Which factors influence the virulence of entomopathogenic fungi? Effect of spore type, oosporein, application method, and pathway of entry on the infectiveness of *Beauveria brongniartii* against *Melolontha melolontha*

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Abstract

Aims

The control of the common cockchafer *Melolontha melolontha* using the entomopathogenic fungus (EPF) *Beauveria brongniartii* is one of the most successful biological control systems. This study aimed to identify factors influencing the outcome of laboratory bioassays, which are crucial early steps in the development of biocontrol products, by using this system as a role model.

Methods and Results

We combined spray and injection applications of conidio- and blastospores of the host-specific pathogen *B. brongniartii* BIPESCO2 (Bip2) and the generalist EPF *Metarhizium brunneum* Ma 43 and applied the treatments to cockchafer adults and larvae. Furthermore, the mycotoxin oosporein was tested alone or with Bip2 blastospores, as well as Bip2 conidiospores, in immersion, spray, and injection treatments of larvae. The most efficient spore suspension was applied to different larval body parts and to their food.

Bip2 and Ma 43 infected adults frequently, but larvae resisted topical spray applications. Injection treatments revealed that adult cuticles offered limited protection, whereas the larval cuticle acted as an effective barrier. Larval thorax and legs, with articulations and intersegmental membranes, were more susceptible than the abdomen. Oosporein synergized with blastospores in larval immersion treatments, but alone had no effect. We propose that oosporein's antibiotic activity disrupts the larval cuticle microbiome, facilitating infection.

Conclusion

Contrary to the assumption that laboratory bioassays overestimate EPF performance under field conditions, we found the opposite. We therefore argue that more elaborate studies are required for realistic evaluation of candidate biocontrol agents, considering host–pathogen traits and test conditions.

Impact Statement: Biological control of insect pests is essential for achieving sustainable agriculture, with entomopathogenic fungi as promising candidates. This study evaluates key factors influencing the outcomes of virulence testing, providing valuable insights to optimize laboratory practices. These findings have significant implications for the efficient development and standardization of biocontrol products using entomopathogenic fungi.

1 Introduction

Entomopathogenic fungi (EPF) are among the most important biocontrol agents on the market and are commercially applied worldwide (de Faria and Wright, 2007; Hajek and Delalibera; 2010; Thakur et al., 2020). Many products are based on fungal strains of the genera *Beauveria*, *Metarhizium*, *Isaria* and *Lecanicillium* and have proven to be effective against a variety of insect pests from the orders Lepidoptera, Coleoptera, and Diptera (Lacey et al., 2015). Today, EPF are essential in integrated pest management (Skinner et al., 2014; Kumari et al., 2022). However, the development of EPF-based products is complex, and the identification of fungal strains with appropriate biocontrol properties is challenging (Butt and Goettel, 2000; Lacey et al., 2015). The efficacy of EPF in laboratory bioassays often does not translate into efficient control in the field (Butt and Goettel, 2000; Lacey et al., 2015; Graf et al., 2023). Nevertheless, there are successful examples of EPF as biocontrol agents (Wraight et al., 2001) which can be used to study factors that enhance the success of the infection process.

Here, we study the infection of the common cockchafer, *Melolontha melolontha* (L., Coleoptera: Scarabaeidae), by the entomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (Hypocreales: Cordycipitaceae), a pathogen from the order Hypocreales, known to infect particularly species of the Melolonthinae subfamily (Townsend et al., 1995; Keller et al., 2003; Srikanth et al., 2006; Zimmermann, 2007a; Nong et al., 2011; Goble et al., 2012). In Europe, *B. brongniartii* is known to specifically infect *Melolontha* spp. and is considered the most important pathogen of the common cockchafer, causing epizootics in larval populations (Keller et al., 2003; Zimmermann, 2007a). There is a strong correlation between the occurrence of *B. brongniartii* and common cockchafer larvae (Keller et al., 2003; Kessler et al., 2004) and this well-known host-pathogen relationship is successfully used to control the common cockchafer in Europe (Dufour, 1894; Ferron, 1967; Fornallaz, 1992; Kessler et al., 2004).

The common cockchafer is a scarab beetle native to Europe and distributed across large parts of the continent (EPPO, 2018). Adult beetles have a short flight period of around four weeks in spring and subsist on leaves of deciduous trees and shrubs (Popov, 1960; Büchi et al., 1986). They mainly feed at forest edges and occasionally cause damage when they infest orchards in large numbers (Wiesmann and Gasser, 1950; Büchi et al., 1986). From their feeding grounds, females fly to nearby meadows, orchards, or plantations to deposit their eggs in the soil (Popov, 1960; Büchi et al., 1986). The larvae are white grubs which spend two to three years below ground feeding on roots (Popov, 1960; Büchi et al., 1986) and cause major damage to low-crown fruit trees, vineyards, and other high-value crops, such as strawberry plantations, as well as in forestry (Büchi et al., 1986; Benker and Leuprecht, 2005; Keller and Zimmermann, 2005; Malusá et al., 2010; Niemczyk et al., 2019; Szmidla et al., 2019). In mountainous areas of Central Europe, the main damage is caused in meadows where larvae detach the sward from the soil (Büchi et al., 1986; Benker and Leuprecht, 2005). Apart from the loss of forage crops, the destruction of the sod can lead to secondary damage from landslides (Büchi et al., 1986; Benker and Leuprecht, 2005). In the European Union as well as Switzerland, no chemical pesticides are authorized for the control of common cockchafer larvae (Directive 2009/128/EC), hence farmers rely on the application of EPF (Kessler et al., 2004).

The state-of-the-art control of common cockchafer larvae is based on the increase of infective propagules of *B. brongniartii* in infested meadows to induce epizootics. For this, *B. brongniartii* is grown on sterilized barley kernels and these so-called fungus colonized barley kernels (FCBK) are applied with a no-till seeder into cockchafer infested meadows (Keller, 2000; Kessler et al., 2004). Thus, densities of *B. brongniartii* conidiospores are increased in the treated soil below the sward and foraging cockchafer larvae which come into contact with the spores, are subsequently infected and killed by the fungus within weeks or months (Kessler et al., 2004; Dolci et al., 2006). However, the exact mechanism of how larvae become infected in the soil and what factors account for the success of this type of treatment is unclear.

Like other hypocrealean EPF, *B. brongniartii* infects its host via cuticle penetration (Ferron, 1967; Hajek and St. Leger, 1994). Conidiospores attach to the surface of the insect host and germinate under favorable conditions (Hajek and St. Leger, 1994; Ortiz-Urquiza and Keyhani, 2013). The germinated spore forms an appressorium and mechanical force as well as degrading enzymes help breach the cuticle (Hajek and St. Leger, 1994; Ortiz-Urquiza and Keyhani, 2013). Once in the hemolymph, fungi form blastospores and exploit the insect's nutrients to multiply and further colonize the host (Hajek and St. Leger, 1994; Ortiz-Urquiza and Keyhani, 2013). After the insect's death, the fungus grows out of the cadaver and forms new conidiospores on the surface, which serve as new inoculum for the next infection cycle (Hajek and St. Leger, 1994; Ortiz-Urquiza and Keyhani, 2013).

EPF release secondary metabolites when penetrating the cuticle or multiplying as blastospores within the insect to suppress their host's immune response and to prevent secondary infection of the insect cadavers by saprophytes (Hajek and St. Leger, 1994; Strasser at al., 2000; Vey et al., 2001; Feng et al., 2015; Fan et al., 2017). Thus, those secondary metabolites influence the virulence of fungal strains (Hajek and St. Leger, 1994; Feng et al., 2015; Mc Namara et al., 2019). Oosporein, a red pigment with antimicrobial and antiviral properties, is the most important secondary metabolite of *B. brongniartii* (Terry, 1992; Kögl and van Wessem, 1944; Strasser et al., 2000; Fan et al., 2017). It has been shown to be slightly toxic to different cell types (Semar, 1993) and at high concentrations to chicken broilers (Pegram and Wyatt, 1981) but does not accumulate in soil nor in potatoes treated with *B. brongniartii* (Abendstein et al., 2000). As such, the application of *B. brongniartii* which produces oosporein is considered as environmentally safe (Zimmermann, 2007a).

Besides the common cockchafer, many other native and invasive scarab beetles are causing substantial damage worldwide (Jackson and Klein, 2006). Many of them exhibit similar life history traits, e.g., soil-dwelling grub stages that feed on roots of meadow plants (Jackson and Klein, 2006). Therefore, it seems promising to adapt the well-established biological control strategy of the common cockchafer to other scarab beetles, such as the invasive Japanese beetle, *Popillia japonica* Newman (Coleoptera: Scarabaeidae). However, as promising results from laboratory bioassays often do not translate into efficient control in the field (Butt and Goettel, 2000; Lacey et al., 2015; Graf et al., 2023), this study investigates various factors which have the potential to substantially influence the outcome of bioassays, thus contributing to a more coherent interpretation of contradictory results from different laboratory virulence tests.

First, we investigated the influence of the host's developmental stage on the success of the infection process in our model system. We hypothesized that the specialized and co-evolved pathogen *B. brongniartii* will readily infect both larval and adult stages of its preferred host insect. Second, we tested differences in the success of a specific versus a more generalist pathogen, by comparing applications of the specialized *B. brongniartii* Bip2 and *Metarhizium brunneum* Petch (Hypocreales: Clavicipitaceae) Ma 43, which is known to have a broad host range (Zimmermann, 2007a; Zimmermann, 2007b). We used different spore types of Bip2 and Ma 43 for our inoculation experiments and hypothesized that the generalist *M. brunneum* will need more time to successfully infect the host than the specialist *B. brongniartii* and generally exhibit a lower infection rate. Furthermore, we tested the secondary metabolite oosporein of *B. brongniartii* separately and in combination with Bip2 blastospores. We expected the spores in combination with oosporein to have a more severe effect on the host insect, than both separately.

Furthermore, we were interested in the importance of the insect's cuticle as a physical barrier which the fungus must overcome during the infection process. Thus, we included spray and dip inoculations as well as spore injection treatments. We anticipated that overriding the external defense mechanisms by injecting spores into the host's hemolymph will increase infection success, especially in the case of the generalist *M. brunneum*.

Finally, we applied *B. brongniartii* Bip2 externally to various parts of the cockchafer larva's body, to compare the vulnerability of those body parts to fungal attack. We hypothesized that infection occurs primarily via the cuticle and is more successful when thin joint membranes (e.g. intersegment membranes of leg articulations) are exposed to fungal spores. In contrast, oral uptake should not play a role.

2 Material and Methods

We carried out three interrelated laboratory experiments on adults and larvae of the common cockchafer. In experiment 1, we compared the effects of the host-specific fungal pathogen of the common cockchafer, *B. brongniartii* (Bip2), and a generalist pathogen, *M. brunneum* (Ma 43), on cockchafer adults and larvae. Different spore types of the EPF as well as application methods were assessed. In experiment 2, we tested the effect of oosporein isolated from Bip2 cultures on larvae, applied alone and in combination with spores. In experiment 3, we assessed the virulence of Bip2 on larvae, depending on different pathways of entry.

2.1 Insects

Common cockchafer (*M. melolontha*) adults and third instar larvae were collected from wild populations in mountainous areas in Switzerland in the northern part of the Alps. Adults were shaken from shrubs and trees during their flight period in May, put in plastic boxes containing moist peat and twigs of host plants as food, and kept at 5 - 6°C until the start of the experiments (between 3 and 10 days). Larvae were collected from infested sods of alpine meadows, placed individually into 90 mL plastic tubes filled with moist peat and slices of carrots as food, and kept at 22°C and 60 % RH in the dark for 5 to 10 weeks. The long quarantine of the larvae ensured that larvae infected by naturally occurring EPF could be excluded from the experiments. Larvae were cooled to 5-6°C for approximately 1 day reduce their activity before inoculation. Larvae used in experiment 1 came from a different population than larvae used in experiment 2 and 3.

2.2 Fungal strains

We used the two commercially available fungal strains, *B. brongniartii* BIPESCO2 (Bip2; isolated in Austria from *M. melolontha*; Strasser et al., 2003) and *M. brunneum* Ma 43 (BIPESCO5 / F52; isolated in Austria from *Cydia pomonella* (L., Lepidoptera: Tortricidae); European Food Safety Authority, 2012) in our experiments. To ensure the fitness of the fungal strains, we used EPF that passed through host insects recently. Conidiospores of *M. brunneum* Ma 43 were isolated from mycosed cadavers of *P. japonica* and those of *B. brongniartii* Bip2 from *M. melolontha* larvae from last year's inoculation experiments. Isolates were plated on selective medium plates (SM: sabouraud 2 % glucose agar (SDA) with cycloheximide (0.05 g L^{-1}), streptomycin sulfate (0.6 g L^{-1}), tetracycline (0.05 g L^{-1}), and dodine (50 mg L^{-1}); modified from Strasser et al., 1996), grown for two weeks at 22°C and 80% RH in darkness, and stored at 5°C after the F2 generation sporulated on the plates. We used these F2 generation plates as starting material for all spore suspensions described below.

2.3 Spore suspensions and oosporein production

Conidiospore suspensions were produced by plating the F2 generation on SM plates. Conidiospores of the F3 generation were washed off the plates with 15 mL of sterile Tween 80 solution (0.1% v/v), and mycelium was removed by vacuum-filtration (Miracloth filter, Merck, Darmstadt, Germany). The final spore concentration was adjusted to 10^7 spores mL^{-1} by adding deionized water after counting spores with a hemocytometer.

Blastospores and oosporein were produced in liquid medium (3% sucrose, 2.5% yeast extract, 1% peptone and 1% barley flour in 500 mL deionized water), which was inoculated with six to eight 7 mm-diameter plugs from F2 generation plates. The liquid cultures were incubated on an orbital shaker (Multitron Orbital Shaker, Infors HT, Switzerland) at 250 rpm at 25°C for four days and at 28°C for three days for Bip2 and Ma43, respectively.

The culture broth from the liquid fermentation process was used in different grades of purity (see "suspension types" in Table 1). It was used directly as suspension containing the (1) unfiltered culture broth with mycelial particles and oosporein, as (2) blastospores and oosporein suspension, which consists of the filtered culture broth, as (3) purified oosporein solution without blastospores, or as (4) filtered and washed blastospores suspension (without oosporein). The culture broth was filtered through Miracloth to remove residues of the medium and mycelial particles (suspension 2). Subsequently, the remaining blastospores and oosporein solution were separated by centrifugation (1174g for 20 min). The supernatant was used for oosporein treatments (solution 3), and the pellet was dissolved in deionized water for blastospore treatments (suspension 4). Filtration and centrifugation were repeated once to remove residues. Spore concentrations of all blastospore suspensions were determined using a hemocytometer and adjusted to 10^7 spores mL^{-1} by adding deionized water. For the blastospore injections, 1 mL of pure blastospore suspension was diluted to 10^6 spores mL^{-1} and 10^7 spores mL^{-1} for experiment 2 and 3, respectively.

We checked the germination rates of all spore suspensions by pipetting three samples 50 µL of each suspension on complete medium plates (CM: 10 g glucose, 0.36 g KH_2PO_4, 1.78 g Na_2HPO_4, 1 g KCl, 0.6 g $MgSO_4 7H_2O$, 0.6 g NH_4NO_3, 5 g yeast extract, 20 g agar per 1 L distilled water; Riba and Ravelojoana, 1984), which were incubated with conidiospores for 24 h and blastospores for 12-18 h. 100 spores of each sample were examined for germination tubes with a binocular microscope (M20, Wild Heerbrugg, Switzerland) at 40x magnification. The germination rate of all spore suspensions exceeded 95%.

2.4 Oosporein concentration

We determined the oosporein concentration for the suspensions used in experiment 2 using HPLC. For this we prepared the suspensions as described above in three replicates. All suspensions were purified by vacuum filtration through cellulose acetate filters (0.45 µm pore size, Satorius Stedim Biotech GmbH, Germany). Filtrates were diluted with Britton – Robinson methanol buffer and analyzed in duplicate using the method described by Seger et al. (2005) with small modifications (Waters 2695 separation module with a 2996 diode array detector, Phenomenex Synergi 150 mm x 2.0 mm Hydro-RP 80A column with 4 µm particle size, Phenomenex, Torrence, USA). The binary elution gradient consisted of water (solvent A) and acetonitrile (solvent B), both containing 0.1% (v/v) acetic acid and 0.9% (v/v) formic acid. The course of the gradient was 5 to 60% B in 6 min, followed by 60 to 98% B in 2 min and kept constant for a further 5 min at a flow rate of 0.3 ml min^{-1}. The column was kept at 23°C. Between analyses, the column was re-equilibrated for 7 min. The injection volume was 5 µl and chromatograms were recorded at 287 nm (sample bandwidth 4 nm).

Calibration curves were obtained for BR5.5-MeOH dilution series of oosporein. All calibration levels were measured three times. The calibration function was obtained by linear regression of the respective oosporein concentration against peak height. Limit of quantification (LOQ) for oosporein was 350 µg L^{-1} (< 1,14 µM).

2.5 Experimental design and procedure

In experiment 1 (Table 1) we combined spray and injection applications of conidio- and blastospores of Bip2 and Ma 43 and applied the treatments to cockchafer adults and third instar larvae. In experiment 2 (Table 1), we applied oosporein alone or in combination with Bip2 blastospores, as well as Bip2 conidiospores to cockchafer larvae as dip, spray, and injection treatments. In experiment 3 (Table 2), we chose the most efficient spore suspension of experiment 2 (combination of Bip2 blastospores and oosporein, applied as dip treatment) and applied it to different body parts of cockchafer larvae and to their food. Experiments with adults were carried out in May (flight period of beetles) and experiments with larvae in September and October. We used 15 insects for each treatment and each experiment consisted of five repetitions.

For all spray treatments, we sprayed each insect with one burst per side using a 30mL manual spray bottle. After this treatment, 37.8 ± 17.2 µL of spore suspension remained on adult individuals and 45.6 ± 18.9 µL on larvae (Supplementary Figure 1), which corresponds to about $3.8 \times 10^5 \pm 1.7 \times 10^5$ and $4.6 \times 10^5 \pm 1.9 \times 10^5$ spores per adult and larva, respectively. Injection treatments consisted of injection of 0.2 µL blastospore suspension or sterile deionized water behind the third leg of each insect (Experiment 1: ~200 spores per insect; Experiment 2: ~2000 spores per insect). For dip treatments, each insect was immersed for 3 – 5 seconds into the respective suspension holding it with tweezers.

After treatment application, insects were held individually in 90 mL plastic tubes filled with moist peat and hazelnut leaves (adults) or carrot slices (larvae) as food. We stored all tubes from one treatment and one repetition together in a plastic box and arranged those boxes randomly on racks in a climate-controlled room (experiment 1, 22°C, 60% RH, day-night cycle 16:8 h; experiments 2 and 3, 22°C, 60% RH, in darkness). We assessed the mortality and checked for visual symptoms of infection with *Beauveria* sp. or *Metarhizium* sp. weekly during a period of 4 weeks for adults and 8-10 weeks for larvae. Mycosed cadavers were removed from plastic boxes and stored at 5°C in the dark until further analysis.

2.6 Genetic analysis

To verify the identity of the fungal isolates growing on insect cadavers, we analysed selected isolates of all different treatments of experiment 1 by SSR (simple sequence repeats) analyses. We propagated the isolates on CM plates covered with filter paper and harvested the mycelia from the filter papers after 4-5 days of incubation. The mycelia were transferred to 2 mL Eppendorf tubes, and lyophilized after freezing them at -70°C. To disrupt the cells of the dry mycelia, we used the FastPrep-24 (MP Biomedicals, Eschwege, Germany; 25 s at 6 m s^{-1}) and glass beads (3 mm and 1 mm) and extracted the DNA with the sbeadex plant kit and King Fisher Flex Purification system (Thermo Fisher Scientific, Waltham, Massachusetts). DNA was standardized to 5 ng μL^{-1} for the multiplex PCRs followed by fragment size analysis (Mayerhofer et al., 2015a; Fernández-Bravo et al., 2021). We used six SSR markers in two primer pair sets (Bb1F4, Bb2A3, Bb2F8, Bb4H9, Bb5F4, Bb8D6 for *B. brongniartii*; Enkerli et al., 2001; Ma2049, Ma2054, Ma2063, Ma2287, Ma327, Ma195 for *M. brunneum*; Oulevey et al., 2009; Mayerhofer et al. 2015a) for each fungal species and included three reference strains for *Metarhizium* spp. (*M. brunneum* ARSEF7524, *Metarhizium robertsii* ARSEF7532 and Ma 43) and two for *B. brongniartii* (Bip2 and *B. brongniartii* BIBESCO 4 also referred to as ART546).

2.7 Data analysis

We used discrete-time hazard models to analyze insect mortality. Mortality measured during time intervals of 7 days was used as dependent variable in a binomial generalized linear model with a complementary log-log link (ASReml-R V4 package, VSNi, Hemel Hempstead, UK). As fixed effects, we fitted interval, experimental treatments, and the interaction between those terms. In this model, an interaction between interval and treatments indicates non-proportional hazards. We fitted box, which was the level of replication and harbored the 15 insect tubes with the treated individuals, as random effect.

To analyze data at specific time points we used subsets of the data and fitted it into quasibinomial generalized linear models to account for the effect of the box that induced a slight overdispersion. In experiment 2, we excluded one replicate of the water injection treatment due to unnaturally high mortality resulting in a strong overdispersion. We decomposed the experimental treatments into a series of individual contrasts and interactions with time intervals to analyze the effects. See results for details.

3 Results

3.1 Experiment 1 – *B. brongniartii* Bip2 and *M. brunneum* Ma 43 treatments

3.1.1 Adults

For adults, the application of *B. brongniartii* Bip2 or *M. brunneum* Ma 43 blasto- and conidiospores increased the mortality of the beetles compared to all control treatments (fungal treatments ↔ control treatments; $F_{3, 33.1} = 38.94$, $p < 0.001$; Figure 1A and B). While nearly all fungus treated adults were dead on day 14, the mean mortality in the control groups was below 50%. We did not find any differences among control treatments (untreated control ↔ Tween 0.01% spray ↔ H_2O injection; $F_{2, 32.8} = 0.35$, $p > 0.1$). Mortality rates changed with time (interval: $F_{3, 100} = 32.44$, $p < 0.001$) and the fungal spore treatments were time-dependent (time × Bip2, Ma 43 treatments; $F_{9, 100} = 3.83$, $p < 0.001$), primarily because mortality effects were more variable in the first interval (days 0 – 7).

Bip 2 spray treatments were fastest in killing adult cockchafers. While around 80% of adults sprayed with Bip2 blastospores and approx. 50 % sprayed with Bip2 conidiospores were dead on day 7, in all other spray treatments mortality was between 10 and 20% (Bip2 spray ↔ Ma 43, Tween 0.01% spray: $F_{1, 39} = 47.79$, $p < 0.001$; Bip2 blastospore spray ↔ Bip2 conidiospore spray:$F_{1, 36} = 14.15$, $p < 0.001$). Ma 43 blastospore spray, Ma 43 conidiospore spray and Tween 0.01% spray did not differ from each other on day 7 ($F_{2, 36} = 1.72$, $p > 0.1$). Furthermore, Bip2 blastospore injection caused a higher mortality rate (approx. 90% vs. approx. 60%) than Ma 43 blastospore injection at day 7 ($F_{1, 36} = 9.02$, $p < 0.01$).

We found mycosed cadavers among all Bip2 or Ma 43 treated adults; between 47% and 77% of the cadavers showed signs of mycosis of the applied fungal species, and all tested fungal isolates matched genetically with Bip2 or Ma 43, respectively (Supplementary Tables 1, 3 and 4). Mycosis of *Beauveria* sp. and *Metarhizium* sp. found in the different control treatments were mostly caused by natural infection with strains differing genetically from Bip2 and Ma 43 (Supplementary Tables 1, 3 and 4).

3.1.2 Larvae

Larval mortality rates varied with time ($F_{9, 422} = 3.39$, $p < 0.001$), but effects of the treatments were time-independent (time × treatment: $F_{72, 350} = 0.20$, $p > 0.5$). The spray treatments, including the Tween 0.01% control application, did not differ from each other (Figure 1C, $F_{4, 71.5} = 0.31$, $p > 0.5$) and the Tween 0.01% spray did not differ from the untreated control ($F_{1, 422} = 1.22$, $p > 0.1$). The mortality rate stayed below 15% in all spray treatments after 10 weeks of incubation. The injection treatments slightly increased mortality (Figure 1D, injection treatments ↔ other treatments; $F_{1, 28.7} = 22.4$, $p < 0.001$) with a trend towards higher mortality in injection treatments with 200 blastospores of either Bip2 or Ma 43 compared to the H_2O injection ($F_{1, 20.7} = 3.08$ $p = 0.09$). This resulted in a higher number of dead larvae in the spore injection treatments than in the H_2O injection treatment at the end of the experiment (Bip2, Ma 43 injection ↔ H_2O injection; $F_{1, 36} = 4.92$, $p < 0.05$). However, none of the injection treatments had a mean mortality rate of over 40% after 10 weeks of incubation. In line with the low mortality of larvae, we found only a few mycosed cadavers in the Bip2 and none in the other spray treatments (Supplementary Tables 2 and 3). By contrast, 50% of the larval cadavers injected with blastospores of either Bip2 or Ma 43 were mycosed with the respective fungal species and strain. One larval cadaver injected with water was mycosed by *B. brongniartii* (Supplementary Tables 2, 3 and 4).

3.2 Experiment 2 – Effects of oosporein and application methods

3.2.1 Oosporein concentration of different suspension types

In all *B. brongniartii* Bip2 liquid cultures, with one exception, oosporein could be detected as a secondary metabolite according to the sample preparation (Table 3). The highest concentration of oosporein was detected in the supernatant (2.18 mM ± 0.4 mM), i.e., in the suspension, that was completely free from fungal biomass after filtration and centrifugation. The second highest concentration was detected in the filtered blastospore suspension. In the unfiltered culture broth, only 22 % of the actually produced oosporein could be detected. The conidiospore suspension was enriched with a very low concentration of oosporein (0.042 mM). The cleaned blastospore suspension, which was resuspended in water after the washing procedure, showed no oosporein contamination (below the detection limit).

3.2.2 Mortality of larvae

The injection of 2000 blastospores of *B. brongniartii* Bip2 per larva, compared to the injection of 200 blastospores per larva in experiment 1 (see 3.1.2), was very efficient in killing common cockchafer larvae. Between 80% and 100% of the larvae died within the first 7 days, and 100% mortality was reached at day 21 in all replicates. The injection of H_2O killed only a few larvae: we found one dead larva at day 7 and three dead larvae in total at day 21 among all replicates. Thus, the blastospore injection was the most successful treatment here, followed by the dip and then the spray treatments (Fig. 2).

Combined application of blastospores with oosporein resulted in higher larval mortality in the dip treatments but not in the spray treatments (Fig. 2). Dip treatments with blastospores and oosporein (mean mortality rates of approx. 80 %) differed significantly from blasto- and conidiospore dip treatments without oosporein (mean mortality rates of approx. 45% and 40%; $F_{1,58} = 25.03$, $p < 0.001$). For the spray treatments, we found that blastospores with and without oosporein (mean mortality rates of approx. 50% and 45%) killed more larvae than conidiospores (mean mortality of approx. 30%; $F_{1,57} = 4.74$, $p < 0.05$) but there was no synergistic effect of oosporein and blastospores ($F_{1,57} = 1.97$, $p > 0.1$). Oosporein without blastospores had no significant effect on mortality. There was no difference between the untreated control, the Tween 0.01% application controls and the oosporein treatments without spores, irrespective of the application method (mean mortalities between 5% and 15%; $F_{4,56} = 1.14$, $p > 0.1$).

Additionally, dip treatments with conidiospores and blastospores without oosporein differed significantly from the Tween 0.01% control ($F_{1, 57} = 17.51$, $p < 0.001$) and the conidiospore spray treatment killed more larvae than Tween 0.01% spray ($F_{1, 56} = 10.61$, $p < 0.01$).

3.3 Experiment 3 – Pathways of entry: Infection via different body parts of the host and insect feed

We did not find differences between dipping the thorax and head, the thorax and the abdomen, or the whole larvae into the spore suspension ($F_{2, 32} = 1.67$, $p > 0.1$). However, we found significantly more dead larvae in treatments where the thorax and legs were immersed into the spore suspension (mean mortality rates between 60% and 75%) as compared to the abdomen (mean mortality rate of approx. 30%; $F_{1, 34} = 19.45$, $p < 0.001$; Figure 3). Nevertheless, dipping the abdomen of the larvae into the spore suspension killed significantly more larvae than immersing the whole larvae into water as control (mean mortality rate of approx. 5%; $F_{1, 32} = 10.19$, $p < 0.01$).

Carrots treated with blastospores and oosporein did not induce a significant higher mortality in larvae (mean mortality rate of approx. 15%) than carrots dipped in water (mean mortality rate of approx. 10%; $F_{1, 32} = 1.86$, $p > 0.1$). The number of dead larvae did not differ between control treatments and was generally low (mean mortality rates between 5% and 10%; untreated control ↔ H_2O larvae ↔ H_2O carrot; $F_{2, 32} = 0.11$, $p > 0.1$).

4 Discussion

The control of common cockchafer larvae with *B. brongniartii* is one of the most successful biological control systems, showing long term control effects for several years (Kessler et al., 2004; Mayerhofer et al., 2015b). To adapt this strategy to other pest insects, a better understanding of this host-pathogen relationship is needed, and key factors affecting the infection process have to be identified. Here, we tested the infection of the common cockchafer with *B.*

brongniartii Bip2 in the laboratory, a strain known to be virulent under field conditions (Laengle et al., 2005; Mayerhofer et al., 2015b), and with the generalist entomopathogenic fungus *M. brunneum* Ma 43.

We found that both fungal strains, the specialist *B. brongniartii* Bip2 and the generalist *M. brunneum* Ma 43, readily infected cockchafer adults but not larvae when applied as topical spray. These results were surprising and do not support our hypothesis. We expected larvae to be susceptible to *B. brongniartii* Bip2, since this fungal strain shows good control effects in field applications (Kessler et al., 2004; Laengle et al., 2005; Mayerhofer et al., 2015b). Furthermore, laboratory experiments conducted under optimal conditions for fungal infection (high moisture level, stable temperature) normally result in higher control effects than field experiments (Lacey et al., 2015), which is not the case here. It is important to note that on average $3.8 \times 10^5 \pm 1.7 \times 10^5$ and $4.6 \times 10^5 \pm 1.9 \times 10^5$ spores were applied to the surface of adults and larvae, respectively. This makes it unlikely that the difference in susceptibility of the two stages may be explained by different amounts of inoculum applied. More likely, the method of topical spray applications of conidio- and blastospores is inefficient to infect cockchafer larvae. This result matches the findings of similar experiments conducted with Japanese beetle adults and larvae (Graf et al., 2023). While adult Japanese beetles were efficiently killed and infected by Ma 43 and Bip2, larvae were not affected by topical spray applications. Besides the low susceptibility of larvae to spray treatments in general, we found significant differences in control efficacy between two experiments. In the second experiment, we found a control effect of around 50% of *B. brongniartii* Bip2 in the spray treatments with blasto- and conidiospores, while there was almost no effect in the first experiment. Materials used and test procedures were similar in both experiments, with the exception that the larvae for experiment 1 and 2 originated from different populations. It has been shown that different populations can differ substantially in fitness and susceptibility to fungal pathogens (Keller et al., 1999), which is confirmed by our data.

Dip treatments with blasto- and conidiospores of *B. brongniartii* Bip2 also reached a control effect of around 50%. Even though we assume that spore dose was higher in dip treatments than in spray treatments, because larvae are completely immersed in the spore suspension, and spores may reach all vulnerable sites of the larval bodies, mortality was not clearly enhanced compared to the spray treatments. However, when we did not remove the oosporein from the blastospore suspensions, mortality of more than 80% was reached in the dip treatments, while it stayed around 50% in the spray treatments. Thus, oosporein did not enhance the control effect of the EPF in spray treatmemts. In contrast, our hypothesis that oosporein enhances the control effect of *B. brongniartii* is met for the dip treatments. Oosporein on its own had no effect on the mortality of common cockchafer larvae. Other studies have shown a similar effect: oosporein had no or only little effect on insects' mortality, but it acted synergistically with *Beauveria* sp. spores and enhanced insect control (Amin et al., 2010; Mc Namara et al., 2019).

There are two possible explanations for this kind of synergistic interaction. It was shown that the presence of exogenous oosporein stimulates the synthesis of oosporein by *Beauveria caledonica* (Mc Namara et al., 2019) and that the ability of an EPF to synthesize oosporein is connected to its virulence (Feng et al., 2015). This way, the combined application of spores and oosporein may have enhanced the virulence of our EPF. However, Fan et al. (2017) suggested that oosporein is normally only produced during late stages of the infection, and is neither involved in the attachment of spores nor the penetration or proliferation within the host. They only detected increasing oosporein levels within the host 24-48 hours after death and postulate that the function of oosporein is supporting sporulation on the host cadaver by reducing competition with bacteria (Fan et al., 2017).

The second explanation is that antibiotic properties of oosporein, shown by the study of Fan et al. (2017), may adversely affect the protective microbiome on the insects' cuticle. Instead of altering the virulence of the EPF, the combined application of spores and oosporein may have enhanced the susceptibility of the host, and, thus, helped *B. brongniartii* Bip2 to infect common cockchafer larvae, when applied exogenously. Most insects carry symbiotic bacteria on their cuticle, present in high numbers on intersegmental membranes, mouth parts and the anus region which are also preferred body parts of entomopathogenic fungi for penetration (Boucias et al., 2018). It has been shown that those symbionts help the insect to fight fungal infections by producing antifungal compounds which hinder spore germination and fungal growth (Kaltenpoth, 2009; Chevrette et al., 2019; Batey et al., 2020; Pessotti et al., 2021; Hong et al., 2022; Wang et al., 2022). Oosporein shows antimicrobial activities against a variety of bacteria and hinders the growth of, among others, Pantoea, Staphylococcus, Stenotrophomonas, Bacillus, and Acinetobacter (Semar, 1993; Alurappa et al., 2014; Fan et al., 2017). Fan et al. (2017) found that 100 µg mL^{-1} of oosporein was sufficient to inhibit growth for more than 90% of bacteria, and 50 µg mL^{-1} already inhibited bacterial growth for 50% or more. In our experiment, we had oosporein concentrations of around 50 µg mL^{-1} in the unfiltered blastospore suspensions and more than 200 µg mL^{-1} in the filtered blastospore suspensions. Thus, oosporein concentrations were high enough to efficiently suppress symbiotic bacteria on the cuticle of common cockchafer larvae. The supernatant with very high oosporein concentrations of 395-630 µg mL^{-1}, but no spores, had no adverse effect on the larvae, which gives evidence that oosporein itself is non-toxic to cockchafer larvae. Furthermore, feeding assays with oosporein did not show adverse effects on cockchafer larvae, *Galleria mellonella* L. (Lepidoptera: Pyralidae) or *Hylobius abietis* L. (Coleoptera: Curculionidae; Abendstein et al., 2001; Mc Namara et al., 2019).

Finally, we cannot rule out that oosporein may have a sublethal effect, which might reduce the fitness of the cockchafer larvae. Again, we assume that this sublethal effect would be stronger for larvae immersed in the suspensions and thus, increase the infection rate and mortality for those treatments as compared to the spray treatment. It was shown that oosporein is slightly toxic towards different cell types, *Daphnia magna* and chicken broilers (Pegram and Wyatt, 1981; Semar, 1993; Jeffs and Khachatourians, 1997; Favilla et al., 2006) and had a weak insecticidal effect on the whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae; Amin et al., 2010).

However, if at all, oosporein acts as a week toxin against other organisms than bacteria, and it is considered as environmentally safe (Zimmermann, 2007a).

Differences in the susceptibility of common cockchafer adults and larvae to an exogenous application of EPF is, however, only one part of the story. Cockchafer adults and larvae also differed in their susceptibility to *B. brongniartii* Bip2 and *M. brunneum* Ma 43 when we bypassed the cuticle as barrier and injected the blastospores directly into the hemolymph. While cockchafer adults were readily killed by the injection of 200 blastospores of Bip2 and Ma 43, larvae were only efficiently infected by Bip2 when we injected 2000 blastospores in the second experiment. Similar to above, the injection of 200 blastospores of either Ma 43 or Bip2 killed adult Japanese beetles within a few days (Graf et al., 2023). Larvae of *P. japonica* also turned out to be significantly less susceptible to blastospore injection than adults, however, about half of the *P. japonica* larvae still died from this treatment in the above-mentioned study. The differences in susceptibility to fungal spore injection between the larvae of these two host insects may simply be a matter of size or mass. The head capsule of L3 Japanese beetle larvae is round 3.1 mm (Fleming, 1972), that of L3 common cockchafer larvae is 6.4 – 7.0 mm (Büchi et al., 1986) which relates well to their differences in body size and mass.

In common cockchafer adults, we found that *B. brongniartii* Bip2 showed a higher mortality rate than *M. brunneum* Ma 43. Bip2 caused higher mortality in the first 7 days than Ma 43 in all treatments with fungal spores (conidiospore spray, blastospore spray, and blastospore injection). This difference was not found for Japanese beetles (Graf et al., 2023). Thus, these results support the hypothesis that the specialized *B. brongniartii* can overcome the cuticle and immune response of cockchafer adults more easily than the generalist *M. brunneum*. Furthermore, blastospores of *B. brongniartii* Bip2 showed a faster speed of kill of cockchafer adults than conidiospores. Generally, the thin-walled blastospores germinate faster than conidiospores (Butt and Goettel, 2000), thus we expected blastospore treatments to exhibit a faster speed of kill than conidiospore treatments under optimal laboratory conditions. Surprisingly, it did not matter whether we injected or sprayed Bip2 blastospores to adult cockchafers. This indicates that the cuticle of cockchafer adults is not efficient at all in protecting the beetles from Bip2 infections.

Taking into account the results presented here and those from the previous study with Japanese beetle larvae and adults (Graf et al., 2023), we hypothesize that stage-specific differences in susceptibility to EPF are common in scarab beetles. We found evidence that the cuticle of the scarabs plays a major role as physical barrier to protect larvae from fungal attack. In contrast, adults are not well protected by their cuticle and readily infected by EPF. Intersegmental membranes are generally known as vulnerable sites of the cuticle (Butt and Goettel, 2000) and other studies already showed that *Metarhizium anisopliae* (Metchnikoff) Sorokin (Hypocreales: Clavicipitaceae) and *Beauveria bassiana* (Bals.-Criv.) Vuill. (Hypocreales: Cordycipitaceae) preferably penetrate their host cuticles through those membranes (Zhang et al., 2010; Chouvenc et al., 2009; Baek et al., 2022). We postulate that adult scarabs, with an exoskeleton of inflexible, chitinized sclerites, possess more areas with thin intersegmental membranes than larvae in order to retain mobility. Larvae are mostly covered with a flexible cuticle, which implies that thin intersegmental membranes are present in the joints of the legs and mouthparts only. As a consequence, fungal spores would reach vulnerable infection sites in adult hosts more easily than in larvae. However, we cannot exclude that behavioral differences of larvae and adults, the chemical compositions of the different cuticles, or a different cuticular microbiome also impact the susceptibility of the two developmental stages to EPF. Furthermore, our results indicate that larvae can encapsulate or eliminate blastospores of *M. brunneum* and *B. brongniartii* in the hemolymph more efficiently than adults. Stage specific differences in susceptibility to pathogens are known from other insect species, especially when adults and larvae do not share the same habitats (Kim et al., 2020; Beak et al., 2022,) which is also the case for most scarab beetles (Jackson and Klein, 2006). Thus, we suggest testing and elaborating control methods targeting adult scarab beetles with EPF (Wey et al. 2025).

Additionally, we found that the upper body parts of larvae, including the thorax and legs, are more susceptible to fungal infection than the abdomen. We did not find differences between the immersion of the whole larvae, the abdomen and thorax without head, and the head and thorax. This indicates that contact of the head (and mouthparts) to the spores of the EPF was less important, but it was important that the thorax, and with this also the legs, were immersed into the spore suspension. We therefore consider the joints of the legs as the main entry points for fungal infection. Our hypothesis contrasts that of Delmas (1973) who found that common cockchafer larvae were most susceptible for infection with *B. brongniartii* (called *Beauveria tenella* in this study) at the labium and the anal region. However, they did point inoculations with conidiospores, not topical sprays or immersions, and they did not include inoculations of legs. We did not find the anus region to be susceptible as the immersion of the abdomen lead to lower mortality than all other dip treatments. We assume that behavioral defense mechanisms of the cockchafer grubs may be responsible for the low infection rate via the anus region, e.g., retraction of potentially susceptible intersegmental membranes, or cleaning behavior.

Finally, we did not find elevated mortality in larvae fed with fungus-contaminated carrot slices. This confirms earlier findings that *B. brongniartii* does not infect common cockchafer larvae through the gut (Ferron, 1967). In addition, these results also give evidence that contamination of the insects' mouthparts with fungal spores during feeding is not sufficient to provoke elevated levels of infection. Again, behavioral defense mechanisms, like cleaning behavior, may play a role.

In conclusion, we observed significant differences in the efficacy of EPF against this scarab host, influenced by spore types, application methods, and developmental stages of the target insect. The fungal metabolite oosporein, produced by *B. brongniartii* during fermentation and insect colonization, notably affected virulence outcomes. While we anticipated effective control of common cockchafer adults and larvae using *B. brongniartii* Bip2 conidio- and blastospores, this was only achieved for adults. The specialist *B. brongniartii* Bip2 outperformed the generalist *M.*

brunneum Ma 43 in infecting adults but not larvae. Additionally, larval susceptibility varied by body part; inoculation of the thorax (including legs) resulted in higher susceptibility compared to the head and abdomen.

Based on these findings, we doubt that standard virulence test results are a suitable tool to forecast EPF performance under field conditions. Contrary to several studies suggesting that laboratory tests overestimate EPF efficacy (Butt and Goettel, 2000; Lacey et al., 2015), our findings indicated a lower susceptibility of common cockchafer larvae to fungal inoculum, despite the proven efficacy of *B. brongniartii* in the field. Notably, the highest control effects were observed when oosporein was retained in blastospore solutions during laboratory tests. Future research should explore the potential synergistic effects of oosporein with conidiospores.

Our findings have significant implications for laboratory methods in biocontrol product development. Virulence testing under optimized conditions may have limited applicability, and results may not be reproducible in semi-field or field settings. We advocate for more comprehensive studies to ensure the successful development of new biocontrol products, taking into account host and pathogen-specific characteristics across various experimental conditions.

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6 Conflict of Interest

No conflict of interest declared.

7 Data Availability

Data is available on request.

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9 Ethical approval statement

This study did not involve research on humans or vertebrate animals. Experiments were conducted exclusively with insects (*Melolontha melolontha*), which are not subject to ethical approval requirements. All procedures complied with institutional biosafety and research integrity guidelines.

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11 Authors contribution

TG: conceptualization, formal analysis, investigation, methodology, visualization and writing – original draft. FS: investigation and formal analysis. HE: investigation, formal analysis and writing – original draft, PAN: conceptualization, formal analysis, validation and writing – review and editing. GG: funding acquisition, project administration, conceptualization, supervision, validation and writing – review and editing.

Table 1 Overview of treatments and application methods of experiments 1 and 2.

	Treatment	Suspension type	Application method
Experiment 1	Control	No suspension	None
	Tween	0.01% Tween	Spray
	H_2O	Deionized water	Injection
	Bip2 conidiospores	Spores in 0.01% Tween	Spray
	Bip2 blastospores	Spores in deionized water	Spray
			Injection
	Ma 43 conidiospores	Spores in 0.01% Tween	Spray
	Ma 43 blastospores	Spores in deionized water	Spray

			Injection
Experiment 2	Control	No suspension	None
	Tween	0.01% Tween	Spray
			Dip
	H_2O	Deionized water	Injection
	Oosporein	Supernatant of filtered Bip2 culture broth	Spray
			Dip
	Bip2 conidiospores	Spores in 0.01% Tween	Spray
			Dip
	Bip2 blastospores	Spores in deionized water	Spray
			Dip
			Injection
	Bip2 blastospores + oosporein	Spores in filtered culture broth	Spray
			Dip

	Bip2 blastospores + oosporein + mycelium	Spores in unfiltered culture broth	Dip

Table 2 Overview of treatments and application methods in experiment 3. Different parts of the body of the larvae, or carrots as their food, were dipped into the suspensions.

Treatment	**Suspension type**	**Dipping method**
Control	No suspension	None
H_2O	Deionized water	Head + thorax + abdomen
		Carrot
Bip2 blastospores + oosporein	Spores in filtered culture broth	Head + thorax + abdomen
		Thorax + abdomen
		Head + thorax
		Abdomen
		Carrot

Table 3. HPLC analysis of oosporein in different *B. brongniartii* Bip2 suspensions. The liquid cultures were harvested by filtration and or centrifugation, the blastospores were also washed and resuspended. All suspensions were diluted in BR5.5-MeOH buffer solution and immediately analysed (n=3; two repetitions). Limit of quantification (LOQ) for oosporein was 350 µg L^{-1} (< 1.14 µM).

Suspension Type	**Oosporein concentration (mM)**
Blastospores filtered (filtered culture broth)	1.088 ± 0.21 mM
Blastospores unfiltered (culture broth)	0.245 ± 0.16 mM
Blastopores washed	below LOQ (< 0.001 mM)
Conidiospores	0.0422 ± 0.0002 mM
Supernatant	2.181 ± 0.432 mM

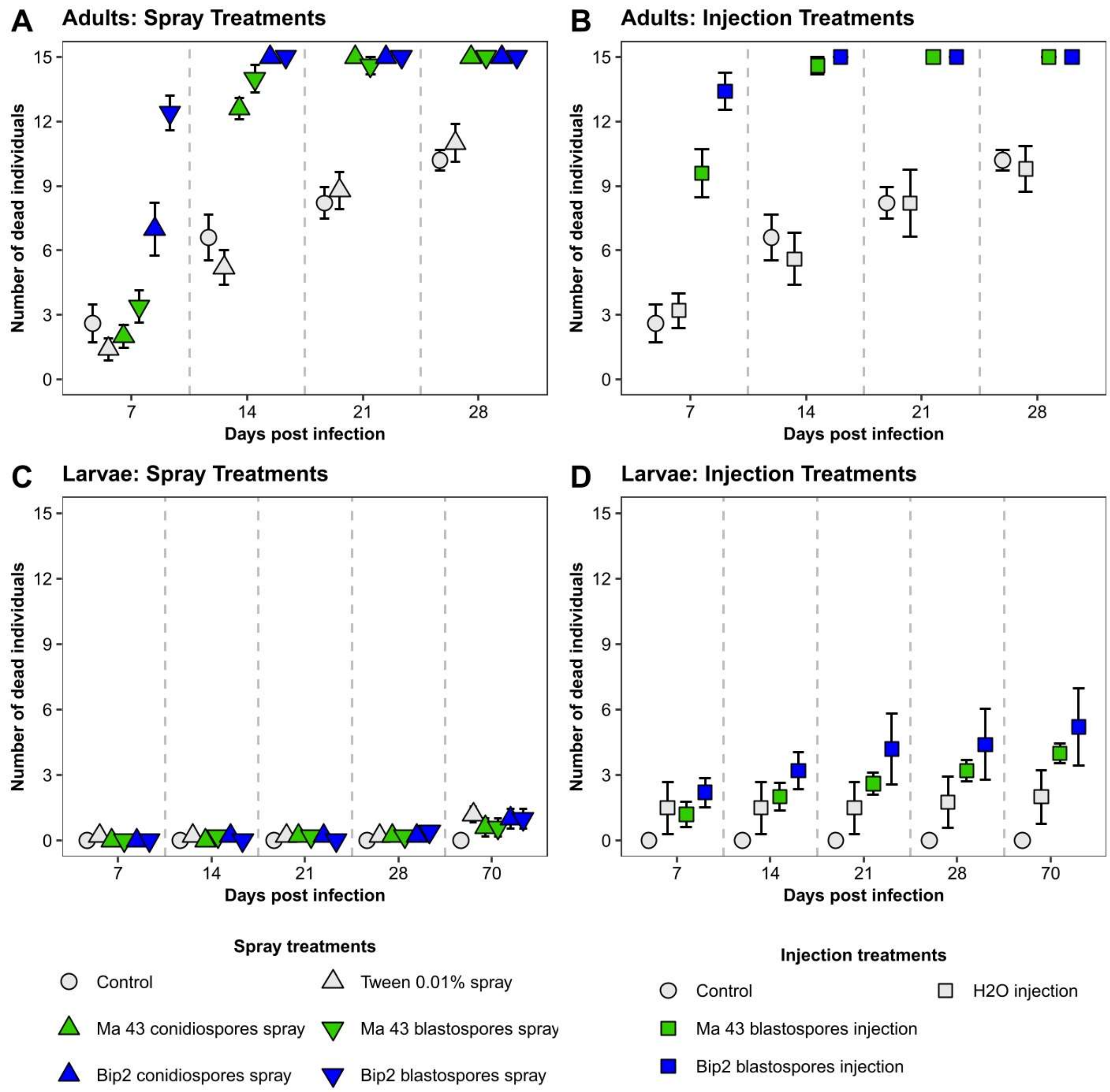

Figure 1 Cockchafer mortality (number of dead insects in cohorts of 15) depending on spray (A, C) and injection (B, D) treatments, for adults (A, B) and larvae (C, D). Spray treatments were applied in H_2O (blastospores) or 0.01% Tween (conidiospores), blastospore injections were suspensions in H_2O. Measurements were made weekly, but data are not shown for weeks 5-9 for the larval data. Control: untreated controls (neither sprayed nor injected). mean±s.e. (n=15).

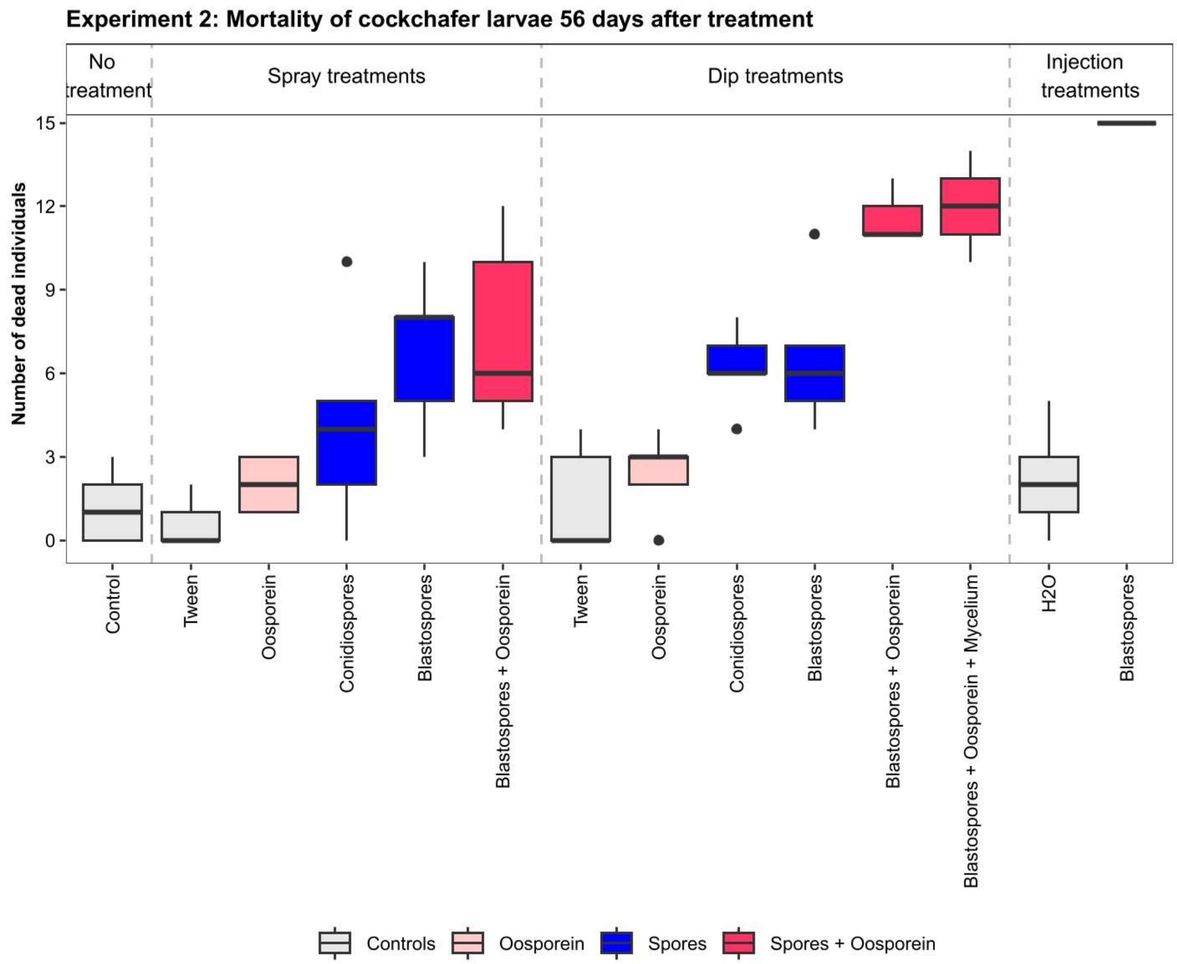

Figure 2 Number of dead cockchafer larvae (in cohorts of 15) after dip-, spray-, and injection treatments with different spore types and oosporein. Mortality was assessed for 56 days in intervals of 7 days, displayed are the cumulated results at the last sampling date. Boxplots show the median and the quartiles of the five replicates per treatment. Treatments are grouped according to the application method and colors as well as horizontal lines delimit different suspension types. Grey: control treatments without spores and oosporein, light pink: treatments with oosporein only, blue: *B. brongniartii* Bip2 treatments with blasto- or conidiospores without oosporein, pink: *B. brongniartii* Bip2 treatments with blastospores and oosporein.

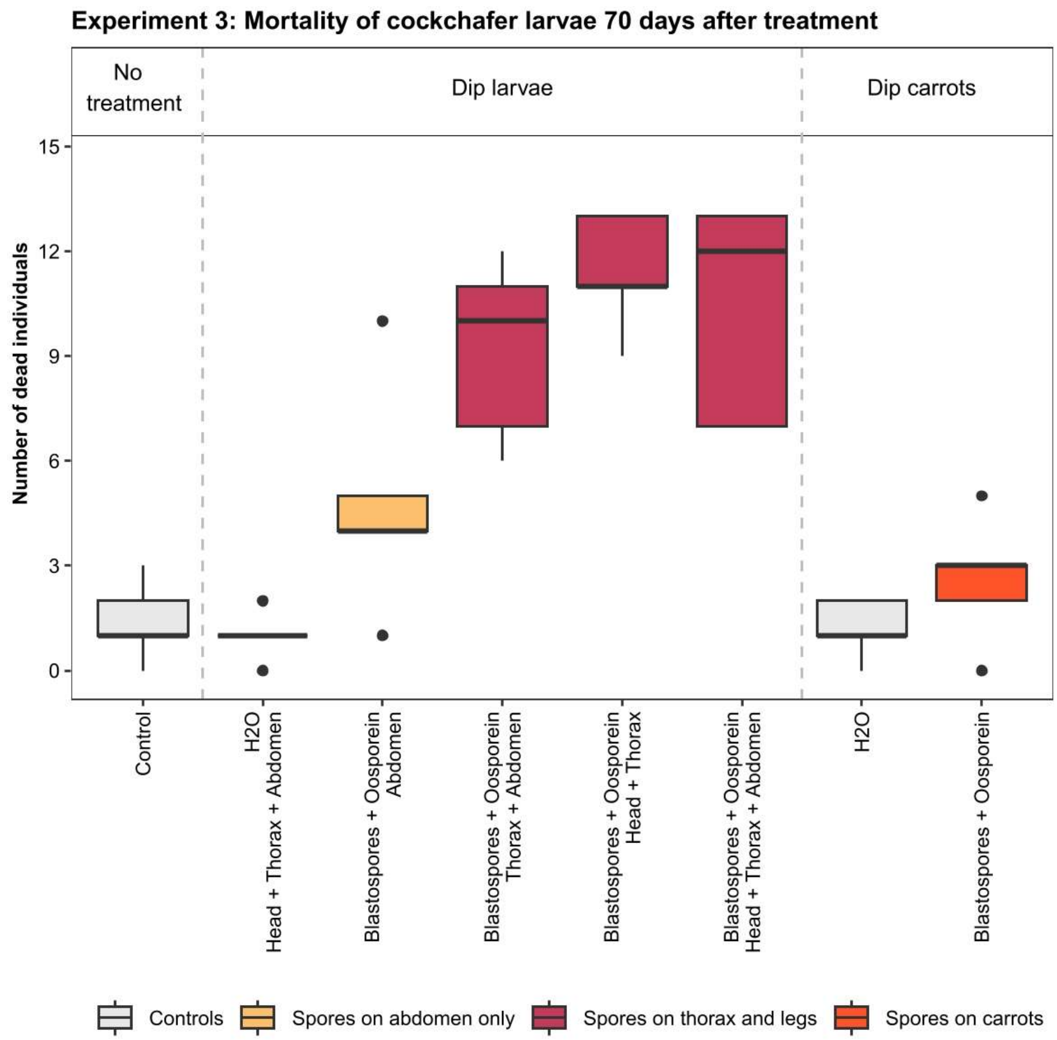

Figure 3 Number of dead cockchafer larvae after dipping different body parts or their feed into a blastospore suspension with oosporein. Mortality was assessed for 70 days in intervals of 7 days, displayed are the cumulated results of the last sampling date. Boxplots show the median and the quartiles of the five replicates per treatment. Horizontal lines group the treatments according to similarities in the treatments. Grey: control treatments without spores and oosporein, pink: Bip2 treatments with blastospores and oosporein.