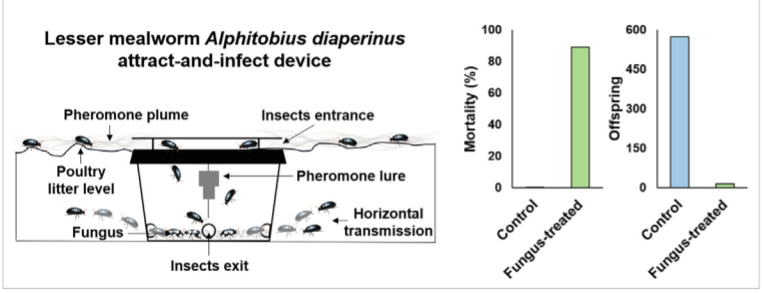
# Development of an attract-and-infect device for biological control of lesser ⊤ mealworm, Alphitobius diaperinus (Coleoptera: Tenebrionidae) in poultry houses

Marla J. Hassemer[a,b], Rogerio B. Lopes[b], Miguel Borges[b], Luis F.A. Alves[c], David M. Withall[d], John A. Pickett[e], Raul A. Laumann[b], Michael A. Birkett[d], Maria C. Blassioli-Moraes[b],

[a] Department of Zoology, Institute of Biological Sciences, University of Brasília, Campus Universitário Darcy Ribeiro, 70910-900, Brasília, DF, Brazil
[b] Embrapa Genetic Resources and Biotechnology, Parque Estação Biológica, PqEB, Av. W5 Norte (final), 70770-917, Brasília, DF, Brazil
[c] Western Paraná State University (Unioeste), Laboratory of Agricultural Biotechnology, Rua Universitária, 2069, 85814-110, Cascavel, PR, Brazil
[d] Department of Biointeractions and Crop Protection, Rothamsted Research, West Common, Harpenden, Hertfordshire AL5 2JQ, UK
[e] School of Chemistry, Cardiff University, CF10 3AT, Wales, UK

GRAPHICAL ABSTRACT

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ABSTRACT

The lesser mealworm, Alphitobius diaperinus Panzer (Coleoptera: Tenebrionidae), is the most important pest in poultry production systems worldwide. Adults and larvae serve as alternative low-quality food for chickens and as a vehicle for animal pathogens resulting in significant economic losses for farmers. Control of this pest is usually performed through insecticide application during the interval between chicken flocks. However, due to the cryptic behaviour of this pest, chemical control is usually not effective. With the aim of developing a sus-tainable approach to control A. diaperinus, we evaluated integration of the A. diaperinus aggregation pheromone with a highly virulent strain (Unioeste 04) of the fungus Beauveria bassiana (Bals.) Vuill. (Ascomycota: Cordycipitaceae) using an attract-and-infect device. Significant levels of A. diaperinus mortality and lower numbers of offspring (larvae) were observed in laboratory experiments using Unioeste 04. Horizontal trans-mission of the Unioeste 04 strain through contaminated living A. diaperinus and cadavers was also observed. In field experiments in commercial poultry houses, a significant level of A. diaperinus mortality was observed in Unioeste 04-treated arenas. The results show that the A. diaperinus aggregation pheromone can be used as an attractant in a device impregnated with infective fungal propagules, increasing adult infection and enhancing disease spread. In addition, the use of the microbial control agent inside the device allows the fungus to remain effective for longer periods in the adverse conditions of the poultry house environment.

Corresponding author.
E-mail address: carolina.blassioli@embrapa.br (M.C. Blassioli-Moraes).

## 1. Introduction

The lesser mealworm, Alphitobius diaperinus Panzer (Coleoptera: Tenebrionidae), is the most important pest occurring in poultry pro-duction systems worldwide (Axtell and Arends, 1990). Adults and larvae serve as alternative low-quality food for chickens and as a ve-hicle for animal pathogens, resulting in significant economic losses for farmers (Despins and Axtell, 1994; Despins and Axtell, 1995; Hazeleger et al., 2008). Control of this pest is usually performed through in-secticide application, mainly pyrethroids, during the interval between chicken flocks. However, due to the cryptic behaviour of this pest, chemical control is usually not effective (Japp et al., 2010). The en-vironmentally friendly and safer characteristics of biological control agents make them candidates for the management of lesser mealworm populations in poultry houses.

In commercial poultry houses, the natural occurrence of in-vertebrate pathogenic fungi Beauveria bassiana (Bals.) Vuill. (Ascomycota: Cordycipitaceae) and Metarhizium anisopliae (Metsch.) Sorok. (Ascomycota: Clavicipitaceae) has already been reported, along with their enzootic activity in lesser mealworm populations (Steinkraus et al., 1991; Geden et al., 1998; Alves et al., 2004, 2005). Previous studies have suggested that selected strains of B. bassiana are more ef-ficient in controlling A. diaperinus in Brazil (Rohde et al., 2006). However, in commercial facilities, the use of these microorganisms directly on the poultry litter decreases their activity, since the litter may have fungistatic or fungicidal properties (Bacon, 1985; Geden et al., 1998; Geden and Steinkraus, 2003; Alexandre et al., 2006; Alves et al., 2008). For instance, the presence of ammonia in the poultry litter in-hibits the germination of spores (Bacon, 1985). Studies have shown that the efficiency of B. bassiana in controlling the lesser mealworm is re-duced to nearly 50% when this fungus is applied directly to the poultry litter after one or more chicken flocks compared to a new litter (Steinkraus et al., 1991; Crawford et al., 1998; Alves et al., 2008, 2015).

In order to minimize the antagonistic effects of poultry litter, studies have focussed on different fungal formulations and different delivery strategies to increase microorganism survival (Alves et al., 2015; Rice et al., 2019). In this sense, the association of pheromones and en-tomopathogenic fungi in an attract-and-infect device is a promising strategy for A. diaperinus management. This strategy was already evaluated against other insect pests, including Coleopterans such as the grain borer Prostephanus truncatus Horn (Bostrichidae) (Smith et al., 1999), the banana weevil Cosmopolites sordidus Germar (Curculionidae) (Tinzaara et al., 2004; Tinzaara et al., 2007; Lopes et al., 2014) and the green beetle Agrilus planipennis Fairmaire (Buprestidae) (Lyons et al., 2012). The combination of pheromones and entomopathogenic fungi in attract-and-infect devices increases infection rates, since the traps en-sure fungal survival. Additionally, pheromones work as attractants, enhancing the number of contaminated individuals, thereby increasing the chances of horizontal infection and the development of an epizootic disease in the target population (Klein and Lacey, 1999; Shah and Pell, 2003).

Previously, the aggregation pheromone produced by males of A. diaperinus was identified for two distinct populations from North America and Brazil, showing quantitative and qualitative differences in their pheromonal blends (Bartelt et al., 2009; Hassemer et al., 2016). Both blends showed good results in the attractiveness of males and females in the laboratory and in the field for their respective popula-tions (Bartelt et al., 2009; Singh and Johnson, 2012; Cossé and Zilcowski, 2015; Hassemer et al., 2016; Hassemer et al., 2019). Given the enzootic activity of B. bassiana against A. diaperinus, the purpose of this study was to evaluate the potential use of B. bassiana in association with the A. diaperinus aggregation pheromone (Brazilian blend), as well as to evaluate the horizontal transmission of B. bassiana by A. diaper-inus, with the aim of developing an attract-and-infect device for the management of this pest in commercial poultry houses.

## 2. Material and methods

### 2.1. Insects

Adults of A. diaperinus were obtained from a commercial poultry farm in Brasília, DF, Brazil (15°59′40.5″ S 47°37′22.8″ W) in 2013/ 2014. The insects were reared at Embrapa Genetic Resources and Biotechnology, Brasília, DF, Brazil in plastic boxes (40 cm length × 20 cm width × 15 cm height) containing a fine layer of sterilized wood shavings (100 g) and corrugated cardboard on top. Adults and larvae were fed with a sterilized commercial chicken feed (100 mg, composition: ground corn, soybeans, wheat, rice meal, meat meal, limestone, salt, vitamin premix, probiotics, and minerals) three times a week and were provided with water daily. Boxes were kept in an environmentally controlled room [(27 ± 2 °C, 60–80% relative humidity (RH), and 16:8h (D/L) photoperiod]. For bioassays, pupae were separated by sex and placed in new boxes. The genders were distinguished by differences in pupal abdominal appendages (Esquivel et al., 2012).

### 2.2. Origin and culturing of B. bassiana strains

The B. bassiana strain Unioeste 04, which has a high potential to infect A. diaperinus adults and larvae, was originally isolated from A. diaperinus in Cascavel, Paraná, Brazil (Rohde et al., 2006). The B. bassiana strain CG82 was originally isolated from an infected co-leopteran in Tucuman, Argentina. Strains were kept in the Invertebrate-Pathogenic Fungal Collection at Embrapa Genetic Resources and Bio-technology, Brasília, and in the Collection of Entomopathogenic Fungi at the Western Paraná State University, Cascavel. Conidia were in-oculated on Potato Dextrose Agar medium (PDA) (Difco Laboratories, Detroit, MI, USA) and incubated for 12–15 days at 25 ± 0.5 °C and 12:12 h (D/L) photoperiod. Wet conidia and hyphal fragments were scraped from medium surface with a spatula and dried over calcium sulphate for 4 days at 25 ± 2 °C before use in the experiments. The number of conidia in the dried powder was determined by suspending 0.01 g powder in 9.99 mL of $H_2O$ + Tween 80® (0.05%) and counting the conidia with the aid of a Neubauer chamber after serial dilution. Dried powder of each strain was weighed and $1 \times 10^9$ viable conidia were placed at the bottom of each trap (weight ranging from 30 to 50 mg). Conidial viability was evaluated by suspending a small portion of the powder in 10 mL of $H_2O$ + Tween 80® (0.05%). Suspensions were homogenized for 30 s in a vortex and 20 μL were poured into Petri dishes containing PDA medium. Inoculated Petri dishes were sealed with sealing film (Parafilm M), and then incubated for 18 h at

26 ± 0.5 °C (12:6h light:dark). Each strain (CG82 and Unioeste 04) was replicated three times, and a total of 100 conidia in different mi-croscopic fields at 400× magnification were scored in each replicate. Conidia were considered germinated when the germ tube length was at least the same length as the diameter of the ungerminated conidia. Conidial viability was above 94% by the time they were used.

### 2.3. Pheromone lures

Rubber septa (10 mm, Sigma-Aldrich) were cleaned by Soxhlet ex-traction (twice) with hexane for 4 h, followed by drying at 40 °C overnight in a gravity convection oven (Precision, Chicago, IL, USA). For formulation of the synthetic aggregation pheromone, the six

aggregation pheromone components were added to each rubber septum in the same ratio produced by males, with the total combined amount of the six compounds being 1 mg (Hassemer et al., 2016). A solution with (R)-limonene (230 μg), (E)-ocimene (160 μg), 2-nonanone (40 μg), (S)-linalool (260 μg), (R)-daucene (80 μg) and (E,E)-α-farnesene (230 μg) in hexane (200 μL) was prepared and used. The lures were prepared fol-lowing the description of Hassemer et al. (2019), by adding 200 μL of the pheromone solution (1 mg/mL of all six components) to each septum. After addition, the septa were left in a laminar flow hood at room temperature to allow solvent evaporation for 8 h. Pheromone baited septa were then stored in a sealed aluminium storage bags and stored in a freezer at −20 °C until required for use in release rate ex-periments.

### 2.4. Chemicals

Hexane (HPLC grade, ≥97%) and diethyl ether were purchased from Sigma-Aldrich (Steinheim, Germany) and re-distilled before use. (R)-Limonene (95%) was purchased from TCI-America (Portland, USA). 2-Nonanone (99%) was provided by Jeffrey R. Aldrich Consulting LLC (Santa Cruz, USA). (E)-Ocimene (98%), (E,E)-α-farnesene (greater than 95%), (S)-linalool (98%) and (R)-daucene (87%) were synthesized as previously reported (Hassemer et al., 2016, 2019).

### 2.5. Attract-and-infect device

For laboratory and field experiments, pitfall traps were designed, comprising of cylindrical plastic boxes (5 cm height × 8 cm diameter) adapted from Hassemer et al. (2019). For each trap, a 3-cm diameter hole was drilled into the lid, through which a rubber septum im-pregnated with synthetic aggregation pheromone could be placed in-side the trap environment. Each septum was suspended by a wire that was attached to a round cardboard cover, which was connected to the plastic lid using screws (Hassemer et al., 2019) (Fig. 1). The cardboard was attached such that a 1-cm gap between it and the lid of the plastic box could be maintained, sufficient to allow insects to move into the trap. Each trap was buried in poultry litter up to the level of the lid, taking care not to cover the top completely, leaving space for the pheromonal plume to disperse above and through the poultry litter. In order to allow the insects to exit the traps, four equidistant holes were drilled in the wall of each trap, at a height of 5 mm from of the bottom. The time that adults (males and females) remained in the pitfall traps after they fell through the lid hole was estimated. For this, a single insect was introduced inside the trap (N = 40, 20/sex) which was placed in a plastic tray (15 cm width × 40 cm length × 10 cm height) filled with wood shavings (see above) and commercial chicken feed (100 mg, see above). The time that all insects took to leave the traps by the holes in the wall was counted. The experiment was conducted in an acclimatized room (27 ± 2 °C, 60 ± 10% RH) in the dark.

### 2.6. Laboratory attract-and-infect bioassays

Laboratory experiments were conducted to evaluate the effective-ness of the attract-and-infect device in controlling A. diaperinus popu-lations. The experimental unit consisted of a plastic tray (15 cm width × 40 cm length × 10 cm height), filled with wood shavings (50 g) and a commercial chicken feed (100 mg, same composition as described above), that was covered by a fabric cloth (2 mm mesh) to allow air exchange. Once poultry litter was constantly moistened by chicken excrement, water was provided three times a week by spraying 10 mL of distilled water per unit. At the centre of each unit, an attract-and-infect trap containing a pheromone septum and ca. $1 \times 10^9$ conidia (CG82 or Unioeste 04) was buried until the trap lid into the wood shavings. The control treatment was a trap containing only a pher-omone septum without fungus. In each experimental unit, 50 (10–50 days old) virgin insects (25 males and 25 females) were re-leased. After 15 days the number of dead insects were recorded, both inside and outside the traps, as well as the number of larvae that re-sulted from mating during the experiment. Five replicates were con-ducted for each treatment and each experiment was performed twice on different dates and with independent fungal batches. The experiments were conducted in an environment-controlled room (25 ± 3 °C and 12:12 h (D/L) photoperiod) with an exhaust system to avoid pher-omone saturation (air-conditioning provided circulating air at 21 $m^3$/ min, LG 24,000 BTU/h).

Conidia viability was evaluated as described above by suspending a small portion collected in the traps in 10 mL of $H_2O$ + Tween 80® (0.05%) after the end of the experiment (15 days). The dead insects were placed in moistened chambers to confirm death by B. bassiana while the living insects were kept in plastic boxes (containing new wood shavings and chicken food) for a further 10 days, i.e., 25 days after the experiment started, when the number of dead insects and number of larvae were recorded again. To evaluate the susceptibility of male and females to fungal infection the sex of the remaining A. dia-perinus adults was determined at the end of the experiments.

### 2.7. Horizontal transmission of B. bassiana by A. diaperinus

Experiments were carried out only using the Unioeste 04 strain of B. bassiana due to the efficacy of this strain against adult A. diaperinus in

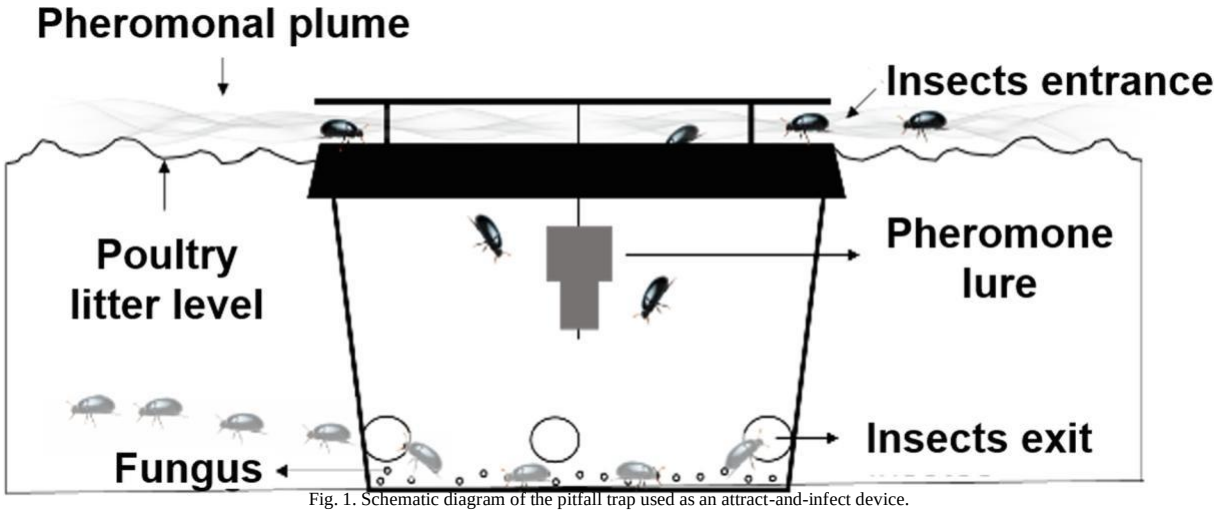

Fig. 1. Schematic diagram of the pitfall trap used as an attract-and-infect device.

laboratory attract-and-infect bioassays (see above). The experimental unit consisted of a plastic container (5 cm high × 8 cm in diameter), where a total of 20 virgin adults of A. diaperinus (10 females and 10 males) ranging in age from 10 to 50 days were placed.

In the first experiment, adult A. diaperinus were marked in the final portion of the right elytra with odour-free acrylic paint. The marked insects were contaminated by contact with B. bassiana conidia, using the trap described above, at a concentration of ca. $1 \times 10^9$ conidia/ trap. The insects were held for 10 min in the traps, as this was de-termined to be the mean time that it took insects to leave the traps. Then the inoculated (marked) insects were transferred to the experi-mental unit in proportions of 10, 20, 30, 40 and 50% in relation to the total number of insects. The control treatment consisted of only 20 healthy insects. Three replicates for each treatment were conducted. The experiment was performed twice on different dates and with in-dependent fungal batches. Fifteen days after insect release, the total number of dead insects (marked and unmarked) was counted, as well as the number of larvae resulting from the mating occurring throughout the experiment as previously described. The dead insects were kept in a moistened chamber for confirmation of death by the Unioeste 04 strain.

In the second experiment, a group of 80 insects of each gender were marked with different colours and contaminated as already described. Seven days after inoculation, dead insects were transferred to a mois-tened chamber for another 3 days to allow for fungal growth and conidiation. The amount of conidia produced on the cadaver was esti-mated by washing a sample of dead insects (six independent replicates of groups of two individuals, completely randomized) in each experi-ment in 1 mL of $H_2O$ + Tween 80® (0.05%). The suspension was vortexed for 20 s, followed by an ultrasonic bath for 2 min and again vortexed in for another 20 s. The number of conidia per cadaver was estimated by counting the cells in a Neubauer chamber under an optical microscope. Other colonized cadavers in the moistened chamber showing similar colonization were then selected and transferred to the experimental unit containing healthy adults in proportions of 10, 20, 30, 40 and 50% of cadavers in relation to total number of insects. The control treatment consisted of only 20 healthy insects. Each treatment had three replicates and experiment was performed twice on different dates and with independent fungal batches. Fifteen days after cadaver release, the total number of dead insects was counted, as well as the number of larvae resulting from the mating occurring throughout the experiment as already described. The dead insects were stored in a moistened chamber for confirmation of death by the Unioeste 04 strain.

### 2.8. Field test with the attract-and-infect device

Experiments were conducted in two commercial poultry houses (120 m length × 12 m width) located in Cascavel, Paraná state, Brazil (24°49′22.2″S 53°24′19.3″W). In each building, 12 circular PVC arenas (80 cm diameter × 1 m height) were installed every 10 m in two rows (Fig. 2). The arenas were buried to a level of approximately 20 cm in the ground to avoid entry of insects from outside and escape of insects from inside. In addition to natural infestation of poultry litter by A. diaperinus (non-quantified population), 1000 additional adults were released in each arena to guarantee insect presence. In the centre of each arena, a pitfall trap (10 cm height × 14 cm diameter) was in-stalled. Six arenas received only pheromonal traps and the other six received pheromonal traps treated with ca. $2.2 \times 10^9$ conidia of B. bassiana strain Unioeste 04. The treatments were intercalated both within and between the rows (Fig. 2). Samples of 200 living insects were collected inside each arena, close to the arena limits (25 cm far from the trap), 2 and 5 days after the initiation of the experiment. Additionally, the viability of conidia collected from traps at the end of the experimental period (5 days) was determined as previously de-scribed. During the experimental period the poultry litter temperature inside each arena was registered daily using a digital soil thermometer (TA290, Hong Kong Lover Technology Co. Ltd., China). The insects were transferred to the laboratory and incubated at 26 ± 0.5 °C, L:D

12: 12 for 15 days. Dead insects were stored in a moistened chamber for confirmation of death by the Unioeste 04 strain.

### 2.9. Statistical analysis

The number of dead A. diaperinus adults and the number of larvae counted per treatment in each experiment were compared by ANOVA followed by Tukey's test in multiple comparisons for parametric data ($p \leq 0.05$). For non-parametric data, Kruskal-Wallis ANOVA followed by Mann-Whitney test was applied for multiple comparisons ($p \leq 0.05$). The number of males and females alive in the lab bioassays was compared by Student t-test ($p \leq 0.05$). The number of adults captured per treatment in the field experiments was compared by Fisher's exact test ($p \leq 0.05$). For the bioassays that were repeated twice in time, the data were analysed together. All the analyses were performed using the statistical program R 2.14.0.35 at 5% significance (R Development Core Team, 2011).

## 3. Results

### 3.1. Laboratory attract-and-infect bioassays

Significantly higher mortality of adult A. diaperinus was observed after exposure to either Unioeste 04 or CG82 strains of B. bassiana in aggregation pheromone-baited traps, compared to aggregation pher-omone-baited traps alone ($\chi^2 = 24.13$; df = 2; $p < 0.001$) (Fig. 3A). Comparing the two strains, mortality was significantly higher in the first 15 days for the Unioeste 04 strain than for the CG82 strain. The same pattern was observed for accumulated mortality after 25 days ($\chi^2 = 24.47$, df = 2, $p < 0.001$) (Fig. 3A). None of the dead insects were found inside the traps and all the cadavers were infected with

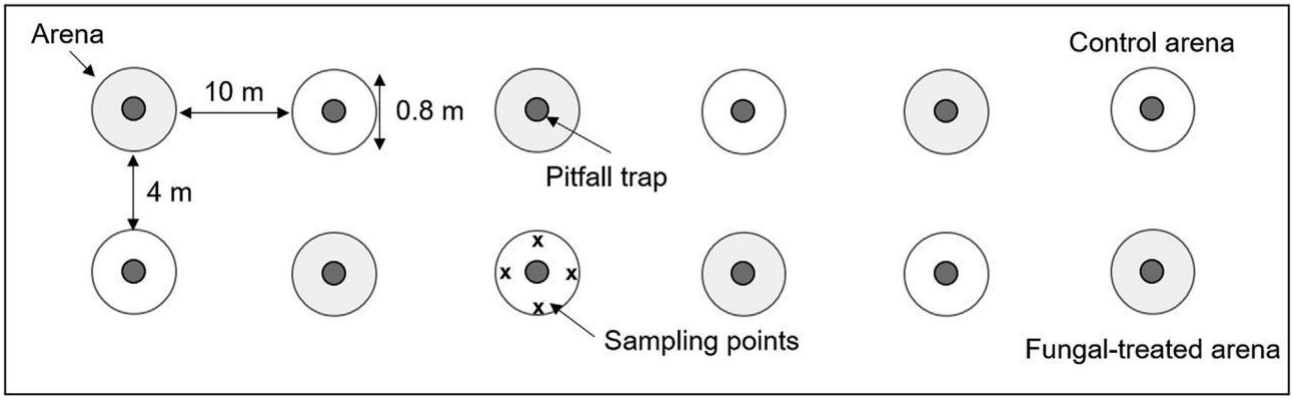


Fig. 2. Schematic diagram of the PVC arenas and pitfall traps distributed inside the poultry houses.

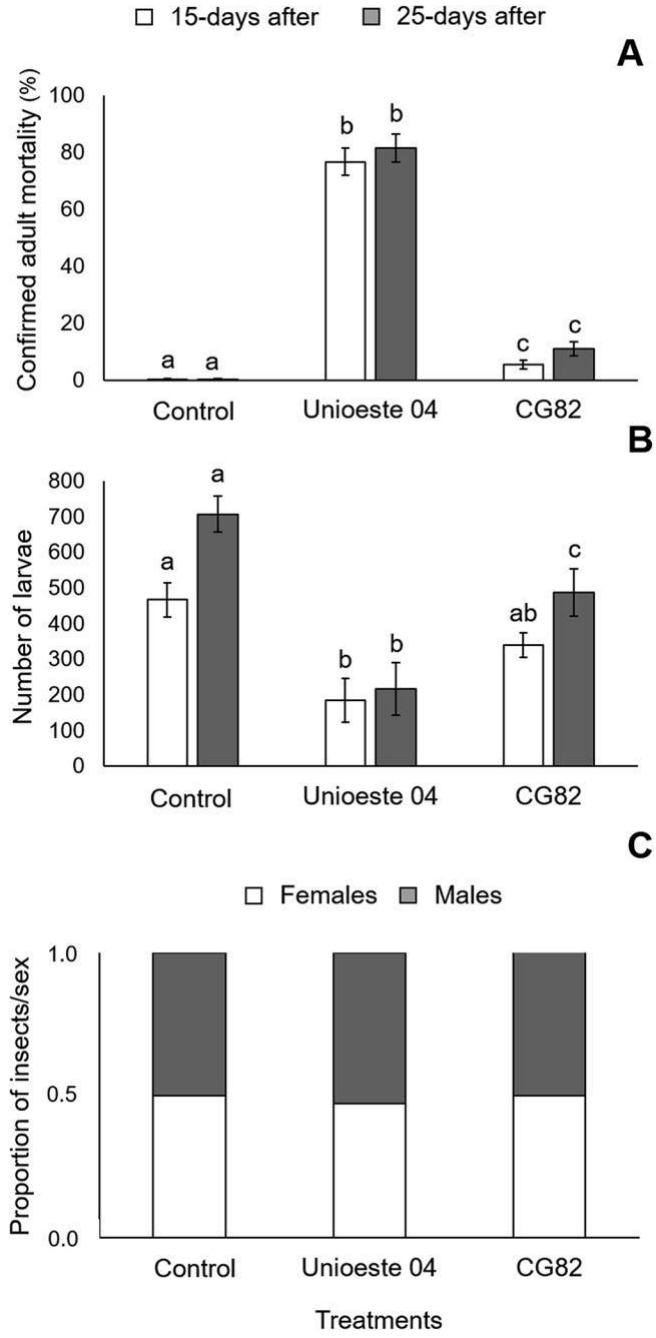


Fig. 3. Confirmed mortality (%) ± standard error adults of Alphitobius dia-perinus exposed to the isolates of Beauveria bassiana Unioeste 04, CG82 and control (without fungus) evaluated 15 and 25 days after exposure, N = 50 (A); and Alphitobius diaperinus offspring (number of larvae ± standard error) 15 and 25 days after exposure (B). Bars followed by distinct letters within the same evaluation period (A and B) differ significantly by the Kruskal-Wallis test ($p \leq 0.05$). Proportion of living males and females of Alphitobius diaperinus 15-days after fungus exposure (C).

fungi. The mean time that insects remained in the traps after falling through the lid hole was 10 ± 1.2 min. The number of larvae produced by A. diaperinus also differed between treatments after the first 15 days ($\chi^2 = 8.75$, df = 2, p = 0.012) and after 25 days ($\chi^2 = 14.71$, df = 2, p < 0.001) (Fig. 3B). After the first 15 days, the number of larvae produced after exposure to Unioeste 04 was significantly lower than for the control ($p < 0.001$), while the number of larvae produced after exposure to CG82 did not differ from Unioeste 04 (p = 0.070) and from the control (p = 0.155) (Fig. 3B). After 25 days, the number of larvae produced after exposure to Unioeste 04 was significantly lower than for both CG82 (p = 0.04) and the control ($p < 0.001$). Significantly fewer larvae were found after CG82 treatment compared to the control (p = 0.03) (Fig. 3B). There were no differences in the mortality of males and females for all the treatments (Control: t = -0.6, df = 1, p = 0.555; Unioeste 04: t = 0.30, df = 1, p = 0.765; and CG82: t = 0.55, df = 1, p = 0.587) (Fig. 3C). At the end of the 15-day ex-perimental period, both B. bassiana strains showed conidial viability greater than 90 ± 4%.

### 3.2. Horizontal transmission of B. bassiana by A. diaperinus

In the first experiment, no mortality was observed for A. diaperinus in the control treatment, therefore this treatment was excluded from statistical analysis. There were no significant differences in the trans-mission of the Unioeste 04 strain when using different proportions of infected insects ($\chi^2 = 1.71$, df = 4, p = 0.18) (Fig. 4A). Considering the number of A. diaperinus offspring produced during the experiment, all infected-healthy adult ratios produced significantly fewer larvae than the control treatment ($\chi^2 = 13.44$, df = 5, p = 0.019) (Fig. 4B). The mortality of adults previously contaminated with the Unioeste 04 (marked) strain was 90% and all the dead insects showed fungal colo-nization. In the second experiment, each colonized cadaver produced an average of $3.8 \times 10^6 \pm 0.1$ conidia. Mummified cadavers were also able to transmit the fungus to the healthy individuals (up to 19%). No mortality was observed in the control treatment and no differences in the transmission rates for the different proportions of infected insects were detected ($\chi^2 = 2.40$, df = 4, p = 0.08) (Fig. 5A). The number of A. diaperinus offspring during the experiment was similar among all cadaver-healthy adult ratios. With exception of the treatment with 20% of dead insects, all other Unioeste 04 treatments differed from the control treatment ($\chi 2 = 13.43$, df = 5, $p < 0.019$) (Fig. 5B).

### 3.3. Field test with the attract-and-infect device

A significant difference in the total number of dead A. diaperinus between Unioeste 04-treated and untreated arenas was observed 2 days after initiation of the experiment (F = 4.31, df = 11, p = 0.022). The number of dead insects after the incubation period was twice as high in the Unioeste 04-treated arena (Fig. 6A). A difference between these treatments was also observed after the confirmation of death by Uni-oeste 04 (F = 120.60; df = 11, $p < 0.001$) (Fig. 6B). After five days, the same pattern was observed, with significant differences between treatments both in the total number of dead (F = 8.93, df = 11, p = 0.001) (Fig. 6A) and in the number of colonized cadavers (F = 549.86, df = 11, $p < 0.001$) (Fig. 6B). Initial conidia viability was 90.8 ± 4.1%, and after five days of exposure inside the trap, viability decreased to 78.9 ± 4.4%. The average temperature in the litter during the experimental period was of 33.2 °C, and peaks of 38.2 °C were registered. For both sampling times (2 and 5 days), insects killed by Unioeste 04 were also observed in very low levels (< 0.5%) in untreated arenas.

## 4. Discussion

Our results show that combining an aggregation pheromone for adult A. diaperinus and a highly virulent strain of B. bassiana in an at-tract-and-infect device has the potential for controlling lesser meal-worms in poultry houses. Our results confirmed the high infectious potential of B. bassiana Unioeste 04 strain on A. diaperinus, as pre-viously reported by Rohde et al. (2006) and the horizontal transmission of the fungus from infected to healthy insects. Moreover, no negative effects of the volatile components of the aggregation pheromone of A. diaperinus on fungal viability were detected, enabling this combination to be used in an attract-and-infect device. The association of the ag-gregation pheromone of A. diaperinus with B. bassiana in traps enhanced

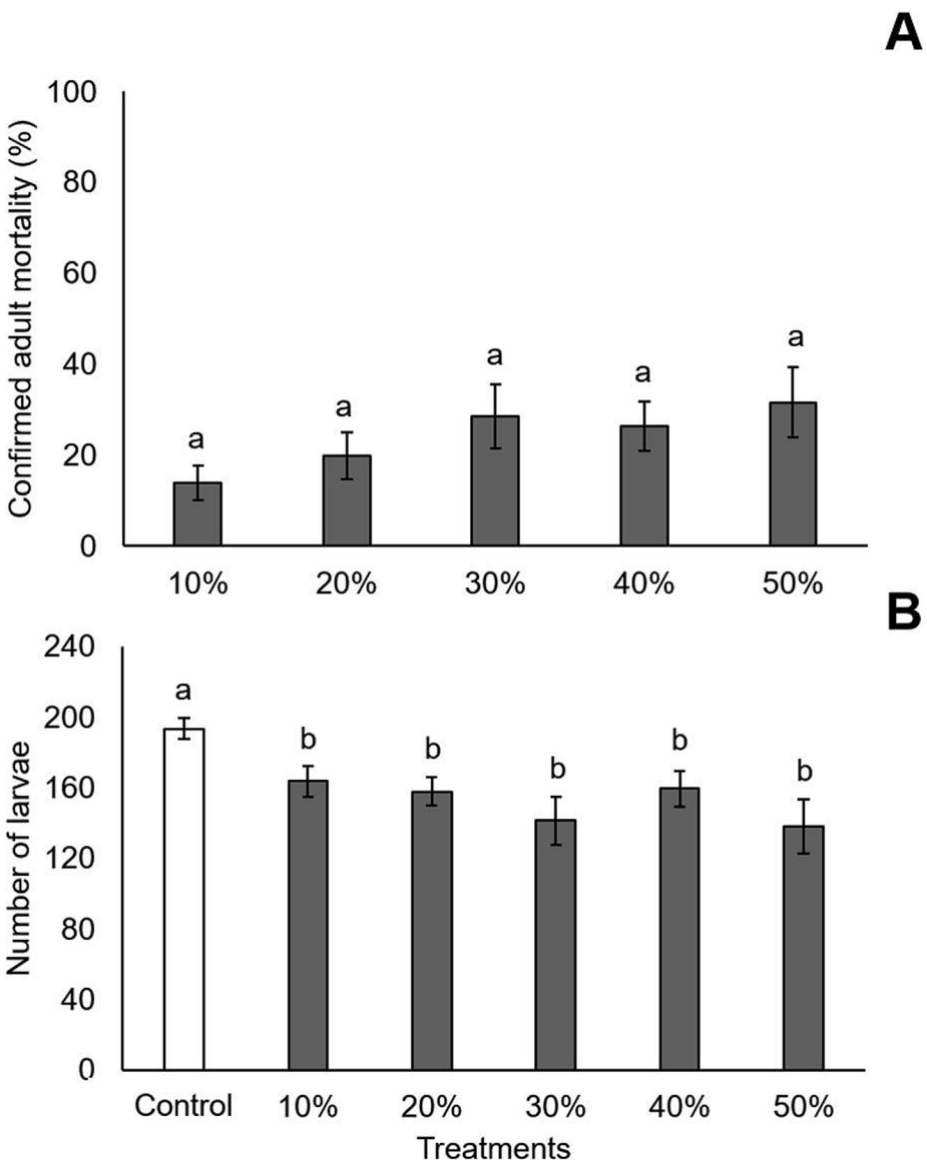


Fig. 4. Confirmed mortality (%) ± standard error of untreated Alphitobius diaperinus adults infected by exposure to 10%, 20%, 30%, 40% and 50% of contaminated insects (A). Alphitobius diaperinus offspring (number of larvae ± standard error) in the same treatments (B). Bars followed by distinct letters differ significantly by ANOVA followed by the Tukey test for multiple comparisons in (A) and Kruskal-Wallis test followed by the Mann-Whitney test for multiple comparisons in (B) ($p \leq 0.05$).

the number of infected insects and consequently its mortality in field conditions compared to control traps. The association of pheromones and entomopathogenic fungi in attract-and-infect device is a viable and promising strategy for pest control that has already explored for other cryptic insects (Vega et al., 2007; Lopes et al., 2014; Lacey et al., 2015; Yusef et al., 2018), especially when applications directed to the target are impracticable. The combination of a pheromone with an in-vertebrate-pathogenic fungus brings specificity and increases the effi-cacy of entomopathogenic fungi, once a significant number of specific target pests enter the trap and are contaminated by the fungus. In our study, the experiments showed that the use of the volatile pheromone of A. diaperinus with fungi did not change the viability of the fungi during 15 days of evaluation.

The auto-inoculation device proposed here was able to attract and kill more than 80% of insects by fungal infection under laboratory conditions for the strain Unioeste 04. As a consequence of this high mortality, there was also significant reduction in A. diaperinus offspring, being three times less than the treatment without fungus (negative control). The reduction in the number of larvae may also reflect in lower infestation rates and contribute to overall population control in repeated applications. Even when ca. $1 \times 10^9$ of CG82 conidia was used inside the trap for small insect populations in laboratory experi-ments, this less virulent strain was shown to be a less effective candi-date for an attract-and-infect strategy, although some reduction in in-sect reproduction was observed.

Another important aspect in the development of the disease in the target population is the horizontal transmission potential of the mi-croorganism (Long et al., 2000). The transmission of B. bassiana among healthy and contaminated individuals has been reported in other Co-leoptera, such as the spruce bark beetle Ips typographus Linnaeus (Co-leoptera: Curculionidae), the banana weevil Cosmopolites sordidus Germar (Coleoptera: Curculionidae) and the bark beetle Ips sexdentatus Boerner (Coleoptera: Curculionidae) (Kreutz et al., 2004; Lopes et al., 2011; Kocaçevik et al., 2016).

In the present study, it was observed that A. diaperinus under la-boratory conditions was able to infect up to 32% of healthy insects through direct contact with living contaminated insects. Given that the insects used in experiments were virgin and sexually mature,

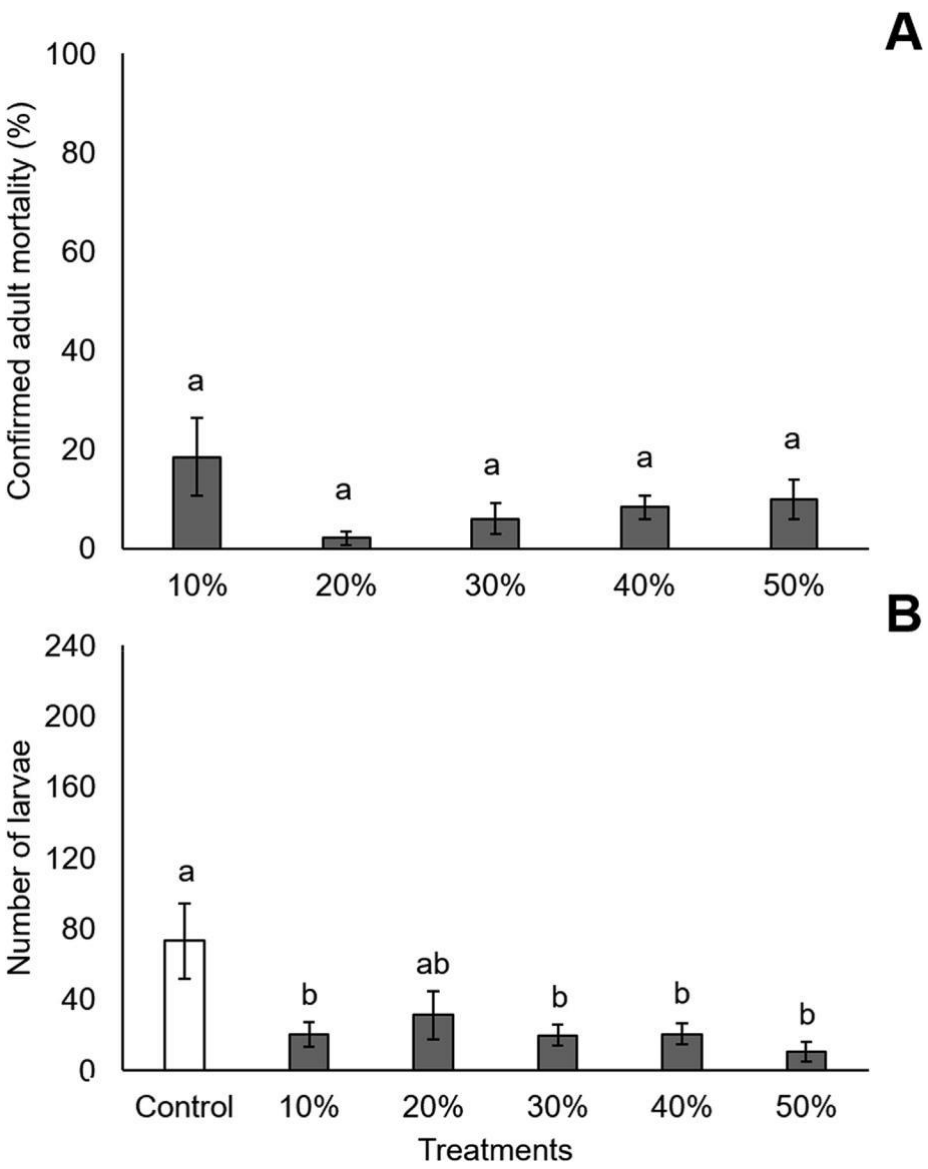


Fig. 5. Confirmed mortality (%) ± standard error of untreated Alphitobius diaperinus adults infected by exposure to 10%, 20%, 30%, 40% and 50% of colonized cadavers (A); Alphitobius diaperinus offspring (number of larvae ± standard error) in the same treatments (B). Bars followed by distinct letters differ significantly by ANOVA followed by the Tukey test for multiple comparisons in (A) and Kruskal-Wallis test followed by the Mann-Whitney test for multiple comparisons in (B) ($p \leq 0.05$).

transmission may have occurred through copulation. This would justify the non-increase of the horizontal transmission with the increase of the number of contaminated insects in the population, once the chance of contaminated insects copulating with each other also increased. It is possible that volatiles naturally produced by males of A. diaperinus in-side or around the traps after aggregation allows the contamination of more individuals by B. bassiana. However, further studies are still needed to confirm that what happened to the bed bug Plautia stali Scott (Hemiptera: Pentatomidae), where both infected genders with B. bassiana are capable to transmit the fungus through copulation with non-contaminated partners (Tsuda et al., 1997), also happens for A. diaperinus. In addition, transmission may also have occurred indirectly through the deposition of conidia from the body of individuals exposed to the fungus in the wood shavings. For A. diaperinus, the mixture of B. bassiana Unioeste 04 dry conidia with wood shavings promoted high mortality of both larvae and adults (Alves et al., 2008).

When colonized A. diaperinus cadavers were used as the inoculum source, transmission rates up to 19% to healthy individuals were ob-served. According to Luz and Fargues (1998) the amount of conidia per cadaver, the temperature and the humidity are factors that influence the entomopathogenic fungi transmission. For comparison purposes, it is noteworthy that the amount of conidia applied in each trap ($1 \times 10^9$ viable conidia) that caused an average adult mortality rate near to 80% was 25 times greater than the amount produced by all cadavers in the treatment with the highest proportion of colonized insects (50%). Therefore, even lower number of conidia cadavers can be an important source of inoculum. Recent studies with B. bassiana suggest that A. diaperinus larvae are more susceptible than adults (Rohde et al., 2006; Rezende et al., 2009), and secondary transmission via cadavers to larvae may be more important in field populations. For confirmation of this hypothesis future transmission tests between insects colonized by the fungus and larvae must also be performed. This was the first time that horizontal transmission of B. bassiana through contaminated living insects and cadavers was reported for A. diaperinus.

In the field experiment, insects contaminated by B. bassiana in the traps were recovered inside the arenas (ca. 3% of the collected in-dividuals, N = 200). These results are promising because infestation rates in the arenas were high (natural population +1000 delivered

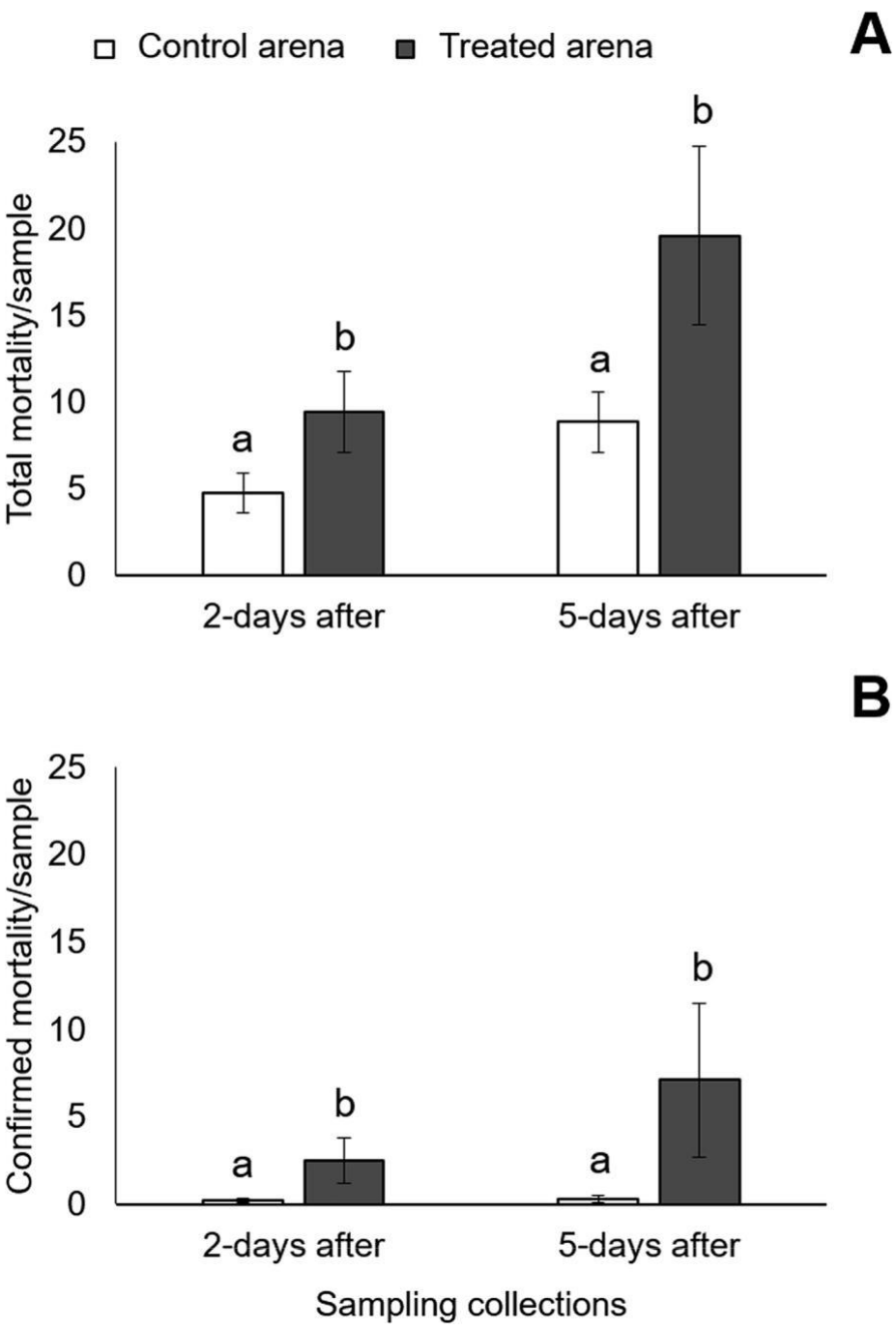


Fig. 6. Number of dead adults ± standard error (A) collected in samples of 200 insects in the control arena and fungal treated arena and kept in lab, 2 and 5 days after device installation. Confirmed mortality ± standard error (B) of Alphitobius diaperinus by Beuaveria bassiana in the control arena and fungal treated arena, 2 and 5 days after device installation (N = 200).

adults) and the amount of conidia in each trap was low. After 5 days in the field, most of the conidia powder vanished from the trap, indicating high insect activity inside the arenas. It is already known that the poultry litter affects B. bassiana conidia negatively because of the fun-gistatic and fungicidal activity of certain compounds present in this material (Bacon, 1985; Geden et al., 1998; Geden and Steinkraus, 2003; Alexandre et al., 2006; Alves et al., 2008). Temperature promoted by microbial activity in the poultry litter is another factor that may in-fluence fungal activity in the field. Levels of mycosis in A. diaperinus decrease as temperatures increase, affecting physiological interactions between the host and pathogen at temperatures over 32 °C (Alexandre et al., 2006; Rice et al., 2019). The average temperature in the litter reached 33.2 °C in our field trial, but the lack of direct contact of the inoculums with the litter and the ventilation provided by the trap probably reduced the heat stress. We can assume that compared to poultry litter, the trap gives a protected place for the fungus, confirmed by the low decrease in the viability of conidia (from ca. 90% to 78%) collected from the traps after 5 days. Furthermore, the conidia powder used in our experiments faded after 24 h, indicating that with time, insects that go into the trap at a later timepoint have less contact with the conidia spores, and this could explain the low horizontal trans-mission. Experiments testing different formulations to obtain improved adhesiveness of the fungus for better horizontal transmission are being conducted in our laboratory (Lopes et al., 2011). A long-term study will

be set up to verify if A. diaperinus populations will remain low or stable over time.

## 5. Conclusions

In summary, the aggregation pheromone of A. diaperinus attracts insects into traps and enhances the probability of adult contamination with a virulent strain of B. bassiana. This allows a lower amount of fungus to be used, and protects the fungus from adverse conditions encountered in avian environments. Unlike the routine use of chemical pesticides, this new attract-and-infect strategy does not pose a risk to the animals. Previously it was shown that when chickens were fed with B. bassiana conidia, neither their health nor development were nega-tively affected, and there were no residues in the meat products (Haas-Costa et al., 2010; Hassemer et al., 2016). The attract-and-infect device could be deployed throughout the production period, and, for the management of migrant insects, at locations outside of the production facilities. Between chicken flocks, B. bassiana could also be applied in a "push-pull-infect strategy", combining the A. diaperinus alarm pher-omone, to promote the movement of insects from their hidden places, with the attract-and-infect device, increasing the number of con-taminated insects and the likelihood of epizoonosis (Hassemer et al., 2015, 2019). Studies are being conducted to evaluate the influence of field conditions on the viability of the fungus within the trap and on the spread of the fungus, and to evaluate the long-term reduction of A. diaperinus populations. The attract-and-infect device proposed here not only could improve the security of poultry production, but could also enhance new industries, such as farmers growing plants as a natural source of semiochemicals to be used in agriculture, the fine chemicals industry to produce synthetic semiochemicals, and biological control companies to produce fungus strains. Three of the semiochemicals present in the aggregation pheromone of A. diaperinus, R-limonene, S-linalool and R-daucene can be obtained from plants (Hassemer et al., 2016), 2-nonanone is commercially available and at low cost. Only the sesquiterpene (E,E)-α-farnesene requires synthesis, since, until now, we do not know a natural source that produces this compound in sig-nificant quantities for extraction. Therefore, our approach is a low-cost, affordable technology that can generate new opportunities in several industries.

## CRediT authorship contribution statement

Marla J. Hassemer: Conceptualization, Methodology, Software, Formal analysis, Writing - original draft, Writing - review & editing. Rogerio B. Lopes: Conceptualization, Methodology, Software, Supervision, Writing - review & editing. Miguel Borges: Conceptualization, Formal analysis, Resources, Funding acquisition, Writing - review & editing. Luis F.A. Alves: Conceptualization, Writing
- review & editing. David M. Withall: Methodology, Software, Writing
- review & editing. John A. Pickett: Conceptualization, Writing - re-view & editing. Raul A. Laumann: Conceptualization, Formal analysis, Writing - review & editing. Michael A. Birkett: Conceptualization, Writing - review & editing. Maria C. Blassioli-Moraes: Conceptualization, Methodology, Software, Formal analysis, Resources, Funding acquisition, Supervision, Writing - review & editing.

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