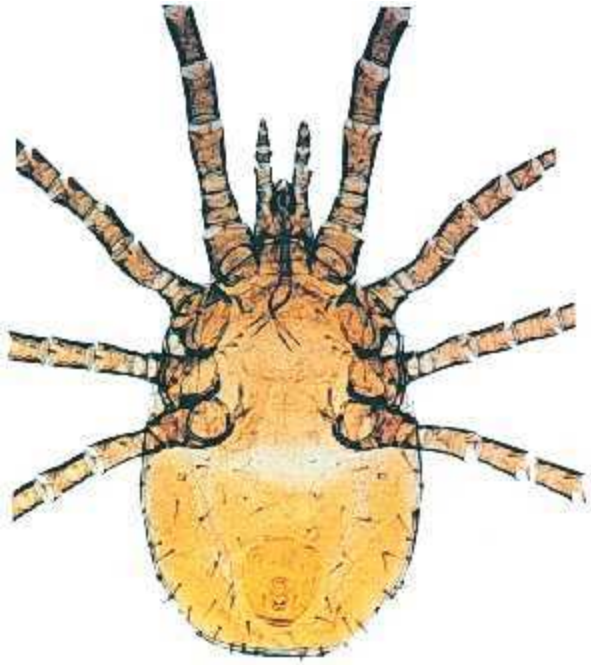
WAGENINGEN UNIVERSITY
LABORATORY OF ENTOMOLOGY

The effect of *B. bassiana* on behaviour and reproduction of the poultry red mite *Dermanyssus gallinae* and the attractivity of a synthetical chicken odour mix



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Contents

Abstract

Dermanyssus gallinae infestions have become a serious pest in the poultry industry in recent years. In order to combat the mites, biological control measures are under investigation, like the attract- and kill method. In this study, mites were tested for mortality, odour attractiveness and reproduction after infection. Mites were infected with *Beauveria bassiana* (1×10^6, 1×10^8 and 1×10^{10} conidia/m^2) and exposed for 10 sec., 3 min., 30 min. or 10 days to the fungus. Mortality after exposure for 3 min. was higher for all concentrations compared to the control ($p < 0.01$) and mortality after exposure to 1×10^{10} conidia/m^2 was higher compared to 1×10^6 and 1×10^8 conidia/m^2 ($p < 0.01$). Continuously, mites were infected with 1×10^{10} conidia/m^2 for 3 min. or left uninfected, and exposed to different odours in a two- choice y-tube olfactometer set-up (attract part). The odours involved are chicken feathers (kairomones) or mite aggregations (pheromones), as well as different synthetic compounds originated from odours relased by human skin bacteria and chicken feather bacteria. Uninfected, unfed mites were attracted to fed mite aggregations or chicken feathers compared to clean air, but not to either of these two odour sources when they were combined. This suggests that both host-seeking and aggregation pheromones are involved and that uninfected mites do not discriminate between them when offered both odour sources (i.e. they are both evenly attractive). In contrast, infected mites were not interested in seeking contact with conspecifics ($p > 0.5$,) but instead preffered host odours ($p = 0.001$,) in the combination with clean air, *and* when offered both choices in the y-tube olfactometer ($p < 0.01$). This indicates that infected mites are more attracted to the host, possibly for taking a blood meal for reproduction. For all combinations tested, no significant differences between responses of uninfected and infected mites was stated (binary logistic regression). Twenty-one synthetic compounds from chicken feather bacteria, identified by GC-MS, were significantly attractive for mites in the combination with paraffin oil only, in two out of three dilutions used (1/100 and 1/1.000.000; the third dilution used was 1/10.000). Because a subdivision of the total set into 10 compounds (also identified from human skin bacteria by GC-MS) or 11 did not show any significant attraction, this suggests that the 21 compounds are only attractive as a total set.
After fed mites were infected, no differences in number of laid eggs, hatched eggs or egg development time were found between the two groups. Apparently, before *B. bassiana* was able to penetrate and benefit from its host, the eggs were able to develop normally in the mean time. However, only eggs directly infected with *B. bassiana* showed significantly less hatching ($p < 0.01$,) than uninfected eggs, suggesting that the fungus was able to inoculate and penetrate the egg before egg development was completed.

Keywords:, *Dermanyssus gallinae*, *Beauveria bassiana*, host-seeking, aggregation, behaviour, Y-tube olfactometer, fecundity

1. Introduction

The poultry red mite *Dermanyssus gallinae* (Order: Acari, suborder: Mesostigmata) has become a serious ectoparasitic pest in the poultry industry worldwide in the last couple of years (Sparagano *et al*., 2009). Mite infestations occur in all kinds of poultry houses: broilers, free-range, barns and biological farming systems. The prevalence of *D. gallinae* infestation in the Netherlands has been estimated at 82% and 83% in cages and barns, respectively (Sparagano *et al*., 2009).
Dermanyssus gallinae feeds on the blood of chickens to complete its life cycle within seven days, when conditions are optimal (first described by Wood, 1917). It stays on the chicken to feed for approximately 0.5-1.5 h (Chauve, 1998). The 6-legged larvae hatch in 2-3 days from the eggs and need 1-2 days to become the first nymph stage, which depends on blood feeding to survive. This so-called protonymph subsequently develops into a deutonymph in another 1-2 days. Before developing into an adult within 1-2 days, it needs again blood feeding. After mating, adult females generally lay eggs within 12 hours after blood feeding; with 3-8 eggs per clutch (Chauve, 1998; Emous *et al*., 2005). A total of 50-60 eggs can be laid in one lifetime, which is about 20 days on average (Chauve, 1998; Emous *et al*., 2005).
A naturally occurring behaviour of mites is the formation of clumps or aggregations by fed adults and to a lesser extent also by fed proto- and deutonymphs (Entrekin and Oliver, 1982). Mite aggregations are present on bars and conveyor belts in poultry houses, and as a consequence can cause severe complications to chickens. Chickens' welfare and health has decreased by apparent irritation, restless behaviour, anaemia and even a possible death eventually (Emous *et al.*, 2005; Kilpinen *et al*., 2005). Next to this, poultry farmers suffer economic losses due to reduced egg production and blood-stained eggs (especially in battery systems where the eggs roll over mite aggregations on the conveyer belt, Emous *et al.*, 2005).
Farmers have used acaricides for decades to combat the mite infestations, but the problem is that they cannot reach the mites effectively, because the mites hide in small cracks and crevices in the chicken cages, especially at daytime. In addition, the problem of pesticide resistance in the mites makes the chemical acaricides, like pyrethroids for example, less effective (Marangi *et al.,* 2009; Thind and Ford, 2007). Instead, other possibilities like the use of biological agents are under investigation, for example the combined application of volatiles and a killing agent in the *attract and kill method* (Koenraadt and Dicke, 2009).
Two different fungi species have been investigated for their pest control potential: *Metharhizium anisopliae* and *Beauveria bassiana*. Strain V245 of the former fungus killed up to 100% of adult *D. gallinae* at day 8, at a concentration of 1×10^6 conidia/ml. The effectiveness of three strains tested (V245, 685 and 715C) seemed to be dependent on the concentration and the exposure time (Tavassoli *et al*., 2008). *Beauveria bassiana* has proven its effect in the control of the banana weevil *Cosmopolites sordidus* by the use of the fungus in combination with aggregation pheromone-baited traps (Tinzaara *et al.*, 2007). Horizontal transmission occurred in *C. sordidus* from inoculated individuals towards uninoculated weevils. Fungi can also be effective in the control of mosquitoes and ticks. Three different *Aspergillus spp.* strains out of 11 strains tested resulted in 80% larval mortality in *Aedes fluviatilis* and *Culex quinquefasciatus* at a concentration between 10^5 and 10^6 conidia/ml (Moraes *et al.*, 2001). In ticks, infection with *B. Bassiana* or *M. anisopliae* (after exposure of only 3 s) in adults and nymphs reached at least 40% mortality (dependent on the fungal formulation used) at a concentration of 10^9 conidia/ml after four weeks in the field (Kaaya and Hassan, 2000).
However, despite the seemingly effective use of fungi in the control of different pests, mites included, little is known about the behaviour of mites after infection with the specific fungus applied in the *attract and kill method*. Different types of behaviour of other insects towards entomopathogenic fungi are reported (Baverstock *et al.*, 2010). The tawny mole cricket *Scapteriscus Vicinus* and the Southern mole cricket *Scapteriscus Borellii* both prevented contact with *B. bassiana* by reduced surface tunnelling along a perimeter in a container treated with the DB-2 strain (Thompson and Brandenburg, 2005).
It is important to know a possible influence of a *B. bassiana* infection on the induction of mite behaviour; in the first place because mites have to be attracted towards the trap with the killing agent, and in the second place when they transfer the killing agent to the rest of the population by horizontal transmission (Koenraadt *et al.*, unpublished data).

Because *D. gallinae* occasionally bites humans (Haag-Wackernagel, 2005; Rosen *et al.*, 2002), a volatile that is attractive for mosquitoes can be attractive for mites as well. A synthetic blend, consisting of 10 compounds, which has its origin from human skin bacteria, (identified by GC-MS) has been shown to be attractive for *Anopheles gambiae* (Verhulst *et al.*, 2009). It appeared that these 10 compounds were also, including with 11 other compounds, isolated from a bacterial headspace and identified by GC-MS from chicken feather bacteria. It is important to know which compounds have its origin from chicken feather bacteria and which are presumably attractive for the red poultry mite.

The aims of this study are to 1) determine the optimal concentration of fungal spores and exposure time to fungal spores for killing *D. gallinae*, 2) investigate whether a *B. bassiana* infection influences attraction of *D. gallinae* towards conspecifcs or the host, 3) investigate the influence of a *B. bassiana* infection of eggs and on the females' fecundity (egg laying, hatching and egg development time) and 4) to test the response of *D. gallinae* towards synthetic compounds from human skin bacteria and chicken feather bacteria.

Infected mites are expected to show different behaviour compared to their healthy counterparts, in the first place because of their weakened physiological status but also possibly because of their changed function in the population. The changed physiological condition may reduce contacts with conspecifics, either because of reduced pheromone production or the absence of perception and thus a lack of response to its healthy conspecifics in the population.
If infected mites are physiologically weakened by the fungus, the egg production and the number of hatching eggs may be reduced. Directly, it is likely that *B. bassiana* inoculated eggs do not hatch or show a hampered or minimized development into the first larval stage.
Considering the last aim, it is expected that uninfected mites are attracted towards 21 compounds isolated from chicken feather bacteria, of which 10 compounds have been shown to be attractive for the malaria mosquitoe *Anopheles gambiae* (Verhulst *et al.*, 2009). It is assumed that the chicken feather bacteria compounds are more attractive than the odours which have its origin from human skin bacteria. This is because it is expected that mosquitoes are more easily attracted towards humans as a host odour than mites, which preferably feed on chickens, and rarely on humans (Kirkwood, 1971).

2. Materials and Methods

2.1 *D. Gallinae* mites and chicken feathers

The mites were obtained from a chicken livestock at the animal (poultry) accomodation De Haar, Department of Animal Sciences, Wageningen University, The Netherlands. Mites were collected in ventilated 50 ml plastic tubes out of small tube traps (see figure 1). Only fresh adult mites were used. Poultry feathers were also collected after 4-days in the sawdust and put into clean 50 ml tubes every test day.

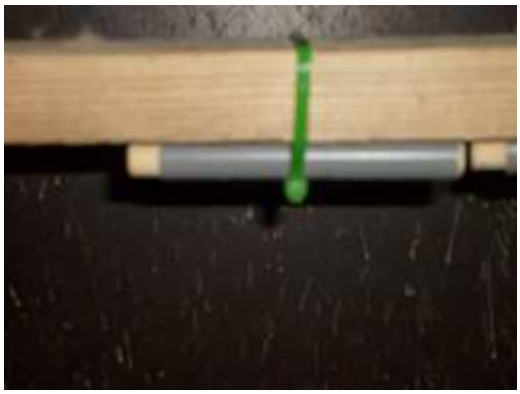


Figure 1. The attachment of a tube trap beneath the chicken perch from where mites are collected.

2.2 The fungus *Beauveria bassiana*

Dry conidia of *B. bassiana* (Vuillemin isolate IMI 391510) are suspended in Shellsol T-mineral oil (Shell Shellsol T®, Shell, The Netherlands). Spore concentration of the stock solution was determined by counting spores (by using an appropriate dilution) in a volume of 0.1 mm^3 with a Bürker-Türk haemocyte counter (W. Schreck, Hofheim/TS, see annex 1) at 400x magnification. The solution was diluted several times in Shellsol T-mineral oil solution to obtain three different concentrations for the infection of the mites: 6,25 x 10^4, 6.25 x 10^6 and 6.25 x 10^8 conidia/ml, leading to 1 x 10^6, 1 x 10^8 and 1 x 10^{10} conidia/m^2 (when 0.6 ml solution is pipetted on 15 x 25 cm proofing paper, see 2.3), respectively.

2.3 Pilot exp. Mortality of mites after *B. bassiana* infection

To determine an optimal fungus concentration- and exposure time at which the mites are infected (for the next experiments, see below), the following pilotexperiment was done.
Mites were exposed to three different fungus concentrations, 1 x 10^6, 1 x 10^8 and 1 x 10^{10} conidia/m^2. Every concentration was combined with four different exposure times: 10 sec., 3 min., 30 min., and continuous exposure (10 days). 10 mites were used per concentration x exposure combination, except the control (only 7 mites were included). Exposure occured in petri-dishes with grooving paper coated with the *B. bassiana* solution. The solution was evenly distributed over a 15 x 25 cm surface on a 18 x 31 cm grooving paper by pipetting 0.6 ml of the solution on a piece of grooving paper and spread with a grooved green K bar (0.31 mm wire diameter, RK Print Coat Instruments Ltd., UK; Farenhorst and Knols, 2010). The papers were dried for 24h. Mites were kept in ventilated 50 ml plastic tubes (20 mites per tube) at 70% RH and 27 °C for the next 10 days. Four plastic tubes containing control mites underwent the same exposure times, but with Shellsol T-mineral oil solution only. In every tube, 100 µl Of water was supplied onto a 2x2 cm piece of paper inside the tube, which was exchanged for another wet paper each 2 days. Mite mortality was checked daily by gently touching them with a small brush. Mites that did not move after touch were declared death.

2.4 Behavioural response to odours of *D. gallinae* in the Y-tube olfactometer

Exp. I: Effect of a B. bassiana infection on host seeking and aggregation behaviour of D. gallinae

The response towards host odours or kairomones, i.e. 4-days old poultry aged feathers (Koenraadt and Dicke, 2009), and aggregation odours (pheromones) of both infected and uninfected mites will be investigated in an Y-tube olfactometer (Figure 2). Air is filtered through activated charcoal, humidified and controlled by a constant flow rate via two gas flowmeters. A humidity of about 70% and a temperature of 26 ℃ was handled. Air is led through glass jars (with synthetic odours, Exp. II) or 50 ml tubes (with feathers or 200 fed mites) with the odor sources into the right and left arm of the Y-tube. Mites were infected at a certain exposure time and fungus concentration that was most appropriate according to the pilot study (see 2.3).
Mites were released at the down-wind site of the Y-tube and had 5 minutes total to walk along the wire into

the glass tubes to choose one of the two odor sources. When they fell of the wire within the first 30 seconds, they were placed back on top of the wire. If they stayed at least 1 minute in one of the two arms of the Y-tube, this was recorded as 'final choice'. All other situations were denoted as `no choice`. After 10 mites were tested, the complete Y-tube was cleaned with hot water and dried. Sixty mites were tested per treatment. Infected and uninfected mites were tested 4 days after the last blood meal (thus considered unfed) and exposed to the fungus or Shellsol T mineral oil only, respectively.
Experiments were randomized over time and days and mites were tested under dark conditions with the help of a red light.

After 4 days of inoculation, infected mites were given two choices at the start of the Y-tube wire: clean air vs clean air; 200 adult fed mites vs a clean air; aged feathers vs clean air; 200 adult fed mites vs aged feathers (both possibilities were presented in 50 ml plastic tubes). The behaviour of the infected mites was compared with the uninfected mites at day 4, which were given the same combinations of two choices (control). The first test combination (clean air vs clean air) was included to test air alone against itself (as a control to exclude a natural bias for the direction in which the mites walk).

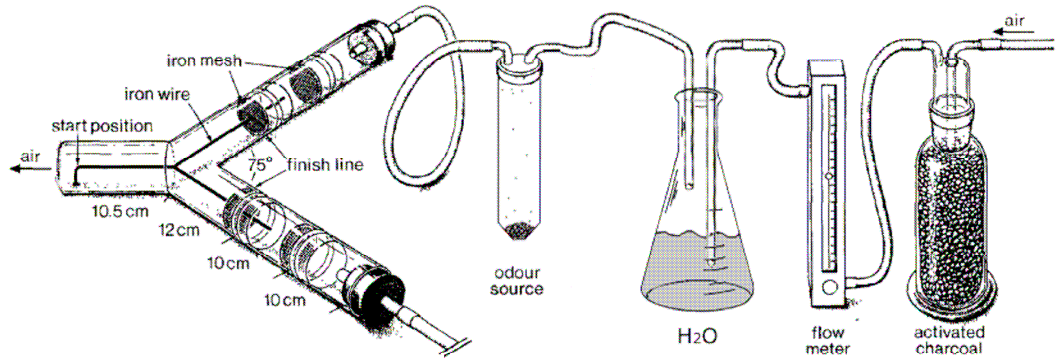


Figure 2. The Y-tube olfactometer. Air is filtered by activated charcoal and guided towards the odor sources by a constant flow rate of 2 l/min into each of the arms of the Y-tube. Glass jars or plastic tubes containing the odor sources are connected by tubes to the right and left arm of the Y-tube.

Exp. II Response of D. gallinae towards chicken feather bacterial odours
The response of adult fed (uninfected) mites (they were tested on the same day as when the mites were collected in the morning) towards 21 volatiles of chicken feather bacteria was also tested in the Y-tube olfactometer. See table 1 for an overview of all the compounds involved.
Three different dilutions of the volatiles were tested: 1/100, 1/10.000, and 1/1.000.000. Compounds were diluted in paraffin oil on weight by using an analytical balance (Mettler AC 100, Mettler Instrument bv., Arnhem) in a fume hood. After vortexing, 100 µl of every compound was pipetted separately into a 0.2 mm LDPE (Low Density PolyEthylene) sachet. Sachets were sealed tightly consequently. Sachets were presented on a "volatile tree": the trunk is a thick wire with a hook on the top, consisting of 5 or 6 smaller wires each attached to the trunk in a horizontal position, with one wire bearing two sachets on either side. The volatile tree was presented in a glass jar before the air flow entered the Y-tube olfactometer.

Different combinations of the compounds are tested. Because 10 of the 21 volatiles are also derived from human skin bacteria (tested in mosquitoe research, Verhulst *et al.*, 2009), these substances are tested against the total set of 21 compounds, to know towards which compounds the mites are attracted the most. Other combinations included are: Clean air vs clean air, empty LDPE sachets vs LDPE sachets with paraffin oil, 11 compounds (see last 11 compounds from bottom till top in table 1) in LDPE vs 11 x LDPE with paraffin oil, 21 compounds in LDPE vs 21 x LDPE with paraffin oil. Gloves are used when the volatile trees are put into the glass jars before testing.
Experimental conditions and set-up are as described in Exp. I.

Compound	Supplier	Purity	HSB origin	CFB Origin
1-butanol	Sigma	> 99%	x	x
2,3-butanedione	Fluka	> 99%	x	x
2-methyl-1-butanol	Sigma	> 99%	x	x
2-methylbutanal	Sigma	~ 95%	x	x
2-methylbutanoic acid	Sigma	~ 98 %	x	x
3-hydroxy-2-butanone	Sigma	≥ 97 %	x	x
3-methyl-1-butanol	Fluka	≥ 99.8 %	x	x
3-methylbutanal	Fluka	≥ 98 %	x	x
3-methylbutanoic acid	Sigma	~99%	x	x
Benzeneethanol	Fluka	≥ 99 %	x	x
Isobutyraldehyde	Sigma	> 99%		x
Ethyl acetate	Sigma	~99.7%		x
2-methyl-1-propanol	Sigma	> 99%		x
2,3-pentanedione	Sigma	~ 97%		x
3-methyl-2-buten-1-ol	Sigma	> 98%		x
2,3-butanediol	Sigma	> 99%		x
Butylester acetic acid	Sigma	> 99.5%		x
1-Butanol-3-methyl-acetate	Sigma	~ 97%		x
Benzaldehyde	Sigma	> 99%		x
Benzylacohol	Sigma	> 99%		x
Decanal	Sigma	~ 95%		x

Table 1. The compounds (21 in total) derived from chicken feather bacteria (CFB) and identified by GC-MS. The first 10 compounds also have its origin from human skin bacteria (HSB) and have been tested in odour-based trapping systems for *An. Gambiae* (Verhulst *et al.*, 2009).

2.5 Exp. III. Sublethal effects of a *B. bassiana* infection in *D. gallinae*

The influence of a *B. bassiana* infection in adult *D. gallinae* on egg-laying, egg hatching and egg development time of female *D.gallinae* was investigated. First, fed adult mites were infected with the most optimal fungus concentration at a certain exposure time (see 2.3). Sixty-four infected mites were placed individually in 0.5 ml tubes, which were ventilated by making 4 small holes in the lid with a needle. Sixty-four uninfected mites received the same treatment (control). The amount of laid eggs (visible under the microscope) were counted every day as long as egg-laying lasts after one blood meal (presumably up to 3 days; Nordenfors *et al*., 1999). Eggs were carefully removed from the tubes with a small brush and transferred to a petri-dish with 400 µl of water supplied on a filter paper. Eggs were counted for their hatchability for up to 7 days.

Whether the hatchability of *D. gallinae* eggs is influenced by infecting the eggs directly with *B. bassiana* was also tested. Female mites were collected out of our chicken cage at *De Haar* and put together in 50 ml tubes. The next day, eggs were carefully transferred out of the tubes onto grooving papers impregnated with either 24h incubated Shellsol or the fungus solution. An amount of 32 eggs were left uninfected but exposed to Shellsol impregnated on paper for 10 sec. only. Another 32 eggs were directly infected with the same concentration used for the mites, but for only 10 sec. in a petri-dish and subsequently transferred individually to another petri-dished (with 400 µl of water) and checked for hatching in the following 3 days.

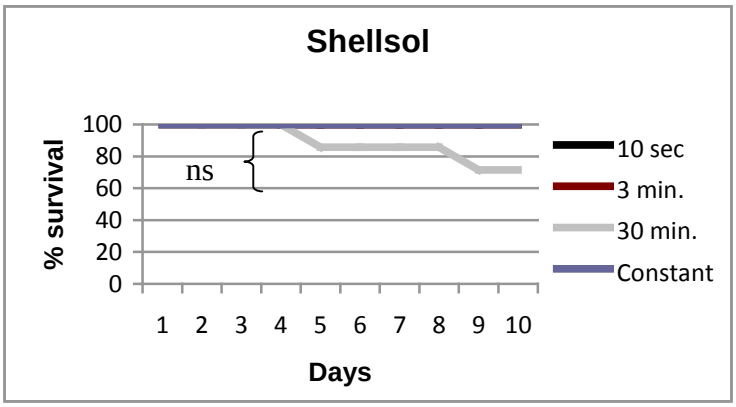
3. Data Analysis

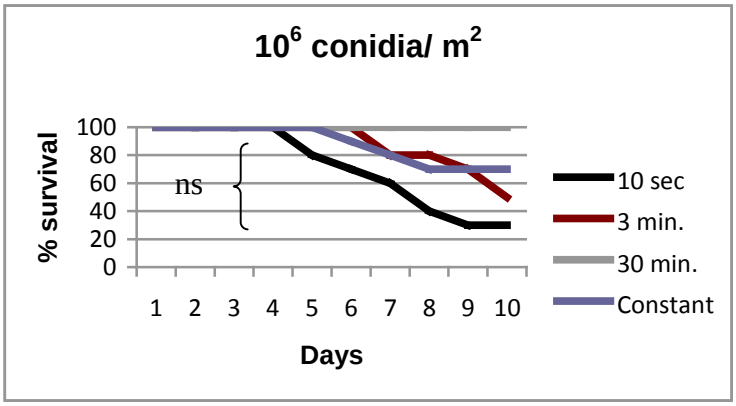
Survival of the mites in the mortality pilot study is analysed by the statistical test Kaplan-Meier Survival Analysis (KMSA). Time is used as a duration variate and the grouping of concentration and exposure time was set as a stratification variable. Because no other covariate effects are possible with KMSA, Cox Regression was also used. Categorical codings in Cox Regression are treatment (infected of uninfected), exposure time and concentration. Survival curves are plotted and tested for significance at a confidence interval of 95%.
Responses of the infected and uninfected mites in the Y-tube olfactometer will be tested with a two-sided binomial test. This is done to check for a deviation from the 50:50 distribution. A 50:50 distribution is expected when a two-choice experiment has been set-up. Binary logistic regression is used to check for differences between infected and uninfected mites. Mites are tested for the relative attractiveness (number of mites choosing one of the two Y-arms out of all reponses, so not-responding mites are excluded in this analysis).
Data from the experiments on egg laying, hatchability and egg infectivity are tested with an independent sample T-test, to check for differences between uninfected and infected mites. Eggs were also tested individually for the specific days they were laid, hatched and the egg development time (day of hatching minus the day of laying the egg by the female mite).
Tests are run using the statistical program PASW Statisctics v17.0.

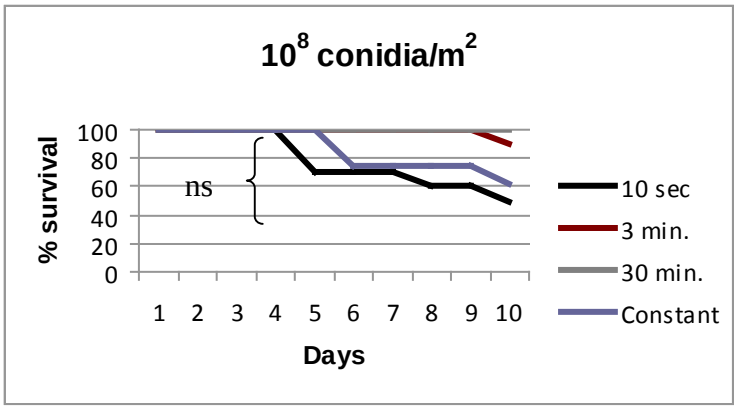
4. Results

4.1 Infection of *D. gallinae* with concentration dependent *B. bassiana* solutions and different exposure times

In the control situation, all mites, except 2 mites (28.6% of total) exposed to Shellsol mineral oil for 30 min.,survived during the following 10 days (see figure 3).







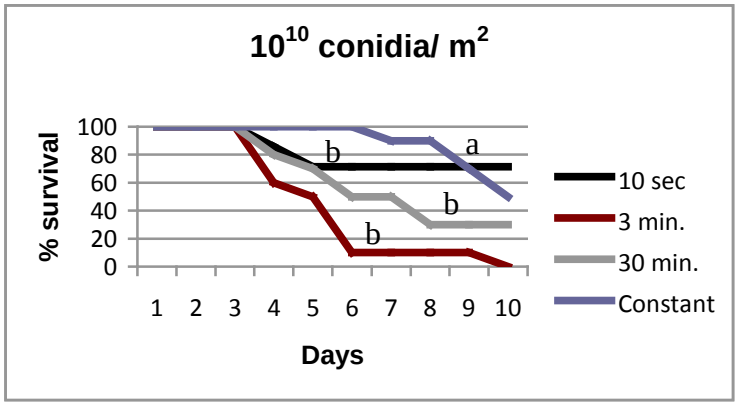


Figure 3-6. The survival curves of mites which were exposed to only Shellsol mineral oil solution (3), 10^6 conidia/ m^2 (4), 10^8 conidia/m^2 (5) or 10^{10} conidia/ m^2 (6) for 10 sec., 3 min., 30 min. or constant for 10 days, *ns*= not significant.

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Comparing the 16 treatment groups (concentration x exposure time) with Log Rank (Mantel-Cox) in the Kaplan- Meier test revealed an overall significant difference between groups (df= 15, $p < 0.001$) in time to event. This effect was especially due to the different concentrations used, which were also significantly different in determining the survival time of the mites (df=3, $p< 0.001$).

4.1.1 Effect of exposure time

In the control situation, 10-days exposure to only shellsol was not significantly different from the other times (df= 3, $p= 0.942$, figure 3).The 10 sec. exposures used in the three spore concentrations were not significantly different from the control (df=3, $p= 0.658$), neither was 30 min. exposure (df=3, $p=0.424$) nor the constant exposure (df=3, $p=0.949$). However, the 3 min. exposure time was significantly different between control and the three spore concentrations (df=3, $p=0.001$, figure 3-6).
At a concentration of 1 x 10^6 conidia/m^2 or 1 x 10^8 conidia/m^2 (Figure 4-5), the different exposure times were not significantly different from each other (df=3, $p=0.340$ and df=3, $p= 0.350$, respectively). However, the highest concentration of fungus was enough for a significant effect between a constant exposure with all other times (df=3, $p= 0.009$) and specifically between the constant and the 3 min. exposure (df=3, $p=0.004$, Figure 6).

4.1.2 Effect of concentration

The mites which were exposed to the lowest concentration of conidia/m^2 started to die on day 5 (20% died) when contact with the fungus had been 10 seconds on day 0 (Figure 4). On day 10, 70%, 50% and 30% of the mites died exposed to the fungus for 10 sec., 3 min. and constant, respectively. Mites exposed to the fungus for 30 min. all survived during the time of the experiment.
At a concentration of 1 x 10^8 conidia/m^2, 70% of the mites exposed to 10 seconds was still alive on day 5, down to 50% on day 10. After 3 min., 30 min. or constant exposure, 90%, 100% and 62.5% was still alive on day 10, respectively (Figure 5).

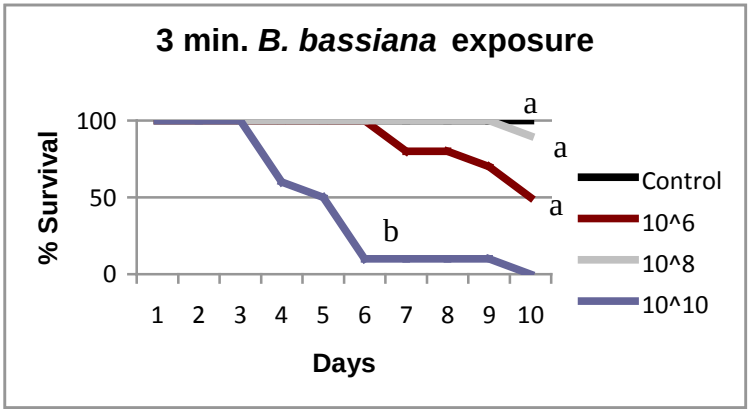

Figure 7-8. Mites were exposed for 3 min. to Shellsol only (control) or different concentrations of *B. bassiana* (in conidia/m^2) (7) and (8) a picture of a mite infected with the highest concentration (10 days after infection).

When mites were exposed to the highest fungus concentration used (Figure 6), mites died on day 4 already. On day 4, 14.3%, 40%, 20% and 0% died after 10 sec., 3 min., 30 min. and constant exposure, respectively. No mites were alive on day 10 which received the 3 min. treatment. Figure 7 shows a picture of a mite infected with 1 x 10^{10} conidia/m^2 after 10 days, note the overwhelming hyphae of *B. bassiana* on the cuticle.
When testing for a concentration effect between 10 sec., 30 min. and constant, no significance was found (df=3, $p=0.658$; $p=0.424$ and $p=0.949$, respectively). Only the 3 min. exposure (Figure 8) showed a significant effect between 1 x 10^{10} conidia/m^2 and both 1 x 10^6 conidia/m^2 and 1 x 10^8 conidia/m^2 (df=3, $p=0.001$). The Expression (B) or Hazard Ratio (HR) was < 1 for both 1 x 10^6 conidia/m^2 and 1 x 10^8 conidia/m^2 (HR=0.150 and HR=0.025), meaning that these groups had a less likely shorter time to event than the tested reference group (in this case the highest concentration of 1 x 10^{10} conidia/m^2).

For continuous experiments, it was decided that mites were infected with 1 x 10^{10} conidia/m^2 at 3 min. exposure time.

4.2 Response of mites towards host-seeking and aggregation odours

In the control situation, where mites were exposed to clean air on both sides of the Y-tube olfactometer, no significant difference was shown in either uninfected (p= 0.720) or infected (p= 0.523) mites (Figure 9). However, when one of the two sides was exchanged for feathers instead, mites preferred this host-seeking odour significantly, in both uninfected (p= 0.047) and infected (p= 0.008) *D. gallinae*. Uninfected mites were significantly attracted towards 200 mites, whereas the infected mites were not (p= 0.019 and p=0.585, respectively).
Infected mites preferred host odours above aggregation odours (p= 0.001) while the uninfected mites did not (p= 0.296).
Although differences in responses between infected and uninfected mites seem to exist with the Binomial test, they are not significant between the two groups in either odour combination in Binary Logistic Regression.

4.3 Response of mites towards chicken feather bacterial odours

In this experiment only 20 uninfected adult fed mites are used and tested for the attractiveness to different odours (21 in total, figure 10). When the two subsets were included in the test separately (10 and 11, see table 1 of the compounds), this led to insignificant results. Specifically, the 11 odours tested against paraffin oil only did not show a significant result for either 1/100 , 1/10.000 or 1/1.000.000 dilution (p=0.210, p=0.143 and p=0.804, respectively). The 10 odours tested against the total set of compounds, did not show a significant result for either 1/100 , 1/10.000 or 1/1.000.000 dilution (p=1.000, p=0.791 and p=0.454). When the total set of compounds was tested against only paraffin oil, two out of three dilutions of the compounds showed a significant result (p=0.019 for 1/100 and p=0.035 for 1/1.000.000).

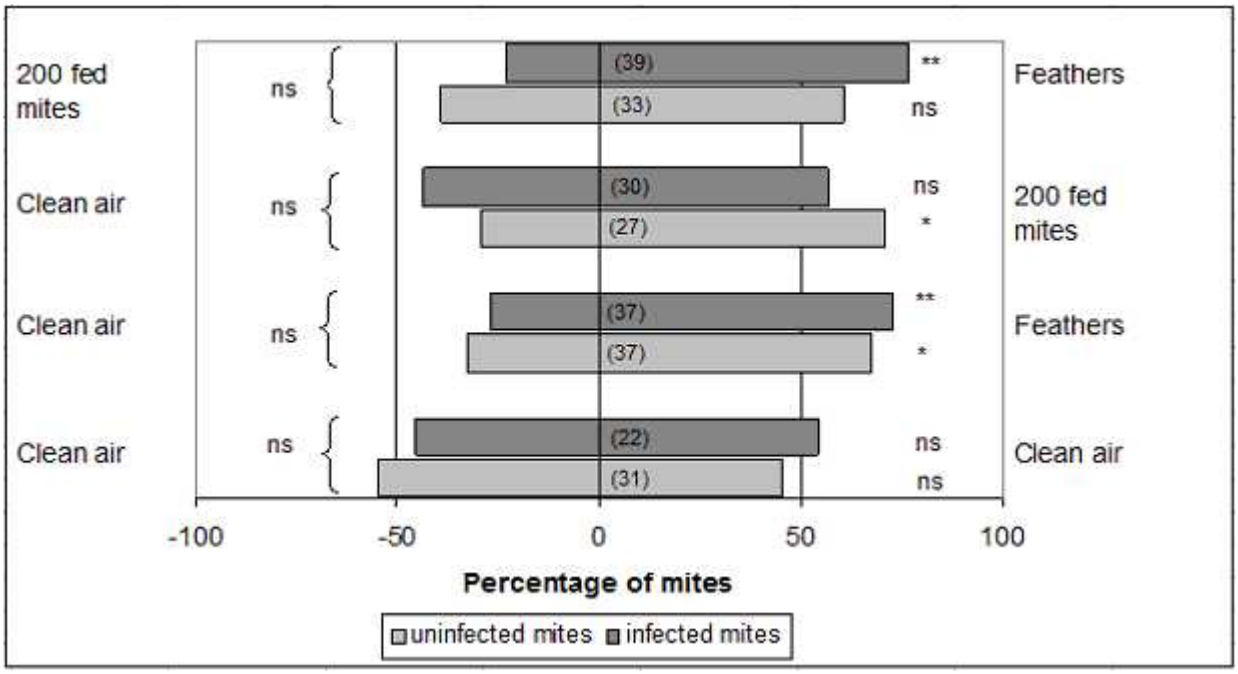

Figure 9. Mites, infected (dark grey) or uninfected (light grey), were tested in the Y-tube olfactometer and exposed to different two-choice odour combinations (N=*60*, * $p < 0.05$ and ** $p < 0.01$, ns= not significant). The total number of responding mites (out of total tested) is shown between parentheses.

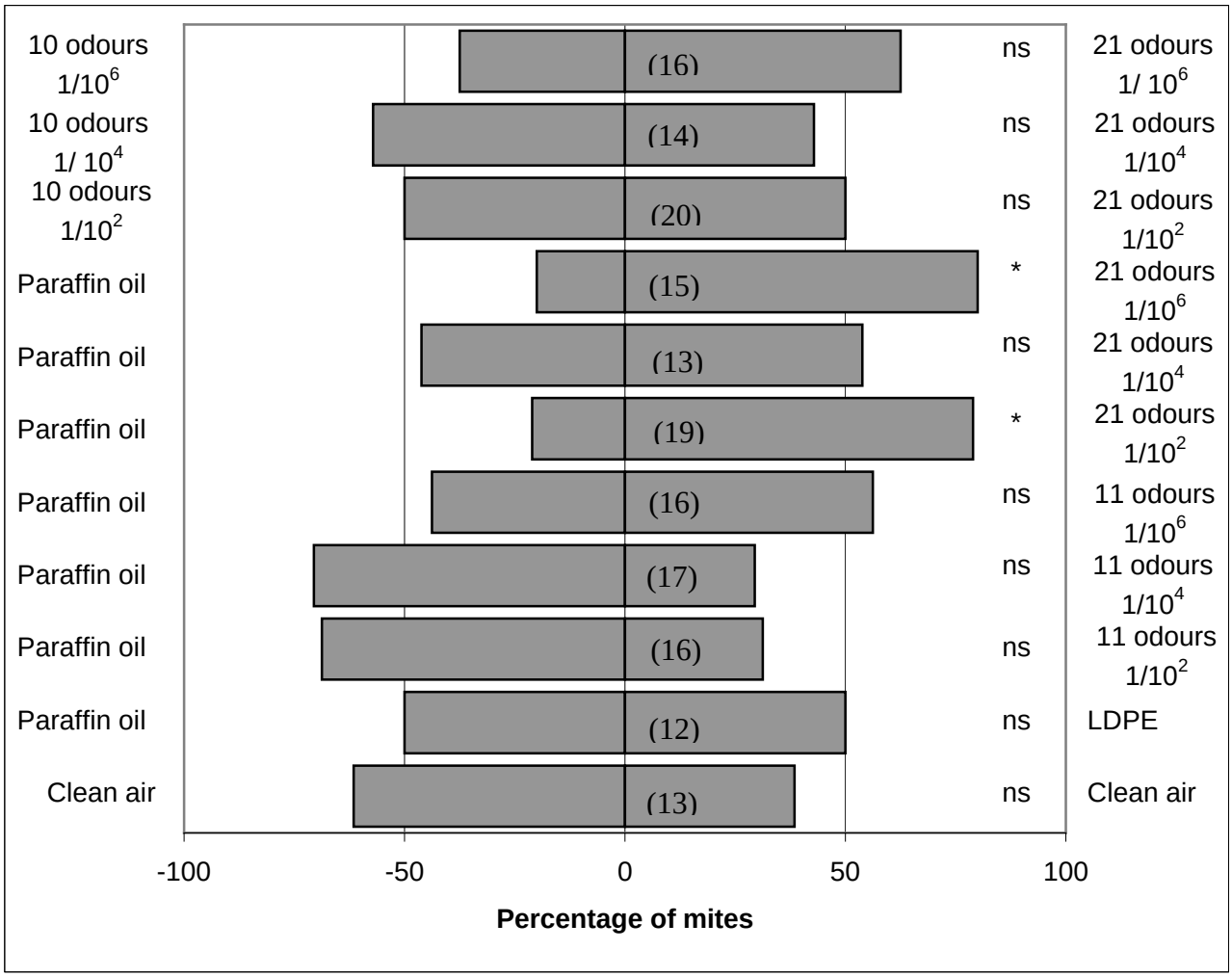

Figure 10. LDPE sachets were used filled with compounds that were diluted with paraffin oil for three times ($1/10^2$, $1/10^4$ and $1/10^6$). Mites could choose for either the total set of compounds (21) or paraffin oil, or two subsets (10, see top or 11, see bottom) also combined with the total set (10) or paraffin oil (11). The number of mites responding out of the total tested (*N*=20) is shown between parentheses.

4.4 Egg clutches and amount of hatches after *B. bassiana* mite and egg infection

In total, 397 eggs were laid by the uninfected mites and 388 by the infected mites (resulting in a mean of 6,27 and 6,07 eggs, respectively) up to four days after the last blood meal. On day 1, 400 eggs were laid out of the 785 eggs in total (with a mean of 3,25 and 3,00 eggs for uninfected and infected mites, respectively), see figure 11. On day 2, a mean of 3,54 eggs were laid by both mite groups. Because some mites escaped during the counting or egg removal (when the PCR tube was opened), this resulted in 124 mites at the end of the experiment. The means have been corrected for this lower amount after day 3 (on this day the first mite escaped).

No significant difference was found in the amount of eggs laid when mites were infected with *B. bassiana* compared with uninfected mites (p= 0.670, figure 12). All mites laid eggs on the first two days of the experiment, except 1 mite, which laid no eggs during the total time (7 days) of the experiment and was presumably a male mite.

No significant difference was found between groups for laid eggs between days (p= 0.296, figure 12), egg hatches between days (p= 0.759) or egg development time (p= 0.962, see figure 13).
Up to 7 days after treatment, the eggs that were directly infected showed no hatching in 20 out of 32 eggs (62,5%). All 32 eggs which were left uninfected, hatched normally after two days of the treatment (p= 0.000 between treatments, see figure 14).

Up to 7 days the amount of dead mites was counted (to check for the effect of *B. bassiana*) and resulted in 49.21% of deaths in the 64 infected mites and 13.12% in the 64 uninfected mites.

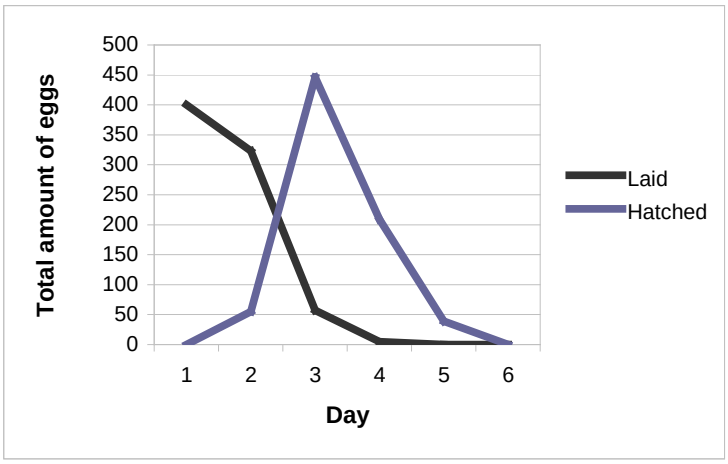

Figure 11. Eggs laid and hatched of 128 mites in total over a time period of 6 days after one blood meal.

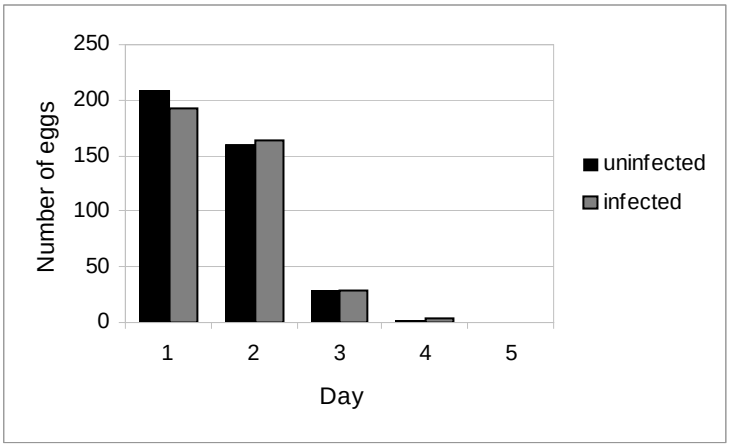

Figure 12. Egg production of mites infected with *B. bassiana* and uninfected mites

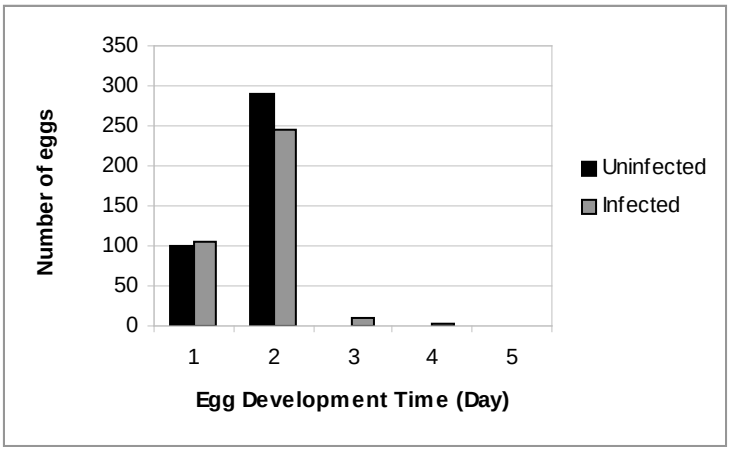

Figure 13. Development time of eggs laid by mites infected with *B. bassiana* and uninfected mites

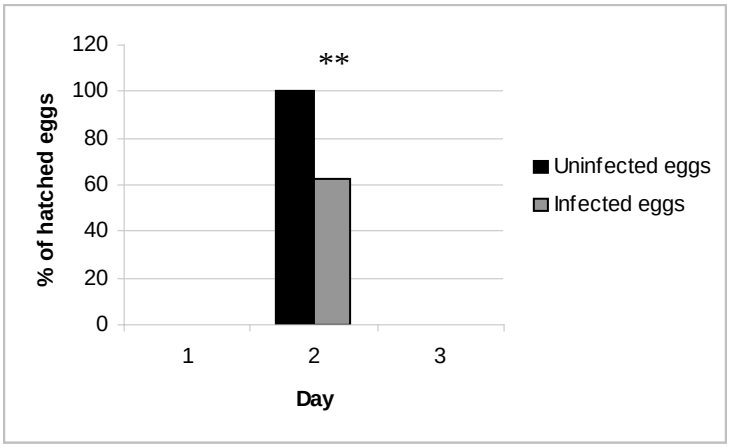

Figure 14. Eggs were infected directly or left uninfected and checked for hatching. P**< 0.01.

5. Discussion

Dermanyssus gallinae showed a higher mortality when exposed to 10^{10} conidia/m^2 of *B. bassiana* compared to the control, suggesting that this fungus is effective in killing the mite. Specifically, when mites were infected with 10^{10} conidia/m^2 of *B. bassiana* for 3 min. in a petri-dish, this was significantly different from the other exposure times, *and* from the other two concentrations. It was expected that longer exposure times would also lead to higher mite mortality, but this has to do with the used method presumably. For the constant exposure a Shellsol proofing paper coated with the fungus was provided into the 50 ml tube, however, mites were still able to walk beneath the paper or in the tip of the bottom of the tube, so without direct contact with the fungus. It is possible that some mites did hardly pick up any conidia from the paper, although they were all placed on the paper inside the tube at the start of the experiment. Tavassoli *et al* .(2008) found that 39.2% of adult *D. gallinae* died after 6 days of exposure to 1 x 10^6 conidia/ml of strain 685 of *M. anisopliae*. In this study, a constant exposure of 6 days with 6.25 x 10^6 conidia/ml (= 1 x 10^8 conidia/m^2) led to 25% of dead mites. However, the method they used was different (a permanent exposure of the mites with the fungus in a petri-dish) and results were very strain-specific within *M. anisopliae*. Studies in which *B. bassiana* has been used for *D. gallinae* infection are absent. Furthermore, it is unknown whether those two different fungi can have different effects on the immune system of the mite, supposedly this is related to the extracellular chitinases which are used as chitin degradation products to penetrate the cuticle of the mite (Leger *et al.*, 1996). *Metarhizium anisopliae* produced acidic isozymes in this process, whereas *B. bassiana* did not.

Exposure of the mites to host-seeking and aggregation odours did not reveal significant differences in responses between uninfected and infected mites, indicating that *B. bassiana* infection did not alter responses. However, within the groups, uninfected unfed mites were significantly more attracted to incubated feathers and aggregations of fed mites, in the combinations with clean air only. This is in consistency with the findings from Koenraadt and Dicke (2009). In contrast, infected mites were attracted to feathers, in combination with clean air only as well as with fed mite aggregations. This presumes an interest in host-seeking above making contact with conspecifs, but not necessarily host-seeking only (because uninfected mites also walked more to feathers in the same two-choice set up). Apparantly, a fungus infection makes the mites more likely to search for a host, possibly because taking a blood meal is necessary for reproduction for *D. gallinae* (Oliver, 1966). Because of their physiological weakened status, they could also have a higher demand for energy supply, possibly also for the immune system of the mite (Gillespie *et al.*, 2000).

Because infected mites avoided contact with conspecifics (in the combinations with clean air or feathers) this could mean that infected mites are unable to detect aggregation pheromones, or do not respond to them when they are detectable. This indicates a changed physiological state inside the mite, either because of weakness or because the mite prevents walking to healthy conspecifics. In termites, it is known that *M. anisopliae* infected *Reticulitermes flavipes* received defensive reactions of uninfected individuals. Uninfected termites emitted also alarm pheromones and they displayed aggregation behaviour. After 24 minutes, this was followed by grooming, biting and burial of the infected mite (Myles, 2002). However, it is unknown if mites can show avoidance behaviour to fungi.

Unfortunately, the result of this study would mean that infected mites in poultry houses would prevent contact with its healthy counterparts. Practically this would bring limitations because of limited possible horizontal transmission between mites, which was shown to be possible in the laboratory (Koenraadt *et all* unpublished data) . However, it has to be emphasized that unfed mites are only tested in this study, which were not observed in the poultry house (personal observation). So in the case of fed mites, the result could be different in that they are more attracted to conspecifics to aggregate, whether infected or not. In fact, a lot of studies report the higher incidence of fed mites to aggregate compared to unfed mites (Entrekin and Oliver, 1982; Koenraadt and Dicke, 2009). It would be promising to infect mites after allowing the mites to feed *in vitro* systems which have been shown to be efficient for *D. gallinae*, for example the *in vitro* feeding device with chicken skin membrane (Arkle *et al.*, 2010; Bruneau *et al.*, 2001).

Fed mites were not able to discriminate between the total set of synthetic feather bacteria compounds or a subset of 11 compounds and neither attracted to the other subset of 10 compounds, but preferred odours of 21 synthetic substances of feather bacteria significantly above paraffin oil only in two out of three dilutions (1/100 and 1/1.000.000). This suggests that the 21 odours belong to a complex odour formation that have to be used together. For future experiments it is important to keep in mind that isolated compounds can give different results than the combination of all compounds together, which at least has been shown in this study. The total set mimics the volatiles released by chicken feather bacteria, thus can be seen as an alternative for the attraction to feathers only, which was significant for fed mites as well in the study of Koenraadt and Dicke, 2009. Moreover, because only 20 mites were tested in this study, results are more dependent on the amount of responding mites (which was even less than 20 tested).

A clear observation during the test was the movement of the forelegs of *D. gallinae* while walking on the Y-tube wire. The poultry red mite has a sensory field in the tip of the foreleg existing of 10 sensillae in total, of which three porous medium sized setae on the tarsus are believed to play a role in chemoreception (Cruz

et al., 2005).

No effect of a *B. bassiana* infection on amount of eggs laid, hatched or egg development time was found. This could be due to the fact that mites were infected *after* they had taken the blood meal and were only followed for one oviposition period for up to 5 days after this last blood meal. Thus, before *B. bassiana* was able to penetrate and benefit from its host, the eggs were able to develop normally in the mean time. Pathogenicity of the fungus starts with germination, followed by penetration by the fungal germ tube and finally death of the host. Different studies indicate a relation between the degree of pathogenicity of *B. bassiana* and the commencement of mortality (Abood *et al.*, 2010; Adane *et al.*, 1996). Because mites started to die on day 4 in the egg-lay experiment of this study, this could mean that the virulent effets of the used *B. bassiana* strain on egg production did not occur before day 4. Mites stopped laying eggs just 4 days after the last blood meal as well (see figure 11), strengthening the possibility of the absence of the influence of the fungus on egg production. Moreover, it is known that virgin *D. gallinae* can partially develop eggs already before mating or taking a blood meal (Oliver, 1966), however mating and engorgement are both necessary for oviposition in this species (Hutcheson and Oliver, 1988). So eggs could have been partially or even totally developed already before mites were taken off the perches, and because the mites just fed before testing, the eggs were laid normally after one day already. After the 4^{th} and 5^{th} blood feeding, mean number of eggs laid were 3.71 and 3.95 (Oliver, 1966) per female, which is not in consistency with this study (mean of 6.27 eggs were produced after x times of feeding). This could be due to the lower sample size of *D. gallinae* in the study of Oliver (amounts were around 11-13 females) compared to this study, or to a higher age and amount of feedings in this study before mites were tested. However, the duration of oviposition (3 days) is similar to the current study. In the study of Nordenfors *et al.* (1999) a total of 295 eggs were laid by 77 females at 25 ℃, with a mean of 3.50 eggs after 1.8 days. This is comparable to the results of this study: 3.54 mean eggs per female after 2 days. Eggs hatched after approximately 2 days into larvae after eggs were deposited for up to three days by 247 female *D. gallinae*, which is also similar to this study (the most eggs that were laid hatched after 2 days, see figure 14) (Hutcheson and Oliver, 1988).
Eggs directly infected showed significantly less hatching, suppossedly because the fungus was able to inoculate and penetrate the egg shell before egg development was completed. Indeed, hyphae were clearly visible 3 days after infection. One dead larvae from an infected egg was observed in the petri-dish, indicating that not only hatching could possibly be influenced by *B. bassiana*. Further research is needed to conclude this observation.
With the current method, no difference in fecundity could be observed between uninfected and infected mites, and therefore, it would be wise to repeat the experiment with fed mites that become infected while still able to have multiple feedings (for example in the feeding device of Arkle *et al.*, 2010). It would be better to let infected mites feed on chickens after they have oviposited for the first time after infection in the laboratory, to ensure that *B. bassiana* infection is completed and that it could have an influence on the fecundity of the female mite.
In conclusion, this study revealed that mites infected with *B. bassiana* were likely to be in search for a host, instead of seeking contact with conspecifics.It also seemed that infection with *B. bassiana* did not have an influence on the fecundity of *D. gallinae*, measured in egg numbers, hatches and the egg development time. However, eggs directly infected with *B. bassiana* showed decreased hatching compared to uninfected eggs.
Whereas attraction to the 21 feather bacteria compounds was significant compared to the preference to two subsets of these compounds, this has to be confirmed by including more mites.

This study has revealed new information about the behaviour and fecundity of *D. gallinae*, which could be relevant in the importance of developing new control methods for the poultry red mite in the poultry industry.

Acknowledgement

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References

1. Abood F., Bajwa G.A., Ibrahim Y.B., Sajap A.S. (2010). Pathogenicity of Beauveria bassiana against the Tiger Moth , atteva sciodoxa (Lepidoptera: Yponomeutidae) J. Entomol. **7**: 19-32.

2. Adane K., Moore D., and Archer S.A. (1996). Preliminary studies on the use of Beauveria bassiana to control Sitophilus zeamais (Coleoptera: curculionidae) in the laboratory. J. Stored. Prod. Res. **32**: 105-113.

3. Arkle S., George D.R., Guy J.H., Sparagano O.A.E. (2010). Comparison of *in vivo* and *in vitro* survival and fecundity rates of the poultry red mite, *Dermanyssus gallinae*. Research in Veterinary Science **88:** 279–280.

4. Baverstock J., Roy H.E., Pell J.K. (2010). Entomopathogenic fungi and insect behaviour: from unsuspecting hosts to targeted vectors. BioControl **55**: 89-102.

5. Bruneau A., Dernburg A., Chauve C., Zenner L. (2001). First in vitro cycle of the chicken mite, Dermanyssus gallinae (DeGeer 1778). utilizing an artificial feeding device. Parasitology **123**: 583–589.

6. Chauve C. (1998). The poultry red mite *Dermanyssus gallinae* (De Geer, 1778): current situation and future prospects for control. Vet. Parasit. **79**: 239-245.

7. Cruz S. M.D., Vega Robles M.C., Jespersen J.B., Kilpinen O., Birkett M., Dewhirst S., Pickett J. (2005). Scanning electron microscopy of foreleg tarsal sense organs of the poultry red mite, *Dermanyssus gallinae* (DeGeer) (Acari: Dermanyssidae). Micron **36**: 415-421.

8. Emous R.A , Van Niekerk T.G.C.M.F., Mul M.F. (2005). Bloedluizen (vogelmijten) op papier en in de praktijk—red mites in theory and practice. Animal Sciences Group/Praktijkonderzoek, Lelystad, 44 pp.

9. Entrekin D.L. and Oliver J.H. (1982). Aggregation of the Chicken Mite *Dermanyssus Gallinae* (Acari: Dermanyssidae). J. Med. Entomol. **19**: 671-678.

10. Farenhorst M. and Knols B. (2010). A novel method for standardized application of fungal spore coatings for mosquito exposure bioassays. Malaria Journal **9**:27.

11. Gillespie J.P., Bailey A.M., Cobb B.,Vilcinskas A. (2000). Fungi as Elicitors of Insect Immune Responses. Archives of Insect Biochemistry and Physiology **44**:49–68.

12. Haag-Wackernagel D. (2005). Parasites from feral pigeons as a health hazard for humans. Ann. Appl. Biol. **147**: 203-210.

13. Hutcheson H.J. and Oliver Jr. J.H. (1988). Spermiogenesis and Reproductive Biology of Dermanyssus gallinae (DeGeer) (Parasitiformes: Dermanyssidae). J. Med. Entomol. **25**: 321-330.

14. Kaaya G.P. and Hassan, S. (2000). Entomogenous fungi as promising biopesticides for tick control. Exp. and Appl. Acarol. **24**: 913–926.

15. Kilpinen O., Roepstorff A., Permin A., Norgaard-Nielsen G., Lawson L.G., Simonsen H.B. (2005). Influence of *Dermanyssus gallinae* and *Ascaridia galli* infections on behaviour and health of laying hens (*Gallus gallus domesticus*). Br. Poult .Sci. **46**:26–34.

16. Kirkwood A.C. (1971). In vitro feeding of *Dermanyssus gallinae*. Exp. Parasit. **29**:1–6.

17. Koenraadt C.J.M., Dicke E.M. (2009), The role of volatiles in aggregation and host-seeking of the haematophagous poultry red mite *Dermanyssus gallinae* (Acari: Dermanyssidae), Exp. Appl. Acarol., published online.

18. Leger R.J.St., Joshi L., Bidochka M.J., Rizzo N.W. & Roberts D.W. (1996). Characterization and ultrastructural localization of chitinases from *Metarhizium anisopliae*, *M. flavoviride*, and *Beauveria bassiana* during fungal invasion of host (*Manduca sexta*) cuticle. Applied and Environ. Microbiol. **62**: 907-912.

19. Marangi M., Cafiero M.A., Capelli G., Camarda A., Sparagano O.A.E., Giangaspero A. (2009). Evaluation of poultry red mite (*Dermanyssus gallinae*, Acarina: Dermanyssidae) susceptibility to some acaricides in a field population from Italy. Exp. Appl. Acarol. **48**: 11-18.

20. Moraes de, A.M.L. , Costa da, G.L., Barcellos de, M.Z.B., Oliveira de, R.L., Oliveira de, P.C. (2001). The entomopathogenic potential of *Aspergillus* spp. in mosquitoes vectors of tropical diseases J. Basic Microbiol. **41**(1): 45–49.

21. Myles T.G. (2002). Alarm, aggregation, and defense by *Reticulitermes flavipes* in response to a naturally occurring isolate of *Metarhizium anisopliae*. Sociobiology **40**:243–255.

22. Nordenfors H., Glund H.J. and Uggla A. (1999). Effects of Temperature and Humidity on Oviposition, Molting, and Longevity of *Dermanyssus gallinae* (Acari: Dermanyssidae). J. Med. Entomol. **36**: 68-72.

23. Oliver J. H., Jr. (1966). Notes on reproductive behavior in the Dermanyssidae. J. Med. Entomol. **3**: 29-35.

24. Rosen S., Yeruham I., Braverman Y. (2002). Dermatitis in humans associated with the mites *Pyemotes tritici*, *Dermanyssus gallinae*, *Ornithonyssus bacoti* and *Androlaelaps casalis* in Israel. Med. Vet. Entomol. **16**: 442-444.

25. Sparagano O., Pavlicevic A., Murano T., Camarda A., Sahibi H., Kilpinen O., Mul M., Emous van R., le Bouquin S., Hoel K., CaWero M.A. (2009). Prevalence and key figures for the poultry red mite *Dermanyssus gallinae* infections in poultry farm systems. Exp. Appl. Acarol. **48**: 3–10.

26. Tavassoli M. Ownag A., Pourseyed S. H. and Mardani K.(2008). Laboratory evaluation of three strains of the entomopathogenic fungus *Metarhizium anisopliae* for controlling *Dermanyssus gallinae*. Avian Pathol. **37**:259–263.

27. Thind B.B. and Ford, H.L. (2007). Assessment of susceptibility of the poultry red mite *Dermanyssus gallinae* (Acari: Dermanyssidae) to some acaricides using an adapted filter paper based bioassay. Vet. Parasit. **144**: 344-348.

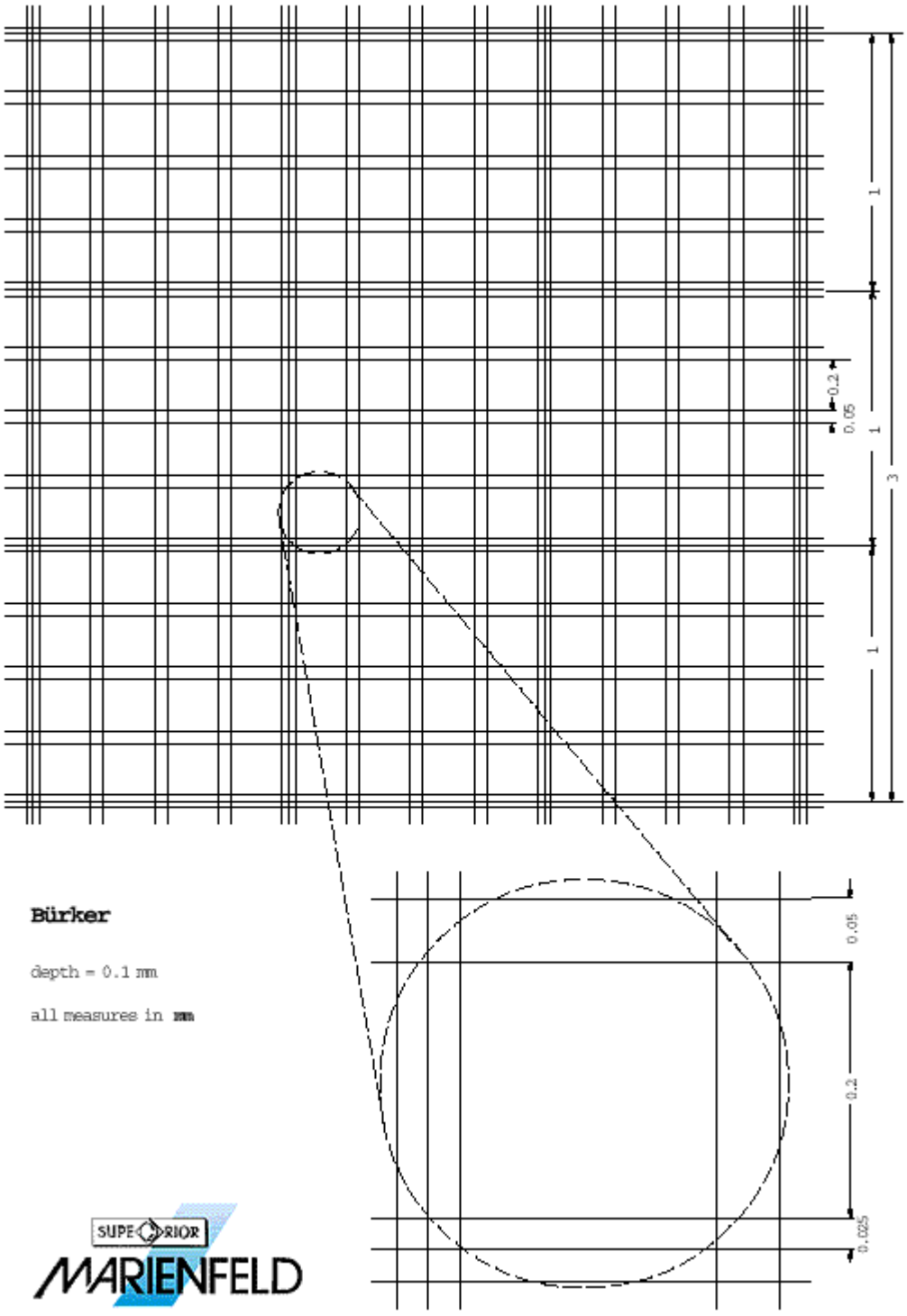
28. Thompson S.R., Brandenburg R.L. (2005). Tunnelling responses of mole crickets (Orthoptera: Gryllotalpidae) to the entomopathogenic fungus, Beauveria bassiana. Environ. Entomol. **34**:140–147.

29. Tinzaara W., Gold C.S., Dicke E.M., Huis van A., Nankinga C.M., Kagezi G.H., Ragama P.E. *et al.* (2007). The use of aggregation pheromone to enhance dissemination of Beauveria bassiana for the control of the banana weevil in Uganda. Biocontrol. Sci. Technol. **17**:111–124.

30. Verhulst N., Beijleveld O.H., Knols B.G.J., Takken W., Schraa G., Bouwmeester H.J. and Smallegange R.C. (2009). Cultured skin microbiota attracts malaria mosquitoes. Malaria Journal **8**: 302.

31. Wood, H.P. (1917). The chicken mite: Its life history and habits. U.S. Department of Agriculture, Bulletin **553**: 1-14.

Annex 1. The Bürker-Türk Haemocyte counter



B. Bassiana conidia were counted in the middle square of 0.1 x 1.0 x 1.0 mm, consisting of 16 smaller square parts, of which one is zoomed in on.