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THÈSE présentée par :

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Découverte et pathologie de virus associés à la mouche soldat noire (*Hermetia illucens*)

Discovery and pathology of novel viruses associated with the unexplored model, *Hermetia illucens* (black soldier fly)

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El Dr. Salvador Herrero Sendra, Catedràtic del Departament de Genètica de la Facultat de Ciències Biològiques de la Universitat de València y la Dra. Elisabeth A. Herniou, Directora de investigación del Centre National de la Recherche Scientifique (CNRS)

Informen:

Que el Sr. Robert Daniel Pienaar, Graduat en CC. Biològiques per la Rhodes University (República de Sudàfrica), ha realitzat sota la nostra direcció el treball d'investigació recollit en esta tesi doctoral que porta por títol "Discovery and pathology of novel viruses associated with the unexplored model, *Hermetia illucens* (black soldier fly)", per tal d'optar al Grau de Doctor per la Universitat de València.

I per fer constància, d'acord amb la legislació vigent, signa la present a Burjassot, a 12 de juliol de 2024.

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Summary in English

Sustainability has become such a rising concern at the global scale over the last two decades that the United Nations has deconstructed global anthropogenic sustainability into 17 ambitious broad development goals, that it aims to achieve by 2030. Alongside this topic, more people are beginning to understand that insects are valuable and should not only be considered as detrimental pests and disease vectors. It has become more apparent that they could play in helping the world achieve *all* 17 development goals. The insect mass-rearing industry is one area of human-insect interactions that is growing exponentially in this capacity.

The species *Hermetia illucens*, also known colloquially as Black soldier flies or BSF, was recently included in the mass-rearing industry for its multiple applications, such as waste management, pharmaceutics and even for being great sources of nutrients for animal feed. They have become a major player in insect mass-rearing industry and boost the economic growth of this blossoming industry. Black soldier flies are relatively easy to rear, especially since it was able to naturalise broadly on every continent, apart from Antarctica. The insect-mass rearing industry in general is not without its issues. Rearing facilities typically make perfect living conditions for pathogens, which have caused disastrous outbreaks for the mass-rearing industry. The knowledge surrounding pathogens can differ significantly for each reared insect, with a poor/no knowledge for most reared species, and a mountain of knowledge for insects such as honeybees and moths. This is surprising, since many core principles of epidemiology were developed using pathogens in insect colonies. Black soldier flies are one such reared insect where nothing was known about their pathogens, particularly viruses, and was believed to not be very susceptible to pathogens, despite a high exposure given their lifestyle. However, there is a growing body of evidence that BSF are in fact impacted by pathogens, and this is starting to gain attention.

The main goal of this thesis was to investigate the virome of BSF for viruses which may infect them and may have the capacity to be pathogenic to them. By finding such viruses, this thesis could develop a foundational library of viruses which could be studied and to better understand the risks that these may pose for BSF.

The **General Introduction** places the rearing of BSF in the context of global sustainability and how the One Health concept also applies to BSF rearing. Initially the different types of rearing

facilities are introduced to give a scope of the different factors that reared BSF can face and are compared to other farmed animals to outline the risk of disease outbreaks. Stemming from this, insect pathology is introduced and how this links to challenges experienced by the mass-rearing industry due to pathogens. I then reviewed basic virome of dipteran species mass-reared for food and feed, and waste management, showing an incredible diversity of viruses and antiviral immunology. I then summarize the basic concepts and caveats of virus discovery using wet laboratory techniques, next-generation sequencing high-throughput approaches and how paleovirology can help us understand what viruses may have infected an organism before. Finally, I provide the brief history of entomopathogen-related studies in BSF before I started my thesis and detailed aims and objectives of this thesis.

In **Chapter 1**, the results focused on the first study to search BSF for viruses. It was published under title "First Evidence of Past and Present Interactions between Viruses and the Black Soldier Fly, *Hermetia illucens*". Using paleovirological approaches, we screened BSF genomes for endogenized viral elements (EVEs) to uncover and characterise what potential insect-infecting viral families could be found associated to BSF. The results showed that BSF as a species had diverse previous interactions with viruses from *Partitiviridae*, *Parvoviridae*, *Rhabdoviridae*, *Totiviridae* and *Xinmoviridae*. In parallel, we also performed screening of BSF metatranscriptomes to find any viruses related to the EVEs and found that a virus was closely related to the EVEs related to *Totiviridae* (TotiEVEs) and we called this *Hermetia illucens* toti-like virus 1 (HiTV1). We then tried to partially characterise HiTV1 and its relationship with the TotiEVEs, and also to demonstrate that HiTV1 is an exogenous virus that infects BSF.

Since there was a reasonable diversity of EVEs the BSF genome, **Chapter 2** saw an optimized a dual *de novo* virus screening approach to help dive deeper into the BSF virome to perform a deep screen for novel exogenous viruses and develop screening tools for them. It is a chapter written as a scientific article in preparation to be published under the title "Optimization of screening methods leads to the discovery of new viruses in black soldier flies (*Hermetia illucens*)". By using a *de novo* screening pipeline called "PoolingScreen" designed to screen a large collection of metagenomic and then metatranscriptomic datasets simultaneously, we were able to find five additional BSF-associated viruses belonging to the families *Dicistroviridae*, *Iflaviridae*, *Inseviridae*, *Rhabdoviridae* and *Solinviviridae*. As part of the dual *de novo* approach, I also implemented Lazypipe2, a comprehensive virus discovery pipeline to screen the same datasets to determine if there were other viruses that PoolingScreen had missed. While

Lazypipe2 did not find additional viruses, these five novel viruses brought the total number of viruses associated with BSF to eight. For the viruses *Hermetia illucens* cripavirus (HiCV), *Hermetia illucens* iflavirus (HiIfV), *Hermetia illucens* insevirus (HiInV), *Hermetia illucens* lebotiviruses (HiLbV, *Lebotiviridae*, formerly HiTV1), *Hermetia illucens* sigmavirus (HiSgV) and *Hermetia illucens* solinvivirus (HiSvV), we had developed RT-PCR and RT-qPCR protocols that could be used to screen BSF samples for these viruses. We then used read-based mapping to screen all available BSF datasets and combined this with RT-qPCR data and found that there was a wide distribution of most viruses across BSF colonies screened from multiple countries. There were also high coinfection rates with HiInV and HiSgV among 40% of the colony collections and only five out the 25 colony collections screened were free of detectable viruses. Although we screened BSF from rearing facilities and laboratories from countries widely distributed across the Northern Hemisphere, we found that most belonged to the same mitochondrial cytochrome c oxidase subunit I haplotype. This study showed that there is a high diversity and prevalence of exogenous viruses in reared BSF, with at least four being closely related to other dipteran and insect pathogens.

Since multiple exogenous viruses were discovered in BSF, in **Chapter 3**, we sought to isolate and characterise the infection interactions for one of the novel viruses predicted to be pathogenic to BSF, HiSvV (*Solinviviridae*). This chapter was also written in preparation to be published as a scientific article titled “A solinvivirus reduces the lifespan of adult *Hermetia illucens* (black soldier flies)”. *Solinviviridae* are novel group of viruses which are known to be entomopathogens or suspected to be pathogenic to arthropods. While not estimated to be widespread among reared BSF, its was detected in a colony free of other detectable viruses which had reports of increase adult mortality and a reduction in fecundity. In this chapter, I isolated HiSvV and reinfected BSF prepupae using an injection-based inoculation and then successfully inoculated adult flies by orally feeding them a virus inoculum. Additionally, we assessed the ability of HiSvV to transmit between BSF by performing cohabitation experiments between males and females. We found that not only did non-infected individuals have similar levels of HiSvV as their pre-infected cohabitors after 15 days of cohabitation showing horizontal transmission, but also HiSvV was detected in egg clusters from infected parents, showing the capacity for vertical transmission. Survival assays showed shorter lifespans of adults infected with HiSvV. Lastly transcriptomic analysis of BSF showed that BSF did not have an RNA silencing response to HiSvV, but a broader immune response related to transcriptional regulation, autophagy pathways and an upregulation of a defensin and cecropin

during an HiSvV infection. Lastly, there was a sex related response showing that the female immune response was more specific than males to an HiSvV infection and that females could mount a strong RNA silencing response to an infection with HiInV (*Inseviridae*), regardless of a coinfection or HiSvV or HiSgV (*Rhabdoviridae*). These results demonstrate that HiSvV is pathogenic to BSF.

In the **Final Discussion**, I summarise the implications of the general findings by the thesis chapters and tie together how they are important in the context of the BSF rearing industry. I also contrast my results with regards to other pathology studies in BSF. I also tie in how HiSvV infections are related to other *Picornavirales*, but predominantly with other *Solinvivirdiae* and how epidemiology can be used to model the impacts of BSF pathogens like HiSvV and where the future of lies for BSF and pathogens within the BSF mass-rearing industry in the context of One Health. I then highlight the similarities between BSF industry with other large scales animal productions and the lessons that could be learned from the previous mistakes. I conclude with some brief suggestions for viral disease management and prevention to improve the relations between the BSF industry and academia.

Résumé en Français

Au cours des deux dernières décennies, le développement durable est devenu une telle préoccupation à l'échelle mondiale que les Nations Unies l'ont décomposé en dix-sept objectifs de développement ambitieux qu'elles visent à atteindre d'ici 2030. Parallèlement à ce sujet, de plus en plus de gens commencent à comprendre que les insectes sont précieux et ne devraient pas être considérés uniquement comme des nuisibles et des vecteurs de maladies. Il est devenu plus évident qu'ils pourraient jouer un rôle important pour aider le monde à atteindre les 17 objectifs de développement durable. En ce sens, l'industrie de l'élevage intensif d'insectes est un domaine d'interaction homme-insecte qui actuellement connait une croissance exponentielle.

L'espèce *Hermetia illucens*, également connue sous le nom de mouches soldats noires (BSF), a été récemment incluse dans l'industrie de l'élevage intensif pour ses multiples applications, telles que la gestion des déchets, la pharmacie et même comme source importante de nutriments pour l'alimentation animale. Elles sont devenues incontournable pour l'élevage d'insectes et stimulent la croissance économique de cette industrie florissante. Les mouches soldats noires

sont relativement faciles à élever, d'autant plus qu'elles ont pu se naturaliser largement sur tous les continents, sauf l'Antarctique. Cependant, l'élevage d'insectes n'est en général pas sans problèmes. Les installations d'élevage créent en effet des conditions de vie parfaites pour les agents pathogènes, et des épidémies désastreuses ont déjà touché l'industrie de l'élevage d'insectes. Les connaissances sur les agents pathogènes peuvent différer considérablement pour chaque insecte élevé, allant de peu ou pas de connaissances pour la plupart des espèces élevées, à une somme importante de connaissances pour les insectes tels que les abeilles et les papillons. C'est surprenant, car de nombreux principes fondamentaux de l'épidémiologie ont été développés en utilisant des agents pathogènes dans des colonies d'insectes. Les mouches soldats noires sont un de ces insectes pour lesquels aucun agent pathogène n'était connu, en particulier les virus, et que l'on croyait peu sensible aux agents pathogènes, malgré une exposition élevée étant donné leur mode de vie. Cependant, il y a de plus en plus d'indices que les BSF sont en fait impactées par des agents pathogènes, et cela commence à attirer l'attention.

L'objectif principal de cette thèse était donc d'étudier le virome des BSF pour détecter des virus susceptibles de les infecter et de déterminer leur potentiel pathogène. La découverte de tels virus au cours de cette thèse permettrait de développer une première liste de virus à étudier afin de mieux comprendre les risques qu'ils pourraient poser pour les BSF.

Introduction Générale L'introduction situe l'élevage des BSF dans le contexte du développement durable mondial et explique comment le concept One Health s'applique également à l'élevage des BSF. Initialement, les différents types d'installations d'élevage sont présentés pour donner une idée des différents facteurs auxquels les BSF d'élevage peuvent être confrontées et sont comparés à d'autres animaux de rente pour souligner le risque d'épidémies. Ensuite, la pathologie des insectes est introduite ainsi que les défis rencontrés par l'industrie de l'élevage massif à cause des agents pathogènes. J'ai de plus passé en revue le virome de base des espèces de diptères élevées en masse pour l'alimentation et la gestion des déchets, montrant une incroyable diversité de virus et de réponse antivirale. Ensuite, je décris les concepts de base et les mises en garde de la découverte de virus en utilisant des techniques de laboratoire, des approches de séquençage de nouvelle génération à haut débit et comment la paléovirologie peut nous aider à comprendre quels virus ont pu infecter un organisme par le passé. Enfin, je fournis un bref historique des études liées aux entomopathogènes chez les BSF et finalement détaille les les objectifs de ma thèse.

Chapitre 1 Dans ce chapitre, les résultats portent sur la première étude de virus chez les BSF. Elle a été publiée sous le titre "First Evidence of Past and Present Interactions between Viruses and the Black Soldier Fly, *Hermetia illucens*". En utilisant des approches paléovirologiques, nous avons examiné les génomes des BSF à la recherche d'éléments viraux endogénisés (EVE) pour découvrir et caractériser quelles familles virales potentiellement infectieuses pour les insectes pouvaient être associées aux BSF. Les résultats ont montré que les BSF en tant qu'espèce avaient eu diverses interactions antérieures avec des virus des familles *Partitiviridae*, *Parvoviridae, Rhabdoviridae, Totiviridae* et *Xinmoviridae*. En parallèle, nous avons également effectué un criblage des métatranscriptomes des BSF pour trouver des virus apparentés aux EVE et avons découvert un virus était proche des EVE associés aux *Totiviridae* (TotiEVE) et nous l'avons appelé Hermetia illucens toti-like virus 1 (HiTV1). Nous avons ensuite caractérisé HiTV1 et sa relation avec les TotiEVE, et aussi de démontrer que HiTV1 est un virus exogène qui infecte les BSF.

Chapitre 2 Étant donné qu'il y avait une diversité importante d'EVE dans le génome des BSF, le Chapitre 2 a vu l'optimisation d'une approche bioinformatique de double criblage de novo de virus pour explorer plus en profondeur le virome des BSF et pour approfondir la recherche de nouveaux virus exogènes. C'est un chapitre écrit sous la forme d'un article scientifique pour être publié sous le titre "Optimization of screening methods leads to the discovery of new viruses in black soldier flies (Hermetia illucens)". En utilisant un pipeline de criblage de novo appelé "PoolingScreen" conçu pour cribler simultanément une grande collection de jeux de données métagénomiques puis métatranscriptomiques, nous avons pu trouver cinq virus supplémentaires associés aux BSF appartenant aux familles *Dicistroviridae, Iflaviridae, Inseviridae, Rhabdoviridae* et *Solinviviridae*. Dans le cadre de la double approche de novo, j'ai également mis en œuvre Lazypipe2, un pipeline complet de découverte de virus pour cribler les mêmes jeux de données afin de déterminer s'il y avait d'autres virus que PoolingScreen aurait manqués. Bien que Lazypipe2 n'ait pas trouvé de virus supplémentaires, ces cinq nouveaux virus ont porté le nombre total de virus associés aux BSF à huit. Pour les virus Hermetia illucens cripavirus (HiCV), Hermetia illucens iflavirus (HiIfV), Hermetia illucens insevirus (HiInV), Hermetia illucens lebotivirus (HiLbV, *Lebotiviridae*, anciennement HiTV1), Hermetia illucens sigmavirus (HiSgV) et Hermetia illucens solinvivirus (HiSvV), nous avons développé des protocoles RT-PCR et RT-qPCR qui pourraient être utilisés pour cribler des échantillons de BSF à la recherche de ces virus. Nous avons ensuite utilisé la cartographie basée sur les lectures pour cribler tous les jeux de données disponibles de BSF et

avons combiné cela avec les données RT-qPCR et avons trouvé qu'il y avait une large distribution de la plupart des virus dans les colonies de BSF testées dans plusieurs pays. Il y avait également des taux élevés de coinfection avec HiInV et HiSgV dans 40 % des collections de colonies et seulement cinq des 25 collections de colonies criblées étaient exemptes de virus détectables. Bien que nous ayons criblé des BSF provenant d'installations d'élevage et de laboratoires de pays largement répartis dans l'hémisphère nord, nous avons trouvé que la plupart appartenaient au même haplotype de la sous-unité I de la cytochrome c oxydase mitochondriale. Cette étude a montré qu'il y a une grande diversité et prévalence de virus exogènes chez les BSF élevées, avec au moins quatre étroitement liés à d'autres agents pathogènes de diptères et d'insectes.

Chapitre 3 Étant donné que plusieurs virus exogènes ont été découverts chez les BSF, dans le Chapitre 3, nous avons cherché à isoler et à caractériser les interactions d'infection pour un des nouveaux virus potentiellement pathogène pour les BSF, HiSvV (*Solinviviridae*). Ce chapitre a également été rédigé pour être publié en tant qu'article scientifique intitulé "A solinvivirus reduces the lifespan of adult Hermetia illucens (black soldier flies)". Les *Solinviviridae* sont un nouveau groupe de virus connus pour être des entomopathogènes ou soupçonnés d'être pathogènes pour les arthropodes. Bien qu'il ne soit pas estimé être largement répandu parmi les BSF élevées, il a été détecté dans une colonie libre d'autres virus détectables mais qui avait signalé une augmentation de la mortalité des adultes et une réduction de la fécondité. Dans ce chapitre, j'ai isolé HiSvV et réinfecté des prénymphes de BSF en utilisant une inoculation par injection, puis inoculé avec succès des mouches adultes en leur donnant un inoculum viral par voie orale. De plus, nous avons évalué la capacité de HiSvV à se transmettre entre les BSF en réalisant des expériences de cohabitation entre mâles et femelles. Nous avons constaté que non seulement les individus non infectés avaient des niveaux similaires de HiSvV que leurs cohabitants pré-infectés après 15 jours de cohabitation, montrant une transmission horizontale, mais aussi que HiSvV était détecté dans les masses d'œufs des parents infectés, montrant la capacité de transmission verticale. Les essais de survie ont montré des durées de vie plus courtes des adultes infectés par HiSvV. Enfin, l'analyse transcriptomique des BSF a montré que les BSF n'avaient pas de réponse de silençage de l'ARN à HiSvV, mais une réponse immunitaire plus large liée à la régulation transcriptionnelle, aux voies de l'autophagie et une régulation positive d'une défensine et d'une cécropine pendant une infection par HiSvV. Enfin, il y avait une réponse liée au sexe montrant que la réponse immunitaire des femelles était plus spécifique que celle des mâles à une infection par HiSvV et que les femelles étaient capables

de monter une forte réponse de silençage de l'ARN à une infection par HiInV (*Inseviridae*), indépendamment d'une coinfection par HiSvV ou HiSgV (*Rhabdoviridae*). Ces résultats démontrent que HiSvV est pathogène pour les BSF.

Discussion Finale Dans la discussion finale, je fait la synthèse des résultats experimentaux issus des chapitres de la thèse et lie leur importance dans le contexte de l'industrie de l'élevage des BSF. Je compare également mes résultats à d'autres études de pathologie chez les BSF. Je discute également les infections par HiSvV au regard d'autres *Picornavirales*, et *Solinviviridae*, et comment l'épidémiologie peut être utilisée pour modéliser les impacts des agents pathogènes des BSF comme HiSvV et où se situe l'avenir pour les BSF et les agents pathogènes dans l'industrie de l'élevage massif des BSF dans le contexte de One Health. Ensuite, je souligne les similitudes entre l'industrie des BSF et d'autres productions animales intensives et les leçons qui pourraient être tirées des erreurs précédentes. Je conclue par quelques suggestions brèves pour la gestion et la prévention des maladies virales afin d'améliorer les relations entre l'industrie des BSF et le milieu académique.

General introduction: Why investigate viruses in black soldier flies?

Ubuntu – "I am because we are" is not just an African mantra from the perspective of human interactions, but also simplifies a fundamental aspect of biology, ecology and sustainability because everything is connected to something

1. Where mass-reared insects fit into One Health, a global initiative.

1.1. What role do black soldier flies play in global sustainability?

The idea of global sustainability has garnered more support over the years, particularly within the scope of the United Nations (UN). The UN have assembled a plan outlining 17 umbrella sustainability development goals (SDGs) to be achieved by 2030 to promote sustainability within a global context (Dangles and Casas, 2019). This is monumental in scale but some important players, such as insects, have only being highlighted recently (Figures GI.1 and GI.2). The role of insects, from the human perspective, can be placed in three dynamic categories, "enemies", "allies" and "solutions", usually gaining attention as enemies.

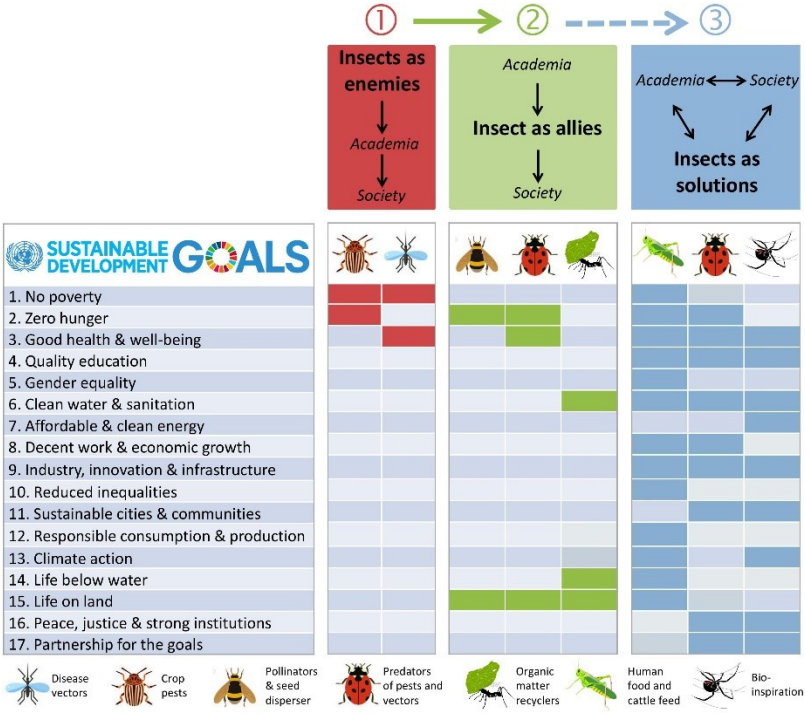


Figure GI.1 Progression of insect and arthropod relations with humanity and how the progression can be aligned with the UN's sustainability goals (From Figure 1 in Dangles and Casas, (2019)).

In a drive to promote the value of insects in global sustainability, Dangles and Casas, (2019) pinpointed that insects can actually be involved in all 17 SDGs, and that they could be useful tools to help progress these goals. This reinforces the inclusive concept behind *Ubuntu* and similar philosophies, which are being increasingly embraced by global entities such as the UN

and the World Health Organization (Graness, 2015; United Nations, 2015; Mwipikeni, 2018; Ewuoso and Hall, 2019; Walls and Vogel, 2023).

Black soldier flies (Diptera, Stratiomyidae, *Hermetia illucens* L. 1758) have been suggested to be a potential player in the global initiative to reach the UN sustainability goals (Figures GI.1 and GI.2B). Dangles and Casas, (2019) suggested that BSF would fit within goal number 12 which aims to promote consumption at sustainable levels and sustainable means of production. Yet, BSF farming could play roles in at least 10 of the 17 goals (Figures GI.1 and GI.2B), such as goals surrounding health, education, equality and sanitation (2 to 6, 10), Industry and sustainable communes (9 and 11) and even in attempts towards peace in some regions (16) (Barragán-Fonseca *et al.*, 2020; Surendra *et al.*, 2020; Tanga *et al.*, 2021; van Huis *et al.*, 2021; Tettamanti and Bruno, 2024). BSF are one of the most intensely studied insects in terms of bioconversion, however research into the use of BSF has grown into many domains (Shelomi, 2020; Tettamanti and Bruno, 2024).

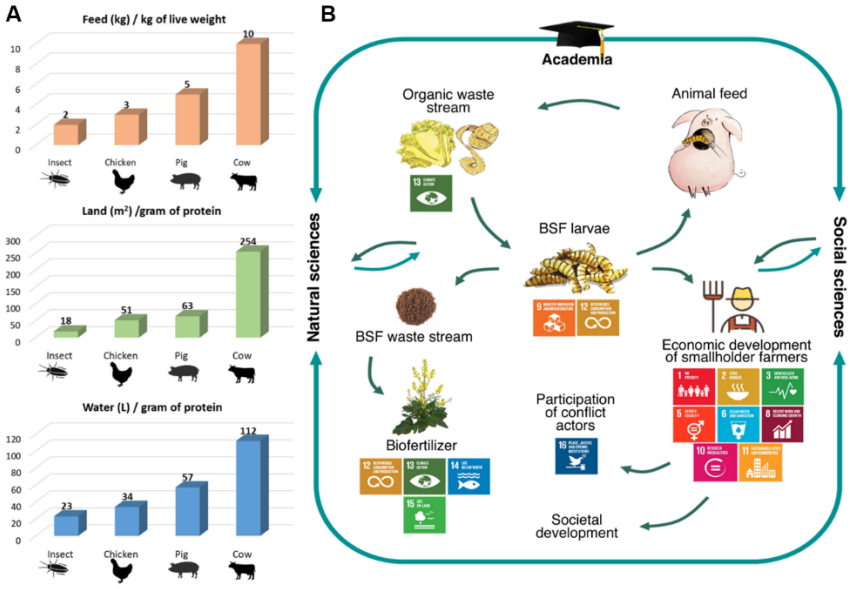


Figure GI.2 Putting the farming of beneficial insects into context of sustainability. A) Comparison of production requirements for edible farmed animals (Adapted from Figure 2 in Guiné *et al.*, (2021)). B) The potential roles of BSF in production and everyday life as part of the Insects for Peace initiative (From Figure 2 in (Barragán-Fonseca *et al.*, 2020)).

Although BSF were first described by Carl Linnaeus in 1758, records show that research focusing directly on BSF or their use has been published as early as 1916. Before 2000, only 35 academic publications were found where "Hermetia illucens" included, "Black soldier fly" only and "Black soldier flies" only were in the titles. Research into black soldier flies really started to take off around 2008 where the number of publications had reached 71. Using Google

Scholar and PubMed search tools, academic publications which included the term "Black soldier fly" in their title yielded as many as 1703 and 113 respectively (Figure GI.3). This increased drastically for both search tools when "*Hermetia illucens*" was also included in the title of the publications with PubMed search obtaining 886 and Google Scholar 3006 with majority being published from 2019. While not depicted in Figure GI.3, the search term "Black soldier flies" in the titles yielded 46 publications by 2024. All in all, this brings the total number of publications, predominantly in English, that include the search terms found using PubMed and Google Scholar to at least 1006 and 4755 by 28th March 2024, respectively.

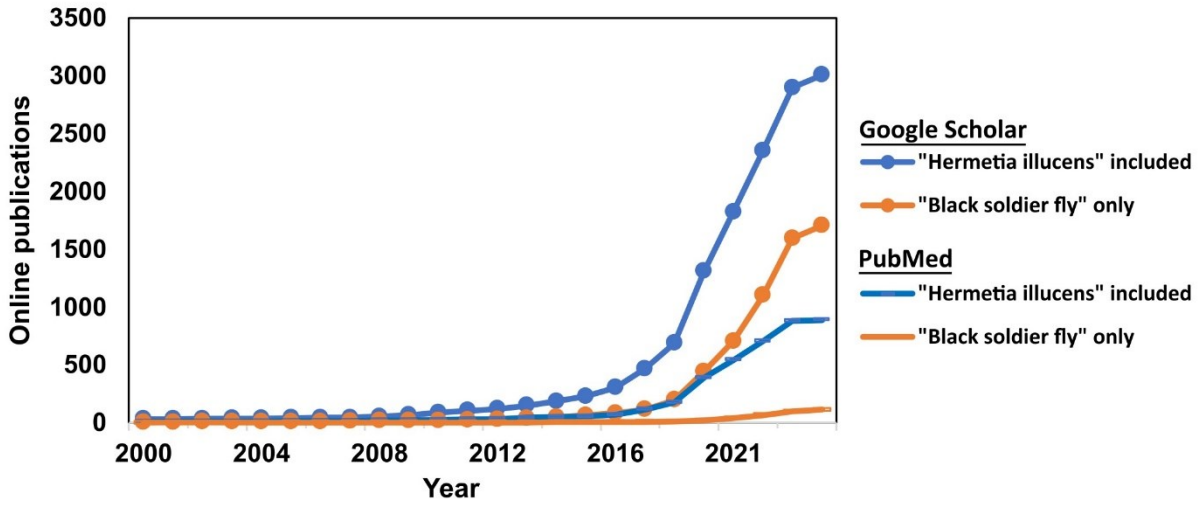


Figure GI.3 Accumulation of scientific publications found using PubMed and Google Scholar between 2000 and 2024 focusing on BSF. Three different searches were used, one with the inclusion of 1) "*Hermetia illucens*", 2) only with the phrase "black soldier fly", and 3) only with the phrase "black soldier flies". For Google Scholar, the search mode was set to not include citations and only search the titles of publications.

Within the last few years, the farming of BSF has spread across the globe with commercial and subsistence farming appearing in Africa, Asia, Europe, North and South America and Oceania. Between 2013 and today, at least 100 countries have or have had some form of BSF rearing (Figure GI.4). It is difficult to know the full scope of BSF farming around the globe since not every person/group rearing BSF may post their rearing experience online, this is something that was reiterated particularly about BSF farming companies in Africa by Tanga and Kababu, (2023). However, while there is an emphasis on established companies, hobbyists should not be forgotten since they can also play a role in awareness and citizen science, particularly when promoting sustainability (Dangles and Casas, 2019; Barragán-Fonseca *et al.*, 2020). While it is impressive that the use of BSF has spread so widely, it is not surprising given that its

documented distribution already spans all continents except Antarctica (Kaya *et al.*, 2021; Inventaire National du Patrimoine Naturel, https://inpn.mnhn.fr/; 31st March 2024).

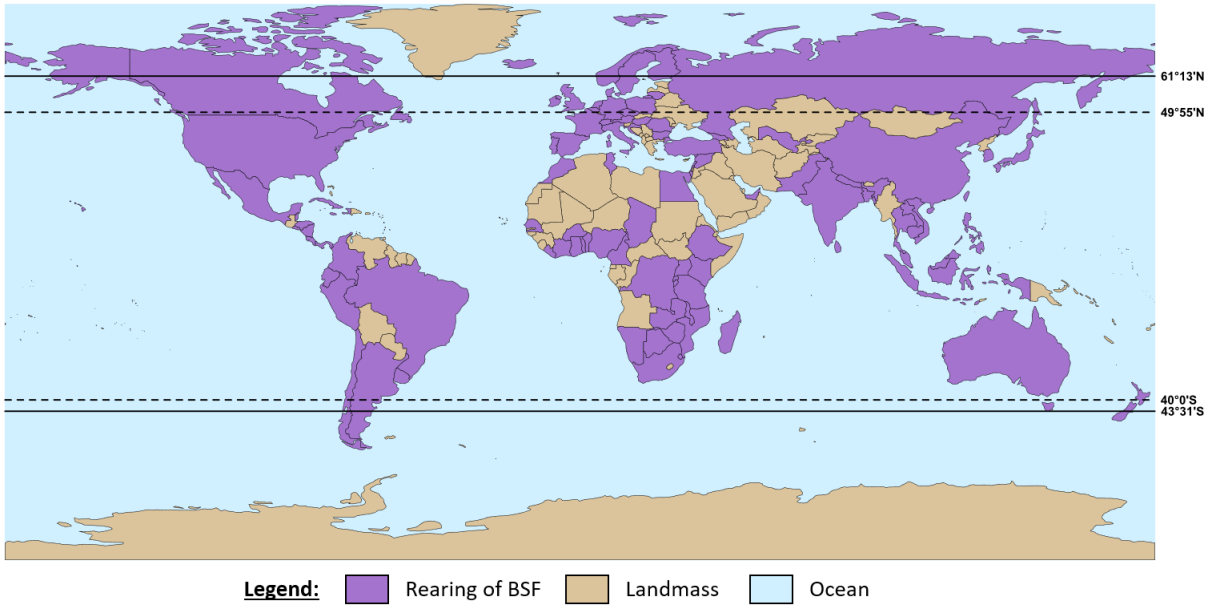


Figure GI.4 Countries highlighted around the world where black soldier fly rearing has been observed searching facebook, Twitter, press releases, scientific articles, commercial rearing facility websites and YouTube in English. For BSF observed in the wild, dashed lines indicate the most northern and southern records of BSF according to Sheppard *et al.*, (1994) and Roháček and Hora, (2013). Solid lines show an updated latitudinal distribution of naturally observed BSF as of March 2024 (https://inpn.mnhn.fr/espece/cd_nom/217341/tab/carte, accessed online : 31st March 2024). Map projection: WGS84.

On the topic of where BSF are being reared, there has been a drastic increase of BSF rearing worldwide (Tomberlin and Huis, 2020), but there has also been an increase in reports of BSF observed in the outdoors. Although already reported widely within a latitudinal belt approximately between 40°S and 45°N in 1994, by 2013, BSF were reported in as far north as 49°55'N (Figure GI.4). In 2015, Marshall *et al.*, (2015) discussed the first record of BSF in Canada being close the Canadian border next to the city of Detroit (USA) in 2007, since then, multiple sightings with southern Canada have been recorded. This was of importance since Canada was looking into the investing in commercial BSF mass-rearing, since it was already being done in Costa Rica, Europe, South Africa and the USA. Naturally, determining where BSF were already naturalised becomes a focus due to the concern of accidental spill-over from farming colonies to the ecosystems, given that BSF have been able to establish so widely (Marshall *et al.*, 2015; Berggren *et al.*, 2019; Maquart, Richard, *et al.*, 2020; Shelomi, 2020). Fast forwarding to 2024, BSF now have been reported as far north as Anchorage (Alaska, USA, 61°13'N) and as far south as Christchurch (New Zealand, 43°31'S) (Figure GI.4). Conservation

and biosecurity are a global concern, even in regions with relatively little human interaction (Greve *et al.*, 2017). However, the overall theme of conservation is not the focus of this story. The natural distribution of BSF in relation to where they are reared prompted us to have a more specific area of concern, the potential crossover of BSF-specific pathogens, parasites and pests. To further understand why, we need to look at different broader types of facilities used for BSF farming.

When it comes to the location of BSF rearing, whether large-scale or small-scale, the rearing system can be simple open compost heaps in someone's garden, to gigantic high-tech closed facilities such as one developed by Innovafeed in France. Sheppard *et al.*, (2002) found little documentation on artificial rearing techniques surrounding BSF and effectively produced the first artificial rearing protocol. Since 2002 there has been an explosion of scientific articles (Nakamura *et al.*, 2016; Meneguz *et al.*, 2018; Miranda *et al.*, 2020; Van *et al.*, 2022), books (Caruso *et al.*, 2014; Dortmans *et al.*, 2017) as well as general websites and blogs across the internet. There is such a large diversity of rearing approaches and experimental setups (Nayak *et al.*, 2024; Wiklicky *et al.*, 2024), that detailing all the different types of installations from compost heaps to mass-rearing facilities is beyond the scope of this introduction but some of the main features are described below.

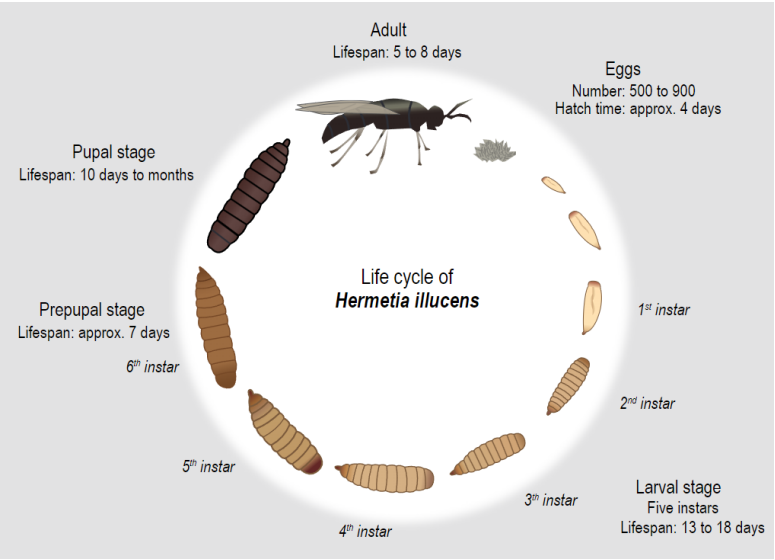


Figure GI.5 Stages of BSF development (From Figure 1 in Lievens *et al.*, (2021), modified from Figure 1 in (De Smet *et al.*, 2018)).

Firstly, there is the life cycle of BSF (Figure GI.5). There are four main stages, 1) Eggs, 2) Larvae, 3) pupae and 4) Adults. The eggs are separated from adult enclosures, left to hatch,

and then placed on seeding substrate. Following, at varying larval ages, they are placed onto a larger substrate batch until pupation. Pupae are often then either filtered out, migrate out of the diet substrate into different collection trays or left to pupate fully in the same substrate. The adults are usually placed in separate reproduction cages from which eggs are collected (Sheppard *et al.*, 2002; Caruso *et al.*, 2014; Nakamura *et al.*, 2016; Jones and Tomberlin, 2020, p. 202; Heussler *et al.*, 2023; Manas *et al.*, 2024). Overall, the full cycle can take around 38 to 50 days, even longer given the possible pupation times (Figure GI.5). Like with other insect rearing, there are three overall types of BSF rearing facilities: open, semi-open and closed (Figure GI.6) (Eilenberg and Jensen, 2018). Each facility type has its pros and cons, but ultimately all three can be utilized for the rearing of BSF depending on the climate where the facility is located.

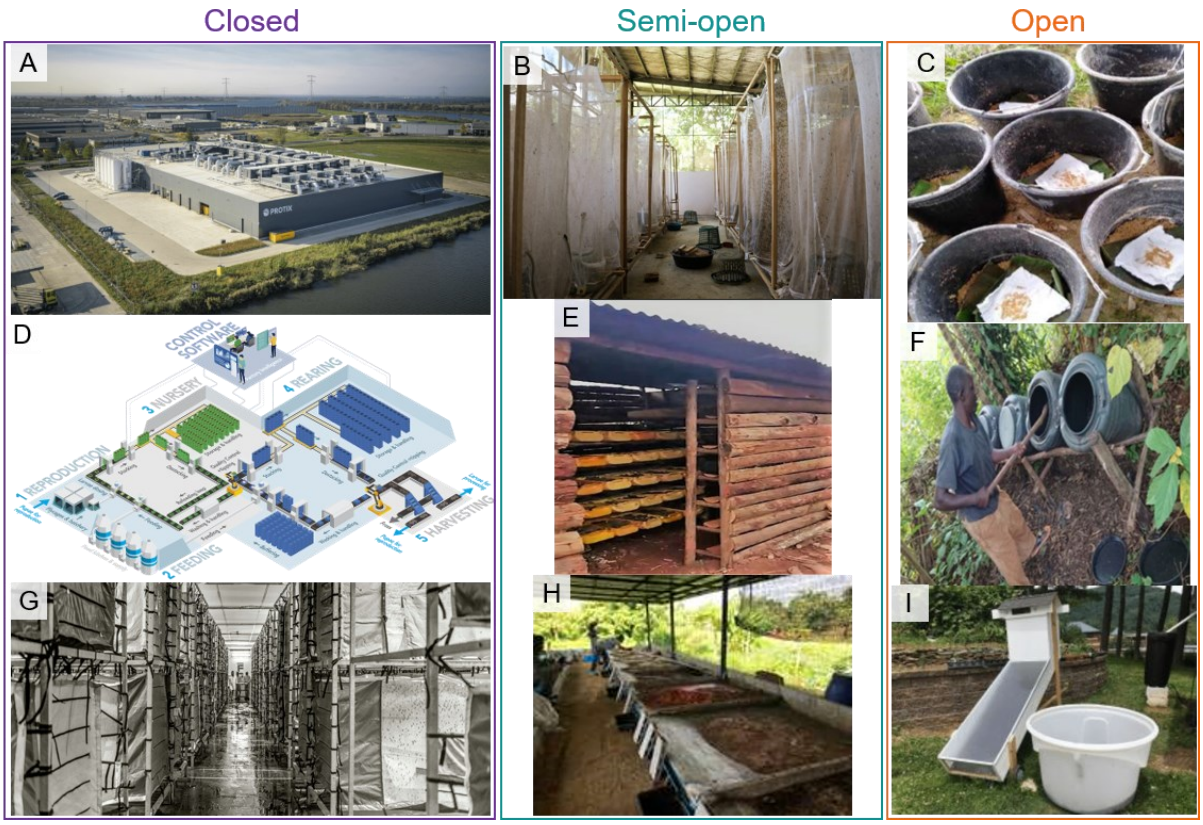


Figure GI.6 General types of BSF rearing facilities independent of scaling. A, D and G are closed facility designs almost completely isolating BSF from the outside. B, E and H show semi-open farming and C, F and I present open style farming. Images A, B, D, G, H and I were obtained from *Insect protein supplier raises €50m for expansion*, 2022, Protix; *How fly larvae can help the Philippines solve landfill, food waste issues*, 2022; *Insect Farming Technology (Black Soldier Fly, Mealworm)*, no date; *Upcycler AgriProtein set to fly with 20 farms in U.S. and Canada*, 2017; Figure 10 in Chalermliamthong *et al.*, 2023 and Figure 2 in Fanatico *et al.*, 2018. C was adapted from Appendix 4 in Rahman, 2022 and E and F from Figure 1 in Abro *et al.*, 2022.

Closed rearing facilities (Figure GI.6A,D & G) are more often large-scale due to cost effectiveness, usually they are climate controlled, have solid barriers and stricter access

(Eilenberg and Jensen, 2018). This can reduce the introduction of commensal species or pathogens/parasites and protects BSF and staff from external weather conditions. When they have zoned layouts, they can also reduce the spread of infectious material or individuals between zones. They do however cost more to run since they use more power to maintain and equipment/water for sanitation purposes. Semi-open facilities (Figure GI.6B, E & H) only provide some protection from the overall climate, similarly open facilities (Figure GI.6C, F & I) are completely exposed with no protection (Eilenberg and Jensen, 2018). Both semi-open and open facilities rely on suitable climates, but are relatively cheap and easy to install and structurally maintain and don't require much in terms of climatization (Eilenberg and Jensen, 2018; Tanga and Kababu, 2023). A downside is that it is easier for pests and other wildlife to pass through these facilities and there is less to protect BSF from their interactions, at the same time, there is a lower emphasis on containing BSF from escaping the facilities. BSF containment however is mainly seen as more of an issue where BSF have not already been naturalised (Marshall *et al.*, 2015). A vital issue that is often overlooked is actually the transfer of pathogens into and out of any type of facility, something that is better but not always addressed in closed facilities (Eilenberg *et al.*, 2015; Destoumieux-Garzón *et al.*, 2018; Eilenberg and Jensen, 2018; Maciel-Vergara *et al.*, 2021). This then raises a concern of pathogen-related health in reared insects.

1.2. One-health

Almost two and a half decades ago, a fundamental shift in the global perspective about the field of disease emergence and epidemiology as a whole started to emerge (Cunningham *et al.*, 2017). The "One-Health" concept posits that the overall health of humans, animals and the environment is all interconnected. While the overall concept has been in the making over a few decades, many of the policies and much of the framework had been drastically developed around 2010 (FAO/OIE/WHO/UNICEF/UNSIC/World Bank, 2008; Zinsstag *et al.*, 2011; Destoumieux-Garzón *et al.*, 2018). Historically, some aspects linked human health to mainly domestic livestock (Bresalier *et al.*, 2015). In light of this, Daszak, *et al.*, (2000) highlighted that the interconnectivity between emerging infection diseases (EID) in different groups of hosts is actually much broader (Figure GI.7A). This perspective also presented a network in which wildlife could act as a reservoir of human and animal diseases that may have spilled over from agricultural installations or human settlements. And thus, how previously managed zoonotic/live-stock diseases (e.g. rabies) could be reintroduced into anthropogenic systems

after silently recovering in the wild ('spill-back'). Additionally, EID migrating through human and farming domains could also negatively impact conservation efforts, as well as threaten wild species (Daszak *et al.*, 2000).

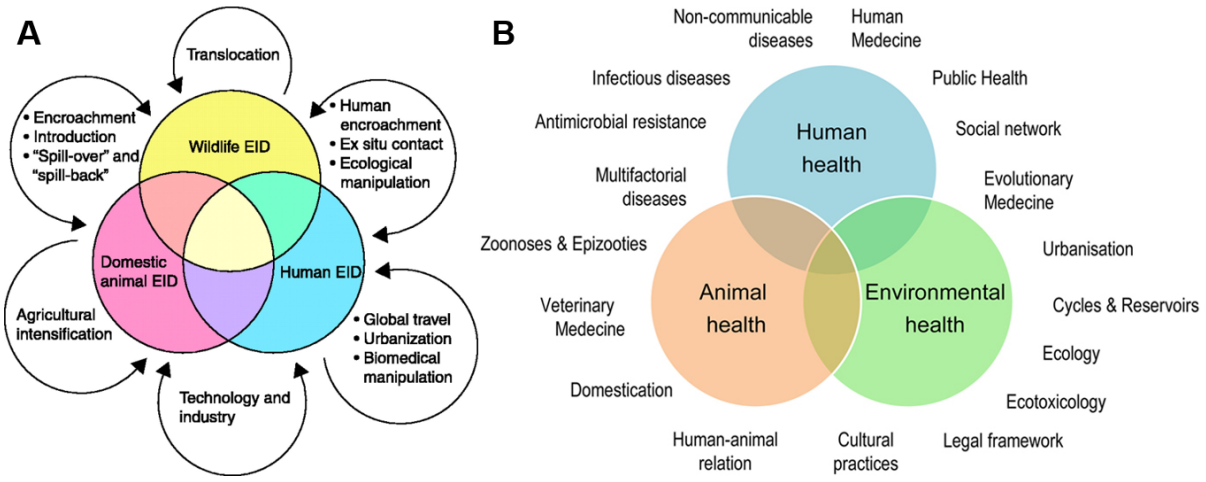


Figure GI.7 Progression of the philosophies and principles encompassing the "One-Health" approach between 2000 and 2018. A) Interactions between emerging infectious diseases (EID) in humans, domestic animals and wildlife (From Figure 1 in Daszak, Cunningham and Hyatt, (2000). B) Relationships between Animal health, Human health and Environmental health incorporated into the One Health framework (From Figure 2 in Destoumieux-Garzón *et al.*, (2018))

The EID network was then reconstructed into the currently used One-Health framework (Figure GI.7B). The One-Health framework prompted a much broader outlook, from looking at the network in the form of EIDs, to general health within each sphere (Bresalier *et al.*, 2015; Destoumieux-Garzón *et al.*, 2018). There are still considerable challenges facing the implementation of the One-Health approach, including changes and adoption at a policy level by many countries, partially driven by economic feasibility in terms of what is considered important by each country's administration (Cunningham *et al.*, 2017). The COVID-19 pandemic is a perfect example of how the global governance and society has been forced into facing some of the pitfalls surrounding the global attempts at the implementation of the One Health agenda (Khanna *et al.*, 2020; Murray, 2020; Pawar, 2020; Lawler *et al.*, 2021).

Epidemics and pandemics of EIDs have been recorded throughout history (Kaur *et al.*, 2020). While not all have been catastrophic, there are a few diseases which have plagued civilizations such as consumption (Tuberculosis, TB), dengue, Ebola, HIV AIDS, malaria, polio, smallpox, "Spanish flu", *Yersinia pestis* plagues (bubonic, septicaemic and respiratory (BSR)) and Zika

(Lönnroth *et al.*, 2009; Gostin *et al.*, 2014; Lucey and Gostin, 2016; Brady and Hay, 2020; Kaur *et al.*, 2020). This list is not exhaustive, and EID outbreaks have not just affected humans, but livestock and wildlife as well by diseases such as African horse sickness, anthrax, blue tongue, foot and mouth, rabies, rinderpest and surra (Vogel, 1996; Maclachlan, 2011; Desquesnes *et al.*, 2013; Jamal and Belsham, 2013; Carpenter *et al.*, 2017; Nagarajan and Rupprecht, 2020; Alam *et al.*, 2022). While some EID outbreaks were catastrophic when they first arose and eventually weaned out (Spanish flu) or eradicated (small pox, rinderpest), others are still with us today. Some diseases have had successful treatments such as the BSR plagues, HIV AIDS, malaria, polio, rabies and Tuberculosis (TB) might not all seem like a threat anymore in some regions (Huremović, 2019; Nagarajan and Rupprecht, 2020; Chumakov *et al.*, 2021; Loddenkemper and Murray, 2021; Nosten *et al.*, 2022). However, in other regions there are regular outbreaks and effective surveillance, and management strategies are the only lines of defence in keeping these pathogens at bay (Cunningham *et al.*, 2017; Destoumieux-Garzón *et al.*, 2018).

While epidemics and pandemics have complex histories relating to each one, there is one simple facet which encompass them all. When any of these disease outbreaks first began, it took some time before the exact etiological agent was recognized, and even longer before control measures or a treatment could be developed (Morse, 2007; Cunningham *et al.*, 2017; Huremović, 2019; Pawar, 2020), in particular when the combinations of symptoms are not already associated with a disease.

1.3. Lessons learnt from the collapses of the global shrimp industry

When looking into livestock epidemics[1], there is a group of livestock which do not receive as much public attention as the cuddlier cows, sheep and chickens. Devastating disease outbreaks have also been experienced within the seafood (crustacean) and insect mass-rearing industries. There are several insect colony collapses which have occurred in insect rearing industry, such as for honey bees, silkworms, crickets (Weissman *et al.*, 2012; Gordon *et al.*, 2014; Tayal and Chauhan, 2017; Duffield *et al.*, 2021; Osterman *et al.*, 2021; Bruckner *et al.*, 2023; Mondal *et al.*, 2024). However, these outbreaks usually are more localized to farms, but have potential to spread between regions as some insect farming can be mobile, or due to the transfer of infected insects between farms or shops. Surprisingly, there is another group of arthropods that have a

[1] The correct term for animals is “epizootics”, but it is still relatively a niche term.

far more menacing tale and lessons to tell. Just as diseases such as foot and mouth, mad cow and rinderpest have caused catastrophic impacts on the cattle farming industry worldwide, the global shrimp farming industry had multiple large crashes principally caused by important EIDs (Feigon, 2000; Boyd *et al.*, 2022). Shrimp farming grew as an industry at exponential rates, due to the development of techniques to farm them in the late 1970s and increasing demand. Unfortunately, overgrowth of the global industry brought a multitude of problems, of which the gravity can be summarize into a few examples. In 1987 the Taiwan shrimp farming industry collapsed almost completely, due to what was thought to be a viral etiological agent (Feigon, 2000). Around 1990, yellow-head virus (YHV) arose in Thailand and eventually decimated livestock in farms around Asia throughout the 1990s (Walker and Mohan, 2009). Then in 1993, mainland China's industry collapsed due to white spot syndrome virus (WSSV), and later the very same pathogen was responsible for a 35% loss in Ecuador's production in 2001 (Boyd *et al.*, 2022). Another major blow to the industry, Thailand lost 46% of their production mass over two years between 2012 and 2014 due to acute hepatopancreatic necrosis disease (AHPND) caused by bacteria (*Vibrio* spp.).

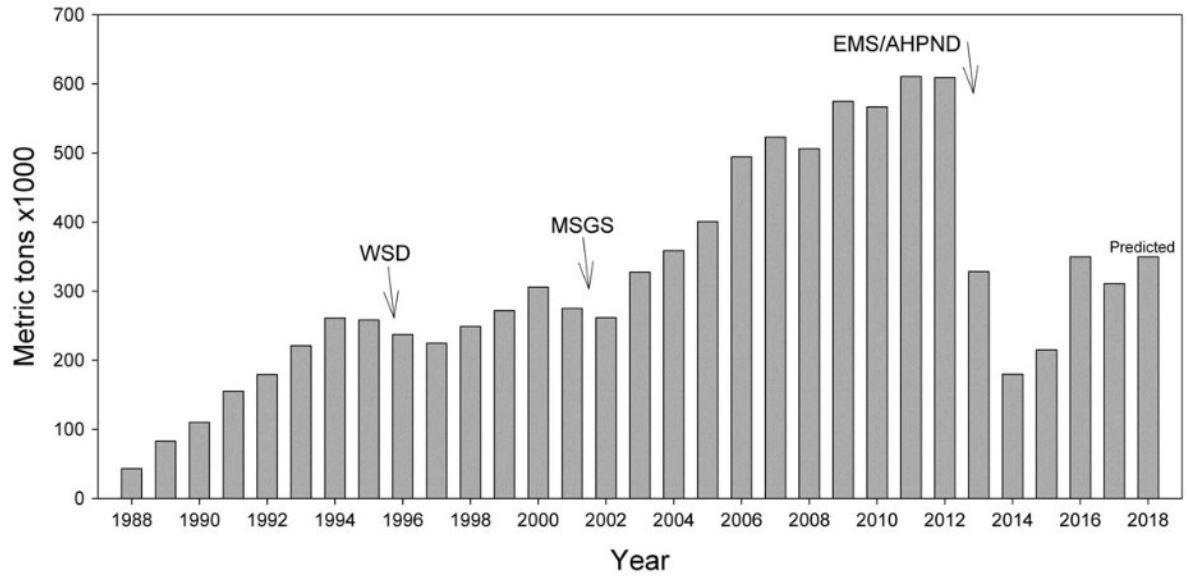


Figure GI.8 Historical documentation depicting the impact of various epidemics on the production of shrimp in Thailand between 1988 and 2018 (From Figure 3 in Flegel, (2019)) Epidemics recorded were acute hepatopancreatic necrosis disease (AHPND), early mortality syndrome (EMS), monodon slow growth syndrome (MSGS) and white spot disease (WSD). The value for the production mass for 2018 was based on a prediction for the publication at the time.

Two factors appear to be predominantly attributed to the sizable impact made by the epidemics experienced by the shrimp industry: 1) The industry growing too fast for proper zoning and

sanitation protocols to be designed and implemented; and 2) Disease research and management was not well implemented within the shrimp industry (Feigon, 2000; Walker and Mohan, 2009; Walker and Winton, 2010; Kumar *et al.*, 2021; Boyd *et al.*, 2022). While the shrimp industry is affected by pathogens on a global scale, efforts at least in Asia have shown promise to help reduce the devastation often associated with shrimp disease outbreaks. Review of scientific literature highlighted disease-related issues, as well as solutions and attempts to create awareness for policy makers and industry (Flegel, 1997, 2012, 2019; Stentiford *et al.*, 2012, 2017; Debnath *et al.*, 2016). By 2016, five diseases were considered "newly emerging" and had become a cause for concern for the industry (Thitamadee *et al.*, 2016). Nonetheless, disease monitoring and response has been better implemented in Asia, allowing the industry to better recover from outbreak events (Figure GI.8) (Flegel, 2019).

These issues have led to changes in policies and awareness, as well as some more focused strategies on protecting industry from harmful pathogens (Flegel, 2019). For example, two previously highly impactful pathogens Taura syndrome virus and infectious hypodermal and hematopoietic necrosis virus, no longer pose a large threat to the shrimp industry since more resistant colonies of *Penaeus vannamei* have been used in commercial production chains. Thus one solution was to makes disease resistant/tolerant strains available for any farm to use to recover from collapses. (Thitamadee *et al.*, 2016). When looking at the attention that shrimp pathogens now receive in Thailand, it's clear that shrimp production will continue to go through disease outbreak cycles (Figure GI.8). Despite this, actions such as industry and political awareness campaigns, transmission reduction and outbreak management within the last 40 years already show that production can recover fairly quickly from these events (Flegel, 2019).

1.4. Brief history of insect pathology

Humans have maintained a direct and beneficial working relationship insects for at least 8000 years (Davidson, 2012). However, records indicate that it was only since about 2500 years ago that we started to acknowledge and develop an understanding about diseases that impact insect populations. Naturally, it was domesticated insects such as silkworms (*Bombyx mori*) and honeybees (*Apis mellifera*) where this attention was concentrated. So much so that it was only in 1602 CE after observation of nematodes in grasshoppers (Orthoptera) that diseases of other insects appeared in historical records (Davidson, 2012). It wasn't until the 1800s when insect pathology really began to take off, however much of the work still focused on moths

(Lepidoptera) and bees (Hymenoptera). Afterwards, the focus started to diversify to other insects and invertebrates since the early 1900s, expanding mainly due to the need to help control insect pests (Davidson, 2012; Hajek and Shapiro-Ilan, 2018). Although it may not be well known, there are strong ties between modern epidemiology and studying pathology in invertebrates. By the 1980s, diseases in invertebrates were being researched in both reared arthropods and those living in the wild. Epidemiologist Roy Anderson and theoretical ecologist Robert May who developed the foundations for epidemiological modelling, which are highly regarded even today, actually developed their mathematical models using disease outbreaks in invertebrates, such as insects before eventually adapting them to human and other vertebrate epidemiology (Heesterbeek and Roberts, 2015; Hajek and Shapiro-Ilan, 2018; Poulin, 2021). This was due to the inherent simplicity of invertebrate responses during infections by pathogens, partly since it was perceived that invertebrates did not typically have an acquired immune response[2] (Anderson and May, 1981). Essentially, Anderson and May, (1981) capitalized on previous notions surrounding epidemiology of microparasites (viruses, bacteria, fungi and nematodes) mainly developed in laboratory conditions (Figure GI.9), and gradually increased the complexity of the models to incorporate external factors which better replicate what can be expected in the environment. The more famous examples by Anderson and May, (1981) among insect pathologists were the modelling of baculovirus infections in insects in wild populations, incorporating a better understanding of dynamics into disease ecology.

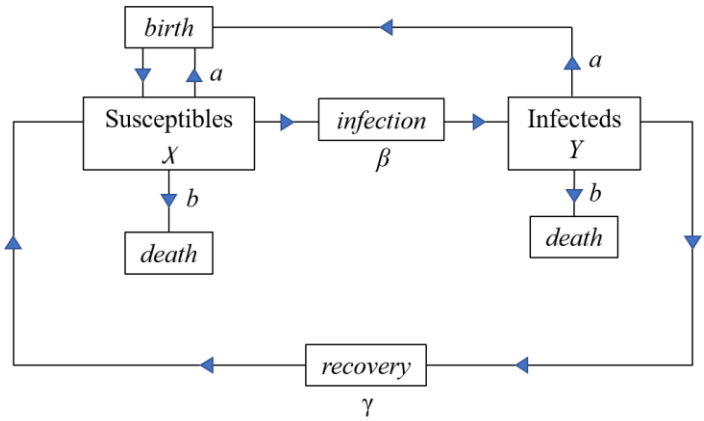


Figure GI.9 A simplified susceptible, infected and recovered (SIR) model proposed by Anderson and May, (1980) based on previous epidemiological assumptions (Adapted from Figure 5). *a* – host birth rate (per individual), *β* – transmission coefficient (only for direct transmission from infecteds to susceptibles), *b* – natural mortality rate of hosts, *Y* – number of infected hosts, γ – rate of host recovery from infection, χ – number of susceptible hosts.

[2]After an infection is cleared, an individual can develop an immune reponse which allows them to tackle reinfections by the same pathogen more easily, often resulting in a faster recovery.

There has been a growing interest in studying invertebrate pathology to improve the health of reared invertebrates, especially more recently since the insect mass-rearing industry has expanded at exponential rates (Lafferty *et al.*, 2015; Eilenberg *et al.*, 2018; Hajek and Shapiro-Ilan, 2018).

1.5. Definition of symptoms and signs

Traditionally, diagnosing diseases relies heavily on the identification of signs and symptoms, which can be misinterpreted (King, 1968; Podder *et al.*, 2024). A symptom is a subjective observation made by the patient about an abnormal sensation or change in their body, such as vertigo or slight deafness. In contrast, a sign is an objective observation made by someone other than the patient, typically a medical professional, like difficulty in walking or issues in communication observed by a doctor. King (1968) emphasized the need for caution in defining these terms to avoid confusion in medical diagnostics, particularly since some signs and symptoms become synonymous by how the observation can be classified, e.g. a doctor witnessing that a deaf patient is not responding when being called (sign), and a patient experiencing deafness (symptom). This complexity highlights the challenge in distinguishing between the two terms, which often overlap and are interdependent in diagnosing conditions. Modern medical practice now attempts to assign appropriate weight to signs and symptoms to improve diagnostic accuracy and reduce confusion in terminology, but challenges still remain (King, 1968; Eriksen and Risør, 2014; Scott-Fordsmand and Tybjerg, 2023).

The predicaments surrounding signs and symptoms did not only affect human patients, but also patients of a more exoskeletal physic, arthropods, more specifically crustaceans and insects. The rules of symptom perception and diagnostics are quite different when dealing with insects, and by extension, arthropods (Kaya and Vega, 2012; Gibbons *et al.*, 2022). Insect cognition, perception and behaviour is much further from our understanding and ability to relate compared to other mammals, particularly domesticate mammals. Thus, different criteria models are needed, not just to categorise the wellbeing and welfare of insects, but also determining what is a symptom and what is a sign. Firstly, in insect pathology, to diagnose a disease/disorder, a syndrome must be established, which is a collection of symptoms and signs bearing significance (Kaya and Vega, 2012; Gibbons *et al.*, 2022). Secondly, since humans do not currently have the capacity to communicate successfully with insects, signs and symptoms are not interchangeable. This is to allow for a less complicated classification of observations and

is essentially one-sided, from the perspective of the insect pathologist. Within insect pathology, to be precise, the currently accepted definition of a "***sign***" in ***invertebrate pathology*** is, "any objective aberration or manifestation of disease indicated by a change in structure". And for a "***symptom***" it is, "any objective aberration in function (including behaviour), indicating disease" (Onstad *et al.*, 2006). Kaya and Vega, (2012) tried to provide a more conceptualised explanation of each, making them easier to incorporate in pathology studies. Signs are rather superficial, what can be seen from looking at the integrity of the anatomy of an insect; i.e. is there a discolouration, is a sturdy-bodied insect now brittle, are ovaries fully developed? On the other hand, a symptom is then an observation in relation to the behaviour of the individual insect/rearing batch or if the bodily functions are well maintained (Kaya and Vega, 2012). However, such as seen in the medical field (King, 1968; Eriksen and Risør, 2014), misuse of the term "symptom" has also occurred in usage, and literature, for example in pathology specific to insects (Schütte *et al.*, 2008; Eilenberg *et al.*, 2015; Maciel-Vergara and Ros, 2017; Eilenberg and Jensen, 2018; Joosten *et al.*, 2020; Maciel-Vergara *et al.*, 2021). This misuse also could be attributed to regular exchanges in publications when describing infection states as "symptomatic" and "asymptomatic" (Eilenberg *et al.*, 2015; Maciel-Vergara and Ros, 2017; Maciel-Vergara *et al.*, 2021), or because it is simpler to stick to one term when the focus is about invertebrate diseases and their role in the ecosystem/rearing facilities and diagnosis is not the main point of the discussion. In this dissertation, the terms "sign" and "symptom" are used independently and from the perspective of an insect pathologist (Onstad *et al.*, 2006). For clarity, the labels "asymptomatic" and "symptomatic" will consistently encompass both signs and symptoms, aligning with standard usage in the literature.

2. Problems encountered in insect mass-rearing

2.1. Brief overview of pathogen surveillance in mass-reared insects

Dangles and Casas, (2019) promoted the idea of implementing an applied entomology focus as part of working towards the UN's 2030 sustainability goals. Part of this involved the inclusion of mass-reared insect species, in particular, mealworms, crickets, black soldier flies. Insect species can offer multiple services beneficial to people, and for example, human consumption of insects alone had been documented in 113 countries by 2013 (Van Huis, 2013; Dangles and Casas, 2019). Like in shrimp aquaculture, insect mass-rearing also contain their fair share of pathogens, many which can cause colony collapse (Thitamadee *et al.*, 2016; Eilenberg and Jensen, 2018; Maciel-Vergara *et al.*, 2021) (Figure GI.10).

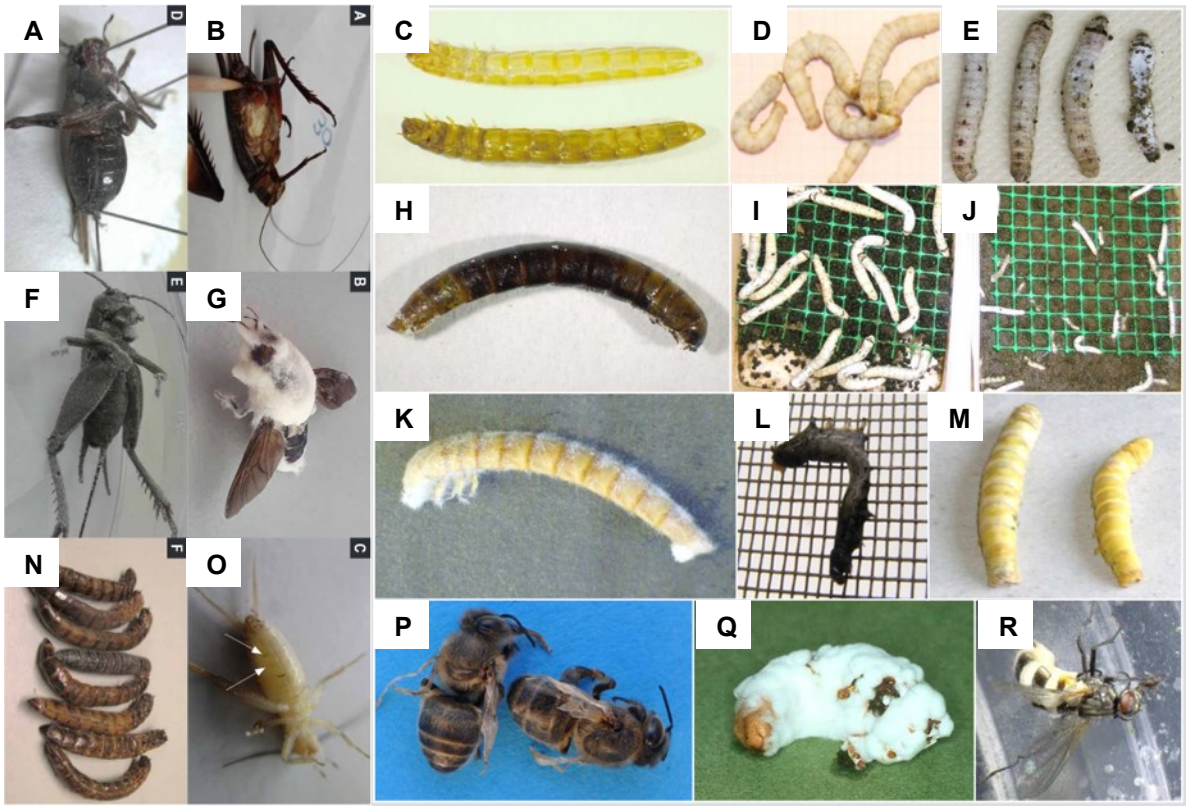


Figure GI.10 Collection of signs of infection in examples of mass-reared insects (Adapted from Figure 1 in Maciel-Vergara *et al.*, (2021) and, Right: from Figure 1 in Eilenberg *et al.*, (2015)) Insect species included in here are A) *Gryllus bimaculatus*, B) *Teleogryllus* sp., C,H,K) *Tenebrio molitor*, D,E,I,J,M) *Bombyx mori*, F) *Modycogryllus* sp., G) *Hermetia illucens*, L) *Spodoptera exigua*, N) *Zophobas morio*, O) *Acheta domesticus*, P) *Apis mellifera*, Q) *Melolontha melolontha* and R) *Musca domestica*.

Some insects such as *Bombyx mori* (silkworms), *Apis mellifera* (honey bees) and *Drosophila sp.* (vinegar flies) are well studied models of mass-reared insect in terms of pathology and pathogens (Imrie *et al.*, 2021; Maciel-Vergara *et al.*, 2021; Wallace *et al.*, 2021). However, there is also currently a growing attention on the pathology of *Tenebrio molitor* (mealworms), *Drosophila sp.* (vinegar flies), *Musca domestica* (house flies) and a collection of Coleoptera (beetles) and Orthoptera (locusts and crickets) (Maciel-Vergara *et al.*, 2021). The host range of pathogens can be specific, restricted to one host species or they can infect multiple hosts (Maciel-Vergara *et al.*, 2021). At the same time, not all pathogens will act strictly as pathogens, some microorganisms can be pathogenic in some conditions, but not in others (Figure GI.11) (Brodeur, 2012; Maciel-Vergara *et al.*, 2021). Here is where a conundrum begins, in terms of pathogen discovery in insects, normally it would take either an interest in pest control or for disease outbreaks to be acknowledged before a pathogen would be studied (Maciel-Vergara *et al.*, 2021). In 2021, at least 325 commercial insect producers were operating and producing

insects for food and feed (F&F) (van Huis *et al.*, 2021). Just in Europe, approximately US$1 billion had been invested into the F&F sector and was estimated to reach somewhere between $4.63 billion and $8 billion by 2030. Given the large investment into the rearing of insects for F&F, waiting until an outbreak occurs to investigate pathogens may not be a reasonable option anymore (Maciel-Vergara *et al.*, 2021; van Huis *et al.*, 2021).

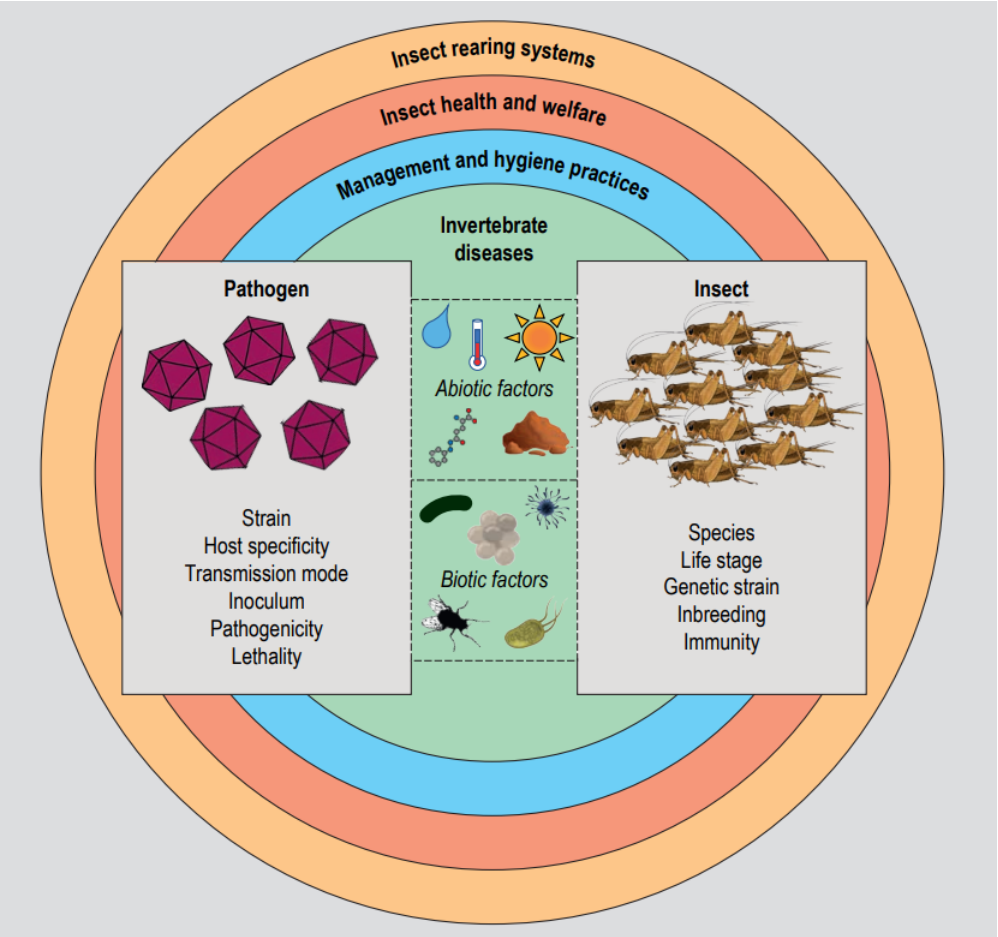


Figure GI.11 Holistic view of factors connected to the triggering of disease outbreaks in colonies within insect rearing facilities (From Figure 2 of Maciel-Vergara *et al.*, (2021))

2.2. Looking into entomopathogenic viruses

Viruses are often overlooked as pathogens and normally not easily detected in rearing colonies. They can difficult to identify since their identification and detection primarily relies on appropriate isolation methods, molecular techniques, high-throughput sequencing and ultra-high magnification microscopy (Chiu, 2013; Maciel-Vergara and Ros, 2017; Cobbin *et al.*, 2021). Yet viruses can be incredibly important economically in mass-reared facilities (Figure GI.12) (Maciel-Vergara and Ros, 2017; Maciel-Vergara *et al.*, 2021). One of the major issues

with viruses is that some viral pathogens can have covert phases which means that no signs or symptoms are readily observed (Maciel-Vergara and Ros, 2017; Williams *et al.*, 2017). Ultimately this opens a gap for viruses to already be present within facilities or be introduced into facilities during colony exchanges. While there are methods in place to perform surveillance on a number of described viruses in reared insects, infections can be missed if tests are not conducted in an appropriate manner or if the virus is unknown (Maciel-Vergara and Ros, 2017; de Miranda, Granberg, Low, *et al.*, 2021).

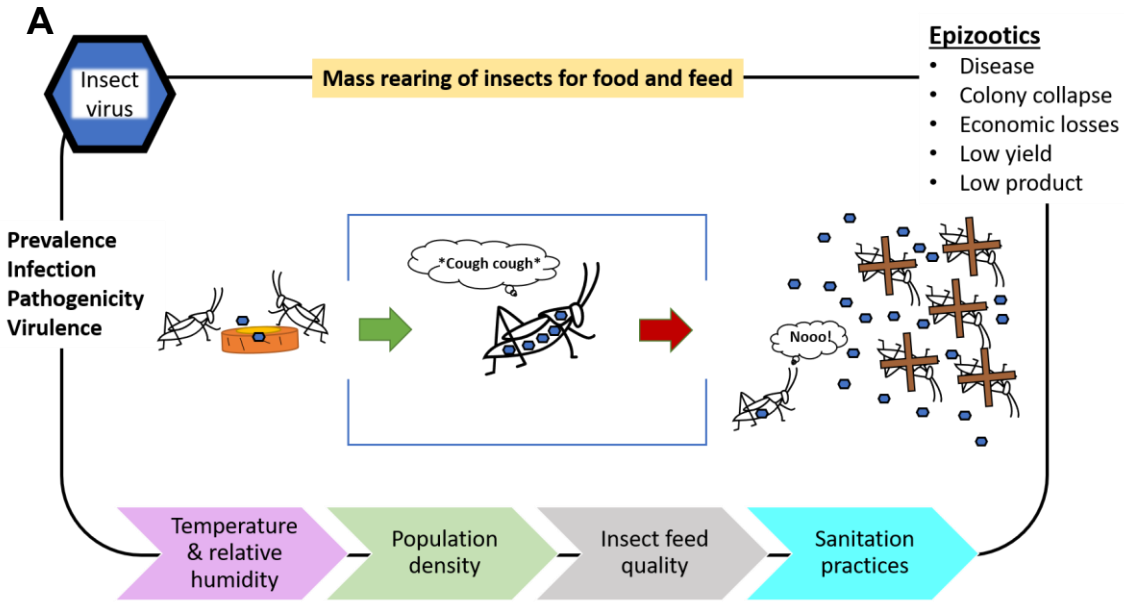


Figure GI.12 Conceptualisation of the impacts that pathogenic viruses can have within insect mass-rearing facilities (**A**) (Adapted from the graphical abstract for (Maciel-Vergara and Ros, 2017)).

Another major issue is that rearing facilities are not just optimal for the insects that they are designed for, they are also optimal for entomopathogens, especially viruses (Eilenberg and Jensen, 2018; Eilenberg *et al.*, 2018). A drawing factor to the sustainability of insect farming is that millions to billions of individuals can be reared in relatively small spaces. As an example, for dipteran larvae, a 6 m^2 tray can easily host 300 000 larvae. When compared to cattle farming, one of the largest farms in 2018 was China Modern Dairy, boasting 4 451 542 ha in size, but only registering 230 000 cows (*Top 10 biggest farms worldwide*, 2018). This is an extreme example but can help put the differences into perspective in terms of space, resource use and feasibility of monitoring to the health of individual insects. Since insect farms tend to take advantage of lower space requirements per individual, this also allows for the build-up of factors such as temperature and humidity within rearing enclosures, potentially facilitating viral pathogen replication and transmission (Figure GI.12). While this is possible to better manage

in closed facilities compared to open and semi-open facilities, this can still be a problem if facilities are not properly equipped, and may also require stable sources of electricity (Maciel-Vergara and Ros, 2017; Eilenberg and Jensen, 2018). If rearing conditions can't be regulated effectively also factoring in pathogens, this can lead to triggering of disease outbreaks which can cause large drops in product yield and potentially colony collapse, leading to large economic losses (Figure GI.11 and Figure GI.12).

2.3. Viruses associated with mass-reared flies

When exploring the NCBI virus database (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/, accessed: 26th April 2024), 1 951 separate viral organism/species (24 388 sequences) were registered on the database with flies (Diptera) listed as the host. Some viruses were listed without knowing the genome type (84), but overall 116 were classified as DNA viruses and 1 751 were classified as RNA viruses). While not all the hosts have been experimentally verified, this still boasts an incredible diversity of viruses found in flies in general. Narrowing the scope, there is a surprising amount of fly species which can be mass reared. To date there are at least 53 species of flies which are artificially reared worldwide (Pascacio-Villafán and Cohen, 2023). Focusing more on flies that are mass-reared for food and feed, as well as their use in waste management, this brings the list down to 15 species currently being used in industry or being researched for their feasibility. In the list, 11 species are used in waste management and seven for food and/or feed, including BSF (Table GI.1).

Knowledge of viruses within mass-reared flies in general is limited to a few species, with majority of the knowledge restricted to selected species like *Drosophila* spp. (112 viruses), *Ceratitis capitata* (13 viruses), and *Bactrocera* spp. (17 viruses) (Table GI.1). This creates a stark contrast to other mass-reared flies, particularly those involved in F&F and waste management, where 12 viruses have been reported, but only in three of the 15 species (Table GI.1). Musca domestica hytrosavirus (MdHV) was found to infect in laboratory experiments, and so far, has not been found to occur naturally in *Musca autumnalis* (Table GI.1) (Geden *et al.*, 2011). Icosahedral virus-like particles (VLPs) were first found in spermatids of *Coelopa frigida*, but were not further characterized (Schrankel and Schwalm, 1975). In light of this, a newly discovered virus, dipteran tombus-related virus OKIAV375 (*Tombusviridae*), was tentatively associated with *C. frigida*. As *Tombusviridae* share a similar morphology to the unclassified VLPs, further work is needed to determine if it is the same virus (Schrankel and Schwalm, 1975; Rochon *et al.*, 2011).

Table GI.1 Estimated number of viruses currently found in flies (Diptera) mass-reared for the purpose of food and feed or waste bioconversion, with the exception of *Drosphila* spp., *Bactrocera dorsalis*, *B. tryoni, Ceratitis capitata* and *Inopus flavus* (Modified from Table A1 in Pascacio-Villafán and Cohen, (2023)).

Family	Species	Food and feed or Waste management*§	Viruses found	Sources
Calliphoridae	*Chrysomya chloropyga*	WM	Unk.	
	C. megacephala	WM	1	NCBI
	C. putoria	WM	Unk.	
	Lucilia sericata	WM	Unk.	
	Protophormia terraenovae	F&F	Unk.	
Coelopidae	*Coelopa frigida*	F&F	2	Schrankel and Schwalm, 1975; Paraskevopoulou *et al.*, 2021
	C. pilipes	F&F	Unk.	
Drosophilidae	*Drosophila* spp.	General research	112	Wallace *et al.*, 2021 ; NCBI
Muscidae	*Musca autumnalis*	WM	1	Geden *et al.*, 2011
	M. domestica	WM; F&F	9	Maciel-Vergara *et al.*, 2021 ; NCBI
Sarcophagidae	*Boettcherisca peregrina*	WM	Unk.	
	Sarcophaga carnaria	WM; F&F	Unk.	
	S. dux	WM	Unk.	
Stratiomyidae	*Hermetia illucens*	WM; F&F	Unk.	
	Inopus flavus	Not mass-reared	9	Asselin *et al.*, 2021; Colmant *et al.*, 2022; Divekar *et al.*, 2024
Syrphidae	*Eristalis tenax*	WM	Unk.	
Tephritidae	*Ceratitis capitata*	Pcon	13	Hernández-Pelegrín *et al.*, 2022
	B. dorsalis	Pcon	9	Qi *et al.*, 2023
	B. tryoni	Pcon	8	

***Some species are not reared for food and feed (F&F) or waste management (WM) but were considered important for the focus of this topic. §Abbreviations used F&F = food and feed, WM = waste management and PCon = Pest control.**

Specific interactions between viruses and flies are poorly studied. Most of the research on the broad viral diversity in flies remains superficial, essentially virome characterization or description (Shi *et al.*, 2016; Webster *et al.*, 2016; Wu *et al.*, 2020; Wallace *et al.*, 2021; Hernández-Pelegrín *et al.*, 2022; Qi *et al.*, 2023; Divekar *et al.*, 2024). There are some species where more in depth studies into host-pathogen interactions have taken place, such as *Drosophila* spp. (Longdon *et al.*, 2009; Tafesh-Edwards and Eleftherianos, 2020; Imrie *et al.*, 2021, 2023), mosquitoes such as *Aedes* spp. and *Culex* spp. (Agboli *et al.*, 2019), *Ceratitis capitata* (Hernández-Pelegrín *et al.*, 2022; Hernández-Pelegrín, García-Martínez, *et al.*, 2024), *Glossina* spp. (Kariithi *et al.*, 2013; Meki *et al.*, 2021) and *Musca domestica* (Moussa, 1978;

Geden *et al.*, 2011). While viral pathology studies are lacking in flies in general, there has been some major strides in fly immunity studies across Culicidae and Drosophilidae. Palmer *et al.*, (2018) laid out a broad plan of the general antiviral immune responses in Diptera. In general, the known innate immunity pathways of flies can be placed into six main categories: Toll pathways, immune deficiency (IMD), JAK-STAT, RNA interference, RNA decay and autophagy (Figure GI.13). Apart from the autophagy pathway controlled by Toll 7, three other immune pathways in flies are receptor-based, JAK-STAT, IMD and other Tolls. Usually, antimicrobial peptides (AMPs) induced by the Toll and IMD pathways target bacteria and fungi (Toll), but there are some which had shown antiviral potential (Palmer, Varghese, *et al.*, 2018; Moretta *et al.*, 2020). But AMPs are not the only outcome of Toll and IMD pathways, phagocyte production and apoptosis and can also be regulated by Toll pathways (Palmer, Varghese, *et al.*, 2018).

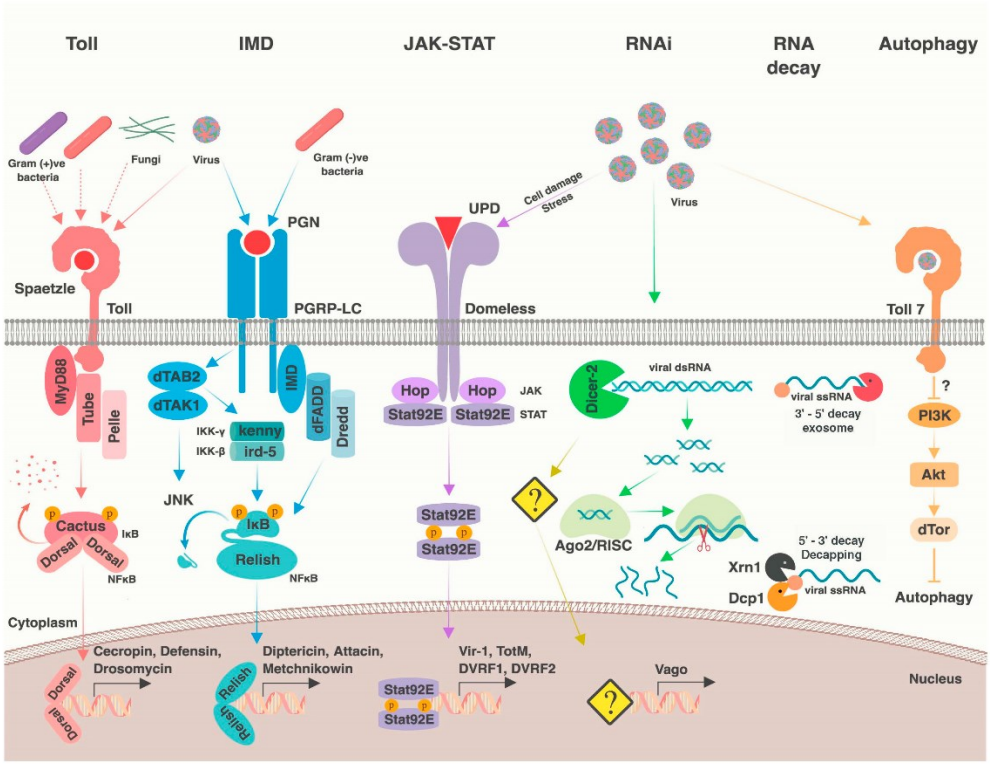


Figure GI.13 Dipteran antiviral immunity in a broad sense (Obtained from Figure 1 in Palmer *et al.*, (2018))

JAK-STAT can play a role in antiviral response, but it is more restricted to certain viruses (Kemp *et al.*, 2013), unlike RNAi (broadest range), Toll and IMD which can target a broader array of viruses (Palmer, Varghese, *et al.*, 2018). JAK-STAT may be more specific since it is suspected that its anti-viral components are actually activated through the Dicer 2 pathway

cascades after recognition of viral double stranded RNA (Kingsolver and Hardy, 2012; Palmer, Varghese, *et al.*, 2018). Within the RNAi pathway, in addition the Dicer proteins, PIWI proteins can also carry a special roll in antiviral response. However, from what has currently been described, the complex PIWI pathway utilises related viral sequences retained within piRNA clusters in the insect genome from previous infections for the PIWI proteins to be effective in viral infections (Cerqueira de Araujo, Huguet, *et al.*, 2022). Notably, insect viruses can also carry genes which can suppress RNAi interactions with dsRNA, promoting the necessity of the other pathways and some level of tailoring to different viruses. *Dicistroviridae* is a group of arthropod-infecting viruses that usually carry an RNAi suppressor (Fareh *et al.*, 2018; Warsaba *et al.*, 2019). Coincidently, the Toll 7 pathway which involves autophagy can be non-specific, but is highly involved in resistance to viruses such as *Dicistroviridae* and *Rhabdoviridae* which can have covert infection states (Longdon *et al.*, 2009; Ferreira *et al.*, 2014; Maciel-Vergara and Ros, 2017; Palmer, Varghese, *et al.*, 2018). There is another RNA specific weapon that can be used against viruses, the RNA decay pathway (Figure GI.13). The antiviral activity of this pathway is more of a secondary benefit, originally, this pathway helps remove unnecessary or harmful RNA floating around the host cell body, but viral RNAs can contain or lack integrity signals used by cell signalling to determine which RNA to keep or remove (Palmer, Varghese, *et al.*, 2018). Flies might not be defenceless against viruses, however the complexity (Figure GI.13) of their immune pathways reiterates how there are virus-specific responses, that may play a role in enforcing the variation of resistance, besides external factors (Palmer, Varghese, *et al.*, 2018).

A Stratiomyidae where the virome has been studied is *Inopus flavus*. Although it is not mass-reared, it was included as a species that is closely related to BSF (Table GI.1). *Inopus flavus* has a diverse virome consisting of nine viruses within a total of eight viral families: *Dicistroviridae*, *Flaviviridae*, *Narnaviridae*, *Orthomyxoviridae*, *Partitiviridae*, *Parvoviridae*, *Totiviridae*[3] and *Xinmoviridae* (Asselin *et al.*, 2021; Colmant *et al.*, 2022; Divekar *et al.*, 2024). Some of these viral families also make a reoccurrence in the viromes of other mass-reared flies mentioned in Table GI.1, such as *Dicistroviridae*, *Iflaviridae*, *Orthomyxoviridae*, *Rhabdoviridae* and *Totiviridae*.

[3] There was recently a massive revision of *Ghabrivirales*, which led to *Totiviridae* being split into 19 families. This is addressed in Chapter 2.

The ecology of some viral families previously studied in Diptera may allow for some level of prediction of interactions (pathogenicity and resistance) in unexplored hosts, in this case BSF, but there are still a couple of considerations to be made. Some immunological work has been done for BSF, nonetheless it can be incredibly difficult to predict how their interactions with viruses may play out. When reviewing dipteran resistance to viruses, Palmer *et al.*, (2018) highlighted five major factors playing a significant role in viral resistance: 1) the genotype, 2) mating status, 3) microbiome, 4) rearing environment, and 5) previous exposure to infections. Currently, a few studies have claimed that BSF are likely more resistant to pathogens in their larval stages (De Smet *et al.*, 2018; Joosten *et al.*, 2020; Lecocq *et al.*, 2020; Moretta *et al.*, 2020; Jensen and Lecocq, 2023). To be brief, this is further supported by some work in models such as *Drosophila melanogaster* which also found sex-based differences in immunity (Kraaijeveld *et al.*, 2008; Fellous and Lazzaro, 2011). I would include a sixth and seventh factor into the mix, life stage and sex, which begins to show how complex antiviral resistance can be. To keep in line with this story, ultimately, no eukaryotic viruses have been isolated from BSF. Thus, to truly understand viral-host interactions, there needs to be some knowledge of viruses present in BSF, before antiviral mechanisms can be explored. In short, BSF viruses first need to be found.

3. General virus discovery

3.1. The basic anatomy of a virus: alive and dead at the same time

While the aim of this thesis was not to define what is a virus, there are some fundamental aspects of viruses that are better reiterated before proceeding with the foundations of virus discovery approaches. Virus genomes can be constructed in multiple ways, and for a long time these constructions have been classified into seven groups under the Baltimore classification system (Figure GI.14) (Koonin *et al.*, 2021). While the genomic structures of each group can vary greatly (Double stranded DNA to Double stranded RNA), ultimately they consist either of DNA or RNA (Hershey and Chase, 1952; Gierer and Schramm, 1956; Rampersad and Tennant, 2018). The length of viral genomes also can vary from approximately 1.7 kb to 2 500 kb and some virus genomes can be multipartite, with some viruses, such as cypoviruses, having as many as ten segments (Breitbart *et al.*, 2017; Legendre *et al.*, 2018; Zhan *et al.*, 2023). The number and type of core genes also vary greatly, with some viruses encoding only two genes, and some well over 1300 genes, with no single universally conserved gene for all viruses (Wickner *et al.*, 2011; Legendre *et al.*, 2018; Obbard, 2018).

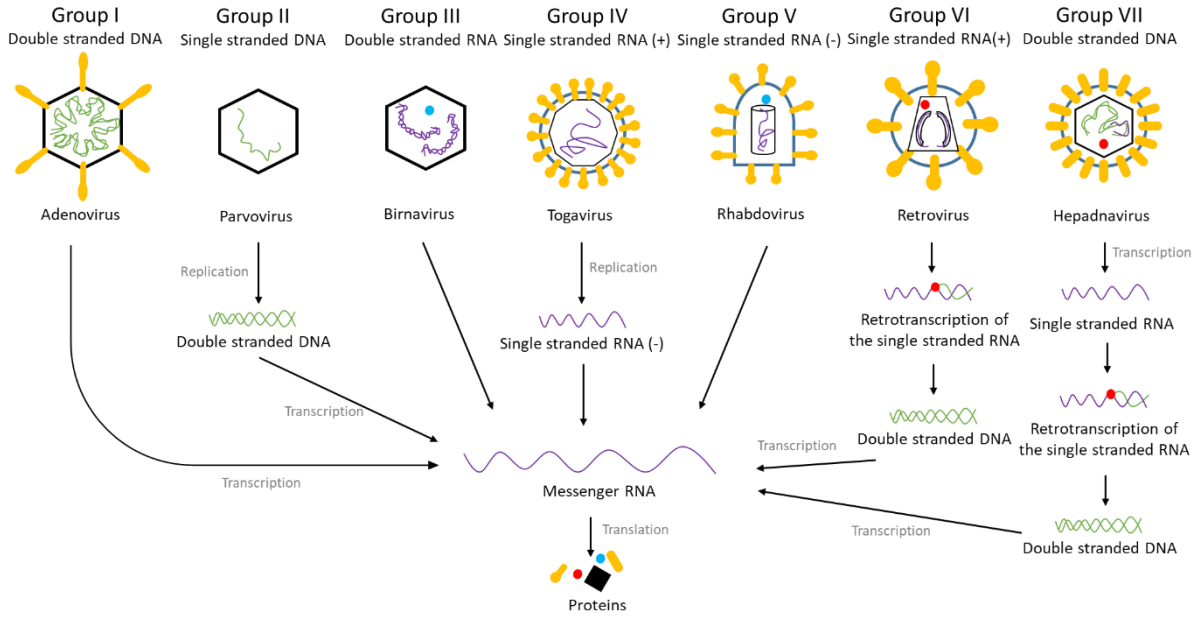


Figure GI.14 Visualisation of the Baltimore classification system for viruses (Used with permission from Alexandra Cerqueira de Araujo).

Viruses typically have a protein coat protecting their genetic material and can also have other structural components such as a membrane (Louten, 2016). They also occur in a variety of shapes and sizes. Essentially, viruses are relatively simple in terms of structure, however, the combination of characteristics can make it complicated when working with unknown viruses. Another factor to consider is that viruses will only replicate when infecting a suitable host, so this adds a layer of complexity when finding viral sequences in samples (Louten, 2016; Obbard, 2018; Koonin *et al.*, 2021).

3.2. Molecular, microscopy and cell culture

Since the discovery of the tobacco mosaic virus in the 1890s, the recognition of viruses as we know them today has evolved significantly (Bos, 2000). Initially hindered by a lack of appropriate isolation and study methods, researchers have since developed several foundational techniques. Prior to the advent of next-generation sequencing, key methodologies included electron microscopy, cell culture, Polymerase Chain Reactions (PCRs), Sanger sequencing, hemagglutination assays, and various protein and immunological assays such as western blots (Baer and Kehn-Hall, 2014; Dolskiy *et al.*, 2020; Reta *et al.*, 2020; Chauhan and Gordon, 2022; Mwanza *et al.*, 2023). In general, plaque assays, western blots, and PCRs have been considered

the gold standards in virus discovery. These methods not only confirm the presence of a virus, as in the case of plaque assays and microscopy techniques like Transmission Electron Microscopy (TEM) and Fluorescence *In Situ* Hybridization (FISH), but also allow for the observation of viral activity and host cell infection (Baer and Kehn-Hall, 2014; Akilesh *et al.*, 2021; Meki *et al.*, 2021).

Techniques such as PCR and FISH require pre-designed probes, meaning the genetic material or proteins must have been previously characterized. Similarly, western blots use specific antibodies, and while they can be developed without extensive prior knowledge of the virus, they do require a suitable host from which antibodies can be isolated (H. Zhu *et al.*, 2020; Liu and Zhang, 2021; Sule *et al.*, 2023). Another technique, Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), aids in antigen isolation to produce antibodies in model organisms like rabbits, and can also provide some information about viral isolates (Wiesner *et al.*, 2021; Wittmeier and Hummel, 2022; Sule *et al.*, 2023). However, like agarose gel electrophoresis, it depends on the molecular weight of the proteins.

While these methods are widely applicable, some require considerable preliminary experimentation. Plaque assays are among the simplest to apply during virus discovery, provided the virus can lyse host cells. Nonetheless, the challenge often lies in the availability of culturable host cells, but cell cultures can sometimes be created by utilizing the appropriate cells of an organism (Saathoff *et al.*, 2024). In some cases, available cell-cultured cells from closely related organism to the suspected host of a virus can be tested as well. This is because a virus host range can be broader in cell culture than in whole organisms (Jiwaji *et al.*, 2016; Elrefaey *et al.*, 2020; Rothenburg and Brennan, 2020). With suitable cells, viral material can be tested in a plaque assay, with results observable from a few hours to overnight. Any resulting plaques can then be analysed using TEM to detect viral-like particles.

3.3. Virus discovery by high-throughput sequencing

Next generation sequencing (NGS) technologies brought a whole new dimension to virus discovery approaches (Greninger, 2018; Shi *et al.*, 2018). High-throughput sequencing (HTS) provides the ability to sequence multiple DNA/RNA sequences at once. There is some confusion however in some literature where the term “metagenomic” sequencing is used regardless of whether the initially extracted material is metagenomic DNA or

metatranscriptomic RNA, thus caution should be taken when reviewing literature (Schoonvaere *et al.*, 2018; Waldron *et al.*, 2018; Bergner *et al.*, 2019; Gebremedhn *et al.*, 2020; Strubbia *et al.*, 2020; Roux *et al.*, 2021). One attempt to move around this is to use the term "Viral metagenomics", however that term is not sufficient to distinguish the exact approach used for "shotgun" meta-omic sequencing studies virome studies. Here we will focus on "shotgun" metagenomic/metatranscriptomic HTS since that is the standard approach for sequencing of virus-related samples (Obbard, 2018; Cantalupo and Pipas, 2019; Cobbin *et al.*, 2021). While this can expedite the discovery process by cutting out the need to isolate viruses before sequencing, there are some processes which need to be understood that will also dictate what bioinformatic approaches can be taken (Louten, 2016; Shi *et al.*, 2018; Cantalupo and Pipas, 2019; Koonin *et al.*, 2021). Since viruses can have either DNA or RNA genomes, performing metagenomics will exclude actively-replicating RNA viruses where there is no DNA intermediary within their replication cycle (Figure GI.14). The natural alternative to this would be to perform metatranscriptomics, however few sequencing technologies available, such as nanopore sequencing, can sequence RNA directly. Normally, for sequencing of RNA (RNAseq), extracted RNA is converted to complementary DNA (cDNA) by poly-A tailing, Oligo dt or random hexamer priming, with each having drawbacks. For instance, not all viruses will have poly-A tails attached to their RNA sequences, so these viruses will not be detected when RNA is isolated or cDNA is synthesised using a poly-A-tailing approach (Parras-Moltó *et al.*, 2018; Wongsurawat *et al.*, 2019). On the other hand, using random hexamer priming will synthesis the total RNA content, but may reduce the reliability of quantification by RNA expression since it will also include non-mRNA (Lowe *et al.*, 2017). However, in this instance mRNA quantification is more important when trying to study the gene expression of the host. The NGS technology used will also play a role in downstream processing since different sequence assembly software or virus discovery pipelines may not be compatible with specific NGS technology. For example, SPAdes can assemble both short and long reads and is compatible with nanopore sequencing data, but Trinity is not optimized very well for long read data (Grabherr *et al.*, 2011; Prjibelski *et al.*, 2020).

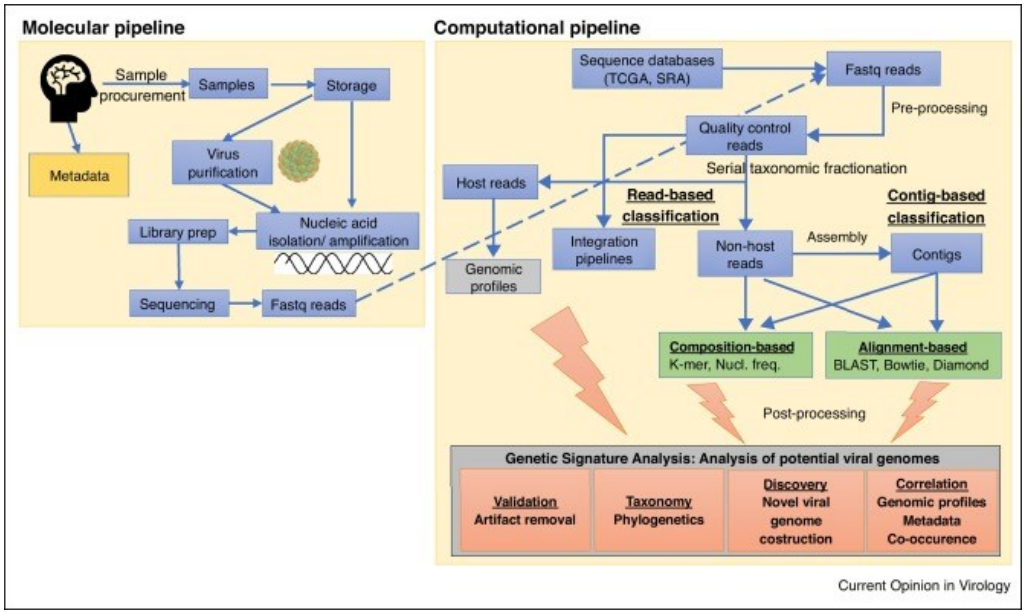


Figure GI.15 The basic workflow of HTS and bioinformatic virus discovery (Obtained from Figure 1 in (Cantalupo and Pipas, 2019)).

Once the sequencing has been performed, for virus discovery, there is a general approach outline that is followed by most virus discovery pipelines (Figure GI.15) (Cantalupo and Pipas, 2019). Sequences are either assembled straight away or reads that map to the host genome are removed before assembly. Then resulting contigs are classified using BLASTx against a small reference database of virus proteins. This is an alignment-based approach, but there is another classification approach which works with K-mer analysis (e.g. using Kraken2 (Lu and Salzberg, 2020)) (Figure GI.15). The final steps are to take the virus-like contigs and then perform a second classification step against a large reference database which has protein sequences from as many organisms as possible, to interrogate and remove any false positive sequences. The resulting viral contigs are then annotated to search for viral genes and then are classified using more comprehensive phylogenetics. Now this is not fool proof as viruses can be too genetically distant to those included in the used databases, or they may not share any genes with viruses that have already been discovered (Krishnamurthy and Wang, 2017; Obbard, 2018; Santiago-Rodriguez and Hollister, 2022). There is also the issue of viruses with segmented genomes which creates a layer of complexity, since classification may be possible for each segment, but then placing all of the segments together is harder when working with diverse samples (Varsani *et al.*, 2018). There are some suggested approaches that can be done bioinformatically, such as phylogenetic placement of each segment or co-occurrence analysis (Varsani *et al.*, 2018; Cantalupo and Pipas, 2019), but these have limits. There is another issue that haunts bioinformatic virus discovery, and that is host determination and replication of

viruses (Webster *et al.*, 2016; Obbard, 2018; Imrie *et al.*, 2023). Determining if a virus is replicating in a host is not straight forward in bioinformatics. For DNA viruses, metagenomics can tell you if a virus is present, but will not easily provide evidence of replication, but if you detect DNA viruses in metatranscriptomic datasets, then you can assume that it is replicating (Obbard, 2018; Porter *et al.*, 2019). For RNA viruses, the opposite approach can be done for viruses with DNA intermediates during replication, but other approaches need to be incorporated if not. For example, searching for negative sense and positive sense strands among the contigs, or comparing viral read abundance or co-occurrence across samples (Cobbin *et al.*, 2021; Warncke and Knudsen, 2022). To verify the host of a virus *in silico*, well designed sampling and small RNA analysis can be useful approaches (Aguiar *et al.*, 2016; Li *et al.*, 2016; Girardi *et al.*, 2018; Obbard, 2018; Obbard *et al.*, 2020). Viral bioinformatics is a rapidly evolving field, new tools are released regularly and already a wealth of information can be generated bioinformatically, but it can also provide a foundation for proceeding with molecular, microscopy and culturing techniques by providing some inference on the biology of the virus.

3.4. Paleovirology using previous viral infections

There are two broad types of virus infections in hosts, exogenous and endogenous (Rasmussen, 1997). Essentially, exogenous infections can transfer horizontally between individuals and endogenous infections are when proviruses transfer vertically in a hereditary fashion between hosts (Eiden, 2008). There are cases where viruses such as the endogenized koala retrovirus in koala bears can also transmit horizontally, producing an exogenous infection between host species (Fiebig *et al.*, 2006; Sornette *et al.*, 2009). Typically, viruses classified within Baltimore classes I to V (Figure GI.14) will produce exogenous infections and we can refer to these as exogenous viruses (EXVs) (Figure GI.16A) (Koonin *et al.*, 2021). Those within classes VI and VII (retroviruses) with the assistance of reverse transcriptases will integrate into the host's genome to become what is called a "provirus", usually followed by the rest of the infection cycle (Figure GI.16B) ((Rasmussen, 1997; Koonin *et al.*, 2021). Since proviruses are integrated into host genomes, when infecting genomes of gametal cells, this can lead to them being passed down to offspring transforming them from regular proviruses to endogenized proviruses (Rasmussen, 1997). Once passed down to the following generations, initially endogenized (pro)viruses can continue to replicate and express, or replicate silently without

expression in a lysogenic stage (Eiden, 2008). After a while, sequences of endogenous viruses can degrade, losing the ability to express and they transform from endogenized viruses to endogenous viral elements (EVEs), eventually losing functionality in all their genes (Katzourakis, 2013; Drezen, Leobold, *et al.*, 2017) (Figure GI.16C). The process of endogenization is not restricted to retroviruses but can also occur to genomes or genome fragments of from all types of viruses via alternative mechanisms (Katzourakis and Gifford, 2010; Holmes, 2011). While not typical of their replication cycle, non-retroviruses can either become proviruses (whole viral genome) or proviral elements (random gene segments) by being incorporated into a host genome (Drezen *et al.*, 2022). Similarly, if these integrations also occur in germline cells, they too can be transferred to offspring and be retained for generations.

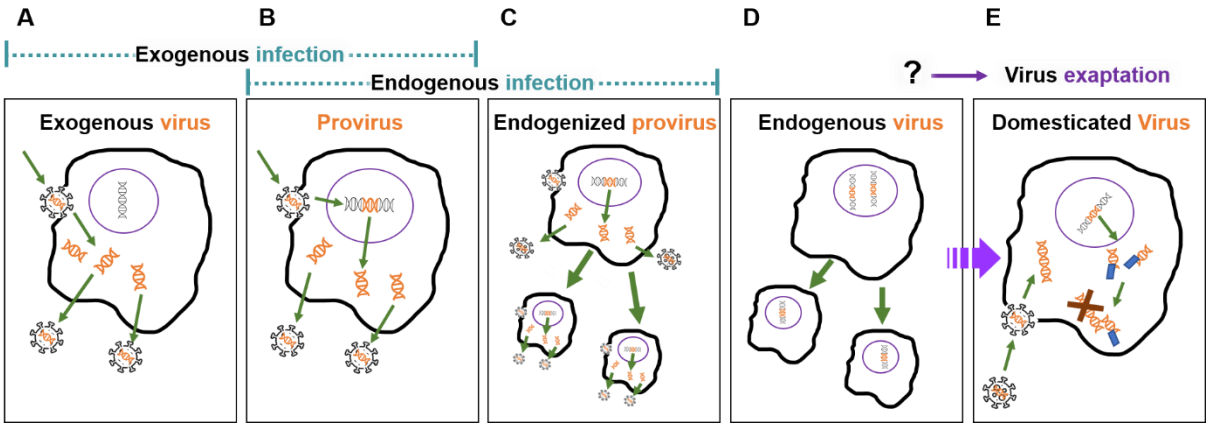


Figure GI.16 Infection type and endogenization of viruses. Viral infections can be considered as exogenous (**A**) and endogenous (**B** and **C**). Both exogenous viruses and proviruses can integrate into germ cell genomes becoming "endogenized proviruses" and passed down to future generations, referred to but sometimes can still be express infectious particles and cause diseases (**C** and **D**). True endogenous viruses often degrade and don't always express as a full virus or independent genes, becoming viral elements (**D**). Sometimes endogenous viruses/viral elements (**D**) can be conserved and domesticated via virus exaptation and expressed as functional genes, often to the advantage of the host (**E**). The orange colour highlights virus-related genetic material (DNA/RNA), while teal indicates infection and purple represents exaptation.

The first discovery of an EVE so to speak occurred in 1966 where capsid (Gag) and envelope (Env) proteins related to Rous sarcoma virus were found in chickens which tested negative for the virus (Weiss, 2013). While the evidence was there, the concept of EVEs was not really accepted until the 1970s with the discovery of reverse transcriptases, despite the fact that prophages had already been discovered in the 1950 (Temin, 1964; Weiss, 2006, 2013). Since then, studies have shown that a number of endogenization events had led to functional EVEs which are utilised by their hosts and have even played roles in the evolution in some hosts (Drezen *et al.*, 2022; Gilbert and Belliardo, 2022). In fact, some EVEs can still retain some

level of function and be used by their host for various applications, this is known as virus exaptation (Figure GI.16D) (Herniou *et al.*, 2013; Drezen *et al.*, 2022). The famous example of this is the development of placenta in mammals, due the integration of EVEs and domestication of Syncytin genes (Cornelis *et al.*, 2013). This has also been well-explored in mosquitos and drosophila flies in terms of immune defences against viruses (for references and examples see PIWI and DICER mentioned in section 2.3). Parasitoid wasps are a different example where nudiviral integrations are utilised in the offspring survival in their hosts (Herniou *et al.*, 2013; Cerqueira de Araujo, Huguet, *et al.*, 2022). Interestingly, in a parasite-host response fashion, there are cases where lepidopteran hosts utilized EVEs to combat parasitisation by parasitoid wasps using "parasitoid-killing factors" (Drezen, Josse, *et al.*, 2017; Gasmi *et al.*, 2021).

All of this falls under the field of paleovirology, where researchers can investigate EVEs hosts to explore host evolution, prehistoric virus-host interactions and the evolution of viruses (Katzourakis, 2017; Gilbert and Belliardo, 2022). Essentially EVEs can be viewed as virus fossils, where it is not required to find exogenous viruses in samples. Given that viruses such as RNA viruses genomes can have a high mutation rate, EVEs mutate more slowly potentially making it easier to make inferences on the evolution of specific viruses (Patel *et al.*, 2011; Aiewsakun and Katzourakis, 2015). In terms of virus discovery, prehistoric-virus host interactions can provide insight into viruses that were previously circulating in host populations. In insects specifically, ter Horst *et al.*, (2019) and Gilbert and Belliardo, (2022) found a plethora of EVEs in insect genomes, showing a diverse set of interactions between insects and viruses, even in insects where exogenous virus interactions have not been previously studied. The tricky part of paleovirology is that it is difficult to work on highly degenerated EVEs or those which are very distantly related to viruses currently infecting organisms. On the bright side, this also means that EVEs can also provide insights into viruses which are currently circulating in hosts, especially when EVE integrations are recent enough that not much genetic degradation has occurred, or in EVEs where they have been relatively well maintained, such as in the cases of domestication (Emerman and Malik, 2010; Patel *et al.*, 2011; Feschotte and Gilbert, 2012; Gilbert and Belliardo, 2022).

4. BSF diseases and pathogens known before 2021

There are already some reviews attempting to cover the welfare and health of BSF, not just in general, but also concerning specific rearing practices and pathogen risks (Joosten *et al.*, 2020; van Huis, 2021; Barrett *et al.*, 2022; Vogel *et al.*, 2022; Kortsmit *et al.*, 2023). On the topic of insects as food and feed, a gap in knowledge that has been highlighted is related to the poorly described pathobiome of BSF. Of the multiple reasons that black soldier flies (BSFs) are heralded as robust and extremely useful in industry and society, one reason that stands out is that they are capability of living on waste and not reported to be commonly affected by pathogens (A. van Huis, 2019; Tomberlin and Huis, 2020; Jensen and Lecocq, 2023). However, due to their life history as decomposers, this means that they are often in substrate that can be riddled with insect pathogens (Joosten *et al.*, 2020). To flourish in the full scope of the journey embarked on in this thesis, I wanted to paint a picture of the knowledge of BSF pathology by discussing entomopathogenic research on BSF until 2021. Despite the uptick in BSF research starting around 2005 (Figure GI.3), by 2015 there still had not been any evidence of BSF disease/associated-pathogens found or reported in scientific literature (Eilenberg *et al.*, 2015). It was only in 2018, where knowledge was released about a 2014 to 2016 survey reporting signs and symptoms occurring within a mass-reared BSF colony (Eilenberg and Jensen, 2018). This survey reported that a rearing facility had experienced "Elongated, rounded mature larvae, moving slowly before dying", and that they were able to resolve the issue by quarantining the affected larvae. At the time, Eilenberg and Jensen, (2018) and the rearing facility suspected that the cause of the signs and symptoms may possibly have been of bacterial origin. With this list in mind, during a search in the literature, four of the observations made about the larvae were similar to signs and symptoms mentioned with a syndrome associated with Idnoreovirus 3 (*Spinareoviridae*) in *Musca domestica* (Moussa, 1978) (Table GI.2). The gut of the BSF was not necessarily checked, and the sign of melanisation observed could have been difficult to spot since mature BSF larvae can normally be much darker in colour (Figure GI.5). While the syndrome observed in the BSF colony could not be fully matched with the syndrome induced by Idnoreovirus 3, there is the possibility that the cause behind the unknown disease, might have been of viral origin. While I would not suggest that it was Idnoreovirus 3 infecting the BSF, should this syndrome (Table GI.2) be observed again in BSF, there would be value in also checking for viruses in affected larvae and in performing post-mortem dissections.

Table GI.2 List of notable signs and symptoms forming a syndrome presented by Moussa, (1978) for Idnoreovirus 3 infection in *Musca domestica* and by Eilenberg and Jensen, (2018) for an unknown disease syndrome in BSF.

	Musca domestica	***Hermetia illucens* (BSF)**
Signs/Symptoms	**Idnoreovirus 3 symdrome**	**Unknown disease syndrome**
Elongated/extended larvae	Yes	Yes
Mature/final Instar death	Yes (Final)	Yes (Mature)
Rounding/abdominal-swelling	Yes (Abdominal)	Yes (Rounding)
Slowed movement/paralysis	Yes	Yes
White integument turning dark brown or black (melanization)	Yes	Unknown
Enlarged/ruptured midgut	Yes	Unknown
Pale brown midgut	Yes	Unknown

While Eilenberg and Jensen, (2018) only reported one occurrence of BSF disease in rearing, there was a video published by Symton BSF on 3rd of July 2016 which reported two different suspected diseases in BSF rearing (*Description of 2 diseases for Black Soldier Fly*, 2016). In the video, there was an effort to describe the signs and symptoms (Table GI.3), which display two different progressions resulting in colony collapse. Referring to the suspected fungal disease, the progression of events was rapid, with collapse occurring within five days after signs and symptoms were first observed, and they were able to successfully induce the same syndrome in more BSF. While it is presented as a fungal disease, when observing the dead larval corpses, they presented signs of decomposition, but no fungal growth was observed on the corpses or dying larvae, something which is often typical of filamentous fungal diseases (Kaya and Vega, 2012; Eilenberg *et al.*, 2015). What's more, is that once larvae hit the pupal stage, they generally developed into adults (*Description of 2 diseases for Black Soldier Fly*, 2016). Turning to the colony collapse where the suspect is unknown, the progression is slower, taking a few weeks but does ultimately end in collapse (Table GI.3). While it did not seem to spread from one container to another, it was striking that infestations of house fly and stable fly larvae were not affected. The observations together do present the possibility that two separate pathogens may be involved, especially since they (at least for the fungal disease) have been reported in other BSF farms, according to Symton BSF. However, before the two syndromes can be confirmed as diseases, Koch's postulates need to be established to verify the etiological causing agent. Symton BSF did claim to be taking further steps, but no reports have since been published.

Table GI.3 Description of signs and symptoms surrounding colony collapses reported by Symton BSF showcasing two suspected diseases experienced in some of their colonies in 2016.

	Signs and symptoms reported	
Observations	**Suspected fungal agent[#]**	**Unknown cause[#]**
Substrate humidity	Doesn't dry, even up to two weeks after larvae have died	-
Larval activity	Become lethargic	-
Location of dead larvae	Mostly on top of substrate	On top and beneath substrate
Spread of symptoms	Yes	No, either present in different containers or not
Rearing collapse	Yes	Yes
Substrate temperature	Cooler than normal	-
Larvae stop eating	Yes	-
Reproducible symptoms	Yes	-
Host specificity	-	Co-habiting house fly and stable fly larvae not affected.
Fungal growth	Colonizing substrate	Not growth observed at all
Fungus colour	White	-
Affected stages	Larval stages between neonates or prepupae	Larvae
Adult emergence	If they reach the prepupal stage	No
Progression after symptoms appear	Most are dead after about five days	Many larvae are still alive after one week.
Size of trays	Less than 1 m^2, unclear number of larvae	Larger bed, about 300 000 larvae
Treatment	Quarantine affected larvae and dispose, then clean equipment	

[#] A single dash indicates that nothing was mentioned in the video.

The first proper experimentation on entomopathogens and BSF can be attributed to (Tourtois *et al.*, 2017). Inadvertently, BSF were tested to see if they could be good hosts to rear nematode[4] species *Steinernema carpocapsae* and *Heterorhabditis bacteriophora*, this was part of the effort to help researchers find more efficient ways to mass-rear these nematodes for use in biological control. Comparing BSF to *Galleria mellonella* (greater wax moth), they looked to see if nematodes would be drawn to BSF when larvae were placed in soil, and found that this was the case for *H. bacteriophora*, but not *S. carpocapsae* (Figure GI.17) (Tourtois *et al.*, 2017). Interestingly, not only did wounding BSF larvae did not improve the host-tracking of *H. bacteriophora*, but it did not appear to increase colonisation of nematodes due to any reason other than allowing more nematodes to enter the body at the same time. The final set of findings was that BSF produced a fraction of nematode juveniles relative to the number produced by *G. mellonella* (Figure GI.17). One thing that can be noted by these findings was that the nematode populations were reared on *G. mellonella* prior to the experiments. When Alonso *et al.*, (2018) included BSF in their broader study including a few different insect host species, they additionally tested if by pre-exposing *G. mellonella* reared nematode juveniles to the homogenate of the different hosts would increase their virulence. While they found that the

[4] Microparasites such as nematodes are included within the term "pathogen" for the context of this thesis.

two tested species of nematode *S. carpocapsae* and *S. feltiae*, performed similarly in BSF after activation, the number of activated infective juveniles required to reach the lethal time needed to kill 50% of the BSF larvae by 5 days post-infection was 100 nematodes, compared to the 2000 and 5000 nematodes required for other species. This could have potential implications for the outcome of the host seeking experiments (Tourtois *et al.*, 2017).

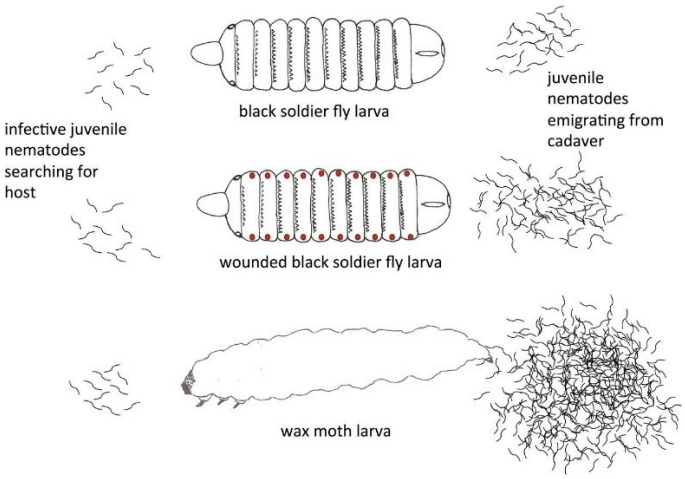


Figure GI.17 Testing the applicability of BSF as hosts for rearing entomopathogenic nematodes (Graphical abstract from Tourtois et al., (2017)).

Two years later in 2019, pathobiome research in BSF had started to gain momentum, beginning with a pamphlet produced by Lecocq, (2019). This provided examples of unexplained malformations in farmed BSF colonies, as well as a lab-based infection of *Beauveria bassiana* (Figure GI.18). There was no explanation behind the disorders, but it was evident at this point that there was a true gap in knowledge of BSF pathogens. Lecocq, (2019) tried to create more awareness that there were some more regular occurrences of disorders in BSF colonies that pointed to a need for research in BSF pathogens. To note, apart from the fungal growth, when looking at the collection of signs provided (Figure GI.18B & D), wing deformities are a notable sign of viruses such as deformed wing virus (Koziy *et al.*, 2019). Further pointing that viruses should be included when exploring cases of BSF disease.

Another two reviews tried to promote an advantage of mass-rearing BSF for food and feed and waste management, particularly highlighting their ability to cut pathogen levels in substrates (A. van Huis, 2019; van Huis *et al.*, 2020). There was still no enquiry if BSF could be affected by pathogens, rather that they seemed to be super tolerant/resistant towards them. For BSF welfare, a review by Joosten *et al.*, (2020) was a major move forward for BSF pathology and health. Not only did they expand on prior questions raised (Eilenberg *et al.*, 2015; Eilenberg and Jensen, 2018; Lecocq, 2019), but they tried to address the gaps in BSF pathogen research

by exploring known pathogens in other Diptera. Overall, a well-rounded perspective was provided on viral, protozoa, fungi and bacteria entomopathogens in other Diptera presented in relation to BSF (Joosten *et al.*, 2020).

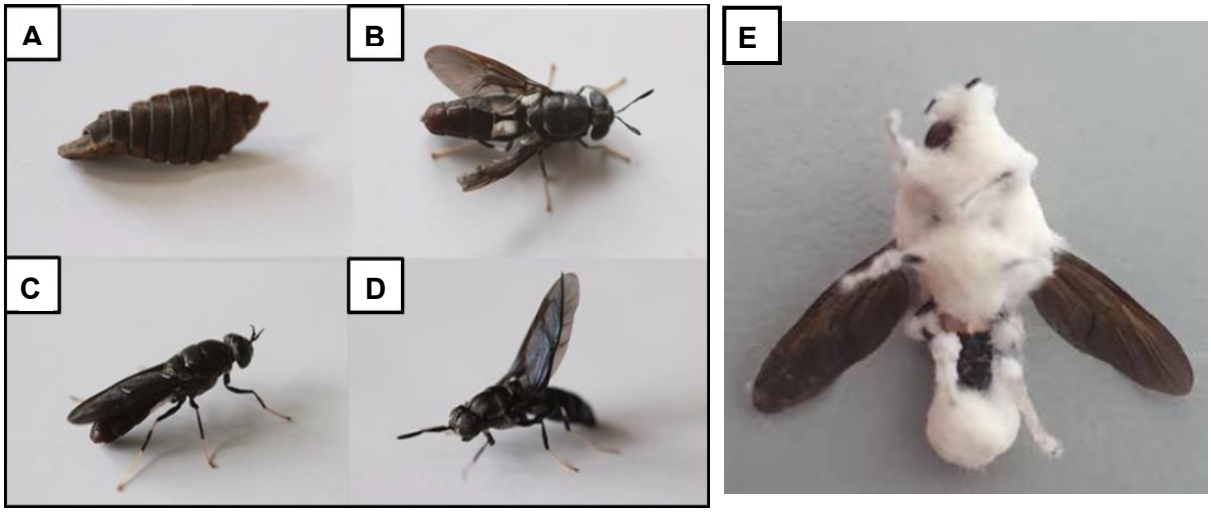


Figure GI.18 Signs of disorders in BSF with unknown cause and sporulation of *Beauveria bassiana*. **A)** Twisted pupa with no successful emergence or resulting in a twisted adult, **B)** Adult with malformation of one or both wings, **C)** Adult with reduced or malformed antennae and **D)** Adult with curved wings or wing-tips (Adapted from Lecocq, (2019)). **E)** Adult BSF that died due to an infection with *B. bassiana* and left post-mortem to observe fungal growth (Adapted from Figure 1 in Lecocq, (2019) and Lecocq *et al.*, (2020))

To examine the effects of a common fungal entomopathogen *Beauveria bassiana* in BSF, Lecocq *et al.*, (2020) performed inoculation and survival assays in adults (Figure GI.18E). They tested adult exposure to two different concentrations of *B. bassiana* conidia using a dip test, and found that an infection using a concentration of 10^8 conidia/ml was able to significantly reduce the lifespan of adults, with both males and females all dying before 12 days post inoculation (Lecocq *et al.*, 2020). It was also found that with the same concentration of conidia, an infection greatly reduced the number of egg clutches laid. However, there was no mention of verifying the mass or number of eggs within each cluster laid. Since BSF can lay eggs gregariously[5] and have multiple oviposition events, it may have been more appropriate to have also quantified the number of eggs within each cluster to cement the difference observed between treatments. Nonetheless, in laboratory conditions, this was the first study to test the effects of an entomopathogen in adult BSF.

[5] When females may lay eggs on top of or alongside egg clusters laid by other females combining them into one large cluster of eggs, usually indistinguishable from each other.

Although outside of the scope of this thesis, following reiterated calls for studies on BSF welfare, I also would like to briefly mention some specific parasitoids and a potential pest also found in BSF colonies when present. What can also be of particular interest is that by 2021, only three parasitoid wasp species were known to parasitise BSF, *Eniacomorpha hermetiae* Delvare 2019 (Delvare *et al.*, 2019), a *Trichopria* sp. Ashmead, 1893 (Bradley *et al.*, 1984; Caruso *et al.*, 2014) and *Dirhinus giffardii* Silvestri, 1914 (Devic and Maquart, 2015). Furthermore, although not much was inferred from these studies other than the identification, these parasitoid species appeared to have an affinity for BSF pupal lifestages and are suspected to potentially pose a hurdle for BSF farms at risk (Delvare *et al.*, 2019; Maquart, Willems, *et al.*, 2020). Lastly, there is not much scientific research into mites in BSF rearing facilities, Reguzzi *et al.*, (2021) noted that one of the mite species found in BSF rearing facilities, *Caloglyphus berlesei* Michael can vector microsporidia. While *C. berlesei* as a pest is not typically considered a principle threat to BSF in farms (Reguzzi *et al.*, 2021), this can open the discussion of disease vector and fomite[6] interactions in BSF farms, however, this is a topic that would be better discussed when more is known about the transmission of BSF-associated pathogens (Joosten *et al.*, 2020).

4.1. Other reports of symptoms in black soldier flies

There is still little public knowledge of reports on disease-like issues observed in BSF rearing facilities. Between 2019 and 2022, members of the Insect Doctors consortium have been contacted by eight rearing facilities reporting signs and symptoms found in their colonies, with some instances leading to high levels of mortality. While many reports were made in confidence, it reinforce claims by Symton BSF about the occurrence of disease-like issues in BSF farms being more regular than previously thought (*Description of 2 diseases for Black Soldier Fly*, 2016). This again emphasizes that is it crucial that research into BSF pathogens continues to be carried out, and since current work has only focused on bacteria, fungi and nematodes, there is much needed attention for virome exploration in BSF.

[6] An abiotic object which can carry a pathogen from one host/source to another.

5. Aims and objectives of thesis

5.1. Thesis outline

The introduction of this thesis has laid a broad foundation on how virus discovery and problems encountered in mass-rearing fit within the One Health narrative and global sustainability while trying to centre this around BSF. With the literature that was reviewed, some areas were found to be in need of attention: 1) Despite some exploration into the microbiome for over 10 years, the virome had been completely overlooked, 2) There is in fact enough evidence to break the anecdotal narrative that BSF are not really affected by pathogens, but little is being done to uncover their pathobiome, 3) The BSF industry is more than 15 years old, but there are few tools/approaches optimized to study interactions between BSF and their pathogens, compared to other models such as crickets, 4) There is no firm pathogen surveillance in BSF, and 5) There has been some work on BSF immunity, but this primarily focuses on bacteria and fungi and most studies focus on one or two immune genes.

Here I try to unravel the BSF virome, focussing on viruses likely to be entomopathogenic[7] and to better understand the interactions between some of these viruses and BSF and finally to develop and optimize techniques that can be used to better study viruses in BSF.

5.2. Research objectives and questions

Firstly, we wanted to know the diversity of viruses which can infect BSF either in natural settings or in BSF rearing facilities. Since no viruses had been previously described in BSF, the first step was the characterize the virome and was done in two parts.

Chapter 1, because there is no guarantee to find actively infecting viruses in BSF samples; we leaned into a paleovirological approach asking if there was evidence of BSF interacting with viruses at some point in their history as a species. Using publicly available BSF genomes, we characterized EVE sequences bioinformatically and also asked what EVEs were related to insect-infecting virus families. At the same time, we were also curious to see if we could find a virus in available BSF transcriptomic datasets that was related to any of the EVEs found in

[7] A pathogen that can cause disease specifically in insects

the process. This was done by using a virus discovery pipeline and then cross referencing viral-like sequences found with the EVE sequences.

Chapter 2, we wanted to evaluate the diversity of exogenous viruses in BSF that could be obtained from various sources and with the potential to be pathogenic to BSF. Originally, we were going to focus on BSF from BSF mass-rearing facilities and perform shotgun sequencing of their RNA but we also exploited a wealth of BSF shotgun HTS datasets available. Thus, I performed data mining to search for exogenous viruses in the metatranscriptomes obtained from BSF found online and also in metatranscriptomes that I had produced from BSF received from mass-rearing facilities. Given some of the challenges in data mining viruses in HTS datasets (discussed in Section 3.3 above) and the high number of BSF datasets, I then wanted to optimize the virus discovery process to decrease the amount of time it would take to screen the results from each BSF dataset for novel and discovered viruses. Additionally, we also wanted to develop cheap molecular tools to screen BSF for any viruses that we had found during the data mining and develop a foundation for viral surveillance of BSF-associated viruses.

Chapter 3, finding viruses and producing screening approaches and tools were not the only goals form this thesis. We also wanted to isolate and demonstrate if any of the discovered viruses could be pathogenic to BSF. With samples collected from BSF rearing facilities, those which tested positive for viruses were then used for virus purification using a general virus prepurification protocol which would allow use to also reinfect BSF with any BSF-associated viruses. Before this PhD, there were no protocols available to infect BSF with viruses. Therefore, we sought to develop reproducible inoculation protocols which would allow for further experiment in BSF to observed virus-host interactions. Using an available candidate, we wanted to observe if the virus would replicate in post inoculation BSF, had specific organ tropism and could transmit naturally between BSF using bioassays.

Because viruses can infect and replicate in hosts without being pathogenic, we would take any virus candidate that would successfully replicate in BSF and perform survival assays in BSF adults to determine if a reduction in adult lifespan could be observed in infected BSF. There was also an interest to test pathogenicity in a BSF separate colony to confirm if the pathogen could be pathogenic in flies from at least two different BSF rearing facilities, to cement that it can act as a natural BSF pathogen. For the final part of my study into BSF-virus interactions,

a transcriptomic study was performed to look at changes at the gene level between infected and non-infected flies. Lastly, to also observe what type of immune response could act against the virus candidate. Chapter 3 summarized the results related to virus-host interactions and pathogenicity testing.

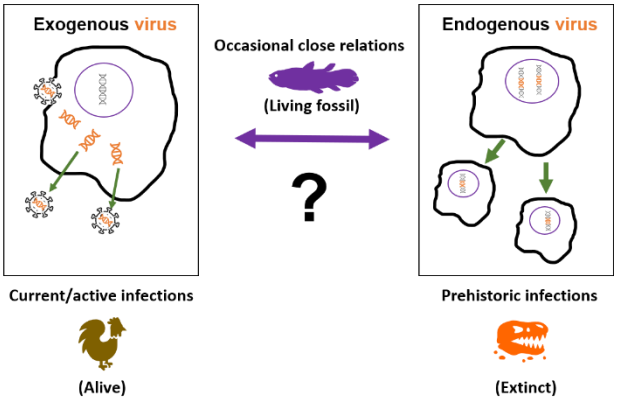
The general discussion highlights how my results integrate into the general context of One Health. I underscore how research in BSF virology has now unlocked a new world in BSF pathology and microbial studies. I then provide an update into BSF pathogen research and how it changes the narrative in world of BSF. It presents how the growth of the BSF industry shares some similarities and differences with the historical growth of the shrimp production industry and how this comparison could help the BSF industry improve current practices to not just increase awareness of BSF health and welfare, but also for the benefit of the BSF industry in the long term. Lastly, I draw into epidemiology/epizoology to explain where BSF research and industry could focus to better understand BSF viruses and the importance of prevention and management approaches within BSF farms.

End of section

Chapter 1: First evidence of past and present interactions between viruses and the black soldier fly, *Hermetia illucens*

Brief overview of chapter

Chapter 1 was published as a scientific article in June 2022 in the MDPI journal *Viruses*. No viruses had been reported in BSF until this study, and no viruses had been isolated from BSF before work for this chapter had started. Here, we primarily focused on identifying endogenous viral elements in three publicly available BSF genomes to understand historical viral interactions using a DIAMOND BLASTx-based EVE discovery pipeline. Independently we developed an exogenous virus discovery pipeline used to screen our own, and also publicly available, BSF metatranscriptomic datasets for viral sequences and to characterize their genomes and determine their phylogeny. We then compared findings from both pipelines and compared a novel *Totiviridae* sequence obtained from the transcriptomes to the *Totiviridae*-like EVEs. We then designed PCR primers to determine if we could find a well-conserved *Totiviridae*-like EVE sequence in the genomes of BSF that we had access to.



I formed the concepts behind this study together with my supervisors and co-author Clèment Gilbert. All the authors had formulated the methodology with Clèment and Carole Belliardo developing the endogenous viral element discovery pipeline and myself developing the exogenous virus discovery pipeline. I performed the bioinformatic analyses and laboratory work, with some assistance from colleague Jirka Manuel Petersen. Together Elisabeth Herniou and I performed the main investigation as well as writing the original draft. Salvador Herrero, Clèment and Elisabeth provided guidance throughout the study and all the authors reviewed and edited manuscript.

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Article: First evidence of past and present interactions between viruses and the black soldier fly, *Hermetia illucens*

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Abstract**:** Black soldier flies (BSFs, *Hermetia illucens*) are becoming a prominent research model encouraged by the insect as food and feed and waste bioconversion industries. Insect mass-rearing facilities are at risk from the spread of viruses, but so far, none have been described in BSFs. To fill this knowledge gap, a bioinformatic approach was undertaken to discover viruses specifically associated with BSFs. First, BSF genomes were screened for the presence of endogenous viral elements (EVEs). This led to the discovery and mapping of seven orthologous EVEs integrated into three BSF genomes originating from five viral families. Secondly, a virus discovery pipeline was used to screen BSF transcriptomes. This led to detecting a new exogenous totivirus that we named hermetia illucens totivirus 1 (HiTV1). Phylogenetic analyses showed this virus belongs to a clade of insect-specific totiviruses and is closely related to the largest EVE located on chromosome 1 of the BSF genome. Lastly, this EVE was found to express a small transcript in some BSFs infected by HiTV1. Altogether, this data mining study showed that far from being unscathed from viruses, BSFs bear traces of past interactions with several viral families and of present interactions with the exogenous HiTV1.

Keywords: black soldier fly; *Hermetia illucens*; *Totiviridae*; virus discovery; endogenous viral elements

1.1 Introduction

Among other entomopathogens, insect viruses appear to have plagued the insect rearing industry for over two centuries (Davidson, 2012). Although well established in Asia, upscaling cricket farming in North America and Europe has been hampered by outbreaks of cricket-infecting viruses belonging to the *Parvoviridae*, *Iflaviridae,* and *Iridoviridae* families that can cause a high level of mortalities and economic losses (Maciel-Vergara and Ros, 2017; Semberg *et al.*, 2019; Reverberi, 2020; de Miranda, Granberg, Low, *et al.*, 2021; de Miranda,

Granberg, Onorati, *et al.*, 2021). Besides recent virus discoveries in crickets, there is also evidence of a wide variation in viral prevalence among cricket populations (Semberg *et al.*, 2019; de Miranda, Granberg, Low, *et al.*, 2021; de Miranda, Granberg, Onorati, *et al.*, 2021; Duffield *et al.*, 2021). Similarly, in honey bees, *Apis mellifera*, viral pathogens affect both wild hives and apiaries globally (Beaurepaire *et al.*, 2020). These examples highlight the potential threats that insect mass-rearing facilities could encounter from insect-infecting viruses. To rapidly circumvent future epizootics, it is important to improve knowledge on the viruses that could emerge in insect mass-rearing facilities, especially in models for which basic information is lacking (Davidson, 2012; Maciel-Vergara and Ros, 2017).

The black soldier fly (BSF, *Hermetia illucens*, Stratiomyidae) is one such insect species for which mass-rearing is currently undergoing fast worldwide growth (Marshall *et al.*, 2015; Wang and Shelomi, 2017; van Huis, 2020) and from which no viruses have so far been described. Most research on BSFs focuses on rearing optimization and application as a prominent source of proteins for the food and feed industry (Marshall *et al.*, 2015; Wang and Shelomi, 2017; Joosten *et al.*, 2020; Tomberlin and Huis, 2020), as well as in biotechnology (Joosten *et al.*, 2020; Moretta *et al.*, 2020; Mouithys-Mickalad *et al.*, 2020; Tomberlin and Huis, 2020; D. Zhu *et al.*, 2020; Xia *et al.*, 2021). BSFs appear particularly robust and resistant to diseases. However, experimental laboratory infections using entomopathogenic nematodes, bacteria, and fungi can cause symptoms and mortality in BSFs (Tourtois *et al.*, 2017; Lecocq *et al.*, 2020; Klüber *et al.*, 2022; Manu *et al.*, 2022). But specific pathogens, including viruses, naturally infectious to BSFs have yet to be characterized (Joosten *et al.*, 2020). As reports of BSFs mortality are increasing, they demonstrate the need to investigate the virome of BSFs.

As a first effort to characterize the BSF virome, we searched for virus-derived sequences in publicly available genomic and transcriptomic datasets. Such an approach has proven useful when characterizing new, free circulating, exogenous viruses (EXVs), which may be co-sequenced with that of the host (Webster *et al.*, 2016; ter Horst *et al.*, 2019; Wu *et al.*, 2020; Wallace *et al.*, 2021). Screening host genomes may also lead to identifying endogenous viral elements (EVEs), i.e., complete or fragmented viral genomes that became integrated into the genome in the germline of their host and were vertically transmitted (ter Horst *et al.*, 2019; Gilbert and Belliardo, 2022). The characterization of EVEs within a robust paleovirological framework can yield unique insights into historical host-virus interactions (Patel *et al.*, 2011; Aswad and Katzourakis, 2012; Aiewsakun and Katzourakis, 2015; Barreat and Katzourakis, 2022; Gilbert and Belliardo, 2022). Insect genomes can host numerous and diverse EVEs,

including some domesticated EVEs that now fulfil key cellular functions (Gauthier *et al.*, 2021; Cerqueira de Araujo, Huguet, *et al.*, 2022). Here we took a two-step bioinformatics approach to explore the viruses that BSFs have encountered in the past (EVEs) or that currently infect BSFs (EXV) (ter Horst *et al.*, 2019; Wu *et al.*, 2020; Gilbert and Belliardo, 2022). In particular, this study primarily uses in silico analyses of publicly available BSF genomes and transcriptomes to ask: (1) whether there is evidence of viral endogenization in BSF genomes, and (2) whether any of these endogenized viruses could be related to any exogenous viruses found in BSF transcriptomes.

1.2. Materials and Methods

1.2.1. Datasets and Samples

Publicly available black soldier fly transcriptomes were downloaded from the NCBI SRA database (https://trace.ncbi.nlm.nih.gov/Traces/sra/, accessed between 30 December 2020 and 1 October 2021). Samples were from the following bioprojects: PRJEB19091 (Vogel *et al.*, 2018), PRJEB39181 (Bonelli *et al.*, 2020), PRJNA431833, PRJNA432297, PRJNA506627 (Zhu *et al.*, 2019), PRJNA573413 (Zhan *et al.*, 2020) and PRJNA575900 (Xu *et al.*, 2020). SRA toolkit (v2.10.9, (Sherry *et al.*, 2012)) was used to convert the SRA files and separate their sequence reads into forward and reverse read fastq files. Publicly available BSF genome assembly fasta files GCA_001014895.1 (BGA1) (Vicoso and Bachtrog, 2015), GCA_009835165.1 (BGA2) (Zhan *et al.*, 2020) and GCF_905115235.1 (BGA3) (Generalovic *et al.*, 2021) were also retrieved from NCBI assemblies and RefSeq repositories (Sayers *et al.*, 2022). Of note, BGA3 is assembled at a chromosomal level and includes seven chromosomes and the mitochondrial genome, leading to a total of 1.01 Gb in size and is predicted to be 98.6% complete. The length of the chromosomes themselves ranges from 15.4 to 222.1 Mb, with an N50 value of 180.46 Mb for scaffolds and 16.01 Mb for contigs (Generalovic *et al.*, 2021). Altogether the genome assemblies and transcriptomes represent BSF colonies from widespread origins. They were generated from BSFs reared in China (Zhu *et al.*, 2019; Xu *et al.*, 2020; Zhan *et al.*, 2020), Germany (Vogel *et al.*, 2018), Italy (Bonelli *et al.*, 2020), the United Kingdom (Generalovic *et al.*, 2021), and the United States of America (Vicoso and Bachtrog, 2015). In addition, BGA2 was also generated alongside transcriptomes produced in bioproject PRJNA573413 (Zhan *et al.*, 2020).

1.2.2. Screening BSF Genome for EVEs

Virus-like sequences were screened for in BSF genome assemblies BGA1, BGA2, and BGA3, except for *Retroviridae* and *Hepadnaviridae* using a DIAMOND-python- and R-based pipeline (archived on Zenodo https://doi.org/10.5281/zenodo.6554302). Sequence regions with viral hits according to the NCBI Identical Protein Groups (IPG) database (8 January 2021) were extracted. Endogenous viral element hits from the same family and closer than 50 bp to each other were considered to correspond to a single EVE, and then screened against the full NCBI nr database (22 January 2021) (Sayers *et al.*, 2022). An R-script (R v4.0.3, (R Core Team, 2013)) was used to summarize the results and obtain the taxonomical information of each EVE candidate using the packages 'taxonomizr' (v0.5.3, (Sherrill-Mix, 2019)) and 'data.table' (v1.13.6, (Dowle *et al.*, 2014)). EVE candidates were checked against *Drosophila melanogaster* proteins in the UniProtKB/Swiss-Prot database (30 March 2021) using BLASTx on NCBI for false-positive assessment. Only sequences that did not receive a *D. melanogaster* protein hit were retained as EVE candidates. Afterwards, to determine EVE locations on the BSF chromosomes, they were mapped onto BGA3 using the in-house mapping software of Geneious Prime (v2021.1-2022.02, https://www.geneious.com). To assess the level of identity between related EVE sequences, if one EVE sequence mapped to a chromosome after the first round of mapping, 20 kb regions which contained the EVE site were extracted from the genome sequence and then the EVEs were remapped to each of the extracted regions. The Geneious mapping parameters were set to the highest sensitivity, but also allowing for any structural variants, short insertions and deletions of any size while excluding any fine-tuning. Finally, to confirm the genomic origin of the EVE sequences, the EVE sequence outputs from the EVE pipeline were mapped to their originating contigs/scaffolds to obtain flanking sequences from the BSF genome of at least 50 bp, depending on contig size. To assess orthology of EVE locations, a megaBLAST on Geneious Prime was then used to determine if the EVE sequences and their flanking regions were found on BGA1, BGA2 and BGA3.

1.2.3. Exogenous Virus Discovery Using Transcriptomic Data

Quality checking of forward and reverse reads was performed using FASTQC (v0.11.9, (Andrews, 2010)). Trimmomatic (v0.39, (Bolger *et al.*, 2014)) was then used to filter reads and remove Illumina adapters. Contigs were then assembled using rnaSPAdes (v3.15.2, (Bushmanova *et al.*, 2019; Prjibelski *et al.*, 2020)). Virsorter2 (v2.1, (Guo *et al.*, 2021)) was used to identify and extract viral-like sequences from the SPAdes assembled contigs,

followed by using CheckV (v0.7.0, (Nayfach *et al.*, 2020)) to assess the estimated completeness and accuracy of the viral-like sequences. CheckV results were searched for sequences that firstly had no warning messages, and for sequences with predicted virus genes. BLASTx (RRID:SCR_001653) was then used on sequences that were considered to be viral by CheckV to screen for false positives and to identify the closest hits against the NCBI 'Non-redundant protein sequences' database (Sayers *et al.*, 2022). The default settings were used: 100 Max target sequences, parameters automatically adjusted for short input sequences, an expected threshold of 0.05, word size of 6, BLOSUM62 matrix, gap costs of existence: 11 and extension: 1 and with a conditional compositional score matrix adjustment and a filter for low complexity regions.

Open reading frames (ORFs) were annotated using Geneious Prime. These ORFs were translated to proteins, and conserved regions were searched using BLASTp (RRID:SCR_001010) with the same parameters as BLASTx, but without filtering for low complexity regions. To identify conserved regions, the E-value threshold was set to 0.01 with a maximum number of hits set to 500 against the CDSEARCH/CDD database.

1.2.4. Phylogeny of Totiviridae

On Geneious Prime, the GAG and POL ORFs of the selected totiviruses were reannotated as per the majority of annotated genomes. Afterwards, the translated amino acid (AA) residues of the POL and GAG ORFs were aligned using the MAFFT aligner (v7.45, (Katoh and Standley, 2013)) using the G-INS-i algorithm, a BLOSUM62 scoring matrix, and the default values of 0.123 for offset value and 3 for the gap open penalty. The alignments were trimmed at both ends, and alignment columns were retained if at least 10% of the sequences had an amino acid at that position. The alignments were concatenated. A maximum likelihood phylogenetic tree was reconstructed using the IQ-TREE 2 software (v2.1.3, (Minh *et al.*, 2020)), which allows for the selection of the best-fit evolutionary model for the data using the automated ModelFinder (Kalyaanamoorthy *et al.*, 2017) and robustness assessment by ultrafast (UF) bootstrap (1000 iterations) (Minh *et al.*, 2013) and Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT) (Guindon *et al.*, 2010). The tree was visualized using Geneious Prime.

1.2.5. Molecular Validation of EVE

Genetic material from three BSF larvae originating from three rearing facilities was extracted using the ZymoBIOMICS DNA/RNA Miniprep Kit (cat. R2002, ZYMO Research, Freiburg im Breisgau, Germany), and DNA was used for the PCR screening of TotiEVE and

to target the region which appears in some transcriptomes. To determine whether the endogenized *Totiviridae* sequence was present in available BSF colonies, the TotiEVE sequences, and hermetia illucens toti-like virus 1 (HiTV1) contigs were mapped to the BSF reference genome (GCF_905115235.1). Primers were designed using Primer 3 (v2.3.7, (Untergasser *et al.*, 2012)) on Geneious Prime with settings for Tm between 50 and 58 °C and GC content between 40 to 60 %, and GC clamp to 1 (Table S1.1). The max size of the target regions searched was 1000 bp, and 650 bp for the region where the short transcripts align (TotiEVE-STs). Amplification reactions were set up with 2.3 µL of 10x Diamond *Taq*® reaction buffer, 1.5 mM of Diamond *Taq*® $MgCl_2$ solution, 1 U of Diamond *Taq*® (TAQ-I021, Eurogentec, Liège, Belgium), 0.3 µM per each of forward and reverse primers, 5 µmol of each dNTP (NU-0010, Eurogentec, Liège, Belgium), 7 to 15 ng of DNA template, and filled to a total volume of 25 µL with RNase/DNase Molecular grade water, which was also used as a negative control. Thermocycling was as follows: Initial denaturing of 95 °C (5 min), then 30 cycles of 94 °C (30 sec), 50/52 °C (30 sec), and 72 °C (1 min), followed by a final extension of 72 °C (7 min) (Table S1.1).

Amplified samples were migrated on an E-Gel® EX 1% Agarose gel alongside a 1 kb Plus Express DNA Ladder (G401001 and 10488091, Invitrogen, Waltham, MA, USA). The bands of the anticipated size were cut out and extracted using the Thermo Scientific™ GeneJET Gel Extraction Kit (K0692, Thermo Fisher Scientific, Waltham, MA, USA). Gel purifications were quantified on a Qubit™ 2.0 Fluorometer with the 1X dsDNA High Sensitivity (HS, Q33231, Invitrogen, Waltham, MA, USA). From the extracted products, 0.41 to 7.76 ng of amplified product DNA underwent a second PCR amplification using the same PCR conditions as mentioned above. Unpurified products from the second PCR amplification were sent to Eurofins Genomics (Konstanz, Germany) for multi-directional sequencing on an ABI 3730XL sequencer (Thermo Fisher Scientific, Waltham, MA, USA). The raw sequence chromatograms were curated on Geneious Prime by aligning them to the reference genome for BSFs, interrogating any mixed peaks, and cross-referencing the bases with the alignments.

1.3. Results and Discussion

1.3.1. Orthologous EVE Sequences Found in Three BSF Genomes

Screening the three BSF genome assemblies using our bioinformatic pipeline revealed 27 viral sequences with close BLASTx hits to members of six viral families (Tables 1.1, S1.2, and S1.3). The size of the viral sequence hits ranged between 148 and 3750 nucleotides. All three BSF genome assemblies received hits related to *Partitiviridae* (5), *Parvoviridae* (7),

Totiviridae (10), and *Totiviridae*-like (3) viruses, while a hit related to either *Rhabdoviridae* or *Xinmoviridae* was found in BGA2 and BGA3 respectively. The pipeline also identified six sequences of porcine reproductive and respiratory syndrome virus (*Arteriviridae*) in the data from BGA2, but these were discarded because they were on contigs that all lacked flanking insect sequences and therefore likely resulted from sequence contamination.

The viral hit retrieved by the pipeline corresponded mostly to *capsid* (17) and *RNA-dependent RNA polymerase* (*RdRP*) (8) genes (Tables 1.1 and S1.2). There was a high nucleotide identity between the EVEs of the same viral families found in the three genomes (between 50 and 100%; Table S1.2). This suggested that some EVEs may be orthologous and predate the separation of the BSF populations from which the genomes originated. As BGA3 was assembled to chromosome level (Generalovic *et al.*, 2021), it was possible to infer the precise location of each of the nine EVEs on the BSF genome (Figure 1.1, Table S1.2). Overall, the EVEs were distributed on four of the seven BSF chromosomes (Figure 1.1; Tables 1.1 and S1.2).

Table 1.1 Summary of EVE sequences found in three BSF genomes.

Viral Family	EVE		BGA	Best Viral Hit	Viral Hit Similarity		Coordinates on BGA Contigs [§]
	Name	Location [†]			AA (%) [¤]	Protein [#]	
Partitiviridae	PartitiEVE	PT	1	Atrato Partiti-like virus 2	44.6	Capsid	JXPW01014295.1:343-1512
		np*	1		49	Capsid	JXPW01121853.1:492-1707
		np*	2		47.5	Capsid	VFFH01000694.1:2871386-2872788
		PT	2		54.8	Capsid	VFFH01002716.1:5535-6390
		PT	3		54.1	Capsid	LR899010.1:144460311-144461333
Parvoviridae	ParvoEVE	PR1	2	Clinch densovirus 1	66.7	Capsid	VFFH01002420.1:17403-17642
		PR1	3	*Densovirinae* sp.	39.2	Capsid	LR899010.1:21909312-21909550
		PR2	1	Haematobia irritans densovirus	45.3	Capsid	JXPW01295709.1:732-1063
		np*	2		62.5	ORF1	VFFH01002716.1:27731-27993
		np*	2		33.5	Capsid	VFFH01002716.1:22618-23330
		PR2	3		45.4	Capsid	LR899010.1:144484849-144485180
		PR3	3	Lone star tick densovirus 1	45.8	ORF1	LR899014.1:3976151-3976299
Rhabdoviridae	RhabdoEVE	Rh	2	Entomophthora rhabdovirus A	55.1	RdRP	VFFH01000694.1:2885224-2885413
Totiviridae	TotiEVE	T1	1	Leptopilina boulardi Toti-like virus	54.8	RdRP	JXPW01175605.1:2029-3362
		T2	1		34.6	Capsid	JXPW01052892.1:5735-9302
		T1	1		28.6	Capsid	JXPW01318472.1:69-1591
		T3	1		33.2	Capsid	JXPW01168285.1:326-1578
		T1	2		53	RdRP	VFFH01002277.1:524489-528239
		T2	2		30.4	Capsid	VFFH01001437.1:1443067-1446431
		np*	2		38.5	Capsid	VFFH01001390.1:32171-33459
		T3	2		36.7	Capsid	VFFH01001777.1:322470-323680
		T2	3		30.4	Capsid	LR899013.1:31694664-31698028
		T3	3		36.8	Capsid	LR899014.1:10621516-10622714
		np*	1	Linepithema humile toti-like virus 1	38.9	Capsid	JXPW01318876.1:130-1861
		T1	3	Dumyat virus	35.4	RdRP	LR899009.1:97208286-97212028
		np*	1		39.9	RdRP	JXPW01237450.1:1885-3346
Xinmoviridae	XinmoEVE	Xi	3	Lepidopteran anphe-related virus OKIAV50	61.6	RdRP	LR899013.1:111581535-111582826

[†] Location refers to the mapping location on the BGA3 genome with the names PartitiEVE (PT), ParvoEVE (PR1-3), TotiEVE (T1-3), RhabdoEVE (Rh) and XimnoEVE (Xi), as illustrated in Figure 1.1. [§] Coordinates on the contigs on the BSF genome assembly (BGA) from which each EVE originated. [#] The protein hit was named according to the type of protein that the original hit was associated with. A more comprehensive set of information and sequences can be found in Tables S1.2 and S1.3d, respectively. [¤] AA stands for amino acid

similarity. np (no position) indicates viral-like sequences related to other EVEs, but that could not be located on BGA3.*

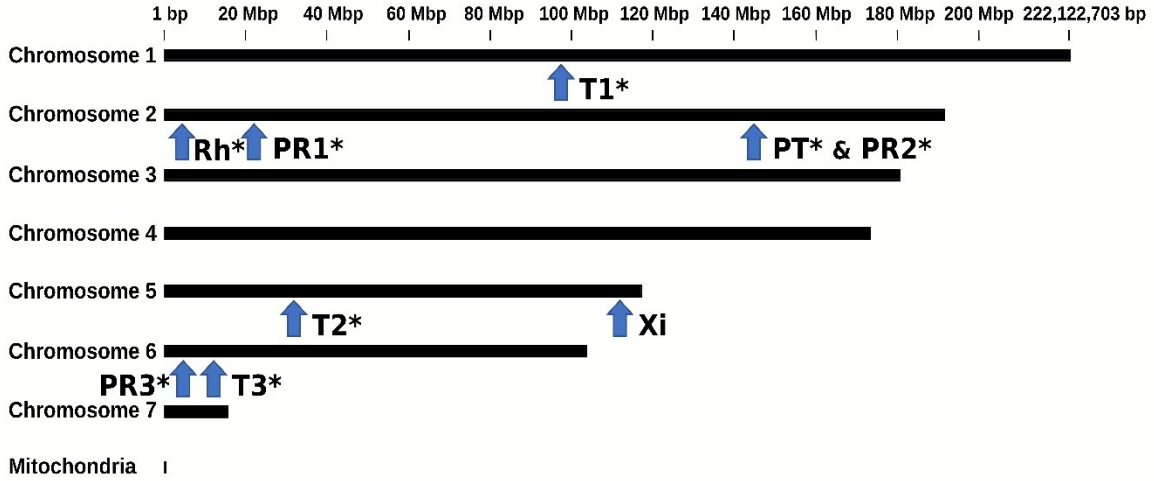

Figure 1.1 Positions of EVE candidates in the genome of BSF, assembled at the chromosome level (BGA3). Letters represent specific sites of related EVE sequences. The letters represent sites where certain EVE sequences obtained from BGA1, 2 & 3 that were related to *Partitiviridae* (PT), *Parvoviridae* (PR1, PR2, PR3), *Rhabdoviridae* (Rh), *Totiviridae* (T1, T2, T3) and *Xinmoviridae* (Xi) could be mapped. The asterisks indicate which EVE site was found on at least two of the three BGAs. Sequences can be found in Table S1.3.

Mapping showed that EVE sequences with above 98% identity to TotiEVE T1 were flanked by the same BSF genome sequences (Table S1.2). Furthermore, successful PCR amplifications were obtained from DNA of larvae coming from three independent BSF colonies using primers to target regions within TotiEVE T1, outside of TotiEVE T1, and overlapping TotiEVE T1 and its flanking region (Figure 1.1). Between data mining analyses and molecular analyses, this totivirus EVE TotiEVE T1 was found in BSF strains reared in the United States of America, United Kingdom, France, and China. This strongly suggests that TotiEVE T1 is present and orthologous in several reared BSF populations.

Likewise, EVE orthology in the three BSF genomes was confirmed through megaBLAST analyses of EVE sequence contigs with their 5′/3′ flanks, as well as the nine BGA3 20 kb BSF regions, including each EVE. TotiEVE T1, T2 and T3, PartitiEVE PT, and RhabdoEVE Rh were orthologous in the three BSF genomes (Figure 1.1). ParvoEVE PR 1 and PR3 were shared at orthologous positions in BGA 2 and 3, while ParvoEVE PR2 showed orthology for BGA 1 and 3. XimnoEVE Xi was only found in BGA3 (Tables 1.1 and S1.2). An additional six virus-like sequences (PartitiEVE2G1, TotiEVE1G1, TotiEVE2G1, TotiEVE10G2, ParvoEVE3G2, PartitiEVE3G2) were found in BGA1 and 2, but could not be located on BGA3, although they were 50 to 75% identical to some of the BGA3 EVEs. They could either be specific to the genomes they were found in or have been removed from the final BGA3 assembly.

This paleovirological analysis shows that the ancestor of the BSF populations from which the data originated (USA, China, UK) already harboured at least nine EVEs from the

Totiviridae, Parvoviridae, Partitiviridae, Rhabdoviridae, and *Xinmoviridae* families. These are genomic traces of past infections showing that BSFs have had interactions with exogenous viruses from these families. As three EVE loci each were detected for parvoviruses and totiviruses, and since the relatively low similarities between them (up to 75%) suggest independent endogenization, interactions between BSFs and exogenous viruses from *Parvoviridae* and *Totiviridae* may have been recurrent over evolutionary time (Figure 1.1, Tables 1.1 and S1.2).

1.3.2. Description and Phylogeny of HiTV1, an Exogenous Totivirus

As with 3750 nucleotides in length, TotiEVE T1 was the largest EVE we found in the BSF genome and comprised partial sequences of both capsid (GAG) and RDRP (POL) genes. We hypothesized that BSF interactions with totiviruses may have happened more recently than with other viruses. We thus investigated the presence of TotiEVE T1 relatives in BSF transcriptomes. The exogenous virus discovery pipeline provided a list of virus candidate contigs, which had leptopilina boulardi toti-like virus (LbTV) as the closest related hit, and this was validated by reciprocal BLASTx. These contigs were labelled as sequences of a new virus candidate, hermetia illucens toti-like virus 1 (HiTV1).

In the transcriptome SRR14339788, derived from larval gut (Table 1.2), a HiTV1 contig 1 of 7247 nt in length was found and subsequently used as the reference sequence for annotation (Figure 1.2) and phylogeny (Figure 1.3). *Totiviridae* genomes are double-stranded RNA and normally between 4.6 to 7 kbp in length (Wickner *et al.*, 2011), although genomes of *Totiviridae* such as LbTV (NC_025218.2) and papaya meleira virus (NC_028378.1), can be as long as 8021 bp and 8768 bp, respectively.

Table 1.2 RNAseq datasets containing HiTV1 contigs longer than 5 kb.

HiTV1 Contig	SRA Number (Bioproject)	Sample	Reference
contig 1	SRR14339788 (PRJNA573413)	Midgut of four-day-old larvae reared on food waste	Zhan *et al.*, 2020
contig 2	SRR10158821.1 (PRJNA573413)	Midgut of four-day-old larvae reared on cow manure	Zhan *et al.*, 2020
contig 3	SRR14339795 (PRJNA573413)	Midgut of eight-day-old larvae reared on cow manure	Zhan *et al.*, 2020
contig 4	ERR1801992.1 (PRJEB19091)	Five individual larvae	Vogel *et al.*, 2018
contig 5	SRR8242288 (PRJNA506627)	Egg Mass	Zhu *et al.*, 2019

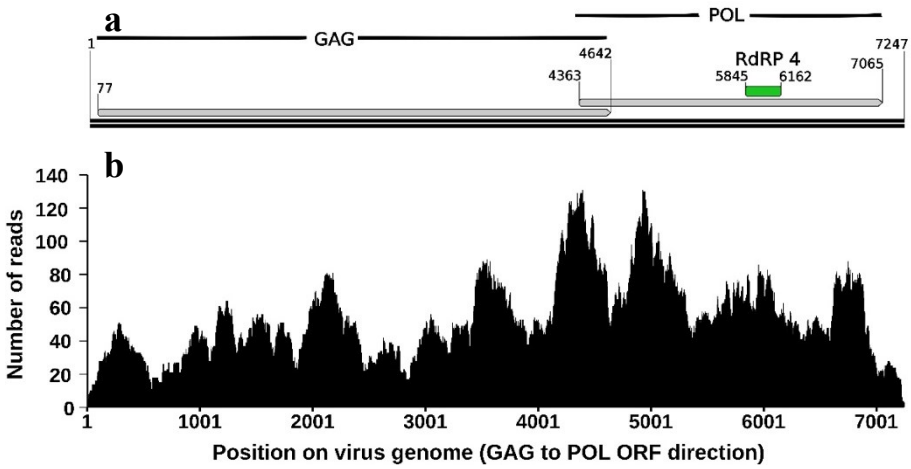

Figure 1.2 Annotation of HiTV1 genome sequence (a) and read coverage (b). (a) Numbers represent the nucleotide position, and two long horizontal black lines represent the double-stranded RNA sequence. A conserved RdRP 4-like domain (E-value of 1.46×10^{-4}, PF02123) was annotated in green. (b) Using Bowtie2 (Langmead and Salzberg, 2012) and SAMtools (Li *et al.*, 2009), raw sequence reads from the transcriptome SRR14339788 were mapped onto the HiTV1 contig 1, which resulted from a SPAdes assembly of SRR14339788 reads. The average coverage of the reads across the contig without gaps was 53.79x.

Like most *Totiviridae* (Wickner *et al.*, 2011), HiTV1 has a simple genome consisting of a GAG and a POL ORF, which encode for a capsid protein and an RdRP protein (Figure 1.2). The POL ORF contains an RdRP 4-like domain (PF02123), typically associated with viral families such as *Totiviridae* (Bruenn, 1993; Lu *et al.*, 2020). Read mapping of transcriptome SRR14339788 onto the HiTV1 contig 1 showed an average coverage of 53.79x across the length of the viral genome (Figure 1.2), which gives high confidence in the HiTV1 assembly.

Out of the 65 BSF RNAseq datasets screened, HiTV1 was found as nearly complete genome contigs in five transcriptomes from three distinct bioprojects, and as shorter contigs in another 48 transcriptomes (Table S1.3f). This finding suggests that HiTV1 is not a sequence contaminant but rather a genuine virus that may be found in particular BSF colonies. As these transcriptomes originated from BSFs reared in Germany, Italy, and China, HiTV1 appears globally distributed. (Tables 1.2 and S1.3a). Moreover, HiTV1 was found in the gut of individual BSF larvae reared under different conditions, a pool of five larvae, and even BSF eggs and adult antennae (SRR10233312.1).

The amount of HiTV1 RNA, which corresponds to the viral genome titre plus viral genome expression, was evaluated by mapping RNAseq reads. TPM values for *gag* and *pol* were quite similar in each sample, showing that both HiTV1 genes were present at similar levels, although more often slightly higher for *pol* (Table 1.3). Comparing these values to that of the *Actin-5C* gene, which is constitutively expressed in BSFs, showed that in all larval samples (contigs 1 to 4), HiTV1 only reached a ratio of 0.002–0.009 of RNA abundance, whereas in the egg mass (contig 5) this ratio reached 0.369–0.434 (Table 1.3). Several hypotheses could be invoked to

explain the different read counts and the ratio between larval and egg stages: (1) viral titre might be higher in the BSF colony the egg mass came from; (2) viral infection might be restricted in particular cells and thus viral reads diluted in larval BSF transcriptomes; (3) generally, the level of actin expression might be lower in the eggs compared to fully active larvae, and this could inflate the ratio (4) HiTV1 particles might accumulate in the eggs. Further work would be needed to determine the tissue tropism of HiTV1, although it was already found in dissected larval midguts, adult antennae, and egg masses.

Table 1.3 Sequence abundance of HiTV1 compared to BSF Actin-5C in different transcriptomes.

Name	Transcripts Per Million (TPM) [§]				
	HiTV1 Contig 1	**HiTV1 Contig 2**	**HiTV1 Contig 3**	**HiTV1 Contig 4**	**HiTV1 [†] Contig 5**
HiTV1 *pol*	9343	6192	3416	3590	204488
HiTV1 *gag*	6848	3473	2302	2494	240733
Actin-5C	983809	990335	994282	993916	554779
Ratio *pol*/*Actin-5C*	0.009	0.006	0.003	0.004	0.369
Ratio *gag*/ *Actin-5C*	0.007	0.004	0.002	0.003	0.434

[§] Transcriptomic reads mapped to each gene and virus contig using HISAT2 (Kim *et al.*, 2015) were filtered using SAMtools, and the TPM values were calculated for CDS regions using Geneious Prime.
[†] Contig 5 was found in the transcriptome of an egg mass, suggesting low cellular activity based on the number of reads found for *Actin-5C*.

Phylogenetic analyses based on the concatenated alignment of the GAG and POL ORFs show that HiTV1 belongs to a clade comprising previously discovered insect-associated totiviruses (Figure 1.3), including LbTV1 (from *Leptopilina boulardi*, wasp), LhTV1 (from *Linepithema humile*, ant), SoMIV (from *Solenopsis invicta*, ant) and ShoTV (from a pool of insects) (Martinez *et al.*, 2016; Lester *et al.*, 2019; ter Horst *et al.*, 2019; Baty *et al.*, 2020). This clade is highly supported and may constitute a new totivirus genus. It is related to PMCLV, which is found in fish, and to the *Giardiavirus* genus, within a larger group of totiviruses predominantly associated with arthropods (Figure 1.3). This group falls outside the clade formed by the genera *Totivirus*, *Trichomonasvirus*, *Victorivirus,* and *Leishmaniavirus*—which mainly infect protozoa or fungi (Wickner *et al.*, 2011). The host range of *Totiviridae* was historically thought to be restricted to fungi and protozoa but is continuously expanding with the discovery of novel *Totiviridae*-like sequences in Arthropoda (Wickner *et al.*, 2011; Nebbak *et al.*, 2021). This is also pushed by the growing association with the prevalence of *Totiviridae*-like contigs appearing in arthropod (and bat faeces) transcriptomes sequenced from samples collected from multiple sites and conditions (Zhai *et al.*, 2010; Yang *et al.*, 2012; Huang *et al.*, 2018; ter Horst *et al.*, 2019; Li *et al.*, 2020), as experienced in this study. Furthermore, experimental studies have demonstrated that arthropod hosts, either in cell culture or whole

models, can be infected by totiviruses (Zhai *et al.*, 2010; Yang *et al.*, 2012; Fauver *et al.*, 2016; Martinez *et al.*, 2016; Li *et al.*, 2020). Altogether, the phylogenetic analyses support the fact that HiTV1 is a totivirus that belongs to a clade that infects insects, and therefore it is highly likely that it infects BSF.

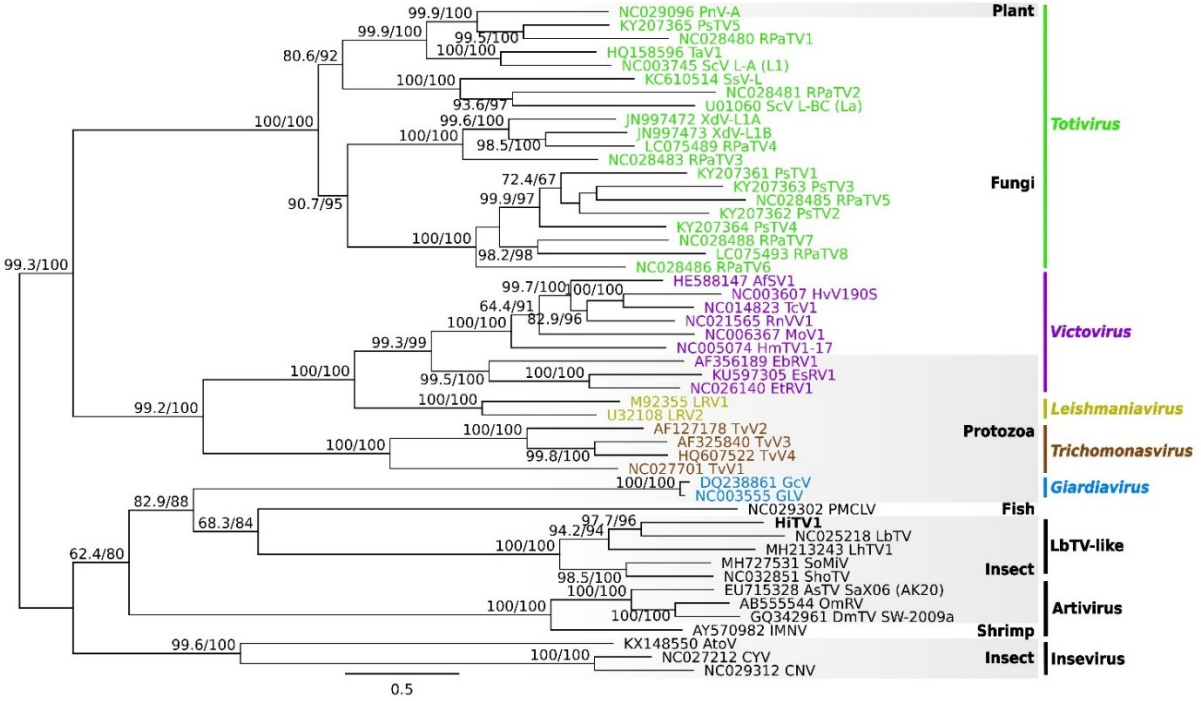

Figure 1.3 Phylogenetic relationships of HiTV1 within the family Totiviridae. The maximum likelihood tree is based on the concatenated alignment of POL and GAG ORF sequences. Hermetia illucens toti-like virus 1 is highlighted in bold. Phylogenetic robustness was assessed using Shimodaira Hasegawa-like approximate likelihood ratio test and UF bootstrap; values are reported for each node. Host groups and virus genera are reported to the right side of the tree. The text for virus genera which are currently accepted by the International Committee on Taxonomy of Viruses (ICTV) and related abbreviated virus names are coloured. Full names of abbreviations can be found in Table S1.4.

1.3.3. Expression of the Endogenous TotiEVE T1

Once they have been integrated into the genome of a host, EVE sequences usually remain as degraded fossil traces of past infections. However, some EVEs may become domesticated or exapted, in which case they can be expressed by the hosts (Katzourakis and Gifford, 2010; Iwasaki *et al.*, 2015; Gauthier *et al.*, 2021; Cerqueira de Araujo, Huguet, *et al.*, 2022; Drezen *et al.*, 2022; Gilbert and Belliardo, 2022). Short transcripts, termed TotiEVE-ST were found in some transcriptomes (Table S1.3a,b). Mapping these 243 to 395 nucleotide-long transcripts on the BGA3 genome showed that they derived from the RdRP region of the TotiEVE in locus T1 (Figure 1.4). These TotiEVE-ST sequences, except TotiEVE-ST2 and 5, completely overlapped (Figure 1.4). In mosquitoes, EVEs have been found to regulate viral infections through the piRNA system (Cerqueira de Araujo, Huguet, *et al.*, 2022). In BSFs, the TotiEVE-ST was only expressed in transcriptomes where HiTV1 contigs were also found, including as

contigs shorter than 5 kb (such as in transcriptomes SRR10158821, SRR14339789, SRR14339790, SRR14339791, SRR14339793, SRR14339795). However, these short transcripts only had an average identity of 71.15% to HiTV1 (Figure 1.5). Therefore, in the absence of experimental evidence, it is unclear whether they may exert any specific anti-viral activity against this totivirus.

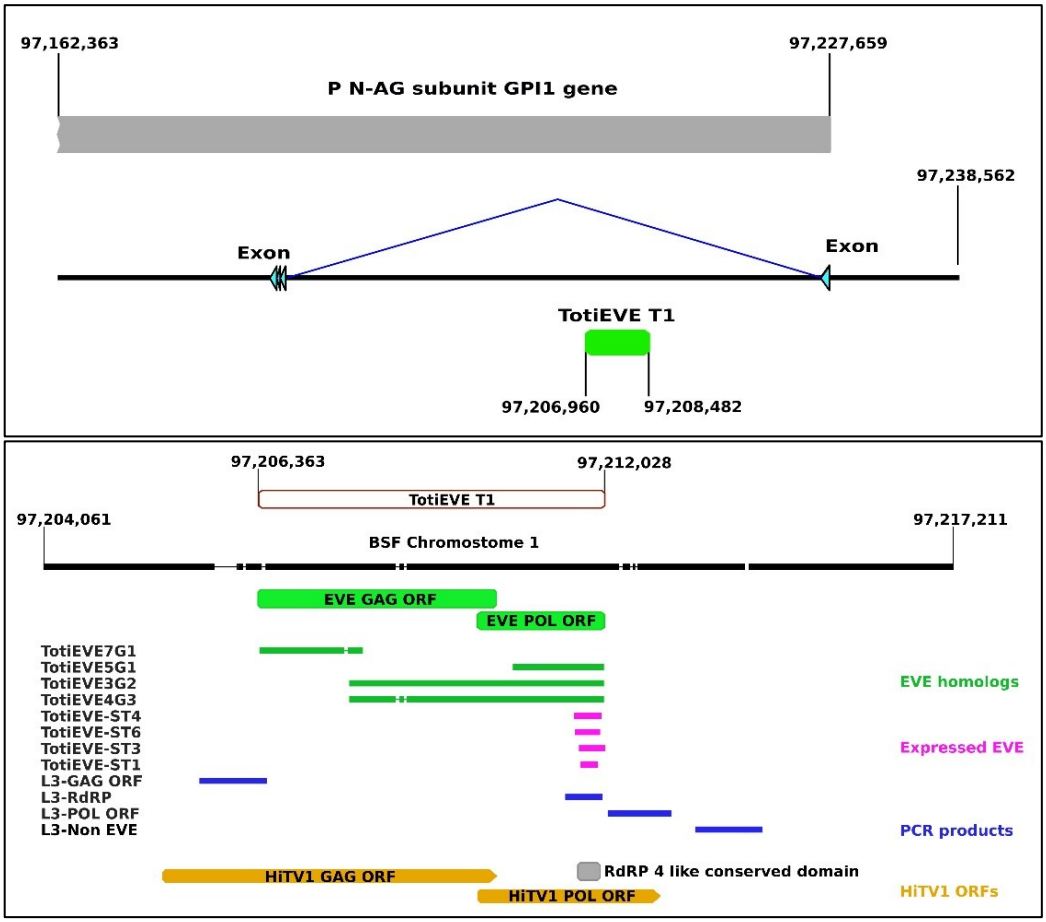

Figure 1.4 Mapping of Totiviridae-related sequences to TotiEVE T1 on chromosome 1 (black) on the BSF genome. TotiEVE T1 (green) is located between two exon sequences (light blue) of the BSF *Phosphatidylinositol N-acetylglucosaminyltransferase subunit GPI1* gene (P N-AG subunit GPI1 gene) (XM_038053365.1) (grey) (**top**). In the lower panel, a closeup of TotiEVE T1 (brown) displaying the mapping of the orthologs found in BGA1, 2, and 3 (green), the expressed short transcripts (pink), the amplified PCR products (dark blue), and an overlay of the HiTV1 ORFS showing that the EVE is shorter than the exogenous virus (orange) and that the expressed EVE aligns to the RdRP conserved domain (grey). The sequences were mapped to a 20 001 nt sequence that flanks TotiEVE T1. The sequences can be found in; Table S1.3.

1.3.4. Phylogenetic Relationships between the Exogenous HiTV1 and TotiEVEs

To investigate the genetic diversity found in the TotiEVE of locus T1 in relation to HiTV1, all the sequences detected in the genomes and transcriptomes through our bioinformatics pipeline or by PCR were included in a single alignment to determine their interrelationships regarding other totiviruses (Figure 1.4). The phylogenetic analysis revealed that HiTV1 was the sister group of all the EVE sequences, which were more than 97% identical to one another,

apart from TotiEVE-ST4, which was 92% identical to the others (Figures 1.4 and 1.5). This result suggests that HiTV1 is closely related to the exogenous ancestor of the orthologous TotiEVEs located at the locus T1 on chromosome 1 of genomes in all the BSF populations investigated.

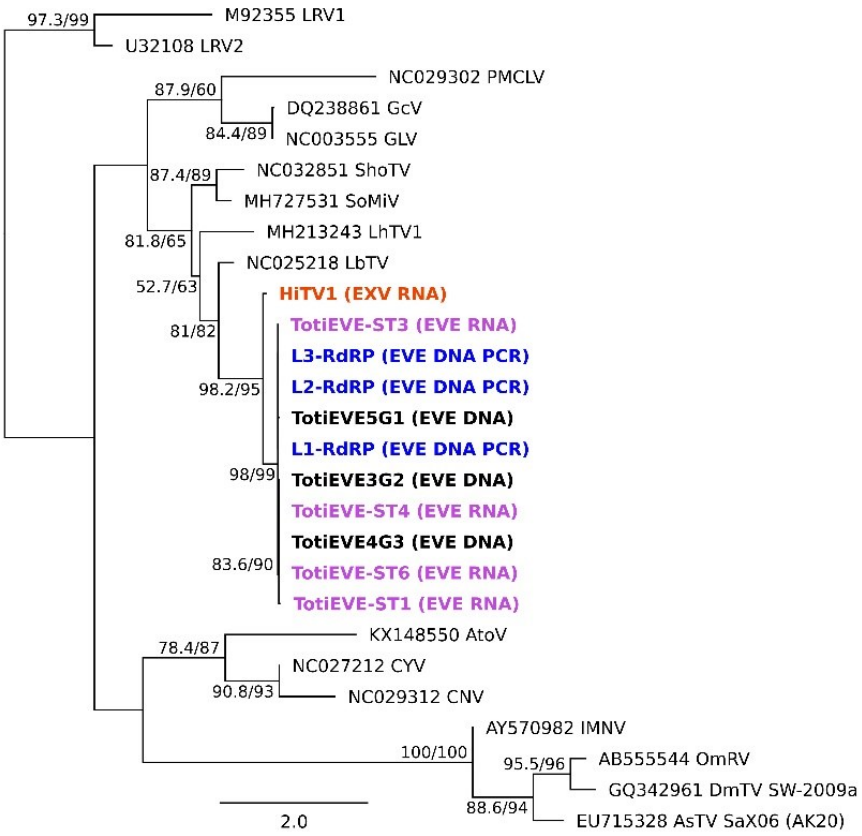

Figure 1.5 Phylogeny of HiTV1 and endogenous Totivirus sequences located in T1 on BSF genomes. Sequences in bold are associated with this study. HiTV1 is in orange, TotiEVEs located in T1 in the three genomes in black, the expressed TotiEVE ST found in four transcriptomes in purple, and PCR products of T1 from independent BSF samples. The outgroup consisted of LRV1 and LRV2 of the genus Leishmaniavirus within the Totiviridae family. Branch support for the maximum likelihood tree was in the order of SH-aLRT and UF bootstraps. Node values scoring lower than 50 were not displayed.

1.4. Conclusions

As the capacity for rearing black soldier flies develops worldwide, epidemiological models predict it is likely that pathogen outbreaks, including viruses, will occur (Anderson and May, 1981). No viruses have so far been discovered in BSFs. However, there is already a wealth of genomic and transcriptomic data that has been generated by different studies and is publicly available. Using public data as a starting point for metagenomic and metatranscriptomic approaches for discovering viruses has led to the discovery of a large wealth of RNA viruses (ter Horst *et al.*, 2019; Wu *et al.*, 2020; Edgar *et al.*, 2022), including in insects (Webster *et al.*, 2016; Gebremedhn *et al.*, 2020; Wallace *et al.*, 2021). One major point arising from these

discoveries has been the difficulty in determining if viruses can infect the organism in which they were discovered in the absence of small RNAseq data or laboratory experiments (Obbard, 2018; Wu *et al.*, 2020; Wallace *et al.*, 2021). Exploring EVEs present in the BSF genome offered additional insights into the virome of BSFs (Emerman and Malik, 2010; Patel *et al.*, 2011; Gilbert and Belliardo, 2022). The EVE results showed that members of five different viral families which are known to infect insects (Walker *et al.*, 2011; Martinez *et al.*, 2016; Cotmore *et al.*, 2019; Cross *et al.*, 2020; da Silva Ferreira *et al.*, 2020; Faizah *et al.*, 2020; Brinton *et al.*, 2021) have interacted with BSFs in the past. The clustering of HiTV1 among arthropod infecting *Totiviridae* and its presence across BSFs under different rearing conditions and locations provides strong evidence that BSFs are the natural hosts of this virus. Remarkably, the TotiEVE was found to produce a short transcript. The function of this TotiEVE-ST remains unclear. However, its presence alongside infections of HiTV1 could indicate that it might be involved in the immune response of BSFs against HiTV1. In conclusion, this study presents the first evidence of past and present virus interactions with BSFs.

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Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14061274/s1, Table S1.1 (Table S1) Details of primers used to amplify regions of the TotiEVE T1 (HITE) on chromosome 1 of BSFs; Table S1.2 (Table S2) Description of BSF EVE candidate sequences found in BSF genomes using the EVE pipeline. and their relation to EVE sites on the BSF genome; Table S1.3 (Table S3) List of sequences resulting from study and related transcriptome list.; Table S1.4 (Table S4): Abbreviations of virus names or accepted virus species names of those used in phylogenetic trees and paper.; Figure S1.1 (Figure S1) Short fragment detection of TotiEVE sequence and expressed TotiEVE-ST region by PCR amplification of BSF larvae extracted DNA (L) from three different rearing facilities.

Author Contributions: Conceptualization: C.G., E.A.H., R.D.P., and S.H. Methodology: C.B., C.G., E.A.H., R.D.P., and S.H. Investigation: E.A.H. and R.D.P. Investigation Guidance: C.G., E.A.H., and S.H. Writing-original draft preparation: E.A.H. and R.D.P. Writing-review and editing: C.B., C.G., E.A.H., R.D.P., and S.H. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Not applicable

Informed Consent Statement: Not applicable.

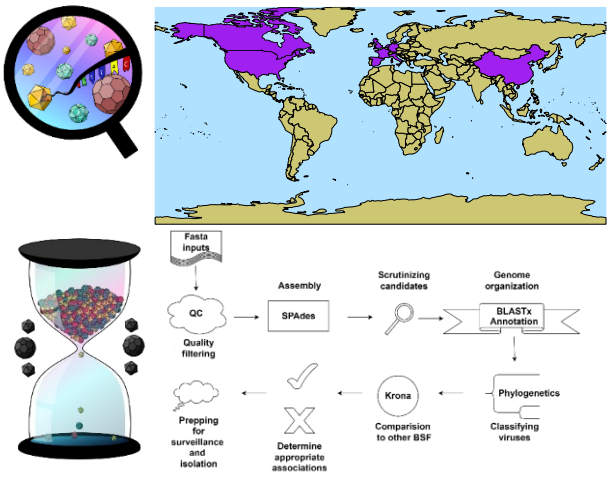
Data Availability Statement: Data in this study was mainly generated from publicly available NCBI bioprojects (https://www.ncbi.nlm.nih.gov/bioproject/, accessed on 16 April 2022). The sequence for HiTV1 contig 1 found in Table S1.3a, is also available in the Third Party Annotation Section of the DDBJ/ENA/GenBank databases under the accession number TPA: BK061373

Conflicts of Interest: The authors declare no conflict of interest.

Chapter 2: Optimization of screening methods leads to the discovery of new viruses in black soldier flies (*Hermetia illucens*)

Brief overview of chapter

This chapter was setup in the format of the Journal of Invertebrate Pathology where it will be submitted for review and publication. Chapter 1 found a diverse set of previous interactions with viruses and also led to the discovery of the first virus found in BSF, hermetia illucens toti-like virus 1. With a large amount of bioinformatic data still to be mined, this study sort to make a more comprehensive data sweep to search for more viruses associated with BSF. Upon realization that there were no available tools that could screen a large number of datasets simultaneously and efficiently for novel viruses, this study also aimed to improve currently available screening approaches to help alleviate this bottlenecking. Through this approach we found five new viruses which allowed us to develop preliminary cost-effective and rapid molecular screening tools for six viruses in BSF. This study also performed a semi global-scale screening of BSF using sequence read mapping and RT-qPCR approaches



Elisabeth and I developed the story behind this study, with inputs from Salvador Herrero. I wrote most of the scripts with some assistance from Fang Shiang Lim and co-author Alexandra Cerqueira de Araujo, who wrote the phylogenetic tree and sequence annotation visualization scripts. I performed the bioinformatic analyses with consultation input from Alexandra and Elisabeth. I performed the laboratory work and received guidance from Salvador and Annie Bezier when developing the qPCR assays. Co-authors Adly M. M. Abd-Alla and Frank Krupa provided sequencing data and BSF homogenate and in which they had detected viral sequence hits and assisted in review of the manuscript. They also performed some microscopy on their sample, but this was not included in the final manuscript. I wrote the original draft of the manuscript with guidance of Elisabeth, and input from Alexandra. Elisabeth and Salvador helped me to finalize the manuscript for the thesis and it was reviewed by all of the co-authors.

Article: Optimization of screening methods leads to the discovery of new viruses in black soldier flies (*Hermetia illucens*)

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Keywords: virus discovery, metatranscriptomics, mass-reared insects, insects as food and feed

Abstract

Virus discovery in mass-reared insects is a growing topic of interest due to outbreak risks and for insect welfare concerns. In the case of black soldier flies (BSF), pioneering bioinformatic studies have uncovered exogenous viruses from the orders *Ghabrivirales* and *Bunyavirales*, as well as endogenous viral elements from five virus families. This prompted further virome investigation of BSF metagenomes and metatranscriptomes, including from BSF individuals displaying signs and symptoms of disease. In this study, we describe five newly discovered viruses from the families *Dicistroviridae*, *Iflaviridae*, *Rhabdoviridae*, *Solinviviridae*, and *Inseviridae*. These viruses were detected in BSF from multiple origins, outlining a diversity of naturally occurring viruses associated with BSF. This viral community may also include BSF pathogens. The growing list of viruses found in BSF allowed the development of molecular detection tools which could be used for viral surveillance, both in mass-reared and wild populations of BSF.

2.1 Introduction

Recent research advances in virus discovery have underlined the vast diversity and potential impact of viruses, particularly in insects. Notably, high-throughput sequencing (HTS) has significantly enhanced our understanding of the insect virome and its rich diversity with over 2 600 unique viruses discovered in insects (Shi *et al.*, 2016; Wu *et al.*, 2020). However, insect viromes remain largely unexplored, including in economically important species like the black soldier fly (BSF, *Hermetia illucens* L. 1758) (Joosten *et al.*, 2020; Jensen and Lecocq, 2023). This gap is especially critical given the rising use of BSF for sustainable waste management and for food and feed, as viral infections within insect mass-rearing facilities could pose a significant risk to productivity and sustainability (Maciel-Vergara and Ros, 2017; Bertola and Mutinelli, 2021; de Miranda, Granberg, Onorati, *et al.*, 2021). Viruses have indeed caused mortalities in cricket mass-rearing facilities in which they are widespread, often hiding in the form of covert infections (de Miranda, Granberg, Low, *et al.*, 2021; de Miranda, Granberg, Onorati, *et al.*, 2021; Duffield *et al.*, 2021; Takacs *et al.*, 2023). In this context, expanding viral surveillance tools is essential to prevent disease risks.

Although BSF are considered resilient against pathogens, they could harbour viruses detrimental to their health (Jensen and Lecocq, 2023). Recently, paleovirological evidence has shed light on past virus interactions with BSF, with the identification of endogenous viral elements related to the families *Parvoviridae, Partitiviridae, Rhabdoviridae, Totiviridae* and *Ximnoviridae* (Pienaar *et al.*, 2022). Data mining of BSF transcriptomes also revealed three exogenous viruses (HiTV1, *Totiviridae* and two *Bunyavirales*) infecting BSF (Pienaar *et al.*, 2022; Walt *et al.*, 2023), although their impact remains undetermined. A recent taxonomical revision of viruses related to totiviruses, now places HiTV1 within the family *Lebotividae* (Sato *et al.*, 2023). There is still a need for further characterization of the BSF virome. Perusing deep-sequencing data outputs from established virus discovery pipelines is usually time-consuming and requires specific expertise. This leads to viral sequences being overlooked, a recurring issue in virus discovery (Obbard, 2018; Cobbin *et al.*, 2021; Waite *et al.*, 2022). A comprehensive high-throughput approach for screening deep-sequenced HTS samples could improve the efficiency and accuracy of viral sequence determination. One method involves screening datasets using mapping and cluster-based approaches, and then performing virus discovery on datasets positive for particular conserved viral domains, such as the RNA-dependent RNA polymerase (Wu *et al.*, 2020; Charon *et al.*, 2022; Edgar *et al.*, 2022; Olendraite *et al.*, 2023; Walt *et al.*, 2023). However, this approach initially restricts the search to few hallmark genes, which are not universally present in viruses and relies on the presence

of enough viral-like sequences in the datasets to be clustered within current software limitations (Li and Godzik, 2006; Edgar, 2010). Other dipteran models such as *Ceratitis capitata* (Hernández-Pelegrín *et al.*, 2022; Hernández-Pelegrín, Ros, *et al.*, 2024) and *Drosophila* spp. (Webster *et al.*, 2016), host fairly diverse viromes compared to BSF. This prompted for a more comprehensive search of BSF datasets and broadened the exploration to BSF from different sources.

This paper thus primarily aims to expand on the diversity of exogenous virus candidates potentially pathogenic to BSF and their prevalence across different BSF populations. To achieve this, we sought to (1) optimize approaches for more comprehensive screenings for viruses in large HTS dataset batches, (2) identify infectious candidates among virus circulating in BSF, (3) determine the prevalence of these viruses in different BSF colonies, and (4) develop PCR and qPCR screening methods for these new BSF viruses. By doing so, this study intends to contribute to sustainable BSF health in insect farming and develop approaches that could be applied across different mass-reared insect models with or without reference genomes.

2.2 Materials and methods

2.2.1 BSF Sampling

A total of 74 BSF transcriptomes were newly produced during this study from mass-reared colonies (NCBI bioprojects PRJNA1079553 and PRJNA841369). This includes 25 samples from company/research facilities in France, the Netherlands and Spain, including some BSF at various life stages displaying signs and symptoms of disease (Table S2.1). Additionally, 49 samples were obtained from three research colonies reared separately at IRBI (Université de Tours, France) and at CBP (Universitat de València, Spain).

Furthermore, 167 sequence read archive (SRA) datasets from 15 bioprojects containing metatranscriptomic and metagenomic data obtained from BSF were retrieved form NCBI using the keywords “Hermetia+illucens” and “black+soldier+fly” (22nd October 2023, Table S2.1).

2.2.2 Extraction and sequencing of genetic material

Total RNA and DNA were extracted from BSF larvae and adults with the ZymoBIOMICS DNA/RNA Miniprep Kit (cat. R2002, ZYMO Research, Freiburg, Germany). The DNA and RNA were quantified using the Qubit™ 2.0 Fluorometer (Invitrogen, Waltham, MA, USA). Total RNA preparations underwent sequencing, wherein poly-A containing mRNA molecules were purified and fragmented. Subsequently, a strand-specific cDNA library was prepared and sequenced either on a SP4 flow cell (2x 150 bp, paired-end) on a NovaSeq 6000 (Illumina, San

Diego, USA) at to Eurofins Genomics Germany GmbH (Ebersberg, Germany), or using a 101 bp paired-end read configuration (SRR28596310) at Macrogen, Inc (Seoul, Republic of Korea). These transcriptomes are archived in the NCBI bioproject PRJNA841369. Furthermore, 24 datasets comprising LncRNA and mRNA were sequenced together on a NovaSeq 6000 (2x 150 bp, paired-end reads) by Novogene (Beijing, China), and can be found under the NCBI bioproject PRJNA1079553.

Additional BSF samples were prepared for RT-qPCR analyses using Tripure (ref. 11667157001, Roche, Basel, Switzerland) according to the Trizol (Invitrogen, Waltham, MA, USA) manufacturer's protocol, with the exception that the RNA pellet was centrifuged at 7600 × g for 10 minutes during the 75% ethanol step. The RNA was quantified either by Qubit or Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

2.2.3 Bioinformatic analyses pipelines

2.2.3.1 Virus and host database construction (PoolingScreen)

Taxonkit (v0.3.0, Shen and Ren, 2021) and SeqKit (v0.10.0, Shen *et al.*, 2016) were used to extract viral (TaxID: 10239) and BSF (TaxID: 7108) proteins from the NCBI nr or nt database and create a host/viral database.

2.2.3.2 Virus discovery (PoolingScreen – a result collating screening pipeline and Lazypipe2)

A virus discovery pipeline (referred to as PoolingScreen) was adapted to incorporate elements of the endogenous viral element discovery pipeline (Pienaar *et al.*, 2022) and to improve the processing time of searching for viruses within a large collection of metatranscriptomic and metagenomic datasets (Figure 1). These adaptations included using DIAMOND BLASTx (v2.0.15.153, Buchfink, Reuter and Drost, 2021) instead of Virsorter2 (Guo *et al.*, 2021) to classify contigs against a BSF protein and viral protein database generated from the NCBI nr database (downloaded 15th May 2022). Contigs with viral-like hits were extracted using an AWK script and reclassified against the entire NCBI nr database (downloaded 15th May 2022). Krona (Ondov *et al.*, 2011) was used to visualize lineages of classified contigs after each classification step. Finally, sequences of contigs with viral hits were concatenated from each sample dataset into one file and assessed with CheckV (Nayfach *et al.*, 2020) (Figure 2.1, Figure S2.1).

Following this, metagenomic and metatranscriptomic datasets were processed using Lazypipe2 (Plyusnin *et al.*, 2023), which was originally coded to work with metagenomes, and was here

modified to process metatranscriptomes as well. This was done by modifying an option to use rnaSPAdes instead of the SPAdes and incorporating the output files of rnaSPAdes.

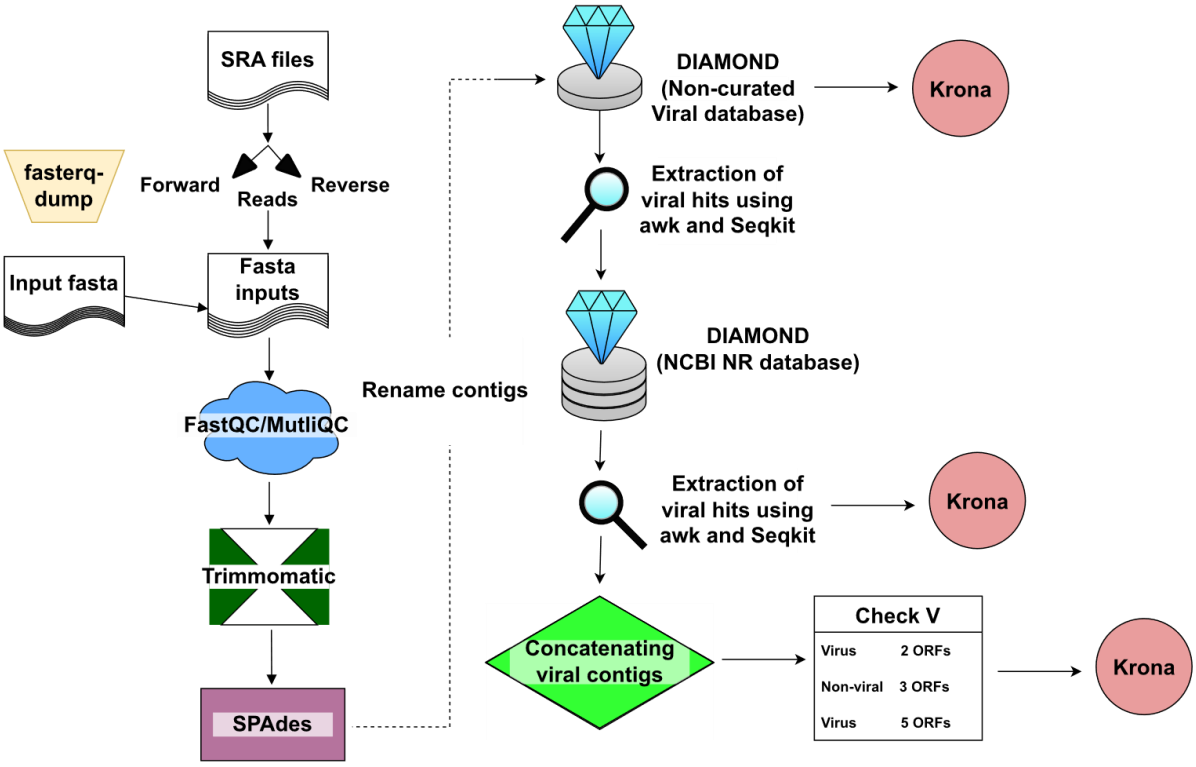

Figure 2.1 Flowchart of the approach followed by PoolingScreen pipeline. Presenting the succession of software and scripts used in this pipeline, as well as the different outputs that can be obtained.

2.2.3.3 Mapping for coverage and plotting heatmaps

To assess prevalence and coverage of viral candidates in BSF datasets, representative sequences were selected for each virus candidate. Fastq files for each metatranscriptomic /metagenomic dataset were compressed to "GNU zip" format and the reads were trimmed using fastp v0.23.2 (Chen *et al.*, 2018). Then the reads were mapped to the representative viral sequences using Bowtie2 v2.4.2 (Langmead and Salzberg, 2012). The mapped reads were imported into BAM format, sorted and indexed to extract mapped reads using samtools v1.9 (Li *et al.*, 2009). Using R v4.2.2 (R Core Team, 2013), an R-script was used to create a heatmap visualising the location of mapped reads of all the datasets simultaneously and the number of mapped reads, separately for each virus. For a virus to be considered present within a sequencing dataset, a threshold of 10 reads had to map across ORF regions. Another R-script was then used to generate a heatmap displaying the presence/absence of viruses within inspected BSF colonies and the output from the script was adjusted using Inkscape v1.1 to 1.2

(Harrington, B. et al (2004-2005), available at: inkscape.org). Finally, a co-occurrence analysis was performed in R to estimate the co-circulation of viruses within BSF colonies.

2.2.4 Viral genome annotation

Viral consensus sequences were annotated using the same approach as (Pienaar *et al.*, 2022) employing ORF finder on Geneious Prime v2021.1- 2023.1.1 (https://www.geneious.com) and BLASTp (RRID:SCR_001010). Additionally, Geneious InterProScan v2.0 and v2.1.0 (Quevillon *et al.*, 2005) plugins and HHpred (Söding *et al.*, 2005; Zimmermann *et al.*, 2018) were used to cross-check BLASTp results. For the virus genome contigs, the mapped reads were viewed on Geneious Prime and the mean coverage was calculated by Geneious Prime. The annotations were exported as GFF files and the coverage plots values were exported as csv files and imported into R to plot the genome annotations and coverage maps.

2.2.5 Phylogenetic analyses of viruses and BSF

The *Ghabrivirales* sequences and alignments were prepared using the same approach as (Pienaar *et al.*, 2022), although BLOSUM30 was used. For the *Picornavirales* tree, the RNA-dependent RNA polymerase (RDRP) conserved domain amino acid sequence from all the viral sequences was used to generate phylogenetic trees. The sequences were selected from the ICTV pages for *Dicistroviridae* (Valles *et al.*, 2017a), *Iflaviridae* (Valles *et al.*, 2017b), *Solinviviridae* (Brown *et al.*, 2019) by the15th August 2021. For the *Rhabdoviridae* tree, the untrimmed L open reading frame (ORF) sequences collection was downloaded from ICTV (https://ictv.global/, downloaded: 31st January 2024) resources webpage for *Rhabdoviridae* and aligned to the L ORF of the *Rhabdoviridae* virus. The alignments for the *Picornavirales* and *Rhabdoviridae* trees were obtained using MaFFT v7.45 (G-INS-I, BLOSUM62) (Katoh and Standley, 2013). The maximum-likelihood trees were inferred using IQ-TREE 2 software v2.1.3 (Guindon *et al.*, 2010; Minh *et al.*, 2013, 2020; Kalyaanamoorthy *et al.*, 2017). For all of the trees, IQ-TREE 2 chose "Q.pfam+F+I+G4" as the model of best fit. All the trees were visualized using a R-script.

2.2.6 Molecular detection assays

RT-qPCR and RT-PCR protocols were designed to detect BSF viruses. Primer sets for both RT-qPCR setups (Table S2.2) were designed using Primer3Plus (Untergasser *et al.*, 2012) with the default setting for RT-qPCR. The product size was set between 100 and 200 bp, and the GC clamp was set to 1. The thermodynamic parameters were followed the (Breslauer *et al.*,

1986) method and the salt correction was set to (Schildkraut and Lifson, 1965). The primer sizes were between 18 and 23 nucleotides, GC content was between 40% and 60% and the minimum primer melting temperature was set to 60 °C and the maximum to 65 °C.

When possible, log10 primer efficiencies for RT-qPCR were calculated using 5 to 7 dilution points of purified RT-PCR products (~1 kb in size) for detected virus candidates. Log2 dilutions were used for HiSV since its abundance was relatively low. Additionally, log10 efficiencies were calculated using 7 dilution points for samples that tested positive for the corresponding virus candidate (Table S2.2B). For viruses detected by RT-qPCR, a representative product for each virus underwent Sanger sequencing at STABvida (Caparica, Portugal) to confirm positive detection of the target sequence. This allowed optimisation of RT-qPCR conditions (Table S2.3). Primer sets used for RT-PCR assays were designed as in (Pienaar *et al.*, 2022) using Primer3 v2.3.7, (Untergasser *et al.*, 2012) (Table S2.4) to amplify ~1kb fragments using conditions summarized in Table S2.5.

2.2.7 Data and scripts availability

The versions of the scripts (including R scripts) used can be found on Zenondo (https://zenodo.org/doi/10.5281/zenodo.12740863). For R, the following packages were used: ape v5.7-1 (Paradis and Schliep, 2019), aplot v0.1.10 (Yu, 2023), BiocManager v1.30.21.1 (Morgan and Ramos, 2023), Biostrings v2.66.0 (Pagès *et al.*, 2022), broom v1.0.4 (Robinson *et al.*, 2023), ComplexHeatmap v2.14.0 (Gu *et al.*, 2016; Gu, 2022), cowplot v1.1.1 (Wilke, 2020), data.table v1.14.8 (Barrett *et al.*, 2024), devtools v2.4.5 (Wickham *et al.*, 2022), dplyr v1.1.0 (Wickham, François, *et al.*, 2023), GenomicAlignments v1.34.1 (Lawrence *et al.*, 2013), GenomicRanges v1.50.2 (Lawrence *et al.*, 2013), ggnewscale v0.4.9 (Campitelli, 2023), ggplot2 v3.4.1 (Wickham, 2016), ggtree v3.6.2 (Yu, 2020, 2022; Yu et al., 2018, 2017), grid v4.2.2 (R Core Team, 2022), gridExtra v2.3 (Auguie, 2017), phytools v1.9-16 (Revell, 2012), plyr v1.8.8 (Wickham, 2011), plotly v4.10.1 (Sievert, 2020), readxl v1.4.2 (Wickham and Bryan, 2023), Rsamtools v2.14.0 (Morgan *et al.*, 2022), reshape2 v1.4.4 (Wickham, 2007), svglite v2.1.1 (Wickham, Henry, *et al.*, 2023), tidytree v0.4.2 (Yu, 2022), treeio v1.22.0 (Wang et al., 2020; Yu, 2022), viridisLite v0.4.1 (Garnier *et al.*, 2022), writexl v1.4.2 (Ooms, 2023).

2.3 Results

2.3.1 BSF host diverse RNA viruses

Candidate virus sequences were obtained from screening 199 metatranscriptomic and 4 metagenomic samples collected from different BSF life stages, anatomy, or frass (Table S2.1). Sequencing depth ranged from 323K (SRR9068903) to 99M reads (SRR18283696) with an average of 34M reads. The mean percentage of BSF reads within each dataset was 90.05% of the total reads, but seven datasets (SRR21686212, SRR21686214, SRR21686215, SRR9068902, SRR9068904, SRR9068905 and SRR9068906) had fewer than 1% (Table S2.1). Virus screening using the PoolingScreen pipeline retrieved contigs for five novel insect viruses: *Hermetia illucens* insevirus (HiInV), *Hermetia illucens* cripavirus (HiCV), *Hermetia illucens* iflavirus (HiIfV), *Hermetia illucens* solinvivirus (HiSvV) and *Hermetia illucens* sigmavirus (HiSgV) (Table 2.1). Additionally, contigs related to BSF uncharacterized bunyavirus-like 1 (BuBV1) were obtained, as well as those matching *Hermetia illucens* lebotivirus (previously identified as *Hermetia illucens* toti-like virus 1 in (Pienaar *et al.*, 2022). Near-complete genomes were assembled for HiInV (5 839 nt), HiCV (9 364 nt), HiSvV (10 861 nt) and HiSgV (11 727 nt), but not for HiIfV, for which only a partial RdRP fragment (1 127 nt) was obtained.

Table 2.4 List of exogenous virus sequences* found in BSF metatranscriptomes

Virus name†	Acronym	Isolate	Viral Order	Viral Family	Genome type	Genome completeness	Length (nt)	Average coverage
Hermetia illucens lebotivirus§	HiLbV		*Ghabrivirales*	*Lebotiviridae*	dsRNA	Near complete	7 247	54
***Hermetia illucens* insevirus**	**HiInV**	**EU**	***Ghabrivirales***	***Inseviridae***	**dsRNA**	**Near complete**	**5 839**	**111**
***Hermetia illucens* cripavirus**	**HiCV**	**CHN**	***Picornavirales***	***Dicistroviridae***	**+ssRNA**	**Near complete**	**9 364**	**622**
***Hermetia illucens* iflavirus**	**HiIfV**	**EU**	***Picornavirales***	***Iflaviridae***	**+ssRNA**	**Short fragment**	**1 127**	**12**
***Hermetia illucens* solinvivirus**	**HiSvV**	**EU**	***Picornavirales***	***Solinviviridae***	**+ssRNA**	**Near complete**	**10 861**	**147 343**
***Hermetia illucens* sigmavirus 1**	**HiSgV**	**EU**	***Mononegavirales***	***Rhabdoviridae***	**-ssRNA**	**Near complete**	**11 727**	**105**
BSF nairovirus-like 1#	BNaV1		*Bunyavirales*	*Nairoviridae*	-ssRNA	Three segments	922 – 4 543	N.P.
BSF uncharacterized bunyavirales 1#	BuBV1		*Bunyavirales*	Non-assigned	-ssRNA	Two segments	853 – 5 696	N.P.

** The nearest relative of these virus candidates was an arthropod/insect infecting virus. †Virus candidates detected in this study are mentioned in bold. Sequences obtained previously §identified as Hermetia illucens toti-like virus 1 in* (Pienaar *et al.*, 2022)*, #*(Walt *et al.*, 2023).

2.3.2 Structure of virus genomes and coverage maps

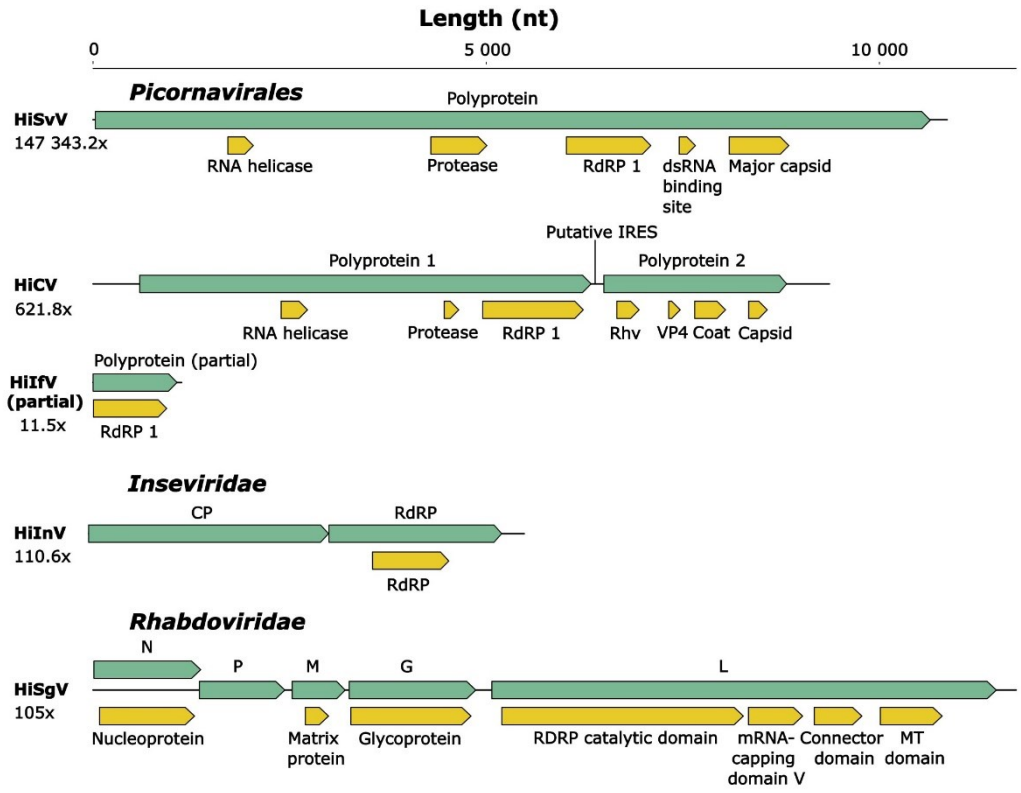

Figure 2.2 Genomic annotation of five newly discovered viruses. Green arrows indicate open reading frames (ORFs) and yellow boxes represent protein families and conserved domains of putative proteins.

Hermetia illucens iflavirus had an 11.5x sequence coverage from which a genome fragment could be assembled (Figure 2.2). The remaining contigs for each of the other four virus candidates had mean coverages ranging from 105x (HiSgV) to 147 343x (HiSvV) (Figure 2.2, Figure S2.2 and Figure S2.3). Apart from the HiInV and HiIfV contigs, multiple putative proteins and conserved domains were annotated for HiSvV, HiCV and HiSgV (Figure 2.2). Most conserved viral protein domains and families were found within the ORFs using InterProScan for HiInV, HiCV, HiIfV and HiSvV (Figure 2.2). However, the putative major capsid-like protein region of HiSvV (probability: 99.21%, E-value: 7.8e-10) was found using HHpred. For HiSgV, domains were found in the L and N ORFs, while matrix protein and glycoprotein family hits were found in ORFs M and G, respectively. Although no protein domain or family could be detected for the ORF in between ORFs N and M, it was assigned as the "P" ORF after cross-referencing the sigmavirus genome structure on ICTV. While an additional ORF (X) can be found in some other sigmavirus genomes (Walker *et al.*, 2022), it was not observed in the genome sequence of HiSgV. For HiCV, a short motif "UGAUCU" 36

nt upstream of a "UUAC" motif suggests the presence of an internal ribosome entry site (IRES), typical of cripaviruses, in the untranslated region between the two polyprotein ORFs (Valles *et al.*, 2017a) (Figure 2.2).

2.3.3 Phylogeny of candidate BSF viruses

Phylogenetic trees were inferred to determine the relationship of the newly discovered virus sequences and to assign them to taxonomical classification if possible (Figure 2.3, Figure 2.4 and Figure 2.5).

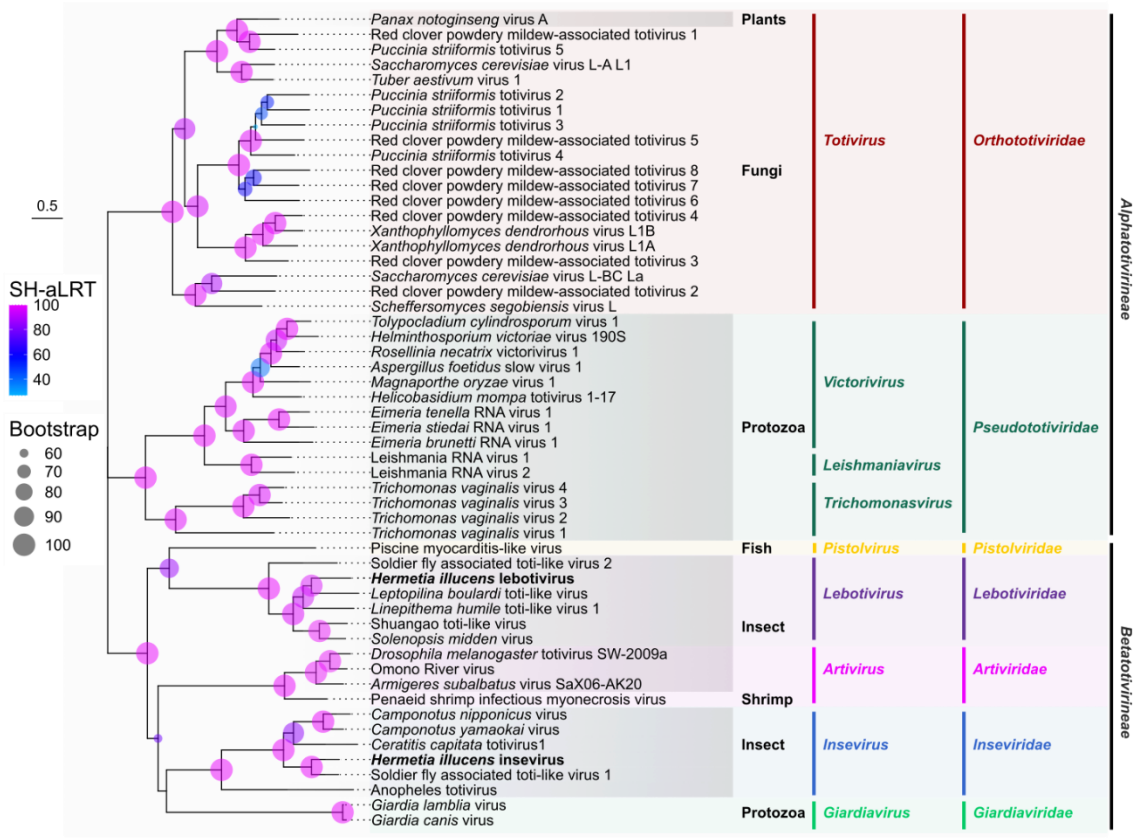

Figure 2.3 Phylogeny of the order *Ghabrivirales* showing the relationships of the two viral sequences found in BSF (in bold). Branch supports are given by bootstrap (node circle size) and Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; node circle colour) values. Sequence accession numbers can be found in Table S2.6.

Focusing on the two BSF viruses related to the order *Ghabrivirales*, we found that they belong to two recently established viral families *Lebotiviridae* (HiLbV) (Pienaar *et al.*, 2022) and *Inseviridae* HiInV (Figure 2.3). Both BSF viruses show significant sequence divergence and can be assigned to new viral species. These 2 families are associated with insect hosts (Zhang *et al.*, 2018; Sato *et al.*, 2023).

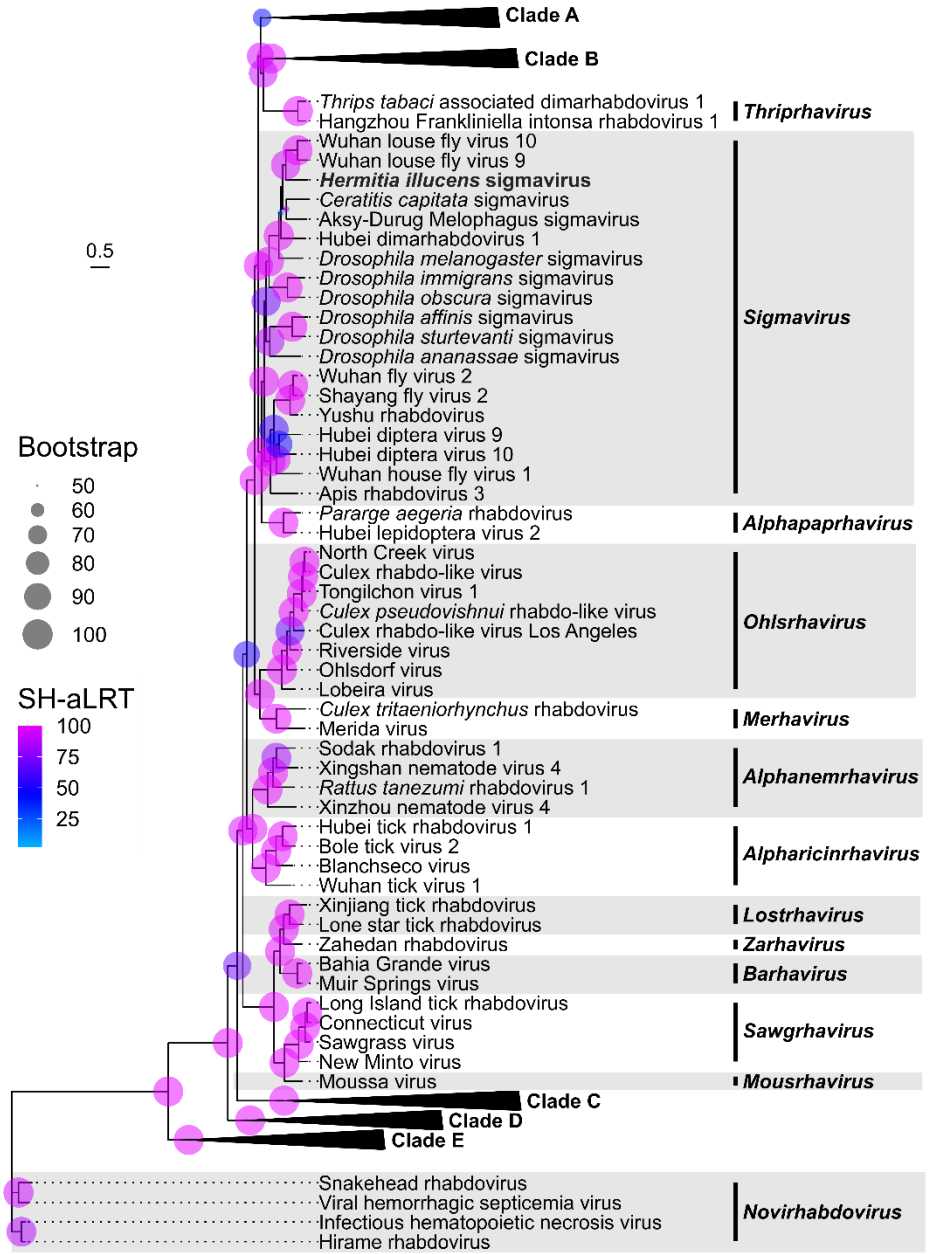

Figure 2.4 Phylogeny of *Hermetia illucens* sigmavirus relative to *Rhabdoviridae*. The sequences were rooted using the *Novirhabdovirus* clade. Branch supports are given by bootstrap (node circle size) and Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; node circle colour) values. Accession numbers of the sequences used and taxa within the collapsed clades can be found in Table S2.7.

Regarding the rhabdovirus contig, *Hermetia illucens* sigmavirus was the only member of *Mononegavirales* among the BSF virus candidates. The placement of HiSgV within the monophyletic *Sigmavirus* clade was well supported by bootstrap and SH-aLRT values (>90 and >80, respectively) (Figure 2.4). The HiSgV is most closely related to other sigmaviruses infecting flies, but branch length suggests it belongs to a new species.

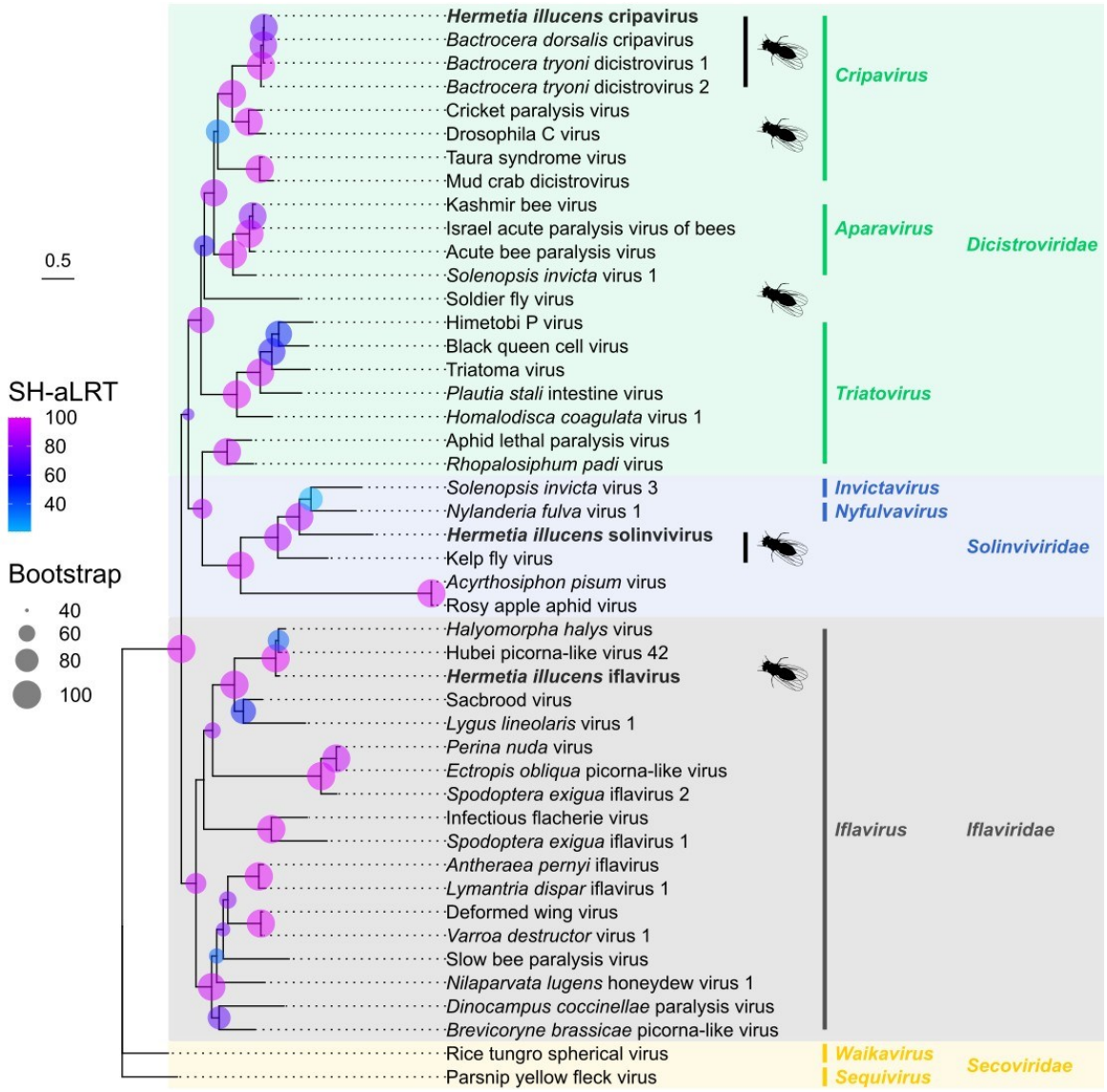

Figure 2.5 Phylogeny of the *Picornavirales* showing the relationship of three BSF viruses to arthropod infecting *Dicistroviridae*¸ *Iflaviridae* and *Solinviviridae* families. *Secoviridae* were used as outgroup. Names of viruses found in BSF are in bold. Viruses with dipteran hosts were highlighted using a silhouette of a fly. Branch supports are given by bootstrap (node circle size) and Shimodaira-Hasegawa-like approximate likelihood ratio test (SH-aLRT; node circle colour) values. All displayed Bootstrap values are higher than 40. Accession numbers of the sequences used can be found in Table S2.8.

Three of the BSF virus candidates, HiCV (*Dicistroviridae*), HiIfV (*Iflaviridae*) and HiSvV (*Solinviviridae*) are distantly related to each other but were all within the order *Picornavirales* (Figure 2.5). Within the *Solinviviridae*, HiSvV was monophyletic with the type species of the family, *Solenopsis invicta virus 3* and *Nylanderia fulva virus 1*, supporting its classification within the family. High bootstrap and SH-aLRT values supported the placement of the HiCV within the *Cripavirus* genus of the *Dicistroviridae*, HiIfV within the genus *Iflavirus* and HiSvV within the family *Solinviviridae,* hence we named it solinvivirus. HiCV showed close

relationship to *Bactrocera dorsalis* cripavirus (BdCV), and *Bactrocera tryoni* dicistrovirus (BtDV) 1 and 2, with branch lengths of less than 0.15 (Figure 2.5) suggesting that these isolates could belong to the same species based on current species demarcation criteria for *Dicistroviridae*. The translated ORFs of BdCV genome (9 117 nt) was > 95% similar at the amino acid level to those of HiCV (9 364 nt), despite the genome being shorter by 247 nt. Both HiIfV and HiSvV are more distantly related to their closest relatives, indicating they likely represent new viral species.

2.3.4 Widespread screening of BSF virus candidates and colony haplotyping

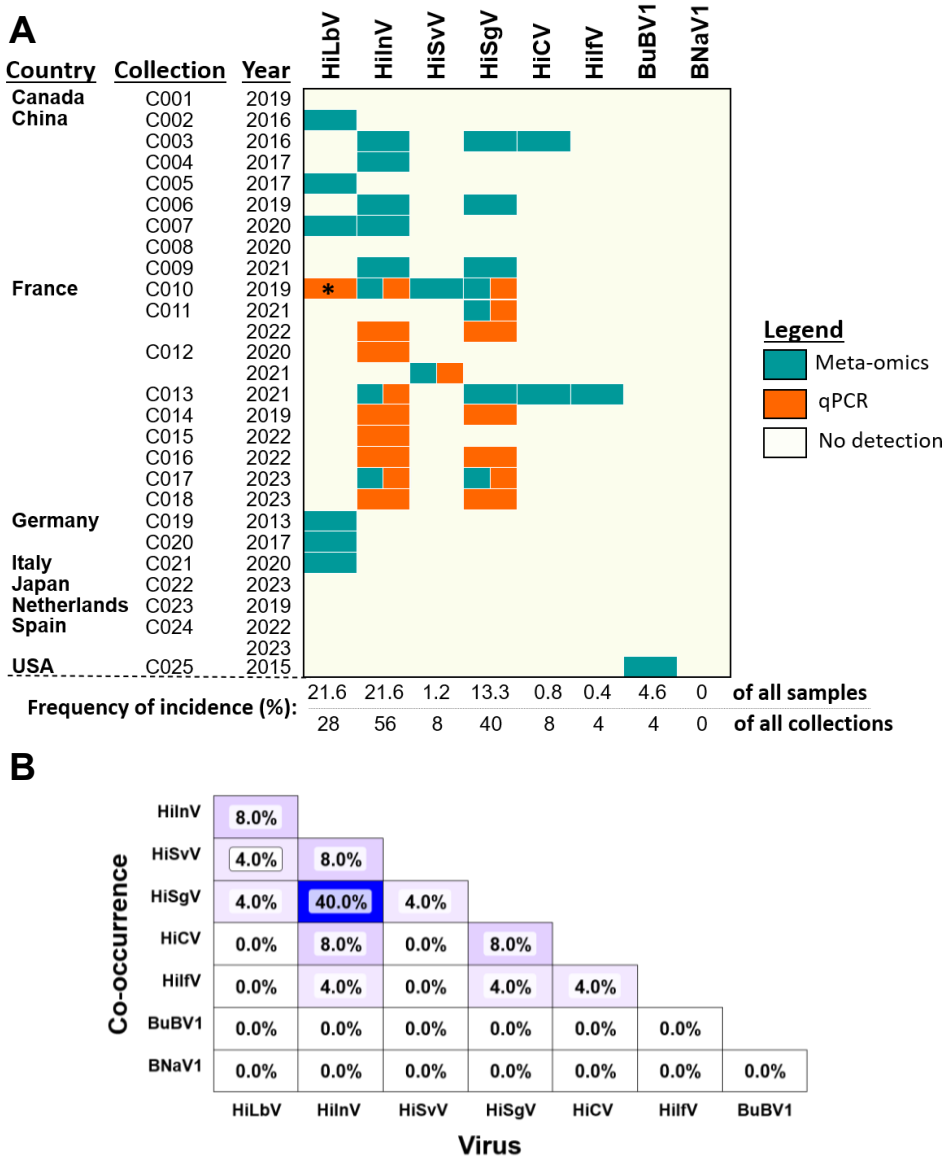

Figure 2.6 Screening of BSF virus candidates occurring naturally in different fly colonies. (A) Presence/absence screening of viral-like sequences in BSF. In green are mapped reads found in metatranscriptomes and metagenomes from different bioprojects and in orange are positive results obtained using RT-qPCR detection. "C###" represented the colony number. The asterisk denotes that HiLbV in C010 was only detected by a positive

RT-qPCR. (B) Co-occurrence analysis of viruses detected in BSF datasets and RT-qPCRs. Two hundred and forty-one BSF datasets were screened in total (including 22 solely by RT-qPCR).

Following the identification of eight viruses associated with BSF HTS data, it was then essential to determine their global prevalence in BSF colonies (Figure 2.6 and Figure S2.4 to S2.7). Two approaches were undertaken, firstly mapping the eight virus genomes against 219 BSF HTS datasets, and secondly screening available samples by RT-qPCR. It was found that HiLbV and HiInV were the most prevalent across samples, each with an incidence of 21.6% (Figure 2.6). However, HiInV and HiSgV were the most widespread across the colonies, present in 56% and 40% of the colonies, respectively. While HiSgV, HiLbV and HiInV were more globally widespread, HiSvV and HiIfV were only detected in datasets collected in France. The sample where the solinvivirus HiSvV was detected for C010 was also tested by RT-qPCR, but the virus could not be detected using this method (Figure 2.6A). *Hermetia illucens* cripavirus was found in two different colonies, one in China and one in France (C003 and C013), sampled five years apart. For the bunyaviruses, only BuBV1 was detected exclusively within the USA (Figure 2.6A and Figure S2.7).

Combining meta-omic and RT-qPCR data, viruses screened in this study were detected in BSF colonies from China, France, Germany, Italy and the USA, but not in colonies from Canada, Japan or the Netherlands. Initially, no viruses were detected in samples from colony C024 obtained in 2022 from a colony in Spain (Figure 2.6A). However, HiInV was detected in two metatranscriptomic datasets, SRR28204394 (C024) and SRR28204391 (C024), related to a 2023 infection experiment with a different virus using BSF from colonies C024 and C017 (PRJNA1079553) (Figure 2.6A and Figure S2.6B). The presence of HiInV genetic material was confirmed by RT-qPCR only for SRR28204391 (Figure S2.6B). A co-occurrence analysis of the samples highlighted that both HiInV and HiSV were not only widely distributed across different colonies, but also co-infected BSF in 40% of the colonies (Figure 2.6B). Although HiLbV is fairly widespread across the collections (28% incidence, Figure 2.6A), it infrequently co-infected BSF alongside HiInV (8%) and even less frequently with HiSgV (4%) (Figure 2.6B). Conversely, neither HiLbV nor HiSvV co-occurred with HiCV or HiIfV showing minimal co-infection with other viruses. Additionally, BuBV1 did not co-occur with any of the other viruses.

2.4 Discussion

This study brought the total number of BSF-associated exogenous viruses to eight candidate species belonging to the orders *Ghabrivirales* (*Inseviridae* and *Lebotiviridae*), *Picornavirales* (*Iflaviridae, Solinviviridae* and *Dicistroviridae*), *Mononegavirales* (*Rhabdoviridae*), and *Bunyavirales* (*Nairoviridae*, and unclassified). From our new datasets, a single near-complete contig for HiSgV was assembled encompassing all five ORFs, confirming a previous report of this BSF virus in the USA (Walt *et al.*, 2024). This study also introduced alternative *in silico* high-throughput screening approaches enabling the detection of seven of the eight virus sequences in all currently available datasets. Additionally, this allowed us to develop RT-PCR and RT-qPCR protocols to survey for six of these viruses. We found that BSF viruses were widely distributed with only five out of the 25 examined colonies testing negative for all of the eight viruses.

2.4.1 Virome diversity and novel discoveries

A large-scale and comprehensive screening of BSF datasets has affirmed seven of the eight RNA virus candidates which can be considered to infect BSF, including five newly discovered viruses. The identification of an exogenous rhabdovirus (HiSgV) and an insevirus (HiInV) parallels the previous finding of endogenous rhabdovirus and totivirus-like sequences in BSF genomes (Pienaar *et al.*, 2022). This suggests recurring interactions between these virus families and BSF. It is noteworthy that while the endogenized RhabdoEVE sequence showed close relatedness to Entomophthora rhabdovirus A than to any known member of the *Sigmavirus* genus, HiSgV represents a distinct *Rhabdoviridae* species from the previously endogenized RhabdoEVE.

The identification of a cripavirus (HiCV, *Dicistroviridae*), iflavirus (HiIfV, *Iflaviridae*) and a solinvivirus (HiSvV, *Solinviviridae*) added the order *Picornavirales* to the virome of BSF. Although the HiIfV contig was short, it was included in the BSF virome as it contained the RdRP region, which would allow for future screening activity. Phylogenetically, HiCV is very close to BdCV, BtDV1 and BtDV2. According to the ICTV demarcation criteria for a new cripavirus species, the amino acid similarity between capsid ORFs must be less than 90% (Valles *et al.*, 2017a). Therefore, HiCV probably belongs to the same virus species as BdCV, which could suggest ecological interactions between the hosts BSF and *Bactrocera dorsalis* and these viruses. However, further investigations are needed as the consensus genome of HiCV is longer than the reference genomes of the viruses found in *Bractocera* spp.

2.4.2 Potential pathogenicity of identified viruses

The viral families *Dicistroviridae*, *Iflaviridae*, *Rhabdoviridae* and *Solinviviridae* contain many members described as insect pathogens (Valles *et al.*, 2017b, 2017a; Brown *et al.*, 2019; Walker *et al.*, 2022). Notably, HiSvV was found in colonies in which signs of disease (e.g. high mortality) were being reported at the time of sampling. Nevertheless, infections for most of the members of these viral families remain latent and unnoticed until certain events trigger high levels of mortality (Maciel-Vergara and Ros, 2017; Martin and Brettell, 2019). While the triggers of disease outbreaks are not well understood for *Dicistroviridae*, *Iflaviridae* and *Solinviviridae*, in general, high viral loads within the host has been associated with disease signs and symptoms (Allen and Ball, 1996; de Miranda *et al.*, 2010; Valles and Porter, 2015; Martin and Brettell, 2019). Management strategies for these viruses could focus on maintaining low virus loads in the infected colonies. Since the transmission of these viruses may be both horizontal and vertical (Valles and Hashimoto, 2009; de Miranda *et al.*, 2010; Valles *et al.*, 2016; Morrow *et al.*, 2023), this should be taken into account when mitigating disease outbreaks.

In *Drosophila* spp, sigmavirus infections can increase sensitivity to CO_2, and can become overt after exposure to increased CO_2 levels, causing visible signs such as mortalities (Lhéritier, 1958; Longdon *et al.*, 2009). Moreover in *Drosophila,* sigmaviruses (*Rhabdoviridae*) are only known to transmit vertically, and infections can remain asymptomatic (Longdon, Wilfert, Obbard, *et al.*, 2011; Longdon, Wilfert, Osei-Poku, *et al.*, 2011; Longdon *et al.*, 2017). Although more investigations are required on HiSgV, monitoring and controlling CO_2 levels could be beneficial to BSF colony health.

While little is known about inseviruses and lebotiviruses, there are some reports of pathogenic interactions within the *Betatotivirinae* (*Ghabrivirales*). For example, the pistolvirid piscine myocarditis virus, causes mortality in salmon. Additionally, four other viruses have been found to co-occurring with mortalities in aquaculture fish (Haugland *et al.*, 2011; Louboutin *et al.*, 2023). This suborder can also cause disease and mortalities in arthropods such as shrimp (artivirid, paneid shrimp infectious myonecrosis virus) and crayfish (cherax giardiavirus-like virus) (Edgerton *et al.*, 1994, 2002; Edgerton and Owens, 1999; Poulos *et al.*, 2006). In contrast, lebotiviruses have been described to co-occur with some benefits to insect hosts, such as increased offspring survival of *Leptopilina boulardi* (Martinez *et al.*, 2016). Studies so far suggest that transmission of *Ghabrivirales* primarily occurs vertically rather than horizontally

(Martinez *et al.*, 2016; Zhang *et al.*, 2018). Since these viruses can cause asymptomatic and symptomatic infections, they should not be overlooked when found in diseased individuals.

2.4.3 Efficiency of screening and diagnostic approaches

Virome work in BSF is still in its early stages; however, foundational knowledge of diverse interactions with viruses has been established (Pienaar *et al.*, 2022; Walt *et al.*, 2023, 2024); this study). Here, the dual de novo-based strategy, using PoolingScreen and Lazypipe2, was instrumental in identifying five novel virus candidates and confirming the two of the already partially characterized viruses (HiLbV and BuBV1). PoolingScreen enabled for the detection of HiSgV, HiLbV and HiInV fragments across datasets which did not contain more universal hallmark genes, such as the RdRP and were therefore missed by Lazypipe2. By relaxing the virus database restriction to include genes other than the viral hallmark genes/protein domains, PoolingScreen broadens the range of potential viral-like sequences. Although this methodology initially introduces a higher number of false positive hits, it significantly expands the spectrum of detectable novel viruses, underscoring the delicate balance between sensitivity and specificity in virus detection. Of note, no insect-associated DNA viruses were so far found to infect BSF. This could result from an analytical bias, but PoolingScreen was able to detect both RNA and DNA viruses already identified in wild bees transcriptomes (PRJNA411946; Schoonvaere et al., 2018). Otherwise, this could reflect the low prevalence of such viruses in BSF populations. Indeed, in *Drosophila melanogaster* the first naturally occurring large dsDNA virus was only discovered in 2015 (Webster *et al.*, 2015) and found to occur at relatively low prevalence in natural populations (Wallace *et al.*, 2021). It is therefore possible that DNA viruses could be found in BSF with increased sampling effort, including by surveying wild populations.

One of the prominent challenges in virome description lies in the initial detection of viral-like sequences. However, genetic databases are becoming well-populated and are regularly updated (Cobbin *et al.*, 2021). This can help to improve the scope of virus detection pipelines, as observed by (Wu *et al.*, 2020). Many virus discovery pipelines still require subsequent characterization of viral-like sequences to ascertain their viral origins, even comprehensive virus discovery pipelines such as Lazypipe2 (Plyusnin *et al.*, 2023), Cenote-taker2 (Michael J. Tisza *et al.*, 2020), VirSorter2 (Guo *et al.*, 2021), VPipe (Wagner *et al.*, 2022) and VirIdAI (Budkina *et al.*, 2021), which can provide fewer false positives. In all cases, confirming the presence of core viral genes, such as the replicative polymerases and capsid

related ORFs, is essential before confidently validating a novel virus candidate. This is important since virus screening is only the first step of viral characterisation.

Computational resources can be a limiting factor for many facilities. However, the human-based hands-on time required to parse through comprehensive outputs can be a more confounding factor as the expected throughput of virus screening increases (Moshiri *et al.*, 2022). More recent developmental approaches of virus pipelines have aimed to address issues observed in HTS virus discovery approaches, balancing accuracy with computational resource and processing time (Budkina *et al.*, 2021; Mastriani *et al.*, 2022). While the sensitivity of pipeline approaches is constantly being improved (Michael J. Tisza *et al.*, 2020; Mastriani *et al.*, 2022; Wu *et al.*, 2023; Hegarty *et al.*, 2024), some pipelines have tried to simplify the exploration of output results (Plyusnin *et al.*, 2023). During this study we coded the option to combine the output of multiple datasets into one or two files which is easier to parse. Usually, output results have to be individually scanned, to traceback resulting viral-like reads to the original sample. While it is possible to combine input datasets for many virus discovery pipelines, this can increase computational requirements for the first few steps and does not allow for dataset traceability if done before contig assembly. Although PoolingScreen used a routine approach for QC, assembly and obtaining sequences with viral hits, it increased the efficiency of viewing the output data to search multiple samples that have potential viruses by simple concatenation of final output results with direct sample traceability. This step greatly reduced user handling time from several days to a few minutes (Figure S2.1). The PoolingScreen approach could thus accelerate analysis turnaround in line with ever-improving time efficiency of sequencing technology and growing plethora of dataset libraries (Goodwin *et al.*, 2016; Moshiri *et al.*, 2022).

In addition to the approach followed by PoolingScreen, the semi-automatic mapping-based screening pipeline can also help rapidly screen samples for known viruses and verify if mapped reads are spanning CDS regions of viral sequences (Figure S2.4 to S2.8) to confirm their genuine presence within datasets. This was observed when screening for BuBV1 and BNaV1, both of which have some regions with a high level of identity with other organisms (Figure S2.8, Tables S2.9 to S2.11), which could induce false positives.

2.4.4 Surveillance and sustainable application

While the susceptibility of BSF to viral diseases remains an open question (Jensen and Lecocq, 2023), the new list of the exogenous viruses and screening tools can promote viral surveillance in BSF. The high-throughput PoolingScreen approach is valuable for underexplored mass-

reared models for which HTS data is available. However, HTS technology is still not cost-efficient enough for routine screening. Therefore, we further developed more cost-effective RT-PCR and RT-qPCR screening tools for BSF viruses.

BSF viruses are widely distributed in rearing facilities across the Northern Hemisphere, and will likely be found on all continents as more data becomes available. An interaction between virus prevalence and the genetic background of hosts could be expected. However, regardless of their geographical origin, most of the BSF colonies screened belonged to the same haplotype (Figure S2.8), as previously established (Ståhls *et al.*, 2020; Kaya *et al.*, 2021; Guilliet *et al.*, 2022). The viral distribution pattern was therefore not influenced by the genetic background of the flies. This further demonstrates that viruses are naturally occurring in rearing facilities, and that better surveillance and management networks should be implemented within the BSF industry. Furthermore, this highlights the need to increase sampling effort as other BSF populations may host and co-evolve with different viruses, which could one day be transferred to the mass-reared colonies.

2.4.5 Conclusion

This study provides a diverse library of eight viruses likely infecting BSF and lays the foundation for viral surveillance in large-scale BSF rearing facilities. However, further studies are required to determine the impact of these viruses on BSF health both in a mass-rearing context and in the wild. A long-standing issue in mass-rearing facilities is that pathogenic agents may not cause disease in all cases of infections. The development of routine diagnostic tools would accelerate our understanding of disease etiology in BSF.

Acknowledgements

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Supplementary data

The supplementary data can be found in Appendix 1

Chapter 3: A solinvivirus reduces the lifespan of adult *Hermetia illucens* (black soldier flies)

Brief overview of chapter

This chapter was written as a draft to be submitted to the Journal of Virology. The previous two chapters and work by an American team found a total of eight viruses in BSF. Through the broad screening approaches made in Chapter 2, disease samples received from a BSF rearing facility led to the discovery of hermetia illucens solinvi-like virus 1 (HiSvV1) (*Solinviviridae*). This study isolated HiSvV1 and tested its ability to infect BSF and its transmission by performing Koch's postulates. This study also developed injection and oral feeding bioassays that could be used to test the effects of viruses and other pathogens in adult BSF and avenues of transmission. Using small RNAseq and total RNAseq, the responses of BSF immune pathways against HiSvV1 infection were analysed. This study also used bioassays to assess some fitness costs in adult BSF by HiSvV1 through bioassays.

Salvador Herrero and I formulated the frame of the study with input from Elisabeth Herniou. Salvador and I developed most of the experiments together and I performed all the experimentation, with some assistance from Pablo García-Castillo for the cohabitation, transmission and infection bioassays. Together co-authors Pablo, Harmony Piterois and I developed and optimized the droplet feeding protocol. Harmony, Valentin Pressoir and Perrine Lutanie helped me perform the Oral replication bioassays. I performed the bioinformatic analyses with some guidance from Elisabeth, Salvador and Alexandra Cerqueira de Araujo. I wrote the original draft of the chapter with guidance from Salvador. Salvador did the main review of the chapter for the thesis and all the co-authors will review the manuscript for the journal submission. I received brief assistance for some bioassays by Melissa Lloyd, Corentin Clave, Carole Labrosse, Thibaut Jossé, Carlos Lopez-Vaamonde and Ariel Muñez Sánchez.

A solinvivirus reduces the lifespan of adult *Hermetia illucens* (black soldier flies)

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[Note the lack of specific mailing information, e.g., postal codes and street addresses. Place each department or affiliation on its own line.]

Running Head: Pathogenicity of solinvivirus on *H. illucens*

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Abstract

Black soldier flies (BSF) have been shown to host multiple viruses, but no studies have isolated these viruses and studied their effects in BSF. Metatranscriptomic analysis of samples collected from a BSF colony with signs of disease in adult BSF were found to contain large quantity of reads belonging to a recently describe virus, the *Hermetia illucens* solinvivirus (HiSvV). We successfully isolated HiSvV from diseased BSF and characterized its structure, transmission, and pathological effects on its host. Our study developed multiple approaches to studying virus interactions in adult BSF. These methods confirmed that HiSvV infects and replicate in BSF causing premature mortality in inoculated adults from two independent BSF colonies. Experiments showed that HiSvV was able to transmit both horizontally and vertically, eliciting a broad immune response in infected BSF. The findings from this study established HiSvV as pathogenic to BSF, leading this to be the first known viral entomopathogen to infect and cause disease in BSF.

3.1 Introduction

Black Soldier Flies (BSF), *Hermetia illucens*, are increasingly used in waste management and as a source of protein in animal feed, making its health and productivity crucial for these industries (Joosten *et al.*, 2020; van Huis, 2021; van Huis *et al.*, 2021; Jensen and Lecocq, 2023; Tanga and Kababu, 2023). Studies on entomopathogens affecting BSF are limited, presenting a gap in BSF pathology that has been highlighted on multiple occasions (Eilenberg *et al.*, 2018; Pienaar *et al.*, 2022; Jensen and Lecocq, 2023; She *et al.*, 2023; Walt *et al.*, 2023). Although there has been a growing number of studies on the effect of more general insect pathogens (Tourtois *et al.*, 2017; Lecocq *et al.*, 2020; Klüber *et al.*, 2022; Manu *et al.*, 2022; Shah *et al.*, 2023), the first natural pathogen of BSF was only recently described in 2023 (She *et al.*, 2023). The authors described a *Paenibacillus* sp.-related pathogen causing the death of BSF with a syndrome previously described as "soft rot". To date, the ITN INSECT DOCTORS programme (https://cordis.europa.eu/project/id/859850) has received twelve confidential reports of issues affecting BSF in rearing facilities with the potential to be disease related.

Issues in mass-rearing can also arise from a less conspicuous agent, viruses (Maciel-Vergara and Ros, 2017; Maciel-Vergara *et al.*, 2021). Often, the presence of viruses can be missed, particularly since signs can mimic those of sub-optimal rearing practices, such as smaller larvae, lower feeding rates, and reduced fecundity (Maciel-Vergara and Ros, 2017; Eilenberg and Jensen, 2018; Maciel-Vergara *et al.*, 2021). In other cases, viral infections may remain invisible until certain factors trigger the infection to produce symptoms such bloated individuals, even death (de Miranda, Granberg, Low, *et al.*, 2021; Duffield *et al.*, 2021; Maciel-Vergara *et al.*, 2021; Takacs *et al.*, 2023). Since 2022, eight exogenous viruses associated with BSF have been discovered (Chapter 2; Pienaar *et al.*, 2022; Walt *et al.*, 2023, 2024). Among them, four were classified into the families *Dicistroviridae, Iflaviridae*, *Rhabdoviridae* and *Solinviviridae* and are closely related to other known insect pathogens (Valles *et al.*, 2017b, 2017a; Brown *et al.*, 2019; Maciel-Vergara *et al.*, 2021; Walker *et al.*, 2022). Previous observations found *Hermetia illucens* lebotivirus (*Lebotiviridae*) and insevirus (*Inseviridae*) and *Hermetia illucens* sigmavirus (HiSgV, *Rhabdoviridae*) as a co-infection in a colony with issues of larval mortality, though HiInV and HiSgV have also been found as co-infections in other colonies without reported symptoms (Chapter 2). One virus, *Hermetia illucens* solinvivirus (HiSvV, *Solinviviridae*), was initially detected in individuals collected from a colony where a sudden increase in premature mortality and a decrease in fecundity was

reported (Chapter 2). Despite this, none of the BSF-related viruses have been isolated and studied further and current studies on viral pathogens in BSF focused on the stability of mammal-infecting pathogenic viruses in BSF larvae (Lecocq *et al.*, 2023; Olesen *et al.*, 2023).

Solinviviridae is a recently described virus family of the *Picornavirales* with non-segmented, linear, positive-sense RNA genomes of approximately 10-11 kb, suspected of including an array of arthropod pathogens (Brown *et al.*, 2019). According to ICTV (ictv.global) and NCBI (ncbi.nlm.nih.gov), there are about 80 viral sequences closely related to *Solinviviridae*, but not yet accepted. Despite this high number, few studies describe the actual viruses or their interactions with their hosts (Valles and Hashimoto, 2009; Valles *et al.*, 2016; Cruz-Flores *et al.*, 2022; Ryabov *et al.*, 2023). The most well described viruses in the *Solinviviridae* are *Solenopsis invicta* virus 3 (SINV3), *Nylanderia fulva* virus 1 (NfV1), and two closely-related viruses, *Apis mellifera* solinvivirus 1 (AmSV1) and *Penaeus vannamei* solinvivirus (PvSV), which are not yet formally accepted as *Solinviviridae*. For SINV3, high virus titre has been linked to mortality and can cause colony collapse and decreased fecundity in ants (Valles and Hashimoto, 2009; Valles and Porter, 2015; Valles, 2024). The ecology of the *Solinviviridae* is not very well established, since the bulk of the family characterization work has been done for SINV3 and NfV1 (Valles and Hashimoto, 2009; Valles, 2012, 2021, 2023; Valles and Oi, 2014; Valles *et al.*, 2014, 2016; Valles and Porter, 2015, 2019; Brown *et al.*, 2019; Valles and Rivers, 2019; Allen, 2020; Arnold *et al.*, 2021; Ryabov *et al.*, 2023). A common feature in *Picornavirales* (including *Iflaviridae* and *Dicistroviridae*) closely related to *Solinviviridae* is that infections in insect rearings can go unnoticed until an outbreak occurs, often resulting in a strong display of symptoms and colony collapse (Allen and Ball, 1996; Valles and Porter, 2015; Maciel-Vergara and Ros, 2017; Martin and Brettell, 2019; de Miranda, Granberg, Low, *et al.*, 2021).

The goal of this study was to analyse sick individuals in search of a pathogenic virus candidate. After identifying HiSvV as being linked to the mortality experienced in a BSF colony, efforts were made to isolate and further characterize the virus. Additionally, to understand the interaction between HiSvV and BSF, we studied its transmission, pathology, and transcriptomic response of BSF to the infection. This comprehensive approach allowed us to better understand HiSvV and assess its risk and relevance to the BSF mass-rearing industry.

3.2 Methods and materials

3.2.1 Rearing of BSF colonies used in the study.

Two colonies, sharing the same genetic haplotype, were used for experimentation during this study and were previously screened for known BSF viruses. For the first colony, referred to as "colony CBP", a starter colony was provided by Entomotech S.L (Almeria, Spain) and the rearing was originally installed at the CBP research group (Spain) in 2022. This colony was considered as "virus free" after initial screenings and was used as the main colony for HiSvV infection studies. The second colony from IRBI (France) has circulating HiInV and HiSgV infections and was used to study virus interactions. The IRBI colony was reared using conditions reported in Manas *et al.*, (2024).

The CPB Colony was reared as followed. The individuals were reared at 28 °C on Gainsville diet (50% Wheat germ, 30% Alfalfa and 20% corn flour) in a similar manner to Deruytter *et al.*, (2023), but adding 150% MilliQ water to dry diet mix (0.3 kg of diet to 0.45 L of water). Essentially, 0.3 L (100 %) of water was initially added to 0.3 kg diet and was kept at 4 °C overnight to allow the diet to absorb the moisture. Before the diet was provided to the neonates or larvae, the remaining 0.15 L (50%) of water was mixed into the premoistened diet. Eggs laid were collected and incubated in parafilm sealed petri dishes, until the neonates hatched and were then placed onto 60g of diet into 0.5 L container. Five to 7 days old larvae were then moved onto 250 g of fresh diet within a 2 L container until pupation. The prepupae and pupae were manually sorted from the substrate and placed into the 2 L containers with a dry sheet of paper towel until emergence. Adults were then placed in 47.5^3 cm cages and a cotton ball soaked in MilliQ water was maintained every two days.

3.2.2 HiSvV detection in transcriptomic data.

Reads from two metatranscriptomic datasets (NCBI sequence read archives SRR28203243 and SRR28203244) obtained from BSF adults originally received from a BSF rearing facility experiencing unexplained mortalities, where analysed for the presence of *Hermetia illucens* solinvivirus (HiSvV). To do that, raw reads were trimmed using fastp v0.23.2 (Chen *et al.*, 2018) and mapped to the reference genome of HiSvV (Appendix 1 list 1). Bowtie2 v2.4.2 (Langmead and Salzberg, 2012) and samtools v1.9 (Li *et al.*, 2009) were used to map and filter the unmapped reads from the BAM files. The mean coverage was then assessed using Geneious Prime v2021.1 (https://www.geneious.com) and the coverage plots were generated using the same script as previously described (Chapter 2).

3.2.3 Virus purification and TEM of HiSvV.

Pools of BSF individuals (either pupae or adults) were homogenized using a 1 ml/5 ml pipette and tip inside a 1.5 ml centrifuge tube/50 ml falcon tube. Then virus particles were partially purified using the virus prepurification protocol used by Hernández-Pelegrín *et al.*, (2022), however, centrifugations were performed at 10 000x *g*. When needed, a concentration step was performed by PEG precipitation and the pellet was resuspended in in 100 µl of PBS. For transmission electron microscopy (TEM), prepurified virus (PPV) underwent negative staining using 2% phosphotungstate (PTA) after samples were fixed to a carbon-coated grid. The grids were visualized and captured using an HT7800 *RuliTEM* 120 kV transmission electron microscope (Hitachi, Chiyoda City, Japan). The mean size of the viral-like particles was measured using 30 particles using ImageJ v1.54g (Schneider *et al.*, 2012).

3.2.4 Virus detection by quantitative RT-PCR (RT-qPCR).

RNA extractions were performed as in Chapter 2. Post extraction, a DNase treatment was performed on the RNA using the DNase I, RNase-free kit (EN0521, Thermo Fisher Scientific, Waltham, MA, USA), followed by cDNA synthesis using the PrimeScript RT Reagent Kit (Perfect Real Time) (TAKRR037A, Takara Bio, Kusatsu, Japan). The reverse transcription quantitative PCRs (RT-qPCRs) were performed as in Chapter 2. The relative abundance of the viral targets was obtained using the *RPL8* housekeeping gene as described in Herrero *et al.*, (Herrero *et al.*, 2019). The plots were generated using the relative abundance values and ggplot2 v3.5.0 (Wickham, 2016) in R.

3.2.5 Infecting BSFs with HiSvV using prepupal injection and droplet feeding adults.

Effect of infection on the CBP colony was initially assessed by viral injection on the prepupal stages. For that, four experiments were performed on different generations using 100 prepupae for each treatment. Prepupae were sorted from substrate and washed 3x with MilliQ water and placed on paper towel to dry before being placed into two 2 L pupation boxes. During inoculation, the needle was inserted dorso-laterally by the end at an angle close to 90° into the tegument connecting the second and third segments from the posterior end (Figure 3.1).

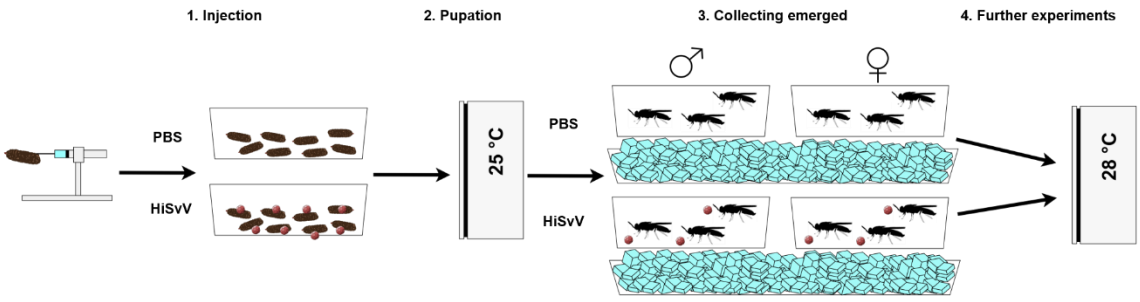

Figure 3.1 Outline of HiSvV inoculation by injection of prepupae. After injection, prepupae were pooled by treatment and pupated at 25 °C. Emerged adults were sexed on ice and separated incubated at 28 °C.

Individuals were injected with 5 µl of either 1x PBS solution or PPV containing 4 x 10^8 HiSvV genomic equivalents/µl. Post-injection, the prepupae were placed into 2L pupation boxes but without any substrate and left to pupate at 25 °C with a lighting routine set to 12 hr/12h hr. From nine days post injection, the containers were checked daily for emerged adults. Once emerged, to collect adults, the containers were placed at 5 °C until the adults fell asleep (maximum of 10 minutes) and quickly, the adult males and females were placed separately into fresh containers precooled on ice for counting and confirmation of sex. Afterwards, they were placed at 28 °C for further experiments and were maintained in complete darkness except adults selected for cohabitation experiments. The rate of emergence and sex was recorded and a Kruskal-Wallis rank sum test (Kruskal and Wallis, 1952) was used to test the overall statistical differences between emergences.

3.2.6 Droplet-feeding inoculation of BSF adults.

Adults were orally infected by feeding them a solution containing prepurified HiSvV (Tettamanti *et al.*, 2022). To do that, adults were collected within 24 hours post-emergence and males and females were retained in two separate dehydration boxes overnight (Figure 3.2A). Each dehydration box would contain a 1 to 2 cm deep layer of wood shavings. For the inoculation, individuals were held inside a P20 pipette tip by gently applying pressure to their thorax against the lip of the tip opening with their wings outside and allowed to drink (Figure 3.2B). A minute portion of the dose droplet solution was first introduced to the proboscis to see if the fly was receptive and then allowed to continue drinking. The abdomen windows were observed for visible dye to confirm that the flies were drinking (Figure 3.2C). The inoculation solution was preprepared as follows per 4 µl dose: 1 µl 5% sucrose water, 1 µl part blue food colouring and 2 µl HiSvV PPV/1x PBS. Each experiment consisted of two treatments, HiSvV inoculated and mock infection (PBS control). Inoculated adults were placed individually in 120

ml cups and 1 ml of MilliQ water was provided directly to the cotton ball placed in between the cup lid a piece of paper towel (Figure 3.2A). The cups were stored in an incubator at 25 °C with a 12 hr:12 hr (light:dark) lighting regime.

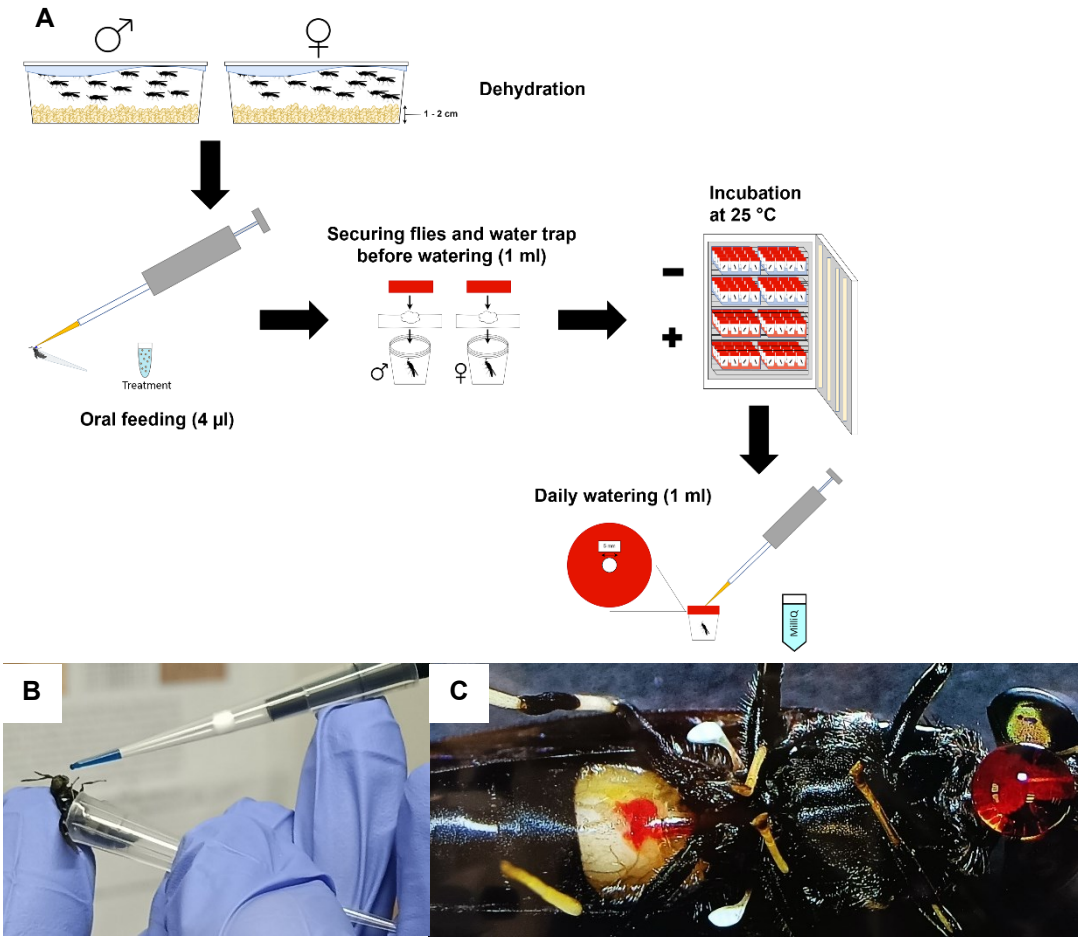

Figure 3.2 Droplet feeding of BSF adults. A) Visualisation of the droplet feeding bioassay starting with the dehydration process and then oral feeding by pipette, placing the dosed flies into cups and providing water, followed by the incubation period and daily watering. B) An adult held steady in a P20 pipette tip while receiving a droplet of virus inoculum. C) Red dye visible in the midgut of an adult after ingestion of solution.

3.2.7 Replication of HiSvV in BSF adults.

The replication of HiSvV was monitored in adults for both the prepupal injection assays and for oral injection assays. For prepupae inoculated by injection, adult males and females from the CBP colony were collected separately post-emergence (PE) for each experiment at the following timepoints: newly emerged (< 1 day), 3 days and 5 days. Afterwards an extra collection was made from adults during cohabitations that had died between 8 and 13 days PE. Pools consisting of two individuals were made during each collection.

To observe if HiSvV titre increased over time after oral inoculation in both the IRBI and CBP colonies, 24 individuals (12 males and 12 females) were orally inoculated with HiSvV and another set for with a mock-infection using PBS. Two males and two females were collected and each sex pooled 1 hour after dosing, then again at three-, five-, seven- and nine days post-inoculation. The experiment was performed for adults from both the CBP and IRBI colonies and the titre of HiSvV, HiSgV and HiInV were quantified using RT-qPCR as described above.

3.2.8 Tropism of HiSvV in adult BSF.

For three of the four experiments using injected prepupae, one male and female each were collected at three days post emergence. Adults were washed with TE buffer (10mM Tris-HCl pH 8-8.6, 1mM EDTA) beforehand, then the head, wings and legs were removed and placed into tubes according to body parts. Afterwards, the corpse was submerged into TE buffer and from the abdomen, the reproductive organs were removed, followed by the fat bodies, mid- and hindgut with malpigian tubules, and lastly the thoracic muscles. Each piece of anatomy was rinsed three times in TE buffer before being submerged in 300 µl of TRItidy G.

3.2.9 Cohabitation experiments.

Fifteen individuals of each sex were selected to place 30 adults in a 25^3 cm cage. This step was repeated four times each in different generations. Male and females emerging within 24-48 hours were used for each experiment and adults inside cages were maintained in a 12-hour light and 12-hour darkness regime. The cages were checked daily for eggs and dead adults, which were subsequently stored at -80 °C to test for horizontal and vertical transmission of HiSvV using RT-qPCR as described above.

3.2.10 Adult BSF survival bioassays after HiSvV infection.

Adults from the CBP and IRBI colonies were orally infected as described above. Mortality was monitored and 1 ml of MilliQ water was provided daily. The adults were again incubated at 25°C for up to 30 days. The experiments were performed separately for each colony using two different concentrations of HiSvV PPV, $1.6x10^1$ genome equivalents/µl and $1.6x10^4$ genome equivalents/µl. Each concentration was tested on separate occasions, including a negative control treatment using 1x PBS in the sucrose solution as a mock infection. Thirty females and 30 males were used for each treatment. Survival curves were plotted using survfit from the survival v3.5.8 R package (Therneau and Grambsch, 2000; Therneau, 2024) and ggsurvplot

from the ggplot2 R package. Type II ANOVAs from the car v3.1.2 R package (Fox and Weisberg, 2019) were then performed on Cox proportional hazard models (survival R package) to test factors such as sex, concentration and colony and the summaries of the statistics were checked.

3.2.11 RNA extraction and sequencing of HiSvV oral infection time course.

The extraction and quantification of the RNA was previously described in Chapter 2 but using TRItidy G (AppliChem GmbH, Darmstadt, Germany) for adults from adults from both the IRBI and CBP colonies. Additionally, small RNA sequencing was performed with the same RNA material as the samples produced for NCBI bioproject PRJNA1079553. Small RNA libraries were generated and single-end 50 bp reads were produced using an Illumina NovaSeq 6000 sequencer (Illumina, San Diego, USA). The library preparations and sequencing procedure was made by Novogene (Beijing, China).

3.2.12 Viral small RNA Profiling .

The small RNA sequencing datasets were trimmed and mapped to HiSvV (Appendix 1 list 1), HiInV (PP626330) and HiSgV (PP626331) and processed using the same approach. Using sRNAplot (Lewis *et al.*, 2018) to generated sRNA profiles from the mapped and filtered bam files, the script was modified to also produce a csv datafile output. An Rscript then used the csv file to generate the profile plots and collations.

3.2.13 Differential expression analysis of BSF based on transcript abundance.

To obtain BSF transcripts, long non-coding RNA (LncRNA) and messenger RNA (mRNA) datasets were first trimmed, and poor-quality reads were removed using fastp. Then the reads were mapped to the BSF reference genome (GCF_905115235.1) using HISAT2 v2.2.1 (Kim *et al.*, 2019). Using samtools, the mapped reads were sorted, filtered and SAM files were converted to BAM files.

An R script from Cerqueira de Araujo et al., (Cerqueira de Araujo, Leobold, *et al.*, 2022) utilizing featureCounts from the Rsubread R package v2.8.2 (Liao *et al.*, 2019) was used in combination with a GTF file for the BSF reference genome to obtain a gene count table from the BAM files. R was then used to generate a PCA for the count table. After, the count table was normalized to the sample totals, then transformed by square root and a dissimilarity matrix was calculated using Bray-Curtis (Bray and Curtis, 1957), before performing pairwise Analysis of Similarities (ANOSIM) on the factors sex and treatment (Clarke and Green, 1988). A

combination of the EggNOG mapper webserver (Huerta-Cepas *et al.*, 2019; Cantalapiedra *et al.*, 2021); accessed 29th February 2024) and findings from Zhan et al., (Zhan *et al.*, 2020) were used to extract a total of 896 immune-related genes from an annotation by the PANNZER2 webserver (Törönen *et al.*, 2018; Törönen and Holm, 2022); accessed: 5th March 2024).

The DESeq2 R package v1.38.3 (Love *et al.*, 2014) was used to contrast treatments HiSvV and mock (PBS) and obtain the log 2 fold changes (L2FCs) of genes using the design model ~"Treatment + Sex + Colony". The gene counts were normalized using DESeq and the counts of genes with a significant L2FC (*padj* < 0.05) were extracted. The normalized counts were then log2 transformed and a heatmap with complete row clustering and row scaled Z-scores plotted using pheatmap v1.0.12 (Kolde, 2019). Significantly differentially expressed genes (DEGs) were also examined by contrasting according to sex and then colony separately. Significant DEGs related to immunity were extracted using the list of 896 immune-related genes (described above). The biological processes (BP) of DEGs with an L2FC greater than 2 or less than -2 were annotated using the gProfiler webserver (biit.cs.ut.ee/gprofiler/gost). The gene ontology was screened against all known genes in the gProfiler BSF database using NCBI gene IDs and g:SCS threshold with user threshold set to 0.05. The top 70 BP results were then plotted using an R script.

3.2.14 Data and scripts availability.

The versions of the scripts used for the analyses can be found archived on Zenondo (https://zenodo.org/doi/10.5281/zenodo.12740863). The transcriptomic data associated with the oral replication experiments can be found in the NCBI bioproject PRJNA1079553.

References for software and packages used in scripts not cited intext: broom v1.0.4 (Robinson *et al.*, 2023), cluster v2.1.4 (Maechler *et al.*, 2022), dplyr v1.1.4 (Wickham, François, *et al.*, 2023), ggforce v0.4.2 (Pedersen, 2024), ggfortify v0.4.17 (Tang *et al.*, 2016; Horikoshi and Tang, 2018), ggplot2 v3.5.0 (Wickham, 2016), htmlwidgets v1.6.4 (Vaidyanathan *et al.*, 2023), plotly v4.10.4 (Sievert, 2020), reshape2 v1.4.4 (Wickham, 2007), svglite v2.1.3 (Wickham, Henry, *et al.*, 2023), tidyverse v2.0.0 (Wickham *et al.*, 2019), vegan v2.6-4, (Oksanen *et al.*, 2022), Writexl v1.5.0 (Ooms, 2023).

3.3 Results

3.3.1 High abundance of HiSvV reads in the diseased colony

A deep-sequencing shotgun transcriptomic approach was employed to analyse the presence of virus-related reads in a BSF colony that had high-levels of premature adult mortality. Two types of samples were analysed: an asymptomatic and freshly emerged adult fly (HA; healthy adult) and an adult fly that had died prematurely (DA, diseased adult). In the transcript data of the BSF adults, other than bacteriophage reads, only viral reads related to HiSvV were detected for both types of samples, although with different abundance. When mapping the reads to the reference sequence for HiSvV (Appendix 1 list 1), the profiles were similar for both datasets and spanned the entire reference sequence (Figure 3.3) although the coverage was drastically different between samples. A much higher coverage was observed in the transcriptome of the DA (147 343x) compared to the HA (296x) (Figure 3.3). This higher viral abundance in the sample derived from the dead adult suggests HiSvV as responsible of the premature mortality observed in some adults.

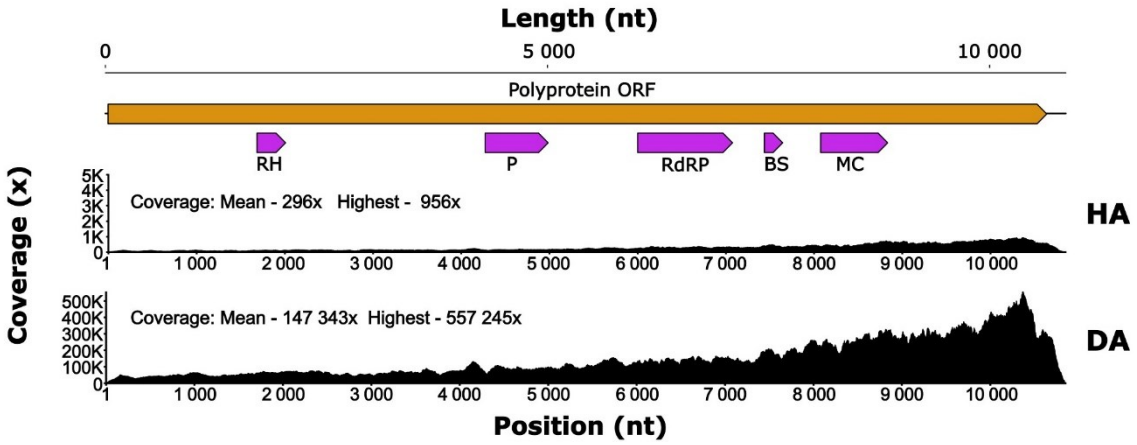

Figure 3.3 Coverage profile of HiSvV reads from the HA and DA samples. Annotated polyprotein ORF (orange) with putative conserved regions of proteins (purple) consist of an RNA helicase (RH), protease (P), RNA-dependent RNA polymerase 1 (RdRP), dsRNA binding site (BS) and a major capsid (MC). The upper coverage panel displays the HiSvV coverage in the healthy adult (HA), and the lower coverage panel shows the coverage of HiSvV in the dead adult (DA). Note the difference in Y-axis scales for the HA and DA samples.

3.3.2 Icosahedral-like viral particles were identified in HiSvV samples

Next, in order to confirm the viral nature of the HiSvV sequence and further characterize its viral structure, transmission and pathogenicity, we partially purified the viral particles. Homogenates (FH) from infected adults were processed to discard cellular debris and generate a partially purified sample (PPV) suitable for electronic microscopy and further infection

experiments (see material and methods). According to the viral quantification, the purification procedure enriched the sample in about 3.5-fold with an increase in the relative abundance of HiSvV from 8679 to 37152 (Figure 3.4A). Given the experimental procedure used in the viral prepurification, finding an enrichment of HiSvV genomes was indicative of the viral nature of the viral sequences and the presence of viral particles in the PPV sample.

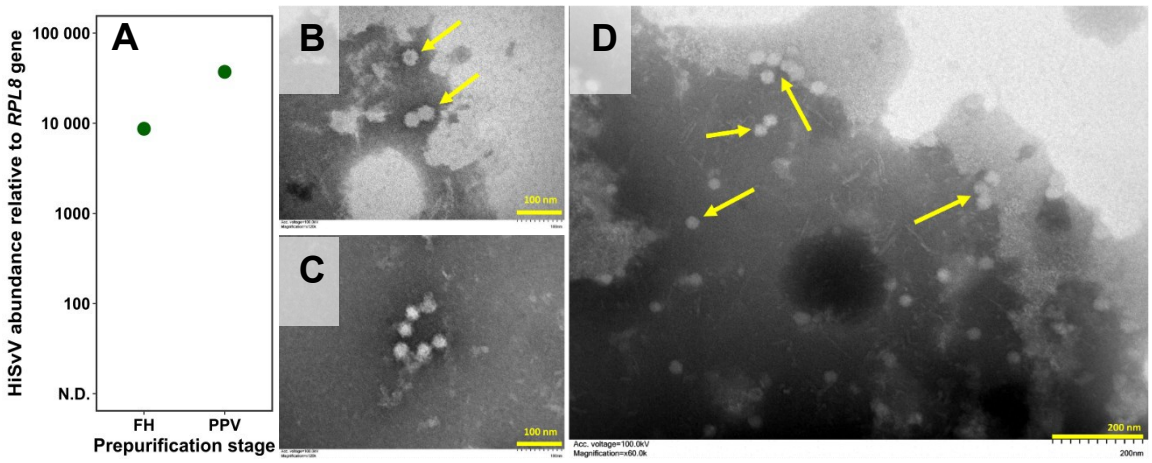

Figure 3.4 HiSvV purification and visualization by TEM. Enrichment of HiSvV genomes after virus partial purification (A). Stages of the prepurification protocol compared were initial homogenate filtered through gauze (FH) and partially purified virus (PPV). TEM observation of HiSvV particles in the PPV samples (B to D). The scale bars in the bottom right corner coloured in yellow represent 100 nm (B and C) and 200 (D).

Further exploration of the PPV sample using TEM (Figure 3.4B-D) led to the visualisation of particles resembling in size and structure other members of the *Solinviviridae* family (Brown *et al.*, 2019). These particles were observed isolated or aggregating together in a chain-like fashion and presented a similar morphology to other insect-infecting solinviviruses (Valles and Hashimoto, 2009; Valles *et al.*, 2016; Yang *et al.*, 2016; Brown *et al.*, 2019). The capsid structure appears to be spherical with an icosahedral-like shape and a mean diameter of 33.9 ± 3 nm (Figure 3.4B-D).

3.3.3 HiSvV replicates in BSF and has a systemic tropism

Initial trials to orally infect BSF larvae with PPV were unsuccessful and HiSvV was not detected in adults derived from two and seven days-old larvae exposed to $6x10^9$ and $1.5x10^8$ HiSvV genomic equivalents/g of diet, respectively. In an alternative approach, BSFs in the

prepupal stage were injected with PPV, and the viral abundance and tissue tropism was determined subsequently by RT-qPCR in adults which emerged on average 17 days after injection (Figure 3.5). HiSvV was detected consistently across the lifespan of both male and female adults. Despite this, there was no drastic increase in the level of HiSvV genetic material within 13 days post emergence (dpe) (Figure 3.5A). Mock-infected adults showed no HiSvV presence when sampled within 24 hours or five dpe, confirming the specificity of the viral detection (Figure 3.5A).

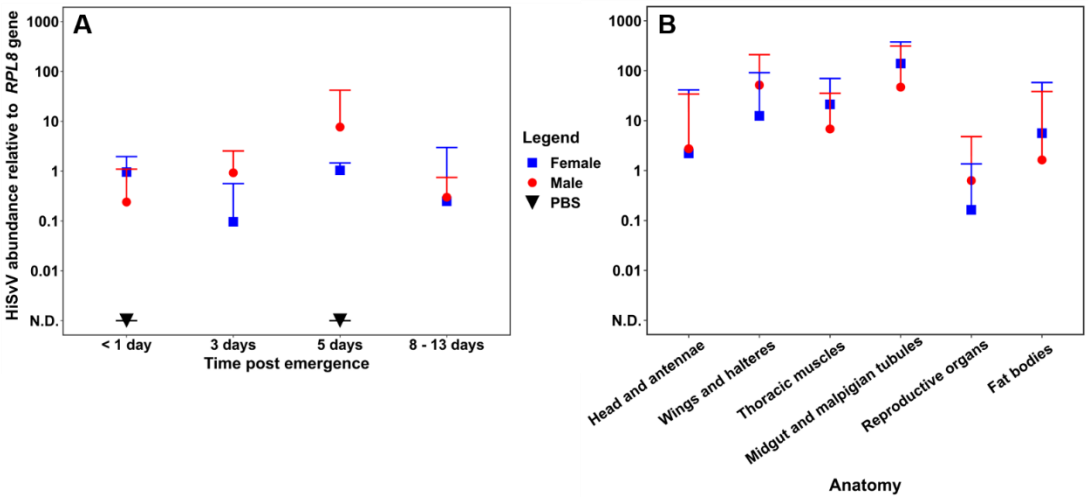

Figure 3.5 Abundance of HiSvV in virus-injected adults. Viral relative abundance at different days after emergence from pupae (A) and detection of HiSvV in different tissues of adults processed three days after emergence (B). The lower threshold for detection of HiSvV in samples was indicated by "N.D.". Individuals injected with PBS were used as negative controls during inoculation. The error bars represent standard deviation (SD) of the mean on a log scale. Only the upper bars and caps of the SD are shown.

Following these observations, adults were sampled at three dpe to assess HiSvV tropism in various tissues (Figure 3.5B). Although HiSvV was found in all examined tissues, it was less prevalent in reproductive tracts (including testis and ovaries). These results indicate a systemic infection, consistent with findings from similar studies on other *Solinviviridae* (Valles and Hashimoto, 2009; Yang *et al.*, 2016).

3.3.4 Adult BSFs are orally infected with HiSvV

Additionally, oral infection of adults was assessed using a droplet feeding method. Two colonies, one devoid of any known RNA viruses (colony CBP) and one having a persistent infection with two additional RNA viruses (HiSgV and HiInV) (colony IRBI) were infected. Viral abundance increased after inoculation in both colonies reaching the maximum value at

seven days post inoculation (Figure 3.6A & B). This increase in HiSvV abundance after inoculation of more than 3 orders of magnitude reflects the active replication of the virus in orally infected adults. The presence of HiInV and HiSgV did not appear to affect the replication of HiSvV (Figure 3.6B). Similarly, the abundance of HiInV was not affected by the infection with HiSvV (Figure 3.6B).

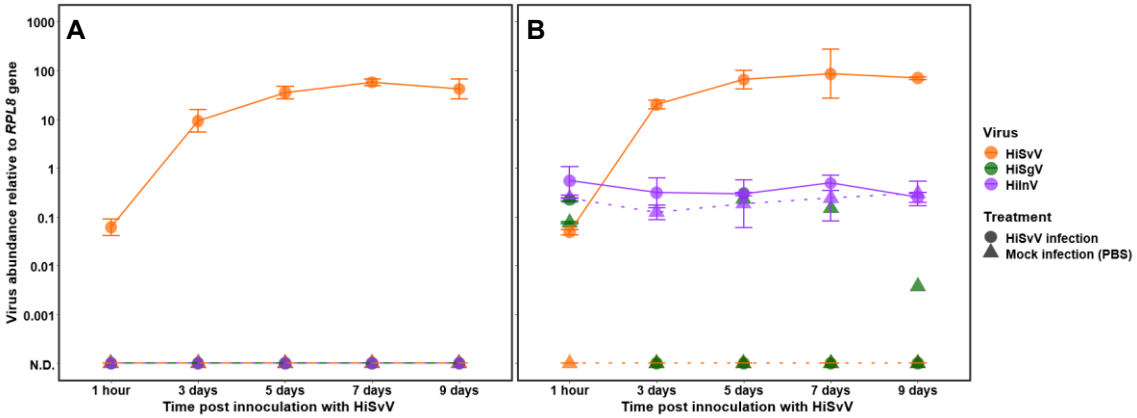

Figure 3.6 Abundance of HiSvV in orally infected adults. Relative viral abundance in adults from colony CBP (free of known BSF viruses) (A) and in adults from colony IRBI where HiSgV and HiInV is prevalent (B). Different colours represent the different viruses HiSvV (orange), HiSgV (green) and HiInV (purple). The circle and triangle shapes are used for the HiSvV- and mock-infected individuals, respectively. Lines were also used to indicate HiSvV (solid) and mock infections (dotted). A line was not included for HiSgV since it was not detected in all individuals. The error bars represented standard deviations.

3.3.5 HiSvV is horizontally transmitted between adults and likely vertically transmitted

Cohabitation experiments were performed using adults emerged after HiSvV injection to determine if HiSvV could be vertically transmitted to the offspring and horizontally transmitted between adults (Figure 3.7). Infected males were reared with non-infected females and *vice versa.* The relative abundance of HiSvV was analysed in the eggs from these crosses as well as in post-mated adult cadavers collected between 14 and 19 dpe.

Hermetia illucens solinvivirus was not detected in the egg clusters from non-infected parents (Figure 3.7B). In contrast, HiSvV was detected in egg clusters where both parents were infected with HiSvV before cohabitation, but also in egg clusters where at least one parent had been infected with HiSvV before cohabitation, independently of the infected sex (Figure 3.7B). These cohabitation results are indicative of the horizontal transmission of HiSvV between BSF adults. In addition, since HiSvV was detected in egg clusters, the results also reveal probable maternal as well as paternal vertical transmission.

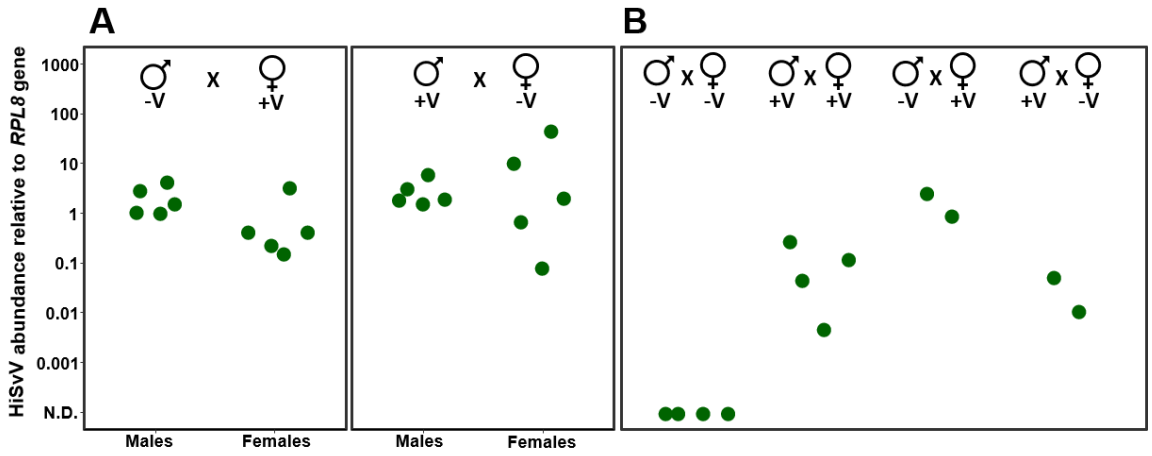

Figure 3.7 Horizontal and vertical transfer of HiSvV in BSF. Adults and eggs were obtained during cohabitation experiments and the level of HiSvV was assessed in the (A) adults and (B) egg clusters. The green points represent single individuals (in A) or single egg clusters (in B) tested. Individuals inoculated with HiSvV during prepupal stage are indicated using "V+", while "V-" specifies mock-infected adults. "N.D." on the y-axis indicates that HiSvV genetic material was not detected during qPCR.

3.3.6 Adult emergence was not affected by HiSvV infection

To further evaluate the impact of HiSvV infection on the BSF, we analysed the emergence rates and potential sex-ratio biases in adults subjected to both HiSvV and control treatments (Figure 3.8). Four independent experiments were carried out. Among the different experiments, the percentage of adult emergence ranged from 79 to 85% and from 58 to 93% across the PBS and HiSvV treatment groups, respectively. Although there was a significant variation in emergence between experiments (χ^2 = 115.07, df = 3, p < 2.2e-16), no statistically significant differences in adult emergence were associated to the viral infection (χ^2 = 0.82, df = 1, p = 0.37), neither when sex was included (χ^2 = 4.26, df = 3, p = 0.23) (Figure 3.8). Among the emerged adults, the difference between males and females ranged between 1 to 8%, presenting a low sex-ratio bias between emerged individuals regardless of treatment. Overall, no visible effect of an HiSvV infection itself on the rate of adult emergence was observed.

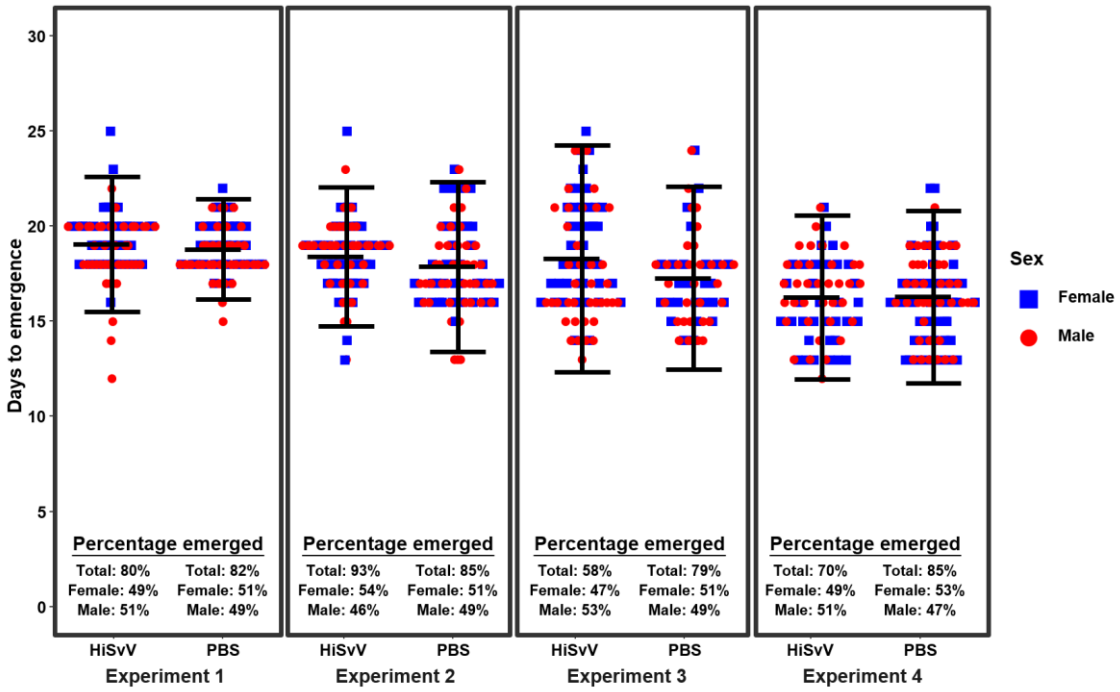

Figure 3.8 Effect of HiSvV infection on adult emergence. Time and emergence rate after viral infection (by injecting prepupal stages). Results correspond to four independent experiments. Each dot corresponds to a single adult. The sex of the obtained adults is indicated with different colours. The error bars indicate the standard deviation of the mean within each treatment group.

3.3.7 HiSvV infection negatively impacts the survival of BSF adults

Since premature mortality of BSF adults was one of the symptoms originally observed, the pathogenic nature of HiSvV was assessed by monitoring the survival of adults infected with HiSvV (Figure 3.9). Adults from two colonies were orally infected 1 dpe and the viral effect on their lifespan was assessed daily. Independent of the infective status and colony, females naturally exhibit shorter lifespans than males (about 5.5 days less on average) (Figure 3.9 and Table S3.1). Survival analysis showed that in both colonies and for both sexes, HiSvV infections reduced adult lifespans. The strongest effects of HiSvV infection (higher hazard ratios, HRs) were observed in the CBP colony (males and females) with HRs ranging from 3.5 to 4.9. For the IRBI colony, HRs where slightly lower with values ranging from 1.6 to 4.3 (Table S3.1D). A reduction in lifespan associated to the viral infection was observed for the two tested concentrations, although a dose effect was only clearly observed for the CBP colony (Table S3.1D & E). In summary, the survival analysis confirmed the reduction in adult lifespan associated to the HiSvV infection and the CBP colony, devoid from other viruses, being more susceptible than the IRBI colony (Figure 3.9, Table S3.1 and Table S3.2).

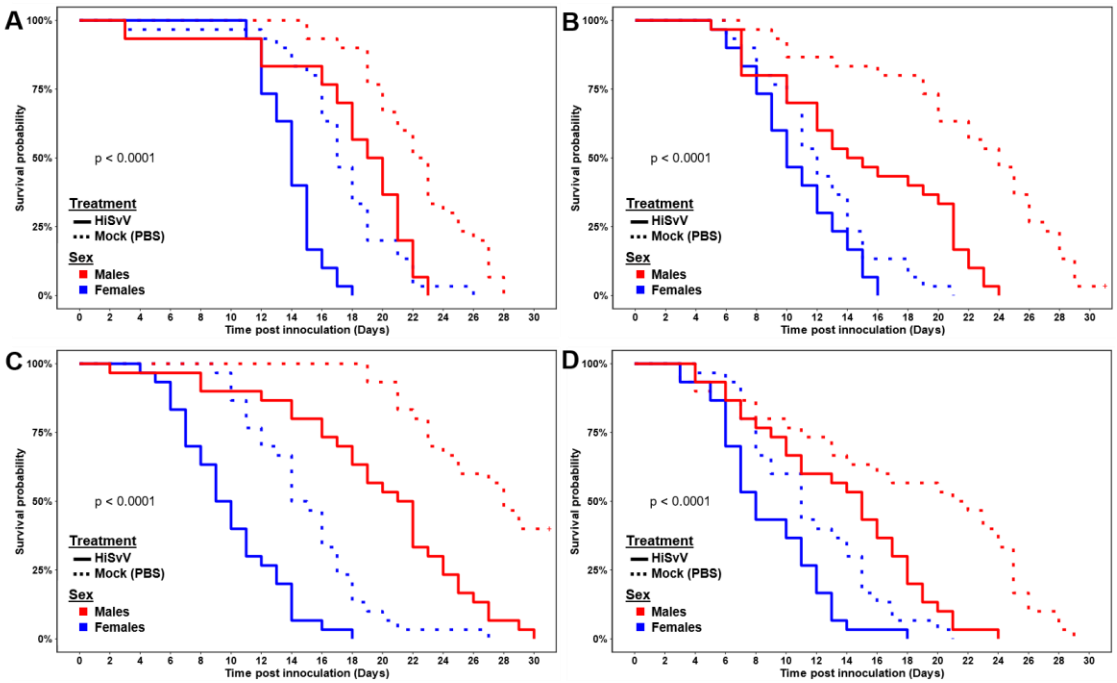

Figure 3.9 Effect of viral infection on adult lifespan. Adults from the colony CBP (A and C) and colony IRBI (B and D) were orally infected with HiSvV PPV at two concentrations: $1.6x10^4$ genome equivalents/µl for experiment 1 (A and B) and $1.6x10^1$ genome equivalents/µl for experiment 2 (C and D). Males and females are differentiated by the colours red and blue respectively and a solid line separate HiSvV-infected from mock-infected (PBS). The p-value within each plot was established by global comparison of strata within each experiment, indicating a statistical difference between all strata.

3.3.8 Absence of siRNA response to HiSvV infection in adults

To check if HiSvV (and HiInV, HiSgV) viral infections were triggering the small interference (siRNA) responses in BSF adults, the small RNA (sRNA) profiles were examined for both colonies (CBP and IRBI) after infection with HiSvV (Figure 3.10). In colony CBP, mapping of sRNA reads to the HiSvV genome showed an increase in abundance from 3 to 7 days post infection (dpi) (Figure 3.10A), supporting active viral infection as detected earlier (Figure 3.6). A broad size range from 19 to 31 nt, targeting the HiSvV viral genome without a prominent peak at 21 nt suggests an absence of typical siRNA antiviral pathway activation. The widespread distribution of the sRNA profile would be indicative of the degradation of HiSvV genomic material and suggests an alternative defence response. The sRNA profiles in colony IRBI for HiSvV saw the same trend (Figure 3.10A), suggesting that the presence of additional viral infections does not change the defence response against HiSvV.

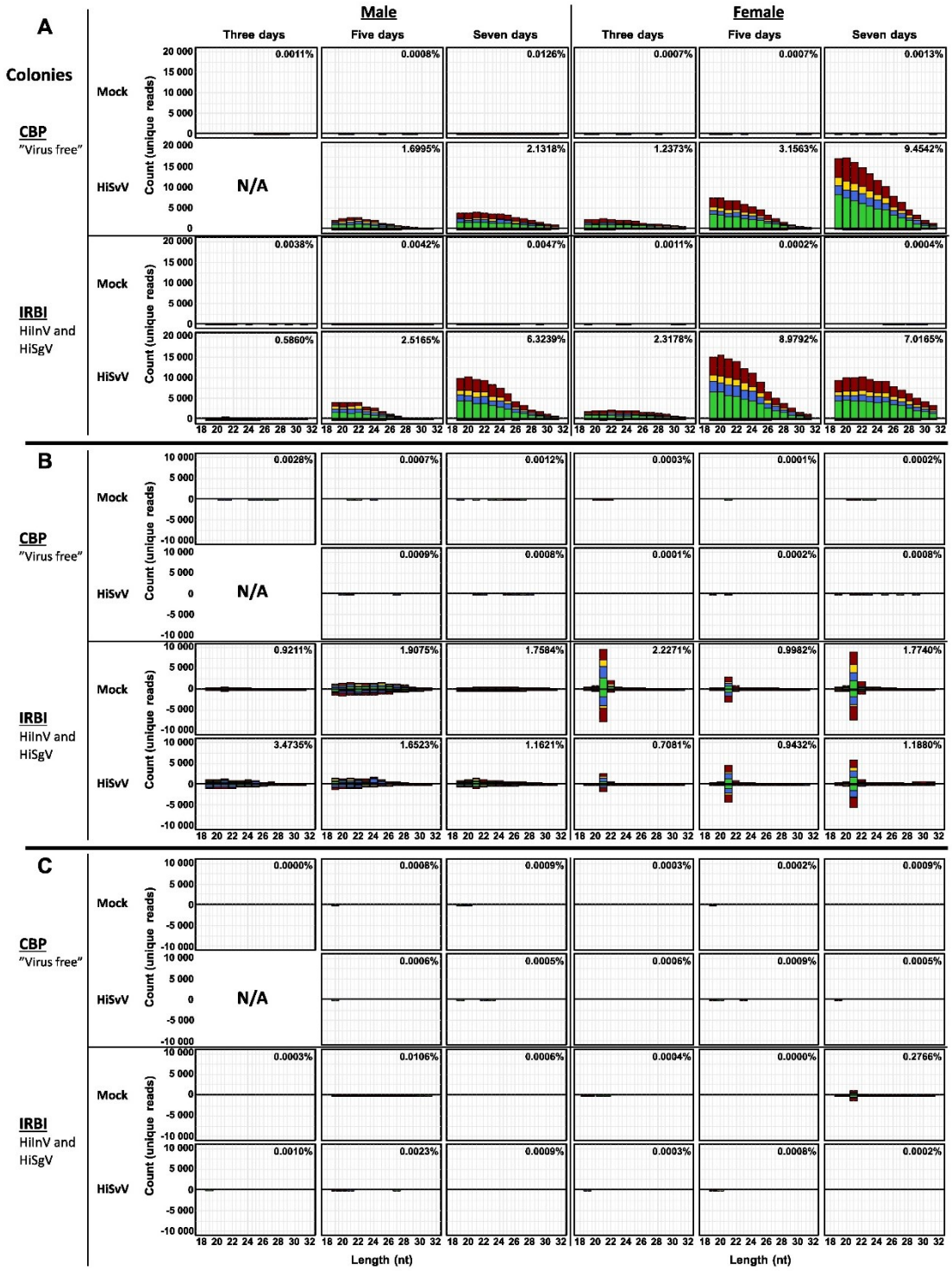

Figure 3.10 Viral small RNA profiling in BSF adults. Reads were mapped to genomes of A) HiSvV, B) HiInV and C) HiSgV s in HiSvV-inoculated and mock-inoculated BSF adults. Profiles were obtained for adults collected at different times post-infection and in males and females. Small RNA sequences which started with an adenine were coloured in green, uracil in blue, thymine in red, guanine in yellow and ambiguous "N" in black. The percentage of mapped sRNA reads to the total number of reads in each sample was indicated in the top right corner of each subplot the bars above zero represent positive sense RNA mapping and the bars below represent negative sense mapping.

Independent of the presence of HiSvV and HiSgV in colony IRBI, both male and female profiles showed a broad range of sRNA sizes mapping to both HiInV strands. However, a 21 nt peak was evident in females, suggesting active siRNA defence responses, which was less apparent in males (Figure 3.10B). For HiSgV infection, a small but clear peak at 21 nt was observed in one female sample collected at 7 dpi but was not clearly detected in other samples, likely due to low infection levels as seen in previous experiments (Figure 3.10C and Figure 3.6B). In summary, these results confirmed the ability of BSF adults to specifically activate the siRNA defence mechanisms in a virus-dependent manner suggesting a complex interplay between host defences and viral evasion strategies.

3.3.9 HiSvV infection triggered a broad immune response in adult male BSF

To shed light into the BSF response to an HiSvV infection, the transcriptional regulation, with special emphasis on the immune-related genes, was analysed (Figure 3.11, Figure S3.1 and Table S3.3). An overall comparison of the transcriptional changes after viral inoculation in males and females of the two BSF colonies was obtained by PCA and ANOSIM analyses of the transcriptomic data. Firstly, major transcriptional differences were attributed mainly to sex ($R = 0.958$, $p = 0.001$) and the clustering was supported in 55.7% of the total variations (Figure 3.11A). Secondly, although the differentiation is weak statistically ($R = 0.510$, $p = 0.058$), HiSvV-infected males tend to cluster away from mock-infected males (Figure 3.11A). However, the HiSvV-infected female clustering intertwined with mock-infected females with no differentiation observed ($R = -0.052$, $p = 0.577$).

A comparison between male and female gene expression revealed 3,479 genes (22.6% of all expressed genes) significantly ($p < 0.05$) regulated with a log2 fold-change (L2FC) of more than 2 (Figure 3.11B). However, this number decreased to 310 genes (2% of expressed genes) when comparing HiSvV-infected to mock-infected flies. Focussing on the immune-related genes, only 128 immune-related genes with significant differential expression ($p < 0.05$) in HiSvV-infected flies were identified, forming four major clusters (Figure 3.11C, Figure S3.1 and Table S3.3). Z-score examination showed that clusters II and IV exhibited increased L2FCs in HiSvV-infected flies, indicating a strong immune response (Figure 3.11C). In contrast, the response was weaker in females, with all clusters showing similar trends, except for cluster IV, which was less affected in females. Markedly, females from colony IRBI exhibited a weaker

response compared to those from colony CBP, highlighting sex and colony-based variations in the immune response to HiSvV infection.

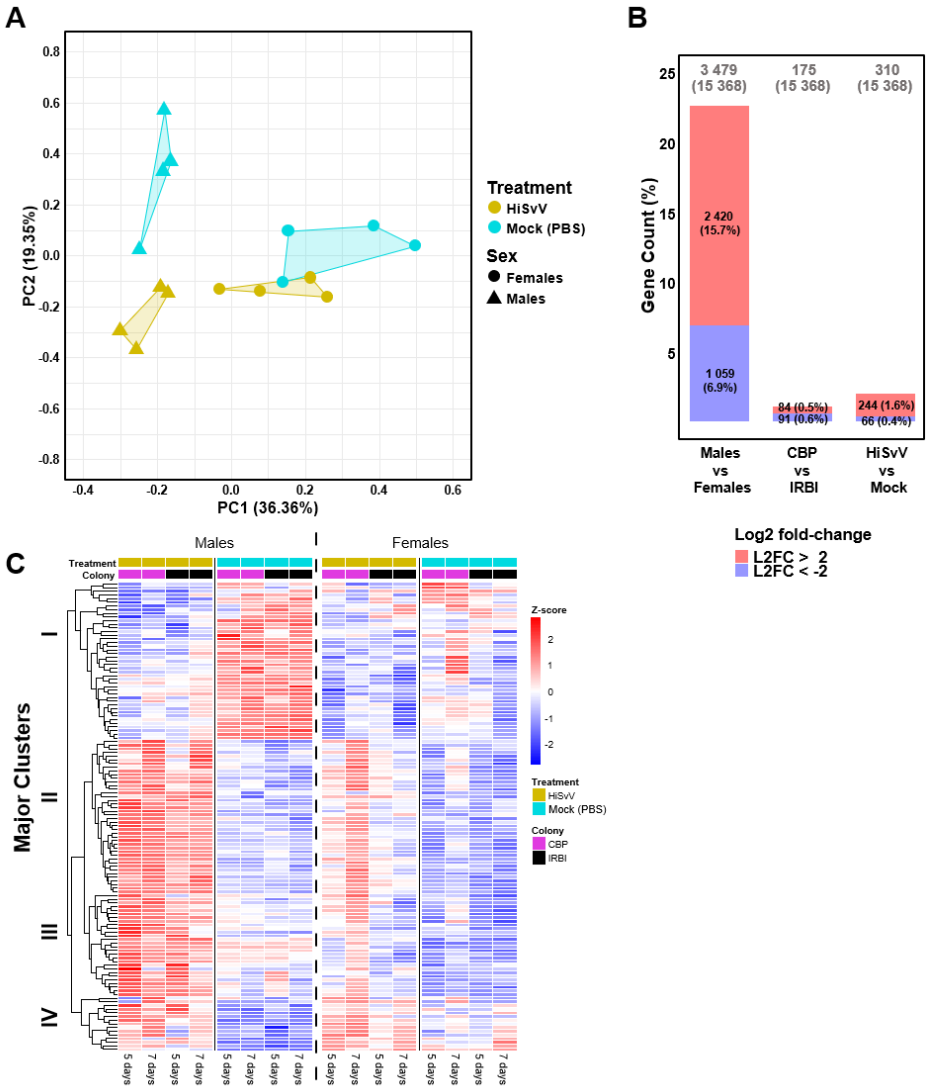

Figure 3.11 Transcriptomic analysis of adult BSF orally infected with HiSvV. A) Principal component analysis of whole transcriptomic data. B) Number of all significant up and down regulated genes in the whole transcriptome and only immune-related genes when contrasted within each grouping and the L2FC was greater than 2-fold. The DESeq2 calculated L2FC was considered significant if the adjusted p-value was less than 0.05. The percentage for immune-related genes was calculated relative to the total number of significant genes with a L2FC greater than 2-fold in their grouping. C) Heatmap of immune-related genes with a significantly different log2 fold-change (L2FC) contrasting HiSvV- vs Mock (PBS)-infected adults.

Focusing on immune-related genes that displayed an L2FC greater than 2-fold, 26 out of 27 of these genes were upregulated (Table 3.1). Predominantly, these upregulated genes are part of three main immune processes: signalling and regulation, antimicrobial peptide (AMP) synthesis, and autophagy. Signalling and regulation processes dominate these selected genes

(10 genes out of 26) with the gene E3 ubiquitin-protein ligase XIAP-like gene (apoptosis regulation) being the most upregulated (L2FC = 9.55). Followed by autophagy-related lysozyme-like genes (5 genes) and Lysozyme 1-like LOC119654544 (L2FC = 5.22) as the second most upregulated gene. These responses are not very target-specific, but there were genes which were more specific such as AMP-related genes (Table 3.5). Although one AMP gene (Cecropin-like peptide 1 LOC119657830) was well downregulated (L2FC = -2.45), three other AMP genes were upregulated with L2FCs above 2, which were Attacin-B-like (L2FC = 5.02), Cecropin-like peptide 1 LOC119657589 (L2FC = 3.2) and Defensin-like (2.15) genes.

Table 3.5 Description of genes with a significant log2 fold-change (L2FC) above 2 and adjusted p-values (pAdj) contrasting between HiSvV- and Mock (PBS)-infected BSF adults. Genes coloured in red were up-regulated and genes coloured in blue were down-regulated according to the mean.

Cluster	GeneID (LOC#)	Description	L2FC	pAdj
II	119648531	E3 ubiquitin-protein ligase XIAP-like	9,55	8,95E-34
II	119654544	Lysozyme 1-like	5,22	2,26E-08
II	119657249	Attacin-B-like	5,02	3,99E-14
II	119654410	Lysozyme-like	4,85	1,68E-20
III	119655009	Lysozyme 1-like	4,73	1,69E-02
II	119660505	Uncharacterized LOC119660505	4,13	3,34E-18
II	119646131	Myb-like protein Q	3,70	9,21E-16
II	119655940	Tetraspanin-2A	3,68	6,84E-32
II	119648931	Baculoviral IAP repeat-containing protein 3-like	3,59	5,68E-12
II	119652145	Peroxisomal acyl-coenzyme A oxidase 3	3,34	8,27E-27
III	119661601	Protein nubbin-like	3,31	7,62E-12
II	119655169	Transferrin-like	3,29	3,25E-18
IV	119657589	Cecropin-like peptide 1	3,20	4,18E-02
II	119649011	Death-associated inhibitor of apoptosis 1-like	3,10	2,84E-04
III	119661286	Tetraspanin-11-like	2,85	9,31E-05
II	119659886	Integrin alpha-PS3-like	2,82	1,40E-14
II	119654408	Uncharacterized LOC119654408	2,58	5,29E-08
II	119654625	Lysozyme-like	2,55	4,74E-05
II	119651771	Adenosine deaminase 2-like	2,50	8,26E-03
III	119659850	GATA-type transcription factor SRE1	2,50	1,97E-07
II	119651604	Protein draper-like	2,48	1,10E-06
II	119650124	Death-associated inhibitor of apoptosis 1-like	2,34	3,59E-14
II	119654409	Uncharacterized LOC119654409	2,32	5,72E-10
II	119654476	Rho-related protein racB-like	2,30	1,41E-06
III	119654947	Uncharacterized LOC119654947	2,22	2,91E-06
IV	119654016	Defensin-like	2,15	4,31E-02
I	119657830	Cecropin-like peptide 1	-2,45	3,92E-02

Despite signalling and regulation factors being the main response, the high L2FC of the Attacin-B-like gene suggests that this AMP is involved in the immune response alongside lysoymic activity (autophagy) against HiSvV (Table 3.5).

3.4 Discussion

Pathology research in mass-reared insects for food and feed has garnered more attention within the last few years, and BSFs are no exception (Eilenberg and Jensen, 2018; Eilenberg *et al.*, 2018; Joosten *et al.*, 2020; Bertola and Mutinelli, 2021; Maciel-Vergara *et al.*, 2021; Jensen and Lecocq, 2023). While Research is beginning to make progress for insects such as crickets and mealworms, it has taken slightly longer for interest to develop in BSF health (Duffield *et al.*, 2021; Maciel-Vergara *et al.*, 2021; Jensen and Lecocq, 2023; Slowik *et al.*, 2023; Takacs *et al.*, 2023; Lim *et al.*, 2024). This study was the first to isolate a virus from diseased BSFs and demonstrated its pathogenicity in this species. This finding is significant since BSFs have become a major economically important species (Tomberlin and Huis, 2020; van Huis, 2021).

We received BSF adult samples from a BSF rearing facility experiencing high levels of adult mortality and reduced egg production. We linked the genetic presence of HiSvV with the presence of viral particles, confirming it as a viral agent with a morphology consistent with members of the *Solinviviridae* family (Brown *et al.*, 2019). We observed oral infection in BSF adults suggesting the horizontal transmission was possible. This was also seen when mock- and HiSvV-infected males and females were cohabiting, although further confirmation (a negative strand qPCR) of the active replication of HiSvV in the reciprocating non-infected individuals would provide additional evidences. Nevertheless, the reasonably high titre in individuals that were initially free of HiSvV and the detection of HisvV in egg clusters support horizontal transmission. This study did not show continuity of HiSvV infection through the whole lifecycle, which is still debated for other *Solinviviridae* such as SINV3 (Valles and Hashimoto, 2009; Valles *et al.*, 2014, 2016). Although like for SINV3, further work is needed to clarify whether the HiSvV can truly replicate in larvae (Valles *et al.*, 2014), These results did suggest the potential for vertical transmission of HiSvV. Injected HiSvV1 displayed systemic tropism, favouring the digestive tract, suggesting natural infection occurs mainly through the oral-faecal route, confirmed by oral inoculation of adults, which was also found for SINV3 (Valles, 2012; Mondotte and Saleh, 2018).

An oral infection of adults by HiSvV clearly reduced their lifespan, inducing premature mortality. This decrease in lifespan was observed for both males and females, with a higher impact in males. In female BSF, the results suggested that a coinfection with HiInV might mitigate the impact of HiSvV, dampening its effects. This study is the first to examine the

effect of a solinvivirus infection on sex, contrasting prior studies focused on unsexed larvae of shrimp or direct effects on female castes and larvae in ants and bees (Valles and Porter, 2015; Valles *et al.*, 2016; Cruz-Flores *et al.*, 2022; Ryabov *et al.*, 2023). Comparing the outcomes of infections by other Solinviviridae, such as SINV3, AmSV1, and PvSV, reveals that premature mortality caused by these viruses played a major role for colony collapse in ants, honeybees, and whiteleg shrimp. (Valles and Porter, 2015; Cruz-Flores *et al.*, 2022; Ryabov *et al.*, 2023).

In Solenopsis invicta, SINV3 also induced changes in host behavior and immunity, with infections affected by environmental conditions like temperatures above 28°C hindering SINV3 infection (Valles and Oi, 2014; Valles *et al.*, 2014; Valles and Porter, 2019; Arnold *et al.*, 2021; Holmes and Johnston, 2023; Valles, 2023, 2024). While these additional interactions are not yet studied for HiSvV, they provide a targeted direction for future research for *Solinvivirdae* in general. Notably, SINV3 was found to decrease the fecundity of infected queens by severely reducing the number of fully developed eggs (Valles *et al.*, 2013). This could also be a factor for HiSvV infection and colony collapse, but would only be answered if future HiSvV studies include the effect in adult fecundity and fertility. In addition, the risk of colony collapse due to HiSvV infection may be reinforced if BSF are dying before mating or oviposition, particularly if virus levels are allowed to build up within the rearing facility (Maciel-Vergara *et al.*, 2021). This risk would be more pertinent for continuous rearing setups, as mating can start at least three to four days post-emergence (Lemke *et al.*, 2023; Munsch-Masset *et al.*, 2023).

To study BSF immune responses against HiSvV infection and survival, we performed an sRNA analysis and DEG analysis using two different BSF colonies with similar genetic backgrounds but different viral infections: one “virus-free” and the other hosting HiInV and HiSgV infections (Chapter 2). For all the HiSvV-infected flies, the broad profiles observed for HiSvV in the sRNA analysis revealed that BSFs did not present a targeted silencing response against HiSvV. Despite this, the sRNA machinery could still be utilised for other viruses, such as was observed against HiInV, particularly in females. The broad profiles for HiSvV indicate that HiSvV may be able to evade the sRNA response pathways. Viruses such as *Dicistroviridae* and *Solinviviridae* can evade RNAi silencing due to a dsRNA binding domain on their genomes (Valles *et al.*, 2016; Fareh *et al.*, 2018; Palmer, Varghese, *et al.*, 2018; Warsaba *et al.*, 2019). The same dsRNA binding domain can be found for HiSvV and likely explains the broad sRNA profile, indicating general viral genetic material degradation rather than specific RNAi or PIWI

responses. This was supported by DEG analysis, which showed significant transcriptional changes in immune-related genes (Figure S3.1 and Table S3.3).

In BSFs from both colonies, males exhibited a broader immune response regardless of HiInV and/or HiSgV infection. However, females from the CBP colony had a broader immune response compared to IRBI females, who showed a more specific DEG response to HiSvV, possibly due to HiInV co-infection, suggesting an HiInV-induced basal activation of antiviral defence pathways which may also target HiSvV. This specificity in response aligns with the lower hazard ratios observed in IRBI females during HiSvV survival assays. While males generally lived longer in survival assays, this effect was attributed to sex and colony-based differences. This antiviral immunity dimorphism has also been found in other flies, such as *Drosophila* spp, suggesting the need for additional studies in insects (Kelly *et al.*, 2018; Palmer, Medd, *et al.*, 2018; Palmer, Varghese, *et al.*, 2018; Oku *et al.*, 2019; Belmonte *et al.*, 2020; Frangeul *et al.*, 2020).

Comparatively, Walt *et al.*, (2024) examined the broader immune response in BSF infected with HiSgV (HiSV1) and HiInV (HiTV2) and found that only eight genes out of 210 candidate antiviral genes were significantly upregulated, part of the IMD, JAK/STAT, and AMP pathways. Although they did not suspect RNAi silencing involvement, our sRNA analysis indicated RNAi involvement for both HiSgV and HiInV. Walt *et al.*, (2024) identified vir-1 among the upregulated genes, which, while a product of the JAK/STAT pathway, is related to the *Dicer*-2/*vago* cascade and stimulated by virus replication (Kingsolver and Hardy, 2012; Kingsolver *et al.*, 2013). When *Dicer*-2 detects viral dsRNA, it uses *vago* to prompt the JAK/STAT pathway to produce *vir*-1 (Kingsolver and Hardy, 2012; Palmer, Varghese, *et al.*, 2018). Although the exact mechanism of action by *vir*-1 is not well described, its involvement with *Dicer*-2 and *vago* suggests a potential connection to RNAi (Kingsolver *et al.*, 2013; Wang, 2021). Our sRNA analysis and findings by Walt *et al.*, (2024) point to the antiviral response against HiSgV and HiInV as part of the basal innate immunity, more than immune priming or specialized pathways such as the pathways associated with PIWI.

Differentially expressed gene analysis for HiSvV showed significant upregulation for genes related to autophagy (Lysosomal response), AMP (Attacin-B-like, Cecropin-like peptide 1, and Defensin-like), as well as signaling and regulation (including apoptosis inhibition). While not all AMPs are able to work against viral infections, some cercropins, attacins and defensins can be involved in antiviral response (Palmer, Varghese, *et al.*, 2018; Moretta *et al.*, 2020). However, the apparent higher number of genes related to the autophagy response indicates that this is likely playing a large role in the general degradation of HiSvV, likely

explaining the degraded sRNA profiles. Autophagy, a general pathway suitable for pathogens evading RNAi and AMPs, is particularly relevant for insect-infecting picorna-like viruses such as *Dicistroviridae* and *Solinviviridae*. It remains to be determined if any upregulated AMPs are directly involved in the host response to HiSvV since there is the possibility of them being used to control any opportunistic microbiota (Valles *et al.*, 2016; Palmer, Varghese, *et al.*, 2018; Warsaba *et al.*, 2019; Klüber *et al.*, 2022).

Previously it was widely thought that BSF would be resistant to viruses. This study showed that a recently discovered virus, HiSvV, is able to significantly shorten the lifespan of adult BSF from distinct colonies and is likely the culprit behind a reported loss of production within a BSF farm. We also found that pathology and immune response differed depending on the resident virome. Since HiSvV has the capacity to decrease production and prompts the need to develop management plans and surveillance tools to prevent future outbreaks and interfacility transfer. This work is an important milestone in BSF pathology and mass-rearing since it is the first study to show that viruses can be a threat to BSF.

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Supplementary data

The supplementary data can be found in Appendix 1

Final discussion: BSF have viruses, now what?

1. BSF pathogens since 2022 – Are BSF Hercules or Achilles?

There is still today, the idea that BSF are the Hercules of the mass-reared insects (Tomberlin and Huis, 2020; Jensen and Lecocq, 2023). Yet, with the work done during my PhD, and also the growing number of studies in BSF pathology, perhaps Achilles may be a more fitting comparison for this increasingly endearing mass-reared species. Before tying together the overarching themes surrounding the BSFs and their associated viruses and what this means for the future of the BSF rearing industry, we need to contrast my findings on BSF viruses with other recent pathology work.

2. BSF and viruses, should we care?

My thesis aimed to optimize virus discovery and develop methods to perform viral pathology studies in BSF. In essence, the thesis started with a paleovirological approach to examine the diversity of historical virus interactions in BSF genomes and linked one EVE to an exogenous virus when compared with current infections circulating in BSF metatranscriptomes. Following this in Chapter 2, a virus discovery pipeline was optimized to screen a large number of BSF datasets in a high-throughput manner and this led to the discovery of five more viruses. Using these six viruses, a rapid screening by mapping high-throughput visualization tool was developed along with RT-PCR and RT-qPCR virus detection assays. Lastly, in Chapter 3, one of the viruses was isolated and its pathology and pathogenicity in BSF was studied, which saw the first viral pathogen of BSF to be identified.

3. BSF host a diverse array of viruses, both currently and historically

Chapters 1 and 2 revealed a diverse set of viruses that could infect BSF, identifying 11 different families within the BSF virome: *Dicistroviridae*, *Iflaviridae*, *Inseviridae* (Formerly *Totiviridae*), *Lebotiviridae* (Formerly *Totiviridae*), *Partitiviridae*, *Parvoviridae*, *Rhabdoviridae*, *Solinviviridae* and *Xinmoviridae*. *Parvoviridae* are DNA viruses, and although DNA viruses were not excluded from the scope of this project, so far, the only transparent evidence of interactions between BSF and DNA viruses is paleovirological. Among the meta-omic datasets analysed for extant viruses, four were metagenomic (MGC), while others were metatranscriptomic (MTC). Despite the challenges in detecting DNA viruses through MTC datasets due to truncation of genetic material between genes, expressed genes of DNA viruses are still identifiable. However, the complexity of DNA virus detection increases when auxiliary

genes are shared with non-viral organisms, a prime example being the case of baculoviruses (Gilbert *et al.*, 2016). To overcome these challenges, PoolingScreen and Lazypipe2 were employed in conjunction with each other. PoolingScreen, detailed in Chapter 2, provides a basic overview of the data, but does not annotate ORFs in its results. Lazypipe2, however, facilitates a more nuanced dissection of the data by providing additional analytical depth. If contig lengths exceeding 1 kb were detected but not confirmed by Lazypipe2, they were considered false positives. While I didn't confirm that there were DNA viruses outside of the BSF genome, given what was found with finding endogenous and exogenous *Totiviridae* (now *Lebotiviridae*) and *Rhabdoviridae*, we do hypothesize that BSF host exogenous DNA viruses, as endogenous *Parvoviridae*-related viruses were found in its genome.

Two *Bunyavirales* viruses, BSF nairovirus-like 1 (BNaV1, *Nairoviridae*) and BSF uncharacterized bunyavirus-like 1 (BuBV1, unclassified *Bunyavirales*), can be considered as part of the BSF virome, since the virome consists of ALL viruses that can at least be found in within the anatomy of the individual (Smith *et al.*, 2022). However, whether they can infect BSF is yet to be determined, particularly since the majority of the datasets where the virus was found were obtained from BSF frass (Walt *et al.*, 2023, 2024; Chapter 2). Having said this, there were some datasets obtained from BSF larvae where at least BuBV1 was detected, albeit normally at often lower levels than in the frass datasets. Tying things back to the NGS-based viruses discovery caveats presented in the General Introduction, the datasets surrounding the discovery of BNaV1 and BuBV1 had a very low sequencing depth, topped with a high percentage of reads mapping to BSF in larval samples. This low depth and proportion of non-BSF reads may present an obstacle in the detection of the BuBV1 and BNaV1 in the BSF larval datasets (Walt *et al.*, 2023, 2024; Chapter 2). The fact that the study bioprojects were obtained a few years apart does also pose a question of whether there is an external reintroduction of the viruses, or they were indeed circulating with the colony. With their recent preprint, Walt *et al.*, (2024) may have partially answered the question of the origin of BuBV1 (Figure D.1). Essentially, all of the datasets where BuBV1 and BNaV1 are detected were obtained from frass (Figure D.1). It should be noted that for BuBV1 it was also found in diet without any contact with BSF larvae, and only one occurrence in actual larval samples. This is in stark contrast to what was found for *Hermetia illucens* toti-like virus 2 (*Hermetia illucens* insevirus, HiInV) and *Hermetia illucens* sigma-like virus 1 (*Hermetia illucens* sigmavirus, HiSgV) which were only found in BSF larval samples, with one exception (Figure D.1).

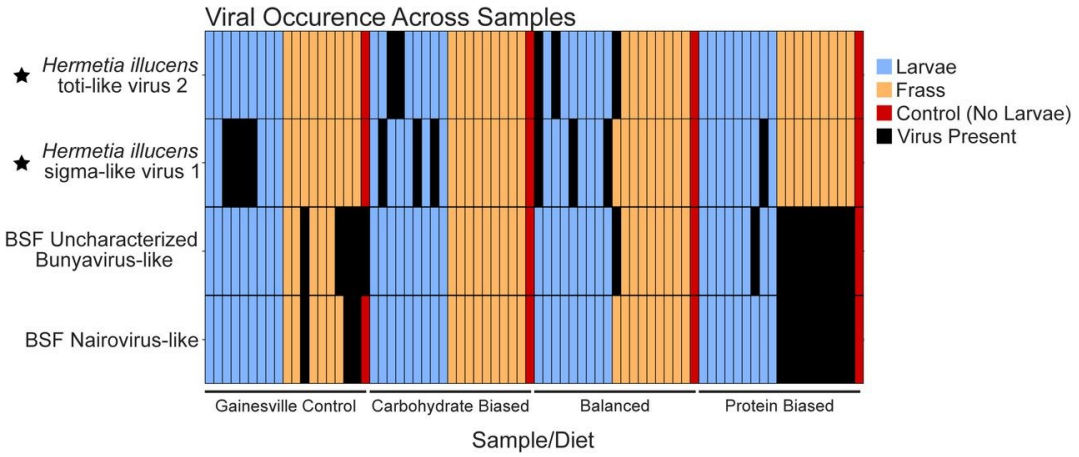

Figure D.1 Presence of mapped viral reads across BSF larval and diet datasets for BSF nairovirus-like 1, BSF uncharacterized bunyavirus-like 1, *Hermetia illucens* toti-like virus 2 (*Hermetia illucens* insevirus) and *Hermetia illucens* sigma-like virus 1 (*Hermetia illucens* sigmavirus) (Obtained from Figure 3 in Walt *et al.*, (2024)).

Additionally, in this set of experiments BNaV1 was only ever detected alongside BuBV1. This in interesting, because when using the high-throughput (HT) mapping-based virus screening pipeline (details in Chapter 2) for datasets from NCBI bioproject PRJNA542977, I also found that some datasets should a similar trend (BNaV1 co-occurring with BuBV1) when using the same viral presence demarcation criteria as Walt *et al.*, (2024), which is a minimum of 10 mapped viral reads within a dataset. However, when examining the distribution of mapped reads across the virus genomes using HT mapping heatmaps (Figure S2.7), two things can be observed. 1) There is a short, conserved region on the L segments of BNaV1 and BuBV1, and one short region on the S segment of BuBV1 where many reads were mapping. 2) When BNaV1 was "detected" in the same datasets as BuBV1, all of the reads were concentrated on this short region on the L segment. On closer inspection accompanied by a BLAST analysis, these regions were joining the edges of ORF sequence with non-coding sequence and BLAST results revealed that the regions were reasonably well-conserved at the nucleotide level across a broad range of other groups of viruses, and also taxonomic kingdoms such as plants and animals (Tables S2.9, S2.10 & S2.11). Walt *et al.*, (2024), mentioned that the negative control sample here BuBV1 was detected only contained a fragment which was similar, thus this along with only finding BuBV1 reads in BSF frass in their second study led them to conclude that BuVB1 may not infect BSF. Without Walt *et al.*, (2024), providing any further information, such as confirming where the fragment mapped to on the BuBV1 genome, it may have been a similar case to what was observed in Tables S2.9, S2.10 & S2.11, possibly being a false positive

identification. More evidence should be presented before confidently presenting BSF as a host of BuBV1 and BNaV1, as these viral sequences might represent commensals.

In terms of viral surveillance in BSFs, this project in conjunction with (Walt *et al.*, 2023, 2024) only managed to survey BSF datasets within selected countries, leaving a huge gap in countries that farm BSF (see Figure GI.4), predominantly in the eastern and southern hemispheres (Figure D.2). Given the diversity of viruses already found to be associated with BSF, this promotes a need to greatly expand the survey and should encourage other countries to also share genetic data to allow for a more collaborative effect in virus research in BSF.

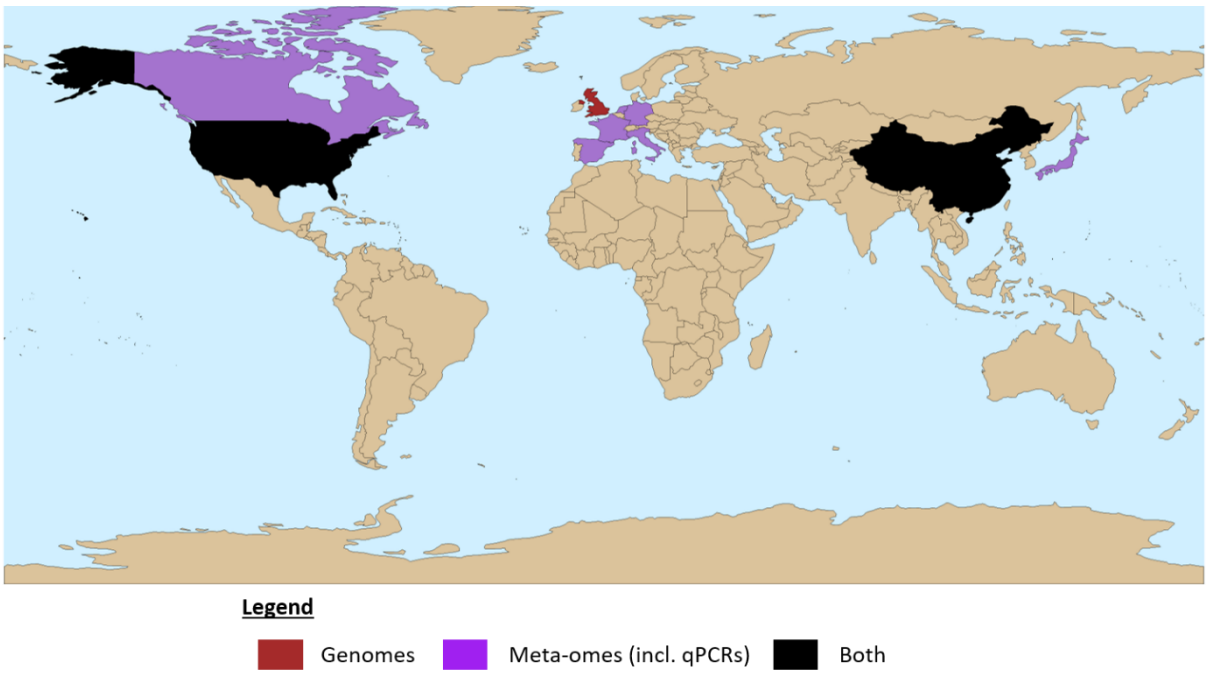

Figure D.2 Global map indicating which countries where BSF genetic material have been screen for viruses. The grouping "Meta-omes" is an umbrella term for metatranscriptomes and metagenomes, but also includes data from RT-qPCRs generated from BSF received from BSF industrial farms and research laboratories. The RT-qPCR data only involved BSF samples received from inside the European Union.

Putting the virome of BSF into a greater context with other flies. For mass-reared flies involved in food and feed, waste management and other selected species (Section 2.3 in the General Introduction), most of the flies screened hosted around nine different exogenous viruses, with the exception of *Drosophila* spp. and *Ceratitis capitata*. With eight viruses found so far in BSF, this shows that the number of viruses found is not overestimated. While virus discovery studies still focus more on mosquitos and *Drosophila* spp., the increase in virus diversity found in other diptera does show promise for more viruses waiting to be discovered. Looking at the closest-

related fly to BSF that has been studied, *Inopus flavus*, nine viruses have been uncovered (Asselin *et al.*, 2021; Colmant *et al.*, 2022; Divekar *et al.*, 2024). There is a high number of virus families (five) shared between the two flies, *Dicistroviridae*, *Iflaviridae*, *Inseviridae* (formerly *Totiviridae*), *Lebtoviridae* (formerly *Totiviridae*) and *Rhabdoviridae*. This was also found for other fly species (Section 2.3 in the General Introduction). This trend could allow for further prediction that BSF may in fact also host a virus from the family *Orthomyxoviridae* since it was another virus family commonly associated with other flies. Something that was intriguing when explore the BSF paleovirome and the exogenous virome of *I. flavus*, is that all the BSF EVEs were related to virus families found in the *I. flavus* exogenous virome. While further work is needed, we can hypothesize for now that the virus families associated with BSF may have an intertwined relationship with the soldier fly family (Stratiomyidae).

In terms of the discovery of viruses in the BSF virome, recent work has stressed that EVEs may be detected more often than previously realised in metatranscriptomic data, thus affecting exogenous virus discovery by data mining (Rozo-Lopez *et al.*, 2023; Brait *et al.*, 2024). It was found that the EVE HiLbV (formerly HiTV1) was expressed in some BSF metatranscriptomes (Pienaar *et al.*, 2022), additionally this was also observed for some EVEs related to *Parvoviridae* (data not shown). Despite this, the exogenous BSF viruses were either too distant from EVEs falling within the same viral family, or were not related to any of the EVEs found. Thus, this reinforces that the eight viruses found in BSF datasets are truly exogenous.

4. BSF are susceptible to viral pathogens and present both specific and general antiviral responses

Since a selection of viruses had been found, the next step was to isolate one and test its pathogenicity and virulence in BSF. In this thesis, Chapter 3 shed light on virus interactions between BSF and a disease which was reported to result in premature mortality and reduce fecundity in BSF mass-rearing. One of the newly discovered BSF viruses, *Hermetia illucens* solinvivirus (HiSvV, *Solinviviridae*, *Picornavirales*), was found in a colony experiencing these signs of disease. Since there had not been any virus work performed on BSF until recently, there were no optimized virus infection protocols. This posed a challenge for attempting to study HiSvV either *in vivo* (whole insect) or *in vitro* (cell culture). There was no preestablished cell culture, thus I tried two different approaches of *in vivo* inoculation, subcutaneous injection and oral feeding, to assess the ability of HiSvV to infect BSF. Once the oral feeding protocol was established and reproducible in adults, it allowed us to setup a scalable bioassay which

could test longevity of BSF adults alone in small containers without being in contact with other BSF. This was a step forward, since studies typically investigate BSF adult performance whilst cohabiting in cages (Chia *et al.*, 2018; Bruno *et al.*, 2019; Lecocq *et al.*, 2020; Liu *et al.*, 2022; Munsch-Masset *et al.*, 2023; Manas *et al.*, 2024).

After isolating HiSvV from infected BSF and performing oral feeding bioassays, this thesis was able to demonstrate Koch's postulates with HiSvV in BSF, verifying the first known viral pathogen in BSF (Chapter 3). We were also able to show that both male and female adult BSF were dying sooner as a result of an HiSvV infection. This is particularly important because even though concerns of viral pathogens have been raised explicitly in scientific literature (Joosten *et al.*, 2020; Bertola and Mutinelli, 2021; Pienaar *et al.*, 2022; Jensen and Lecocq, 2023; Walt *et al.*, 2023), there are still fair number of medium to large scale industry players who are under the impression that BSF are resilient towards viruses in general or have become desensitized to novel virus discovery research in insects since many virome studies don't follow through with Koch's postulates or further interaction research. This was even more apparent after I had presented some work on HiSvV at a BSF-focused conference, BSF Con in Cambridge, UK (September 2023). But our research did not stop at determining infectivity and pathogenicity of HiSvV in BSF.

Even though we did not currently investigate further signs and symptoms of the disease caused by HiSvV, we did study interactions between HiSvV and BSF at the transcriptional level (Chapter 3). While studying the transcriptional responses, we found that there appears to be a broad antiviral immune response to HiSvV infection. Not only that, but that there was evidence indicating a level of sexual dimorphic immune responses in BSF (Kelly *et al.*, 2018; Shepherd *et al.*, 2021). In the case of BSF, males tended to have a broader range of upregulated immune genes than females. This could be related to where females prefer to allocate resources, but further experimentation is required to confirm this. Looking into virus interactions is not a simple affair, one reason being that it is not always just one virus or pathogen which is infecting the host at the same time (Chapter 2; Gasmi *et al.*, 2018; Deschodt and Cory, 2022; Du *et al.*, 2022; Hernández-Pelegrín *et al.*, 2022; Lin *et al.*, 2023; Slowik *et al.*, 2023). One of the advantages of us finding a colony of BSF which was free of detectable viruses was that we were able more accurately observed the impact of HiSvV on BSF. Having said this, coinfections can affect how viruses interact with the host (Gasmi *et al.*, 2018; Du *et al.*, 2022). So, we also explored an HiSvV infection using a BSF colony where we had previously detected

a coinfection with HiInV and HiSgV. While we didn't see an impact on HiSvV replication by the coinfection, we did observe that the mortality of females with this coinfection was less impacted when also infected with HiSvV. In these same females, we saw that fewer immune genes were upregulated than in females which were not coinfected with HiInV and HiSgV. Combined with the specific sRNA response to HiInV by female BSF, we hypothesize that this is likely due to Immune basal activation. It is already known that dsRNA can lead to upregulation of *vir*-1 as observed for *Drosophila* in response to an infection with Drosophila C virus (DCV, *Dicistroviridae*, *Picornavirales*), a single stranded RNA virus that has a dsRNA binding domain (Kingsolver and Hardy, 2012; Kingsolver *et al.*, 2013; Palmer, Varghese, *et al.*, 2018; Warsaba *et al.*, 2019; Wang, 2021). Immune basal activation by a dsRNA virus such as HiInV, which clearly has more specific responses, could lead to other antiviral responses that may make BSF females more tolerant to HiSvV infection (Chapter 3; Fareh *et al.*, 2018; Palmer, Medd, *et al.*, 2018; Palmer, Varghese, *et al.*, 2018; Warsaba *et al.*, 2019; Walt *et al.*, 2024).

Our results also suggest that since there was broad immune response which was not necessarily directly interacted with phenoloxidase (PO) pathways or was much broader than just one or two AMPs such as defensins or cecropins. Thus, there is further incentive to include a broader variety of immune genes into studies which are more targeted qPCR approaches to assessing general or antiviral immune responses in BSF in studies assessing multiple pathogens together (González-Santoyo and Córdoba-Aguilar, 2012; Kingsolver and Hardy, 2012; Palmer, Varghese, *et al.*, 2018; Moretta *et al.*, 2020; Bruno *et al.*, 2021; Vogel *et al.*, 2022; Cho and Cho, 2024; Shah *et al.*, 2024). This is important where studies focusing on BSF interacting with multiple pathogens (Candian *et al.*, 2023). It is also incredibly relevant since a few studies have been testing mainly PO or AMPs independently as a proxy when measuring BSF immune response performance (Opare *et al.*, 2023, 2024). This is crucial for BSF colonies that have not been tested for BSF-associated viruses, given the widespread viral prevalence in domesticated colonies (Chapter 2).

To date, there are multiple tools and protocols to study virus and other pathogens in BSF. From this thesis alone, bioassay protocols and viral screening tools were produced. Other work has produced bioassay protocols to study BSF pathogens strictly in BSF larvae (Lecocq *et al.*, 2023; Shah *et al.*, 2023; She *et al.*, 2023). There is even a publicly available BSF cell line which could be used to test viruses and other intracellular pathogens (Saathoff *et al.*, 2024). This

development can only benefit future BSF studies as our understanding of BSF pathology continues to grow and approaches are constantly improved.

5. A diverse array of non-viral pathogens could also impact BSF

Since 2022, research on pathogens, parasites, and pests affecting BSF has expanded. Klüber *et al.*, (2022) took a less conventional route and explored the pathogenicity of culturable BSF gut microbiota. They obtained around 69 bacterial and 24 fungal isolates and tested six of them in BSF larvae after a literature search had shown that they could be pathogenic in other insects. Injection assays in BSF larvae tested a relatively high dose of 10^6 conidia or colony forming units, but did show that *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Microbacterium thalassium* and *Fusarium solani* could reduce the number of larvae to below 50% within 1 to 4 dpi (Figure D.3A). Two of the isolates, *P. aeruginosa* and *K. pneumoniae* were able to reduce BSF larvae survival to 20 % and 16.7% respectively by 7 days, and *F. solani* killed all larvae within 1 dpi. This shows that these specific microbiota could act as facultative pathogens, particularly since the two others tested (*Diutina rugosa* and *Alcaligenes faecalis*) did not reduce the larval survival lower than 75% (Figure D.3A) (Klüber *et al.*, 2022).

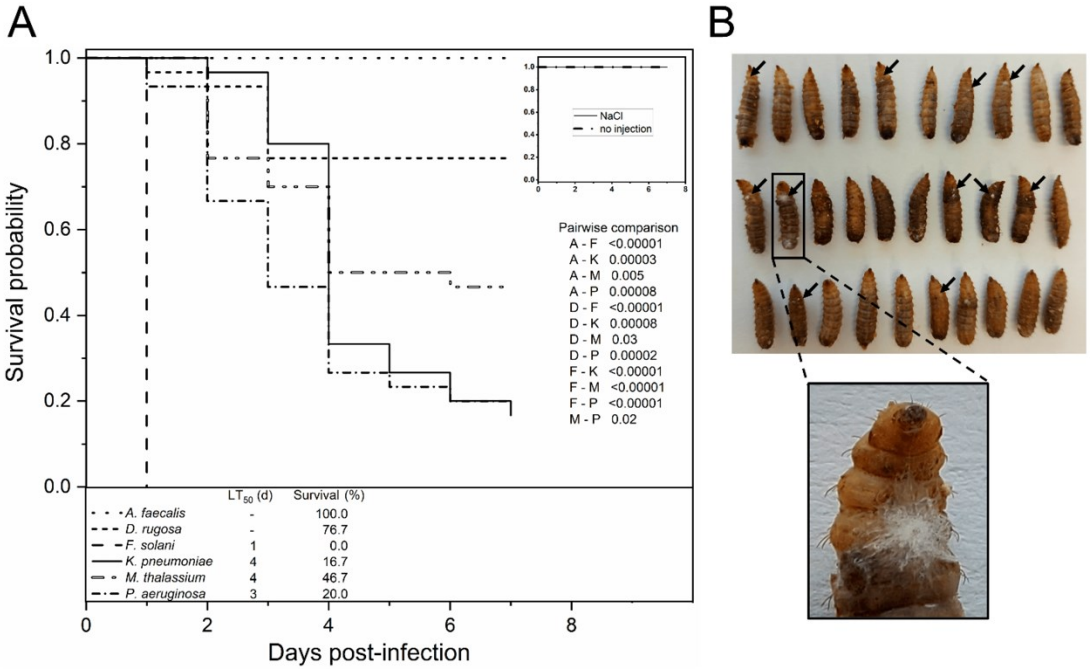

Figure D.3 Injection assays in BSF using isolated BSF gut microbiota. A) Survival curves of BSF larvae inoculated with bacterial isolates identified as *Alcaligenes faecalis*, *Klebsiella pneumoniae*, *Microbacterium thalassium* and *Pseudomonas aeruginosa*, a yeast isolate identified as *Diutina rugosa* and a filamentous fungal isolate identified to *Fusarium solani*. B) Larvae infected with *F. solani* collected post-mortem. (Obtained from Figure 6 in (Klüber *et al.*, 2022)).

Caution should be taken since the doses used for bacteria or fungi injection were relatively high, and possibly resulted in a septic shock-like effect due to instant microbiota disequilibrium rather than true pathogenesis. For instance another recent study by Shah *et al.*, (2023) demonstrated that injecting with approximately 3 bacterial cells of *Pseudomonas protegens* Pf-5 into five days-old BSF larvae reduced the survival of larvae by 50% before 3 dpi. This shows that *P. protegens* Pf-5 can have a clear pathogenic and highly virulent effect on BSF larvae when injected. Nevertheless, it is not improbable that BSF larvae could encounter much higher levels of these bacteria and fungi in mass-rearing facilities (Eilenberg *et al.*, 2015; Eilenberg and Jensen, 2018; Vogel *et al.*, 2022).

The first natural bacterial pathogen[8] of BSF found is *Paenibacillus thiaminolyticus* GX6 and was first isolated in 2023. This was the first published investigation into signs such as BSF mortality and development issues at a multiple BSF farms associated with the term "soft rot" (Figure D.4). She *et al.*, (2023) were able to successfully replicate symptoms in BSF larvae when mixing *P. thiaminolyticus* cells in the diet, thus demonstrating Koch's postulates for this pathogenic bacterium. They also found that the syndrome was more prevalent in higher temperatures (above 27 °C) and when the humidity was between 50 to 60%. Interestingly, they found that inoculating BSF with *P. thiaminolyticus* spores did not yield visible signs of disease, only when adding vegetative cells, suggesting that the endospores may not be easily disturbed when passing through the BSF gut (She *et al.*, 2023).

Figure D.4 A comparison of corpses in one of the facilities affected by soft rot (Obtained from Figure 1 in She *et al.*, (2023))

[8] A pathogen that has been found to cause infections/outbreaks outside of experimental settings and uses traditional infection/transmission routes (Baker, 1998).

While any BSF pathogen reviews tended to focus on published scientific literature, a BSc Honours student mini-thesis attempted to investigate a few incidences of suspected diseases and pest interactions found in very exposed open BSF rearing (Rahman, 2022). While it was difficult to ascertain an accurate understanding of this work, originally written in Indonesian, what is clear that there was some potential in observations made, particularly in an apparent fungal attack on BSF larvae and what appears to be cases of "soft body/rot" (Figure D.5) (Rahman, 2022). Although a thorough testing of Koch's postulates should be demonstrated from these cases, it still points to the potential of disease states in BSF larvae. While it is not clear if a specific introduced pathogen was behind the main cause of death, there is room to suspect that the conditions may have allowed for a disruption to the symbiosis of the BSF microbiome, leading to the rise of opportunistic pathogens resulting in the death and rapid decomposition of the BSF larvae. Once again, while work by Klüber *et al.*, (2022) may not have shown acute pathogenesis in culturable BSF microbiota (General Introduction), these and other observations documented by Rahman, (2022) may have demonstrated the opportunistic potential laid out by Klüber *et al.*, (2022), and this still can pose a concern for BSF farmers, at least in open and semi-open facilities.

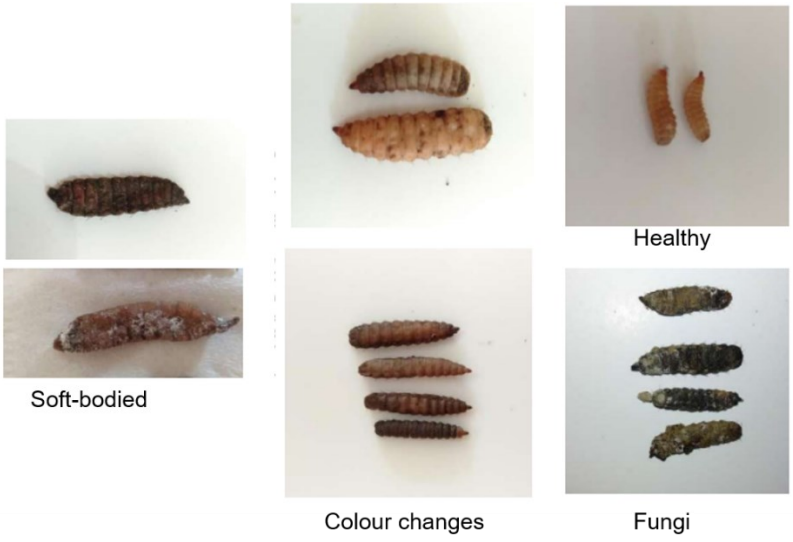

Figure D. 5 Different abnormalities or suspected signs of disease in BSF larvae (modified from Appendix 6 in Rahman, (2022)).

Further work on *Beauveria bassiana* has provided a deeper insight into this fungus' interaction with BSF (Mani *et al.*, 2023). Firstly, when dipping larvae into a highly concentrated *B. bassiana* suspension, they did not appear to observe a significant display of signs of disease.

However, by just allowing the larvae to feed on *B. bassiana* spores mixed in the diet, they observed a stark reduction in fifth-instar larval weight, adult emergence and adult weight. This upholds results by Lecocq *et al.*, (2020), and also greatly expands on their work. Mani *et al.*, (2023), also delved into the metabolic interactions surrounding *B. bassiana* infection and found that manipulation of protein synthesis was suspected to play a role in the pathology of the infection. Caution should be taken however when trying to reproduce work by Mani *et al.*, (2023) as they did not provide any conditions related to the rearing of the BSF at their laboratory, nor provide at least the temperature during the experimentation. They also miscited Lecocq *et al.*, (2020) when justifying the concentration of conidia used during experimentation (10^7 conidia/ml), using a concentration 10x less than used by Lecocq *et al.*, (2020). Whether this would have changed the overall outcome of the study, however, remains to be seen, particularly since a concentration of 10^7 conidia/ml is still a high concentration.

Branching away from microbial work, more parasitoid wasps have been found to actively parasitise BSF, this now brings the current list to seven species; *Calyoza hermetiae* (Bethylidae), *Dirhinus anthracia* Silvestri, 1914 (Chalcididae), *Eniacomorpha bouceki* Delvare 2019 (Chalcididae), *Spalangia cameroni* (Spalangiidae), *S. obscura* (Spalangiidae), *S. simplex* (Spalangiidae) and a species of *Trichopria* Ashmead, 1893 (Diapriidae) (Maquart, Willems, *et al.*, 2020; Binoy *et al.*, 2023). There is currently some hesitation about the list of parasitoids known to prey on BSF since a few *Dirhinus* spp, for example *D. giffardii*. may be synonymous to *D. anthracia*. Lastly, *Dermanyssus gallinae*, the poultry red mite, has also been recorded to negatively impact BSF colonies and should be monitored for in production (Mahmoud *et al.*, 2023)

6. Reproducibility and understanding pitfalls in BSF research

The ever increasing research of microbiomes in insects has cemented the idea that insects also need to be treated as holobionts[9] when studying host-pathogen interactions (Dheilly, 2014; Simon *et al.*, 2019; Savio *et al.*, 2022; Biggs *et al.*, 2023). This is why we were fortunate to be able to obtain a BSF colony which was free of detectable viruses and pathogens, when

[9] A concept similar to the "super organism" theory (Wilson and Sober, 1989), the holobiont includes the individual organism (host) and all the other organisms that live inside and direct exterior of the host.

investigating the effects of HiSvV in BSF (Thomas *et al.*, 2003; Hamelin *et al.*, 2019; Barrera *et al.*, 2021; Barreat and Katzourakis, 2022; Venter *et al.*, 2022). During this thesis, when performing oral-inoculated HiSvV viral replication and survival bioassays, we did encounter a level of variability in the experiments between the two colonies (Chapter 3). The two colonies are part of the same major haplotype (Figure S2.8) and the rearing approaches and diet were similar enough that the main factor which concerned us was that one colony had circulating viruses and the other did not (C017 and C024, Figure 2.6A). Initially, regardless of sex, the mock-infected (PBS treatment) flies from the IRBI colony were dying approximately 2.16 times faster than those from the CBP colony under the same bioassay conditions (Table S3.1A). We know that sex as a factor had a strong influence of the BSF lifespan in the experiments, but this was also influenced by which colony they had originated from (Table S3.1A). Further examination found that both sexes in the CBP colony and the males from the IRBI colony had hazard ratios within a similar range of each other (between 3.04 and 4.9, Table S3.1D) Since the females from the IRBI colony had much lower HRs compared to the others (1.6 to 2.03, Table S3.1D), this gives a different view of HiSvV infection in BSF. Had we just relied on the IRBI colony, we would have likely inferred that HiSvV was more pathogenic to males but did not impact females nearly as much. Therefore, we would have missed that a coinfection with HiInV can dampen the effect of an HiSvV infection on female mortality. Although better studied with other microparasites, this has been observed with other virus coinfections in insects (Goenaga *et al.*, 2015; Seabloom *et al.*, 2015; Pauli *et al.*, 2018; Patterson *et al.*, 2021), highlighting the importance of knowing the virome in experimental colonies and realizing that even viruses considered as non-pathogenic could positively or negatively impact the pathogenic state[10] and/or virulence [11]of a pathogen.

While our two colonies are in the same haplotype, research studies have shown that BSF can have a higher genetic variation than previously thought (Ståhls *et al.*, 2020; Kaya *et al.*, 2021; Guilliet *et al.*, 2022; Generalovic *et al.*, 2023). As highlighted by (Maciel-Vergara *et al.*, 2021), genetics can play a role not just in response to the virulence of a pathogen, but also whether the infection can be suppressed into a non-pathogenic phase. This is not just a factor in rearing facilities, but also in small-scale experiments. Some strains, if more exposed to pathogens, or have been interbred with other stains that were, could host some PIWI clusters which allow the immune response to activate against certain viruses being tested, which is potential the case for

[10] Pathogen infections can have infection phases where they do not illicit pathogenic effects in a host.
[11] How capable a pathogen is to cause pathogenic effects in a host, and how severe the effects can be.

HiSgV, HiLbV and HiInV, given their wide prevalence (Figure 2.6A). Given the arsenal of immune responses in BSF (De Smet *et al.*, 2018; Moretta *et al.*, 2020; Zhan *et al.*, 2020; Generalovic *et al.*, 2021), it is possible that some genes may have different basal activation or transcription regulation could differ slightly. Nonetheless, there are multiple factors which can affect infections, and these should be considered when planning experimental designs or which stains/colonies to use. Lastly, because the BSF rearing facilities can differ vastly in size and complexity, the scalability of experimental setups needs to be factored in to better reflect conditions which may be experienced by pathogens, vectors and BSF (Box 1).

Box 1: Strengths and weaknesses of experimental scaling

Experiments can occur at different levels of complexity. This also includes factors likes the size of the experimental setup, the number of individuals, density, ability to interact with other members of the subject species and vectors or additional species. Here is a list of some strengths and weaknesses for running experiments at different sizes and complexities which should be considered based on the questions and hypotheses being tested.

Experiment scale	Strengths	Weaknesses
Single individual experiments	• Easier to track effects at the genetic or anatomical level • Easy to control variables • Easier to ensure that all individuals are inoculated	• No interactions with other individuals, e.g. can't factor in group immunity or rate of transmission • More time needed per observation point
Paired and pooled experiments	• Some interaction information such as rate of transmission • Reproduction and generation studies • Can still be run by one person • Still able to control climatic conditions through the entire mesocosm	• Individuals need to be tracked and caught to be examined, disturbing others. • Multiple climate chambers may be required depending on size. • Effects no longer unaffected by contact with other individuals
Large-scale pooled experiments	• Easier to see the effects of a disease at the colony scale, e.g. colony collapse • Closer to industrial set ups	• Easy for pathogen infections to lose control if the experiment is not well quarantined. • Social immunity of the host. • Harder to measure symptoms. • Hard to ensure that all individuals are infected. • Difficult to track individuals • Hard to control for opportunistic pathogens. • Easy for uncontrollable factors to interfere with the experiment. • Multiple people required to run the experiment • The results are more variable, thus will need more replicates

The increasing discussion around reproducibility in BSF research in general can be encapsulated with one keyword: "variable". Recent studies have highlighted significant issues with reproducibility, where findings from similar studies often differ (Bosch *et al.*, 2020; Hopkins *et al.*, 2021; Deruytter *et al.*, 2023; Nayak *et al.*, 2024; Wiklicky *et al.*, 2024). This variability is not necessarily due to poor scientific standards but one suspicion is rather the complexity and inexperience in BSF experimentation (Wiklicky *et al.*, 2024). Even with established protocols, significant variation—up to 60% between different facilities and 24% within the same site—can occur (Deruytter *et al.*, 2023). This poses challenges, particularly when working with genetically diverse populations (Bosch *et al.*, 2020; Hopkins *et al.*, 2021; Deruytter *et al.*, 2023). Wiklicky *et al.*, (2024) suggested implementing positive and negative control bioassays before proceeding with experiments to establish a baseline for natural variation which can then incorporated when assessing the number of replicates needed and comparing results between experiments or to other studies. Since we saw a high variation when comparing the adult emergence rates post-injection between the four experiments performed on different generations of BSF, this suggestion may have potentially benefited our HiSvV injection bioassays (Figure 3.8). While this approach has been applied primarily to bioconversion studies, it could benefit pathology studies as well. Given the nascent stage of BSF pathology research, adopting lessons from bioconversion studies could foster a coordinated approach (Bosch *et al.*, 2020; Hopkins *et al.*, 2021; Deruytter *et al.*, 2023; Wiklicky *et al.*, 2024).

The issue of reproducibility has also begun in pathogen studies, and this can be observed by looking into fungal and nematode work. (Tourtois *et al.*, 2017; Lecocq *et al.*, 2020; Manu *et al.*, 2022; Mani *et al.*, 2023; Opare *et al.*, 2023). When determining if BSF could be productive hosts for the mass-rearing of entomopathogenic nematodes, Manu *et al.*, (2022) observed that the number of infective juvenile (IJ) nematodes emerging from 4th instar BSF larvae cadavers was half the number from final instar Galleria mellonella larvae. They concluded that BSF are suitable for industrial rearing for nematode *Heterorhabditis bacteriophora*, which is the opposite of what was concluded by Tourtois *et al.*, (2017). Manu *et al.*, (2022) tested a more susceptible and softer-bodied lifestage of BSF than Tourtois *et al.*, (2017), who tested the 5th instar and found ten times fewer nematodes emerging from BSF larvae compared to *Galleria mellonella* larvae. Despite potentially finding a more suitable lifestage of BSF, Manu *et al.*, (2022) did not provide sufficient experimental details, such as the origin or pretreatment

of nematodes, which results by Alonso et al. (2018) suggested that the pretreatment of nematodes could have an influence.

Box 2: The known pathogenic status of microorganisms that have been found in BSF or are general pathogens tested in BSF.

Taxonomical group	Organism	Capability to infect BSF	Pathogenic or just watchlist*	Study determining pathogenicity
Virus	*Hermetia illucens* solinvivirus	Isolated from diseased BSF and tested in laboratory	Pathogenic	This thesis
	Hermetia illucens insevirus	Circulating in BSF	Watchlist	
	Hermetia illucens sigmavirus			
	Hermetia illucens lebotivirus			
	Hermetia illucens cripavirus	Associated with BSF		
	Hermetia illucens iflavirus			
	BSF nairovirus-like 1	Associated with BSF through frass and sporadic presence		Walt *et al.*, 2023, 2024
	BSF uncharacterized bunyavirales 1			
Fungi	*Beauveria bassiana*	Laboratory infection	Pathogenic	Lecocq *et al.*, 2020; Mani *et al.*, 2023
	Diutina rugosa	BSF microbiome and laboratory infection	Watchlist	Klüber *et al.*, 2022
	Fusarium solani		Pathogen (opportunistic)	
Bacteria	*Klebsiella pneumoniae*			
	Pseudomonas aeruginosa			
	Microbacterium thalassium			
	Alcaligenes faecalis		Watchlist	
	Paenibacillus thiaminolyticus GX6	Isolated from disease BSF and tested in laboratory	Pathogenic (opportunistic)	She *et al.*, 2023
	Pseudomonas protegens Pf-5	Laboratory infection	Pathogenic	Shah *et al.*, 2023
Nematoda	*Heterorhabditis bacteriophora*		Potential to parasitize BSF and induce mortality§	Tourtois *et al.*, 2017; Alonso *et al.*, 2018; Manu *et al.*, 2022
	Steinernema carpocapsae			
	Steinernema feltiae			

Watchlist microorganisms, may either have already been studied in BSF but more work is needed to confirm if they are pathogenic to BSF or not. § Nematodes tested in experiments were able to infect BSF and induce mortality, but did not necessarily show sufficient host seeking behaviour during the infective juvenile stage.

Furthermore, when Mani *et al.*, (2023) found that dipping BSF larvae in *B. bassiana* did not promote infection, contrasting what was suggested by other studies (Lecocq *et al.*, 2020; Opare *et al.*, 2023). However, Mani *et al.*, (2023) optimized feeding BSF larvae a diet laced with *B. bassiana* for over 24 hours, and this resulted in stronger infection effects. In the case of Mani *et al.*, (2023), when trying to reproduce previously used inoculation methods and other pathogenicity studies, care should be taken in exploring pathogen effects on hosts. Using BSF from a company for experimentation is acceptable with appropriate controls, but comparing

different colonies would be advisable for inferring reproducible interactions with fewer limitations. Laboratory colonies may yield observations not replicable in natural conditions but have fewer influencing factors. Replicating measures is crucial when testing inoculation strategies to determine their sufficiency. (Bosch *et al.*, 2020; Hopkins *et al.*, 2021; Deruytter *et al.*, 2023; Nayak *et al.*, 2024; Wiklicky *et al.*, 2024). Mani *et al.*, (2023) did make a fair point however, different techniques have merits depending on experimental aims. To simulate natural infections and characterize symptoms, short exposure times or low concentrations might not depict the full infection outcome due to uneven substrate distribution and potential spore loss. These factors should be considered in BSF pathological experiments. Nonetheless there is now a broad list of viruses and other microorganisms that are pathogenic, opportunistic or are on a watchlist to be further studied in BSF (Box 2).

7. The growing relevance of epidemiology in the insect industry and insect health

There are two main approaches to virus discovery in insects: (1) using culturing, microscopy, molecular techniques, and typically first-generation sequencing[12], and (2) using next-generation sequencing (NGS) and bioinformatics (General Introduction). Both approaches are best used in conjunction when possible, even so, discovery and surveillance is only the first step of virus hunting[13] (Figure D.6). The complexity of virus hunting in insects will differ depending on the end goal. There are a number of studies which focus mainly on characterizing the virome of insects, where the analysis stops once viral sequences are annotated and the viruses have been classified (Roberts *et al.*, 2018; Schoonvaere *et al.*, 2018; Gebremedhn *et al.*, 2020; Li *et al.*, 2020; Wu *et al.*, 2020; Konstantinidis *et al.*, 2022; Divekar *et al.*, 2024). Then there are pathogen studies which proceed with demonstrating Koch's postulates to determine if any of the viruses found were the pathogenic agent behind a disease syndrome (Valles and Hashimoto, 2009; Valles *et al.*, 2016; Martemyanov *et al.*, 2023). Other studies try to perform surveillance, or better understand the pathology of a viral infection, sometimes to also find means of prevention and management of outbreaks (de Miranda, Granberg, Low, *et al.*, 2021; Duffield *et al.*, 2021; Zanella-Saenz *et al.*, 2022; Takacs *et al.*, 2023). There is one aspect however that few studies assess which is vital in the mass-rearing industry, and that is upscaling of the virus discovery and research approaches and outputs to better integrate with

[12] First generation sequencing encompasses methods which only sequence PCR/clonal DNA products (Heather and Chain, 2016).
[13] Virus hunting as the name states is the search for viruses either through blind exploration or searching for a viral cause to a disease outbreak.

the mass-rearing industry (Eilenberg and Jensen, 2018; Eilenberg *et al.*, 2018; Bertola and Mutinelli, 2021; Maciel-Vergara *et al.*, 2021; Herren *et al.*, 2023).

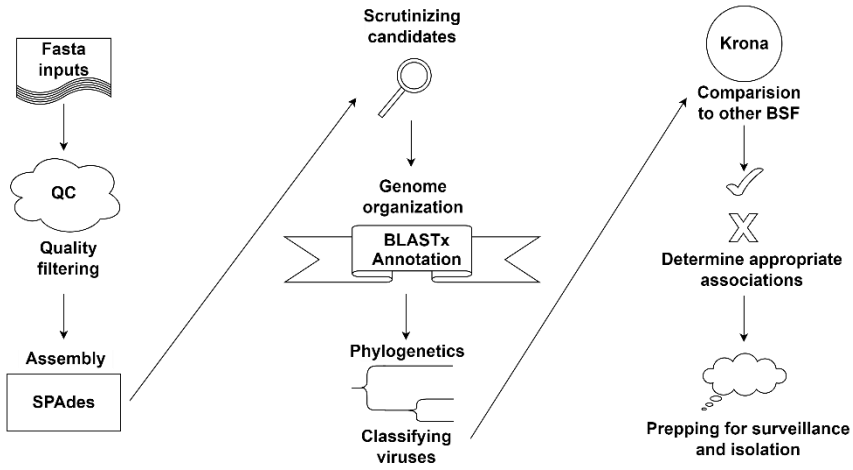

Figure D.6 Outline of bioinformatic virus discovery from sequencing data to virus candidates selected for surveillance.

Now that entomopathogens have been found to infect BSF and cause disease, the next step would be to determine their epidemiological importance. The focus of this thesis is not the intrinsic details of epidemiological mathematical modelling (Hajek and Shapiro-Ilan, 2017; Eilenberg *et al.*, 2018; Poulin, 2021; Saldaña *et al.*, 2024), but there is some context that can help one to better understand why a viral pathogen such as HiSvV would be important in BSF rearing. This can be complex since conditions can differ widely not just between wild environments and insect rearing farms, but also between different types of rearing farms (Anderson and May, 1981; Eilenberg and Jensen, 2018; Herren *et al.*, 2023; Saldaña *et al.*, 2024). Once the epidemiology of a pathogen has been appropriately studied, this can allow for more detailed outcome predictions during and before outbreaks occur (Maciel-Vergara and Ros, 2017; Maciel-Vergara *et al.*, 2021; Plowright *et al.*, 2024). This can be further supplemented since it may also allow the impacts of outbreaks, such as mortality rates, development time and fecundity to be factored into production models, such as the bio-economic model for BSF facilities (Peeler and Otte, 2016; Plowright *et al.*, 2017; Zaalberg *et al.*, 2024) to measure the potential economic impact of an outbreak in a colony.

Utilizing some of the more complex susceptible, infected and recovered models (SIR) developed by Anderson and May, (1981) (Figure D.7), we can try to better understand the impact that HiSvV may have in a BSF rearing. Anderson and May, (1981) developed their SIR models around certain factors such as vertical transmission, latent periods of infection, stress,

host density, host reproduction and active life stages of microparasites which occur outside the host (free-living) (Figure D.7B).

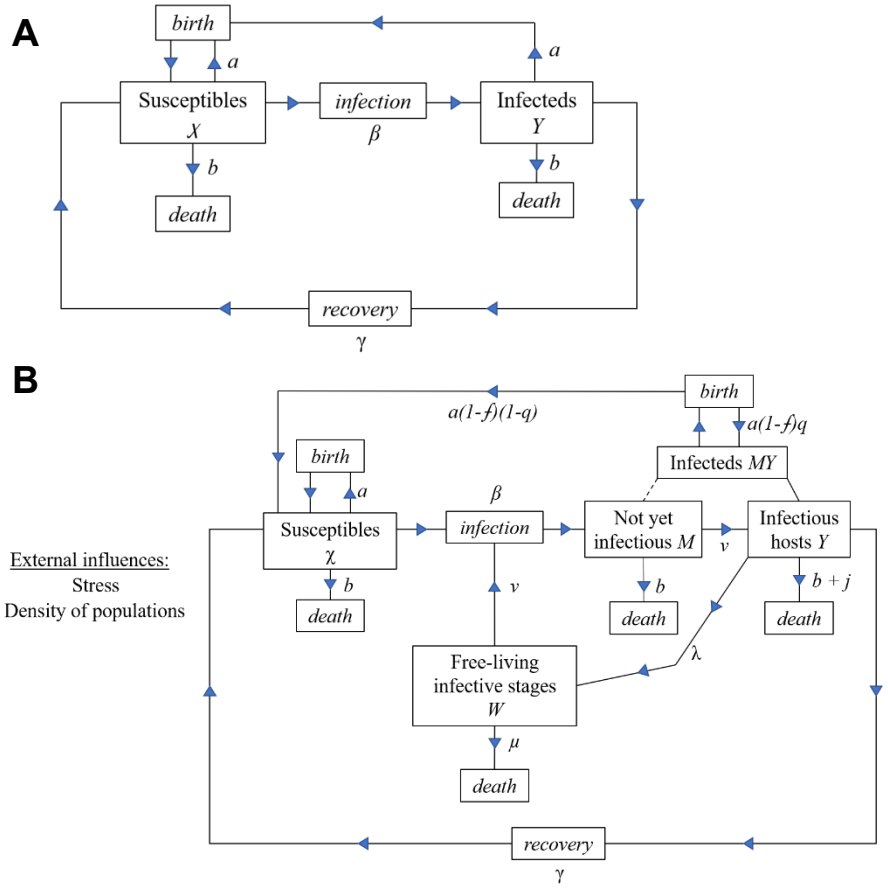

Figure D.7 Infectious disease modelling of invertebrate pathogens. A) A simple schematic of a basic dynamic infection model proposed by Anderson and May, (1980) based on previous epidemiological assumptions (Adapted from Figure 5). B) A combined visual representation of the models presented by Anderson and May, (1980) (Adapted from Figures 5, 8, 9 and 15). Definitions of the mathematical symbols as provided by Anderson and May, (1980): a – host birth rate (per individual), β – transmission coefficient (only for direct transmission from infecteds to susceptibles), b – natural mortality rate of hosts, M – number of infected, but not yet infectious, (latent) hosts, Y – number of infectious hosts, MY – number of hosts either infectious or not infectious, j (α) – disease-induced mortality rate, W – number of free-living infective stages of parasite, v – rate at which of infective stages produced per infected host, on average, μ – mortality rate of free-living infective stages, λ – rate of production of infective stages, per infected host, γ – rate of host recovery from infection, χ – number of susceptible hosts, q – proportion of offspring of infected hosts acquiring infection by vertical transmission, and f – fractional decrease in birth rate of infected hosts.

Turning back to HiSvV as an example, there are already factors which could be taken into consideration for understanding the epidemiology of HiSvV and its infection (description in Chapter 3). Currently, it has been determined that HiSvV can infect BSFs (susceptible individuals "χ", infections "β", and infected individuals "Y") and increase the mortality rate in adults ("b"), and differing immune responses between males and females shows the potential for recovery ("γ") from an HiSvV infection (Figure D.7A). These factors set the foundation for

a basic susceptible, infected and recovered (SIR) model (Figure D.7A) to be used to model the outcomes of an HiSvV outbreak. To keep it simple, we will maintain the perspective that the goal of a host against an infection is the eradication of the pathogen or suppression of the pathogen levels to survive (Anderson and May, 1981). Since medium- to large-scale BSF farming, like other mass-rearing facilities can create an environment more dynamic than in laboratory experiments (Maciel-Vergara *et al.*, 2021; Herren *et al.*, 2023), the density of the adults and any other stressors (e.g. climatic, CO_2 levels, disturbance, competition or mating) need to be factored into the SIR model (Figure D.7B). It should also be noted that we do not know how long it takes ("*v*") for infected but non-infectious BSF adults ("*M*") to become infectious ("*Y*").

Considering horizontal transmission of HiSvV, viruses don't have what is considered as a "free-living" stage (living outside the host) (Forterre, 2016). However, their tested persistence in the environment and retention of the capability of each virus particle to infect new or reinfect hosts at specific timepoints could potentially be substituted into the variable "*W*" in the SIR model (Figure D.7B). This is something that could be important when the appropriate decontamination methods are not sufficient to treat the entire exposed space within a facility, or even to consider which cleaning/sanitizing protocols would be necessary during an outbreak or during preventative maintenance. Albeit, this does not replace another epidemiological variable, the basic reproductive number (R_0), which is already involved in transmission outside of SIR modelling (Delamater *et al.*, 2019). This leads to the next phase of SIR modelling, we now know that HiSvV could be vertical transmitted (represented as "*q*"), and the effect of HiSvV on fecundity and larvae survival from infected parents ("$a(1-f)$") needs to be tested (Figure D.7B). Currently this is centred primarily on direct transmission, but once a pathogen like HiSvV is better understood, disease vectors and fomites could and should also be factored into modelling which may be more often applicable in semi-open and open rearing setups.

Having a simple SIR model to understand or better measure the impact of an outbreak in an insect colony is unfortunately only just the starting point. As the rearing and farming facilities increases in size and complexity in organization, so does the complexity of factors affecting the epidemiology of the pathogen interactions with BSF. Generally, a pathogen host already has a mix of factors impacting their exposure and reception to the pathogens (Figure D.8). For example, the distribution of sources such as reservoirs or other hosts can already impact the expected level of exposure to a pathogen that an individual host can have, how easily

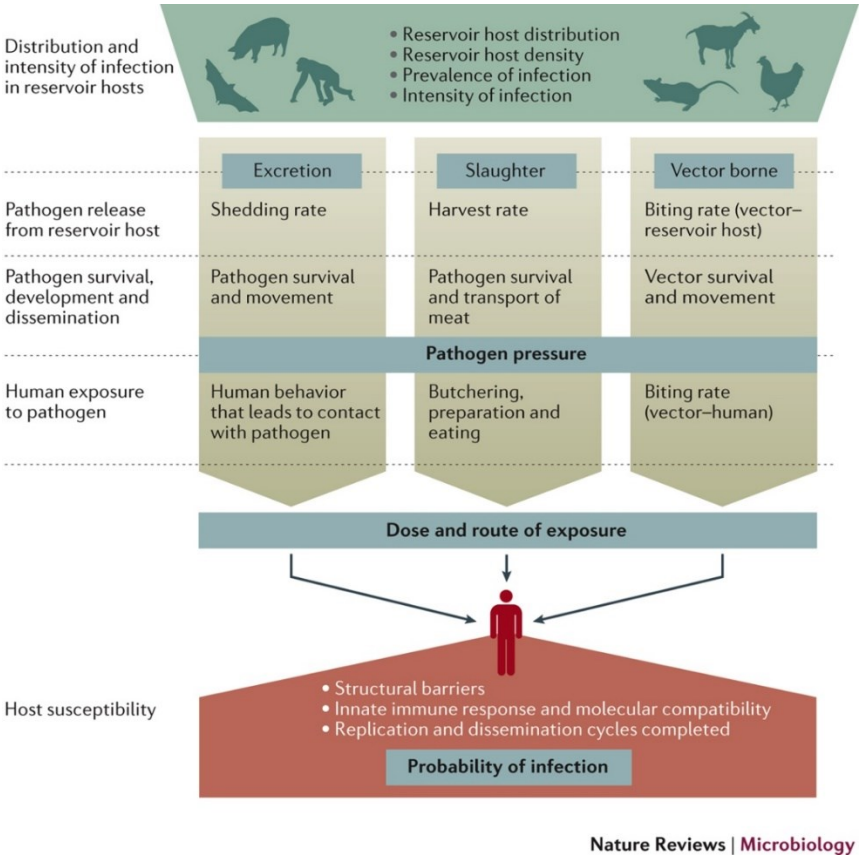

Figure D.8 Factors that can affect the chances of exposure to and infection by a pathogen depending on its ecology. (Obtained from Figure 1 in (Plowright *et al.*, 2017)).

transmitted the pathogen is (Plowright *et al.*, 2017; Saldaña *et al.*, 2024). This can be further extended if the pathogen is either very prevalent among sources or has a very sporadic prevalence. To expand on things in the context of BSF, BSF can live for more than 20 days under the right rearing conditions (Bruno *et al.*, 2019). From a research perspective, this means that there is an opportunity to exploit their immune response and monitor their ability to recover from pathogens during their adult stage, since there are pathogens which can kill insect hosts within less than five days post infection (Maciel-Vergara and Ros, 2017). This is also applicable for BSF rearing that follows a continuous introduction of freshly emerged adults into a single cage, without removing older adults first. This setup can promote a large variation in the number of infected hosts between any given points in time and allows for changes in density. Why this is important? Pathogen levels will fluctuate within a colony, and their fluctuation may drive colonies through symptomatic and asymptomatic phases (Anderson and May, 1981; Maciel-Vergara *et al.*, 2021). If a pathogen within a BSF rearing persists due to difficulties in eradicating it or lack of resistance developing within a colony, this could also lead to the assumption that a colony is being reinfected by external sources (e.g. fresh batches

of diet or vectors/fomites) on multiple occasions. Including these factors into an epidemiological framework starts to make sense when upscaling from simple laboratory experiments to medium-scale and large-scale rearing facilities. Currently there is no treatment or certified management strategies for viral pathogens in BSF such as HiSvV. All these factors combined, highlight that better understanding the epidemiology behind BSF pathogens will help improve prevention and management approaches, and that further research on HiSvV would help reduce the risk of it becoming a persistent problem within rearing facilities if it is able to enter these facilities.

8. One Health, BSF farming distribution, wild distribution and pathogen transfer

The BSF mass-rearing industry shares several similarities with the shrimp production industry SPI. Both industries experienced exponential growth within a decade of developing artificial rearing techniques, with significant livestock sharing between farms, especially in the early stages (Lightner *et al.*, 1997; Feigon, 2000; Tomberlin and Huis, 2020; Kaya *et al.*, 2021; Generalovic *et al.*, 2023). Like the SPI, the BSF industry expanded rapidly before comprehensive sanitary and rearing regulations were established. However, associations such as IPIFF have laid strong foundations for BSF regulations and practices much faster, within 10 to 17 years compared to the 20 to 40 years for the SPI (Feigon, 2000; Arnold van Huis, 2019; Flegel, 2019; Alagappan *et al.*, 2022; Boyd *et al.*, 2022; Caparros Megido *et al.*, 2024).

A key difference between the two industries is the diversity of purposes for which BSF are farmed, including waste management, animal feed, and pharmaceutical applications, compared to shrimp being primarily produced for human consumption (Flegel, 2019; Tomberlin and Huis, 2020). This diversity creates a more complex regulatory environment, as different regulations apply to different sectors (Derrien and Boccuni, 2018; Alagappan *et al.*, 2022; Caparros Megido *et al.*, 2024). Additionally, BSF farming varies widely in scale, from large industrial farms to small academic or non-commercial operations (Figure GI.6). This variability can lead to genetic mixing and transfer between facilities (industrial, academic and private), contributing to a global network of BSF colonies (Ståhls *et al.*, 2020; Kaya *et al.*, 2021; Generalovic *et al.*, 2023; Tanga and Kababu, 2023). For example, there is a market in BSF egg/adult production to either seed, maintain, modify colonies at both commercial and non-commercial BSF farms at all scales of production and purposes (Farrugia, 2024).

While core rearing techniques exist, each BSF farm may have unique conditions that could influence pathogen dynamics (Vogel *et al.*, 2022). According to Maciel-Vergara *et al.*, (2021) specific conditions at one facility could trigger a covert infection to become overt, leading to disease outbreaks (Figure GI.11). This means that while some pathogens present in a BSF colony are not causing issues at facility A, specific conditions at facility B could act as a catalyst for a covert infection by a pathogenic microorganism to change to an overt infection triggering a disease outbreak at facility B.

Biosecurity concerns are relevant to all types of BSF facilities—open, semi-open, and closed. Despite the perception that closed facilities are more secure, they are not immune to pathogen spill-over and spill-back, especially in regions where BSF populations have naturalized (Marshall *et al.*, 2015; Eilenberg and Jensen, 2018; Joosten *et al.*, 2020; Bang and Courchamp, 2021; Kaya *et al.*, 2021; Tanga *et al.*, 2021; Tanga and Kababu, 2023). Interactions between wild and farmed BSF populations, and associated parasites can pose significant biosecurity risks, highlighting the need for robust containment and management practices across all farming setups. There is also the issue that some pathogens, such as viruses have the capability to jump from closely related host. So regular entry and exit of diseased insects or other arthropods in facilities can promote exposure to pathogens which may jump to the reared insect colonies. In terms of HiSvV, it was very rare in tested colonies. The family *Solinviviridae*, is associated with a wide array of insects, and being RNA viruses, they naturally mutate at faster rates than DNA viruses or other pathogens like bacteria and fungi (Combe and Sanjuán, 2014; Wongsurawat *et al.*, 2019). Thus, there is the possibility that it may have jumped from a pest within the rearing facility at some point. But until more farms are screened or until it is found in another host, this is only a small possibility.

There are various paths for pathogens such as viruses to enter mass-rearing facilities, food, entry points, fomites like staff clothing and shoes, air vents (Eilenberg and Jensen, 2018; Joosten *et al.*, 2020; Maciel-Vergara *et al.*, 2021). There is no fortress that is impenetrable, but identifying these points can help reduce the risk of pathogens, and also help track down the source if an outbreak occurs. Facilities need to be of the understanding that outbreaks are likely to occur at some point. But this does mean that if the effort is put into monitoring and managing at least weak points of pathogen control and surveillance, that faster responses can be implemented when needed.

9. Does it reward to work pre-emptively?

Comparing pathogen surveillance and prevention and management (P&M) strategies, and lack of, across the honeybee, shrimp, cricket, and silkworm industries reveals significant economic impacts when outbreaks occur (Smith *et al.*, 2013; Flegel, 2019; de Miranda, Granberg, Low, *et al.*, 2021; Duffield *et al.*, 2021; Osterman *et al.*, 2021; Kim *et al.*, 2024). The extent of damage and recovery time are crucial factors, but with recent improvements, surveillance networks have contributed to shorter recovery periods, at least in shrimp and silkworms (Peeler and Otte, 2016; Stentiford *et al.*, 2017; Tayal and Chauhan, 2017; Flegel, 2019; Mondal *et al.*, 2024).

For novel or unknown pathogens, identification and characterization, as well as the development of preventative and management strategies, can take over two years of active research. For instance, our experience with HiSvV1 began in early 2021, and it took nearly three years to isolate, characterize, and confirm its pathogenicity to BSF by the end of 2023. While the COVID-19 pandemic demonstrated that such processes could be expedited to months, several limitations exist in BSF pathogen research: 1) outbreak timescales, 2) available resources (financial, human, time, materials), 3) prior knowledge of the insect model, 4) ease of industry-academic collaboration, and 5) the availability of tools and techniques (Khanna *et al.*, 2020; Murray, 2020; Khan *et al.*, 2021).

When looking at financial reasons, there is a wealth of research on pathogens in insects which are not mass-reared. Or like for viruses like *Baculoviridae*, hosts such as *Spodoptera exigua* are mass-reared but as hosts to produce more viruses that can be used for biological control of pest *S. exigua* on crop farms (Hussain *et al.*, 2021). Relating this to BSF using HiSvV as an example. One solinvivirus SINV3 has been fairly well studied, one of the main reasons so is that its host *Solenopsis invicta* the invasive red fire ant is a major global pest and by 2003, it was estimated that *S. invicta* was costing the USA around $6 billion a year (Valles and Hashimoto, 2009). A major concern was the lack of entomopathogens associated with *S. invicta*, so this boosted efforts to find and study them in order to assess their capability as a biological control agent. *Solenopsis invicta* virus 3 was first detected in 2009 and there are many studies surrounding the virus including its ability as a biocontrol agent (Valles *et al.*, 2022).

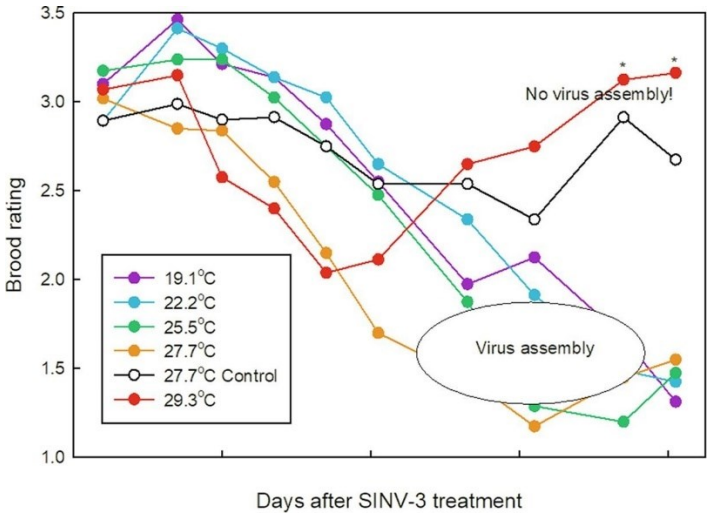

Figure D.9 Impact of rearing temperature on SINV3 infection in Solenopsis invicta (Obtained from the graphical abstract in Valles and Porter, (2019))

After 15 years of active research, SINV3 still is has not entered the biological control market and is not directly contributing to any economy. Unfortunately, biological control research can take decades before biocontrol agents are used commercially, often due to safety concerns. The BSF market is estimated to reach up to $8 billion (US) by 2030 (Chapter 1). BSF pathogens are now only receiving much attention, BSF viruses, less so. Now that BSF viruses have been established, it may benefit the industry financially to push research into these viruses to better understand them before they are a problem. *Hermetia illucens* solinvivirus has the capability to cause colony collapse, which would have huge financial implications on badly impacted BSF production facilities. Research has shown that infections with SINV3 are known to treatable/manageable using temperature control (Figure D.9) (Valles and Porter, 2019). Investing in research on HiSvV, for example, to answer questions like these would be beneficial to the industry.

When I began my research, there were no known viruses, optimized virus purification methods, established viral bioassays, or rapid diagnostic tests for BSF. Now, protocols exist to test virus pathogenicity in BSF upon receiving samples, significantly reducing the waiting period for farmers from three years to between two weeks and three months in well-equipped labs. We have also performed these and discovered a novel viral entomopathogen in BSF. This advancement highlights the importance and benefits of pre-emptive research and development in managing pathogen outbreaks in the insect industry.

10. Final conclusions and perspectives

To recap, my thesis pursued an investigation into the virome of BSF, a model which has taken over the insect mass-rearing by storm. The primary objectives were: 1) to identify viruses associated with BSF, 2) to develop screening methods and experimental tools for studying these viruses, and 3) to isolate and analyse a virus and its interactions with BSF. This research was motivated by the rapid growth of the BSF market, accompanied by increasing reports of pathogen outbreaks in BSF colonies.

Our findings revealed that BSF host a wide diversity of exogenous RNA viruses, adding to the historical evidence of interactions with DNA viruses. While exogenous DNA viruses have yet to be discovered in BSF, it is anticipated that further investigations will uncover more viruses, including those already known to infect other hosts.

A significant advancement in BSF research was demonstrating the pathogenicity of HiSvV to BSF, which has important implications for the mass-rearing industry. Additionally, exploring the interactions of HiSvV with BSF and other viruses such as HiInV and HiSgV has provided novel insights into BSF immunology and opened new avenues for virology research in BSF.

Given the spread of BSF viruses, it is crucial to include wild BSF populations in virus screening efforts. This thesis underscores the importance of incorporating the One Health concept into BSF welfare. Although the BSF mass-rearing industry is unique, valuable lessons can be drawn from other mass-reared species to guide the industry towards better health management practices.

A concerning disconnection between academia and the industry has been identified. To address this, general suggestions for pathogen research and management in BSF are proposed (Box 3).

The myth of BSF resistance to viruses has been debunked, marking the beginning of a new chapter in BSF research. There is much still to explore, and this work lays the foundation for future studies on BSF and their viral associates.

Box 3: Propositions for viral disease management and prevention

Joosten *et al.*, (2020) outlined epidemiological management practices for BSF farms, primarily based on approaches effective for bacteria and fungi. While doing so, they focused on the design of facilities and processes to reduce the introduction of pathogens into the facilities. They then expanded on transmission within the facility, and the importance of implementing of internal barriers, cleaning properly in between batches and controlling environment conditions in a manner that is less conducive to pathogen survival/growth. Below, I present some propositions which fall under continuous surveillance and collaboration for improved prevention and management strategies, employable for closed, semi-open and open BSF farming facilities.

1. **Timepoint sampling and monitoring**:
    - Sample BSF strategically every two months and discard those older than six months. This approach helps create a timestamp indicating when a pathogen might have been introduced. It also provides a reference picture of the colony when the pathogen was likely absent.
    - This can also better help understand pathogenic thresholds of microbes.
2. **Safe and recovery BSF strains**:
    - Initially pathogen free populations could be found and made available.
    - Breed BSF genetic populations that are free from specific pathogens, and more resistant to pathogens (viruses), as recovery colonies for farms impacted by pathogens, like the shrimp industry does to recover from collapse. (Thitamadee *et al.*, 2016)
3. **Understanding of BSF biology**:
    - While understanding of BSF biology is improving, more research is needed (Lemke *et al.*, 2023). It is important for academia and industry to fully understand BSF biology.
    - Previous research speculated that adult BSF don't have mouthparts and don't feed, implying they cannot transmit diseases (Newton *et al.*, 2005; Koutsos *et al.*, 2022). However, Bruno and colleagues demonstrated that BSF adults have fully functioning digestion (Bruno *et al.*, 2019).
    - The mindset that pathogens are not present if not checked needs to be discouraged.
4. **Interactions between industry and academia**:
    - Industry could strive for greater transparency and cooperation with academia.
    - Academia could be more open-minded to industry experience, challenges and goals.
    - Understanding the time it takes for virus discovery and study is crucial.
    - Developing management strategies promptly is essential.
5. **Role of mass-rearing facilities in surveillance**:
    - Surveillance is critical for early detection and management of pathogens.
    - Consider the impact of pathogen findings on company reputations. Does discovering pathogens affect them negatively?
    - Videos and reports, like *Description of 2 diseases for Black Soldier Fly*, 2016, can significantly aid in surveillance and discovery efforts. Farms often communicate among themselves, but this information may not always reach scientists who focus on published journal articles.
    - Encouraging citizen science, such as using a symptom description document template available in multiple languages, could streamline surveillance efforts.
6. **Promoting honesty and proactivity**:
    - Establishing a reputation for honesty and proactive issue resolution can benefit the industry. For example, a company could state, "We found this pathogen, but managed the outbreaks effectively, making our strains more resilient." This proactiveness could be seen in a more positive light and allow for better trust between BSF rearing facilities and cliental. Especially in facilities that provide live BSF as a product.

Resumen extendido en Castellano

El desarrollo sostenible se ha convertido en una preocupación creciente a escala mundial durante las dos últimas décadas, tanto que las Naciones Unidas han desglosado la sostenibilidad antropogénica global en 17 ambiciosos objetivos de desarrollo que pretenden alcanzar para 2030. Paralelamente, cada vez más personas están comenzando a entender que los insectos son valiosos y no deben ser considerados únicamente como plagas perjudiciales y vectores de enfermedades. Se ha hecho más evidente que podrían desempeñar un papel importante para ayudar al mundo a alcanzar los 17 objetivos de desarrollo. La industria de la cría masiva de insectos es un área de interacción entre humanos e insectos que está creciendo exponencialmente en esta capacidad.

La especie *Hermetia illucens*, también conocida coloquialmente como moscas soldado negras (BSF), fue recientemente incluida en la industria de la cría masiva por sus múltiples aplicaciones, tales como la gestión de residuos, la farmacia e incluso como una gran fuente de nutrientes para la alimentación animal. Se han convertido en un actor principal en la industria de la cría masiva de insectos y en un impulso para el crecimiento económico de esta industria floreciente. Las moscas soldado negras son relativamente fáciles de criar, especialmente desde que han podido naturalizarse ampliamente en todos los continentes, excepto en la Antártida. De hecho, al menos alguna forma de cría de BSF ha ocurrido en al menos 100 países, desde Alaska hasta Nueva Zelanda, demostrando que realmente pueden criarse en una variedad de condiciones. La industria de la cría masiva de insectos en general no está exenta de problemas. Las instalaciones de cría suelen crear condiciones de vida perfectas para los patógenos, lo que ha causado brotes desastrosos para la industria de la cría masiva. Los conocimientos sobre los patógenos pueden diferir significativamente para cada insecto criado, con poco o ningún conocimiento para la mayoría de las especies criadas, y una montaña de conocimientos para insectos como las abejas y las polillas. Esto es sorprendente, ya que muchos de los principios fundamentales de la epidemiología se desarrollaron utilizando patógenos en colonias de insectos. Las moscas soldado negras son uno de esos insectos criados sobre los que no se sabía nada acerca de sus patógenos, y se creía que no eran muy susceptibles a los patógenos, a pesar de una alta exposición dada su estilo de vida. Sin embargo, hay un creciente cuerpo de evidencia que muestra que las BSF de hecho están afectadas por patógenos, y esto está comenzando a ganar atención. El trabajo limitado sobre patógenos que se había realizado en BSF se centró en bacterias, hongos y nematodos entomopatógenos, dejando un vacío en el conocimiento sobre virus en BSF.

Resumen extendido en Castellano

El descubrimiento de virus se realiza a menudo utilizando tres enfoques diferentes. El primer enfoque se basa en técnicas de laboratorio, como la centrifugación para aislar virus, examinándolos con microscopios especializados como los microscopios electrónicos de transmisión. El segundo enfoque se basa en la secuenciación de alto rendimiento y análisis bioinformáticos. Estos dos primeros enfoques tienen sus propias advertencias. Esto lleva a desafíos al trabajar con organismos sobre los que se sabe poco acerca de su biología y virus, ya que múltiples técnicas de laboratorio requieren conocimientos previos del virus para ser utilizados y pueden requerir una gran cantidad de trabajo para realizar en múltiples muestras. Luego está el problema de usar la secuenciación de alto rendimiento para el descubrimiento de virus, es difícil determinar si los virus descubiertos están activos, son capaces de infectar y causar enfermedades en los organismos en los que se encuentran. Para este propósito, es mejor combinar los dos enfoques para permitir que una investigación se centre en muestras que fueron preseleccionadas para virus y permitir que se genere algún conocimiento de antemano. Luego proceder con los experimentos más laboriosos en muestras seleccionadas. El desafío restante es que trabajar con virus es difícil en organismos no modelos, como las BSF.

El objetivo principal de esta tesis fue investigar el viroma de las BSF para detectar virus que pudieran infectarlas y determinar si tienen la capacidad de ser patógenos para ellas. Al encontrar tales virus, esta tesis podría desarrollar una biblioteca fundamental de virus que podrían ser estudiados y comprender mejor los riesgos que estos pueden suponer para las BSF. Esta tesis utilizó un enfoque paleovirológico para investigar las interacciones históricas de virus dentro de las BSF mediante el análisis de elementos virales endogenizados (EVEs) en los genomas de las BSF y la identificación de familias de virus que infectan insectos, mientras que simultáneamente buscaba virus activos en los conjuntos de datos transcriptómicos disponibles de BSF. Evaluó la diversidad de virus exógenos en las BSF de diversas fuentes, incluidas instalaciones de cría masiva y conjuntos de datos disponibles públicamente, mediante la minería de datos para identificar virus y optimizar el proceso de descubrimiento de virus. Se desarrollaron herramientas moleculares para el cribado rentable de virus asociados a las BSF. El estudio tenía como objetivo desarrollar ensayos experimentales que pudieran usarse para estudiar virus aislados y determinar la patogenicidad de al menos uno de los virus descubiertos mediante la purificación de virus, experimentos de reinfección, ensayos de supervivencia y estudios transcriptómicos, identificando cambios a nivel de genes y respuestas inmunitarias en BSF infectadas.

La **Introducción General** sitúa la cría de BSF en el contexto de la sostenibilidad global y cómo el concepto de One Health también se aplica a la cría de BSF. Inicialmente se presentan los diferentes tipos de instalaciones de cría para dar una idea de los diferentes factores a los que pueden enfrentarse las BSF criadas y se comparan con otros animales de granja, como los camarones, para resaltar el riesgo de brotes de enfermedades. A partir de esto, se introduce la patología de los insectos y cómo esto está relacionado con los desafíos experimentados por la industria de la cría masiva debido a los patógenos. Luego revisé el viroma básico de las especies de dípteros criadas en masa para la alimentación y la gestión de residuos, mostrando una increíble diversidad de virus y una compleja inmunología antiviral. A continuación, resumo los conceptos básicos y las advertencias del descubrimiento de virus utilizando técnicas de laboratorio húmedo, enfoques de secuenciación de nueva generación de alto rendimiento y cómo la paleovirología puede ayudarnos a entender qué virus pueden haber infectado un organismo anteriormente. Finalmente, proporciono un breve historial de estudios relacionados con los entomopatógenos en las BSF antes de comenzar mi tesis y los objetivos detallados de esta.

En el **Capítulo 1**, los resultados se centraron en el primer estudio de búsqueda de virus en las BSF. Se publicó bajo el título "First Evidence of Past and Present Interactions between Viruses and the Black Soldier Fly, *Hermetia illucens*". Utilizando enfoques paleovirológicos, examinamos los genomas de las BSF en busca de elementos virales endogenizados (EVE) para descubrir y caracterizar qué familias virales potencialmente infectantes de insectos podían encontrarse asociadas a las BSF. Los resultados mostraron que las BSF tenían nueve integraciones diferentes de EVE en el genoma, con ocho de los EVEs ortólogos en al menos dos de los tres genomas de BSF analizados. Los resultados también mostraron que, como especie, las BSF habían tenido diversas interacciones previas con virus de las familias *Partitiviridae*, *Parvoviridae*, *Rhabdoviridae*, *Totiviridae* y *Xinmoviridae*. Todas estas familias virales contienen virus que pueden infectar insectos, y al menos *Parvoviridae* y *Rhabdoviridae* contienen virus que son patógenos para insectos. *Totiviridae* contiene miembros que son patógenos, pero no se sabe si la familia contiene virus que sean patógenos obligados para insectos. Algo más que fue interesante fue que la mayoría de las secuencias de EVE provienen de genes de cápside de virus, con algunas provenientes de la ARN polimerasa dependiente de ARN (RdRP). En paralelo, también realizamos un cribado de metatranscriptomas de BSF para encontrar virus relacionados con los EVEs y encontramos que un virus estaba estrechamente relacionado con los EVEs relacionados con *Totiviridae* (TotiEVE) y lo llamamos Hermetia

illucens toti-like virus 1 (HiTV1). Al analizar cruzadamente HiTV1 con los TotiEVE, se hizo evidente que HiTV1 podría clasificarse como un virus exógeno, y no como un producto expresado de los TotiEVE. Luego intentamos caracterizar parcialmente HiTV1 y su relación con los TotiEVE. Encontramos que uno de los TotiEVE estaba tan bien conservado que aún pudimos mapear ambos marcos de lectura abiertos de HiTV1 al EVE, lo que sugiere que esta fue la integración más reciente en el genoma de las BSF. Luego intentamos ver la prevalencia de HiTV1 en los metatranscriptomas disponibles públicamente de BSF, así como en nuestra propia colección. Encontramos que HiTV1 estaba presente en colonias de BSF en múltiples países y tenía una distribución amplia en colonias de Francia, China, Alemania e Italia. Además, los metatranscriptomas provenían de colonias criadas bajo diferentes condiciones y HiTV1 se detectó en diferentes anatomías de BSF y en todas las etapas de vida de BSF, incluidos los huevos. Con una prevalencia así, pudimos inferir que las BSF son un huésped de HiTV1. Finalmente, en algunos metatranscriptomas detectamos contigs de secuencia corta que estaban estrechamente relacionados con HiTV1, abarcando la región del gen RdRP que codifica el dominio conservado de la RdRP. Estos contigs cortos estaban lo suficientemente distantes para mostrar que no eran del HiTV1 exógeno, sino que eran casi idénticos a dos de los TotiEVE, lo que indica que provienen de los TotiEVE y no del HiTV1 exógeno. Se desconoce la función de los contigs cortos expresados, pero existe la posibilidad de que puedan estar involucrados en el silenciamiento de ARN de HiTV1.

Dado que había una diversidad razonable de EVE en el genoma de las BSF, el **Capítulo 2** vio la optimización de un enfoque dual de cribado de novo de virus para ayudar a explorar más a fondo el viroma de las BSF para realizar un cribado profundo de nuevos virus exógenos y desarrollar herramientas de cribado para ellos. Es un capítulo escrito como un artículo científico en preparación para ser publicado bajo el título "Optimization of screening methods leads to the discovery of new viruses in black soldier flies (*Hermetia illucens*)". Utilizando un pipeline de cribado de novo llamado "PoolingScreen" diseñado para cribar simultáneamente una gran colección de conjuntos de datos metagenómicos y luego metatranscriptómicos, pudimos encontrar cinco virus adicionales asociados a las BSF pertenecientes a las familias *Dicistroviridae*, *Iflaviridae*, *Inseviridae*, *Rhabdoviridae* y *Solinviviridae*. Como parte del enfoque dual de novo, también implementé Lazypipe2, un pipeline integral de descubrimiento de virus para cribar los mismos conjuntos de datos y determinar si había otros virus que PoolingScreen había pasado por alto. Aunque Lazypipe2 no encontró virus adicionales, estos cinco nuevos virus elevaron el número total de virus asociados a las BSF a seis. Para los virus

Hermetia illucens cripavirus (HiCV), *Hermetia illucens* iflavirus (HiIfV), *Hermetia illucens* insevirus (HiInV), *Hermetia illucens* lebotivirus (HiLbV, *Lebotiviridae*, anteriormente HiTV1), *Hermetia illucens* sigmavirus (HiSgV) y Hermetia illucens solinvivirus (HiSvV), desarrollamos protocolos RT-PCR y RT-qPCR que podrían usarse para cribar muestras de BSF en busca de estos virus. Recibimos muestras de otros laboratorios y granjas industriales y las cribamos utilizando las pruebas RT-qPCR. Durante este trabajo, otro grupo descubrió dos virus adicionales, BSF uncharacterized bunyavirus-like 1 (BuBV1) y BSF nairovirus-like 1 (BNaV1) pertenecientes al orden *Bunyavirales*, pero no se diseñaron pruebas moleculares para ellos. Luego utilizamos la cartografía basada en lecturas para cribar todos los conjuntos de datos disponibles de BSF contra los ocho virus y combinamos esto con datos de RT-qPCR y encontramos que había una amplia distribución de la mayoría de los virus en las colonias de BSF cribadas en varios países, incluidos Estados Unidos, Canadá, Francia, Alemania, Italia, China, Japón, España y Países Bajos. Los virus HiLbV y HiInV (21,6%), seguidos de HiSgV (13,3%) fueron los más prevalentes en las muestras analizadas, pero HiInV fue el más extendido en las diferentes colonias (56%). También hubo altas tasas de coinfección con HiInV y HiSgV en el 40% de las colecciones de colonias y solo cinco de las 25 colecciones de colonias cribadas estaban libres de virus detectables. Sorprendentemente, no detectamos ninguno de los dos virus *Bunyavirales* en colonias fuera de Estados Unidos. Aunque cribamos BSF de instalaciones de cría y laboratorios de países ampliamente distribuidos en el hemisferio norte, encontramos que la mayoría pertenecía al mismo haplotipo de la subunidad I de la citocromo c oxidasa mitocondrial. Este estudio mostró que hay una gran diversidad y prevalencia de virus exógenos en las BSF criadas, con al menos cuatro estrechamente relacionados con otros patógenos de dípteros e insectos.

Dado que se descubrieron múltiples virus exógenos en las BSF, en el **Capítulo 3**, buscamos aislar y caracterizar las interacciones de infección para uno de los nuevos virus previstos para ser patógeno para las BSF, HiSvV (*Solinviviridae*). Este capítulo también fue escrito en preparación para ser publicado como un artículo científico titulado "A solinvivirus reduces the lifespan of adult *Hermetia illucens* (black soldier flies)". Los *Solinviviridae* son un nuevo grupo de virus conocidos por ser entomopatógenos o sospechosos de ser patógenos para los artrópodos. Algunos de los virus mejor estudiados fueron capaces de colapsar colonias de abejas, camarones y hormigas, pero no se sabía que fueran muy patógenos en larvas de hormigas. Aunque no se estima que estén ampliamente distribuidos entre las BSF criadas, se detectó en una colonia libre de otros virus detectables que había informado de un aumento en

la mortalidad de adultos y una reducción en la fecundidad. En este capítulo, aislé HiSvV y reinfecté prénifas de BSF utilizando un enfoque de inoculación por inyección. Luego lo reasillé y lo caractericé más utilizando microscopía electrónica de transmisión, proporcionando evidencia empírica de que estábamos aislando y trabajando con un virus funcional. Debido a que no se había realizado trabajo experimental con virus antes en BSF, tuvimos que desarrollar y optimizar bioensayos que nos permitieran estudiar las interacciones entre HiSvV y BSF. Al inyectar HiSvV en prénifas de BSF, encontramos que después de que los adultos emergieron, la infección por HiSvV era sistémica, pero con niveles elevados en el intestino de los adultos. Sin embargo, no vimos un impacto en la tasa de emergencia de los adultos por una infección con HiSvV. Además, evaluamos la capacidad de HiSvV para transmitirse entre las BSF realizando experimentos de cohabitación entre machos y hembras. Encontramos que no solo los individuos no infectados tenían niveles similares de HiSvV que sus cohabitantes pre-infectados después de 15 días de cohabitación, mostrando transmisión horizontal, sino también que HiSvV se detectaba en los grupos de huevos de los padres infectados, mostrando la capacidad de transmisión vertical. Trabajamos con múltiples diseños de bioensayos, uno de estos diseños nos permitió inocular con éxito moscas adultas alimentándolas oralmente con un inóculo viral. Al infectar BSF adultas con HiSvV a través de la alimentación con gotas, los ensayos de supervivencia mostraron vidas más cortas de los adultos infectados con HiSvV. Debido a que tuvimos acceso directo a una segunda colonia de BSF, que era genéticamente similar a la colonia original con la que estábamos trabajando, pero esta colonia tenía coinfecciones con HiInV y HiSgV, esto nos permitió estudiar también las interacciones entre HiSvV y los otros dos virus. Decidimos repetir los ensayos de infección de supervivencia con la segunda colonia y encontramos que una infección por HiSvV también redujo la vida útil de los adultos de la segunda colonia, confirmando el hecho de que HiSvV es patógeno para las BSF. Trabajando nuevamente con ambas colonias de BSF, realicé dos enfoques transcriptómicos diferentes para estudiar las interacciones inmunes relacionadas entre BSF y HiSvV. En primer lugar, realicé un análisis de perfiles de ARN pequeño (sRNA) para determinar si había patrones de perfiles específicos que pudieran observarse para infecciones con HiSvV, HiSgV y HiInV. Encontramos que las hembras tienen una respuesta de sRNA muy clara y específica para HiInV y había alguna evidencia de esto para HiSgV, aunque pocos de los individuos analizados también tenían una coinfección con HiSgV. Esto fue muy diferente en comparación con lo observado para los perfiles de sRNA para HiSvV. Para HiSvV, hubo una amplia gama de tamaños para los perfiles de sRNA, que van desde 19 hasta 32 nucleótidos de longitud. Esta amplia gama de tamaños indica que no hay silenciamiento de HiSvV por las

vías de ARN pequeño. HiSvV, otros *Soliviviridae* y otras familias *Picornavirales* como Dicistroviridae tienen un dominio de ARN de doble cadena especial en sus genomas, que se ha encontrado que ayuda a los virus a evitar las defensas de silenciamiento de ARN del huésped, como las vías de ARN pequeño. Esto nos llevó a nuestro segundo enfoque, realizar un análisis de genes diferencialmente expresados (DEG) en transcriptomas producidos utilizando ARN largo no codificante y ARN mensajero. Trabajando con las mismas moscas para el análisis de ARN pequeño, utilizamos DESeq2 para analizar los cambios de pliegue en los genes relacionados con el sistema inmunitario de BSF. Encontramos que las BSF machos adultos infectados con HiSvV tenían un mayor número de genes inmunitarios sobreexpresados en comparación con las hembras, lo que indica una respuesta inmune más amplia en los machos. Además, las hembras que fueron infectadas con HiInV antes de ser coinfectadas con HiSvV tenían un número mucho menor de genes inmunitarios sobreexpresados en comparación con las hembras sin una coinfección con HiInV. En general, la respuesta inmune más amplia observada durante el análisis DEG mostró genes sobreexpresados relacionados con la regulación transcripcional, las vías de autofagia y dos péptidos antimicrobianos como una defensina y una cecropina durante una infección por HiSvV. Estos resultados demuestran que HiSvV es patógeno para las BSF.

En la **Discusión Final**, resumo las implicaciones de los hallazgos generales de los capítulos de la tesis y cómo son importantes en el contexto de la industria de cría de las BSF. También contrasto mis resultados con respecto a otros estudios de patología en las BSF. También vinculo cómo las infecciones por HiSvV están relacionadas con otros *Picornavirales*, pero principalmente con otros *Solinviviridae*, y cómo la epidemiología puede usarse para modelar los impactos de los patógenos de las BSF como HiSvV y dónde se sitúa el futuro para las BSF y los patógenos dentro de la industria de cría masiva de BSF en el contexto de One Health. Luego, destaco las similitudes entre la industria de las BSF y otras producciones animales a gran escala y las lecciones que podrían aprenderse de los errores anteriores. Concluyo con algunas sugerencias breves para la gestión y prevención de enfermedades virales para mejorar las relaciones entre la industria de las BSF y el ámbito académico.

Conclusiones de la tesis

Para recapitular, mi tesis ha investigado el viroma de las BSF, un modelo que ha revolucionado la cría masiva de insectos. Los objetivos principales fueron: 1) identificar virus asociados a las BSF, 2) desarrollar métodos de cribado y herramientas experimentales para estudiar estos virus, y 3) aislar y analizar un virus y sus interacciones con las BSF. Esta investigación fue motivada por el rápido crecimiento del mercado de BSF, acompañado de un aumento en los informes de brotes de patógenos en colonias de BSF.

Nuestros hallazgos revelaron que las BSF albergan una amplia diversidad de virus de ARN exógenos, sumándose a la evidencia histórica de interacciones con virus de ADN. Si bien aún no se han descubierto virus de ADN exógenos en las BSF, se anticipa que futuras investigaciones descubrirán más virus, incluidos aquellos que ya se sabe que infectan a otros huéspedes.

Un avance significativo en la investigación de BSF fue demostrar la patogenicidad de HiSvV para las BSF, lo que tiene importantes implicaciones para la industria de la cría masiva. Además, explorar las interacciones de HiSvV con las BSF y otros virus como HiInV y HiSgV ha proporcionado nuevos conocimientos sobre la inmunología de las BSF y ha abierto nuevas vías para la investigación virológica en las BSF.

Dada la propagación de los virus de BSF, es crucial incluir las poblaciones silvestres de BSF en los esfuerzos de cribado de virus. Esta tesis destaca la importancia de incorporar el concepto de Salud Única en el bienestar de las BSF. Aunque la industria de la cría masiva de BSF es única, se pueden extraer valiosas lecciones de otras especies criadas en masa para guiar a la industria hacia mejores prácticas de gestión de la salud.

Se ha identificado una desconexión preocupante entre el ámbito académico y la industria. Para abordar esto, se proponen sugerencias generales para la investigación y gestión de patógenos en las BSF (Cuadro 3).

El mito de la resistencia de las BSF a los virus ha sido desacreditado, marcando el comienzo de un nuevo capítulo en la investigación de las BSF. Aún queda mucho por explorar, y este trabajo sienta las bases para futuros estudios sobre las BSF y sus virus asociados.

Cuadro 3: Propuestas para la gestión y prevención de enfermedades virales

Joosten *et al.*, (2020) delinearon prácticas de gestión epidemiológica para granjas de BSF, principalmente basadas en enfoques efectivos para bacterias y hongos. Al hacerlo, se centraron en el diseño de instalaciones y procesos para reducir la introducción de patógenos en las instalaciones. Posteriormente, expandieron sobre la transmisión dentro de la instalación y la importancia de implementar barreras internas, limpiar adecuadamente entre lotes y controlar las condiciones ambientales de manera que sean menos propicias para la supervivencia/crecimiento de patógenos. A continuación, presento algunas propuestas que se enmarcan en la vigilancia continua y la colaboración para mejorar las estrategias de prevención y gestión, aplicables a instalaciones de cría cerradas, semiabiertas y abiertas de BSF.

1. **Muestreo y monitoreo en puntos específicos:**
    - Tomar muestras de BSF estratégicamente cada 2 meses y descartar aquellas de más de 6 meses. Este enfoque ayuda a crear un registro temporal que indique cuándo podría haberse introducido un patógeno. También proporciona una referencia de la colonia cuando es probable que el patógeno estuviera ausente.
    - Esto también puede ayudar a comprender mejor los umbrales patogénicos de los microbios.
2. **Cepas de BSF seguras y de recuperación:**
    - Podrían encontrarse y ponerse a disposición de los productores de poblaciones inicialmente libres de patógenos.
    - Criar poblaciones genéticamente distintas de BSF que estén libres de patógenos específicos y sean más resistentes a patógenos (virus), como colonias de recuperación para granjas afectadas por patógenos, similar a lo que hace la industria del camarón para recuperarse de colapsos (Thitamadee *et al.*, 2016)
3. **Comprensión de la biología de BSF:**
    - Aunque la comprensión de la biología de BSF está mejorando, se necesita más investigación (Lemke *et al.*, 2023). Es importante que el ámbito académico y la industria comprendan plenamente la biología de las BSF.
    - Investigaciones anteriores especularon que los adultos de BSF no tienen partes bucales y no se alimentan, lo que implica que no pueden transmitir enfermedades (Newton *et al*., 2005; Koutsos *et al.*, 2022). Sin embargo, Bruno y colegas demostraron que los adultos de BSF tienen una digestión completamente funcional (Bruno *et al.*, 2019).
    - Es necesario desalentar la mentalidad de que los patógenos no están presentes si no se verifica.
4. **Interacciones entre la industria y la academia:**
    - La industria podría esforzarse por una mayor transparencia y cooperación con la academia.
    - La academia podría ser más abierta a la experiencia, desafíos y objetivos de la industria.
    - Es crucial comprender el tiempo que lleva el descubrimiento y estudio de virus.
    - Desarrollar estrategias de gestión de manera oportuna es esencial.
5. **Papel de las instalaciones de cría masiva en la vigilancia:**
    - La vigilancia es crítica para la detección temprana y la gestión de patógenos.
    - Considerar el impacto de los hallazgos de patógenos en la reputación de la empresa. ¿El descubrimiento de patógenos les afecta negativamente?
    - Videos e informes técnicos (como *Description of 2 diseases for Black Soldier Fly*, 2016) pueden ayudar significativamente en los esfuerzos de vigilancia y descubrimiento. Las granjas suelen comunicarse entre sí, pero esta información no siempre llega a los científicos que se centran en artículos publicados en revistas.
    - Fomentar la ciencia ciudadana, como usar una plantilla de documento de descripción de síntomas disponible en varios idiomas, podría agilizar los esfuerzos de vigilancia.
6. **Promoción de la honestidad y la proactividad:**
    - Establecer una reputación de honestidad y resolución proactiva de problemas puede beneficiar a la industria. Por ejemplo, una empresa podría decir: "Encontramos este patógeno, pero gestionamos los brotes de manera efectiva, haciendo nuestras cepas más resistentes". Esta proactividad podría verse de manera más positiva y permitir una mejor confianza entre las instalaciones de cría de BSF y la clientela, especialmente en instalaciones que proporcionan BSF vivas como producto.

El recuento de palabras para el resumen ampliado y las conclusiones de la tesis en Castellano es **4067**

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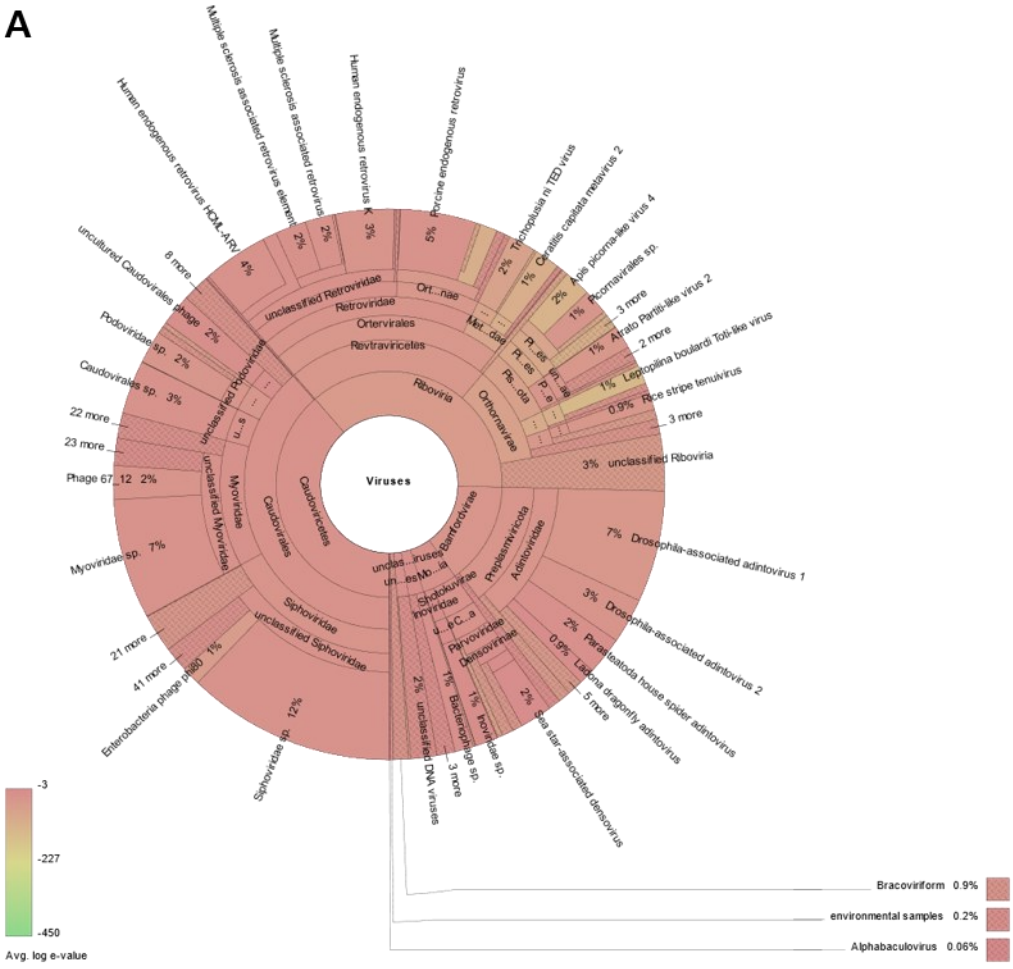
Appendix 1

A



B

Project	Sample	Contig/transcript name
PRJNA573413	SRR10158821v1	NODE_491_length_6298_cov_11.663936_g315_i0
PRJNA573413	SRR10158821v1	NODE_57228_length_243_cov_1.594118_g52214_i0
PRJNA573413	SRR14339787	NODE_16611_length_694_cov_3.840580_g12128_i0
PRJNA573413	SRR14339787	NODE_12465_length_1031_cov_3.869520_g8434_i0
PRJNA573413	SRR14339783	NODE_19019_length_431_cov_1.474860_g16065_i0
PRJNA573413	SRR14339788	NODE_493_length_7247_cov_25.114441_g327_i0
PRJNA573413	SRR14339784	NODE_2343_length_3297_cov_5.388337_g1530_i0
PRJNA573413	SRR14339789	NODE_34338_length_341_cov_2.250000_g29042_i0
PRJNA573413	SRR14339790	NODE_27994_length_371_cov_2.493289_g23215_i0
PRJNA573413	SRR14339795	NODE_662_length_6569_cov_29.114070_g398_i0
PRJNA573413	SRR14339795	NODE_30861_length_357_cov_3.221831_g25256_i0
PRJNA573413	SRR14339791	NODE_24059_length_395_cov_2.152174_g19626_i0
PRJEB19091	ERR1801986	NODE_5359_length_1710_cov_3.772589_g4016_i0
PRJEB19091	ERR1801988	NODE_11056_length_863_cov_3.619701_g8563_i0
PRJEB19091	ERR1801990	NODE_3219_length_2159_cov_3.854147_g2431_i0
PRJEB19091	ERR1801991	NODE_1602_length_2920_cov_3.607555_g1234_i0
PRJEB19091	ERR1801992	NODE_128_length_6578_cov_9.578947_g87_i0
PRJEB19091	ERR1801993	NODE_25300_length_234_cov_2.959538_g23680_i0
PRJEB19091	ERR1801994	NODE_8493_length_964_cov_4.624585_g6964_i0
PRJEB19091	ERR1801995	NODE_5186_length_1758_cov_5.045374_g3935_i0
PRJEB19091	ERR1801996	NODE_841_length_3897_cov_5.045620_g636_i0
PRJEB19091	ERR1801998	NODE_1057_length_3436_cov_5.831704_g805_i0

Figure S2.1 Krona output visualization of PoolingScreen results after CheckV processing. A) Snapshot of the Krona chart displaying virus hits and colouring to indicate the average log e-value. B) Krona list of contigs with the closest-hit classified within *Totiviridae*.

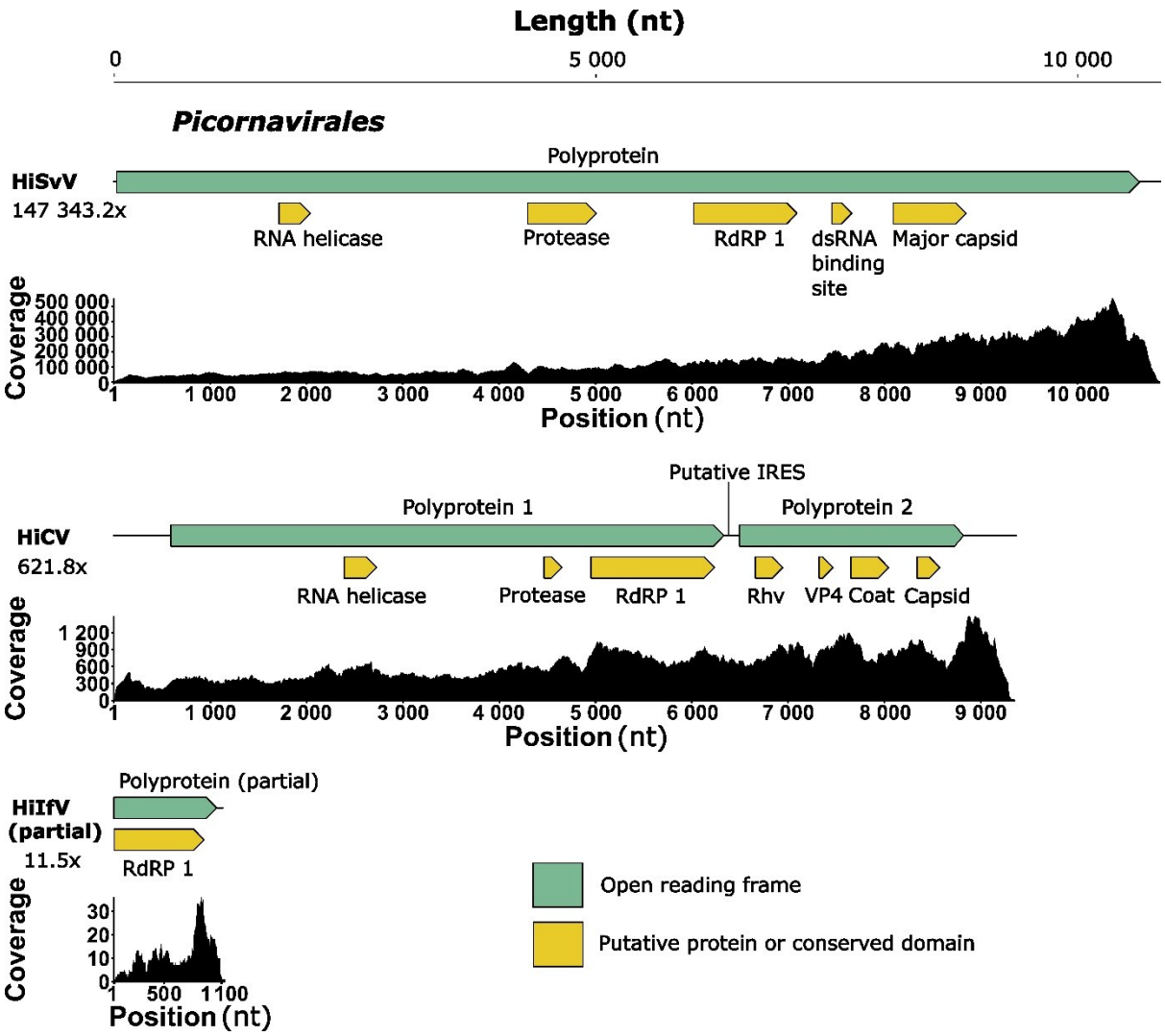


Figure S2.2 Profiling of reads mapping to *Picornavirales* virus candidates HiSvV, HiCV and HiIfV.

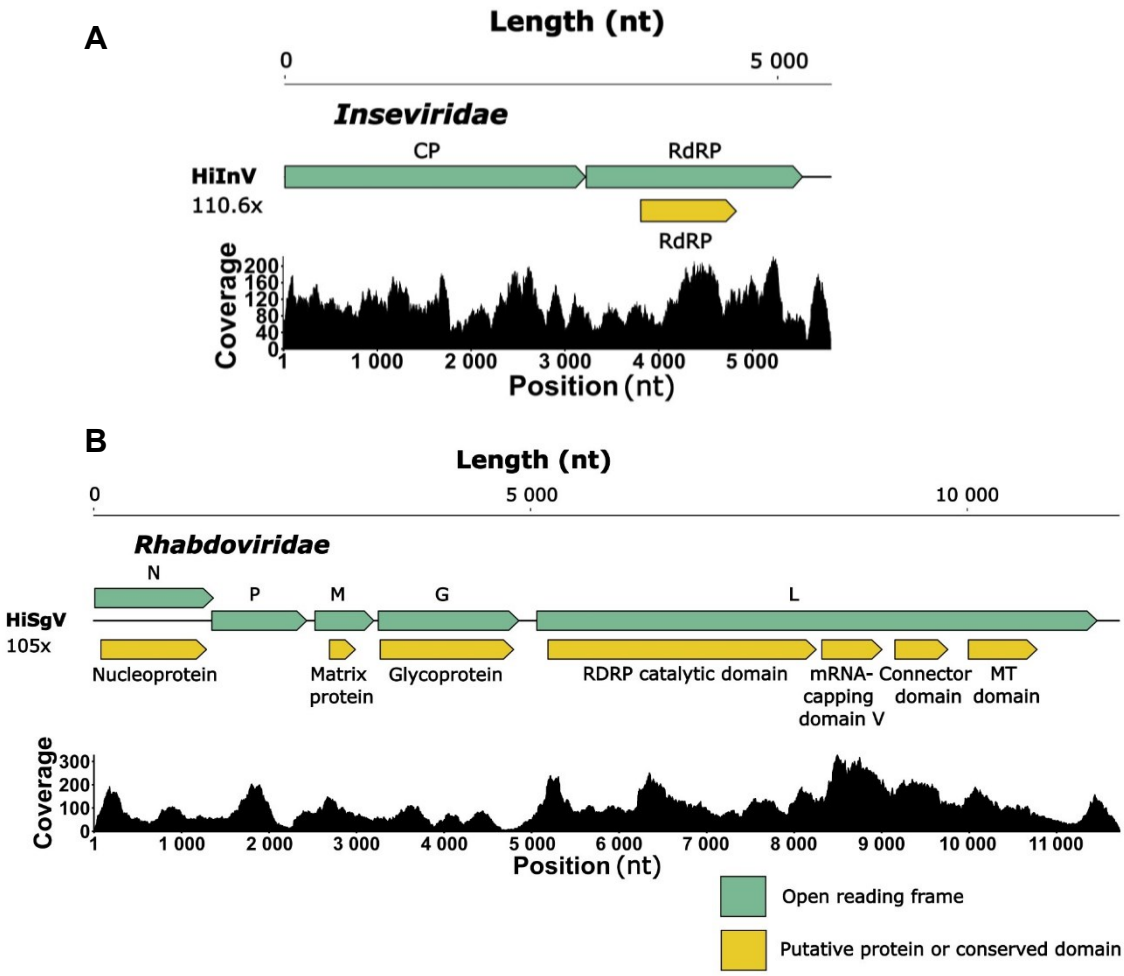


Figure S2.3 Coverage graphs of HiInV (A) and HiSgV (B).

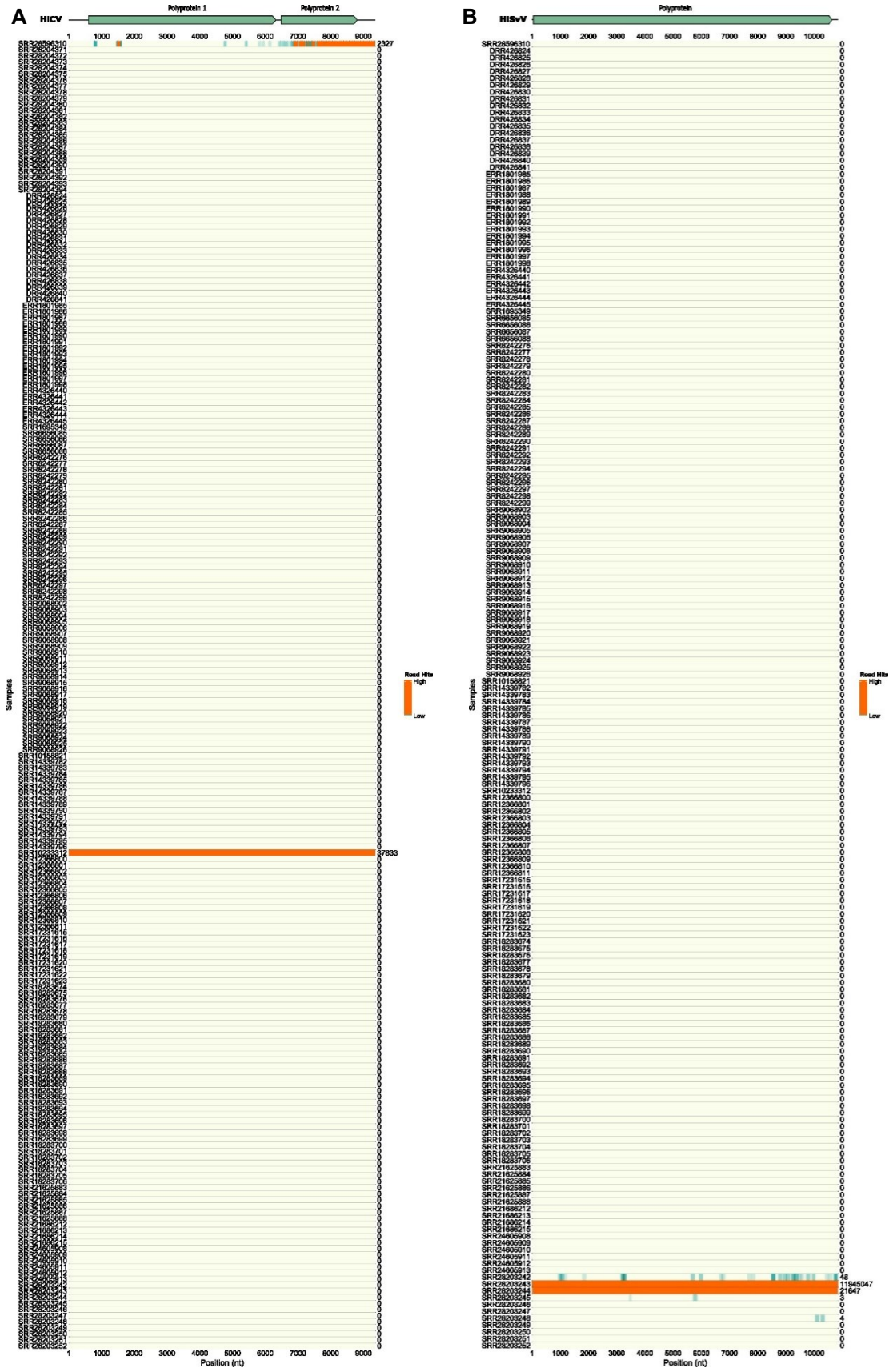


Figure S2.4 Count and position of reads mapped to (A) HiCV and (B) HiSvV. The colour scale maximum was set to 10 counts per nucleotide position. The total number of mapped reads is displayed on the right-hand side of the plot. Samples from PRJNA1079553 were not included for HiSvV as they were from an HiSvV infection experiment.

Figure S2.5 Count and position of reads mapped to (A) HiIfV and (B) HiSV. The colour scale maximum was set to 10 counts per nucleotide position. The total number of mapped reads is displayed on the right-hand side of the plot.

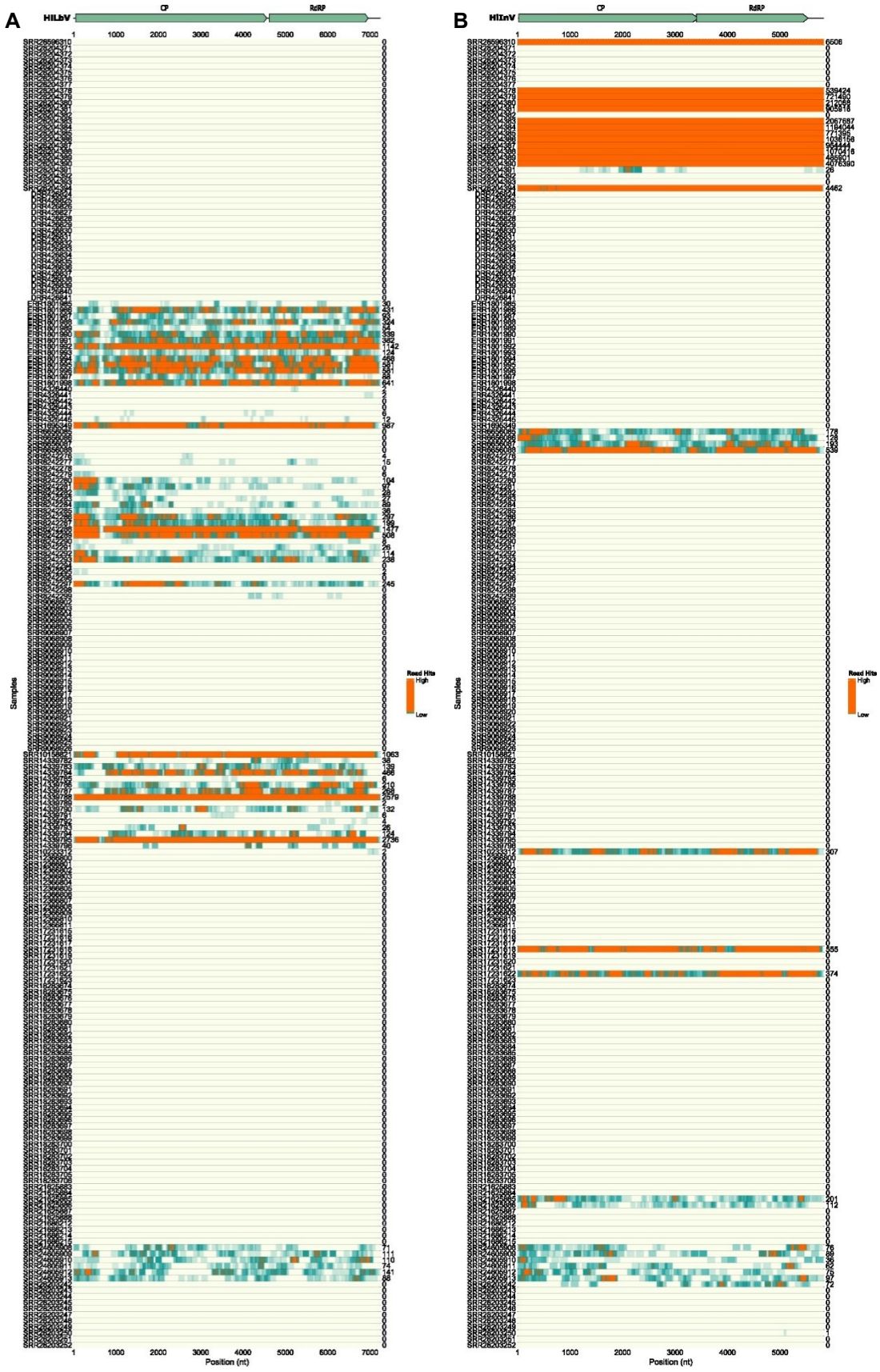


Figure S2.6 Count and position of reads mapped to (A) HiLbV and (B) HiInV. The colour scale maximum was set to 10 counts per nucleotide position. The total number of mapped reads is displayed on the right-hand side of the plot.

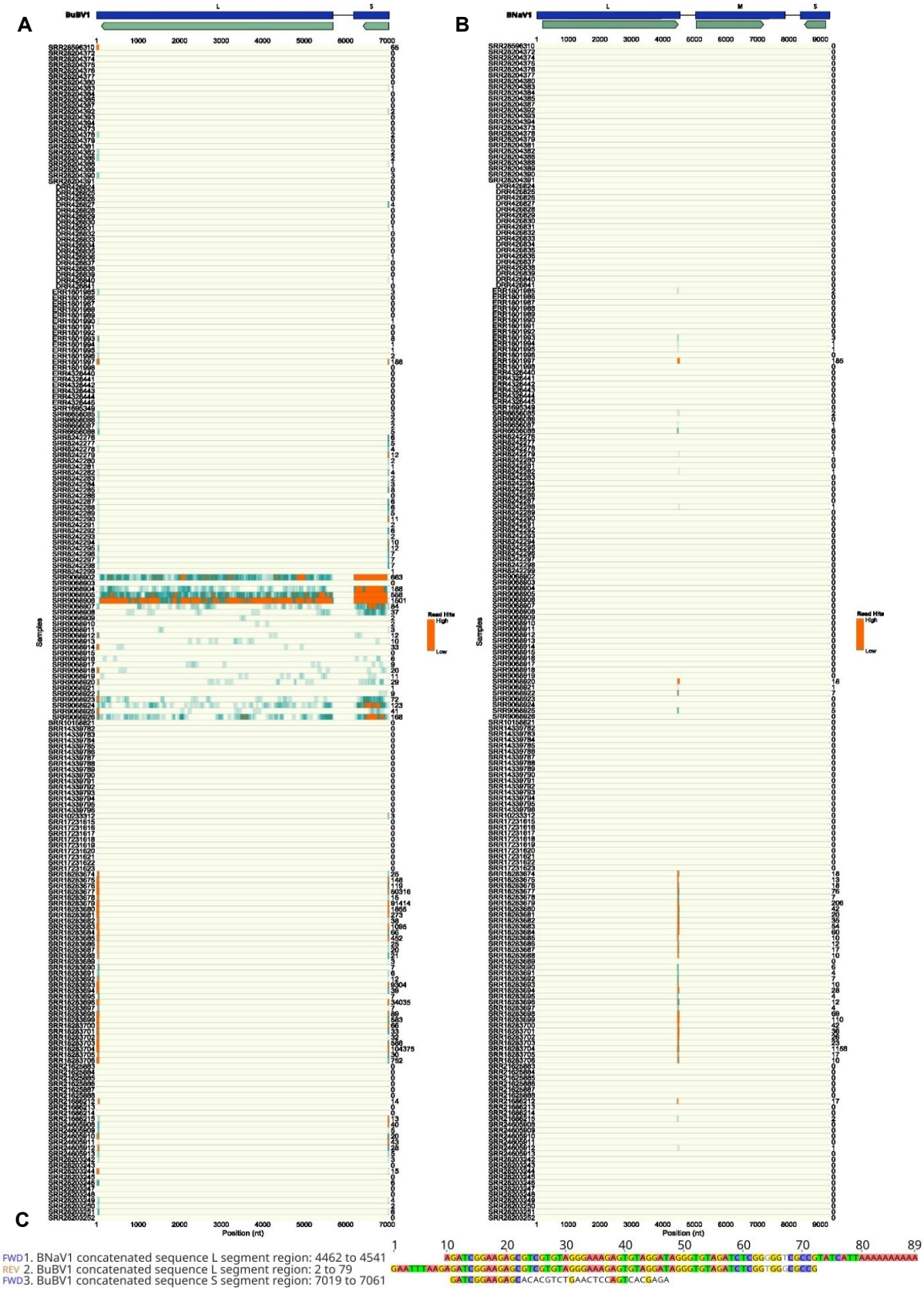


Figure S2.7 Count and position of reads mapped to (A) BuBV1 and (B) BNaV1. The colour scale maximum was set to 10 counts per nucleotide position. The total number of mapped reads is displayed on the right-hand side of the plot. For the sequences annotations in blue indicated the boundaries of each genomic segment and those in a pale green represented the predicted ORFs using the Geneious Prime ORF finder. (C) Alignment of regions with short gaps removed on BuBV1 and BNaV1 where reads mapped in negative samples Using Geneious aligner on default settings with global free end gaps and identity set to 1.0/0.0.

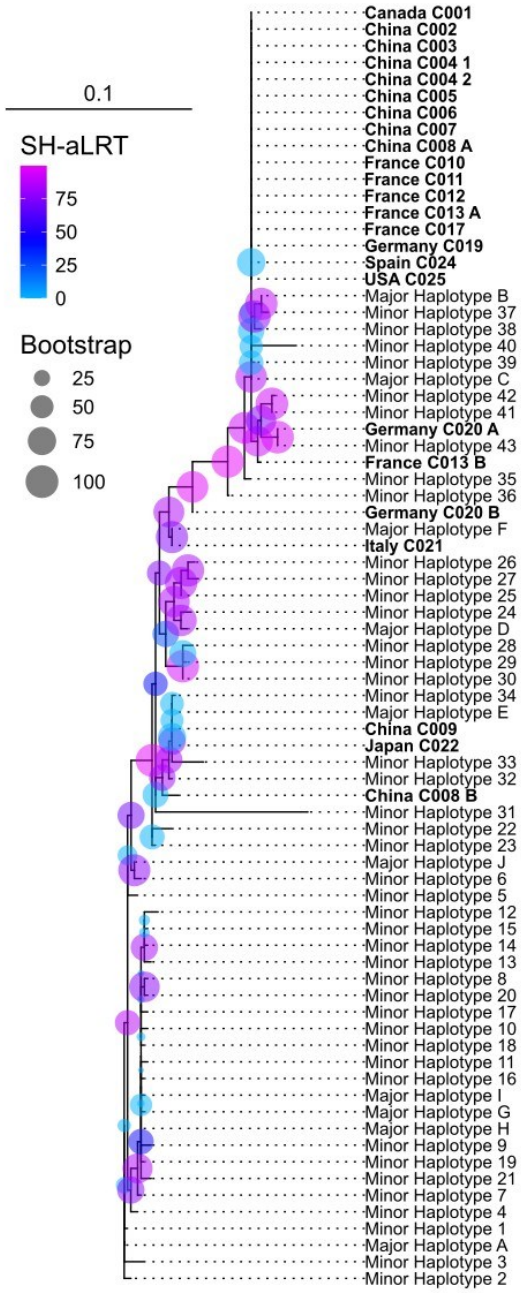


Figure S2.8 Phylogenetic relationships of BSF used to generated metatranscriptomes and metagenome relative to BSF CO1 haplotypes assigned by Guilliet *et al.*, (2022). Tip labels in bold indicate which CO1 PCR regions were added in this study. The suffix A or B indicates if there was a sequence variant detected using freebayes within the PCR region of the consensus sequence associated with each HTS dataset. Raw reads from representative HTS datasets from each bioproject/colony were mapped using HISAT2 v2.2.1 (Kim *et al.*, 2019) to the CO1 sequence extracted from the BSF mitochondria genome sequence (NCBI Acc: NC_035232.1). The resulting consensus sequences were then calculated in Geneious Prime and extract as done by Guilliet et al., (2022), and any ambiguous bases were corrected to the most common nucleotide within the column. Following a modified approach of Guilliet *et al.*, (2022), the CO1 primers were used to extract a 658 nt region. For each extracted region of the consensus sequences, if observed, two "variant" versions following the freebayes results were used to construct a phylogenetic tree with the representative sequences from each of the 53 haplotypes found by (Guilliet *et al.*, 2022). A maximum likelihood tree was generated using IQ-TREE 2 using the model "TN+F+I+G4" and visualized in Geneious Prime. To account for any potential CO1 variation in the consensus sequences within individual datasets, freebayes v1.1.0 (Garrison and Marth, 2012) was used with the default settings, including a minimum alternate fraction of 0.2 and ploidy set to 2.

Table S2.1 Simplified metadata and sequencing depth of metatranscriptomic and metagenomic datasets obtained from 16 NCBI bioprojects NCBI and resulting from this study.

Bioproject	Colony proxy	Accession number	Sample type	Sequencing depth (reads)	Reads mapped to BSF genome (%)	Country	cDNA	Year collected	Publication
PRJNA506627									Zhu *et al.*, 2019
	C002	SRR8242276	Prepupa	29 443 458	97.84	China	mRNA	2016	
		SRR8242277		27 187 908	97.65				
		SRR8242278	Whole larva	26 191 323	97.68				
		SRR8242279		26 795 391	97.81				
		SRR8242280	Pupa	29 917 867	97.75				
		SRR8242281		29 586 915	97.70				
		SRR8242282	Prepupa	29 029 502	97.57				
		SRR8242283		29 669 635	96.79				
		SRR8242284	Pupa	32 771 049	98.01				
		SRR8242285		31 753 348	98.09				
		SRR8242286	Eggs	30 853 375	97.66				
		SRR8242287		27 796 603	97.60				
		SRR8242288		36 514 341	97.65				
		SRR8242289		30 425 319	97.77				
		SRR8242290	Whole larva	28 957 803	97.76				
		SRR8242291		39 622 374	97.54				
		SRR8242292		29 398 605	97.70				
		SRR8242293		31 396 047	97.45				
		SRR8242294		24 715 817	96.66				
		SRR8242295		33 091 023	97.83				
		SRR8242296		33 364 804	98.25				
		SRR8242297		32 261 093	98.02				
		SRR8242298		32 051 870	98.05				
		SRR8242299		37 790 807	98.20				
PRJNA575900									Xu *et al.*, 2020
	C003	SRR10233312	Antennae	59 278 973	95.67	China	Total RNA	2016	
PRJNA431833									No publication
	C004-1	SRR6656085	Adult head	23 704 759	95.80	China	Total RNA	2017	
		SRR6656086		21 319 145	97.26				
PRJNA432297									No publication
	C004-2	SRR6656087	Antennae	21 141 460	96.05	China	Total RNA	2017	
		SRR6656088		23 131 206	97.64				
PRJNA573413									Zhan *et al.*, 2020
	C005	SRR10158821	Whole larva	29 290 360	98.29	China	Undefined	2017	
		SRR14339782		24 724 657	98.61				
		SRR14339783		28 560 697	98.17				
		SRR14339784		31 414 788	98.03				
		SRR14339785		31 993 623	98.28				
		SRR14339786		30 979 571	98.07				
		SRR14339787		31 953 058	98.12				
		SRR14339788		35 146 620	97.78				
		SRR14339789		32 365 467	98.44				
		SRR14339790		26 212 503	98.44				
		SRR14339791		29 867 346	98.42				
		SRR14339792		26 167 690	97.07				
		SRR14339793		26 377 257	96.61				
		SRR14339794		23 943 532	98.33				
		SRR14339795		26 364 920	98.55				
		SRR14339796		28 650 717	97.63				
PRJNA882227									No publication
	C008	SRR21686212	Larval Gut	18 779 090	0.55	China	Metagenome	2020	
		SRR21686213		18 756 736	39.36				
		SRR21686214		27 211 337	0.09				
		SRR21686215		22 631 970	0.18				
PRJNA788971									Jin et al., 2022
	C009	SRR17231615	Whole larva	26 633 800	97.62	China	mRNA	2021*	
		SRR17231616		30 30 9183	98.15				
		SRR17231617		32 617 703	98.44				
		SRR17231618		24 289 935	98.23				
		SRR17231619		27 136 876	98.34				
		SRR17231620		31 513 777	98.08				
		SRR17231621		33 062 079	98.62				
		SRR17231622		32 742 965	96.87				
		SRR17231623		19 476 580	98.16				
PRJEB39181									Bonelli *et al.*, 2020
	C021	ERR4326440	Larval midgut	49 353 774	95.61	Italy	mRNA	2020	
		ERR4326441		48 867 550	95.58				
		ERR4326442		46 361 984	95.60				
		ERR4326443		46 762 074	95.54				
		ERR4326444		33 758 406	95.56				
		ERR4326445		33 170 998	95.62				

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PRJNA267949									https://1kite.org/
	C019	SRR1695349	Whole adult	9 479 964	98.80	Spain	mRNA	2013	
PRJEB19091									Vogel *et al.*, 2018
	C020	ERR1801985	Five pooled	9 538 398	84.68	Germany	Total RNA	2017	
		ERR1801986	whole larvae	15 957 613	95.92				
		ERR1801987		14 238 939	89.79				
		ERR1801988		11 752 326	95.48				
		ERR1801989		13 637 624	93.00				
		ERR1801990		15 318 559	92.86				
		ERR1801991		17 622 048	87.14				
		ERR1801992		16 047 887	94.38				
		ERR1801993		16 474 035	90.61				
		ERR1801994		14 488 091	93.31				
		ERR1801995		19 221 342	92.36				
		ERR1801996		11 943 784	91.01				
		ERR1801997		14 755 342	93.54				
		ERR1801998		12 668 993	95.06				
PRJNA841369									This study
	C010	SRR28203242	Whole larva	1 588 742	88.56	France	mRNA	2019	
		SRR28203250		783 188	87.90				
	C011	SRR28203252		1 715 902	30.20			2021	
		SRR28203251		1 059 143	84.38				
		SRR28203249		2 694 502	90.76				
		SRR28203248		35 268 732	96.24				
		SRR28203247		12 317 595	95.29				
	C012	SRR28203246		47 209 175	94.65			2020	
		SRR28203245	Whole adult	10 824 660	94.48				
		SRR28203244		47 015 739	95.58			2021	
		SRR28203243		12 614 673	68.47				
	C013	SRR28596310	Viral prepurification	43 228 709	35.97		Total RNA	2020	
PRJNA1079553									This study
	C024	SRR28204394	Two whole	24 917 409	96.18	France	Long non-coding and mRNA	2023	
		SRR28204393	adults	28 050 612	95.40				
		SRR28204382		22 827 940	96.63				
		SRR28204377		23 833 594	98.05				
		SRR28204376		26 961 631	98.10				
		SRR28204375		24 512 170	96.24				
		SRR28204374		22 377 924	83.27				
		SRR28204373		22 297 961	69.64				
		SRR28204372		21 896 534	74.11				
		SRR28204371		27 331 015	96.56				
		SRR28204392		26 167 866	88.49				
		SRR28204391		24 533 304	77.95				
	C017	SRR28204390		24 232 324	78.03				
		SRR28204389		22 129 668	95.20				
		SRR28204388		20 282 150	95.32				
		SRR28204387		24 489 237	96.14				
		SRR28204386		18 429 236	94.91				
		SRR28204385		26 882 020	96.47				
		SRR28204384		33 253 558	91.93				
		SRR28204383		34 134 018	82.10				
		SRR28204381		19 649 192	68.76				
		SRR28204380		17 211 470	95.69				
		SRR28204379		21 564 498	91.09				
		SRR28204378		21 401 813	90.67				
PRJNA814308									Auger et al., 2023
	C001	SRR18283674	Whole larva	45 614 145	94.59	Canada	mRNA	2019	
		SRR18283675		90 950 462	95.64				
		SRR18283676		75 523 286	94.95				
		SRR18283677		60 609 125	93.23				
		SRR18283678		84 026 755	96.77				
		SRR18283679		88 491 495	96.17				
		SRR18283680		77 959 173	95.86				
		SRR18283681		78 120 441	96.04				
		SRR18283682		76 735 717	94.98				
		SRR18283683		74 792 527	93.80				
		SRR18283684		76 390 159	92.96				
		SRR18283685		73 391 823	96.22				
		SRR18283686		77 645 498	96.12				
		SRR18283687		86 749 789	96.60				
		SRR18283688		62 872 366	95.56				
		SRR18283689		68 703 092	96.15				
		SRR18283690		66 526 957	96.26				
		SRR18283691		65 826 037	96.58				
		SRR18283692		80 094 923	96.40				
		SRR18283693		47 559 089	97.30				
		SRR18283694		91 091 680	96.27				
		SRR18283695		80 809 502	97.27				
		SRR18283696		99 269 907	96.23				

		SRR18283697		68 904 415	94.94			
		SRR18283698		77 908 467	92.97			
		SRR18283699		80 804 635	91.14			
		SRR18283700		76 182 997	95.40			
		SRR18283701		73 298 911	94.72			
		SRR18283702		53 377 559	96.16			
		SRR18283703		67 713 623	93.42			
		SRR18283704		83 230 688	94.38			
		SRR18283705		94 756 026	94.43			
		SRR18283706		44 995 934	94.93			
PRJDB14676								Nakagawa et al., 2023
	C022	DRR426824	Larval fat bodies	49 737 338	95.89	Japan	Total RNA	2021
		DRR426825		41 492 723	95.29			
		DRR426826		44 382 459	96.46			
		DRR426827		45 190 527	95.89			
		DRR426828		47 414 750	95.37			
		DRR426829		43 327 090	96.10			
		DRR426830		47 386 750	96.49			
		DRR426831		47 158 915	95.68			
		DRR426832		45 968 814	95.61			
		DRR426833		46 719 890	95.05			
		DRR426834		49 149 556	96.72			
		DRR426835		44 166 742	95.93			
		DRR426836		48 353 292	93.88			
		DRR426837		52 132 901	95.23			
		DRR426838		45 014 071	95.32			
		DRR426839		45 465 692	95.09			
		DRR426840		44 245 512	95.32			
		DRR426841		55 465 079	95.77			
PRJNA882017								No publication
	C006	SRR21625883	Whole larva	62 972 282	96.58	China	Undefined	2019
		SRR21625884		65 528 314	96.70			
		SRR21625885		62 991 758	96.75			
		SRR21625886		65 442 644	97.15			
		SRR21625887		62 993 392	96.45			
		SRR21625888		65 341 384	96.55			
PRJNA973253								Luo et al., 2023
	C007	SRR24605908	Whole larva	27 684 450	94.83	China	mRNA	2020
		SRR24605909		31 481 117	95.29			
		SRR24605910		27 221 789	95.96			
		SRR24605911		26 873 641	92.84			
		SRR24605912		29 172 225	96.01			
		SRR24605913		24 439 143	95.45			
PRJNA542977								Walt *et al.*, 2023
	C025	SRR9068902	BSF larva frass	775 489	0.42	USA	Total RNA	2015
		SRR9068903		322 536	35.34			
		SRR9068904		2 174 526	1.25			
		SRR9068905		914 572	6.13			
		SRR9068906		1 834 432	0.49			
		SRR9068907	Whole larva	4 349 955	89.69			
		SRR9068908		3 326 402	90.88			
		SRR9068909		4 096 154	94.26			
		SRR9068910		1 746 081	93.47			
		SRR9068911		1 988 087	93.87			
		SRR9068912		1 517 604	93.87			
		SRR9068913		4 994 568	93.68			
		SRR9068914		6 069 492	95.41			
		SRR9068915		377 798	91.88			
		SRR9068916		4 056 683	93.17			
		SRR9068917		2 835 020	92.11			
		SRR9068918		1 222 809	90.31			
		SRR9068919		1 175 953	89.27			
		SRR9068920		1 814 611	91.34			
		SRR9068921		575 831	88.52			
		SRR9068922		730 986	89.65			
		SRR9068923		1 856 297	88.63			
		SRR9068924		4 597 058	92.65			
		SRR9068925		2 823 365	91.46			
		SRR9068926		5 273 027	87.35			

*The collection year was not specified on NCBI or in the publication, thus the year when the data was uploaded to NCBI was used.

Appendix 1

Table S2.2 Details of qPCR primers used for RT-qPCR assays. A) Organism, sequence and target of the primer pairs. B) Efficiencies of qPCR primer pairs under two different qPCR conditions.

A

Organism	Target	Primer name	Primer sequence (5'-3')	Target size (bp)	Reference
Hermetia illucens	*RPL8* gene (Housekeeping)	RPL8 F RPL8 R	TTG ACT GTC GAA GCC TTA CC GCC GTG CAT ACC ACA AAT AC	130	Gao et al., 2019
Hermetia illucens lebotivirus	POL ORF	HiLbV F HiLbV R	GCG AGG ACA GGT ATT TAG ACC CAC TCT TCT ACT GTA GG	100	This study
Hermetia illucens insevirus	POL ORF	HiInV F HiInV R	GAG ATA CTC CTC AGA ACA CG AGC TTT ACC CTC TCC TTC	100	
Hermetia illucens sigmavirus	RdRP	HiSV F HiSV R	CTA TTA TGC AGG GAG AGG CTA ATC CTC CTG CTT GAC	104	
Hermetia illucens solinvivirus	Major capsid	HiSvV F HiSvV R	AGG TGT AGC TTA GAC CCT AG GAG TTC CAA TAG CTC CAG	127	
Hermetia illucens cripavirus	RdRP	HiCV F HiCV R	CTA GAG TGA TGA CGT ACG AG CGC TCT TAC CAG GTA AAG	135	
Hermetia illucens iflavirus	RdRP	HiIfV F HiIfV R	TTC TGT AGC ACC TGA ATA TG TCT AAA TGG TGT GAC TTC TG	118	

B

Primer pairs	Conditions set 1 Efficiency (%)	Expected melt curve peak Tm (°C)	Conditions set 2 Efficiency (%)	Expected melt curve peak Tm (°C)
RPL8 F & RPL8 R	74.03	~85	103.5	~85
HiLbV F & HiLbV R	N.Y.D.	N.Y.D.	N.Y.D.	N.Y.D.
HiInV F & HiInV R	84.48	~80	100§	~80
HiSV F & HiSV R	96.10*	~77	100§	~77
HiSvV F & HiSvV R	74.33	~81	100.97	~81
HiCV F & HiCV R	N.Y.D.	N.Y.D.	N.Y.D.	N.Y.D.
HiIfV F & HiIfV R	N.Y.D.	N.Y.D.	N.Y.D.	N.Y.D.

*The efficiency was calculated using a log 2 dilution of a positive sample.
N.Y.D. (not yet determined) was used to indicate features where the primer qPCR product sequences could not be successfully verified by sequencing or no qPCR positive sample has been obtained yet.
§ *Efficiencies failed and need to be redone for the publication and defence, so they were calculated using the efficiency value 100%*

Table S2.3 Thermocycling conditions used for qPCRs. A) First set of conditions used for original testing of the primers and for basic detection with the thermocycling conditions at the top and PCR mix at the bottom. B) Optimized thermocycling conditions for the primer pairs and sample material, top, and an optimized qPCR mixture for the assays, bottom.

A

Cycles	Temperature	Time	Stage
1x	95 °C	15 min	Holding
40x	95 °C	15 sec	Cycling
	60 °C	20 sec	
	72 °C	20 sec	
1x	95 °C	15 sec	Melting
	60 °C	1 min	curve
Increments of +0.3 °C up to 95 °C		15 sec per	

Reagent	Per tube (µl)
Taq mastermix*	4
Primers (F & R mix, 0.25 µM each)	2
Water	10
cDNA template/ Water (Negative control)	4 (~47.3 ng)
Total volume	20

*Taq mastermix - 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia)

B

Cycles	Temperature	Times	Stage
1x	50 °C	2 min	Holding#
	95 °C	10 min	
40x	95 °C	15 sec	Cycling#
	60 °C	1 min	
1x	95 °C	15 sec	Melting curve
	60 °C	1 min	
Increments of +0.05 °C/s up to 95 °C		15 sec per	

#Increments of 1.6 °C/s during holding and cycling stages.

Reagent	Per tube (µl)
Taq mastermix*	2.6
Primers (F & R mix, 0.3 µM each)	0.39 each
Water	7.62
cDNA template/ Water (Negative control)	2 (~12 ng)
Total volume	13

*Taq mastermix - 5 x HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia)

Table S2.4 Primer information for RT-PCR primers which can be used to create qPCR target template controls for testing and quantification

Species	Target	Primer name	Primer sequence (5'-3')	Target size (bp)	Reference
Hermetia illucens	*RPL8* gene (Housekeeping)	RPL8 F RPL8 R	TCG CAA GCA TGT GTA AAA C CTA TAC TGC GAC ACT GAA CC	963	This study
Hermetia illucens lebotivirus	POL ORF	HiLbV F HiLbV R	TGT TAC AGG AAC ATG GAG TC GAA CAG AGT ACT CAG ACG AC	1000	
Hermetia illucens insevirus	POL ORF	HiInV F HiInV R	TCA CGC GCA AAA TAA AGA AC CTT TCT TCA AAG GCA GGT AAC	1000	
Hermetia illucens solinvivirus	Major Capsid	HiSvV F HiSvV R	TTG AGG AAA ATT AAC CAG CC CAT TGT AGT GCA CTG TTG AG	1 000	
Hermetia illucens cripavirus	Pro and RdRP	HiCV F HiCV R	CAA GCA CTA TGG CTG TTA TG AAC CGT GTT TTT CCT TGT TC	1 000	
Hermetia illucens iflavirus	RdRP region	HiIfV F HiIfV R	GAG TCT TTT GTA ATG GAC CG AAT TTC AGA CCA CTG CAA AG	950	

Table S2.5 Thermocycling conditions and PCR mix for the RT-PCR primer pairs.

Temperature	Time	Cycles
95 °C	3 min	1x
94 °C	30 sec	35x
55 °C	30 sec	
72 °C	30 sec	
72 °C	7 min	1x
4 °C	Until stopped	

Reagent	Per tube (µl)
Taq mastermix*	25
Primer F (0.3µM)	1.5
Primer R (0.3 µM)	1.5
Water	18
cDNA template/ Water (Negative control)	4 (~47.3 ng)
Total volume	50

*NZYTaq II 2x Green Master Mix (2x) (NZYTech, Lisbon, Portugal)

Appendix 1

Table S2.6 Virus list and accession numbers of sequences used for the *Ghabrivirales* phylogenetic analysis.

Virus name	**Family**	**Genus**	**Accession**
Omono River virus	*Artiviridae*	*Artivirus*	AB555544
Drosophila melanogaster totivirus SW-2009a	*Artiviridae*	*Artivirus*	GQ342961
Armigeres subalbatus virus SaX06-AK20	*Artiviridae*	*Artivirus*	EU715328
Penaeid shrimp infectious myonecrosis virus	*Artiviridae*	*Artivirus*	AY570982
Giardia canis virus	*Giardiaviridae*	*Giardiavirus*	DQ238861
Giardia lamblia virus	*Giardiaviridae*	*Giardiavirus*	NC003555
Anopheles totivirus	*Inseviridae*	*Insevirus*	KX148550
Camponotus yamaokai virus	*Inseviridae*	*Insevirus*	NC027212
Camponotus nipponicus virus	*Inseviridae*	*Insevirus*	NC029312
Hermetia illucens insevirus	*Inseviridae*	*Insevirus*	Appendix 1 list 1
Ceratitis capitata totivirus1	*Inseviridae*	*Insevirus*	OL957313
Soldier fly associated toti-like virus 1	*Inseviridae*	*Insevirus*	PP410011
Hermetia illucens lebotivirus	*Lebotiviridae*	*Lebotivirus*	Appendix 1 list 1
Leptopilina boulardi toti-like virus	*Lebotiviridae*	*Lebotivirus*	NC025218
Linepithema humile toti-like virus 1	*Lebotiviridae*	*Lebotivirus*	MH213243
Solenopsis midden virus	*Lebotiviridae*	*Lebotivirus*	MH727531
Shuangao toti-like virus	*Lebotiviridae*	*Lebotivirus*	NC032851
Soldier fly associated toti-like virus 2	*Lebotiviridae*	*Lebotivirus*	PP410012
Tuber aestivum virus 1	*Orthototiviridae*	*Totivirus*	HQ158596
Saccharomyces cerevisiae virus L-A L1	*Orthototiviridae*	*Totivirus*	NC003745
Puccinia striiformis totivirus 5	*Orthototiviridae*	*Totivirus*	KY207365
Red clover powdery mildew-associated totivirus 1	*Orthototiviridae*	*Totivirus*	NC028480
Panax notoginseng virus A	*Orthototiviridae*	*Totivirus*	NC029096
Scheffersomyces segobiensis virus L	*Orthototiviridae*	*Totivirus*	KC610514
Red clover powdery mildew-associated totivirus 2	*Orthototiviridae*	*Totivirus*	NC028481
Saccharomyces cerevisiae virus L-BC La	*Orthototiviridae*	*Totivirus*	U01060
Xanthophyllomyces dendrorhous virus L1A	*Orthototiviridae*	*Totivirus*	JN997472
Xanthophyllomyces dendrorhous virus L1B	*Orthototiviridae*	*Totivirus*	JN997473
Red clover powdery mildew-associated totivirus 4	*Orthototiviridae*	*Totivirus*	LC075489
Red clover powdery mildew-associated totivirus 3	*Orthototiviridae*	*Totivirus*	NC028483
Puccinia striiformis totivirus 1	*Orthototiviridae*	*Totivirus*	KY207361
Puccinia striiformis totivirus 3	*Orthototiviridae*	*Totivirus*	KY207363
Puccinia striiformis totivirus 2	*Orthototiviridae*	*Totivirus*	KY207362
Puccinia striiformis totivirus 4	*Orthototiviridae*	*Totivirus*	KY207364
Red clover powdery mildew-associated totivirus 5	*Orthototiviridae*	*Totivirus*	NC028485
Red clover powdery mildew-associated totivirus 6	*Orthototiviridae*	*Totivirus*	NC028486
Red clover powdery mildew-associated totivirus 7	*Orthototiviridae*	*Totivirus*	NC028488
Red clover powdery mildew-associated totivirus 8	*Orthototiviridae*	*Totivirus*	LC075493
Piscine myocarditis-like virus	*Pistolviridae*	*Pistolvirus*	NC029302
Eimeria brunetti RNA virus 1	*Pseudototiviridae*	*Victorivirus*	AF356189
Eimeria stiedai RNA virus 1	*Pseudototiviridae*	*Victorivirus*	KU597305
Eimeria tenella RNA virus 1	*Pseudototiviridae*	*Victorivirus*	NC026140
Aspergillus foetidus slow virus 1	*Pseudototiviridae*	*Victorivirus*	HE588147
Helminthosporium victoriae virus 190S	*Pseudototiviridae*	*Victorivirus*	NC003607
Tolypocladium cylindrosporum virus 1	*Pseudototiviridae*	*Victorivirus*	NC014823
Rosellinia necatrix victorivirus 1	*Pseudototiviridae*	*Victorivirus*	NC021565
Helicobasidium mompa totivirus 1-17	*Pseudototiviridae*	*Victorivirus*	NC005074
Magnaporthe oryzae virus 1	*Pseudototiviridae*	*Victorivirus*	NC006367
Leishmania RNA virus 1	*Pseudototiviridae*	*Leishmaniavirus*	M92355
Leishmania RNA virus 2	*Pseudototiviridae*	*Leishmaniavirus*	U32108
Trichomonas vaginalis virus 2	*Pseudototiviridae*	*Trichomonasvirus*	AF127178
Trichomonas vaginalis virus 3	*Pseudototiviridae*	*Trichomonasvirus*	AF325840
Trichomonas vaginalis virus 4	*Pseudototiviridae*	*Trichomonasvirus*	HQ607522
Trichomonas vaginalis virus 1	*Pseudototiviridae*	*Trichomonasvirus*	NC027701

Table S2.7 Virus list and accession numbers of sequences used for the *Rhabdoviridae* phylogenetic analysis.

Virus name	Genus	Accession	Collapsed clade
Aruac virus	*Arurhavirus*	KM204987	Clade A
Inhangapi virus	*Arurhavirus*	KM204991	Clade A
Santa Barbara virus	*Arurhavirus*	KM350503	Clade A
Xiburema virus	*Arurhavirus*	KJ636781	Clade A
Caligus rogercresseyi rhabdovirus	*Caligrhavirus*	KY203909	Clade A
Lepeophtheirus salmonis rhabdovirus 127	*Caligrhavirus*	KJ958536	Clade A
Lepeophtheirus salmonis rhabdovirus 9	*Caligrhavirus*	KJ958535	Clade A
Dolphin rhabdovirus	*Cetarhavirus*	KF958252	Clade A
Harbour porpoise rhabdovirus	*Cetarhavirus*	MN103537	Clade A
Adelaide River virus	*Ephemerovirus*	JN935380	Clade A
Berrimah virus	*Ephemerovirus*	HM461974	Clade A
Bovine ephemeral fever virus	*Ephemerovirus*	AF234533	Clade A
Hayes Yard virus	*Ephemerovirus*	MH507506	Clade A
Kimberley virus	*Ephemerovirus*	JQ941664	Clade A
Koolpinyah virus	*Ephemerovirus*	KM085029	Clade A
Kotonkan virus	*Ephemerovirus*	HM474855	Clade A
New Kent County virus	*Ephemerovirus*	MF615270	Clade A
Obodhiang virus	*Ephemerovirus*	HM856902	Clade A
Porcine ephemerovirus 1	*Ephemerovirus*	OK086697	Clade A
Porcine ephemerovirus 2	*Ephemerovirus*	OK086698	Clade A
Puchong virus	*Ephemerovirus*	MH507505	Clade A
Yata virus	*Ephemerovirus*	KM085030	Clade A
Bangoran virus	*Hapavirus*	MW491752	Clade A
Flanders virus	*Hapavirus*	KM205002	Clade A
Gray Lodge virus	*Hapavirus*	KM205022	Clade A
Hart Park virus	*Hapavirus*	KM205011	Clade A
Holmes Jungle virus	*Hapavirus*	KY421919	Clade A
Joinjakaka virus	*Hapavirus*	KM205016	Clade A
Kamese virus	*Hapavirus*	KM204989	Clade A
La Joya virus	*Hapavirus*	KM204986	Clade A
Landjia virus	*Hapavirus*	KM205010	Clade A
Manitoba virus	*Hapavirus*	KM205008	Clade A
Marco virus	*Hapavirus*	KM205005	Clade A
Mosqueiro virus	*Hapavirus*	KM205014	Clade A
Mossuril virus	*Hapavirus*	KM204993	Clade A
Ngaingan virus	*Hapavirus*	FJ715959	Clade A
Ord River virus	*Hapavirus*	KM205025	Clade A
Parry Creek virus	*Hapavirus*	KM204988	Clade A
Porton virus	*Hapavirus*	MW491751	Clade A
Wongabel virus	*Hapavirus*	EF612701	Clade A
Eel virus European X	*Perhabdovirus*	FN557213	Clade A
Lake trout rhabdovirus	*Perhabdovirus*	MN963997	Clade A
Leman virus	*Perhabdovirus*	MN963996	Clade A
Perch rhabdovirus	*Perhabdovirus*	JX679246	Clade A
Scophthalmus maximus rhabdovirus	*Scophrhavirus*	HQ003891	Clade A
Wuhan redfin culter dimarhabdovirus	*Scophrhavirus*	MG600013	Clade A
Eelpout rhabdovirus	*Siniperhavirus*	KR612230	Clade A
Siniperca chuatsi rhabdovirus	*Siniperhavirus*	DQ399789	Clade A
Pike fry rhabdovirus	*Sprivivirus*	FJ872827	Clade A
Spring viremia of carp virus	*Sprivivirus*	U18101	Clade A
Almpiwar virus	*Sripuvirus*	KJ399977	Clade A
Chaco virus	*Sripuvirus*	KM205000	Clade A
Charleville virus	*Sripuvirus*	MH899109	Clade A
Cuiaba virus	*Sripuvirus*	MH89911	Clade A
Hainan black-spectacled toad rhabdovirus	*Sripuvirus*	MG600016	Clade A
Niakha virus	*Sripuvirus*	KC585008	Clade A
Sena Madureira virus	*Sripuvirus*	KM205004	Clade A
Sripur virus	*Sripuvirus*	KM205023	Clade A
Bimbo virus	*Sunrhavirus*	MW491756B	Clade A
Boteke virus	*Sunrhavirus*	MW491753	Clade A
Burg el Arab virus	*Sunrhavirus*	MW491759	Clade A
Dillard's Draw virus	*Sunrhavirus*	MG251664	Clade A
Garba virus	*Sunrhavirus*	KM204982	Clade A
Harrison Dam virus	*Sunrhavirus*	KJ432573	Clade A
Kolongo virus	*Sunrhavirus*	MW491757	Clade A
Kwatta virus	*Sunrhavirus*	KM204985	Clade A
Matariya virus	*Sunrhavirus*	MW491760	Clade A
Nasoule virus	*Sunrhavirus*	MW491755	Clade A
Oak Vale virus	*Sunrhavirus*	JF705876	Clade A
Ouango virus	*Sunrhavirus*	MW491758	Clade A
Sandjimba virus	*Sunrhavirus*	MW491754	Clade A
Sunguru virus	*Sunrhavirus*	KF395226	Clade A
Walkabout Creek virus	*Sunrhavirus*	KJ432572	Clade A
Bas-Congo virus	*Tibrovirus*	JX297815	Clade A

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Beatrice Hill virus	*Tibrovirus*	KY073943	Clade A
Coastal Plains virus	*Tibrovirus*	GQ294473	Clade A
Ekpoma virus 1	*Tibrovirus*	KP324827	Clade A
Ekpoma virus 2	*Tibrovirus*	KP324828	Clade A
Mundri virus	*Tibrovirus*	OM320812	Clade A
Sweetwater Branch virus	*Tibrovirus*	KM204997	Clade A
Tibrogargan virus	*Tibrovirus*	GQ294472	Clade A
Durham virus	*Tupavirus*	FJ952155	Clade A
Klamath virus	*Tupavirus*	KM204999	Clade A
Tupaia rhabdovirus	*Tupavirus*	AY840978	Clade A
Wenzhou Myotis laniger tupavirus 1	*Tupavirus*	OM030290	Clade A
Wufeng Rhinolophus pearsonii tupavirus 1	*Tupavirus*	MZ328291	Clade A
American bat vesiculovirus	*Vesiculovirus*	JX569193	Clade A
Carajas virus	*Vesiculovirus*	KM205015	Clade A
Chandipura virus	*Vesiculovirus*	GU212856	Clade A
Cocal virus	*Vesiculovirus*	EU373657	Clade A
Isfahan virus	*Vesiculovirus*	AJ810084	Clade A
Jinghong bat virus	*Vesiculovirus*	MF279192	Clade A
Jurona virus	*Vesiculovirus*	KM204996	Clade A
Malpais Spring virus	*Vesiculovirus*	KC412247	Clade A
Maraba virus	*Vesiculovirus*	HQ660076	Clade A
Mediterranean bat virus	*Vesiculovirus*	MW557336	Clade A
Mejal virus	*Vesiculovirus*	MW798173	Clade A
Morreton virus	*Vesiculovirus*	KM205007	Clade A
Perinet virus	*Vesiculovirus*	HM566195	Clade A
Piry virus	*Vesiculovirus*	KU178986	Clade A
Radi virus	*Vesiculovirus*	KM205024	Clade A
Vesicular stomatitis Alagoas virus	*Vesiculovirus*	EU373658	Clade A
Vesicular stomatitis Indiana virus	*Vesiculovirus*	AF473864	Clade A
Vesicular stomatitis New Jersey virus	*Vesiculovirus*	JX121110	Clade A
Wufeng Myotis altarium vesiculovirus 1	*Vesiculovirus*	OM030292	Clade A
Yinshui bat virus	*Vesiculovirus*	MN607594	Clade A
Yug Bogdanovac virus	*Vesiculovirus*	JF911700	Clade A
Barur virus	*Ledantevirus*	KM204983	Clade B
Bughendera virus	*Ledantevirus*	MT325641	Clade B
Fikirini virus	*Ledantevirus*	KC676792	Clade B
Fukuoka virus	*Ledantevirus*	KM205001	Clade B
Kanyawara virus	*Ledantevirus*	KY385390	Clade B
Kern Canyon virus	*Ledantevirus*	KM204992	Clade B
Keuraliba virus	*Ledantevirus*	KM205021	Clade B
Kolente virus	*Ledantevirus*	KC984953	Clade B
Kumasi rhabdovirus	*Ledantevirus*	KJ179955	Clade B
Le Dantec virus	*Ledantevirus*	KM205006	Clade B
Longquan Niviventer coninga ledantevirus 1	*Ledantevirus*	MZ328293	Clade B
Mount Elgon bat virus	*Ledantevirus*	KM205026	Clade B
Nishimuro virus	*Ledantevirus*	AB609604	Clade B
Nkolbisson virus	*Ledantevirus*	KM205017	Clade B
Oita virus	*Ledantevirus*	KM204998	Clade B
Taiyi bat virus	*Ledantevirus*	MN607592	Clade B
Vaprio virus	*Ledantevirus*	MG021441	Clade B
Wenzhou Rhinolophus pusillus ledantevirus 1	*Ledantevirus*	OM030289	Clade B
Wuhan louse fly virus 5	*Ledantevirus*	KM817654	Clade B
Yongjia tick virus 2	*Ledantevirus*	KM817662	Clade B
Arboretum virus	*Almendravirus*	KC994644	Clade C
Balsa virus	*Almendravirus*	KX228198	Clade C
Coot Bay virus	*Almendravirus*	KX228196	Clade C
Menghai virus	*Almendravirus*	KX785335	Clade C
Puerto Almendras virus	*Almendravirus*	KF543749	Clade C
Rio Chico virus	*Almendravirus*	KX228197	Clade C
Xiangshan rhabdo-like virus 1	*Almendravirus*	OK491499	Clade C
Frog lyssa-like virus 1	*Amplylivirus*	MK473367	Clade D
Aravan virus	*Lyssavirus*	EF614259	Clade D
Australian bat lyssavirus	*Lyssavirus*	AF081020	Clade D
Bokeloh bat lyssavirus	*Lyssavirus*	JF311903	Clade D
Duvenhage virus	*Lyssavirus*	EU292119	Clade D
European bat lyssavirus 1	*Lyssavirus*	EF157976	Clade D
European bat lyssavirus 2	*Lyssavirus*	EF157977	Clade D
Gannoruwa bat lyssavirus	*Lyssavirus*	KU244266	Clade D
Ikoma lyssavirus	*Lyssavirus*	JX193798	Clade D
Irkut virus	*Lyssavirus*	EF614260	Clade D
Khujand virus	*Lyssavirus*	EF614261	Clade D
Lagos bat virus	*Lyssavirus*	EU293108	Clade D
Lleida bat lyssavirus	*Lyssavirus*	KY006983	Clade D
Mokola virus	*Lyssavirus*	YO9762	Clade D
Rabies virus	*Lyssavirus*	M13215	Clade D
Shimoni bat virus	*Lyssavirus*	GU170201	Clade D
Taiwan bat lyssavirus	*Lyssavirus*	MF472710	Clade D
West Caucasian bat virus	*Lyssavirus*	EF614258	Clade D
Anole lyssa-like virus 1	*Replylivirus*	BR001666	Clade D
Wenling crustacean virus 10	*Alphacrustrhavirus*	KX884450	Clade E

Wenling crustacean virus 11	*Alphacrustrhavirus*	KX884456	Clade E
Shayang fly virus 3	*Alphadrosrhavirus*	KM817636	Clade E
Wuhan house fly virus 2	*Alphadrosrhavirus*	KM817649	Clade E
Hymenopteran rhabdo-related virus 109	*Alphahymrhavirus*	MT153372	Clade E
Hymenopteran rhabdo-related virus 38	*Alphahymrhavirus*	MT153454	Clade E
Hymenopteran rhabdo-related virus 46	*Alphahymrhavirus*	MW314718	Clade E
Lasius neglectus virus 2	*Alphahymrhavirus*	MH477288	Clade E
Agave tequilana virus 1	*Alphanucleorhabdovirus*	BK014297	Clade E
Artemisia capillaris nucleorhabdovirus 1	*Alphanucleorhabdovirus*	OM372677	Clade E
Constricta yellow dwarf virus	*Alphanucleorhabdovirus*	KY549567	Clade E
Eggplant mottled dwarf virus	*Alphanucleorhabdovirus*	KJ082087	Clade E
Joa yellow blotch associated virus	*Alphanucleorhabdovirus*	MW014292	Clade E
Maize Iranian mosaic virus	*Alphanucleorhabdovirus*	MF102281	Clade E
Maize mosaic virus	*Alphanucleorhabdovirus*	AY618418	Clade E
Morogoro maize-associated virus	*Alphanucleorhabdovirus*	MK063878	Clade E
Peach virus 1	*Alphanucleorhabdovirus*	MN520414	Clade E
Physostegia chlorotic mottle virus	*Alphanucleorhabdovirus*	KX636164	Clade E
Potato yellow dwarf virus	*Alphanucleorhabdovirus*	GU734660	Clade E
Rice yellow stunt virus	*Alphanucleorhabdovirus*	AB011257	Clade E
Taro vein chlorosis virus	*Alphanucleorhabdovirus*	AY674964	Clade E
Wheat yellow striate virus	*Alphanucleorhabdovirus*	MG604920	Clade E
Hymenopteran rhabdo-related virus 23	*Betahymrhavirus*	MW314717	Clade E
Hymenopteran rhabdo-related virus 24	*Betahymrhavirus*	MW039260	Clade E
Hubei rhabdo-like virus 9	*Betanemrhavirus*	KX884448	Clade E
Shayang ascaridia galli virus 2	*Betanemrhavirus*	KX884414	Clade E
Bird's-foot trefoil nucleorhabdovirus	*Betanucleorhabdovirus*	MH614262	Clade E
Alfalfa-associated nucleorhabdovirus	*Betanucleorhabdovirus*	MG948563	Clade E
Apple rootstock virus A	*Betanucleorhabdovirus*	MH778545	Clade E
Asclepias syriaca virus 2	*Betanucleorhabdovirus*	BK014299	Clade E
Bacopa monnieri virus 2	*Betanucleorhabdovirus*	BK014480	Clade E
Blackcurrant-associated rhabdovirus	*Betanucleorhabdovirus*	MF543022	Clade E
Cardamom vein clearing virus	*Betanucleorhabdovirus*	MN273311	Clade E
Cnidium virus 1	*Betanucleorhabdovirus*	MZ983390	Clade E
Datura yellow vein virus	*Betanucleorhabdovirus*	KM823531	Clade E
Plectranthus aromaticus virus 1	*Betanucleorhabdovirus*	BK014300	Clade E
Rhododendon delavayi virus 1	*Betanucleorhabdovirus*	BK014301	Clade E
Sonchus yellow net virus	*Betanucleorhabdovirus*	L32603	Clade E
Sowthistle yellow vein virus	*Betanucleorhabdovirus*	MT185675	Clade E
Zhuye pepper nucleorhabdovirus	*Betanucleorhabdovirus*	MH323437	Clade E
Lepidopteran rhabdo-related virus 34	*Betapaprhavirus*	MT153466	Clade E
Spodoptera frugiperda rhabdovirus	*Betapaprhavirus*	KF947078	Clade E
Blacklegged tick rhabdovirus 1	*Betaricinrhavirus*	MF360790	Clade E
Chimay rhabdovirus	*Betaricinrhavirus*	MF975531	Clade E
Curionopolis virus	*Curiovirus*	KM204994	Clade E
Iriri virus	*Curiovirus*	KM204995	Clade E
Itacaiunas virus	*Curiovirus*	KM204984	Clade E
Rochambeau virus	*Curiovirus*	KM205012	Clade E
Actinidia virus D	*Cytorhabdovirus*	MW550041	Clade E
Alfalfa dwarf virus	*Cytorhabdovirus*	KP205452	Clade E
Anthurium amnicola virus 1	*Cytorhabdovirus*	BK014302	Clade E
Asclepias syriaca virus 1	*Cytorhabdovirus*	BK014298	Clade E
Bacopa monnieri virus 1	*Cytorhabdovirus*	BK014479	Clade E
Barley yellow striate mosaic virus	*Cytorhabdovirus*	KM213865	Clade E
Bemisia tabaci-associated virus 1	*Cytorhabdovirus*	BK014303	Clade E
Cabbage cytorhabdovirus 1	*Cytorhabdovirus*	KY810772	Clade E
Chrysanthemum yellow dwarf associated virus	*Cytorhabdovirus*	MW039593	Clade E
Colocasia bobone disease-associated virus	*Cytorhabdovirus*	KT381973	Clade E
Cucurbit cytorhabdovirus 1	*Cytorhabdovirus*	MT381995	Clade E
Glehnia littoralis virus 1	*Cytorhabdovirus*	BK014304	Clade E
Gymnadenia densiflora virus 1	*Cytorhabdovirus*	BK014305	Clade E
Kenyan potato cytorhabdovirus	*Cytorhabdovirus*	MN689395	Clade E
Lettuce necrotic yellows virus	*Cytorhabdovirus*	AJ867584	Clade E
Lettuce yellow mottle virus	*Cytorhabdovirus*	EF687738	Clade E
Maize yellow striate virus	*Cytorhabdovirus*	KY884303	Clade E
Maize-associated cytorhabdovirus	*Cytorhabdovirus*	KY965147	Clade E
Northern cereal mosaic virus	*Cytorhabdovirus*	AB030277	Clade E
Nymphaea alba virus 1	*Cytorhabdovirus*	BK014307	Clade E
Papaya virus E	*Cytorhabdovirus*	MH282832	Clade E
Paper mulberry mosaic associated virus	*Cytorhabdovirus*	MN872813	Clade E
Persimmon virus A	*Cytorhabdovirus*	AB735628	Clade E
Raspberry vein chlorosis virus	*Cytorhabdovirus*	MK240091	Clade E
Rice stripe mosaic virus	*Cytorhabdovirus*	KX525586	Clade E
Rose virus R	*Cytorhabdovirus*	MT952336	Clade E
Strawberry crinkle virus	*Cytorhabdovirus*	MH129615	Clade E
Strawberry virus 1	*Cytorhabdovirus*	MK211271	Clade E
Tagetes erecta virus 1	*Cytorhabdovirus*	BK014308	Clade E
Tomato yellow mottle-associated virus	*Cytorhabdovirus*	KY075646	Clade E
Trachyspermum ammi virus 1	*Cytorhabdovirus*	BK014309	Clade E
Trichosanthes associated rhabdovirus 1	*Cytorhabdovirus*	BK011194	Clade E
Trifolium pratense virus A	*Cytorhabdovirus*	MH982250	Clade E

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Trifolium pratense virus B	*Cytorhabdovirus*	MH982249	Clade E
Wuhan insect virus 4	*Cytorhabdovirus*	KM817650	Clade E
Wuhan insect virus 5	*Cytorhabdovirus*	KM817651	Clade E
Wuhan insect virus 6	*Cytorhabdovirus*	KM817652	Clade E
Yerba mate chlorosis-associated virus	*Cytorhabdovirus*	KY366322	Clade E
Yerba mate virus A	*Cytorhabdovirus*	MN781667	Clade E
Citrus chlorotic spot virus	*Dichorhavirus*	KY700686	Clade E
Citrus leprosis virus N	*Dichorhavirus*	KX982179	Clade E
Clerodendrum chlorotic spot virus	*Dichorhavirus*	MG938507	Clade E
Coffee ringspot virus	*Dichorhavirus*	KF812526	Clade E
Orchid fleck virus	*Dichorhavirus*	AB244418	Clade E
Maize fine streak virus	*Gammanucleorhabdovirus*	AY618417	Clade E
Allium angulosum virus 1	*Varicosavirus*	BK059208	Clade E
Alopecurus myosuroides varicosavirus 1	*Varicosavirus*	LN713933	Clade E
Brassica rapa virus 1	*Varicosavirus*	BK014310	Clade E
Lettuce big vein-associated virus	*Varicosavirus*	AB075039	Clade E
Lolium perenne virus 1	*Varicosavirus*	BK014312	Clade E
Melampyrum roseum virus 1	*Varicosavirus*	BK014314	Clade E
Morning glory varicosavirus	*Varicosavirus*	MW922438	Clade E
Pinus flexilis virus 1	*Varicosavirus*	BK014316	Clade E
Red clover associated varicosavirus	*Varicosavirus*	MF918568	Clade E
Vitis varicosavirus	*Varicosavirus*	LC604719	Clade E
Xinjiang varicosavirus	*Varicosavirus*	MW897032	Clade E
Zostera-associated varicosavirus 1	*Varicosavirus*	BK014484	Clade E
Rattus tanezumi rhabdovirus 1	*Alphanemrhavirus*	MT085340	
Sodak rhabdovirus 1	*Alphanemrhavirus*	MT875151	
Xingshan nematode virus 4	*Alphanemrhavirus*	KX884459	
Xinzhou nematode virus 4	*Alphanemrhavirus*	KX884462	
Hubei lepidoptera virus 2	*Alphapaprhavirus*	KX884415	
Pararge aegeria rhabdovirus	*Alphapaprhavirus*	KR822826	
Blanchseco virus	*Alpharicinrhavirus*	MN025503	
Bole tick virus 2	*Alpharicinrhavirus*	KM817629	
Hubei tick rhabdovirus 1	*Alpharicinrhavirus*	MW721934	
Wuhan tick virus 1	*Alpharicinrhavirus*	KM817660	
Bahia Grande virus	*Barhavirus*	KM205018	
Muir Springs virus	*Barhavirus*	KM204990	
lone star tick rhabdovirus	*Lostrhavirus*	KU127239	
Xinjiang tick rhabdovirus	*Lostrhavirus*	MH688524	
Culex tritaeniorhynchus rhabdovirus	*Merhavirus*	AB604791	
Merida virus	*Merhavirus*	KU194360	
Moussa virus	*Mousrhavirus*	FJ985748	
Hirame rhabdovirus	*Novirhabdovirus*	AF104985	
Infectious hematopoietic necrosis virus	*Novirhabdovirus*	L40883	
Snakehead rhabdovirus	*Novirhabdovirus*	AF147498	
Viral hemorrhagic septicemia virus	*Novirhabdovirus*	Y18263	
Culex pseudovishnui rhabdo-like virus	*Ohlsrhavirus*	LC514057	
Culex rhabdo-like virus	*Ohlsrhavirus*	MF176333	
Culex rhabdo-like virus Los Angeles	*Ohlsrhavirus*	MH188003	
Lobeira virus	*Ohlsrhavirus*	MK780203	
North Creek virus	*Ohlsrhavirus*	KF360973	
Ohlsdorf virus	*Ohlsrhavirus*	KY768856	
Riverside virus	*Ohlsrhavirus*	KU248085	
Tongilchon virus 1	*Ohlsrhavirus*	KU095840	
Connecticut virus	*Sawgrhavirus*	KM205020	
Long Island tick rhabdovirus	*Sawgrhavirus*	KJ396935	
New Minto virus	*Sawgrhavirus*	KM205009	
Sawgrass virus	*Sawgrhavirus*	KM205013	
Aksy-Durug Melophagus sigmavirus	*Sigmavirus*	OL420709	
Apis rhabdovirus 3	*Sigmavirus*	MZ822104	
Ceratitis capitata sigmavirus	*Sigmavirus*	KR822825	
Drosophila affinis sigmavirus	*Sigmavirus*	GQ410980	
Drosophila ananassae sigmavirus	*Sigmavirus*	KR822812	
Drosophila immigrans sigmavirus	*Sigmavirus*	KX884434	
Drosophila melanogaster sigmavirus	*Sigmavirus*	GQ375258	
Drosophila obscura sigmavirus	*Sigmavirus*	GQ410979	
Drosophila sturtevanti sigmavirus	*Sigmavirus*	KR822816	
Hermetia illucens sigma-like virus 1	*Sigmavirus*	Appendix 1 list 1	
Hubei dimarhabdovirus 1	*Sigmavirus*	KX884431	
Hubei diptera virus 10	*Sigmavirus*	KX884433	
Hubei diptera virus 9	*Sigmavirus*	KX884429	
Shayang fly virus 2	*Sigmavirus*	KM817635	
Wuhan fly virus 2	*Sigmavirus*	KM817646	
Wuhan house fly virus 1	*Sigmavirus*	KM817648	
Wuhan louse fly virus 10	*Sigmavirus*	KM817657	
Wuhan louse fly virus 9	*Sigmavirus*	KM817656	
Yushu rhabdovirus	*Sigmavirus*	MW826525	
Hangzhou Frankliniella intonsa rhabdovirus 1	*Thriprhavirus*	MZ209657	
Thrips tabaci associated dimarhabdovirus 1	*Thriprhavirus*	MN714687	
Zahedan rhabdovirus	*Zarhavirus*	KJ830812	

Table S2.8 Virus list and accession numbers of sequences used for the *Picornavirales* phylogenetic analysis.

Virus name	**Family**	**Genus**	**Accession**
Hermetia illucens cripavirus 1	*Dicistroviridae*	*Cripavirus*	Appendix 1 list 1
Bactrocera dorsalis cripavirus	*Dicistroviridae*	*Cripavirus*	MN738553
Bactrocera tryoni dicistrovirus 1	*Dicistroviridae*	*Cripavirus*	MW208808
Bactrocera tryoni dicistrovirus 2	*Dicistroviridae*	*Cripavirus*	MW208809
Cricket paralysis virus	*Dicistroviridae*	*Cripavirus*	NC003924
Drosophila C virus	*Dicistroviridae*	*Cripavirus*	NC001834
Mud crab dicistrovirus	*Dicistroviridae*	*Aparavirus*	NC014793
Taura syndrome virus	*Dicistroviridae*	*Aparavirus*	NC003005
Israel acute paralysis virus of bees	*Dicistroviridae*	*Aparavirus*	NC009025
Kashmir bee virus	*Dicistroviridae*	*Aparavirus*	NC004807
Acute bee paralysis virus	*Dicistroviridae*	*Aparavirus*	NC002548
Solenopsis invicta virus 1	*Dicistroviridae*	*Aparavirus*	NC006559
Aphid lethal paralysis virus	*Dicistroviridae*	*Cripavirus*	NC004365
Rhopalosiphum padi virus	*Dicistroviridae*	*Cripavirus*	NC001874
Homalodisca coagulata virus 1	*Dicistroviridae*	*Triatovirus*	NC008029
Black queen cell virus	*Dicistroviridae*	*Triatovirus*	NC003784
Triatoma virus	*Dicistroviridae*	*Triatovirus*	NC003783
Himetobi P virus	*Dicistroviridae*	*Triatovirus*	NC003782
Plautia stali intestine virus	*Dicistroviridae*	*Triatovirus*	NC003779
Soldier fly virus	*Dicistroviridae*	Unassigned	MW357714
Nilaparvata lugens honeydew virus 1	*Iflaviridae*	*Iflavirus*	NC038302
Lymantria dispar iflavirus 1	*Iflaviridae*	*Iflavirus*	NC024497
Antheraea pernyi iflavirus	*Iflaviridae*	*Iflavirus*	NC023483
Varroa destructor virus 1	*Iflaviridae*	*Iflavirus*	NC006494
Deformed wing virus	*Iflaviridae*	*Iflavirus*	NC004830
Brevicoryne brassicae picorna-like virus	*Iflaviridae*	*Iflavirus*	NC009530
Slow bee paralysis virus	*Iflaviridae*	*Iflavirus*	NC014137
Lygus lineolaris virus 1	*Iflaviridae*	*Iflavirus*	NC038301
Hubei picorna-like virus 42	*Iflaviridae*	*Iflavirus*	NC032769
Hermetia illucens iflavirus 1	*Iflaviridae*	*Iflavirus*	Appendix 1 list 1
Halyomorpha halys virus	*Iflaviridae*	*Iflavirus*	NC022611
Sacbrood virus	*Iflaviridae*	*Iflavirus*	NC002066
Dinocampus coccinellae paralysis virus	*Iflaviridae*	*Iflavirus*	NC025835
Spodoptera exigua iflavirus 1	*Iflaviridae*	*Iflavirus*	NC016405
Infectious flacherie virus	*Iflaviridae*	*Iflavirus*	NC003781
Spodoptera exigua iflavirus 2	*Iflaviridae*	*Iflavirus*	NC023676
Ectropis obliqua picorna-like virus	*Iflaviridae*	*Iflavirus*	NC005092
Perina nuda virus	*Iflaviridae*	*Iflavirus*	NC003113
Parsnip yellow fleck virus	*Secoviridae*	*Sequivirus*	NC003628
Rice tungro spherical virus	*Secoviridae*	*Waikavirus*	NC001632
Nylanderia fulva virus 1	*Solinviviridae*	*Nyfulvavirus*	NC030651
Solenopsis invicta virus 3	*Solinviviridae*	*Invictavirus*	NC012531
Hermetia illucens solinvivirus 1	*Solinviviridae*	Unassigned	Appendix 1 list 1
Kelp fly virus	*Solinviviridae*	Unassigned	NC007619
Acyrthosiphon pisum virus	*Solinviviridae*	Unassigned	NC003780
Rosy apple aphid virus	*Solinviviridae*	Unassigned	DQ286292

Appendix 1

Table S2.9 Distribution of the top hits by megaBLAST on the 10th April 2024 on the extracted region (position 4462 to 4541) of the L segment of the concatenated BNaV1 genomic sequence.

Organism	Blast name	Score	Number of hits	Description
root			100	
-Viruses	viruses		44	
--Severe acute respiratory syndrome coronavirus 2	viruses	121	41	Severe acute respiratory syndrome coronavirus 2 hits
--Enterococcus phage SSMH01	viruses	106	1	Enterococcus phage SSMH01 hits
--Human alphaherpesvirus 2	viruses	97.1	1	Human alphaherpesvirus 2 hits
--human gammaherpesvirus 4	viruses	69.4	1	human gammaherpesvirus 4 hits
-Asarum shuttleworthii	flowering plants	119	1	Asarum shuttleworthii hits
-Asarum satsumense	flowering plants	115	1	Asarum satsumense hits
-Lasthenia californica	eudicots	108	1	Lasthenia californica hits
-Helicobacter pylori	e-proteobacteria	104	2	Helicobacter pylori hits
-Yersinia enterocolitica	enterobacteria	102	1	Yersinia enterocolitica hits
-Synthliboramphus antiquus	birds	99	2	Synthliboramphus antiquus hits
-Salmonella sp.	enterobacteria	97.1	1	Salmonella sp. hits
-Ralstonia solanacearum	b-proteobacteria	75	1	Ralstonia solanacearum hits
-Cyprinus carpio	bony fishes	71.3	1	Cyprinus carpio hits
-Phallusia mammillata	tunicates	65.8	1	Phallusia mammillata hits
-uncultured Aenigmarchaeota archaeon	archaea	65.8	1	uncultured Aenigmarchaeota archaeon hits
-Larimichthys crocea	bony fishes	65.8	1	Larimichthys crocea hits
-Heterocephalus glaber	rodents	65.8	42	Heterocephalus glaber hits

Table S2.10 Distribution of the top hits by megaBLAST on the 10th April 2024 on the extracted region (position 2 to 79) of the L segment of the concatenated BuBV1 genomic sequence.

Organism	Blast name	Score	Number of hits	Description
root			100	
-cellular organisms			33	
--Eukaryota	eukaryotes		28	
---Mesangiospermae	flowering plants		4	
----Lasthenia californica	eudicots	95.3	1	Lasthenia californica hits
----Asarum satsumense	flowering plants	93.5	1	Asarum satsumense hits
----Asarum shuttleworthii	flowering plants	89.8	1	Asarum shuttleworthii hits
----Oryza rufipogon	monocots	67.6	1	Oryza rufipogon hits
---Danio rerio	bony fishes	75	1	Danio rerio hits
---Cyprinus carpio	bony fishes	73.1	19	Cyprinus carpio hits
---Synthliboramphus antiquus	birds	73.1	2	Synthliboramphus antiquus hits
---Phallusia mammillata	tunicates	67.6	1	Phallusia mammillata hits
---Heterocephalus glaber	rodents	67.6	1	Heterocephalus glaber hits
--Helicobacter pylori	e-proteobacteria	89.8	2	Helicobacter pylori hits
--Yersinia enterocolitica	enterobacteria	78.7	1	Yersinia enterocolitica hits
--Streptococcus suis	firmicutes	69.4	1	Streptococcus suis hits
--uncultured Aenigmarchaeota archaeon	archaea	67.6	1	uncultured Aenigmarchaeota archaeon hits
-Severe acute respiratory syndrome coronavirus 2	viruses	91.6	44	Severe acute respiratory syndrome coronavirus 2 hits
-Enterococcus phage SSMH01	viruses	86.1	1	Enterococcus phage SSMH01 hits
-human gammaherpesvirus 4	viruses	73.1	1	human gammaherpesvirus 4 hits
-synthetic construct	other sequences	71.3	17	synthetic construct hits
-Human alphaherpesvirus 2	viruses	69.4	1	Human alphaherpesvirus 2 hits
-Brassica yellows virus	viruses	67.6	1	Brassica yellows virus hits
-Caudoviricetes sp-	viruses	67.6	2	Caudoviricetes sp. hits

Table S2.11 Distribution of the top hits by megaBLAST on the 10th April 2024 on the extracted region (position 7019 to 7061) of the S segment of the concatenated BuBV1 genomic sequence.

Organism	Blast name	Score	Number of hits	Description
root			102	
-Viruses	viruses		65	
--Riboviria	viruses		56	
---Pisoniviricetes	viruses		54	
----Severe acute respiratory syndrome coronavirus 2	viruses	69.4	51	Severe acute respiratory syndrome coronavirus 2 hits
----Arabis mosaic virus	viruses	63.9	1	Arabis mosaic virus hits
----Physalis rugose mosaic virus	viruses	62.1	2	Physalis rugose mosaic virus hits
---Diabrotica undecimpunctata virus 1	viruses	63.9	1	Diabrotica undecimpunctata virus 1 hits
---Puma lentivirus	viruses	62.1	1	Puma lentivirus hits
--Caulobacter phage Ccr29	viruses	65.8	1	Caulobacter phage Ccr29 hits
--Mycobacterium phage Kashi_RDG1	viruses	65.8	1	Mycobacterium phage Kashi_RDG1 hits
--Paenibacillus phage Pahemo	viruses	63.9	1	Paenibacillus phage Pahemo hits

--Caudoviricetes sp-	viruses	63.9	2	Caudoviricetes sp. hits
--macacine betaherpesvirus 3	viruses	63.9	1	macacine betaherpesvirus 3 hits
--Salmonella phage TS13	viruses	62.1	1	Salmonella phage TS13 hits
--human gammaherpesvirus 4	viruses	62.1	2	human gammaherpesvirus 4 hits
-Cyprinus carpio	bony fishes	65.8	2	Cyprinus carpio hits
-Candidatus Stammera capleta	enterobacteria	65.8	1	Candidatus Stammera capleta hits
-Fargesia denudata	monocots	63.9	2	Fargesia denudata hits
-Vector pPTK-BC-IPR	other sequences	63.9	1	Vector pPTK-BC-IPR hits
-Cloning vector pCA-DEST2430	other sequences	62.1	1	Cloning vector pCA-DEST2430 hits
-Cloning vector pCA-DEST2420	other sequences	62.1	1	Cloning vector pCA-DEST2420 hits
-Methylomonas koyamae	g-proteobacteria	62.1	1	Methylomonas koyamae hits

Appendix 1 list 1 List of viral sequences used for the analyses of the BSF associated viruses.

BNaV1: BSF Nairovirus-like 1 genomic L, M and S segments concatenated

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Appendix 1

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AUACCAUUUCAAUGUAUGUACAUUCUGAUAUUCCAAUAAUGUUUCAAACAUUUGAUUAUAUUUCCCUUCUAAAUGACUCAAACUUAUGUAA
AACGAUUUGAUAGAAAGGUUUGGCAAGUAUACUUCAUCAUAGAUCUAAUAUAAUAAAUGAUCAAUACUAAGCAAUUCUAAUAUAAUAGCUU
UAACAACAUGCAUUAUUAUUUAUGCAUGAUGAUUGUAGUGGGUUUCAGUUGAACAUAAGAAGAAGACAGAUUUAAGUAUGAGUUUCGCAA
UUAAAGUUACAAGUCAUGCUCAGAGUUAAGGUUUAAUAAGAAGAUUAUAAGGUUUUACUAUAAAGUUUUUGGCCCCUACUGGUUGAUAGU
UUAAAAACAAUUUAAAUUUAUUAAUAUCGAAUGUAUUCACUUACAAUCACAGCAUGUUGAAGGAAAAGUUAAAUUCAAAUUUAAGAUAUUA
AAAUAUUAUUUGAUAGUGCUAACCUCUAACAGAGAGGGAGCGGGGGUGGUNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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UUAUCUAGUACAACUGACUGAUUUAUGAAUAAAUUGAAAAUGUAAUUUAUUUCUUUAAACAGAAGACAAAUUCUGAUUAUGUGUAUUGGU
GAUCUCAAUAAUUUCAGAGAUAAAGAUAAUGUUUGAUUGGAAUCUUCUAGUGCCAAUGUAGCCAUAGAGAUUGCGCAAGAACUUUGAUUC
AAUCUCACUUGCAUCAUCAACACUAUCAAUAUACUGGACCAGCUCAAACUUUUCUUGAUCUGAGAGACGGAUGAAGUAAAAUGCUAAGCU
CAUGUUCUUACAUGUCCUGUAUAGACUUUGUUCUUCCUCAGAAAUAGAAUCACGCUUUGUUGCAUCAGACAUCUUUGAUGUAAUUGCUU
UAAGAGCUCUAAUCAUUUUCACUAUAAGCUCAUUCAUCAGGCUUGGGCCAAAUGUCUUUUUCACUAAAUUGACAUCCUUCUUGUCAUCUG
CUGUUAUGGAAUCUAAUGCUGCUGCUGCUUCAUCACCAAUAAGAUUCAAAAACUUCGAUCUAAGAUCAUUUAAGGACAUAAGAAAAUUCU
UAUCUAAGAAUUUUGACAAAUCAACAUGCUUUGCAUUGAACCUGAACACAGGCUUUGGAUUUACAGCUGUUUGUGAUGAUUGUCUCGGU
CUUUUGAUACCUGAGCUAGAAGAUUUAGACAUGUUAUCAGGUAAAGAUAUUUGGAAAACUGAAUUCGGAUCAUUGCUGCCUUUUGAGUC
CAUUUAUCUGAAAAUGUUGAAUUCUAAGAUGAGUAGAAUAAUAAAUAAUAAAAGACAUAAUGUAAGCUUGUUUCAGUUCAUGAGAUUAACA
GUUUUAGUGGAUAGUUCUUUUCCCAAAGGGGACGAGGUAU

BuBV1: BSF uncharacterized bunyavirus-like 1 genomic L and S segments concatenated

CCGGCGCCCACCGAGAUCUACACCCUAUCCUACACUCUUUCCCUACACGACGCUCUUCCGAUCUCUUAAAUUCCUUCUCCAUCAUUGUG
UCUCAGGCUUUGAUCUUAGGUGACCUAGAAAAACCCUUCUGAGAUAUAGUCAUUGUCUCCAAAUGUAAUAUCUAAUCCAUCUAACUUGUU
CAUGAUGUCAGUAAACACUGAGACCUCGUUGAAAUCAUCUGGGUUAACAUCCAGCUCUAAUACGGCAUCAAUAUGGGGCUUGGAUUCCA
GAAUGGAUUGUUUGUCUUUCAAUUCUCCCAUUAUAGGGGAUUUGAAUAGGACCAUCUCAAUGUCAUCAAAGUUGCCCUUUUUAAUCUCA
UCAUAAACUGCGAAGUUUGCUCUGCUGUGACAGAAGCUUCUGAGCGAUUCAGCAACUGACUGCAGGUUAUCGGUAAACCCGGCUGCUAC
CAUGAAAGCAGGUCCUUUGUCAGAAUUAAGAAAUGAUCUCACUAUUAGACUGAUCUCAUCAUCUUCUUCAGGUAGAUCUUCCUCAAGGUG
GAUGCAGCCGCAUCUGUAAUUGGCUAUAAGGAGAGCUGAUACCAGGAGCUUGUGAGACACUUUAGCGUUCGACACUGACAUGUAUCUUG
GUGCUGAGACAAUGACUAAAACAUUGCUUUUCUCCUGUUGGCUGGUGUCCAUUCCAUCAAAUAUGUCAUCAAGAUCUAACAUCUUUGAC
GUUGCAGACAUAUCCAUUGCUUCUCUAACGUCUAUGUCUUCGAUUAUUUUGCUAUCAUCCGGAUUUAUUCCCUCUAGCAUGCUCAGAAA
CUCAAAGAAGCCUGCAUCAUGACUCUUCUGUGAUUGGUUCUGUCUUUUAGCCUUUUCUUCCUUUAUUCUCCUCACGAUUUCCUUUGGUA
CGCUCAUUUCAGCUGUCACGAAAUAAUUUUCCAAGUCACCGUUUUCUAGCAUGACUUCAUAAGCUUUUUGGAAGUCACCCCUUGUUAGU
GUCACGUCAGUCAGAGAAGUAGUUGCUACUAGAGUUGAGCCAUGGUUGAAUGAGGACCUAUAAGUAUUCCCCUUGUUGAAGCGCAUUGU
GCAGGUUCCCUUGUCUUUCUUAGUCUGGAAGGAUGAAAUGUAGGGCAUUUUGCUAGUCCACGAUAUCUCUGAUAUCAGGCUCAGUGCU
GACACGUAUAUUUUCUCAAUUACCUUCUCAGUUUCAUUAUACUCUAGUCUGGAGACAUUUCCUCUGCUAGCUUGAAACGUUCUCACCAAU
UGCUCUAUCACAAAGUAUCUCUCUGCCAUCGUGUUGUUGGAAAUUUCGUCUGGAAGAGUAACGGAAAGUUUAGACGAAAAGUGCCUCAU
CACAUGUCGUACUAUUUCCUCUAAGGAUGAAUUUUUGUUCACUGUAGGUAAGGACUCAUAUGUGGACAAUAGCAAGGAAAUUGUGGACA
UAAGAUUUCUGUCUGAUUGCCUAUAAGUGACUUUCUUGUGACCAGGGCUCACUCUCUCUACCUUAUGCAUGAAGAACCCCUCAAUGCUA
GCUUUACCCUCGAUCAUCUUAGCGUCAAACGCUGUCGUCGAUCCUGAUCUGGAUAGUUCUUCGACAAGUGCCUCUUUCUCGACUUUCUU
UCUCGACAUUGGUGUAGUGGCUUCGUACCUCUCCGCAAUUUUCCUCUGUAGAUACACUGCUCUCAAGGGGCCAGCCAGUCUCUGUGCA
UUGCGAAGGUUAUCUGACAUCACCUUGAAUAUUCCUUUCGAUAUAGGAUCGCUGGACAUCUCAGAUAAUUCUAGAGCUAUGGAAUUCAU
UUUUUGGUAAGUCUCAGACUUCAAUACGGGUCUAUGCUGAAGAACAGCUUCUGGUAUCAGAUCCACUUUAGGCUUGAGUCCUCUUUUCA
GUAUCUUAUGUUGUAGGAUUGGCACUUUCCUCACGUCGAGUCCCCAUCUGAGACAUAGCAGGCACAGAUUCAUGUAGUAGACUAAAUAU
GCCUCACUAUCUGUUAAUCCAGAAUUGAUUCCAUCUGAGUAGGAUGACAUGACAGAUCGAUAAUCUAGUAUAACAUCGCCUGUGGUCUU
AGGGCUUAUGAGGGAAUUCGUGAACUUCAGUGAGUUCGAUCCAACUGACUCAGGAGCUAAGAAUAUUGAAUUGAAUUCCACAAGUUUCU
CUGAGGCCAUGUUCUUAUAAUCACUCGAUUUGAUGUUGAAAUAUCUGAAAAAGCAGCGAGUGGCUGAGAAUAGGAACUUCCCUAUUUUG
AUUUUAAUCUCACUUUUGAUCUCGUCCGAUAUUGGCACUGCUUCGUCAGAUGAAUUGCACGAUAUGAAGGUAGCUAAGUCAUCACUCGU
GACCAUAUGGUUGAUUUCUACAUCCAUACCUGGCUCCUUGAUGCAUUCGGUCAAUACACUAGACAUCACUUUAACUACCAUACUGUGAUA
CAAUGAGGAUGUGUAAUGGAAUAUUCCUUGCGCCAUAUGAUACUUACCUAAUCUUCCGCAUAGUGUAUAGGACUCGGUUGCUUCCUCUU
UCAACUCAGGGUAUGGUAAACUCUCAAAUAUUUUGAACUCCGACAUGAAGCAUGUUAACAUGGGAAUGAAGAAUUCUGUUGAAUGAUUUG
ACAAAUAAGUCAUCAUGUAUCCAAAUAGAGUUGUGUUGAAGUUCGGUCCCCAUCUGGUCUGAUCAACCGUUAGCAUCAUGCCGACUUCG
CUGCUCAAGGUUUUAACGUAAUGUUGGGUGAGUAUGACUUCUUUAUCUUUGUUGGCUAACAUGUCAAUACCAGUGUAUGAGCCUAGUUU
CUUGCUUAACUUCUCUGUUAUAGACUGUAGAAUUCUAAAUUGUGAGGACAAUAUGGAAAUCUCCCUGUCUCCUCCCACCUGAUCCUUGU
CGAACAUUCUCAUUGACAAAUCCACAAAGUUCGAGGCUUCAAGUAUUGAGAAUGCUAACUGCUUCACUGAAGGGCACUCAUGAUGUUGUA
CUAGAUUUCUCAUGGAUUCCAUGGCUUUGCAAGCUUUGACUUUCACGGGACAAUAAGAUGCCUUCAUUGUACUAAGACUGGACAGAGUU
GGUAGUGUUCCGUUGAAGGAUCGAACCGUAUCCAUGAGAUUGUCGAGGUGUGUAGACAUCAUGUCGAUGGAUAUUGGAGAGUAUGUGA

AUCUACCAUCGGUCUUGAUAGCUAGUCUGUCUAUCAUAUCAAAAUGUUCUCUAACUUUGUCGAUUGUUGGCUCACCGAGGGUCUCGACG
UAAUGAGAAUGUAGCUCCAGUACAUCCCUCUCAUGUUGGAUAGAUAGAUUUAUUUCAGAAUAUAAUUCCUUCAAGACGUUGACUCUGUGC
CUCCUCUUACCGUAUGUCUCGGGUGGACAGAGGUUAACCAGGAACGCUUCAUAACCUAUUUGAGAUUCUGGUAAGUUAAAUAAGGGUCC
UUUUCCGAGUGUUUGUGGUUGGUAAAGAUUGAAAAGAAGCCUCAAGGACGGUUUCAUGUACAUUGCUUUUACUUUCCCGCUAUCAUCCA
AGAGUUCUUUCUUUACUUUCUUAAACUGUUCAUAAAGAAGAGGAGAAUUGAAGAAAUGACCAGUGAUCAAGUACCUGAAUGGCUUGAGAA
GCUUGGAUACUCCCCAGGAAUUCUCGUAUAUUAGAGCUCCAUAUAGAUCAAUCAUGCUUUCUCUGUGCUUAUCUGAGCAAUGUUUGGCA
GCAGAAACUACACAUGAUAUGAAUUGAUUUUUUGCUACAAGAAAUCGGGCAAUAUCGGGUUGUCUCCACUUUUCAAACCUGACUAGCUUG
CCGUCCUGAGCGUAGGAUACGAAGAGCACACCUGAAUCUGUUGUUCUAAUUAGACCUCUGGCAUGCAUGACGAUCGAUAACCCUUCAAG
CUCAGCUCUUAAAACCACACACUGAUCGUCUCUGAGAUUCAUCGCGGCAUGGGAGAUCUUCUGCUUAAUUCUUAACUCAUGGUAAUAUU
UGCUUGUCUGGAUGAGUUUCUUGAGAUUCGGGAUGCCAGCGCUGCAAAUUGGUCUCACGUCCAGAGGAUCUAGUGGUAUUCCUUCCAC
UCUGUUGCUGACAUCGGUGGCCUUCUCCCUGUGCAAUUUCCUGAACGUGUACAUAUCGUAGUUGUAUUCGCGAACAAUGGGAAUCACUG
AAUCCAUAUCAUCUAGUUUGAGAACUCUCUUUAUUGAUGGUUUAAGCAGGUACUCUUUAUACUCAGGUCUUGCAUCAGAGUUUAUUAUU
GCCAGAACAUAAUCUCUAUCUUCCACCAGGGAUUUUAUCUCAGAGUUUAAUUCAUGAGGAGCUAACAAAGCUUUGGUAUUGGUCGUCCA
GUACAUGAUCCUCCCUUCUUGGUCAUGGUGAAUAGGGUCUAAGGUUAUACCAGUUUGAGUUUUGAUCUCAGAGCCAACGGUUAGAUAGU
UUCCUCGAUAAGAUGAUUCUGAGGUGCUCAUCUCAUAGAAAACAUGGGGCUUGGAUCUACUAACAACGUUGUACUUAUCUUUGAGAAAG
UGAUGGCUCCAAACUUCCAUGAGAUCUUCCCUUAUGUCUAAGACAGUUUCUGAGAUGGCUGAGAUGUUCUGAUCUACCUGUAUUGAUUU
UGACACACUAUCCUGGACUCUCUCCACGAGGACUUGUUGAGCUAGCUUCCCAAGAUUGCGAACCGAUUCUUUGUCCUUCAUAUGCUCUG
CUAUUCUUCUAUCAAAAGUUGAAUGAAAGCUAGCUUGCGAACCUUCGUUUCUAUUUAGGAAUCUCAUCUGAUUGUUGAUUUUGGAUUGA
AUGUAAGAAGUGUCGCAGAUAUCAGUCGCAUAGGAUAGGUUGAUCAUUGAUACGGUGCUAUCGUCCAUGACAUAGGGUAUCAUGUACUU
GUUGAUUGACACUAUUGGGCUGCAGGAGGAAUUCACCUUGUUCAAUAGCUUUUCAACCUUCCUUCGCGUAUUGGAAGUUGUGGUGUCAA
GCAGAAUCUCACACGUGUCAGAUUGCCUGAUUAUUGAGUCAAACCUAUGUCCAUGUCUGAUUUCCUCUAUCGAAAAGUUAAACCCGAGC
GACAUAGAGAAGGCUAUGGUGGCUGUCUGGUGUAGCGAGAGAGAAGCGCUGUUACUAGUAUUUUCAGAUGUUAUCUCAUUAUGAUUCG
CAGUUAGAGCCACAUCCCAGUUGUAGCUAGGAUCAGUCAUUCGUAUAGUACCUAUGUCAUGAGAGGAAUGUCCUAGUAACUCAUUGCGA
UCAGUUCUUAUCUCAAAUAGUCCUCUCACCUUGAUAAAUCUGAGAGUACUGAUUGGAAAUUUCCUGACACUGAUCUCAGGGUUUAGCAC
UGGUGACAUCGUUUCAAGCUUCGAUGUAGUCAGAUGAAUGGUUUCUCCUUGAAUGAAGUGCAUGAUCUGCUUCGAGCUAAGAUCGUCAU
UCGUAUAUAAUUUGAGCAUGUUCGCUAUUUCCUUGACUUUGGUUAGGCUGAAGAGCAUCGGUGUUUUAGAAACCCAACAGUACUUUCGC
AAAGCCACAAUGUUAUCUUUGAGAAUCUGUACAUUGGUGGGAUCCUCGAAAUCAUGGUACUGAAGAUGGUUGUAUGGUGUUAUCUGCAC
AUGGUACUCUGAGUCCUCAGUAAGAAUUUCCAUCUUCCCCUUGAUCCACGAGAAGGGUAUAGCUCUUUUGGAGUCAAGUAUUACUGGUU
UGUAGGGAGCAUGACUUUUGAUAAAUCUCCUCAGAGCUCCAGUCAGCUUUUGAGAUUUUGAUUUAUCUGAUUGCAUGCUUACCGCNNNN
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNUAAAAAUUGAAAAACGCAACAUUCUGAUCUCAUUCAGAC
AUAGUCGAAGUGCCUAUAUCUGUUGUCAGACAUAGAUCUCUAAGACUAGAAGCUGAGAUUCAGAAUUACGAUUCCAAUACUAUAUGGAUA
GGCUUUCUUGUGGAAAAACUAAGUUUUUCAGACGGAACUAAAAUGUCACAUGACUAAACACCAUGGUCUAGCAAUCAGUCUCUUUUGCCC
UGAAGUCCAAUUUAAGGAGAUUGUGGUGGAGUUUGCAGGUUCUGAUCAUCAUUUCCAUCGUCAUCGUUUGAAGAUUGCUGGUUGUCGU
CUUGGUCAUCAUCAUCUGAGGCAUCAUUGGUGGAAAGAAUAUAUUGGUAGAAUUUAUUGUUAAUUUCCCAUCGUUUGGUUCCGAAGUAG
UCGAUAUAAUUACGAUCUCUGCGACUAAGGCUGGUAAUGAAAUUAUCCUUCUGAAGAGGAUUGAGAUCCCGAUAUUGACUCACCAUGUU
ACGGAUUCUAGUUUUCUCGUUAUCAUCCAUCUUCCUGUAUUGAUGAACUAAUGCCAUAUUCUUCACCGAUCGAAAGAAUCCAUCCUCUCU
CCUAAUGAUGAGAUUUGCAUUGACCGCAUUGAUUUCUCGAUCUACAACUGCCUUAAACGAAUCAUAGUCAGUUGCAUCACGAUCGAAGAA
UCGUUCAACAUCAACUUGGGGGUCUUCCUUGAGCAUGAAUCCAUCUAAUGUUGUACCAGUUAAGUGCUCAUCUGUGGGAACCAAGCUCU
CGAUUUGGGCUAUAAGAUCUUCUACGUUGAGAUUGAAUGCCAUAAGCGAAUUGGAUUGGGUAGAUCGGAAGAGCACACGUCUGAACUCC
AGUCACGAGA

HiIfV: *Hermetia illucens* iflavirus (reversed)

UAACCUUAAAGAUGAAAAGAGACCAGUUGAAAAACUUCUCCAAUUAGGAGGUACUCGAGUCUUUUGUAAUGGACCGCUACAUAAUGUAUU
GUUAGUACGUAAGUAUUUUUUGCAUUUUAUAGCAGCAUUUAUGAAAAAUAGACACCAAUUGCUCCACGCUGUUGGAGUUAACGCUUUAAG
CGAUGAGUGGUCACGCAUUGCAAAUUCACUACUAAAACAAAGUACCAACGCAUCAACACUUGAUUACCAUAAUUUUGGAGCCGGUUUUUC
GGCCUUAGUAGCACAAAAAGCUUAUGAGUUAAUUUUAAGAUGGACAAAACAACACGUUAAGGAAUUAGAUGGCACUGAUCUGGAUAUGUG
CAUACUUACAGCUUUGACACAAGAUUGUCUAAACUCAACACAUAUUGUGAACAACACAGUUUACAUGCAAGGAUGUGGUUCCCCUAGUGG
AUCGGUUUUUACAACUACUAUUAAUACAAUGGUCAAUAUAUUAUAUUUGUUUUUGUCAGUAAAACACUAUUAUAAACCUAACCCUAACGAU
UAUAUGCACCCAGUAACUUUUAUUAAGCGCAACUUGUCAAUUUAUGCUUAUGGAGAUGAUCUGAUAUUUUCUGUAGCACCUGAAUAUGCU
GAAUUGUUAAACUCAAUUACAAUAUCCAAAUAUUUGGCAGAAUAUAAUAUUGUUGCAACAGAUGCUAUUAAAACUGCAGAAGUCACACCAU
UUAGACCUCUCUUAGAAAGCACCUUCCUUAAAAGGAGGUUUUAUCCCCAUCCCAAAAGAAGUGGACUAUUUCUUAGUCCUUUAGAAGAGU
AUAGUAUAAAAUCUUGUACUCAGUGGGUGUGGAAAUCUUCUAAUAAAUCAUUAGCUACUAGAGUUAAUGCUGCAGCUGCCUUACUGAAUG
CACAUGGUUGGGGACCAAAAUAUUACGCCAAUUUUAAACAUUUAUUGAAUAACGCUUUGAAUAAAAAACACAUCGAGCCACUAGCUUUGC
AGUGGUCUGAAAUUGAUGAACAGUUUUUUAGUACAGGGCUAGAUAGUAUGUAUGAUGAUAUUAUUAAUGAUAUAUAAUACUUUAUGCACA
UGUAUUGUUUUAUUAGAUUAGUCUACCUUGCAUGAUGAAUCAAUA

HiInV: *Hermetia illucens* insevirus

GACGCGACAUUUACGAUAUACUUACUGACACAGUUCACCGCAGUGAUAUGGAUAGCAACGACAAAAAGCAACCAGAAAACAGCGAAAAAG
AACAAACAGAACAGACAGACGGACCAAAAUGGACGAACAUCGACAAUAUUUACUACAAUCAAGUAGUGAAGAAGACAUUUUCCGAAUGGG
UGAUAGACUACCCAUUCGACGUAGGACAGACUCUAACCGGAAUUCAGAGUUUACCCUGUGGGAUAGACACCGCAGGACAGCCAGGAUGU
ACCUCCCAAACAUAUACAUCAUUCAACUGCUUGUCUCGAUCAUACUUAUGCUCAUUUGCUACGUAUACCGACCCUGGAGCAAAGAAACAA
CGCGCCCUACUGCCAGCGAGGAAGAUAUCUAUGGUAGACAAAGAGUACAAGACCAAAGACCCCAAGUUGCGCAAAAUAAUAAAAGAGGGU
ACGCGAAUCCCAGUAAUAGGGAUGGGCCUGGAGAGCCUCCCCCGCUUGAUAAAUCAGGGGAUGGUUUCACCGGAGUCUACAUCAUCUAA
UCUGUUACCAACGCCCAAAUACUUCGAUAUGUUGGCUAGUACAUACACGCCUCGAUACUUUGAAAUCCUAUCCUCAUAUAUCCACUCCAC
CAUUGGAGGGUCAAGGGUGAAAAUAAUGGAAGAGUUGAGGGAUGCCAGCAUAAAGCAUGAUGUUUACAAUACGUACAAAAGCAUGCUAU
CUGGAGACGGAGCAGAGACAUCUACUCAAACUCCAGCGCAACCAGAUGAACCACCAACCUCCACUCCGGAAAUAAUUACGCCCACAGACA
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CAAUUUGAGAAGCCAGCACUGUCUCCAAUGUACAUACAAUUCGCCAACAGACUCAAGGAUACAACCCAGACAGGUACAUUAGGAAGUAUA
GUCAAGGGGGCGAUGUUAUUAGACGGUACGUCUAUAAUGUCCUUGGUUGAUACACUGGAAAAGACUUCGGGAUCCACUAGAGAGGUGU
CAGCGCAUCUAAGGCUCAUGUGCAUGGCCAUGAGCCUGGACGAACAGUCUCACCUAAACGAUCCAGUAGAUUUUCAAUUUGCUCGUAGG
UACAGCAGGUUGAGCAAAUGGGACAUGCCAAUGAAUGAGCAAAUCCGGGAUUUCACCAUCAUAACAUCAACCCCUUCCUUCCCUAAGGCA
AACAUAUCAGCCAUAACUGUUGACAAAUUUCUAGCAUGGCAAAAAGGGGUCUGUGAACUAACAGAUGUCACUUGGCAACGACAGGGAAUA

Appendix 1

GAUAAAGACUGGUCGGUAGUACCCGUUAGGUCAGCAUAUUUGUCAAACCGCUCUAUAAUCCCAUACAUUUUUUCUUUUGUCCACAGUCA
UUGGUGGGCUGGUACCGUGAACCAUUUUACUUAUGGUGAAUAUAGCAACCUGAAAAACUUUCCACAAAAGACAUCUGUACAUACUUUGAU
GCCUUCAGAAAAUUCGGUAGUUAUUCCGGCCCCCGACAGCGUUUUGCUGGUUAUUACUGACAUGUCCUCCACCAGUCGGACACCAGAAG
GAGUGGUGUUGAUGGGUGUAAAUGUGCCAUGGGCGGAUGCGCCACUGGAGGAAAACGACCAAGGUAUAAUUCAGCCAAAUACGCCCACC
CCUAUGGAUAUGCUGUGGUCUAGAUGUUGGAAAACAUCUGAGAUACCAAAUUUGAAACAUAGUUUGGUCGGGGCAUUAAGAGAACUUAC
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AAACAGCGAUCAUGCAAUCAACGUACAUACAGCUCUUUGGUGACAUGAUUCAGUAUGUUAAGGUCAAGGAUAUGGUGGACUCUUGGACC
GAGUGGGAUGAGUCUGUGAUUAUGGACUAUUGGGGUAUGGAUCCGUUUACAGAUAUAGAUUGGCUCAGUUAUUCACCAGUACCAACCCA
UCUAAGUCUACAAUGGAUAUUUAAAUUGGACCUAUCCAACAUGGACCACGAGUAUACAAACAGGAUGUACAAAUAUUCUUACGUAUACGA
GGACAUAGAUGACGUCAGCCAUACAGUUAGAAUGGACCUGAUGGGGCUUGAUUUAGACAACAAAGCAGACCUGUCUAAAAUUCUUGCAA
UUACUACCAUCAAUUUUGAGCAAUACGCCCCAAUCGUGGUGAAUGUGUACGGACCUAGAGGACCGCAUACCUAUGGGAUGUGGCUAGAU
CAAUGGGCAUAUAUAUCCAAUUCAGCGAUGGGUAAGGGACAUGUACAGCCCAGCCAACAGUAUUUCGAAUCUUUGACACCUACUAUAUCC
CCGGUUAAUACAGACAUAGCCUACAUGGAGGAGGAUAGAGUCGUGAUACUGAAUUCCAGAGCAGAGAAGAUUAACACCAAACUUCUAAAA
AUAAACAUAUCCGACAUACGCUGGCCUGACCCACCUAAGGUUAGGAAUAACAUAACAUCAUCGAUGGCAACCGCAACACCAGCUGAAGCG
UUGCUGUCACAAGGACAGAAACUGCCAGAAUCUACAGAAAGCAAAACAGAAGCCAAGGCAGAUUCGGGGGAACAGCAGCCCCCAACAGAU
ACAGCCUGAACAAUAACAGCAGGUUGGGCACUCUCACGCGCAAAAUAAAGAACAGAGUGCCCGCAACAGGGAUGAGCGCGCCUUACAAU
GGAUCGUGUCCGGUUUAUCCUGCUAUGUUAGACACUCAUGUUGUAAGUGGGUUUUCAGAACCCACCGAGUUGAGACCAAAGUCUGAUAA
GCUGGAAAAUUUGAUGCGAUUUAGAUGUAGGAUCCAAAGAAUUGGGUCAUCCCCAAGAGAGUUAGCUGUUGUCGAUCAUAUAUAU
AUAUCUCUUGUCUCUAUUCUUGUGGGCAGAAGAACGUAGCCCGAAACCUAACAUGGUACCUGGGUACUAUCAAAAAUAGGUUCGUUCGU
UUUCUAAAAAAGAAUAAGAGGUAUUCCUCAGAACACGAAGAUGACUAUUAUCGCCACAUGCUCAAGAAAUUAAGCGCAUGGGCCUUUCCA
CAAAUAUUUGAAGGAGAGGGUAAAGCUGUAUACAUGUUAUACUGCCAUUUACACCACCUAACAGGCCGAGCGACAUUUACUAAAGAGCAG
AUACACUCUGACAUAUCAACAUGGGUCGGGGAUAAGAACACAGAAAACGCGAGCACAAAGAAGAGAAUAGAUAUGAAACAUUUCUCAUCG
UAUAUGGACAGAGUUUUCUCUGAAUGGUAUAGAGGUGAAUUUACAGGGCACCUCAAUUUCAGAGAAUUCGCUAAUGAUUAUCUUAGAUG
GGGCACAUCGGGCGGGGCUCCUAAAGUCGAAUUGUUUCAAGGAGGCACUUACAGAACUAAGUGGGCUUGGGCUCUUGCCAAUCCCACC
AACGAAGAUGGUUCCCUUAAAGAAAACUACGACUUAUGGAGACAAGCGAAAACCGAUUCCAAUCAGUAUGCUAGCGUUGCACUAAAGGAG
GAGUCUGCCAAAACGAGAGAAGUAAUCACCACACCUAUGACAUCUUACAUUAGACAAAGCUACCUACUGUAUCGUUGGGGGAAAAUUAAU
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CGACUGGAGUGUACCCAAACAGGCUGUUAUUGAAUUUCUAGAGAGAUUAGGCAGUCUUGACGCAGAAUGCCGCUUGGUCGCUGACGAG
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GACUGGGAGUCCUGGUUGUGCACCCCUCAAUCAGCUGGAGGAGGGGGAUGUAGAGAGUGGAUGAACCCGAACAAGAGAUGGGUAACAC
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CGACCUUUAAGCAUGAAGCGAACAUAACAGAAACUAUUUUCAGCUUGAUGUAUGGGUCAAUUUCCAUCAAUACAUUGAACAGCAGGCUAA
CCAGGAAGAUUCCAUCAUAUAUGAGACUUUCCAGUAGAACGCGAUUAAUUGAUUUCUUGCUUUCUGGGUCCAAGAAAUCCACGUUCUGC
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UGGGUCAUACACUAGACUUGACUGUCAUUCACAGGUUGGGCAGUUAUGCGGUGUAUGAGGACAGAAAACGUCCCUGUGACAGAAACUUG
UACGCUUAGUAGGUAACGUGGCAACGUGCAAGUUUGGACUUCUUGUAAGUAAGCCACACCGUAUUUAUCAGCGAAUGCCCCAGAAGCCC
UACGGGGGGUGUGGGGUGUCCCGC

HiLbV: *Hermetia illucens* lebotivirus

GUCCAAGUGAUUUGAAAACCGACUCGCUAGUUCAGCACCGAUAACCUUCGUGGGGAACGUGCCGUAACGUAGCGAGAUGAAGCGUAAAA
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HiSgV: *Hermetia illucens* sigmavirus (reversed)

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Appendix 1

CAUCAAUCAUCCGAAAAAAUCUCAAGCGAUAUUCGACUCCUCUAGUAGUGUUAAGCAGAAUUAUAAGAUAGAUUUAACCUUUAUAGAUUC
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CAAAUUAUGCAAAUUUAUUAGCGAGUGAUUUGGCUUUUAUAGUUAUUAAAGAUCAAUUUAAGACUAAAAAAUUAUGGCCGGUCGAUCCCA
AAAAACUAUCAUCAGACCACAUGUUGUACAAAUAUAUAGUCAAAGGAACAUGGCCCAACAAUAGUGUAAUCGAAGCCUUUGGUCCUCACU
GGCAUGAGCUUCCGUUAAUAAAAUGUUUUGACAUUCCCGAUGUCAUCGACCCUUCACUGAUUUAUUCUGAUAAAUGUCAUUCUUUAAGUA
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UUAUGGGACAGUUUCUGGGUUAUCCUAAUCUAAUUACAAGAACACAUGAAAUUUUUGAAAAAUCAUUAAUCUAUUAUGCAGGGAGAGGAG
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GUUUCUUUAUAAUUUCUAUGGACACUUGGUUAGAAUAAUCAAUGAGAUUCAUAACCCGCACUAAGAGGACCUGUCAAGAUAUAUUGAA
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UGUCAGAAGAAGUCCAAUGUGGUCAUGUUCUGCUUCUCAUGCAGAUCAAUUAAGAAGACAAUCUUGGGGUGAAAAGGUCUAUGGAGCCA
CUGUUCCGCAUCCCGCUGAAAUGUUUGGAACACCGGAAGAAUCCAAAGUUCGAUGUUCUAACUGUAAUCAGAGUUUUCCACAUUCAUUA
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GAAUGUUAGCUACAACAAACAACUUAGCUGAUUUGGGUGAUACAAAUUAUGAUUUUAUGUAUCAAGCCUGUUUGUUAAAUGCCUUGCUGU
CAACCGGCGAAAUUCAUAAUGAAAAUAAAGGUCAAGGAUAUUAUCACCAACAUCUCAAAUGCAAAGAUUGUUUAAGACCGAUUGAGGAAAU

CUACAUCAACACAUCAUUGCCCUAUAAACAUCCAGACGUUUCAAAUGAGCUCAAACACUGGAAACCUGAAGAAACUCCUUGGUCUGUAUU
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AGUUUCCUUCCAUAAAAUCAAUCAAUAAAAUGUGUGAAUUCUUUAAUAGAGGAGUUACAUAUAAAUCAUUUGAUAGCGGAACUGGACUUA
UAUCCCUAUUACGAACUGAGGAAAACUUGUCAACUAGUGGCUCUGUUAAGAAAAUUUCCUUGUUAAUAGAGGGGAAAGAGAAAACAAUAG
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UCAAUUUUCAUUUGUUUGAGAAUUUUAAUAUUAUAUCAGGAUUAAUUUAAUUUAUGUUUUUGAAAUAAACUAAGUUAUUGUAUAAUUUGU
AUUAAUGUAGUUUCUGG

HiSvV: *Hermetia illucens* solinvivirus (reversed)

UAUAAACUACGUAAUUGUUAAAACGGAUAAAUGUAUACAAUUAUGCAGCCUAUAAACGAAAAGACCUUAGCCAAGGCAAAGCGAAGUGGA
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GACCCAAAAGAGUGUGACAAGCGCUUGCUUUCAUCAAUUUAUUUAACAGCAAAUACCUUUAGGUUGGAAGUGGAACGUACUCUGCAAGC
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CCCGAUACAUUGUUGUCACGUCUUGCUGAACUUUCUGACCAAAGCAGCAUCGAACCUACAUGCUCUAGAAUAACAUGGUUUAAGUGUGU
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GUUCCUUACAACUUGAGAGUCAGUAUACAUUGGACUUGAAUGAGCCAGGUGUCUUAAGAAGAAUGGGAUUUACAGGAAAUUUCUUAGAA
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AACCUAGCUAUAAACCUGCUUGCAUCCCUGUUUUCAAUUUACAUUUGUGUACCAUUCGUCCUUGUUAGUAUUGUGUAUAUAUAUUAUCAA
UUAUACUAUACUUUUGAGGAGAAAUAUGAUAUCACCAAAUGUAAGGAAUACAUCUUUAAGAAAUAUCAGGACUAUUUAAAAUUCUACAAUG
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GUUUAUCAAAGUAACUUUGGAGGCGAUGCUCGGUUCAGUGACUUUACAGUUCAAGAAACAAUUAGUGGAUUCGAUUGCUCCUAUAAUUU
CGAUGGAAAACCAGUUUCUCGUUCAUUCUCAUAUGCAAACAUAUCCAACUAUCUUUUCCGUGGUGAACCACUAUUGGACUUAACGAAUAU
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GAUUUUAGUUGGAAUUGUUGCAAUUUUGUUGAGUGUCUUAACUGUCUACUCUCUUAAGAAAAUCUUCACCGAAAGCUCAGAUUCAGAAG
AUGACAAGUGUGAUUAUCAAAAGAAACCACGAAUGAAAGGAAGAAGGCGAGAAGACUCUGGCGAAGAAGGAAGAGUAAACAGAUUGAGAG

Appendix 1

GACCAAGAGUCAAAGGGAAACGACGAGAAGUAUCAGAUGACGAUGAGCGUAGAAACAAAAGACGACAGCAAUAUUCUAGUGAUGAAAGCG
AGGAUGAUACUCUUGAACAACACAAACCUCGUGUAAAAGGUCGUCGACGCGACAUAUCAGAUGAUGAACAAAAAUAUGACAAACAAGUCC
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AAACAAUAGAACAAAGAAAUGAAUUUUUAUCUGGACAAGCAAGAUGUUAUGGAUUGUUUGUUAGAGGCAGAACUCUUGUCACUGUAAAUC
ACGCUGCGGACUUACCUAUUUACGUUGCUUGUUCAGAAUUUGGAACAAACGUAUACAGGGCUACUAUUAUCAAGAGAUUCACUAAAGAAU
GUCGUGAUUUUUGCAUAUUAACUCUAGACAAAAAUCAGUACAAGAACAACAUCCAGAACUUCUCAGACAUAACUAAAAAUUUCCAGCGAAA
ACGGAACUUAGAUGACGAACAUGUUGAGUGCAUUUUAAAAAGGUAUAGUGAUAGUGUAUCAAAGAAAAAUCACGAACAAUAUCUCCAUGG
CGUUAUGUAUUAUUAUCAUGACGAAUUGAGCAGUUACAUGGAUAAUGGAGAAGCUUUCUCUAUAUCAAAUUUUGGAUAUGUUAGUUUCU
CCAGUUCAAAACUAGCAUUGACAACAUAUGGAGAUUGCGGGUUACCGUACUAUUGCUAUAACUUAAAUGAAAAUGGCACUCCAACAAUUU
AUCUAGGGGGUAUUCAUUGUAUGGGAAAUGUGAGUAGAACAUAUUCAAUUGGUAUUGCUGCGCUUAUUUACCAAGAAGAUUUUGCUUGU
AUGCACCAACAAUCUGAGAAAUGUGUAUGGUGUACAGAUAACAUCAGCAUAAAAGAUCCGUUAUCUGAGAAGAAACAAUGUGAGGGACAC
AAGAUCGUUUGGAAUAAUGAACAUUUCACCAGUCUUCUCUCUUAUCUCCGUGAACUAAAGUACUAUUUCGUACAAUAUCCAAAAUUCUAU
GGAUCCAUACGUAAAAGUUCAGGAAGUUCUUCUCAAGGUAGUGUUGAGCAUUCACACACCCACUUUAUGCCCGAACUAGAGACAAAAUUC
UCUUAUCCCCAAGAUUUUAAAUGGCGUGAACAUGAGCACUCAAAGUAUGGUAAAUGUUUAACUCUCAUUUUUAAGAGUCAAAAUGUUCGC
UCUUUGGAAAAGAUCUUAUCUAAACUGGAUUUUGUUGAUGAAUUUGUCAUCUAUUCGCAAUACUUUAAAGAACAACAGACGAGUAAUAAG
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AUCAACGACAAAUAGACCCUGAAUUGGAUGAAUAUGUUUACGAAGGUCUACGAGACUUUGAGAUUCAAAUUUCACAAUCCAUUUUACCGG
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CUUAAACAACGUGUGAUGAAAAACUAUCAAAAACAUAUCAACACUUCUUAUGCAGAUUGUAGAGAACUUACUGAUCAUGAAGUAUUCUACG
GACUAUCAGGACCACUAGCCAAUGGAAUGAAUGGAAUAGAUGUGAAUAAAUCUGUUGGAUUUACUCUUUCUAAUCUGUUUUAUGUCACCA
CAAAGAGUGAUGUAUUAGAAUUAUCAGAAACUGGCAAGUAUAAGUGGGCUGAUAAUGAAGCUAGCGAGUUUCUCCAAGAUUGGUAUAAAA
AUUCCAAGAAAGCAAUUAGAGAAGGUAAAACUGUCAGGACUUACUAUUUGGACUUAGUAAAAAUGGAAAAACUAAAACGAGAAAAGAAUUU
CCUUGGACGAAUAUUUUGUAAUUGUGAUUUUAAUGAAUUGCUUUUGCAACGUAAAUUUUUGGGAAUGUUCCUAGCAAAAUCAACUGUUU
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GAUUAAACCAAAAUUACAUUUGUGCCUCGCCUAUUGCUAUAGAGGACACACCACGUAACAUAAGUACGUUACCAUUGGAUAAGUACCUUG
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CAGGGAAUUUUGAUCCAAAGUUUCUUGCAUCCAACAUUUGUAAUUACUCAACAGUGCACUACAAUGUGGAUGACAGUACAGUAUACGUAG
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HiCV: *Hermitia illucens* cripavirus (reversed)

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Appendix 1

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Table S3.1 Statistical analyses models inferred from survival of BSF over 30 days when infected with HiSvV. A) Mock infection (PBS) survival between colony CBP and colony IRBI (n = 60). B) Adults infected with a $1.6x10^4$ genome equivalents/µl concentration of HiSvV PPV between colony CBP and IRBI (n = 30). C) Adults infected using HiSvV PPV with a concentration of $1.6x10^1$ genome equivalents/µl between colony CBP and IRBI (n = 30). D) The comparison between treatments separated by colony, sex and experiment. E) Adult males and females from colony CBP and IRBI infected with concentrations $1.6x10^4$ and $1.6x10^1$ genome equivalents/µl of HiSvV PPV between dose concentrations (n=30).

A

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Sex	**130.69**	**1**	**2.90e-30**	***
Experiment	0.07	1	7.96e-01	
Colony	**26.69**	**1**	**2.39e-07**	***
Sex:Experiment	**4.61**	**1**	**3.17e-02**	*
Sex:Colony	1.51	1	2.19e-01	
Experiment:Colony	**5.95**	**1**	**1.47e-02**	*
Sex:Experiment:Colony	**16.72**	**1**	**4.34e-05**	***

Hazard ratio of coxph model:	**Surv(death, status) ~ sex + experiment + colony**					
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
Females	**1.97**	**7.15**	**0.17**	**11.31**	**< 2e-16**	***
Experiment 1	0.01	1.01	0.13	0.07	9.45e-01	
Colony IRBI	**0.77**	**2.16**	**0.14**	**5.58**	**2.39e-08**	***

B

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Sex	**44.28**	**1**	**2.85e-11**	***
Colony	**5.98**	**1**	**1.45e-02**	*
Sex:Colony	1.94	1	1.64e-01	

Hazard ratio of coxph model:	**Surv(death, status) ~ sex + colony**					
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
Females	**1.59**	**4.89**	**0.25**	**6.26**	**3.96e-10**	***
Colony IRBI	**0.46**	**1.58**	**0.19**	**2.46**	**1.40e-02**	*

C

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Sex	**53.03**	**1**	**3.28e-13**	***
Colony	**12.44**	**1**	**4.19e-04**	***
Sex:Colony	**6.67**	**1**	**9.83e-03**	**

Hazard ratio of coxph model:	**Surv(death, status) ~ sex + colony**					
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
Females	**1.68**	**5.38**	**0.24**	**7.02**	**2.21e-12**	***
Colony IRBI	**0.69**	**2.00**	**0.20**	**3.52**	**4.37e-04**	***

D

Hazard ratio of coxph model:		**Surv(death, status) ~ Treatment**						
Colony	**Sex**	**Dose (GE/µl)**	**Coef.**	**HR**	**SE(coef.)**	**z**	***p*-value**	
CBP	**Males**	**$1.6x10^4$**	**1.26**	**3.52**	**0.31**	**4.06**	**5.02e-05**	***
		$1.6x10^1$	**1.43**	**4.19**	**0.31**	**4.57**	**4.96e-06**	***
	Females	**$1.6x10^4$**	**1.59**	**4.89**	**0.32**	**4.96**	**6.89e-07**	***
		$1.6x10^1$	**1.25**	**3.48**	**0.28**	**4.38**	**1.19e-05**	***
IRBI	**Males**	**$1.6x10^4$**	**1.47**	**4.33**	**0.33**	**4.39**	**1.12e-05**	***
		$1.6x10^1$	**1.11**	**3.04**	**0.32**	**3.51**	**4,56E-04**	***
	Females	$1.6x10^4$	0.44	1.55	0.27	1.63	1,03E-01	
		$1.6x10^1$	**0.71**	**2.03**	**0.27**	**2.6**	**9,32E-03**	**

E

Hazard ratio of coxph model:		**Surv(death, status) ~ concentration**						
Colony	Dose (GE/µl)	Sex	**Coef.**	**HR**	**SE(coef.)**	**z**	***p*-value**	
CBP	**$1.6x10^4$**	**Males**	**0.78**	**2.19**	**0.30**	**2.64**	**8.28e-03**	**
		Females	**-0.91**	**0.40**	**0.27**	**-3.44**	**5.92e-04**	***
IRBI	$1.6x10^4$	Males	-0.36	0.70	0.27	-1.36	1.73e-01	
		Females	-0.42	0.66	0.26	-1.60	1.10e-01	

Table S3.2 Hazard ratios and deviance-based analysis of HiSvV infection in adult BSF. Colony CBP (A and C) and colony IRBI (B and D) when infected with two concentrations of HiSvV PPV: $1.6x10^4$ genome equivalents/µl (A and B) and $1.6x10^1$ genome equivalents/µl (C and D).

A

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Treat	**42.73**	**1**	**6.28e-11**	***
Sex	**58.27**	**1**	**2.28e-14**	***
Treat:Sex	1.82	1	1.77e-01	

Hazard ratio of coxph model: Surv(death, status) ~ treatment + sex						
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
HiSvV	**1.42**	**4.14**	**0.22**	**6.44**	**1.19e-10**	***
Females	**1.71**	**5.52**	**0.23**	**7.59**	**3.30e-14**	***

B

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Treat	**17.01**	**1**	**3.71e-05**	***
Sex	**47.33**	**1**	**6.00e-12**	***
Treat:Sex	**7.74**	**1**	**5.41e-03**	**

Hazard ratio of coxph model: Surv(death, status) ~ treatment + sex						
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
HiSvV	**0.83**	**2.30**	**0.20**	**4.09**	**4.35e-05**	***
Females	**1.62**	**5.06**	**0.25**	**6.51**	**7.71e-11**	***

C

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Treat	**41.78**	**1**	**1.02e-10**	***
Sex	**99.33**	**1**	**2.14e-23**	***
Treat:Sex	0.03	1	8.59e-01	

Hazard ratio of coxph model: Surv(death, status) ~ treatment + sex						
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
HiSvV	**1.36**	**3.90**	**0.21**	**6.43**	**1.25e-10**	***
Females	**2.56**	**12.90**	**0.27**	**9.52**	**< 2e-16**	***

D

Analysis of Deviance Table (Type II tests)				
LR	**Chi²(χ^2)**	**Df**	***p*-value**	
Treat	**17.82**	**1**	**2.43e-05**	***
Sex	**35.36**	**1**	**2.74e-09**	***
Treat:Sex	1.73	1	1.88e-01	

Hazard ratio of coxph model: Surv(death, status) ~ treatment + sex						
	Coef.	**HR**	**SE(coef.)**	**z**	***p*-value**	
HiSvV	**0.85**	**2.34**	**0.20**	**4.20**	**2.70e-05**	***
Females	**1.29**	**3.64**	**0.22**	**5.85**	**4.98e-09**	***

Table S3.3 Description of genes with a significant log2 fold-change (L2FC) and adjusted *p*-values (pAdj) contrasting between HiSvV- and Mock (PBS)-infected BSF adults. Genes coloured in red were up-regulated and genes coloured in blue were down-regulated according to the mean.

Cluster	GeneID (LOC#)	Description	L2FC	pAdj	Rank
I	119650043	Ras-related protein Rab-43	-0,58	2,80E-02	92
I	119651397	Tribbles homolog 2-like	-0,40	2,72E-02	86
I	119648825	Digestive cysteine proteinase 1-like	-0,98	1,12E-02	104
I	119648192	PXMP2/4 family protein 3	-0,72	3,32E-02	96
I	119654891	Extracellular superoxide dismutase [Cu-Zn]	-0,91	6,68E-03	102
I	119659877	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	-1,11	3,38E-02	107
I	119657102	Peptidoglycan-recognition protein 2-like	-1,38	9,61E-03	122
I	119657830	Cecropin-like peptide 1	-2,45	3,92E-02	128
I	119647832	Hydroxyacylglutathione hydrolase, mitochondrial	-0,50	3,36E-02	89
I	119659474	Hydroxysteroid dehydrogenase-like protein 2	-0,53	3,44E-02	90
I	119647248	Glycogen phosphorylase	-1,56	7,34E-04	125
I	119654901	Peroxisome assembly factor 2	-0,83	4,11E-02	99
I	119654977	Death-associated inhibitor of apoptosis 2	-0,57	4,37E-02	91
I	119649642	Gram-negative bacteria-binding protein 3-like	-0,84	4,37E-02	100
I	119650287	Aspartate aminotransferase, cytoplasmic	-0,41	3,90E-02	87
I	119658003	Neuroglian	-0,46	4,20E-03	88
I	119659632	Beta-1,3-glucan-binding protein-like	-1,86	7,64E-04	127
I	119661266	(Lyso)-N-acylphosphatidylethanolamine lipase	-0,66	1,39E-02	94
I	119647270	D-aspartate oxidase	-0,73	1,76E-02	97
I	119650135	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	-1,21	4,66E-03	115
I	119655170	Beta-1,3-glucan-binding protein-like	-1,42	4,68E-02	123
I	119656730	Transferrin	-1,17	4,71E-02	112
I	119648026	Tyrosine 3-monooxygenase	-1,29	3,98E-03	119
I	119652475	Annulin	-1,15	3,39E-02	111
I	119661275	Probable peroxisomal acyl-coenzyme A oxidase 1	-1,31	5,85E-03	120
I	119651539	Indole-3-acetaldehyde oxidase-like	-1,23	3,87E-04	116
I	119649890	Peroxiredoxin-6-like	-1,14	6,63E-03	110
I	119650936	Phosphatidylethanolamine-binding protein homolog F40A3.3-like	-1,19	1,05E-02	114
I	119649948	Prostatic acid phosphatase	-0,65	3,69E-02	93
I	119650467	FH1/FH2 domain-containing protein 3	-0,91	4,88E-02	103
I	119656886	Hemocyte protein-glutamine gamma-glutamyltransferase-like	-1,12	4,49E-03	108
I	119648725	Beta-1,3-glucan-binding protein-like	-1,32	2,41E-02	121
I	119655509	Beta-1,3-glucan-binding protein-like	-1,74	1,17E-02	126
I	119660508	Acyl-CoA synthetase short-chain family member 3, mitochondrial	-1,18	1,89E-02	113
I	119648611	Peroxisomal multifunctional enzyme type 2	-0,81	4,22E-02	98
I	119646430	Uricase	-1,24	3,59E-02	117
I	119649978	Catalase	-1,08	4,38E-03	106
I	119646980	Putative fatty acyl-CoA reductase CG5065	-1,07	1,76E-02	105
I	119657808	Protein spaetzle-like	-0,85	2,22E-02	101
I	119647193	Malate dehydrogenase, cytoplasmic	-1,27	6,86E-03	118
I	119647814	UTP--glucose-1-phosphate uridylyltransferase	-0,72	3,40E-02	95
I	119657036	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	-1,12	5,88E-03	109
I	119657963	NADH-quinone oxidoreductase subunit B 2	-1,43	1,58E-03	124
II	119661333	F-actin-capping protein subunit beta	0,71	4,02E-03	74
II	119656546	Ras-related protein Ral-a	0,94	2,59E-04	65
II	119656668	Integrin beta-PS	0,74	1,40E-02	71
II	119658136	NF-kappa-B essential modulator	0,84	1,17E-02	68
II	119654625	Lysozyme-like	2,55	4,74E-05	18
II	119661335	23 kDa integral membrane protein-like	0,99	1,76E-02	63
II	119661779	Integrin beta-nu	1,82	5,66E-06	33
II	119655700	Uncharacterized LOC119655700	1,41	4,40E-03	42
II	119654410	Lysozyme-like	4,85	1,68E-20	4
II	119659057	Uncharacterized LOC119659057	1,92	3,34E-07	28
II	119660505	Uncharacterized LOC119660505	4,13	3,34E-18	6
II	119654476	Rho-related protein racB-like	2,30	1,41E-06	24
II	119653883	Endoribonuclease Dicer	1,21	1,71E-05	52
II	119658783	Ras-like GTP-binding protein RhoL	1,19	3,30E-05	54
II	119651771	Adenosine deaminase 2-like	2,50	8,26E-03	19
II	119657249	Attacin-B-like	5,02	3,99E-14	3
II	119649969	Platelet-derived growth factor receptor alpha	0,51	1,71E-02	84
II	119648034	Hybrid signal transduction histidine kinase A	1,29	1,86E-04	48
II	119661747	Myosin heavy chain, non-muscle	1,05	1,03E-04	60
II	119647439	Heat shock protein 83	0,55	1,10E-02	83
II	119648694	DE-cadherin	1,01	3,30E-05	62
II	119648931	Baculoviral IAP repeat-containing protein 3-like	3,59	5,68E-12	9
II	119649011	Death-associated inhibitor of apoptosis 1-like	3,10	2,84E-04	14
II	119647505	Spectrin beta chain, non-erythrocytic 1	1,29	6,77E-08	47
II	119650124	Death-associated inhibitor of apoptosis 1-like	2,34	3,59E-14	22
II	119646131	Myb-like protein Q	3,70	9,21E-16	7

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II	119649596	Dual specificity protein phosphatase Mpk3	1,62	1,24E-11	38
II	119660353	Uncharacterized LOC119660353	1,78	5,66E-08	34
II	119654408	Uncharacterized LOC119654408	2,58	5,29E-08	17
II	119654409	Uncharacterized LOC119654409	2,32	5,72E-10	23
II	119651604	Protein draper-like	2,48	1,10E-06	21
II	119656637	ETS-like protein pointed	1,44	2,81E-06	40
II	119648531	E3 ubiquitin-protein ligase XIAP-like	9,55	8,95E-34	1
II	119654544	Lysozyme 1-like	5,22	2,26E-08	2
II	119650013	Protein D2-like	1,24	8,27E-05	51
II	119656959	Peptidoglycan-recognition protein LE	1,99	2,77E-07	27
II	119659746	Cytokine receptor-like	1,89	1,02E-11	30
II	119646505	Uncharacterized LOC119646505	1,90	9,88E-09	29
II	119655169	Transferrin-like	3,29	3,25E-18	12
II	119652145	Peroxisomal acyl-coenzyme A oxidase 3	3,34	8,27E-27	10
II	119655940	Tetraspanin-2A	3,68	6,84E-32	8
II	119659886	Integrin alpha-PS3-like	2,82	1,40E-14	16
III	119646589	Juvenile hormone epoxide hydrolase 1-like	1,32	1,90E-02	46
III	119661230	H(+)/Cl(-) exchange transporter 7	0,88	3,53E-04	67
III	119661286	Tetraspanin-11-like	2,85	9,31E-05	15
III	119646962	Epidermal growth factor receptor	0,61	9,69E-03	82
III	119654947	Uncharacterized LOC119654947	2,22	2,91E-06	25
III	119658693	Peptidoglycan-recognition protein LB-like	1,63	1,85E-04	37
III	119646152	Matrix metalloproteinase-2	1,53	4,07E-07	39
III	119658691	Peptidoglycan recognition protein 3-like	1,19	4,08E-02	53
III	119650712	Protein late bloomer-like	1,14	3,72E-03	55
III	119656560	Cation-independent mannose-6-phosphate receptor	0,66	4,63E-02	79
III	119646668	Inhibitor of nuclear factor kappa-B kinase subunit beta	1,13	1,98E-04	57
III	119649577	Laminin subunit alpha	0,68	3,68E-03	76
III	119652596	Cell death abnormality protein 1-like	1,10	1,17E-02	59
III	119653794	Putative fatty acyl-CoA reductase CG5065	1,36	1,98E-04	45
III	119654916	Insulin-like growth factor 2 mRNA-binding protein 1	0,68	9,69E-03	77
III	119656558	Protein toll-like	1,12	9,95E-03	58
III	119657852	Uncharacterized LOC119657852	1,03	2,58E-02	61
III	119647972	Ets DNA-binding protein pokkuri	0,62	4,59E-02	81
III	119648719	Toll-like receptor 6	1,14	1,29E-02	56
III	119652202	Fibrillin-1-like	1,38	2,63E-02	44
III	119655009	Lysozyme 1-like	4,73	1,69E-02	5
III	119650821	Uncharacterized LOC119650821	1,86	1,14E-02	31
III	119655711	Down syndrome cell adhesion molecule-like protein Dscam2	0,88	4,37E-02	66
III	119656559	Protein toll-like	1,84	2,23E-03	32
III	119651157	Non-lysosomal glucosylceramidase	0,73	5,41E-03	72
III	119648853	Death-associated inhibitor of apoptosis 1-like	1,63	2,49E-07	36
III	119659850	GATA-type transcription factor SRE1	2,50	1,97E-07	20
III	119661601	Protein nubbin-like	3,31	7,62E-12	11
IV	119654016	Defensin-like	2,15	4,31E-02	26
IV	119657589	Cecropin-like peptide 1	3,20	4,18E-02	13
IV	119658627	F-actin-uncapping protein LRRC16A	0,71	3,95E-02	73
IV	119650564	Serine-arginine protein 55	0,70	2,30E-02	75
IV	119652268	Alkyldihydroxyacetonephosphate synthase	1,25	9,30E-03	50
IV	119660152	Uncharacterized LOC119660152	1,71	1,07E-06	35
IV	119657747	Cap-specific mRNA (nucleoside-2'-O-)-methyltransferase 1	0,81	1,45E-03	69
IV	119659367	Caspase-8	1,40	5,64E-09	43
IV	119650343	ADP-ribosylation factor 1	0,45	3,18E-02	85
IV	119658474	Uncharacterized LOC119658474	1,41	1,97E-02	41
IV	119659114	Cytokine receptor-like	0,75	1,47E-03	70
IV	119655536	Uncharacterized LOC119655536	1,27	1,12E-02	49
IV	119661013	Ras-related protein Rap1	0,67	2,53E-02	78
IV	119658945	F-actin-capping protein subunit alpha	0,65	3,02E-02	80
IV	119659285	E3 SUMO-protein ligase PIAS3	0,95	3,32E-02	64

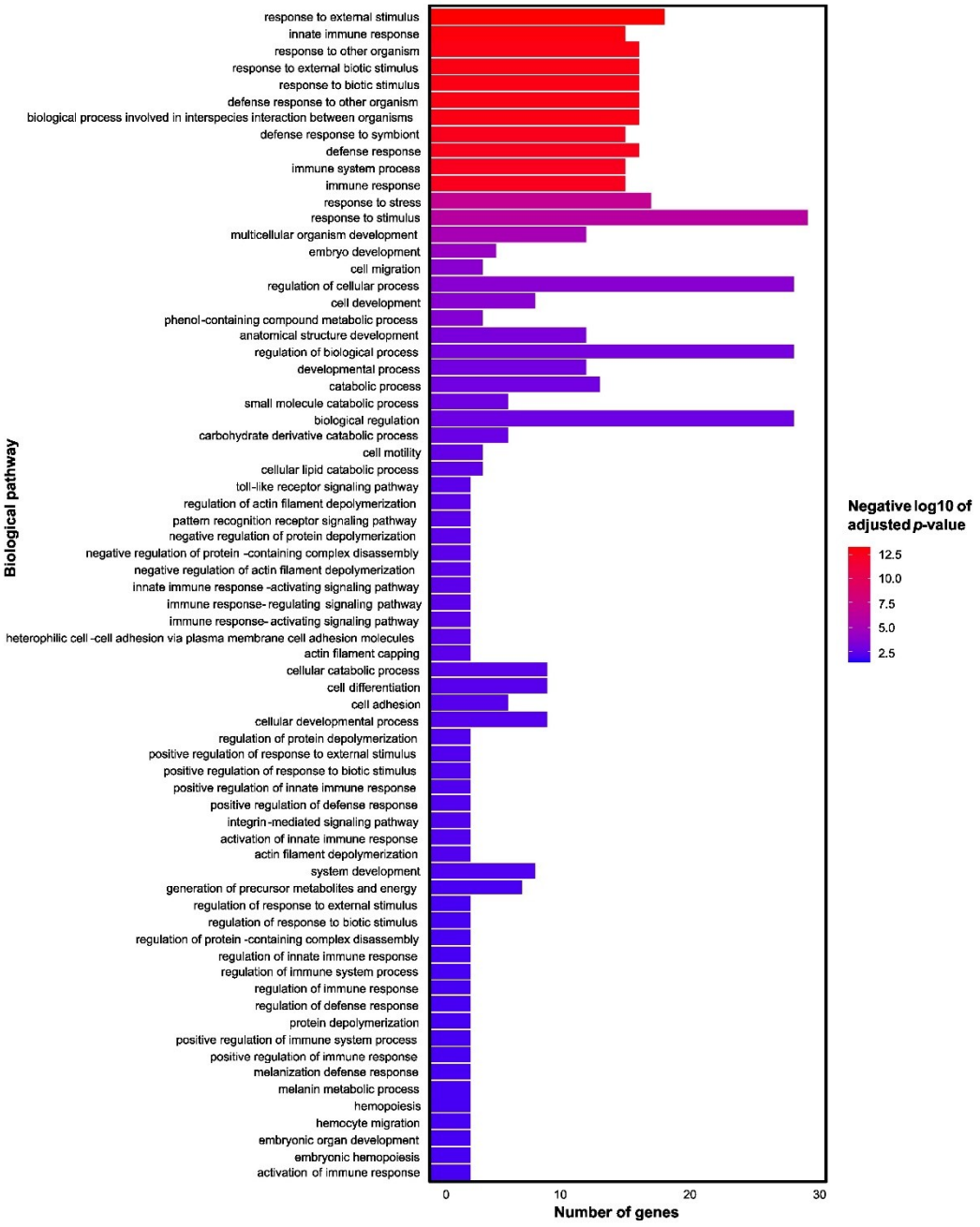

Figure S3.1 Top 70 biological process (BP) gene ontology terms related to 128 immune genes with a significant DESeq2 log2 fold-change contrasting between HiSvV- and Mock (PBS)- infected BSF adults. The following terms were excluded since they were broad umbrella terms: "biological process", "metabolic process", "cellular process", "cellular metabolic process". All terms had an adjusted *p*-value < 0.05 and the *p*-values were converted to negative log10.

Robert Daniel PIENAAR

Découverte et pathologie de virus associés à la mouche soldat noire (*Hermetia illucens*)

Résumé en français :

Cette thèse a investigué le virome des mouches soldats noires (BSF, *Hermetia illucens*), une espèce clé dans l'industrie de l'élevage massif d'insectes. Avant cette étude, les connaissances sur les virus infectant les BSF étaient limitées. Initialement, nous avons recherché des éléments viraux endogènes dans les génomes de BSF, découvrant neuf régions d'ADN d'origine virale liées à cinq familles de virus infectant les insectes : *Partitiviridae*, *Parvoviridae*, *Rhabdoviridae*, *Lebotiviridae* (anciennement *Totiviridae*) et *Xinmoviridae*. Nous avons ensuite examiné les ensembles de données métatranscriptomiques et métagénomiques de BSF, identifiant six virus exogènes appartenant à six familles : *Discistroviridae*, *Iflaviridae*, *Inseviridae*, *Lebotiviridae*, *Rhabdoviridae* et *Solinviviridae*. Cela a conduit au développement d'outils bioinformatiques et moléculaires pour le criblage à haut débit des virus, révélant une présence virale répandue dans les colonies de BSF à travers le monde, y compris dans les fermes industrielles et les laboratoires académiques. Certaines colonies étaient simultanément infectées par plusieurs virus. Pour évaluer la pathogénicité, nous avons isolé *Hermetia illucens* solinvivirus (HiSvV) et mené des bioessais. Nous avons constaté que les adultes BSF infectés par HiSvV mouraient beaucoup plus tôt que les non-infectés, confirmant la pathogénicité de HiSvV. Les expériences ont montré la transmission de HiSvV entre les BSF et l'infection par voies orale et sous-cutanée. De plus, nous avons étudié les réponses des gènes immunitaires chez les BSF lors de l'infection par HiSvV et dans les populations avec des co-infections naturelles de *Hermetia illucens* sigmavirus (HiSgV) et *Hermetia illucens* insevirus (HiInV). Cette thèse démontre que les BSF hébergent divers virus depuis longtemps. Ce travail fournit de nouveaux outils et des méthodes efficaces pour détecter les virus connus dans les colonies de BSF, soulignant la nécessité d'établir de nouvelles pratiques dans les installations d'élevage de BSF pour gérer et prévenir les infections virales.

Mots clés : Mouche soldat noire, Découverte de virus, Métatranscriptome, Métagénome, Entomoculture, Bioinformatique

Discovery and pathology of novel viruses associated with the unexplored model, *Hermetia illucens* (black soldier fly)

Summary :

This thesis investigated the virome of black soldier flies (BSF, *Hermetia illucens*), a key species in the insect mass-rearing industry. Prior to this study, there was limited knowledge about viruses infecting BSFs. Initially, we searched for endogenized viral elements in BSF genomes, discovering nine DNA regions of viral origin related to five insect-infecting virus families: *Partitiviridae*, *Parvoviridae*, *Rhabdoviridae*, *Lebotiviridae* (formerly *Totiviridae*), and *Xinmoviridae*. We then examined BSF metatranscriptomic and metagenomic datasets, identifying six exogenous viruses from six families: *Discistroviridae*, *Iflaviridae*, *Inseviridae*, *Lebotiviridae*, *Rhabdoviridae*, and *Solinviviridae*. This led to the development of bioinformatic and molecular tools for high-throughput virus screening, revealing widespread viral presence in BSF colonies globally, including industrial farms and academic labs. Some colonies were simultaneously infected with multiple viruses. To assess pathogenicity, we isolated *Hermetia illucens* solinvivirus (HiSvV) and conducted bioassays. We found that HiSvV-infected BSF adults died significantly sooner than non-infected ones, confirming HiSvV's pathogenicity. Experiments showed HiSvV transmission between BSFs and infection via oral and subcutaneous routes. Further, we studied immune gene responses in BSFs during HiSvV infection and in populations with natural co-infections of *Hermetia illucens* sigmavirus (HiSgV) and *Hermetia illucens* insevirus (HiInV). This thesis demonstrates that BSFs host diverse viruses and have done so for a long time. This work provides new tools and cost-effective methods for detecting known viruses in BSF colonies, highlighting the need for inclusive BSF welfare practices in rearing facilities to manage and prevent viral infections.

Keywords : Black soldier fly, Virus discovery, Metatranscriptome, Metagenome, Insect farming, Bioinformatics

Elisabeth Herniou