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Nephridiophagids (Chytridiomycota) reduce the fitness of their host insects

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ABSTRACT

Nephridiophagids are unicellular fungi (Chytridiomycota), which infect the Malpighian tubules of insects. While most life cycle features are known, the effects of these endobionts on their hosts remain poorly understood. Here, we present results on the influence of an infection of the cockroach *Blattella germanica* with *Nephridiophaga blattellae* (Ni = *Nephridiophaga*-infected) on physical, physiological, and reproductive fitness parameters. Since the gut nematode *Blatticola blattae* is a further common parasite of *B. germanica*, we included double infected cockroaches (N + Ni = nematode plus Ni) in selected experiments. Ni individuals had lower fat reserves and showed reduced mobility. The lifespan of adult hosts was only slightly affected in these individuals but significantly shortened when both *Nephridiophaga* and nematodes were present. Ni as well as N + Ni females produced considerably less offspring than parasite-free (P-free) females. Immune parameters such as the number of hemocytes and phenoloxidase activity were barely changed by *Nephridiophaga* and/or nematode infections, while the ability to detoxify pesticides decreased. Quantitative proteomics from hemolymph of P-free, Ni, and N + Ni populations revealed clear differences in the expression profiles. For Ni animals, for example, the down-regulation of fatty acid synthases corroborates our finding of reduced fat reserves. Our study clearly shows that an infection with *Nephridiophaga* (and nematodes) leads to an overall reduced host fitness.

1. Introduction

Parasitism is one of the most common lifestyles on Earth, causing a large proportion of species diversity and biomass (Dobson et al., 2008). Widely distributed in all prokaryotic and eukaryotic kingdoms, a parasitic lifestyle is also frequently observed in a group of phylogenetically early-diverging, zoosporic fungi known as Chytridiomycota or 'chytrids'. Parasitic chytrids have been shown to infect a broad range of host taxa such as algae, plants, amphibians or insects (Fisher et al., 2009; Frenken et al., 2017; Greenberg and Palen, 2019; Ivanić, 1937; Obidiegwu et al., 2014; Ormières and Manier, 1973; Radek et al., 2011). In addition to other parasites such as gut nematodes, chytrids affiliated to the genus *Nephridiophaga* (Nephridiophagales or 'nephridiophagids'; Voigt et al., 2021) are often found in cockroaches and other insects infecting the Malpighian tubules (Fabel et al., 2000; Purrini and Rohde, 1988; Purrini and Weiser, 1990; Radek and Herth, 1999). Different life cycle stages predominantly colonize the lumen of these excretory organs (Woolever, 1966). The life cycle consists of a vegetative merogony phase with multinucleated plasmodia that divide into oligonucleate and uninucleate cells, and a sporogonic phase, in which plasmodia internally form tiny, uninucleate spores (5–10 μm length). Oral uptake of mature spores, which have been released with the faeces, ensures parasite transmission between host individuals (Radek and Herth, 1999; Radek et al., 2002; Woolever, 1966). Poor morphological characteristics have led to several misidentifications of nephridiophagids as Microsporidia or Haplosporidia (e.g., Lange, 1993; Perrin, 1906; Purrini and Weiser, 1990; Sprague, 1970). Their affiliation to the Chytridiomycota has only recently been revealed by molecular phylogenetic analyses (Strassert et al., 2021).

Although nephridiophagids are generally referred to as parasites, knowledge about their impact on the fitness of the host insects is scarce. Different fitness components may be affected, e.g., reproductive success, physical fitness, and physiological fitness parameters such as immunocompetence, functionality of the Malpighian tubules, and susceptibility

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to chemical insecticides. So far, only a reduced activity has been reported as an external sign for diminished physical fitness due to *Nephridiophaga* infection (Purrini and Weiser, 1990). In some host species, infected Malpighian tubules showed hypertrophy or even partial destruction (Ivanić, 1937; Purrini et al., 1988; Purrini and Rohde, 1988; Purrini and Weiser, 1990; Radek and Herth, 1999).

Different hemocyte types in the hemocoel of insects drive cellular immune processes such as phagocytosis or encapsulation of foreign particles. In the cockroach *Blattella germanica*, these functions are mainly achieved by granulocytes (granular cells), which make up to 96% of all hemocytes (Chiang et al., 1988). The immune response of insects also consists of humoral processes that kill pathogens through lysis or melanization using antimicrobial peptides, cytotoxins, and various enzymes (e.g., Ali Mohammadi Kojour et al., 2020; Lavine and Strand, 2002; Rosales, 2017; Siva-Jothy et al., 2005; Tsakas and Marmaras, 2010; Whitten and Ratcliffe, 1999). The latter regulates coagulation or melanization of hemolymph via a complex cascade (Lavine and Strand, 2002). A key enzyme of this cascade is the phenoloxidase, whose conversion from a proenzyme into its active form is induced even by low amounts of for example lipopolysaccharides, peptidoglycan or glucans from microorganisms (Söderhäll and Cerenius, 1998). To what extent nephridiophagids drive any of the described cellular and humoral immune responses is currently completely unknown.

To uncover the potential impact of a *Nephridiophaga* infection on the host insect's fitness, we compared the body weight, fat content, and muscle mass of infected and uninfected *B. germanica* cockroaches. We further investigated the mobility and reproductivity of individuals from these two populations, and — in order to shed light on the immunocompetence — compared the number of hemocytes in a defined volume of hemolymph and phenoloxidase activity. By using insecticides, we also tested a potential reduced detoxification capability of the Malpighian tubules. Finally, we studied physiological differences between infected and non-infected populations using quantitative proteomics. During our studies, we found numerous *Nephridiophaga*-infected individuals to be co-infected with the gut nematode *Blatticola blattae*; a parasite that is prevalent in *B. germanica* populations and is transmitted easily by oral uptake of durable eggs (Adamson and Waerebeke, 1992; Bozeman, 1942; Kobayashi et al., 2021; Tsai and Cahill, 1970). Since co-infecting parasites may facilitate or antagonize each other by different mechanisms such as suppressing the immune system, increasing host tolerance, or competing for space or energy resources (Rovenolt and Tate, 2022), we included these double-infected hosts in some experiments as an additional batch next to the parasite-free and *Nephridiophaga*-infected cockroaches.

2. Material and methods

2.1. Blattella germanica populations

Experiments were conducted with the German cockroach *Blattella germanica*. Colonies of this species can easily be established due to a relatively short reproduction time, and they are regularly and heavily infected with *Nephridiophaga blattellae*. Individuals of *B. germanica* were obtained from the German Environment Agency (UBA). They were cultured at 24 °C in vase-shaped glass containers (~5,300 cm^3) equipped with filter paper under the supply of water, apples, and dry food (64.5% ground dog biscuit [Sniff Spezialdiäten GmbH, Soest] with 32.3% oat flakes and 3.2% dry yeast). Individuals of three different batches (hereafter 'populations') were used. i) Parasite-free (P-free): oothecae were isolated and washed (2 s 70% ethanol, 10 min 5% NaClO, 3 min $Na_2S_2O_3$, 3 times 2 s distilled water) and hatched individuals were cultured in clean containers; the parasite-free status was controlled by light microscopy. ii) *Nephridiophaga*-infected (Ni): as i), but hatched individuals were fed with Malpighian tubules extracted from other individuals that were highly infected with *Nephridiophaga*. iii) Nematodes + Ni (N + Ni): as obtained from the German Environment Agency or unintentionally nematode-infected Ni cultures.

2.2. Physical fitness: Body weight, fat content, and muscle mass

The following procedures are based on the methods described by Marden (1987, 1989). At first, the fresh weight was determined. To determine fat and muscle masses, 20 males and 20 non-ootheca bearing females of each P-free and Ni animals were sacrificed by freezing for 1–2 min at −18 °C. Fat determination: since most body fat is stored in the abdomen, separated abdomens were weighed (fresh weight) and dried for 48 h at 60 °C (dry weight) in Eppendorf tubes. Then, 1.5 ml chloroform ($CHCl_3$) was added to each tube, and closed tubes were shaken for 24 h at room temperature to dissolve the fat. After aspirating the lipid-contaminated chloroform, the body parts were dried again for 24 h at 60 °C (dry weight without fat). The fat content was calculated by subtracting the fat-free dry weight from the dry weight of the abdomen. Muscle weight: the highly muscular thoraxes were used. Fragile parts, which could easily break off, such as wings and fine parts of the legs, were removed before determining the fresh weight (legs were broken off at the femur-tibia joint). The thoraxes (plus femora) were then dried for 48 h at 60 °C and weighed a second time (dry weight) in Eppendorf tubes. 1.5 ml 1.4% NaOH was added to each tube, and closed tubes were shaken for 24 h at room temperature to dissolve the proteins. After sucking off the alkali solution, the empty cuticle envelopes of the thoraxes were dried again for 24 h at 60 °C and weighed (dry weight without muscle). The muscle mass of the thorax was calculated by subtracting the protein-free dry weight from the dry weight of the untreated thorax.

2.3. Physical fitness: Catching trial

Ninety males, 30 each of P-free, Ni, and N + Ni, were placed individually in a plastic container (400 cm^2, with fluon-coated walls), and caught with forceps after briefly nudging each individual 1–2 min after its transfer. Catching times were recorded. In a second trial, 30 males of each P-free and Ni animals were caught and the Malpighian tubules of all Ni individuals were checked for spores under the light microscope. To discriminate between infection intensities, categories were built: low = more than 10 s necessary to find spores; moderate = <10 s necessary to find spores; heavy = few spores seen instantly; and very heavy = numerous spores seen instantly. Dependence between infection intensity and catching time was tested with the Pearson correlation coefficient.

2.4. Function of Malpighian tubules: Sensitivity towards insecticides

Sixty males, 30 each of P-free and N + Ni, were anaesthetized in a beaker on crushed ice. Single individuals were taken out, put on their back and supplied with 2 × 1 µl of 0.5 µg/µl Bendiocarb (Sigma) dissolved in acetone between their forelegs (topical application). The number of dead animals was recorded at 24 h intervals.

2.5. Lifetime and reproductivity

Forty females, 20 each of P-free and Ni, were isolated 0–3 days after the final molt (note, due to limited laboratory access because of the COVID-19 pandemic, cockroaches could be checked twice a week only). Females were then kept separately under identical conditions (24 °C, water and food supply) in a Petri dish together with a single male. In case the male died before the female, it was replaced by a new one. Petri dishes were regularly replaced when mold infestation (due to supplied apple pieces) became too heavy. For each female, the numbers of oothecae and offspring over lifetime were recorded (both were continuously removed from the Petri dish). At the end of the experiment, all cockroaches were dissected to control whether they were still free of parasites (P-free) or infected with *Nephridiophaga* (Ni). Nine females of the Ni group had acquired an additional gut nematode infection during

the long cultivation time and were thus included in the analysis as N + Ni group.

2.6. Immune reaction: Hemocyte number

Hemolymph from 20 individuals (10 males, 10 females) from each of the three populations (P-free, Ni, N + Ni) was collected by cutting through a femur of an ice-cooled cockroach and sucking up the extruding fluid with a cooled capillary. For counting the number of hemocytes, ten reaction fields of an ice-cooled microscopic slide (Marienfeld GmbH & Co. KG) were equipped with 20 µl of a 20 mmol/l Bis-Tris buffer and 1 µl of 1 µg/ml 4′,6-diamidine-2-phenylindole (DAPI; labels DNA; final concentration 0.045 ng/ml). Before that, 1 µl of hemolymph from different individuals has been added to each field. The cooling largely prevented the coagulation of the hemocytes. Air-drying for several hours in darkness allowed a reliable counting of the cells. The cells were registered by their DAPI-labeled nuclei using a fluorescent microscope (Zeiss Axiophot with UV-excitation) with a 40 × objective. Ten images per cockroach sample were used to count the cell number with the program ImageJ. For this, records were converted into black/white binary images by defining a certain threshold. Partly overlapping cells were separated using the function 'watershed' with the option 'binary'. Regions with clumped cells were counted manually. Absolute numbers were not relevant per se but the comparison allowed inferences concerning the cellular immune activity (high number = high activity).

2.7. Immune reaction: Phenoloxidase activity

Sixty cockroaches with cut forelegs, 20 (10 males, 10 females) each of P-free, Ni, and N + Ni, were centrifuged for 1 min at 300 *g* and 2 °C. For this, individuals were put in 0.5 ml tubes with openings at the tips, which were placed in collection tubes. The volume of gained hemolymph was determined by weighing the collection tube before and after centrifugation. To 1 µl hemolymph, 8 µl acetone was added and briefly vortexed to activate the prophenoloxidase (Fisher and Brady, 1983). The mixtures were then pipetted into wells of a cooled 96-well plate of a spectrophotometer (BioTek Synergy HT) before 20 µl 20 mmol/l Bis-Tris buffer was added, and 60 µl saturated (4 mg/ml) DOPA (L-Dopa-(phenyl-d3)) as substrate for phenoloxidase. The control contained no hemolymph but only 60 µl saturated DOPA plus 50 µl of the Bis-Tris buffer. The optical density was recorded every minute for 2 h at 490 nm (maximal absorption of melanin) and 30 °C. To measure the phenoloxidase activity, the slope (V_{max}) during the linear phase of the reaction, in which the enzyme catalyses the transition from DOPA to dopachrome, was used (De Block et al., 2007). The rising values of the optical density were used as measure for enzyme activity.

2.8. Quantitative proteome analysis by label-free liquid chromatography–mass spectrometry (LC–MS).

Hemolymph of P-free, Ni, and N + Ni populations was used. 1 µl hemolymph from each of 25 males was collected by centrifugation as described in paragraph *2.7.* For each population, five samples containing the pooled hemolymph of five individuals were prepared. 5 µl hemolymph per pool was transferred to a tube containing 20 µl urea denaturing buffer (6 M urea, 2 M thiourea, and 10 mM HEPES; pH 8.0). Sample preparation for LC-MS was carried out according to Rodríguez-Rojas and Rolff (2020) with minor modifications. In short, disulfide bonds from the hemolymph proteins were reduced by adding 1 µl dithiothreitol (10 mM) and incubated for 30 min at room temperature. Thereafter, the samples were alkylated by adding 1 µl iodoacetamide (55 mM) solution and incubated at room temperature for another 30 min in the dark. Samples were diluted with four volumes of ammonium bicarbonate buffer (40 mM) and digested overnight at 37 °C by adding 1 µl trypsin protease (1 µg/µl). The samples were acidified by adding 5% acetonitrile and 0.3% of trifluoroacetic acid (TFA; final concentration), and subsequently desalted using C18 StageTips with Empore™ C18 Extraction Disks (Rappsilber et al., 2007). Peptides eluted from the StageTips were dried by vacuum centrifugation.

Peptides were reconstituted in 20 µl of 0.05% TFA, 4% acetonitrile and 1 µl of each sample was applied to an Ultimate 3000 reversed-phase capillary nano liquid chromatography system connected to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Samples were injected and concentrated on a PepMap100 C18 trap column (3 µm, 100 Å, 75 µm inner diameter [i.d.] × 2 cm; Thermo Fisher Scientific) equilibrated with 0.05% TFA in water. After switching the trap column inline, LC separations were performed on an Acclaim PepMap100 C18 capillary column (2 µm, 100 Å, 75 µm i.d. × 25 cm; Thermo Fisher Scientific) at an eluent flow rate of 300 nl/min. Mobile phase A contained 0.1% (v/v) formic acid in water, and mobile phase B contained 0.1% (v/v) formic acid and 80% (v/v) acetonitrile in water. The column was pre-equilibrated with 5% mobile phase B followed by an increase to 44% mobile phase B over 100 min. Mass spectra were acquired in a data-dependent mode, utilizing a single MS survey scan (m/z 350–1650) with a resolution of 60,000, and MS/MS scans of the 15 most intense precursor ions with a resolution of 15,000. The dynamic exclusion time was set to 20 s, and the automatic gain control was set to 3×10^6 and 1×10^5 for MS and MS/MS scans, respectively.

MS and MS/MS raw data were analyzed using the MaxQuant software package (version 2.0.2.0) with implemented Andromeda peptide search engine (Tyanova et al., 2016a). Data were searched against the *B. germanica* reference proteome downloaded from Uniprot (29,020 proteins, last modified February 26, 2021) using the default parameters and enabling the options label-free quantification (LFQ) and match between runs. Filtering and statistical analysis were carried out using the Perseus software (Tyanova et al., 2016b). Only proteins that were identified and quantified with LFQ intensity values in at least three (out of five) replicates (within at least one of the three experimental groups) were used for downstream analysis. Missing values were replaced from normal distribution (imputation) using the default settings (width 0.3, down shift 1.8). Mean log2-fold differences between Ni and N + Ni populations each against P-free control were calculated in Perseus using Student's *t*-tests. Proteins with a minimum 2-fold intensity change compared to P-free control (log2-fold change ≥ 1 or log2-fold change ≤ -1) and p-value ≤ 0.05 were considered significantly affected by the infections.

2.9. Statistical analysis and data handling.

All datasets were tested for normality (Kolmogorov-Smirnov test), and Levene's test was used to assess the equality of variances. For two-sample comparison, Welch's *t*-test was performed except for proteomics analysis, where classic Student's *t*-test was used instead. If multiple comparisons were necessary, one-way ANOVA was used to detect if at least any mean differed. Because ANOVA tests were not significant, there was no need to perform any multiple comparisons of means.

3. Results

3.1. Body weight, fat content, and muscle mass

The fresh weight of intact bodies of P-free and Ni cockroaches differed significantly in male and in female animals. P-free *B. germanica* males had an average body fresh weight of 47.28 mg in contrast to Ni males, which had only a mean weight of 44.12 mg (*t*-test, $p < 0.001$; Fig. 1a). The females were generally heavier than the males but also showed a reduced weight in infected individuals. P-free females had an average fresh weight of 97.32 mg in contrast to only 74.34 mg in the infected ones (*t*-test, $p < 0.001$; Fig. 1a). The dry muscle weight (of the thorax) was only slightly reduced in Ni cockroaches (males: mean P-free/Ni 2.92/2.75 mg; *t*-test, $p = 0.44$, females: mean P-free/Ni 3.88/

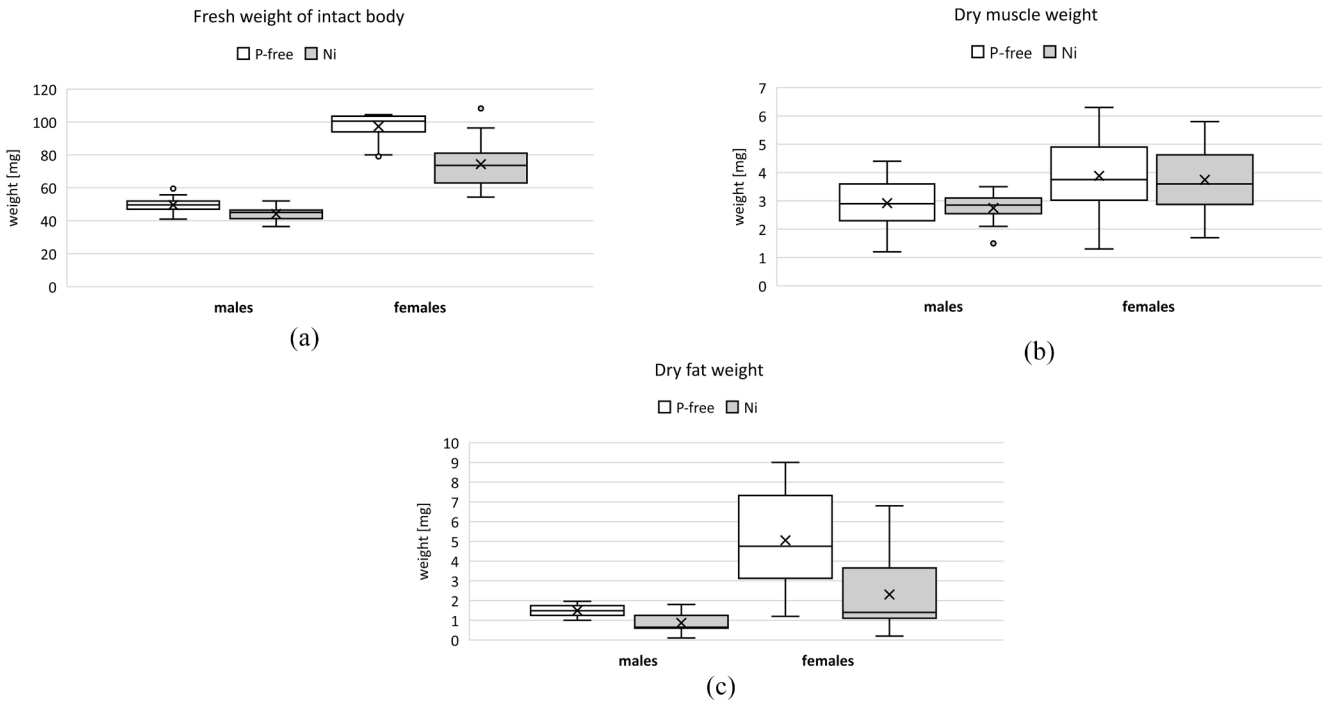

Fig. 1. Weights of P-free and Ni *B. germanica* males and females. **a.** Fresh weight of the intact body, **b.** dry muscle weight (thorax), **c.** dry fat weight (abdomen). In males and females, Ni individuals had a lower fresh weight and lower fat mass ($p < 0.001$).

3.74 mg; *t*-test, $p = 0.70$; Fig. 1b). In contrast, there was a highly significant difference in the fat content of non-infected and infected cockroaches. The abdomens of males had a mean dry fat weight of 1.50 mg in P-free and 0.87 mg in Ni individuals ($p < 0.001$; Fig. 1c). The abdomens of non-ootheca-bearing females weighed on average 5.05 mg in P-free and 2.30 mg in Ni individuals (*t*-test, $p < 0.001$; Fig. 1c). Regardless of whether the individuals were free of parasites or infected with *Nephridiophaga*, females always had a higher muscle and fat weight than males.

3.2. Physical fitness: Catching trials

In a first trial, the catching time for 90 males, 30 each of P-free, Ni, and N + Ni, was compared (Fig. 2a). Due to significant higher mobility of P-free individuals, it took about double the time to catch them (mean 4.79 s) in comparison to Ni (mean 2.61 s) and N + Ni (mean 2.68 s) individuals ($p < 0.01$). A nematode infection in addition to *Nephridiophaga* did not significantly change the catching time compared to Ni individuals. In a second catching trial, the influence of different degrees of *Nephridiophaga* infection from a Ni population was compared. Again, the longest average catching time was observed for P-free males with 4.31 s, while increasing infection intensity led to shorter catching times (Fig. 2b). Dependence between infection intensity and catching time was confirmed by a Pearson correlation coefficient of −0.78 ($r(28) = -0.78$, $p < 0.001$).

3.3. Function of Malpighian tubules: Sensitivity towards insecticides

The effect of a topical application of the pesticide Bendiocarb was strongest during the first day and did not vary much between the test groups P-free and N + Ni (Fig. 3). In P-free cockroaches, 50% of the individuals died, and in N + Ni cockroaches, 56.6% of the individuals died. However, in the following, only N + Ni cockroaches died, while the number of survivors in the P-free trial remained constant. After six days, only 10% of the initial 30 N + Ni individuals were still alive. The difference in the survival rate between P-free and N + Ni groups was significant ($p = 0.012$).

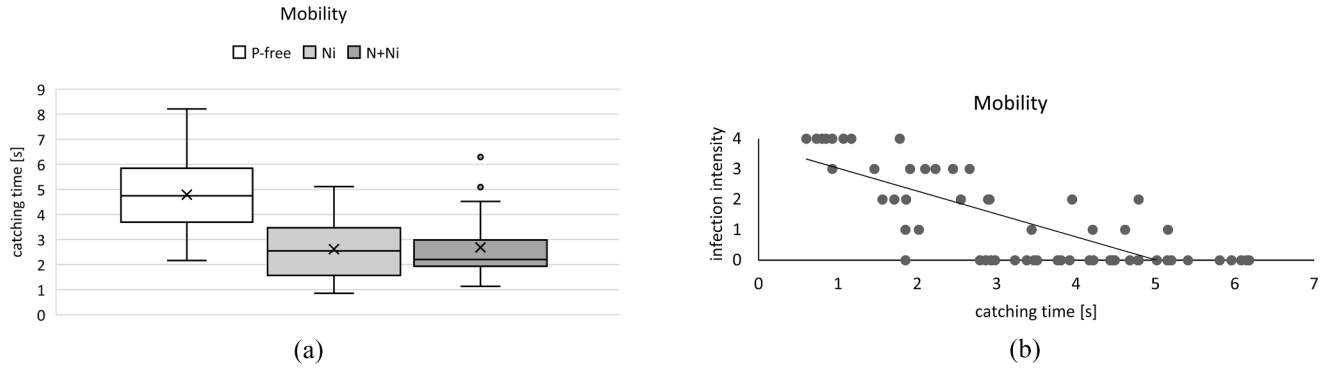

Fig. 2. Physical fitness: mobility. **a.** Catching time for P-free, Ni, and N + Ni *B. germanica* males. The mobility of P-free individuals was considerably higher than in the parasitized individuals ($p < 0.001$). **b.** Correlation between *Nephridiophaga* infection intensity and time it takes to catch *B. germanica* males (all free of nematodes). The higher the *Nephridiophaga* infection, the shorter was the catching time ($p < 0.001$).

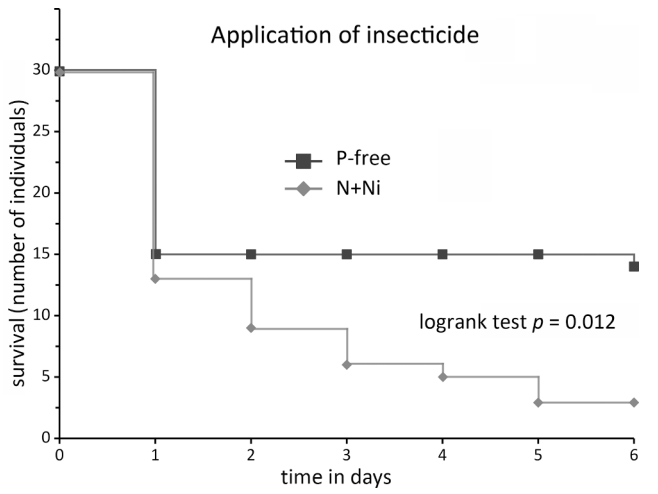

Fig. 3. Kaplan-Meier curve showing the survival rate of *B. germanica* males after topical application of the insecticide Bendiocarb. While half of the P-free individuals recovered, most of the N + Ni individuals died within six days ($p < 0.05$).

3.4. Lifetime and reproductivity

The most durable adult females of the P-free and Ni groups lived about six months and produced egg-cases. Dead females were dissected and checked for parasites. Whereas all P-free females were free of parasites, about half of the Ni females showed an additional infection with the gut nematode *Blatticola blattae* (a co-infection, which was hard to prevent during the long cultivation time). We therefore compared the lifetime and reproductivity of 20 P-free to 10 Ni and 9 N + Ni individuals (the infection status of one Ni individual remained unclear). The lifetime of adult *B. germanica* females was significantly shorter in the N + Ni group (*t*-test, $p < 0.05$). Whereas the lifetime, after reaching the adult stadium, averaged 167 days for P-free individuals, it averaged only 120.5 days for N + Ni individuals (Fig. 4). There was only weak evidence that a *Nephridiophaga* infection alone reduces the lifetime. With an average of 156.7 days, Ni individuals lived nearly as long as P-free individuals (Fig. 4).

The reproductivity of parasitized hosts was significantly reduced from on average 96 offspring for P-free females to 49 in the Ni group and 43 in the N + Ni group (*t*-test, $p < 0.05$; Fig. 5). On a per month calculation basis, there was a tendency of offspring-reduction only in N + Ni cockroaches compared to P-free ones, but there was no statistical proof (*t*-test, $p = 0.08$). The individual breeding success differed. While 50% of the Ni and 55% of the N + Ni females did not produce fertile oothecae, only 25% failed in the P-free group. In successfully produced

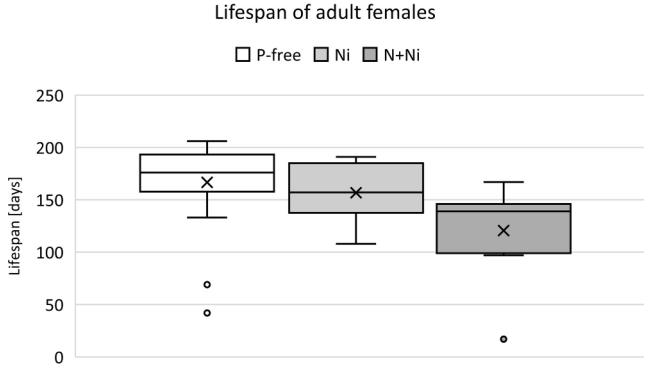

Fig. 4. Lifespan of adult P-free, Ni, and N + Ni *B. germanica* females. The lifetime of N + Ni individuals was significantly shorter than that of P-free or Ni individuals ($p < 0.05$).

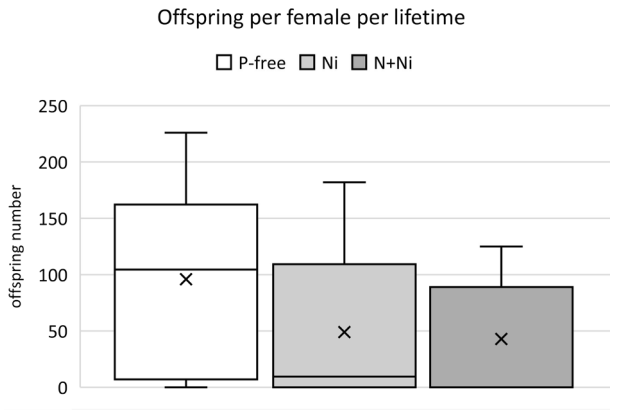

Fig. 5. Offspring number per lifetime of adult P-free, Ni, and N + Ni *B. germanica* females. The two parasitized groups had significantly less progeny ($p < 0.05$).

fertile oothecae, the number of hatched larvae was comparable between the different groups (means: P-free = 33.6, Ni = 35, N + Ni = 29.7). The minor differences of the P-free group to the other two groups were not significant (*t*-test, $p = 0.07–0.57$).

3.5. Immune reaction: Hemocyte counts

The means of the relative hemocyte number from 10 male and 10 female individuals per population were as follows: P-free = 2278 (standard deviation (sd) = 1182), Ni = 2089 (sd = 2044), N + Ni = 2398 (sd = 968) (Fig. 6). There was only week evidence for differences in the number of produced hemocytes between the three populations (ANOVA $F(2, 57) = 0{,}219$, $p = 0{,}804$). The mean hemocyte numbers in the three populations sorted by sex did not reveal significant differences, either (females ANOVA $F(2, 27) = 0{,}938$, $p = 0{,}404$; males ANOVA $F(2, 27) = 1{,}315$, $p = 0{,}285$). In P-free populations, the females had less hemocytes (1901.1) than males (2655.3), while in parasitized populations, the males (Ni = 1849.2 and N + Ni = 1889.5) had less than females (Ni = 2328.9 and N + Ni = 2899.8).

3.6. Immune reaction: Phenoloxidase activity

Hemolymph phenoloxidase activity was registered as the difference between optical density measured at the beginning and after two hours of reaction. There were only moderate differences in phenoloxidase activity between the three populations (ANOVA $F_{(2,57)} = 3.114$, $p = 0.052$). While Ni individuals showed a slightly lower phenoloxidase activity than P-free ones, N + Ni individuals showed higher activity. The means for the combined sexes were as follows: P-free = 0.544 (sd = 0.349), Ni = 0.454 (sd = 0.389), N + Ni = 0.760 (sd = 0.453) (Fig. 7).

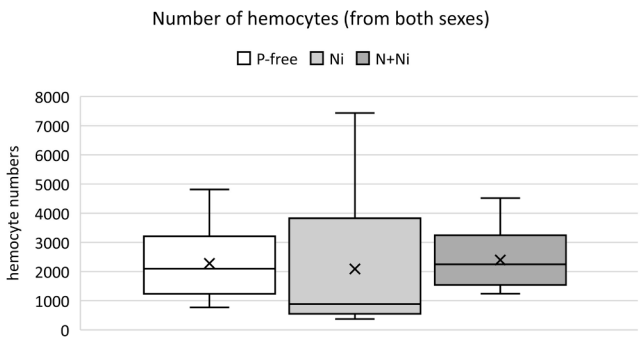

Fig. 6. Mean of hemocytes in hemolymph samples from P-free, Ni, and N + Ni *B. germanica* individuals (males and females). There was no significant difference in hemocyte numbers (*p* greater than 0.05).

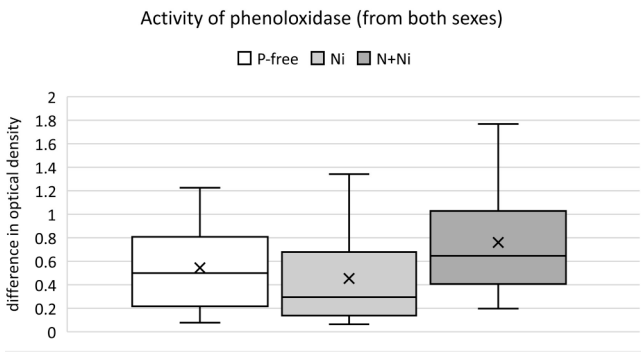

Fig. 7. Activity of phenoloxidase in the hemolymph of P-free, Ni, and N + Ni *B. germanica* individuals (males and females). N + Ni individuals showed a moderate higher activity ($p = 0.052$).

The data for only males or females were both not significant. The statistical analysis for the males showed that the empirical F value exceeded the critical F value of $F_{crit} = 3.354$ (ANOVA $F_{(2, 27)} = 5.639$, $p = 0.090$). Thus, although there was a noticeable difference in the individual mean values of the groups (P-free = 0.453, Ni = 0.255, and N + Ni = 0.817), the error level alpha 0.05 was exceeded and the result was therefore not significant. In females, the mean values were quite similar: P-free = 0.606, Ni = 0.653, and N + Ni = 0.645 (ANOVA $F_{(2, 27)} = 0.033$, $p = 0.968$).

3.7. Quantitative proteomics

Protein intensities of hemolymph samples from P-free, Ni, and N + Ni populations were compared by LFQ to explore differences evoked by the parasites. In total, 412 proteins were identified and quantified. Several proteins were significantly up- or down-regulated with at least a 2-fold change in relative intensity (52 for Ni *vs* P-free, 112 for N + Ni *vs* P-free, and 200 for N + Ni *vs* Ni; Suppl. Table 1). Although there was some variation between the five replicates within the same experimental group, different groups were clearly separated by the principal component analysis (PCA), indicating different gene expression patterns for P-free, Ni, and N + Ni populations (Fig. 8). A considerable amount of identified proteins is still uncharacterized, but several other significantly affected genes were coding for proteins with known functions (Fig. 9). In comparison to P-free cockroaches, the fatty acid-binding protein (*FABPM_0*; hereafter, the corresponding gene names are given in parentheses), which functions as a lipid chaperon in fatty acid trafficking

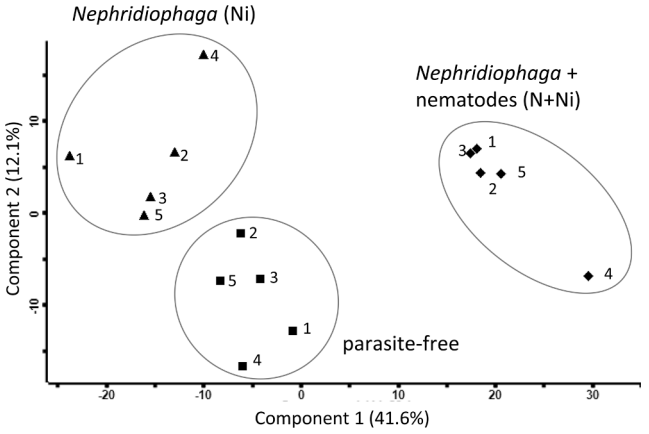

Fig. 8. Principal component analysis of the protein biosynthesis patterns of P-free, Ni, and N + Ni *B. germanica* males showing the segregation of the three analyzed groups by their protein intensity profiles detected by label-free quantitative mass spectrometry.

in cells, and fatty acid synthase (*Fasn*) were both significantly less abundant in Ni individuals. The synthesis of components of the muscle motor protein myosin, i.e., myosin heavy chain (*Mhc*) and myosin light chain (*Mlc-c*), and paramyosin (*Prm*; organization of actomyosin structure) was also down-regulated. In contrast, the synthesis of hemolymph lipopolysaccharide-binding proteins (*LPSBP_1*) was slightly up-regulated upon *Nephridiophaga* infection (Fig. 9A).

An infection not only with *Nephridiophaga* but also with gut nematodes led to a different expression profile. Down-regulated upon infection were for example the genes coding for chitinase (*Chit1_1*), endoglucanase (*celD*), hexamerin (*HEXA*), and several lipopolysaccharide-binding proteins, while others were up-regulated, such as those coding for fatty acid synthase (*Fasn*), other lipopolysaccharide-binding proteins, heat-shock protein (*Hsp83*), and ATP citrate lyase (*ACLY*) (Fig. 9B).

4. Discussion

4.1. Lifetime and reproductivity

In contrast to females double-infected with nematodes and *Nephridiophaga* (N + Ni), females infected with *Nephridiophaga* only (Ni) did not show a shortened lifetime. We therefore believe it is reasonable to assign the effect of lifetime reduction to the nematode infection. The number of ootheca-producing females, on the other hand, was already highly reduced in the Ni population (25% less) and did not change much upon double infections (N + Ni; 30% less). Thus, we conclude that *Nephridiophaga* is the major driver of the strong negative effect on the egg-case production. This is in agreement with findings by Müller-Graf et al. (2001), who reported a rather slight decrease (7% less) of ootheca production in females that were infected with nematodes. The highly reduced offspring number in the Ni group, for which the lifetime was nearly the same as for the P-free group, indicates a crucial negative effect of *Nephridiophaga*. In case of additional nematode infection, the slightly exacerbated total offspring reduction can be explained by the shortened lifetime.

The ability to reduce the egg-production/fecundity of insects has already been documented for other unicellular early-branching fungi, such as diverse microsporidia species (Becnel and Andreadis 2014; Preston et al., 2020). Also, it has been shown for *B. germanica* that feeding and drinking behavior reach their peaks during the egg maturation period (Cochran, 1983). Although food supply was unlimited in our experiments, the presence of parasites may have reduced resources available for the host and hence reduced the production of progeny.

4.2. Physical fitness and behavior

Ni individuals had a significantly reduced fresh weight compared to P-free individuals. This was due to a reduced fat mass. The muscle mass was not affected. The down-regulation of fatty acid synthases observed in the proteomic study of Ni animals is in line with our observation of a reduced fat content in individuals of this population. The down-regulation of the fatty acid-binding protein, which among other cellular functions is involved in uptake and transport of long-chain fatty acids, may also play a role in the disturbance of the fatty acid metabolism (Zimmerman and Veerkamp, 2002). Apparently, the strong infection led to an increased consumption of nutrients by the nephridiophagids causing a reduced lipid storage in the fat body's adipocytes of their hosts. Such a reduced fat storage in Ni individuals (both sexes) could explain the poorer reproduction ability since the fat body of females mobilizes energy reserves for the ovaries and produces the vitellogenin for the eggs (Arrese and Soulages, 2010; Lambiase et al., 2000).

Mobility is vital for survival in order to find food and mating partners, and escape from predators. Thus, a reduced muscle mass would represent a selection disadvantage. Despite similar muscle masses in P-free and Ni groups, the movement behavior of Ni animals was strongly

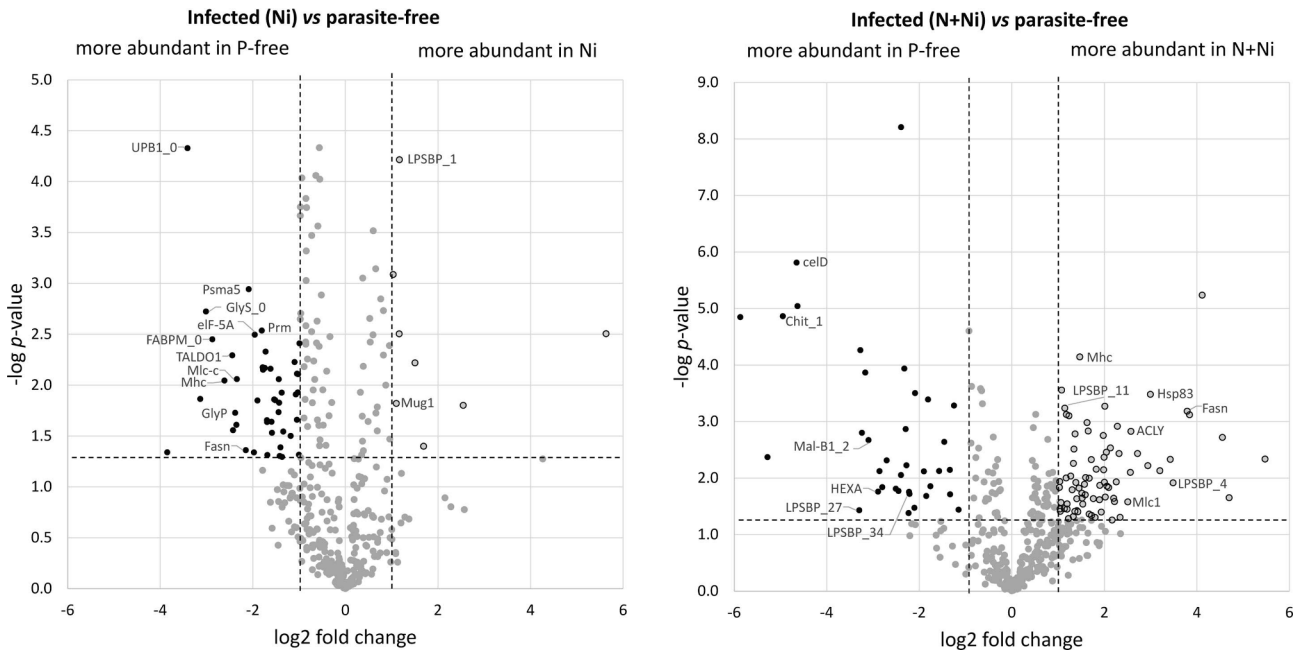

Fig. 9. Volcano plots. -log *p*-values *vs* log2 fold change of protein intensities quantified by LC-MS of hemolymph obtained from *B. germanica* males infected with *Nephridiophaga* (Ni) (**a**) and males infected with *Nephridiophaga* plus nematodes (N + Ni) (**b**), each compared to parasite-free (P-free) males as control. Selected genes coding for significantly up- or down-regulated proteins with known functions are labeled. Dashed horizontal lines represent *p*-value thresholds (significant hits with $p < 0.05$ are located above). Dashed vertical lines at −1 and + 1 represent a 2-fold change in protein abundance between the pairwise comparisons of experimental groups. Proteins left to −1 have at least a 2-fold higher abundance in P-free controls, while the proteins right to + 1 have at least a 2-fold higher abundance in the infected individuals.

affected. A reduced mobility was already noticed in other cockroach species (Purrini et al., 1988) or beetles (Purrini and Weiser, 1990) being infected with nephridiophagids. This phenomenon may be partially explained by an altered nervous control, since Ni animals not only showed a reduced movement but needed a stronger impulse to start moving at all. It is known that some parasites can change the behavior of their host, e.g., in order to facilitate the completion of the parasitic life cycle or to immobilize living insects as food for the progeny (Libersat et al., 2009). Documented interference with the host's neuroendocrine signaling systems in some of these cases reinforces the idea of an altered nervous control (Adamo and Shoemaker, 2000; Helluy and Thomas, 2003). The role of myosin in the reduced motility remained unclear. While our proteomic study revealed a down-regulation of myosin heavy (*Mhc*) and light chains (*Mlc-c*) and paramyosin (*Prm*) in Ni animals, some of these proteins were up-regulated in N + Ni animals, although the escape reaction was similarly slow. Further potential reasons for a reduced mobility of parasitized insects may be ascribed to a direct damage caused by the pathogen, adaptive sickness behaviors that allow animals to conserve energetic resources during infection, or energetic expenditure arising from performing an effective immune response (Vale et al., 2018).

4.3. Immune parameters

The presence of parasites (Ni and N + Ni) did neither show a significant impact on the host's hemocyte number nor on the phenoloxidase activity of the hemolymph. Whereas lack of impact on the latter has already been shown for several insects infected with viruses, bacteria or fungi (González-Santoyo and Córdoba-Aguilar, 2012), the number of hemocytes was found to be positively correlated with the immune resistance in some of these hosts (e.g., *Drosophila*; Eslin and Prévost, 1998). The immune system reacts very sensitively to foreign antigens and injuries. Therefore, the strong dispersion of the measured values may be influenced, e.g., by the intensity of parasitization or the age of the animals — factors that were not controlled in our experiments.

Only the multiple infected N + Ni individuals showed a close to significant higher phenoloxidase activity. Thus, additional nematodes but not *Nephridiophaga* alone seem to activate this immune parameter, at least to a certain extent. Melanization by phenoloxidase takes place at the end of an enzyme cascade. We artificially forced all prophenoloxidases of the hemolymph samples to convert into phenoloxidase and could not find an effect by *Nephridiophaga*. However, an immune-modulating effect may occur already in the earlier steps of activation. Another unicellular fungal parasite, the microsporidium *Nosema bombycis*, suppresses melanization by inhibiting host serine protease (that typically initiates the activation cascade within the hemocytes) with serpins (Bao et al., 2019). Our proteomic study did not show a raised number of serpins in Ni individuals but an up-regulated muroglobin-1 synthesis (*Mug1*; Fig. 9). This protein is also a potent inhibitor of serine protease (Kato et al., 2001) and thus might have prevented an increased synthesis of prophenoloxidase in the Ni animals. However, in the double infected N + Ni animals, muroglobin-1 was not among the significantly up-regulated proteins. To what extent nematodes may hamper the effect of nephridiophagids is currently unknown.

Another reason for the indifferent hemocyte numbers and phenoloxidase activity may be that neither *Nephridiophaga* nor the nematodes per se live in the hemocoel. Only in case of a damaged gut wall or a damaged epithelium of the Malpighian tubules, the parasites would directly contact the hemocoel and its effector systems. This may happen occasionally as severe *Nephridiophaga* infection damages the Malpighian tubules (Ivanić, 1937; Purrini et al., 1988; Radek and Herth, 1999), which could allow a leakage of parasite cells into the hemocoel. A simultaneous presence of different foreign organisms may also obscure clear immunological effects by undermining the activity of the immune system (Rovenolt and Tate, 2022). To further clarify the influence of the parasites on the immune system, additional components and organs need to be analyzed. For example, the Malpighian tubules and the gut of insects produce antimicrobial peptides (AMPs), and *B. germanica* possesses at least five AMP gene families to fight against microbial

pathogens (Buchon et al., 2014; Silva et al., 2020). The infection of these organs by the parasite may thus directly influence the release of AMPs.

4.4. Malpighian tubules: Insecticide

Most of the insecticide-treated N + Ni individuals died, while the P-free individuals, which had survived the first day, were able to detoxify the insecticide. This finding can be explained by a reduced ability to remove toxins due to a *Nephridiophaga* infection. Malpighian tubules heavily infected with *Nephridiophaga* hypertrophy and can even be destroyed so that their function in excretion and transport of toxic compounds may be disturbed (Aparecida Pacheco et al., 2014; Chahine and O'Donnell, 2011; Ivanić, 1937; Purrini and Weiser, 1990; Radek and Herth, 1999). The co-infecting nematodes, which live in the gut of the cockroaches, are not expected to cause such an effect on Malpighian detoxification.

4.5. Quantitative proteomics

Non-overlapping protein expression patterns in the PCA indicated clear differences in the protein biosynthesis between P-free, Ni, and N + Ni populations. In Ni individuals, the expression of *LPSBP_1* was slightly up-regulated. Hemolymph lipopolysaccharide-binding proteins are known to bind to the LPS of gram-negative bacteria and by this activate the immune system (Koidzumi et al., 1997). Jomori and Natori (1991) proposed a function of LPSBPs from the American cockroach in trapping or neutralizing symbiotic bacteria from leaky mycetomes. In the here studied German cockroaches, a partial destruction of Ni Malpighian tubules or penetration of the gut wall by migrating stages could have led to an additional contamination of the hemolymph with gut bacteria that needed to be eliminated (Castillo et al. 2011, Cooper and Eleftherianos 2016). The potential roles of further up- or down-regulated genes in the Ni population have been discussed above in the context of special fitness parameters.

A nematode infection in addition to a *Nephridiophaga* infection increased the number of proteins that showed significant changes compared to P-free cockroaches. Further, a comparison of Ni and N + Ni populations resulted in an even more deviating pattern showing that a double infection not only added up- or down-regulated proteins but also completely changed the expression profile. For example, the expression of the chitinase gene *Chit1_1* was drastically down-regulated in the N + Ni group. Chitinases are generally found in insects since they need to reshape their chitinous cuticle during the molt, but the enzymes are also used for pathogen defense and digestion. The cuticle of juvenile and adult nematodes does not contain chitin (except in the pharynx) but the eggshells do (Zhang et al. 2005). Thus, chitin hydrolysis may lead to laying defective eggs or embryonic death (Cheng and Peng, 2019). In addition to the gene coding for chitinase, the expression of the endoglucanase gene *celD* was drastically down-regulated. Endoglucanases have been shown to give synergistic effects to chitinases, and multifunctional cellulases also bind and degrade chitin (Ekborg et al., 2007; Philip et al., 2020). Yet, whether or not the gut nematodes are capable to suppress the host's chitinase or endoglucanase synthesis in order to protect their eggs remains speculative at this point.

The expression of the hexamerin gene *HEXA* was also highly down-regulated in N + Ni individuals. Hexamerins are storage proteins that may reach high concentrations in the hemolymph (Telfer and Kunkel, 1991). The presence of parasitic nematodes (and *Nephridiophaga*) reduced the amount of this storage protein, maybe by its own utilization or disruption of fat body function. Since hexamerins also support egg formation (Pan and Telfer, 1996), its shortage may be co-responsible for the low number of egg-case producing parasitized females in our reproduction experiments. These results indicate that parasitism impacts fitness by compromising both reproduction and survival.

5. Conclusions

The only noticeable impact of an infection with nephridiophagids and gut nematodes in the German cockroach is a reduced mobility in animals with a high burden of nephridiophagids. However, by scrutinizing physical, physiological, and reproductive fitness parameters, we revealed more detriments for infected cockroaches such as a reduced fat body or reproduction, whereby the severity of impairment by *Nephridiophaga* or nematodes differed depending on the respective parameter.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2022.107769.

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