Norwegian University
of Life Sciences

Master's Thesis 2018 60 ECTS

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Bed bug activity during heat treatments, and physiological effects among surviving individuals

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Preface and acknowledgments

This thesis was written as a part of an ongoing research program on bed bugs at the Department of Pest Control at the Norwegian Institute of Public Health (NIPH). Thanks to NIPH for use of laboratories, experimental animals and equipment. Especially thanks to my supervisors at NIPH Anders Aak and Bjørn Arne Rukke for guidance throughout the whole thesis, with topic for the thesis, creation of the bioassay, execution of the experiments, and helpful advices on the thesis structure and contents. I also thank my supervisor at NMBU Tone Birkemoe, and students Martin Paliocha and Kristian Seres for helpful comments on the thesis. Lastly, thanks to Ingunn Ruud for help with grammar and orthography.

Abstract

Simulated heat treatments with different heating rates were performed in experimental climate chambers to study bed bug behavior during rising heat. In addition, detrimental effects of these short time exposures to sub-lethal heat were recorded. On average, the bed bugs started to move as a reaction to the heat at 40°C. Some bed bugs reacted as early as 37°C, and others as late as 42°C. Heating rate did not affect the temperature that caused the initial movement response to the heat. Activity, measured as number of bed bugs walking at the same time increased from 37 to 42°C, and lower rate of temperature increase in the heat treatments reduced activity. As a consequence of the heat treatment, the bed bugs had a reduction in eggs per female, a reduction in hatching success of the eggs and reduced feeding abilities. The results from my thesis show that during heat treatments, pest controllers should be aware of bed bug activity when the temperature in the harborages passes 38°C, and increasing activity from this point.

Figure 0: Thesis word cloud.

Keywords: Bed bugs; *Cimex lectularius*; heat treatment; sub-lethal heat; behavioral response; heat avoidance; physiological heat response; pest control

Introduction

1.1 Bed bugs

The common bed bug (*Cimex lectularius*) (Hemiptera: Cimicidae) is a flightless nocturnal insect, and an ectoparasite that feeds on human blood (Usinger, 1966). Bed bugs are an increasing problem all over the developed world (Doggett et al., 2012). Norway has had a six-fold increase in treated infestations the last ten years, with 500 in 2006 and 3000 treated infestations in 2016 (Norwegian Institute of Public Health, 2017). The cryptic nature of bed bugs makes control harder, as bed bugs can hide in places that are difficult to get to during a treatment of the infestation (Doggett et al., 2012). Bed bugs have evolved both behavioral and physiological resistance to insecticides (Romero et al., 2007, Dang et al., 2017), which has made bed bug control more difficult. Infestations also have increased due to more international and local travel; bed bugs can disperse passively through human clothing (Hentley et al., 2017) and luggage (Reinhardt and Siva-Jothy, 2007). As bed bugs can live in furniture, acquisition of second hand items such as beds or upholstered furniture can contribute to bed bug dispersal (Potter, 2006). Bed bugs are wingless, but can also disperse actively through walking (Cooper et al., 2015). Walking may be the most important form of dispersal between rooms in the same building (Reinhardt and Siva-Jothy, 2007). Bed bug infestations often start with only a few individuals (Saenz et al., 2012, Fountain et al., 2014), and due to swift population growth, a small bed bug infestation can increase exponentially to a huge bed bug population, with a 35-fold increase per generation, which is just three months (Polanco et al., 2011).

Bed bugs can affect people in many negative ways. These include discomfort, mental health issues (Doggett et al., 2012), and both public (Hwang et al., 2005) and private expenditure (Reinhardt and Siva-Jothy, 2007). Serious mental health issues as insomnia, anxiety, and post-traumatic stress disorder can occur during or after a bed bug infestation (Goddard and de Shazo, 2012, Susser et al., 2012). Pathogen transmission by bed bugs has been demonstrated in laboratory experiments (Goddard and deShazo, 2009, Salazar et al., 2015, Leulmi et al., 2015), however, public health reports have not found any evidence of bed bugs being responsible for any outbreak of infectious diseases (Lai et al., 2016, Delaunay et al., 2011).

Like most other mobile animals, activity in bed bugs is controlled by many biotic and abiotic factors, such as mate location, foraging, and circadian rhythms. Mate location in bed bugs occurs generally over a short range, as bed bugs live in clusters (Usinger, 1966). Foraging and host location is short range, compared to other ectoparasites such as the tsetse fly (*Glossina pallidipes*) (Voskamp et al., 1998). Bed bugs are nocturnal, and is much more active in the night than the day (Romero et al., 2010), and human stimulus increase the bed bugs activity (Aak et al., 2014). Abiotic factors as temperature has a big impact on activity for insects (Chown and Nicolson, 2004).

1.2 Temperature limitations for insects

Temperature affects biochemical reactions, protein structure, metabolism, and other physiological traits in all living organisms (Garrity et al., 2010). In insects, temperature also controls important life-history traits, such as development time (Regniere et al., 2012, Campbell et al., 1974). Three general temperature ranges are used to describe the insect's performance over a range of

temperatures. These are the optimal range, the sub-optimal range and the lethal range (Fields, 1992). The optimal temperature range is in the middle, were the insects have the best performance, with sub-optimal- and lethal temperature ranges for both colder and hotter temperatures on each side. The general ranges can be divided further; the sub-lethal range is in the end of the sub-optimal range, just before the lethal range starts.

Behavior related to optimization of body temperature is called behavioral thermoregulation. Air temperature has a huge impact on an insects body temperature as insects are poikilothermic (Gullan and Cranston, 2010). Because the lack of internal regulation of body temperature, insects must control their body temperature by other means. Body temperature of ectotherms is mainly controlled by seasonal and daily temperature change, habitat choice, selection of microhabitat (sun/shade, burrowing, climbing, and grouping), postural adjustments and physiological adaptions (muscle activity, panting, urination, etc.) (Stevenson, 1985). In addition, some insects use behavioral thermoregulation, such as the bumblebee (*Bombus* spp.) uses flight muscles to increase body temperature (Heinrich, 1975).

The temperature range an insect species can tolerate varies with species. Insects have smaller variance in upper temperature limits, compared to lower limits (Neven, 2000). However, smaller variance in upper lethal temperatures does not mean that certain insects cannot survive in extremely high temperatures. Ants in the genus *Cataglyphis* can tolerate extremely high temperatures, both because of physiological and behavioral adaptions; species in this genus can tolerate body temperatures around 53-55°C during scavenging mid-day in the Saharan desert (Moseley, 1997). The critical maximum temperature for *Cataglyphis bicolor* is 55°C (Gehring and Wehner, 1995), 8°C higher than the average reported for other insects (Addo-Bediako et al., 2000). The temperature range an insect can tolerate generally increases with latitude, (Deutsch et al., 2008), consequently the tropical bed bug (*Cimex hemipterus*) has a shorter temperature range than the closely related common bed bug (Benoit, 2011).

Too high temperatures can decrease the fitness of the insects by reducing the number of offsprings (Silbermann and Tatar, 2000) and heat stress may increase the resources needed to survive. The heat can be fatal by denaturizing proteins, by causing cellular or tissue damage, and by destabilizing phospholipid membranes, which are central components in stable functioning of the nervous system (Stevenson, 1985, Gullan and Cranston, 2010, Fields, 1992).

Heat shock proteins (HSPs) can increase the thermal tolerance of insects (Gullan and Cranston, 2010, Moseley, 1997). Production of heat shock proteins are induced by several stress-factors, among them heat, cold, and starvation (King and MacRae, 2015), and a few heat specialist insects can produce heat shock proteins even prior to heat exposure (Gehring and Wehner, 1995). Heat shock proteins increase thermal tolerance because it prevents denaturation of other proteins, and contributes to protein refolding (King and MacRae, 2015, Denlinger and Yocum, 1998, Tatar, 1999). Generally, for insects, Hsp70 is the most important heat shock protein associated with thermal protection (Chown and Nicolson, 2004, Denlinger and Yocum, 1998); for bed bugs, both Hsp70 and Hsp90 are highly produced during heat exposure, and associated with thermal protection (Benoit et al., 2009).

1.3 Thermal tolerance for bed bugs

High temperatures cause heat stress also in bed bugs. Critical lethal temperatures are much studied for bed bugs (DeVries et al., 2016a, Kells and Goblirsch, 2011, Loudon, 2017, Naylor and Boase, 2010, Pereira et al., 2009). Heat-related mortality for bed bugs is a function of temperature and exposure time (Appendix, Table I). Other factors such as development stage (Kells and Goblirsch, 2011, Olson et al., 2013), heating rate (Kells and Goblirsch, 2011) and feeding status (DeVries et al., 2016a) may affect time and temperature requirements for bed bug mortality. Relative humidity is shown to have an effect on heat-related mortality in stored-product insects (Fields, 1992), but this effect was marginal in bed bugs (Rukke et al., 2015), possibly because of bed bugs having good water retention abilities (Benoit et al., 2007).

The optimal temperature range for bed bugs is 25-32°C (Benoit, 2011), whereas 32-38°C can be defined as sub-optimal. Within the sub-optimal temperature range the bed bugs performances will be reduced, but rapid death will not occur in a few days (Rukke et al., 2018). Prolonged exposure to temperatures from 39-42°C can be lethal (Rukke et al., 2015, Pereira et al., 2009), but rapid death will not occur in a few hours. As bed bugs in this thesis are exposed to heat for a short time period, the sub-optimal temperature range for bed bugs will be extended, and 39-42°C will be defined as sub-lethal. Temperatures >43°C is lethal in a short time period (Pereira et al., 2009). In summary, defined in this thesis for short time exposure to heat, the optimal range is 25-32°C, sub-optimal range is 32-38°C, sub-lethal range is 39-42°C and the lethal range is >43°C. Prolonged exposure (days/weeks) to sub-optimal temperatures can influence bed bugs negatively by increasing mortality, reducing feeding abilities, reducing egg production, reducing egg-hatching success and have negative offspring effects (Rukke et al., 2015, Rukke et al., 2018).

1.4 Pest control

A half century ago, pesticides alone were the main method to control bed bugs (Potter, 2011), but concerns for health issues for people and the environment, combined with increased resistance in the bed bugs towards the pesticides has limited the use and efficiency of the pesticides (Potter, 2011, Dang et al., 2017, Romero et al., 2007). Today, integrated pest management (IPM) is the preferred treatment method for pest control. IPM combines more than one treatment method and is commonly used by pest control firms in Norway to treat bed bug infestations. Common treatment methods included in IPM today are pesticides, physical removal of bed bugs (vacuuming) or infested furniture, laundering, steaming, desiccant dust and use of lethal temperatures (Koganemaru and Miller, 2013).

Understanding the bed bugs behavior towards the methods used for treatment may increase the efficiency for the pest control. For instance, bed bugs use CO_2 as a signal for host localization and the use of host signals can improve the use of desiccant dust in the field (Aak et al., 2017). In contrast, bed bugs have evolved behavioral resistance to many pesticides, were they have developed the ability to avoid or reduce exposure to pesticides (Dang et al., 2017). Knowledge of the bed bugs behavior towards different treatments may improve the efficiency of the treatment.

Heat treatments are one of the methods that can be applied in IPM. Heat treatments are commonly utilized to control pest insects in crops after harvest (Lurie, 1998, Fields, 1992),

stored animal product (Rajendran and Parveen, 2005), and control for pest insects in wood (Lewis and Haverty, 1996, Pinto et al., 2007, Hansen et al., 2011). In some instances, as in artifacts in museums, chemicals used for pest control may be damaging for the artifacts, and heat treatments or cold could be used to preserve the artifacts in good condition (Strang, 1992, Beiner and Ogilvie, 2005). The interest in heat treatments of insects in crops is increasing because of growing demand from consumers to reduce chemicals used on the crops (Lurie, 1998).

The use of lethal temperatures is a frequently used method to remove bed bugs. Boiling water, steam and air heat was used in the late 1800s and early 1900s (Potter, 2011). Today, heat treatments are used on small objects as well as on entire structures/rooms. It is very effective on a small scale, for example when treating luggage and exposed laundry. At the right time and temperature combination, clothes can be washed and/or tumble-dried, and luggage can be freezed in a household freezer to kill the bed bugs and prevent settlement at home (Naylor and Boase, 2010, Olson et al., 2013, Rukke et al., 2017). Obtaining lethal temperatures is harder when treating rooms or houses; bed bugs may find refuge from the heat in cracks or other heat-isolated areas.

The use of heat treatments is one method with both benefits and disadvantages. Benefits include environmental friendly, fast and localized treatment, and good results if done properly (Pinto et al., 2007, Müller et al., 2014). High cost and possible damage to furniture and other heat sensitive objects are some possible disadvantages when using heat treatments, compared to other methods (Pinto et al., 2007, Müller et al., 2014). Pest control firms in Norway that use heat treatments for bed bug control claim to have good results, with few recalls for additional treatments (pers. comm. Espen Roligheten, Boligbygg Oslo).

1.6 Objective of the thesis

In this thesis, I investigated bed bug behavior (movement) during heat treatments with different types of heat raise from normal temperature conditions until the end of the sub-lethal temperature range at 42°C. Additionally, I study the physiological effects of heat treatments, on adult bed bugs, and their offsprings, when time and temperature conditions for mortality is not met. The aim of the thesis is to gain knowledge about behavior and physiology that can be applied to heat treatments, to improve bed bug control. In this thesis, I will study and discuss:

At what temperature does bed bugs start movement as a response to heat and is this starting point dependent on the rate of temperature increase?
At what temperatures during heating are bed bugs most active, and is activity dependent on rate of temperature increase?
What are the physiological consequences of short time exposure of sub-lethal heat for the bed bugs?

Implications of the findings are discussed in relation to applied aspects of heat treatments in bed bug control.

Materials and methods

2.1 Experimental animals and feeding

Stock culture bed bugs were collected from hotels in Oslo in 2009 and have been kept in stock at NIPH (Norwegian Institute of Public Health) since then. The bed bugs are kept in 140 mL polyethylene boxes (VWR, Oslo, Norway) with metal mesh screen in the lid

(Burmeister AS, Oslo, Norway) and pieces of folded paper towels inside as harborages (3x3cm). Boxes are kept in climate chambers (Sanyo versatile environmental test chambers, Panasonic Healthcare Co, Japan) with photoperiod 16:8/light:dark (night 17:00-01:00), at 22°C at 65% humidity (Standard breeding conditions). Feeding of the bed bugs was performed according to Aak and Rukke (2014); the bed bugs were fed with human blood. 9 ml blood was drawn into each of two vacutainers and 0.1 ml heparin (Heparin LEO, 5000 IE/mL; Orifarm A/S, Odense, Denmark) was added to each vacutainer immediately after donation, then the vacutainers were stirred. Three feeding bags were made of stretched parafilm m® (VWR International, Oslo, Norway) (Figure 1) and 6 ml blood was added to each. An unstirred water bath (JB AQUA S2 PLUS, Grant Instruments, Cambridge, UK) was heated to a water temperature of 40°C. Feeding bags were placed on top of the racks in the water bath on paper towels that just touched the water surface. The water temperature at 40°C gives a heat in the feeding bags around 37°C. I exhaled into the box through the mesh screen to activate feeding with CO_2 host signal, and then boxes with bed bugs were placed on top of the feeding bags and fed for 7 minutes. The bed bugs fed through the mesh and the parafilm.

2.2 Bioassay

A bioassay was created to study bed bug behavior when exposed to increasing heat. The heat treatments were done in climate chambers. Two climate chambers were used, with capasity for two arenas per climate chamber, giving a maximum of four replicates each experimental day. Inside of the climate chambers, the arenas for the bed bugs were built (Figure 2). The edges of an A3 paper were bordered with insect trap coating (Tangle-Trap® company, Michigan, U.S.A.) to prevent escape from the arena. A plastic box (31x22.5x6.5 cm, Ultra-Plast A4/60, VWR, Norway) was placed on top of the A3 paper. The top 1cm of the plastic box was painted with INSECT-a-SLIP (BioQuip Products, California, U.S.A), to serve as a difficult barrier to pass for the bed bugs. A4 self-adhesive paper (Herma, Filderstadt, Germany) was pasted on the base of the plastic box, to make it easier for the bed bugs to walk on. A LEGO® brick frame (11x11x2cm) was placed in the middle of the plastic box. The frame also serves as a barrier for the bed bugs. As bed bugs are relatively inefficient climbers (Hinson et al., 2017), the hard plastic is difficult for the bed bugs to climb on, however, quite an easy challenge compared to the obstacle of the plastic box with INSECT-a-SLIP. These barriers divide the arena into three zones: central zone (1), inner zone (2) and outer zone (3) (Figure 3). To allow observations without opening the inner glass doors of the climate chamber, an angled mirror was installed to get bird's-eye view over the arena. Maximum heating capacity for the climate chambers was 1°C $2min^{-1}$. Because the integrated thermometer in the climate chamber was not accurate enough to precisely measure the actual temperature in the arenas, SL52T iButtons (Signatrol, Tewkesbury, UK, Software for iButtons: TempIT4-Pro) were used to give accurate temperatures in each arena. The iButtons were programmed to high resolution (0.07°C) and sample rate at one recording per min. The climate chambers used in the heat treatments held the same circadian rhythm as the climate chamber with stock cultures. All treatments were done in the daytime, in artificial light. The A3 paper with insect trap coating, INSECT-a-SLIP and the self-adhesive paper were replaced each week, between each different heat treatment.

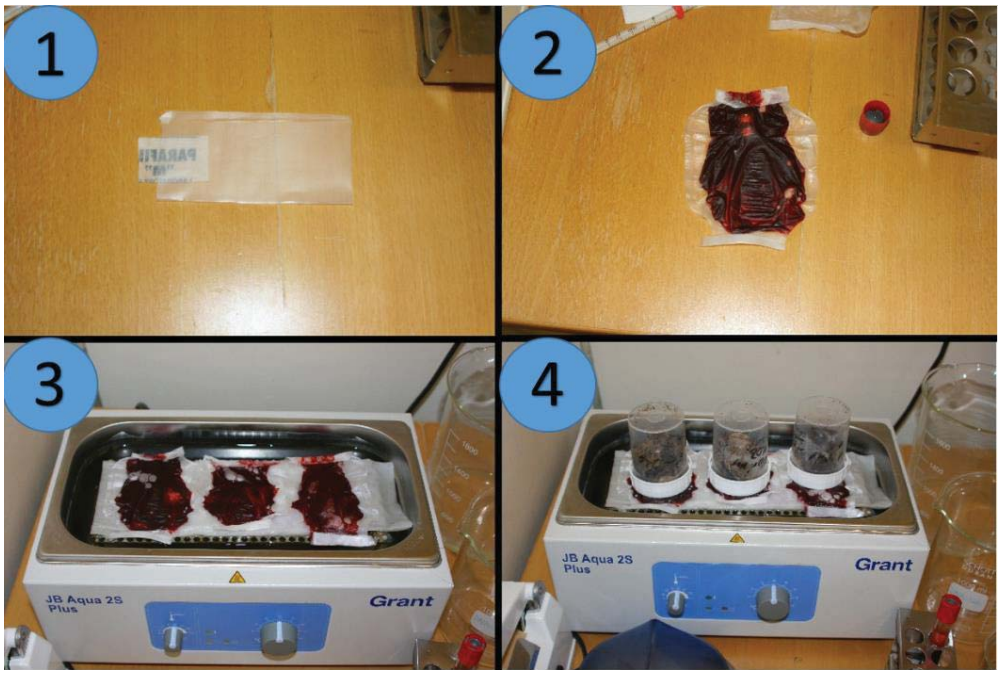

Figure 1: Pictures from the feeding of the bed bugs: (1) Unstretched parafilm m®. (2) A finished feeding bag made of stretched parafilm m® with 6 ml human blood. (3) Heating of the feeding bags on a rack in a water bath. (4) The boxes with bed bugs are placed with the lid down, which allowed the bed bugs to feed through the metal mesh screen. Stock cultures bed bugs are fed in this picture. Photos: Marius Saunders.

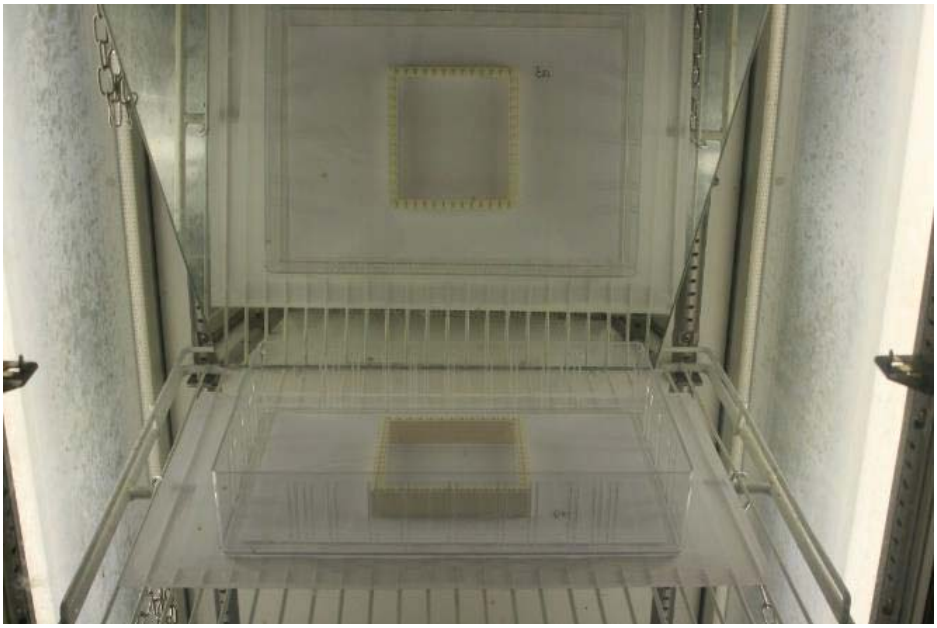

Figure 2: Picture from one of the climate chambers with an arena. A mirror was installed to get bird's-eye view over the arena when looking into the climate chamber. The arena consists of an A3 paper with insect trap coating, a plastic box on top of the paper, and the central LEGO® brick frame. Bed bugs and iButton is not included in the picture. Photo: Marius Saunders.

2.3 Experimental protocol

The experiments were initiated by picking out 5th instar nymphs from the stock cultures and feeding them on day one (Table 1). The nymphs were given two weeks to molt into adults. On day 15, newly molted adults were put into separate boxes, with three males and three females in each box (6 bed bugs, sex ratio 1:1), and then fed. Adults that did not feed were discarded, and replaced with fully engorged adults. These six adults stayed together as one experimental unit without any contact with other bed bugs until the end of the experiment. Each bed bug was only used once. As there was only opportunity for four replications each day, bed bugs were introduced into the arena three, four or five days after last feeding. The bed bugs were placed in the middle of central zone in the arena, together with the iButton at approximately 18:00 the day before the heat treatment to settle down. The next day the heat treatment started. To register movement of bed bugs during the heat treatments, pictures were taken manually with a camera (Canon EOS 400D, Canon Inc., Tokyo, Japan). Immediately after the heat treatments, the surviving bed bugs were collected from the arena and placed in new polyethylene boxes with a 3x3 cm piece of a paper towel. Boxes were put into standard breeding conditions for two weeks. On day 34, the adults were removed from the boxes and given two attempts to feed, just minutes apart. After feeding, the adults were discarded. Eggs stayed in the original box for another fortnight, giving all laid eggs time to hatch. On day 48, 1st instar nymphs were fed. Nymphs and the eggs were killed in a freezer before the experiment was terminated.

2.4 Experimental heat treatments

Each heat treatment had two arenas per climate chamber and two climate chambers each day spread over three days. This gives a total of 12 arenas and 72 bed bugs per heat treatment. Four different temperature regimens were chosen to see if rate of temperature increase had an effect on activity. All temperature regimens had a rapid temperature increase (1°C $2min^{-1}$) from 22-34°C, then rate of temperature increase was changed to provide (1) rapid, (2) intermediate, (3) slow, and (4) extra slow increase of temperature (Table 2, Appendix table IV). In all the heat treatments, the arenas were heated to 42.5°C. The control treatment was held constantly at 22°C.

Based on results from the initial heat treatments, an additional temperature regimen was designed to test a potential practical solution for bed bug control that could limit activity. This heat treatment was maintained at a higher temperature during the night, therefor titled (5) the overnight heat treatment. It started at 22°C as the other temperature regimens, but the temperature was rapidly increased to 36°C immediately after the release into the arena, and then held at 36°C for 20 hours (Table 2, Appendix table IV). After 20 hours, the temperature was rapidly increased to 42.5°C.

Table 1: The activities in the experimental protocol from start until the end of the experiment. At day 15 the bed bugs were separated into 12 experimental units with 6 bed bugs in each unit for each of the 6 different treatments. The 12 experimental units were exposed to heat treatments on three separate dates the following week, symbolized with the separation in the original column. Color in the treatment columns symbolize which activity that were done on each day.

Day	Activity	Control			Rapid			Intermediate			Slow			Extra slow			Overnight		
1	Feeding of 5th instar nymphs																		
2	Molting period																		
3																			
4																			
5																			
6																			
7																			
8																			
9																			
10																			
11																			
12																			
13																			
14																			
15	Feeding adults, adults in new boxes n= replicates	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4	n= 4
16	Rest and digestion period																		
17																			
18	Release into the arena																		
19	Control / Heat treatment																		
20	Egg laying period																		
21																			
22																			
23																			
24																			
25																			
26																			
27																			
28																			
29																			
30																			
31																			
32																			
33																			
34	Separation of eggs and adults. Feeding of adults. Adults discarded.																		
35	Egg hatching period																		
36																			
37																			
38																			
39																			
40																			
41																			
42																			
43																			
44																			
45																			
46																			
47																			
48	Feeding of 1st instar nymphs. Nymphs and eggs killed.																		
Total	Replicates	n=12			n=12			n=12			n=12			n=12			n=12		

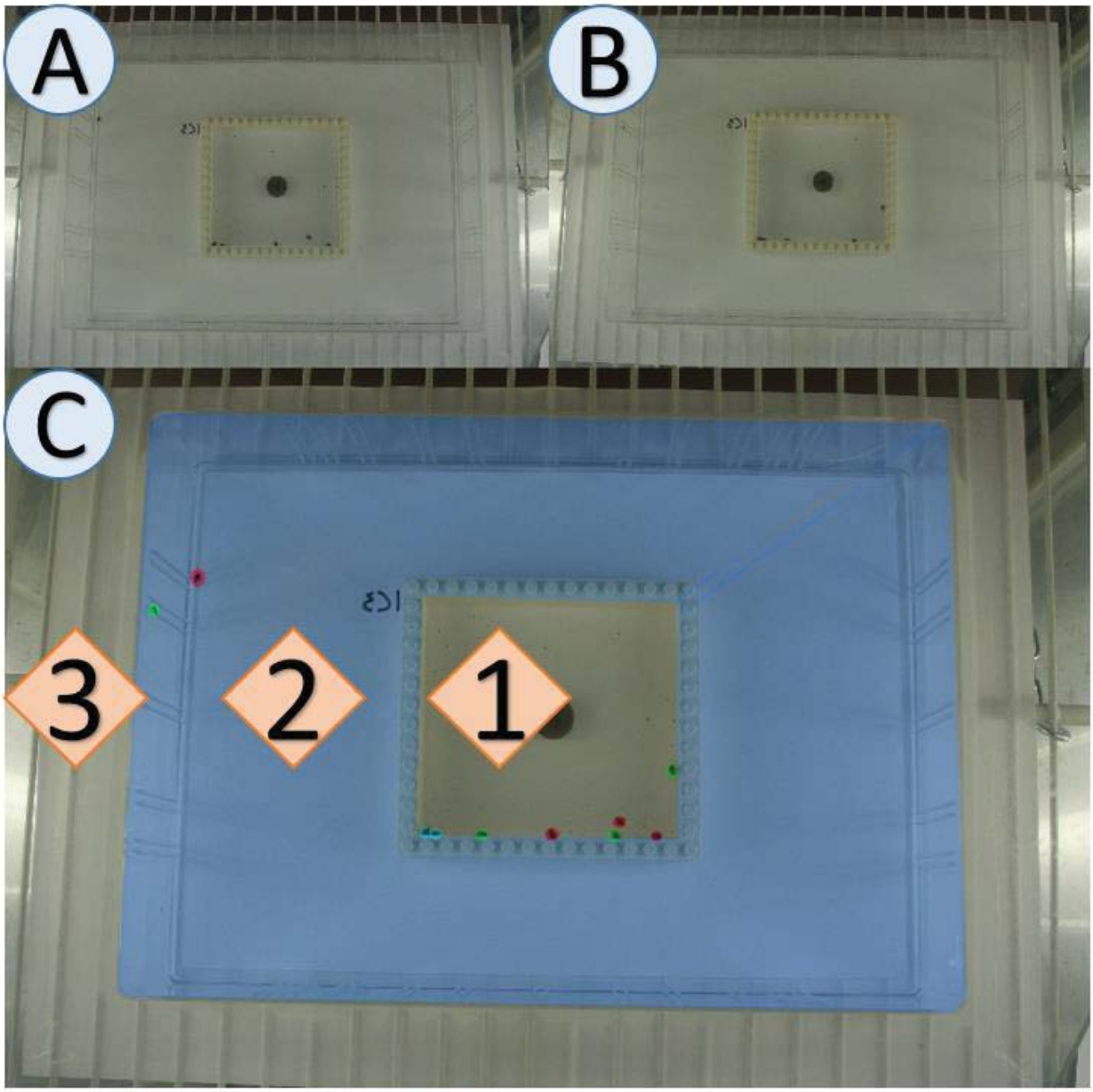

Figure 3: Description of registration of activity for the bed bugs during heat treatment, and the different zones in the arena. **A:** Picture taken at 15:08. **B:** Picture taken the minute later at 15:09. **C:** Merging of the two previous photos (A&B). Blue marked bed bugs did not move within the minute, green and red marked individuals moved, red was the first position, green was the new position one minute later. In this observation, 4 were active, whereas 2 were not active, this gives a bed bug activity at 4/6 (0.67). The central zone (1) is the area inside of the LEGO® brick frame. The inner zone (2) is marked in blue, and is the area outside of the LEGO® brick frame, but within the plastic box. The outer zone (3) is outside of the plastic box on the A3 paper. Movement outside from zone 3 is impossible because of the insect trap coating on the edges. Photos: Marius Saunders.

Table 2: Overview over the differences between the different temperature regimens.

Temperature regimen	Control	Rapid	Intermediate	Slow	Extra slow	Overnight
Rate of temperature increase until 34°C	Constant at 22°C	1°C 2min^{-1}	1°C 2min^{-1}	1°C 2min^{-1}	1°C 2min^{-1}	1°C 2min^{-1}
Rate of temperature increase after 34°C	Constant at 22°C	1°C 2min^{-1}	1°C 16min^{-1}	1°C 32min^{-1}	1°C 64min^{-1}	1°C 2min^{-1}
Overnight temperature	22°C	22°C	22°C	22°C	22°C	36°C
Heat treatment start	15:00	15:00	13:15	11:00	06:44	18:00 (22-36°C) 15:20 (36-42.5°C)
Heat treatment end	15:43	15:43	15:56	15:56	15:56	15:38
Picture intensity before 34°C	1 picture per min	1 picture per min	1 picture per min	1 picture per min	1 picture per min	No data
Picture intensity after 34°C (One pair of pictures taken one minute apart).	No data	1 picture per min	1 pair per 8th min	1 pair per 16th min	1 pair per 32th min	1 picture per min (Starts at 36°C)
Relative humidity in the climate chambers during the heat treatment	60%	60%	60%	60%	60%	60%

2.5 Response variables and collection of data

First responses to heat, sustained activity and dispersal during heat treatments.

Behavioral data was collected by comparing positions of the bed bugs in the pictures taken during the heat treatments. One picture was taken every minute from 22-34°C. From 34°C pictures were taken in pairs, one minute apart for every 0.5°C increase (Table 2).

The first time each bed bug moved, the exact temperature at that time was registered. This was done to find the temperature that initiated movement, and the percentage of bed bugs that have reacted to the heat at different temperatures during a heat treatment.

To find the proportion of bed bugs with sustained activity during a heat treatment, the bed bugs' positions were compared to the bed bugs' position one minute earlier and the proportion of bed bugs changing position was registered (Figure 3).

To connect activity to escape abilities of the bed bugs during a heat treatment, I compared the position of the bed bugs in the arena zones, at release, before the heat treatment started, and after the heat treatment ended.

Long-term detrimental effects after the heat treatments.

Long-term effects were quantified by registering changes in life history traits after the heat treatment. Two weeks after the heat treatment, the proportion of adults that fed was registered (Table 1), and four weeks after the heat treatment, the number of hatched/unhatched eggs and proportion of nymphs that fed were registered.

2.6 Statistical analysis

R-studio, R-commander (NMBU), and SigmaPlot10 were used for statistical analysis. ANOVAs and t-tests assumption were inspected with residual vs fitted plots and normal q-q plots for variance and normality (Appendix, Figure I). If any of the statistical requirements were not met, non-parametric were used.

First responses to heat.

Average temperature that caused first movement response for the bed bugs was analyzed with one-way ANOVA and contrast t-tests. Each arena was given one out of 12 different temperatures from 36.5-42°C, to get independent observations throughout the heat treatments, and percentage of the bed bugs in each arena that had moved heretofore at the given temperature were used to make a

regression line with binomial logit GLM. The percentage of bed bugs that had moved is the response variable and the temperature is the explanatory variable. The variable of different temperature regimens was removed, as it did not provide a better fit of data (Deviance test, x^2=0.488, df=3, p=0.92). The temperature regimen with high overnight temperature was ignored in the regression to generate an accurate estimation for heating from normal room temperature (22°C).

Sustained activity during heat treatments.
Two-way ANOVA and post-hoc Tukey at α=0.05 were used for analysis of the bed bugs' sustained activity in the heat treatments. Temporal pseudo-replication may be a problem with the data, as observations were made in the same experimental unit (arena) more times during the heat treatment, and are therefore not statistically independent (Hurlbert, 1984). However, there were very small differences between the simple two-way ANOVA model and the complex model that accounted for pseudo-replication (two-way nested ANOVA with interaction); therefore, a simplified ANOVA was used. Defense for the statistical model chosen is written in the appendix. In the two-way ANOVA, activity was the response variable. Temperature was set to a fixed factor for the nearest 1°C. The temperatures, the temperature regimens and the interaction were the explanatory variables. One arena with bed bugs was excluded, as four bed bugs were inside the LEGO® bricks. The control treatment and temperatures without activity (22-36°C) were excluded in the analysis.

Dispersal during heat treatments.
Paired t-test was used to analyze if the dispersal from the central zone during heat treatment differed normal dispersal occurring in the arenas until temperature increase started. Kruskal-Wallis H-tests were used to analyze if there was a different number of bed bugs that dispersed from the central zone between the different temperature regimens.

Long-term detrimental effects after heat treatments.
All binomial data (Feeding, Yes/No. Egg hatching, Yes/No) were analyzed with Pearson's chi-square test, and post hoc tested with chi-squared pairwise nominal independence tests. P-values for the post hoc tests were adjusted with Bonferroni correction to correct for multiple testing. For the binomial data, all data within each temperature regimen was pooled to be used for analysis. Three boxes in the intermediate heat treatment were excluded for nymphs feeding, as a mistake during the feeding did not give the nymphs in these boxes the opportunity to feed.
Since two females died during the heat treatment, the average number of eggs per female in each box were analyzed by one-way ANOVA and post hoc tested with Tukey groups at α=0.05. The male deaths did not have a statistical impact on number of eggs (one-way ANCOVA, F=1.17, p=0.28), and there were at least two males in each experimental unit. Therefore, number of males were not included in the analysis.
Additionally, a harm index was calculated with [(Proportion adults feeding) × (Number of eggs per female) × (Proportion of hatched eggs) × (Proportion of nymphs feeding)] for each experimental unit, and analyzed with one-way ANOVA and post hoc tested with Tukey groups at α=0.05 to compare how harmful the different temperature regimens were for the bed bugs.

Results

3.1 Behavioral responses to heat

The average temperature that started movement during heat treatments was 39.87°C [median: 40.01, n=281, SE: ±0.06] when heated from room temperature (22°C). There were some individual differences when the bed bugs reacted to the heat (SD±1.09). First movement response for the bed bugs was independent of temperature regimen for those whom had 22°C over the night (One-way ANOVA, temperature regimen with 36°C night temperature excluded, df=277, F=1.31, p=0.27). In the temperature regimen with 36°C over the night, the bed bugs had an earlier first reaction to the heat at 39.35°C [median: 39.32, n=72, SD±1.10, SE: 0.13], than the bed bugs in the other temperature regimens (One-way ANOVA with all temperature regimens, df=348, F=4.15, p=0.002, contrast t-test, temperature regimens with 22°C night temperature vs temperature regimen with 36°C night temperature, df=348, t=3.546, p<0.001, figure 4).

The regression line shows that most bed bugs reacts to the heat within a relatively short temperature range during increasing heat (Figure 5, Table 3) and predicts that 10%, 50%, and 90% of bed bugs in a population have moved as a response to the heat at 38.5°C, 39.8°C, and 41.2°C, respectively.

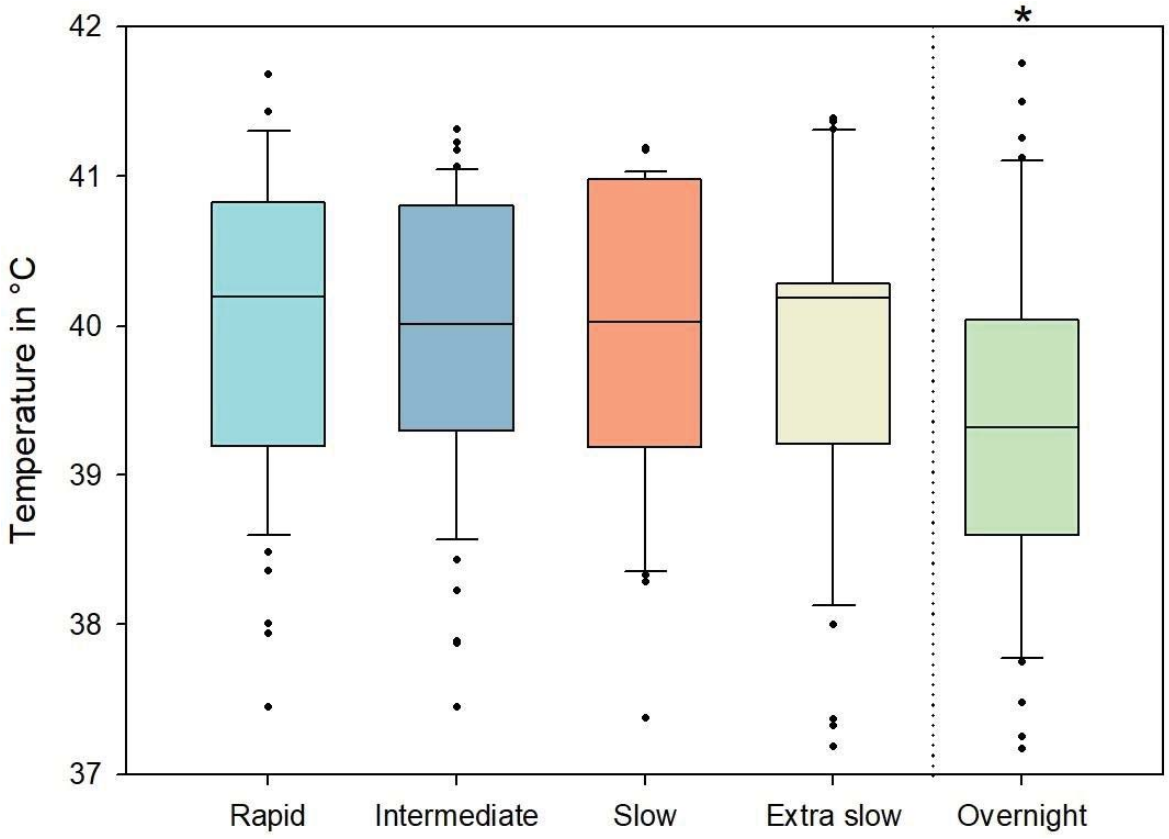

Figure 4: Boxplot with median, 10%, 25%, 75% and 90% quantiles for the temperature the bed bugs had a first movement response to the heat for the different heat treatments. Dots are outliers of the quantiles. * indicates significant difference from the other heat treatments at p<0.05.

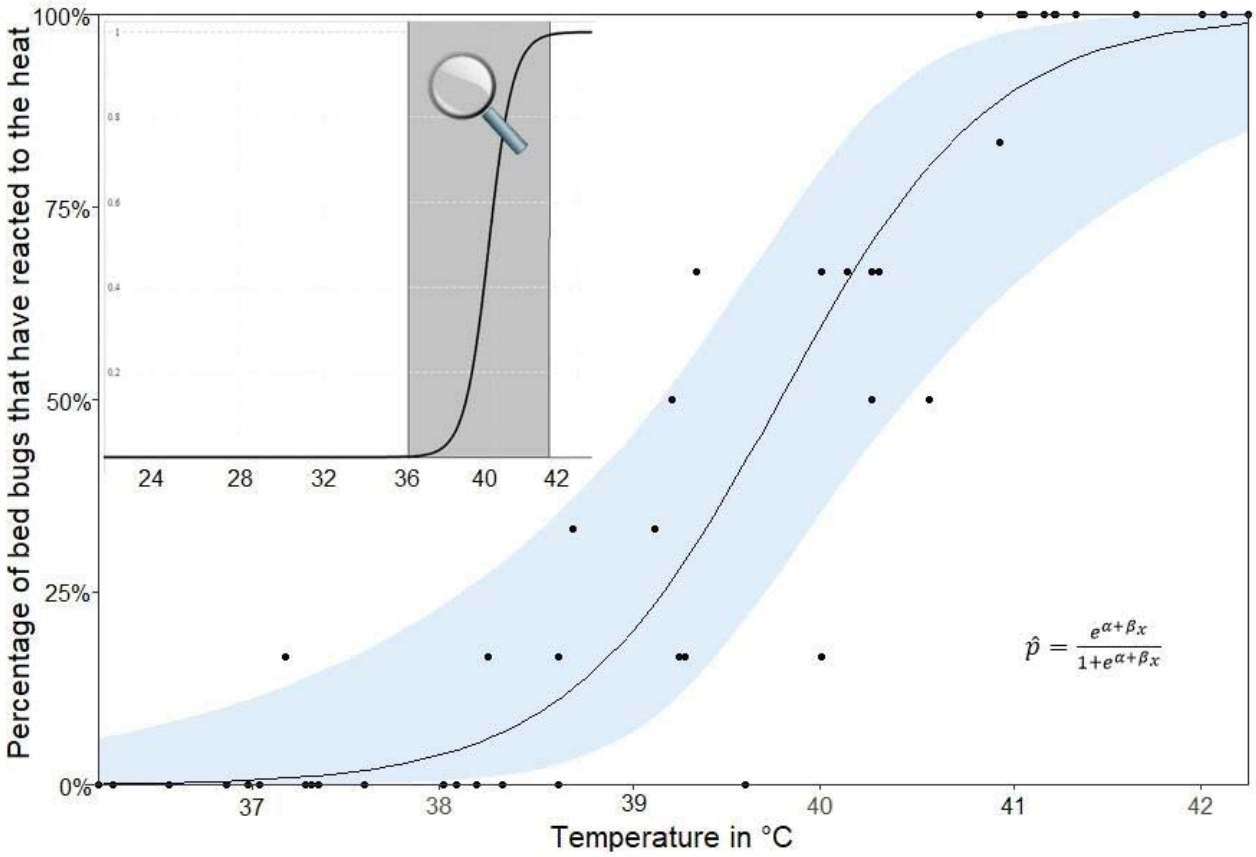

Figure 5: Percentage of bed bugs (y-axis) that had moved in response to the heat (x-axis). The regression line is fitted with binomial GLM, and the blue area is a 95% confidence interval. Dots are single observations. The formula in the figure is the binomial GLM regression model.

Table 3: Parameters and test scores for the parameters used for the regression model in figure 5.

	LR x^2	P(>x^2)	Parameter (α=intercept, β=slope)	Estimate	Standard Error	z-value	P(>|z|)
Temperature	35.98	<0.001	Intercept	-69.23	19.23	-3.60	<0.001
			Slope	1.74	0.48	3.58	<0.001

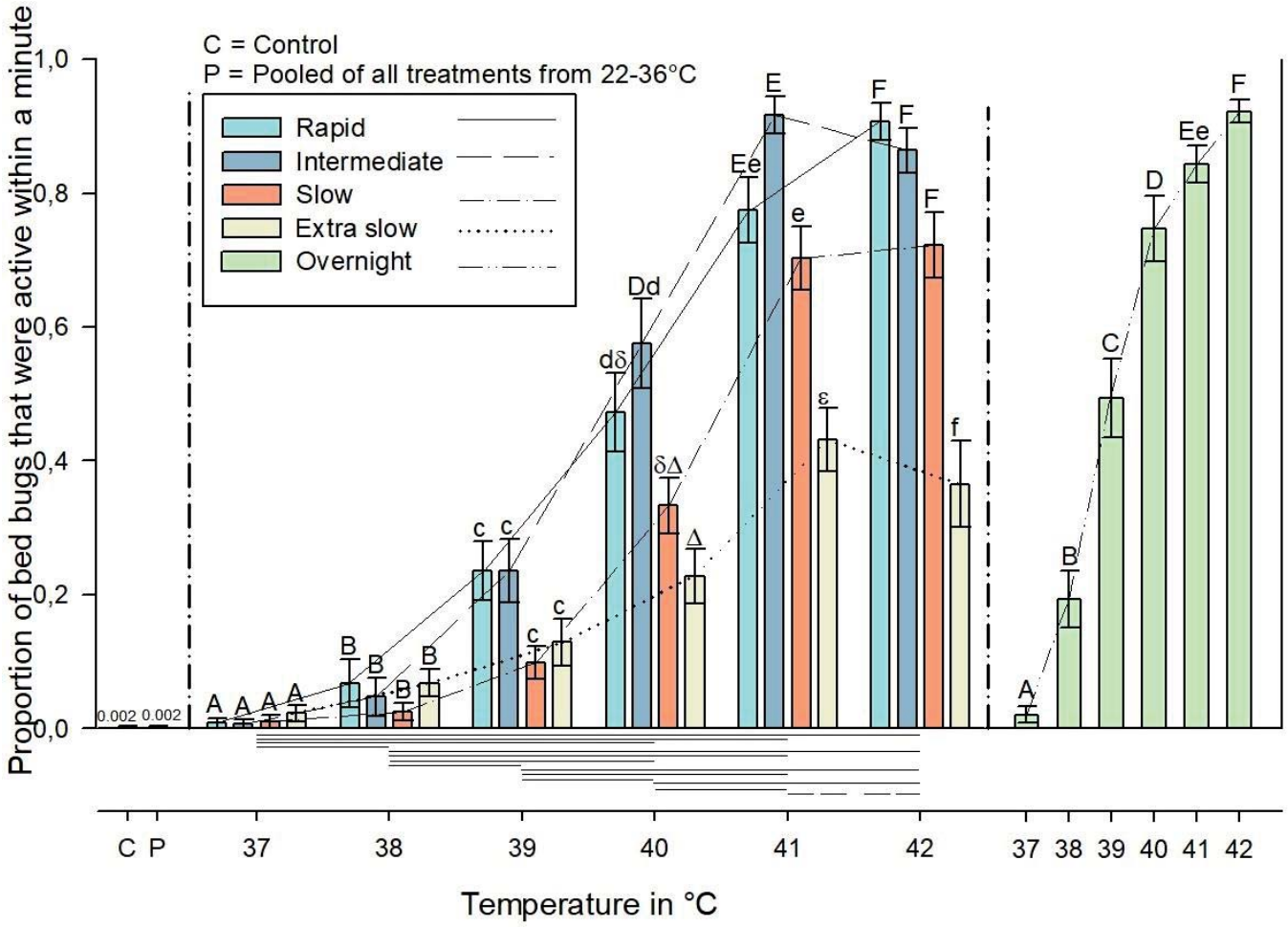

Figure 6a: Activity level for the bed bugs (y-axis) at different temperatures (x-axis) and in different temperature regimens (different colored bars). Activity level is defined as proportion of bed bugs that changed position within a minute. The temperature regimen with higher overnight temperature was separated from the others in the figure, to easier visualize the decreasing activity level as the heat treatments got longer. Different letters show significant difference (α=0.05) between the different temperature regimen within same temperature. On the x-axis, C is the control at 22°C, and P is all temperatures from 22-36°C polled from all heat treatments. Error bars are standard error.

Figure 6b: Different lines show significant difference (α=0.05) in activity between the different temperatures, from 37-42°C. Solid lines show a difference in activity between the temperatures, whereas dotted line shows equal levels of activity between the temperatures.

Table 4: ANOVA-table on activity level. Temperature and temperature regimen are fixed factors, with inclusion of the interaction.

	df	SS	F	P(>F)
Temperature	5	362.7	363	<0.001
Temperature regimen	4	64.7	64.6	<0.001
Interaction	20	8.4	8.4	<0.001
Residuals	670	22.9		

The different temperatures, the different temperature regimens and the interaction between those had an effect on the sustained activity (Figure 6, Table 4). In all treatments, there is an increase in activity from 37°C to 41°, whereas 41°C and 42°C has equally high levels of activity (Post hoc Tukey, 41 vs 42, t= -1.28, p=0.79, Figure 6b). The overall sustained activity decreased when the temperature regimens had a slower heating (Tukey groups, α=0.05, Overnight > Rapid = Intermediate > Slow > Extra slow). When the bed bugs were not exposed to heat, activity was less than 0.3% in both the control and in the range from 22-36°C.

Movement out of the central zone was higher during the heat treatment than during the night prior to the heat treatment (paired t-test, t=10.16, df=58, p<0.001). At the start of the heat treatments 4 bed bugs were found in outer zone, 10 in the inner zone and 346 in the central zone. At the end of the heat treatments that was changed to 7, 83 and 270, respectively (Figure 7). However, the tendency to move from the central zone did not differ between the temperature regimens (Kruskal-Wallis, H=7.27, df=4, p=0.12).

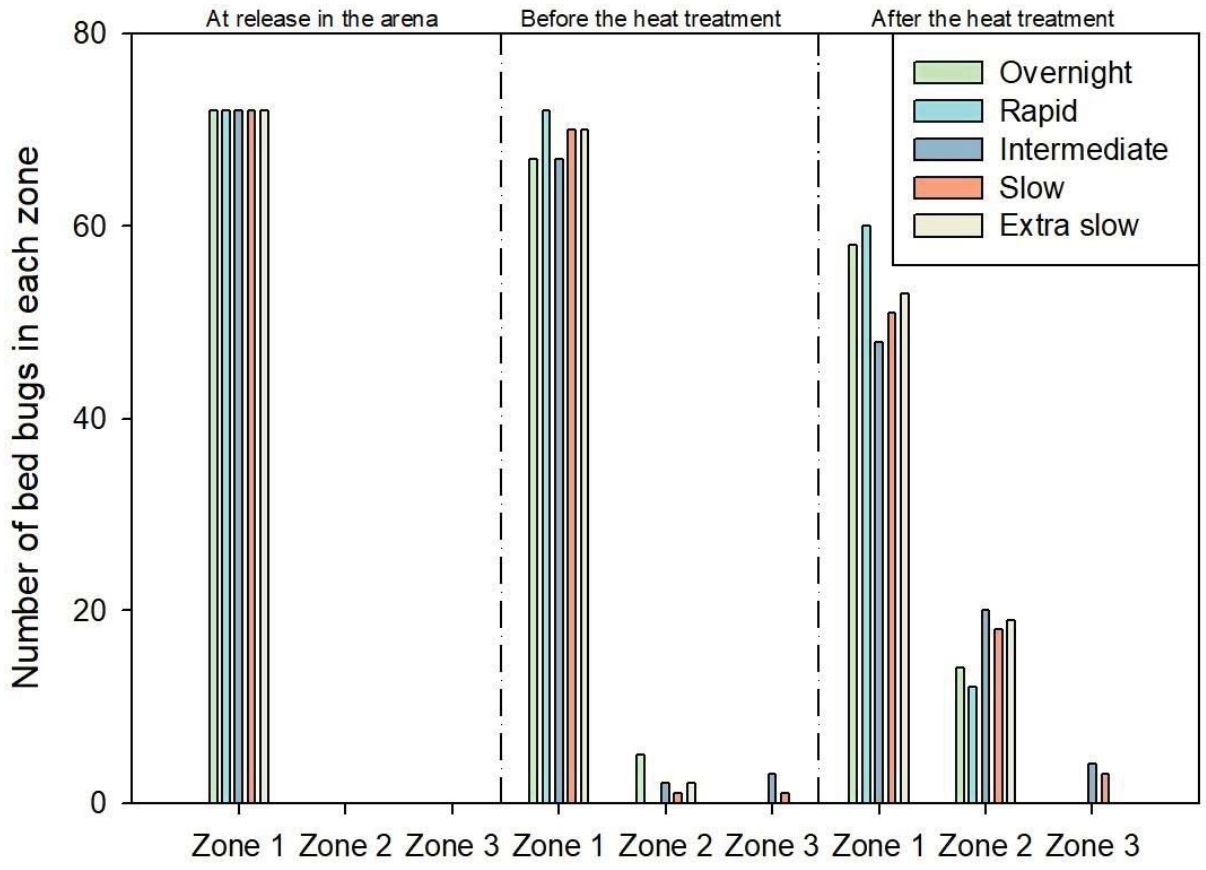

Figure 7: Position of total number of bed bugs in each of the arena zones. At release into the arena, the day after, before the heat treatment started, and after the heat treatment was finished.

3.2 Long-term physiological damage caused to bed bugs by a short heat treatment

All the bed bugs were alive after the heat treatment; however, a few got discarded as they got stuck in the insect trap coating (three in the intermediate, and two in the slow heat treatment), and a few died during the two weeks after the heat treatment (one in the slow, and five in the extra slow heat treatment).

The heat treatments affected the adult bed bugs ability to feed (Pearson's Chi-squared test, x^2=177.17, df=5, p<0.001, Figure 8). The two temperature regimens with slowest warmup had a clear reduction in adults that had a blood meal compared to from the control (Pairwise nominal independence test, control: slow, p<0.001, control: extra slow, p<0.001).

The number of eggs deposited in the two weeks after the treatment was affected by the heat treatments (One-way ANOVA, df=66, F=4.91, p<0.001, Figure 9). The bed bugs showed a reduction in fecundity in all the heat treatments compared to the bed bugs in the control; however, there was no significant difference in number of eggs per female between the different temperature regimens (Post hoc Tukey groups at α=0.05). The hatching-success of the eggs laid after the heat treatment were affected (Pearson's Chi-squared test, df=5, x^2=131.04, p<0.001, Figure 9). All the heat treatments, except the rapid heat treatment (Pairwise nominal independence test, control: rapid, p=0.23) had a significant reduction in successfully hatched eggs compared to the control.

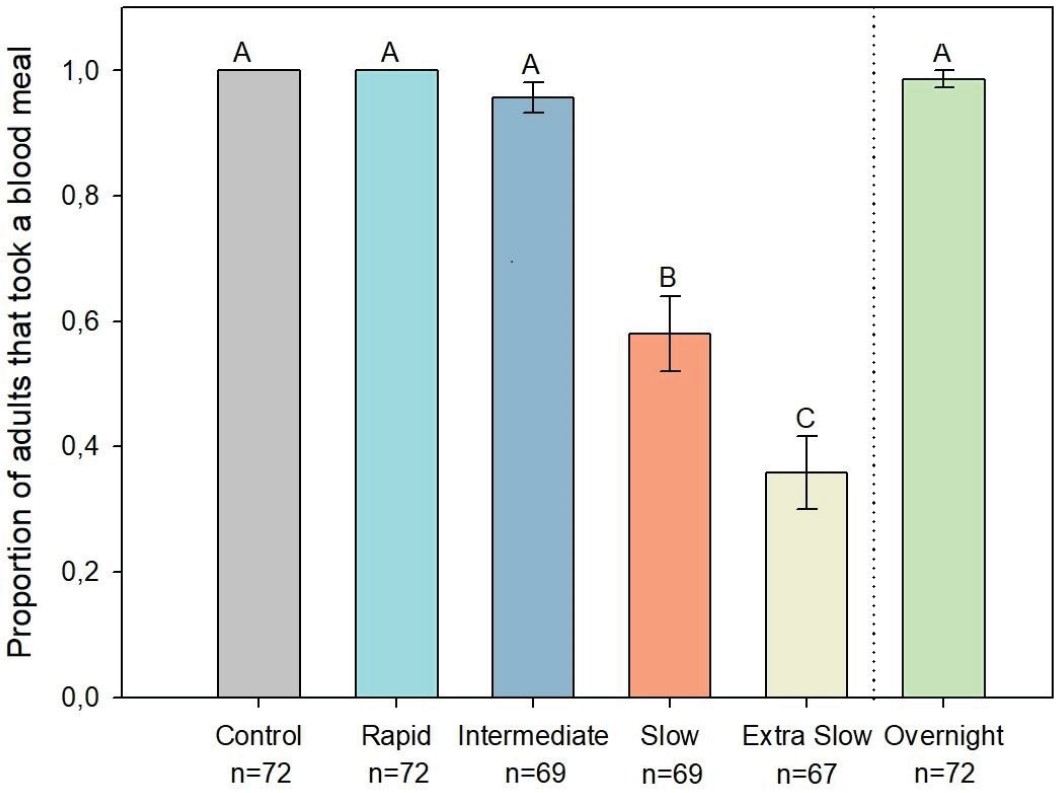

Figure 8: Proportion of adults that took a blood meal after two trails, with heat treatments and control. n is total of bed bugs that survived until the feeding, all treatments started with 72 adults. Different letters symbolize significant difference at p<0.05. Error bars are standard error.

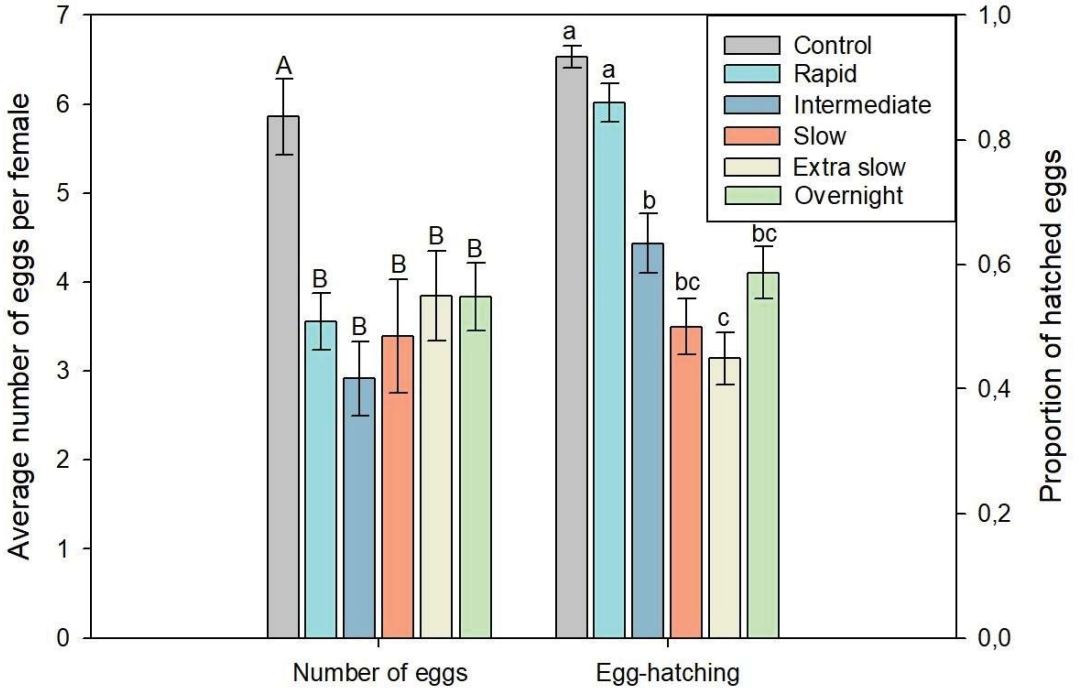

Figure 9: Average number of eggs laid per female on left Y-axis and proportion of hatched eggs on right Y-axis. Different letters display significant difference at $p<0.05$. Error bars are standard error.

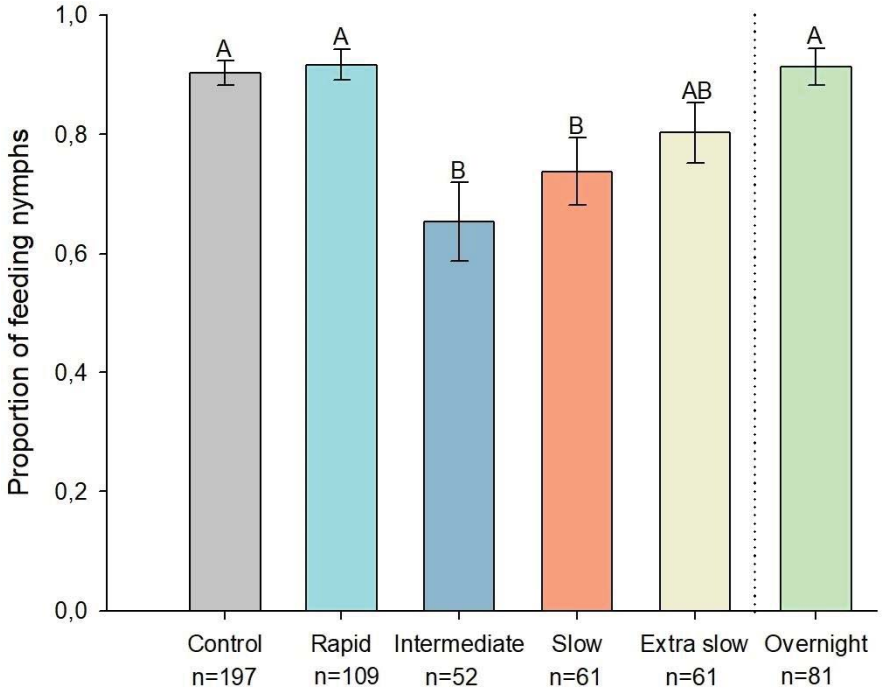

Figure 10: Proportion of feeding nymphs, born by heat-treated adults. Different letters display significance at $p<0.05$. Error bars are standard error.

Table 5: Harm index and summary of the long-term detrimental effects the heat treatment had on the bed bugs. The harm index is an assembled approximation on how harmful the heat treatments were for the bed bugs and is calculated with: (Proportion adults feeding) × (Number of eggs per female) × (Proportion of hatched eggs) × (Proportion of nymphs feeding). For the summary, green cells do not differ from control, red cells differ from control at α=0.05. For the harm index (±SE), different colors are given to symbolize post hoc Tukey groups, were different colors/letters is statistical significant at α=0.05. A=Green, B=Yellow, C=Orange, D=Red.

	Control	Rapid	Intermediate	Slow	Extra Slow	Overnight
Adults feeding	1	1	0.957	0.579	0.358	0.986
Number of eggs	5.86	3.55	2.92	3.39	3.85	3.83
Egg-hatching	0.933	0.859	0.634	0.5	0.449	0.587
Nymphs feeding	0.904	0.917	0.654	0.738	0.803* *p=0.11	0.914
Harm index	4.94±0.42 A	2.80±0.29 B	1.22±0.28 CD	0.73±0.19 D	0.53±0.15 D	2.02±0.32 BC

The heat treatments did have an influence on the feeding ability of nymphs born by heat-treated adults (Pearson's Chi-squared, df=5, x^2= 34.59, p<0.001, Figure 10). Nymphs born from adults in the intermediate and slow heat treatment showed a reduction in feeding ability, compared to the control (Pairwise nominal independence test, Control: Intermediate, p<0.001, Control: Slow, p=0.007). The extra slow heat treatment had a reduction in the proportion of feeding nymphs, however, this reduction was not statistical significant (Pairwise nominal independence test, Control: Extra slow, p=0.11).

From the calculated harm index assembling the different long-term effects, bed bugs in all treatments show a reduction in performance compared to the control (One-way ANOVA, df=66, F=32.8, p<0.001, post hoc Tukey groups at α=0.05, Table 5). Performance was greatest reduced in the intermediate, slow and extra slow heat treatment.

Discussion

4.0 Key results for discussion

We found that the temperature that initiated movement for the bed bugs was just under 40°C. During the heat treatments, higher temperatures caused more activity, and temperature regimens with slower heating decreased sustained activity. We also found that even short-time exposure to sub-lethal temperatures reduced fitness, through reduction of feeding ability, reduction in egg number and hatching success.

4.1 Bed bugs responses to heat, activity and dispersal during a heat treatment

Temperature related behavior is much studied in form of thermoregulation (Chown and Nicolson, 2004). Insects can use daily temperature changes to behaviorally avoid harmful temperatures (Dahlgaard et al., 2001), but behavior related to sudden repellent

temperatures and heat escape is not widely documented for insects (Ma and Ma, 2012b).

The lack of difference between the temperature regimens in the temperature that initiated movement suggests that there is a critical temperature around 40°C that induces behavioral stress in bed bugs. However, when the bed bugs were hold at 36°C during the night, the temperature of initial movement was slightly lower. Similar results are found in the English grain aphid (*Sitobion avenae*) and the bird cherry-oat aphid (*Rhopalosiphum padi*), where heat acclimation at 36°C decreased the temperature that caused escape from the heat with about 1° (Ma and Ma, 2012b). Temperature history may have reduced the threshold needed to react to the heat to reduce heat damage (Ma and Ma, 2012b).

In the heat treatments, majority of the bed bugs responded to the heat with movement between 38 and 42°C. Studies on bed bugs' thermal orientation found bed bugs could differentiate temperatures of 2°C (Rivnay, 1932, DeVries et al., 2016b). 2°C may be bed bugs' temperature resolution, this converges with the range 38 to 42°C, around 40°C. 2°C may be the difference needed for a bed bug to sense differences in temperature, explaining the individual differences in first movement response.

The first movement appear in a temperature range which not only is harmful for a prolonged time, but deadly (Rukke et al., 2015). In an evolutionary perspective, we should expect the temperature that started movement to be lower, as even prolonged exposer to temperatures as low as 34°C decreases relative fitness and the optimal temperature range ends at 32°C for bed bugs (Rukke et al., 2015, Benoit, 2011). However, selection of traits relevant to heat tolerance may conflict with others traits which are more directly linked to fitness, and therefore may not be highly selected in natural populations (Fasolo and Krebs, 2004). Occasionally, heat stress related behavior is directly linked to fitness, as rice folder moths (*Cnaphalocrocis medinalis*) that have experienced heat stress, changes place for oviposition to the lower side of the leaf (Bodlah et al., 2017), therefore such behavior is more likely to be highly selected. For bed bugs, high selection pressure for traits relevant to heat escape from high ambient temperatures seems unlikely, as stable indoor temperatures rarely reach harmful temperatures for bed bugs.

Avoidance of critical temperatures is essential for survival. In our heat treatments, the bed bugs had a first movement response, on average about 5°C before knockdown temperature. Similar results are found in the English grain aphid (*S. avenae*). The English grain aphid (*S. avenae*) normally sits still to reduce dangers of predation and to save energy, but has a first movement response to increasing heat about 5°C before knockdown temperature (Ma and Ma, 2012a). To escape the heat, they jump off the host plant (Ma and Ma, 2012a).

Physiological mechanisms responsible for behavioral heat escape is not fully understood for insects (Hamada et al., 2008). In fruit flies (*Drosophila* spp.), warmth-activated anterior cell neurons localized in the brain is responsible for behavioral avoidance of harmful upper temperatures (Hamada et al., 2008). These neurons are activated just over the optimal temperature for fruit flies (*Drosophila* spp.), and is dependent on an ion-channel (TRPA1) (Hamada et al., 2008). In the

yellow fever mosquito (*Aedes aegypti*), the same ion-channel is used for thermoregulation and host-seeking behavior (Corfas and Vosshall, 2015). This ion-channels role in bed bugs has not yet been studied, but if present, we might speculate that it has a similar role in bed bugs as in other insects, and may contribute to host location (Corfas and Vosshall, 2015), thermoregulation (Rosenzweig et al., 2005), or avoidance of harmful heat (Hamada et al., 2008). It is possible, but unlikely that the high temperatures in the heat treatments were not as repellent because of bed bugs' attraction to heat for host location. This seems unlikely as bed bugs sense the differences between target- and ambient temperature, and not an absolute temperature of the target (DeVries et al., 2016b).

Heat treatments increase activity in the red flour beetle (*Tribolium castaneum*) (Semeao et al., 2013). The heat treatments caused increased activity in the bed bugs and when the heat treatments got longer, the bed bugs were less active; the reduced activity may be explained by the longer time and temperature combination. We found similar increases in activity in the sub-lethal temperature range for bed bugs, as has been shown for the dusty brown beetle (*Gonocephalum simplex*) (Klok et al., 2004). In both studies, the insects showed a higher level of activity when the temperature got closer to critically high temperatures. The increasing activity as a response to the heat seems to be skewed approximately 5°C towards higher temperatures for the dusty brown beetle (*G. simplex*) relative to bed bugs, which may be explained with the difference in the critical temperatures for the species. (DeVries et al., 2016a, Klok et al., 2004). The increasing activity as temperature is reaching critical temperatures suggests a higher level of behavioral stress when the heat gets more dangerous.

Bed bugs can disperse actively between room and apartments in room temperature (Cooper et al., 2015), but heat treatments effects on dispersal for bed bugs are poorly understood. In Norway, whole compartment heat treatments are abundantly used in Oslo, with few recalls for additional treatments (pers. comm. Espen Roligheten, Boligbygg Oslo). From actual bed bug control, it does not seem that heat treatments increase dispersal of bed bugs between rooms or apartments compared to normal dispersal, but to get a successful heat treatment, meticulous work must be done (pers. comm. Espen Roligheten, Boligbygg Oslo). The heat treatments in our study caused an increasing number of the bed bugs to cross the barrier from the central zone. However, it is not clear if this was a result of random movement caused by panic, or if this was an active search for lower temperatures. Red flour beetles (*T. castaneum*) normally walks in mills, but have the ability to fly. For the red flour beetle, the ability of behavioral avoidance from high temperatures, and search for lower temperatures during heat treatments is limited, and heat treatments does not increase dispersal, compared to normal dispersal (Semeao et al., 2013). If this is also true for bed bugs, heat treatments should not increase dispersal. This is supported from field experience from actual bed bug control (pers. comm. Espen Roligheten, Boligbygg Oslo). The capability for bed bugs to search for lower temperatures to escape the heat during a heat treatment is unknown. The fact that bed bugs

need to be less than 3 cm from a heated target for direct movement towards the target (DeVries et al., 2016b), suggests that finding lower temperatures may also be limited to very close areas.

4.2 Possible mechanisms behind the physiological damage

The time and temperature combination in the heat treatments was not lethal; however, the heat treatments had several detrimental effects that reduced fitness for the bed bugs. Heat related mortality for bed bugs is dependent on both time and temperature (Kells and Goblirsch, 2011). Both starting temperature and rate of temperature change have effects on critical temperatures that are needed to harm insects (Terblanche et al., 2007). Slower rate of temperature increase reduces the critical upper temperatures, whereas higher starting temperatures increase the critical upper temperatures (Terblanche et al., 2007). In our experiments, some of the detrimental effects done to the bed bugs were independent of the different temperature regimens, whereas other effects got more harmful as the temperature regimens had a slower warmup and the heat treatment got longer.

Normally, adult bed bugs feed weekly at room temperature (Usinger, 1966), but some of the heat treatments caused a reduction in feeding two weeks after the heat exposure. The proportion of adult bed bugs feeding declined as the temperature regimens got a slower heating. This can be explained as when the rate of temperature increase got slower, the bed bugs got much more time in the harmful heat. Heat can cause damage to metabolism, nervous system and the endocrine system (Neven, 2000), which could lead to a reduction in feeding. Hormones might contribute to regulation of digestion in some insects (Okasha, 1968). During heat exposure, whole tissues are more sensitive to heat damage than single cells and macromolecules (Neven, 2000). The heat could damage tissues in the mouthpart, in form of mouthpart deformities. To protect themselves from the heat, bed bugs produce heat shock proteins during heat exposure (Benoit et al., 2009). Continuous expression of the same heat shock proteins has been shown to cause mouthpart deformities in harlequin fly larvae (*Chironomus riparius*) (Park and Kwak, 2008). However, for bed bugs, production of heat shock proteins seems like an unlikely explanation for mouthpart deformities, since the temperature regimen which had a temperature at 36°C over the night should had activated high levels of heat shock protein production (Benoit et al., 2011), and this temperature regimen did not have a reduction in feeding adults. It is clear that longer time in harmful heat reduces feeding for bed bugs, therefore reduces fitness, however, since damage to the nervous system, endocrine system and mouthparts was not inspected, the explanation for the reduction in feeding stays unclear.

High temperatures can reduce egg production for insects, but the mechanisms behind the reduction is not fully understood (Mahroof et al., 2005). All heat treatments in our study showed a decrease in number of eggs per female compared to the control. Temperatures from 36°C over a longer period of time has been shown to cause a reduction in number of eggs for bed bugs (Rukke et al., 2018, Rukke et al., 2015). One possible explanation for the egg reduction is loss of endosymbionts, as prolonged temperatures at 36°C gives a loss of

endosymbionts, and loss of endosymbionts can cause reduction in egg production for bed bugs (Chang, 1974). The reduction in laid eggs, due to loss of symbionts, as a result of heat has also been shown for two types of stink bugs (*Acrosternum hilare* and *Murgantia histrionica*) (Prado et al., 2010).

Synthesis of heat shock proteins may be an important factor to explain the reduction of successfully hatched eggs. A few hours in sub-lethal heat (40°C) reduces egg hatchings success for the diamondback moth (*Plutella xylostella*) (Zhang et al., 2013), and heat shock protein production reduces egg hatching success for fruit flies (*D. melanogaster*) (Silbermann and Tatar, 2000). A shorter heat treatment (30 min) causes less expression of heat shock proteins, compared to a longer treatment (60 min) for fruit flies (*D. melanogaster*) (Silbermann and Tatar, 2000). All heat treatments in our study had a reduction in egg hatching success, except the rapid heat treatment. The rapid heat treatment in our experiments only had 14 min over 36°C, therefore little time for synthesis of heat shock proteins, and this may explain why this treatment was not different from the control. The overnight heat treatment, which otherwise had very similar detrimental effects as the rapid heat treatment, showed a clear decline in egg-hatching success, which the rapid heat treatment did not, supporting the hypothesis that expression of heat shock proteins reduces egg-hatching success also in bed bugs.

Heat is not only harming the bed bugs, but can also have negative consequences for microorganisms within the insects. Some insects can search for higher temperatures heat get rid of pathogens (Wojda, 2017), but heat can also be harmful for bacterial symbionts. *Wolbachia* is an important bacterial symbiont for bed bugs, especially for provisioning of B vitamins (Nikoh et al., 2014). Sub-lethal temperatures for bed bugs can reduce *Wolbachia* in the adults (Li et al., 2014, Jia et al., 2009). A reduction in *Wolbachia* can lead to a reduction in egg-hatching success for bed bugs (Hosokawa et al., 2010), which may be another factor explaining the observed reduction in egg-hatching success in our experiments. *Wolbachia* is transferred from mother to offspring in an early stage of the embryogenesis (Hosokawa et al., 2010), therefore a reduction in *Wolbachia* in adults can reduce the *Wolbachia* in nymphs. This could lead to problems with nutrient production by symbionts for the nymphs. This could be one of the reasons nymphs that are born by long-term heat treated adults in sub-lethal temperatures have a reduction in molting ability (Rukke et al., 2018). In our experiment, nymphs in some of the heat treatments had a reduction in feeding. DNA is vulnerable to heat damage (Neven, 2000), which may be part of the explanation on negative offspring success, together with the loss of symbionts.

4.3 Relevance to bed bug control

From the result, I cannot conclude if fast or slow heat increase is the best when using heat treatments for bed bugs; a quick rise in temperature would reach critical temperatures faster, however, the increased overall activity may affect dispersal in field conditions. It is not clear from our results if high activity for a short period or low activity for a long period would lead to the lowest risk of dispersal during a heat treatment.

There were some individual differences for when the bed bugs started to move as a

response to the heat; hence, pest controllers should expect that some bed bugs start to move at 38°C, with only a few bed bugs reacting to the heat before that temperature. After the bed bugs first movements as a response to the heat, pest controllers should be aware of increasing activity as temperature approaching critical temperatures that causes knockdown.

During a heat treatment, the harborages where the bed bugs hide may have much lower temperature (>10°C), than the room temperature (Pereira et al., 2009). When the bed bugs in the isolated harborages (e.g. the bed mattress) first reacts to the heat, room temperature may be deadly within few minutes (Pereira et al., 2009, Loudon, 2017). Therefore, the bed bugs have very low chances to escape from central objects in the room during a heat treatment. Thus, moveable infested objects should be in the middle of the room during heat treatments.

Our results showed a decrease in activity as the heating rate got slower and none of the bed bugs reacted to heat under 37°C. From these results, we tried to make a practical solution to reduce bed bug activity during a heat treatment. The idea behind the overnight heat treatment was that the bed bugs were heated to a temperature that was harmful and exhausting, but that temperature also had to be lower than the temperature that caused movement as a response to the heat. We expected that the bed bug would sit still in 36°C, slowly being exhausted over the night without moving, and have little activity as the temperature increased past 38°C and upwards. However, results were not as expected. This treatment had the highest activity, and harm done on the bed bugs was low. The overnight heat treatment also had a significant earlier first movement response to the heat, however, a discrepancy of 0.5°C has no significance in practical bed bug control. The temperature at 36°C may have been too low to cause exhaustion for the bed bugs.

Our results show a clear reduction in overall fitness for the bed bugs after heat treatment. Other bed bug studies in the sub-lethal heat also shows a clear reduction in population fitness after exposure to long time sub-lethal temperatures (Rukke et al., 2018, Rukke et al., 2015). The reduction in population fitness caused by both short, and long time exposure to sub-lethal heat could be used to weaken the bed bug population in combination with other treatment methods.

Conclusion and future directions

We found that most bed bugs first react with movement to temperatures between 39-41°C, and that this response was independent from rate of temperature increase. Pest controllers should be aware of some bed bug starts to move as a response to the heat at 38°C, and increasing levels of activity from this point. More research on internal sensors that controls thermoregulation and heat avoidance are needed to explain the observed behavioral response to the heat. The TRPA1 ion-channel role in temperature dependent behavior is not yet studied for bed bugs, and should be investigated further.

Laboratory experiments should use labyrinth studies to determine bed bugs' capability to escape in specific directions, but more importantly, the ability for bed bugs to actively disperse in a response to heat treatments should be studied in the field as the practical application of our results are difficult to determine.

We found that bed bugs were less active when the rate of temperature increase was low. A reduction in activity is favorable from a pest controller's view, and methods to reduce activity even more should be investigated further. Our practical solution may have had a too low overnight temperature to decrease activity for the bed bugs; raise and hold strategy at higher temperatures from 37 to 39°C could be investigated further.

The bed bugs were negatively affected by the heat treatments, with reduction in feeding and fecundity. The relatively short heat treatments had similar effects as long time exposure to sub-lethal heat (Rukke et al., 2018, Rukke et al., 2015). Possible mechanisms behind the reduced fitness as physiological damage, cost of thermal protection, and loss of symbionts could be investigated further to better the theory of heat damage towards insects. Even though the mechanisms behind the reduced fitness is unclear, even short time exposure to sub-lethal heat can reduce the bed bugs' fitness, which can be applied in bed bug control.

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Appendix

Defense for use of not optimal statistical model

The model chosen for sustained activity analysis (two-way ANOVA with interaction) is not optimal, because of unbalanced dataset, pseudo-replication (Hurlbert, 1984) and the explanatory variable is not continuous (Activity range 0-1). However, the model gives a good fit for the data, and fulfill the ANOVA requirements of normality and constant variance. The model does not fulfill the assumption of independent observations, as observations were made in each arena more than one time. The control and observations within the range from 22-36°C had no activity, and were ignored in the analysis, as it would conflict with the assumption of equal variance. The model (two-way ANOVA with interaction) were compared to a more complex statistical model that accounts for pseudo-replication (two-way nested ANOVA with interaction, with each arena set to a factor within each heat regimen, table II, table III). The nested ANOVA can explain differences in activity between different temperatures, but not differences between the heat regimens, as arena is a random factor. The simplified model was chosen, as it give a good fit for our data, as R^2 values were similar between the two models, and most importantly, as post hoc testing for differences between heat regimens is possible in the regular two-way ANOVA, but not in the nested ANOVA. The model may have some problems with estimations, with over predicting activity when the activity is low, and under predicting activity when activity is high. The model parameters give good fit for our data, but should not be used on new datasets.

Table I: Time requirements for mortality in different studies. Bed bugs were directly put into these temperatures.

Temperature	Time	Unit of measurement	Study
38.5°C	9 days	100% mortality	(Rukke et al., 2015)
40°C	2 days	100% mortality	(Rukke et al., 2015)
41°C	100 min	100% mortality	(Pereira et al., 2009)
43°C	25 min	100% mortality	(Pereira et al., 2009)
45°C	94.8 min	$LTime_{99}$	(Kells and Goblirsch, 2011)
47°C	2 min	100% mortality	(Pereira et al., 2009)
49°C	60 sec	100% mortality	(Pereira et al., 2009)
50°C	45 sec	100% mortality	(Loudon, 2017)
65°C	10 sec	100% mortality	(Loudon, 2017)

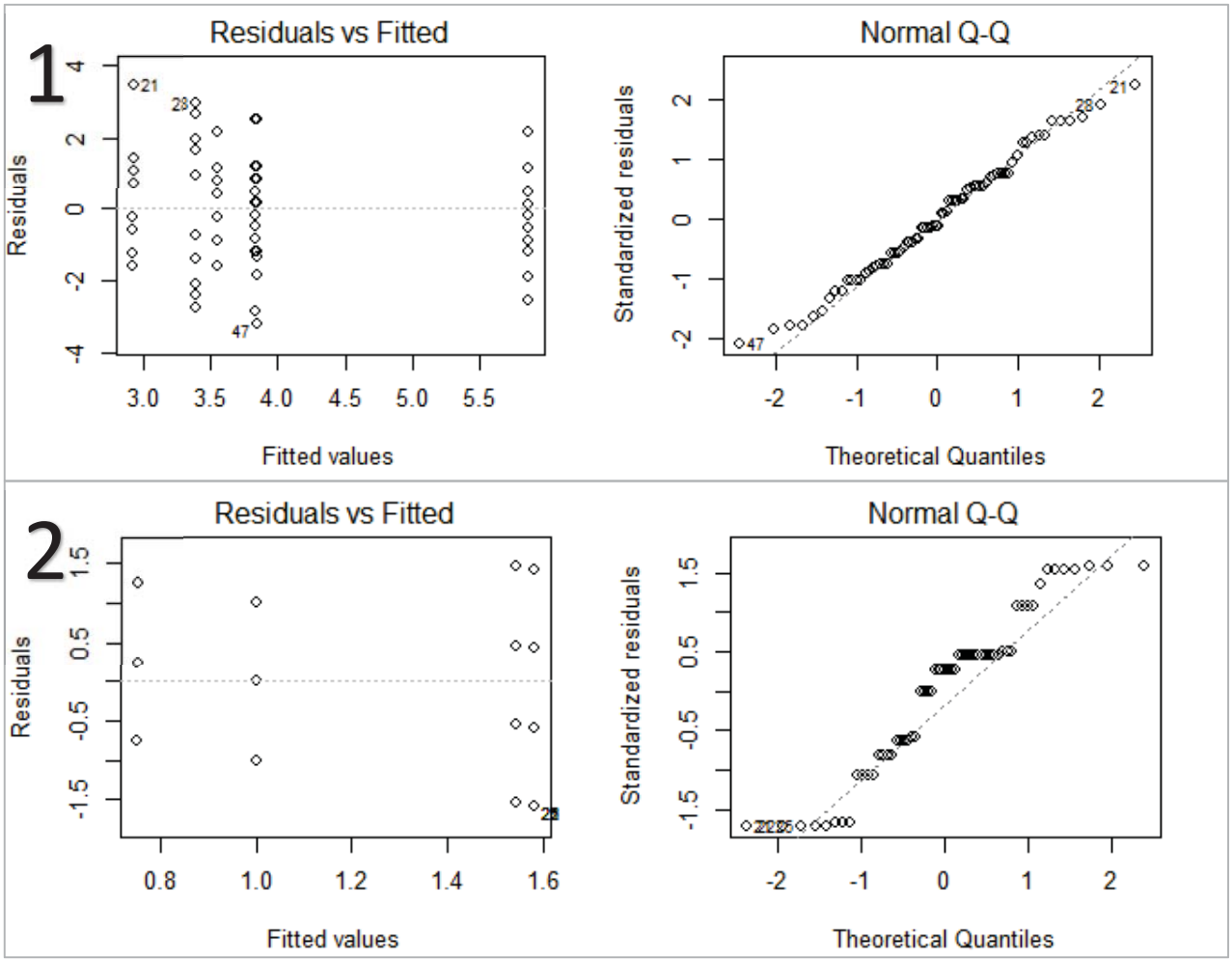

Figure I: (1) Residual vs fitted plot, and normal Q-Q plot for eggs per female by temperature regimen. Assumptions of equal variance and normality are fulfilled. ANOVA was used.
(2) Residual vs fitted plot, and normal Q-Q plot for number of bed bugs dispersing from the central zone during the heat treatments. Assumptions of equal variance is acceptable, however, the assumption of normality is not. Kruskal-Wallis H-test was used.

Table II: ANOVA table for two-way nested ANOVA with interaction. Temperature set to a fixed factor for the nearest 1°C, temperature regimen set to a fixed factor, arena set to a random factor within each temperature regimen, and the interaction between temperature regimen and temperature.

	Df	SS	F	P(>F)
Temperature	5	58.71	375.45	<0.001
Temperature regimen	4	8.35	13.93	<0.001
Arena within temperature regimen	54	2.21	2.21	<0.001
Temperature : temperature regimen interaction	20	5.96	9.52	<0.001
Residuals	616	19.27		

Table III: Comparison between Two-way ANOVA and nested ANOVA.

	Two-way ANOVA	nested ANOVA
R^2 adjusted	0.7604	0.7816
R^2 predicted	0.7491	0.7515
Standard deviation	0.1853	0.1768

Table IV: Sanyo versatile environmental test chambers programmed for the experimental weeks. It has a maximum of 12 steps. Chambers were programmed to endless cycles, so the last step continued until 1:00 the next morning, giving the experimental chambers the same day cycle as in the breeding chambers.

Rapid												
Time	01:00	15:00	15:43									
Temperature(°C)	22	43	22									
Light intensity	2	2	0									
Humidity	60	60	60									
Intermediate												
Time	01:00	13:15	13:40	13:56	14:12	14:28	14:44	15:00	15:16	15:32	15:48	15:56
Temperature(°C)	22	34	35	36	37	38	39	40	41	42	43	22
Light intensity	2	2	2	2	2	2	2	2	2	2	2	0
Humidity	60	60	60	60	60	60	60	60	60	60	60	60
Slow												
Time	01:00	11:00	11:24	11:56	12:28	13:00	13:32	14:04	14:36	15:08	15:40	15:56
Temperature(°C)	22	34	35	36	37	38	39	40	41	42	42.5	22
Light intensity	2	2	2	2	2	2	2	2	2	2	2	0
Humidity	60	60	60	60	60	60	60	60	60	60	60	60
Extra slow												
Time	01:00	06:44	07:08	08:12	09:16	10:20	11:24	12:28	13:32	14:36	15:40	15:56
Temperature(°C)	22	34	35	36	37	38	39	40	41	42	42.5	22
Light intensity	2	2	2	2	2	2	2	2	2	2	2	0
Humidity	60	60	60	60	60	60	60	60	60	60	60	60
Overnight												
Time	01:00	15:20	15:38	18:00								
Temperature(°C)	36	43	22	36								
Light intensity	2	2	2	0								
Humidity	60	60	60	60								
Control												
Time	01:00	17:00										
Temperature(°C)	22	22										
Light intensity	2	0										
Humidity	60	60										

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