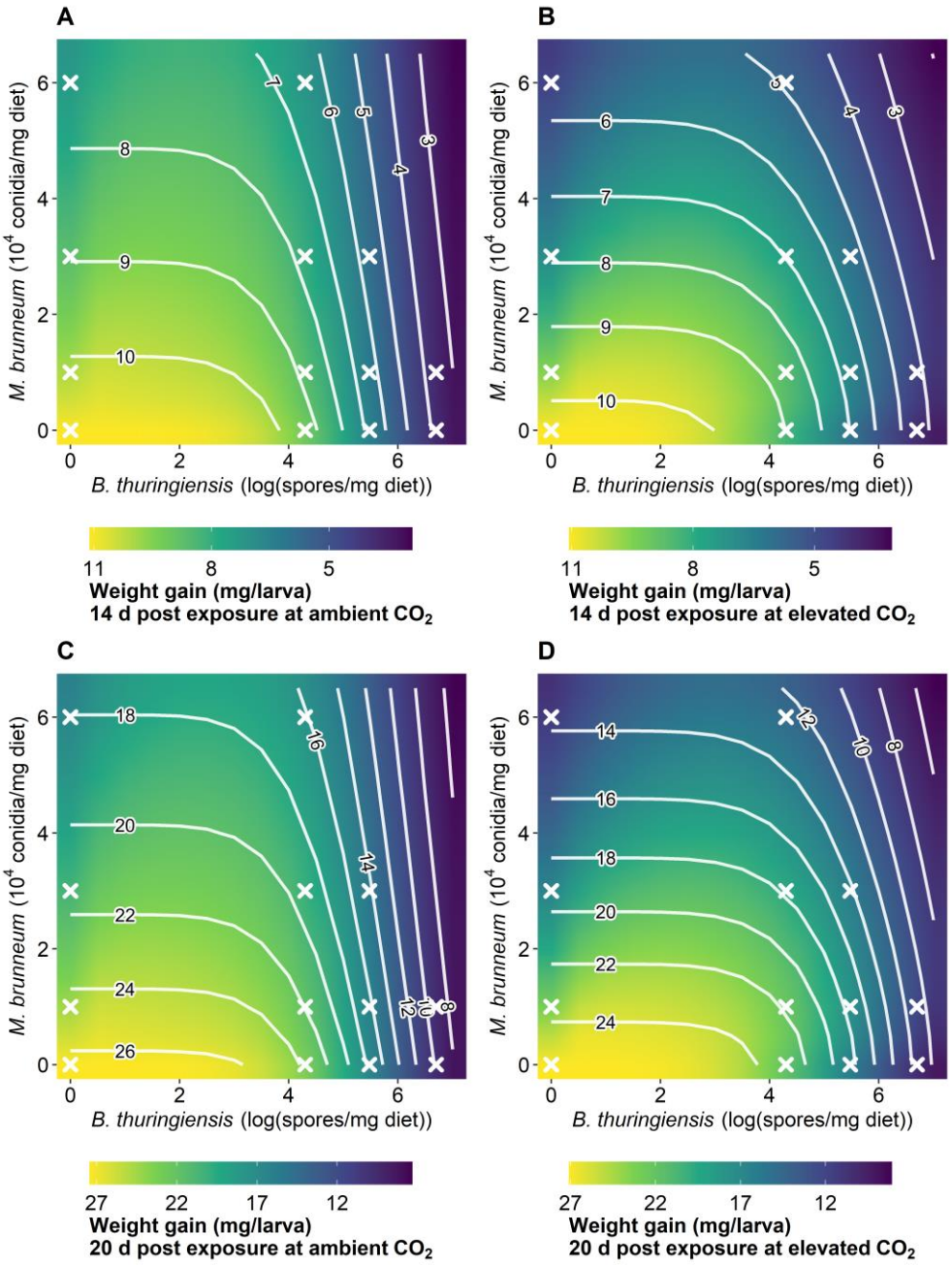

Figure 5.5 Experimental repetition 1. Contour plots of the endpoint weight gain (mg/larva).

The *B. thuringiensis* concentrations are plotted on the x-axes, the *M. brunneum* concentrations are plotted on the y-axes. The white isobole lines show independent action (IA) prediction of the joint effects, whereas the colour gradients show the outcome of the models that describe the data best (Table 5.2). The white crosses show the tested individual (along x- and y-axis) and mixture (inside the plots) concentrations. **A** 14 days post exposure to pathogens at ambient CO_2. **B** 14 days post exposure to pathogens at elevated CO_2. **C** 20 days post exposure to pathogens at ambient CO_2. **D** 20 days post exposure to pathogens at elevated CO_2.

5.4.3 *In vitro* experiments

The addition of different concentrations of *M. brunneum* conidia did not affect the viability of *B. thuringiensis* spores (p = 0.205, Tables 5.3, S5.5). However, elevated CO_2 decreased the viability of *B. thuringiensis* spores significantly (p <0.001, Tables 5.3, S5.5). The addition of different concentrations of *B. thuringiensis* spores to *M. brunneum* had a significant effect on their germ tube length (p <0.001, Table 5.3). The subsequent Tukey HSD test, however, did not reveal any significant differences between mixture treatments (Table S5.6). Furthermore, the germ tubes were significantly longer at elevated CO_2 (p <0.001, Tables 5.3, S5.6).

Table 5.3 Results of two-way ANOVAs when *M. brunneum* (*Mb*) conidia were added to a fixed concentration of *B. thuringiensis* (*Bt*) spores (first row) and when *Bt* spores were added to a fixed concentration of *Mb* (second row).

	Mixture			CO_2			Mixture × CO_2		
	F	*p*	d.f.	*F*	*p*	d.f.	*F*	*p*	d.f.
Mb added to *Bt*: viability of *Bt* spores (cfu/ml)	1.43	0.205	6	16.96	<0.001	1	1.07	0.383	6
Bt added to *Mb*: *Mb* germ tube length (µm)	7.72	<0.001	5	608.61	<0.001	1	1.46	0.201	5

5.5 Discussion

Our results show that in this set of experiments, synergism between *M. brunneum* and *B. thuringiensis* in *T. molitor* larvae only occurs when the mixture is dominated by *B. thuringiensis*. However, most of the areas that the model described as synergism were outside the tested mixture treatments. The majority of the interactions were modelled as either antagonistic or additive at ambient and elevated CO_2 concentrations, indicating that the risk does generally not increase by mixed exposure to the two pathogens at the tested CO_2 concentrations. This is contrary to previous findings, which have demonstrated strong synergistic interactions between *B. thuringiensis* and other fungal pathogens in other insect species [17-19, 22, 23]. The methods used to analyse the interactions, however, varied widely between the studies making it difficult to compare them directly to our results. In two studies ANOVAs were used [18, 22], in one study no statistical analysis was provided [19],

and only two studies used reference models to compare with the observed effects [17, 23]. A number of authors suggested that the delayed development or starvation of insects exposed to *B. thuringiensis* could be responsible for synergistic interactions during mixed exposure with a fungal pathogen [18, 22], with smaller larvae generally being more susceptible to pathogens [28]. However, even though exposure to *B. thuringiensis* significantly reduced larval biomass and individual weight gain in our experiments, the interactions with *M. brunneum* were mostly antagonistic or additive. Antagonism has been reported in a system in which chickpea (*Cicer arietinum*) plants were inoculated with *B. bassiana* as an endophyte and combined with *B. thuringiensis*. In these experiments, the mixture of pathogens led to synergistic, antagonistic, or additive interactions in larvae of the lepidopteran cotton bollworm (*Helicoverpa armigera*), depending on the *B. thuringiensis* dose applied [29]. Our study is, to our knowledge, the first report of antagonistic interactions between *B. thuringiensis* and a fungal pathogen in a coleopteran species.

Our results on the effect of CO_2 concentrations on larvae exposed to *M. brunneum* on survival and larval biomass are inconclusive, with differing results from the two experimental repetitions of the bioassay. In a previous study we conducted with single concentrations of the two pathogens we found increased survival of larvae exposed to elevated CO_2 (unpublished data, chapter 4). Despite the same CO_2 concentrations being tested in both the current and previous study, the data are not directly comparable because different pathogen concentrations and data analysis methods were applied. The *in vivo* interactions between the two pathogens were relatively stable at the two time points (14 and 20 days after exposure). At ambient CO_2 there were slight changes between the two time points from S/A with antagonism at 14 days to DR with major antagonism at 20 days for larval biomass and vice-versa for survival in the experimental repetition 1. In the experimental repetition 2, there was one change from IA to S/A with antagonism for larval biomass. Furthermore, there was a change from DR to IA for individual weight gain at elevated CO_2 in the experimental repetition 2. Similarly, there was no general trend when comparing the *in vivo* interactions at the two CO_2 concentrations

suggesting that the tested CO_2 concentrations only have little or no effect on the pathogen interactions.

Our results from the *in vitro* interactions experiments between *B. thuringiensis* spores and *M. brunneum* conidia on artificial media are comparable with a previous study in which the authors also found no effect of *B. thuringiensis* on the development and growth of *M. anisopliae* or *B. bassiana* [19]. Even though we found a significant effect of the mixture treatment on conidia germ tube lengths, there were no effects detected after the post-hoc test. Interestingly, a previous study found that more conidia of *M. robertsii* germinated on the cuticles of *L. decemlineata* larvae infected with *B. thuringiensis*, than on the cuticles of control larvae [17]. This is, however, likely to be an indirect effect of *B. thuringiensis* through the insect host because the two pathogens did not come into direct contact in this experiment [17]. Moreover, Kryukov et al. [19] found that *M. anisopliae* weakly inhibited *B. thuringiensis* growth but this effect waned after 15-20 h [19]. In our experiment, we measured spore viability of *B. thuringiensis* 16 h after application on artificial media, which could be an explanation why we did not find an inhibitory effect of *M. brunneum* on *B. thuringiensis* spore viability.

We found a significant increase in the conidial germ tube lengths at elevated CO_2 concentration, which is in accordance with a previous study that showed faster germination of *M. brunneum* conidia at elevated CO_2 (unpublished data, chapter 4). Increased germination of conidia at elevated CO_2 has also been described for another entomopathogenic fungus (*Entomophaga maimaiga*) [30]. Furthermore, we found a tendency for lower spore viability of *B. thuringiensis* at elevated CO_2, which is comparable to a previous study (unpublished data, chapter 4).

The fact that we found antagonistic interactions between pathogens but no evidence of direct inhibition between the pathogens in the *in vitro* experiments, suggests that there is an indirect interaction via the insect host. In the insect immune system, the Toll pathway is induced in response to fungal pathogens and gram-positive bacteria [31]. Medina Gomez et al. [32] suggested that the Toll pathway in response to *B.*

thuringiensis and *M. anisopliae* is highly expressed in *T. molitor* larvae [32]. The exposure to the faster-acting *B. thuringiensis* therefore might have induced the Toll pathway, which could lead to better protection from infection by *M. brunneum*, a hypothesis that remains to be tested.

The method we describe here to examine interactions between pathogens allows a thorough analysis of potential interactions, which might be dose-level or dose-ratio dependent [24]. Using the MIXTox model affords an opportunity to test interactions between different pathogen species in *T. molitor* or other economically important insect species. In addition, this model could also be used to understand interactions between different environmental stressors or between pathogens and environmental stressors. As the field of insect mass-production is moving forward, methods to quantify risks of different stressors on insect health are essential to protect reared insects and ensure optimised production. Although interactions for different endpoints can be quantified by using the MIXTox model, additional methods are needed to understand the mechanisms underlying the outcomes, such as the measurements we made of direct interactions between pathogens *in vitro*. Further studies could focus on the innate immune response, following mixed infections to shed light on the mechanism or mechanisms involved. Moreover, the interactions between entomopathogens may be time-dependent, and sequential occurrence of different pathogens might result in altered interactions compared to simultaneous occurrence [33].

Our findings demonstrate that interactions between *M. brunneum* and *B. thuringiensis* can range from synergism to antagonism. Notably, most combinations do not significantly increase the risk of disease measured as survival, or total or individual larval biomass in *T. molitor* larvae. The MIXTox model proves to be a powerful tool to investigate co-exposure to pathogens and quantifying risks in mass-reared insects, and identify high risk combination ratios where treating for one of the pathogens would have a higher relative benefit than treating for the other. In addition, this tool may potentially benefit other fields of research, such as biological

control of insect pests, offering potential use in evaluating, and optimising the efficacy of mixed pathogen applications.

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6. Chapter: General discussion

6.1 Outline

In this thesis, I aimed to investigate the impact of the environment on host-parasite interactions in mass-reared insects. I studied the effect of different abiotic and biotic stressors in combination, and provided recommendations for the mass production of insects with a focus on the rearing of *Tenebrio molitor*.

Here, I discuss the findings and challenges of this work in a broader context, and suggest future work directions based on my findings (section 6.2). I offer recommendations for the insect mass-rearing industry for managing environmental conditions and reducing the impact of parasite infection in production systems (section 6.3), and finally provide the key conclusions of this thesis (section 6.4).

6.2 Multiple stressors in mass-reared insects

In chapter 2, I reviewed the literature on environment-host-parasite interactions in mass-reared insects. A major finding of this review was that the environment affects the susceptibility of mass-reared insects to parasites in various ways ranging from positive, neutral, to negative effects. We should thereby aim to take advantage of the positive effects of the environment to reduce insect susceptibility to parasites. Therefore, I developed a workflow, which helps to identify optimised environmental conditions that reduce the impact of parasite infection in mass-reared insect populations. It is important to note that ‘optimum’ is not a straightforward term and it needs to be defined what exactly is optimised, especially as different host and parasite traits may have different ‘optimal’ environmental conditions. Finally, I identified three outstanding questions based on the current knowledge of environment-host-parasite interactions [1]:

1. *Does increased investment into immunity following environmental stress lead to negative impacts in mass-produced insects?*

2. *Can mismatches in parasite and host performance under different environmental conditions (specifically humidity, moisture content, and gas concentrations) be utilised to alter infection outcomes in mass-reared insects?*

3. *How do co-infections alter host and parasite performance under different environmental conditions? What are the implications for mass-rearing insects?*

Each of my experimental studies (chapters 3 – 5) tackles one of the above questions, which I discuss in the following sections.

6.2.1 Heat stress has immediate and persistent effects on immunity and development of *Tenebrio molitor*

Does increased investment into immunity following environmental stress lead to negative impacts in mass-produced insects?

In chapter 3, I aimed to answer the first question by exploring whether heat stress in the larval stage has immediate and persistent impacts on host immunity, development, reproduction, and susceptibility to parasite infection (measured as mortality). One of the key findings of this chapter is that heat stress indeed leads to increased investment into immunity in *T. molitor* larvae, correlating with an increased survival probability after exposure to *Metarhizium brunneum*. This result adds to the current knowledge of the effects of short durations of heat stress on immunity and host-pathogen interactions in insects, which previously mainly have been tested in the lepidopteran *Galleria mellonella* [2-5]. The larvae also had a reduced weight gain in the following five days, which will have a negative impact for mass-rearing. However, the reduction in weight gain was compensated for ten days after the heat stress, and there were no differences in survival probability to *M. brunneum* when exposed five days after the heat stresses, indicating that overall, the effects were only of short duration. This result has practical implications because it

shows that the application of the tested heat stresses are not a useful method to persistently increase the immune response and thereby decrease the susceptibility of mass-reared *T. molitor* larvae to pathogens as previously suggested by other authors [6, 7]. I also found transgenerational effects, such that a short heat stress during the larval stage increased the weight of their subsequent offspring. This work generates new questions. For the understanding of transgenerational effects, it would be beneficial to understand if the differences in weight of the offspring persist during the life cycle of these insects. Furthermore, the hypothesis that higher investment into growth of the offspring may lead to lower investment into immunity and therefore higher susceptibility to pathogens should be tested in the future.

6.2.2 The effect of CO_2 concentrations on entomopathogens and insect-pathogen interactions

Can mismatches in parasite and host performance under different environmental conditions (specifically humidity, moisture content, and gas concentrations) be utilised to alter infection outcomes in mass-reared insects?

In chapter 4, I aimed to answer parts of the second question on how CO_2 affects *M. brunneum*, *Bacillus thuringiensis*, and *T. molitor*. Here, I assessed the effects of elevated CO_2 on the pathogens individually and on the *in vivo* interactions after exposing *T. molitor* larvae to the pathogens. The main goal was to understand if the industrially relevant CO_2 concentrations occurring in mass-rearing systems of *T. molitor* are disadvantageous, beneficial, or irrelevant in regards to the impacts of the pathogens on the larvae. The key finding here is that larvae reared at elevated CO_2 concentrations were less susceptible (measured as mortality) to the two tested pathogens. This result has practical implications for the production of *T. molitor* as it shows that the elevated CO_2 concentrations in mass-rearing systems of *T. molitor* are not disadvantageous for the larvae when exposed to the two tested pathogens. Moreover, no sublethal effects caused by the elevated CO_2 were detected. The increase in germination of *M. brunneum* conidia, and the decrease of viability and persistence of *B. thuringiensis* spores caused by elevated CO_2, did not predict their

virulence when tested *in vivo*. This demonstrates how important *in vivo* bioassays are to measure interactions, but it also shows that we do not currently understand the mechanism of the altered susceptibility of *T. molitor* larvae exposed to elevated CO_2. Future research should therefore investigate if the exposure to elevated CO_2 affects the immune response or the microbiota of the larvae, which may result in altered susceptibility of these insects to parasites. Understanding these mechanisms could improve the optimisation of environmental conditions in mass-rearing systems and understanding of how gases affect the microbiota could improve the way microorganisms used as probiotics are used in mass-reared insects [1, 8].

6.2.3 Double trouble? Quantifying the risk from co-exposure to multiple pathogens in *Tenebrio molitor* at different CO_2 concentrations

How do co-infections alter host and parasite performance under different environmental conditions? What are the implications for mass-rearing insects?

In chapter 5, I aimed to answer the third of the abovementioned questions using *T. molitor* larvae co-exposed to *M. brunneum*, *B. thuringiensis*, and elevated CO_2. Specifically I wanted to understand if the risk of reduced larval mass and mortality changes under co-exposure by using an ecotoxicological MIXTox model.

The outcomes of co-infections have been studied before using various approaches. For example, several authors have compared survival probability curves of insects exposed to individual and mixed pathogen doses together with measuring the relative reproduction of the co-infecting pathogens [e.g., 9, 10]. This approach can be useful when empirically testing biological concepts related to pathogen-pathogen competition and virulence, which previously have been described with theoretical models [11]. Important concepts in this regard are the ‘competitive exclusion principle’ (i.e., “in the competition between two sympatric non-interbreeding populations over the same ecological niche, one will displace the other” [12]) and the ‘trade-off hypothesis’ (i.e., “a trade-off between pathogen traits, in particular, virulence and transmission, such that more rapid host exploitation, which results in

higher transmission, comes at the cost of increasing host mortality, which would curtail the transmission process" [13]).

To understand the actual effects of co-exposure to entomopathogens on the insect hosts, different authors have used the co-toxicity co-efficient ($\frac{observed\ mortality - predicted\ mortality}{predicted\ mortality} \times 100$), which determines the interaction between pathogens based on the mortality of the insects [e.g., 14, 15]. Moreover, the visual comparison of the independent action reference model to the confidence intervals of the observed mortality over time has been successfully used to investigate interactions between a fungal entomopathogen and an insecticide [16]. The comparison of isoboles is another method to evaluate overall interactions between different entomopathogens [17]. These approaches are useful, but they do not detect dose-dependency or switches between antagonism and synergism, which is important in the risk assessments of multiple stressors. The MIXTox model used in chapter 5, in contrast, can be used to detect dose-ratio and dose-level dependencies by analysing the responses from different individual and mixture treatments simultaneously.

The first key finding of this chapter is that the MIXTox model is a useful tool to study interactions between entomopathogens and reveals complex interactions between entomopathogens. This model has previously been used to study the effects of chemical mixtures but is for the first time applied here to understand interactions between pathogens in *T. molitor*. The second key finding is that co-exposure to the tested abiotic and biotic stressors generally did not increase the risk of dying or having a reduced larval biomass in early-stage larval *T. molitor* populations. Interestingly, synergistic interactions between the two pathogens, as described in other coleopteran species [18-21], were only found in very few circumstances in this study. Generally, antagonistic or additive interactions between the pathogens were detected in chapter 5, which adds to the current knowledge of interactions between *B. thuringiensis* and fungal pathogens suggesting that the mechanisms of interaction between the two pathogens is different in *T. molitor* compared to other coleopteran species. Moreover, the *in vitro* interactions between the different stressors did not

predict what happened *in vivo*. Further research is needed to disentangle the mechanism that leads to antagonism between *M. brunneum* and *B. thuringiensis*. Gram-positive bacterial and fungal entomopathogens induce the Toll pathway in the insect's immune response [22, 23] and future studies should test if the faster acting *B. thuringiensis* induces this pathway, which then potentially decreases the susceptibility of *T. molitor* larvae to *M. brunneum*. Understanding the mechanism could then potentially be used to predict which other pathogen combinations result in antagonism. Related to that question is how the interactions change during sequential application of the pathogens. In other systems, it has been shown that the sequential application of pathogens can alter their interactions. In larvae of the velvet bean moth (*Anticarsia gemmatalis*) and the fall armyworm (*Spodoptera frugiperda*), for example, the application of a nucleopolyhedrovirus (NPV) two days before or after the application of *Metarhizium rileyi* generally led to an antagonistic interaction. However, applying the two pathogens simultaneously led to an additive interaction [24]. The hypothesis to be tested in future studies on the system described in chapter 5 of this thesis would be that application of the slower acting *M. brunneum* shortly before *B. thuringiensis* does not result in antagonism.

6.2.4 General findings, challenges, and future directions

We typically think that stressors have negative impacts on organisms, leading to even worse effects when combined with other stressors. For example, in a recently published meta-analysis of combined effects caused by parasites and chemicals in arthropods, 77% of all the combinations showed synergism (i.e., higher mortality than predicted from the reference models) [25]. Reporting bias might be one of the reasons why synergistic interactions between stressors were found in this meta-analysis, with most studies investigating interactions between chemicals and parasites with the aim of reducing the use of chemicals to control pest insects. Moreover, the authors found that the experimental designs used had a larger impact on finding synergistic interactions than biological factors [25]. In all the experimental studies conducted in this thesis, either abiotic (chapters 3 and 4) or biotic (chapter 5) stressors led to cross-protection (i.e., a stressor triggers a response that benefits the host when confronted with another stressor [26]) from lethal or

sublethal pathogen infection. The different outcomes between the results in this thesis and the meta-analysis, might be explained by the different stressors (i.e., chemicals) used in the studies investigated in the meta-analysis. Cross-protection caused by environmental stressors protecting insect hosts from parasite infection seems to be a common phenomenon. Of all the studies that are presented in Table 2.1 (chapter 2), 50% (10 out of 20) reported at least one evidence of cross-protection [1]. Similarly, in a literature search conducted on multiple stressors (abiotic and biotic) of agricultural pest insects 58% (76 out of 130) of the reported experiments revealed cross-protection [27] demonstrating the importance of these protective effects derived from a number of different stressors.

In relation to parasite infection, a central mechanism of cross-protection is the induction of immune signalling pathways by primary stressors as discussed in chapter 2 and shown in chapter 3. However, other mechanisms are also important when it comes to cross-protection in insect hosts. Firstly, primary stressors might induce various biochemical responses, for example as in the induction of antioxidant, cryoprotectant, or heat shock protein (HSP) production [1, 27]. Cytochromes (e.g., cytochrome P450s monooxygenases) and esterases are crucial for the breakdown of pesticides and other xenobiotics [27, 28]. HSPs mediate processes that refold or degrade misfolded proteins and they are induced as a response to various different stressors such as heat, cold, pesticides, crowding [27, 29] but also parasite infection, as introduced in chapter 2. Secondly, environmental stressors may induce epigenetic modifications (i.e., heritable alterations of the gene function or expression without altering the DNA sequence) through DNA methylation, microRNAs, or histone modifications [27]. Epigenetic modifications induced by different stressors might overlap and thereby result in cross-protection [27]. To improve our understanding of the mechanisms behind environment-host-parasite interactions it would be of great value to test the effect of the environmental conditions on biochemical and epigenetic modifications, which then in turn might help to predict how certain parasite species are impacted by the environmental conditions.

The high number of possible interactions between environment, hosts, and parasites may be one of the explanations why it can be difficult to obtain the same results when repeating certain experiments (i.e., conducting the same experiment at different time points using the exact same methods), which was one of the challenges throughout the experimental studies in this thesis. There is therefore a clear need for standardisation of the methods. When working with pathogens, this includes avoiding changes of virulence. Attenuation of virulence occurs in many entomopathogenic fungi species when subcultured on artificial media, for certain strains even after the first subculture [30]. The approach chosen to avoid attenuation of virulence over time was to keep a stock fungal culture at -80˚C, which was consistently used for subcultures for experimental work. Insect cultures, however, have to be reared continually. This might explain why the results of repeated *in vitro* experiments testing the entomopathogens on artificial media were generally more consistent than experiments involving host and parasites. Especially in chapter 5, in which two pathogen species in combination with one environmental stressor were tested at the same time, the two independent experimental repetitions did not result in the same outcomes, even though the trends were largely similar. When investigating the effects of co-exposure of insects to different microorganisms, other authors have also found different outcomes when repeating experiments [e.g., 31, 32]. One way to deal with a lack of repeatability between different experimental repetitions is to report all the repetitions separately, which then show the variation. This is the approach chosen in the cited literature above and in chapter 5.

However, standardisation of experimental conditions and methods is not only important for repeating the same experiment but it would also be beneficial to establish standardised experimental conditions when working with *T. molitor* or other insect species produced for food and feed. Such standard guidelines exist, for example, for research on honeybees [33] or the testing of chemicals on their effects on organisms in the environment [34]. Having standardised protocols for experiments with mass-reared insects would improve the replicability (i.e., “the ability to produce a consistent result with an independent experiment asking the same scientific question” [35]) of experiments, simplify the comparison of outcomes

between different studies, and possibly make the study results more relevant for industrial applications.

Another challenge faced when conducting multiple stressor studies, is that experiments can become very large when testing different concentrations and all the combinations therein. For example, if the aim would be to test the impact of three different stressors with each of them having two concentrations and one control, a full-factorial experiment would consist of 27 treatments (3^3). By adding an additional concentration to each stressor, the full-factorial experiment already consists of 64 treatments (4^3). On the one hand, this forces us to formulate clear hypotheses before designing the experiments because it is often not possible to test all the possible stressor combinations due to time restrictions and logistical constraints for setting up and monitoring of experiments. On the other hand, it limits the conclusions that can be gained from experiments because the interactions are often dependent on the stressor concentrations used as shown in chapter 5. For host-parasite studies, it is therefore crucial to develop methods that allow for efficient exposure of the hosts to the parasite. I exposed the larvae of *T. molitor* individually to *M. brunneum* in chapter 3. However, for the studies described in chapters 4 and 5, I had to develop an alternative method of exposure (i.e., mixing the pathogens directly into the diet) because of the high number of insects and treatments tested. In the future, the monitoring of experiments might be facilitated by new technologies. In a recent study, Majewski et al. [36] presented an artificial intelligence (AI) monitoring system for the mass-rearing of *T. molitor* [36]. Besides delivering data on size of individual insects, this system can also detect dead larvae, pests, remaining feed, chitin, and frass [36]. Using AI would be a way to make data collection in multiple stressor studies more efficient and thereby the number of treatments could be increased while decreasing the amount of manual labour.

The use of novel technologies together with innovative experiments will improve the understanding of host-parasite interactions in mass-reared insects in the future. This knowledge will in turn help to optimise environmental conditions and protect mass-reared insect populations from parasite infection.

6.3 Recommendations for the industry of mass-rearing insects

One of the aims of this thesis was to provide recommendations for the industry of mass-rearing insects (in particular for the production of *T. molitor*). Here I list the specific recommendations gained from the literature review (chapter 2) and the studies on *T. molitor* (chapters 3 – 5):

- The risk of parasite infection in insect mass-rearing systems can be decreased using multiple options such as managing hygiene, quarantine facilities, and selective breeding of insects. However, if these tools are not feasible or too expensive for certain parasite groups or species, optimising the environmental conditions to reduce the risk of parasite infection can be used as an additional tool. The workflow described in chapter 2 (Figure 2.2) can be used to identify optimal environmental conditions.

- Exposure of *T. molitor* larvae to one occurrence of heat stress (2 or 14 hours at a temperature of 38˚C; 10˚C above rearing temperature) is not a useful approach to persistently increase the immunity of *T. molitor* larvae and it is not recommended to be used for this purpose (chapter 3).

- The current maximal allowed CO_2 concentrations for human safety of 5,000 ppm (tested concentrations were 4,500 ± 500 ppm) increase the survival probability of early-stage *T. molitor* larvae exposed to *M. brunneum* or *B. thuringiensis*. It is therefore recommended to keep these larvae at the mentioned elevated CO_2 concentrations (chapter 4).

- The ecotoxicological MIXTox model is a powerful tool to study the interactions between different stressors in a structured and efficient manner. Using this approach is recommended for the risk assessment of multiple stressors occurring at the same time (chapter 5).

6.4 Conclusions

The environmental conditions that mass-reared insects are exposed to play a central role in how the insect hosts interact with parasites. The research presented in this thesis advances our understanding of the impact and mechanisms of environment-host-parasite interactions based on a broad literature review and experimental studies on *T. molitor* larvae. These findings illustrate that the impact of co-occurring stressors should be studied in combination as different stressors interact with each other. The key conclusions from this thesis are:

- By affecting host-parasite interactions, the environment can change the productivity (either measured as survival or larval biomass) of insect mass-rearing systems. The environment affects parasites directly but also indirectly via the host's immune response, development, and microbiota (chapters 2 – 4).

- The adjustment of environmental conditions can be used as a tool to reduce the impact that parasites have in mass-reared insects. This can be done by establishing performance curves for different host and parasite traits over a range of different environmental conditions (chapter 2). Alternatively, specific relevant environmental conditions can be chosen to understand the risk (chapters 3 – 5).

- Environmental stressors (i.e., short heat stress and elevated CO_2) led to cross-protection from pathogen infection shown as decreased mortality of *T. molitor* larvae exposed to entomopathogens (chapters 3 and 4).

- Heat stress during the larval stage has persistent impacts leading to reduced hemocyte concentrations of larvae exposed to *M. brunneum* one week after exposure and increased weight of offspring larvae (chapter 3).

- Cross-protection depends on the duration of the environmental stress and likely on the pathogen dose. A short heat stress (2 h) led to cross-

protection from fungal infection whereas a long heat stress (14 h) did not lead to cross-protection (chapter 3). Elevated CO_2 concentration led to decreased mortality of larvae exposed to either *M. brunneum* or *B. thuringiensis* (chapter 4). However, these results were not reproduced later when using different pathogen doses (chapter 5).

- Elevated CO_2 increases the time to germination of *M. brunneum* conidia and it decreases the viability and persistence of *B. thuringiensis* spores (chapters 4 and 5). However, the indirect effect of CO_2 via *T. molitor* seems to be more relevant for the infection outcome, with larvae reared at elevated CO_2 showing an increased survival probability when exposed to the two pathogens separately (chapter 4).

- The outcome of parasite infection does not only change by adding environmental stressors, but also by adding additional parasite species. Interactions between *M. brunneum* and *B. thuringiensis* on larval survival, biomass, and individual weight gain were mostly antagonistic or additive indicating that the risk of dying and reduction of biomass under co-exposure does not increase (chapter 5).

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Appendices to Chapter 2

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Review

Environment–host–parasite interactions in mass-reared insects

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The mass production of insects is rapidly expanding globally, supporting multiple industrial needs. However, parasite infections in insect mass-production systems can lower productivity and can lead to devastating losses. High rearing densities and artificial environmental conditions in mass-rearing facilities affect the insect hosts as well as their parasites. Environmental conditions such as temperature, gases, light, vibration, and ionizing radiation can affect productivity in insect mass-production facilities by altering insect development and susceptibility to parasites. This review explores the recent literature on environment–host–parasite interactions with a specific focus on mass-reared insect species. Understanding these complex interactions offers opportunities to optimise environmental conditions for the prevention of infectious diseases in mass-reared insects.

Highlights

Mass-reared insects are kept in artificial environments different from their natural habitats. Additionally, insect populations kept at high densities are generally more susceptible to parasites, which can have devastating impacts on insect mass-rearing systems.

Environmental conditions affect parasites directly and indirectly by altering insect immunity, microbiota, development, and reproduction, which are all important aspects in combatting parasites. In this way, host–parasite interactions are altered by the environment.

The environmental conditions in mass-rearing systems can often be precisely controlled. Optimising environmental conditions in insect rearing is therefore a promising tool to reduce the risks caused by parasites in combination with existing hygiene practices.

Parasites and environmental stress in mass-reared insects

For thousands of years, humans have mass-reared domesticated insects such as honeybees and silkworms. The mass rearing of insects on an industrial scale, however, is a relatively new concept, and this burgeoning industry is vital in producing insects for research, pollination services, and biological control of pests and vectors [1,2]. The most recent development is the production of insects, such as flies, mealworms, crickets, and locusts, as a protein source for aquaculture, livestock, and human consumption [1,3,4].

A key threat to insect rearing is the risk of infection by parasites (we use the word 'parasite' to refer collectively to microbial pathogens, macroparasites, and parasitoids). Parasites might be present as covert infections in mass-reared insect populations [5] or they might be introduced via the feed, addition of insect stocks, the air, or wild insects [6]. High prevalence and transmission of parasites are more likely in mass culture than in natural populations as insects are reared at very high densities [3]. Parasites can cause lethal or sublethal effects leading to substantial economic losses [3,7–11] (Box 1).

Mass-reared insects are also exposed to a range of abiotic environmental stressors. High insect densities can lead to elevated temperatures due to **metabolic heat production** (see Glossary), and this may be exacerbated by low air exchange [12–14]. In addition, high insect densities lead to accumulation of carbon dioxide (CO_2) [15–17] and other gases [17] due to respiration. Moreover, relative humidity and moisture content might be increased when insects are kept at high densities [5]. Certain insects (e.g., dipteran, hymenopteran, or orthopteran species) require a supplementary controlled lighting supply during the day [18–20], which can become stressful if the intensity or duration of light exposure are unsuitable. Rearing processes often include transport, handling, and sieving of insects, which result in mechanical vibrations [21]. In addition, ionising radiation is used in the **sterile insect technique (SIT)** which is employed to control insect pests and vectors of human diseases [1,22,23].

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Box 1. Examples of economic losses caused by parasites in mass-reared insects

- Several parasites have caused losses in silkworm production systems since their establishment more than 6000 years ago in China [10]. In more recent times, the largest economic impact on the silkworm industry was caused by the microsporidium *Nosema bombycis* causing the highly lethal disease 'pébrine' in the domestic silkworm (*Bombyx mori*) [90]. *N. bombycis* was first described in 1857, and in 1865 it caused the annihilation of the French and Italian silkworm industries [9,10]. To this day, *N. bombycis* is considered to be the main risk in silkworm production systems [90,91]. The severe impact of this parasite has led to the implementation of hygienic measures and the keeping of eggs and early instars in specialised well-equipped facilities to reduce the risk of infection [10].
- The mite *Varroa destructor* is considered to be the parasite with the biggest economic impact on colonies of the Western honeybee (*Apis mellifera*) [64]. This parasite suppresses its host's immune system, and is a vector of several viruses such as deformed wing virus (DWV) [92]. *V. destructor* came to Europe in the 1970s, and in the 1980s it was found in the USA [93]. Together with other interacting factors, such as pesticides, climate change, and other parasites, *V. destructor* is likely one of the major causes of colony collapse disorder (CCD) [92,94]. A variety of methods are nowadays used to control *V. destructor* ranging from the application of chemical treatments to hygienic practices [10].
- *Acheta domesticus* densovirus (AdDV) can severely affect mass-reared house crickets (*A. domesticus*). This virus was first identified in 1977 in a Swiss mass-rearing facility of *A. domesticus* [95] and has frequently led to devastating epidemics in European mass-rearing facilities [7,11], forcing many producers to discontinue *A. domesticus* production [11]. Since 2009, severe outbreaks of AdDV have also been recorded in Northern America, with losses of hundreds of millions of dollars in the production of *A. domesticus* as pet feed [7]. It is suggested that the virulence of AdDV is increased when the crickets are exposed to other stressors such as crowding, high relative humidity, or temperatures above 35°C [11].

The susceptibility of insect hosts and the virulence of their parasites depend on, and may be altered by, environmental conditions. Because hosts and parasites often have different environmental conditions at which performance is maximised, the impacts of the environment on host–parasite interactions are not trivial [24]. For example, if a host and a parasite have different thermal optima, there will be a mismatch of thermal performance, making the outcome of infection dependent on temperature [24,25]. Different host traits (e.g., growth and immune response) often have different environmental performance curves [26]. In mass-reared insect cultures, these trade-offs need to be considered when optimising environmental conditions for the most relevant host traits.

It is critical to understand how the environment affects host–parasite interactions to avoid losses caused by parasites (Box 1). The effects of abiotic environmental conditions on parasites of mass-reared insects have recently been reviewed [5]. Our aim here, by contrast, is to describe the impact of abiotic environmental conditions on different aspects of the insect host performance and how that may alter the outcomes of host–parasite interactions in mass-reared insects (Figure 1). We identify implications for the mass production of insects and knowledge gaps in this area. We focus predominantly on insect species that are commonly mass reared [1,4], using literature from other insect species in some cases to give a better overview of possible interactions.

Environment and immunity

Different parts of the innate immunity and the behavioural immunity in insects act specifically against different parasite groups (Figure 1). The relationship between environmental conditions and innate immunity can be a result of crosstalk (parasite infection and environmental stress induce the same signalling pathway) or cross-tolerance (the same mechanism protects from both parasite infection and damage by environmental stress) [27]. Cross-tolerance appears to be important in mass-reared insects in relation to temperature as outlined in the following examples.

Effects of temperature on innate immunity

Temperature is the environmental condition most frequently studied in relation to insect immunity (Table 1 and Figure 1). A temperature change can increase or decrease the insect's innate immune

Glossary

Antimicrobial peptide (AMP): a heterogeneous group of short-chained amino acids involved in the insect humoral immune response active against a broad range of parasites.
Blastospores: asexual fungal spores formed from hyphae inside the insect host during the infection process.
Diapause: the period in which insect development is delayed due to adverse environmental conditions, such as cold temperatures during winter.
Ectotherm: an organism that depends on the environmental conditions to regulate its body temperature.
Haemocytes: cells involved in the immune response of insects. Haemocyte concentrations can increase in response to infection in order to encapsulate, phagocytose, or lyse parasites.
Haemolymph: fluid in invertebrates analogous to blood in vertebrates. It contains and transports haemocytes, nutrients, and other compounds.
Heat shock proteins (HSPs): a family of proteins expressed after stress to protect denaturation of polypeptides or helping other proteins to refold.
Host microbiota: the community of microorganisms that exists inside or on the host.
Melanisation: a process involved in parasite encapsulation, formation of cytotoxic components, and wound healing, which results in dark pigmentation of melanised areas.
Metabolic heat production: generation of heat due to physiological processes of the insects.
Phenoloxidase: a key enzyme in the cascade leading to melanisation. Phenoloxidases occur in the haemolymph as prophenoloxidases before they are activated.
Phototaxis: movement of an organism towards a light source.
RNAi: a process in eukaryotic cells in which double-stranded RNA molecules suppress mRNA either for host gene regulation or as a defence mechanism against parasites.
Sterile insect technique (SIT): a method in which mass-reared males of an insect species are treated with ionizing radiation and thereby sterilised. The sterile males are thereafter released in massive numbers to mate with wild females, which then cannot produce offspring.

Symbiont: one partner in an intimate ecological relationship (symbiosis). The relationship can be either mutualistic, commensal, or parasitic.

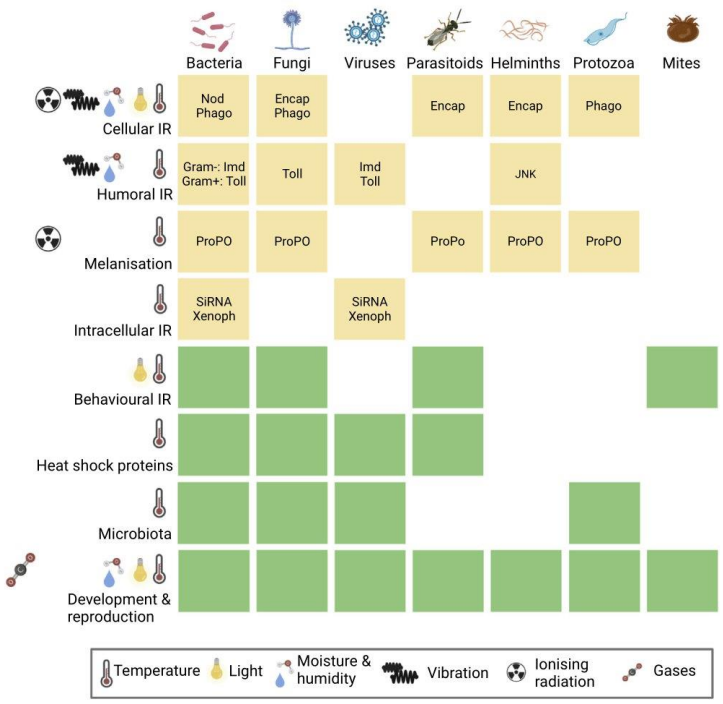

Figure 1. Possible effects of the environment on host–parasite interactions. Different parasites are known to interact with innate immune responses (shown as yellow boxes) and other components (shown as green boxes) of mass-reared insects. Conversely, environmental stressors (left panel, explained in legend) are known to affect the insect hosts, and they can thereby affect the infection outcome. The cellular immune response involves different types of haemocyte. The differentiated haemocytes are formed from stem cells called prohaemocytes, which are released into the haemolymph [68]. Smaller targets can be engulfed by single haemocytes through phagocytosis (Phago). For various bacteria, several haemocytes form nodules (Nod) surrounding the targets, and for larger parasites this process is called encapsulation (Encap). The humoral immune response involves the production of antimicrobial peptides. Different parasites trigger different signal transduction pathways – immunodeficiency (Imd), Toll, and c-Jun-N-terminal kinase (JNK) pathways [99,100]. The cellular and humoral response are both involved in the melanisation process, which is initiated by the activation of the prophenoloxidase (ProPO) pathway to produce phenoloxidase. As a result, the dark pigmented melanin is produced surrounding a parasite in the encapsulation process or around a wound [68,100]. Intracellular immune responses, such as RNAi and xenophagy (Xenoph), are active against viruses and intracellular bacteria [100]. RNAi is regulated by the siRNA pathway [99] and it silences essential parasite genes by producing small RNA sequences by the host, which interfere with the parasite RNA [100].

response, which often correlates with altered susceptibilities to parasites (Table 1). Temperature stress may also have transgenerational effects. Greater wax moth (*Galleria mellonella*) larvae in **diapause** induced by low temperature, for example, had a reduced encapsulation rate in the **haemolymph**, which coincided with a decreased survival probability when exposed to the fungal parasite *Cordyceps militaris* [28]. By contrast, elevated temperature frequently increases innate immune responses, such as **antimicrobial peptide (AMP)** production, increasing survival probabilities of *G. mellonella* larvae when exposed to parasites [29,30]. Interestingly, temperature stress can also induce transgenerational changes in innate immune responses; cold stress applied to the parental

Table 1. Overview of recent studies focusing on the effects of environmental stress on the innate immune response and the impact on susceptibility to parasites of mass-reared insect species[a]

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Refs
Elevated temperature	*Drosophila melanogaster* (common fruit fly)	Female and male larvae and pupae	*Leptopilina boulardi* (parasitoid wasp) strain ISm	↔ Encapsulation	Susceptibility to becoming parasitised: ↔	[67]
			L. boulardi strain ISy	↓ Encapsulation	Susceptibility to becoming parasitised: ↔	[67]
		Female and male adults	*Pseudomonas aeruginosa* (bacterium)	↓ Cuticular melanisation	↑	[48]
		Female adults	*P. aeruginosa*	Expression of immune response genes (↔ Pgrp-LC, ↔ relish, ↔ diptericin)	↔	[65]
			Lactococcus lactis (bacterium)	Expression of immune response genes (↓ spatzle, ↔ cactus, ↔ metchnikowin)	↔	[65]
	Galleria mellonella (greater wax moth)	Female and male larvae	*Candida albicans* (fungus)	↑ Haemocyte conc., ↑ AMP gene expression (gallerimycin, transferrin, inducible metalloproteinase inhibitor, galiomicin)	↓	[29]
			Malassezia furfur (fungus)	↓ Melanisation	↔	[96]
			Metarhizium robertsii (fungus)	↑ Lysozyme-like activity, ↑ PO, ↔ encapsulation, ↓ AMP gene expression (galiomicin and gallerimycin)	↓	[36]
			Streptococcus agalactiae (bacterium)	↔ Cuticular melanisation	↔	[97]
	Megachile rotundata (alfalfa leafcutting bee)	Female and male larvae	*Ascosphaera aggregata* (fungus)	↑ Overall expression of immune response genes	↓	[19]
Reduced temperature	*D. melanogaster*	Female and male larvae and pupae	*L. boulardi* strain ISm	↔ Encapsulation	Susceptibility to becoming parasitised: ↔	[67]
			L. boulardi strain ISy	↑ Encapsulation	Susceptibility to becoming parasitised: ↓	[67]
		Female adults	*P. aeruginosa*	Expression of immune response genes (↑ Pgrp-LC, ↔ relish, ↔ diptericin)	↔	[65]
			L. lactis	Expression of immune response genes (↔ spatzle, ↔ cactus, ↔ metchnikowin)	↔	[65]
			Metarhizium anisopliae (fungus)	↔ Haemocyte conc., ↔ PO, expression of immune response genes (↔ drosomycin, ↔ defensin,↔ diptericin, ↑ Turandot-A, ↔ cecropin, ↔ metchnikowin, ↔ drosocin, ↔ vir-1), ↔ wound-induced melanisation	↔	[98]

(continued on next page)

Table 1. (continued)

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Refs
	G. mellonella	Female and male larvae	*Aspergillus fumigatus* (fungus)	24, 48, or 72 h after stress: ↓↔↔ haemocyte conc., expression of immune response genes (↔↔↔ apolipophorin, ↔↔↔ arylphorin, ↔↔↔ prophenoloxidase)	↑ When exposed to parasite 24 h after stress, ↔ when exposed to parasite 72 h after stress	[54]
			Steinernema feltiae (nematode)	↓ PO, ↑ lysozyme activity	↔	[66]
			Steinernema carpocapsae (nematode)	↓ PO, ↑ lysozyme activity	↓	[66]
			Heterorhabditis bacteriophora (nematode)	↓ PO, ↑ lysozyme activity	↓	[66]
			Bacillus thuringiensis (bacterium)	↓ PO, ↑ lysozyme activity	↑	[66]
			C. albicans	↑ Haemocyte conc., ↑ AMP gene expression (gallerimycin, transferrin, inducible metalloproteinase inhibitor, galiomicin)	↓	[29]
			Cordyceps militaris (fungus)	↓ Antifungal peptide gene expression, ↑ antibacterial peptide gene expression	↑	[73]
			C. militaris	↓ Encapsulation, ↔↓ PO (depending on temp.)	↑	[28]
	M. rotundata	Female and male larvae	*A. aggregata*	↑ Overall expression of immune response genes	↓	[19]
Short (<2 h) elevated temperature	*G. mellonella*	Female and male larvae	*B. thuringiensis*	↑ Antimicrobial activity of larval haemolymph, ↑ expression of gallerimycin, cecropin and galiomicin in the fat body, ↔ expression of the metalloproteinase inhibitor-IMPI	↓	[30]
			Beauveria bassiana (fungus)	↓ Expression of gallerimycin and galiomicin, ↑ lysozyme-like activity, ↑ antifungal activity of haemolymph	↓	[87]
	Tribolium castaneum (red flour beetle)	Female and male adults (exposed to stress), adult offspring (effects measured)	*B. thuringiensis*	↓ PO	↔	[31]
Short (<2 h) reduced temperature	*D. melanogaster*	Female adults	*M. anisopliae*	↑ Haemocyte conc., ↔ PO, expression of immune response genes (↔ drosomycin, ↔ defensin,↑ diptericin, ↑ Turandot-A, ↔ cecropin, ↔ metchnikowin, ↔ drosocin, ↔ vir-1), ↔ wound-induced melanisation	↔	[98]

Table 1. (continued)

Environmental stress	Host species	Host sex and life stage	Parasite species	Stress increases (↑), decreases (↓), or does not affect (↔) immune response	Stress increases (↑), decreases (↓), or does not affect (↔) susceptibility (i.e., susceptibility to death unless otherwise stated) to parasites	Refs
	T. castaneum	Female and male adults (exposed to stress), adult offspring (effects measured)	*B. thuringiensis*	↑ PO	↓	[31]
Fluctuating temperature	*Apis mellifera* (Western honeybee)	Female pupae and adults	*M. anisopliae*	↑ PO	↑	[34]
Vibration	*G. mellonella*	Female and male larvae	*A. fumigatus*	24, 48, or 72 h after stress: ↑↑↔ haemocyte conc., expression of immune response genes (↑↔↔ apolipophorin, ↑↑↔ arylphorin, ↔↔↔ prophenoloxidase)	↓ When exposed to parasite 24 h after stress, ↔ when exposed to parasite 72 h after stress	[54]
			C. albicans	↑ Haemocyte conc., ↑ AMP gene expression (galiomicin, inducible metalloproteinase inhibitor), ↔ AMP gene expression (transferrin, gallerimycin)	↓	[37]
Ionising radiation	*Ceratitis capitata* (Mediterranean fruit fly)	Female and male larvae	*Diachasmimorpha longicaudata* (parasitoid wasp)	↓ Encapsulation	↑	[18]

[a]Abbreviations: AMPs, antimicrobial peptides; conc., concentration; PO, phenoloxidase activity.

generation of red flour beetles (*Tribolium castaneum*) increased **phenoloxidase** activity in their offspring and decreased mortality of the offspring when exposed to a bacterium (*Bacillus thuringiensis*). Moreover, *T. castaneum* larvae from cold-stressed parents had an increased development time until pupation compared with larvae from parents that did not receive a cold stress, which indicates a trade-off between immune response and development [31]. The effect of temperature on intracellular immunity has been studied in mosquitoes (*Aedes aegypti*). **RNAi** was hindered in adult mosquitoes reared at 18°C compared with those reared at 28°C, which coincided with elevated infection levels of chikungunya virus (CHIKV) and yellow fever virus at low temperatures [32].

The investment in immune responses requires energy. However, energy for immune responses has been suggested to be limited when insects cope with thermal stress [33,34], and potentially other environmental stresses. Adult fruit flies (*Drosophila melanogaster*) kept at 18°C, as opposed to 25°C, downregulated the expression of AMP genes. By inducing downregulation in mutant flies independently of temperature, the authors demonstrated that AMP downregulation led to prolonged lifespans and augmented stress resistance, for example, in the case of starvation [35]. Thermal stress can also have the opposite effect, resulting in increased immunity [19,29,30,36,37]. One possible mechanism for this phenomenon is cross-tolerance. Tang *et al.* [38] found that the expression of **heat shock proteins (HSPs)** in housefly (*Musca domestica*) larvae increases after heat stress but also after bacterial challenge (*Escherichia coli* or *Staphylococcus aureus*). Interestingly, a lack of these HSPs (due to silencing of HSP gene expression using RNAi) then led to lower survival after bacterial infection or heat stress, which

proves that the same mechanism protects from heat stress and infection [38]. Similarly, HSP gene expression has been shown to increase after exposure of *D. melanogaster* to RNA viruses [Drosophila C virus (DCV), Cricket paralysis virus (CrPV), or Invertebrate iridescent virus (IIV-6)] [39]. HSPs are traditionally not considered as part of innate immunity in insects. Nevertheless, we know from other invertebrates that HSPs can enhance phagocytosis, increase phenoloxidase production, and protect host protein denaturation during parasite infection [40]. An upregulation of HSP gene expression after infection is thought to be beneficial to the host in silkworm (*Bombyx mori*) eggs parasitised by a parasitoid wasp (*Telenomus theophilae*) [41] or in *G. mellonella* infected with the fungus *Conidiobolus coronatus* [42]. In *B. mori* cells, however, HSPs support the proliferation of *B. mori* nucleopolyhedrovirus (BmNPV) [43,44], demonstrating that the role of HSPs is not always beneficial to the insect host.

Temperature might also affect insect immunity through its effect on the insect cuticle. Yellow mealworm (*Tenebrio molitor*) larvae reared at 28°C had darker cuticles (higher degrees of cuticular **melanisation**) as adults, compared with adults that had been reared at lower temperatures (18°C or 23°C) during their larval stage [45]. In addition, darker beetles also had an increased survival probability compared with lighter beetles when exposed to a fungal parasite (*Metarhizium anisopliae*) [46]. However, it is unclear if this was a result of decreased ability of the fungus to penetrate highly melanised cuticles or if it was because increased melanisation correlates with other innate immune responses [47], leading to the lower mortality in beetles exposed to *M. anisopliae* [46]. In adult *D. melanogaster,* darker cuticles correlated with increased survival when exposed to a bacterium (*Pseudomonas aeruginosa*) [48], a parasite that does not infect the host by penetrating the cuticle. Here, on the contrary, higher temperatures led to lighter cuticles [48]. Melanisation as a result of temperature change possibly demonstrates cross-tolerance [48], as cuticular melanisation is known to play a role in thermoregulation [49] but also in immunity.

Effects of ionising radiation, humidity, light, and vibration on innate immunity

In contrast to our knowledge of the effect of temperature, our understanding of the effects of other environmental conditions on innate immunity in mass-produced insects is limited. The effect of ionising radiation on the immune response has been studied in Mediterranean fruit flies (*Ceratitis capitata*), which are commonly reared for SIT. In contrast to control larvae, irradiated larvae (40 gray – the SI unit of ionising radiation dose) did not accumulate phenoloxidase over the course of development [22]. Similarly, encapsulation rates and adult emergence decreased in *C. capitata* with increasing ionising radiation when parasitised by a parasitoid wasp (*Diachasmimorpha longicaudata*) [18]. Ionising radiation is thought to affect the cellular immune response of insect hosts directly; as **haemocytes** lack pigmentation, they are thought to be sensitive to this type of radiation [23]. This highlights the need to optimise the ionising radiation dose, taking into account sterility, fitness, and other traits.

Humidity affects parasite survival outside the host as well as both parasite virulence [5,50] and host susceptibility in insects. For example, in larvae of the lepidopteran pest species beet armyworm (*Spodoptera exigua*), increasing relative humidity led to decreased antioxidant activities, which correlated with decreased survival when exposed to a nucleopolyhedrovirus (SeNPV) [51]. Conversely, the larvae of the Mediterranean flour moth (*Ephestia kuehniella*) showed increased nodulation at an elevated relative humidity (85%) when infected with *B. thuringiensis* compared with larvae kept at a low relative humidity (43%) [52]. However, the effects of humidity on the immune responses of mass-reared insects remain to be investigated more thoroughly.

The effects of artificial light have been studied in the Australian black field cricket (*Teleogryllus commodus*), which is not commonly mass-reared but used as a model organism. Durrant *et al.* [53] found that dim artificial light during the night decreased haemocyte concentration in the

haemolymph, which could have negative effects on infection outcome in case of an exposure to parasites [53].

In contrast to the decreased immune responses observed in response to ionising radiation and light, short periods of vibration have been shown to increase haemocyte concentrations and the expression of several AMP genes in *G. mellonella* larvae, coinciding with lowered mortalities when exposed to the fungal parasites *Candida albicans* [37] and *Aspergillus fumigatus* [54].

Effects of the environment on behavioural immunity

Environmental conditions are also of high relevance in behavioural immunity (host behaviours that lead to the avoidance or mitigation of parasite infection [55,56]). When given the choice, certain insect species increase their body temperature by seeking places with elevated temperatures to suppress the development of parasites [20,56–58], a phenomenon called behavioural fever or fevering. Adult migratory locusts (*Locusta migratoria*) infected with *M. anisopliae*, for example, had an 85% higher survival rate when given the opportunity to increase their body temperature for at least 4 h per day compared with adults that were not enabled to fever [56]. The reduced mortality rate due to behavioural fever is potentially a combined effect of parasite inhibition at elevated temperatures and the triggering of the immune response of the host (Figure 1). Sangbaramou *et al.* [20] found that *L. migratoria* nymphs exposed to the fungal parasite *Beauveria bassiana* had an elevated haemocyte concentration and an absence of circulating fungal **blastospores** in their haemolymph when given the opportunity to increase their body temperature behaviourally. By contrast, nymphs kept at constant temperatures had fewer haemocytes, and circulating fungal blastospores were observed. This correlated with higher mortality in nymphs kept at constant temperatures compared with fevering nymphs [20].

It is important to note that behavioural fever is not exhibited against all parasite species. Adamo *et al.* [59] found that house crickets (*Acheta domesticus*) increased their body temperature when infected with the Gram-negative bacterium *Rickettsiella grylli*, leading to increased survival. However, when the crickets were infected with another Gram-negative bacterium (*Serratia marcescens*), they did not increase their body temperature. Moreover, increasing the temperature artificially did not increase survival when exposed to *S. marcescens*, demonstrating that behavioural fever can be targeted by the insects against parasites that are affected by the temperature change (i.e., *R. grylli*) but not against parasites that remain unaffected by the temperature increase (i.e., *S. marcescens*) [59]. In adults of *M. domestica*, the intensity of the behavioural fever was found to be positively correlated with the dose of *B. bassiana* they received [60], indicating that insects can optimise the costs and advantages of behavioural fever.

In contrast to increasing the body temperature by fevering, buff-tailed bumblebees (*Bombus terrestris*) actively lower their body temperature by remaining in the field overnight when parasitised with parasitoid conopid flies to delay parasite development [61]. Similarly, adult *D. melanogaster* infected with a fungal (*Metarhizium robertsii*) or bacterial (*P. aeruginosa*) parasite exhibited a preference for cooler temperatures compared with uninfected flies; the survival probabilities of the flies when infected was increased under these cooler temperatures [62,63]. This demonstrates that the ability to raise and to lower temperature behaviourally can be beneficial for infected hosts.

A change in temperature could also act as a cue for parasitism, allowing social insects to react to infested broods. Brood cells of the Western honeybee (*Apis mellifera*) parasitised by mites (*Varroa destructor*) have a higher temperature than nonparasitised cells. Bauer *et al.* [64] therefore suggested that worker bees might use these elevated temperatures as a cue to remove infested

brood cells, a hygienic behaviour that can decrease parasite dispersal inside the beehive. The causal relationship between parasitism, elevated temperature, and brood removal however remains to be demonstrated [64]. A further form of behavioural immunity through **phototaxis** has been shown recently in adult flies (*Drosophila nigrospiracula*) being infested by mites (*Macrocheles subbadius*) whereby the risk of mite infestation is higher in the dark than in the light. Hence, flies demonstrated avoidance behaviour, spending more time in lightened areas than in dark areas when mites were present [55].

Investigating the effects of environment on immunity is complex

To deal with the complexity of the insect immune system, several immune responses can be studied simultaneously and over time [54,65] in combination with experimentally establishing the susceptibility of hosts to their parasites, as done in the studies presented in Table 1. Several factors will define how the environmental conditions affect immune responses and susceptibility to parasites of insect hosts. Different genetic strains of *C. capitata* show, for example, different parasitoid encapsulation rates [18]. Furthermore, immune responses can depend on species (Table 1) and on sex, as shown in *T. molitor* with increased encapsulation rates in females [45] and in *T. commodus* with increased haemocyte concentrations in females [53]. In social insects, the interactions between temperature and immune response are even more complex, as different castes are adapted to different environments. When worker bees, queens, and drones of *A. mellifera* were exposed to a heat stress during the pupal stage, the phenoloxidase activities in the adult stages were either increased, decreased, or remained unchanged, respectively [34]. Last, the effect of the environment on host immunity also depends on the parasite species [66], and the genetic strain of the parasite [67].

Environment, host microbiota, and parasites

Protection from parasites in insects derives not only from their own immune system, but also from their associated **host microbiota** [68], for example, by the production of antimicrobials by bacterial **symbionts** [69]. Additionally, the host microbiota can increase the ability of the host to cope with environmental stress. Adults of *D. melanogaster* exposed to the nonparasitic fungus *Aspergillus oryzae* had an increased survival probability under heat stress [70]. The parasites in turn can also affect the host microbiota. The toxins of *B. thuringiensis*, for example, can alter the gut bacterial community composition and reduce the total bacterial load in the guts of *L. migratoria* [71]. In a recent review, Savio *et al.* [72] found that members of the bacterial genus *Lactobacillus* appear to be of great importance in decreasing susceptibility of mass-reared insects to fungal and bacterial parasites, whereas members of the genera *Wolbachia* and *Spiroplasma* reduce susceptibility to viral infection [72].

Temperature affects host microbiota and thereby infection outcome

Thermal stress can act directly on parasites, but it can also affect the host's microbiota and thereby affect the outcome of infection by parasites. Studies of these interactions need to contend with complex systems and understanding of multiple interactions. For example, bacterial symbionts are suggested to have a temperature-dependent effect on the infection by a fungal parasite (*C. militaris*) in *G. mellonella* larvae. Mortality caused by *C. militaris* infection at high temperature (25°C) was reduced in comparison to lower temperature (15°C). This coincided with an increased abundance of enterococci and enterobacteria (both of which have inhibitory effects on *C. militaris in vitro*) in the haemolymph and in the gut at high temperatures in response to infection [73]. In addition, the host responses to different parasites were also temperature dependent. Expressions of lysozyme genes, which play a role in the Toll and prophenoloxidase pathways mainly active against bacteria and fungi, were increased. However, the expressions of cecropin genes, which play a role in the Imd pathway against Gram-negative bacteria (Figure 1), were decreased

at high temperatures potentially favouring the Gram-negative enterobacteria [73]. Similarly, in common Eastern bumblebees (*Bombus impatiens*), increasing temperature leads to decreased infection intensity of a trypanosomatid parasite (*Crithidia bombi*) [74]. At high temperatures (above 32°C), this can be explained by a direct growth inhibition of *C. bombi* [75]. However, at temperatures below 32°C the parasite is not directly inhibited by temperature and indirect inhibition might stem from acid-producing bacteria [74] (many bacterial gut symbionts of bees transform carbohydrates into short-chained fatty acids, acidifying the gut [76]), which increase their metabolic rates with increasing temperatures [75]. However, the relationship between temperature, host microbiota, and gut pH remains poorly understood [74]. These examples demonstrate that different infection outcomes in response to temperature may result from multiple interactions.

Environment, host development, reproduction, and parasites

Two of the most important parameters in the mass production of insects are development and reproduction, which define the productivity of the systems. As **ectotherms**, the physiology of insects is directly linked to the environmental temperature. Altered development rates due to environmental conditions might lead to a trade-off with immunity [31] as discussed before. However, highest growth rates under conditions optimised for growth, may lead to lowest susceptibility to infection because the parasite dose per host mass decreases with increasing body mass. For example, larger larvae of *G. mellonella* showed decreased mortality when exposed to the bacterium *S. aureus*, which correlated with increased lipid weights in larger larvae [77]. A similar effect might occur when optimising other environmental conditions for higher growth rates. For example, *T. molitor* larvae show increased growth rates when kept in darkness versus alternating light/dark conditions [78] and grow faster with increasing relative air humidity [79]. Larger *T. molitor* larvae show higher survival probabilities than smaller larvae when exposed to *B. thuringiensis* [80], yet the causal relationship between environment, insect body mass, and parasite susceptibility remains to be investigated.

The interaction between reproduction and immunity of the insect host when affected by the environment can lead to different outcomes. First, different energy investments under altered environmental conditions can lead to trade-offs between reproduction and immunity. In brown-banded cockroaches (*Supella longipalpa*) parasitised by an acanthocephalan parasite (*Moniliformis moniliformis*), for example, elevated temperatures led to decreased reproduction compared with healthy cockroaches, whereas at lower temperatures both parasitised and healthy cockroaches had similar reproductive outputs [81]. One possible explanation might be that the immune response is increased at elevated temperature (e.g., in response to increased parasite performance), which leaves less energy for reproduction. Second, reproduction and immunity may both be increased or decreased under environmental stress. *B. impatiens* queens exposed to a short CO_2 narcosis showed increased reproduction and at the same time an increased ability to eliminate bacteria (*Providencia rettgeri*) in their haemolymph [82], which may result in trade-offs with other energy-consuming processes that were not measured. Finally, trade-offs between immunity and reproduction depend on the parasite species. In Texan field crickets (*Gryllus texensis*), differing responses to temperature were shown when infected by different parasites; control crickets kept 7°C above average field temperature showed higher reproduction, as did crickets exposed to a sublethal dose of the Gram-negative bacterium *S. marcescens*. By contrast, when exposed to a sublethal dose of the Gram-positive bacterium *Bacillus cereus*, the elevated temperatures did not lead to higher reproduction [83]. This could not be explained by different thermal optima of the parasites, but the authors suggest that the immune response against *B. cereus* was more energy intensive than that against *S. marcescens,* leading to lower investment in reproduction [83]. These examples demonstrate that the effects of environmental conditions on potential trade-offs between immunity and reproduction and

other important energy-consuming processes need to be carefully assessed before choosing environmental rearing conditions (Figure 2).

Implications and applications for mass rearing of insects

Mass-rearing conditions are set to maximise productivity (i.e., output of nondiseased insects). However, parasitic infections can lead to devastating losses in production systems. Environmental conditions play an essential role in the defences of mass-reared insects to parasites (Figure 1 and Table 1). Adjusting these conditions to increase defences against parasites should therefore be considered when choosing environmental conditions (Figure 2), which will in turn lower the risk of lethal and sublethal effects caused by parasites and maintain productivity of insect mass rearing.

The environmental conditions can be constant or fluctuating throughout the mass-rearing process. Extreme environmental conditions can also appear as 'shocks' (i.e., short-term changes

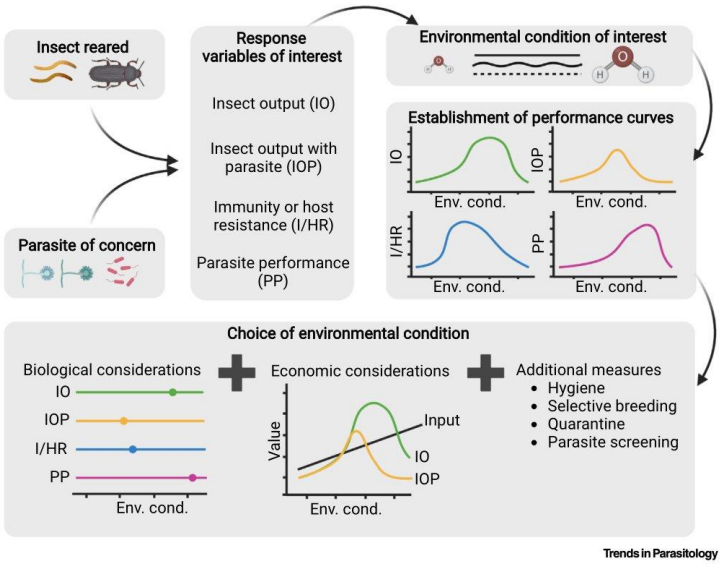

Figure 2. Workflow for choosing environmental conditions in mass-rearing systems. The insect (species, strain, and life stage) reared and the parasite (species and strain) of concern define the response variables to be measured. The response variables should at least consist of a measure of insect output (e.g., insect biomass) when the population is parasite-free and when parasitised, a measure of host resistance or immunity (e.g., encapsulation), and a measure of parasite performance (e.g., germination of fungal spores). Thereafter, the environmental condition of interest is chosen (e.g., humidity is illustrated here). Environmental conditions can remain constant over time (continuous line), occur as fluctuations (wavy line), or as shocks (broken line). The chosen response variables are measured over a range of levels of the environmental condition to establish performance curves (shown as hypothetical curves). Finally, choosing the environmental condition is based on biological and economic considerations, and additional measures that are feasible. Different response variables potentially have different peak performances in response to environmental conditions, which allows choosing a condition that is furthest away from peak parasite performance but closest to maximised insect immunity and output. For the economic considerations, the risk of parasite presence needs to be assessed. In a situation of low parasite infection risk (influenced by additional measures), maximised insect output (green curve) and monetary input value are used for calculating the optimal environmental condition. In a situation of high parasite infection risk (e.g., because of costly additional measures), maximised insect output with parasite infection (yellow curve) and input value are used to identify the optimal environmental condition.

of conditions) (Figure 2). These different regimes of environmental conditions can either be a result of intentional measures to increase productivity or they can be a result of the rearing process itself. Fluctuating temperatures in an innocuous thermal range are often beneficial for a variety of different response variables of insects (e.g., reproduction, growth, thermal tolerance, and development) [26]. Spring field crickets (*Gryllus veletis*) that were acclimatised to fluctuating temperatures had higher survival probabilities when infected with the fungal parasite *Metarhizium brunneum* compared with crickets kept at constant temperatures [25]. Similarly, *T. molitor* larvae had increased antibacterial activity in their haemolymph when exposed to a fluctuating temperature regime (±8°C) compared with larvae kept at constant temperatures [84]. Interestingly, fluctuating temperatures have also been found to mediate the course of coinfections. The fungus *M. anisopliae* is highly virulent to desert locusts (*Schistocerca gregaria*) under constant temperatures (30°C). However, under a fluctuating temperature regime (20–42°C) the survival of *S. gregaria* infected with *M. anisopliae* decreased only when they were additionally exposed to another fungus (*B. bassiana*). This is interesting as *B. bassiana* on its own did not decrease survival under fluctuating temperatures compared with uninfected locusts [85]. In certain systems, the outcomes of host–parasite interactions under fluctuating temperatures can be predicted by calculating the averages of the outcomes at constant maximal and minimal temperatures [25], although this is not always possible in other systems [86].

Insects kept at high densities produce metabolic heat leading to temperature gradients in rearing containers, with the highest temperatures in the centre, compared with the lowest temperatures at the edges [12–14]. Such temperature gradients provide the opportunity for mass-reared insects (i.e., insects of the orders Orthoptera, Diptera, and Hymenoptera) to exhibit behavioural fever or cooling that may lead to avoidance or suppression of parasites. To our knowledge, there have been no studies to date that explore temperature selection in response to parasite infection in mass-rearing settings.

Short thermal or physical shocks can occur during handling of mass-reared insects, such as transportation or during sieving processes. These shocks had beneficial effects on the host immunity and survival when exposed to parasites [29,30,37,87]. Browne *et al.* [54] found that the beneficial effects of thermal and physical shocks peak 24 h and diminish 72 h after the stress [54], which indicates that these short exposures might not be useful to increase immunity over a prolonged period. The regulation of immune response gene expression depends on duration and frequency of thermal shocks [88]. However, the frequent applications of thermal and physical shocks on the immune response and susceptibility of mass-reared insect species have, to our knowledge, not been tested thus far. Such studies would be essential to understand the long-term effects and potential trade-offs in reproduction, growth, or other traits. Furthermore, transgenerational trade-offs between immune response and development are possible [31] and should be considered in the context of using environmental stress to decrease offspring susceptibility to parasites.

In order to make informed decisions regarding choice of environmental conditions, performance curves of relevant response variables are needed. In addition to biological considerations, economic considerations need to be taken into account in any mass-rearing system that is commercially producing insects [26]. Depending on the degree of risk of parasite infection, the chosen environmental conditions may differ to mitigate against infection, whilst maintaining biological and economic optimal outcomes (Figure 2).

Concluding remarks

Environmental conditions affect host–parasite interactions in mass-reared insects directly or indirectly by changing immunity, microbiota, development, and reproduction of the insect hosts. Optimising environmental conditions merely for increased production (i.e., growth and reproduction)

Outstanding questions

Does increased investment into immunity following environmental stress lead to negative impacts in mass-produced insects?

How do coinfections alter host and parasite performance under different environmental conditions? What are the implications for mass-rearing insects?

Can mismatches in parasite and host performance under different environmental conditions (specifically relative humidity, moisture content, and gas concentrations) be utilised to alter infection outcomes in mass-reared insects?

may lead to higher susceptibility to parasites as energy investment into a certain trait (e.g., reproduction) can reduce energy investment into another trait (e.g., immunity) [83]. However, environmental conditions can also be optimised to reduce the risk of parasite infection in mass-rearing systems (Figure 2) whilst maintaining an adequate level of insect quality.

Although our knowledge of how the environment affects host–parasite interactions in mass-reared insect species has expanded recently, several key questions remain (see Outstanding questions). Additional efforts are needed to understand sublethal effects (e.g., effects on weight gain or reproductive output) of environment–host–parasite interactions, as they have a tremendous potential to reduce productivity in the mass production of insects in the long term. Moreover, efforts are needed to investigate key mass-reared insect species as well as the parasite species that are challenges in mass-production systems, as our current knowledge stems from a few model organisms (Table 1). We also need to acknowledge that the environment in which insects are reared is a combination of both differing environmental conditions and potentially multiple parasites infecting hosts simultaneously. Future research should therefore consider how different environmental stressors and parasites interact with each other, as the outcomes of such interactions might not be predictable by studying the stressors or parasites individually [85,89]. There remains a significant dearth of knowledge on how moisture content, gas concentrations, and relative humidity affect host–parasite interactions in mass-reared insect species and how the host microbiota is affected by different environmental conditions. Finally, it should be considered that optimising environmental conditions is one of many options available to maintain insect health in mass-reared systems. The simultaneous use of other interventions and tools (Figure 2) will all help to keep parasites under control in insect mass-rearing systems.

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Declaration of interests

The authors declare no competing interests.

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Appendices to Chapter 3

Assessment of EC_{50} values

Tenebrio molitor larvae were exposed to *M. brunneum* doses (2×10^2, 2×10^3, 2×10^4, 2×10^5, and 2×10^6 conidia per larva) or Triton-X (0.05% v/v) control in the same way as described in chapter 3 either at the start of the experiment or five days after the start of the experiment. Seven replicates (one replicate consisting of one cup with ten larvae) per fungal dose or control were used in this experiment. The survival of larvae 12 days after exposure to *M. brunneum* was analysed using a three-parameter log-logistic model as described in the Appendix of Chapter 5 (Assessment of EC_{50} values). The results are shown in Figure S3.1 and Table S3.1.

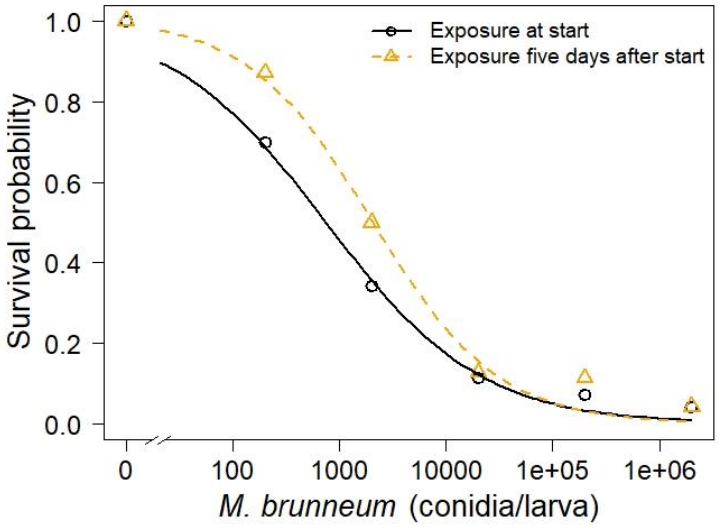

Figure S3.1 Assessment of EC_{50} values.
Three-parameter log-logistic models of *T. molitor* survival when exposed to *M. brunneum* either at the start of the experiment (black, circles) or five days after the start of the experiment (orange, triangles).

Table S3.1 Assessment of EC_{50} values.
EC_{50} values, slopes (β) and upper limits (μ_{max}) of three-parameter log-logistic models of *T. molitor* survival when exposed to *M. brunneum* either at the start of the experiment or five days after the start of the experiment. SE, standard error.

	Exposure at start	Exposure five days after start
[1]$EC_{50} \pm$ SE	738.52 ± 125.31	2005.29 ± 303.13
$\beta \pm$ SE	0.60 ± 0.06	0.74 ± 0.08
$\mu_{max} \pm$ SE	1.00 ± 0.03	1.01 ± 0.03

[1]conidia/larva

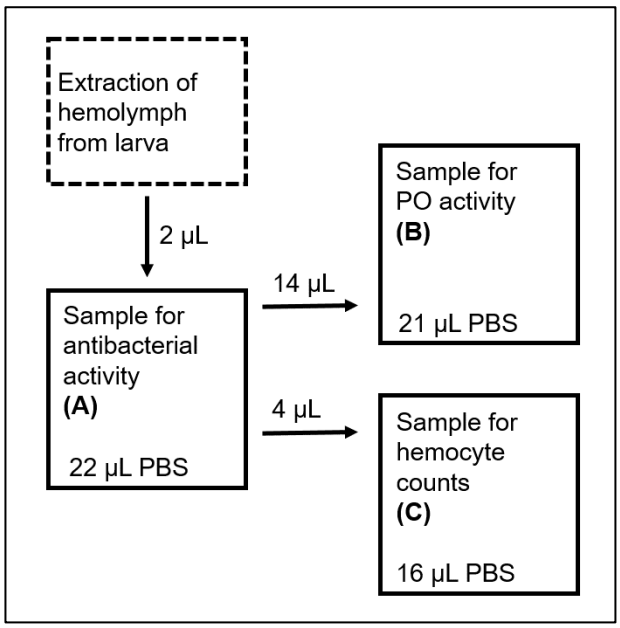

Figure S3.2 Schematic representation of the hemolymph extraction.
From each larva 2 µl was extracted and added to 22 µl PBS **(A)**. This sample was later used for the analysis of antibacterial activity. Thereafter, 14 µl of the diluted sample **(A)** was added to 21 µl PBS **(B)** for the analysis of phenoloxidase activity. Another 4 µl of the diluted sample **(A)** was added to 16 µl PBS **(C)** for the counting of hemocytes. Samples **(A)** and **(B)** were put into liquid nitrogen and subsequently stored at -80°C, whereas sample **(C)** was used immediately after extraction.

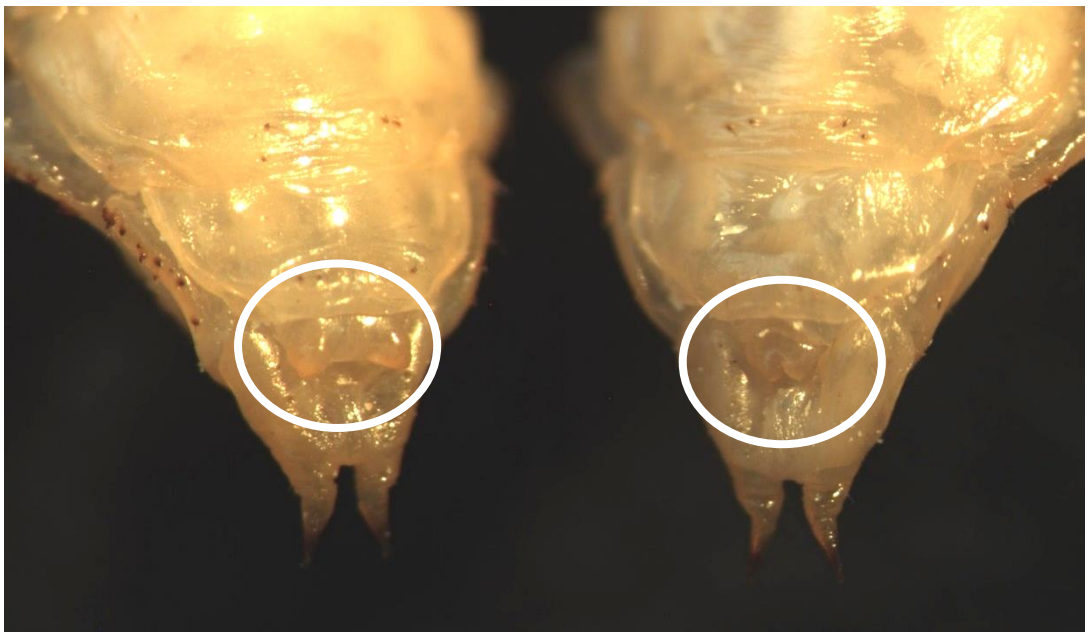

Figure S3.3 Difference between female and male pupae (white circles).
The female pupa (left) has a pair of clearly separated papillae on the seventh visible sternite, whereas the male pupa (right) only has a small swelling on the same location.

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RESEARCH ARTICLE

Heat stress has immediate and persistent effects on immunity and development of *Tenebrio molitor*

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Abstract

The yellow mealworm (*Tenebrio molitor*) is a promising insect species for mass-rearing for the production of feed and food. In mass-production systems, insects may be exposed to abiotic stressors such as heat stress as well as potentially lethal pathogens. To ensure mass-reared *T. molitor* populations are healthy and productive there is a need to understand both the risks, and potential benefits of heat stress, on the fitness of insects and their susceptibility to pathogens. In this study, we investigated the effects of a short (2 h) or a long (14 h) heat stress (38 °C) exposure on the susceptibility and the immune responses of *T. molitor* larvae exposed to a fungal pathogen (*Metarhizium brunneum*). Larvae were exposed to the pathogen either immediately or five days after the heat stress treatments. The development of heat stressed larvae and their offspring was also assessed. A short heat stress immediately before exposure to *M. brunneum* increased the survival probability of *T. molitor* larvae, which correlated with increased antibacterial activity in the hemolymph. The exposure of larvae to short, or long heat stresses five days before pathogen exposure did not affect their survival, despite a temporary lowered body mass gain of heat stressed larvae. However, heat stressed larvae showed decreased hemocyte concentrations when exposed to *M. brunneum*. We also found an increased body weight in larval offspring of females that had been exposed to a short heat stress as larvae themselves. These findings demonstrate the importance of understanding the effects of heat stress in the long-term. The beneficial effects of heat stress on pathogen susceptibility in *T. molitor* and the negative effects on body mass gain are only transient, whereas negative effects on immune response (hemocyte concentrations) persist over an extended period.

Keywords

immune response – *Metarhizium brunneum* – pathogen – parasite – yellow mealworm

1 Introduction

The mass-rearing of insects for human consumption and livestock feed is a growing sector as insects are a promising sustainable source of protein (Van Huis, 2021). Insects generally are more efficient than conventional livestock in converting their feed substrate into body mass and they require less water to produce an equivalent mass of protein (van Huis, 2013). Moreover, insects produce less greenhouse gases than for example

pigs and chickens, and they are able to convert organic materials from waste streams into proteins (Sogari *et al.*, 2019; Van Huis, 2021).

The yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), is one of the most important species currently mass-produced for food and feed purposes. In addition, it is an important model organism (Grau *et al.*, 2017), a pest of stored grains (Vigneron *et al.*, 2019), and it has been shown to be useful for biodegradation of plastics (Brandon *et al.*, 2018; Bulak *et al.*, 2021).

A major challenge when mass-rearing *T. molitor* is the risk of infections by entomopathogens (Eilenberg *et al.*, 2015; Slowik *et al.*, 2023). Such infections may be lethal or sublethal for the insects, and can lead to significant and severe economic losses when insects are being mass-reared for food and feed (Eilenberg *et al.*, 2015). High prevalence and spread of entomopathogens are more likely in cultured insects than in the wild, due to high rearing densities which facilitate transmission between infected and non-infected hosts (Eilenberg and Jensen, 2018; Shapiro-Ilan *et al.*, 2012).

In addition to biotic stressors [we use the term 'stressor' to describe a "variable that causes a quantifiable change, irrespective of its direction (increase or decrease), in a biological response" (Orr *et al.*, 2020)], mass-reared insects are also exposed to environmental stressors (Herren *et al.*, 2023). Temperature is one of the most important environmental factors determining insect growth (Cossins and Bowler, 1987), metabolic rate, and body composition (Bjorge *et al.*, 2018). Mass-reared insects are kept at high densities and they can therefore be exposed to elevated temperatures due to accumulated metabolic heat (Deruytter *et al.*, 2022; Morales-Ramos and Rojas, 2015). Furthermore, *T. molitor* can absorb water vapour from the environment (Hansen *et al.*, 2004), which is an energy-consuming process that also produces metabolic heat (Hansen *et al.*, 2006). The highest growth rates in *T. molitor* larvae were recorded at a temperature of 31 °C (Bjorge *et al.*, 2018). However, it has been reported that at high densities *T. molitor* larvae elevate the temperature inside rearing containers by 5-10 °C (Morales-Ramos and Rojas, 2015) or even 14 °C above the set rearing temperature (Deruytter *et al.*, 2022). Such hotspots of temperature may be present in a rearing crate for a short amount of time (e.g. when detected quickly and the densities in the rearing crates are adjusted) or the conditions can occur for a prolonged period (e.g. when remaining undetected overnight).

Besides affecting the growth and metabolism, temperature has also been shown to affect both immune response and susceptibility to pathogens in insects (Browne *et al.*, 2014; Kryukov *et al.*, 2018; Sheehan *et al.*, 2020; Vogel *et al.*, 2022). Insect immunity can be broadly divided into cellular and humoral immune responses. Hemocytes are the main component of the cellular response to infection in insects. These cells can kill pathogens through encapsulation or phagocytosis (Lundgren and Jurat-Fuentes, 2012). Oenocytes (a type of hemocyte) produce prophenoloxidase (Vigneron *et al.*, 2019), which is the zymogen of phenoloxidase, an important enzyme in the melanisation process, which begins when prophenoloxidase turns into phenoloxidase, only minutes after infection (Royet, 2004). Thereafter, plasmatocytes (a type of hemocyte) are recruited to the pathogen. Through lysis of the plasmatocytes, melanin is released and encapsulates the pathogen (Castrillo, 2018). Another important part of insect immunity is the humoral immune response, which involves the production of AMPs (antimicrobial peptides) that are active against bacteria and fungi (Castrillo, 2018). *Tenebrio molitor* larvae kept at 30 °C were found to have increased phenoloxidase activity and antibacterial responses compared to larvae kept at 10 or 20 °C (Catalán *et al.*, 2012). Moreover, increasing temperature (18, 23 and 28 °C) led to an increased cuticle darkness (an index of melanisation of the cuticle) of adult *T. molitor* (Prokkola *et al.*, 2013), which previously has been linked to increased resistance to the entomopathogenic fungus *Metarhizium anisopliae* (Barnes and Siva-Jothy, 2000). Increased cuticular melanisation also correlated with increased phenoloxidase activity and increased hemocyte concentrations in the hemolymph (Armitage and Siva-Jothy, 2005). The effects of temperatures above 30 °C on immune response and host-pathogen interactions in *T. molitor* are currently not known. However, the effect of heat stress has been studied in the lepidopteran species *Galleria mellonella*, which has a similar optimal temperature for larval development (29-33 °C) as *T. molitor* (Wojda *et al.*, 2020). In *G. mellonella*, heat stress generally leads to an increased immune response and decreased susceptibility to different fungal and bacterial pathogens (Browne *et al.*, 2014; Mowlds and Kavanagh, 2008; Wojda and Taszłow, 2013).

Because of the potential beneficial impacts of temperature stress on the immune response of insects, some authors have suggested using temperature stress to increase the immunity of mass-reared insects (Grau *et al.*, 2017; Maciel-Vergara *et al.*, 2021). However, it remains unclear the time for which any beneficial effects of heat stress on immunity persist. Moreover,

temperature can affect host-pathogen interactions indirectly by affecting the development of insects and thereby influencing their susceptibility to pathogens. In *G. mellonella*, for example, the susceptibility to pathogens decreases with increasing body mass and length of the larvae (Hesketh-Best *et al.*, 2021). Hence, insects that have a slower body mass gain due to temperature stress might be at risk over a longer period to acquire a lethal infection. Furthermore, temperature can affect the timing, as well as the number of moults in the larval development of *T. molitor* (Ribeiro *et al.*, 2018). Moulting may reduce the risk of infection following exposure to fungal pathogens, because conidia are removed with the moulted exoskeleton before they can penetrate the cuticle (Kim and Roberts, 2012; Vandenberg *et al.*, 1998; Vestergaard *et al.*, 1995). However, after moulting the new cuticle might be more susceptible to fungal penetration until it is completely sclerotized and melanised (Kanyile *et al.*, 2022).

We hypothesized that heat stress will have immediate beneficial effects on immunity and pathogen susceptibility of *T. molitor* larvae, as shown in other insect species. Furthermore, we hypothesized that the increased energy investment in immunity after heat stress will have persistent negative effects on fitness, pathogen susceptibility, and immunity of the larvae. In this study, the generalist fungal entomopathogen *Metarhizium brunneum* was used to test pathogen susceptibility of *T. molitor* larvae. *Tenebrio molitor* larvae can be naturally infected by fungi of the genus *Metarhizium* (Steinwender *et al.*, 2014). Wakil *et al.* (2014) found that these fungi can be present in stored grains (Wakil *et al.*, 2014), which makes them important pathogens in the production process of *T. molitor*, because stored grains are commonly utilised for feed in mass-rearing facilities of *T. molitor* (Cortes Ortiz *et al.*, 2016).

The research reported here documents the susceptibility (i.e. survival) of *T. molitor* larvae when exposed to a fungal pathogen immediately after the exposure to either of two heat stresses (short 2 h or long 14 h) or exposed to the pathogen five days following exposure to the heat stresses. Secondly, the immune responses (phenoloxidase activity, hemocyte concentration and antibacterial activity of the hemolymph) and moulting of larvae exposed to the same combination of fungal pathogen and heat stresses are measured. Finally, the development (i.e. weight gain, pupal weight, time until pupation, and number of exuviae) of heat stressed larvae and their offspring (i.e. number of offspring and weight of larval offspring) are assessed.

2 Materials and methods

Experimental design overview

The larvae of *T. molitor* were exposed to *M. brunneum* immediately, or five days after exposure to a short (2 h), or a long (14 h) heat stress (38 °C) (Figure 1). All insects were constantly kept at 28 °C (except during the heat stress treatments) for the remaining duration of the experiment. One group of larvae was exposed to a lethal dose (LD_{50}, previously established in pre-experimental bioassays) of *M. brunneum* immediately after the heat stress treatments. A control group of larvae was not exposed to *M. brunneum*. Similarly, two other groups of larvae were exposed to either *M. brunneum* (LD_{50}) or no *M. brunneum* five days after the heat stress treatments. Subsequently, survival was assessed in all groups daily for 12 days. To compare the immune responses in the different treatment groups, measurements of hemocyte concentration, phenoloxidase activity, and antibacterial activity were made two days after *M. brunneum* or control exposure in all four groups. Similarly, shed exuviae were counted two days after exposure to *M. brunneum* or control treatments.

To compare the development and reproduction of larvae exposed to heat stress (without *M. brunneum* treatment), larval mass (one day before, and five and 10 days after heat stresses), moulting frequency, development duration until pupation, pupal weight, number, and weight of offspring were recorded.

Insect culture

Tenebrio molitor larvae were initially sourced from the company Ÿnsect (Evry, France). The insects were kept in continual laboratory culture over more than five generations before the start of the experiments. Adult *T. molitor* (40 females and 40 males) were kept in 750 ml plastic containers (15 cm × 9.5 cm = 142.5 cm^2) containing 100 g diet for one week. The diet was provided by Ÿnsect and consisted of wheat bran (35.9%), corn dried distillers grains (30%), wheat (23.7%), and beer yeast (10.4%). The adults were fed with 5 g bacteriological water agar (1% w/v) twice a week. The containers containing eggs and hatching larvae received 10 g bacteriological water agar (1% w/v) twice a week starting one week after removal of the adults. Three weeks after removal of adults, 50 g of diet was added to each container. All breeding and rearing took place at 28 °C (±0.5 °C) in complete darkness. Open containers with water (560 cm^2 surface) were placed in the top of the incubator to maintain a relative humidity of approximately 65% (±5%) and filled with fresh water once a

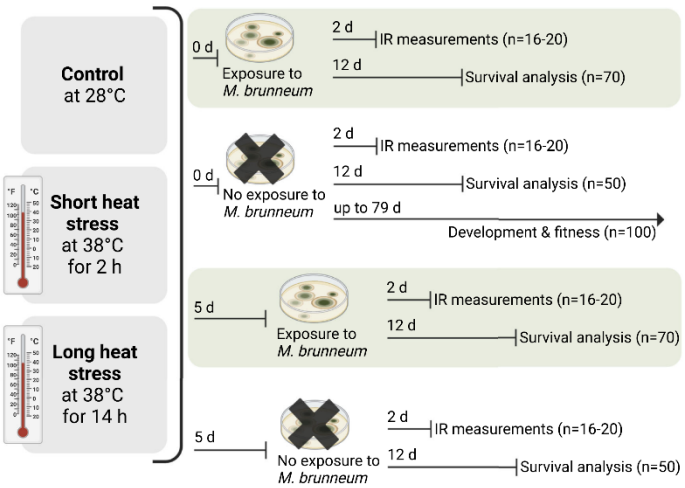


FIGURE 1 Schematic representation of the experimental design. *Tenebrio molitor* larvae were either exposed to a control (28 °C), a short (2 h), or a long (14 h) heat stress (38 °C) and all groups then remained at 28 °C. Two groups of larvae were either exposed to a lethal dose of *M. brunneum* or a control treatment immediately after the heat stress treatments. Similarly, two other groups were exposed to the lethal *M. brunneum* dose or control treatment five days after the heat stress treatments. The subsequent survival was assessed daily for 12 days in all groups and immune responses (IR) in the different treatments were measured two days after the *M. brunneum* exposure together with the measurement of moulting. Additionally, development and fitness were measured in a group of larvae exposed to the different heat stress treatments but not exposed to *M. brunneum*.

week. The temperature and relative air humidity in the incubators were monitored every 15 min using EasyLog™ EL-SIE-2 dataloggers (Lascar Electronics Ltd., Wiltshire, UK) throughout the breeding, rearing, and during experiments.

Exposure of larvae to heat stress

The larvae for experiments were chosen based on age and individual weight, rather than larval instars, because it has been shown that common methods to determine instars are unreliable in *T. molitor* (Morales-Ramos *et al.*, 2015). Larvae (29 days after removal of adults), weighing 40-65 mg/larva, were placed in groups of 10 larvae in transparent cups (5.2 cm diameter) each containing 6 g diet and 1 g bacteriological water agar (1% w/v) for one day at 28 °C (±0.5 °C). Thereafter, the larvae were exposed to either a short heat stress [38 °C (±0.5 °C) for 2 h, 65% RH (±5%)], a long heat stress [38 °C (±1 °C) for 14 h, 65% RH (±5%)], or no heat stress [constant 28 °C (±0.5 °C), 65% (±5%)]. All groups of larvae were immediately returned to an incubator at 28 °C (±0.5 °C) after treatments were applied.

Exposure to Metarhizium brunneum

Metarhizium brunneum isolate KVL12-30 (culture collection of the Department of Plant and Environmental Sciences, University of Copenhagen, Copenhagen, Denmark) was grown on Petri dishes (9 cm diameter, triple vented, Thermo Fisher Scientific, Waltham, MA, USA) containing Sabouraud Dextrose Agar (SDA) media (65 g/l) at 23 °C (±0.2 °C) in complete darkness for 21 days and sealed with Parafilm™ (Merck, Rahway, NJ, USA). These were considered the first subcultures (one transfer since stock culture). To harvest conidia, 10 ml of Triton-X (0.05% v/v, Merck, Rahway, NJ, USA) was added to each of five Petri dishes, and conidia were harvested using a Drigalski spatula (Heathrow Scientific, Vernon Hills, IL, USA). The subsequent suspension containing the conidia was then poured into a 50 ml Falcon tube and centrifuged at 3,000 rpm (1,872 *g*, centrifuge 5810 R, Eppendorf, Stevenage, UK) for 3 min. Afterwards, the supernatant was discarded and 20 ml of Triton-X (0.05% v/v) was added to the Falcon tube and the centrifuging step was repeated with the settings as above. The supernatant was again discarded and another 20 ml of Triton-X (0.05% v/v) was added to the tube. The

obtained stock suspensions were then diluted ten times by adding 0.1 ml of each stock suspension to 0.9 ml Triton-X (0.05% v/v). This was repeated twice to obtain a 1000-times diluted suspension. From this suspension, 20 µl was added to a 0.2 mm Fuchs-Rosenthal hemocytometer (Thermo Fisher Scientific, Waltham, MA, USA) and the conidia were counted under a light microscope, at 400 times magnification.

Conidial viability was assessed by spreading 100 µl of 10^6 conidia/ml on each of three Petri dishes with SDA, which were incubated at 28 °C (±0.5 °C) for 18 h. Thereafter, 300 conidia were counted on each Petri dish and the numbers of germinated and un-germinated conidia were noted. A conidium was considered as germinated when it had a germ tube at least as long as the smallest diameter of the conidium.

The cups containing larvae were checked for shed exuviae before putting the larvae in new cups (5.2 cm diameter) in groups of 10 (same groups as before) containing a filter paper in the bottom (3 × 3 cm) moistened with 0.15 ml sterile water and a soaked filter paper (2 × 5 cm) on the inner side of the cup. The larvae that were exposed to *M. brunneum* immediately after the heat stress received 2 µl of 3.5×10^5 conidia/ml suspension (7×10^2 conidia per larva), directly applied on the metathorax of each larva using a pipette. The larvae in the control treatments were exposed in the same way to 2 µl Triton-X (0.05% v/v). The cups were closed with ventilated lids to ensure high air humidity. After 24 h, the larvae (same groups as before) were moved to new cups containing 6 g diet and 1 g bacteriological water agar (1% w/v) without lids. The cups were checked daily for shed exuviae during the first two days. Twice a week, 1 g bacteriological water agar (1% w/v) was added to each cup.

The larvae that were exposed to *M. brunneum* five days after the heat stress were treated in the same way as the larvae that were exposed to *M. brunneum* immediately after the heat stress, except that they received 2 µl of 10^6 conidia/ml suspension (2×10^3 conidia per larva) instead of 3.5×10^5 conidia/ml. These different *M. brunneum* doses, at different time points, were chosen based on data from previous pre-experimental bioassays to achieve similar survival rates in the *M. brunneum* groups not exposed to heat stress treatments.

Survival of larvae

The survival of the larvae was checked daily for 12 days and dead larvae were removed from cups. To check the infection status of the larvae, cadavers were surface sterilized by dipping them in a Sodium Hypochlorite (NaClO) solution (2-3% v/v) for 20 s. They were then rinsed two times in sterile water and put individually in Petri dishes (3 cm diameter) for 24 h. Next, wet filter papers were added inside the lids of the Petri dishes and they were sealed with Parafilm™. The cadavers were checked visually for fungal outgrowth and sporulation every second day.

Immune response measurements

Extraction of hemolymph from larvae

Hemolymph samples were taken from larvae two days after exposure to *M. brunneum*, which is long enough to allow the fungus to germinate, penetrate the cuticle, and cause an immune response, but before any mortality occurs in the larvae. The hemolymph samples were used to measure antibacterial activity, phenoloxidase activity, and hemocyte concentration. Three Eppendorf tubes (0.5 ml, Merck, Rahway, NJ, USA) were prepared for each hemolymph sample. In the first Eppendorf tube 22 µl PBS (phosphate-buffered saline; 0.01 M) (A), in the second Eppendorf tube 21 µl PBS (B), and in the third Eppendorf tube 16 µl PBS (C) was added (Supplementary Figure S1). All Eppendorf tubes were kept on ice during hemolymph extraction.

The larvae were held using forceps so that they could not move, and the tibia of the posterior left leg was severed using forceps so that a droplet of clear hemolymph formed. Immediately afterwards, 2 µl of hemolymph was extracted and added to Eppendorf tube (A) using a pipette. The Eppendorf tube (A) was vortexed and afterwards 14 µl of this sample was added to Eppendorf tube (B), which was put immediately in liquid nitrogen to analyse phenoloxidase activity later. Another 4 µl from Eppendorf tube (A) was added to Eppendorf tube (C). Eppendorf tube (A) was then put in liquid nitrogen to analyse antibacterial activity and the sample in Eppendorf tube (C) was used for counting hemocytes (Supplementary Figure S1). Once all the samples were collected, the Eppendorf tubes (A) and (B) were transferred from liquid nitrogen to a freezer at –80 °C.

A total of 20 hemolymph samples were extracted in each treatment originating from larvae from four different cups (five larvae per cup). If samples contained additional tissue fragments smaller than hemocytes, they were considered most likely not pure and they were therefore excluded from analysis resulting in variable sample sizes (hemolymph from 16-20 larvae per treatment was analysed).

Antibacterial activity

The antibacterial activity of hemolymph is an indication of the content of AMPs in a sample (Haine *et al.*, 2008; Hultmark *et al.*, 1982). The method was adapted from Haine *et al.* (2008). This method is used to measure relative antibacterial activity in hemolymph, expressed as the diameter of inhibited growth of the bacterium *Arthrobacter globiformis*. The squared diameter of the inhibited zone is linear to the log of AMP concentration in the hemolymph (Hultmark *et al.*, 1982). However, Haine *et al.* (2008) suggested measuring antibacterial activity as the diameter of the inhibited zone "to avoid compounding marginal measurement errors by multiplying them" (Haine *et al.*, 2008).

One colony of *A. globiformis* isolate 20124 previously grown on solid P1 media (15 g bacteriological agar, 10 g peptone, 5 g yeast extract, 5 g glucose, and 5 g NaCl in 1 l dH_2O) at 30 °C was added to a 250 ml Erlenmeyer flask containing 25 ml liquid P1 media (10 g peptone, 5 g yeast extract, 5 g glucose and 5 g NaCl in 1 l dH_2O). The Erlenmeyer flask was incubated on a platform shaker at 200 rpm at 30 °C in complete darkness. After 24 h, the bacterial suspension was poured into a 50 ml Falcon tube and centrifuged for 10 min at 3,000 rpm (1,872 *g*, centrifuge 5810 R) at 4 °C. The supernatant was discarded and 20 ml sterile dH_2O was added. The centrifuging step was repeated with the settings mentioned above. The supernatant was again discarded and another 20 ml sterile dH_2O was added to the Falcon tube. The obtained stock suspension was serially diluted ten times by adding 0.1 ml of the stock suspension to 0.9 ml sterile dH_2O to obtain 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 times dilutions. Subsequently, 10 µl of each of the six latter dilutions were pipetted on solid P1 media (three dilutions per Petri dish). By tilting the Petri dish (9 cm diameter, triple vented) on one side, the suspensions ran down on the media forming straight lines. Three replicates per dilution were prepared. The Petri dishes were incubated at 30 °C and the stock suspension was kept in the fridge in the meantime. After 48 h, the colony forming units (cfu) from at least three different dilutions were counted and the concentrations in the different dilutions were calculated using the formula:

$$\frac{cfu}{ml} = \frac{Counted\ cfu}{0.01\ ml} * Dilution\ factor$$

Thereafter, the average of at least three concentrations was calculated. Solid P1 medium was autoclaved and afterwards put in a water bath at 45 °C. A bacterial suspension of *A. globiformis* containing 10^7 cfu/ml was prepared in dH_2O from the stock suspension. Once the P1 medium reached 45 °C, 10 µl of the prepared bacterial suspension was added per ml of medium. Thereafter, the medium containing the bacterial suspension was put on a magnetic stirrer and Petri dishes (9 cm diameter, triple vented) with 5 ml medium were prepared. In the meantime, the samples from Eppendorf (A) were thawed on ice. Once the Petri dishes were dry, a pipette with a 2 ml tip was used to form eight holes (2.5 mm diameter) in the medium within each Petri dish. Thereafter, the holes were labelled on the outside and 2 µl of the hemolymph samples was added to each hole (2 µl of PBS was added to a hole of each Petri dish as a control; seven hemolymph samples and one PBS control per Petri dish). Two technical replicates (originating from the same biological replicate, in this case the same larva) were prepared on two different Petri dishes and the Petri dishes were sealed with Parafilm™. After incubation at 30 °C for 48 h, two perpendicular diameters of the inhibited zones were measured using a digital caliper. The inhibited zone was calculated as the average of the two diameters minus the average of the two diameters of the well of the control of the same plate. The average of the two technical replicates was used as one data point.

Measurement of phenoloxidase activity

The samples in Eppendorf tubes (B) were stored at −80 °C for at least 24 h. L-DOPA (L-3,4-dihydroxyphenylalanine; 20 mM; 4 mg/ml) was prepared in dH_2O and put on a magnetic stirrer until it dissolved completely. Thereafter, frozen samples were thawed on ice for 30 min. Each sample was vortexed and afterwards centrifuged for 10 min at 6,500 rpm (3,873 *g*, Microfuge 20R, Beckman Coulter Ltd., High Wycombe, UK) at 4 °C. This step disposed of cell debris in the samples, which potentially could inhibit the reaction between phenoloxidase and L-DOPA. Afterwards, two technical replicates per sample were prepared on the same 96-well plate by pipetting 10 µl of the sample per well. Next, 10 µl of PBS was added in each of five control wells per 96-well plate. A volume of 90 µl of L-DOPA was added to each well of the hemolymph sample and PBS controls. The 96-well plate was read using a Synergy™ HT MultiDetection Microplate Reader (BioTek®, Winooski, VT, USA) with Gen5 software (BioTek®, Winooski, VT, USA). The OD (optical density) was measured every minute for 90 min using a wavelength of 490 nm at 30 °C. The Vmax (maximal velocity of the reaction) was measured as the slope during 15 min of the linear phase. Vmax was

expressed as ΔOD490 = milliunit/min. The average of the two technical replicates was used as one data point.

Counting of hemocytes

The samples from Eppendorf tubes (C) were vortexed and 14 µl of the sample was immediately pipetted on a Fast-read102 counting slide (Immune Systems Ltd., Devon, UK). The hemocytes were left to settle for 1 min and afterwards, the hemocytes from all ten squares (one square containing 16 grids) were counted on the counting slide. The resultant hemocyte concentration was calculated using the formula:

$$Hemocytes/\mu l = \frac{Average\ number\ of\ hemocytes\ per\ square}{0.1\,\mu l} * 60$$

The volume above each square is 0.1 µl and the sample was 60 times diluted. The average of the counts from the ten squares was used as one data point. Even though there are multiple types of hemocytes present in the hemolymph of *T. molitor*, we decided to focus on the total number rather than distinguishing between the different types.

Development and fitness measurements

Larvae from each of the heat treatments were weighed per cup (10 larvae together) one day before then five and 10 days after exposure to the heat stress treatments. The weight gain per 10 larvae was calculated as the weight of the larvae five, or 10 days after the heat stress minus the weight one day before the heat stress. During the larval development, 1 g bacteriological water agar (1% w/v) was added twice a week. The shed exuviae were counted and removed every second day and larvae were also checked for pupation every second day. Newly emerged pupae were weighed and the sex of each pupa was noted. Males and females were distinguished by examination of the developing genital structures on the ventral side of the eighth abdominal segment. The male has only a small swelling on the seventh visible sternite, whereas the female has a pair of clearly separated papillae on the sternite (Bhattacharya *et al.*, 1970) (Supplementary Figure S2). The first 25 male and female pupae in each heat stress treatment were put individually in separate 50 ml Falcon tubes, each containing 1 g of diet. These pupae were checked every second day for adult emergence after which they remained in their tubes for four to eight days. Thereafter, one male and one female beetle of the same heat treatment (n = 25) were put together in a 50 ml Falcon tube containing 7 g of diet. The pairs were moved to new Falcon tubes with 7 g diet every week for three weeks and they were provided with 0.5 g bacteriological water agar (1% w/v) twice a week. After three weeks, the adult beetles were removed. The larvae in each Falcon tube were counted and weighed in groups, 23 days after the adult beetles were removed.

Statistical analysis

The experimental investigation of survival, immune responses, and moulting of larvae after exposure to *M. brunneum* and heat stresses were performed on two independent occasions (included as random effects in all statistical models), whereas development and fitness measurements of larvae only exposed to heat stresses were performed once. Differences were considered significant at $P < 0.05$. Data was only subjected to one- or two-way ANOVAs (analysis of variances) when normality (QQ-plots) and homogeneity of variances (Levene test, $P > 0.05$) assumptions were satisfied. All statistical analyses were performed with R v. 4.1.0 (R Core Team, 2021), Figure 1 was prepared with Biorender (Anonymous, 2022a), and Figures 2-6 were prepared with GraphPad Prism version 9.3.1 (Anonymous, 2022b).

Survival analysis

Survival analyses of larvae exposed to *M. brunneum* immediately and five days after heat stress were performed separately using the survival (Therneau, 2021), and the coxme (Therneau, 2020) packages in R. Only the treatment groups that showed any mortality were analysed using the Cox model, because in the groups with no mortality (treatments without *M. brunneum* exposure) the Cox model resulted in degenerate estimates. A mixed effects Cox model was used to analyse the survival data. The cups, initially containing 10 larvae per cup, as well as the experiments (repetition on two independent occasions) were included as random effects. Pairwise comparisons of the heat stress treatments were carried out using Tukey contrasts with single step adjustment for multiple testing in the multcomp package (Hothorn *et al.*, 2008). To confirm that *M. brunneum* exposure resulted in different survival to no exposure to *M. brunneum*, the survival was additionally analysed using a log-rank test in the survival package (Therneau, 2021). In addition, a mixed effects Cox model (with cup and experimental repetition as random effects) was used to analyse the effect of using different pathogen doses at 0 or five days after heat stress (analysis only done on larvae that were not exposed to heat

stress but to *M. brunneum*) to understand if both doses achieved comparable mortality rates.

Immune responses, moulting during two days, and mycosis

Immune response and moulting data assessed two or seven days after heat stress were analysed independently. To compare hemocyte concentrations across heat treatments and exposure to *M. brunneum*, a generalized linear mixed model with a negative binomial error distribution (used for overdispersed count data) was implemented. To compare antibacterial and phenoloxidase activity across heat treatments and exposure to *M. brunneum*, generalized linear mixed models with gamma error distributions (used for right-skewed data) were implemented. To compare the number of shed exuviae two days after *M. brunneum* exposure across heat treatments, a generalized linear mixed model with a Poisson error distribution was implemented. To compare the proportion of *M. brunneum* exposed larvae showing mycosis across heat treatments, a generalized linear mixed model with a Binomial error distribution was implemented. All above models were implemented using the lme4 package (Bates *et al.*, 2015). Experiment (repetition on two independent occasions) and cups were included as random effects in all models. Models were selected using the drop1 function and removing non-significant terms, and pairwise comparisons were performed using the emmeans package (Lenth, 2022). Statistics for terms that were excluded in the final models were extracted from the analysis prior to their elimination.

Development analysis

Data on weight gain five and 10 days after the heat stress, number of exuviae until pupation, and number of offspring over three weeks were subjected to one-way ANOVAs using Tukey's HSD (Honestly Significant Difference) tests to separate the means. Pupal weights were analysed using a two-way ANOVA with sex and heat treatments as fixed effects and cup as random effect. Data on duration of development from heat stress until pupation were analysed using a mixed effects Cox model with sex, heat stress treatment, and their interaction as fixed effects. Moreover, the cups were added as a random effect. The analysis was performed using the coxme package (Therneau, 2020). The data on the average larval weight of each mated pair per week were subjected to one-way ANOVA using Tukey's HSD tests to separate the means. Data from one mated pair in the group that received a long heat stress was excluded because the female beetle died in the first week of egg laying.

Results

Short-term effect of heat stress on pathogen susceptibility

To test the short-term effect of heat stress on pathogen susceptibility of *T. molitor*, larvae were exposed to a lethal dose of *M. brunneum* immediately after a short (2 h), long (14 h), or no heat stress. There was no difference in survival probabilities of control larvae exposed to *M. brunneum* 0 or 5 days after the heat stress, indicating that the two tested pathogen doses achieved comparable mortality rates (Table 1). The germination rates of *M. brunneum* conidia in all experiments were >99%. *Metarhizium brunneum* exposure had a significant effect on survival (log-rank test: $\chi^2 = 84.4$; 1, 718; $P < 0.001$). A short and a long heat stress treatment had a significant effect on the survival probabilities of larvae exposed to *M. brunneum* (Table 1). After post-hoc tests the survival probability of larvae exposed to *M. brunneum* was still significantly increased when they received a short heat stress compared to larvae that were constantly kept at 28 °C ($P < 0.001$; Figure 2) but not compared to larvae that received a long heat stress ($P = 0.279$; Figure 2). The survival probability of larvae exposed to a long heat stress was not significantly different compared to larvae that were kept constantly at 28 °C ($P = 0.057$; Figure 2). There was no effect of heat stress on mortality in larvae that were not exposed to *M. brunneum* (Figure 2), indicating that all the mortality observed in the pathogen treatments can be attributed to exposure to *M. brunneum*. Of all the cadavers, 76, 100, and 84% showed mycosis (visible fungal outgrowth) in no, short, and long heat stress treatments, respectively, without a significant effect of heat stress (Table 2). Subsequently, all of these cadavers sporulated, producing typical green conidia of the *Metarhizium* genus.

To measure the short-term effect of heat and pathogen stress on immune responses, hemolymph samples of larvae were extracted two days after a short, long or no heat stress with either exposure to a lethal dose of *M. brunneum* or no pathogen exposure. Exposure to *M. brunneum* had no significant effect on antibacterial activity (Figure 3A, Table 2) and there was no significant pathogen exposure*heat stress interaction (Figure 3A, Table 2). The heat stress treatment alone had a significant effect on antibacterial activity (Figure 3A, Table 2) and post-hoc tests revealed that both a short

TABLE 1 Results of the statistical analyses using mixed effects cox proportional hazards models to analyse survival and duration until pupation of *T. molitor* larvae (results of pairwise comparisons for survival analyses are given in the text)

	Hazard ratio ± standard error	Wald statistic	Degrees of freedom (d1, d2)	*P*-value
Survival of control larvae exposed to *M. brunneum* immediately or five days after heat stress				
M. brunneum dose	1.087 ± 0.196	0.420	1, 278	0.670
Survival of larvae exposed to *M. brunneum* immediately after heat stress				
Short heat stress	0.324 ± 0.308	−3.660	1, 278	<0.001
Long heat stress	0.532 ± 0.275	−2.290	1, 278	0.022
Survival of larvae exposed to *M. brunneum* five days after heat stress				
Short heat stress	1.126 ± 0.235	0.500	1, 278	0.610
Long heat stress	1.303 ± 0.231	1.140	1, 278	0.250
Duration until pupation after heat stress				
Short heat stress	1.132 ± 0.189	0.660	1, 198	0.510
Long heat stress	1.017 ± 0.188	0.090	1, 198	0.930
Sex	1.402 ± 0.203	1.660	1, 298	0.097
Short heat stress*Sex	0.925 ± 0.287	−0.270	2, 198	0.780
Long heat stress*Sex	0.729 ± 0.289	−1.090	2, 198	0.270

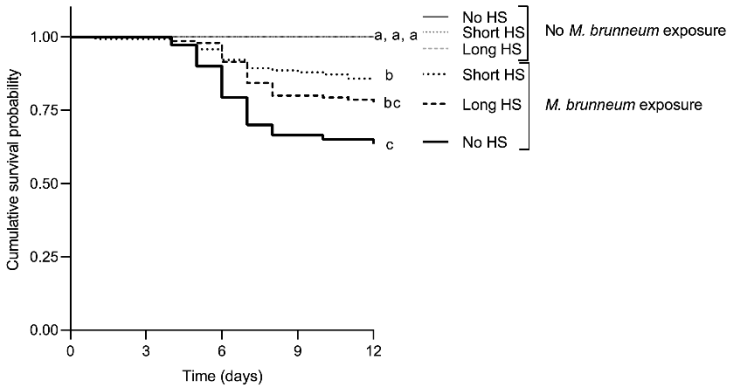


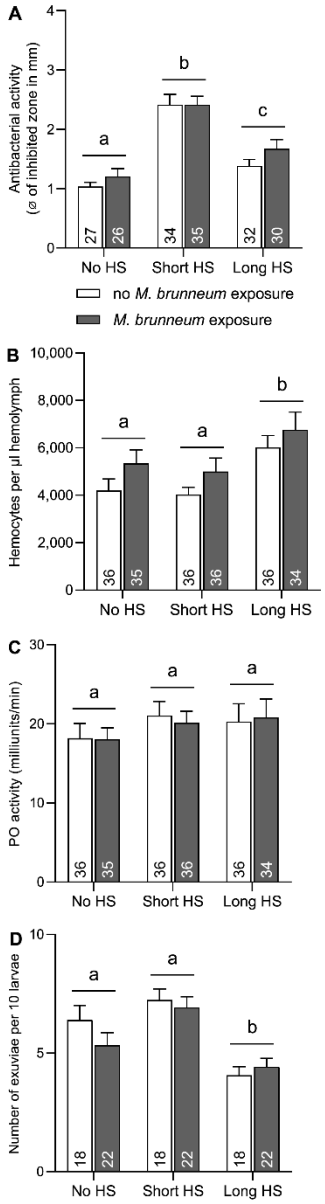
FIGURE 2 Cumulative survival probabilities of *T. molitor* larvae exposed to *M. brunneum* (7×10^2 conidia per larva) immediately after a short (2 h), long (14 h) or no heat stress (HS). The results for each treatment are reported as the median values of two independent experiments. Each group in each experiment consisted of seven cups with 10 individuals per cup resulting in n = 140. Letters at the end of the curves designate significant differences between the three treatment groups exposed to *M. brunneum* (pairwise comparisons of means, $P < 0.05$). Larvae only exposed to no, short, and long heat stresses without exposure to *M. brunneum* were included in both experiments and showed 100% survival in all treatments. Each of these control groups in each experiment consisted of five cups with 10 individuals per cup resulting in total n = 100.

and a long heat stress increased antibacterial activity in the hemolymph significantly compared to no heat stress (both $P < 0.001$; Figure 3A).

Hemocyte concentration was significantly affected by exposure to *M. brunneum* (Figure 3B, Table 2), although no significant differences could be identified when treatment combinations were compared with each other individually in the post-hoc tests. Furthermore, heat stress treatments had a significant effect on hemocyte concentration (Figure 3B, Table 2); a

TABLE 2 Results of statistical analyses using generalized linear mixed models and general linear models (results of pairwise comparisons are mentioned in the text). Bold terms were retained in the final models

Purpose	Test	Independent variable	χ^2 (chi-square)	Degrees of freedom (d1, d2)	*P*-value
Compare **mycosis** between heat treatments of larvae exposed to *M. brunneum* immediately after heat stress	Generalized linear mixed model with a binomial error distribution	Heat	0.389	2, 101	0.533
Compare **mycosis** between heat treatments of larvae exposed to *M. brunneum* five days after heat stress	Generalized linear mixed model with a binomial error distribution	Heat	0.009	2, 191	0.926
Compare **hemocyte concentrations** between heat treatments and exposure to *M. brunneum* two days after heat stress	Generalized linear mixed model with a negative binomial error distribution	**Heat** **Pathogen** Heat*Pathogen	**10.901** **6.359** 0.472	**2, 208** **1, 208** 2, 208	**<0.001** **0.012** 0.790
Compare **hemocyte concentrations** between heat treatments and exposure to *M. brunneum* seven days after heat stress	Generalized linear mixed model with a negative binomial error distribution	**Heat** Pathogen **Heat*Pathogen**	**39.793** 2.617 **14.044**	**2, 211** 1, 211 **2, 211**	**<0.001** 0.106 **<0.001**
Compare **antibacterial activity** between heat treatments and exposure to *M. brunneum* two days after heat stress	Generalized linear mixed model with gamma error distribution	**Heat** Pathogen Heat*Pathogen	**91.647** 3.461 1.895	**2, 179** 1, 179 2, 179	**<0.001** 0.063 0.388
Compare **antibacterial activity** between heat treatments and exposure to *M. brunneum* seven days after heat stress	Generalized linear mixed model with gamma error distribution	Heat Pathogen Heat*Pathogen	2.985 0.032 1.135	2, 197 1, 197 2, 197	0.084 0.858 0.567
Compare **phenoloxidase activity** between heat treatments and exposure to *M. brunneum* two days after heat stress	Generalized linear mixed model with gamma error distribution	Heat Pathogen Heat*Pathogen	2.242 0.002 0.175	2, 208 1, 208 2, 208	0.134 0.963 0.916
Compare **phenoloxidase activity** between heat treatments and exposure to *M. brunneum* seven days after heat stress	Generalized linear mixed model with gamma error distribution	**Heat** Pathogen Heat*Pathogen	**5.123** 1.084 3.912	**2, 211** 1, 211 2, 211	**0.024** 0.298 0.141
Compare the **number of shed exuviae** during two days after *M. brunneum* exposure between heat treatments (immediately after heat stress)	Generalized linear mixed model with a Poisson error distribution	**Heat** Pathogen Heat*Pathogen	**27.535** 0.613 1.764	**2, 115** 1, 115 2, 115	**<0.001** 0.434 0.414
Compare the **number of shed exuviae** during two days after *M. brunneum* exposure between heat treatments (five days after heat stress)	Generalized linear mixed model with a Poisson error distribution	Heat Pathogen Heat*Pathogen	0.230 1.363 5.776	2, 115 1, 115 2, 115	0.631 0.243 0.056



long heat stress increased hemocyte concentration in the hemolymph compared to the short heat stress ($P < 0.001$; Figure 3B) and no heat stress ($P = 0.003$; Figure 3B). There was no significant pathogen exposure*heat stress interaction on hemocyte concentration (Figure 3B, Table 2). The phenoloxidase activity was not affected by exposure to *M. brunneum*, heat stress, or pathogen*heat stress interaction (Figure 3C, Table 2).

The number of exuviae shed per 10 larvae over the two days after heat and pathogen exposure was significantly affected by heat stress treatments (Figure 3D, Table 2) but not by exposure to *M. brunneum* (Figure 3D, Table 2) or by the pathogen exposure*heat stress interaction (Table 2). The number of exuviae per replicate was significantly lower in larvae that received a long heat stress compared to larvae that did not receive a heat stress ($P = 0.006$; Figure 3D) or a short heat stress ($P < 0.001$; Figure 3D). There was no significant difference in the number of exuviae between short and no heat stress ($P = 0.071$; Figure 3D). The number of exuviae shed during the first day (during which larvae were exposed to the heat treatments and before exposure to *M. brunneum*) were <0.1 exuviae per replicate on average in each treatment. Moulting during the heat treatments was therefore disregarded.

Persistent effects of heat stress on pathogen susceptibility

To test the persistent effects of heat stress on pathogen susceptibility of *T. molitor*, larvae were exposed to a lethal dose of *M. brunneum* five days after a short, long or no heat stress. The germination rates of *M. brunneum* conidia in all experiments were >99%. *Metarhizium brunneum* exposure had a significant effect on survival (log-rank test: $\chi^2 = 157.0$; 1, 718; $P < 0.001$). Of all the cadavers from the pathogen exposed treatment, 93,

FIGURE 3 Immune responses and moulting of larvae two days after no, short or long heat stress (HS) either control (white) or exposed to *M. brunneum* (grey). The results are based on two independent experiments. (A) Mean (+SEM) antibacterial activity (diameter of inhibited zone in mm). (B) Mean (+SEM) hemocytes per µl hemolymph. (C) Mean (+SEM) phenoloxidase (PO) activity (milliunits/min). (D) Mean (+SEM) number of exuviae per replicate during two days after pathogen exposure. Different lowercase letters denote statistically significant differences between heat stress treatments (pairwise comparisons of means, $P < 0.05$). (A-C): number of samples (individuals) per treatment are given in each bar. (D): number of replicates (each containing 10 larvae) are given in each bar.

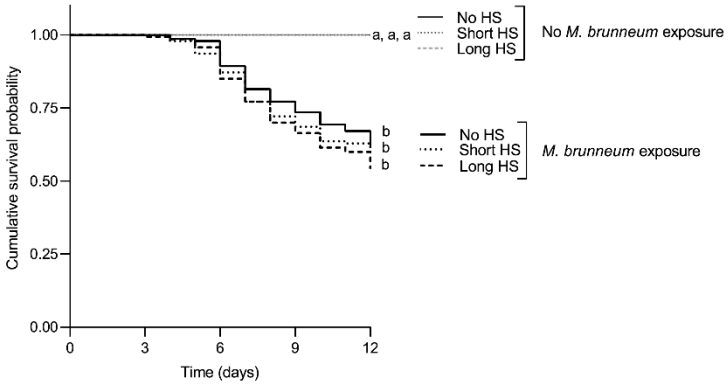


FIGURE 4 Cumulative survival probabilities of *T. molitor* larvae exposed to *M. brunneum* (2×10^3 conidia per larva) five days after a short (2 h), long (14 h) or no heat stress (HS). The results for each treatment are reported as the median values of two experiments. Each group in each experiment consisted of seven cups with 10 individuals per cup resulting in n = 140. Pairwise comparisons of means showed no significant differences between the treatment groups ($P < 0.05$). Larvae only exposed to no, short, and long heat stresses without exposure to *M. brunneum* were included in both experiments and showed 100% survival in all treatments. Each of these control groups in each experiment consisted of five cups with 10 individuals per cup resulting in total n = 100.

84, and 94% showed mycosis in no, short, and long heat stress treatments, respectively (Table 2), with all these cadavers also showing sporulation. A short or a long heat stress treatment had no significant effect on the survival probability of larvae exposed to *M. brunneum* (Table 1 and Figure 4). Larvae that were not exposed to *M. brunneum* but only the short, long or no heat stress treatments did not show mortality.

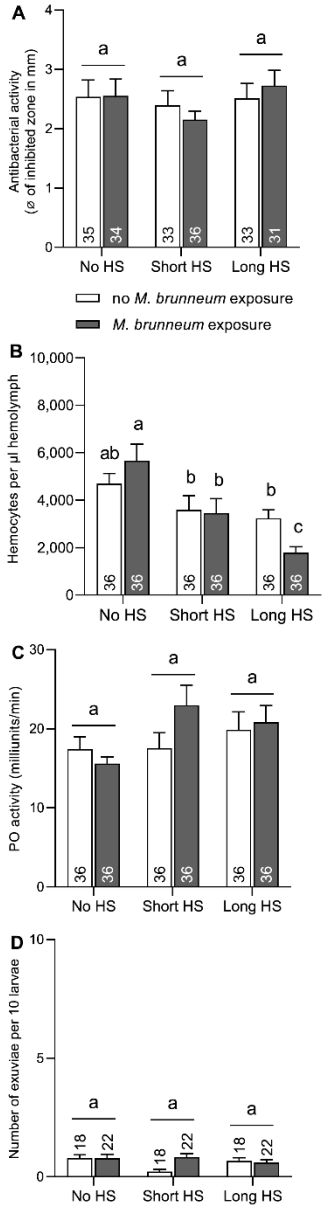
To measure the persistent effects of heat and pathogen stress on immune responses, hemolymph samples of larvae were extracted seven days after a short (2 h), long (14 h) or no heat stress with either exposure to a lethal dose of *M. brunneum* or no pathogen exposure (two days before). The exposure to *M. brunneum*, heat stress or their interaction did not have a statistically significant effect on antibacterial activity (Figure 5A, Table 2). The interaction between *M. brunneum* exposure and heat stress had a significant effect on hemocyte concentration in the hemolymph (Figure 5B, Table 2) and was therefore retained in the model (consequently treatments were compared with each other individually). In the larvae exposed to *M. brunneum*, increasing durations of heat stress treatments led to significantly decreasing hemocyte concentrations in the hemolymph (Figure 5B).

Phenoloxidase activity was significantly affected by heat stresses (Figure 5C, Table 2), although significant differences could not be identified when treatments were compared with each other individually in the post-hoc test. There was no significant effect of exposure to *M. brunneum* on phenoloxidase activity (Figure 5C, Table 2) and there was no significant pathogen exposure*heat stress interaction (Figure 5C, Table 2). The number of exuviae shed during the two days after *M. brunneum* exposure was generally very low and there was no significant effect of exposure to pathogen, heat, or their interaction (Figure 5D, Table 2).

Effect of heat stress on development and fitness

To examine the effects of heat stress on development and reproduction, larval weight gain, development duration until pupation, pupal weight, and the number and weight of offspring of females that had been exposed to heat stress as larvae were measured. Heat stress treatments had a significant effect on larval weight gain in the first five days (Tables 3 and 4). Post-hoc tests revealed that weight gain was highest in the control treatments, and lowest in the long-heat stress treatment (Tables 3 and 4). After 10 days, the larvae that had been exposed to either a short or long heat stress had both compensated the weight gain and there were no significant differences between treatments anymore (Tables 3 and 4).

Heat stress treatments had a significant effect on the total number of exuviae shed until pupation per replicate (Tables 3 and 4). Post-hoc tests revealed that a short heat stress increased the total number of exuviae shed in comparison to no heat stress (Tables 3 and 4) and long heat stress (Tables 3 and 4). The duration of the larval development until pupation was not affected by heat



stress treatments or by sex (Tables 1 and 4). Similarly, the heat stress treatments and sex did not affect the pupal weights (Tables 3 and 4). The number of offspring per female over a three week duration was not affected by the heat treatments (Tables 3 and 4).

The number and weight of offspring from heat stressed parents were measured to assess the effect of heat stress during the larval stage on reproductive success and offspring. Heat stress treatments experienced as larvae had a significant effect on the weight of their larval offspring (Table 3). A short heat stress increased the weight of their offspring compared to the offspring of larvae that were not exposed to a heat stress (in weeks 2 and 3) and compared to those exposed to a long heat stress (in weeks 1-3) (significant p-values are shown in Figure 6).

3 Discussion

In this study, we demonstrate that *T. molitor* larvae exposed to a short heat stress have a higher survival probability when exposed to *M. brunneum* as hypothesised. This result correlates well with our finding that short heat stressed larvae had an increased antibacterial activity in their hemolymph, indicating an increased concentration of AMPs in the hemolymph that can fight fungal cells. In contrast, we found that the survival probability of larvae receiving a heat stress treatment five days prior to pathogen exposure was no different to that of non-heat stressed larvae despite the reduced weight gain of the heat stressed larvae.

Our result on increased survival after a pathogen exposure in combination with a short heat stress is in

FIGURE 5 Immune responses and moulting of larvae seven days after no, short, or long heat stress (HS) either control (white) or exposed to *M. brunneum* (grey). The results are based on two independent experiments. (A) Mean (+SEM) antibacterial activity (diameter of inhibited zone in mm). (B) Mean (+SEM) hemocytes per µl hemolymph. (C) Mean (+SEM) phenoloxidase (PO) activity (milliunits/min). (D) Mean (+SEM) number of exuviae per replicate during two days after pathogen exposure. Different lowercase letters denote statistically significant differences between heat stress treatments or individual treatments used for hemocyte per µl hemolymph because interaction between *M. brunneum* and heat stress exposure was significant (pairwise comparisons of means, $P < 0.05$). (A-C): number of samples (individuals) per treatment are given in each bar. (D): number of replicates (each containing 10 larvae) are given above each bar.

TABLE 3 Results of statistical analyses using analysis of variances

Purpose	Test	F-value	Degrees of freedom (df1, df2)	*P*-value
Compare weight gain during five days after heat stress between heat treatments	One-way ANOVAs using Tukey's Honestly Significant Difference (HSD) tests to separate the means	116.300	2, 27	ANOVA: <0.001 Tukey's HSD: long-control: <0.001 short-control: 0.0273 short-long: <0.001
Compare weight gain during 10 days after heat stress between heat treatments	One-way ANOVAs using Tukey's Honestly Significant Difference (HSD) tests to separate the means	2.347	2, 27	ANOVA: 0.115
Compare number of exuviae until pupation between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	6.075	2, 27	ANOVA: 0.007 Tukey's HSD: long-control: 0.700 short-control: 0.007 short-long: 0.045
Compare number of offspring during three weeks between heat treatments	One-way ANOVA	0.007	2, 71	0.993
Compare pupal weights between sexes and heat treatments	Two-way ANOVA	Treatment: 1.857 Sex: 0.194 Treatment:Sex: 1.759	Treatment: 2, 293 Sex: 1, 293 Treatment:Sex: 2, 293	Treatment: 0.158 Sex: 0.660 Treatment:Sex: 0.174
Compare weights of offspring from heat-stressed parents from first week between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	3.494	2, 71	ANOVA: 0.036 Tukey's HSD: long-control: 0.481 short-control: 0.302 short-long: 0.028
Compare weights of offspring from heat-stressed parents from second week between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	15.010	2, 71	ANOVA: <0.001 Tukey's HSD: long-control: 0.939 short-control: <0.001 short-long: <0.001
Compare weights of offspring from heat-stressed parents from third week between heat treatments	One-way ANOVA using Tukey's Honestly Significant Difference (HSD) tests to separate the means	11.150	2, 71	ANOVA: <0.001 Tukey's HSD: long-control: 0.466 short-control: 0.003 short-long: <0.001

accordance with studies conducted on *G. mellonella* larvae (Browne *et al.*, 2014; Mowlds and Kavanagh, 2008; Wojda and Taszłow, 2013). It has been suggested that temperature stress might induce immune priming in insects (Browne *et al.*, 2014; Grau *et al.*, 2017), a form of innate immune memory (Melillo *et al.*, 2018; Vigneron *et al.*, 2019), which protects insects from pathogens when they have been previously exposed to a sub-lethal dose of the pathogen (Little and Kraaijeveld, 2004). It is however important to note that the term

TABLE 4 Larval weight gain, number of exuviae shed until pupation, development duration until pupation, pupal weight and number of offspring per female that were exposed to no, short (2 h), or long (14 h) heat stress (HS) as larvae[1]

	Weight gain 5 d after stress per 10 larvae (mg) (n = 10)	Weight gain 10 d after stress per 10 larvae (mg) (n = 10)	Number of exuviae per 10 larvae (n = 10)	Days until pupation		Weight per pupa (mg)		Number of offspring per female during 3 w (n = 24-25)
				♀ (n = 54-61)	♂ (n = 39-45)	♀ (n = 54-61)	♂ (n = 39-45)	
No HS	475.9 ± 5.5a	637.6 ± 11.2a	18.6 ± 0.9a	23.3 ± 0.5a	22.4 ± 0.4a	142.4 ± 2.5a	140.9 ± 3.1a	129.6 ± 9.1a
Short HS	443.3 ± 8.5b	675.9 ± 10.5a	21.9 ± 0.5b	23.0 ± 0.4a	22.4 ± 0.4a	148.3 ± 2.4a	145.7 ± 3.3a	130.3 ± 7.4a
Long HS	305.7 ± 9.4c	617.1 ± 28.1a	19.4 ± 0.6a	23.5 ± 0.4a	23.3 ± 0.5a	140.9 ± 2.8a	148.3 ± 3.5a	128.7 ± 10.5a

[1]Means (±SEM) followed by different letters within a column indicate significant differences among the treatments (pairwise comparisons of means, $P < 0.05$).

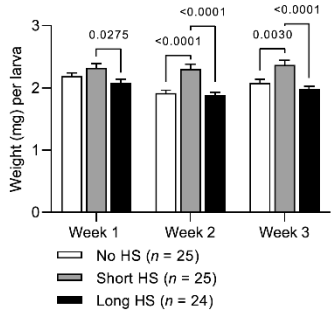


FIGURE 6 Mean (±SEM) weight (mg) per larva of parents that received no (white), a short (grey) or a long (black) heat stress (HS). The eggs were laid in three consecutive weeks by the same females. Larvae were weighed 23 days after removal of parents. Significant differences between heat stress treatments per week are denoted by *P*-values.

immune priming should not be used to describe the immediate effect of a stress on an infection as this does not involve any form of immune memory. Previous studies have only investigated larval susceptibility to pathogens immediately after the heat stress (Browne *et al.*, 2014; Mowlds and Kavanagh, 2008; Wojda and Taszłow, 2013). In contrast, we exposed the larvae to the pathogen both immediately after and five days after the heat stress. Although we found an increase of survival probability and antibacterial activity immediately after the short heat stress, we could not detect within-generation immune priming caused by heat stress, as larvae exposed to the pathogen five days after the heat stress were equally susceptible compared to larvae that were kept at constant temperatures. Moreover, heat stressed larvae did not show any increase in the measured immune responses after seven days.

In addition to key immune responses, we also measured the number of shed exuviae after heat and pathogen exposure. This is an important parameter to measure in experiments involving fungal pathogens and *T. molitor* larvae; *T. molitor* can have a variable number of moults (Ribeiro *et al.*, 2018) and moulting might affect the ability of the fungus to penetrate the insect's cuticle (Kim and Roberts, 2012). Larvae exposed to a long heat stress moulted less during the first two days compared to larvae exposed to no or short heat stress. Larvae that received a long heat stress, therefore, may have had a higher probability of becoming infected by the fungus, which could be an additional explanation why their survival probability after pathogen exposure was not as high as that of the short heat stressed larvae. This is, however, only speculative because the direct relationship between moulting and fungal penetration through the cuticles was not measured in this study.

To understand the potential long-term benefits and risks related to heat stress in *T. molitor* production systems we also tested the effect on reproduction and body mass of the offspring. We found increased body mass in offspring of females that had been exposed to a short but not to a long heat stress as larvae. This indicates that heat stress duration on the parental generation might follow a hormetic response, with short heat stress having a beneficial impact on body mass of offspring. However, in other insect species, heat stress on the parental generation decreases body mass of offspring (Jeffs and Leather, 2014) and increases the developmental time of offspring (Eggert *et al.*, 2015). To confirm the long-term implications of heat stress on the body mass of offspring, further studies should compare the pupal mass of offspring, adjusted for their developmental time.

Mass-reared *T. molitor* larvae can be exposed to elevated temperatures due to metabolic heat production

(Deruytter *et al.*, 2022; Morales-Ramos and Rojas, 2015). For the mass-rearing of these insects, it is therefore important to understand the impact of heat stress, to determine if short durations of heat stress should be avoided or even facilitated, for example, to increase resistance to diseases as previously suggested (Grau *et al.*, 2017; Maciel-Vergara *et al.*, 2021). The intensities of the tested heat stresses in this study did not result in a persistent increase of disease resistance. Only immediate exposure to the fungal pathogen after the heat stress led to increased survival probability. To use heat stress as a method to decrease mortality in cultured *T. molitor* populations, the exact timing of pathogen exposure would be needed, which does not seem feasible. It is, however, important to note that in the current study, insects were exposed to a single heat stress and repetitive or pulsed exposures to heat stresses might occur when *T. molitor* larvae are mass-reared, which would be relevant to investigate in future studies.

Future work should also investigate if there are transgenerational effects on immune responses such that *T. molitor* larvae from heat stressed parents express altered immune responses as shown in other insect species (Eggert *et al.*, 2015). Furthermore, the infection outcome after heat stress might differ when testing other strains of *T. molitor* and different pathogen species. The infection outcome following heat stress could also be affected by symbionts with different thermal requirements. *Gregarina* spp., frequent commensals in the gut of *T. molitor* (Sumner, 1933; Valigurová, 2012), for example, do not survive temperatures over 36-37 °C (Clopton *et al.*, 1992), hence temperature stress might be disadvantageous to these species. Therefore, more research is required to fully understand the effects of heat stress on immunity, insect health, and resistance to disease infections.

In conclusion, we show that heat stress has an impact on host-pathogen interactions in *T. molitor*. Not only the duration of the heat stress but also the timing of the exposure to the pathogen affects the outcome of infection and immune responses. The results in this study show that increased survival after heat stress comes with a cost (i.e. decreased hemocyte concentrations at a later stage of growth and slower body mass gain). The persistent effects are, however, not as severe as hypothesised (i.e. no difference in survival of larvae exposed to the pathogen five days after heat stress). Moreover, we show that heat stress can affect the body mass of the offspring, which demonstrates the importance of investigating effects of temperature in the long-term and not only in the short-term. Beneficial effects on pathogen susceptibility wane rapidly and the reduction in weight gain after heat stress is only temporary. However, there are negative prolonged effects of heat stress on immune responses, which, together with the impact of repeated exposures to heat stress, should be investigated in further studies. This understanding will help safeguard insect health in mass-reared populations, which is crucial for the rapidly growing industry of rearing insects for feed and food.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.24421828

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Author contributions

Conceptualisation and design: PII, IIII, AMD, NVM; Data collection, curation, and analysis: PH; Investigation: PH; Writing – original draft: PH; Writing – reviewing, critiquing, and editing: PH, HH, AMD, NVM.

Conflict of interest

The authors declare no conflicts of interests.

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Appendices to Chapter 4

Assessment of lethal pathogen concentrations

Tenebrio molitor larvae were exposed to different concentrations of *B. thuringiensis* (2×10^7, 8×10^8, 2×10^9, 4×10^9, and 8×10^9 spores/ml) or *M. brunneum* (10^5, 10^6, 10^7, 10^8, and 3×10^8) or water controls as described in Chapter 4. Four replicates (one replicate consisting of one cup with 30 larvae) per pathogen concentration or control were used in this experiment. The survival of larvae 14 days after exposure to the pathogens was analysed using a three-parameter log-logistic model as described in the Appendix of Chapter 5 (Assessment of EC_{50} values). The results are shown in Figure S4.1 and Table S4.1.

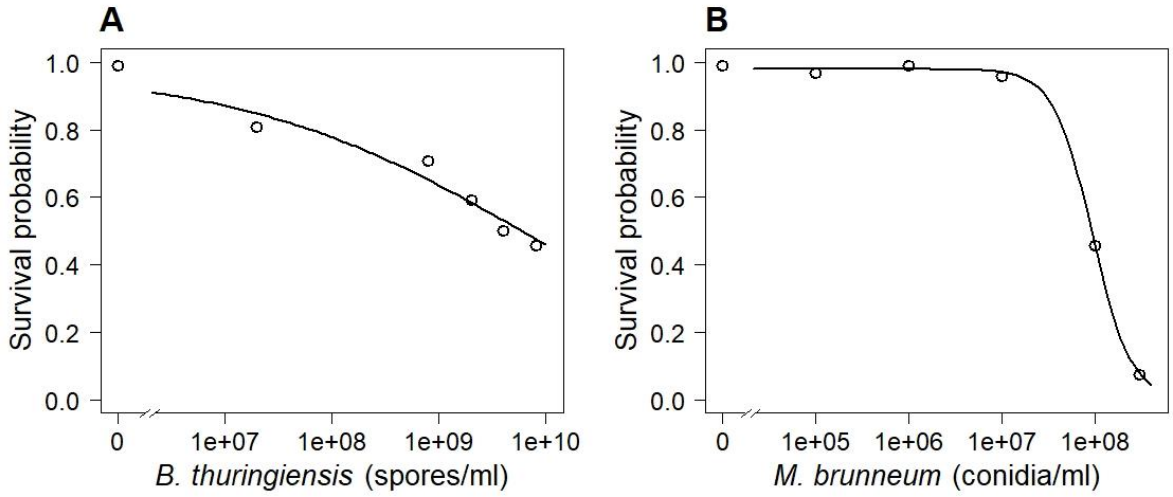

Figure S4.1 Assessment of EC_{50} values.
A Three-parameter log-logistic models of *T. molitor* survival when exposed to *B. thuringiensis*. **B** Three-parameter log-logistic models of *T. molitor* survival when exposed to *M. brunneum*.

Table S4.1 Assessment of EC_{50} values.
EC_{50} values, slopes (β) and upper limits (μ_{max}) of three-parameter log-logistic models of *T. molitor* survival when exposed to *B. thuringiensis* or *M. brunneum*. SE, standard error.

	B. thuringiensis	*M. brunneum*
[1]$EC_{50} \pm$ SE	$6.82 \times 10^9 \pm 3.09 \times 10^9$	$9.38 \times 10^7 \pm 5.92 \times 10^6$
$\beta \pm$ SE	0.32 ± 0.09	2.10 ± 0.38
$\mu_{max} \pm$ SE	0.98 ± 0.05	0.98 ± 0.01

[1]spores/ml for *B. thuringiensis* and conidia/ml for *M. brunneum*

Appendices to Chapter 5

Assessment of EC_{50} values

The larvae were distributed in groups of 30 to empty 60-ml cups to starve for 24 hours, 17 days after removal of the adults. Spore suspensions of *B. thuringiensis* (5×10^7, 5×10^8, and 5×10^9, and 1.4×10^9 spores/ml) and conidia suspensions of *M. brunneum* (10^7, 5×10^7, 10^8, 2×10^8, and 3.5×10^8 conidia/ml) were prepared in H_2O. Afterwards, 100 µl of the pathogen suspension were added to 100 mg diet inside 60-ml cups (n = 4). Furthermore, 100 µl of dH_2O were added in the same way as controls (n = 5). The diet and suspension were mixed thoroughly with a sterile inoculation loop. The larvae were then added to these cups. After two days, the larvae (in groups) and the leftover diets were weighed. The larvae were moved to fresh 60-ml cups containing diet (0.6 × fresh weight of larvae) and water agar (1% w/v; 0.6 × fresh weight of larvae). The larvae were weighed thereafter every second day for 14 days in total. At the same time new diet and water agar was added as described above and dead larvae were removed and counted.

The effect of the different pathogen concentrations on larval biomass (mg/cm^2), survival, and weight gain (mg/larva) was described using a three-parameter log-logistic model given in equation S5.1:

$$y = \frac{\mu_{max}}{1+\left(\frac{c}{EC_{50}}\right)^{\beta}} \qquad Equation\ S5.1$$

where y is the response (either larval biomass, survival, or weight gain), μ_{max} is the upper limit (i.e., response when the pathogen concentration is 0), c is the pathogen concentration, EC_{50} is the pathogen concentration that results in 50% reduction of μ_{max}, and β is the slope parameter using the drc package [1].

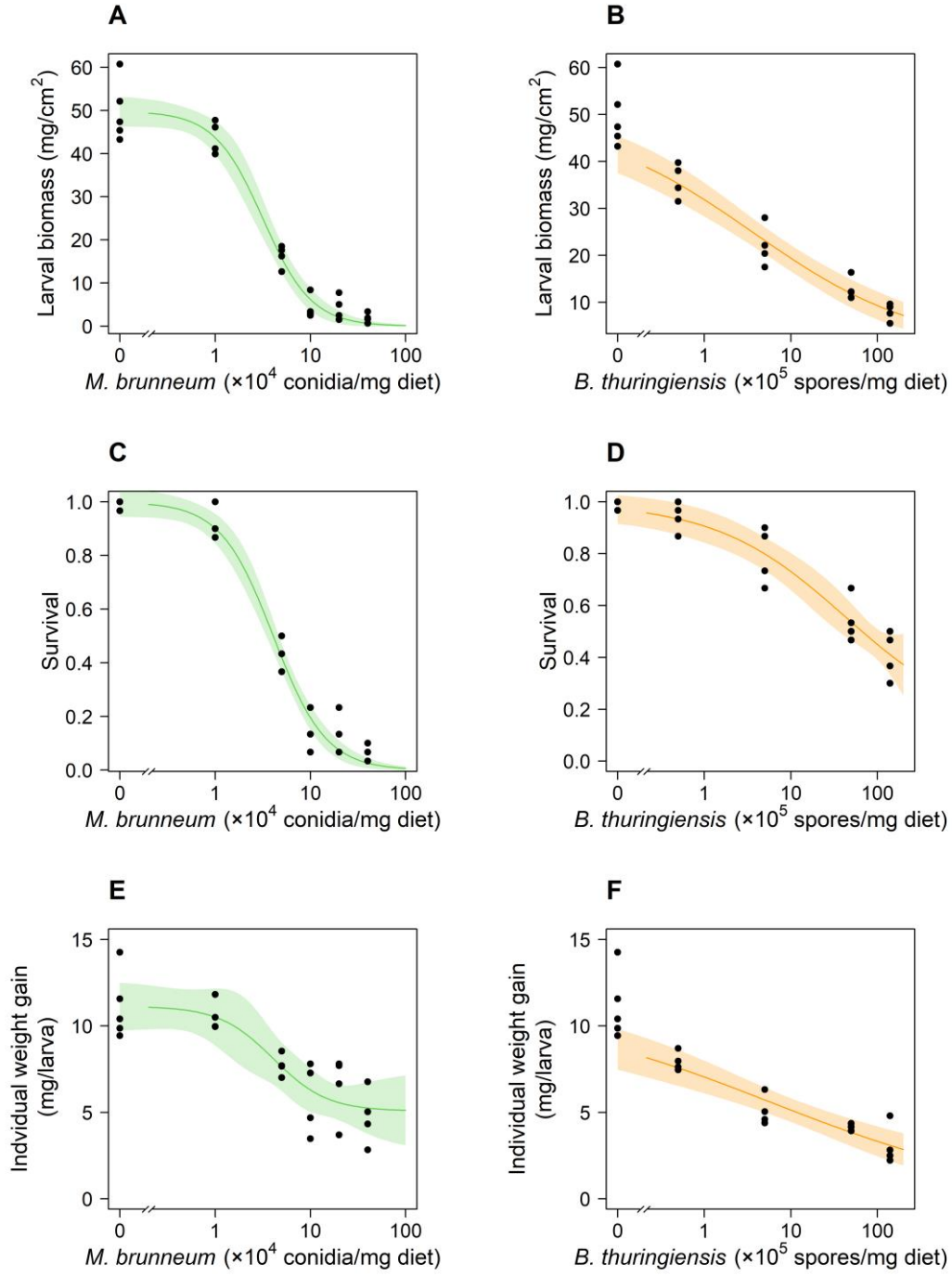

Figure S5.1 Initial assessment of EC_{50} values.
Three-parameter log-logistic models of larval biomass, survival, and individual weight gain of larvae exposed to individual concentrations of either *M. brunneum* (shown as green lines) or *B. thuringiensis* (shown as orange lines) at ambient CO_2 concentration 14 days after exposure. Shaded areas show 95% confidence intervals, dots show data points. **A** Larval biomass (mg/cm^2) after *M. brunneum* exposure. **B** Larval biomass (mg/cm^2) after *B. thuringiensis* exposure. **C** Survival after *M. brunneum* exposure. **D** Survival after *B. thuringiensis* exposure. **E** Individual weight gain (mg/larva) after *M. brunneum* exposure. **F** Individual weight gain (mg/larva) after *B. thuringiensis* exposure.

Table S5.1 Initial assessment of EC_{50} values.

EC_{50} values, slopes (β) and upper limits (μ_{max}) of three-parameter log-logistic models of larval biomass (mg/cm^2), survival, and individual weight gain (mg/larva) of larvae exposed to individual pathogen (*M. brunneum* or *B. thuringiensis*) concentrations at ambient CO_2 concentrations 14 days after exposure. SE, standard error.

	Larval biomass (mg/cm^2)	Survival	Individual weight gain (mg/larva)
M. brunneum			
[1]EC_{50} ± SE	3.03 ± 0.38	3.88 ± 0.35	3.88 ± 1.68
β ± SE	1.70 ± 0.24	1.58 ± 0.18	1.54 ± 1.12
μ_{max} ± SE	49.81 ± 1.74	1.00 ± 0.03	5.08 ± 1.11
B. thuringiensis			
[2]EC_{50} ± SE	3.03 ± 1.00	37.14 ± 68.37	4.61 ± 2.48
β ± SE	0.44 ± 0.06	0.58 ± 0.24	0.30 ± 0.06
μ_{max} ± SE	49.88 ± 1.91	1.00 ± 0.03	11.14 ± 0.52

[1]× 10^4 conidia/mg diet

[2]× 10^5 spores/mg diet

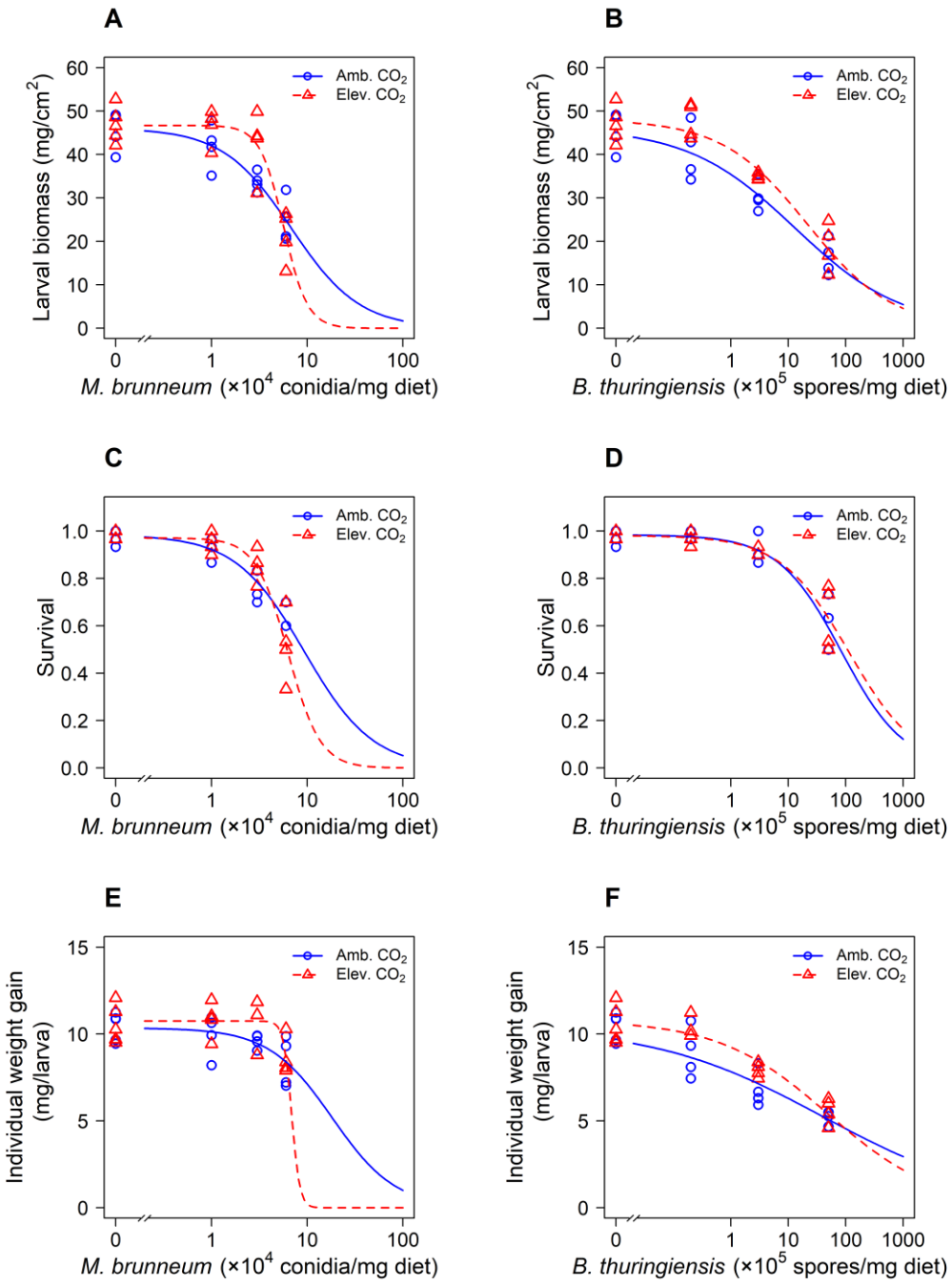

Figure S5.2 Experimental repetition 2. Three-parameter log-logistic models of larval biomass, survival, and individual weight gain of larvae exposed to individual pathogen concentrations at either ambient (Amb.; blue, circles) or elevated (Elev.; red, triangles) CO_2 concentrations 14 days after exposure.
A Larval biomass (mg/cm^2) after *M. brunneum* exposure. **B** Larval biomass (mg/cm^2) after *B. thuringiensis* exposure. **C** Survival after *M. brunneum* exposure. **D** Survival after *B. thuringiensis* exposure. **E** Individual weight gain (mg/larva) after *M. brunneum* exposure. **F** Individual weight gain (mg/larva) after *B. thuringiensis* exposure.

Table S5.2 Experimental repetition 2. EC_{50} values, slopes (β) and upper limits (μ_{max}) of three-parameter log-logistic models of larval biomass (mg/cm^2), survival, and individual weight gain (mg/larva) of larvae exposed to individual pathogen (*M. brunneum* or *B. thuringiensis*) concentrations at either ambient (Amb.) or elevated (Elev.) CO_2 concentrations 14 days after exposure.
EC_{50} values followed by different letters for the same endpoint and pathogen indicate significant differences among the treatments. SE, standard error.

	Larval biomass (mg/cm^2)		Survival probability		Individual weight gain (mg/larva)	
	Amb. CO_2	Elev. CO_2	Amb. CO_2	Elev. CO_2	Amb. CO_2	Elev. CO_2
M. brunneum						
[1]EC_{50} ± SE	6.83 ± 1.16a	5.69 ± 0.40a	9.33 ± 1.24a	6.31 ± 0.45a	18.03 ± 19.28a	7.09 ± 3.46a
β ± SE	1.21 ± 0.33	3.51 ± 1.17	1.21 ± 0.21	2.59 ± 0.68	1.33 ± 1.26	8.54 ± 24.93
μ_{max} ± SE	46.07 ± 1.85	46.71 ± 1.87	0.98 ± 0.02	0.97 ± 0.03	10.35 ± 0.46	10.75 ± 0.32
B. thuringiensis						
[2]EC_{50} ± SE	13.10 ± 5.13a	5.69 ± 0.4a	83.83 ± 18.78a	112.63 ± 37.54a	41.13 ± 26.64a	49.83 ± 19.42a
β ± SE	0.46 ± 0.09	3.51 ± 1.17	0.79 ± 0.22	0.74 ± 0.24	0.29 ± 0.07	0.46 ± 0.08
μ_{max} ± SE	46.14 ± 1.95	46.71 ± 1.87	0.98 ± 0.02	0.98 ± 0.02	10.47 ± 0.43	10.80 ± 0.37

[1]× 10^4 conidia/mg diet
[2]× 10^5 spores/mg diet

Table S5.3 Results of comparison of EC50 values between ambient and elevated CO2 concentrations for experimental repetitions (Exp. rep.) 1 and 2. Data shown in Table 5.1 for experimental repetition 1 and S5.2 for experimental repetition 2.

	Larval biomass (mg/cm^2)		Survival		Individual weight gain (mg/larva)	
	t	*p*	*t*	*p*	*t*	*p*
Exp. rep. 1						
M. brunneum	2.872	0.008	3.568	0.001	1.431	0.164
B. thuringiensis	-0.538	0.595	-0.987	0.332	-0.393	0.698
Exp. rep. 2						
M. brunneum	0.832	0.413	1.743	0.092	0.523	0.605
B. thuringiensis	-0.995	0.328	-0.702	0.489	-0.273	0.787

Table S5.4 Experimental repetition 2. Results of MIXTox models for larval biomass (L. biom. in mg/cm^2), survival (Surv.), and individual weight gain (Ind. wg. in mg/larva) at ambient and elevated CO_2 either 14 or 20 days after pathogen exposure. Models that described the data best are highlighted in green. IA, independent action; S/A, synergistic/antagonistic; DR, dose ratio-dependent; DL, dose level-dependent.

	14 days								**20 days**							
	Ambient CO_2				**Elevated CO_2**				**Ambient CO_2**				**Elevated CO_2**			
	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL
L. biom.																
μ_{max}	45.08	46.33	46.15	46.59	48.12	47.94	48.09	47.97	101.66	104.73	104.45	105.25	111.18	110.93	111.31	110.98
β_{Bt}	7.44	6.56	6.59	6.12	7.28	7.40	7.35	7.36	7.71	6.69	6.72	6.17	7.50	7.58	7.50	7.54
β_{Mb}	1.30	1.21	1.25	1.13	3.11	3.27	3.54	3.23	1.42	1.28	1.34	1.17	3.45	3.55	3.75	3.51
[1]$EC50_{Bt}$	6.19	6.05	6.08	6.06	6.10	6.13	6.28	6.13	6.22	6.06	6.08	6.07	6.15	6.18	6.30	6.18
[2]$EC50_{Mb}$	8.28	6.92	6.92	6.92	5.82	5.92	5.65	5.92	8.03	6.66	6.55	6.79	5.63	5.69	5.49	5.70
a	NA	1.06	1.51	2.48	NA	-0.27	2.80	-0.20	NA	1.21	1.73	2.93	NA	-0.18	2.35	-0.09
b	NA	NA	-0.84	1.09	NA	NA	-5.69	-0.71	NA	NA	-0.94	1.14	NA	NA	-4.67	-2.14
Residuals	1184.07	1102.49	1100.62	1093.15	1488.38	1482.69	1376.13	1482.46	6527.19	5982.15	5971.72	5916.68	9176.49	9164.29	8785.58	9163.10
R^2	0.79	0.81	0.81	0.81	0.82	0.82	0.83	0.82	0.78	0.80	0.80	0.80	0.80	0.80	0.81	0.80
[3]$p(X^2)$	<0.001	0.052	0.764	0.502	<0.001	0.652	0.125; 0.047[4]	0.929	<0.001	0.032	0.761	0.445	<0.001	0.791	0.135	0.934
Surv,																
μ_{max}	0.98	0.98	0.98	0.98	0.97	0.97	0.97	0.97	0.97	0.98	0.97	0.98	0.97	0.96	0.96	0.97
β_{Bt}	12.23	11.53	11.72	11.28	8.78	9.03	9.36	8.74	10.96	10.15	10.33	9.83	9.01	9.16	9.44	8.99
β_{Mb}	1.45	1.42	1.45	1.39	2.39	2.55	2.86	2.36	1.47	1.43	1.46	1.39	2.45	2.59	2.86	2.44
[1]$EC50_{Bt}$	6.91	6.87	6.89	6.88	7.00	7.02	7.12	7.04	6.93	6.89	6.92	6.91	6.93	6.95	7.05	6.96
[2]$EC50_{Mb}$	9.74	8.41	8.41	8.41	6.27	6.41	6.10	6.59	9.57	8.18	8.18	8.18	6.14	6.13	5.93	6.32
a	NA	1.07	1.93	1.44	NA	-0.47	1.72	-0.01	NA	1.17	2.06	1.72	NA	-0.27	1.73	-0.00
b	NA	NA	-1.55	0.76	NA	NA	-4.22	-119.85	NA	NA	-1.64	0.89	NA	NA	-3.92	-158.10
Residuals	75.37	70.34	70.04	70.27	81.99	81.01	78.60	80.62	72.71	66.61	66.27	66.48	82.59	82.16	80.03	81.89
R^2	0.76	0.78	0.78	0.78	0.79	0.79	0.80	0.80	0.76	0.78	0.78	0.78	0.79	0.79	0.80	0.79
[3]$p(X^2)$	<0.001	0.025	0.586	0.795	<0.001	0.322	0.121	0.535	<0.001	0.014	0.558	0.719	<0.001	0.511	0.145	0.607

Table S5.4 (continued)

	14 days								20 days							
	Ambient CO_2				Elevated CO_2				Ambient CO_2				Elevated CO_2			
	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL	IA	S/A	DR	DL
Ind. wg,																
μ_{max}	10.05	10.48	10.46	10.48	10.81	10.83	10.97	10.82	25.44	26.27	26.24	26.28	28.34	28.38	28.57	28.37
β_{Bt}	4.38	3.95	3.98	3.71	5.94	5.71	5.18	5.10	4.59	4.10	4.12	3.87	5.49	5.28	4.86	5.06
β_{Mb}	3.66	1.08	1.10	1.07	13.21	12.16	8.90	12.59	3.99	1.27	1.28	1.26	21.61	20.49	17.16	20.73
[1]$EC50_{Bt}$	6.71	6.58	6.62	6.66	6.62	6.56	6.77	6.68	7.05	6.95	6.98	7.04	7.08	7.02	7.23	7.08
[2]$EC50_{Mb}$	1.00	2.28	2.28	2.28	7.08	7.08	7.08	7.08	1.00	2.10	2.10	2.10	6.55	6.55	6.55	6.55
a	NA	1.35	3.06	3.53	NA	0.44	5.90	1.72	NA	1.30	2.46	2.85	NA	0.36	3.50	0.78
b	NA	NA	-2.30	1.79	NA	NA	-8.658	2.91	NA	NA	-1.59	1.82	NA	NA	-5.18	2.25
Residuals	51.40	47.96	47.87	47.63	68.90	68.47	60.27	67.43	308.12	292.68	292.37	291.57	512.31	510.20	488.75	509.56
R^2	0.73	0.75	0.75	0.75	0.72	0.72	0.75	0.72	0.69	0.71	0.71	0.71	0.61	0.62	0.63	0.62
[3]$p(X^2)$	<0.001	0.055	0.755	0.755	<0.001	0.562	0.029; 0.009[4]	0.369	<0.001	0.094	0.813	0.653	<0.001	0.640	0.131	0.796

[1]in log(spores/mg diet)
[2]in 10^4 conidia/mg diet
[3]resulting from F-test for IA (IA vs Null model), or from Chi-square tests (X^2) for S/A (IA vs S/A), DR (DR vs S/A), and DL (DL vs S/A).
[4]comparison with IA; comparison with S/A

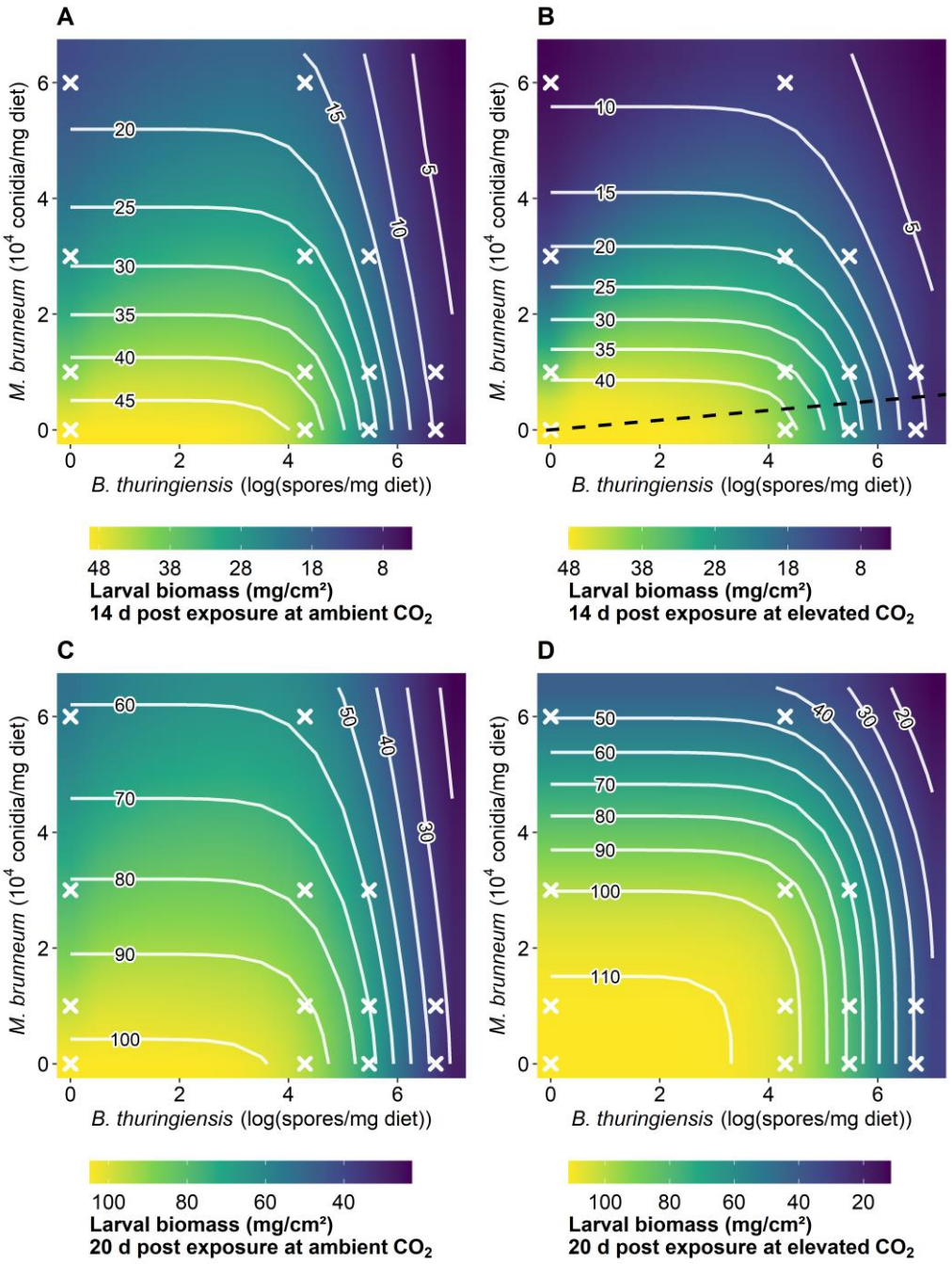

Figure S5.3 Experimental repetition 2. Contour plots of the endpoint larval biomass (mg/cm^2). The *B. thuringiensis* concentrations are plotted on the x-axes, the *M. brunneum* concentrations are plotted on the y-axes. The white isobole lines show independent action (IA) prediction of the joint effects, whereas the colour gradients show the outcome of the models that describe the data best (Table S5.4). The white crosses show the tested individual (along x- and y-axis) and mixture (inside the plots) concentrations. **A** 14 days post exposure to pathogens at ambient CO_2. **B** 14 days post exposure to pathogens at elevated CO_2. **C** 20 days post exposure to pathogens at ambient CO_2. **D** 20 days post exposure to pathogens at elevated CO_2.

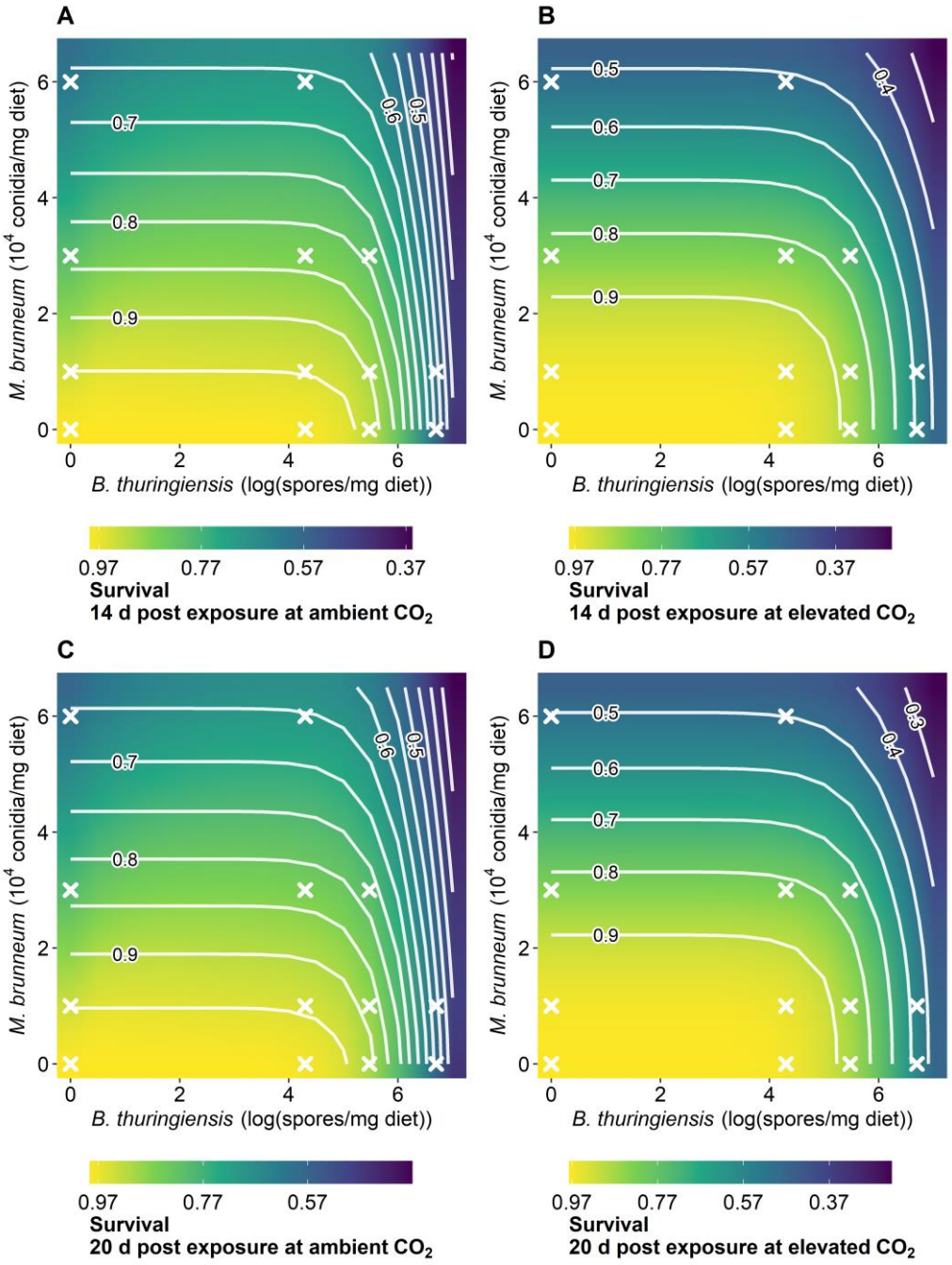

Figure S5.4 Experimental repetition 2. Contour plots of the endpoint survival of larvae. The *B. thuringiensis* concentrations are plotted on the x-axes, the *M. brunneum* concentrations are plotted on the y-axes. The white isobole lines show independent action (IA) prediction of the joint effects, whereas the colour gradients show the outcome of the models that describe the data best (Table S5.4). The white crosses show the tested individual (along x- and y-axis) and mixture (inside the plots) concentrations. **A** 14 days post exposure to pathogens at ambient CO_2. **B** 14 days post exposure to pathogens at elevated CO_2. **C** 20 days post exposure to pathogens at ambient CO_2. **D** 20 days post exposure to pathogens at elevated CO_2.

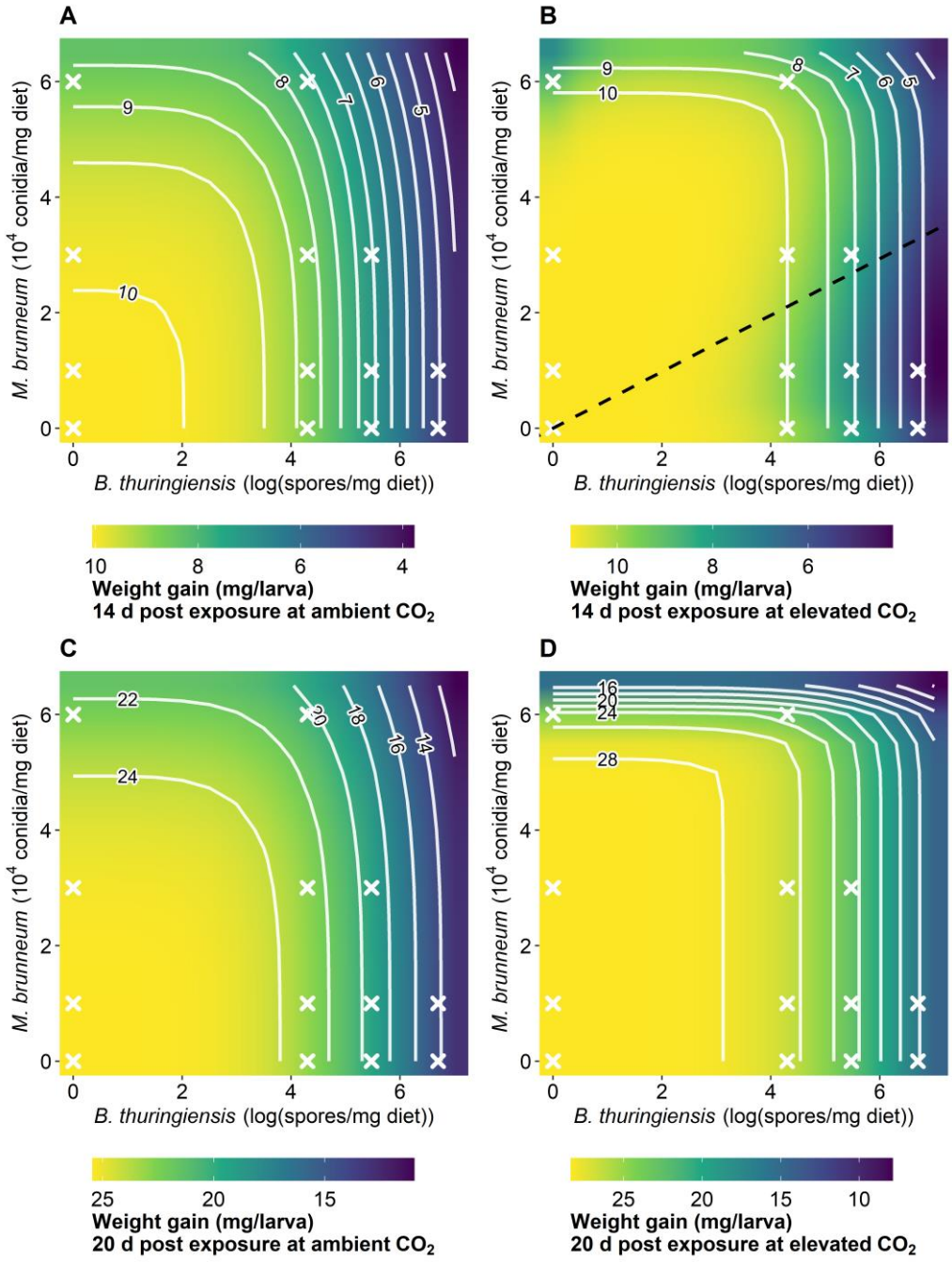

Figure S5.5 Experimental repetition 2. Contour plots of the endpoint weight gain (mg/larva). The *B. thuringiensis* concentrations are plotted on the x-axes, the *M. brunneum* concentrations are plotted on the y-axes. The white isobole lines show independent action (IA) prediction of the joint effects, whereas the colour gradients show the outcome of the models that describe the data best (Table S5.4). The white crosses show the tested individual (along x- and y-axis) and mixture (inside the plots) concentrations. The black dotted line marks the shift from antagonism (above the dotted lines) to synergism (below the dotted lines) for data that was best described using dose ratio-dependent deviation (DR). **A** 14 days post exposure to pathogens at ambient CO_2. **B** 14 days post exposure to pathogens at elevated CO_2. **C** 20 days post exposure to pathogens at ambient CO_2. **D** 20 days post exposure to pathogens at elevated CO_2.

Table S5.5 Colony forming units (cfu) per ml of *B. thuringiensis* spores exposed to different concentrations of *M. brunneum* conidia at either ambient or elevated CO_2. Different letters indicate significant (p <0.05) differences.

	B. thuringiensis : *M. brunneum*						
	1:0 (control)	1:3	1:1.5	1:0.5	1:0.1	1:0.033	1:0.002
Ambient CO_2	1,217 ± 123ab	1,478 ± 92a	1,250 ± 73ab	1,328 ± 84ab	1,094 ± 70ab	1,367 ± 93ab	1,283 ± 63ab
Elevated CO_2	1,078 ± 66ab	1,150 ± 75ab	1,000 ± 79b	1,144 ± 87ab	1,156 ± 456ab	1,127 ± 82ab	983 ± 101b

Table S5.6 Germ tube lengths of *M. brunneum* conidia exposed to different concentrations *of B. thuringiensis* spores/crystals at either ambient or elevated CO_2. Different letters indicate significant (p <0.05) differences.

	M. brunneum : *B. thuringiensis*					
	1:0 (control)	1:30	1:10	1:2	1:0.66	1:0.33
Ambient CO_2	41.8 ± 0.9a	41.1 ± 1.6a	41.8 ± 1.2a	43.8 ± 1.0a	41.4 ± 1.1a	40.2 ± 1.2a
Elevated CO_2	56.7 ± 1.1b	60.8 ± 1.5b	60.0 ± 1.1b	58.6 ± 1.2b	59.6 ± 1.1b	56.5 ± 1.4b

Reference

1. Ritz, C., Baty, F., Streibig, J.C., and Gerhard, D., Dose-response analysis using R. Plos One, 2015. 10. https://doi.org/10.1371/journal.pone.0146021