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# **Insect pathogenic fungi and desiccant dust against heat stressed bed bugs – maternal effects and response regulation from feeding**

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## ABSTRACT

The development of novel control options is a prerequisite for improved management of the insecticide resistant common bed bug *Cimex lectularius*. This laboratory study investigated the killing ability of insect pathogenic fungi *Beauveria bassiana* or desiccant dust (syloid) on bed bugs after having been exposed to sub-lethal heat stress at 32°C, 34°C and 36°C for 5 continuous days. The first instar nymphs were only indirectly exposed to sub-lethal heat through their parents. Heat stressed adults and their nymphs were exposed to fungi or syloid for 5 minutes or 3 days respectively. Maternal effects from heat stress passed to the next generation and drastically impacted the survival of first instar nymphs without showing any immediate effect on adults. Syloid caused 100% mortality within 3 days for adults or 2 days for nymphs whereas fungi had a slower impact with only 60% mortality after 20 days. The exposure to fungi or syloid appears to override the effect from previous heat stress and no striking synergistic or additive effects were found in susceptibility. Indications of a temperature dependent increase in mortality were observed in most treatments, but this was only significant among the offspring. Access to blood increased survival among the offspring, fungi treated offspring and syloid treated adults compared to starved individuals. The practical use of the result seems limited as no major synergies related to application of fungi or desiccant dust were detected, but it is shown that, heat stress in combination with denial of blood may improve the overall treatment success through reduced performance among adults and their offspring.

## INTRODUCTION

### The bed bug problem

Bed bugs have been a persistent pest in human societies (Usinger, 1966) for over 3000 years as evidenced by their retrieval from Egyptian archeological sites (Panagiotakopulu and Buckland, 1999). After the Second World War and to the beginning of 21century the bed bug problem was highly reduced in developed countries due to efficient insecticides and improvement of socio-economic conditions (Doggett et al., 2012, Koganemaru and Miller, 2013). From approximately year 2000, the bed bug has reoccurred as a major pest with multiple reports of their comeback in published literature, popular mainstream and social media (Boase, 2001, Doggett et al., 2018 in press, Doggett et al., 2012). Globalization and insecticide resistance are believed to be the main reasons for their recent resurgence (Davies et al., 2012, Romero et al., 2007, Seong et al., 2010, Gordon et al., 2014). Changes in pest control practices, lack of bed bug awareness and inadequate control measures are also important factors responsible for a wide spread bed bug outbreak (Doggett et al., 2018 in press).

Recent bed bug infestations have been reported from residential areas, hotels, schools, health care facilities, laundries, theaters, retail outlets and even at public transportations within communities. The flightless bed bugs are only able to move actively within apartments or between buildings in the proximity of their harborage (Cooper et al., 2015, Wang et al., 2010) whereas long-distance passive dispersal occurs by accidental transfer of human belongings with back packs, luggage, clothes, furniture etc. (Fountain et al., 2014). Human localities with brief occupancy and high turnover rates are more susceptible to bed bug outbreaks (Boase, 2001, Reinhardt and Siva-Jothy, 2007).

Bed bugs are an important cause of concern in public health. Infestation may cause skin reactions (itching, rash, and dermatitis), allergic hypersensitivity and secondary bacterial infections from scratching of bites. In rare cases, fever (Doggett et al., 2012) or asthma (Ashcroft et al., 2015) can also occur. The severity of skin reaction depends on the immune response of host and the number of repeated bite episodes (Araujo et al., 2009). Repeated bites can also result in anemia and iron deficiency (Pereira et al., 2013). Apart from physical stress, bed bugs may also cause anxiety, sleeplessness, discomfort, fear, delusions, nightmares, nervousness and agitation (Susser

et al., 2012, Ashcroft et al., 2015). Bed bug infestation can cause social embarrassment, social disgrace and social isolation (Usinger, 1966). Until recently it was believed that bed bugs do not spread pathogenic or blood-borne diseases; however, controlled laboratory studies have shown that bed bugs are capable of transmitting the parasite *Trypanosoma cruzi*, causative agent of Chagas disease (Salazar et al., 2014), and the bacteria *Bartonella quintana*, etiologic agent of Trench fever (Leulmi et al., 2015).

Bed bug infestations can cause huge financial loss in private and commercial households, hospitality industry and other sectors by litigation claims, complete replacement of infrastructure, productivity loss, costs from pest control, etc. (Reinhardt and Siva-Jothy, 2007, Doggett et al., 2012, Davies et al., 2012). The hospitality and housing industry fears bed bug infestation as it comes with the risk of adverse publicity and consequent legal action. Treating heavily infested households requires huge financial support that is nonetheless a problem for low-income communities due to financial limitedness and refusal to discard infested furniture (Wang et al., 2009, Singh et al., 2013). Housing organizations serving low-income populations are suffering most due to inadequate financial resources (Bennett et al., 2016). Improper management of bed bugs through inappropriate inspections, inefficient eradication attempts and lack of knowledge in multifamily settings increases the risk of further spreading of bed bugs and assist establishment to adjacent apartments (Bennett et al., 2016).

### **Conventional control strategies**

Once bed bugs are introduced, it can be very difficult to get rid of this pest depending on the intensity of infestation (Doggett et al., 2012). Heavy infestations require different control measures than light infestations. Management of all different stages of infestation (introduction, establishment, growth and spread) is equally important to achieve efficient control of bed bugs in an area (Doggett et al., 2012). Identification and inspection, management efforts and follow-up preventive measures are important for bed bug control in residential areas (Kells, 2006).

Regardless of various preventive methods (Vaidyanathan and Feldlaufer, 2013); active and passive monitoring traps, visual inspection and trained canines are useful for bed bug

identification (Bennett et al., 2016). Indications of bed bug infestation may include dark fecal marks on beddings, reactions from insect bites and of course spotting bed bugs themselves. Bed bugs exist in small numbers when they are first introduced in a new environment. Bed bugs hiding behavior and lower population size often makes the visual inspection difficult (Pinto et al., 2007). However, early identification is advantageous as bed bug control at this point is comparatively easier and requires no insecticide treatment at all (Cooper et al., 2016). In contrast, long-term, undetected and untreated populations lead to heavy infestations that are costly and difficult to eradicate. Delayed identification also poses higher risk of bed bug dispersal to neighboring apartments.

After identification, chemical and non-chemical control methods are important for effective bed bug control (Doggett et al., 2012). Bed bugs have mainly been treated with direct application of **insecticides** (Pinto et al., 2007) due to its widespread availability, easy usage, and low cost. Among insecticides the most common are – pyrethroids, carbamates, insect growth regulators, silicates, organophosphates, arylpyrroles, chlorfenapyr and neonicotinoids that are generally available in different forms as liquid, dust or spray. Studies have shown that over exploitation of insecticides and cross-resistance has induced multiple resistance mechanisms in most bed bug populations over time (Romero et al., 2007, Zhu et al., 2010). Behavioral mechanism of insecticide avoidance in bed bugs may lead to avoid area of insecticide treated harborage and consequently reduce the risk of lethal insecticide exposure (Romero et al., 2009). Physiological mechanism also contributes to resistance in bed bugs through the process of increased metabolic detoxification, reduced cuticular penetration, and decreased target-site sensitivity (Dang et al., 2017). In addition to bed bug resistance, insecticide application is also problematic due to health risks (Davies et al., 2012). Direct and continuous exposure to insecticides in and around bedrooms and living places is very unhealthy and poses risk to human health. Insecticide application should be kept at minimum level to avoid any possible negative health impacts on humans. Development of new insecticide with new modes of action, different formulation, and target-specific cuticular penetration should be pursued to potentially ease bed bug control.

Non-chemical control method includes physical and mechanical approaches. **Destroying infested furniture and equipment, reducing clutter and sealing cracks and crevices** are some fast and primary actions to reduce bed bug infestations. Hot steamers, heating bags, or heating

the entire room can be utilized for heat treatments. In case of **whole room heating**, some cold spots can arise where the core lethal temperature could be lacking and provide refuges for bed bugs (Koganemaru and Miller, 2013). **Hot steaming** is labour intensive and proper steaming often requires professionals (Kells, 2006). **Laundering and drying** of bedcovers and cloths at 60°C can kill all stages of bed bugs (Naylor and Boase, 2010). For cold temperature treatment, **liquid CO<sub>2</sub>, liquid nitrogen or freezer** (–20°C for 2 days) can be used to kill bed bugs (Benoit et al., 2009). It is also important to assure that bed bugs are not hiding in the areas where lethal core cold temperature is absent. **Bed encasement** seals an infestation inside. The **vacuuming** can help to remove many bed bugs from exposed refuges and keep the infestation low. However, vacuuming usually does not remove eggs properly as eggs are glued to the substrate. **Traps** are helpful for monitoring and to suppress the population when combined with other control measures. All these methods are important to reduce infestations, but repeated treatments are often necessary for effective control (Kells, 2006).

**Integrated Pest Management (IPM)** combines chemical, physical and mechanical approaches. IPM is considered as the best method for successful suppression of bed bug infestations (Bennett et al., 2016) because single method is usually not sufficiently efficient (Singh et al., 2013). The principles in IPM also include a combined package of prevention and or early detection, identification, education, treatment and monitoring that is highly important to fight bed bugs within a community (Koganemaru and Miller, 2013). IPM can reduce the risk of spreading and lower the cost of bed bug eradication by identification in early stages. Community wide awareness and continuous education is also important in an IPM method to reduce the risk of spreading, effective eradication, increase cooperation and to prevent misuse of insecticides. After eradication, a follow-up inspection is important to evaluate the efficacy of the control method as well as to identify any possible sign of existing infestation or re-infestation.

Apart from these control measures, predators and diseases can contribute for biological control of bed bugs. Many terrestrial arthropods may predate upon bed bugs such as spiders, ants, mites, pseudoscorpions etc. (Usinger, 1966) but predators as a control method has no practical application. In laboratory study, fungus and bacteria have been shown to cause mortality in bed bugs (Usinger, 1966, Barbarin et al., 2017, Barbarin et al., 2012, Aak et al., 2017).

In recent times, there is no single effective, cheap, easy and safe methods available to fight bed bugs and satisfactory competence of conventional control methods are occasionally lacking. Bed bug control is usually time consuming, energy demanding, labourious and expensive. Increasing bed bug problem demands invention of new cost-effective bed bug control methods. As bed bugs inhibit human dwellings, it is also important to develop control methods that poses minimal risk to human health. Since bed bugs develop resistance to insecticides, use of a wide range of control methods are necessary and research to increase promising techniques are highly needed (Wang et al., 2009, Rukke et al., 2015). Sub-lethal heat, insect pathogenic fungi and desiccant dusts are potential candidates to contribute towards effective bed bug control.

### **Sub-lethal heat**

Temperature is one of the most important factors that regulate insect's growth, development, activity and reproduction. Insects exposure to high temperature might cause desiccation, damage DNA molecules, denature proteins, alter pH, impair cuticle, deprive nutrients, and produce intoxicants that can lead to behavioral and physical impairment, and death (Chown and Nicholson, 2004, Kells and Goblirsch, 2011, Harrison et al., 2012). High temperature can also cause heat-knock down in bed bugs. Currently, temperature is considered as one of the major non-chemical approaches for bed bug control (Kells and Goblirsch, 2011, Doggett et al., 2012). Stress from thermal exposure for more than 24 hours may be considered as sub-lethal at 32 – 38°C, critical at 38 – 42°C and lethal at 43 – 49°C for bed bugs (Usinger, 1966, Pereira et al., 2009, Kells and Goblirsch, 2011, Rukke et al., 2015). Mortality from thermal stress strictly relies on both temperature and exposure time as recent studies revealed that a few minutes exposure to 60°C, an 1 hour exposure to 48°C, 2 days exposure to 40°C and a month exposure to 37°C can kill bed bugs efficiently (Omori, 1941, Benoit et al., 2009, Rukke et al., 2015). Commercial pest managers use temperature above 55°C for effective killing of all life stages of bed bugs within a few hours (Pereira et al., 2009, Kells and Goblirsch, 2011). Such elevated temperature treatments are highly energy demanding and may damage objects. Prolonged exposure to sub-lethal heat can be an additional bed bug control option since it bears the potential to provide a stressful environment to bed bugs outside their optimum temperature zone. Sub-lethal effects are reported



in several insects (Mahroof et al., 2005), however, effects from this stressor have not been much investigated for bed bugs.

## Insect pathogenic fungi

Insect pathogenic fungi act as a biologically based pesticide against a vast number of insect pests, including some blood sucking species (i.e. mosquitoes, tsetse fly, and ticks). *Beauveria bassiana*, *Aspergillus flavus*, *Isaria fumosoroseus*, and *Lecanicillium muscarium* were experimentally tested to control bed bugs under laboratory conditions and the first two have been found fatal for bed bugs (Barbarin et al., 2012, Aak et al., 2017). Conidia usually invade within 24 hours through mouth parts, inter-segmental folds and spiracles and induce infection. Infection begins by activation, germination and initial cuticular penetration and produce toxins to kill bed bugs (Hayek and St Leger, 1994). Temperature and humidity are both crucial for insect pathogenic fungal activity (Jaronski, 2010). *B. bassiana* has a distinctive mode of action with no known resistance or cross-resistance in bed bugs and also very effective on pyrethroid-resistant groups (Barbarin et al., 2017). It may also be horizontally transferred to individuals hidden in harborages and thus is a good candidate for bed bug control (Aak et al., 2017). Further studies are needed to understand the interaction between bed bugs and *B. bassiana* in terms of physiology, reproduction and behavior to reveal benefits and limitations of this potential killing agent.

## Desiccant dust

Desiccant dust products include either synthetic SiO<sub>2</sub> (CimeXa) or diatomaceous earth (Mother Earth) as an active ingredient and are generally effective against bed bugs. Direct contact to desiccant dust is important as dust would not act as a killing agent unless it adheres to the bed bugs cuticle (Anderson and Cowles, 2012, Akhtar and Isman, 2013). Bed bugs have the unique quality to preserve water through their epicuticular lipid that protects them against environmental stress (Ebeling, 1971). Desiccant dusts can destroy the protective wax layer from the waterproof cuticle to increase desiccation and ensure rapid death (Anderson and Cowles,

2012, Akhtar and Isman, 2013, Potter et al., 2014). Desiccant dust also works on insecticide resistant groups (Anderson and Cowles, 2012). However, desiccant dust tends to lose its effectiveness in the presence of moisture. Normally, desiccant dusts are recommended to use on specific locations such as – wall voids, cracks and crevices, furniture stands, and beneath carpets due to precautionary measures to minimize human exposure (Koganemaru and Miller, 2013).

### **Potential benefits of using multiple stressors**

Individual stressor may be functional with lower efficacy but the sequential exposure effects from different stressors may put a stronger negative pressure upon the population. This is often necessary for effective control. Sub-lethal heat may have the potential to make bed bugs more susceptible for other conventional control measures (Benoit, 2011, Koganemaru and Miller, 2013). Thermal stress may increase desiccation, cuticular permeability and metabolic rate in insects. Elevated metabolic rate may also increase depletion of food reserve and respiration (Hansen et al., 2011). These factors can be detrimental for bed bugs if utilized with further exposure of killing agents after sub-lethal heat stress. Elevated heat stress may increase bed bugs movement and assist them to come in contact with killing agents. Multiple stressors can also suppress the detoxification mechanism and increase toxicity in bed bugs. Earlier studies also suggest that bed bugs are more susceptible to insecticides after being stressed (Doggett et al., 2012). Therefore, addition of killing agent to sub-lethal heat stressed bed bugs may increase mortality by being additive or synergic (more than additive) – and if so, such combined efforts can be a valuable management tool. Bed bugs have not been subjected to such investigations prior to this study.

### **Study aim**

The aim of this study was to investigate if it is possible to enhance the mortality of bed bugs using multiple stressors. The adults and their first instar nymphs were exposed to insect pathogenic fungi or desiccant dust after having been exposed to sub-lethal heat treatment (the nymphs were only indirectly exposed to sub-lethal heat through their parents) and the following predictions were made:

- 1) the bed bugs will suffer no mortality with 5 days exposure to sub-lethal heat treatment at 32°C, 34°C, and 36°C;
- 2) the treatment by fungi after exposure to sub-lethal heat will reduce the survival of bed bugs more than fungi treatment alone;
- 3) the treatment by desiccant dust after exposure to sub-lethal heat will reduce the survival of bed bugs more than desiccant dust treatment alone;
- 4) the lack of feeding may increase mortality among bed bugs compared to fully fed individuals.

## MATERIALS AND METHODS

### Study species

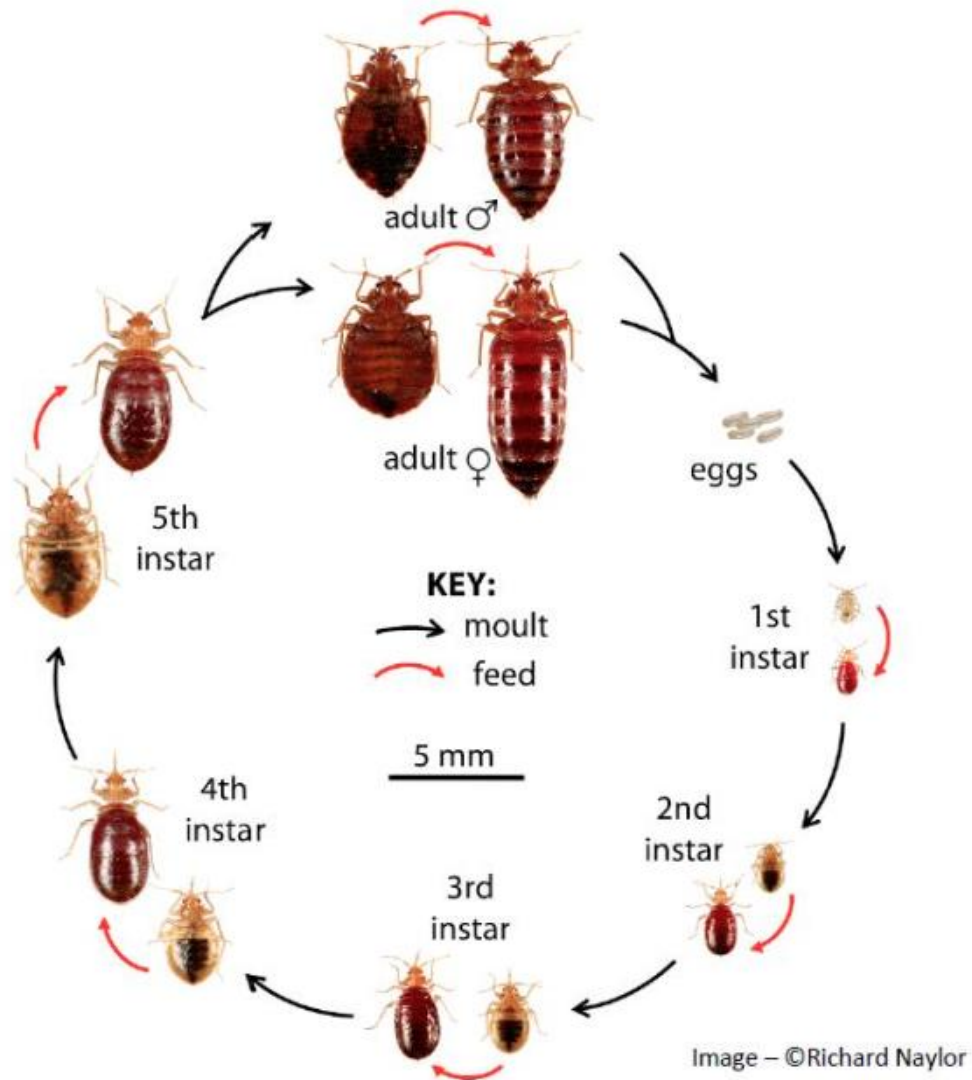
The bed bug belongs to the order Hemiptera and the family Cimicidae. This family represents more than 90 blood sucking ectoparasites and most are associated with birds or bats. Only three species of cimicids have made the switch to using humans as the primary hosts; namely – the common bed bug *Cimex lectularius*, the tropical bed bug *Cimex hemipterus* and the bat bug *Leptocimex boueti*. However, this paper will only focus on the common bed bug.

Bed bugs are flightless, chestnut brown colored and dorso-ventrally flattened small insect. Adults are approximately 5 mm long and weigh up to 5 mg. After a blood meal, the length may increase by 30 – 50% and the weight by 150 – 200% (Usinger, 1966, Araujo et al., 2009). Females are oval in shape while males are slender and pointy at the end. Adult males actively seek out freshly fed females for mating and deliver sperm inside the female body cavity instead of utilizing the genital tract (Reinhardt and Siva-Jothy, 2007). This insemination process is called traumatic insemination. Adult female lays 200 – 500 eggs during its life time mainly in and around their harborages and egg takes 4 – 21 days to hatch at optimum temperature (20 – 22°C) (Harlan, 2006). Bed bugs possess a total of 5 nymphal stages, each of the instars needs at least one blood meal to go for the next level and finally moult to the adult stage (Omori, 1941). The first instar nymph is translucent and approximately one millimeter long whereas the fifth instar

nymph is more identical in appearance with adults. The average life span of adults is 6 – 12 months (Usinger, 1966).

Bed bugs usually reside in groups in partly dark, dry, rough and protective harborages near their host. The different hiding places usually includes mattresses, all kind of furniture, crevices and cracks of the walls, curtains, electrical boards, floors, underneath carpets etc. Bed bugs can locate these sites by the presence of chemical cues such as aggregation pheromones (Reinhardt and Siva-Jothy, 2007, Romero et al., 2009). Typical harborages contain adults, nymphs, eggs, eggshells, moulted skins, and feces. Bed bugs leave their harborages in search of food and return to it after ingesting their blood meal (Reinhardt and Siva-Jothy, 2007, Aak et al., 2014).

Blood feeding is obligatory for mating, egg production, longevity and completion of entire life cycle (Figure 1) (Usinger, 1966, Reinhardt and Siva-Jothy, 2007, Benoit et al., 2007). Generally, bed bugs are active during nights searching for a blood meal when the host is asleep (Araujo et al., 2009). In absence of humans, they may feed on birds, poultry, pets, mice, rats and bats (Rozendaal, 1997). Adult bed bug requires 10 – 20 minutes for full engorgement (Usinger, 1966). After having blood meal bed bugs gather inside their harborages and remain aggregated during the digestion period. Females are more operative and quick to responds to host signals (Aak et al., 2014). Bed bugs detect hosts mainly by body heat and carbon dioxide (Usinger, 1966), but odors from the skin plays a minor role (Reinhardt and Siva-Jothy, 2007). Once fed bed bug does not require another blood meal for a week and can live for several months without a host by their particular adaptation of preserving water within the body (Usinger, 1966, Benoit et al., 2007). This unique feature also provides bed bugs with a strong stress tolerance that enables them to persist for a long time in storage, inside furniture or in vacant rooms (Reinhardt and Siva-Jothy, 2007, Benoit, 2011).



**Figure 1.** The life cycle of the common bed bug (*Cimex lectularius*) showing eggs, five nymph instars, and both sexes (all stages in fed and unfed conditions) (The illustration was taken from *The Bed bug*, 2013, European code of practice, version 2).

## Bed bug cultures

The laboratory strain of bed bugs was obtained from Oslo, Norway. The population has been cultured in the laboratory since 2009. The stock is maintained systematically by providing appropriately heated human blood in every 2 weeks (Aak and Rukke, 2014). The population was nurtured at a suitable temperature of 22°C with 60% relative humidity (RH) at a daily photoperiod of 16:8 (L/D).

## Climate chambers, experimental boxes and technical equipment

The whole experiment was conducted inside Sanyo climate chambers (MLR-351H, Medinor ASA, Oslo, Norway). The four climate chambers were set at 22°C, 32°C, 34°C, and 36°C. The 22°C is hereafter denoted as room temperature. The temperature and humidity were monitored by tiny tag data loggers (Presisjonsteknik, Oslo, Norway). Experimental bed bugs were kept inside 140 ml polyethylene boxes (VWR straight sample container, VWR, Oslo, Norway) throughout the study. Each experimental box had a lid with a circular opening of 40 mm diameter perfectly secured by a metal mesh screen (0.25 mm openings; Burmeister AS, Oslo, Norway) that allows aeration and blood feeding without handling the bed bugs. A folded filter paper (Whatman No.1, 47 mm) was used in each box to provide a substrate for the bed bugs and to allow deposition of eggs.

## Killing agents

The insect pathogenic fungi was obtained from the product BotaniGard 22WP (*B. bassiana* strain GHA,  $2 \times 10^{13}$  cfu/kg, Laverlam International, USA). Conidia was suspended in water according to the manufacturer's instructions to provide a 0.02% conidia suspension. To make a substrate that could be used to expose bed bugs to conidia, circular patches of Jersey knit cotton with a diameter of 47 mm were dipped into the fungal solution. The patches were allowed to dry on petri dishes for 3 – 4 days at room temperature. To confirm functionality of the product all bed bugs that died after exposure to conidia were checked for mycosis by drying over silica gel for 5 days (Lacey, 2012, Navon and Ascher, 2000). Then dried carcasses were transferred onto a wet filter paper (Whatman No.1, 90 mm), covered with a transparent lid, sealed by parafilm and kept at room temperature for few days. White fungal growth on bed bugs indicated that the cause of death was fungi.

The desiccant dust product Syloid 244 FP (GRACE GmbH & CO, Germany) (hereafter denoted as syloid) was used as the second killing agent. Syloid is synthetic amorphous silica powder (99.6% SiO<sub>2</sub>) with a particle size of 5.5 µm. Syloid is comparable to the commercial product CimeXa.  $0.62 \pm 0.04$  mg of syloid was used as a coating on fresh filter papers (Whatman No.1,

47 mm) to produce an effective dose of 0.18 g/m<sup>2</sup>. Fresh filter papers were put into these petri dishes and shook very well to ensure even and proper coating of dust.

## Experimental protocol

Prior to the onset of the experiments, fifth instar nymphs were collected from the stock cultures and fed for 15 minutes. Fully engorged nymphs were transferred to new boxes and kept in room temperature for 2 weeks to moult. Freshly molted adults were sexed by checking their genitalia before a blood meal was offered. Only fully fed individuals were included in the experiment. During day 1 – 5 of the experiment, the bed bugs received temperature stress according to the experimental design (Table 1) and on day 6 a new blood meal was offered to half of the bed bugs in each temperature treatment, while the rest remained starved. On day 7, the bed bugs were exposed to the killing agents or simply kept on clean filter papers to measure the separate effect from the heat stress. For fungi exposure, bed bugs were allowed to crawl over the conidia loaded substrates for 5 minutes before being put back into new boxes with clean filter papers. For syloid exposure, bed bugs were released to syloid coated filter papers and allowed to crawl on them until the end of the experiment. During the following 20 days, mortality in this *adult experiment* was registered on a daily basis for 10 days and then every other day until day 27 of the experiment. Bed bugs that appeared dead were checked by exhaling a breath of air into the polyethylene boxes. If they did not move, a soft brush was used to check if they were still alive. The adult experiment was terminated on day 28, but at this stage, the bed bugs in the control and the bed bugs having received only heat stress had produced offspring. Their first instar nymphs were collected and transferred from one box to another by a soft brush with extreme caution. Half of these nymphs were fed to full engorgement and the rest remained starved. These nymphs were kept at room temperature and consequently never received any direct heat stress. The nymphs either were kept on clean filter paper or were exposed to the two killing agents in the same way as the adults. During the following 20 days, the mortality in this *offspring experiment* was registered on a daily basis for 10 days and then every other day until day 50 of the experiment.

## Experimental treatments

A total of 1200 bed bugs were used in the experiment and the gender and fed – unfed ratio was perfectly balanced between all treatments (Table 1). 208 experimental boxes were used and each box contained either 3 males and 3 females (*adult experiment*) or 6 first instar nymphs (*offspring experiment*). Each experimental box represents a replicate. The design reflects a subset of a population that includes parents and second-generation nymphs, and provides 72 – 120 individuals in each experimental condition. All adult individuals were fed initially, while only half of the individuals received blood during the experiment.

**Control:** A total of 6 adult boxes and 6 offspring boxes were used as the control. They were kept at room temperature (22°C) for the entire experiment, provided blood or remained starved, and handled in parallel with other experimental treatments.

**Sub-lethal heat stress:** A total of 6 adult boxes and 6 offspring boxes were used to measure the effect from heat stress alone. Adults received heat stress for 5 continuous days at either 32°C, 34°C, and 36°C inside the climate chambers, whereas offspring effects was measured on first instars produced by the same adults after the heat stress.

**Mortality from baseline fungi:** A total of 10 adult boxes and 10 offspring boxes were used to provide measures of the effect from fungi alone. The bed bugs were exposed to the fungi and kept at room temperature (22°C) for the entire experiment, provided blood or remained starved, and handled in parallel with other experimental treatments.

**Mortality from baseline syloid:** A total of 10 adult boxes and 10 offspring boxes were used to provide measures of the effect from the syloid alone. The bed bugs were kept at room temperature (22°C) for the entire experiment, provided blood or remained starved, and handled in parallel with other experimental treatments.

**Fungi exposure after sub-lethal heat stress:** A total of 10 adult boxes and 10 offspring boxes were used to test the efficacy of fungi in a population having experienced heat stress. Adults received heat stress for 5 continuous days at either 32°C, 34°C, and 36°C inside the climate chambers before being exposed to the fungi. Offspring produced by heat treated adults



were similarly exposed to the fungi to measure any indirect effects from the heat on the new generation.

**Syloid exposure after sub-lethal heat stress:** A total of 10 adult boxes and 10 offspring boxes were used to test the efficacy of syloid in a population having experienced heat stress. Adults received heat stress for 5 continuous days at either 32°C, 34°C, and 36°C inside the climate chambers before being exposed to syloid. Offspring produced by heat treated adults were similarly exposed to syloid to measure any indirect effects from the heat on the new generation.

**Table 1.** Experimental design showing fungi or syloid treatments on *C. lectularius* after being directly (adults) or indirectly (first instar nymphs) heat stressed. Code denotes \* = survival measures, blood = 15 minutes access to blood, fungi = 5 minutes exposure to conidia, and syloid = cronic exposure to syloid at a rate of 0.18 g/m<sup>2</sup>.

Day	Control		Fungi		Syloid		Heat stress							
	blood	22°C	blood	22°C	blood	22°C	blood	32°C	34°C	36°C	blood	32°C	34°C	36°C
0														
1														
2														
3														
4														
5														
6														
7														
8														
9														
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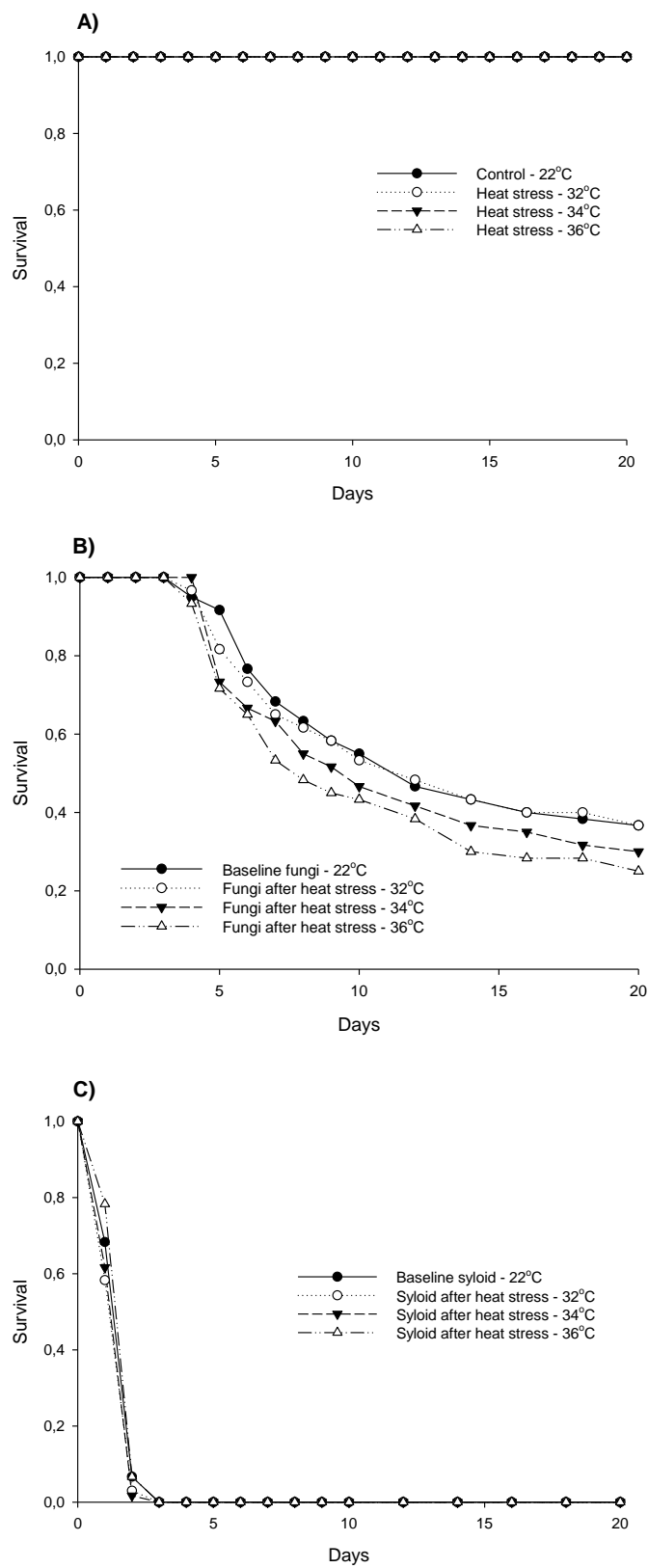
## Statistical methods

Analysis were performed using SigmaPlot, 13.0 (Systat Software, San Jose, CA, USA) and JMP Pro 13.0.0 (SAS institute, Cary, NC, USA). The Kaplan-Meier product limit method with the log-rank test between groups was used for survival analysis. It was also used to check if there was any effect from previous heat stress in the fungi or syloid treatments. The data were checked for normality, and multiple comparisons were performed using two-way ANOVA and pairwise comparisons were performed using t-tests. If tests for normality failed, the nonparametric alternatives Mann-Whitney rank sum test was used for t-test. The level of significance was set to 0.05 for all tests. The averages are always given with  $\pm$  standard error (SE).

## RESULTS

### The effect of heat, fungi or syloid on adults

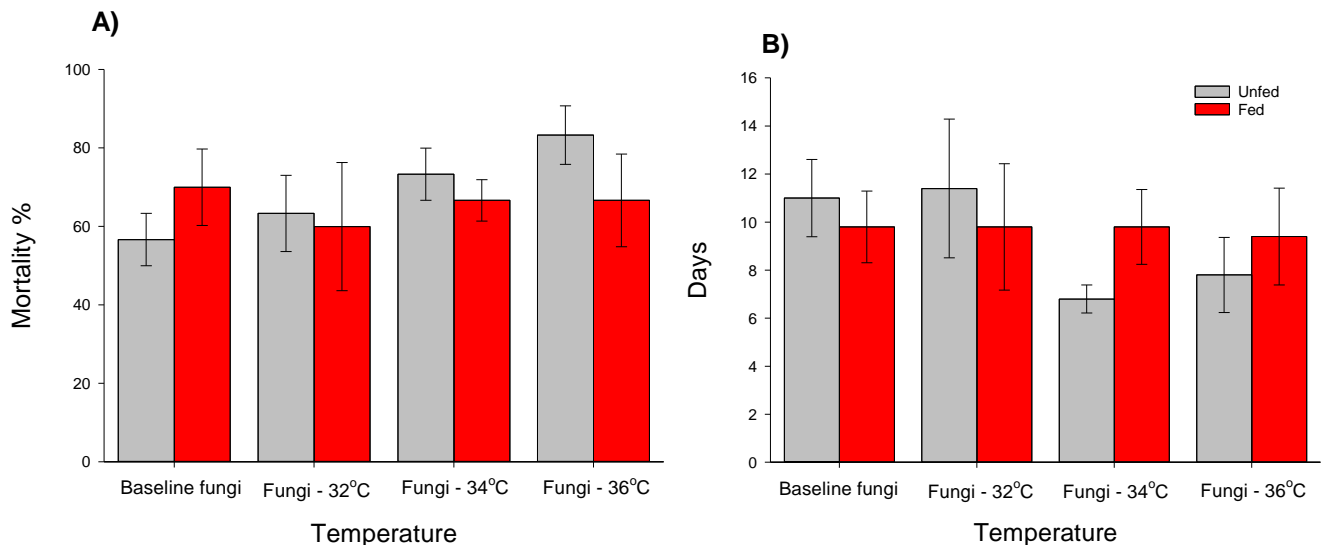
No deaths occurred when adults were treated with sub-lethal heat at 32°C, 34°C, and 36°C and there was no adult mortality in the control at 22°C (Figure 2A). The introduction of either fungi or syloid induced significant lower survival relative to the untreated control for all temperature regimens (Kaplan-Meier log-rank test (*only least significant test shown*); *baseline fungi - 22°C vs control - 22°C*:  $\chi^2 = 35.13$ ,  $df = 1$ ,  $p \leq 0.001$ , Figure 2B vs 2A; *baseline syloid - 22°C vs control - 22°C*:  $\chi^2 = 89.34$ ,  $df = 1$ ,  $p \leq 0.001$ , Figure 2C vs 2A). There was observed a slow reduction in survival from fungi where the first adult died at day 4 and 50% mortality was reached at day 12 and at the end of the experiment less than 40% survived (Figure 2B). A rapid decrease in survival was observed among adults at day 1 and 100% died within day 3 when syloid was the cause of death (Figure 2C).



**Figure 2.** Survival of adult *C. lectularius* after exposed to treatment at A) sub-lethal heat stress, B) fungi after sub-lethal heat stress, and C) syloid after sub-lethal heat stress.

## The effect of fungi after sub-lethal heat stress on adults

Survival was comparable between the adult bed bugs that were exposed to fungi and those that were exposed to fungi after sub-lethal heat stress. No significant difference in survival were found between different temperatures (Kaplan Meier log-rank tests:  $\chi^2 = 3.56$ ,  $df = 2$ ,  $p = 0.16$  (*only most significant test shown*), Figure 2B) but the final survival after 20 days was slightly lower among heat stressed adults and ranged from 22% to 38%. As there appeared to be subtle temperature dependent differences in final survival and in the time of death, these minor effects were investigated by separating fed and unfed individuals across the temperature stress. No significant differences were found in the final mortality (two-way ANOVA, Table 2A) when temperature and feeding were included as single factors with interaction, but unfed adults showed a tendency for a mortality gradient of 64%, 74%, and 84% according to 32°C, 34°C and 36°C, respectively, as opposed to the fixed mortality of approximately 60% among the fed individuals (Figure 3A). The time it took to reach 50% mortality was not significantly different for fed and unfed adults (two-way ANOVA, Table 2B), but a potential temperature dependency was also observed here, as unfed bed bugs reached 50% mortality approximately 2 - 4 days earlier than the fed bed bugs at 34°C and 36°C (Figure 3B).



**Figure 3.** Effects of starvation on *C. lectularius* adults exposed to fungi after sub-lethal heat stress showing A) final mortality, and B) time to reach 50% mortality.

**Table 2.** Mortality of *C. lectularius* adults exposed to fungi after sub-lethal heat stress. Two-way analyses of variance were used to test the effects of temperature, feeding and their interactions showing A) final mortality, and B) 50% mortality.

A)						B)					
Source	DF	SS	MS	F	P	Source	DF	SS	MS	F	P
Temp	2	905,505	452,753	0,865	0,434	Temp	2	27,800	13,900	0,599	0,558
Feeding	1	592,474	592,474	1,132	0,298	Feeding	1	12,033	12,033	0,518	0,479
T x F	2	240,393	120,196	0,230	0,796	T x F	2	31,267	15,633	0,673	0,519
Residual	24	12,557,585	523,233			Residual	24	557,200	23,217		
Total	29	14,295,957	492,964			Total	29	628,300	21,666		

### The effect of syloid after sub-lethal heat stress on adults

Because the syloid was highly efficient and 100% mortality was reached in all treatments within 3 days, any minor differences from temperature stress was difficult to identify. There was no significant difference between temperatures among fed (Kaplan Meier log-rank test (*only largest difference shown*); *baseline syloid vs syloid after heat stress at 32°C*:  $\chi^2 = 0.22$ ,  $df = 1$ ,  $p = 0.63$ , or unfed adults *baseline syloid vs syloid after heat stress at 36°C*:  $\chi^2 = 0.06$ ,  $df = 1$ ,  $p = 0.8$ ). Temperature was consequently removed as a factor by pooling all temperature treatments within the same group and investigating survival for day 1, 2 and 3 according to feeding status (Table 3). A significant effect from feeding on survival was found among adults at day 1 (t-test,  $t = 3.16$ ,  $p = 0.004$ ) and at day 2 (Mann-Whitney rank sum test,  $T = 195$ ,  $p = 0.018$ ), showing that fed individuals lasted slightly longer.

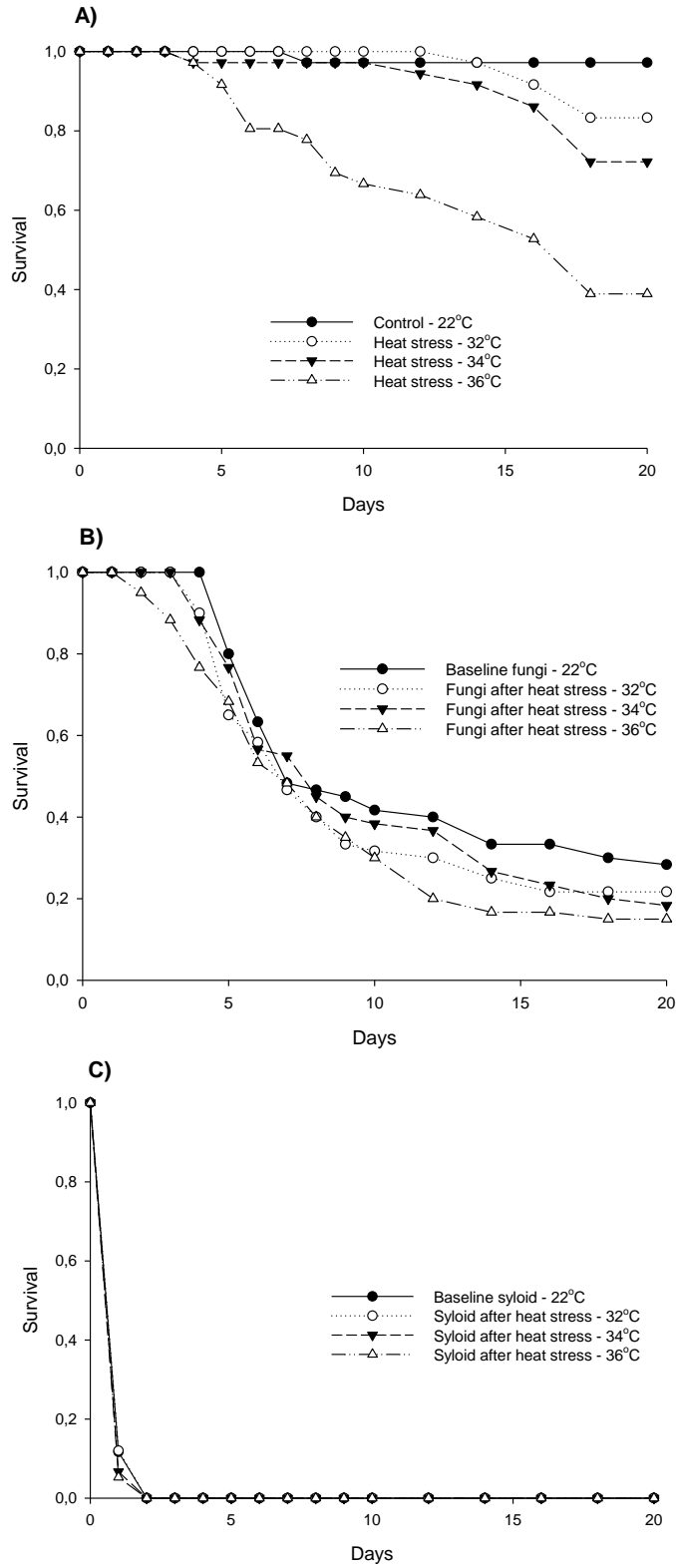
**Table 3.** Average  $\pm$  SE percentage survival of *C. lectularius* adults exposed to syloid after sub-lethal heat stress showing access to feeding increased survival among the fed compared to unfed individuals.

Days	Fed	Unfed
1	80.0 $\pm$ 5.4 %	52.2 $\pm$ 6.8 %
2	8.0 $\pm$ 3.2 %	0.0 $\pm$ 0.0 %
3	0.0 $\pm$ 0.0 %	0.0 $\pm$ 0.0 %

## The effect of maternal heat stress on offspring

The nymphs who indirectly experienced the heat through their parents showed reduced survival with increasing heat and sub-lethal stressors at two uppermost temperatures were significantly different from the control (Kaplan-Meier log-rank tests;  $\chi^2 = 3.74$ ,  $df = 1$ ,  $p \leq 0.05$  (*only least significant test shown*), Figure 4A). Only a single nymph died in the control at 22°C, whereas the temperature stressed adults produced nymphs with more than 18% final mortality (Figure 4A).

Fed and unfed offspring were compared across temperatures and both feeding and temperature was found to be significant factors (two-way ANOVA, Table 4). Unfed nymphs showed a significant mortality gradient of 34%, 56%, and 100% according to the indirect heat stress at 32°C, 34°C, and 36°C respectively whereas only 23% of the fed individuals died at 36°C indirect heat stress (Figure 5).

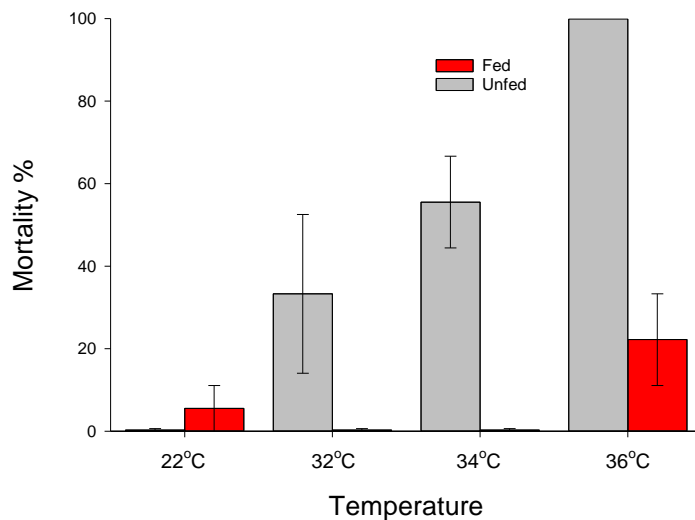


**Figure 4.** Survival of first instar *C. lectularius* nymphs after exposed to treatment at A) indirect sub-lethal heat stress, B) fungi after indirect sub-lethal heat stress, and C) syloid after indirect sub-lethal heat stress.



**Table 4.** Final mortality of first instar *C. lectularius* nymphs exposed to indirect sub-lethal heat stress. Two-way analysis of variance was used to test the effects of temperature, feeding and their interactions.

Source	DF	SS	MS	F	P
Temp	2	6,420,025	3,210,012	10,403	0,002
Feeding	1	13,887,778	13,887,778	45,005	<0,001
T x F	2	1,481,852	740,926	2,401	0,133
Residual	12	3,702,963	308,580		
Total	17	25,492,617	1,499,566		



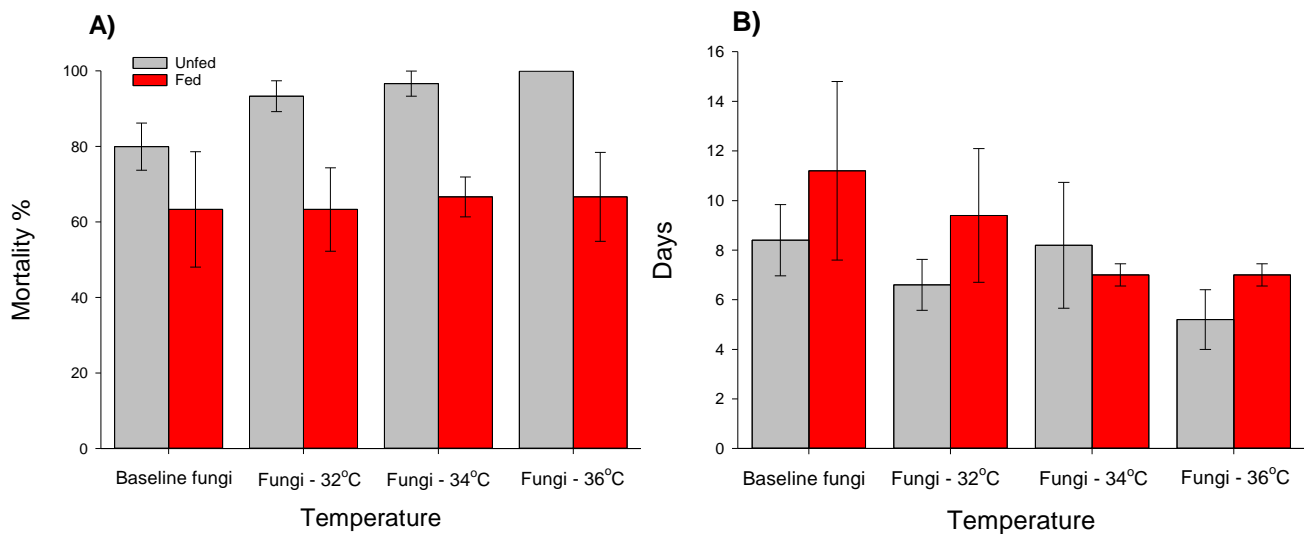
**Figure 5.** Effects of starvation on first instar *C. lectularius* nymphs exposed to indirect sub-lethal heat stress through their parents showing final mortality.

### The effect of fungi or syloid on offspring

The introduction of either fungi or syloid on nymphs induced significant lower survival relative to the untreated control (Kaplan-Meier log rank test (*only least significant test shown*); *baseline fungi – 22°C vs control - 22°C*:  $\chi^2 = 40.5$ ,  $df = 1$ ,  $p \leq 0.001$ , Figure 4B vs 4A; *baseline syloid vs control – 22°C*:  $\chi^2 = 102.83$ ,  $df = 1$ ,  $p \leq 0.001$ , Figure 4C vs 4A). There was observed a slow survival rate from fungi and a rapid reduction in survival from syloid (Figure 4B and Figure 4C) but nymphs died 1-2 days earlier, as compared to the adults.

## The effect of fungi on indirectly heat stressed offspring

Effects of fungi on offspring produced by heat stressed adults did not differ significantly from the fungi treatment (Kaplan-Meier log rank test; (*only least significant test shown*) *baseline fungi vs fungi on indirectly heat stressed nymphs at 36°C*:  $\chi^2 = 3.71$ ,  $df = 1$ ,  $p = 0.053$ , Figure 4B). By separating fed and unfed individuals, feeding was found to be a significant factor among offspring (two-way ANOVA,  $F = 27.51$ ,  $df = 1$ ,  $p \leq 0.001$ , Table 5A) that reduced final mortality from more than 90% among the unfed, to approximately 60% among the fed individuals (Figure 6A). However, the time it took to reach 50% mortality was not significantly different for fed and unfed nymphs (two-way ANOVA, Table 5B) but an overall tendency towards reduced survival time with increasing temperatures could be observed (Figure 6B).



**Figure 6.** Effects of starvation on *C. lectularius* nymphs exposed to fungi after indirect sub-lethal heat stress showing A) final mortality, and B) time to reach 50% mortality.

**Table 5.** Mortality of first instar *C. lectularius* nymphs exposed to fungi after sub-lethal heat stress. Two-way analyses of variance were used to test the effects of temperature, feeding and their interactions showing A) final mortality, and B) 50% mortality.

A)						B)					
Source	DF	SS	MS	F	P	Source	DF	SS	MS	F	P
Temp	2	129,670	64,835	0,246	0,784	Temp	2	20,067	10,033	0,725	0,494
Feeding	1	7,260,607	7,260,607	27,512	<0,001	Feeding	1	9,633	9,633	0,696	0,412
T x F	2	18,515	9,257	0,0351	0,966	T x F	2	21,667	10,833	0,783	0,468
Residual	24	6,333,667	263,903			Residual	24	332,000	13,833		
Total	29	13,742,459	473,878			Total	29	383,367	13,220		

## The effect of syloid on indirectly heat stressed offspring

The highly efficient syloid induced 100% mortality within 2 days in all offspring produced by heat stressed adults. Even when temperature was removed as a factor by pooling all temperature treatments within the same group, rapidly dying nymphs showed no significant difference in survival according to feeding status at day 1 (Mann-Whitney rank sum test,  $T = 119$ ,  $p = 0.58$ ) or at day 2 (Table 6).

**Table 6.** Average  $\pm$  SE percentage survival of first instar *C. lectularius* nymphs exposed to syloid after indirect sub-lethal heat stress showing access to feeding failed to increase survival among the fed compared to unfed individuals.

Days	Fed	Unfed
1	5.0 $\pm$ 2.5 %	2.0 $\pm$ 1.8 %
2	0.0 $\pm$ 0.0 %	0.0 $\pm$ 0.0 %

## DISCUSSION

This study is the first report to investigate the potential of bed bug control methods using insect pathogenic fungi or syloid after sub-lethal heat stress. The effects from these stressors were analyzed individually and sequentially on heat stressed parents and their first instar nymphs, where half of the individuals got access to blood and the rest remained starved. Several important

aspects derive from this study. First, it was observed that sub-lethal heat in the range of 32 – 36°C for 5 days causes no mortality, but induces negative effects that flow towards the next generation. Second, syloid was confirmed as an efficient low-dose bed bug killing agent that causes faster mortality than fungi. Third, the exposure to fungi or syloid appears to override any additive or synergistic effects from heat stress to leave only minor differences between these three temperatures. Finally, feeding is crucial for bed bugs as an aid against stressors since starvation induces faster mortality and more final deaths compared to fed individuals.

Recent studies have investigated the direct heat exposure effect on adults at 34 – 40°C for an exposure time of 3, 6 and 9 days (Rukke et al., 2015), and 34 – 38°C constant or disrupted exposure for two or three weeks (Rukke et al., 2017). Both studies also examined the maternal effects regarding feeding, fertility and development on offspring. This study investigated the effects of the temperature at 32 – 36°C with exposure for 5 continuous days and also examined the maternal effects on second generation offspring. Bed bugs exposed to 34°C, 35.5°C and 37°C for 3 days show no observable effect on adults, but 6 days exposure had distinct effect on fecundity of adults; and offspring from heat treated adults show impaired moulting and die long before reaching the adulthood (Rukke et al., 2015). The present study found no immediate effect on adults after 5 days exposure at 32 – 36°C, but their offspring experienced significant maternal effects, reflected by a remarkable failure in the survival especially at the two uppermost temperatures. If nymphs would have received direct heat exposure, the effects on a population level may have been even stronger. This can potentially limit population growth and confirms that the intensity of exposure influences the fecundity and survival of their offspring. In Rukke et al (2017), heat treatment at 36°C and 38°C for 3 weeks induced higher mortality than the 2 weeks treatments at the same temperature. Also, the time-temperature interaction induced a limited level of mortality at 34°C after 3 weeks of exposure and significant mortality above 38°C after 2 weeks of exposure. This reveals that treatment length at sub-lethal heat exposure is important to induce mortality among adults and exposure in the range of 32 – 36°C may become lethal if the treatment duration is extended.

Stress from heat can cause significant metabolic changes in bed bugs during or immediately after the exposure to protect the individuals against the stress (Benoit, 2011). Expression of heat shock proteins (HSPs) in bed bugs appears to be most important in this regard as these proteins prevent

and repair damage to proteins and consequently minimize detrimental effects from heat stress and restrain mortality (Chown and Nicholson, 2004). It has been documented in earlier studies that one hour exposure to 44°C yields an up-regulation of HSPs in bed bugs (Benoit et al., 2009). Heat exposure for 5 continuous days in the thermal range of 32 – 36°C in my study seems to allow adult bed bugs to produce heat shock proteins and consequently they were able to withstand the thermal stress and no detrimental physiological effects appeared among adults during the recovery period. It may also be possible that the adults in this study handled these time-temperature combinations without any help from HSPs. Since the physiological sub-lethal heat resilience among bed bugs is improperly understood, either of the above-mentioned two possibilities can be true. Earlier studies confirm that bed bugs are indeed well adapted to high temperature (Usinger, 1966) and sub-lethal heat exposure below the critical temperature level of 37°C has only limited impact on survival of adults (Benoit et al., 2009, Benoit, 2011, Rukke et al., 2015). Possible explanation for this may include bed bugs exposure to human blood at 37°C thermal stress during feeding that may trigger adaptation in physiological responses due to countermeasures against this stress through generations.

The offspring from heat stressed parents may show more susceptibility, faster mortality and more final deaths than the adults as the surface to volume ratios decrease as bed bugs progress towards adults (Benoit et al., 2007, Benoit et al., 2009). The observed maternal effects in first instar nymphs with a normal appearance and behavior indicate no dysfunction in the nymphs yet they died without direct exposure to the sub-lethal heat. This implies an impact from the obligate mutualistic *Wolbachia* bacteria. Bed bugs get essential B vitamins and other nutrients from these symbionts. Earlier studies reported that sub-lethal heat significantly reduces symbionts from the mycotomes of bed bugs (Chang, 1974). It is possible that the parents suffer from malnutrition and failed to supply essential nutrients and symbionts to their eggs (Nikoh et al., 2014) after exposed to sub-lethal heat. Consequently, first instars offspring may have suffered due to imbalanced relationship with these helpful bacteria that led to severe deficiency of essential nutrients and caused death. However, the temperatures and exposure times used in this study should not severely affect the symbionts and most of the negative effects are likely a result of reduced nutrition through the eggs. The maternal effect is important when considering long-term population dynamics and both population limiting characteristics and ecological demands requires further investigations.

Level of infection among bed bugs exposed to insect pathogenic fungi appears to be influenced by conidial concentration, length of exposure, substrate type, environmental conditions, behavior, horizontal transfer and temporal distribution of contagious individuals (Barbarin et al., 2012, Aak et al., 2017, Ulrich et al., 2015). Barbarin et al (2012) investigated the residual biopesticide treatments of *B. bassiana* where bed bugs were exposed to conidial oil formulation at a high dose for 1 hour and found rapid mortality within 3 – 5 days. The present study exposed bed bugs to *B. bassiana* at a low dose for only 5 minutes and revealed that a brief exposure can kill more than 60% bed bugs within 20 days in a laboratory setting. The insect pathogenic fungi have been found as an effective killing agent for bed bug control in earlier studies (Barbarin et al., 2012, Aak et al., 2017), but the reduced success of the fungi in this study is likely a result of the short exposure period. The method still appears to be a good approach to reduce bed bug infestation. The gradual and slow mortality observed from fungi treatment in this study is also identical with low doses in previous studies (Aak et al., 2017) and if the exposure time had been increased, the efficiency is likely to be elevated. This study also investigated the potential synergistic or additive effect of exposure to fungi after sub-lethal heat stress on adults and their first instar nymphs, but no remarkable differences in susceptibility were found. There was only observed a slight increase in mortality at the higher temperature treatment. This subtle variation in mortality in different temperature regimen indicates that there might have been some additional effects on bed bugs from earlier heat stress, but it could have been masked by the mortality from fungi, as bed bugs cannot die twice. Heat stress might still be relevant as it may have an impact on the population dynamics.

Previous studies have investigated the control potential of desiccant dust in combination with CO<sub>2</sub> as a bed bug activity stimulant (Aak et al., 2016). They examined the dust-dose responses at four different concentrations of 3.0, 1.0, 0.3, and 0.1 g/m<sup>2</sup> to identify the pure effect of desiccants. Syloid was found effective and killed all bed bugs within 5 days at the rate of 0.1 g/m<sup>2</sup> and increasing application rate significantly reduced the time to achieve 100% mortality. In the present study, I used 0.18 g/m<sup>2</sup> syloid. But, acute mortality of all adults or offspring within only 3 or 2 days made it difficult to investigate the individual or sequential effects of syloid and heat stress in survival analysis. It could have been a wise step to set only a few hours gap between mortality registrations instead of 24 hours. Also, the mortality could have been slowed down by applying even lower dose of syloid but this is demanding as it is difficult to apply

extremely small amounts of spore on filter papers. A shorter exposure time, such as the 5 minutes used for the fungi exposure would have been a better solution. It is also likely that the use of less efficient dusts could have revealed synergistic and additive effects.

Recent studies have investigated the effects of denial to blood feeding as a stressor among bed bugs (Benoit et al., 2007, Benoit et al., 2009). This study investigated if starvation decreases bed bugs survival while they are exposed to multiple stressors. Heat stressed adults were starved for 5 days or offered a blood meal the day before being exposed to fungi or spore. The freshly hatched nymphs from unfed or fed parents were collected and immediately exposed to fungi or spore. In most treatments, access to feeding helped bed bugs to withstand the stressors compared to starved individuals. The increased survival time may provide reproduction opportunities to adults in a natural environment that may provide more viable eggs and more nymphs, and consequently assist in continuation of life cycle. As long as a host is absent and not provide blood, denial to feeding will decrease the population survival in bed bugs. It would consequently be beneficial if treated rooms are kept vacant and the bed bugs do not get a chance to feed. Bed bugs and the first instar nymphs in particular, will die quicker in hot and dry environments without access to feeding (Benoit et al 2007, Benoit et al 2009, Rukke et al, 2017). My results also followed this general trend because unfed first instar nymphs were found more susceptible to stressors. Feeding is especially important for first instars nymphs to counter desiccation and starvation as they lose water at a faster rate than the adults (Usinger, 1966, Benoit et al., 2007). Access to feeding in bed bugs also increased the survival after being exposed to neuro-toxic insecticides and induces resilience to desiccant dust (Singh et al., 2016b, Choe and Campbell, 2014). In my study, the fed bed bugs were also able to withstand the desiccant dust for at least one additional day compared to unfed individuals. As adults requires an additional blood meal to reproduce, denial of feeding can also effect reproduction and decrease nymphs count which is beneficial to lower the population size in the long run. The heat treated adults also showed indications of impaired feeding before being exposed to killing agents but it was not shown in the results since the uptake of blood is difficult to measure scientifically. It would have been interesting to quantify the amount of blood taken by individual bed bugs to assess if sub-lethal heat treatment impaired the feeding capability of the adults or their first instar nymphs, but the experimental population size made it difficult keeping track of sex of adults or count the

offspring number. Multiple shifts from one petri dish to another could also cause additional stress or injury to bed bugs. Considering all these shortcomings, it was decided not to follow the feeding measurements in the present study. However, the reason behind reduction of feeding ability after heat stress is unknown, but the inability to feed is an important management tool for bed bug control as it may affect the population dynamics.

The sub-lethal heat range for this study was chosen under consideration of consumer's benefits during practical field application. These temperature regimens do not require high energy and is quite inexpensive. People can easily create certain desired temperatures within apartments by using two 1500W electrical ovens and fans (Rukke et al., 2015). Another benefit of sub-lethal heat is that it bears minimal risk of heat damage to objects (woods, concrete, plastic) within a room setting. The commercial use of heat treatment can often reach above 60°C and bears potential risk of damage to objects through the process of irreversible deformation, unequal extension, and reduction of strength. Moreover, such elevated temperature might trigger bed bugs escape behavior and they disperse purposefully towards cooler hiding areas. This comes with the risk of spreading bed bugs to neighboring apartments through cable shafts and pipes. It is also possible that they may find cold spots where they can avoid the heat. All these factors should be carefully considered before the field application of sub-lethal heat.

As the preparation, application and maintenance of *B. bassiana* are easy and thus advantageous for field application (Barbarin et al., 2012, Aak et al., 2017); it is an outstanding potential candidate for bed bug control. The fungus *B. bassiana* depends on bed bug movement over the substrate to pick up spores on their tarsi and different body parts. Since bed bugs are highly gregarious, horizontal transfer could be highly beneficial to deliver fungi in concealed harborages and assist to effective control (Aak et al., 2017). In real field situation, it is possible that, bed bugs may get more exposed to the fungi as opposed to the fixed time and concentration in a laboratory setting. Gradual collection of new conidial spores can be carried out by individuals and restocked in the harborages over time. Several exposures may possibly increase the mortality rate to a higher level. In field settings, bed bugs may experience even slower mortality at lower doses via transient exposures, or at the time when fungal residues will be decaying. Low dose and slower activity would be problematic to suppress the population and



achieve proper eradication. Fungi usually act slowly which is usually problematic. However, this problem can be solved if additional control tool is utilized along with the fungi. Another problem is the dry indoor environment that may reduce the efficacy of the fungi (Jaronski, 2010, Lacey et al., 2015). Exposure to high amount of fungal spores in and around human households certainly holds higher risk of spore inhalation (Madsen, 2011) that could trigger various respiratory and allergic reactions (Mendell et al., 2011, Jaakkola et al., 2013). The possible negative effects from fungal exposure should be considered before the application. More studies are certainly needed to accomplish a better understanding of this established killing agent to control bed bug.

Implementation of desiccant dust is often a better solution for certain places in the households where use of liquid or spray insecticide is not appropriate as it may cause damage to objects, such as – electric equipment. Dust formulations may provide longer residual protection than chemical sprays as they are more readily picked up by bed bugs opposed to dry spray residues (Singh et al., 2016a). It is unlikely that bed bugs will continuously stay on a dust-treated surface if they have a choice to avoid the contact in a natural setting. Silica gel is effective for horizontal transfer that can improve the spread of insecticide into hard to reach hiding places and lead to overall success of bed bug control (Singh et al., 2013). Some hiding places may require higher doses of syloid than others and application of syloid in all possible harborages is equally important. Syloid is physically stable and works efficiently as long as it is dry. Moist environment during syloid application and treatment therefore must be avoided. As silica gel formulations can be equally toxic for minimum one month or up to a year after one single application in different sites (Potter et al., 2014), thus longer residual activity prevents repeated application. Syloid is also a product without neuro-toxicants and is advantageous as it slows down the development of insecticide resistance in bed bugs (Potter et al., 2014). Syloid can also be advantageous as it can be used as preventive measure to prohibit bed bugs active dispersal. Syloid consists of small particle and bears low toxicity to humans but they may have unwanted impact on human skin, airways and mucous membranes through the process of limited desiccation and irritation. Since the long or short-term exposure effect to this product is not yet fully been investigated, it is necessary to use the lowest possible concentration of syloid. Using low amount of syloid is also beneficial when it comes to cleaning out the residue after extermination of bed bug infestation to minimize any potential health risk.

## CONCLUSIONS AND FUTURE DIRECTIONS

This study represent that the effect of sub-lethal heat alone is limited and is unlikely to eradicate an infestation but sub-lethal heat stress at this level mostly affect the second generation and therefore holds a potential to limit population growth in the long run. This study proved that, in completely controlled laboratory situation, syloid has a high potential to control bed bugs compared to fungi. New approaches are continuously given emphasis in IPM method where each method contributes partial treatment and together provide an elevated success. Sub-lethal heat, insect pathogenic fungi and syloid all are potential candidates for efficient future bed bug control and can be utilized as an important management tool in an IPM setting. More studies are needed to investigate the prolonged effect of sub-lethal heat on bed bug populations. Several different time-temperature setting can be evaluated to induce even more detrimental maternal effects, reduce egg production, hatching success, feeding, development, and moulting capability to limit the population growth instantaneously. It would be particularly interesting to investigate parallel stress from multiple stressors instead of sequential exposure. Studies of the physiological and behavioral differences between fed and unfed bed bugs can also be pursued to elucidate the defense mechanisms against stressors. For all above-mentioned approaches, it is equally important to investigate the effects in a field setting to check the feasibility of maintaining thermal stress and if it can reach inside all harborages. It will also be important to examine the field efficacy of the killing agents to quantify how bed bugs will react to multiple stressors in an IPM setting.

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