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MINI REVIEW

Review of insect pathogen risks for the black soldier fly (*Hermetia illucens*) and guidelines for reliable production

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Abstract

Black soldier fly [BSF; *Hermetia illucens* L. (Diptera: Stratiomyidae)] larvae are very effective in transforming low-grade food waste into valuable high-end proteins and fat, in intensive production facilities. The production output of this species is growing quickly, but upscaling brings risks to the health status of the reared insects. Until now, not a single major case of disease outbreak caused by a pathogen in a BSF production unit has been reported. This contrasts with data on other species of mass-produced insects, which have experienced various disease outbreaks, indicating that BSFs are comparatively resistant to insect diseases. Further, there are no records of natural infections caused by entomopathogens in BSF. In this review, the known entomopathogens of Diptera, especially BSF, and their potential risks for causing disease in these insects are summarized.

Introduction

Insects are increasingly being used as mainstream sources for food and feed in the developed world. In 2018, more than $300 million was invested in expansion of this industrial sector and large insect production facilities were opened (Reidy, 2019). *Hermetia illucens* L. (Diptera: Stratiomyidae), the black soldier fly (BSF), has drawn interest because the larvae of this fly consume a wide range of organic waste material and have an advantageous nutrient profile (Sheppard et al., 1994, 2002). As production of this species is upscaled, the economic risk of disease in the production stock caused by entomopathogens increases. Aquaculture is a relevant point of reference as it also concerns the growth of huge numbers of cold-blooded animals in controlled environments. In 2014, the FAO estimated that aquaculture produced revenues of $144 billion, and that disease was responsible for $6 billion in losses, about 4% of the value of the industry (Brummett et al., 2014; FAO, 2014). Furthermore, these losses tended to be concentrated, such as the Chilean infectious salmon anemia outbreak of 2007, which cost the Chilean aquaculture industry $2 billion (i.e., one-third of annual losses due to disease) and 20 000 jobs (Brummett et al., 2014).

Research on entomopathogens in BSF has not yet received much attention compared to other species of Diptera. This is partly because, until recently, BSF was not a commercially important species. Another reason is that the larvae of the species are anecdotally known to be highly resistant to infection and disease. The absence of any documented disease outbreak in BSF production is surprising, as other insect species for food and feed, such as *Tenebrio molitor* L., have experienced various disease outbreaks caused by entomopathogens in production (Eilenberg et al., 2015). Whereas the BSF industry is still relatively small, monitoring for pathogens may prevent their establishment and diagnostic protocols are important in order to react accordingly if a production stock becomes infected. Types of entomopathogens that may infect BSF need to be identified and characterized, to ensure that

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disease is prevented or controlled, and further spread avoided.

In this review we first outline the immunology of Diptera, focusing on species close to BSF. Then candidate dipteran entomopathogens that could affect BSF are treated, such as fungi, viruses, protozoa, and bacteria, followed by diagnostic and screening methods, including pathogen isolation and identification. After an assessment of risks and possible management strategies regarding insect pathogens and diseases in Diptera and BSF, the review concludes with strategic advice for professionals (farmers, veterinarians, and diagnosticians) and topics for future BSF research.

Immunology

Insect defense against invading entomopathogens includes behavioral and/or innate immune responses. The immune system of insects is exclusively innate, as opposed to vertebrates that also have an adaptive system with specific recognition and memory. However, the innate immune responses in insects are well-developed, with cell-mediated and humoral responses. The immune responses to infections are mediated by hemocytes, the fat body, the midgut, and the salivary glands, among other tissues (Hillyer, 2016).

New research indicates that exposure to sub-lethal doses of pathogens can give added protective effects (Cooper & Eleftherianos, 2017). In the evolutionary arms race between insect host and pathogen, three possible outcomes are most likely: (1) the host evolves an effective immune system resisting infections or eventually eliminating the pathogen; (2) the pathogen overcomes the host immune response and invades the host tissue causing lower fitness of the infected host, and in the worst case death; or (3) co-existence between pathogen and host develops, to the benefit of both (Wang et al., 2019).

The first line of defense against invading pathogens is the physical barrier, the exoskeleton, which includes a hard cuticular lining, and some general anti-microbial secretions against non-specific infections. After the physical barrier is passed, the cell-mediated and humoral defense mechanisms are activated through at least four major interconnected routes. The humoral response is characterized by binding pathogen-associated molecular patterns to host-derived pattern recognition receptors and by the secretion of antimicrobial peptides (AMPs) into the hemolymph (Hillyer, 2016; Rosales, 2017). This causes activation of immune signals, which amplify the immune response, inducing the production of antimicrobial factors and activating effector pathways. The result is that the pathogen is killed via hemocyte-mediated responses, such as phagocytosis, melanization, cellular encapsulation, nodulation, lysis, RNAi-mediated virus destruction, autophagy, and apoptosis (Hillyer, 2016; Lecocq et al., 2019). In response to parasites, the immunological reaction consists of recognizing signals from the invading parasites, modulation and amplification of the recognition signal, and effector metabolites (e.g., AMPs), directly involved with parasite inhibition (Lemaitre & Hoffmann, 2007), and subsequent encapsulation.

Immunology in Diptera

The immunology of the fruit fly *Drosophila melanogaster* Meigen and the house fly *Musca domestica* L. is well-studied and may provide various leads for research on BSF. Immune responses of dipteran species are often multifunctional. Biochemical analysis of the hemolymph of *D. melanogaster* and other Diptera found seven groups of AMPs, which show a wide variety of actions against microorganisms. They were grouped into three families based on their main biological targets: (1) against Gram-positive bacteria: defensins; (2) against Gram-negative bacteria: cecropins, drosocin, attacins, and diptericin; and (3) against fungi: drosomycin and metchnikowin (Imler & Bulet, 2005).

In dipteran species, recent results suggest that host-specific fungal pathogens evolved in response to the immune system of different hosts (de Fine Licht et al., 2017). This suggests that the host's immune system can, to some extent, be linked to the level of specialization. Wang et al. (2019) studied pattern recognition receptors (PRRs), which form an expansion of the insect immune system, by adapting its immune system to a changing environment. The PRRs can work against all types of entomopathogens: viruses, fungi, bacteria, etc.

Many species of microbes symbiotically exist with species of Diptera; for example, endosymbiotic bacteria of the genera *Wolbachia* and *Rickettsia* may assist *Drosophila* spp. in developing resistance to fungal and viral infections (Liu & Guo, 2019).

Immunology in black soldier flies

Knowledge of the immunology of BSF remains very limited. In one study, larvae challenged with Gram-negative and Gram-positive bacteria showed a significant increase in phenoloxidase activity compared to controls (Zdybicka-Barabas et al., 2017).

Black soldier fly larvae express a wide spectrum of AMPs, several of which are induced by high bacterial loads in the diet (Vogel et al., 2018). Such a wide spectrum of response is a rich area for future research. Indeed, BSF larvae seem to be well adapted to combating microorganisms in general, a characteristic that can have a broad

significance with regard to protection from infections caused by opportunistic microorganisms in their growth medium. In a study by Lalander et al. (2015), the presence of BSF larvae actually lowered the concentration of *Salmonella* spp. in their substrate. Overall, we therefore hypothesize that BSF larvae are in general rather resistant to the microflora in their substrates, and can inactivate microorganisms, but more research is needed on this subject.

Entomopathogenic fungi

Most entomopathogenic fungi belong to the orders Hypocreales (several genera), Entomophthorales and Neozygitales (both Entomophthoromycota), and Onygenales (genus *Ascosphaera*) (Boomsma et al., 2014). Insect pathogenic fungi are transmitted from an infected to a susceptible host by spores. Many species are widely distributed in the environment and can be found in soil, water, and air but also on plant surfaces and in infected insects. When spores (mostly conidia) reach a suitable host, they adhere to the exoskeleton, germinate, and penetrate through the cuticle (Hajek & St. Leger, 1994). Here, we focus on insect pathogenic fungi in terrestrial and semi-aquatic environments, as fungal infection in aquatic larvae are of little relevance for BSF. Furthermore, we focus on natural primary infections by entomopathogenic fungi recorded on dipterans. Thereby we exclude opportunistic generalist fungi that mostly cause secondary infections, such as those of the genera *Aspergillus*, *Penicillium*, and *Fusarium*.

Tables 1 and 2 list some examples from the literature in which entomopathogenic fungi – Entomophthorales and Hypocreales, respectively – were documented infecting Diptera species during various life stages and at different locations. There is no published record of entomopathogenic fungal infections in BSF in nature. It would be valuable to execute specific field studies on this. Such research could isolate, culture, and identify specific entomopathogenic fungi infecting BSF and test them for pathogenicity under laboratory conditions. There are some records of entomopathogenic fungi of other Stratiomyidae species (Table 2) but information is still very scarce compared to various other groups of dipterans.

Entomophthorales are highly specialized pathogens of Diptera (Jensen & Eilenberg, 2001). Interestingly, by host manipulation, infected adults often seek elevated positions that promote transmission through better spore dispersal (Roy et al., 2006) and cause epidemics in adult flies such as *M. domestica*, even in production facilities (Eilenberg et al., 2015). There are no published records of infections by Entomophthorales in Stratiomyidae and related families such as Bombilidae, Therevidae, and Asillidae (Wiegmann et al., 2011), although a photo of a dead fly from Stratiomyidae in Eilenberg (2000) appears to show an early-stage fungal outgrowth caused by an entomophthoralean fungus. Unfortunately, this soldier fly and the fungus were never identified. Also, Bałazy (1993) mentions briefly a record from East Europe or USSR of an *Erynia* species infecting an unidentified stratiomyid fly (or flies). There are a few records of Entomophthorales infections in Tabanidae and Rhagionidae (Keller, 2007a, 2008).

Many of the Hypocreales species are opportunists and/or generalists, occurring in soil, water, on plants, and on several arthropod species. In this way insect pupae and larvae can easily encounter spores of these fungal species (Table 2). The number of fungal species found on Stratiomyidae is relatively low compared to other more studied families, such as Anthomyiidae and Muscidae, probably due to under-studying and under-sampling rather than to the non-existence of these fungal species in Stratiomyidae. Studies on entomopathogenic fungal infections have usually been limited to insect species that are pests or are important beneficial species such as insects used for biological control or production. However, it is likely that almost all insect species collected intensively will be found to be a natural host of several entomopathogenic fungi species (Meyling & Eilenberg, 2007). Hypocreales have in general a broader host range and infect various life stages in the same insect species, a large difference with Entomophthorales species, which in most cases infect only the adult of the host or only the larval instar. Because some hypocrealean entomopathogenic fungi are used as biocontrol agents for insects, there is a potential risk of spill-over from the environment into production facilities. In future research it would be interesting to study the effect of these pathogenic biological control agent species on various BSF development stages. It is also highly relevant to sample for natural fungal infections in Stratiomyidae and explore pathogenicity and virulence of such fungi against BSF.

Entomopathogenic viruses

Insect viruses are small infectious agents that only replicate inside living cells. They include DNA viruses such as baculoviruses, nudiviruses, hydrosaviruses, iridoviruses, and densoviruses and RNA viruses such as dicistroviuses, iflaviruses, and reoviruses (Maciel-Vergara & Ros, 2017). Insect viruses can be transmitted horizontally, for example by oral uptake, or via wounds, or they can be transmitted vertically, that is, to the next generation (transovarial, transoval). Oral uptake often occurs by larval ingestion of virus material excreted from infected individuals or released from cadavers (Lietze et al., 2009; Harrison &

Table 1 Representative list of natural infections in Diptera caused by entomopathogenic fungi from the Entomophthorales

Entomophthorales spp.	Diptera group	Diptera family	Development stage	Continent/ region	Source
Batkoa spp.	Nematocera	Cecidomyiidae	Adult	Europe	Leatherdale (1970)
		Chironomidae	Adult	Europe	Keller (2008)
		Limoniidae	Adult	Europe	Barta & Cagáň (2006); Keller (2007a, 2008)
		Simuliidae	Adult	Europe	Keller (2008)
		Tipulidae	Adult	Europe	Keller (2007a, 2007b); Keller (2008); Tkaczuk et al. (2011)
	Brachycera	Empididae	Adult	Europe	Keller (2008)
		Rhagionidae	Adult	Europe	Keller (2008)
	Cyclorrhapha	Psilidae	Adult	Europe	Eilenberg & Philipsen (1988); Keller (2008)
Conidiobolus spp.	Nematocera	Chironomidae	Adult	Europe	Keller (2007a)
		Culicidae	Adult	Europe	Keller (2007a)
		Limoniidae	Adult	Europe	Bałazy et al. (2011)
		Tipulidae	Adult	Europe	Keller (2007a); Bałazy et al. (2011)
	Cyclorrhapha	Psilidae	Adult	Europe	Eilenberg (1988)
Entomophaga spp.	Nematocera	Chironomidae	Adult	Europe	Keller (2008)
		Limoniidae	Adult	Europe	Humber (1989) in Barta & Cagáň (2006); Keller (2007a, 2007b), Keller (2008); Bałazy et al. (2011)
		Ptychopteridae	Adult	Europe	Jensen & Eilenberg (2001); Keller (2007a)
		Tipulidae	Adult	Europe	Keller (2007a, 2007b), Keller (2008); Tkaczuk et al. (2011); Bałazy et al. (2011)
	Brachycera	Rhagionidae	Adult	Europe	Keller (2007b, 2008)
		Tabanidae	Adult	Europe	Keller (2007a)
	Cyclorrhapha	Calliphoridae	Adult	Europe	Keller (2007a)
Entomophthora spp.	Nematocera	Cecidomyidae	Adult	Europe	Keller (2007a, 2008); Bałazy et al. (2011); Tkaczuk et al. (2011)
		Chironomidae	Adult	Europe	Jensen & Eilenberg (2001); Keller (2007a, 2008)
		Culicidae	Adult	Europe	Keller (2007a, 2008)
		Sciaridae	Adult	Europe	Leatherdale (1970); Keller (2007a, 2008)
		Simuliidae	Adult	Europe	Leatherdale (1970); Keller (2007a, 2008)
		Tipulidae	Adult	Europe	Leatherdale (1970)
	Brachycera	Empididae	Adult	Europe	Leatherdale (1970)
		Hybotidae	Adult	Europe	Leatherdale (1970); Keller (2008)
	Cyclorrhapha	Anthomyiidae	Adult	Europe, North America	Carruthers et al. (1985); Eilenberg et al. (1994); Klingen et al. (2000); Pell et al. (2001); Jensen & Eilenberg (2001); Jensen et al. (2006, 2009); Keller (2007a, 2008); Bałazy et al. (2011)
		Calliphoridae	Adult	Europe, North America	Steenberg et al. (2001); Jensen et al. (2006); Keller (2008)
		Chloropidae	Adult	Europe	Leatherdale (1970)
		Fanniidae	Adult	Europe	Mullens et al. (1987); Steenberg et al. (2001)
		Muscidae	Adult	Europe, North America	Mullens et al. (1987); Watson & Petersen (1993); Jensen & Eilenberg (2001); Steenberg et al. (2001); Skovgård & Steenberg (2002); Jensen et al. (2006, 2009); Keller (2008)
		Psilidae	Adult	Europe	Eilenberg & Philipsen (1988); Keller (2007b, 2008)
		Scatophagidae	Adult	Europe	Steenberg et al. (2001); Jensen et al. (2006); Keller (2007a, 2007b), Keller (2008)
		Syrphidae	Adult	Europe	Leatherdale (1970); Jensen & Eilenberg (2001); Steenberg et al. (2001); Keller (2007b, 2008)

Table Table 1 Continued

Entomophthorales spp.	Diptera group	Diptera family	Development stage	Continent/ region	Source
Erynia sensu lato (*Erynia*, *Pandora*, *Furia*)	Nematocera	Bibionidae	Adult	Europe	Keller (2007a)
		Cecidomyiidae	Adult, pupae	Europe	Leatherdale (1970)
		Chaoboridae	Adult	North America	Cuebas-Incle (1992)
		Chironomidae	Adult	Europe, North America	Cuebas-Incle (1992); Keller (2007a, 2008)
		Culicidae	Adult	Europe	Keller (2007a)
		Limoniidae	Adult	Europe	Keller (2007a, 2007b), Keller (2008)
		Psychodidae	Adult	Europe	Leatherdale (1970); Keller (2007a, 2008); Bałazy et al. (2011)
		Simuliidae	Adult	Europe, North America	Keller (2007a, 2008)
		Sciaridae	Adult	Europe	Leatherdale (1970); Keller (2007b, 2008); Tkaczuk et al. (2011); Ruszkiewicz-Michalska et al. (2012)
		Tipulidae	Adult	North America, Europe	Leatherdale (1970); Cuebas-Incle (1992)
	Cyclorrhapha	Anthomyiidae	Adult	Europe	Eilenberg et al. (1994)
		Calliphoridae	Adult	Europe	Leatherdale (1970); Keller (2007a, 2007b), Keller (2008); Bałazy et al. (2011); Tkaczuk et al. (2011)
		Chloropidae	Adult	Europe	Leatherdale (1970)
		Lauxaniidae	Adult	Europe	Keller (2007a, 2008)
		Muscidae	Adult	Europe	Leatherdale (1970); Steenberg et al. (2001); Keller (2007b, 2008)
		Psilidae	Adult	Europe	Eilenberg & Philipsen (1988)
		Scatophagidae	Adult	Europe	Bałazy (1993); Bałazy et al. (2011)
Strongwellsea spp.	Cyclorrhapha	Anthomyiidae	Adult	Europe	Eilenberg et al. (1994); Eilenberg & Michelsen (1999); Klingen et al. (2000); Keller (2007a, 2008)
		Calliphoridae	Adult	Europe	Eilenberg & Michelsen (1999)
		Fanniidae	Adult	Europe, North America	Humber (1976); Eilenberg & Michelsen (1999); Keller (2007a)
		Muscidae	Adult	Europe	Eilenberg & Michelsen (1999); Keller (2007b, 2008)
Zoophthora spp.	Nematocera	Sciaridae	Adult	Europe	Keller (2007a, 2007b), Keller (2008); Ruszkiewicz-Michalska et al. (2012)
		Tipulidae	Adult	Europe, North America	Keller (2007a); Hajek et al. (2016)
	Brachycera	Opomyzidae	Adult	Europe	Tkaczuk et al. (2011)
	Cyclorrhapha	Dryomyzidae	Adult	Europe	Keller (2007a); Bałazy et al. (2011)

Hoover, 2012). Many insect viruses are host specific and will only infect one host species or a few closely related species. However, there are exceptions.

Viruses of Diptera

Various types of insect viruses are known from Diptera, but so far none have been described for BSF. Viruses classified in the family Hytrosaviridae are known to infect tsetse flies, such as GpSGHV in *Glossina pallidipes* Austen, and cause reduction in the host's fecundity (Abd-Alla et al., 2008). *Musca domestica* can become infected with the related MdSGHV, which causes lowered fitness of its host and eventually the infected host dies (Lietze et al., 2011). Idnoreovirus 3 (Reoviridae) infections in house

Table 2 Representative list of natural infections in Diptera caused by entomopathogenic fungi from the Hypocreales

Hypocreales spp.	Diptera group	Diptera family	Development stage	Continent/ region	Source
Beauveria spp.	Cyclorrhapha	Ephydridae	Adult, pupae	North America	Castrillo et al. (2008)
		Heleomyzidae	Adult	Europe	Leatherdale (1970); Steenberg et al. (2001)
		Muscidae	Adult	Europe	Steinkraus et al. (1990); Steenberg et al. (2001)
Harposporium spp.	Cyclorrhapha	Drosophilidae	Adult	Europe	Leatherdale (1970)
Hirsutella spp.	Cyclorrhapha	Heleomyzidae	Adult	Europe	Leatherdale (1970)
Isaria spp.	Nematocera	Bibionidae	Adult	Europe	Bałazy et al. (2011)
		Cecidomyiidae	Adult	Europe	Leatherdale (1970)
		Psychodidae	Adult	Europe	Leatherdale (1970)
		Tipulidae	Adult	Europe	Leatherdale (1970)
	Brachycera	Stratiomyidae	Adult	Europe	Leatherdale (1970)
	Cyclorrhapha	Agromyzidae	Unknown	Europe	Smith (1993) in Zimmermann (2008)
		Glossinidae	Unknown	Europe	Smith (1993) in Zimmermann (2008)
		Muscidae	Adult	Europe	Skovgård & Steenberg (2002)
		Tachinidae	Unknown	Europe	Smith (1993) in Zimmermann (2008)
Lecanicillium spp.	Nematocera	Sciaridae	Adult, larvae	Australia	Tkaczuk et al. (2011)
	Cyclorrhapha	Muscidae	Adult	Europe	Steenberg et al. (2001); Skovgård & Steenberg (2002)
Metarhizium spp.	Cyclorrhapha	Anthomyiidae	Larvae	Europe	Klingen et al. (2002)
		Lonchaeidae	Adult	Europe	Leatherdale (1970)
		Muscidae	Adult	Europe	Skovgård & Steenberg (2002)
Ophiocordyceps spp.	Cyclorrhapha	Muscidae	Adult	Europe	Leatherdale (1970)
Polycephalomyces spp.	Cyclorrhapha	Heleomyzidae	Adult	Europe	Leatherdale (1970); Matočec et al. (2014)
Tolypocladium spp.	Cyclorrhapha	Anthomyiidae	Larvae, pupae	Europe	Klingen et al. (2002)

flies cause swollen abdomen, cessation of feeding, paralysis, and finally death (Moussa, 1978). New detection tools make the discovery of new types of viruses easier and faster every year.

Virus interactions with insects

The interactions between an insect virus and its host are complex and new studies will shed light on new ecological aspects, which may be of high significance in rearing. Especially covert virus infections may pose a high danger to insects in production (Williams et al., 2017). Infections may be present in an insect production facility, yet remain undetected due to the lack of apparent symptoms. However, the virus can be activated during increased levels of stress in the insect and may cause significant damage. As most viruses have a narrow host range, it is important to keep wild BSFs and closely related species outside a BSF production factory to prevent introduction of potentially harmful viruses into the rearing facilities.

Screening wild BSF populations and current production colonies for virus infections is important and would be a good way to gain knowledge on the risks and current status in the sector.

Entomopathogenic protozoa

Protozoans are several groups of taxonomically unrelated organisms. Among protozoa, several species from various taxa can infect insects: Sarcomastigophora (flagellates and amoebae), Microspora (microsporidia), Apicomplexa (gregarines, neogregarines, and coccidia), and Ciliophora (ciliates) (Undeen & Vávra, 1997).

Trypanosomatids are the most commonly occurring flagellate protozoa in insects, including Diptera. They live in the gut lumen or attached to epithelial cells. More rarely, they may also infect the insect's salivary glands or mouth parts. Those which penetrate to the salivary gland via hemolymph tend to be more virulent to the insect host

(Undeen & Vávra, 1997). Trypanosomatid parasites of Brachycera flies might be more widely distributed than previously thought (Wilfert et al., 2011; Týč et al., 2013). The parasitic relationship between the trypanosomatids and their hosts is largely unexplored, but *D. melanogaster* exposed to trypanosomatids can have delayed pupation (Ebbert et al., 2003). There are only six entomopathogenic amoebae species described, and none of them have been reported from Diptera hosts (Lange & Lord, 2012).

Microsporidia infect practically all tissues of all stages of the insect host. The two most commonly infected tissues are the fat body and midgut epithelium. Microsporidians generally cause chronic infections with slow-acting and progressive severity, but they rarely cause acute infections. The symptoms associated with microsporidiosis in insects range from obvious tissue manifestations to abnormal developmental and behavioral changes (Becnel & Andreadis, 2014). Five microsporidian species – *Amblyospora* sp., *Edhazardia aedis*, *Parathelohania* sp., *Nosema algerae*, and *Vavraia culicis* – have been targeted for biological control programs of aquatic dipteran (*Aedes* spp., *Anopheles* spp., and *Culex* spp.) (Solter & Becnel, 2007). The effectiveness of the parasitoid *Muscidifurax raptor* Girault & Sanders, a commercially produced biological control agent, is seriously reduced by *Nosema muscidifuracis* infections causing reduced lifespan and reproduction capacity (Geden et al., 1995). Recently a new microsporidium parasite infecting a laboratory colony of *D. melanogaster* was documented (Franzen et al., 2005). Under conditions such as over-crowding and stress, microsporidia can devastate mass-reared colonies of insects by inducing prolonged development, physical deformations, reduced fecundity, and reduced longevity (Bjørnson & Oi, 2014; Stentiford et al., 2016).

Eugregarines, neogregarines, and coccidia all belong to Apicomplexa. They are unicellular and spore-forming and include insect parasitic forms. The transmission of all apicomplexa occurs through ingestion of contaminated food (Lange & Lord, 2012). The eugregarines are relatively large organisms (often 50–200 μm) and the only cell divisions occur during formation of oocysts, which often takes place outside of the host cells, normally in the gut. They are then released back into the environment via the feces, which would be a risk to BSF larvae which are reared in substrate consisting for a large part of their feces. Their direct effect on the host is mostly minimal, except for forms that occupy the Malpighian tubules (Lange & Lord, 2012). Eugregarines are often seen in *T. molitor* rearing and production and have also been reported in sandflies and mosquitoes (Young & Lewis, 1977; Ostrovska et al., 1990). The eugregarinae *Diplocystis tipulae* is a common pathogen of *Tipula paludosa* Meigen (Carter, 1976) and laboratory experiments have shown that infections may cause a reduced larval size (Er & Gökçe, 2005).

The neogregarines have a very complex lifecycle, which includes extensive multiplication within the insect host; therefore, they are normally more virulent than eugregarines, resulting in lethal infections. New hosts are infected when they eat food contaminated with oocysts, either through scavenging, predation, or the breakdown of cadavers (Lange & Lord, 2012). Neogregarines have been reported from 11 insect host orders, including Diptera, but there is a lack of adequate descriptions and affiliations with the hosts (Lange & Lord, 2012).

Coccidia are mainly parasites of vertebrates and only 1% are known to infect insects. Species within the genera *Ithania* and *Rasajeyna* have been reported to infect midgut cells of craneflies and the oocysts are believed to be released with the feces, as with the eugregarines (Lange & Lord, 2012).

Ciliates are mostly free-living organisms, but two parasitic genera exist in aquatic Diptera (Culicidae, Chironomidae, and Simuliidae). *Lambornella clarki* causes increased mortality of mosquito adults and parasitic castration of its female hosts (Egerter & Anderson, 1985).

To our knowledge, protozoan infections in BSF have not yet been documented. The ecological host specificity of protozoa is generally considered to be narrow, but laboratory experiments have shown that the physiological host specificity may expand (Solter & Maddox, 1998). Therefore, in the case of BSF, risks of transmission could occur through infected feed or accidental ingestion of cadavers or feces from invasive infected dipterans.

Entomopathogenic bacteria

Bacteria are prokaryotic microorganisms of a few micrometers long. They may have the form of spheres, rods, or spirals. They may be aerobic or anaerobic, motile or nonmotile, and some genera like *Bacillus* spp. may produce resistant endospores to enter a dormant stage (Nicholson et al., 2000). Ubiquitous in natural water and soils, they engage in numerous and multifaceted interactions with dipterans.

Both internally and externally, bacteria can be mutualistic (beneficial), parasitic (pathogenic), or commensal (harmless in normal situations). Beneficial bacteria may assist in digestion or reproduction, or have positive effects on the immune system, among others. At the opposite end, bacterial entomopathogens have either obligate or facultative relationships with their hosts. For example, *Paenibacillus* spp. only proliferate within the insect host, whereas *Serratia* spp. can also grow in the environment outside the host. Bacterial transmission or infection is

most often through oral ingestion although infection through other openings, such as wounds, is possible via injured cuticula (Jurat-Fuentes & Jackson, 2012; Lecocq et al., 2019). Nematodes or parasitoids can also be vectors of pathogenic bacteria for insects (Eilenberg et al., 2015). In general, bacterial entomopathogens first enter the host hemocoel and avoid the insects' immune system, to proliferate and produce virulence factors which cause disease, and which ultimately kill the host (Aronson et al., 1986). Upon host death, bacteria use the carcass as a nutrient source until the formation of dormant life stages, such as endospores in the case of *Bacillus* spp., or they infect a new host after transmission, possibly via vectors (Glare et al., 2017).

Entomopathogenic bacteria are widely popular as biocontrol agents of pests. They make up the greatest contribution to research into new types of biopesticides and can control a wide range of insect taxa (Ruiu, 2015). As a result, many studies investigating the susceptibility of insects, of the order Diptera, to bacterial pathogens, aim at controlling pests or vectors of diseases. The aim of those agents is to kill their host, and their use implies a potential risk of spill-over of bacteria from a target insect to BSF. Among those, *Bacillus thuringiensis* is the most commonly used biopesticide. It has been used to control a wide range of organisms and especially insect pests from lepidopterans to coleopterans and dipterans. Host susceptibility to *B. thuringiensis* is varied and diverse due to a high number of subspecies, strains, or formulations. *Bacillus thuringiensis israelensis* was the first subspecies found to be toxic to dipteran larvae (Margalith & Ben-Dov, 2000; Ben-Dov, 2014). The bacterium is pathogenic only through oral intake: a highly dense protein crystal (Bt toxin) destroys epithelia cells of the insects' mid-gut, allowing mid-gut bacteria to enter the hemocoel and then the rest of the body (Raymond et al., 2010). Another bacterium, *Lysinibacillus sphaericus*, has been reported as an effective biological control agent against various Diptera, often in combination with *B. thuringiensis* (Berry, 2012).

Other generalist bacteria that can kill insects from various orders, such as *Pseudomonas fluorescens* and *P. entomophila*, have also been shown to infect and kill *M. domestica* (Padmanabhan et al., 2005; Dieppois et al., 2015), potentially posing a risk to BSF production. Similarly, species of *Serratia* are ubiquitous in the environment and several of the 14 species in this genus have been found associated with diseased and dead insects (Grimont & Grimont, 1978). *Serratia marcescens* has been reported as a potential or facultative pathogen and following oral ingestion may cause disease in the blow fly *Lucilia sericata* Meigen (O'Callaghan et al., 1996).

Studies investigating the internal and external microbiome of healthy insects and their substrate showed strong links between the diversity of bacterial species in the insects and the type of substrate used. Most research focuses on the use of molecular methods, such as 16S rRNA gene sequencing and pyrosequencing, to detect and identify the bacteria present (Forster et al., 2007; Jeon et al., 2011; Zheng et al., 2013a; Kim et al., 2014; De Smet et al., 2018; Wynants et al., 2019). Zheng et al. (2013b) found many potentially pathogenic bacteria in all BSF stages, such as Xanthomonadales. To a smaller extent they also found opportunistic pathogens, for example, *Lysobacter* spp., Burhholderiales, *Bacteroides* spp., *Clostridia*, and *Bacillus* spp. (Zheng et al., 2013b). Research is needed on the pathogenic effects of these bacteria to BSF.

A number of studies showed that BSF is able to reduce the bacterial load of food-safety related bacteria such as *Escherichia coli*, *Salmonella* spp., and *Enterococcus* spp. (Erickson et al., 2004; Liu et al., 2008; Lalander et al., 2013), and this is promising. Whereas other dipterans have been known to carry or vector these bacteria, such an effect in BSF should be explored. Nonetheless, given the apparent link between the bacterial flora in dipterans and the substrate in which they develop and feed (Jeon et al., 2011), more research is needed on the pathogenesis, pathology, and epidemiology, including the risk of bacterial infections in BSF production.

Diagnosis and identification of pathogens

The transmission or infection routes of insect pathogens vary. The entry that a pathogen uses to infect an insect impacts its diagnosis and identification.

Fungal infections

The infection route of most fungal pathogens is through the cuticle followed by growth in the insect hemolymph. The first diagnosis of a fungal infection is through the observation of mycelium structures, conidia, and conidiophores on a dead insect. To stimulate the development of fungal diagnostic features, the sample can be placed in an incubator under humid conditions for 48 h. Microscopy remains the most common method for the identification of the most important genera and main species of fungal pathogens infecting insects (Humber, 2012). However, advances in molecular techniques imply that DNA extraction, polymerase chain reaction (PCR), and DNA sequencing are more and more commonly used to identify fungal species, and MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) may be used as a protein-based identification method for fungi as well (Bader, 2017).

Virus infections

Typical clinical symptoms of insect virus infections include reduced weight gain, delayed molting, or oviposition, translucent thorax, swollen abdomen, enlarged brownish or milky midgut or hindgut, watery feces, paralysis, and finally death (Lacey & Brooks, 1997). To detect and identify viruses, PCR-based methods and sequencing are widely used. Electron microscopy, DNA restriction endonuclease analysis, serology, and histopathology are also applied to a lesser extent (Harrison & Hoover, 2012). Identifying RNA viruses may require more attention than detecting DNA viruses and methods include total RNA isolation and reverse transcriptase (RT)-PCR in addition to some of the above-mentioned tools. Guidelines for insect virus detection that are more specific need to be developed, to facilitate the use of some of these techniques in standard diagnostic protocols (Maciel-Vergara & Ros, 2017; Lecocq et al., 2019).

According to the OIE-World Organization for Animal Health (OIE, 2019), six bacterial or parasitological diseases of bees are notifiable, but none of the insect viral diseases are so far notifiable. This may change in the near future, as the insect farming sector is growing fast, with growing research and knowledge on the impact of serious veterinary pathogens, including viruses.

Protozoan infections

Symptoms of protozoan infections include a decrease in fitness parameters of the insects, whereas external features may not be present. To detect their presence and abundance in the insect, dissection of the insect and microscopy of the gut fluid is commonly used as a first step in diagnosis. Furthermore, protozoa can be identified to their major groups using morphological characters. Many of these pathogens are host specific, meaning that in some cases, a short identification key in addition to the host-species information can be a good starting point for their identification (Solter et al., 2012). Fresh preparations of gut contents and body cavity fluids and examination under the microscope (light and transmission electron microscopy) using a variety of staining techniques is common. Once again, for closer inspection, DNA extraction, general PCR, and sequencing for single or partial genes have become more widely used in recent years.

Bacterial infections

Discoloration and/or darkening in infected hosts are often a reliable, first criteria for diagnosis of bacterial diseases. Isolation of pathogenic bacteria is carried out by aseptically extracting a hemolymph sample and plating it onto artificial growth media for cultivation. These plates then are incubated during 2–7 days at a temperature comparable to that of the insect culture facility, often ca. 28 °C. Alternatively, cultivation-independent methods such as DNA extraction and 16S sequencing are used. Identification of pathogenic bacterial species are nowadays performed by the protein-based method MALDI-TOF (Carbonnelle et al., 2011) starting with a pure colony of the bacterium, or by 16S rDNA gene sequencing (Fisher & Garczynski, 2012).

A protocol for the diagnosis and identification of a pathogen in BSF populations depends on the first symptoms observed. Firstly, if sub-lethal reduction in fitness parameters are observed, such as decreased fecundity, size, or lengthened development time, with no apparent increase in mortality, the focus should be on evaluating changes in abiotic management factors such as humidity, temperature, airflow, or insect density/starvation. Secondly, when physical symptoms are apparent, such as a change in smell of the insects, behavior, discoloration, lethargy, or body form (swollen abdomen), samples should be collected immediately. These potentially infected insects need to stay alive until specimens for bacteriology are taken, and if the insect is already dead the fresher the sample, the better (i.e., within 1 h after death), to avoid post-mortal contamination of bacteria and fungi. Following sampling (inoculation on specific growth media and/or sampling for molecular testing), the samples should be processed according to standard operating procedures (SOPs) of general bacteriology (Barrow & Feltham, 1993) or specialized publications, or according to manufacturer's directions, as SOPs for diagnosis for insects are in development.

Epidemiology and management

An insect production facility is basically like a fortress, preventing entry of diseases into its walls and restricting their spread within them. To design a successful fortress, it is essential to understand ways an enemy can penetrate frontline defenses, and to know how to control the movement of the enemy once they are inside the walls. Two types of facilities are currently used to produce BSF: semi-open facilities, where it is relatively easy for animals and the elements to enter and exit freely, and closed facilities. Both types of facilities present pathways for disease to enter and spread.

Frontline defense against introduction of insect pathogens

Entomopathogens are diverse and small enough to exploit virtually any entry point into a facility. However, two vectors for entomopathogens are particularly effective at transmitting diseases into facilities: insect feed and insects from outside the facility.

Black soldier fly is primarily cultivated using low-value feed from by-products or food waste. Consequently, the feed may contain fungi and bacteria, or their spores, and it may contain live insects that carry potential entomopathogens. Several pre-processing procedures can mitigate these risks. The simplest is visual screening and rejection of feed that shows signs of infestation or unfamiliar decomposition. However, this step is insufficient to detect entomopathogenic fungi or bacteria, including those which are commercially deployed on crops as pesticides. Heating feed is a straightforward way to eliminate most vectors, but it is energy intensive and costly, especially when feed has a high water content. Furthermore, depending on the temperature, it does not kill spores of some fungi and bacteria. Grinding or milling, for example, with an impact mill, can effectively kill insects. Radiation technologies may also be a way to kill pest insects and pathogens in bulk feed deliveries (Josephson & Peterson, 1982). These technologies are already well developed for treatment of bulk commodities, treatment of pollen for bees (Yook et al., 1998), and for carrying out sterile insect programs such as those carried out by the International Atomic Energy Agency (Vienna, Austria).

Disease prevention in BSF farms

Another source of insect-borne disease could be brood stock from suppliers. Eggs of BSF purchased from a source outside of the production facility or captured from the wild should come from a 'specific pathogen free' (SPF) approved source or otherwise be subjected to proper screening procedures during weeks of quarantine, as is advised for other animal husbandry branches (OIE, 2019).

Vectors of insect pathogens

All animals, but especially insects, from outside the facility are potential vectors for entomopathogens. Escapees from BSF production facilities, or their descendants, would be the most effective insect vectors because they are the same species as the production animals and thus can vector even the highly host-specific pathogens. Semi-open BSF facilities with a large number of escapees face a risk that those will leave the facility for mating, come in contact with diseased flies, and bring the pathogens back into the facility when searching for the right oviposition site. The adult BSF stage will likely be the only insect stage to make trips into and out of the facility due to the limited mobility of the other life stages. Closed facilities can be designed to eliminate this risk. Mesh can be applied to the small openings where incursion is possible, and the building is typically sealed unless people or materials are entering or exiting. These types of precautions prevent insects from escaping the facility and entering.

Other ways of transmission of insect pathogens

Other entry and transmission methods for pathogens are via air, water, or pathogen-carrying materials that are brought in by employees. Counteracting airborne and waterborne pathogens requires diagnosing the likelihood and type of threat. Thus, depending on the severity and form of the pathogen, steps for air filtration and water purification can be taken. These will be most effective in closed facilities. To reduce the risk of pathogens traveling on workers and materials into the facility, the use of procedures common in other livestock cultivation are recommended. These include work-specific clothing, restriction of access between sectors in the facilities, and washing procedures (IPIFF, 2019; OIE, 2019).

Our understanding of how entomopathogens exploit each of the vectors described above is still in its infancy. Although common sense and experience from other livestock provide guidance for how to reduce the risk of insect-borne pathogens, feed-borne pathogens, and pathogens entering through the elements like wind and rain, specific understanding of infection pathways is still missing. Regarding insect-borne pathogens, we lack both detailed knowledge on the diseases that would follow a given pathway as well as on their hosts. The latter, especially, could be particularly useful for location-specific defenses, as BSF is now cultivated across the globe. Feed sources also differ widely. As more knowledge is gained about what the main classes of feed are, specific research into how each of these can harbor pathogens and pest insects is necessary.

Transmission of insect pathogens within facilities

Once a pathogen has entered a facility, multiple pathways exist for its spread. Infected insects may transmit and thereby spread disease to one another through various channels. Feed-borne pathogens may come into direct contact with insects, or they may multiply in the feed and become endemic in the facility. Spores of fungi especially may be transmitted through air flows. A robust system for controlling internal spread of pathogens will reduce the risk to all production activities in the event that a pathogen enters. In addition to protecting production animals, such a system may also prevent the spread of non-entomopathogens that are problematic for food and feed production.

Insects that spread disease by carrying it on their body should be prevented from moving freely inside the facility. This can be accomplished by reducing their mobility with effective barriers between facility sectors, or by trapping them. Different species are susceptible to different trapping methods, but glue traps and bug zappers, widely used in food production facilities, are effective baseline

technologies. A major source of insect vectors is the production population itself. Stressed animals, such as those that have received too little food, may become weak, and this will lower their immune status and make them susceptible to opportunistic pathogens causing disease. Infected animals then serve as feed sources for opportunistic pathogens to multiply, which increase the infection pressure on the healthy animals. Horizontal transmission between animals in production has been demonstrated by Maciel-Vergara et al. (2018), who showed that stressed larvae of *Zophobas morio* Fabricius are cannibalistic and rapidly spread the pathogen *Pseudomonas aeruginosa*. The BSF larvae could also resort to cannibalism under adverse conditions, as known from *M. domestica* (Lam et al., 2007), so this pathway of transmission could also be a risk for this species. Proper treatment of the insects is the best defense against spread of pathogens as animals with healthy immune systems and low aggression are less likely to harbor or spread diseases.

Tactics to prevent spread within the insect farm

Eilenberg et al. (2018) highlights two ways to reduce the likelihood of disease transmission inside insect facilities. First, when possible, set the environment in the facility to humidity and temperature values that are not conducive to fungi and bacteria. Doing so in advanced, closed facilities is easier if they are properly designed. For example, there is a method describing a mating cage for flies with the ability to deliver precisely controlled climate that can presumably induce optimal production behavior in the animals, but also control the multiplication of pathogens in the mating cage (Patent NL2020175B1). However, closed facilities with a poorly designed air-control system or layout may form pockets of climate that are exceptionally undesirable because the closed design of the building may magnify climate trends. In such instances, the simpler semi-open design could be more robust.

The second tactic for containing the spread of pathogens inside facilities that Eilenberg et al. (2018) highlight is to practice good hygiene in the facilities. Water and detergent cleaning are advised for controlling and preventing bacterial and fungal pathogens, whereas UV light is proposed as an effective way of controlling viruses. These types of sterilization are useful for preventing both horizontal and vertical spread of disease. Batch-wise production and cleaning of growth spaces have the benefit that diseases that develop in a production generation are disinfected in a cleaning step before another generation is introduced into the contaminated growth space. The industry has signaled the importance of hygienic practices in production facilities, primarily driven by the desire to comply with food and feed regulations. Although hygiene has typically been achieved by manual cleaning, publicly available sources show that companies are also developing automated systems to keep insect facilities clean. An interesting method describes a cage system for BSF mating which is capable of cleaning itself between each cycle (Patent NL2020154B1).

Cleaning is particularly important in controlling pathogens introduced into the facility via feed. The BSF production usually makes use of crates and troughs in which larvae are grown in the feed. Some processes operate batch-wise, whereas others use continuous processes in troughs. Without regularly cleaning the crates, pathogens introduced by feed potentially multiply to levels that are overwhelming for the larval immune system. By operating with a batch-wise process, grow containers are cleaned after each generation, and contaminated feed from a delivery is purged from the system. Regular cleaning of the storage infrastructure, such as silos, is also advisable for this reason. General recommendations for cleaning and disinfection are given by OIE (2019) in chapter 4.14 of the Terrestrial Code, and in manufacturer guidelines of commercial disinfectants.

In addition to proper climate control, air filters, and hygiene, spread of disease can also be reduced by physical barriers. In BSF production, modular production units are a useful means for maintaining physical barriers. Individual growth crates for larvae, housed in separated rooms per production batch reduce the risk of disease spreading between them (Patent NL2010666C). Isolated cages reduce the risk of disease spreading between mating cages. In addition to making it more difficult for diseases to spread by location, modular structures like these can also be designed to high climate controllability and cleanability specifications.

Conclusions and future research

As the BSF industry progresses into industrial scale production, the trend of increasingly sophisticated and controlled growth solutions will likely continue. Advanced facilities with capabilities to prevent infections from entering and stop any spread of infection within facilities will be utmost important. Insect pathogen defense and control solutions developed for advanced facilities may eventually trickle down in part to less-advanced facilities. However, semi-open facilities may still be preferable if a properly designed advanced solution is not possible, as the robustness of these simpler facilities is preferable to a badly designed closed facility, in which insect pathogens could amplify to high numbers. The most immediate action that any BSF farmer can take in his/her insect disease management is to send live insect samples to an insect disease

laboratory for diagnosis and specific advice. Second to this, buying a basic stereo-microscope and developing in-house dissection skills is a very low-cost step to having the ability to independently perform basic diagnoses and take measures. Each BSF farm should have a specialized veterinarian or biologist for regular preventive farm monitoring on insect diseases.

Fundamental research on insect pathogens potentially infecting BSF is vital. Black soldier flies, suggested to be native to North and/or South America, can now be found across tropical and temperate regions all over the world (Wang & Shelomi, 2017). Although their natural history precludes them from vectoring specific known pathogens to humans, there remains a risk of transmission from wild to cultured populations and therefore a need for more in-depth risk assessment. Future research should focus on discovery of potential pathogens in wild BSF and other Stratiomyidae populations, in order to more accurately predict the dangers to production facilities. Specialized diagnostic laboratories with specific tests are required, for metagenomics to discover relevant pathogens on diseased insects. Moreover, these laboratories also need to test for presence and transmission of potential pathogens from other (pest) insects to BSFs in production. Many production facilities are being developed in proximity to agricultural production and therefore, the risk of spill-over of biological control agents needs to be assessed alongside their effect on BSFs. Furthermore, the substrates used to feed the flies could themselves harbor potential pathogens endangering productivity. An interesting and relevant prevention topic is to apply or promote specific beneficial microorganisms in the process to suppress diseases. Introduction of beneficial microbes may be carried out to create a synergistic biome for the larvae, as described in Grau et al. (2017). As either a post-treatment step or as a standalone procedure, probiotic treatments may be effective at reducing the presence of pathogens as the standalone result was already achieved in Grau et al. (2017) for a model insect species. However, implementation of radiation pre-treatments and use of probiotics in BSF production facilities are so far unexplored and in an early stage, respectively. The above-mentioned studies could be used to support a proper epidemiological risk assessment throughout the production chain of insects, from substrate to end product.

Overall, we would like to stress the importance of collaboration and the coordination of international efforts to diagnose and manage potential diseases in BSF production systems. As is the case for most domesticated species, new diseases emerge over time. Research as discussed above will enable the industry to be better prepared with respect to prevention, early detection, and appropriate control.

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