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Hermetia illucens adults are susceptible to infection by the fungus *Beauveria bassiana* in laboratory experiments

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RESEARCH ARTICLE

Abstract

Rearing of the black soldier fly, *Hermetia illucens*, in mass production systems is increasing. Its use as both a bioconverter of organic waste and as feed for other livestock has transformed it into one of the most produced insects in the world. As a result, new research is needed to evaluate the risk of insect diseases affecting it and thus productivity. While some studies have focused on the larval stage of the flies, to date, few have assessed risks to the adult stage, vital to the production system. In this study, the susceptibility of adult black soldier flies to the entomopathogenic fungus, *Beauveria bassiana* KVL 03-122 was evaluated in laboratory experiments by quantifying mortality, egg-laying capacity, and sporulation when the flies were subjected to two concentrations of the fungus. The findings showed that adult flies are susceptible to the biocontrol agent *B. bassiana*, with high mortality and low egg count in the high dose treatment. Our results confirmed that adult black soldier flies appear susceptible to a fungal pathogen and we discussed the findings in relation to consequences for production.

Keywords: insect pathology, black soldier fly, infection

1. Introduction

The black soldier fly (BSF), *Hermetia illucens* (Diptera: Stratiomyidae), has been mass reared since around the mid-1990s as a method for managing vast amounts of organic waste and residuals from the agriculture and food product industry (Sheppard *et al.*, 1994). Since then, it has become one of the most important insects in the world for bioconversion and is being reared by multiple companies on an industrial scale (De Smet *et al.*, 2018). Its relatively high protein levels (from 37 to 63% dry matter) (Barragan-Fonseca *et al.*, 2017) as well as recent developments in the EU, authorising the use of BSF proteins as a feed ingredient for aquaculture has only increased the growth of the industry. However, such rapid expansion, from small-scale facilities to industrial scale production, means that several important aspects of BSF biology and its susceptibility to insect pathogens are still unknown. One concern is that pathogens that can result in partly or complete mortality of larval or adult populations remain unidentified (Tomberlin and Cammack, 2017). An understanding of the susceptibility of this insect species to potentially damaging insect pathogens is thus essential to avoid sudden serious damage in production just when other industries start to depend on them. Professional prevention measures, such as monitoring, are the first necessary tool to minimise insect pathogen introduction into the facilities (Lecocq *et al.*, 2019). Monitoring is to be accompanied with diagnostics, to react adequately, if a production batch anyway becomes infected, to prevent an outbreak throughout the facility.

The types of insect pathogens that may potentially infect BSF need to be identified and characterised, to ensure that these diseases can be prevented or controlled, and further spread avoided. Studies on fungal infections in insects have usually been limited to insect species that are pests (in agriculture or as vectors of vertebrate diseases) or are important beneficial species such as insects used for biological control or pollination. There are no published records of fungal infections for BSF in nature or in the laboratory.

Insect pathogenic fungi produce infective stages (spores, mostly conidia) that are released from infected insect cadavers (Gottwald and Tedders, 1982). When conidia reach a suitable host, they adhere to the exoskeleton, germinate, and penetrate through the cuticle (Boomsma *et al.*, 2014; Hajek and St Leger, 1994). The number of conidia released per host is dependent on fungus species, host species, and host size (Meyling and Eilenberg, 2007). Furthermore, the warm and humid conditions typical for BSF production systems, are ideal for the development of fungal epizootics (Carruthers and Soper, 1987; Eilenberg *et al.*, 2018).

Several isolates of the insect pathogenic fungus *Beauveria bassiana* (Ascomycota; order Hypocreales), are used as biological control agents against urban pests and agricultural pests in crop fields, forests, and greenhouses (Pell *et al.*, 2001). This species is an opportunist and a generalist, infecting many insect host species. The fungus occurs in or on several substrates and living organisms and even as a plant endophyte (Inglis *et al.*, 2001; Vega *et al.*, 2012). Natural dispersal of *B. bassiana* conidia occurs by wind, rain and insect activity (Hajek 1997; Inglis *et al.*, 2001; Meyling and Eilenberg, 2007; Shah and Pell, 2003). Dipteran species found naturally infected by *B. bassiana* include several fly species from the family Muscidae occurring indoor in high numbers at cattle farms: *Musca domestica*, *Musca autumnalis*, *Stomoxys calcitrans*, *Haematobia irritans*, *Haematobia stimulans*, *Hydrotaea* spp. and *Morellia* spp. (Skovgård and Steenberg, 2002; Steenberg *et al.*, 2001; Steinkraus *et al.*, 1990). The use of *B. bassiana* in biological control in agriculture and the occurrence of naturally infected insects in the environment may increase the risk of this fungus entering a BSF production facility via infected insects and other overspill from the environment.

The BSF has never been challenged with an insect pathogenic fungus such as *B. bassiana* so there is no available information on the effect of infections on BSF mortality and the consequences for egg laying. In this study, we report a new, adapted bioassay for assessing the susceptibility of BSF adults to the fungal pathogen *B. bassiana*. The specific strain used in this study was selected based on two main factors: 1) The *B. bassiana* isolate originated from a dipteran insect; 2) The isolate originated from Europe. The rationale follows from two studies that showed that *Beauveria* spp. infections in silk moth production facilities in China were due to local strains, which were unrelated to an exotic strain of *B. bassiana* used for biocontrol in forests of the same regions (Chen *et al.*, 2015, 2016).

2. Materials and methods

The flies

Pupae of BSF, *H. illucens*, were provided by Protix, Dongen, the Netherlands. All pupae were placed in an incubator at 30 °C and 60% R.H. until emergence. Within 24 h of emergence, flies were lightly sedated with CO_2, sexed and separated into individual plastic medicine cups (4×4 cm), ready for fungal exposure as detailed below. Once exposed to their respective treatment, 25 males and 25 females were grouped together and placed in a large Plexiglas cage (30×30×30 cm) and given *ad libitum* access to water in a falcon tube (Figure 1). The Plexiglas cages were also kept in the incubator at 30 °C and 60% R.H.

The procedure was repeated on three separate occasions, using a new batch of pupae every time. This resulted in a total of 450 flies in the experiment.

Fungal strain and conidia suspension

B. bassiana strain KVL 03-122, isolated from *Pegoplata aestiva* (Diptera, Anthomyiidae) collected from a Danish agroecosystem, was kept in the culture collection at the Department of Plant and Environmental Sciences, University of Copenhagen, Denmark, at -80 °C. It was cultivated on Sabouraud dextrose agar (SDA) and incubated at 23 °C for 14 days to allow for sporulation. Conidia was harvested by scrapping the surface of the culture with a sterile loop in 10 ml sterile water and subsequently the solution was filtrated over three layers of sterile gauze to eliminate hyphae and agar. The concentration of conidia was assessed with a haemocytometer (Neubauer improved) and the concentration was adjusted in sterile water to 10^5 conidia/ml (low concentration) and 10^8 conidia/ml (high concentration). A germination test was conducted to test the viability of the conidia by plating out 100 µl of the 10^5 conidia/ml (low concentration) on a SDA plate. After incubation for 24 h at 23 °C, the germination of 3×100 conidia was assessed. Only batches with conidia germination rates over 95% were used.

Bioassay

The flies were exposed to three treatments: control with demineralised water; water + low concentration of *B. bassiana* (10^5 conidia/ml); and water + high concentration of *B. bassiana* (10^8 conidia/ml). We used water for the suspensions for two reasons: first, initial trials proved that using Triton resulted in a very high mortality (almost instant after dipping in solution); second, the use of water better mimics a real situation in an insect production unit.

Exposure was carried out by gently picking the flies with soft tweezers and dipping them in the solution of their

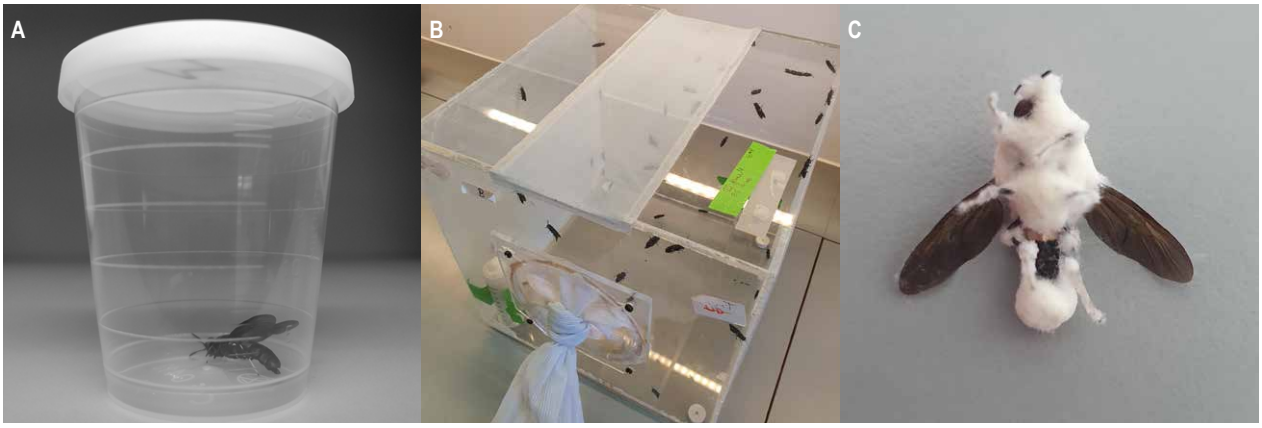

Figure 1. (A) Newly emerged flies were lightly sedated with CO_2, sexed and placed in individual medicine cups prior to exposure to fungal treatment. (B) Once all flies were exposed to the fungal pathogen or control treatments, they were released into Plexiglas cages and provided with water for the duration of the experiment. (C) Once an adult fly died, it was removed from the cage daily and stored individually in a moisty cup so an infected fly would sporulate.

respective treatments. After dipping, the flies were placed back inside a 30 ml medicine cup until all flies had been treated. Once all the flies were treated, they were placed in their respective Plexiglas cages as previously described. On the third day of the bioassay, an egg tray provided by Protix was placed above larval feed and placed inside the cages. Feed was composed of one-part laying hen pellets to 0.94 parts bran and 3.75 parts warm water. All egg trays were removed after 48 h (day 5) and the number of egg masses was counted. Mortality was recorded daily. Dead flies were removed and placed in individual 30 ml cups, lined with wet filter paper to ensure high humidity, for observation until the appearance of fungal spores.

Statistical analysis

The effect of the *B. bassiana* treatments on adult BSF mortality was assessed using Kaplan-Meier curves, with control, low concentration and high concentration as treatments and sex (M or F) as strata. A log-rank test was used to assess statistical differences between groups. The effect of the fungi on the fecundity as measured by the number of egg masses was assessed using a generalised linear mixed model with a Poisson distribution, with treatment as a fixed effect and replicate as a random effect. Multiple comparisons were adjusted using a sequential Bonferroni correction. Significance was measured at $P<0.05$. All tests and graphs were carried out in SPSS Version 25 (IBM Corp., Armonk, NY, USA).

3. Results

Effect of *Beauveria bassiana* on adult mortality

The effect of *B. bassiana* (Bb) on adult BSF mortality was assessed by collecting all the dead flies daily until the last fly died (Figure 2). There was a significant effect of both our treatments (LowBb/HighBb) and sex (F/M) on fly mortality, except in the high concentration treatment. While males lived significantly longer than females in the control treatment (X^2=20.2, df=5; $P<0.001$) and in the LowBb treatment (X^2=10.3, df=5; P=0.001), this effect disappeared in the HighBb treatment (X^2=1.1, df=5; P=0.285). Specifically, control flies (mean lifespan = 14.8±0.4 days) lived significantly longer (X^2=232.7, df=2; $P<0.001$) than HighBb flies (mean lifespan = 6.1±0.1 days) and longer (X^2=10.1, df=2, P=0.001) than LowBb flies (mean lifespan = 12.8±0.4 days).

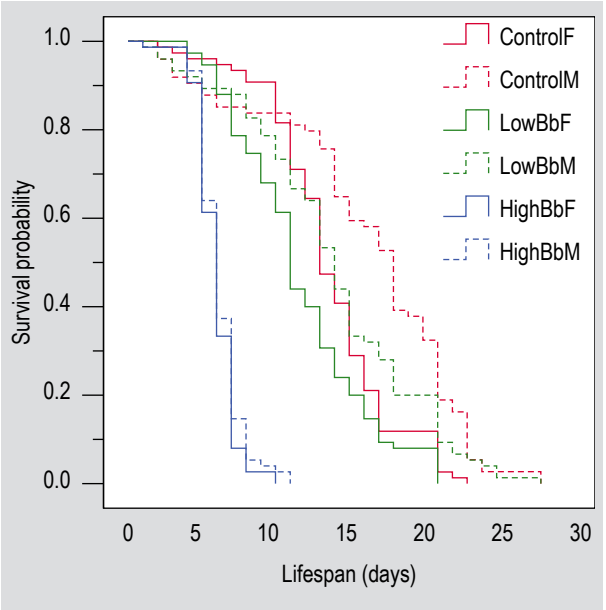

Figure 2. Survival probability of the adult BSF flies in the control, LowBb (10^5 conidia/ml) and HighBb (10^8 conidia/ml) treatments. There were significant differences between treatments and males (dashed lines) lived significantly longer than females (full lines) except in the HighBb treatment.

Sporulation of *Beauveria bassiana* on adult flies

Successful infection of the flies by the fungi was confirmed by observing sporulation from dead flies (Figure 1C). Only one fly was suspected to have been infected by the fungus in the control group because of potential contamination, resulting just in 0.7±0.6% overt infections. In the low concentration and high concentration treatments, we observed respectively, very successful infections, with an average of 36% and 96% of the flies sporulating (Figure 3).

Effect of *Beauveria bassiana* on egg laying capacity

Egg masses laid over the course of 48 h, between day 3 and 5, for each of the treatments were collected (Figure 4). We found overall significant differences based on treatment (F=4.3, df=2, *P*=0.005). With a mean of 3.3±1.1 egg masses, the females in the HighBb treatment laid significantly fewer egg masses than those in both the Control treatment (mean = 15.3±2.5, F=12.0, df=2, *P*=0.011) and the LowBb treatment (mean = 12.3±2.2, F=9.0, df=2, *P*=0.018). There were no statistical differences between the number of egg masses laid by the Control treatment and the LowBb treatment.

4. Discussion

In this study, the aim was to test the susceptibility of adult BSF to a fungal pathogen and its effect on the egg laying capacity of the females. To this end, we developed a bioassay method and demonstrated, for the first time, that adult BSF are susceptible to the fungus *B. bassiana* (strain KVL 03-122), in concentrations of 10^5 conidia/ml and 10^8 conidia/ml. We found that both the lower and higher concentrations had significant effects on mortality in the adult flies, ranging between 36 and 96%, respectively. This result is in line with

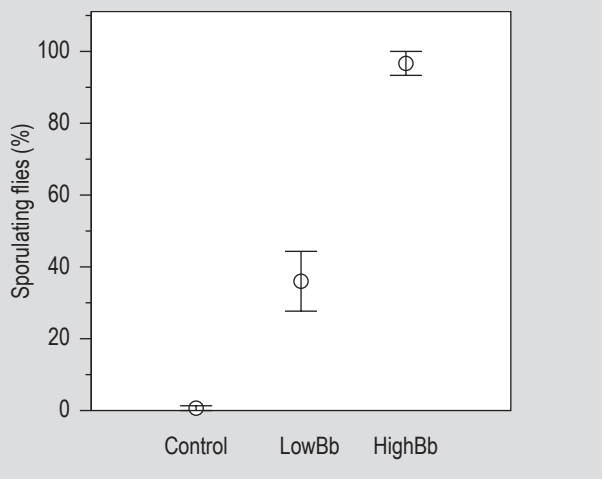

Figure 3. Average percentage of adult BSF flies with sporulation of *B. bassiana* after death, in the Control, LowBb (10^5 conidia/ml) and HighBb (10^8 conidia/ml) treatments. Bars represent ± 1 se from the mean.

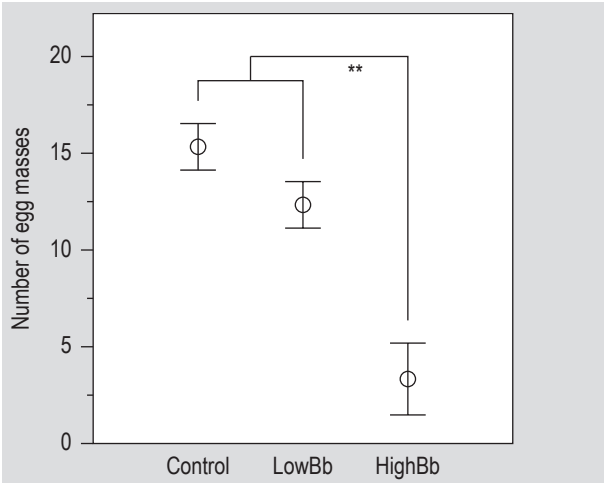

Figure 4. Average number of egg masses laid by female flies over the course of 48 h in the Control, LowBb (10^5 conidia/ml) and HighBb (10^8 conidia/ml) treatments. Bars represent ± 1 se from the mean. ** Denotes significant difference at <0.05.

other studies that tested the mortality of the adult stage of several dipteran species (although none from Stratiomyidae) caused by other strains of *B. bassiana* (De la Rosa *et al.*, 2002; Quesada-Moraga *et al.*, 2006; Watson *et al.*, 1995). In those studies mortality ranged between 70 and 90%, at a concentration of 10^8 conidia/cm^2, in the house fly and stable fly (Watson *et al.*, 1995) and between 30 and 100%, at concentrations ranging from 10^5 to 10^8 conidia/ml, in the Mediterranean fruit fly, *Ceratitis capitata* (Quesada-Moraga *et al.*, 2006). The differences in longevity observed between the sexes are also in accordance with recent research showing that BSF males tend to outlive female flies (Bertinetti *et al.*, 2019; Jucker *et al.*, 2019; Nakamura *et al.*, 2016). As with other fly species such as *Drosophila melanogaster*, this effect can be explained in terms of a cost associated with egg laying in females and affecting their longevity (Partridge *et al.*, 1987). However, in our case we found that such eventual difference was masked in the high concentration treatment since 96.7% of the flies showed overt symptoms post-death. This means that regardless of sex, the fungus was able to kill close to 100% of the flies in around 6 days. In addition, we found that female BSF exposed to the treatment with 10^8 conidia/ml were severely affected with regards to their egg-laying capacity. This is consistent with research in other dipteran species. For example, Quesada-Moraga *et al.* (2006) found that fecundity of the Mediterranean fruit fly was reduced by 50.8%, six days after treatment of both males and females (n=5 replicates containing 5 females and 5 males) with *B. bassiana* (10^6 conidia/ml). However, it is worth noting that Castillo *et al.* (2000) found no negative effect of *B. bassiana* infection on the survival and fecundity of females of the same species of Mediterranean fruit fly. Castillo *et al.* (2000) inoculated 2-day-old male and female flies while in our study, and Quesada-Moraga *et al.* (2006) newly emerged adult males and females were treated.

The application of *B. bassiana* as a biological control agent close to BSF facilities could have effects on the BSF industry. Indeed, house flies and stable flies, naturally infected by *B. bassiana* (Skovgård and Steenberg, 2002; Steenberg *et al.*, 2001), are potential pest species entering BSF production facilities. Therefore, closed facilities need to be designed to eliminate contamination risks. Furthermore, healthy flies interacting with cadavers, spores-contaminated drinking water, or spores-contaminated cages could be at a greater risk of infection than through vertical transmission by mating since females mostly mate only ones (Toledo *et al.*, 2014). Recently, Giunti *et al.* (2018) described BSF females as monogamist since multiple mating attempts were not observed by mated females in the presence of virgin males. Females contaminated with spores could also contaminate the oviposition sites where other females follow. However, more field-realistic studies still need to be conducted including determination of the minimum concentrations of spores required for such infections. Companies need to practice good hygiene in facilities, preferably use closed systems and batch-wise production (Eilenberg *et al.*, 2018). Water and detergent cleaning of all breeding facilities are advised for controlling and preventing fungal pathogens before a new generation is introduced into the breeding cages. While manual cleaning is in most cases the current practice, publicly available sources show that companies are also developing automated cage systems for BSF mating which is capable of cleaning itself between mating cycles (Jansen *et al.*, 2017).

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Conflict of interest

The authors declare no conflict of interest.

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