Viral and fungal pathogens in house cricket rearing:

Effects of temperature, density and co-infection

Propositions

1. Biotic and abiotic stressors need to be examined jointly to optimise house cricket rearing.
   (this thesis)

2. The Acheta domesticus densovirus is transmitted both horizontally and vertically.
   (this thesis)

3. Changes in the food culture of westernised societies are needed to achieve the Sustainable Development goals set by the UN.

4. Integration of artificial intelligence into everyday life requires clear regulations.

5. Change in attitude regarding the environment starts with the education of the youngest generations.

6. The habit of solving complex issues by simple solutions is destined to fail.

Propositions belonging to the thesis entitled:

Viral and fungal pathogens in house cricket rearing: effects of temperature, density and co-infection

Jozsef Takacs
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Viral and fungal pathogens in house cricket rearing: effects of temperature, density and co-infection

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Viral and fungal pathogens in house cricket rearing: effects of temperature, density and co-infection

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Thesis

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CHAPTER 1

General introduction

1. Challenges in the food chain

The world population is expected to reach 10 billion by 2050 [1]. Agricultural production should increase by 60% compared to the current production rates to provide sufficient calories, nutrients and raw materials to match this expected increase in the population [2]. The current agricultural production requires large energy, water and fertilizer investments to obtain high yields, but these also cause a large carbon footprint [3-5]. Lowering the carbon footprint of food production is currently on the agenda of most governments and companies, partly due to the pressure from the society. However, the problem is not solely in the carbon footprint of food or more broadly in agricultural production, but also in the losses along supply chains and in the waste of end products [6]. A well-known example of this concerns waste of safe and nutritious food, which is not consumed at the table and therefore gets discarded [7]. An alarming volume of food ends up as food waste: for instance in the Nordic countries this amounts to 10-30% of the purchased food [8], but globally, one third of all the food produced will not be utilized as intended [9]. This also involves a major economic impact as the total value of food wasted can reach up to 750 billion USD, bringing not just an economic but a societal and environmental impact as well [10]. While the westernized parts of the world have easy access to the majority of the available resources, in many parts of the world everyday access to food or food resources can be an issue. The current production could already be sufficient to feed the world, however, hunger and malnutrition occurs in several regions on a regular basis, which is due to the unequal distribution of the resources [11]. What needs to change in our food systems to ensure the eradication of hunger and in the meantime, how can we stop any further over-exploitation of our environment? The solution to the problem might come from increasing the efficiency of the current supply chains. If we can reduce the resources that are required per unit of output and reduce the loss of recourses between the producer and the consumer, the goal to feed 10 billion people might be achieved. How and what is being produced and consumed needs to go through a transition and alternatives to the current protein sources are required. Edible insects are proposed to be a sustainable, promising alternative to the traditional animal based proteins [12], but the environmental impact of eating insects will only be considerable if they are consumed widely and regularly. Currently, approximately 2000 insects species are eaten at various places around the world, but the majority of these are collected from nature [13]. Only a minor fraction of those species are currently mass-reared as food, concerning beetles of the Tenebrionidae *Tenebrio molitor*, *Alphitobius diaperinus*, and *Zophobas morio,* the orthopterans *Acheta domesticus*, *Gryllodes sigillatus*, *Gryllus bimaculatus,* and *Locusta migratoria* and the lepidopterans *Galleria mellonella* and *Bombyx mori* [14]. It is important to mention that in the European Union not all of these species are approved as food, only four of these, *T. molitor, A. diaperinus, A. domesticus* and *L. migratoria*, are currently authorized in certain forms and after certain processing [15].

2. Crickets as food

Several species of crickets are among the 2000 insect species that are consumed by humans, mainly the adult stage[16]. Crickets can be either mass-produced (Fig. 1) or collected from nature. Species collected from nature include *Anabrus simplex* and *Acanthoplus discoidalis* [17], both the collection and consumption of these species can be an additional source of income or can be used as provision when food shortages occur. Nevertheless, the majority of crickets consumed are produced in production facilities of various sizes, which range from smallholder farms to large scale, highly automated, industrial facilities. Cricket farmers focus mainly on the rearing of a few species such as *A. domesticus* and *G. bimaculatus*. Among crickets, the house cricket *A. domesticus* is traditionally the most commonly reared species, which can be attributed to the relative ease of rearing, fast growth, good taste and nutritional values, such as the high protein content of 60-70% (based on dry matter) [18] and their optimal fatty acid ratio and profile [19-21]. The majority of commercial scale house cricket production facilities for human consumption are located in

Asia and Africa [22], but throughout the world there are commercial producers also for zoo and pet feed purposes and for research use in laboratories. Certain house cricket-derived products received approval from the European Food Safety Authority (EFSA) in 2022 to be used in products intended for human consumption [23]. This important milestone can boost the production of house crickets in Europe and can motivate producers elsewhere to get their products approved by EFSA to be allowed on the EU market. Because of the good nutritional profile and functional properties in food [24], house cricket-derived products have already been incorporated into various food products [17, 25]. High quality food products are one of the important factors, which can shift house crickets from the delicacy category to an everyday food ingredient. Consumer acceptance in the western world and price also play key role in this process.

3. The house cricket

The house cricket *A. domesticus* L. (Orthoptera: Gryllidae) is native to Asia, but nowadays it is widely spread around the world. House crickets grow up to ~2-3 cm in length and can weigh between 0.3-0.6 grams, having a sandy light brown colour at the early instars, which gradually darkens as they reach adulthood. The life cycle of crickets ranges from 30-50 days [16, 26], but this can vary by the environmental or rearing conditions, some papers report 57 days as the life cycle [20]. Crickets have a hemimetabolous life cycle with eggs, nymphs and adults, so without diapause or a pupal stage. The house cricket life cycle starts with the egg phase, which last between 8-12 days, from which the first nymphal instars (also called pinheads) hatch. The nymphal phase can last between 35-50 days, depending on temperature, feed quality and cricket density. The nymphs moult seven times, the last moult giving rise to adults. It is noteworthy that mortality is highest during the first three nymphal stages [20].

Cricket production

Beside the willingness from the consumers, production volumes of house crickets also need to meet the demand in order to secure their position on the market. House cricket production involves a wide range of different scale production units, which are also often referred to as cricket farms. Halloran [22] described the different aspects of small- and medium-sized cricket farms throughout Southern Asia and Africa. Small-scale production or laboratory rearing is mainly done with the use of plastic containers [20, 27]. These allow easy manipulation and stacking of the production units. Temperature control can be done by placing the containers into climate controlled chambers or rooms or in incubators for a more precise control. The hot-spot of small scale and semi-industrial production is in Thailand, where approximately 20.000 farmers rear crickets [28] at different scales in concrete “pools” [28, 29] (Fig. 1). Larger number of crickets can be housed in these units compared to plastic containers and the rearing units are cheap to build. However, the temperature is mainly dependent on the environmental temperature and these units are exposed to pests, predators and various microorganisms, which can easily enter the rearing. A few companies run industrial scale, highly controlled and automated rearing facilities (Fig. 1). The costs to build and run these facilities are quite high [30], but their production output is also higher than the small and medium scale farming. Most of the large rearing facilities are established in North America and in Europe and produce crickets as feed for the pet industry or as ingredients for food [26]. House crickets can be fed with various feedstock, such as chicken feed, fruits and vegetables and various agricultural by-products [16, 31]. Special attention needs to be paid to the price of the diet provided [32] as this is a major component in the costs of cricket

rearing, but the cricket feed also needs to have a proper nutritional composition [33], otherwise cannibalism can be induced [20].

Figure 1. Different scales of cricket production facilities . Left-hand: Small-scale cricket production (Photo: Pieter Rouweler), middle: Semi-industrial cricket production in Thailand (Photo: Afton Halloran) and right-hand: Industrial scale cricket production (Photos: Andrea Liceaga & Aspire Food Group, with permission from Elsevier).

4. Challenges in cricket production – biotic and abiotic stressors

Biotic and abiotic stresses can threaten house cricket production, inducing for example cannibalism, disease symptoms or mortality. Biotic stressors include predators and microorganisms, such as bacteria, viruses, fungi, protists and parasites, while abiotic stressors include temperature, humidity, and noise. Eilenberg et al. [34] summarized the possible disease threats in commercial insect rearing systems and Eilenberg and Jensen [35] provided information on the possible methods on disease prevention and management, which also applies to house cricket rearing facilities.

Biotic stressors

Viruses

Among the microorganisms that can infect the house cricket, viruses represent one of the main threats [36]. House crickets are known to harbour various viruses, such as the Acheta domesticus densovirus (*Parvoviridae*), Acheta domesticus mini ambidensovirus (*Parvoviridae*), Acheta domesticus volvovirus (unclassified), invertebrate iridescent virus 6 (*Iridoviridae*), Acheta domesticus iflavirus (*Iflaviridae*) and cricket paralysis virus (*Dicistroviridae*) [36-40]. Most of these viruses were detected with the use of PCR and qPCR techniques. This gives a limited overview of the viruses that are present, as only known viruses and those for which primers are readily available can be targeted via these techniques. Over the past decade with the advancement of new generation sequencing (NGS), viral discovery in insects developed rapidly [41], but viral detection with sequencing was lacking until recently in house crickets. Miranda et al. [37], from the listed house cricket viruses, only found one DNA virus, the invertebrate iridescent virus 6. Cholleti et al. [42] found none of the earlier listed cricket pathogens to be present. Acheta domesticus densovirus (AdDV) is responsible for most of the losses in house cricket rearing facilities. This virus can jeopardize the entire house cricket production by causing epizootics in the rearing leading to major losses in the production or even to colony collapses [26, 27, 37, 43-46]. The first case description of an AdDV outbreak dates back to 1977, when an epizootic was described in a Swiss rearing facility [47], but also in the United

States, already in the late 1980s the first occurrence of the virus was reported [48]. Throughout the early 2000s various cases were reported from North America and Europe [26, 45, 49] and a report from 2013 in Japan [49] shows the global occurrence of AdDV. AdDV belongs to the *Densovirinae* subfamily of the family *Parvoviridae*. Densoviruses include non-enveloped isometric, linear, single-stranded DNA viruses with a diameter of approx. 25 nm and a genome size of 4-6 kb [26, 43, 50]. Viruses belonging to the subfamily of the *Densovirinae* are known to be highly virulent towards their hosts [51].

The substantial economic losses that can be caused by AdDV urge the research towards a better understanding of the underlying mechanism of virus infection and transmission and of disease outbreaks and disease monitoring, which will all aid in developing optimized prevention strategies. Currently there are no known AdDV-free cricket colonies, although there are stocks, which are claimed to be AdDV free. Whether these cricket stocks are actually AdDV-free is questionable, since negative testing might be due to the use of mismatching primers, as currently five genetically distinct AdDV strains are known (Fang-Shiang Lim, pers.com.). Additionally, successful detection of all of these AdDV strains by PCR requires primers based on conserved regions (Fang-Shiang Lim, pers. com.). Most cricket colonies seem to carry a covert AdDV infection, not showing any visible symptoms. The current knowledge on AdDV is insufficient to understand how covert infections become active, overt infections leading to disease outbreaks and mortality in the cricket rearings. Covert infections are not clearly recognizable, as the symptoms of the disease might not be prevalent or only include mild symptoms, such as diminished feeding or reduced growth, life span and fecundity. Active infections show clear symptoms, the hind legs of the crickets get paralyzed, their abdomen becomes swollen, whitish and milky as the virus liquefies the organs [26]. The virus is likely transmitted horizontally, via sexual transmission, body contact, surfaces or faeces and research suggests that even the air filters of the rearing facilities can contain AdDV [26]. In addition, it is also possible that the virus is vertically transmitted (from parents to offspring). Currently, prevention of disease outbreaks depends on frequently monitoring the rearing. However, this might be too late for an effective intervention to prevent economic loss. Therefore, additional knowledge is needed on the dynamics of AdDV outbreaks, to be able to predict potential outbreaks.

Technological advancements allow modern diagnostic tools such as new generation sequencing (NGS) and quantitative real time polymerase chain reaction (qPCR) to be used by the producers to identify and predict diseases. However, these methods can best be utilized in combination. For instance, detection of a pathogen by NGS does not necessarily mean an active or even a covert infection. However, if the information of NGS is combined with qPCR, information on the upregulation of host anti-microbial peptides (AMPs) and with certain predictive production parameters (such as the survival rates, developmental time, biomass, and reproductive rate) and producers can make fact-based decisions on how to react. By collecting data on the abiotic factors influencing the rearing, other patterns like changes in temperature, feed quality, air pressure etc. can be also identified and the possible triggers of AdDV outbreaks can be linked to the epizootics. However, disease outbreaks should not only be treated with curative methods, but should rather be avoided with the use of preventive methods, further limiting the economic damage. One way to allow for prevention of AdDV outbreaks would be through the establishment of AdDV-free breeding stocks of house crickets, but this requires a more in-depth understanding of the vertical transmission of AdDV.

Entomopathogenic fungi (EPF)

Several taxa within the Fungi are capable of killing insects [52, 53]. Some entomopathogenic fungi (EPF) are widely used for biological control purposes to control for instance agricultural pests [54] or mosquitoes vectoring diseases [55]. Isolates belonging to the order Hypocreales, in particular of the genera *Beauveria* and *Metarhizium*, are the most commonly used. Most EPF infect insects by penetrating their cuticle, and

thus are not dependent on an oral mode of infection [56]. The infection cycle starts with adhesion of conidia to the cuticle followed by germination and penetration of the cuticle. After a successful penetration of the cuticle the fungus will grow in the hemolymph, and eventually it will result in the death of the host due to starvation, organ destruction or toxin production. After the death of the host, the fungus produces new infective spores called conidia externally [56]. *Beauveria bassiana* can infect various insect hosts, and there are several isolates, some of which are highly host specific. Similarly to *Beauveria*, the genus *Metarhizium* contains both host-specific species, such as *Metarhizium acridum* which only infect species from the order Orthoptera [57], and more generalist species, such as *Metarhizium brunneum* which is known to infect a broad range of insect species across different orders. Entomopathogenic fungi can be introduced into cricket rearings in various ways. In open rearing facilities, infected insects can vector the fungi into the rearing space. Since many of the EPFs mentioned earlier are widely used as biocontrol agents, these EFPs can enter the cricket rearings by drift from nearby sprayings, or via feedstock, which was earlier treated with EPFs.

Abiotic stressors

Insect farming involves a complex system where a wide range of parameters interacts with each other. The main components are the insects themselves, the feedstock provided for their growth and the environmental conditions in which they are kept.

Temperature is a crucial factor in insect production systems, since insects are poikilothermic organisms, meaning that their body temperature is dependent on the ambient temperature [58]. The developmental rate, which is strongly correlated with growth rate, is dependent on the temperature, with a minimum and maximum in the curve [59, 60]. For house crickets, the optimum rearing temperature ranges between 28-30 °C [61]. Deviations from the optimal temperature can result in temperature stress, leading to negative physiological effects [62]. One possible reason for temperature deviation is the application of curative treatments against pathogens, e.g. by transient exposure to high temperature [63]. Crickets are known to exhibit the phenomenon of behavioural fever [64], choosing areas with elevated temperatures upon infection by bacteria.

Density of crickets in the rearing is a crucial factor to avoid crowding-related stresses. Density has been well studied for other insect species [65, 66], but in the case of crickets, only a few studies address the topic [67-69]. During the development from pinheads to fully-grown adults, the cricket body mass increases from 0.7 mg to 300 – 500 mg of adult live body weight. As this is an increase of ~400 - 700 fold, the occupied space by each individual also increases in the rearing space. The proportionally reduced individual-to-surface ratio leads to enhanced contact and interactions among crickets, which can facilitate the spread of disease-causing agents and which can create stress in the population. The cricket rearing area is often reported as volume per individual, but this methodology can lead to false conclusions since most cricket rearing facilities provide some kind of internal structure in the rearing units (Fig. 1) to increase the surface area within. Structures in use are egg trays, paper barriers, and others with the intention to create additional surface area to allow the crickets to exhibit their natural behaviour.

Feed used in most cricket rearings is chicken feed [33, 70]. Further research aiming to optimize rearing substrates to the dietary needs of the house crickets is needed, similar to the work done by Morales-Ramos et al. [32] for the selection of feed ingredients. A diet formulation providing all the necessary nutrients of house crickets also can contribute to a robust immune system of the insects, since lacking nutrients are known to interfere with immune capacities [71, 72]. A robust immune system is especially important for house cricket rearing, because high rearing densities are known to be a highly conducive environment for disease epidemics [37, 73]. However, sustainable insect farming should aim to feed the insects with non-

utilized by-products of the food chain. On a farm level it could be that other than mixing feed ingredients for a balanced diet, little can be done with respect to optimization of feed.

5. Research objectives and thesis outline

The central objective of this thesis is to study the effects of key stressors associated with house cricket rearing that could affect cricket survival and biomass growth and/or that could be triggers of AdDV outbreaks. These stressors include density, temperature and two fungal pathogens that the crickets can be exposed to. A better understanding of the relation of AdDV levels and these stressors can be used to implement changes in the rearing setups with the aim to reduce the chances of AdDV outbreaks and losses related to this pathogen, or other suboptimal conditions. Some of the stressors had been evaluated individually [61, 67, 68, 74]. Various immune challenge experiments were conducted with crickets, examining the phenoloxidase enzyme activity, lysozyme-like enzyme activity, the number of circulating hemocytes, and encapsulation ability [64, 75-77]. These experiments mainly used single stressors such as nylon filament insertion [77, 78] or bacterial injections [64, 76]. Combined stressors affecting the cricket simultaneously under rearing conditions received little attention so far. In this thesis, several combined stressor experiments were conducted to assess the effect on the survival, biomass and AdDV levels.

Rearing temperature and cricket density were selected as two key factors in successful house cricket rearing (**Chapter 2).** Three different temperatures in combination with three different rearing densities are tested. The effects on cricket survival, on male and female individual weight and on total biomass obtained are investigated. This is the first study to examine the combined effect of these two stressors.

In **Chapter 3**, a selection of *Beauveria* and *Metarhizium* isolates are tested for their pathogenicity to house crickets. Subsequently, one isolate from each genus is tested in combination with three different rearing densities to evaluate the combined effect of these biotic and abiotic stressors on cricket survival, individual weight and the total biomass obtained.

In **Chapter 4**, I examine the tissue tropism of AdDV and determine its presence and levels in reproductive tissues and the midgut of house cricket females and males. The effect of mating on virus levels is also tested. In addition, I measured the change in virus levels throughout the rearing cycle of crickets. The results provide fundamental information to understand viral transmission and disease outbreaks, and to design optimized preventive measures.

Although AdDV is considered the most prevalent disease in house cricket rearing, we cannot ignore potential other viruses to be present in crickets, and the possible effects that those viruses could have on cricket fitness and on disease outbreaks. In **Chapter 5**, I identify which DNA and RNA viruses are present in three different house cricket colonies. This is the first study to use NGS on pooled samples obtained from various crickets stocks to report both DNA and RNA viruses present.

In **Chapter 6**, the findings of the research chapters in the thesis are integrated. I discuss how cricket rearing is affected by multiple stressors, which need to be examined in combination to design optimal cricket rearing strategies. Furthermore, I elaborate on the need for complex diagnostic methods integrating modern technologies and classical bioassays. In the end of the chapter, suggestions for future research are given.

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CHAPTER 2

2

Effects of temperature and density on house cricket survival and growth and on the prevalence of Acheta domesticus densovirus

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Abstract

The house cricket, *Acheta domesticus*, is a commonly reared insect for food and feed purposes. In 1977, a report described a colony collapse, which was caused by the single-stranded DNA virus Acheta domesticus densovirus (AdDV). Currently, there are no confirmed *A. domesticus* colonies free of AdDV, and viral disease outbreaks are a continuous threat to *A. domesticus* mass rearing. Correlations between cricket rearing density or temperature and AdDV abundance have been hypothesized, but experimental evidence is lacking. Optimised rearing conditions, including temperature and density, are key to cost-effective cricket production. In this study, house crickets were subjected to different combinations of rearing density (10, 20, 40 crickets per box) and temperature (25, 30, 35 °C) to study the effect on cricket survival, biomass, and AdDV abundance. Rearing temperature affected had a minor effect on survival, which ranged between 80 and 83%. Total cricket biomass increased with higher temperatures and higher densities. Viral abundance in crickets at the end of the rearing period was variable; however, high rearing density seemed to result in higher AdDV abundance. At 35 °C, a temperature considered suboptimal for house cricket production, viral abundance tended to be lower than at 25 or 30 °C.

Keywords: *Acheta domesticus*; house cricket; insect production; insects as food and feed; cricket viruses; entomopathogenic viruses; mass rearing

1. Introduction

The continuing increase in the global human population urges for better use and distribution of resources, such as food, feed, and raw materials produced by agriculture [74]. Mass rearing of insects is proposed as a solution for better utilization of organic matter from the side and residual streams required to produce a sufficient amount of good quality food and feed [13]. Over the last decade, several companies started industrial-scale insect rearing to produce insects for food and feed purposes [75]. The insect-rearing industry aims to utilize and upcycle (low) value organic resources [31], thereby producing alternatives to the current protein sources with a relatively lower environmental impact [76]. For several decades, the house cricket *Acheta domesticus* L. has been reared as a pet/zoo feed in Europe and North America and as a food source in Asia [17, 29]. This cricket has a favourable nutritional profile [77, 78], which allows its direct utilization by the food industry as an ingredient of various products [24, 25]. However, the mass production of this species can be easily jeopardized by the Acheta domesticus densovirus (AdDV), of which the first report dates back to 1977, describing a colony collapse due to a disease caused by this virus [26]. AdDV can cause massive disease outbreaks in *A. domesticus* colonies, causing high mortality and the loss of almost entire production batches [26, 36, 43-45]. At present, there are no confirmed virus-free *A. domesticus* colonies known. AdDV is a single-stranded DNA virus from the insect-specific *Densovirinae* subfamily of the *Parvoviridae* family [43]. Viruses from this subfamily are known to be highly virulent for their host organisms [51]. AdDV also infects other cricket species, but in these species, the infection does not result in mortality [45]. Cricket-rearing facilities are conducive for virus transmission due to the high degree of crowding, the high humidity, and the high temperature, conditions set to promote biomass output [27]. However, it is not yet understood if AdDV is mainly present in a covert state, in which the symptoms are not clearly visible and may include mild symptoms such as diminished feeding. Currently, there is no information on how the virus can switch from a covert state into an active, overt state, with apparently paralyzed and swollen crickets. The literature available on AdDV mainly covers its detection and quantification [26, 46], but studies examining the transmission between individuals and different generations of crickets are lacking. During the growing phase of the crickets, several possible stress factors can be identified, such as non-optimal temperature, too high humidity, high rearing densities, or the presence of various entomopathogens [34]. These factors are hypothesized to interact with both each other and the host organism, making them possible triggers for symptomatic AdDV outbreaks. Temperature is a crucial factor in insect production systems since insects are poikilothermic organisms, meaning their body temperature is dependent on the ambient temperature [58]. Insect developmental rate depends on temperature according to an optimum curve delimited by a minimum and maximum [59, 60]; for house crickets, the optimal range is between 28 °C and 30 °C [61]. When the temperature in the rearing facility deviates from the optimal range, it can result in temperature stress, which can lead to negative physiological effects [62]. The infrastructure required for heating and cooling in rearing facilities is one of the major costs involved in both the construction and the operation of an insect production facility [79]. To achieve maximal efficiency in the production unit, it is, therefore, logical to aim for the highest possible rearing densities in order to increase productivity per surface area. In most cases, growers use egg trays and paper corridors to increase the surface area within the production unit and to provide dark hiding areas as a resting space for the crickets [80]. During the growth phase, house crickets increase in biomass from approximately 0.7 mg as pinhead (freshly hatched nymphs) to 300–500 mg of adult weight, an increase of ~500–750-fold (Jozsef Takacs, personal observations). Consequently, with the increase in body volume, the resting space will become smaller, and crowding can be observed in the production units.

Optimal densities combined with optimal growth temperatures resulting in the highest yields per batch are favourable from a commercial production perspective. Unlike other mass-reared insect species [65-67, 81], only a few studies investigated optimal rearing densities and temperatures for house crickets [67, 68]. To

date, it is unknown if there is a correlation between rearing density, temperature, and the abundance of the AdDV. Optimising rearing conditions for the best possible yields while maintaining low disease incidence is crucial for the economic viability of cricket mass production. Therefore, we investigated the possible consequences of different temperatures and densities on survival, biomass production, and AdDV abundance.

2. Materials and Methods

Cricket rearing and experimental units

The house cricket colony kept at the Laboratory of Entomology, Wageningen University since 2006 was reared in plastic containers (40 L, containing ca. 500 adults) in a climate-controlled room at ~28 °C [32] and crickets were provided with chicken feed (Kuiken Opfokmeel, Kasper Fauna Food, Woerden, The Netherlands), carrot slices and water from water dispensers on a daily basis. For the experiment, 20-day-old crickets (calculated from the date of hatching) were randomly selected from the main rearing. The experimental units consisted of plastic boxes with a volume of 900 cm^3 (10 × 15 × 6 cm) in which 10, 20, or 40 crickets were housed per box. Each box received a piece of cardboard egg tray (5 × 5 × 7 cm), water gel (DCM Aquaperla®, De Ceuster Meststoffen N.V., Grobbendonk, Belgium), and feed trays (3.5 × 0.2 cm) to provide the crickets with hiding space, water, and feed sources, respectively. In the 10 and 20 crickets per box densities, one feed tray was used, while in the 40 crickets per box density, two feed trays were used to avoid the possible stress caused by the lower individual-to-feeding surface ratio expressed as the number of individuals per unit of volume. The lowest of the three densities we tested corresponded with the density commonly used in house cricket lab colonies, including the colony from which our experimental crickets originated. Our experimental design was aimed at testing density as a possible stress factor; hence we tested 2× and 4× higher densities. The boxes were observed daily, and water and feed were replenished when required to allow *ad libitum* drinking and feeding. The boxes were kept in incubators set to the corresponding treatment temperature of 25, 30, or 35 °C, all at 12:12 L:D period and on average 75–85% relative humidity.

The experiments were finished when the first chirp sounds appeared, signalling male maturity. This was at varying time points due to the different temperatures resulting in different developmental durations (15–16 days at 35 °C, 21–22 days at 30 °C, and 30–31 days at 25 °C). At the end of the experiments, the number of crickets per cage was counted and considered as survivors; missing crickets were considered dead. When crickets were observed dead, they were not removed.

Three different temperatures combined with three different densities were tested, creating nine different treatments, with five replicate boxes per treatment. The experiment was serially repeated three times (Experimental runs 1, 2, and 3); thus, the total number of crickets used in the experiments was 3150. The three experimental runs were carried out between May and November 2022.

DNA extraction and qPCR analysis

At the end of the experiments, five adult crickets (three females and two males) were taken from every experimental unit (box) and stored at −20 °C until DNA extraction. At the start of each experimental run, cricket samples were also taken from the main rearing to determine the initial viral load since a negative control with virus-free crickets was not available. Homogenization of the crickets sampled was conducted by pestle and mortar with the addition of 0.5 mL of phosphate-buffered saline (PBS) solution as the first step of the DNA extraction. The homogenized samples were centrifuged for 15 min at 8000 RPM, after which the supernatants were removed and used for DNA extraction. The DNA extraction was carried out

using the Qiagen dNeasy® Blood and Tissue kit (Qiagen, Hilden, Germany), following the Total insect DNA extraction protocol, except for sample homogenization, which was conducted as described above. Quality control of the DNA samples was carried out using Nanodrop® ND1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Relative quantitative PCR (qPCR) was performed to quantify the number of AdDV viral copies in the samples. The *Acheta domesticus* elongation factor 1 alpha gene, partial coding sequence (Genbank access. nr. EU414685.2) with an amplicon size of 199 base pairs was used as host reference gene (see Table 1 for primer sequences). For the primer design, Primer3 (version 4.1.0) was used [82]. The AdDV primers targeted a gene encoding for a non-structural protein with the amplicon size of 96 base pairs and were adopted from the publication of Duffield et al. [27] (Table 1). Reactions were performed with SYBR™ Select Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA) with a total reaction volume of 20 µL containing 3 µL template DNA, 5.4 µL autoclaved water, 10 µL SYBR™ Select Master Mix, 0.8 µL forward and 0.4 µM reverse primers. The protocol for the thermal cycling was as follows: UDG activation for 2 min at 50 °C and denaturation for 2 min at 94 °C, followed by 40 cycles of 30 s at 95 °C, 45 s at 59 °C and 1 min at 72 °C. Plate reads to measure fluorescence were conducted after every extension step. A visual inspection of the melting curve with a temperature gradient of 0.5 °C/5 s for the interval of 55 to 95 °C was carried out as quality control to ensure that only specific products were amplified [83]. Primer efficiencies were calculated by incorporating a standard curve for every plate individually and were in the range of 95–105% for both the host and the viral primers. The amplification was carried out using a CFX96 Touch Real-Time PCR Detection System and the CFX Maestro 5.3 software, both from Bio-Rad Laboratories, Inc., Hercules, CA, USA. The threshold cycle (Ct) for each well was determined by the CFX Maestro software using a single CQ (Ct) threshold determination mode.

Raw abundance data from the qPCR runs were analysed using the qbase+ Version: 3.2 (Biogazelle, Zwijnaarde, Belgium—www.qbaseplus.com, accessed on 5 July 2022) software, with target and run specific amplification efficiencies. The abundance of AdDV was expressed as Calibrated Normalized Relative Quantities (CNRQ) [84]. This is achieved by first normalizing the CQ values to the host reference target (the host gene) and then by scaling the fold of change to the reference group of the start values (samples taken at the start of the experiments to determine initial viral abundance). After a logarithmic transformation, the CNRQ values were exported for further analyses in R Studio.

Table 1. Primers used in qPCR (EF1- Elongation factor 1, and AdDV96- Acheta domesticus densovirus).

Name	Reference	Primer sequence 5′ to 3′ (Forward/Reverse)	Amplicon Size (bp)	Gene Target
EF1	This study	GGAAATCAAGAAGGAAGTCAGC/GGCATCCAAAGCCTCAATAAG	199	*A. domesticus* elongation factor 1 alpha gene
AdDV96	Duffield et al. [18]	GCGAGCAATCCCGACTACTA/CGCGTTGTTGATGTCCTTCC	96	Non-structural protein

Data analyses and visualization

Data were analysed using R, version 4.2.2 (31 October 2022) [85] and R Studio Team (2020).

To test if the data sets were normally distributed, a Shapiro–Wilk test of normality was used [86]. If the data met the assumptions of normality and homogeneity of variance, analysis of variance (ANOVA) was used to test the differences between the treatments. The following variable met the assumption of normality: relative abundance data.

If the data was not normally distributed, generalized mixed effect models (GLM) were built using the lme4 package of R [87]. The following variables did not meet the assumption of normality: cricket survival, total biomass, and female and male individual weight. The GLM models were compared based on the Second-order Akaike information criterion (AIC) values, and the best-fitting models with the lowest AIC were selected [88]. In the case of the total biomass, female and male individual weight data, the model including an interaction between temperature and density was the model best fitting the data; the experimental run included a random factor. A GLM with binomial distribution was used to compare survival. The best-fitting model for the survival data used temperature as a fixed factor and the experimental run as a random factor.

In case a factor had a significant effect, pairwise comparisons were carried out with the Package 'emmeans,' version 1.8.4-1 from R [89] or with Tukey's honest significant test (HSD) test. Data were visualized using the R package ggplot2 [90].

3. Results

The following experiments combined three densities and three temperatures into nine treatments to test their effect on cricket survival, total biomass, and relative viral abundance.

Cricket survival

The temperature had a main effect on cricket survival (Figure 1, Supplementary Table S3). No effect of density or interaction between temperature and density was detected on survival. Survival was higher for crickets reared at 25 °C than for those reared at 30 °C ($p = 0.0111$). No difference in cricket survival was seen between 25 °C and 35 °C or 30 °C and 35 °C ($p = 0.5210$ and 0.1693, respectively).

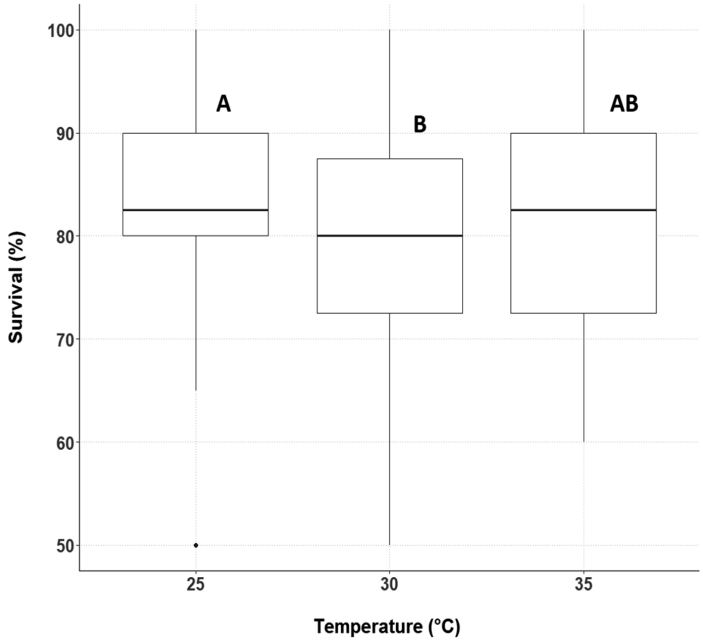

Figure 1. Median cricket survival calculated over the three experimental runs. Temperatures tested are indicated along the X-axis. The Y-axis represents survival (%), horizontal lines depict the median, boxes indicate the lower and upper quartiles, vertical lines extend to minimum and maximum values, black dots represent outliers. Boxplots that have no letters in common differ significantly.

Cricket biomass

Total biomass of the surviving individuals

The total harvested biomass of surviving crickets differed significantly between treatments; there was an interaction between rearing temperature and rearing density (Figure 2, Supplementary Table S2). The three treatments with the lowest rearing density of 10 individuals per box resulted in significantly lower produced biomass compared to all other treatments. Between the three treatments with density 10, at 25 °C, a significantly lower weight was produced compared to the 30 °C and 35 °C treatments (p = 0.0002 and 0.0004, respectively). There was no significant difference at density 10 between the temperatures 30 °C and 35 °C (p = 1.000). At the density of 20 individuals per box, the pattern was similar to density 10; at the two higher temperatures of 30 °C and 35 °C, significantly higher biomass was recorded than at 25°C (p = 0.0098 and 0.0375, respectively). The biomass at 30 °C and 35 °C for density 20 did not differ significantly from each other (p = 1.000). Among all treatments, those with a density of 40 individuals per box resulted in the highest average total harvested biomass. However, total biomass at the density of 40 individuals per box was similar at the three tested temperatures (p = 0.1112, 0.4317, and 0.9988, respectively). The exact p values for the pairwise comparisons can be found in Supplementary Table S2.

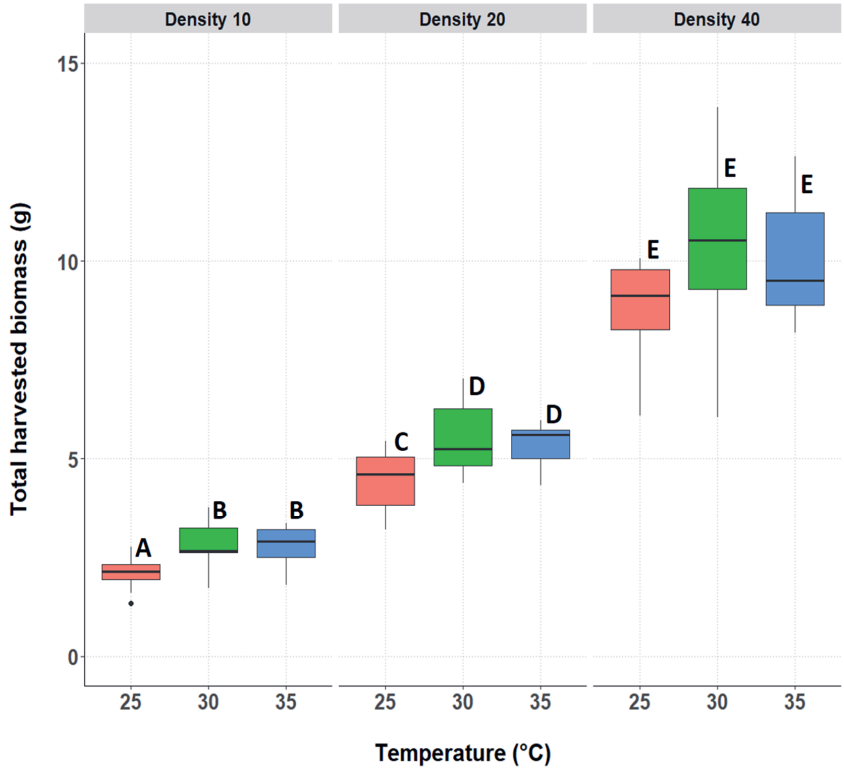

Figure 2. Total harvested biomass (g) for each treatment group. Temperatures are indicated along the X-axis per density tested, indicated above the figure (D10, D20, or D40). The boxplots show the median total biomass (bold horizontal line), boxes depict lower and upper quartiles, vertical lines extend to minimum and maximum values, and black dots represent outliers. Boxplots that have no letters in common differ significantly.

Individual weight of the surviving crickets, separated by genders

The median individual weight of the crickets was significantly affected by both temperatures and by density. Since gender had a significant effect on the individual weight ($p < 0.0001$), with females being heavier than males, the individual weight data for the two genders were analysed separately. The median individual weight of the surviving female crickets differed significantly between treatments (Figure 3 and Supplementary Table S4), showing an interaction between rearing temperature and rearing density. The lowest weight was achieved at 25 °C, which was significantly lower than 30 °C and 35 °C. The female individual weight was significantly higher at 30 °C compared to 35 °C, at the densities of 20 and 40 crickets.

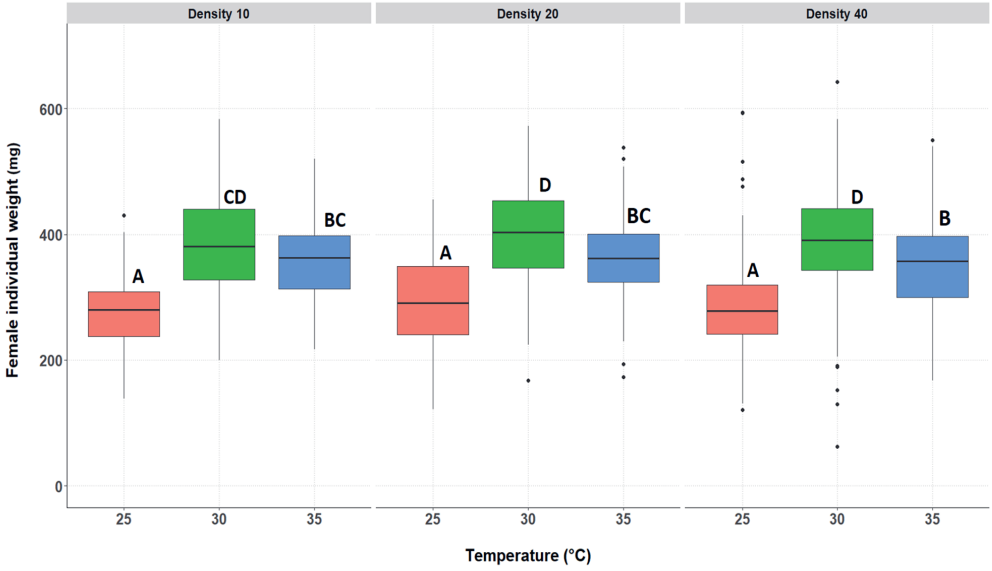

Figure 3. Female individual weight (mg) for each treatment group. Temperatures are indicated along the X-axis per density tested, indicated above the figure (D10, D20, or D40). The boxplots show the median individual weight (bold horizontal line), boxes depict lower and upper quartiles, and vertical lines extend to minimum and maximum values, black dots represent outliers. Boxplots that have no letters in common differ significantly.

The individual weight of the surviving male crickets differed significantly between treatments (Figure 4 and Supplementary Table S5); there was an interaction between rearing temperature and rearing density. Similarly to females, the median male individual weight was the lowest at 25 °C for all densities. Male individual weight differed significantly between 30 °C and 35 °C for the densities of 20 and 40. At 35 °C, the individual weight of males was significantly higher at density 10 compared to 40.

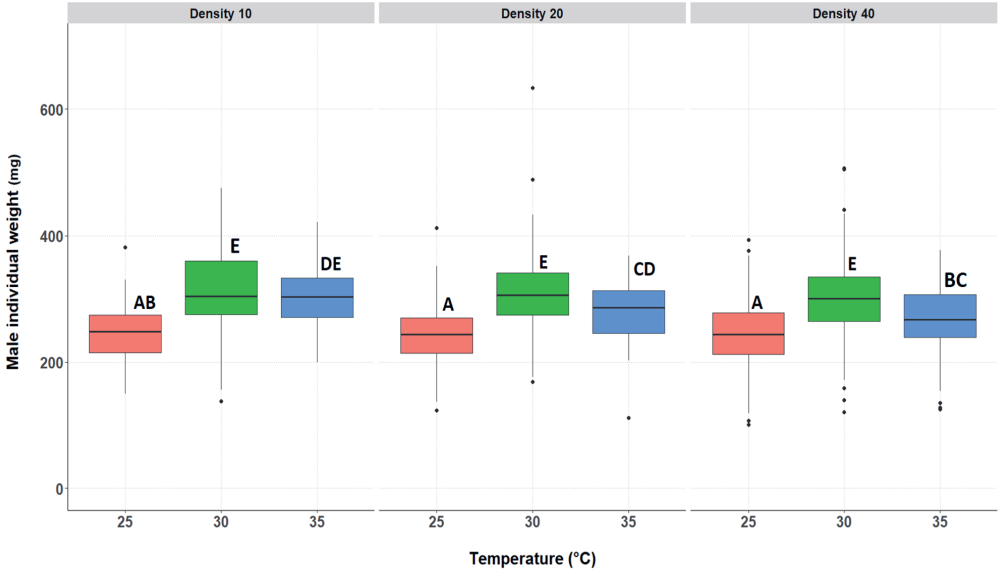

Figure 4. Male individual weight (mg) for each treatment group. Temperatures are indicated along the X-axis per density tested, indicated above the figure (D10, D20, or D40). The boxplots show the median individual weight (bold horizontal line), boxes depict lower and upper quartiles, and vertical lines extend to minimum and maximum values, black dots represent outliers. Boxplots that have no letters in common differ significantly.

Relative abundance of AdDV

The variance analysis showed a major, significant batch effect on the viral abundance data collected at the end of the experimental runs, as confirmed by a one-way ANOVA ($p = 2 \times 10^{-16}$). Therefore, the three experimental runs were analysed separately. The initial viral load of the three batches of nymphs was similar (ANOVA, $p = 0.773$). There was no significant interaction effect between temperature and density on the relative viral abundance for any of the three experimental runs ($p = 0.1635$; 0.0866 and 0.5902, respectively) (Suppelementary Table S1). In the case of experimental run 1, only temperature had a significant effect on the viral levels ($p = 0.0405$), being higher at 25 °C than at 35 °C; however, no effect of density was observed ($p = 0.7845$). For experimental run 2, neither an effect of temperature nor density was observed ($p = 0.8391$ and 0.2092, respectively). However, in experimental run 3, both temperature and density significantly affected viral abundance ($p = 0.0024$ and 0.0047, respectively). Among the three experimental runs (Figure 5), only in the first run a high fold of change in viral abundance was observed; the second and third runs resulted in minimal overall changes. In the case of experimental runs 1 and 3, rearing at 35 °C tended to result in lower relative viral abundance.

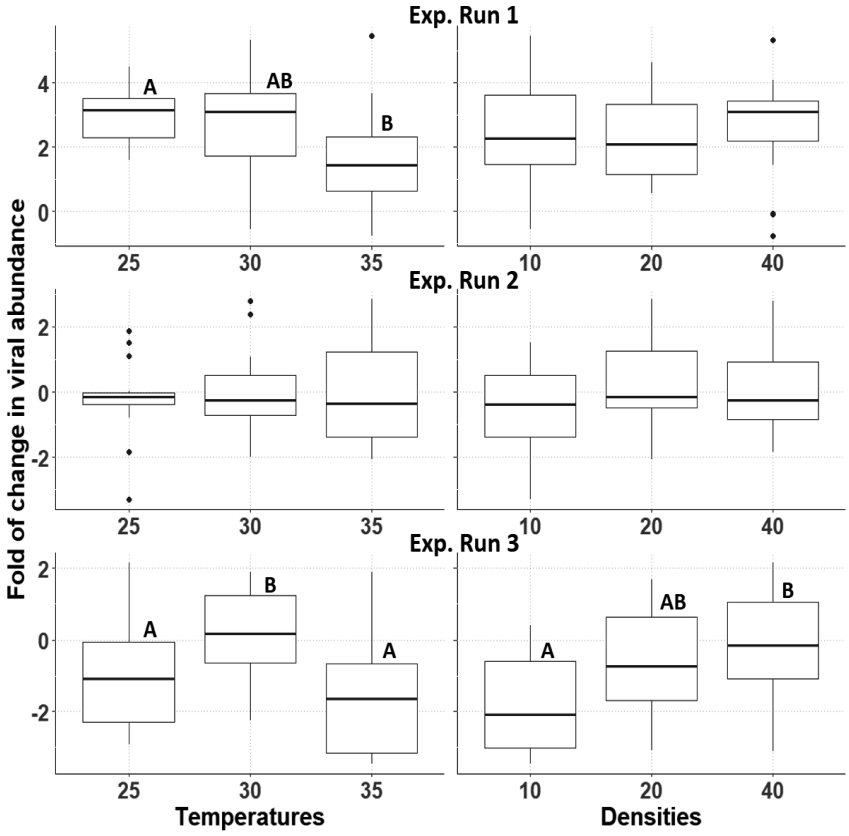

Figure 5. Relative viral abundance in the three experimental runs. The Y-axis represents the fold of change in viral abundance, relative to the start levels, while the X-axis represents the different temperatures (three left−hand panels) and different densities (three right-hand panels). The boxplots show the lower and upper quartiles; vertical lines extend to minimum and maximum values, black dots represent outliers. Boxplots that have no letters in common differ significantly.

4. Discussion

For cold-blooded animals and especially insects, the environmental temperature has a strong impact on survival, developmental rate, reproduction, and behaviour [91-93]. The ambient temperature can be relatively easily manipulated within the cricket-rearing facilities using ventilation systems. To decide on an optimal temperature, it is advised to test a range of potential temperatures; however, this did not receive focus from research so far.

In this study, the temperatures of 25 °C, 30 °C, and 35 °C were selected to cover the likely range of cricket production in a semi- or fully industrialized facility. Significant differences were observed for the biomass produced for the different rearing temperatures and densities tested. Harvested cricket biomass showed a minor effect of rearing at temperatures 30 °C and 35 °C with densities of 10 and 20 crickets per box. At the rearing density of 40 crickets per box, there were no differences in total biomass between the three temperatures tested. Another important aspect is the individual body weight of the crickets. Minor differences in individual body weight between the three densities tested were found. The temperatures of 30 °C and 35 °C resulted in higher body weight for both females and males. A higher individual body weight is profitable for producers. In addition, higher female body weight can be important for the breeding stock of an insect-rearing facility, as it will result in higher egg yields [94, 95]. A recent study from 2023 showed that higher rearing densities could reduce the survival of house crickets significantly [68]. From the productivity perspective, the question might be raised: are the losses due to mortality caused by high rearing densities outweighed by the higher cricket biomass yield? In the results presented in this article, the increase of cricket rearing density interacted with the tested rearing temperatures, resulting in a clear increase in the total harvested cricket biomass. Further research should address the maximum rearing density that prevents competition for feed and space that may lead to reduced biomass harvest and to an increased hazard of disease epidemics since high densities are conducive for disease epidemics [37, 73].

The economic impact of viral disease outbreaks is well known for several mass-reared insect species [96, 97], and there are also references directly connecting densovirus outbreaks with economic losses in cricket production facilities [36]. Decreased rearing densities are hypothesized as a possible solution in house cricket rearing to avoid viral disease outbreaks, but experimental data supporting this practice is lacking. The viral abundance data of the experiments described in this article did not unequivocally confirm the hypothesis of higher rearing densities resulting in higher viral abundance over the density range studied, although a significantly higher abundance was found at density 40 in one of the three batches. However, this observation could be due to the high variation of the final viral loads between the experimental runs. High variation of viral levels is prevalent in the rearing, not just over time but also over life stages simultaneously present in the facilities. Insect densities in mass-rearing of house crickets are poorly documented in the literature; expressions are found as number of individuals per volume unit of rearing container or per surface unit [32] which complicates comparisons among studies. Moreover, in practice, the surface area in rearing containers is often substantially enlarged by adding carton material, e.g., carton previously used for holding chicken eggs, as applied in our study. Extrapolation of the densities we studied to mass-rearing scale is not straightforward and requires additional experiments. Additionally, our data showed that viral abundance was not impacted by density, but mainly by rearing temperature. Similarly, there was no effect of rearing density on survival (despite high relative viral loads in some cases), but the rearing temperature of 30 °C resulted in slightly lower survival than a temperature of 25 °C.

With increasing rearing densities, the occurrence of cannibalism should also be monitored. In our density experiments, we did not record if cannibalism occurred, but insect rearing often has to deal with losses caused by cannibalism when the rearing densities are high [81, 98, 99], and crickets are known to be highly

cannibalistic [100]. Cannibalism can also make the insects more vulnerable to opportunistic pathogens since the cuticle, the natural barrier preventing the entry of the pathogens, is penetrated. This way, the first defence of the crickets can be bypassed by pathogens [101]. For densoviruses, however, this might be different since those infect mainly via oral ingestion [102]. To date, no research addressed the correlation between the rearing densities and the incidences of cannibalism in crickets. In the rearing of both Copenhagen University and Wageningen University and Research, personal observations were made of crickets cannibalising on asymptomatic and AdDV symptomatic individuals. When consuming symptomatic crickets, relatively high viral exposure can occur for the cannibalistic crickets, which can lead to a viral infection. However, cannibalism can also suggest feed depletion or may indicate that nutrients are lacking from the diet used in the rearing system [103-105], which can induce healthy individuals to cannibalise (diseased) individuals. This can initiate a chain reaction in the rearing, which might lead to the rapid colony collapses reported as a result of AdDV outbreaks. Lacking nutrients can also result in decreased immune functions of insects [106, 107], making them less resistant to possible pathogen challenges. House crickets are commonly reared on chicken feed [33, 70], but optimised diets should be developed to increase feed conversion efficiency and cost-effectiveness. These studies could also evaluate the effect of different diets on the abundance of AdDV. Furthermore, there are several other factors influencing the crickets during their rearing, such as humidity and light: dark cycles. The presence of other viruses and their interaction with the crickets and with AdDV can also have an impact on AdDV levels. Evaluating these parameters individually and in combination will resolve their effects on cricket production.

5. Conclusions

Temperature and density are key parameters for the cost-efficiency of most animal production facilities. Our experiments aimed at evaluating the possible effects of varying rearing densities and temperatures on survival, total biomass, and AdDV abundance in house cricket rearing. Currently, AdDV is the main threat to house cricket rearing. Whereas a temperature of 35 °C that is considered suboptimal for house cricket rearing does not appear to be a trigger for AdDV outbreak, the highest density tested may trigger higher viral abundance, depending on an unknown factor favouring viral replication. The results presented in this article can be considered as a basis for further research aiming to optimise the production conditions of cricket rearing for maximum efficiency. Based on the economic aspects of the production, producers can evaluate what is more beneficial for their facility and rearing setup. However, the actual cost of heating is mainly dependent on the location and heating, ventilation, and air conditioning systems. Cricket-rearing facilities can be found in all climatic conditions; the local circumstances and costs will determine which temperatures are better suited for the production and/or are more economically viable to maintain in the facility.

Acknowledgments: We would like to thank Monique van Oers and the other program coordinators of the Insect Doctors program, the Insect Rearing Team of the Laboratory of Entomology for maintaining the rearing and providing the crickets for the experiments and Lia Hemerik for advice on the statistical analyses.

Supplementary Tables and Figures

Table S1. Analysis of variance (ANOVA) output tables for the three experimental runs on relative viral abundance data.

Experimental run 1	Analysis of variance				
	Df	SS	MS	F	p-value
Temp	2	12.87	6.437	3.509	0.0405
Dens	2	0.9	0.448	0.244	0.7845
Temp:Dens	4	12,73	3.184	1.736	0.1635
Residuals	36	66.03	1.834		
Experimental run 2	**Analysis of variance**				
	Df	SS	MS	F	p-value
Temp	2	0.61	0.306	0.176	0.8391
Dens	2	5.67	2.835	1.635	0.2092
Temp:Dens	4	15.38	3.845	2.217	0.0866
Residuals	36	62.44	1.734		
Experimental run 3	**Analysis of variance**				
	Df	SS	MS	F	p-value
Temp	2	25.34	12.671	7.151	0.00243
Dens	2	22.16	11.080	6.253	0.00467
Temp:Dens	4	5.04	1.259	0.710	0.59016
Residuals	36	63.79	1.772		

Table S2. P values for the pairwise comparisons for the biomass data

P =	25°C D10	25°C D20	25°C D40	30°C D10	30°C D20	30°C D40	35°C D10	35°C D20	35°C D40
25°C D10		<.0001	<.0001	0.0002	<.0001	<.0001	0.0004	<.0001	<.0001
25°C D20			<.0001	<.0001	0.0098	<.0001	<.0001	0.0375	<.0001
25°C D40				<.0001	<.0001	0.1112	<.0001	<.0001	0.4317
30°C D10					<.0001	<.0001	1.0000	<.0001	<.0001
30°C D20						<.0001	<.0001	1.0000	<.0001
30°C D40							<.0001	<.0001	0.9988
35°C D10								<.0001	<.0001
35°C D20									<.0001

Table S3. Survival, harvested biomass and individual weight of house crickets reared at nine combinations of temperatures and densities

Treatment	Survival (%) (±SE)	Mean harvested biomass (mg) (±SE)	Mean female weight (mg) (±SE)	Mean male weight (mg) (±SE)
25°C D10	79.3 (±7)	2114.467 (±5)	273 (±7.22)	246 (±6.23)
25°C D20	83.0 (±5)	4442.133 (±4.29)	294 (±6.57)	241 (±4.42)
25°C D40	83.3 (±4)	8834.267 (±2.85)	283 (±4.12)	244 (±3.4)
30°C D10	81.3 (±7)	2832.400 (±7.59)	389 (±10.88)	315 (±8.69)
30°C D20	78.3 (±5)	5553.400 (±5.71)	400 (±7.44)	308 (±6.26)
30°C D40	77.0 (±4)	10491.133(±4.02)	391 (±6.44)	301 (±3.53)
35°C D10	84.7 (±8)	2785.933 (±5.55)	359 (±8.02)	302 (±6.02)
35°C D20	82.7 (±5)	5408.000 (±4.4.)	364 (±5.21)	279 (±4.3)
35°C D40	79.0 (±4)	10065.467 (±3.56)	351 (±4.66)	270 (±3.45)

Table S4. P values for pairwise comparisons for female individual body weight.

P =	25°C D10	25°C D20	25°C D40	30°C D10	30°C D20	30°C D40	35°C D10	35°C D20	35°C D40
25°C D10		0.5244	0.9350	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
25°C D20			0.9548	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
25°C D40				<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
30°C D10					0.9940	1.0000	0.2323	0.3979	0.0100
30°C D20						0.9644	0.0038	0.0025	<0.0001
30°C D40							0.0329	0.0293	<0.0001
35°C D10								0.9983	0.9999
35°C D20									0.7292

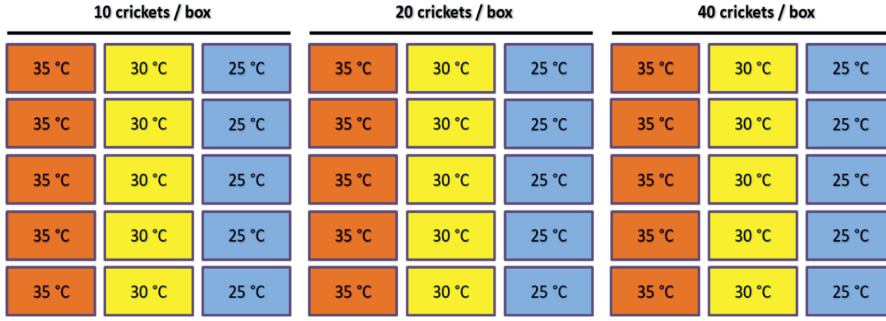

Figure S1. Overview of a single experimental run

CHAPTER 3

3

Density and entomopathogenic fungi as single and combined stressors for *Acheta domesticus*, the house cricket

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Abstract

The house cricket *Acheta domesticus* is a promising candidate for food and feed purposes. Crickets are usually reared in high densities to maximize cost effectiveness, but this may imply stressful conditions affecting cricket fitness and it creates a highly favorable environment for disease outbreaks. In this study, first, we compared the virulence of six isolates of *Beauveria bassiana* and of six isolates of *Metarhizium brunneum* against house crickets. Second, we investigated the effect of simultaneous stressors on cricket health, by exposing crickets to selected fungal pathogens (*B. bassiana* isolate KVL 97-44 or *M. brunneum* KVL 20-01) under three different densities. We recorded the effect of co-stressing on cricket mortality, individual cricket biomass and total cricket biomass. Both the exposure to fungal pathogens and the high rearing density reduced cricket survival and had a negative impact on total biomass production. The average individual biomass of females and males in case of *B. bassiana* exposure was negatively affected, but no effect of *M. brunneum* was observed. The increased cricket densities had a minor negative impact on the individual biomass. The combined effect of the two stressors created a trend towards a lower individual biomass, but with only a minor effect in the case of *B. bassiana* exposure. Producers need to consider the risk of a higher mortality with higher cricket densities, but the increased losses might be outweighed by the higher total biomass output from the same surface area. Fungal pathogens are a major threat if they appear in the rearing area, but only if high spore numbers are present, which are less likely to occur when preventive measures and proper sanitation protocols are established. House cricket producers with good hygiene and rearing setups can overcome the threats, but constant monitoring and, if necessary, intervention is required.

Keywords: house cricket, entomopathogenic fungi, density, mass rearing, insects as food

1. Introduction

In recent years alternative protein sources gained attention and insects, which are promising both for food and feed purposes, also received increasing interest [12]. The interest is based on several attributes, in particular the lower environmental impact during the insect production cycle and the lower amount of resources required per unit of product, when compared to conventional livestock species such as pork, beef, and poultry [108, 109]. Crickets are one of the promising candidates for mass-rearing as an alternative protein source. The house cricket *Acheta domesticus* has a history of traditional small-scale production and is already used as human food widely in Africa and Asia [17]. Targeting the human food market gives the advantage of higher revenues, when compared to insect species that are mainly used as animal feed and therefore have to compete with fishmeal and soymeal prices. House cricket production is already reaching volumes of 7500 tonnes per year in Thailand alone [28] where some 20.000 farmers rear crickets [22]. The reason why house crickets are so popular is partly due to their good nutritional qualities, with fatty acid and amino acid profiles optimal for humans [18-21], and partly due to the relatively uncomplicated production process [17]. Besides fried in whole, house crickets can also be processed and used as food ingredients [24, 25], allowing easy incorporation into the diet in particular in the western world. This use as food ingredient allows crickets to be part of meals on a daily basis instead of being consumed as a delicacy.

Crickets have to face many stressors in the rearing [34]. Biotic stressors include microbial entomopathogens such as viruses, bacteria, and fungi. Entomopathogenic fungi (EPF) enter cricket-rearing facilities from outside, especially in open, semi-industrial facilities, where there are no dedicated heating ventilation and air conditioning systems (HVAC) in place. The HVAC system, if equipped with high efficiency particulate air filter (HEPA), can filter out fungal spores from air [110] and prevent infected or vector organisms from entering the facilities. Infections can still be introduced into the facilities via contaminated equipment, personnel or feedstock.

Up to 700 EPF species have been described belonging to 100 different orders [111]. Only a few of these are described in detail and these are mostly species used for biological control of insects [56]. From these selected species, more than 170 products are registered and sold as biopesticide [112] which currently represent 19 % of the global biopesticide market [53]. The majority of the EPF products are based on species of the genera *Beauveria* and *Metarhizium*, which together represent 70% of the products currently on the market [53, 56, 112]. The success of EPFs to infect their insect hosts depends on various biotic and abiotic parameters [113]. Environmental temperature is one key parameter, for instance most *Beauveria* and *Metarhizium* isolates are effective between temperatures ranging from 20 to 30 °C [114-116]. Upon successful infection of the hosts, the ambient temperatures can also affect the infection rates and the speed of kill [115]. Another key parameter for successful EPF infection is high relative humidity [116], which is a critical factor for fungal spore germination [115]. House cricket rearing facilities are often located in areas with high environmental temperatures and high humidity and therefore provide suitable environments for different EPFs to infect the crickets, which are present in high densities in the rearing units.

Beside microbial pathogens, rearing density as a biotic stressor also plays an important role in disease dynamics in insect rearing systems [35]. Elevated rearing densities have negative effects on production parameters, as shown for other mass-reared insect species such as the black soldier fly [117]. For other species, such as the yellow mealworm (*Tenebrio molitor* L.), the best growth was achieved at a relatively low density (0.5 larvae/cm^3) [66]. Therefore, similarly assessing the possible effects of cricket rearing density on the survival, individual biomass and total biomass produced is highly important to optimize the production. Insect densities in rearing units are usually not reported as the number of individuals per unit of surface area (cm^2), but as number of individuals per unit volume of the rearing space. For cricket rearings,

the fundamental issue with this latter density measure is that almost every cricket production unit is equipped with surface-increasing structures [29], which can be egg trays or carton paper structures, therefore the actual available area for crickets is more appropriately expressed in number of individuals/cm^2. Recent research on cricket rearing densities highlighted the importance of choosing optimal ratios of individuals per surface area to limit mortality [68].

This study aimed to assess the virulence of different *Beauveria bassiana* and *Metarhizium brunneum* isolates by measuring the mortality of house crickets exposed to those isolates. In addition, the study also investigated the combined effect of fungal exposure and cricket density to test potential co-stressing effects. We used the most virulent isolate of each of the fungal species and three cricket densities and measured cricket survival, individual biomass and total biomass.

2. Materials and methods

Cricket rearing

House crickets were reared at Copenhagen University, Section Organismal Biology. The crickets were kept in climate-controlled incubators in plastic containers (32 x 21 x 23 cm), with a 12:12 L:D cycle, at 30 °C with a relative humidity (RH) ranging between 65-70 %. The crickets were fed with grinded chicken feed (Gold 4 Gallico Pellet, Versele Laga, Brough United Kingdom), water gel and organic cucumber slices sourced from local supermarkets. Crickets used for the entomopathogenic fungi exposure experiment were 30 days old at the start of the experiment and were followed for an additional eight days. Crickets used for the co-stressing experiments were 20 days old at the start of the experiments and were followed for an additional 20 days, when they reach the approximate harvest size in industrial conditions.

Spore collection

Entomopathogenic fungi from the culture collection at Section of Organismal Biology, Copenhagen University were used in the experiments. In total, twelve isolates, six *B. bassiana* and six *M. brunneum* (Table 1), were screened for their virulence against the house crickets.

The EPFs were cultured in 90-mm diameter Petri dishes on Sabouraud dextrose agar with yeast added (SDA-Y) [118], at 25 °C in complete darkness for 30-40 days. Conidia were harvested by adding 10 mL 0.05 % Triton-X to a plate, then rubbing with a sterilised Drigalski spatula, avoiding stirring up the agar [113]. Conidia concentrations were determined with a Neubauer haemocytometer (Hausser Scientific Company, Horsham, USA) under phase contrast microscopy and conidia were subsequently diluted with 0.05 % Triton-X to a concentration of 1×10^8 conidia/ml.

EPF virulence

Crickets of 30 days old (counted from hatching) were anesthetized by CO_2 and then submerged into a solution of 1×10^8 conidia/ml for a few seconds. Control treatments were similarly submerged in 0.05 % Triton-X solution. Twenty crickets were used for each isolate and control treatments. Subsequently, the crickets were placed individually in plastic containers (volume 350 ml, diameter: 89 mm, height: 85 mm) in climate-controlled incubators as described above and were checked daily to record survivorship and to provide water and feed. To provide optimal conditions for the spores to germinate and penetrate the cricket cuticle, slices of cucumber were placed in the containers to increase the local RH.

Table 1. EPF isolates used in the virulence screening. (KVL: EPF culture collection at Section of Organismal Biology, University of Copenhagen and CECT: Spanish Culture Type collection)

Nr.	Isolate number	Species	Origin
1	KVL 97-44	*B. bassiana*	Zimbabwe
2	KVL 03-90	*B. bassiana*	Denmark
3	KVL 03-127	*B. bassiana*	Denmark
4	KVL 03-144	*B. bassiana*	Denmark
5	KVL 04-17	*B. bassiana*	Nicaragua
6	KVL 12-20	*B. bassiana*	Colombia
1	KVL 00-89	*M. brunneum*	Denmark
2	KVL 12-18	*M. brunneum*	Switzerland
3	KVL 12-30	*M. brunneum*	Denmark
4	KVL 16-36	*M. brunneum*	Sweden
5	KVL 19-39	*M. brunneum*	Slovenia
6	KVL 20-01 (CECT 20784)	*M. brunneum*	Spain

Co-stressing using EPF and density

For the density and EPF co-stressing experiment, plastic containers of 10 x 14 x 6 cm with a mesh-covered opening added to the lids to allow ventilation were used to house the crickets. Similar to the main rearing, the plastic containers were placed in climate-controlled incubators with 12:12 L:D cycle, at 30 °C with the RH ranging between 65-70 %. A single piece of cardboard egg tray (5 x 5 x 7 cm) was inserted in every box to provide the crickets with hiding space. Grinded chicken feed, water gel and organic cucumber slices were provided as feed and water sources. Crickets were kept in three densities: 10, 20 or 40 individuals in a container. In containers with 40 individuals, two feed trays (3.5 x 0.2 cm) were provided instead of one to prevent possible stress due to limited access to feed [67]. Feed and water were checked daily and were replenished to ensure *ad libitum* availability.

Crickets of 20 days old (counted from hatching) were anesthetized by CO_2, and were then submerged into a solution of 1×10^8 conidia/ml for a few seconds. The most virulent *B. bassiana* isolate (KVL 97-44) and the most virulent *M. brunneum* isolate (KVL 20-01) were selected. For each of the three density groups, 10 containers were used, of which half was exposed to the EPF and half was only exposed to Triton-X as a control treatment. To provide optimal conditions for the spores to germinate and penetrate the cuticle, slices of cucumber were placed in the containers to increase the local RH.

The experiments were finished when the crickets reached the age of 40 days, by which time the majority of crickets had reached adulthood. Surviving individuals were counted, sexed and their biomass was recorded. The *B. bassiana* experiment consisted of three experimental runs, conducted between May and September 2021 and the *M. brunneum* experiment consisted of two experimental runs, conducted between October and December 2021.

Data analyses

Data were analysed with R, version 4.2.2 (2022-10-31) [85] and R Studio (2020). In case of the EPF virulence results, a Cox regression analysis was used [119] to calculate the hazard ratios for each isolate using the survival package. Normality tests were conducted with the Shapiro–Wilk test of normality [86].

When the datasets met the required assumptions, analysis of variance (ANOVA) or Linear mixed effect models (LMER) were used to test the differences between treatments. This was the case for male individual biomass in the *B. bassiana* and the *M. brunneum* experiments. When the assumptions were not met, generalized mixed effect models (GLM) were used, with the lme4 package of R [87]. This was the case for the female individual biomass, cricket survival, total biomass, female individual biomass for both the *B. bassiana* and the *M. brunneum* experiments. The GLM models were compared based on the second-order Akaike information criterion (AIC), the best fitting models with the smallest AIC were selected [88]. A GLM with binomial distribution was used to compare survival of the crickets for both isolates tested in the co-stressing experiments. The best fitting model for the survival data was using temperature as a fixed factor and the experimental run as a random factor. In case of the total biomass data, the model including an interaction between temperature and density was the best model fitting the data, with experimental run included as a random factor. In case the factors had a significant effect, pairwise comparisons were carried out with the Package 'emmeans', version 1.8.4-1 from R [89] or with the Tukey's honest significant difference test (HSD) test. Data was visualized using the R package ggplot2 [90].

3. Results

Screening EPF isolates for virulence

No significant differences in virulence were found between the different *B. bassiana* isolates and the control treatment (Figure 1, Table S5). Although not significantly different from the control, strain KVL 97-44 resulted in the lowest survival probability of 46.5 % after 8 days.

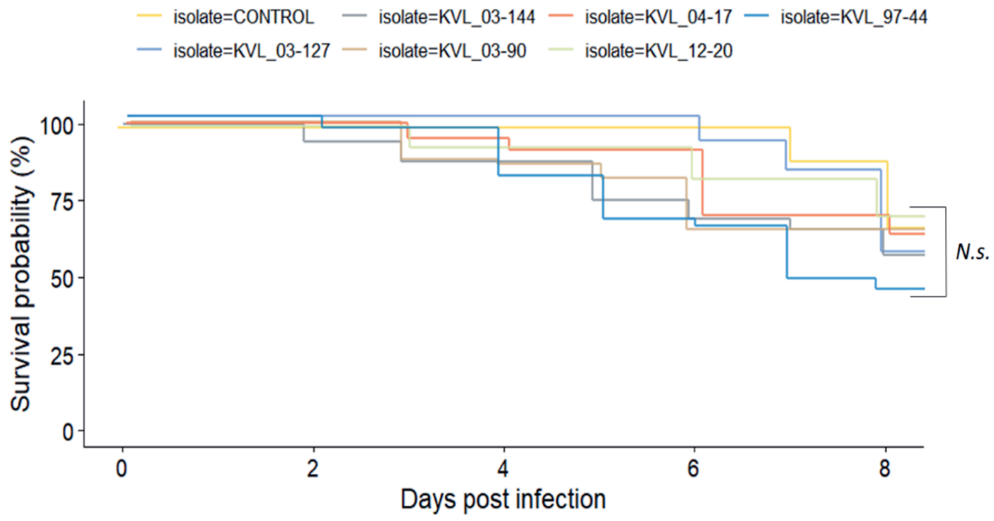
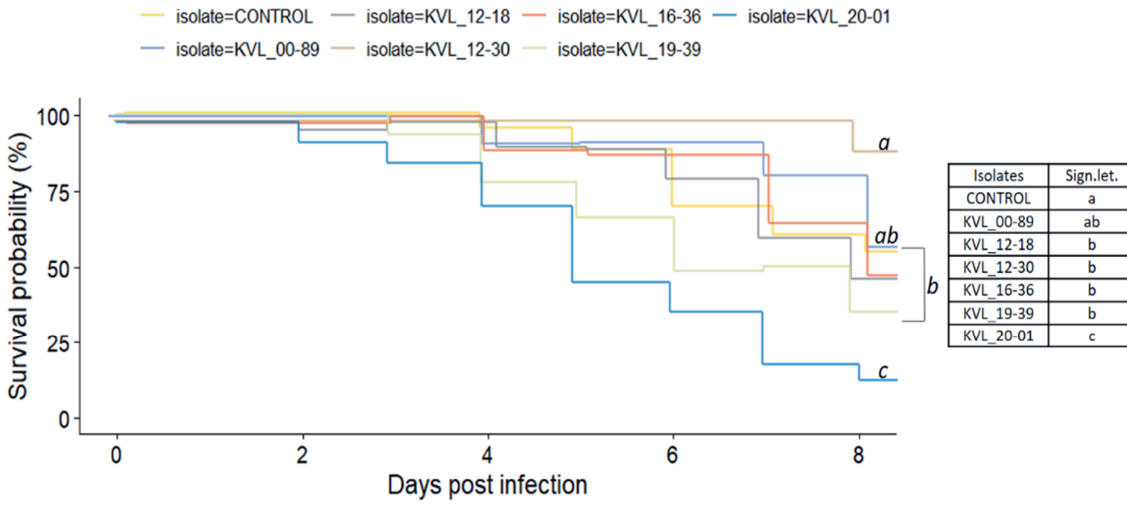


Figure 1. Average survival probability (%) of house crickets exposed to isolates of the entomopathogenic fungus Beauveria bassiana, for different days post exposure, based on Kaplan-Meier estimates. Names of the different isolates are listed above the figure, each corresponding to a different color. N.s.: no significant differences in the time course of survival probability between isolates.

Significant differences in virulence were found between the tested *M. brunneum* isolates (Figure 2 and Table S6) and all the isolates except KVL 00-89 were significantly different from the control. KVL 20-01 was the most virulent isolate, with a survival probability of 10.0 % after 8 days.



Isolates	Sign.let.
CONTROL	a
KVL_00-89	ab
KVL_12-18	b
KVL_12-30	b
KVL_16-36	b
KVL_19-39	b
KVL_20-01	c

Figure 2. Average survival probability (%) of house crickets exposed to isolates of the entomopathogenic fungus Metarhizium brunneum, for different days post exposure, based on Kaplan-Meier estimates. Names of the different isolates are listed above the figure, each corresponding to a different color. If there are no shared letters indicated with the curves at day 8, survival probability differed significantly between isolates.

Combined effect of cricket density and *Beauveria bassiana* exposure

Survival

Cricket survival was significantly affected by cricket density and exposure to *B. bassiana* while there was no interaction between the two factors (Figure 3) ($p > 0.05$). A higher density led to a lower survival, with the survival at density 40 being significantly lower than the survival at both density 10 ($p < 0.0001$) and 20 ($p < 0.0001$). The survival at density 20 was similar to the survival at density 10 (P = 0.2580). Exposure to *B. bassiana* significantly reduced cricket survival compared to no exposure ($p = 0.0004$).

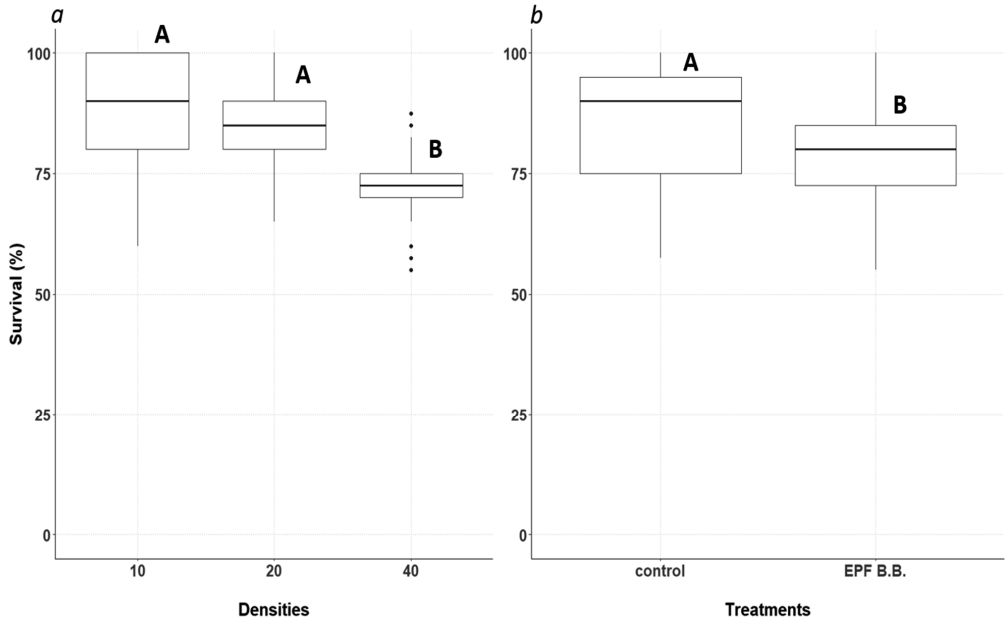


Figure 3. Median cricket survival recorded (%) for three different cricket densities (a) and in response to exposure to spores of Beauveria bassiana (EPF B.B.) or non-exposure (control) (b). In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values. Boxplots that have no letters in common differ significantly.

Female individual biomass

Female individual biomass was affected by an interaction between density and exposure to *B. bassiana* (Figure 4). When exposed to *B. bassiana*, female mean biomass was significantly higher at density 10 than at densities 20 and 40 (p = 0.0088 and 0.0024, respectively) or than density 20 non-exposed (control) (p = 0.0069), but was not significantly higher than the control treatments at density 10 and 40 (p = 0.2051 and 0.3039). There was no significant difference in female mean biomass between any of the non-exposed (control) treatments at any density. There was no significant difference in female mean biomass between exposed or non-exposed (control) treatments at density 20 (p = 1.0000 and 1.0000), or between the density 40 exposed or non-exposed (control) (p = 0.3077).

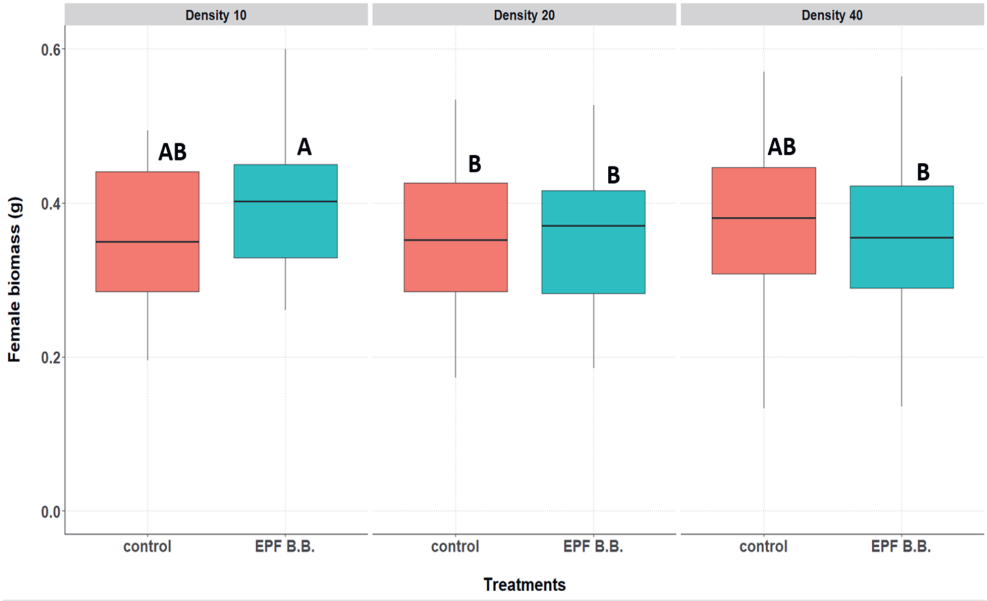


Figure 4. Female biomass (g) for each treatment group. Non-exposure (control) or exposure to Beauveria bassiana (EPF B.B.) is indicated along the X-axis, per density tested, indicated in the top of the figure panel. In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values. Boxplots that have no letters in common differ significantly.

Male individual biomass

Male biomass was affected by both *B. bassiana* exposure and by cricket density and there was an interaction between the two factors (Figure 5). With increasing density male biomass seemed to decrease and this trend was further affected by *B. bassiana* exposure, although not significantly. The individual male body biomass at density 40 with exposure was significantly lower than that observed at density 10 (both exposure and non-exposure (control)) and density 20 control (p = 0.0048, 0.0107 and 0.0170, respectively).

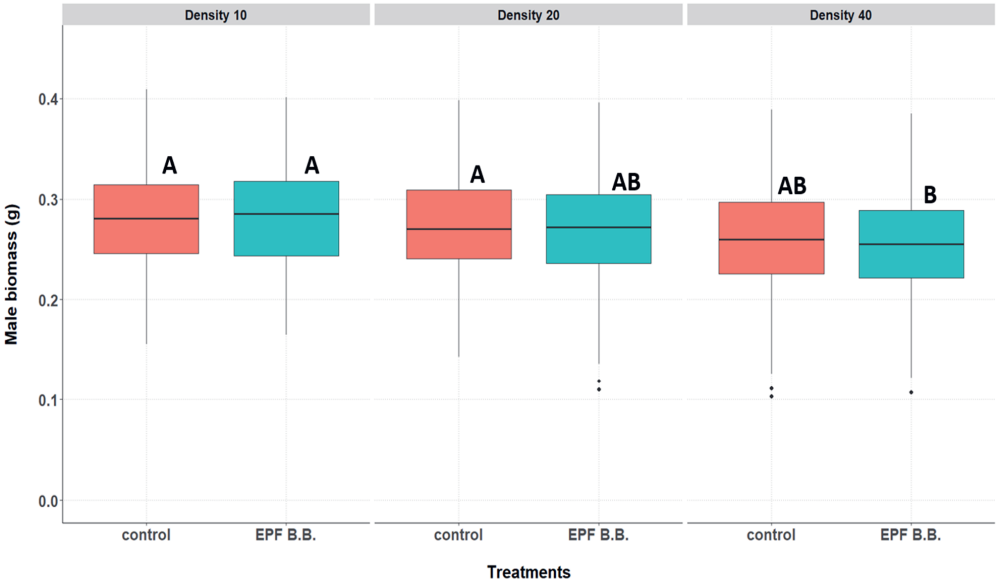


Figure 5. Male biomass (g) for each treatment group. Non-exposure (control) or exposure to Beauveria bassiana (EPF B.B.) is indicated along the X-axis, per density tested, indicated in the top of the figure panel. In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values. Boxplots that have no letters in common differ significantly.

Total biomass

Total cricket biomass was significantly affected by an interaction between *B. bassiana* exposure and cricket density (Figure 6). The lowest density resulted in the lowest biomass, which was significantly lower than for all the other treatments. Exposure to *B. bassiana* did not cause significantly lower biomass than the controls at density 10 ($p = 1.0000$) and density 20 ($p = 0.0979$). At density 20, a significantly lower total biomass was collected than at density 40, and no significant difference was found between the non-exposed (control) and *B. bassiana*-exposed group ($p = 0.0979$). In case of density 40, crickets that had been exposed to *B. bassiana* had a significantly lower total biomass than non-exposed (control) crickets ($p = 0.0173$).

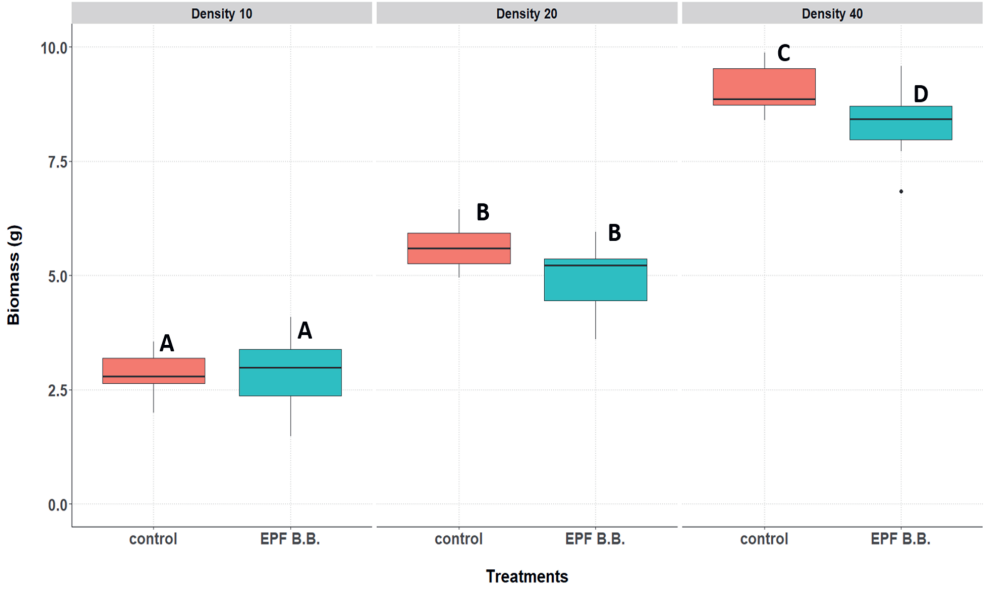


Figure 6. Total harvested biomass (g) for each treatment group. Non-exposure (control) or exposure to Beauveria bassiana (EPF B.B.) is indicated along the X-axis, per density tested, indicated in the top of the figure panel. In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values. Different letters indicate significant differences between the treatments. Boxplots that have no letters in common differ significantly.

Combined effect of cricket density and *Metarhizium brunneum* exposure

Survival

Cricket survival was significantly affected by cricket density and the exposure to *M. brunneum*, while there was no interaction between the two stressors (Figure 7). A higher density led to a lower survival, with the survival at density 40, being significantly lower than at density 10 and 20 ($p < 0.0001$ and $p = 0.0003$ respectively). The survival at density 20 was significantly lower than at density 10 ($p = 0.0187$). Exposure to *M. brunneum* had significantly reduced cricket survival compared to no exposure (control; $p = 0.0003$).

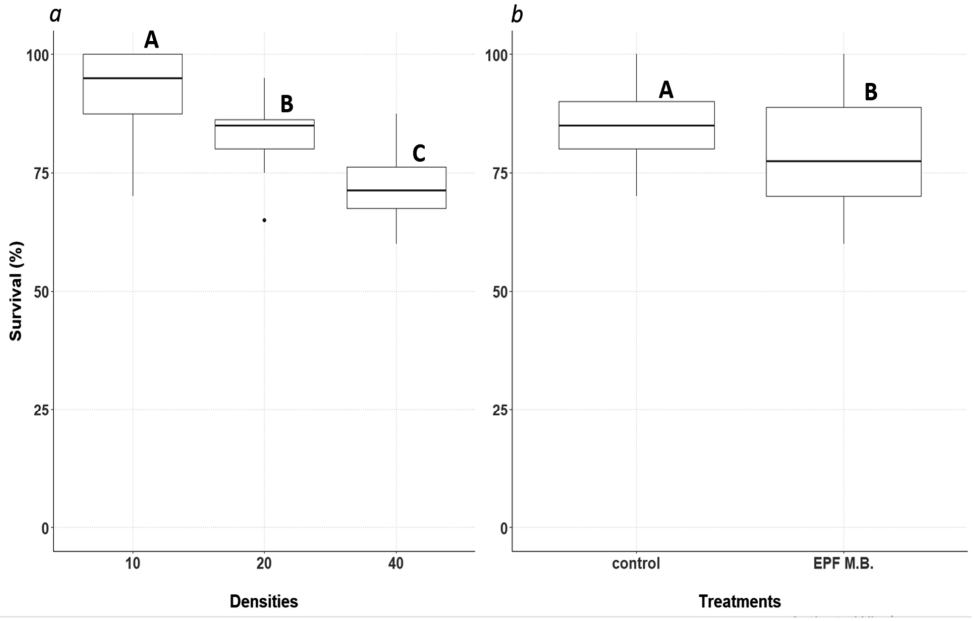


Figure 7. Median cricket survival recorded (%) for three different cricket densities (a) and in response to exposure to spores of the Metarhizium brunneum (EPF M.B.) or non-exposure (control) (b). In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values. Different letters indicate significant differences between the treatments. Boxplots that have no letters in common differ significantly.

Individual female biomass

Individual biomass of female house crickets was not influenced by either density or exposure to *M. brunneum* and there was no interaction between the two stressors ($p > 0.05$; Figure 8). All treatments showed the same average female biomass.

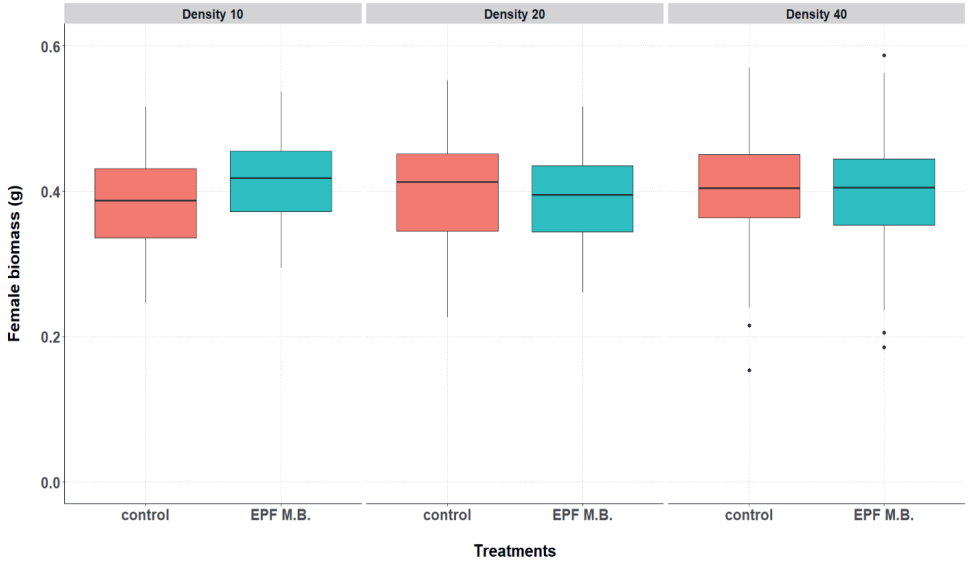


Figure 8. Individual female biomass (g) for each treatment group. Non-exposure (control) or exposure to Metarhizium brunneum (EPF M.B.) is indicated along the X-axis, per density tested, indicated in the top of the figure panel. In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values.

Individual male biomass

Individual biomass of male house crickets (Figure 9) was not influenced by either density ($p = 0.5698$) or exposure to *M. brunneum* ($p = 0.2415$) and there was no interaction between the two terms either ($p = 0.0949$; Figure 9). All treatments showed the same average male biomass.

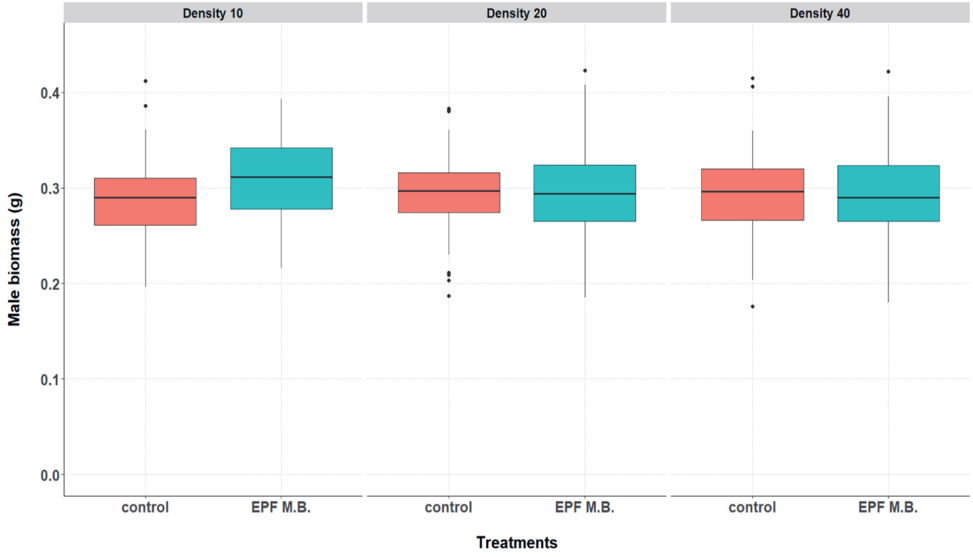


Figure 9. Male biomass (g) for each treatment group. Non-exposure (control) or exposure to Metarhizium brunneum (EPF M.B.) is indicated along the X-axis, per density tested, indicated in the top of the figure panel. In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values.

Total biomass

Total cricket biomass was significantly affected by an interaction between *M. brunneum* exposure and cricket density (Figure 10). Rearing crickets at a density of 10 resulted in the lowest biomasses observed, significantly lower than all the other densities, but the exposure to *M. brunneum* did not cause a significant difference in total biomass at density 10 ($p = 0.9950$). Crickets reared at density 20 had a significantly lower total biomass than crickets reared at density 40, but there was no significant difference between the control and *M. brunneum*-exposed groups at density 20 ($p = 0.1450$). At the density of 40, the total biomass of the control and *M. brunneum*-exposed groups differed significantly ($p < 0.0010$), with the *M. brunneum*-exposed group having a lower biomass.

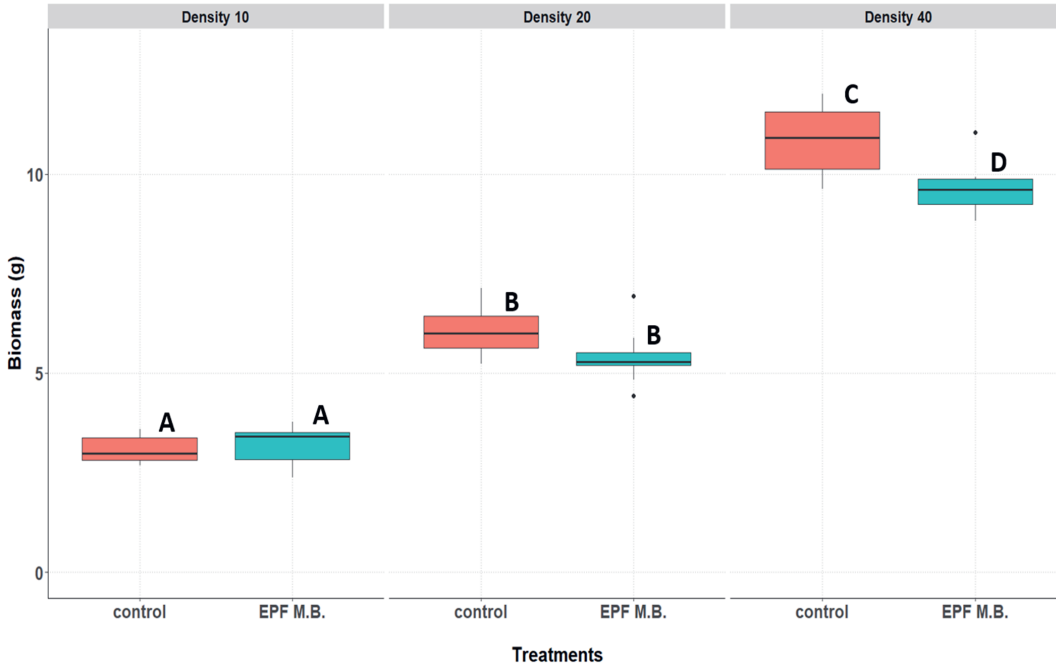


Figure 10. Total harvested biomass (g) for each treatment group. Non-exposure (control) or exposure to Metarhizium brunneum (EPF M.B.) is indicated along the X-axis, per density tested, indicated in the top of the figure panel. In the boxplot, the horizontal line depicts the median; the boxes indicate the lower and upper quartiles and the vertical lines extend to minimum and maximum values.

4. Discussion

House crickets are a promising species to be mass-produced as human food, but various stressors can cause losses in the rearing. These stressors can be temperature [69], rearing density [68] and various entomopathogens such as viruses [36], bacteria [34] or entomopathogenic fungi [34]. In the rearing environment, stressors will be present simultaneously and will interact with each other and the crickets themselves, creating a complex system to examine. Here, we set out to test the effects of a combination of stressors on cricket biomass. After a screening and selection process of strains belonging to the two most commonly known entomopathogenic fungal species, *M. brunneum* and *B. bassiana*, one isolate of each was chosen. The selected isolates were separately combined with three different rearing densities, creating six treatments per isolate, of which half were exposed to the EPF, while density was applied as a single stressor and was regarded as the control treatment.

Variation in host range and virulence has been described for both *Beauveria* and *Metarhizium* [120]. For instance, insects belonging to the orders of Lepidoptera, Coleoptera and Diptera showed different responses to isolates of *B. bassiana* and against each order of insects *B. bassiana* showed differences in virulence and pathogenicity [121]. Several of the screened *B. bassiana* isolates did not induce a significantly different mortality in the exposed crickets compared to the non-exposed control, even with the use of a high conidia concentration (1×10^8 conidia/ml). Other exposure experiments with similar concentrations, resulted up to 70-90% mortality rates for *Schistocerca gregaria, Bemisia tabaci* and *Trialeurodes vaporariorum* [122, 123].

Based on the results of the screening we used the most virulent *M. brunneum* isolate, KVL 20-01, which caused a significantly higher mortality compared to all other *M. brunneum* isolates. For *B. bassiana* isolates, no significant differences were detected, but the isolate KVL 97-44 caused the highest mortality and was selected for use in the co-stressing experiments. The choice of rearing density is a crucial decision for insect rearing [66] and especially for cricket producers. The aim is to maximize the biomass produced in the available rearing space, but at the same time to avoid unnecessary losses due to crowding in the facilities. Establishing the rearing facilities is a major cost in insect mass-rearing [79], therefore the best possible utilization of the area is necessary, finding a balance between a healthy population and biomass production. Increased densities in the experiments resulted in reduced survival, in line with the findings of Mahavidanage et al. [68]. Other parameters, such as appropriate feeding surface, also have to be considered to avoid competition for feed and possible relevant consequences [67]. Producers also need to consider that high-density cricket rearing environments are susceptible to viral disease outbreaks [26, 45] and crickets are known to host a wide range of viruses [17, 36]. In case of biomass production, an interaction between the rearing densities and exposure to EPFs was observed. At the highest rearing densities, the detrimental effect of the EPFs on cricket survival was stronger, indicating the importance to assess the possible stressors in combination. High rearing densities may also come with other challenges for cricket producers, such as increased risk of disease transmission and cannibalism, which is known to occur in house cricket rearing [100]. The occurrence of cannibalism in crickets can also in part explain the decreased survival at higher densities. Although high rearing densities presented here seem favourable from the biomass production perspective, the decreased survival rates show the need for further studies to optimize all aspects of house cricket mass-rearing. Such studies need to examine the diet, temperature, entomopathogens and the interactions between these factors before extrapolating the results presented to a mass-rearing scale.

Combined stresses also can trigger well-known viral pathogens such as *A. domesticus* densovirus (AdDV) and cricket producers need to pay special attention to this risk as well. Further studies should also examine

the effect of exposure to entomopathogenic fungi on the levels of AdDV, to see if the combined effect has a detrimental effect on cricket survival due to virus-induced mortality.

Acknowledgements

We would like to thank Nicolai Vitt Meyling for his input on the selection of fungal isolates, Enrique Quesada Moraga for providing permit to use the KVL 20.01 isolate and Antoine Lecocq for his input and help during the design and experiments.

Supplementary Tables and Figures

Table S1. Pairwise comparison *p* values for the female weight data, for *B. bassiana*.

	D10 Control	D10 EPF BB	D20 Control	D20 EPF BB	D40 Control	D40 EPF BB
D10 Control		0.205	0.990	0.986	0.952	0.986
D10 EPF BB			0.006	0.008	0.303	0.002
D20 Control				1.000	0.274	1.000
D20 EPF BB					0.307	1.000
D40 Control						0.132

Table S2. Pairwise comparison *p* values for the male weight data, for *B. bassiana*.

	D10 Control	D10 EPF BB	D20 Control	D20 EPF BB	D40 Control	D40 EPF BB
D10 Control		1.000	0.976	0.465	0.095	0.004
D10 EPF BB			0.979	0.525	0.140	0.010
D20 Control				0.842	0.292	0.017
D20 EPF BB					0.971	0.380
D40 Control						0.750

Table S3. Pairwise comparison *p* values for the biomass data, for *B. bassiana*.

	D10 Control	D10 EPF BB	D20 Control	D20 EPF BB	D40 Control	D40 EPF BB
D10 Control		1.000	<0.001	<0.001	<0.001	<0.001
D10 EPF BB			<0.001	<0.001	<0.001	<0.001
D20 Control				0.097	<0.001	<0.001
D20 EPF BB					<0.001	<0.001
D40 Control						0.017

	D10 Control	D10 EPF M.B.	D20 Control	D20 EPF M.B.	D40 Control	D40 EPF M.B.
D10 Control		0.995	<0.001	<0.001	<0.001	<0.001
D10 EPF M.B.			<0.001	<0.001	<0.001	<0.001
D20 Control				0.145	<0.001	<0.001
D20 EPF M.B.					<0.001	<0.001
D40 Control						<0.001

Table S4. Pairwise comparison *p* values for the biomass data, for *M. brunneum*

Table S5. Pairwise comparisons of the *B. bassiana* strains using Log-Rank test. *p* value adjustment method: Benjamini-Hochberg (BH).

Isolates	CONTROL	KVL_03-127	KVL_03-144	KVL_03-90	KVL_04-17	KVL_12-20
KVL_03-127	0.87					
KVL_03-144	0.50	0.60				
KVL_03-90	0.66	0.84	0.84			
KVL_04-17	0.58	0.66	0.88	0.87		
KVL_12-20	0.88	0.84	0.50	0.66	0.50	
KVL_97-44	0.50	0.50	0.89	0.84	0.87	0.50

Table S6. Pairwise comparisons of the *M. brunneum* strains using Log-Rank test. *p* value adjustment method: Benjamini-Hochberg (BH).

Isolates	CONTROL	KVL_00-89	KVL_12-18	KVL_12-30	KVL_16-36	KVL_19-39
KVL_00-89	0.651					
KVL_12-18	0.726	0.354				
KVL_12-30	0.001	0.004	0.000			
KVL_16-36	0.844	0.485	0.809	0.000		
KVL_19-39	0.077	0.027	0.205	< 0.0001	0.148	
KVL_20-01	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.008

CHAPTER 4

Viral levels and tissue tropism of Acheta domesticus densovirus throughout the house cricket production cycle

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Abstract

The house cricket, *Acheta domesticus,* is a commonly reared insect for food and feed purposes. *Acheta domesticus* is often infected with Acheta domesticus densovirus (AdDV), a single-stranded DNA virus that can cause high mortality resulting in collapse of house cricket colonies. AdDV infection and disease outbreaks in cricket mass-rearing can be prevented by either obtaining virus-free crickets or reducing virus spread. To limit virus spread, a better understanding of how viral levels vary and how the virus is transmitted during cricket development is needed. Viral levels were measured throughout the rearing cycle using quantitative PCR on samples collected 1) simultaneously from different life stages present in the rearing room at a given time and 2) weekly from a single rearing container that was monitored during the successive stages of development. To study viral tissue tropism and to infer the route of virus transmission, viral levels were measured in various tissues from mated and non-mated adult crickets. Ovaries of both unmated and mated females and the spermatheca of mated females were collected, while testes and accessory glands were collected from unmated and mated males. Guts were collected from both genders. Our results showed that viral levels increased in the course of development of nymphs into adults. Interestingly, for both genders, unmated individuals had significantly lower viral levels than mated individuals. Furthermore, AdDV was present in every tested tissue, but at different levels. In both mated and unmated females, the gut and ovaries showed higher viral levels than the spermatheca of mated females. The dissected male tissues all had similar viral levels. The results presented suggest that AdDV is both horizontally and vertically transmitted among house crickets and provide relevant information for future work on establishing virus-free cricket lines.

Keywords: *Acheta domesticus*, Acheta domesticus densovirus, house cricket, insect production, insects as food and feed, cricket viruses, entomopathogenic viruses, mass-rearing

1. Introduction

The house cricket, *Acheta domesticus* L., has a long history of commercial production as pet and zoo feed [45] as well as a food source, mainly in Asia [29] but also in other parts of the world [17]. House crickets have a favorable nutritional profile [18, 20], making them a good candidate as a food ingredient [78]. In 2022, partially defatted house cricket powder was authorized by the European Food Safety Authority [23] for use as food which is sold in the European Union from early 2023 onwards. This legalization can boost production volumes as crickets and their derived products have already been tested to be used as ingredients in food [24, 25]. The increasing interest in house cricket production also increases the importance of stable, consistent and economically viable production of this species. However, house cricket production can be jeopardized by the Acheta domesticus densovirus (AdDV), which is known to cause epidemics and colony collapse in production facilities [26, 36, 43-45]. AdDV is a single-stranded DNA virus from the *Parvoviridae* family [43, 124], belonging to the insect-specific subfamily *Densovirinae*. Viruses belonging to this subfamily are usually highly virulent to their host [51]. Other cricket species, including the commercially produced *Gryllus bimaculatus* and *Gryllodes sigillatus* [45], can also harbour AdDV, and although AdDV does not cause mortality in these species, it is unknown whether (mild) adverse effects occur [45]. In *A. domesticus*, AdDV is often present in a covert state, not causing any visible symptoms. Knowledge on which factors trigger an AdDV epidemic is currently limited [69], hampering the possibilities of preventive or curative methods. A crucial aspect of virus infection dynamics is the transmission route of the virus, since the mode of transmission influences the spread and prevalence of the virus in the population [125]. The two main types of viral transmission are vertical and horizontal transmission, and both forms can occur simultaneously [126]. Vertical transmission occurs when the parental generation transmits the virus to the offspring, for instance via the eggs or via sperm [125]. Transmission via the eggs can happen either on the egg surface (transovum transmission), or within the eggs (transovarial transmission) [127-130]. Horizontal transmission can take place in many forms: by ingesting contaminated feed [36], via cannibalism [131], or also via the feces. During mating, both horizontal and vertical transmission can take place simultaneously. Males and females can transmit the virus to each other during mating (sexual transmission) [132, 133], which is considered as horizontal transmission. At the same time, the virus can be paternally (via the sperm) or maternally (via the eggs) transmitted to the offspring, which is considered as vertical transmission. House cricket rearing facilities are an ideal environment for virus transmission with the temperature and humidity settings favoring growth of crickets and, with a high cricket density, resulting in frequent contact between individuals, food and feces [27, 34]. Current knowledge on AdDV transmission in crickets is very limited and a better understanding of virus transmission will help to optimize rearing strategies to prevent or reduce transmission and to lower the likelihood of viral outbreaks. For example, viral levels could be reduced by limiting horizontal transmission using disinfection procedures. Alternatively, if transmission is mainly vertical, but the efficiency of vertical transmission is < 100%, virus-free cricket-breeding stocks could be created by selecting uninfected individuals. Virus-free house cricket breeding stocks could be also established by identifying the optimal interference point in the vertical transmission of the virus. Additionally, densoviruses are known to be capable of replicating in many of the insect host tissues including the fat body, gut and ovaries [134, 135]. Measuring viral levels in different tissues can give clues about the mode of transmission: with horizontal transmission, high viral levels are expected in the gut since this is the organ through which the virus enters the body after oral infection, while in case of vertical transmission, high viral levels are expected in the reproductive tissues. Therefore, we investigated viral levels in different tissues of crickets, including mated and non-mated individuals. In addition, we measured viral levels during nymphal development and in the adult stage.

2. Materials and methods

House cricket colony

The *A. domesticus* colony has been kept at the Laboratory of Entomology, Wageningen University & Research, since 2006. Crickets are housed in 40 L plastic containers in a climate chamber at ~28°C with ~50% relative humidity (RH) and are provided with chicken feed (Kuiken Opfokmeel, Kasper Fauna Food, Woerden, the Netherlands), slices of carrot and water from bird water dispensers. At any given time, six different age groups (Table 1) are present simultaneously in the rearing, ranging from the youngest instars to fully developed adults with approximately one week of age difference between them.

Sampling approach

Two sampling approaches were used to investigate viral levels in the different age groups of the house cricket colony, from pinheads to adults. Firstly, a "snapshot" sampling was done. This involved sampling pinheads and nymphs of different ages and adults present in the rearing chamber at a single time point. Five different age groups, which differed a week in age were sampled in the snapshot sampling (Age groups 1, 2, 3, 4 and 6; Table 1). When it was possible to determine the gender (for crickets older than four weeks), equal numbers of males and females were taken. Secondly, a sequential sampling was also done. To this end, rearing containers containing recently hatched pinheads were sampled for six consecutive weeks (age groups 1 to 6; Table 1). Due to the high number of crickets in a container (several hundreds), the removal of 16 individuals per week was assumed not to have an influence on cricket density and virus transmission. The crickets were subsequently stored at -20 °C until DNA extraction.

Table 1. Group numbers with the associated age ranges, average body weight and the samples sizes used in the two types of sampling approaches.

Age group	Age of crickets (days)	Average weight (SD) mg	Snapshot sampling # individuals	Sequential sampling # individuals
1	0-7*	NA	8	8
		NA	8	8
2	7-14	9.718 (2.019)	16	16
3	14-21	47.166 (16.656)	16	16
4	21-28	221.531 (76.119)	16	16
5	28-35**	304.687 (80.465)	-	16
6	35-42***	356.843 (75.983)	16	16

*Only pinheads were sampled **Only the last nymphal stage before adulthood was sampled. ***Only adults were sampled. SD: standard deviation. - No sample taken

Tissue dissections

Tissue dissections were performed on non-mated and mated individuals that were six weeks old. To obtain non-mated adults, five-weeks-old nymphs were collected from the colony. These crickets were separated by gender into different boxes and kept in incubators under similar conditions as in the main rearing until sexual maturity was visible at the age of six weeks. To obtain tissues from mated individuals, six-weeks-old crickets were collected from the main rearing colonies, when crickets are actively mating and depositing eggs. Collected crickets were transferred to incubators but in this case, genders were kept mixed. Next, both

mated and non-mated crickets were starved by removing the feed source and were provided with only a water source 48 h prior to dissection.

Dissections were done under a stereomicroscope in sterile phosphate-buffered saline (PBS). For every sample, tissues of three individual crickets were pooled to create a sample. For every organ, eight samples were collected in total. For mated and non-mated females, the gut tracts, the ovaries with the eggs and the spermatheca (only for mated females; only spermathecae with a spermatophore were collected, indicating that females had mated. For mated and non-mated males, the gut tracts, the testes and the accessory glands were collected. Samples were stored in PBS solution at -20°C until DNA extraction.

DNA extraction and qPCR analysis

After the samples were thawed, they were homogenized with a sterile pestle in a 1.5 ml Eppendorf® tube, which was followed by centrifugation at 14,000 rpm for 10 min. Next, the supernatant was removed and used for DNA extraction. The DNA extraction was done using the Qiagen DNeasy® Blood and Tissue kit (Qiagen, Germany), following the 'Total insect DNA extraction' protocol, except for sample homogenization, which was carried out as described above. Quality control of the DNA extraction was carried out using Nanodrop® ND1000 Spectro-photometer (Thermo Fisher Scientific Inc., United States).

A quantitative PCR (qPCR) was performed to quantify the AdDV viral levels in the samples. To this aim, a 96 base pair (bp) fragment of the AdDV gene encoding the non-structural protein 2 (NS2) was amplified, using primers reported by Duffield *et al.* [27] (Table 1). As a host reference gene, 199 bp of the *Acheta domesticus elongation factor 1 alpha* gene (*EF1a*; Genbank access nr. EU414685.2) was amplified, (Table 2). Reactions were performed with SYBR™ Select Master Mix (Thermo Fisher Scientific Inc., United States), as described by Takacs *et al.* [69].

Table 2. Primers used in qPCR.

Name	Reference	Primer sequence 5' to 3' (forward / reverse)	Amplicon size (bp)	Gene target
EF1	This study	GGAAATCAAGAAGGAAGTCAGC/ GGCATCCAAAGCCTCAATAAG	199	*A. domesticus* elongation factor 1 alpha gene
NS2	Duffield *et al.*, 2021	GCGAGCAATCCCGACTACTA/ CGCGTTGTTGATGTCCTTCC	96	AdDV non-structural protein 2

qPCR data normalization

The normalized viral levels of AdDV in the different samples was calculated by the following formula (after which the outcome of the formula was $\log_{10}$ transformed):

$$E^{-Ct\,(AdDV)} / E^{-Ct\,(EF1a)}$$

in which E represents the amplification efficiency, which equals two in case of 100% efficiency, and Ct represents the threshold cycle value of the qPCR amplification either for the viral (AdDV) or the host (EF) targets [136]. The amplification efficiency was calculated and incorporated for every plate and every primer pair individually and were in the range of 95-105% for both the host and the viral primers, which was confirmed with the use of dilution series to obtain a standard curve.

Data analyses and visualization

All data were analysed using R, version 4.2.2 (2022-10-31) [85], and R Studio. To test if the data were normally distributed, a Shapiro-Wilk test of normality was conducted [86]. For datasets that met the assumptions of normality and homogeneity of variance (Table S3), a two-way analysis of variance (ANOVA) or linear mixed effect models were used to test the effect of the variables and their interaction. If the data did not meet the assumptions of a two-way ANOVA (Table S3), generalized mixed effect models (GLM) were built using the lme4 package of R [87]. The GLM models were compared based on the second-order Akaike information criterion (AIC) values and the best fitting models with the lowest AIC were selected [88]. In case the factors or the interactions of the factors had a significant effect, pairwise comparisons were carried out with the Rpackage ‘emmeans’, version 1.8.4-1 [89] or with the Tukey's HSD (honestly significant difference) test. Data visualization was done using the R package ggplot2 [90].

3. Results

Viral levels throughout nymphal development into the adult stage – snapshot sampling

The snapshot sampling was used to assess the normalized viral levels in five age groups (Table 1). We observed higher viral levels for the older age groups 4 and 6 (Fig. 1, Table S1). The age groups 1 and 2 (p = 0.9316), 1 and 3 (p = 0.9316), and 2 and 3, (p = 0.3633) all had similar viral levels. Age groups 1,2 and 3 viral levels were significantly lower than those of age groups 4 and 6 ($p < 0.0001$ for all comparisons). Viral levels were also significantly higher for the crickets in age group 6 compared to the age group 4 (p = 0.0131).

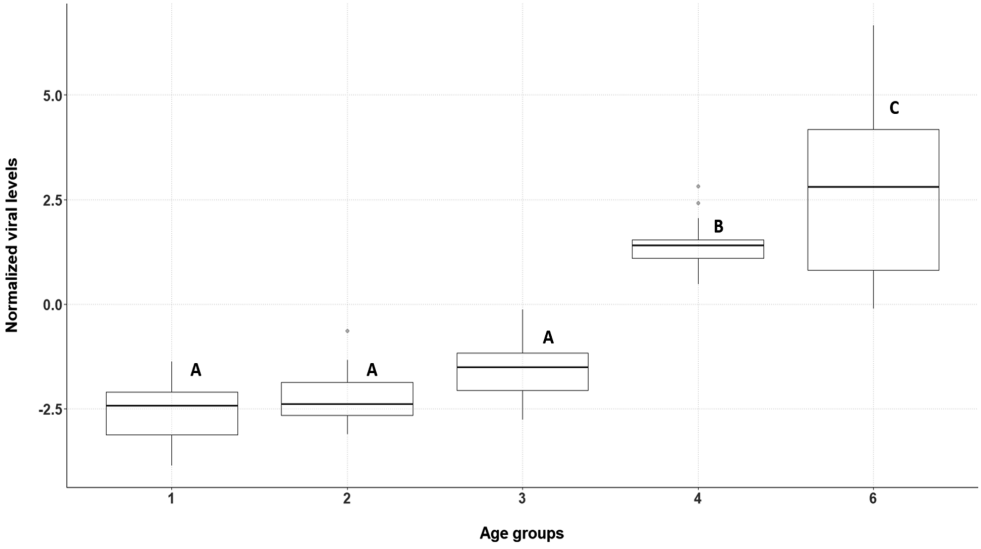

Figure 1. Normalized viral (DNA) levels (relative to the host EF1a gene) for each age groups of crickets sampled from the rearing using snapshot sampling. The Y-axis represents the normalized viral levels, while the X-axis represents the age groups of the crickets (see Table 1). The boxplots show the median (horizontal line), the lower 25th, and the third 75th quartiles; vertical lines extend to the lowest and highest values. Age groups that have no letters in common differ significantly.

Viral levels throughout the cricket nymphal development into the adult stage – sequential sampling

For the sequential sampling method, the selected rearing containers were sampled weekly for six consecutive weeks (representing 6 age groups, Table 1). The lowest viral levels found in the crickets sampled in age group 1, which was significantly lower than found in all other groups ($p < 0.0001$ for all comparisons). In age group 2, a large variation can be observed, which caused this group to have similar viral levels to age group 4 and 5 (Table S2). The highest viral level was observed in crickets sampled from age group 6, which was significantly higher than in crickets sampled for all of the other age groups (Table S2). Overall, a consistent increase in viral levels was observed, similarly as it was found in the snapshot sampling, but the viral levels did not reach similarly high levels like in case of the snapshot sampling.

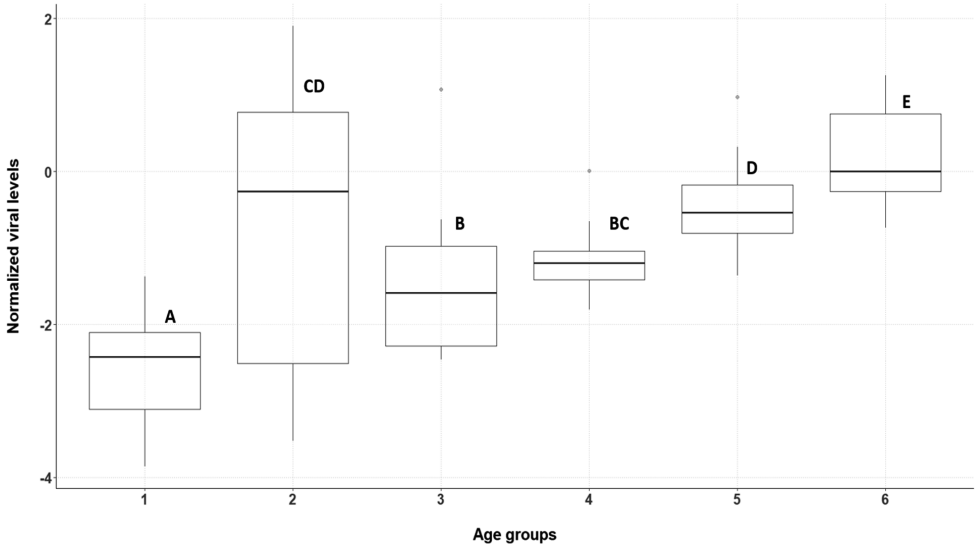

Figure 2. Normalized viral (DNA) levels (relative to the host EF1a gene) for each age groups of crickets sampled from the rearing using sequential sampling. The Y-axis represents the normalized viral levels, while the X-axis represents the sampling age groups (see Table 1). The boxplots show the median (horizontal line), the lower 25th, and the third 75th quartiles; vertical lines show extend to the lowest and highest values. Age groups that have no letters in common differ significantly.

Effect of mating and gender on the viral levels in individual crickets

Both mating status and gender had a significant effect on the viral levels in adult crickets of age group 6, but the best fitting model did not include an interaction term between mating and gender (Fig. 3). A pairwise comparison showed that male crickets had significantly higher viral levels than female crickets ($p = 0.0027$) (Fig. 3A). The viral levels were significantly different between non-mated and mated individuals ($p < 0.0001$) (Fig. 3B).

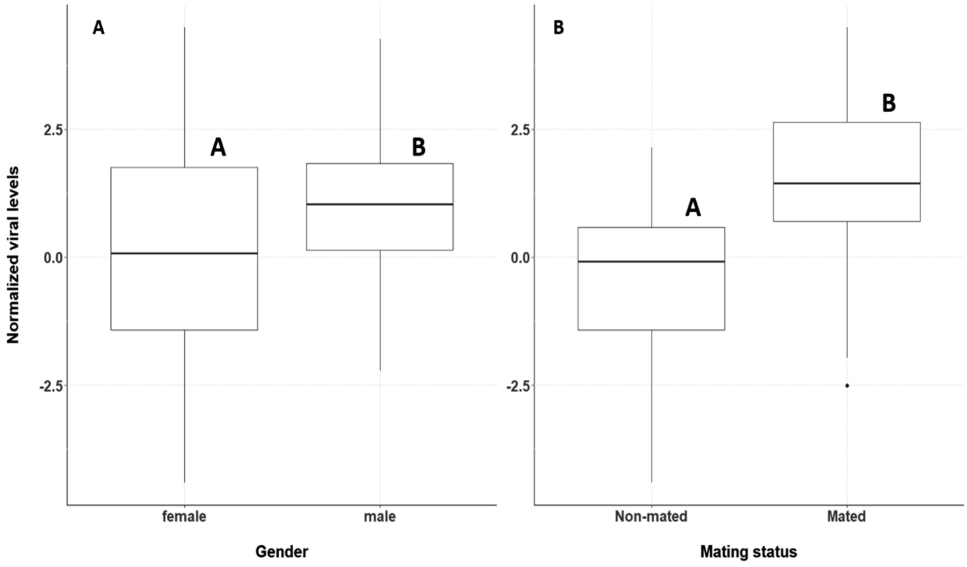

Figure 3. Normalized viral (DNA) levels (relative to the host EF1a gene) levels of males and females (A) and of non-mated and mated adult crickets (B). The Y-axis represents the normalized viral levels (see comment with Fig. 1), while gender and mating status are indicated along the X-axis. The boxplots show the median (horizontal line), the lower 25th, and the third 75th quartiles; vertical lines extend to the lowest and highest values. Boxplots that have no letters in common differ significantly.

Viral tissue tropism in the different genders - females

In female house crickets, the AdDV was detected in all tested tissues. Both the mating status and the tissue type had a significant effect on the viral levels ($p < 0.0001$ and $p < 0.0001$, respectively), but there was no interaction between these factors ($p = 0.8720$) (Fig. 4). Among the tissues, the guts and the ovaries had similar viral levels ($p = 0.9143$), which were significantly higher than found in the spermatheca ($p < 0.0001$ and $p < 0.0001$, respectively) (Fig. 4A). The mated female crickets had significantly higher viral levels than non-mated ones ($p < 0.0001$) (Fig. 4B).

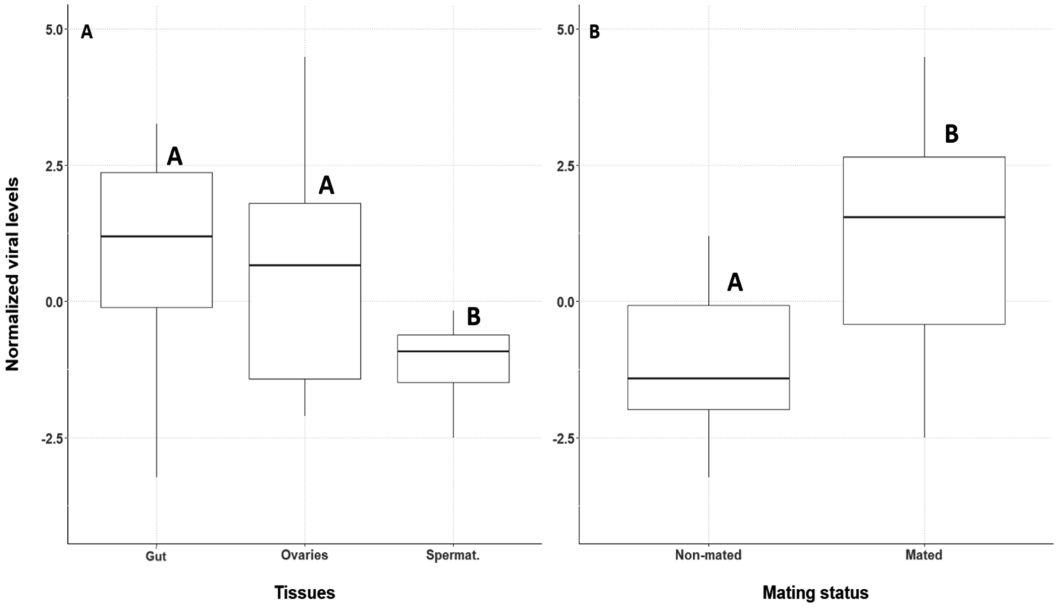

Figure 4. Normalized viral (DNA) levels (relative to the host EF1a gene) in tissues of female house crickets (A) and between non-mated and mated females (B). The Y-axis represents the normalized viral levels, while the X-axis represents the different tissues (A) (Spermat. refers to spermatheca) and the mating status (B). The boxplots show the median (horizontal line), the lower 25th, and the third 75th quartiles; vertical lines extend to the lowest and highest values. Boxplots that have no letters in common differ significantly between the tissues (A) and between mated and non-mated females (B).

Viral tissue tropism in the different genders - males

In male house crickets, all the tested tissues were positive for the presence of AdDV. There was no effect of the tissue type on the viral levels (p = 0.0951) (Fig. 5A), only the mating status had a significant effect on the viral levels (p = 0.0001) and there was no interaction between the mating status and the tissue type (p = 0.4545) (Fig. 5). Mated males had significantly higher viral levels than the non-mated ones. (p = 0.0001) (Fig. 5B).

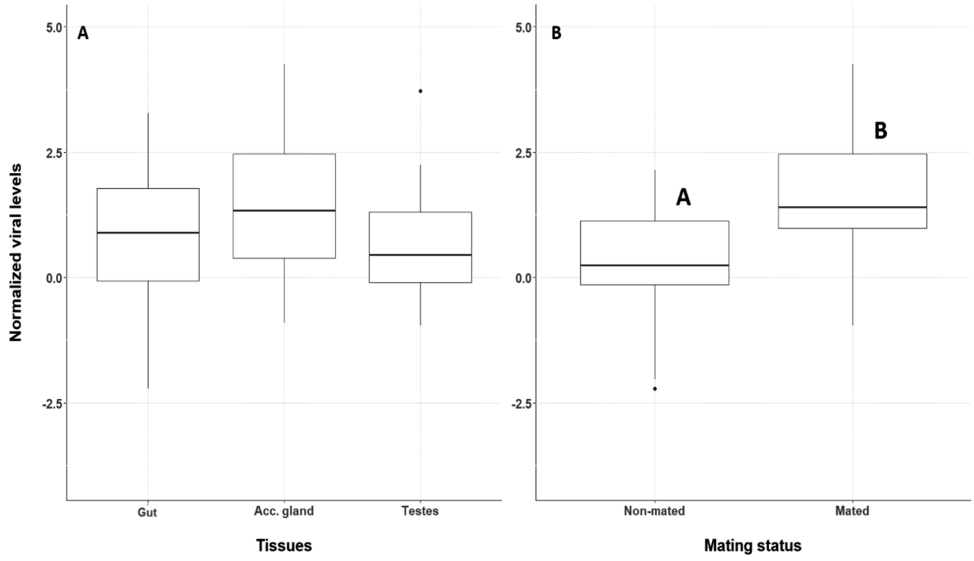

Figure 5. Normalized viral (DNA) levels (relative to the host EF1a gene) in tissues of male house crickets (A) and between non-mated and mated males (B). The Y-axis represents the normalized viral levels, while the X-axis represents the different tissues (A) (Acc. gland refers to accessory glands) and the mating status (B). The boxplots show the median (horizontal line), the lower 25th, and the third 75th quartiles; vertical lines extend to the lowest and highest values. Boxplots that have no letters in common differ significantly between the tissues (A) and between mated and non-mated females (B).

4. Discussion

In this study, we set out to investigate viral levels during the cricket nymphal development into the adult stage, viral tissue tropism and possible viral transmission routes. We found that the level of AdDV increased during nymphal development into the adult stage of the house crickets. Viral levels were higher in males compared to females, and higher in mated adults compared to unmated adults. Furthermore, AdDV was present in every tested tissue of the adult crickets and significant differences in viral levels were found between the different tissues of females, with higher levels in the gut and ovaries compared to the spermatheca. Detailed knowledge on these aspects will help to identify possible interference points with the aim to develop colonies low in viral levels, or completely free of viruses.

Viral levels were found to increase during cricket nymphal development into the adult stage, with the highest levels found in the adult stage. In addition, the virus was detected in both the reproductive tissues and the gut of males and females, indicating that the virus is transmitted horizontally as well as vertically. Since the body size of the nymphs in the rearing increases over time, and the surface area in the rearing container remains the same, the frequency of physical interactions (*e.g.*, direct contact, mating or cannibalism) between individuals will increase, leading to increased probability of horizontal transmission of AdDV. Furthermore, interaction of the crickets with contaminated surfaces, such as the rearing containers, surface-enlarging structures therein, feed, feces and conspecifics exposes the insects to higher quantities of virus. In addition, stress due to physical interactions can have a major impact on the cricket's immune defense, which might lead to increased viral levels in the crickets. An earlier publication on AdDV mentioned that mortality due to AdDV is the most prevalent in the late instars of house crickets [26]. Our findings are in line with this observation and showed an increase in viral levels during cricket development. Szelei *et al.* (2011) found AdDV to be detectable in the air filters of their experimental facilities and suggested that the virus can also spread via the air [26]. This allows for easy spread and contamination with AdDV in the entire rearing facility. The above-mentioned scenarios suggest horizontal transmission of the virus and highlight the difficulty of eradicating the virus once it is already present.

The presence of the virus in the adult reproductive tissues suggests that the virus is also vertically transmitted. Further work should test the role of eggs or sperm in the vertical transmission, with special attention to where and at which levels the virus could be found. In particular, it should be analyzed whether the virus is present inside the eggs or only on the egg surface. If the virus is transmitted on the surface of the eggs, decontamination treatments could be tested [128, 137]. One possible cost-effective surface decontamination method could be for instance exposing the eggs to a controlled dosage of UV light. Preventing vertical transmission from the parental generation to the offspring, or selecting virus-free offspring if vertical transmission is less than 100%, would allow the establishment of virus-free breeding stocks of house crickets.

The increase of AdDV levels happens simultaneously with sexual maturation and mating. We found that mated house cricket exhibited a higher level of AdDV compared to non-mated individuals, and this was seen for both males and females. The mating status is known to have a strong effect on the immune investment of the individual. Tradeoffs are inevitable, with allocation of the available resources to mating, reproduction and associated traits [138]. In most cases, mated females allocate fewer resources to immune defense compared to non-mated counterparts [139-141]. Similar results were shown for female house crickets, where a lower immune response was mounted by mated females upon receiving an immune challenge, compared to non-mated females [142]. This might explain our results of mated females having a significantly higher viral level compared to non-mated ones. Because egg production is a relatively costly process for female insects [141] and since it has been shown to reduce the available sources for the immune system [143], a shift in resource allocation may pave the way for pathogens to increase their levels in

insects. The hormonal changes induced by mating in females can also have an important role. The physiological changes associated with maturation, which are induced by mating, are often controlled by juvenile hormones [141], which were shown to affect immunity [138, 144]. Similarly, re-allocation of resources can be observed for males that need to invest in sperm production and in mating-related traits. It is important to mention that in some cases, mating can also have a positive effect on the immune system. For instance, mated females of *Gryllus texensis* crickets had a higher chance of survival upon a bacterial infection compared to non-mated females [145].

Sperm of insects can be utilized by viruses to 'hitch hike' to females [146]. After mating, female insects store the spermatophore from the males in an organ called the spermatheca. This organ is also responsible for maintaining and releasing the sperm to fertilize the eggs [147]. In the non-mated females, the spermatheca could not be collected (these were too small to be found), therefore, the changes in the viral levels in these tissues induced by mating could not been quantified. However, spermathecae were collected from all mated females (confirming their mated status) and the viral level in the spermatheca was lower than in all the other female organs. Taking into account the high viral levels found in the testes and accessory glands of male crickets, the low levels found in the spermatheca were unexpected. However, as sperm was not collected and tested separately, we cannot conclude if the high viral levels are due to their presence in the testes or in the sperm itself. The role of the male accessory glands is to facilitate transport and maturation for the sperm cells and to provide them with nourishment which is essential for sperm viability [148]. Viruses could utilize the accessory glands for the earlier mentioned hitchhiking [146] with a higher probability of transmission in mated than in non-mated males, which correlates with the high viral levels also found in the crickets tested. Mating may serve as a signal for AdDV that its host is approaching the end of its potential life span and vertical transmission via the sperm enhances transmission of the virus. However, it might be that the virus is present in all tissues of the crickets, and thereby it is difficult to conclude whether presence in the reproductive tissues results in vertical transmission.

Most house cricket colonies are likely to harbor an AdDV infection present in a covert state, which under certain circumstances can be triggered to an overt, lethal infection. Outbreaks that wipe out complete mass-rearings have been reported, resulting in severe financial consequences for the producers [26, 45], endangering the economic viability of house cricket farming operations. To prevent disease outbreaks an in-depth understanding of AdDV-cricket interactions, of AdDV transmission and of rearing conditions triggering lethal outbreaks is needed [69]. Based on the acquired knowledge, effective monitoring methods and preventive and/or curative methods can be developed and utilized [34, 149].

5. Conclusions

House cricket production can be jeopardized by AdDV and information on viral levels and virus transmission during cricket development is currently lacking. This knowledge is indispensable for any preventive treatment aiming to reduce or eradicate AdDV in crickets. We found that viral levels steadily increased over time in the rearing containers, reaching the highest values in adult crickets. This correlates with observations made by the cricket mass-rearing industry and data from earlier publications showing that AdDV-induced mortality is the highest for late instars. Mating was shown to increase AdDV levels for both females and males, suggesting that changes in resource allocation during mating impact cricket immunity and thereby AdDV levels. Additionally, our results suggest that the virus is transmitted both horizontally and vertically. Future research should address in depth how the viral particles are vertically transmitted from the parental generation to the offspring: via sperm, inside the eggs or on their surface. If the viral particles are not inside the eggs, surface sterilization could be applied to the eggs to establish virus-

free breeding stocks. Furthermore, to reduce their losses due to AdDV cricket producers could utilize sanitation treatments that limit horizontal transmission.

Acknowledgments: We would like to thank Monique van Oers and the other program coordinators of the Insect Doctors program, the Insect Rearing Team of the Laboratory of Entomology for maintaining the rearing and providing the crickets for the experiments.

Supplementary tables

Table S1. P values for pairwise comparisons of the snapshot sampling viral level data.

Week nr.	W2	W3	W4	W5
W1	0.9316	0.0758	<0.0001	<0.0001
W2		0.3633	<0.0001	<0.0001
W3			<0.0001	<0.0001
W4				0.0131

Table S2. P values for pairwise comparisons of the sequential sampling viral level data.

Week nr.	W2	W3	W4	W5	W6
W1	<0.0001	0.0001	<.00001	<.00001	<.00001
W2		0.0061	0.2724	0.7691	0.0002
W3			0.6734	0.0001	<.00001
W4				0.0101	<.00001
W5					0.0144

Table S3. Data analysis methods used for the different datasets.

Section of the results	Data analysis method
3.1. Viral levels throughout the cricket development – snapshot sampling	Generalized mixed effect model (GLM)
3.2. Viral levels throughout the cricket development – sequential sampling	Linear mixed effect model (LMER) with line as a random factor
3.3. Effect of mating and gender on the viral levels in individual crickets	Generalized mixed effect model (GLM) with interaction between the gender and mating status
3.4. Viral tissue tropism in females	Two way ANOVA
3.5. Viral tissue tropism in males	Two way ANOVA

CHAPTER 5

5

Discovery of DNA and RNA viruses present in house cricket colonies

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Abstract

The house cricket, *Acheta domesticus,* is a commonly reared insect for food and feed purposes. House crickets are known to be infected by a range of both DNA and RNA viruses. Some of these viruses, such as the Acheta domesticus densovirus (AdDV) can cause massive epizootics in production facilities, which can endanger the economic viability of these enterprises. Various studies identified an array of viruses present in house cricket colonies. However, the majority of these studies used a primer-based PCR approach for detecting viral presence and were limited to the viruses already known to be pathogens of house crickets. With the advancement of next generation sequencing technologies, virus detection and discovery can be carried out *via* an unbiased approach. In this study, total DNA and RNA were sequenced from three different colonies of house crickets, to screen for exogenous DNA and RNA viruses present. In the case of RNA viruses, three putative new virus species so far not associated with house crickets were discovered. In the case of DNA viruses, only AdDV was detected and no other exogenous entomopathogenic DNA viruses were found. Surprisingly, two out of the three-cricket colonies sampled proved to be free of AdDV. Many of the known, earlier described cricket viruses were not present in either of the tested colonies. This analysis shows that an unbiased approach of virus detection in cricket rearing is essential for revealing the potential threats for cricket production systems.

Keywords: *Acheta domesticus*, house cricket, insect production, DNA viruses, RNA viruses, cricket viruses, entomopathogenic viruses, mass rearing

1. Introduction

Over the past decade, the interest in insects as food and feed has gained momentum, resulting in an investment of more than 1 billion euros in the sector by 2021 and this is expected to reach up to 3 billion by 2030 [14]. Insects and products derived from them, can be utilized for various purposes: for pharmaceuticals [150], as animal feed ingredients [151, 152] and for waste valorisation [153]. The market on which the highest profit margins can be reached with insect products is the human food market. Currently in the European Union (EU), four insect species are authorized by the European Food Safety Authority (EFSA) [23] to be used for food production; the grasshopper *Locusta migratoria*, the yellow mealworm *Tenebrio molitor*, the lesser mealworm *Alphitobius diaperinus* and the house cricket *Acheta domesticus*. For a long time, house crickets are traditionally reared and consumed in Asia [17, 22] and with the recent authorization house cricket production is also expected to increase within the EU. House crickets have a favorable nutritional profile and are amenable for mass-rearing [20, 21]. However, there are also major drawbacks related to mass-rearing of crickets; the available research on the nutritional needs of house crickets is limited, but mainly the numerous viruses that infect and can rapidly eradicate colonies are the most concerning. The main viral pathogen of house crickets is the Acheta domesticus densovirus (AdDV), which has a long history of causing losses in cricket production, with the first described case from Europe dating back to 1977 [26] and already by the 1980s AdDV was also present in the United States [44]. Ever since, AdDV has been frequently reported from Europe [26], North America [45] and Asia [49], showing its worldwide status. AdDV belongs to the insect-specific subfamily *Densovirinae* of the family *Parvoviridae*. Viruses belonging to the *Densovirinae* are known to be insect pathogens and some are also used in biological control of insects [51, 154]. Densoviruses are non-enveloped, paraspherical, autonomous viruses, with a diameter ranging between 23 and 28 nm and a single-stranded, linear DNA genome of 4-6 kb [155, 156] and can cause infections in insects of six different insect orders (Blattodea, Diptera, Hemiptera, Hymenoptera, Lepidoptera, and Orthoptera) [135, 155]. In the case of commercially produced insects, besides the earlier mentioned house crickets, densoviruses are known to cause losses in silkworms [157] and in yellow mealworms [156]. In crickets, not only AdDV, but also other viruses can cause disease-related losses [158]. Cricket paralysis virus (CrPV; family *Dicistroviridae*) [159] can cause symptoms similar to those of AdDV and is also considered as a serious pathogen for house cricket farms [36], as well as cricket iridovirus (CrIV) (a strain of invertebrate iridescent virus type 6 (IIV-6); family *Iridoviridae*) [160, 161]. Other viruses include A. domesticus mini ambidensovirus (AdMADV), A. domesticus volvovirus (AdVVV) [39, 40], A. domesticus iflavirus (AdIV) [161] and A. domesticus virus (AdV) [161]. Many studies aimed at monitoring house cricket colonies to detect viruses use PCR- or qPCR-based detection methods [26, 27, 37, 46]. While these techniques are useful to detect and quantify known viruses, they are unsuitable to detect any variants of known species that fail to amplify due to mutations in the primer-annealing sites, or on any completely novel virus species. In contrast, next generation sequencing (NGS) approaches provide an unbiased screening approach, revealing new and unknown viruses that are present in house cricket colonies. The advancement over recent years in the sequencing technologies allowed the costs to decrease, which created an unseen volume of new sequencing data. For insects in general, NGS has revolutionized the field of virus discovery, with a range of metagenomics studies revealing that insects are a major reserve for viruses, with a high virus abundance and diversity [41, 162]. For crickets, so far only two publications are available that used NGS to detect viruses of DNA and RNA origin. Miranda et al. [37] sequenced DNA obtained from frass coming from commercially reared house crickets. However, from the known house cricket viruses, only the invertebrate iridovirus 6 was found. Although many other DNA and RNA viruses were present in the frass, most of those were viruses infecting plants, bacteria and other insects. Furthermore, Cholleti et al. [42] sequenced crickets provided with four different feed treatments but found none of the earlier listed cricket pathogens to be present in the samples

or any new entomopathogenic ones. In our study, we aimed to analyze crickets originating from three different colonies, to i) discover new exogenous viruses, which are currently not known to be associated with house crickets. ii) reveal the difference in exogenous viral pathogens present between different cricket colonies.

2. Materials and methods

Cricket colonies

Samples were taken from three different house cricket colonies. The samples labelled as "WUR" originated from a colony kept at the Laboratory of Entomology, Wageningen University since 2006. Briefly, the house crickets were reared in plastic containers (40 L) in a climate-controlled room, with 16:8 L:D cycle, at ~28 °C with a relative humidity (RH) around 50 % and provided with chicken feed (Kuiken Opfokmeel, Kasper Fauna Food, Woerden, the Netherlands) and carrot slices as feed source and water from dispensers on a daily basis. Samples labelled as "CPH" are originating from a colony kept at Copenhagen University, Section Organismal Biology since 2019. Here, the crickets were kept in plastic containers (32 x 21 x 23 cm) in climate-controlled incubators, with a 12:12 L:D cycle, at 30 °C with a relative humidity (RH) ranging between 65-70 %. The "CPH" crickets are fed with grinded chicken feed (Gold 4 Gallico Pellet, Versele Laga, Brough, United Kingdom), water gel and organic cucumber slices retrieved from local supermarkets. Samples labelled as "COMM" are of retail origin from a commercial local pet shop in the vicinity of Wageningen, the Netherlands. The rearing conditions of this cricket colony were unknown.

DNA and RNA extraction

For each colony, three female and male adults were subjected to DNA extraction, and three female and three male adults were subjected to RNA extraction. For the DNA extractions, samples were homogenized with a sterile plastic pestle in 0.2 mL of sterilized phosphate-buffered saline (PBS) solution in 2 ml Eppendorf tubes. After the homogenization, the samples were centrifuged for 15 min at 8000 rpm to precipitate debris. The supernatant was removed and transferred to a clean tube for DNA extraction. Subsequently, extraction of DNA was carried out using the Qiagen DNeasy® Blood and Tissue kit (Qiagen, Germany), following the 'Total insect DNA extraction supplementary protocol' (except for the sample homogenization stated earlier). For the RNA extractions, samples were homogenized as described before, except that the PBS solution was replaced with TRIzol® (Thermo Fisher Scientific Inc., MA, United States). The samples were split into two separate tubes, to allow the necessary volume of 800 µl TRIzol® reagent and the cricket body mass in one tube, without spillage of the reagent. This step was also important to avoid the clogging of the spin columns in the following steps. After the homogenization, total RNA extraction was carried out using the Direct-zol™ RNA MiniPrep Kit (Zymo Research, CA, United States). Quality control of the DNA and RNA samples was confirmed by a Nanodrop® ND1000 Spectrophotometer (Thermo Fisher Scientific Inc., MA, United States). For each colony, an equal volume of the three male and three female DNA or RNA extractions were pooled into one DNA or RNA sample, respectively, to be submitted for Illumina sequencing. All samples were stored at -80°C until their shipment.

Virus discovery and detection

All samples were sequenced at Macrogen Europe (Amsterdam, The Netherlands). Both DNA and RNA samples were sequenced with the use of the NovaSeq 6000 sequencing platform, with a difference in the library preparation: for the DNA samples the TruSeq Nano DNA Kit was used while for RNA samples the TruSeq Stranded Total RNA LT Sample Preparation Kit (Gold) was used. Before starting the pipeline to detect and discover viruses, the quality of the sequencing reads from both DNA and RNA datasets were checked with fastqc (v0.11.8) [163]. Subsequently, adapters and low-quality reads were trimmed and

removed in all sequencing datasets by running fastp (v0.23.3) [164] with the deduplication mode on (--dedup) and the hash buffer number and buffer size was respectively increased to improve the accuracy of calculating duplication (--dup_calc_accuracy 6). Reads from the DNA and RNA datasets were then mapped on the *Acheta domesticus* genome (GCA_014858955.1) with the programs bowtie2 (v2.2.5) [165] and hisat2 (v2.2.1) [166] respectively, with default parameters. The unmapped reads from the DNA and RNA datasets were extracted via samtools and assembled by running metaspades and rnaviralspades (Spades v3.15.4) [167], respectively. Next, the assembled contigs of viral origin were selected to be viral using VirSorter2 (v2.2.4) [168] with the parameter -includegroups "dsDNAphage,NCLDV,RNA,ssDNA,lavidaviridae". The output of VirSorter2 containing putative viral contigs was evaluated by running tBLASTn against the Refseq protein database of RNA viruses and DNA viruses (downloaded 09/07/23) with the threshold e-value set at 1×10^{-5}. The output of the tBLASTn was filtered manually to remove the putative viral contigs that were not at least 2000 and 1000 base pairs (bp) in length for DNA and RNA viruses, respectively. Finally, in order to remove contigs that were falsely considered as viral but instead were derived from bacteriophage, bacterial, fungal or *A. domesticus* origin, a BLASTx was performed against the complete non-redundant protein database from NCBI (accessed on 15/10/23) with a threshold e-value set at 1×10^{-5}.

Annotation of the viral genomes and phylogenetic analyses

Potential open reading frames (ORFs) found in the new viral candidate segments were defined with the use of the NCBI open reading frame finder (accessed on 01/11/23), with the settings as "ATG" and alternative initiation codons and minimum ORF length as 150 bp. From the ORFs defined for each segment, the protein sequences were individually transferred to Interpro, to identify the protein domains found within the ORFs [169]. From the Interpro output, the defined domains (such as RNA-dependent RNA polymerase (RdRP), methyltransferase, glycoprotein, viral protein) for each ORF were visualized using the gggenes package (0.5.1) [170] within the R software (4.2.2)[90]. For the newly discovered putative viruses, a phylogenetic tree was constructed by selecting the protein sequence of the RdRP gene. This gene was then used as a query for a BLASTp search against the non-redundant database in NCBI (accessed on 02/11/23) to obtain the closest viral ancestors for the putative viruses. The final set of RdRP proteins were aligned using MAFFT (v7.505) [171] with the iterative refinement method E-INS-I in combination with the Smith-Waterman algorithm (--genafpair, --maxiterate 1000 and --localpair). Alignments were then trimmed using Trimal (v1.4.1) [172] using the heurisitic selection method (-automated1). A maximum-likelihood tree reconstruction was created by IQtree (v2.0.3) [173] using the ModelFinder method (-m MFP) [174] in combination with the implementation of an ultrafast bootstrapping with 3000 replicates (-B 3000), the nearest neighbor interchange (-bnni) and aminimum correlation coefficient of 0.99 (-bcor 0.99). The phylogenetic trees were all midpoint-rooted and branches with a bootstrap support less than 50 were deleted. Visualization and annotation of the phylogenetic trees were made using iTOL (v6) [175].In the case of the newly discovered Acheta domesticus nodavirus and cypovirus, an additional BLASTx of the assembled unfiltered contigs was done against the most closely related nodavius (Genbank: GCA_031551785.1) and cypovirus (Genbank: GCA_000850245.1) to complement the viral genomes with the missing segments that were falsely assigned as non-viral.

Viral abundance

For all the detected cricket viruses, viral abundance was calculated by mapping the trimmed reads (described above) on the viral genomic sequences and on the host gene elongation factor 1a (EF1a; JX897494.1) using Bowtie2 (v2.5.2) [165] and samtools (v1.6) [176]. The number of reads mapped on the different viral genomes were normalized with the number of reads mapped on the host gene. Viruses were considered absent when the normalized counts were below $1x10^{-4}$. Log_{10} transformed normalized viral

abundance was visualized by using the heatmap.2 function from the gplots (v3.1.3) package within the R software (v4.2.2) [85].

3. Results

Three newly discovered RNA viruses in *Acheta domesticus*

Our study aimed to explore the RNA viruses present in three different house cricket colonies. After completing the assembly and different BLAST steps, the complete and partial genomes of four different RNA viruses were found in the three cricket colonies. The sequencing data revealed three new putative RNA viruses of which two were single-stranded RNA (+ssRNA) viruses and were named as Acheta domesticus virus 2 (AdV2) (Figure 1) and Acheta domesticus nodavirus 1 (AdNdV1) (Figure 2). The third new virus was a double-stranded RNA (dsRNA) virus, named Acheta domesticus cypovirus 1 (AdCyp1) (Figure 3).

The first putative new virus, AdV2, showed high similarity with the Solenopsis invicta virus 17 (SiV17) that was found in the fire ant, *Solenopsis invicta* [177]. The RdRPs of AdV2 and SiV17 have 99.3% amino acid sequence identity. AdV2 consists of one segment of 10 kb, in which three open reading frames were found. The first and second smaller ORFs contain the domains encoding for glycoproteins, while the third larger ORF encodes a polyprotein containing methyltransferase, helicase and RdRP domains (Figure 1).

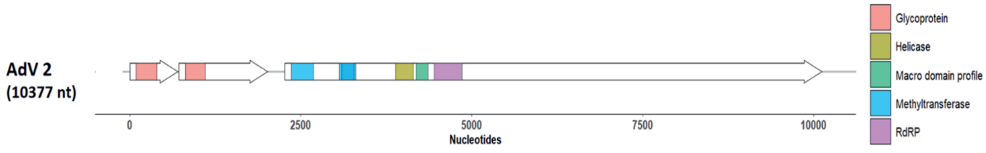

Figure 1. Putative genome organization of Acheta domesticus virus 2 (AdV2). The white arrows represent the open reading frames (ORFs), while the different colored regions represent the different domains found within each of the ORFs.

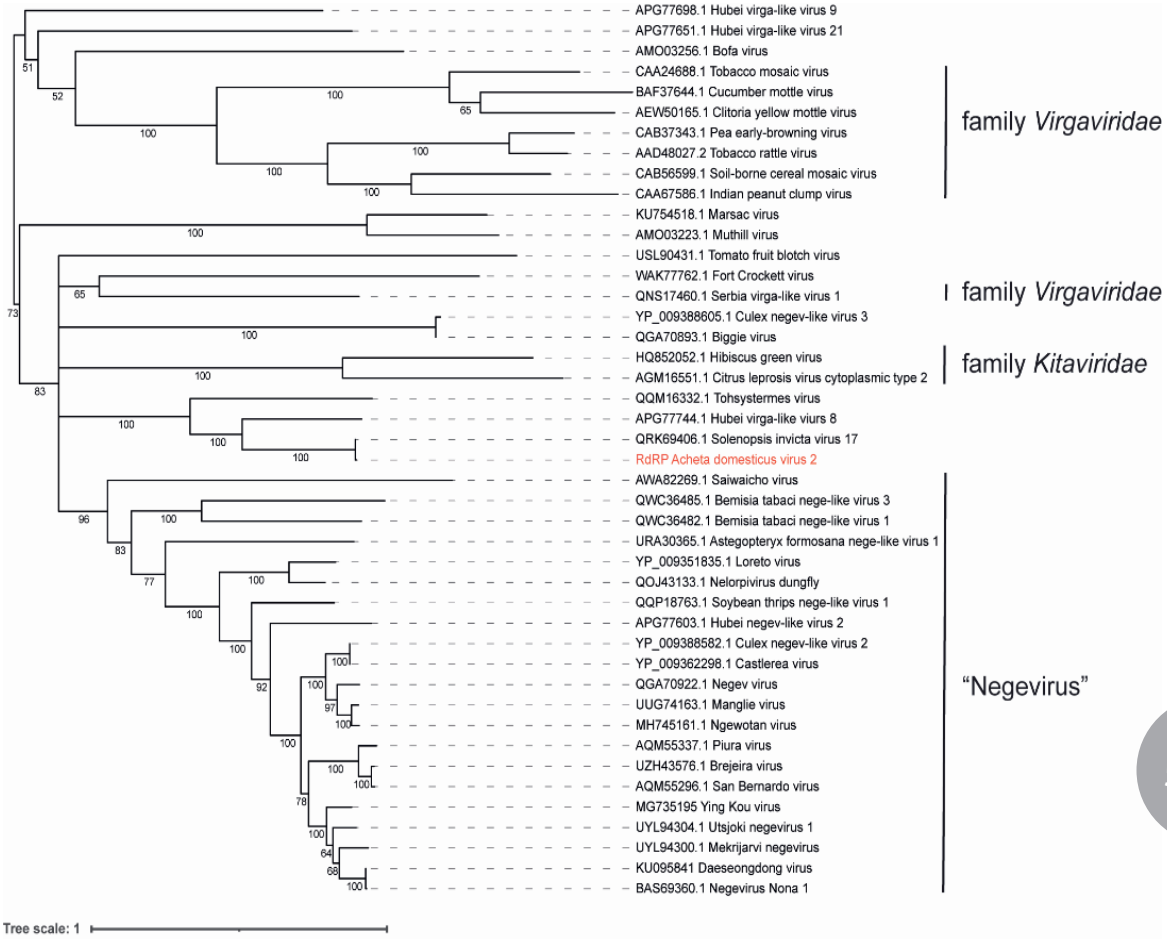

Figure 2. Maximum likelihood phylogeny of the RdRP protein of AdV2 (in red) and related viruses of the order Martellivirales. Numbers below the branches indicate bootstrap values (3000 replicates). Only values larger than 50 are indicated.

Phylogenetic analysis suggested a close relation with the abovementioned Solenopsis invicta virus 17, Hubei virga-like viurs 8 and Tohsystermes virus infecting fire ants, myriapods and termites, respectively (Figure 2). Those viruses are positioned within the order *Martellivirales*, between the negeviruses and viruses of the families *Kitaviridae* and *Virgaviridae* and therefore could not be assigned to a known virus family.

The second new +ssRNA virus was AdNdV1, belonging to the family *Nodaviridae* (order *Nodamuvirales*). Like other nodaviruses, AdNdV1 has a segmented RNA genome consisting of two segments. The first, shorter segment contains the two ORFs encoding for viral coat proteins, while the second and larger segment contains the methyltransferase and RNA polymerase domains (Figure 3). The RdRP of AdNdV1 is closely related to Ceratitis capitata nodavirus, CcaNdV1 (UOI84721) (Figure 4), with a 99.6% amino acid identity score for the RdRP. Viruses clustering with AdNdV1 and CcaNdV1 infect hosts from the insect orders Coleoptera and Diptera and AdNdV1 is the first nodavirus found infecting a host species belonging to the order Orthoptera.

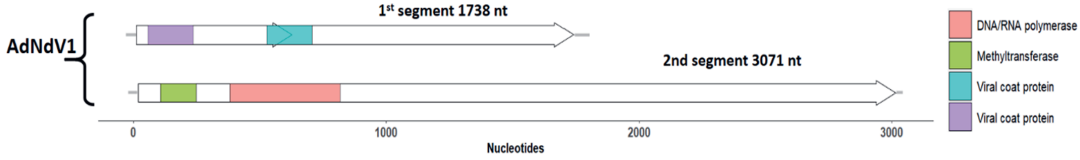

Figure 3. Putative genome organization of Acheta domesticus nodavirus 1 (AdNdV1). The white arrows represent the open reading frames (ORFs), while the different colored regions represent the different domains found within each of the ORFs.

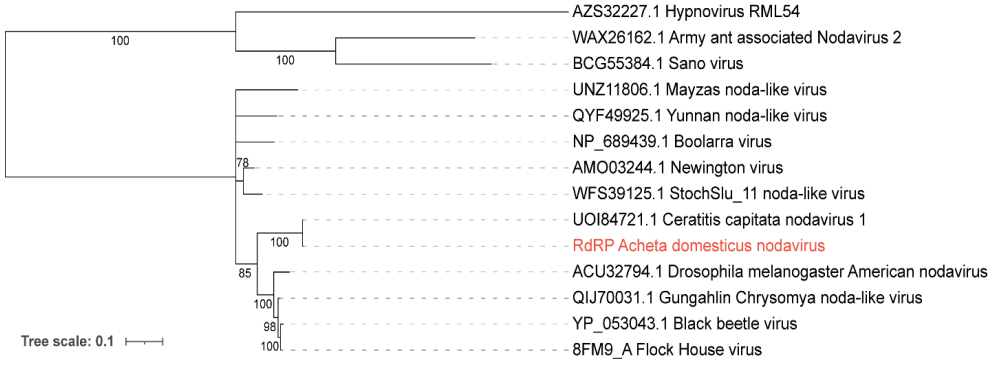

Figure 4. Maximum likelihood phylogeny of the RdRp protein of AdNdV1 (in red) and other nodaviruses. Numbers below the branches indicate bootstrap values (3000 replicates). Only values above 50 are indicated.

The third putative new virus was AdCyp1, belonging to the family *Spinareoviridae* (order *Reovirales*). For AdCyp1, six genome segments were found, ranging in length from 770 to 4445 bp (Figure 5). The first and fifth segment encodes for hypothetical proteins, of which the first segment is likely to contain the RdRp. The second segment contains the methyltransferase domain, while the third segment encodes the capsid protein. The fourth segment contains the viral protein 2 with unknown function and the sixth segment contains the polyhedrin domain. Most likely, additional AdCYp1 segments can be detected in the house cricket, since members of the *Reoviridae* usually contain 10 to 12 genome segments [178]. AdCyp1 clusters with other insect-infecting cypoviruses and dinovernaviruses, however, it does not show a close similarity to any of those viruses (Figure 6).

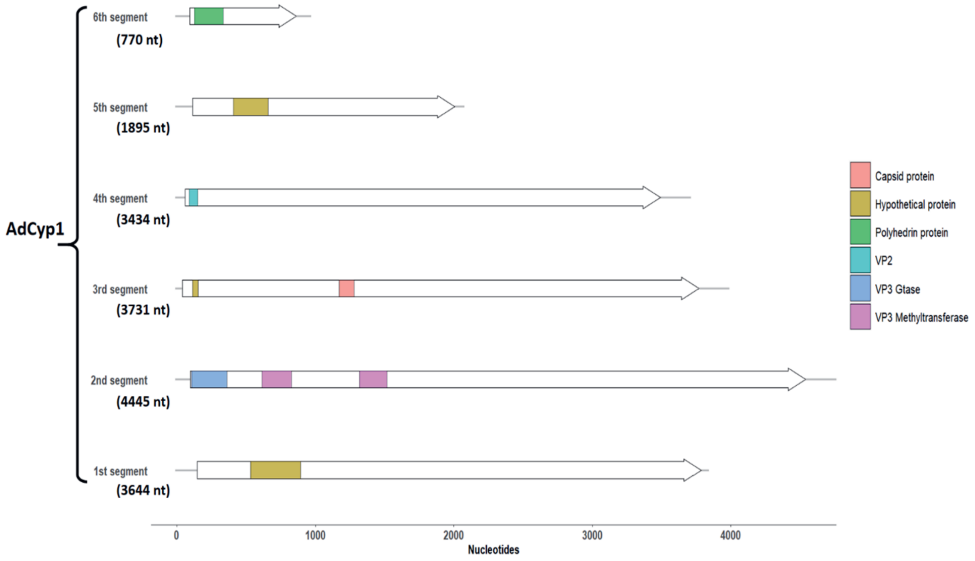

Figure 5. Putative genome organization of Acheta domesticus cypovirus 1 (AdCyp1). The white arrows represent the open reading frames (ORFs), while the different colored regions represent the different domains found within each of the ORFs.

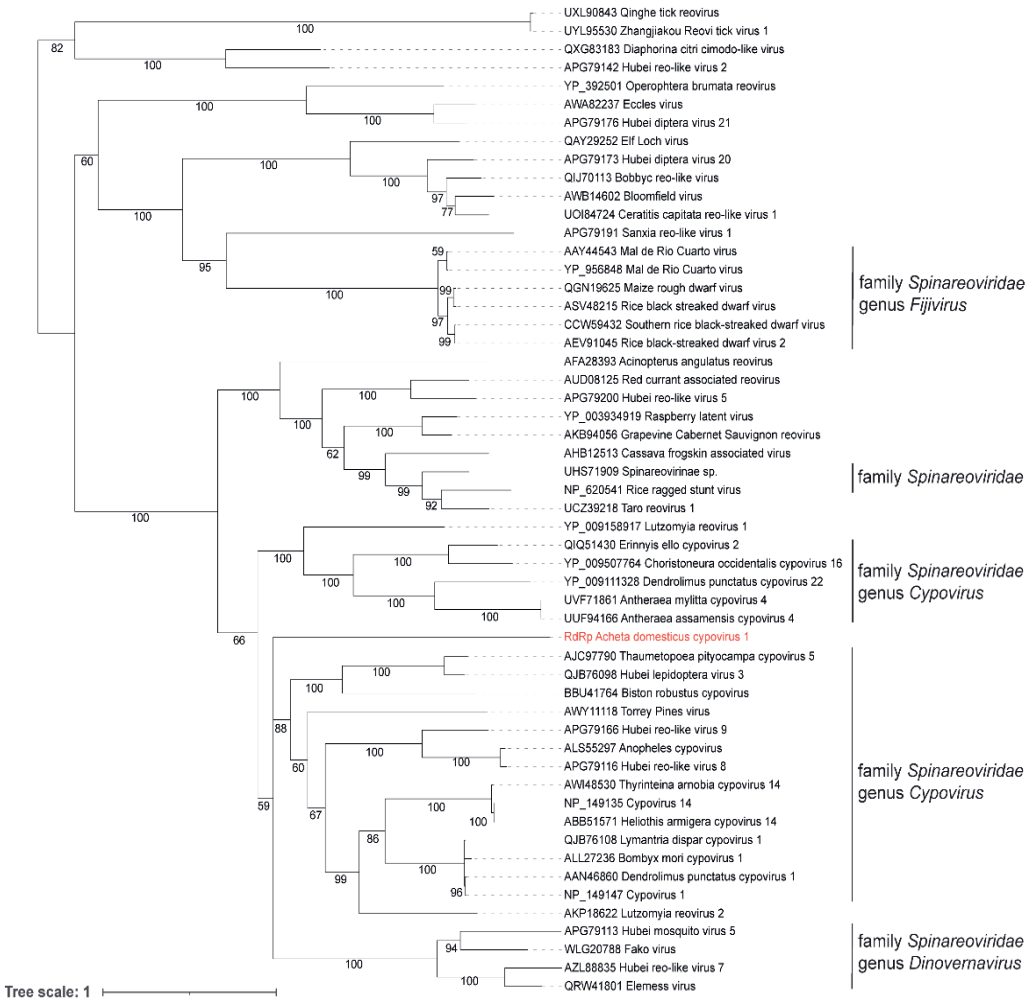

Figure 6. Maximum likelihood phylogeny of the RdRp protein of AdCyp1 (in red) and other viruses of the Reovirales order. Numbers below the branches indicate bootstrap values (3000 replicates). Only values above 50 are indicated.

Screening of the RNA sequencing datasets also revealed the presence of Acheta domesticus iflavirus (AdIV) belonging to the family *Iflaviridae*, which was already described and associated with house crickets in previous studies [161]. However, this viral genome was only partially detected (1 kb segment), while iflaviruses usually have a genome size of 9-11 kb [179].

The Acheta domesticus densovirus is only present in one of the cricket colonies

Only one exogenous DNA virus was detected that matched with sequences in the DNA databases, the already known AdDV. This virus was only detected in the WUR colony. The other colonies, COMM and CPH, did not harbor AdDV or any other exogenous DNA viruses.

The prevalence and viral abundance varies between different cricket colonies

An assessment of the prevalence and viral abundance of the five different viruses detected was carried out on the obtained DNA and RNA sequencing datasets (Figure 7). The WUR colony was infected with all five viruses at the highest viral load found across the three colonies. The highest abundance was found for AdV2, followed by AdCyp1. Both in the COMM and CPH colonies the AdDV, AdIV and AdV2 were not detected. The COMM colony contained two viruses, AdNdV1 and AdCyp1, both at low abundance. The CPH colony only contained AdCyp1 at low abundance.

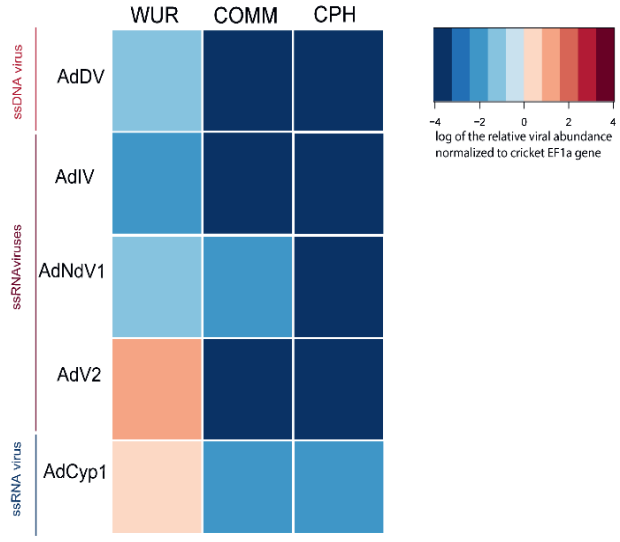

Figure 7. Viral relative abundance in RNA and DNA sequencing datasets. Heatmap of the relative abundance of the four RNA and the one DNA viruses in three cricket colonies. The colors represent the log_{10} of relative viral abundance calculated as the viral read count normalized by the insect hosts EF1a read count.

4. Discussion

The newly emerging insect rearing industry for food and feed purposes can face various pathogen challenges [14, 34], but unlike in the case of other farmed animals like cattle, poultry, honeybee etc., the veterinarian knowledge to identify, treat and prevent the diseases is lacking. House cricket colonies can be infected by numerous viral pathogens [36, 37], resulting in a challenging situation for diagnostics and treatments to prevent or cure viral epidemics. In this study, we identified three putative new RNA viruses found in different house cricket colonies. The new viral candidate genomes were annotated and the RdRp proteins were used to infer their phylogenetic relationship to other viruses. The first new putative virus, AdV2, showed similarities with negeviruses and viruses of the family *Kitaviridae* and *Virgaviridae*. Knowledge about the infectivity, transmission and host range of negeviruses is currently limited [180], but they are commonly found in mosquitoes [181]. The second putative new virus, AdNdV1, is from the family *Nodaviridae*, which is further divided into alphanodaviruses, infecting a wide range of insects and betanodaviruses, infecting fish species and considered as serious pathogens in fisheries [182]. In laboratory conditions, alphanodaviruses caused reduced growth, paralysis and high mortality rates in *Galleria mellonella* [182]. The third putative new virus, AdCyp1, is from the family *Spinareoviridae*. The dsRNA genome of viruses within the *Spinareoviridae* consists of 10-12 segments [178] . Cypoviruses are insect-specific viruses that replicate in the cytoplasm of the infected host. Cypoviruses form polyhedra, which are transmitted between insects [183, 184], and have been found infecting more than 250 insect species [178]. Cypoviruses are used in biological control of insects [185], although in many species they produce only chronic disease without high larval mortality [178]. A fragment of an iflavirus was also found, however, as it was only a small fragment of 1 kb of the 9-11 kb genome size of iflaviruses [186], therefore the presence of the virus cannot be confirmed.

The screening of a limited number of colonies revealed three new viruses, which highlights that the current knowledge on viral pathogens affecting house crickets is limited. Screening in publicly available databases and from other accessible cricket colonies is likely to result in the detection of additional novel viruses in crickets, as insects are considered as a major reserve of RNA viruses [41, 162]. The diversity in viruses creates a challenging situation for cricket farming, as when new viruses are described, those are often not further investigated regarding their infection cycle and the possible effects on their hosts. Therefore these necessary information is not available for producers to possibly identify viral diseases that they might encounter in their stocks. Yet, the knowledge of which viruses could be present in the production facilities therefore requires attention [26, 45]. This allows producers to take measures to prevent disease outbreaks, or to switch to a virus-free breeding stock. In case of described and annotated viruses, primers and non-destructive sampling methods can be developed for screening purposes [46], which allows producers to monitor numerous viruses simultaneously, so a comprehensive picture of all the possible viral pathogens within their colonies can be obtained.

Monitoring and disease prevention or treatment in the facilities is a complex situation [149], which can yield valuable insights for producers. A first step is to use the knowledge generated with NGS and the relevant bioinformatic pipelines to know which viruses could potentially be present in their colonies. A primer-based approach can only detect already known viruses, thus NGS can serve as a better starting point in the discovery of viruses. As a second step, after identification of the viruses present, primer-based viral level measurements can provide important data on the dynamics of the viral levels in the colonies. An increase in mortality and decreases in cricket biomass and reproductive output may be used as warning signals to examine the dynamics of viral levels, therefore monitoring these data is the third step. Creating databases on those traits and cross-validating these with viral level data can point out which viruses are causing the detrimental effects. Additionally, changes in the diet, environmental conditions or the microbiome of the crickets can be evaluated. The gut microbiome of crickets can play an important role

against viral infections, since most insect viruses are orally infectious [187]. Several protective barriers are present in the insect gut against infection by viruses and other harmful microorganisms, such as the cell-intrinsic antiviral immunity, the peritrophic matrix, the mucin layer [188] and the beneficial gut microbiome [189, 190]. This complex system with many simultaneous interactions contributes to prevention of viral infections. However, depending on the content of the gut microbiome, adverse effect can also occur, by bacteria present in the gut facilitating the infection by a viral pathogen [191]. All three new putative viruses detected have a high potential to cause negative effects or losses in house cricket production, highlighting the importance of screening for and describing new insect viruses.

5. Conclusions

Techniques like NGS can serve as an important diagnostic tool to identify the viral pathogens present in insect rearing. This identification is the first step to establish both preventive and curative measures in avoiding production losses caused by these pathogens. However, presence of certain viruses in the colonies does not necessarily mean active infections causing detrimental effects on the crickets. Therefore, further studies should be carried out to determine the viral infection cycle, the mode of transmission and the negative or positive effects on the crickets.

Acknowledgments: We would like to thank Joop van Loon for his contribution in the development and reviewing of this chapter.

CHAPTER 6

6

General discussion

1. Introduction

Governments throughout the world are shifting focus in their agendas towards sustainability of food production and consumption. The food chain is a major contributor to CO_2 emissions, one third of global anthropogenic GHG emissions [192] are associated with the supply chain of our food: production, processing, warehousing and delivery to our tables. Therefore, food production is a major focal point to reduce CO_2 emissions to have a lower carbon footprint associated with our diets. Humans need to eat, but what we eat has a major impact on the associated carbon footprint, as meat and dairy products are the ones that contribute the most to the carbon footprint [193]. There are several alternatives for meat and dairy products, which can offer lower carbon footprints, such as soy, cultured meat, seaweed and insects. Edible insects are known to need the lowest amount of water and smallest land surface per unit of product and have a lower carbon footprint than either beef, pork or poultry [14, 194, 195]. However, what insects are fed with has a crucial role in determining if there is actually a benefit with regard to environmental impact [196]. One clear advantage of industrially farmed insect species is that all of them are poikilotherms, which means they do not use metabolic energy to regulate their body temperature [156]. In addition, unlike the earlier mentioned farmed animals, insects are mainly eaten completely, thus their production results in a smaller proportion of by-products.

The house cricket, *Acheta domesticus,* is mainly eaten in its entirety or the whole crickets are processed into food ingredients. House crickets are fast growing and have good nutritional values [20, 21]. Products derived from house crickets received approval (after certified processing) for human consumption from the European Food Safety Authority (EFSA) in 2022 [23]. This important milestone can boost the production of house crickets in Europe and can motivate producers outside the continent to get their products approved by EFSA to be allowed on the EU market. Consumer acceptance of insects, however, still needs to develop in order to become part of daily diets. Consuming entire insects is more challenging especially for people in the Western world, a possible solution to overcome this issue is to incorporate insect-derived products as food additives or ingredients. There are numerous attributes of house crickets which makes them a great food additive or ingredient, such as the high protein content of 60-70% (based on dry matter) [18] or their favorable fatty acid profile [19, 21]. Because of their good nutritional profile and functional properties, house cricket-derived products have been already incorporated into various food products successfully [24, 25]. High quality food products are important to shift house crickets from the delicacy category to become an everyday food ingredient on a wider scale. An additional condition for transitioning from the delicacy category is production on a sufficiently large scale, for which in which recent years important investments were made. Currently the largest commercial-scale house cricket production for human consumption is located in Asia and Africa [17, 22], but throughout the world there are commercial producers also for zoo and pet food purposes [45] and for research use in laboratories [197]. Recently a large-scale industrial cricket farm was opened in Canada, with 12,000 metric tons annual capacity [198].

Even though house crickets have various positive attributes, their susceptibility to viral and other microbial diseases can compromise the success of cricket farming [17, 36]. The viral and microbial pathogens can have various negative effects, such as reduced productivity, smaller size of individuals and in the worst case entire colony collapses [34] resulting in major economical setbacks. As described in the General introduction (Chapter 1) and in Chapters 2, 4, and 5, house cricket production faces a major threat in the form of the Acheta domesticus densovirus (AdDV) and other viral pathogens. The objective of this thesis was to assess the influence of a combination of possible stressors on AdDV levels and potential outbreaks in crickets and to assess the effect on the major production parameters, including cricket survival and biomass production. The following sections summarize and discuss the main findings presented in this thesis. Furthermore, one additional key element of house cricket rearing, the feeding substrate, is discussed

together with the immune system of insects and more specifically house crickets. The complex topic of insect welfare is also briefly discussed, as mass rearing conditions of insects and possible disease issues in the rearing systems are strongly connected to insect welfare.

2. Combined stress factors: temperature and density

Various abiotic and biotic stressors can play a role in AdDV becoming activated, but the actual disease mechanisms and the triggering stressors are currently unknown. In Chapter 2, the combined effects of two stressors, rearing temperature and cricket density, on cricket survival, cricket biomass and the viral abundance of AdDV were assessed. For optimal house cricket rearing, a high cricket survival and biomass are desired, while the preferred virus levels are low. Efficient use of resources at the production facilities is an aim of the producers for cost reasons. The costs associated with the heating, ventilation and air conditioning (HVAC) systems of insect rearing facilities are major, both for the capital expenditures (CapEx) and for the operating expenses (OpEx) [30, 79]. House crickets like most insect species are unable to regulate their body temperature and therefore are exposed to the ambient environmental temperatures. The optimal temperature range for cricket rearing is reported to be between 28-30 °C [61, 199]. Depending on the region where facilities are located, either heating or cooling might be required to maintain this optimal temperature range, which depending on the type of facilities and local energy costs can be a major cost component in OpEx of the rearing facility. Therefore, three rearing temperatures were selected for testing, including the optimal temperature, 30 °C, and two deviating 5 °C from that: 25°C and 35°C. Since the HVAC-controlled rearing space for industrial scale comes at a high price [30], the best utilization of the available rearing area is also required. Therefore, the highest possible individual/surface ratio is favorable for the producers, as long as there are no negative effects. For crickets, the rearing density is often expressed as volume per individual; however, this can be highly misleading and does not describe the actual rearing environment adequately. Cricket rearing units usually contain some form of surface-increasing devices or structures; these can be egg cartons/trays, paper corridors etc. For sanitation purposes, these structures either need to be cheap and readily available to be replaced between each rearing cycle, or should be easily cleanable in case of reusing them. When these surface-increasing devices are used, the rearing density expressed as volume per individual becomes obsolete and the surface area per individual should be used instead. Few studies examined the rearing densities of house crickets: the study of Tennis et al. [67] highlighted the importance of the available feeding surface, which needs to be increased alongside the rearing densities. A more recent study of Mahavindanage et al. [68] tested the effect of rearing density on growth, survival, and starvation resistance of the crickets. In our experiments, we tested combinations of rearing temperatures and densities, to assess their effect on survival, biomass, individual weight of males and females and on the change in AdDV levels, relative to the start values.

Survival of the crickets was found to be only affected by temperature, no effect of density or interaction between the two factors was found. Highest survival was observed at 25°C, while the 30°C resulted in the lowest survival rates, significantly lower than 25°C . Since other studies found the optimal rearing temperature for house crickets to be between 28-30 °C [61, 199] this result was unexpected. A possible explanation for this phenomenon could be that similarly to crickets, AdDV also may have an optimal temperature range for its replication. Temperatures outside the optimal range can negatively affect the virus, which can result in increased cricket survival due to lower virus multiplication rates. This hypothesis is supported by the results on relative viral abundance. The fold change in increase of viral abundance relative to the starting levels was the lowest at 35°C, significantly lower compared to that found at 30°C in one case and in a second experiment, the fold of change in viral abundance was also the lowest at 35°C, although in this case not significantly. Further experiments could assess the possible use of elevated temperatures to reduce AdDV levels in house crickets. House crickets (and other insects as well) are known to exhibit

"behavioural fever" [64], choosing elevated temperatures upon bacterial infection. Similar studies could be performed to determine if crickets exhibit the same behavior to reduce AdDV replication. A different aspect of cricket rearing is the total cricket biomass obtained, which was found to be affected by an interaction between rearing temperature and density. In case of low (10) and mid (20) densities, crickets reared at 30°C and 35°C showed a significantly higher biomass than at 25°C; at the highest density (40) biomass was also higher although not significantly so. Combined with the differences in developmental times until adulthood (~two weeks at the highest temperature, ~three weeks at the mid temperature and 30-31 days at the lowest temperature), from biomass production perspective the higher rearing temperatures clearly show benefits. Among the tested rearing densities, the highest density of 40 crickets seemed most optimal with respect to total biomass production, with no effect on cricket mortality. Increasing rearing density is favorable from a production perspective, but this cannot be done without a limit. Mortality and starvation resistance are negatively affected above 0.93 cricket/cm^2 according to Mahavindanage [68]. In addition, in our experimental run 3 the highest rearing density resulted in the highest fold change in viral abundance, but this was only found for this experimental run, the other two runs did not lead to differences in viral abundance between the densities. It is also important to mention the large variation between the three experimental runs in viral abundance, for experimental run 1, a much higher fold change was observed when compared to the two other experimental runs. This may have resulted from variation in viral abundance between the rearing batches, however, the initial viral loads at the start of the experiments did not differ significantly between the batches, thus the differences occurred during the rearing cycle.

3. Challenges represented by the co-stressing effect of other microbial pathogens and density

Entomopathogenic fungi (EPF) are commonly used in agricultural insect pest control in integrated pest management (IPM) programs and in organic production systems. In particular, species from the genera *Beauveria* and *Metarhizium* [56, 112] are the most commonly used. Species from these two genera are mostly considered as generalist EPFs; one strain can infect hosts from many different orders. House crickets are often reared in open or semi-open facilities [22], which can facilitate the entrance of EPF via infected individuals or airborne spores. Since EPF are also applied as biopesticides and the spores can persist for a long time, contaminated feed material can also vector EPF into cricket rearing facilities. In Chapter 4, a screening of six *B. bassiana* and six *M. brunneum* isolates was performed. The results were not as expected, since several isolates did not reduce survival of the exposed crickets compared to the control. These results suggest that crickets are quite resistant to these fungal pathogens. In our study, crickets were exposed to a high spore concentration (1×10^8 conidia/ml) [122, 123], arguably higher than concentrations crickets would be exposed to in nature or under mass-rearing conditions during an infection. However, in nature environmental parameters [113] such as temperature [114-116], humidity [116], lower densities and less physical interaction between individuals do not facilitate the spread of infections. Large-scale industrial setups mainly consist of semi- or fully closed spaces where high numbers of individuals, humidity and temperature settings and frequent physical interaction between individuals all create an environment which facilitates the spread of fungal or other entomopathogens [35, 149]. Therefore, even though the tested EPF do not appear to be a major problem, producers should be aware of the threat these can pose. Especially if a prior infection by entomopathogens burdens the immune system of the crickets, secondary infections can be problematic. In addition, prior infections by other pathogens could pave the way for AdDV infections. Also important to take into account is that host range and virulence variation is known to occur for both *B. bassiana* and *M. brunneum* isolates [120]. Virulence of *B. bassiana* for insects from different orders differs significantly [121]. The selected strains to use in combination with different rearing densities were the ones showing the highest negative effect on survival rates of the crickets in the initial screening experiments. Infection by either of these strains decreased the survival of crickets, but there was no interaction with rearing density. Interestingly, in case of the total biomasses obtained, the two stressors showed an

interaction, but this only resulted in decreased biomass production in case of the highest density treatments combined with the exposure to EPFs.

4. AdDV transmission and replication: timing and tissue tropism

AdDV is known for over 50 years now, with the first report in crickets dating back to the 1970s. AdDV can be present in a covert state, i.e. without visible symptoms, but it still can cause diminished feeding and reduced growth of crickets. This can cause losses to cricket producers even without major viral outbreaks. Currently the mechanism and time course of the infection by AdDV is poorly described, mainly limited to observations of the symptoms arising from an overt (lethal) infection [26]. The mode of transmission is a key element in viral infections, because it has a major impact on the spread and prevalence of the virus in the given environment or population [125]. To prevent or limit viral transmission, first the transmission mode has to be identified. The two main routes of viral transmission are vertical and horizontal transmission, and both can occur simultaneously [126]. Vertical transmission occurs when the virus is transmitted from the parental generation to the offspring. Vertical transmission can happen via sperm or via the eggs: when the virus is present on the egg surface it is called transovum transmission, and when it is transmitted within the egg it is called transovarial transmission [127-130]. Horizontal transmission can happen via contaminated feed [36], via cannibalism [131], via feces or during mating (sexual transmission; from female to male or male to female) [132, 133]. So far there was no information available on the transmission and the tissue tropism of AdDV, which is necessary information for designing strategies to limit virus transmission.

In Chapter 3, the first insights into the viral levels over time and into the localisation of AdDV are reported. Tissues selected for testing were the reproductive organs of both genders and the gut tract, collected from mated and unmated individuals. The presence and viral levels in the reproductive organs can help to understand how the virus is vertically transmitted over generations, while the gut tract is where most viruses enter the host, via oral infection with the feed [187] and where viruses can be shed into the feces. Upon maturation, the priority in physiological processes shifts from development to reproduction. This shift also means a change in resource allocation, the immune system might get a lower amount of resources (compared to before maturation) because of re-allocation of resources to the reproductive organs [142]. Mating can also increase susceptibility to diseases as it was shown that both female and male insects allocate a lower amount of resources to immune defenses than the non-mated males [139-142]. In the experiments we found that both female and male house crickets had significantly lower viral levels if they were unmated, when compared to the mated individuals, which is in line with the above-mentioned findings and is suggestive of sexual transmission. Furthermore, AdDV was present in every tested tissue, but at different levels, showing tissue tropism of the virus. In both mated and unmated females, the gut and ovaries showed higher viral levels than the spermatheca of the mated females. The dissected male tissues all had similar viral levels in all tested organs. The shift in resource allocation upon sexual maturation may pave the way for pathogens to increase their abundance in insects; Szelei et al. [26, 157] described AdDV-induced mortality to peak in the older instars. Hormonal changes in the course of development also influence the defense capabilities of insects, since the titers of juvenile hormones decreased [141] and these have an effect on immunity [138, 144]. The results presented in this chapter correlated with these findings, since the viral levels found in the crickets steadily increased throughout cricket development.

5. Widening the perspective: the role that other viruses can play in house cricket epizootics

Insects are considered as a major reserve for viruses [41, 162], which only started to be revealed over the last decade, when the cost of new generation sequencing decreased thereby making this method widely accessible. The ubiquitous occurrence of viruses in insects is supported by the results of the viral screening presented in Chapter 5. Three putative new exogenous RNA viruses were found in the screened colonies, Acheta domesticus virus 2 (AdV2), Acheta domesticus nodavirus 1 (AdNdV1) and Acheta domesticus cypovirus 1 (AdCyp1). However, as only three cricket colonies were screened, extending the search into more colonies and publicly available data will likely lead to more new exogenous viruses. It is important to realize that identification of viruses is only the first step in a long process in the context of disease management in insect rearing facilities. After identification of new, possibly pathogenic, viruses, a next step is to study those viruses in more detail, studying the infection cycle and possible detrimental (or beneficial) effects on the host. One of our newly detected putative viruses, AdV2, is from the family *Iflaviridae*, and viruses from this family have been associated with house crickets before [161]. Members of the *Iflaviridae* often cause asymptomatic infections [200], similarly to AdDV. Asymptomatic viruses pose a threat, since under certain circumstances a covert infection can switch to an overt (lethal) infection. Often the house cricket producers are unaware of the presence of covert virus infections, and upon a viral outbreak, epizootics can develop rapidly [45]. The other two putative new viruses found, AdNdV1 from the *Nodaviridae* family and AdCyp1 from the *Reoviridae* family were not previously associated with crickets. Although viruses from both families are known to infect insects [136, 185], so far no cricket pathogens were described from these families. AdCyp1 is the first cypovirus to infect insects from the order Orthoptera. With the description and annotation of new viruses present in the house cricket, non-destructive detection and screening methods can be developed [46]. The ability to detect viruses is a major step, since this way producers can screen for virus-free crickets either from their own stock or from external sources to establish a virus-free production stock. However, maintaining these stocks to be virus free with influx of people, equipment, air and various feed sources, might prove to be challenging to sustain for longer periods of time. With the knowledge of which viruses can be present in house crickets, experiments can be done to assess how those affect the fitness and immune system of house crickets, for instance which antimicrobial peptides (AMP) they trigger upon infection. Some viruses have been shown to downregulate the gene expression of certain AMPs, and screening for deviations in AMP expression can serve as an early warning signal for producers [201]. Studies also evaluated AMPs, which are often upregulated to combat viral infections. In case an AMP is upregulated against viral infections, they are called antiviral peptides and have been a focus point for antiviral drug research [202]. Monitoring the viral levels in the colonies and the associated AMPs needs to be combined with various preventative measures also to ensure that house cricket colonies are safe from viral outbreaks [35], since preventative treatments are more economically favorable compared to curative treatments, of which few are available for mass-produced insects.

6. What other factors can play a (major) role in disease susceptibility of house crickets?

Optimal development and performance of crickets is highly dependent on various crucial environmental factors as discussed earlier in Chapters 2 and 4. Another important aspect which was not included in the experiments in this thesis, is the feed on which the crickets are reared. The amount and nutritional content of the diet that insects are provided with also plays a vital role [203, 204] both in cricket development and in their resistance against diseases. In nature, insects are exposed to a wide range of feed sources, which carry a highly varying nutritional content, therefore, insects evolved the capability to cope with unbalanced diets successfully. This is achieved by various behavioral and physiological attributes, which altogether

ensure the intake of all the necessary nutrients and calories [33, 205, 206]. Even though these capabilities can ensure survival and reproduction, they might result in longer development, lower body mass or other consequences that are unfavorable from an industrial perspective. In large-scale industrial insect production, producers aim to fulfill all the requirements of the insects produced to ensure good and consistent production. However, for cricket farming the cost of feed can represent half of the OpEx costs [207], thus producers aim to avoid wasting feed and provide only the necessary amount. Beside the right amount, the right nutritional content also has to be delivered via the feed, creating a balanced diet. Unlike for other mass-reared insect species, such as the black soldier fly *Hermetia illucens* (BSF) [65, 208-211] or the yellow mealworm, *Tenebrio molitor* [70], the nutritional needs of the house cricket have not been extensively investigated [32, 33, 61], especially not under mass-rearing conditions. Further research is needed to create nutritious, cost- effective mass-rearing diets for house crickets.

Optimal diets are not just important from the perspective of biomass production, but also a requirement for a healthy population. Access to optimal diets and the proper functioning of the immune system is clearly correlated for insects. The role of the immune system is to prevent the entry, establishment and spread of pathogens and parasites into the host, thereby increasing the likelihood to develop into a reproducing individual [212, 213]. Insects have various immune responses: innate responses are generated by cellular and humoral defense pathways, which together create a highly capable defense system. Upon the entry of the (pathogenic) microbe, the cellular immune responses is activated, which is done either ingestion by hemocytes or encapsulation and nodule formulation in case of larger microbes [214]. The humoral defense starts with the recognition of the pathogen by pattern recognition receptors [214], which is followed by the synthesis of various antimicrobial peptides (AMPs) [215]. When nutritional limitations occur, the immune system can only combat viral infections with reduced efficiency. Infection by a nucleopolyhedrovirus combined with food limitation significantly reduced larval survival, development rate, body mass, fecundity and levels of hemolymph phenoloxidase of *Malacosoma pluviale californicum*, although the number of circulating hemocytes was not affected [72]. Even a short-term deprivation or supplementation can make differences in immune functioning: deprivation of nutrients resulted in down-regulation of the immune system function in *T. molitor* [216] and nutritional supplementation in the diet increased survival rates of *Drosophila melanogaster* when parasitized by the parasitoid *Leptopilina boulardi* [217]. In the case of Mormon crickets, *Anabrus simplex*, nutrient deficiencies were shown to result in lower spontaneous phenoloxidase (PO) activity and also increased susceptibility to *B. bassiana* infections [218]. The immune system of crickets is relatively well described, as they served as model organisms for several immunological studies in the past [64, 213, 219, 220]. The interaction between the host immune system and the virus is a complex process, as the actions by the immune system are counteracted by mechanisms of the virus to escape those [221]. Further research should investigate what immune pathways are activated upon AdDV infections in house crickets. Alternatively, the immune system of crickets could be immune-primed with deactivated viruses to increase resistance to those in later stages, similarly as was done for *Aedes aegypti* mosquitoes [222].

7. Future recommendations

Combining efforts to prevent disease, to develop curative treatment and to select for disease resistance should be the focal point of future research examining diseases of house crickets. The epizootic problems associated with AdDV might be a result of a complex interaction of several factors, leading up to major losses in production facilities. Unfortunately most producers only realize viral problems when visible symptoms of the infection occur, often being too late to prevent an outbreak. To avoid this, the education of insect “veterinarian” professionals is urgently needed. Trained professionals could give education to house cricket producers and screening by reference labs could be made compulsory *via* regulatory measures enforced by local authorities. However, the high costs associated with establishing mass-scale insect farms,

or the opposite, the lack of monetary resources in case of the small-scale farmers, work against application of such solutions. To protect both the industry and the consumers, governmental initiatives, coordination and funding are necessary to establish the bridge between scientific knowledge and the production practice and to create applied research institutes, similar to those that already exist for various other sectors of agriculture.

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References

1. World Population Prospects 2019, United Nations. 2019 [cited 2023. 05.30.]; Available from: Available online at: https://population.un.org/wpp/
2. Scialabba, N., O. Jan, C. Tostivint, A. Turbé, C. O'Connor, P. Lavelle, A. Flammini, J. Hoogeveen, M. Iweins, F. Tubiello, L. Peiser, and C. Batello, Food wastage footprint: Impacts on natural resources. Summary report. 2013.
3. Rotz, C.A., F. Montes, and D.S. Chianese, The carbon footprint of dairy production systems through partial life cycle assessment. Journal of Dairy Science, 2010. **93**(3): p. 1266-82.
4. Nijdam, D., T. Rood, and H. Westhoek, The price of protein: Review of land use and carbon footprints from life cycle assessments of animal food products and their substitutes. Food Policy, 2012. **37**(6): p. 760-770.
5. Knudsen, M.T., A. Meyer-Aurich, J.E. Olesen, N. Chirinda, and J.E. Hermansen, Carbon footprints of crops from organic and conventional arable crop rotations – using a life cycle assessment approach. Journal of Cleaner Production, 2014. **64**: p. 609-618.
6. Rezaei, M. and B. Liu, Food loss and waste in the food supply chain. International Nut and Dried Fruit Council: Reus, Spain, 2017: p. 26-27.
7. Li, B., T.L. Yin, I.A. Udugama, S.L. Dong, W. Yu, Y.F. Huang, and B. Young, Food waste and the embedded phosphorus footprint in China. Journal of Cleaner Production, 2020. **252**: p. 119909.
8. Gjerris, M. and S. Gaiani, Household food waste in Nordic countries: Estimations and ethical implications. Etikk I Praksis, 2013. **7**(1): p. 6-23.
9. Gustavsson, J., C. Cederberg, U. Sonesson, R. Van Otterdijk, and A. Meybeck, Global food losses and food waste. 2011, FAO Rome.
10. Papargyropoulou, E., R. Lozano, J.K. Steinberger, N. Wright, and Z. bin Ujang, The food waste hierarchy as a framework for the management of food surplus and food waste. Journal of Cleaner Production, 2014. **76**: p. 106-115.
11. Holt-Giménez, E., A. Shattuck, M. Altieri, H. Herren, and S. Gliessman, We already grow enough food for 10 billion people … and still can't end hunger. Journal of Sustainable Agriculture, 2012. **36**(6): p. 595-598.
12. Belluco, S., C. Losasso, M. Maggioletti, C.C. Alonzi, M.G. Paoletti, and A. Ricci, Edible insects in a food safety and nutritional perspective: A critical review. Comprehensive Reviews in Food Science and Food Safety, 2013. **12**(3): p. 296-313.
13. Van Huis and J.K. Tomberlin, Insects as food and feed: from production to consumption. 2017, Wageningen, The Netherlands: Wageningen Academic Press.
14. Van Huis, A., Edible insects: Challenges and prospects. Entomological Research, 2022. **52**(4): p. 161-177.
15. Novel Food Regulation, European Union. 2023 [cited 2023 09.07.]; Available from: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32015R2283.
16. Fernandez-Cassi, X., A. Supeanu, M. Vaga, A. Jansson, S. Bogyist, and I. Vagsholm, The house cricket (*Acheta domesticus*) as a novel food: a risk profile. Journal of Insects as Food and Feed, 2019. **5**(2): p. 137-157.
17. Van Huis, Edible crickets, but which species? Journal of Insects as Food and Feed, 2020. **6**(2): p. 91-94.
18. Rumpold, B.A. and O.K. Schluter, Nutritional composition and safety aspects of edible insects. Molecular Nutrition & Food Research, 2013. **57**(5): p. 802-23.
19. Riekkinen, K., K. Vakevainen, and J. Korhonen, The effect of substrate on the nutrient content and fatty acid composition of edible insects. Insects, 2022. **13**(7): p. 590.
20. Collavo, A., R. Glew, Y.S. Huang, L.-T. Chuang, R. Bosse, and M. Paoletti, House cricket smallscale farming, in Ecological implications of minilivestock. Potential of insects, rodents, frogs and snails, M. Paoletti, Editor. 2005, Science Publishers, Enfield N.H.,USA.
21. Zielińska, E., B. Baraniak, M. Karaś, K. Rybczyńska, and A. Jakubczyk, Selected species of edible insects as a source of nutrient composition. Food Research International, 2015. **77**: p. 460-466.
22. Halloran, A., R.C. Megido, J. Oloo, T. Weigel, P. Nsevolo, and F. Francis, Comparative aspects of cricket farming in Thailand, Cambodia, Lao People's Democratic Republic, Democratic Republic of the Congo and Kenya. Journal of Insects as Food and Feed, 2018. **4**(2): p. 101-114.
23. Efsa Panel on Nutrition, N.F., A. Food, D. Turck, T. Bohn, J. Castenmiller, S. De Henauw, K.I. Hirsch-Ernst, A. Maciuk, I. Mangelsdorf, H.J. McArdle, A. Naska, C. Pelaez, K. Pentieva, A. Siani, F. Thies, S. Tsabouri, M. Vinceti, F. Cubadda, T. Frenzel, M. Heinonen, R. Marchelli, M. Neuhauser-Berthold, M. Poulsen, M.P. Maradona, J.R. Schlatter, H. van Loveren, D. Azzollini, and H.K. Knutsen, Safety of partially defatted house cricket (*Acheta domesticus*) powder as a novel food pursuant to Regulation (EU) 2015/2283. EFSA Journal, 2022. **20**(5): p. e07258.

24. Udomsil, N., S. Imsoonthornruksa, C. Gosalawit, and M. Ketudat-Cairns, Nutritional values and functional properties of house cricket (*Acheta domesticus*) and field cricket (*Gryllus bimaculatus*). Food Science and Technology Research, 2019. **25**(4): p. 597-605.
25. Osimani, A., V. Milanovic, F. Cardinali, A. Roncolini, C. Garofalo, F. Clementi, M. Pasquini, M. Mozzon, R. Foligni, N. Raffaelli, F. Zamporlini, and L. Aquilanti, Bread enriched with cricket powder (*Acheta domesticus*): A technological, microbiological and nutritional evaluation. Innovative food science & emerging technologies, 2018. **48**: p. 150-163.
26. Szelei, J., J. Woodring, M.S. Goettel, G. Duke, F.X. Jousset, K.Y. Liu, Z. Zadori, Y. Li, E. Styer, D.G. Boucias, R.G. Kleespies, M. Bergoin, and P. Tijssen, Susceptibility of North-American and European crickets to Acheta domesticus densovirus (AdDNV) and associated epizootics. Journal of Invertebrate Pathology, 2011. **106**(3): p. 394-399.
27. Duffield, K.R., J. Hunt, B.M. Sadd, S.K. Sakaluk, B. Oppert, K. Rosario, R.W. Behle, and J.L. Ramirez, Active and covert infections of cricket iridovirus and Acheta domesticus densovirus in reared *Gryllodes sigillatus* crickets. Front Microbiol, 2021. **12**: p. 780796.
28. Hanboonsong Y, Jamjanya T, and Durst P B, Six-legged livestock : edible insect farming, collection and marketing in Thailand. FAO, Rome, 2013.
29. Halloran, A., Y. Hanboonsong, N. Roos, and S. Bruun, Life cycle assessment of cricket farming in north-eastern Thailand. Journal of Cleaner Production, 2017. **156**: p. 83-94.
30. Kok, R., Preliminary project design for insect production: part 1-overall mass and energy/heat balances. Journal of Insects as Food and Feed, 2021. **7**(5): p. 499-509.
31. Oonincx, D.G., S. van Broekhoven, A. van Huis, and J.J.A. van Loon, Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products. PLoS ONE, 2015. **10**(12): p. e0144601.
32. Morales-Ramos, J.A., M.G. Rojas, A.T. Dossey, and M. Berhow, Self-selection of food ingredients and agricultural by-products by the house cricket, *Acheta domesticus* (Orthoptera: *Gryllidae*): A holistic approach to develop optimized diets. PLoS ONE, 2020. **15**(1): p. e0227400.
33. Bawa, M., S. Songsermpong, C. Kaewtapee, and W. Chanput, Effect of diet on the growth performance, feed Conversion, and nutrient content of the House cricket. Journal of Insect Science, 2020. **20**(2).
34. Eilenberg, J., J.M. Vlak, C. Nielsen-LeRoux, S. Cappellozza, and A.B. Jensen, Diseases in insects produced for food and feed. Journal of Insects as Food and Feed, 2015. **1**(2): p. 87-102.
35. Eilenberg, J. and A.B. Jensen, Prevention and management of diseases in terrestrial invertebrates, in Ecology of Invertebrate Diseases. 2017: Hoboken, New Jersey, USA. p. 495-526.
36. Maciel-Vergara, G. and V.I.D. Ros, Viruses of insects reared for food and feed. Journal of Invertebrate Pathology, 2017. **147**: p. 60-75.
37. de Miranda, J.R., F. Granberg, M. Low, P. Onorati, E. Semberg, A. Jansson, and A. Berggren, Virus diversity and loads in crickets reared for feed: Implications for husbandry. Frontiers in Veterinary Science, 2021. **8**: p. 642085.
38. Maciel-Vergara, G., Diseases and other health issues in cricket rearing. 2020 05 09]; Available from: https://www.insectdoctors.eu/upload_mm/1/5/e/6aca74c9-ce33-4867-a0f8-d140cb34cf50_Cricket%20diagnostics.pdf.
39. Pham, H.T., M. Bergoin, and P. Tijssen, Acheta domesticus Volvovirus, a novel single-stranded circular DNA virus of the house cricket. Genome announcements, 2013. **1**(2): p. e0007913.
40. Pham, H.T., Q. Yu, M. Bergoin, and P. Tijssen, A novel Ambisense densovirus, Acheta domesticus mini Ambidensovirus, from crickets. Genome announcements, 2013. **1**(6): p. e00914-13.
41. Shi, M., X.D. Lin, J.H. Tian, L.J. Chen, X. Chen, C.X. Li, X.C. Qin, J. Li, J.P. Cao, J.S. Eden, J. Buchmann, W. Wang, J. Xu, E.C. Holmes, and Y.Z. Zhang, Redefining the invertebrate RNA virosphere. Nature, 2016. **540**(7634): p. 539-543.
42. Cholleti, H., M. Vaga, A. Jansson, A.L. Blomström, and M. Berg, House crickets (Othroptera: Gryllidae: *Acheta domesticus*) reared in small-scale laboratory conditions harbour limited viral flora. Journal of Insects as Food and Feed, 2022. **8**(10): p. 1149-1155.
43. Liu, K., Y. Li, F.X. Jousset, Z. Zadori, J. Szelei, Q. Yu, H.T. Pham, F. Lepine, M. Bergoin, and P. Tijssen, The Acheta domesticus densovirus, isolated from the European house cricket, has evolved an expression strategy unique among parvoviruses. Journal of Virology, 2011. **85**(19): p. 10069-78.
44. Styer, E.L. and J.J. Hamm, Report of a Densovirus in a commercial cricket operation in the Southeastern United-States. Journal of Invertebrate Pathology, 1991. **58**(2): p. 283-285.
45. Weissman, D.B., D.A. Gray, H. Thi Pham, and P. Tijssen, Billions and billions sold: Pet-feeder crickets (Orthoptera: Gryllidae), commercial cricket farms, an epizootic densovirus, and government regulations make for a potential disaster. Zootaxa, 2012. **3504**: p. 67-88.

46. Semberg, E., J.R. de Miranda, M. Low, A. Jansson, E. Forsgren, and A. Berggren, Diagnostic protocols for the detection of Acheta domesticus densovirus (AdDV) in cricket frass. Journal of Virological Methods, 2019. **264**: p. 61-64.
47. Meynadier, G., G. Matz, J.-C. Veyrunes, and N. Bres. Virose de type densonucleose chez les orthopteres. in Annales de la Société entomologique de France (NS). 1977. Taylor & Francis.
48. Styer, E. and J. Hamm, Report of a densovirus in a commercial cricket operation in the southeastern United States. Journal of Invertebrate Pathology, 1991. **58**(2): p. 283-285.
49. Pham, H.T., H. Iwao, J. Szelei, Y. Li, K. Liu, M. Bergoin, and P. Tijssen, Comparative genomic analysis of Acheta domesticus densovirus isolates from different outbreaks in Europe, North America, and Japan. Genome Announcements, 2013. **1**(4).
50. Tijssen, P. and M. Bergoin. Densonucleosis viruses constitute an increasingly diversified subfamily among the parvoviruses. in Seminars in Virology. 1995. Elsevier.
51. Fédière, G., Epidemiology and pathology of Densovirinae. Contributions to Microbiology, 2000. **4**: p. 1-11.
52. Boomsma, J.J., A.B. Jensen, N.V. Meyling, and J. Eilenberg, Evolutionary interaction networks of insect pathogenic fungi. Annual Review of Entomology, 2014. **59**: p. 467-85.
53. Smagghe, F., R. Spooner-Hart, Z.-H. Chen, and M. Donovan-Mak, Biological control of arthropod pests in protected cropping by employing entomopathogens: Efficiency, production and safety. Biological Control, 2023. **186**: p. 105337.
54. Sinha, K.K., A.K. Choudhary, and P. Kumari, Entomopathogenic fungi, in Ecofriendly pest management for food security, Omkar, Editor. 2016, Academic Press: San Diego. p. 475-505.
55. McNeil Jr, D.G., Fungus fatal to mosquito may aid global war on Malaria. The New York Times, 2005. **10**.
56. Mantzoukas, S., F. Kitsiou, D. Natsiopoulos, and P.A. Eliopoulos, Entomopathogenic fungi: Interactions and applications. Encyclopedia 2022, 2022. **2**(2): p. 646-656.
57. Brunner-Mendoza, C., M.d.R. Reyes-Montes, S. Moonjely, M.J. Bidochka, and C. Toriello, A review on the genus *Metarhizium* as an entomopathogenic microbial biocontrol agent with emphasis on its use and utility in Mexico. Biocontrol Science and Technology, 2018. **29**(1): p. 83-102.
58. Hoffmann, K.H., Environmental Physiology and Biochemistry of Insects. 1985, Springer Berlin Heidelberg: Berlin, Heidelberg.
59. Chown, S.L., S. Chown, and S. Nicolson, Insect Physiological Ecology: Mechanisms and Patterns. 2004, Oxford, United Kingdom: Oxford University Press.
60. Dixon, A.F.G., A. Honek, P. Keil, M.A.A. Kotela, A.L. Sizling, and V. Jarosik, Relationship between the minimum and maximum temperature thresholds for development in insects. Functional Ecology, 2009. **23**(2): p. 257-264.
61. Clifford, C.W. and J.P. Woodring, Methods for rearing the house cricket, *Acheta domesticus* (L.), along with baseline values for feeding rates, growth rates, development times, and blood composition. Journal of Applied Entomology, 1990. **109**(1-5): p. 1-14.
62. Neven, L.G., Physiological responses of insects to heat. Postharvest Biology and Technology, 2000. **21**(1): p. 103-111.
63. Shah, P.N., X. Ruan, J.J.A. van Loon, and M. Dicke, Temperature-modulated host-pathogen interactions between *Hermetia illucens* L. (Diptera: Stratiomyidae) and *Pseudomonas protegens* Pf-5. Journal of Invertebrate Pathology, 2023. **198**: p. 107934.
64. Adamo, S.A., The specificity of behavioral fever in the cricket *Acheta domesticus*. Journal of Parasitology, 1998. **84**(3): p. 529-33.
65. Barragan-Fonseca, K.B., M. Dicke, and J.J.A. van Loon, Influence of larval density and dietary nutrient concentration on performance, body protein, and fat contents of black soldier fly larvae (*Hermetia illucens*). Entomologia Experimentalis et Applicata, 2018. **166**(9): p. 761-770.
66. Deruytter, D., C.L. Coudron, and J. Claeys, The effects of density on the growth and temperature production of *Tenebrio molitor* Larvae. Sustainability, 2022. **14**(10): p. 6234.
67. Tennis, P.S., J.F. Koonce, and M. Teraguchi, The effects of population density and food surface area on body weight of *Acheta domesticus* (L.) (Orthoptera: Gryllidae). Canadian Journal of Zoology, 1977. **55**(12): p. 2004-2010.
68. Mahavidanage, S., T.M. Fuciarelli, X. Li, and C.D. Rollo, The effects of rearing density on growth, survival, and starvation resistance of the house cricket *Acheta domesticus*. Journal of Orthoptera Research, 2023. **32**(1): p. 25-31.
69. Takacs, J., A. Bryon, A.B. Jensen, J.J.A. van Loon, and V.I.D. Ros, Effects of temperature and density on House cricket survival and growth and on the prevalence of Acheta domesticus densovirus. Insects, 2023. **14**(7): p. 588.
70. Harsányi, E., C. Juhász, E. Kovács, L. Huzsvai, R. Pintér, G. Fekete, Z.I. Varga, L. Aleksza, and C. Gyuricza, Evaluation of organic wastes as substrates for rearing *Zophobas morio, Tenebrio molitor,* and *Acheta domesticus* larvae as alternative feed supplements. Insects, 2020. **11**(9): p. 604.
71. Alaux, C., F. Ducloz, D. Crauser, and Y. Le Conte, Diet effects on honeybee immunocompetence. Biology Letters, 2010. **6**(4): p. 562-5.

72. Myers, J.H., J.S. Cory, J.D. Ericsson, and M.L. Tseng, The effect of food limitation on immunity factors and disease resistance in the western tent caterpillar. Oecologia, 2011. **167**(3): p. 647-55.
73. Steinhaus, E.A., Crowding as a Possible Stress Factor in Insect Disease. Ecology, 1958. **39**(3): p. 503-514.
74. Mc Carthy, U., I. Uysal, R. Badia-Melis, S. Mercier, C. O'Donnell, and A. Ktenioudaki, Global food security – Issues, challenges and technological solutions. Trends in Food Science & Technology, 2018. **77**: p. 11-20.
75. Van Huis , A. and D.G.A.B. Oonincx, The environmental sustainability of insects as food and feed. A review. Agronomy for Sustainable Development, 2017. **37**(5): p. 1-14.
76. Smetana, S., A. Mathys, A. Knoch, and V. Heinz, Meat alternatives: life cycle assessment of most known meat substitutes. International Journal of Life Cycle Assessment, 2015. **20**(9): p. 1254-1267.
77. Mariod, A.A., M.E. Saeed Mirghani, and I. Hussein, *Acheta domesticus* House Cricket, in Unconventional oilseeds and oil sources, A.A. Mariod, M.E. Saeed Mirghani, and I. Hussein, Editors. 2017, Elsevier Inc. : Amsterdam, The Netherlands. p. 323-325.
78. Montowska, M., P.L. Kowalczewski, I. Rybicka, and E. Fornal, Nutritional value, protein and peptide composition of edible cricket powders. Food Chem, 2019. **289**: p. 130-138.
79. Kok, R., Preliminary project design for insect production: part 4 – facility considerations. Journal of Insects as Food and Feed, 2021. **0**(0): p. 1-12.
80. Kieruzel, M., The selection of shelter place by the house cricket. Acta Neurobiologiae Experimentalis, 1976. **36**(5): p. 561-80.
81. Kuriwada, T., N. Kumano, K. Shiromoto, and D. Haraguchi, High population density and egg cannibalism reduces the efficiency of mass-rearing in *Euscepes postfasciatus* (Coleoptera: Curculionidae). Florida Entomologist, 2009. **92**(2): p. 221-228, 8.
82. Untergasser, A., I. Cutcutache, T. Koressaar, J. Ye, B.C. Faircloth, M. Remm, and S.G. Rozen, Primer3--new capabilities and interfaces. Nucleic Acids Res, 2012. **40**(15): p. e115.
83. Lee, C., J. Kim, S.G. Shin, and S. Hwang, Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J Biotechnol, 2006. **123**(3): p. 273-80.
84. Hellemans, J., G. Mortier, A. De Paepe, F. Speleman, and J. Vandesompele, qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol, 2007. **8**(2): p. R19.
85. R Core Team, R: A language and environment for statistical computing. R Foundation for Statistical Computing. 2020.
86. Shapiro, S.S. and M.B. Wilk, An analysis of variance test for normality (complete samples). Biometrika, 1965. **52**(3-4): p. 591-611.
87. Bates, D., M. Mächler, B. Bolker, and S. Walker, Fitting linear mixed-effects models using lme4. Journal of Statistical Software, 2015. **67**(1).
88. Burnham, K.P. and D.R. Anderson, Model selection and multimodel inference. 2nd ed. ed. 2004, New York: Springer.
89. Searle, S.R., F.M. Speed, and G.A. Milliken, Population marginal means in the Linear-model - an Alternative to least-squares means. American Statistician, 1980. **34**(4): p. 216-221.
90. Wickham, H., ggplot2: Elegant graphics for data analysis. 2016: Springer International Publishing.
91. Saska, P., W. van der Werf, L. Hemerik, M.L. Luff, T.D. Hatten, A. Honek, and M. Pocock, Temperature effects on pitfall catches of epigeal arthropods: a model and method for bias correction. Journal of Applied Ecology, 2013. **50**(1): p. 181-189.
92. Chia, S.Y., C.M. Tanga, F.M. Khamis, S.A. Mohamed, D. Salifu, S. Sevgan, K.K.M. Fiaboe, S. Niassy, J.J.A. van Loon, M. Dicke, and S. Ekesi, Threshold temperatures and thermal requirements of black soldier fly *Hermetia illucens*: Implications for mass production. PLoS ONE, 2018. **13**(11): p. e0206097.
93. Lazzaro, B.P., H.A. Flores, J.G. Lorigan, and C.P. Yourth, Genotype-by-environment interactions and adaptation to local temperature affect immunity and fecundity in *Drosophila melanogaster*. PLoS Pathogens, 2008. **4**(3): p. e1000025.
94. Richardson, J.M.L. and R.L. Baker, Effect of body size and feeding on fecundity in the damselfly *Ischnura verticalis* (Odonata: Coenagrionidae). Oikos, 1997. **79**(3): p. 477-483.
95. Stahlschmidt, Z.R., N. Rollinson, M. Acker, S.A. Adamo, and L. Martin, Are all eggs created equal? Food availability and the fitness trade-off between reproduction and immunity. Functional Ecology, 2013. **27**(3): p. 800-806.
96. Guo-Ping, K. and G. Xi-Jie, Overview of silkworm pathology in China. African Journal of Biotechnology, 2011. **10**(79): p. 18046-18056.
97. Genersch, E., Honey bee pathology: current threats to honey bees and beekeeping. Applied microbiology and biotechnology, 2010. **87**(1): p. 87-97.

98. Perrin, A., A.-S. Gosselin-Grenet, M. Rossignol, C. Ginibre, B. Scheid, C. Lagneau, F. Chandre, T. Baldet, M. Ogliastro, and J. Bouyer, Variation in the susceptibility of urban *Aedes* mosquitoes infected with a densovirus. Scientific Reports, 2020. **10**(1): p. 18654.
99. Williams, T. and O. Hernandez, Costs of cannibalism in the presence of an iridovirus pathogen of *Spodoptera frugiperda*. Ecological Entomology, 2006. **31**(2): p. 106-113.
100. Grispoldi, L., M. Karama, S. El-Ashram, C.M. Saraiva, J. Garcia-Diez, A. Chalias, S. Barbera, and B.T. Cenci-Goga, Hygienic characteristics and detection of antibiotic resistance genes in crickets (*Acheta domesticus*) breed for flour production. Microbiology Research, 2021. **12**(2): p. 503-512.
101. Maciel-Vergara, G., A.B. Jensen, and J. Eilenberg, Cannibalism as a possible entry toute for opportunistic pathogenic bacteria to insect hosts, exemplified by *Pseudomonas aeruginosa*, a pathogen of the Giant mealworm Z*ophobas morio*. Insects, 2018. **9**(3): p. 88.
102. Wang, Y., A.S. Gosselin Grenet, I. Castelli, G. Cermenati, M. Ravallec, L. Fiandra, S. Debaisieux, C. Multeau, N. Lautredou, T. Dupressoir, Y. Li, M. Casartelli, and M. Ogliastro, Densovirus crosses the insect midgut by transcytosis and disturbs the epithelial barrier function. Journal of Virology, 2013. **87**(22): p. 12380-91.
103. Kortsmit, Y., M. van der Bruggen, B. Wertheim, M. Dicke, L.W. Beukeboom, and J.J.A. van Loon, Behaviour of two fly species reared for livestock feed: optimising production and insect welfare. Journal of Insects as Food and Feed, 2023. **9**(2): p. 149-169.
104. Ye, J.W. and J. Li, Factors affecting cannibalism by *Mallada basalis*. Biocontrol Science and Technology, 2020. **30**(5): p. 442-450.
105. Kakimoto, T., K. Fujisaki, and T. Miyatake, Egg laying preference, larval dispersion, and cannibalism in *Helicoverpa armigera* (Lepidoptera : Noctuidae). Annals of the Entomological Society of America, 2003. **96**(6): p. 793-798.
106. Lee, K.P., S.J. Simpson, and K. Wilson, Dietary protein-quality influences melanization and immune function in an insect. Functional Ecology, 2008. **22**(6): p. 1052-1061.
107. Wilson, J.K., L. Ruiz, and G. Davidowitz, Dietary protein and carbohydrates affect immune function and performance in a specialist herbivore insect (*Manduca sexta*). Physiological and Biochemical Zoology, 2019. **92**(1): p. 58-70.
108. Oonincx, D.G.A.B., J. van Itterbeeck, M.J. Heetkamp, H. van den Brand, J.J.A. van Loon, and A. van Huis, An exploration on greenhouse gas and ammonia production by insect species suitable for animal or human consumption. PLoS ONE, 2010. **5**(12): p. e14445.
109. Van Huis, A., Potential of insects as food and feed in assuring food security. Annual Review of Entomology, 2013. **58**: p. 563-83.
110. Mahieu, L.M., J.J. De Dooy, F.A. Van Laer, H. Jansens, and M.M. Ieven, A prospective study on factors influencing aspergillus spore load in the air during renovation works in a neonatal intensive care unit. Journal of Hospital Infection, 2000. **45**(3): p. 191-7.
111. Augustyniuk-Kram, A. and K. J, Entomopathogenic fungi as an important natural regulator of insect outbreaks in forests (Review), in Forest ecosystems - More than just trees, A.B. Juan and L. Yueh-Hsin, Editors. 2012, IntechOpen: Rijeka. p. 265-294.
112. Faria, M.R.d. and S.P. Wraight, Mycoinsecticides and Mycoacaricides: A comprehensive list with worldwide coverage and international classification of formulation types. Biological Control, 2007. **43**(3): p. 237-256.
113. Seid, A.M., B.L. Fredensborg, B.M. Steinwender, and N.V. Meyling, Temperature-dependent germination, growth and co-infection of *Beauveria* spp. isolates from different climatic regions. Biocontrol Science and Technology, 2019. **29**(5): p. 411-426.
114. Liu, H., D. Abrol, and U. Shankar, Microbial control of crop pests using entomopathogenic fungi. Integrated Pest Management: Principles and Practice. CABI, Jammu, India, 2012: p. 254.
115. Mwamburi, L.A., M.D. Laing, and R.M. Miller, Effect of surfactants and temperature on germination and vegetative growth of *Beauveria bassiana*. Brazilian Journal of Microbiology, 2015. **46**(1): p. 67-74.
116. Acheampong, M.A., C.A. Coombes, S.D. Moore, and M.P. Hill, Temperature tolerance and humidity requirements of select entomopathogenic fungal isolates for future use in citrus IPM programmes. Journal of Invertebrate Pathology, 2020. **174**: p. 107436.
117. Dzepe, D., P. Nana, A. Fotso, T. Tchuinkam, and R. Djouaka, Influence of larval density, substrate moisture content and feedstock ratio on life history traits of black soldier fly larvae. Journal of Insects as Food and Feed, 2020. **6**(2): p. 133-140.
118. Parker, D., N.V. Meyling, and H.H. De Fine Licht, Phenotypic variation and genomic variation in insect virulence traits reveal patterns of intraspecific diversity in a locust-specific fungal pathogen. Journal of Evolutionary Biology, 2023. **36**(10): p. 1438-1454.

119. Therneau, T.M. and P.M. Grambsch, Modeling survival data: extending the Cox model. Statistics for biology and health. 2000, New York: Springer.
120. Shah, P.A. and J.K. Pell, Entomopathogenic fungi as biological control agents. Applied microbiology and biotechnology, 2003. **61**(5-6): p. 413-23.
121. Rohrlich, C., I. Merle, I. Mze Hassani, M. Verger, M. Zuin, S. Besse, I. Robene, S. Nibouche, and L. Costet, Variation in physiological host range in three strains of two species of the entomopathogenic fungus *Beauveria*. PLoS ONE, 2018. **13**(7): p. e0199199.
122. Wakil, W., M.U. Ghazanfar, M. Usman, D. Hunter, and W. Shi, Fungal-based biopesticide formulations to control nymphs and adults of the Desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae): a laboratory and field cage study. Agronomy, 2022. **12**(5): p. 1160.
123. Quesada-Moraga, E., E.A.A. Maranhao, P. Valverde-García, and C. Santiago-Álvarez, Selection of *Beauveria bassiana* isolates for control of the whiteflies *Bemisia tabaci* and *Trialeurodes vaporariorum* on the basis of their virulence, thermal requirements, and toxicogenic activity. Biological Control, 2006. **36**(3): p. 274-287.
124. Penzes, J.J., H.T. Pham, P. Chipman, E.W. Smith, R. McKenna, and P. Tijssen, Bipartite genome and structural organization of the parvovirus Acheta domesticus segmented densovirus. Nature Communications, 2023. **14**(1): p. 3515.
125. Chen, Y., J. Evans, and M. Feldlaufer, Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. Journal of Invertebrate Pathology, 2006. **92**(3): p. 152-9.
126. Cory, J.S., Insect virus transmission: different routes to persistence. Current Opinion in Insect Science, 2015. **8**: p. 130-135.
127. Brito, T.F., V.L. Coelho, M.A. Cardoso, I.A.A. Brito, M.A. Berni, F.L. Zenk, N. Iovino, and A. Pane, Transovarial transmission of a core virome in the Chagas disease vector *Rhodnius prolixus*. PLoS Pathogens, 2021. **17**(8): p. e1009780.
128. Kukan, B., Vertical transmission of nucleopolyhedrovirus in insects. Journal of Invertebrate Pathology, 1999. **74**(2): p. 103-11.
129. Cory, J.S. and J.H. Myers, The ecology and evolution of insect baculoviruses. Annual Review of Ecology, Evolution, and Systematics, 2003. **34**(1): p. 239-272.
130. Akhanaev, Y.B., I.A. Belousova, D.A. Lebedeva, S.V. Pavlushin, and V.V. Martemyanov, A comparison of the vertical transmission of high- and low-virulence nucleopolyhedrovirus strains in *Lymantria dispar* L. Insects, 2020. **11**(7).
131. Huger, A.M., A new virus disease of crickets (Orthoptera: *Gryllidae*) causing macronucleosis of fatbody. Journal of Invertebrate Pathology, 1985. **45**(1): p. 108-111.
132. Knell, R.J. and K.M. Webberley, Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. Biological Reviews of the Cambridge Philosophical Society, 2004. **79**(3): p. 557-81.
133. Werling, K.L., R.M. Johnson, H.C. Metz, and J.L. Rasgon, Sexual transmission of *Anopheles gambiae* densovirus (AgDNV) leads to disseminated infection in mated females. Parasites & Vectors, 2022. **15**(1): p. 218.
134. Altinli, M., J. Soms, M. Ravallec, F. Justy, M. Bonneau, M. Weill, A.S. Gosselin-Grenet, and M. Sicard, Sharing cells with Wolbachia: the transovarian vertical transmission of Culex pipiens densovirus. Environmental Microbiology, 2018. **21**(9): p. 3284-3298.
135. Tijssen, P., J.J. Penzes, Q. Yu, H.T. Pham, and M. Bergoin, Diversity of small, single-stranded DNA viruses of invertebrates and their chaotic evolutionary past. Journal of Invertebrate Pathology, 2016. **140**: p. 83-96.
136. Hernandez-Pelegrin, L., A. Llopis-Gimenez, C.M. Crava, F. Ortego, P. Hernandez-Crespo, V.I.D. Ros, and S. Herrero, Expanding the medfly virome: Viral diversity, prevalence, and sRNA profiling in mass-reared and field-derived medflies. Viruses, 2022. **14**(3): p. 623.
137. Virto, C., C.A. Zarate, M. Lopez-Ferber, R. Murillo, P. Caballero, and T. Williams, Gender-mediated differences in vertical transmission of a nucleopolyhedrovirus. PLoS ONE, 2013. **8**(8): p. e70932.
138. Oku, K., T.A.R. Price, and N. Wedell, Does mating negatively affect female immune defences in insects? Animal Biology, 2019. **69**(1): p. 117-136.
139. Siva-Jothy, M.T., Y. Tsubaki, and R.E. Hooper, Decreased immune response as a proximate cost of copulation and oviposition in a damselfly. Physiological Entomology, 1998. **23**(3): p. 274-277.
140. Rolff, J. and M.T. Siva-Jothy, Copulation corrupts immunity: a mechanism for a cost of mating in insects. Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(15): p. 9916-8.
141. Schwenke, R.A., B.P. Lazzaro, and M.F. Wolfner, Reproduction-immunity trade-offs in insects. Annual Review of Entomology, 2016. **61**: p. 239-256.
142. Bascunan-Garcia, A.P., C. Lara, and A. Cordoba-Aguilar, Immune investment impairs growth, female reproduction and survival in the house cricket, *Acheta domesticus*. Journal of Insect Physiology, 2010. **56**(2): p. 204-11.

143. Short, S.M. and B.P. Lazzaro, Female and male genetic contributions to post-mating immune defence in female *Drosophila melanogaster*. Proceedings of the Royal Society: Biological Sciences, 2010. **277**(1700): p. 3649-57.
144. Schwenke, R.A. and B.P. Lazzaro, Juvenile hormone suppresses resistance to infection in mated female *Drosophila melanogaster*. Current Biology, 2017. **27**(4): p. 596-601.
145. Shoemaker, K.L., N.M. Parsons, and S.A. Adamo, Mating enhances parasite resistance in the cricket *Gryllus texensis*. Animal Behaviour, 2006. **71**(2): p. 371-380.
146. Mao, Q., W. Wu, Z. Liao, J. Li, D. Jia, X. Zhang, Q. Chen, H. Chen, J. Wei, and T. Wei, Viral pathogens hitchhike with insect sperm for paternal transmission. Nature Communications, 2019. **10**(1): p. 955.
147. Pascini, T.V. and G.F. Martins, The insect spermatheca: an overview. Zoology (Jena), 2017. **121**: p. 56-71.
148. Happ, G.M., Structure and Development of Male Accessory Glands in Insects, in Insect Ultrastructure, R.C. King and H. Akai, Editors. 1984, Springer US: Boston, MA. p. 365-396.
149. Eilenberg, J., v.M.M. Oers, A.B. Jensen, A. Lecocq, G. Maciel-Vergara, L.P.A. Santacoloma, J.J.A. van Loon, and H. Hesketh, Towards a coordination of European activities to diagnose and manage insect diseases in production facilities. Journal of Insects as Food and Feed, 2018. **4**(3): p. 157-166.
150. Hahn, T., E. Tafi, A. Paul, R. Salvia, P. Falabella, and S. Zibek, Current state of chitin purification and chitosan production from insects. Journal of Chemical Technology & Biotechnology, 2020. **95**(11): p. 2775-2795.
151. Bosch, G., S. Zhang, D.G.A.B. Oonincx, and W.H. Hendriks, Protein quality of insects as potential ingredients for dog and cat foods. Journal of Nutritional Science, 2014. **3**: p. e29.
152. Barragán-Fonseca, K.B., M. Dicke, and J.J.A. van Loon, Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – a review. Journal of Insects as Food and Feed, 2017. **3**(2): p. 105-120.
153. Gasco, L., I. Biancarosa, and N.S. Liland, From waste to feed: A review of recent knowledge on insects as producers of protein and fat for animal feeds. Current Opinion in Green and Sustainable Chemistry, 2020. **23**: p. 67-79.
154. Li, J., Y. Dong, Y. Sun, Z. Lai, Y. Zhao, P. Liu, Y. Gao, X. Chen, and J. Gu, A Novel Densovirus isolated from the Asian tiger mosquito displays varied pathogenicity depending on its host species. Frontiers in Microbiology, 2019. **10**: p. 1549.
155. Cotmore, S.F., M. Agbandje-McKenna, M. Canuti, J.A. Chiorini, A.M. Eis-Hubinger, J. Hughes, M. Mietzsch, S. Modha, M. Ogliastro, J.J. Penzes, D.J. Pintel, J. Qiu, M. Soderlund-Venermo, P. Tattersall, P. Tijssen, and C. Ictv Report, ICTV Virus taxonomy profile: *Parvoviridae*. Journal of General Virology, 2019. **100**(3): p. 367-368.
156. Armien, A.G., R. Polon, D. Rejmanek, R.B. Moeller, and B.M. Crossley, Outbreak of densovirus with high mortality in a commercial mealworm (*Tenebrio molitor*) farm: A molecular, bright-field, and electron microscopic characterization. Veterinary Pathology, 2023. **60**(5): p. 689-703.
157. Li, Y., Z. Zadori, H. Bando, R. Dubuc, G. Fediere, J. Szelei, and P. Tijssen, Genome organization of the densovirus from *Bombyx mori* (BmDNV-1) and enzyme activity of its capsid. Journal of General Virology, 2001. **82**(Pt 11): p. 2821-2825.
158. Eilenberg, J., S.N. Gasque, and V.I.D. Ros, Natural enemies in insect production systems, in Insects as food and feed : from production to consumption, A. van Huis and J.K. Tomberlin, Editors. 2017, Wageningen Academic Publishers: Wageningen, The Netherlands. p. 201-223.
159. Kerr, C.H., U. Dalwadi, N.E. Scott, C.K. Yip, L.J. Foster, and E. Jan, Transmission of Cricket paralysis virus via exosome-like vesicles during infection of Drosophila cells. Scientific Reports, 2018. **8**(1): p. 17353.
160. Bertola, M. and F. Mutinelli A systematic review on viruses in mass-reared edible insect species. Viruses, 2021. **13**, DOI: 10.3390/v13112280.
161. de Miranda, J.R., F. Granberg, P. Onorati, A. Jansson, and A. Berggren, Virus prospecting in crickets-Discovery and strain divergence of a novel Iflavirus in wild and cultivated *Acheta domesticus*. Viruses, 2021. **13**(3).
162. Li, C.X., M. Shi, J.H. Tian, X.D. Lin, Y.J. Kang, L.J. Chen, X.C. Qin, J. Xu, E.C. Holmes, and Y.Z. Zhang, Unprecedented genomic diversity of RNA viruses in arthropods reveals the ancestry of negative-sense RNA viruses. eLife, 2015. **4**: p. e05378.
163. Andrews, S. FastQC: a quality control tool for high throughput sequence data. 2010 [cited 2023 11.01.]; Available from: http://www.bioinformatics.babraham.ac.uk/projects/fastqc.
164. Chen, S., Y. Zhou, Y. Chen, and J. Gu, fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics, 2018. **34**(17): p. i884-i890.
165. Langmead, B. and S.L. Salzberg, Fast gapped-read alignment with Bowtie 2. Nature Methods, 2012. **9**(4): p. 357-9.
166. Kim, D., J.M. Paggi, C. Park, C. Bennett, and S.L. Salzberg, Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nature Biotechnology, 2019. **37**(8): p. 907-915.
167. Bankevich, A., S. Nurk, D. Antipov, A.A. Gurevich, M. Dvorkin, A.S. Kulikov, V.M. Lesin, S.I. Nikolenko, S. Pham, A.D. Prjibelski, A.V. Pyshkin, A.V. Sirotkin, N. Vyahhi, G. Tesler, M.A. Alekseyev, and P.A. Pevzner, SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biology, 2012. **19**(5): p. 455-77.

168. Guo, J., B. Bolduc, A.A. Zayed, A. Varsani, G. Dominguez-Huerta, T.O. Delmont, A.A. Pratama, M.C. Gazitua, D. Vik, M.B. Sullivan, and S. Roux, VirSorter2: a multi-classifier, expert-guided approach to detect diverse DNA and RNA viruses. Microbiome, 2021. **9**(1): p. 37.
169. Paysan-Lafosse, T., M. Blum, S. Chuguransky, T. Grego, B.L. Pinto, G.A. Salazar, M.L. Bileschi, P. Bork, A. Bridge, L. Colwell, J. Gough, D.H. Haft, I. Letunic, A. Marchler-Bauer, H. Mi, D.A. Natale, C.A. Orengo, A.P. Pandurangan, C. Rivoire, C.J.A. Sigrist, I. Sillitoe, N. Thanki, P.D. Thomas, S.C.E. Tosatto, C.H. Wu, and A. Bateman, InterPro in 2022. Nucleic Acids Research, 2023. **51**(D1): p. D418-D427.
170. Wilkins, D. Gggenes: draw gene arrow maps in 'Ggplot2'. 2023 [cited 2023 01.11.]; Available from: https://CRAN.R-project.org/package=gggenes.
171. Katoh, K., K. Misawa, K. Kuma, and T. Miyata, MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Research, 2002. **30**(14): p. 3059-66.
172. Capella-Gutierrez, S., J.M. Silla-Martinez, and T. Gabaldon, trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics, 2009. **25**(15): p. 1972-3.
173. Minh, B.Q., H.A. Schmidt, O. Chernomor, D. Schrempf, M.D. Woodhams, A. von Haeseler, and R. Lanfear, IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. Molecular Biology and Evolution, 2020. **37**(5): p. 1530-1534.
174. Kalyaanamoorthy, S., B.Q. Minh, T.K.F. Wong, A. von Haeseler, and L.S. Jermiin, ModelFinder: fast model selection for accurate phylogenetic estimates. Nature Methods, 2017. **14**(6): p. 587-589.
175. Letunic, I. and P. Bork, Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Research, 2021. **49**(W1): p. W293-W296.
176. Li, H., B. Handsaker, A. Wysoker, T. Fennell, J. Ruan, N. Homer, G. Marth, G. Abecasis, R. Durbin, and S. Genome Project Data Processing, The sequence alignment/map format and SAMtools. Bioinformatics, 2009. **25**(16): p. 2078-9.
177. Xavier, C.A.D., M.L. Allen, and A.E. Whitfield, Ever-increasing viral diversity associated with the red imported fire ant *Solenopsis invicta* (Formicidae: Hymenoptera). Virology Journal, 2021. **18**(1): p. 5.
178. Ryabov, E.V., Invertebrate RNA virus diversity from a taxonomic point of view. Journal of Invertebrate Pathology, 2017. **147**: p. 37-50.
179. Valles, S.M., Y. Chen, A.E. Firth, D.M.A. Guerin, Y. Hashimoto, S. Herrero, J.R. de Miranda, E. Ryabov, and C. Ictv Report, ICTV Virus taxonomy profile: *Iflaviridae*. Journal of General Virology, 2017. **98**(4): p. 527-528.
180. Patterson, E.I., T.F. Kautz, M.A. Contreras-Gutierrez, H. Guzman, R.B. Tesh, G.L. Hughes, and N.L. Forrester, Negeviruses reduce replication of alphaviruses during coinfection. Journal of Virology, 2021. **95**(14).
181. Shi, M., P. Neville, J. Nicholson, J.S. Eden, A. Imrie, and E.C. Holmes, High-resolution metatranscriptomics reveals the ecological dynamics of mosquito-associated RNA viruses in Western Australia. Journal of Virology, 2017. **91**(17): p. 10.1128/jvi.00680-17.
182. Venter, P.A. and A. Schneemann, Nodaviruses, in Encyclopedia of virology, B.W.J. Mahy and M.H.V. Van Regenmortel, Editors. 2008, Academic Press: Oxford, UK. p. 430-438.
183. King, A.M.Q., M.J. Adams, E.B. Carstens, and E.J. Lefkowitz, Family - Reoviridae, in Virus taxonomy, A.M.Q. King, et al., Editors. 2012, Elsevier: San Diego. p. 541-637.
184. Eberle, K.E., J.T. Wennmann, R.G. Kleespies, and J.A. Jehle, Basic techniques in insect virology, in Manual of techniques in invertebrate pathology, L.A. Lacey, Editor. 2012, Academic Press: San Diego, USA. p. 15-74.
185. Prasad, V. and S. Srivastava, Chapter 13 - Insect Viruses, in Ecofriendly pest management for food security, Omkar, Editor. 2016, Academic Press: San Diego. p. 411-442.
186. Van Oers, M., Genomics and biology of iflaviruses. Insect virology, 2010: p. 231-250.
187. Ros, V.I.D., D. Panziera, R. Nalcacioglu, J.M. Petersen, E. Ryabov, and M.M. van Oers, Viral diseases of insects, in Invertebrate Pathology, A.F. Rowley, C.J. Coates, and M.W. Whitten, Editors. 2022, Oxford University Press. p. 249-285.
188. Franz, A.W., A.M. Kantor, A.L. Passarelli, and R.J. Clem, Tissue varriers to Arbovirus infection in mosquitoes. Viruses, 2015. **7**(7): p. 3741-67.
189. Ma, E., Y. Zhu, Z. Liu, T. Wei, P. Wang, and G. Cheng, Interaction of viruses with the insect intestine. Annual review of virology, 2021. **8**(1): p. 115-131.
190. Yin, C., P. Sun, X. Yu, P. Wang, and G. Cheng, Roles of symbiotic microorganisms in Arboviral infection of arthropod vectors. Trends in Parasitology, 2020. **36**(7): p. 607-615.
191. Wu, P., P. Sun, K. Nie, Y. Zhu, M. Shi, C. Xiao, H. Liu, Q. Liu, T. Zhao, X. Chen, H. Zhou, P. Wang, and G. Cheng, A gut commensal bacterium promotes mosquito permissiveness to Arboviruses. Cell Host Microbe, 2019. **25**(1): p. 101-112 e5.

192. Crippa, M., E. Solazzo, D. Guizzardi, F. Monforti-Ferrario, F.N. Tubiello, and A. Leip, Food systems are responsible for a third of global anthropogenic GHG emissions. Nature Food, 2021. **2**(3): p. 198-209.
193. Gaillac, R. and S. Marbach, The carbon footprint of meat and dairy proteins: A practical perspective to guide low carbon footprint dietary choices. Journal of Cleaner Production, 2021. **321**: p. 128766.
194. Guiné, R.P.F., P. Correia, C. Coelho, and C.A. Costa, The role of edible insects to mitigate challenges for sustainability. Open Agriculture, 2021. **6**(1): p. 24-36.
195. Halloran, A., N. Roos, J. Eilenberg, A. Cerutti, and S. Bruun, Life cycle assessment of edible insects for food protein: a review. Agronomy for Sustainable Development, 2016. **36**(4): p. 57.
196. Lundy, M.E. and M.P. Parrella, Crickets are not a free funch: frotein capture from scalable organic side-streams via high-density populations of *Acheta domesticus*. PLoS ONE, 2015. **10**(4): p. e0118785.
197. Horch, H.W., The cricket as a model organism : development, regeneration, and behavior. 2017, Springer Nature: Tokyo, Japan.
198. Watson, E. Aspire Food Group ramps up world's largest cricket production facility, scouts for 2nd site. 2023 2023.10.17.]; Available from: https://agfundernews.com/aspire-ramps-up-worlds-largest-automated-cricket-production-facility-scouts-for-2nd-site.
199. Booth, D.T. and K. Kiddell, Temperature and the energetics of development in the house cricket (*Acheta domesticus*). Journal of Insect Physiology, 2007. **53**(9): p. 950-3.
200. Parry, R., F. Naccache, E.H. Ndiaye, G. Fall, I. Castelli, R. Luhken, J. Medlock, B. Cull, J.C. Hesson, F. Montarsi, A.B. Failloux, A. Kohl, E. Schnettler, M. Diallo, S. Asgari, I. Dietrich, and S.C. Becker, Identification and RNAi profile of a novel Iflavirus infecting senegalese *Aedes vexans arabiensis* mosquitoes. Viruses, 2020. **12**(4): p. 440.
201. Palmer, W.H., J. Joosten, G.J. Overheul, P.W. Jansen, M. Vermeulen, D.J. Obbard, and R.P. Van Rij, Induction and suppression of NF-kappaB signalling by a DNA virus of *Drosophila*. Journal of Virology, 2019. **93**(3): p. 10.1128/jvi.01443-18.
202. Vilas Boas, L.C.P., M.L. Campos, R.L.A. Berlanda, N. de Carvalho Neves, and O.L. Franco, Antiviral peptides as promising therapeutic drugs. Cellular and Molecular Life Sciences, 2019. **76**(18): p. 3525-3542.
203. Prabhu, V., D. Perez-Staples, and P.W. Taylor, Protein: carbohydrate ratios promoting sexual activity and longevity of male Queensland fruit flies. Journal of Applied Entomology, 2008. **132**(7): p. 575-582.
204. Barragán-Fonseca, K.B., Flies are what they eat : tailoring nutrition of Black Soldier Fly (*Hermetia illucens* L.) for larval biomass production and fitness. 2018, Wageningen University: Wageningen.
205. Behmer, S.T., Insect herbivore nutrient regulation. Annual Review of Entomology, 2009. **54**(1): p. 165-87.
206. Raubenheimer, D. and S.J. Simpson, Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. The Journal of Experimental Biology, 2003. **206**(Pt 10): p. 1669-81.
207. Durst, P.B. and Y. Hanboonsong, Small-scale production of edible insects for enhanced food security and rural livelihoods: experience from Thailand and Lao People's Democratic Republic. Journal of Insects as Food and Feed, 2015. **1**(1): p. 25-31.
208. Chia, S.Y., C.M. Tanga, I.M. Osuga, S.A. Mohamed, F.M. Khamis, D. Salifu, S. Sevgan, K.K.M. Fiaboe, S. Niassy, J.J.A. van Loon, M. Dicke, and S. Ekesi Effects of waste stream combinations from brewing industry on performance of black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). PeerJ 2018 (2018) 11, 2018.
209. Woods, M.J., L.C. Hoffman, and E. Pieterse, Artificial diets for neonatal black soldier fly (*Hermetia illucens*) larvae. Journal of Insects as Food and Feed, 2019. **5**(2): p. 99-105.
210. Diener, S., C. Zurbrugg, and K. Tockner, Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. Waste Management & Research, 2009. **27**(6): p. 603-10.
211. Fischer, H. and N. Romano, Fruit, vegetable, and starch mixtures on the nutritional quality of black soldier fly (*Hermetia illucens*) larvae and resulting frass. Journal of Insects as Food and Feed, 2021. **7**(3): p. 319-327.
212. Rolff, J. and M.T. Siva-Jothy, Invertebrate ecological immunology. Science, 2003. **301**(5632): p. 472-475.
213. Pinera, A.V., H.M. Charles, T.A. Dinh, and K.A. Killian, Maturation of the immune system of the male house cricket, *Acheta domesticus*. Journal of Insect Physiology, 2013. **59**(8): p. 752-60.
214. Satyavathi, V.V., A. Minz, and J. Nagaraju, Nodulation: an unexplored cellular defense mechanism in insects. Cell Signaling Technology, 2014. **26**(8): p. 1753-63.
215. Tanaka, H., J. Ishibashi, K. Fujita, Y. Nakajima, A. Sagisaka, K. Tomimoto, N. Suzuki, M. Yoshiyama, Y. Kaneko, T. Iwasaki, T. Sunagawa, K. Yamaji, A. Asaoka, K. Mita, and M. Yamakawa, A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*. Insect Biochemistry and Molecular Biology, 2008. **38**(12): p. 1087-110.
216. Siva-Jothy, M.T. and J.J.W. Thompson, Short-term nutrient deprivation affects immune function. Physiological Entomology, 2002. **27**(3): p. 206-212.

217. Vass, E. and A.J. Nappi, The effects of dietary yeast on the cellular immune response of *Drosophila melanogaster* against the larval parasitoid, *Leptopilina boulardi*. Journal of Parasitology, 1998. **84**(4): p. 870-2.
218. Srygley, R.B., Diet drives the collective migrations and affects the immunity of mormon crickets and locusts: A comparison of these potential superspreaders of disease. Integrative and Comparative Biology, 2016. **56**(2): p. 268-77.
219. Adamo, S.A., M. Jensen, and M. Younger, Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly G-integer): trade-offs between immunity and reproduction. Animal Behaviour, 2001. **62**(3): p. 417-425.
220. Adamo, S.A., Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. Journal of Insect Physiology, 2004. **50**(2-3): p. 209-16.
221. Feng, M., S. Fei, J. Xia, V. Labropoulou, L. Swevers, and J. Sun, Antimicrobial peptides as potential antiviral factors in insect antiviral immune response. Frontiers in Immunology, 2020. **11**: p. 20-30.
222. Vargas, V., J. Cime-Castillo, and H. Lanz-Mendoza, Immune priming with inactive dengue virus during the larval stage of *Aedes aegypti* protects against the infection in adult mosquitoes. Scientific Reports, 2020. **10**(1): p. 6723.

Summary

The house cricket, *Acheta domesticus*, is a commonly reared insect for food and feed purposes. In 1977, a report described a colony collapse, which was caused by the single-stranded DNA virus, the Acheta domesticus densovirus (AdDV) and ever since, disease outbreaks are a continuous threat to *A. domesticus* mass-rearing. Currently the reasons leading to viral outbreaks are unknown, and in most cases house crickets show no symptoms and only little mortality occurs in the populations. AdDV infection and disease outbreaks in house cricket mass-rearing could be prevented by identifying triggers leading to viral outbreaks and removing or preventing such triggers, by reducing virus spread or by obtaining virus-free crickets. To limit virus spread, a better understanding of how viral levels vary and how the virus is transmitted during the house cricket life cycle is required. The central objective of this thesis was to study the effects of key stressors associated with house cricket rearing that could affect house cricket survival and biomass and/or that could be triggers of AdDV outbreaks. These stressors include cricket density, temperature and exposure to two fungal pathogens. House crickets are usually reared in high densities to maximize cost effectiveness, but this may imply stressful conditions affecting house cricket fitness and creating a highly favourable environment for disease outbreaks. A better understanding of the relation between AdDV levels and these stressors can be used to implement changes in the rearing setups with the aim to reduce the probability of AdDV outbreaks and losses related to this pathogen or other suboptimal conditions. Combined stressors affecting house crickets simultaneously under rearing conditions received little attention so far. In this thesis, several combined stressor experiments were conducted to assess the effects on house cricket survival, biomass and AdDV levels in different tissues.

Correlations between house cricket rearing density or temperature and AdDV abundance have been hypothesized, but experimental evidence is lacking. Optimised rearing conditions, including optimal temperature and house cricket density, are key to cost-effective house cricket production, thus these two key factors were selected (**Chapter 2**). House crickets were subjected to three different temperatures (25, 30, 35 °C) in combination with three different rearing densities (10, 20, 40 crickets per experimental unit). The effects on cricket survival, on male and female individual biomass, on total biomass obtained and on AdDV levels (relative to the starting levels) were recorded and statistically compared. Rearing temperature had a minor effect on cricket survival, which ranged between 80 and 83%. Total cricket biomass increased with higher temperatures and higher densities. Viral abundance in crickets at the end of the rearing period was variable; however, a high rearing density tended to result in a higher AdDV abundance. At 35 °C, a temperature considered suboptimal for house cricket production, viral abundance tended to be lower than at 25 °C or 30 °C. This was the first study to examine the combined effect of these two stressors.

In a next study two other stressors were combined, a microbial pathogen and rearing density (**Chapter 3**). We first compared the virulence of six isolates of *Beauveria bassiana* and six isolates of *Metarhizium brunneum* to house crickets. Subsequently, the most virulent isolate from each genus (*B. bassiana* isolate KVL 97-44 and *M. brunneum* isolate KVL 20-01) were tested in combination with three different cricket densities (10, 20, 40 crickets per experimental unit). The combined effect of these stressors on cricket survival, individual female and male biomass and the total biomass obtained was evaluated. Both the exposure to fungal pathogens and the high rearing density reduced cricket survival and had a negative impact on the total biomass production. The average individual biomass of females and males in case of *B. bassiana* exposure was negatively affected, but no effect of *M. brunneum* exposure was observed. The

increased cricket densities had a minor negative impact on individual biomass. The combined effect of the two stressors resulted in a trend towards a lower individual biomass, but with only a minor effect in the case of *B. bassiana* exposure. Producers need to consider the risk of a higher mortality with higher cricket densities, but the increased losses might be outweighed by the higher total biomass output from the same surface area. Fungal pathogens are a major threat if they appear in the rearing facility, but only if relatively high spore numbers are present, which are less likely to occur when preventive measures and proper sanitation protocols are established. House cricket producers with good hygiene and rearing setups can overcome the threats, but constant monitoring and, if necessary, intervention might be required.

Literature suggest that mortality due to AdDV peaks in the late instars of house crickets, but this has not yet been experimentally examined. In **Chapter 4**, therefore, the changes in viral levels were measured throughout the rearing cycle, using quantitative PCR. Samples were collected 1) simultaneously from different life stages present in the rearing room at a given time and 2) weekly from a single rearing container that was monitored during the successive stages of development. In addition, the tissue tropism of AdDV was examined by determining its presence and levels in different tissues. Viral levels were measured in various tissues from mated and non-mated adult crickets to infer the route of virus transmission. Ovaries of both unmated and mated females and the spermatheca of mated females were collected, while testes and accessory glands were collected from unmated and mated males. Guts were collected from both genders. We found that viral levels are increasing during the life cycle of the house crickets, with older crickets having higher viral levels. Interestingly, for both genders, unmated individuals had significantly lower viral levels than mated individuals. Furthermore, AdDV was present in every tested tissue, but at different levels. In both mated and unmated females, the gut and ovaries showed higher viral levels than the spermatheca of mated females. The dissected male tissues all had similar viral levels. The results presented suggest that AdDV is both horizontally and vertically transmitted among house crickets and provide relevant information for future work on designing optimized preventive measures and on establishing virus-free cricket lines.

Although AdDV is considered the most prevalent virus in house cricket rearing, we cannot ignore other viruses to be potentially present in crickets, and the possible effects that those viruses could have on cricket fitness and on disease outbreaks. Various studies identified viruses present in house cricket colonies, but the majority used a primer-based approach for detecting viruses and were limited to viruses already known to be pathogens of house crickets. With the advancement of next generation sequencing (NGS) technologies, virus detection and discovery can be carried out using an unbiased approach. In **Chapter 5**, DNA and RNA viruses present in house crickets were identified, by analyzing sequencing data from house crickets originating from three different populations. This is the first study to use NGS on pooled samples obtained from various cricket stocks to report both DNA and RNA viruses present. Three putative new RNA viruses are described, which were not yet associated with house crickets before. Two of the new viruses were positive single stranded RNA viruses (+ssRNA) and were identified as the Acheta domesticus virus 2 (AdV2) from the *Iflaviridae* family and the Acheta domesticus nodavirus 1 (AdNdV1) from the *Nodaviridae* family. These viruses were so far not associated with house crickets. The third new virus was a double stranded RNA virus (dsRNA) from the *Reoviridae* family and was identified as Acheta domesticus cypovirus 1 (AdCyp1). As for other DNA viruses, only AdDV was detected and none of the earlier described cricket viruses (Cricket paralysis virus (CrPV) cricket iridovirus (CrIV), the A. domesticus mini ambidensovirus (AdMADV), A. domesticus volvovirus (AdVVV) , A. domesticus iflavirus (AdIV) and the A. domesticus virus (AdV)) were present in either of the tested stocks. Unexpectedly, from the three

tested colonies, only one was infected by AdDV, the other two proved to be free of AdDV. In **Chapter 6**, the findings of the research chapters are integrated. I discuss how cricket rearing is affected by multiple stressors that need to be examined in combination to design optimal cricket rearing strategies. Furthermore, I elaborate on the need for complex diagnostic methods integrating modern technologies and classical bioassays. At the end of the chapter, suggestions for future research are given.

Resumé

Fårekyllingen, *Acheta domesticus*, er et almindeligt opdrættet insekt til fødevare- og fode. Tilbage i 1977 blev den kolonikollaps beskrevet, og det var forårsaget af den enkeltstrengede DNA-virus, Acheta domesticus densovirus (AdDV), og lige siden har sygdomsudbrud været en konstant trussel mod masseopdræt af *A. domesticus.* Årsagerne til virusudbruddene er ukendte, i de fleste tilfælde viser fårekyllingerne ingen symptomer, og der er kun en lille dødelighed i populationerne. AdDV-infektion og sygdomsudbrud i masseopdræt af fårekyllinger kan forhindres ved at identificere de faktorer, der fører til virusudbrud, og fjerne eller forhindre sådanne udløsende faktorer, ved at f.eks. at reducere virusspredningen eller ved at skaffe virusfrie fårekyllinger. For at begrænse virusspredningen er det nødvendigt med en bedre forståelse af, hvordan virusniveauerne varierer og hvordan virussen overføres i løbet af fårekyllingens livscyklus. Det centrale formål med denne afhandling var at undersøge effekten af stressfaktorer i forbindelse med opdræt af fårekyllinger, som kunne påvirke fårekyllingernes overlevelse og biomasse og/eller være udløsende faktorer for udbrud af AdDV. Disse stressfaktorer omfatter tæthed af fårekyllinger, temperatur og eksponering af to insektpatogene svampe. Fårekyllinger opdrættes normalt i høje tætheder for at maksimere omkostningseffektiviteten, men det kan indebære stressende forhold, der påvirker fårekyllingernes fitness og skaber et meget gunstigt miljø for sygdomsudbrud. En bedre forståelse af forholdet mellem AdDV-niveauer og disse stressfaktorer kan bruges til at implementere ændringer i opdrættene med det formål at reducere sandsynligheden for AdDV-udbrud og relateret tab eller andre suboptimale forhold. Flere stressfaktorer kan påvirker fårekyllingerne samtidigt og det har hidtil ikke fået megen opmærksomhed. I denne afhandling blev der udført flere eksperimenter med en kombination af stressfaktorer for at vurdere effekten på fårekyllingernes overlevelse, biomasse og AdDV-niveauer i forskellige væv.

Der er blevet fremsat hypoteser om sammenhænge mellem tætheden af fårekyllinger eller temperaturen og forekomsten af AdDV, men der mangler eksperimentelle beviser. Optimerede opdrætsforhold, herunder optimal temperatur og tæthed af fårekyllinger, er nøglen til en omkostningseffektiv produktion af fårekyllinger, og derfor blev disse to nøglefaktorer valgt (**kapitel 2**). Fårekyllingerne blev udsat for tre forskellige temperaturer (25, 30, 35 °C) i kombination med tre forskellige opdrætstætheder (10, 20, 40 fårekyllinger pr. forsøgsenhed). Virkningerne på fårekyllingernes overlevelse, på hannernes og hunnernes individuelle biomasse, på den samlede biomasse og på AdDV-niveauerne (i forhold til startniveauerne) blev registreret og sammenlignet statistisk. Opdrætstemperaturen havde en mindre effekt på fårekyllingernes overlevelse, som varierede mellem 80 og 83 %. Den samlede fårekyllingebiomasse steg med højere temperaturer og højere tætheder. Virusmængden i fårekyllinger ved slutningen af opdrætsperioden var variabel, men der var en tendens til at høj opdrætstæthed resulterede i en højere AdDV-mængde. Ved 35 °C, en temperatur, der anses for at være suboptimal til produktion af fårekyllinger, var virusmængden generelt lavere end ved 25 °C eller 30 °C. Dette var det første studie, der undersøgte effekten af disse to stressfaktorer i kombination.

I den næste undersøgelse blev to andre stressfaktorer undersøgt, et mikrobielt patogen og opdrætstætheden (**kapitel 3**). Vi sammenlignede først virulensen af seks isolater af *Beauveria bassiana* og seks isolater af *Metarhizium brunneum* på fårekyllinger. Derefter blev det mest virulente isolat fra hver slægt (*B. bassiana*-isolat KVL 97-44 og *M. brunneum*-isolat KVL 20-01) testet i kombination med tre forskellige tætheder af fårekyllinger (10, 20, 40 fårekyllinger pr. forsøgsenhed). Effekt af disse stressfaktorer på fårekyllingernes overlevelse, den individuelle hun- og hanbiomasse og den samlede

biomasse blev evalueret. Både eksponeringen for svampepatogener og den høje opdrætstæthed reducerede fårekyllingernes overlevelse og havde en negativ indvirkning på den samlede biomasseproduktion. Den gennemsnitlige individuelle biomasse af hunner og hanner i tilfælde af *B. bassiana* eksponering blev påvirket negativt, men der blev ikke observeret nogen effekt af *M. brunneum* eksponering. Den øgede tætheder af fårekyllinger havde en mindre negativ indvirkning på den individuelle biomasse. Den kombinerede effekt af de to stressfaktorer resulterede i en tendens til en lavere individuel biomasse, men med kun en mindre effekt i tilfælde af *B. bassiana* eksponering. Producenterne er nødt til at overveje risikoen for en højere dødelighed ved højere tætheder af fårekyllinger, men de øgede tab kan opvejes af det højere samlede biomasseudbytte fra det samme overfladeareal. Svampepatogener er en stor trussel, hvis de forekommer i opdrættene, men kun hvis der er et relativt højt antal sporer til stede. Dette er mindre sandsynligt, da der ofte er etableret forebyggende foranstaltninger og inklusiv sanitetsprotokoller. Fårekyllingeproducenter med god hygiejne kan minimere truslerne, men konstant overvågning og om nødvendigt indgriben kan være påkrævet.

Litteraturen tyder på, at dødeligheden på grund af AdDV topper i de sene stadier hos fårekyllinger, men det er endnu ikke blevet undersøgt eksperimentelt. I **kapitel 4** blev ændringerne i virusniveauerne derfor målt gennem hele opdrætscyklussen ved hjælp af kvantitativ PCR. Prøverne blev indsamlet 1) samtidigt fra forskellige livsstadier, der var til stede i opdrætsrummet på et givet tidspunkt, og 2) ugentligt fra en enkelt opdrætsbeholder, der blev overvåget i løbende henover forskellige successive udviklingsstadier. Derudover blev AdDV's vævstropisme undersøgt ved at bestemme dets tilstedeværelse og niveauer i forskellige væv. Virusniveauer blev målt i forskellige væv fra parrede og ikke-parrede voksne fårekyllinger for at belyse transmission af virusen. Æggestokke fra både uparrede og parrede hunner og spermatheca fra parrede hunner blev indsamlet, mens testikler og accessoriske kirtler blev indsamlet fra uparrede og parrede hanner. Der blev indsamlet tarme fra begge køn. Vi fandt, at virusniveauerne stiger i løbet af fårekyllingernes livscyklus, og at ældre fårekyllinger har højere virusniveauer. Det er interessant, at for begge køn havde uparrede individer signifikant lavere virusniveauer end parrede individer. Desuden var AdDV til stede i alle testede væv, men på forskellige niveauer. Hos både parrede og uparrede hunner viste tarmen og æggestokkene højere virusniveauer end spermatheca hos parrede hunner. De dissekerede væv fra hannerne havde alle lignende virusniveauer. Resultaterne tyder på, at AdDV overføres både horisontalt og vertikalt blandt fårekyllinger, hvilket giver relevant information til fremtidigt arbejde med at designe optimerede forebyggende foranstaltninger og etablere virusfrie fårekyllingelinjer.

Selvom AdDV anses for at være den mest udbredte virus i fårekyllingeopdræt, kan vi ikke ignorere andre vira, der potentielt kan være til stede i fårekyllinger, og de mulige effekter, som disse vira kan have på fårekyllingernes fitness og på sygdomsudbrud. Forskellige studier har identificeret vira i fårekyllingekolonier, men de fleste har brugt en primer-baseret tilgang til at detektere vira og har været begrænset til vira, der allerede er kendt som patogener hos fårekyllinger. Med udviklingen af next generation sequencing-teknologier (NGS) kan detektion og opdagelse af nye virus udføres ved hjælp af en objektiv tilgang. I **kapitel 5** blev DNA- og RNA-virusser i fårekyllinger identificeret ved at analysere sekventeringsdata fra fårekyllinger fra tre forskellige populationer. Dette er den første undersøgelse, der bruger NGS til at undersøge både DNA- og RNA-vira fra forskellige fårekyllingebestande. Tre formodede nye RNA-vira er beskrevet, som endnu ikke har været associeret med fårekyllinger før. To af de nye virusser var positive enkeltstrengede RNA-virusser (+ssRNA) og blev identificeret som Acheta domesticus virus 2 (AdV2) fra Iflaviridae-familien og Acheta domesticus nodavirus 1 (AdNdV1) fra Nodaviridae-familien. Disse vira har hidtil ikke været forbundet med fårekyllinger. Den tredje nye virus var en

dobbeltstrenget RNA-virus (dsRNA) fra Reoviridae-familien og blev identificeret som Acheta domesticus cypovirus 1 (AdCyp1). Hvad angår andre DNA-vira, blev kun AdDV påvist, og ingen af de tidligere beskrevne fårekyllingevira (Cricket paralysis virus (CrPV), cricket iridovirus (CrIV), A. domesticus mini ambidensovirus (AdMADV), A. domesticus volvovirus (AdVVV), A. domesticus iflavirus (AdIV) og A. domesticus virus (AdV)) var til stede i nogen af de testede bestande. Uventet nok var kun én af de tre testede kolonier inficeret med AdDV, mens de to andre viste sig at være fri for AdDV.

I **kapitel 6** integreres resultaterne af forskningskapitlerne. Jeg diskuterer, hvordan opdræt af fårekyllinger påvirkes af flere stressfaktorer, som skal undersøges i kombination for at designe optimale strategier for opdræt af fårekyllinger. Desuden uddyber jeg behovet for komplekse diagnostiske metoder, der integrerer moderne teknologier og klassiske bioassays. I slutningen af kapitlet gives der forslag til fremtidig forskning.

About the author

Jozsef Takacs was born in a small town on the eastern great plain of Hungary. After his graduation from high school, he studied horticulture at the University of Debrecen. Already during his BSc studies, in 2016, he went to Wageningen with the Erasmus+ program, initially for 5 months and now in 2024 he is finishing his “short” stay at Wageningen with obtaining his PhD degree. He started to work with insects and mites during his BSc internship at Wageningen Plant Research. After obtaining his BSc degree, he continued with an MSc in plant pathology and entomology. The European Union Marie Skłodowska-Curie Innovative Training Network ‘Insect Doctors’ gave him the perfect opportunity to further develop his knowledge on diseases and challenges of mass-rearing insects by doing a dual degree joint program at Copenhagen University and Wageningen University. Jozsef continues working in the field of entomology as the R&D manager of a Hungarian black soldier fly mass-rearing company.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review/project proposal (9 ECTS)

- Interaction between pathogens crickets: who paves the way for the other?

Post-graduate courses (17.5 ECTS)

- Concepts and terms in insect pathology and their placement in the general contexts of diseases and health; University of Copenhagen (2020)
- The value and pitfalls of metagenomics in pathogen detection and discovery; INRAE (2021)
- Mixtures and combined stressors: use of multi stressor theory for invertebrate function based and points; CESAM (2021)
- Statistical methods for the biosciences; University of Copenhagen (2021)
- Laboratory methods in insect pathology across organisms; INRAE and CNRS (2021)
- Insects as feed; PE&RC (2022)
- Transdisciplinary training on topics concerning insects as food and feed; INRAE and CNRS (2022)
- BSTS better safe than sorry; WUR (2022)
- Introduction to R and R studio; PE&RC (2022)

Laboratory training and working visits (2 ECTS)

- Insect exposure to bacteria; INRAE (2021)

Invited review of journal manuscripts (2 ECTS)

- Viruses: induction of multiple immune signalling pathways in *Gryllodes sigillatus* crickets during active viral infections (2022)
- Journal Insect as Food and Feed: immunity-related genes can serve as early diagnostic markers for infection in housefly rearing systems (2023)

Competence, skills and career-oriented activities (2.8 ECTS)

- Workshop on the individual research projects and the collaborations within and across WPs; Wageningen University (2020)
- How to write highly cited papers; UK Centre for Ecology and Hydrology (2021)
- Responsible conduct of research; university of Copenhagen (2020)

Discussion groups/local seminars or scientific meetings (6.1 ECTS)

- 34e NEV entomology day (2022)
- Insects doctors and stakeholders symposium (2023)
- Workshop on individual research projects (2020)
- Section of organismal biology seminars; Department of Plant and Environmental Sciences, Copenhagen University (2020, 2021)

International symposia, workshops and conferences (7.3 ECTS)

- AFFIA Conference; oral presentation; online (2021)
- Insects to feed the world; oral presentation; Quebec, Canada (2022)
- 73rd Annual conferences of animal science; oral presentation; Porto, Portugal (2022)
- Society for invertebrate pathology annual conference; oral presentation; Washington, USA (2023)

Lecturing/supervision of practicals/tutorials (3 ECTS)

- Ecological aspects of bio-interactions (2022)
- Insects as food and feed (2023)

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