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Natural infection of a holometabolous insect with gregarines and their detection in pupae

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ABSTRACT

Gregarines are symbionts of the gut or other body cavities associated with numerous insect species. Knowledge about natural gregarine infection levels and across metamorphosis in holometabolous hosts is sparse. Individuals of the mustard leaf beetle (*Phaedon cochleariae*, Coleoptera: Chrysomelidae) were collected from eight populations and dissected to estimate the prevalence and load of infection with gregarines. In the laboratory, the effect of larval density (single individuals versus groups of five) on subsequent larval gametocyst load and adult infection with *Gregarina cochlearium* was tested using a stereomicroscope. The presence of gregarine DNA on the surface and in pupae was investigated by performing PCR on pupal surface swabs, swabbed pupae and washed pupae. Histological sections were used to visualize gregarine life stages in larvae and pupae. Gregarines were found in all of the examined populations, with high variation in prevalence and load among the populations and between the sexes, being higher in female beetles. The likelihood and intensity of gregarine infection in adults was influenced by their age and their experienced larval density. Gregarine DNA was present in pupal surface swabs, swabbed pupae and washed pupae. In the histological sections, gamontocysts (encysted stage of paired gamonts in syzygy) were visible only in the guts of the larvae, while they were not confined to the digestive system in pupae. High prevalence in the field and the presence of gregarines in pupae are indicators of a tight relationship between this gregarine species and its host.

1. Introduction

In nature, organisms constantly interact with other organisms of the same as well as of other species. This can result in various relationships between two organisms, such as symbiosis, which can take the form of mutualism, commensalism or parasitism (Leung and Poulin, 2008). Mutualism and parasitism in particular can play a crucial role in shaping ecological dynamics and community structures (Chomicki et al., 2019, Hatcher et al., 2012). It is therefore important to study host-symbiont relationships. To better understand these relationships, levels of interdependence and specificity between the host and the symbiont should be assessed (Chomicki et al., 2022). To achieve this, the occurrence of such symbioses under natural conditions needs to be explored. When the symbiont lives within the host, the question also arises as to how the symbiont is maintained across the host life-cycle, especially when metamorphosis occurs, as is the case in holometabolous insects.

In arthropods, gregarines are widespread symbionts (Schrével and Desportes, 2016) that are usually highly host-specific (Rueckert et al., 2018). Gregarines belong to the Apicomplexa and can have a wide range of effects on their hosts, which has led to the suggestion to place them on a mutualism-parasitism continuum (Rueckert et al., 2019). The gregarine life cycle starts in the host as a sporozoite that attaches to the gut wall (Boisard and Florent, 2020). The sporozoite takes up nutrients from the host (Valigurová and Florent, 2021) and develops into the

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Table 1
Information on the sampled wild populations of *Phaedon cochleariae*.

Location ID	Location name	Coordinates	Date of collection (day/month/year)	Density (n/m^2)	*Number of collected individuals*
DB	pond "Mühlenmasch" Dornberg	Lat: 52.0650750 Long: 8.5077390	06.06.2023	100	15
LU	Lutter	Lat: 51.9974515 Long: 8.4926818	12.07.2023	30	15
LB	Lichtebach	Lat: 51.9866555 Long: 8.4587729	01.06.2023	50	16
SB	Sprungbach	Lat: 51.9439297 Long: 8.6067379	07.07.2023	30	16
ME	Menkhauser Bach	Lat: 51.935591 Long: 8.624762	28.08.2024	5	12
BO	pond Bokelfenn	Lat: 51.924499 Long: 8.671972	29.08.2024	5	15
EQ	Ems	Lat: 51.8547323 Long: 8.6941063	21.06.2023	40	15
PA	Pleichach, close to Würzburg	Lat: 49.88067 Long: 10.08542	08.04.2024	50	21

trophozoite stage, which lives free in the gut lumen. The trophozoite develops into a gamont that later pairs with another gamont in syzygy, forming the gamontocyst, which further develops into the gametocyst (Boisard and Florent, 2020). The gametocysts are excreted with the host's feces into the environment, releasing spores/oocysts containing sporozoites which can then be taken up again by a new host (Boisard and Florent, 2020). Depending on the host species, the prevalence, i.e. the relative number of infected individuals in a population, as well as the gregarine load, i.e. the number of gregarines in an individual, can differ pronouncedly in wild populations. For example, the prevalence of gregarines in wild *Costelytra zealandica* (Coleoptera: Scarabaeidae) was up to 90 % (Allison, 1969), while it was only 1.8 % in adults of different dragonfly species (Locklin and Vodopich, 2010). The load can range, for example, from 1–135 in *Gammarus fasciatus* (Amphipoda: Gammaridea) (Grunberg and Sukhdeo, 2017) to 6–24 in *Dorypteryx domestica* (Psocodea: Psyllipsocidae) (Rueckert and Devetak, 2017). Within one species, also large differences among individuals exist. These differences can arise, for example, due to seasonal variation and changes in the host's life cycle (Allison, 1969, Grunberg and Sukhdeo, 2017), the sex of the host (Locklin and Vodopich, 2010) and pollutants such as heavy metals (Pižl and Sterzyńska, 1991).

Larvae and adults of the mustard leaf beetle, *Phaedon cochleariae* (Coleoptera: Chrysomelidae), are known to be infected by the gregarine species *Gregarina cochlearium* (Wolz et al., 2022a). A gregarine infection was found to have negative effects on *P. cochleariae*, such as causing a prolonged developmental time or reduced adult beetle mass, no effects at all, or even positive effects such as a higher survival probability (Barber et al., 2025, Barber et al., 2024, Wolz et al., 2022a, Wolz et al., 2022b). These findings were obtained in laboratory populations that had been established from wild-collected beetles and kept in the laboratory for several generations, while outcrossing once a year with beetles taken from the wild. In these laboratory populations, gregarine prevalence and load were high. However, little is known about gregarine prevalence and load in wild *P. cochleariae* populations.

While larvae and adults of *P. cochleariae* show visible gregarine stages when dissected, no gregarine life stages could be found in the light-colored pupae under the stereomicroscope (pers. observation). When keeping gregarine-infected larvae of *P. cochleariae* individually, some of the emerging adults were no longer infected with gregarines, while some showed an infection with trophozoites, though the load was rather low (Barber et al., 2025). In another experiment, larvae were kept in groups of five to ten, with all adults later showing an infection (Wolz et al., 2022b). It was therefore unclear what happens to the gregarines during the pupal stage of *P. cochleariae*. During metamorphosis, the gut structures of different beetle hosts are remodeled (Vommaro et al., 2024) or partly shed (Chiang and Shelomi, 2023), which may pose a challenge to the gregarines. In some Trichoptera species, the life cycle of gregarines is restricted to the larval stage and pupae and adults are not infected (Corallini and Bicchierai, 2016, Moretti and Corallini Sorcetti, 1981). In these species, larvae and adults live in distinct environments, which could be the reason why the gregarines need to complete their life cycle under constant environmental conditions within the larvae. In wild *C. zealandica*, larvae were infected with gregarines, while prepupae, pupae and adult beetles were no longer infected (Allison, 1969). Since in *P. cochleariae* adults are also infected with gregarines and larvae and adults share the same habitat, the question remains whether the beetles reinfect themselves with gregarines after metamorphosis or if the infection is carried over the pupal stage.

In this study we aimed to 1) explore natural infection rates in adults from wild populations of *P. cochleariae*, 2) understand the impact of single versus group living of the host on infection rates of larvae, pupae and adults of different age with *G. cochlearium*, and 3) investigate whether *G. cochlearium* can be found in or on pupae. Therefore, we collected adult beetles from eight natural populations to assess their gregarine prevalence and load. To test for a potential host density effect on gregarine infection, we tracked the gregarine gametocyst excretion by larvae as well as the infection rates of pupae and 10- and 21-day old adult beetles after having kept larvae either individually or in groups of five. To test if gregarine life stages are present in or on the pupae, we performed polymerase chain reaction (PCR) profiling of swabs taken from pupal surfaces, swabbed pupae and surface washed pupae. We hypothesized that the gregarine prevalence in most of the sampled wild beetle populations is high (> 50 %), with no population being completely gregarine-free. The populations were hypothesized to show significant differences in gregarine load, with female beetles having a higher load than males due to their body mass and consumption differences (Tremmel and Müller, 2013). Infection rates of adults in the laboratory were hypothesized to be higher when larvae were kept in groups compared to individually-kept larvae, because of a potentially higher amount of excreted gametocysts allowing for reinfection. Finally, we hypothesized that gregarine DNA would be present in pupal surface swabs and thus on pupae, but not inside pupae such as in washed pupae, as gregarine life stages may be lost due to the major reconstruction during host metamorphosis.

2. Material and methods

2.1. Collection of wild beetles

Wild beetles of *Phaedon cochleariae* were collected from eight populations in Germany from their Brassicaceae host plant *Nasturtium officinale* in creeks and ponds over the course of two years between late spring (April) and late summer (August) (Table 1). At other sites, single beetles and larvae were also seen on *Cardamine amara* (Brassicaceae),

but their numbers were too low to collect individuals from these populations. Several beetle populations were found in nature protection areas. Protected areas were only entered, and beetles were only collected after gaining permission from the competent authority at the environmental agency of the corresponding county. As adults of *P. cochleariae* do not appear to display flying behavior (De Paiva, 1977) and seem to distribute through water currents (pers. observation), populations were defined as being distinct when the water bodies were not connected in the direction of the prevailing current. If two creeks connected into one downstream, beetles that were collected upstream were also considered as separate populations. For each population, beetle density was approximated in one square-meter quadrats and extrapolated. Beetles were only collected if the density was at least 5 individuals/m^2 and no more than 15 % of the population would have been removed. From those populations, beetles (n = 15–21) were put directly into 50 mL Falcon tubes (about ten individuals per tube). As soon as possible (after between 30 min and 2 h), the beetles were placed individually into Petri dishes (5.5 cm in diameter), lined with moist filter paper and supplied *ad libitum* with leaves of watercress (*Nasturtium officinale*) plants grown in a greenhouse (60 % r.h., 16:8 light:dark). Beetles were kept in climate chambers (Binder GmbH, Tuttlingen, Germany; 20 °C, 65 % r.h. 16:8 light:dark) for seven days, before being frozen at −20 °C. This was done to ensure that a possible gregarine infection would be macroscopically detectable; trophozoites are not visible within the first few days after adult beetle eclosion and the age of the wild beetles was unknown. After thawing, the sex of each beetle was determined and individuals were dissected in sodium-phosphate buffer (0.1 M; pH = 7.2) using a stereomicroscope. The head was carefully removed with tweezers, pulling out the gut with it. The gut was then opened along its length and trophozoites were counted. The prevalence was assessed as the relative number of infected beetles within one population while the load was assessed as the number of trophozoites in one individual. Beetles were also visually examined for other symbionts, such as mites or parasitoid larvae, using the stereomicroscope.

2.2. Test for effects of single versus group living on larvae gametocyst excretion and trophozoite numbers in adults of different age

Adult *P. cochleariae* of a laboratory culture were kept in plastic rearing boxes (20 × 20 × 6.5 cm) with about 100–200 individuals per box in climate chambers (same conditions as above) and fed with Chinese cabbage (*Brassica rapa* L. ssp *pekinensis*) grown in the greenhouse (same conditions as above). The beetles had been reared for several years in our laboratory and the gene pool was refreshed almost every year by introducing individuals captured in the area of the Ems (Table 1). At the start of the experiment, beetles were provided with fresh cabbage leaves for oviposition. After 24 h, the leaves with the eggs were transferred into a new rearing box. Hatching larvae were fed with leaves that were already partly consumed by adult beetles for 24 h, to ensure reinfection with gregarines (reinfection leaves). Infected individuals excrete gametocysts with infective spores; it was therefore assumed that these infective spores will be present on the reinfection leaves. Reinfection leaves were offered to the larvae for two days after hatching to ensure an infection with gregarines. On the third day after hatching, larvae were randomly subjected to two different density treatments in order to test whether the subsequent gregarine load depends on host density. Larvae were either kept individually ('single', as previously done in Barber et al. 2025) or in groups of five ('group', as previously done in Wolz et al. 2022b; n = 20 per treatment) in Petri dishes (5.5 cm in diameter), lined with moist filter paper. From the third day onwards, larvae were supplied *ad libitum* with cabbage leaf discs taken from fresh, uncontaminated plants, to avoid introducing further gregarines from outside. Every other day, the number of gametocysts in the Petri dishes was counted before the leaf discs were exchanged with fresh, uncontaminated discs. On the day of pupation, pupae were transferred into new Petri dishes lined with dry filter paper. All individuals were kept individually from the pupation stage onwards. After adult emergence, gametocysts were again counted every other day and the beetles were supplied regularly with uncontaminated cabbage leaf discs. After 10 days of adulthood, half of the individuals of each treatment were frozen at −20 °C. After 21 days of adulthood, the remaining individuals were frozen at −20 °C, following the experimental design of Barber et al. (2025). This was done to test whether gregarine infection might get lost during the adult stage due to the removal of leaf material and therefore gametocysts and infective spores. After freezing, the beetles were dissected as described above to count the number of trophozoites. When host individuals died during the timeframe of the experiment, the gametocyst and trophozoite numbers corresponding to those individuals were not included in the final dataset. In the group treatment, replicates were excluded when three or more individuals died during larval development.

2.3. Test for effects of single versus group living on likelihood of pupal infection

To test whether gregarine DNA can be found in pupae, and if there is a dependence on larval density, larvae were kept either individually or in groups of five as described above (n = 20 Petri dishes per treatment). At the day of pupation, whole pupae were transferred individually into 95 % ethanol (VWR International, Rosny-sous-Bois-cedex, France) and stored at −20 °C. For DNA extraction, the pupae were removed from the ethanol and homogenized with a pistil in 100 µL of extraction buffer [10 mM Tris, 2 mM EDTA with pH = 8, 1 % SDS (all Carl Roth GmbH & Co. KG, Karlsruhe, Germany) and 10 mM NaCl (VWR International, Leuven Belgium)]. After homogenization, another 380 µL of extraction buffer and 20 µL of proteinase K (Carl Roth GmbH & Co. KG) were added. Samples were manually vortexed for 5 s and shaken in a water bath at 56 °C for at least 3 h. After vortexing again, 250 µL NaCl and 700 µL of a trichloromethane/isoamyl alcohol mixture (ROTI®C/I, Carl Roth GmbH & Co. KG) were added to the samples and vortexed manually for 5 s. Samples were centrifuged for 15 min at 16,000 g. The supernatant of the upper phase was transferred into a new Eppendorf tube and 40 µL sodium acetate (VWR International) and 280 µL propan-2-ol (VWR International) were added. Samples were inverted multiple times and then centrifuged again for 20 min at 16,000 g to precipitate the DNA. The supernatant was discarded and 500 µL 70 % ethanol were added to the pellet. Samples were stored at 8 °C overnight. After 10 min at room temperature, the samples were centrifuged again for 10 min at 16,000 g and the supernatant was discarded. The remaining ethanol was allowed to evaporate until the pellet was completely dry. After that, 50 µL of TE-buffer (10 mM TRIS and 1 mM EDTA with pH = 8) were added to the samples and they were stored at 4°C. Two blanks (negative controls, ultrapure water) were treated in the same way to test for a potential contamination, but did not contain any insect material. Two gregarine-infected larvae (positive controls) were extracted in the same way.

2.4. Testing for presence of gregarine DNA in and on the surface of pupae

To test whether gregarine DNA is found on the surface or inside the pupae, three sets of samples were prepared. For that, 30 pupae (max. 6 h old) from rearing boxes (with a density of 150–200 individuals per box) were swabbed with autoclaved cotton swabs by first wetting the swab in 100 µL ethanol and then gently swabbing the dorsal, ventral and lateral surfaces three times each from head to back. The pupal swab (swab taken from the pupae, n = 30) was placed in an Eppendorf tube and the swabbed pupa (n = 30) was placed in another tube. The third kind of sample was washed pupae (n = 30), taken also from the rearing boxes. Therefore, a pupa was held with tweezers and dipped and slightly shaken in deionized water three times. To all of the samples (i.e. swabs, swabbed pupae and washed pupae), 400 µL of 95 % ethanol was added for storage. Swabbed and washed pupae were extracted as described above (2.3). Swabs were not homogenized with a pistil but were

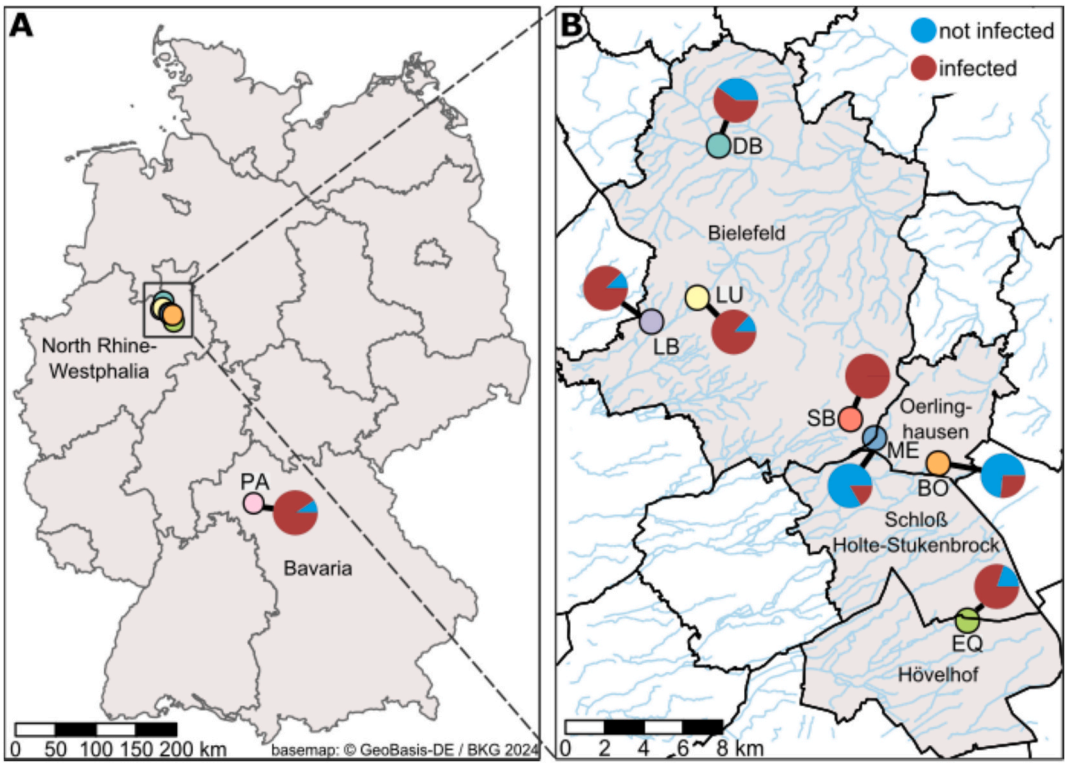

Fig. 1. Map of the populations found in Germany (A) with the insert (B) showing the populations found close to Bielefeld. Proportions of infected to not infected beetles per population are shown as pie charts. Abbreviations and further information about the populations are given in Table 1.

otherwise extracted as described above. For a representation of the types of pupal samples that were screened for gregarine DNA, see Fig. S1.

2.5. PCR reaction, sequencing and verification of gregarine presence in pupal samples

The following forward and reverse primers were designed from the small subunit gregarine rDNA sequence [described in Wolz et al. (2022a), accession number: OM286796.1] using Primer-BLAST (Ye et al., 2012) and used for the PCR reaction: Fgregcoch (5′->3′) GAAATCTCACCAGGCCCGAA and Rgregcoch (5′->3′) TTCCATTGGG-CAGCGAGATG, with an expected product length of 506 bp (both Sigma-Aldrich, St. Louis, Missouri, USA). For the PCR reaction, 9 μL of the master mix and 1 μL DNA-extract were mixed on ice in a PCR-tube. The following final concentrations were prepared for the master mix: 1× PCR buffer (without $MgCl_2$, pH 8.8), 1.5 mM $MgCl_2$, 200 μM of each dNTP, 0.1 μM of each primer (Fgregcoch and Rgregcoch), and 0.25 U SuperHotStart Taq DNA polymerase (all Genaxxon bioscience GmbH, Ulm, Germany). Ultrapure water was added to adjust the volume. A negative control consisting only of mastermix was added for each PCR reaction. The PCR program was set to the following settings: 15 min of initial denaturation at 95 °C, followed by 29 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 1 min and extension at 72 °C for 1.45 min. It was ended with a final extension period at 72 °C for 10 min. For electrophoresis, 1 μL of PCR product was mixed with 1 μL of SafeGel redstain [diluted to 20× final concentration and mixed with loading buffer (pH = 8) after supplier protocol, Genaxxon bioscience GmbH] and 3 μL of ultrapure water. To size the PCR products, a size standard (100 bp DNA ladder, New England Biolabs GmbH, Frankfurt a. M., Germany) was run on each gel. Electrophoresis was performed using 2 % agarose gels in 1:10 diluted Tris-Borate-EDTA buffer [54 g TRIS, 20 mL 0.5 M EDTA with pH = 8, 27.5 g boric acid (Carl Roth GmbH & Co. KG) filled up to 1 L with ultrapure water], with the voltage set to 120 V, and the gels were run for 45 min. Photographs were taken under UV-light. Gel images were inspected visually for presence or absence of bands. To verify the sequence of the bands, a subset of ten bands from whole pupal samples (see section 2.3; consisting of individuals from both single and group treatment) as well as all bands from pupal swabs, swabbed pupae and washed pupae (see section 2.4) were sequenced (seven, nineteen and twelve bands, respectively). Briefly, 5 μL of the PCR product was purified with 1 μL of 10 U exonuclease I and 1 μL of 1 U shrimp alkaline phosphatase (both New England Biolabs GmbH) and sequenced in both directions using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit on a 3730xl DNA Analyzer (Thermo Fisher Scientific: Waltham, Massachusetts, USA). Sequences were identified using BLAST of the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/) against the nt database. All sequences of Fgregcoch were aligned with the original *G. cochlearium* sequence and pairwise distances were computed as the Kimura-2-parameter using MEGA version 12 (Kumar et al., 2024). For four samples the chromatograms of the Fgregcoch sequences did not show clear results. For these samples, sequences were compared and combined with the Rgregcoch sequence and the consensus was used for alignment (see Fig. S2 and S3 for examples of chromatograms). Please note that the PCR protocol was established on *P. cochleariae* larvae that were known to be either gregarine-free or infected with gregarines. DNA (i.e. visible bands in the gel) could only be retrieved from gregarine infected individuals, highlighting the specificity of the primers used.

2.6. Histological sections of larvae and pupae

To visually inspect a gregarine infection of *P. cochleariae*, histological sections were performed on two larvae (2^{nd} instar) and four pupae on the day of pupation. Individuals were taken from the lab rearing and fixed in Bouin's fixative (Carl Roth GmbH & Co. KG, ready to use). Larvae were fixed for two days, pupae were kept in the fixative for 90 minutes, before piercing them with a needle and returning them into the fixative overnight. After fixation, the samples were transferred to 70%

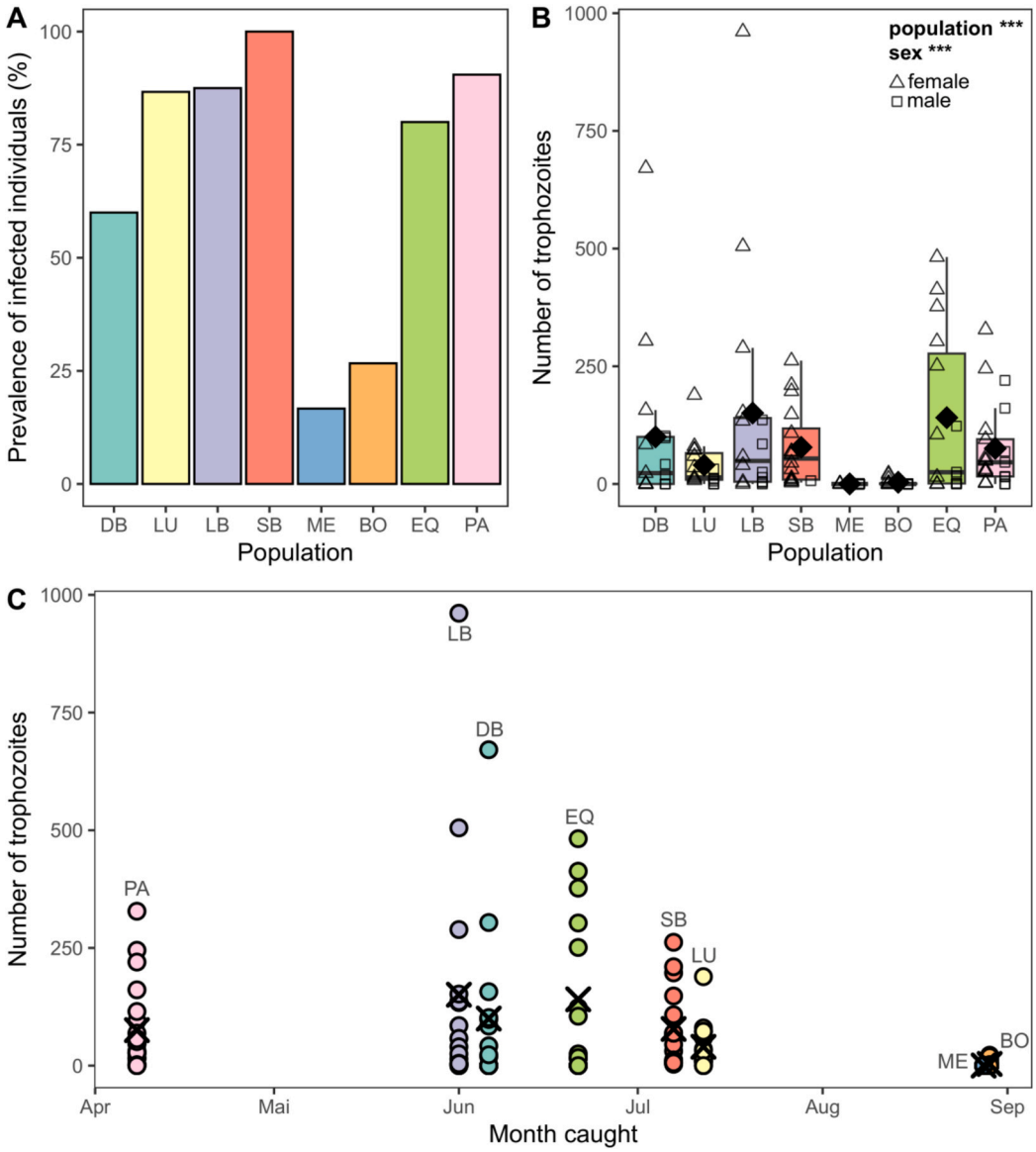

Fig. 2. Prevalence of gregarine infection in adult beetles (A), number of gregarine trophozoites in adult beetles (B) and number of trophozoites per month in which adults were collected (C) from wild *Phaedon cochleariae* populations. For data about prevalence and the number of trophozoites, populations are sorted by latitude (A+B). Data are shown as bar plots (A), box-whisker plots with the box showing the interquartile range, the diamond showing the mean, the horizontal line the median and whiskers extending to the maximum and minimum values within 1.5-fold interquartile range and individual data as symbols (B) and dot plots, with the crosses marking the means (C) (n = 12-21 individuals per population, for exact numbers see Table 1). Text in the plot shows significant effects of the two predictors population and sex at $p < 0.001$ (***, GLM followed by type II Wald χ^2 test).

ethanol. For histological processing, the specimens were dehydrated in an ascending ethanol series, cleared in methyl benzoate and butanol, and embedded in paraffin at 60 °C. Paraffin blocks were trimmed and sectioned at 5 µm using a rotary microtome (Leica RM2165, Leica Microsystems). Sections were mounted on slides, stretched in distilled water, and dried overnight at 40 °C. Paraffin was removed with petroleum benzine. Sections were rehydrated through a descending ethanol series to distilled water, stained with carmalaun, aniline blue, and orange G (all Waldeck GmbH & Co. KG), and additionally treated with phosphotungstic acid (Carl Roth GmbH & Co. KG), and finally transferred back up to 100% ethanol and finally petroleum benzine.

2.7. Statistical analyses

All statistical analyses were carried out with R in RStudio version 4.5.2 (R Core Team, 2025) using the packages *car* (Fox & Weisberg, 2019), *glmmTMB* (Brooks et al., 2017, McGillycuddy et al., 2025), *DHARMa* (Hartig, 2024), *emmeans* (Lenth, 2025) and *exactRankTests* (Hothorn & Hornik, 2022). The effects of population and the sex on the gregarine load of wild beetles was analyzed using a generalized linear model (GLM) with a negative binomial distribution and log link function. Model assumptions and fit were assessed using simulated residual diagnostics in *DHARMa*. A type II Wald χ^2 test was used to test for significant effects of the predictors of the GLM. The effects of the density treatment (singe vs. group) and the time (in dph) as well as their interaction on the number of excreted gametocysts were analyzed using a generalized linear mixed model (GLMM) with a generalized Poisson distribution and log link function, using natural splines (df = 3), Petri dish as a random effect and the number of individuals per Petri dish as an offset. A type III Wald χ^2 test was used to test for significant effects of the predictors of the GLMM. Data from day 5 after hatching were excluded, as they very likely resulted from methodological errors and

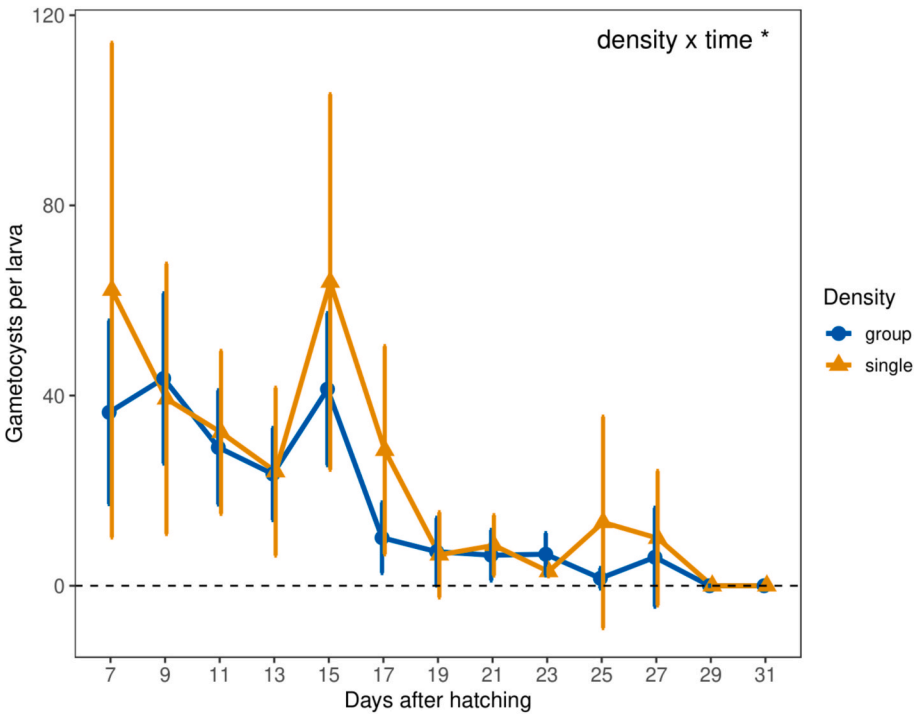

Fig. 3. Numbers of excreted gregarine gametocysts per *Phaedon cochleariae* larva, from day seven after hatching onwards, counted every two days until pupation. Larvae were either kept in groups of five (group) or individually (single). First pupae were found at day 17 after hatching; at day 32 after hatching all individuals had pupated. Data are shown as line-plots with the symbols showing the mean and the error bars showing the standard deviation. Text in the plot shows significant effect of the interaction of the predictors density and time at $p < 0.05$ (*, GLMM followed by type III Wald χ^2 test; $n = 17$-18 per density treatment at the first data point).

because of high variation from the rest of the dataset, as the mean gametocyst count was 27 and 10 standard deviations above the mean of all other days for the group and single treatments, respectively. Therefore, data were analyzed and presented from day 7 after hatching onwards. To test for effects of the density treatment (single vs. group) and the age (10 days old vs. 21 days old) on the proportion of adults that are infected with gregarines (yes/no), a GLM with binomial distribution was used, while a negative binomial distribution was used for the effects of these predictors on the trophozoite number in adults. Type II Wald χ^2 tests were used to test for significant effects of the predictors of the GLMs.

A Fisher's exact test was used to test for differences between density treatments (single vs. group) in the proportion of gregarine DNA present (i.e. visible bands) in whole pupae after PCR.

3. Results

3.1. Gregarine prevalence and load in wild populations

Seven wild populations of *P. cochleariae* were found in close proximity to Bielefeld (North Rhine-Westphalia, Germany), while one population was located farther away in the proximity of Würzburg (Bavaria; Germany) (Table 1, Fig. 1A+B). Populations had approximate densities of between 5 and 100 individuals/m^2. All *P. cochleariae* populations were found at small waterbodies such as creeks or ponds with clear water (often close to springs) and sand-rich waterbeds, where the host plants often grew in muddy patches close to the water's edge.

The prevalence of infected beetle individuals varied greatly among the populations (Fig. 2A). In the SB population, 100 % of the individuals were infected, while in the ME population only 17 % of the individuals were infected. Generally, six out of the eight populations had a prevalence of > 50 %. The number of trophozoites per beetle differed significantly between the populations ($X^2 = 98.974$, $df = 7$, $p < 0.001$) (Fig. 2B). In the LB population, which was the population with the highest mean load, the number of trophozoites was 150.7 ± 246.4 (mean ± sd), while it was 0.2 ± 0.4 in the ME population, which had the lowest load. Over all individuals, the range of the number of trophozoites was 0 to 961. The SB population had the highest prevalence but not the highest load. Likewise, the PA population had the second highest prevalence but a relatively low load. However, the populations ME and BO with the lowest prevalence also had the lowest load. There seemed to be a pattern in the number of trophozoites per beetle over the course of the year, with a peak in June when looking at the range, though this pattern was less pronounced in the means (Fig. 2C). There was no spatial trend in the number of trophozoites per beetle (Fig. S4). Females had a significantly higher load than male beetles ($X^2 = 21.067$, $df = 1$, $p < 0.001$).

While dissecting the beetles, two other parasite species were detected in/on some of the beetles. A freshwater mite species and/or its eggs could be detected under the elytra of some individuals of the populations LB, SB, EQ and PA. The larvae of a tachinid fly were found in a few individuals of the SB and LU population. For exact numbers see Table S1.

3.2. Relationship between gregarine infection and larval density

The number of excreted gametocysts per larva was significantly influenced by the interaction of the group treatment and the time ($\chi^2 = 8.43$, $df = 3$, $p = 0.038$) (Fig. 3). The number of gametocysts per larva tended to decrease after hatching, with the exception of day 15 after hatching, at which the number was increased. After that, it decreased again and stayed relatively low until reaching (close to) zero a few days before pupation, which happened at the latest after 32 days. The proportion of beetles that showed visible trophozoites after dissection was significantly influenced by the age of the beetles ($\chi^2 = 5.2$, $df = 1$, $p = 0.022$) and by trend influenced by the density treatment ($\chi^2 = 3.26$, $df = 1$, $p = 0.071$). The number of visible trophozoites in adults was significantly influenced both by age of the beetle ($\chi^2 = 9$, $df = 1$, $p = 0.003$) and the density treatment ($\chi^2 = 5.83$, $df = 1$, $p = 0.016$) (Fig. 4).

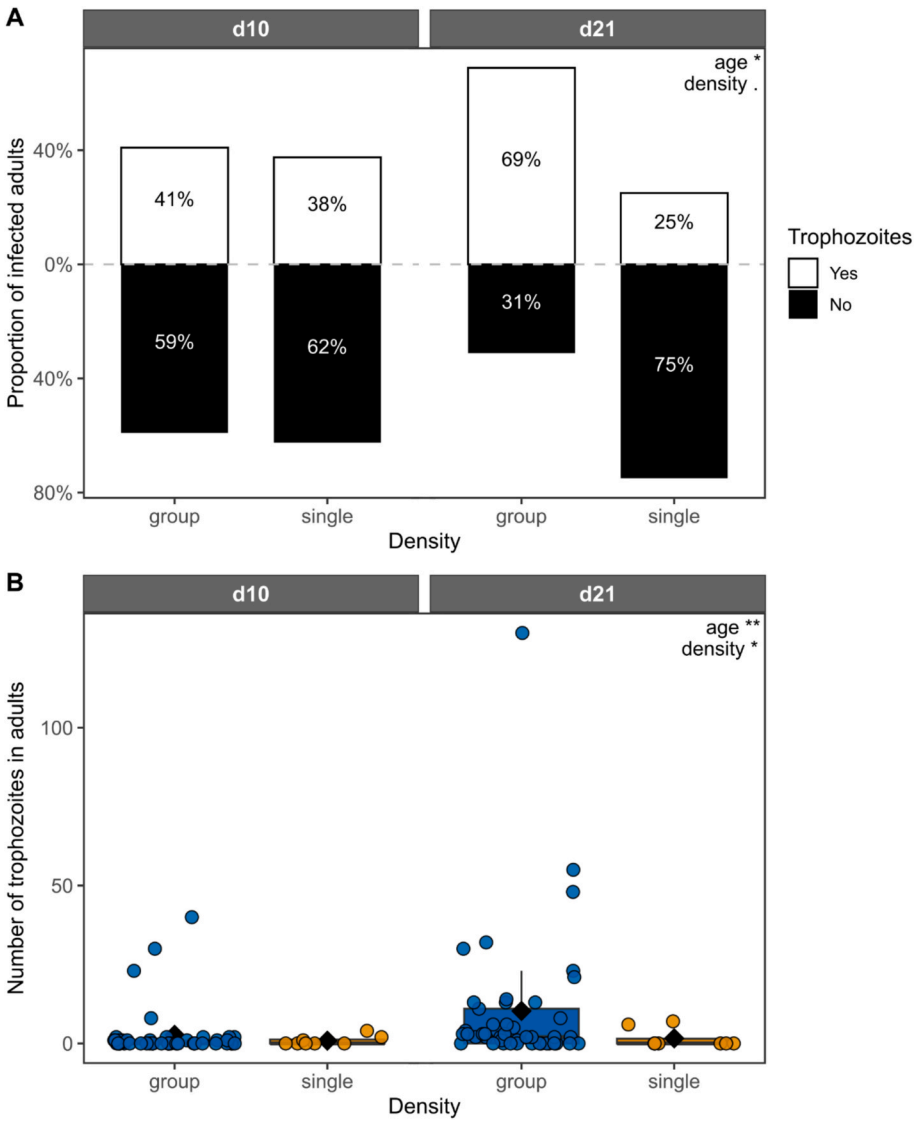

Fig. 4. Proportion of *Phaedon cochleariae* adults with gregarine trophozoites present (yes/no; A) and number of trophozoites found after dissection of adult beetles (B). Beetles were either kept in groups of five (group) or individually (single) as larvae and frozen at either day 10 (d10) or day 21 (d21) of adult age. Data are presented as diverging bar plots (A) and box-whisker plots (B) with the box showing the interquartile range, the diamond representing the mean, the horizontal line the median and whiskers extending to the maximum and minimum values within 1.5-fold interquartile range; individual data points are shown. Text in the plot shows significant effects of the predictors density and age at different levels of significance ** $p < 0.01$, * $p < 0.05$ or a trend with marginal significance "." $p < 0.1$ (GLM followed by type II Wald χ^2 test; group n = 44-45 per age class, single n = 8-10 per age class).

Generally, the number of gametocysts per adult beetle was not higher than two at any time point (with only one exception, where 14 were found for one individual at one time point). In the first six days after adult eclosion, no individuals of the single treatment excreted gametocysts, while there were a few individuals in the group treatment that did (data not shown). In some beetles, no gametocysts were found prior to dissection, but trophozoites were still visible when dissected and vice versa: some adults excreted gametocysts but no trophozoites were visible when dissected (Table S1).

3.3. Gregarine infection in and on the surface of pupae

The proportion of pupae that showed visible gregarine bands in the gel after PCR was significantly higher in pupae that were kept in groups than in pupae that were kept individually as larvae (Fig. 5A; OR = 3.91, 95% CI [1.26, 13.67], p = 0.011). Only in 23 % of the pupal swab samples bands were recovered, while in 63 % of the swabbed pupae and 40 % of the washed pupae bands were present (Fig. 5B). Sequencing of the PCR products confirmed that visible bands in the gels contained the targeted gregarine DNA sequence (Table S1). Sequences showed only little variation from each other (Fig. S5) with pairwise distances ranging from 0.00 – 0.02 (values > 0.01 only in samples where the consensus was used for alignment and sample P49_S; Table S1).

3.4. Presence of gregarines in histological sections of larvae and pupae

In the histological sections of larvae, different gregarine life stages were visible. Trophozoites showed the distinguishable protomerite and deutomerite (Fig. 6 A-B) and could be seen still attached to the gut wall (Fig. 6 C-D). A gamontocyst with still distinguishable gamonts could also be seen in sections of one larva (Fig. 7 A-B). In larval sections, gregarine life stages were only found within the digestive system. In pupal sections, gamontocysts could be found outside of the digestive systems (Fig. 7 C-F). The gut wall appeared to be much thinner in the pupal sections than in the larval sections and even seemed to disintegrate at one point (Fig. 7 E-F).

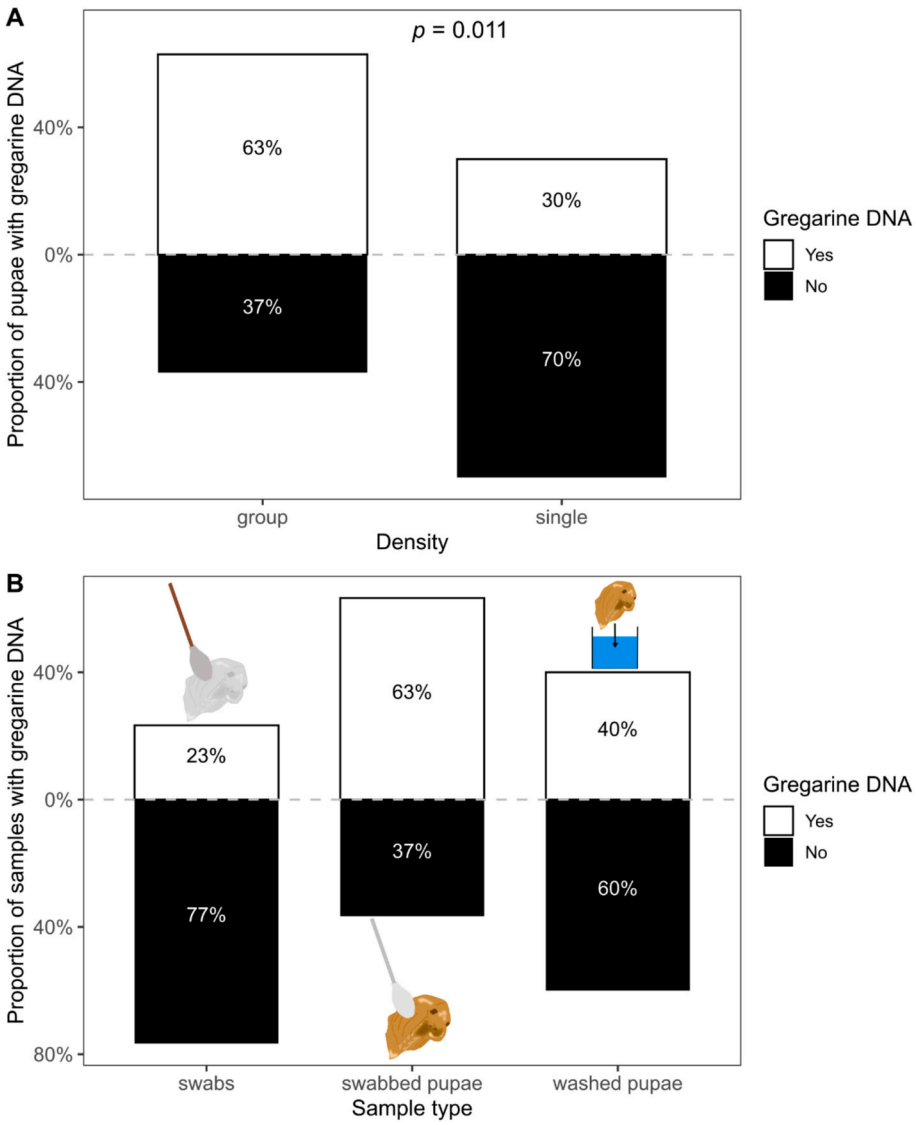

Fig. 5. Proportion of whole *Phaedon cochleariae* pupal samples that contained gregarine DNA (i.e. showed a visible band in the gel after performing a diagnostic PCR for *Gregarina cochlearium*) (A). Beetles were either kept in groups of five (group) or individually (single) as larvae and were stored in ethanol at the day of pupation. Proportion of either swabs taken from pupal surfaces (swabs), swabbed pupae or washed pupae that contained gregarine DNA (i.e. showed a visible band after PCR) (B). Samples were taken from pupae of a rearing box (150-200 individuals per box) that were not older than 24 h. PCR products were confirmed to contain the targeted gregarine DNA by sequencing and alignment with the original *G. cochlearium* sequence. Data are shown as diverging bar plots. The *p*-value is given for A (Fisher's Exact test; single $n = 20$, group $n = 89$). No statistical test was performed for B ($n = 30$ per sample type).

4. Discussion

This study revealed detailed insights into the biology of the gregarine *G. cochlearium* and its holometabolic host, the mustard leaf beetle *P. cochleariae*. In line with our expectation that most of the wild beetles would be infected with gregarines, six of the eight populations had a prevalence above 50 %. Similarly high prevalences were also found for other gregarine species including *C. zealandica* (Allison, 1969), and *D. domestica* (Rueckert and Devetak, 2017). In the closely related species *Phaedon brassicae*, the prevalence was even 100 % at three different locations across multiple sampling time points (Kim et al., 2015). However, the prevalence of infection may vary among species, depending both on the host and gregarine species (Nazimov, 2024). Furthermore, a high prevalence at the population level did not necessarily result in a high load at the individual level in *P. cochleariae*. This may result from individual differences in beetle resistance to the gregarine infection or differences in gregarine virulence among the populations, as could be observed in different populations of *Danaus plexippus* (Lepidoptera: Nymphalidae) (Altizer, 2001).

In the wild *P. cochleariae* populations of the present study, the gregarine load showed a possible temporal, but no spatial pattern, only partly supporting our hypothesis. However, since each population was only sampled once, this finding should be interpreted with care. Temporal differences in gregarine infections were found in other species (Allison, 1969, Grunberg and Sukhdeo, 2017, Locklin and Vodopich, 2010, McKinley et al., 2024). These temporal differences were linked to, for example, moulting processes of the host (Allison, 1969) or seasonal changes in host demography and body mass (Grunberg and Sukhdeo, 2017). In the present study, only adults were examined, excluding the possibility that changes occurred due to differences in developmental stages. Moreover, we found a higher gregarine load in females than in males. Adult females of *P. cochleariae* have a ca. 20 % higher body mass than males and they also consume more food during their development (Tremmel and Müller, 2013), which may enhance the likelihood that females ingest more infective spores. Sex-specific differences in gregarine load were also found in different species of dragonflies (Locklin and

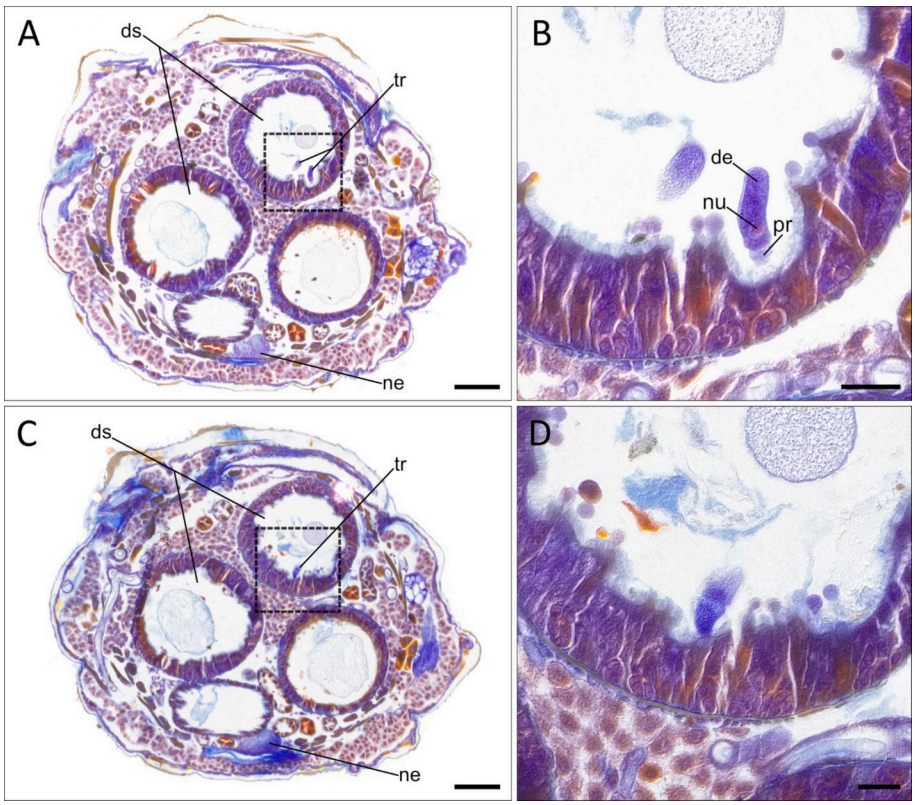

Fig. 6. Histological sections of a *Phaedon cochleariae* larva. Overviews of the whole section (A, C) and close-ups of gregarine trophozoites in the digestive system (B, D) are shown. For one trophozoite, the protomerite and deutomerite are clearly visible (B). Structures are labelled as ds: digestive system of the host, tr: trophozoite, ne: nervous system of the host, de: deutomerite, nu: nucleus of the gregarine, pr: protomerite. Scale bar: 0.5 mm (A, C); 0.1 mm (B, D).

Vodopich, 2010). In *Enallagma boreale* (Odonata: Coenagrionidae), females had a higher chance of being infected (Hecker et al., 2002), while in *Gryllus bimaculatus* it was higher for males, even though there was no difference in body size between the sexes (Simmons and Zuk, 1992).

Other explanations for differences in the prevalence and load of gregarines in their hosts may be differences in population density (Grunberg and Sukhdeo, 2017), in environmental parameters, such as water pH (Hasik et al., 2024), pollutions (Pižl and Sterzyńska, 1991), or sampling over different years (Grunberg and Sukhdeo, 2017). More research is needed to determine which factors drive the variation in gregarine abundance in *P. cochleariae*, but ideally, non-invasive sampling should be used to avoid any impacts on natural populations. One possibility to determine the prevalence non-invasively would be to collect feces of beetles in the field (i.e. by capturing the beetles, collecting their feces in tubes and releasing the beetles again), which could then be tested for presence of gregarine DNA using PCR profiling. In addition, on some *P. cochleariae* beetles collected in the wild, a freshwater mite species and its eggs could be found. In odonates, water mites and gregarines often co-occur (Hasik et al., 2024) and one study even revealed a positive correlation between water mite and gregarine prevalence (Ilvonen et al., 2018).

In our laboratory assays, the number of excreted gametocysts per *P. cochleariae* larva differed between larvae kept singly or in groups over the course of time. Thus, group-keeping likely does not generally lead to a higher gregarine load and thus reinfection rate during development amongst larvae. The number of excreted gametocysts decreased when nearing pupation and was often zero directly before pupation. This may be connected to the consumption by the larvae, which also decreases close to pupation and approaches zero directly before pupation (Barber et al., 2024). During this period, the probability of taking up infective spores thus decreases and, at the same time, the larvae also probably excrete fewer gametocysts.

In line with our hypothesis, the chance of being infected with gregarines and the number of trophozoites showed a tendency towards higher values in adults that were kept in groups as larvae than those kept individually. The absolute number of gametocysts within the same space (Petri dish) was higher for larvae kept in groups, which might result in higher reinfection probabilities in the adults. Interestingly, the age of the beetles also significantly influenced the chance of adults being infected and the number of trophozoites. One explanation for the difference between younger and older adults may be the gregarine life cycle. After pupation, gregarines may need some time to reestablish in the adult beetles and thus gregarine life-stages may be too small to be visible under a stereomicroscope. In contrast, in young larvae (2-3 days old), gregarine trophozoites are already visible (pers. observation). It is still unknown how long the life-cycle of the gregarine species *G. cochleariae* lasts for and whether trophozoites may differ in developmental speed in larvae versus adults. The observation that 21-day old, isolated adults were still infected with gregarines suggests that gregarine infections are not lost during the pupal or adult stage by removing infective stages when removing food material.

The ratio of infected to uninfected 21-day old adults closely corresponded to the ratio of gregarine DNA found in whole and swabbed pupae. This finding demonstrates that there can indeed still be gregarine life stages in and/or on the pupae and that adults might not necessarily need to reinfect themselves after pupation. Concordance between these ratios reinforces the assumption that in 10-day old adults gregarines are probably present, although they may not yet be visible. In future studies, this could be tested with PCR on gut or fecal samples of beetles collected throughout ontogeny. Gregarine DNA was also amplified from all types of pupal samples. Thus, in contrast to our hypothesis, gregarine DNA is most likely present both in the pupa and on the pupal surface, though there seems to be a higher chance of gregarine DNA being present inside the pupa. Gregarine gametocysts are excreted by beetle larvae and

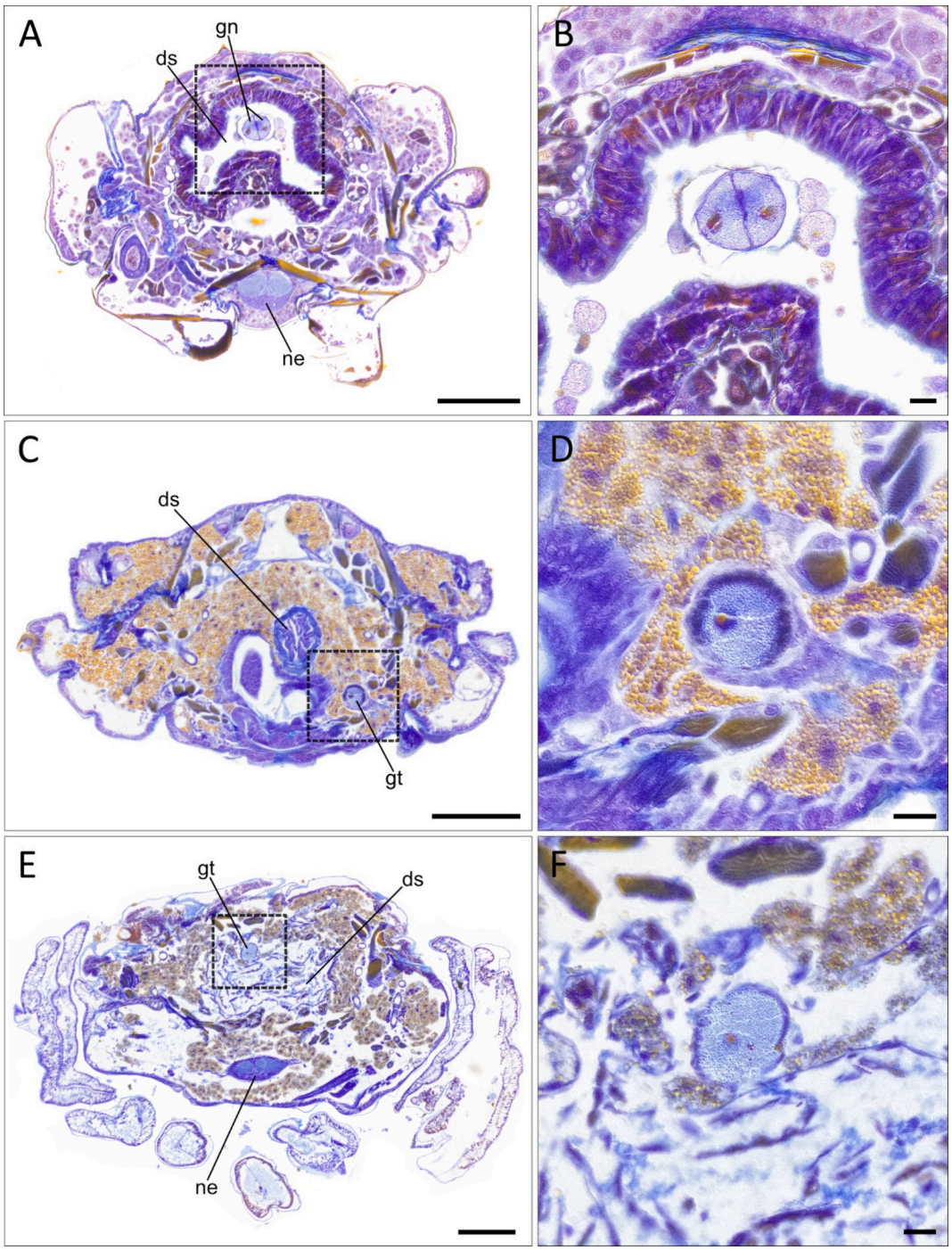

Fig. 7. Histological sections of a *Phaedon cochleariae* larva (A, B) and pupa (C-F). Overviews of the entire section (A, C, E) and close-ups of gregarine gamontocysts (B, D, F) with two gamonts still distinguishable in (B) are shown. Structures are labelled as ds: digestive system of the host, gn: gamont, ne: nervous system of the host, gt: gregarine gamontocyst. Scale bar: 1 mm (A, C, E); 0.1 mm (B, D, F).

adults and release infective spores/oocysts into the environment (Schrével and Desportes, 2016, Wolz et al., 2022a). Since the pupae were held together with many larvae of different stages in this experiment, they likely came into contact with contaminated feces and thus spores/oocysts could adhere on the surface of the pupae. In washed pupal samples, spores adhering to the surface may have been washed off, while other gregarine life stages may still be inside the pupa, nevertheless reducing the overall chance of detecting gregarine DNA inside these pupae. This might explain why gregarine DNA could only be amplified from around 40 % of washed pupae, while the corresponding proportion was 63 % for group-reared and swabbed pupae.

Histological sections confirmed that gregarines are indeed also present inside the pupae of *P. cochleariae*. Gregarine gamontocysts were found in the pupal sections, though unlike in larvae, they were no longer situated in the gut. We also did not find trophozoites in any pupal sections, which could potentially be due to chance. However, during the pupal phase of the host, it may be more advantageous for the gregarine to be in the stage of a gamontocyst rather than a trophozoite. The cyst wall of the gamontocyst may function like a protective coat (Vegni Talluri and Dallai, 1991) that keeps the gregarine stable even outside of the host gut, when host anatomy changes drastically during metamorphosis. How exactly this gamontocyst can reinfect the adult after

emergence is still unclear. In two species of *Aedes* (Diptera: Culicidae), a gregarine infection was also found to persist in the pupal stage with gametocysts forming (Sanders and Poinar, 1973) and damaging the Malpighian tubes (Barrett, 1968). However, species-specific differences were reported concerning eugregarine infection during the pupal stage in various insect species (Nowlin, 1922, Corallini and Bicchierai, 2016, Lantova and Volf, 2014). In different mosquito species, gregarines were found in the Malpighian tubes of the pupae as gametocysts, while in different sandfly species they were present as gamonts in the body cavity or as trophozoites in the gut lumen of the pupae (Lantova and Volf, 2014). Overall, host pupation can pose a considerable challenge to these gregarine species (Lantova and Volf, 2014). For some Coleopteran species, pupal stages were also described as being free of gregarines (Rodriguez et al., 2007, Allison, 1969, Kim et al., 2014). For example, in the rice flour beetle *Tribolium confusum* (Coleoptera: Tenebrionidae), infected larvae that were kept individually and in flour that did not contain gametocysts, hatching adults were no longer infected with gregarines (Thomas and Rudolf, 2010).

Having used various complementary approaches, our study provides insights into natural infection levels of *P. cochleariae* with gregarines and the gregarine status during pupation. We could find gregarine infections in every studied population. Keeping larvae in groups enhanced their likelihood to also be infected as adults. Gregarines can be transmitted in and on the host pupa. Overall, our findings support the notion of a tight relationship of this gregarine and its host.

CRediT authorship contribution statement

Alessa Barber: Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Marco Niekampf:** Methodology, Investigation. **Ira Wachendorf:** Methodology. **Joseph I. Hoffman:** Writing – review & editing, Methodology. **Sonja Rückert:** Writing – review & editing, Methodology, Investigation. **Caroline Müller:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Data curation, Conceptualization.

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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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jip.2026.108663.

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