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Surviving in the fast lane: no increased mortality, but faster growth for pathogen-exposed larvae of a family-living beetle

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Abstract

Animal taxa exhibiting post-hatching care can be found throughout the animal kingdom. During this period, parents aggregate with their offspring and allow them to invest their resources into growth and development as parents take over energy consuming tasks. Studies show that food provisioning and social immunity by parents can alleviate the costs of an offspring's immune response to pathogen exposure. However, this issue has rarely been explored in offspring of species showing plasticity in their dependency on parental care. Here, we raise the question of how offspring are affected by pathogen exposure if they have access to social immunity through a caring parent. Parents of *Nicrophorus vespilloides*, a species exhibiting facultative post-hatching care, control the carcass microbiome via their antimicrobial exudates, stopping further decay and protecting their offspring from potential pathogens. We exposed *N. vespilloides* offspring to a generalist entomopathogenic fungus, *Beauveria bassiana*, while manipulating the post-hatching parental care. We monitored offspring performance throughout their development and found, curiously, larvae showed an increase in mean weight and growth rate after being exposed to the pathogen, regardless of parental care, while their survival and adult immunity were unaffected. Simultaneously, our results indicate that females invest fewer resources into their offspring if they have been exposed to the pathogen. Overall, we show that offspring of facultative subsocial species may not respond differently to pathogen exposure depending on their parents' aid. Additionally, our results indicate that offspring of facultatively subsocial species may adjust their growth rate in response to pathogen exposure.

Keywords: *Beauveria bassiana*, *Nicrophorus*, social immunity, parental care, immunity, larval development, social evolution

Introduction

Parental care is associated with benefits for offspring, enhancing development and survival to adulthood (Klug & Bonsall, 2010). By attending and fostering their brood, parents create a favourable environment, for example by providing food, maintaining and defending a nest or crucial resource, and fending off predators, parasites, or pathogens (Clutton-Brock, 1991; Royle et al., 2012; Trumbo, 1996). By shielding offspring from adverse conditions, this protective buffer not only alleviates immediate costs but may also allow offspring to save precious resources long-term. Instead of requiring investment into defence, immunity, or concealment, offspring receiving parental care may focus on development and long-term fitness benefits (Cotter et al., 2004). Pathogen pressure and immune responses, for instance, are well demonstrated to incur fitness and long-term immunity costs (Brown et al., 2019; Cotter et al., 2004; Kaufmann et al., 2014; Lindström et al., 2004; Tye et al., 2020). Parental care can not only offset these costs by bolstering offspring immunity, e.g., through food provisioning (Boucicot et al., 2024; Saino et al., 1997), but also reduce them directly, e.g., through social immunity.

Social immunity, i.e., any collective or personal mechanism that has emerged and/or is maintained at least partly due to the antipathogen defence it provides to homospecific group members (sensu Van Meyel et al., 2018), is an important factor in the maintenance of complex social structures and a possible driver of early social evolution, such as in facultative families (Cotter & Kilner, 2010a; Cremer et al., 2007, 2018; He et al., 2018; Jones et al., 2018; Meunier, 2015). For instance, faeces of wood cockroaches possess antimicrobial properties, which can sanitize their communal nests and thus lower the microbial threat in their environment (Rosengaus et al., 2013). This phenomenon also occurs in earwigs (Diehl et al., 2015), where maternal post-hatching care affects how offspring alter their gene expression in response to a pathogen (Körner et al., 2020), but does not prevent long-term effects of exposure on offsprings' life-history traits (Vogelweith et al., 2017). In facultatively subsocial burying beetles of the genus *Nicrophorus*, parental care is associated with excretion of antimicrobial exudates on vertebrate carcasses (Cotter & Kilner, 2010a; Degenkolb et al., 2011; Hoback et al., 2004).

It stands to reason that parental deprivation, in turn, could incur costs in terms of immunocompetence to offspring. Indeed, loss of parents can have drastic effects on the developing offspring and their life-history traits as seen in red deer, orcas, and many birds (Andres et al., 2013; Foster et al., 2012; Klug & Bonsall, 2014; Montesdeoca et al., 2017; Royle et al., 2012). Reduced immunocompetence in particular was demon-

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strated in mice and rats (Michaut et al., 1981; von Hoersten et al., 1993), but also in *Nicrophorus vespilloides* burying beetles where larvae showed reduced lytic activity in their excretions when deprived of caring parents (Reavey et al., 2014). Conversely, *N. vespilloides* larvae were also shown to increase expression of several immune genes in absence of maternal care (Ziadie et al., 2019). In family-living earwigs, evidence suggests that maternal care does not shape offspring immunity long-term (Vogelweith et al., 2017) but does affect offspring responses to pathogens in the nest (Körner et al., 2020). These findings indicate that the relationship of parental care on offspring immunity may depend on environmental conditions, such as pathogen prevalence. Even though the role of pathogen pressure is considered to be an important factor in social evolution (Meunier, 2015; Schmid-Hempel, 1998), the interplay between pathogen exposure and the benefits of parental care during early social evolution remains poorly understood. On one hand, pathogen pressure has often been considered a key obstacle in social evolution (e.g., Hughes et al., 2002). On the other hand, microbial and pathogen prevalence have been hypothesized to select for social immunity and intensive brood care (Korb & Heinze, 2016). These are important drivers of the consolidation of facultative family life towards obligate and/or more complex social systems (Jackson & Hart, 2009; Turnbull et al., 2010; Van Meyel et al., 2018). Here, we set out to answer the question how the harmful effects of pathogen exposure during early larval development are influenced and potentially alleviated by access to social immunity in the form of a caring female in *N. vespilloides*, a beetle exhibiting facultative family life.

While *Nicrophorus* offspring show a broad spectrum of dependency on parental care, ranging from facultative to obligate (Capodeanu-Nägler et al., 2016), with intermediate states as seen in *N. vespilloides*, they are not merely passive recipients. Instead, they actively contribute to the creation of their specific niche by producing exudates of their own, supporting parents in their efforts to obfuscate microbe-derived volatiles and toxins and preventing the spread of potential carrion and grave-soil pathogens (Arce et al., 2013; Jacobs et al., 2014; Körner et al., 2023). When a burying beetle pair discovers a suitable carcass, they bury it in the soil and start preparing it for their offspring. Parents face a trade-off between their external and personal immunity, increasing their offspring's survival while decreasing their own future reproductive success with mothers bearing the brunt of antibacterial defence (Arce et al., 2012; Cotter & Kilner, 2010b; Cotter et al., 2010; Jacobs et al., 2014; Steiger et al., 2011). Control of the carcass microbiome is ensured by coating the carcass with their anal and oral exudates, preventing further decay and fighting off pathogens while inoculating the carcass with beneficial microbes for their offspring (Körner et al., 2023; Pukowski, 1933; Scott, 1998; Shukla et al., 2018b). Their exudates contain lytic components that degrade the cell wall of gram-positive bacteria, as well as antimicrobial peptides that inhibit the growth of bacteria, yeasts, and fungi (Cotter & Kilner, 2010b; Cotter et al., 2010; Hall et al., 2011; Hoback et al., 2004; Steiger et al., 2011). Care behaviours after pathogen exposure have been studied, yet the effects of *Nicrophorus*'—and other subsocial species'—social immune defence against dedicated pathogens remain largely unknown (Ratz et al., 2021).

Entomopathogenic fungi, such as *Beauveria bassiana*, have evolved many mechanisms to breach the cuticle, the first layer of defence against pathogens, and take advantage of the nutrient-rich haemocoel of insects after infecting their hosts (Ortiz-Urquiza & Keyhani, 2013). This poses a constant threat for insects, considering that only a small number of antimicrobial peptides show antifungal activities (Ekengren & Hultmark, 1999; Xiao et al., 2012; Xu et al., 2012). *Beauveria bassiana* is an entomopathogenic generalist and one of the most commercially used mycoinsecticides, causing the "white muscarine disease" (Faria & Wraight, 2007; Loreto & Hughes, 2016; Mascarin & Jaronski, 2016; Rehner et al., 2011). Its hydrophobic conidia bind to the cuticle of insect, force their way into the haemocoel of insects through proteases and chitinases and, once inside, they avoid their hosts' immune system (Fang et al., 2009; Holder et al., 2007; Pendland et al., 1993). We decided to exploit *B. bassiana*'s generalist way of infection to challenge the immune system of *N. vespilloides* offspring and investigate how post-hatching care affects offspring after pathogen exposure in a species with facultative family life and potent social immunity. Specifically, we tested how pathogen exposure affects offspring performance during *Nicrophorus* family life, how it shapes offspring immunity long-term, and to what degree these effects depend on post-hatching care.

To this end, we exposed recently hatched larvae of *N. vespilloides* to either spores of *B. bassiana* or a control solution and let them develop with or without post-hatching care. We periodically checked their performance during the crucial early stages of larval development and at the end of the family life (and post-hatching care) period. Additionally, we investigated long-term effects of our treatments by measuring immunity parameters of the resulting adult offspring.

Materials and methods

Beetle husbandry

Nicrophorus vespilloides beetles (Figure 1) used in this experiment were 13th-generation descendants of beetles originally collected near Bayreuth, Germany (49°5510.8″ N 11°3422.0″ E). Beetles were kept under standard laboratory conditions in a climate chamber (20 °C, 16:8 light–dark rhythm) with up to five same-sex siblings in one box (10 × 10 × 6 cm). They were fed twice a week with thawed fly larvae (*Lucilla sericata*), with three to four larvae per beetle. To prevent inbreeding the population was regularly infused with fresh-caught beetles and breeding pairs were chosen to achieve optimal outbreeding using Kinshipper (www.kinshipper.com, Bayreuth).

Spore harvest

To harvest the *B. bassiana* spores, we filled a sterile glass funnel with sterile glass wool (ca. 70 × 70 × 2 mm) and moistened it with 0.5% Tween20. The funnel was placed on top of an Erlenmeyer beaker. *Beauveria bassiana* (Balsamo) Vuillemin strain was isolated from *Liophloeus tessalatus* and has been acquired through DSMZ (DSM 62075; DSMZ, Braunschweig). Spores were grown on sealed Petri dishes containing Agar media. Petri dishes were incubated at 25 °C for 12 days until they were completely covered by the fungus. They were then flooded with 15 ml 0.5% Tween20 and carefully shaken. The spores were then carefully scrapped off with a spatula and were then suspended in the Tween20 solution, together with any mycelium that was also scrapped off. The spore solution was then filled into the funnel. The process was

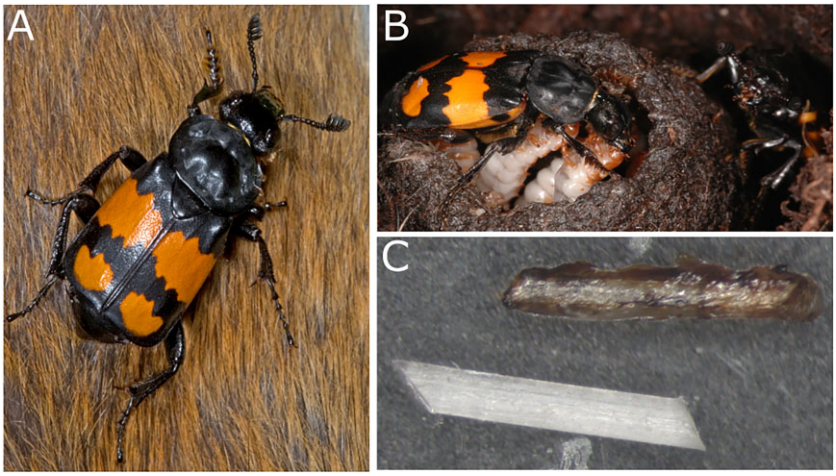

Figure 1. (A) A single *Nicrophorus vespilloides* on an animal carcass. (B) A pair of breeding *N. vespilloides* beetles surrounding the feeding cavity of the prepared animal carcass, with their offspring inside. (C) Comparison between an unused nylon filament (below) and a nylon filament that was recovered from a beetle's body after implantation a day prior. Images for (A) and (B) used with permission from Heiko Bellmann.

repeated before the filtrate was collected and vortexed. This filtration is designed to minimize any residual mycelia in the beetle nursery to expose larvae to spores only. Finally, spore concentration was determined with a disposable hemacytometer (Neubauer Improved, Nanoentek) under a microscope.

Experimental design

To investigate whether exposure to *B. bassiana* affects the larval development of *N. vespilloides*, and to what degree this effect depends on post-hatching care (i.e., the presence of a caring female), we used a 2 × 2 full-factorial design with a total of 60 *N. vespilloides* females (Figure 2). After measuring their pronotum width using a calliper, each female was placed in a plastic box (10 × 10 × 6 cm) with moist coconut peat and a mouse carcass (20.69 ± 1.29 g; www.tropic-shop.de). Breeding pairs remained under light for 3 hr to facilitate carcass burial and were then transferred to a dark climate chamber at 20 °C, where male and female then prepared the carcass for the arrival of the larvae.

Following oviposition and carcass preparation, carcass and females were transferred to a new box with fresh coconut peat, while we discarded the male and kept the original box with laid eggs. Oviposition and embryonic development, until eggs are hatching, takes approximately 3 days (Potticary et al., 2024; Smiseth et al., 2006). *Nicrophorus* females, as many other insects, possess spermathecae and lay their eggs asynchronously over an extended period (on average over 27 hr [Müller, 1987]), resulting in oviposition well after the initial mating event (up to 54 hr), while the stored sperm is fertile up to 3 weeks (Eggert, 1992; House et al., 2007). To separate later laid eggs from the carcass, we moved females and carcass each morning, over a period of 2 days. Females do not continue laying eggs during post-hatching care, as the presence of larvae supresses additional egg laying (Engel et al., 2016). Delayed oviposition after 2 days of carcass transferal never occurred in our study, as freshly hatched larvae would have been easy to distinguish based on size differences and developmental status.

Two days after eggs were laid in the original box, boxes were checked every 2 hr for emerging larvae, which were then collected, placed in a Petri dish containing moist filter paper, and kept at 4 °C to slow larval activity and desiccation. All larvae for this experiment were collected over a period of 6–8 hr, with a maximum age difference of 3 hr at the start. Pooling larvae this way excludes any genetic effect in the later analysis and allows standardization of brood size (the number of larvae a female has to care for). Under natural circumstances, larvae of a single female hatch asynchronously and arrive at the carcass over time, pooling ensure a much closer age at the start than natural circumstance would. Pooling of offspring is a common method in species with no evidence for kin recognition among parents and their offspring, e.g., *Forficula auricularia* (Kramer et al., 2015) and also the burying beetles (Capodeanu-Nägler et al., 2018; Rebar et al., 2020; Smiseth et al., 2003). Furthermore, we cannot rule out that the brief refrigeration period during larval pooling—necessary to halt the larvae's development—prime their immune system, increasing their pathogen resistance.

To ensure larvae from different clutches were thoroughly mixed during our pooling, we collected an excess of larvae (i.e., at least 20). Then, 10 pooled larvae were placed on one carcass and were assigned to one of four treatments, manipulating both post-hatching care (female presence) and pathogen exposure: (a) post-hatching care and exposed to pathogens ($n = 15$), (b) post-hatching care and exposed to control solution ($n = 15$), (c) no post-hatching care and exposed to pathogens ($n = 15$), and (d) no post-hatching care and exposed to control solution ($n = 15$). After pupation, we selected four adult beetles, two male and two female, from each clutch of adult offspring to subject to our immune assays.

Larval exposure and treatment setup

Post-hatching care was manipulated either by removing the female from the prepared carcass or by letting her stay with her prepared carcass, before adding the larvae. This ensured that all larvae fed on carcasses that were prepared by a breeding pair of burying beetles, ensuring that the carcass microbiome is not an additional threat to the developing larvae during the first few days of their live. *Nicrophorus vespilloides* broods are sometimes left with no post-hatching care, although that happens rarely (Müller et al., 2007).

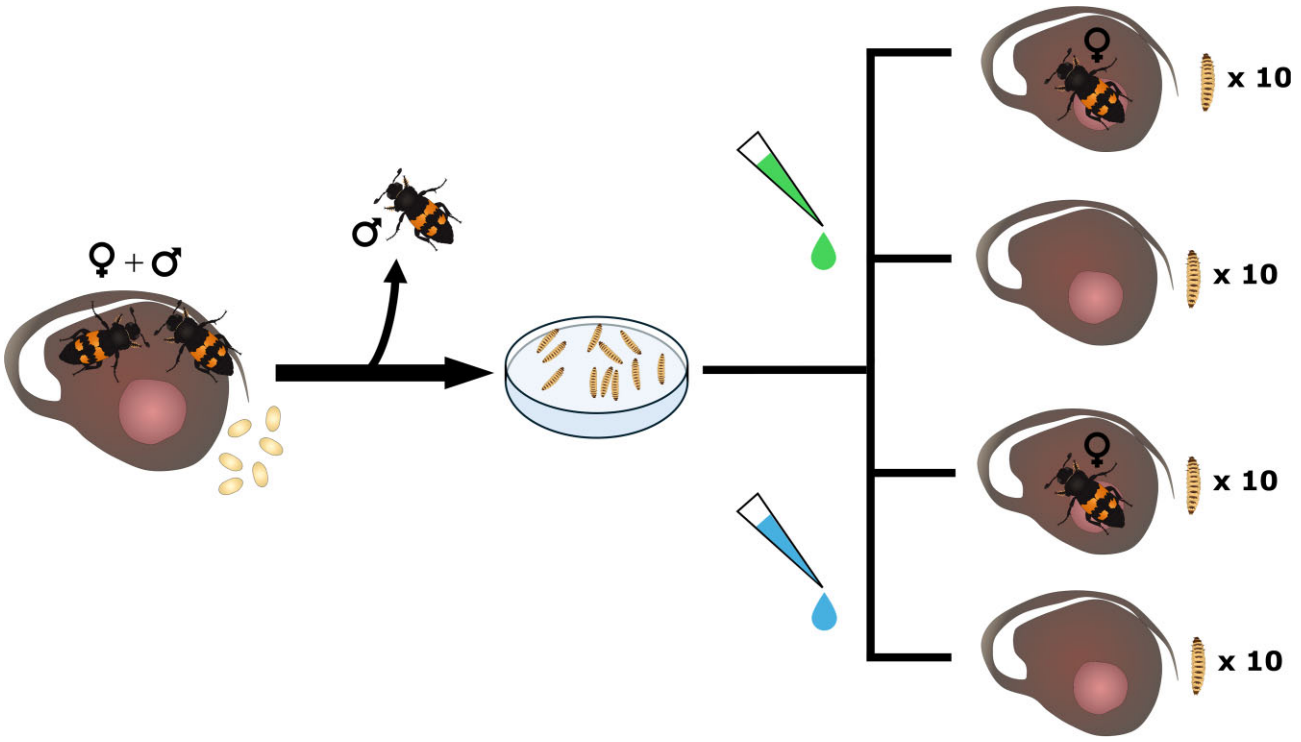

Figure 2. Visual representation of our experimental workflow, indicating how larvae were collected and to which treatments they were subjected to. Green (above) = exposure to *Beauveria bassiana* spores. Blue (below) = exposure to the control solution.

Pathogen exposure was manipulated by submerging each larva into either 0.5% Tween20 solution with 1×10^7 *B. bassiana* spores/ml (pathogen exposure) or 0.5% Tween20 solution with no spores (control). Given the lack of information on fungal pathogen effects on *Nicrophorus*, we chose this concentration to start out investigating *B. bassiana*/*Nicrophorus* interactions on the lower end of the concentration and mortality spectrum of the fungus (Kaay & Hassan, 2000; Wilson et al., 2001).

Larvae were only added to females whose eggs had already hatched to prevent infanticide (Eggert & Müller, 2011). Female *Nicrophorus* beetles do not distinguish between foster offspring and genetically related offspring, if the larvae arrive at the carcass during the correct time (Müller & Eggert, 1990; Rauter & Moore, 2002; Trumbo & Wilson, 1993). In treatments with no female, we created an artificial feeding cavity by cutting through the first layers of skin (~0.5–1 cm deep) until the flesh was visible (Prang et al., 2022). Ultimately, each treatment group consisted of 15 samples total. Carcass weights did not differ between treatments (Kruskal–Wallis, $\chi^2 = 4.045$, $df = 3$, p-value = 0.256). By standardizing carcass and brood size to a relatively large carcass for the number of larvae, we avoided *Nicrophorus* offspring competing for carrion food.

We counted and weighed larvae (and females, if applicable) at 24 and 48 hr after larvae were added to a carcass, and upon larval dispersal, using a precision scale (KERN, ABJ120-4NM). Dispersal is easily recognized in *Nicrophorus* since at this point larvae abandon their aggregation at the mostly or entirely consumed carcass and look to burrow around the edges of the box. Upon dispersal (after 8 ± 2 days) surviving larvae were placed into a soft plastic box (11 × 11 × 6 cm) with compacted coconut peat to pupate (24 hr dark, 20 °C). After 19–21 days, adult beetles eclosed and emerged from the coconut peat. We marked time of eclosion when at least one beetle emerged from the peat. Eclosed beetles were then collected and sexed before placing them in new boxes (10 × 10 × 6 cm) with at most five same-sex siblings. If possible, we avoided to put single beetles into a box to avoid possible effects of total isolation on individual beetles. Beetles were then given food ad libitum and the time to fully melanise over the next 3 days. We then selected one beetle per sex per family for the 24-hr encapsulation assay (see later). A day later, we again selected one beetle from each sex per family to extract haemolymph for immune analyses. If only one beetle of a sex eclosed in a treatment brood, we prioritized the haemolymph extraction over the encapsulation assay. After extracting haemolymph, all beetles were freeze-killed at −20°C before measuring their pronotum width.

Offspring performance

To investigate the offspring performance under our treatment conditions, we counted the number of surviving larvae at 24 hr, 48 hr, and after dispersal, weighing all larvae from each brood simultaneously. By weighing all larvae, we measured the total brood weight of all surviving larvae from the 10 that were added to the carcass at the beginning of the experiment. The total brood weight was then used to calculate the mean larval weight by diving the total brood weight through the number of surviving larvae at the time point in question. We then used the mean larval weight to calculate the relative weight gain, from now on referred to as growth rate (see the *Statistical analysis* section). We recoded the time between start of exposure treatment and dispersal for each brood in days. After eclosion, the emerged beetles were counted again and their pronotum size after complete melanisation was measured as a proxy for body size and an indicator of lifetime re-

productive success (Scott, 1997; Steiger et al., 2012). From all 60 treatment broods, 3 failed to survive until dispersal.

Measurements of immunity

Encapsulation rate

To measure the encapsulation rate of our treated *N. vespilloides* beetles, we first anaesthetized each beetle with CO_2 before puncturing the cuticle between the third and fourth abdominal segments with a sterile syringe needle (Ø 0.6 mm). Into the wound, we inserted a 2-mm-long nylon filament (Shakespeare, Omniflex), which was roughened beforehand with sandpaper. After the implantation, single beetles were placed in new boxes containing a thin layer of soil. One day later, the beetles were freeze-killed at −20 °C and stored for later extraction of the filament. To measure the encapsulation rate, the nylon filament was recovered from the frozen beetles using Dumont tweezers. Recovered filaments were placed on glass slide together with a control filament (unimplanted) and photographed under a stereo microscope (Stemi 508, Carl Zeiss Microscopy GmbH; Figure 1C). We then measured the mean grey value of each filament using ImageJ (Schneider et al., 2012). The encapsulation rate was then calculated by subtracting the grey value of the control from the melanized filaments (Steiger et al., 2011).

Haemolymph extraction

To obtain haemolymph for measuring phenoloxidase (PO) activity and lytic activity, beetles were anaesthetised with CO_2 and haemolymph was extracted by piercing the cuticle between pro- and mesothorax beneath the pronotum on the ventral side of the beetle using a sterile needle. Emerging haemolymph formed a droplet on the cuticle and was collected using a 5-µl glass capillary (Hirschmann, Ringcaps). Extracted haemolymph was then split up into two samples and each was diluted 1:10 with either PBS (Phosphate-Buffered Saline) or 5 mM MOPS (3-(N-Morpholino)-propane sulphonic acid) buffer with protease inhibitor, for use in the lytic activity assay or PO activity assay, respectively. Samples were immediately stored at −80 °C until use. Due to insufficient extracted haemolymph volumes or capillaries blocked by rapid coagulation, we were not able to sample each beetle and had to exclude 65 out of 240 samples from further analysis.

PO activity assay

To measure the PO activity, we added 10 µl of MOPS-diluted haemolymph sample together with 10 µl of MOPS buffer (5 mM, no protease inhibitor) and 10 µl of distilled H_2O to a well of a 96-well plate. Next, we added 180 µl of L-DOPA (l-3,4-dihydroxyphenylalanine) solution (39.4 mg of L-DOPA in 20 ml of 5 mM MOPS buffer, without protease inhibitor) in two steps of 90 µl, using the second one to mix the samples by pipetting up and down. Because L-DOPA is difficult to dissolve, the solution was first vortexed for 15 min and then placed in an ultrasonic bath for 3 min before sterile filtering it. L-DOPA solution was freshly made for each 96-well plate. The PO activity of a sample was assessed by measuring increasing light adsorption of the melanized substrate at 490 nm for 2 hr every 2 min in a microplate reader (Synergy H1 Microplatereader, Biotek). During analysis, we excluded sample curves with an R^2 (goodness of fit) value of less than 0.8.

Lytic activity assay

To measure the lytic activity of the haemolymph, we added 10 µl of a diluted haemolymph sample to a well of a 96-well plate. We then added 90 µl of a *Micrococcus luteus* bacteria solution (3 mg of freeze-dried *M. luteus* in 10 ml of PBS), which was vortexed beforehand. Solutions in each well were mixed by carefully pipetting up and down. The well plate was then placed in a plate reader, at 600 nm for 2 hr, with reads every 2 min, to measure the optical density of the samples. We excluded sample curves with an R^2 value of less than 0.8 from further analysis.

Statistical analysis

Data analysis was conducted in RStudio version 2024.12.0+467 (Posit team, 2024) using R version 4.3.3 (R Core Team, 2024) loaded with packages, *interactions* 1.2.0 (Long, 2024), *DHARMa* 0.4.6 (Hartig, 2022), *lme4* 1.1-35.3 (Bates et al., 2015), and *car* 3.1-2 (Fox & Weisberg, 2019). Data visualization was done using *ggplot2* 3.5.1 (Wickham, 2016), RColorbrewer 1.1-3 (Neuwirth, 2022), and interact_plot from *interactions*. Data organization aids were *tidyr* 1.3.1 and *dplyr* 1.1.4 (Wickham et al., 2023, 2024). To test the effects of our treatments on larval growth rate, larval survival, mean larval weight, total brood weight, dispersal time, and mean pronotum width after eclosion, we used generalized linear models (GLMs) (offspring performance parameter ~ post-hatching care treatment × pathogen exposure). To ensure comparability, we excluded total brood weight and survival data if the surviving larvae were miscounted, e.g., if at a later point we found more than at a previous one (i.e., more larvae at 48 hr than at 24 hr). Growth rate of larvae was calculated using the formula $(lm_X - lm_0) \div lm_0$, where lm_X is the mean larval weight of the time points in question (24 hr, 48 hr, and dispersal) and lm_0 is the mean larval weight before subjecting the larvae to their treatments. Dispersal time was analysed after log-transforming data to ensure homoscedasticity and Gaussian distribution. For larval survival, we employed a Poisson distribution corrected for overdispersion. GLMs for larval growth rate, mean larval weight, total brood weight, and mean pronotum width were all fit using Gaussian distribution.

PO activity, lytic activity, and encapsulation rate were analysed using generalized linear mixed-effect models or GLMers with the interaction of our two treatments, adding offspring ID as a random effect, as one male and one female of each family were used [immune parameter ~ post-hatching care treatment × pathogen exposure + (1|ID)]. PO values were transformed using log(PO) + 0.001 in the model to ensure homoscedasticity and Gaussian distribution. We tested whether offspring sex influences immune parameter by fitting a GLMer with sex as a predictor and offspring ID as a random effect [immune parameter ~ offspring sex + (1|ID)]. Models for lytic activity and encapsulation rate were also fitted using Gaussian distribution.

Following this, we used a GLM to test whether female weight change, over the period of post-hatching care, can be explained by the pathogen treatment (female weight change ~ pathogen exposure). Additionally, we tested wheher larval performance parameters can be predicted by the interaction term of female weight change and pathogen exposure treatment (offspring performance parameter ~ female weight change × pathogen exposure). These two models were fitted

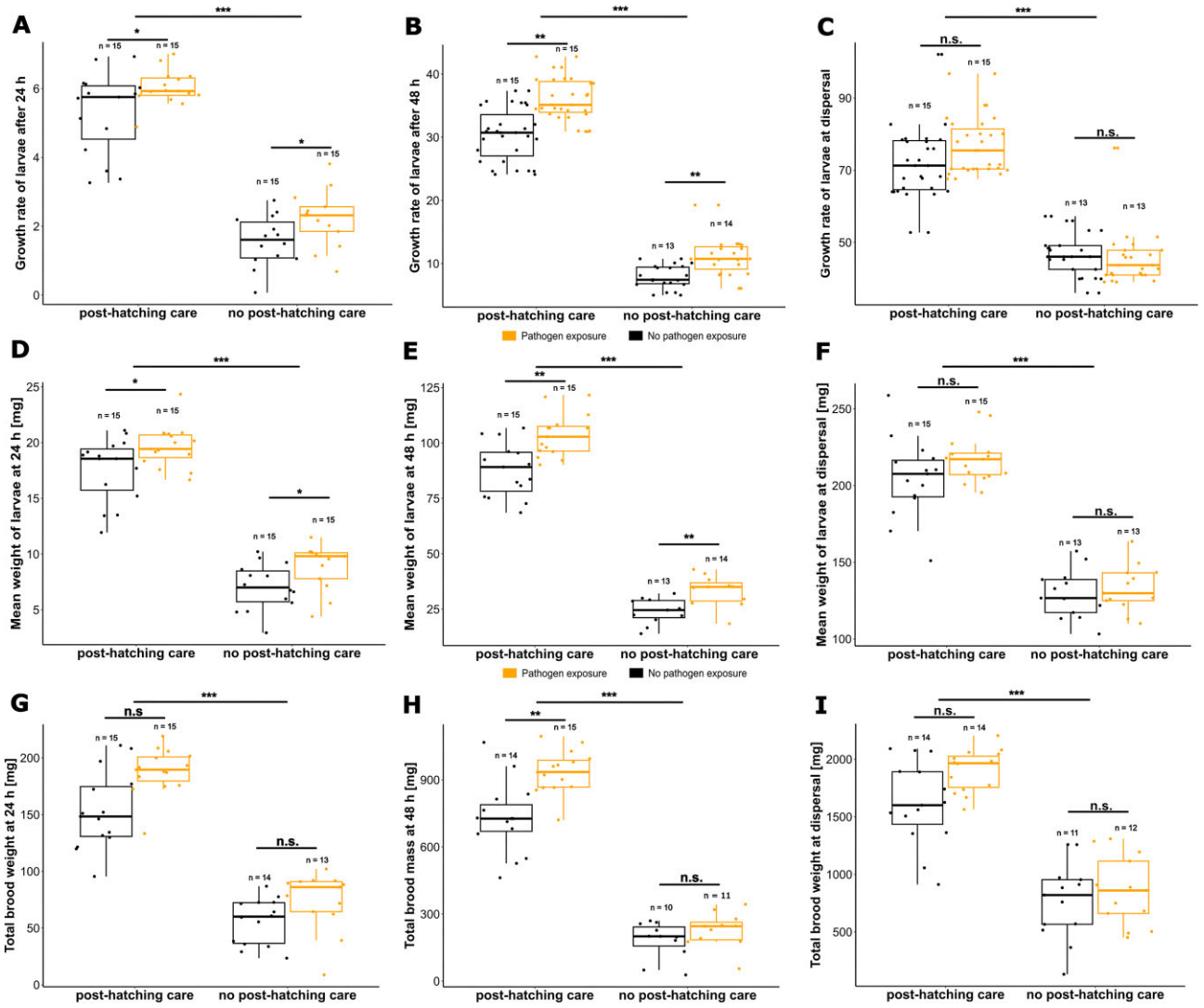

Figure 3. Boxplots of the larval growth, total brood weight, and mean larval weight of *Nicrophorus vespilloides* offspring at different developmental stages, dependent on whether they were exposed to a *Beauveria bassiana* spore solution or the control solution. First row shows the larval growth rate at 24 hr (A), 48 hr (B), and at carcass dispersal (C). Second row shows the mean larval weight at 24 hr (D), 48 hr (E), and at carcass dispersal (F). Third row shows the total brood weight at 24 hr (G), 48 hr (H), and at carcass dispersal (I). Boxplots show median (horizontal line), 25% and 75% quartile (boxes), and 1.5 × interquartile range (whiskers). Statistical differences are indicated by asterisks.

using Gaussian distribution and used only the subset of data where females are present (post-hatching care). The weight change of females during the post-hatching care was calculated similarly to the relative growth rate, where fm_X is the weight of a female of the time point in question (24 hr, 48 hr, and dispersal) and fm_0 is the female's weight before subjecting the larvae to their treatments.

Results

Offspring performance—growth rate and survival

The larval growth rate and mean larval weight at 24 and 48 hr were increased in larvae exposed to the pathogen compared to larvae exposed to the control solution (Figure 3A–D and Table 1) and overall higher in larvae with post-hatching care compared to larvae without post-hatching care (Figure 3A–D and Table 1). Larval growth rate and mean larval weight at 24 and 48 hr were not affected by the interaction of both treatments at these time points (Table 1). At dispersal, larval growth rate and mean larval weight were influenced only by the post-hatching care treatment but not by pathogen exposure or their interaction. Larvae that received post-hatching care showed an increased growth rate and an increased mean larval weight (Figure 3C and F and Table 1).

Total brood weight of larval offspring at 24 hr was influenced only by post-hatching care, with broods receiving post-hatching care showing higher weight (Figure 3G and Table 1). At 48 hr, total brood weight was overall higher in larvae with post-hatching care (Figure 3H and Table 1). In addition, total brood weight at 48 hr was higher in pathogen-exposed larvae, but only in those that had received post-hatching care (Figure 3H and Table 1). At dispersal, total brood weight was higher in larvae that received post-hatching care compared to those without (Figure 3I and Table 1). Finally, we found that the caring females' weight change during post-hatching

Table 1. Effect of pathogen exposure and post-hatching care on larval performance parameters, survival, and dispersal time of *Nicrophorus vespilloides* larvae.

	Larval growth rate 24 hr			Larval growth rate 48 hr			Larval growth rate dispersal		
Predictors	χ^2	*df*	*p*-value	χ^2	*df*	*p*-value	χ^2	*df*	*p*-value
Post-hatching care	149.638	1	**<0.001**	318.50	1	**<0.001**	53.980	1	**<0.001**
pathogen exposure	4.452	1	**0.035**	7.01	1	**0.008**	0.002	1	0.965
Interaction	0.020	1	0.883	1.60	1	0.206	0.940	1	0.332
	Mean larval weight 24 hr			**Mean larval weight 48 hr**			**Mean larval weight dispersal**		
Post-hatching care	171.126	1	**<0.001**	351.77	1	**<0.001**	114.453	1	**<0.001**
Pathogen exposure	5.823	1	**0.016**	8.32	1	**0.004**	0.265	1	0.607
Interaction	0.009	1	0.924	1.38	1	0.240	0.761	1	0.383
	Total brood weight 24 hr			**Total brood weight 48 hr**			**Total brood weight dispersal**		
Post-hatching care	103.312	1	**<0.001**	131.80	1	**<0.001**	41.175	1	**<0.001**
Pathogen exposure	3.461	1	0.063	0.691	1	0.406	0.128	1	0.720
Interaction	1.511	1	0.219	6.949	1	**0.008**	2.175	1	0.140
	Survival 24 hr			**Survival 48 hr**			**Survival dispersal**		
Post-hatching care	2.836	1	0.092	5.158	1	**0.023**	6.697	1	**0.010**
Pathogen exposure	0.173	1	0.677	0.008	1	0.929	0.008	1	0.929
Interaction	0.573	1	0.449	0.160	1	0.689	0.583	1	0.445
	Survival eclosion			**Time of dispersal**					
Post-hatching care	7.164	1	**0.007**	43.296	1	**<0.001**			
Pathogen exposure	0.221	1	0.638	0.186	1	0.666			
Interaction	2.308	1	0.129	0.575	1	0.448			

Note. Significant *p*-values are highlighted in bold.

care had no effect on offspring performance (Figure S1 and Table S1).

Larvae dispersed later from the carcass if they received post-hatching care, but dispersal time was unaffected by pathogen exposure or an interaction of both treatments (Figure 4A and Table 1). Offspring survival was not influenced by either treatment at 24 hr (Figure 4B and Table 1). At 48 hr, dispersal and after eclosion, survival was increased by post-hatching care (Figure 4B and Table 1).

Increasing the concentration of the spore solution did not alter our findings regarding the survival of pathogen-exposed larvae without post-hatching care (Figure S2 and Table S2). During analysis, we excluded a particular outlier that appeared 1.5 times heavier at 48 hr, but not before or after. Exclusion of this outlier revealed a reduced growth rate of pathogen-exposed larvae at 48 hr compared to larvae exposed to the control solution (Figure S2 and Table S2); without exclusion there is no effect on growth rate. Other than this, high concentration pathogen exposure did not affect offspring performance parameters (Figure S2 and Table S2).

Adult offspring performance and immunity

After eclosion, the pronotum width of the now adult offspring was positively influenced by post-hatching care while being unaffected by pathogen exposure or their interaction (Figure 5D and Table 2). Adult offspring immunity parameters were not affected by sex (Table 2); hence, sex was not included in further analyses. Encapsulation rate, PO activity, and lytic activity were not affected by either treatment or their interaction (Figure 5A–C and Table 2).

Pathogen effect on caring female

During the first 24 hr of post-hatching care, female parent weight change was not affected by pathogen exposure to larvae (Figure 6A and Table 3), but after 48 hr and at dispersal, females lost less weight if they cared for larvae that were exposed to the pathogen (Figure 6B and C and Table 3).

Discussion

As individuals form groups and the proximity to other individuals increases, parasites and pathogens can be easily transferred from host to host (Cremer et al., 2007; Godfrey et al., 2006; Pie et al., 2004). While there is evidence of costs of infection and immunity (Brown et al., 2019; Cotter et al., 2004) rising with larger group sizes, social species have evolved different strategies to combat the spread of pathogens in a group, which may alleviate or outright remove these costs (Elliot & Hart, 2010; Wilson et al., 2003). In our study, we addressed the question whether and how post-hatching care in a species with extensive social immunity abilities, showcased by control of the carcass-associated microbiome through their antimicrobial exudates (Körner et al., 2023), can alleviate the consequences of a possible pathogen infection by exposing freshly hatched *N. vespilloides* larvae to spores of the entomopathogenic fungus *B. bassiana*.

Curiously, larvae that were exposed to *B. bassiana* spores showed an increase in growth rate and mean larval weight during their first 48 hr, regardless of the post-hatching care treatment. We are not able to detect any weight differences at the time of dispersal, indicating that the increased growth rate does not persist through all stages. At the same time, their survival was not affected by the presence of the pathogen, although it is a well-known entomopathogenic fungus, able to infect various hosts, including beetles (Mascarin & Jaronski, 2016). Note that we did not control for actual *B. bassiana* infection of larvae, i.e., surface microscopy of cuticle or incubation of dead larvae following pathogen exposure. *Nicrophorus* exudates exhibit antifungal and antibacterial properties (Hwang & Lin, 2013; Rozen et al., 2008; Suzuki, 2001), while larval secretions show the highest antimicrobial

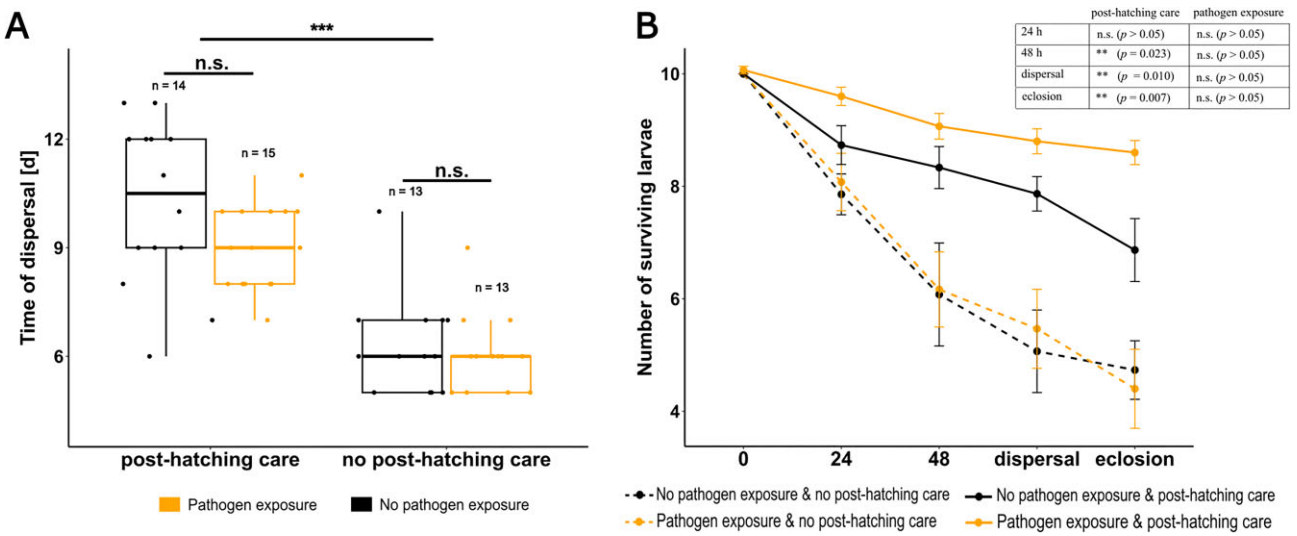

Figure 4. Influence of the treatments on the time *Nicrophorus vespilloides* offspring dispersed from the carcass as well as on their survival till eclosion. (A) Dispersal time of *Nicrophorus* larvae after they were subjected to our treatments. Boxplots show median (horizontal line), 25% and 75% quartile (boxes), and 1.5 × interquartile range (whiskers). Statistical differences are indicated by asterisks. (B) Mean number of surviving *Nicrophorus* larvae at different developmental stages after they were subjected to the different treatment conditions. Whiskers show standard error. For statistical differences, see the included table, as well as Table 1 for additional statistical information.

activity shortly after their arrival at the carcass (Arce et al., 2013), which coincides with the time we exposed them to the fungal spores. Burying beetles change and control the carcass microbiome while inoculating the nursery with their own gut microbiota, thus transferring beneficial bacteria to their offspring (Duarte et al., 2018; Körner et al., 2023; Miller et al., 2019). Beauvericin and oosporein, among other secondary metabolites of *B. bassiana*, show antimicrobial and insecticidal properties (Alurappa et al., 2014; Castlebury et al., 1999; Sood et al., 2017); hence, they might prove beneficial in reducing the overall microbial load of harmful bacteria on the freshly hatched larvae, thus accelerating their growth. Additionally, oosporein has been shown to cause increased feeding behaviour in *Hylobius abietis* and increased expression of a gustatory receptor (McNamara et al., 2019). In their study, McNamara et al. (2019) postulate that the increased feeding after the oosporein treatment reflects an increased energy demand due to the activation of an immune response. It stands to reason that the presence of *B. bassiana* in the carcass nursery of *N. vespilloides* might alter the microbial environment to the benefits of larvae, potentially also influencing their feeding behaviour through oosporein, which affects the larvae's immune system, causing the larvae to meet an increased energy demand by feeding on the carcass more frequently during the first 48 hr of their life.

These first 48 hr represent a critical stage of development for the *Nicrophorus* larvae (Eggert et al., 1998). Hence, an alternative hypothesis to explain the growth acceleration after pathogen exposure is that larvae might try to escape the precarious environmental conditions (e.g., pathogen prevalence) through rapidly developing towards a more resilient stage at which they are less reliant on the social immunity of their parent in fending off the pathogen threat. A similar effect has been shown in *Tribolium castaneum*, where an immune challenge accelerates their development, while frogs enter metamorphosis earlier if environmental conditions are declining (Day & Rowe, 2002; Morey & Reznick, 2000; Roth & Kurtz, 2008; Wilbur & Collins, 1973). Systemic growth and immune response are both energy sinks, resulting in a trade-off between the two processes (Ahmed et al., 2002; Bonneaud et al., 2003). In insects, this trade-off is mediated by the fat body, an organ that stores energy in form of fatty acids and synthesizes haemolymph proteins (Arrese & Soulages, 2010; Delanoue et al., 2010). Mobilization and reallocation of resources to the immune response are initiated by the Toll pathway, as it suppresses insulin signalling in the fat body, stunting growth and decreasing the organism's body weight (Clark et al., 2013; DiAngelo et al., 2009). Upon an immune challenge, production of antimicrobial proteins and haemolymph proteins increases, with the metabolic rate increasing as well (Ardia et al., 2012; Freitak et al., 2003; Han et al., 1999), while the longevity of the organism is decreased after an immune challenge (Armitage et al., 2003). Vice versa, an increase in systemic growth can lead to a reduced immune response, as more resources are used to grow the organism body (Krams et al., 2015). This increased growth stems from elevated metabolic activity, producing more reactive oxygen species (ROS), which damage cells, reduce reproductive success, and negatively impact the organism's lifespan (Monaghan, 2008; Monaghan et al., 2009; Tarry-Adkins et al., 2009), as growth and resistance to ROS appear to be negatively correlated (Kim et al., 2011). ROS are a part of the insect's immune response in the midgut. Their production is induced upon an immune challenge, while the homeostasis of redox balance is controlled by enzymes that can be influenced by *B. bassiana*'s oosporein, causing a reduction of active ROS in the midgut, leading to a subsequent dysregulation of gut immunity and the gut-associated microbiome (Brown et al., 2021; Feng et al., 2015; Ha et al., 2005a, 2005b). Under normal circumstances, the costs of an increased ROS production through enhanced growth under pathogen exposure might be too costly, as larvae under control conditions do not exhibit this additional increase. While this mechanism may explain why larvae would undergo a costly process of accelerated growth, this response behaviour presupposes that *B. bassiana* is indeed a potential threat towards the larvae. Furthermore, there should be costs associated with

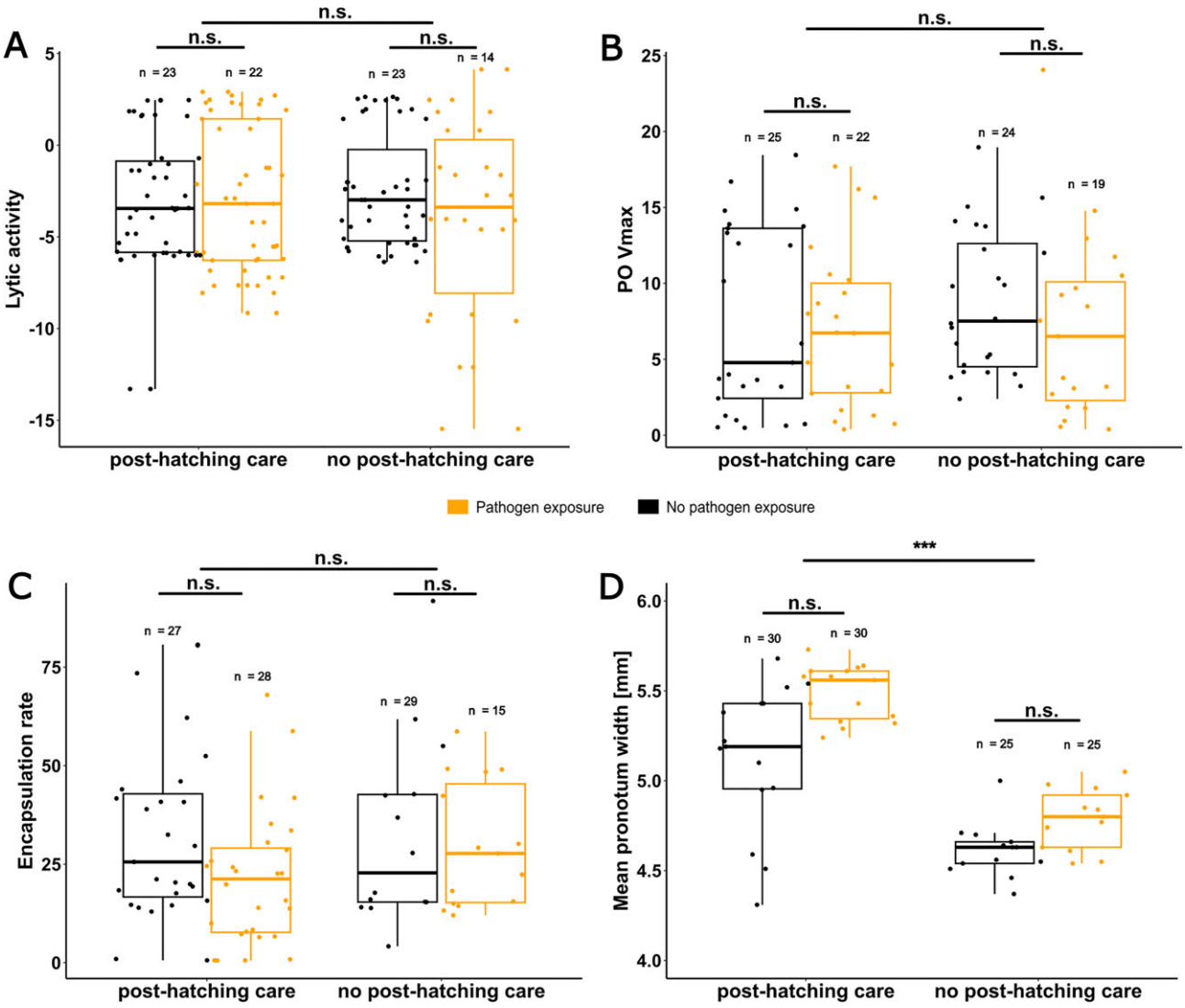

Figure 5. Influence of our treatments on immune parameters and eclosion size of adult *Nicrophorus vespilloides* beetles. (A) Mean pronotum width of adult offspring in (millimetre) after eclosion. (B) Encapsulation rate of *Nicrophorus* beetles after they were subjected to our treatments. (C) Phenoloxidase activity in haemolymph samples of adult *Nicrophorus* beetles after they were subjected to our treatments. (D) Lytic activity in haemolymph samples of adult *Nicrophorus* beetles after they were subjected to our treatments. Boxplots show median (horizontal line), 25% and 75% quartile (boxes), and 1.5 × interquartile range (whiskers). Statistical differences are indicated by asterisks.

increased growth—otherwise, larvae would always take advantage of faster growth, given the ephemeral nature of the resource (Körner et al., 2023).

Since we found no differences in survival between pathogen-exposed and control larvae, we tried to ascertain whether exposed larvae suffer long-term costs of the increased growth by subjecting offspring to several immune assays after they eclosed. We found that neither of our treatments influenced the lytic and PO activity of the beetles' haemolymph, nor was the encapsulation rate affected. In *Drosophila*, infection with *B. bassiana* leads to reduced PO activity and reduced lytic activity as the fungus suppresses the host's immune response (Feng et al., 2015; Matskevich et al., 2010; Roxström-Lindquist et al., 2004), while an early-life immune challenge with the fungus *Metarhizium* in the European earwig *F. auricularia* increased offspring mortality and affected immunity in adulthood (Vogelweith et al., 2017). Given that pathogen exposure did not affect any measured immune parameter, it stands to reason that larvae were able to withstand an exposure to *B. bassiana* during early larval development without measurable costs in adult immunity. During development the cuticle undergoes several changes, hardening and changing its chemical composition (Hepburn & Chandler, 1976; Sahm et al., 2024), potentially making it harder for spores to attach themselves to the outer layer. Indeed, in *Tribolium*, antifungal compounds are imbedded into the cuticle, making it necessary to compromise the cuticle surface composition, so that *B. bassiana* is able to infect and kill the host (Akbar et al., 2004; Mannino et al., 2023; Pedrini et al., 2010). *Nicrophorus vespilloides* larvae receive facultative post-hatching care and are thus under selective pressure to endure greater microbial prevalence in absence of caring parents (Capodeanu-Nägler et al., 2016). It has been postulated that social immunity may be an important component of the parent–offspring conflict in *Nicrophorus* families (Ziadie et al., 2019). Future studies should investigate to what degree larvae alter their metabolism and immune investment under heightened microbial pressure in presence or absence of post-hatching care.

Table 2. Effect of pathogen exposure and post-hatching care on immune parameters of adult *Nicrophorus vespilloides* and their mean pronotum width after eclosion.

	PO activity			Lytic activity			Encapsulation rate		
Predictors	χ^2	*df*	*p*-value	χ^2	*df*	*p*-value	χ^2	*df*	*p*-value
Post-hatching care	2.917	1	0.088	0.304	1	0.581	0.003	1	0.961
Pathogen exposure	2.971	1	0.085	0.958	1	0.328	0.107	1	0.744
Interaction	1.681	1	0.195	0.671	1	0.413	0.876	1	0.350
	Mean pronotum width								
Post-hatching care	30.333	1	**<0.001**						
Pathogen exposure	3.216	1	0.073						
Interaction	1.841	1	0.175						
	PO activity			**Lytic activity**			**Encapsulation rate**		
Sex	1.248	1	0.264	0.028	1	0.868	0.716	1	0.397

Note. PO = phenoloxidase. Significant *p*-values are highlighted in bold.

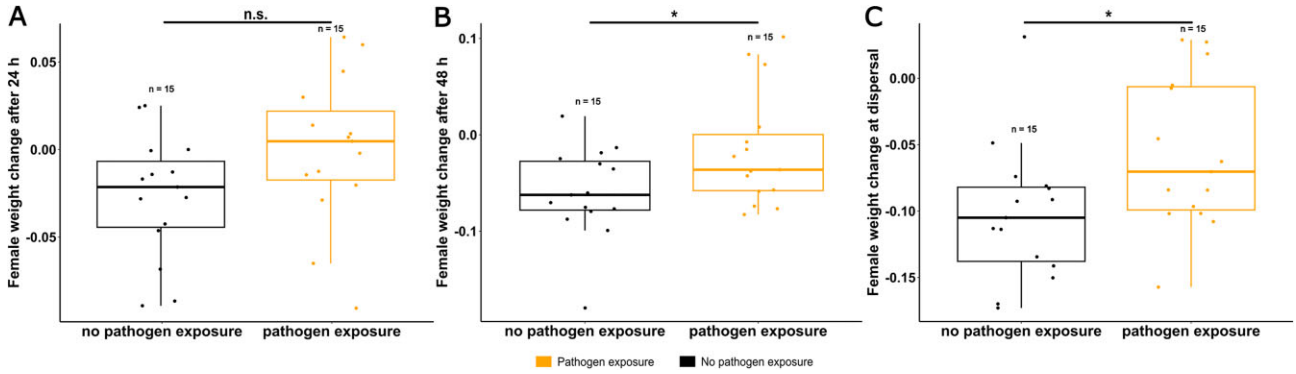

Figure 6. Influence of pathogen exposure on the female weight during post-hatching care. (A) Mean female weight change after 24 hr of post-hatching care. (B) Mean female weight change after 48 hr of post-hatching care. (C) Mean female weight change at dispersal of larvae. Negative values represent a weight loss, whereas positive values a weight gain. Boxplots show median (horizontal line), 25% and 75% quartile (boxes), and 1.5 × interquartile range (whiskers). Statistical differences are indicated by asterisks.

Table 3. Effect of pathogen exposure to larvae on female weight change during the time of post-hatching care in *Nicrophorus vespilloides*.

Female weight change	After 24 hr			After 48 hr			At dispersal		
Predictors	χ^2	*df*	*p*-value	χ^2	*df*	*p*-value	χ^2	*df*	*p*-value
Pathogen exposure	3.643	1	0.056	4.950	1	**0.026**	5.384	1	**0.020**

Note. Significant *p*-values are highlighted in bold.

In our study, we initially used a relatively low concentrated spore solution (1×10^7 spores/ml). As there is no available data on the pathogenicity of *B. bassiana* on *N. vespilloides*, we compared our chosen concentration to those used in similar studies and found reported values ranging from 2.6×10^6 to 1×10^8 spores/ml (Apirajkamol et al., 2025; Chen et al., 2018; Chengxiang et al., 2011; Hou et al., 2014; Vertyporokh et al., 2020). Since we found no effects of the pathogen exposure on survival, we used a higher concentration of 2.33×10^8 spores/ml in a different, independent assay and also found no effects on survival. This suggests that larvae indeed are able to withstand spores of *B. bassiana*. Using the higher concentration, we could not detect the growth rate increasing effects we found in the lower concentration treatment—instead, growth rate was reduced after 48 hr (but not after 24 or 72 hr). This discrepancy might be explained by a dose-dependent effect the spores have on the immune system of the larvae. At lower concentration an escape reaction, like the one we observed in this study, might be feasible as the exposure itself does not represent a lethal threat, if faster growth is less costly than simply enduring the exposure. This might not be possible at higher concentrations, where the threat to larvae is greater and all excess energy must be allocated to immune defence, making growth for escape too costly.

As expected, we found that larvae receiving post-hatching care showed not only a higher survival but also a higher growth rate, as well as a higher mean and total brood weight. These benefits continued through their adulthood after eclosion, reflected by their larger pronotum size (but see Capodeanu-Nägler et al., 2016). Post-hatching care involves

continued carcass maintenance, food provision of larvae with predigested carrion, and protection of the brood and resource (Eggert et al., 1998; Potticary et al., 2024; Scott, 1998). Under laboratory conditions guarding of the carcass is not expected to yield measurable benefits given the lack of invading conspecifics or predators. In the case of *N. vespilloides*, post-hatching care is not obligately required but yields benefits for developing larvae (Capodeanu-Nägler et al., 2016; Eggert et al., 1998). While post-hatching care is known to be often biparental, the female-only post-hatching care in our experiment occurs frequently and performs similarly to biparental care (Potticary et al., 2024; Smiseth & Moore, 2004). Males typically invest less energy into the biparental care (e.g., feed less, desert the carcass earlier, and invest less in social immunity; Cotter & Kilner, 2010b; Müller et al., 2007; Scott & Traniello, 1990; Smiseth & Moore, 2004).

Our results show that the post-hatching care by a female increases offspring survival, while the pathogen exposure had no effect on the survival of the developing larvae. Increased survival under female presence is well known in *N. vespilloides*, as the post-hatching care is beneficial but not necessary to ensure the larvae's survival (Capodeanu-Nägler et al., 2016; Eggert et al., 1998). This effect was present through all stages of larval development and after eclosion. Interestingly, larvae that received post-hatching care dispersed later than those that received no post-hatching care. There are two nonmutually exclusive explanation for this finding. Nematodes, gravesoil bacteria, and their toxic waste products prove detrimental for eggs and larvae; hence, breeding *Nicrophorus* beetles must manage the nursery microbiome very carefully through their exudates to aid their offspring during development (Arce et al., 2012; Jacobs et al., 2014; Körner et al., 2023; Rozen et al., 2008; Shukla et al., 2018a, 2018b; Wang & Rozen, 2019). As larval secretions possess antimicrobial properties themselves, albeit less potent ones compared to adult beetles, we argue that larvae developing on carcasses not continually maintained by a female may have to disperse earlier, unable to supress the proliferation of harmful microorganisms on their own—especially considering the relatively small brood size of 10 larvae provided to each carcass at the start of our experiment, compared to nonstandardized broods (Arce et al., 2013; Prang et al., 2022). Alternatively, larvae may be selected to take maximal advantage of post-hatching care if it is provided, thus delaying their dispersal in favour of prolonged benefits and higher efficiency of growing up with post-hatching care. Note that due to variation in dispersal time across samples, weight at dispersal is conflated with time to dispersal (i.e., larval weight depends on time on carcass). Further experiments are required to fully understand the association of post-hatching care, total development time, and offspring performance. Indeed, our data and past studies show that larvae grow faster and larger when cared for by a female (Capodeanu-Nägler et al., 2016).

During the time of post-hatching care, larvae and their parents closely interact as the mother provisions food by oral trophallaxis as larvae are fed predigested carrion (Potticary et al., 2024). Female weight can be used as an indicator of investment into care, as it is reduced during the time of post-hatching care (Trumbo & Xhihani, 2015; Wang et al., 2021). Female parents in our study overall lost weight during the time of post-hatching care, but this weight loss was reduced if females were caring for larvae that had been exposed to the pathogen as opposed to the control solution. In earwigs, mothers have been shown to adjust their care in response to fungal pathogens (Diehl & Meunier, 2018). *Nicrophorus* females may sense *B. bassiana* spores on the cuticle of the larvae, similarly to how several other insects are known to identify soil, plants, and mycosed insects inoculated with *B. bassiana* spores (Loreto & Hughes, 2016; Meyling & Pell, 2006; Ormond et al., 2011; Rondot & Reineke, 2018). They might then adjust their energy investment, namely reducing their feeding frequency, into the current brood, as they try to balance their future reproductive success with the needs of their present offspring (Haig, 1990). As the females not only feed the larvae but also groom them during post-hatching care (Pukowski, 1933), prolonged close contact between females and their offspring might expose the female to the spores as well. Further studies are needed that take the pathogen transfer between parents and offspring into account while investigating the interplay between pathogen exposure and prevalence of harmful microbes in the nursery with the costs of care in this family system. If pathogens are transmitted, the question remains whether caring females are selected for self-preservation and future reproduction, or whether they would rather terminally invest and stay to care as best they can (as suggested in Ratz et al., 2020, 2021). Our results indicate that females may reduce their efforts towards pathogen-exposed larvae, but unless the mechanism behind increased weight gain of the larvae is determined, it cannot be ruled out to be at work in caring females as well.

Here, we provide first data on the effects of *B. bassiana*, a generalist entomopathogenic fungus, on the offspring of *N. vespilloides*, indicating that early pathogen exposure can increase the development speed of the larvae. We hypothesize that this peculiar effect may be due to secondary metabolites of the pathogenic fungus, or an adaptive response inciting the larvae to hurry through a critical stage in their life cycle. We also found no evidence that this increase in growth rates has long-term consequences for the matured offspring, such as reduced adult immunity or body size. Potential negative long-term effects may be found in reduced fecundity, reduced lifetime, or increased accumulation of ROS, as we have not measured these facets of adult life. Our results also show no increased mortality of *B. bassiana*-exposed larvae, suggesting that *N. vespilloides* is highly resistant against this pathogen. Overall, these findings suggest that in a resource-rich environment, offspring in vulnerable larval stages may be able to accelerate their development at no apparent cost to escape a perceived microbial threat. Our results open interesting questions about the role of the microbiome control and social immunity of parents during post-hatching care, and how larvae respond to pathogens if resources are limited.

Supplementary material

Supplementary material is available at *Journal of Evolutionary Biology* online.

Data availability

All data required to replicate analyses in this manuscript are available for download at Dryad under repository DOI: 10.5061/dryad.7sqv9s54m.

Author contributions

Leon Müller (Data curation [lead], Formal analysis [equal], Investigation [lead], Validation [equal], Visualization [lead], Writing—original draft [lead], Writing—review & editing [equal]), Sandra Steiger (Resources [lead], Supervision [supporting], Writing—review & editing [supporting]), and Maximilian Körner (Conceptualization [lead], Data curation [supporting], Formal analysis [equal], Funding acquisition [lead], Methodology [lead], Project administration [lead], Resources [supporting], Supervision [lead], Validation [equal], Writing—original draft [equal], Writing—review & editing [equal])

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Conflicts of interest

None declared.

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