Behavioral Ecology and Sociobiology (2025) 79:113
https://doi.org/10.1007/s00265-025-03639-8

ORIGINAL ARTICLE

The transition to a social parasitic lifestyle influences maintenance and expression of essential immune defence traits

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Received: 13 March 2025 / Revised: 15 June 2025 / Accepted: 20 August 2025 / Published online: 21 October 2025

Abstract Social parasites and their equally social hosts offer interesting systems to understand gains and losses in evolution. Yet, they have so far rarely been used to study the evolution of immune defence traits. To gain a better understanding of the evolutionary consequences of a transition to a social parasitic lifestyle on immune defence traits, here we investigated the facultative social parasitic ant *Formica sanguinea*, the obligate social parasitic ant *Polyergus rufescens* and their common social host ant species *Formica fusca* in mixed as well as single species experimental setups. We found that during sanitary care of fungal entomopathogen exposed pupae, *F. fusca* performs not only most of the sanitary behaviour of brood grooming but performs brood grooming also according to the degree of social parasite specialization, engaging in four and ten times more brood grooming in mixed setups involving *F. sanguinea* and *P. rufescens*, respectively. We also found that *F. sanguinea* engages in brood grooming and worker allo-grooming irrespective of *F. fusca* presence, while *P. rufescens* never expressed these sanitary behaviours. Consequently, the presence of *F. fusca* delayed fungal outgrowth on brood of *P. rufescence* and diminished its worker mortality risk, while *F. fusca* presence was not similarly beneficial to *F. sanguinea*. Finally, we found that in the absence of *F. fusca*, *F. sanguinea* upregulates the use of venom for sanitary purposes. We conclude that social parasites are interesting systems to study the maintenance and expression of immune defence traits and the evolutionary pressures that shape investment in these traits.

Significance statement The transition to a social parasitic lifestyle may alleviate selective pressures at maintaining or expressing immune defence traits that are essential for free-living species, with social parasites instead relying on immune services provided by their social host. We explored this in two dulotic (aka "slave-making") social parasites that differ in the degree of their dependence on the parasitic mode of life and their common host species during sanitary care of fungus-infected brood. We found that the host showed increased brood grooming rates towards social parasitic brood indicating exploitation of social immune services. We also found that the facultative social parasite has retained immune defence traits, likely to be able to survive without a host, while the obligate social parasite relies on immune services provided by the host and has lost essential immune defence traits, like many other ancestral traits associated with a free-living cooperative life style.

Keywords Social immunity · External immune defence · Behaviour · Slave maker · Venom · Evolution

Introduction

Interactions between species can drive evolutionary change with closely interacting species imposing selection pressures on one another that result in reciprocal adaptations (Thompson 2009). Well known examples include interactions between predators and prey, mutualistic partners or parasites and hosts. Parasitism is a widely distributed lifestyle and evolutionary transitions to a parasitic lifestyle are often characterized by the loss of ancestral traits that are essential for free-living organisms and the gain of new or

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Communicated by B. Baer.

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modified traits to exploit resources from hosts (Poulin and Randhawa 2015; Ebert and Fields 2020).

Insect societies provide unique opportunities to study the evolutionary consequences of a transition to a parasitic lifestyle, as they are host to numerous parasites that have adapted to life in insect societies (Schmid-Hempel 1998; Hughes et al. 2008). Among the many parasites of insect societies are also other social insect species. These social parasites, like avian and fish brood parasites (Sato 1986; Davies 2011; Kilner and Langmore 2011; Thorogood et al. 2019; Langmore et al. 2024), exploit the social behaviour of their host species for their own survival and reproduction, including brood care but also nest construction and defence as well as foraging and feeding of the adult parasite (Rabeling 2020). This form of parasitism evolved several times independently in the social hymenoptera including ants (Hölldobler and Wilson 1990; Buschinger 2009), bees (Smith et al. 2013; Lhomme and Hines 2018) and wasps (Cervo 2006). Coevolution between social parasites and their social hosts has generated a plethora of morphological, physiological and behavioural adaptations in both interacting partners (Brandt et al. 2005; Grüter et al. 2018; Fischer et al. 2020) that can be used to understand gains and losses in evolution and mechanisms of social living (Cini et al. 2015, 2019). For example, recent work has found evidence of relaxed selection in genomes of social parasitic ants and a reduction in gene families important for social living (Schrader et al. 2021; Jongepier et al. 2022; Stoldt et al. 2022; but see Smith et al. 2015). So far, however, social parasites and their hosts have rarely been used to understand the evolution of immune defence traits.

Apart from their body innate immune system, social insects possess a diversity of physiological, behavioural, and organizational adaptations to counteract the risk of disease contraction and spread within their societies (Cremer et al. 2007, 2018; Pull and McMahon 2020). Physiological and behavioural immune defence traits involved in these adaptations are regularly directed at the social environment, including the nest structure, adult nest members and developing brood (Otti et al. 2014; Tragust 2016). For example, to mitigate an impending infection risk of brood after exposure to spores of a fungal entomopathogen, formicine ants use sanitary brood grooming in conjunction with the uptake of their antimicrobial, highly acidic venom during the behaviour of acidopore grooming to reduce fungal spore load and germination ability (Tragust et al. 2013). As social parasites exploit behaviours of a social species to raise their offspring, it is likely that they also exploit immune defence traits and immune defence services of their social host for e.g. sanitary care of their brood (Cotter et al. 2019). Such exploitation will likely alleviate selection pressures at maintaining or expressing immune traits and thus may reveal whether immune traits are costly to deploy or to maintain (McKean et al. 2008; McKean and Lazzaro 2011). For example, previous studies have noted that the metapleural gland, a glandular structure unique to ants whose secretion often exhibits antimicrobial activity (Yek and Mueller 2011; Tragust 2016) and is costly to produce (Poulsen et al. 2002), is either absent or significantly reduced in many ant social parasites compared to their free-living host counterparts (Brown 1968; Sumner et al. 2003a; Yek and Mueller 2011). This suggests that ant social parasites can invest less into the maintenance and deployment of the metapleural gland and/or its antimicrobial secretion as a costly immune defence trait, likely, as they exploit the disease defence system of their host.

The aim of the present study is to gain a better understanding of the evolutionary consequences of a transition to a social parasitic lifestyle on immune defence traits. We hypothesize that the transition to a social parasitic lifestyle will have resulted in a relaxed selection on the maintenance and/or expression of immune defence traits in proportion to the degree of social parasite specialization on the host. To investigate this, we use two different dulotic aka "slave-making" social ant parasites, *Formica sanguinea* and *Polyergus rufescens*, which differ in their degree of specialization on their common social host species *Formica fusca*. Dulosis originated several times convergently across ants in distantly related clades (D'Ettorre and Heinze 2001; Trager 2013; Blaimer et al. 2015; Sanllorente et al. 2018; Romiguier et al. 2018; Borowiec et al. 2020). Dulotic species conduct raids of nearby host colonies to capture their larvae and pupae, which upon adult emergence in the dulotic nest then take over the tasks of foraging, nest maintenance and offspring rearing (Stoldt and Foitzik 2020). *F. sanguinea* is a facultative social parasite that can still maintain a functional colony in the absence of a social host, whereas *P. rufescens* is entirely dependent on a social host for colony maintenance and function (Talbot and Kennedy 1940; Savolainen and Deslippe 1996, 2001; Mori et al. 2001; Rabeling 2020; Stoldt and Foitzik 2020).

To study the consequences of a transition to a social parasitic lifestyle on immune defence traits in *F. sanguinea* and *P. rufescens*, we monitored sanitary brood care against fungal entomopathogen exposed brood in these species and their host species, *F. fusca*, in either mixed species setups (facultative social parasite with social host and obligate social parasite with social host) or single species setups (each species alone). We rationalized that a comparison of functional (host) and subfunctional (parasite) worker in mixed species setups will reveal whether and how immune traits are expressed, while a comparison of subfunctional (parasite) worker in mixed vs single species setups will reveal whether immune traits are conditionally expressed depending on the social environment, i.e. host presence. Finally, we assumed that a comparison of facultative and obligate parasite will reveal to what extent the degree of social parasite specialization on the host influences immune defence trait maintenance and expression.

Material and methods

Experimental setup and procedure

For the experiment, worker and pupae of four colony fragments of the obligate social parasitic ant *Polyergus rufescens* (Pr), and of six colony fragments of the facultative social parasitic ant, *Formica sanguinea* (Fs), together with workers of their common host ant species *Formica fusca* (Ff), were freshly collected in the field (vicinity of Białystok, N-E Poland, 15-20th August 2018) and kept in nests with plaster bottom (2L) until setup of the experiment (end of August 2018). A part of the workers of the host ant, *F. fusca*, as well as *F. fusca* pupae originated from queenright *F. fusca* laboratory colonies established in the lab of Tomasz Włodarczyk at the University of Bialystok.

Small experimental nests (Petri-dishes, Ø 55 mm) with a plaster bottom and a small indentation covered with a sheet of plastic as a nest were used to conduct the experiment. Each experimental nest contained 10% sugar-solution as food and for the duration of the experiment the plaster bottom was continuously kept moist with tap water and the food replenished according to need. Two workers of *P. rufescens, F. sanguinea,* and/or *F. fusca* were put in these experimental nests in the following three single and two mixed species combinations (Fig. 1). Experimental nests of the three single species combinations were prepared with either two workers of *F. fusca* (Ff+Ff, n=36 with six replicates each from the two queenright *F. fusca* laboratory colonies, six replicates each from two colony fragments of *F. fusca* and *F. sanguinea*, and six replicates each from two colony fragments of *F. fusca* and *P. rufescens*), two workers of *F. sanguinea* (Fs+Fs, n=36 with six replicates each from six colony fragments of *F. fusca* and *F. sanguinea*), or two workers of *P. rufescens* (Pr+Pr, n=24 with six replicates each from four colony fragments of *F. fusca* and *P. rufescens*). The two mixed species setups were prepared with either one worker of *F. fusca* and one worker of *F. sanguinea* (Ff+Fs, n=36 with six replicates each from six colony fragments of *F. fusca* and *F. sanguinea*) or one worker of *F. fusca* and one worker of *P. rufescens* (Ff+Pr, n=24 with six replicates each from four colony fragments of *F. fusca* and *P. rufescens*).

After 24 h of worker acclimation, fungus infected pupae were added to the experimental nests. To infect pupae, each pupa was rolled in a droplet of 2 µl of 4×10^8 spores/ml of the entomopathogenic fungus *Metarhizium pingshaense* (strain KVL 02–73, isolated from soil near an *Atta cephalotes* ant nest in Panama, 94% germination rate checked the day before infection) until it was completely coated. Experimental nests containing two workers of the host ant species *F. fusca* in the single species combination Ff+Ff received two infected *F. fusca* pupae. Experimental nests containing workers of the facultative social parasite *F. sanguinea* in single (Fs+Fs) or mixed (Fs+Ff) species combination received two infected *F. sanguinea* pupae and experimental nests containing workers of the obligate social parasite *P. rufescens* in single (Pr+Pr) or mixed (Pr+Ff) species combination received two infected *P. rufescens* pupae (Fig. 1). Pupae and adult workers in experimental nests always originated from a different colony or colony fragment, except for 12 replicates of the combination Ff+Fs and Fs+Fs, in which pupae of the same colony fragment were erroneously assigned to adult workers.

After adding the fungus exposed pupae to the workers, each experimental nest was filmed for 10 min with a Logitech C920 or C910 webcam to record the behaviour of the ants toward infected pupae. Filming for 10 min was repeated another two times within 8 h after infection resulting in three 10 min. videos (30 min. total per experimental nest, see also Supplementary Material 1, Supplementary description of behavioural filming procedure). The next day (24 h after adding the infected pupae to the workers), pupae were removed from the experimental nests and placed in separate petri-dishes (Ø 55 mm) with moistened filter paper to observe outgrowth of the fungal entomopathogen for the following 13 days. Workers remained in the original experimental nests and their mortality was checked daily for the following 13 days. Upon death, workers were removed from the experimental nests, surface sterilized (Lacey and Solter 2012) and placed in Petri-dishes (Ø 90 mm) with moist filter-paper to observe outgrowth of the fungal entomopathogen for at least 10 days after their death.

Behavioural observations

Evaluation of ant behaviours during the 30 min. of filmed video per experimental nest was performed manually noting the occurence (number of events) and duration of the behaviour, pupae grooming, allo-grooming, self-grooming and acidopore grooming. Pupae grooming was defined as active movement of adult worker mouthparts back and forth over the surface of pupae, allo-grooming as reciprocal grooming between the two adult workers within an experimental nest

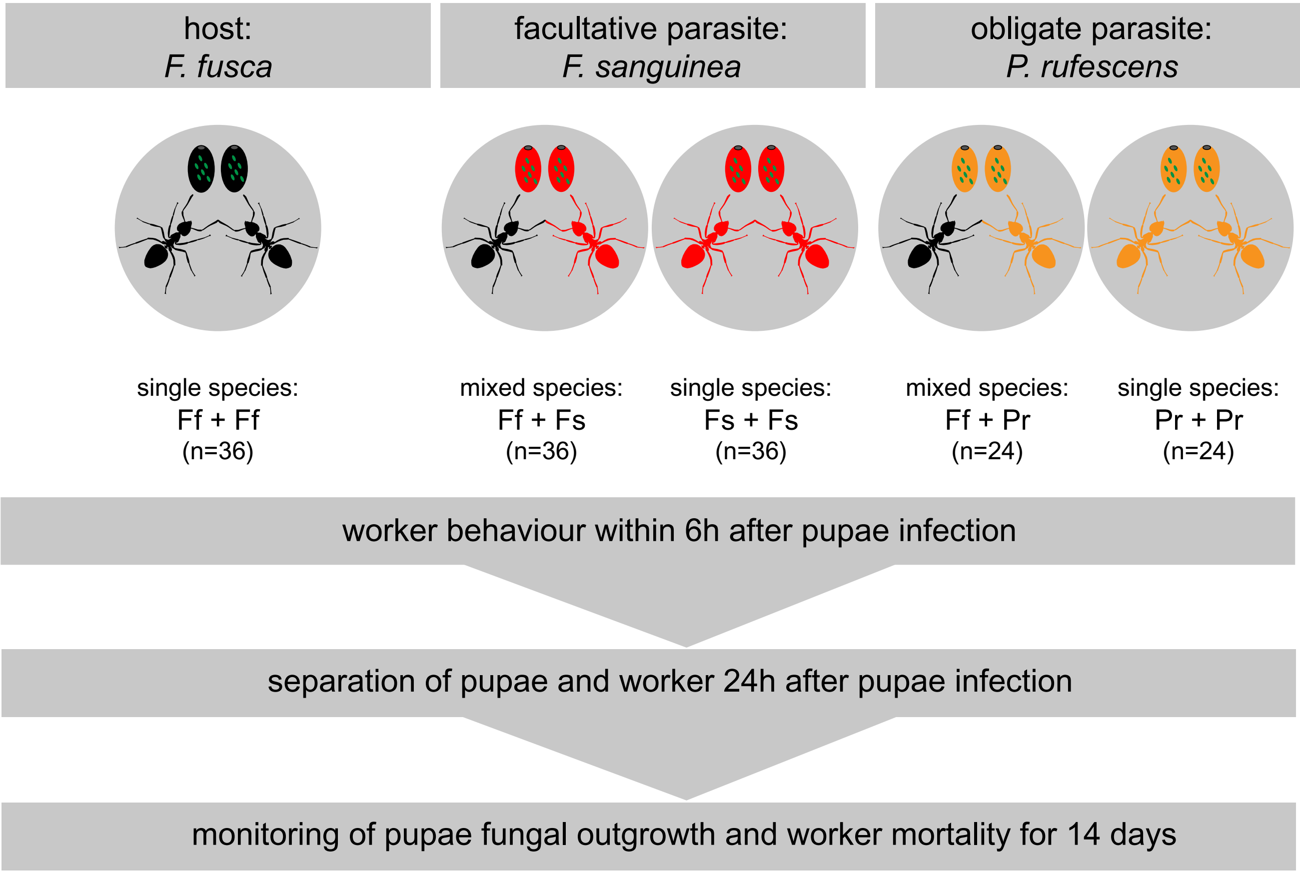

Fig. 1 Schematic drawing of the experimental design used in the present study. Each experimental nest (grey circles, Petri-dishes, Ø 55 mm) either contained the facultative social parasitic ant *F. sanguinea* (red), the obligate social parasitic ant *P. rufescens* (orange) or their common social host ant *F. fusca* (black). Worker and pupae were either combined in the single species combination Ff+Ff (two workers of *F. fusca* and two pupae of *F. fusca*, n=36), Fs+Fs (two workers of *F. sanguinea* and two pupae of *F. sanguinea,* n=36) and Pr+Pr (two workers of *P. rufescens* and two pupae of *P. rufescens*, n=24) or the mixed species combination Ff+Fs (one worker of *F. fusca* and one worker of *F. sanguinea* with two pupae of *F. sanguinea*, n=36) or Ff+Pr (one worker of *F. fusca* and one worker of *P. rufescens* with two pupae of *P. rufescens*, n=24). All pupae received an infection with the entomopathogenic fungus *Metarhizium pingshaense* (2 µl containing $4\times10.^{8}$ spores/ml, green dots on pupae) and the behaviour of workers was observed within 8 h after the infection (30 min. total per experimental nest). After 24 h pupae and workers were separated and the outgrowth of the fungus on the pupae as well as worker mortality was monitored for the next 13 days (total monitoring time 14 days)

and self-grooming as grooming of adult workers toward themselves. The bending of adult worker gaster tips between the legs towards the mouthparts was scored as acidopore grooming behaviour, separately from worker self-grooming. Finally, any contact of workers with pupae, be it active during pupae grooming and carrying of pupae or passive when walking around experimental nests and resting, was scored as pupae contact and served as a proxy for fungus exposure of workers in experimental nests. As workers of *F. fusca*, *F. sanguinea* and *P. rufescens* are morphologically distinguishable, blind evaluation of ant behaviours was not possible. However, to minimize observer bias, evaluation of ant behaviours was carried out by a student that was blind to the specific goals and hypotheses of the experiment.

Statistical analyses

All statistical analyses were performed in R (R Core Team 2024). Analyses were run separately for experimental nests containing pupae of the facultative social parasite *F. sanguinea* (single and mixed species combination Fs+Fs and Ff+Fs, respectively) and pupae of the obligate social parasite *P.s rufescens* (single and mixed species combination Pr+Pr and Ff+Pr, respectively) as pupa species identity differed between these experimental nests and might have influenced our measures of behaviour, pupae fungal outgrowth and adult mortality. Experimental nests containing workers and pupae of the host ant species *F. fusca* in the single species combination Ff+Ff were not included in statistical analyses but served as a point of reference for *F.*

fusca behaviour, pupae fungal outgrowth and adult mortality in the absence of workers and pupae of a social parasite.

Behavioural data (number of occurences and duration of the following behaviours: pupae grooming, worker allogrooming, self-grooming and acidopore grooming as well as pupae contact) was analysed in generalized linear mixed models (function “glmmTMB” in package “glmmTMB”, Brooks et al. 2017) including worker species identity as fixed predictor (three levels: host species *F. fusca* in mixed species setups, social parasite species *F. sanguinea* or *P. rufescens* in mixed species setups and social parasite species *F. sanguinea* or *P. rufescens* in single species setups) and colony or colony fragment origin of worker and pupae as well as experimental nest as random factors. In models involving the social parasite species *F. sanguinea* we also included origin of pupae and adults within the experimental nest (two levels: same or different) as an additional random factor to account for the fact that in 12 replicates of the combination Ff+Fs and Fs+Fs, pupae of the same colony fragment were erroneously assigned to adult workers. Before analyses behavioural data in experimental nests with single species combinations Fs+Fs and Pr+Pr were summed up over both workers and then divided by two to obtain worker behaviours representative of one individual in these single species combinations that are comparable to individual worker behaviours in experimental nests with mixed species combinations. Statistical models on behavioural data were run with either negative binomial (family argument: nbinom1 or nbinom2) or gaussian errors and a dispersion parameter was added according to need. Model assumptions were checked using model diagnostic tests and plots implemented in the package “DHARMa” (Hartig 2024).

Fungal outgrowth on pupae and mortality of adult workers was analysed with Cox-mixed effects models with colony or colony fragment origin of worker and pupae as well as experimental nest and origin of pupae and adults within the experimental nest as random factors (function “coxme” in package “coxme”, Therneau 2024b). Adult worker species identity (three levels: host species *F. fusca* in mixed species setups, social parasite species *F. sanguinea* or *P. rufescens* in mixed species setups and social parasite species *F. sanguinea* or *P. rufescens* in single species setups) served as a fixed predictor for worker mortality while species combination (two levels: single species or mixed species setup) served as a fixed predictor for fungal outgrowth on pupae. Pupae that were opened by workers within the first 24 h of the experiment (Pull et al. 2018; five *F. fusca* pupae from four single species Ff+Ff experimental nests, one *P. rufescens* pupa from one mixed species Ff+Pr experimental nest, and one *F. sanguinea* pupa from one single species Fs+Fs experimental nest) or that hatched during the course of the experiment (four *P. rufescens* pupae from two mixed species Ff+Pr experimental nests) were censored from the day of opening or hatching onwards in models of fungal outgrowth on pupae. The assumption of proportional hazards in Cox-mixed effects models was checked with function “cox.zph” in package “survival” (Therneau 2024a).

In all statistical models, significance of the fixed predictor was assessed by comparing models to null (intercept only) models using a Likelihood ratio test (LR-Test). Pairwise comparisons between factor levels of a significant predictor were performed using post-hoc tests adjusting the family-wise error rate according to the method of Westfall (function “glht” in package “multcomp”, Bretz et al. 2016). For the number of acidopore grooming events in experimental setups involving the social parasite species *F. sanguinea*, the null (intercept only) model resulted in a model convergence error, making it impossible to assess the significance of the fixed predictor via model comparisons using a LR-Test. We thus resorted to assess the significance of the fixed predictor in this model with a Wald-Test (function “glht” in package “multcomp”, (Bretz et al. 2016). Similarly, for the duration of pupae contacts in experimental setups involving the social parasite species *F. sanguinea* the full model resulted in a model convergence error. Inclusion of origin of pupae and adults within the experimental nest (two levels: same or different) as a fixed predictor instead of as a random effect resolved this convergence issue. Significance of the fixed predictor species identity was also in this model assessed with a Wald-Test.

Results

We found that workers of the host species *Formica fusca* perform most pupae grooming behaviour when together with the social parasitic ant species *Formica sanguinea* or *Polyergus rufescens* in mixed species setups (Figs. 2A and 3A). Compared to single species setups involving only *F. fusca* workers and *F. fusca* fungus exposed pupae (Supplementary Material 1, Table S1; mean pupae grooming occurence number ± standard error s.e: 0.53 ± 0.14; mean pupae grooming duration ± s.e.: 8.31 ± 2.83), *F. fusca* workers performed approximately four times more pupae grooming in mixed species setups involving workers and fungus exposed pupae of the facultative social parasitic ant species *F. sanguinea* (mean pupae grooming occurence number ± s.e: 2.31 ± 0.42; mean pupae grooming duration ± s.e.: 41.44 ± 8.26) and approximately ten times more pupae grooming in mixed species setups involving workers and fungus exposed pupae of the obligate social parasitic ant species *P. rufescens* (mean pupae grooming occurence number ± s.e: 5.25 ± 1.01; mean pupae grooming duration ± s.e.: 110.54 ± 33.09). Pupae grooming of *F. sanguinea* on the

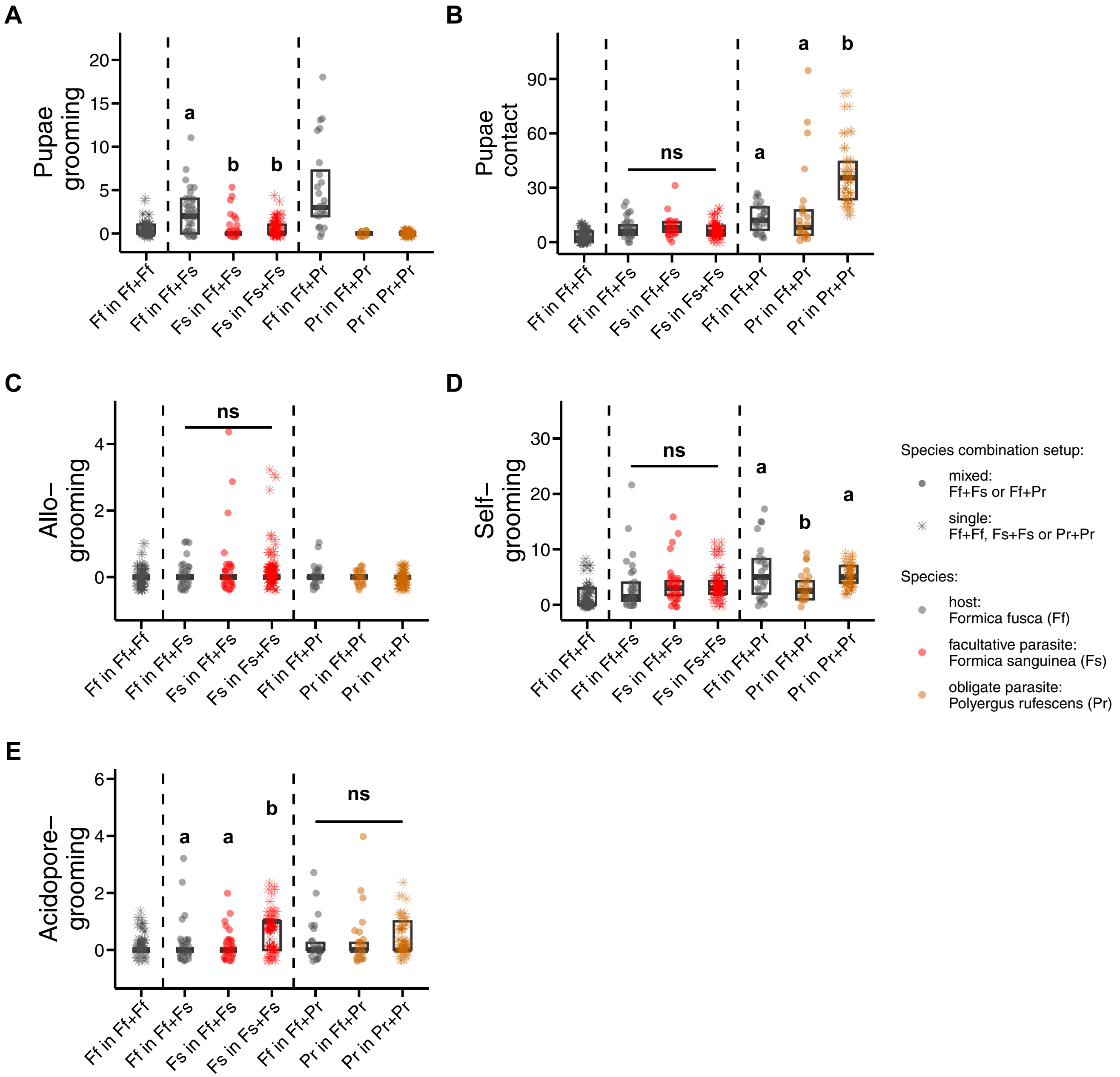

Fig. 2 Number of occurrences of the worker behaviours pupae grooming (A), pupae contact (B), allo-grooming (C), self-grooming (D) and acidopore grooming in the single (star shaped points, Ff+Ff, Fs+Fs or Pr+Pr) and mixed (round points, Ff+Fs or Ff+Pr) species setups of the host species *F. fusca* (Ff, black), the facultative social parasite *F. sanguinea* (Fs, red) and the obligate social parasite *P. rufescens* (Pr, orange). In the mixed species setups Ff+Fs and Ff+Pr the individual behaviour of the workers Ff, Fs or Pr is presented separately, while in the single species setups Ff+Ff, Fs+Fs and Pr+Pr the behaviours of the two workers were summed up and then divided by two to obtain individual worker behaviours that are comparable to individual worker behaviours in mixed species setups. Lower case letters indicate statistically significant differences at α=0.05 between workers separated by dashed lines, while ns denotes no significant differences. Points represent individual measurements and boxplots show median and interquartile range. Note that for pupae grooming (A) and allo-grooming (C) in experimental nests involving *P. rufescens* (Pr, orange), no statistical analysis was conducted, as *P. rufescens* was never observed to perform pupa grooming or allo-grooming

other hand, although being significantly lower than that of *F. fusca*, was not different between mixed and single species setups (Figs. 2A and 3A; Supplementary Material 1, Table S2, Table S3), while pupae grooming of *P. rufescens* was never observed, neither in mixed nor single species setups (Figs. 2A and 3A; Supplementary Material 1, Table S1, Table S4, Table S5).

Despite a higher pupae grooming effort in mixed species setups involving the facultative social parasitic ant *F. sanguinea* (combined *F. fusca* and *F. sanguinea* mean

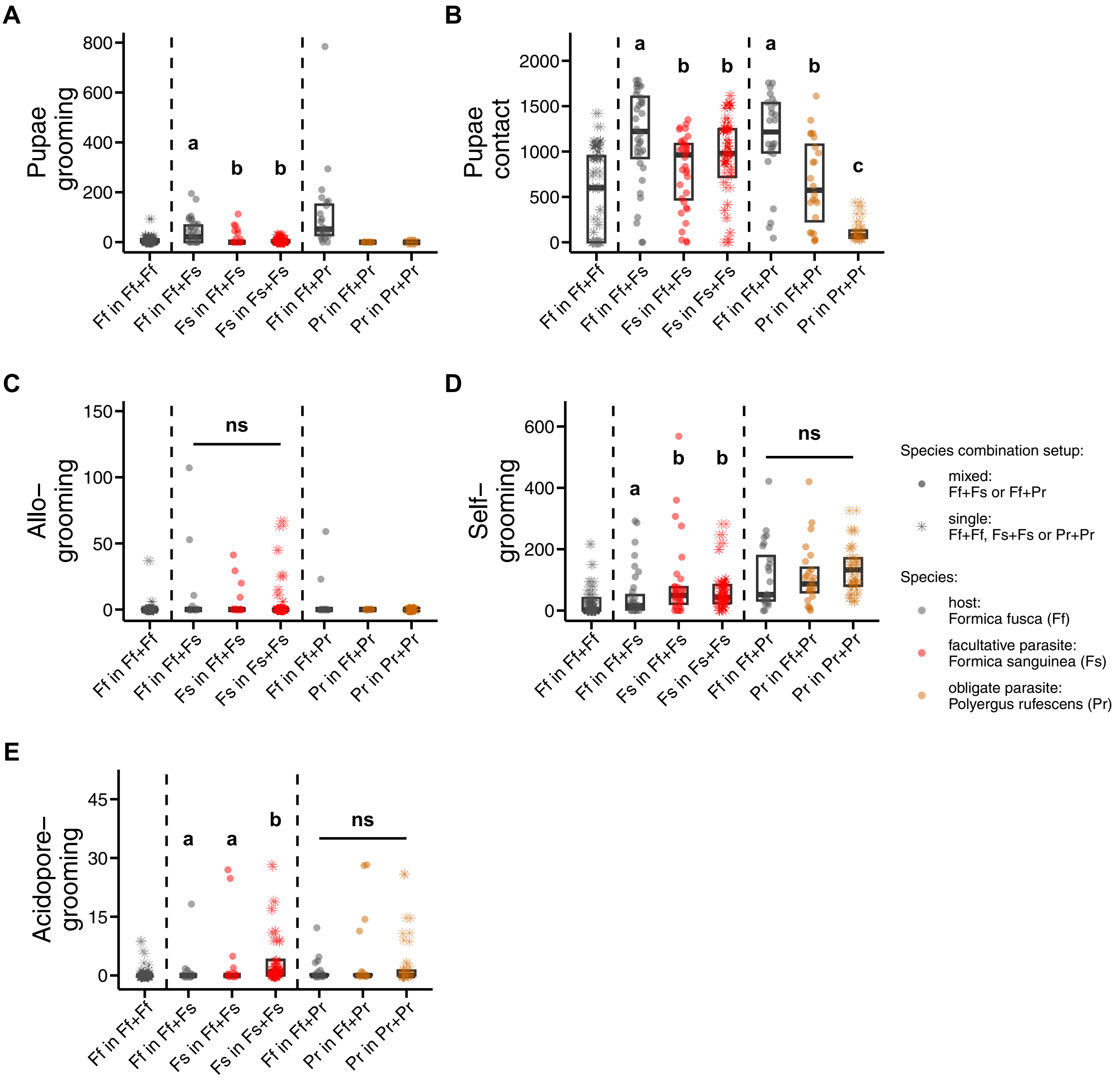

Fig. 3 Duration of the worker behaviours pupae grooming (A), pupae contact (B), allo-grooming (C), self-grooming (D) and acidopore-grooming in the single (star shaped points, Ff+Ff, Fs+Fs or Pr+Pr) and mixed (round points, Ff+Fs or Ff+Pr) species setups of the host species *F. fusca* (Ff, black), the facultative social parasite *F. sanguinea* (Fs, red) and the obligate social parasite *P. rufescens* (Pr, orange). In the mixed species setups Ff+Fs and Ff+Pr the individual behaviour of the workers Ff, Fs or Pr is represented separately, while in the single species setups Ff+Ff, Fs+Fs and Pr+Pr the behaviours of the two workers were summed up and then divided by two to obtain individual worker behaviours that are comparable to individual worker behaviours in mixed species setups. Lower case letters indicate statistically significant differences at α = 0.05 between workers separated by dashed lines, while ns denotes no significant differences. Points represent individual measurements and boxplots show median and interquartile range. Note that for pupae grooming (A) and allo-grooming (C) in experimental nests involving *P. rufescens* (Pr, orange), no statistical analysis was conducted, as *P. rufescens* was never observed to perform pupa grooming or allo-grooming

pupae grooming occurence number ± s.e.: 1.44 ± 0.26; mean pupae grooming duration ± s.e.: 26.38 ± 4.96) compared to *F. sanguinea* single species setups (combined *F. sanguinea* and *F. sanguinea* mean pupae grooming occurence number ± s.e.: 0.69 ± 0.15; mean pupae grooming duration ± s.e.: 5.92 ± 1.50; Supplementary Material 1, Table S1), fungal outgrowth probability on *F. sanguinea* pupae was not significantly different between the care by one *F. fusca* and one *F. sanguinea* worker in mixed species setups or by two *F. sanguinea* workers in single species setups (Fig. 4; COXME,

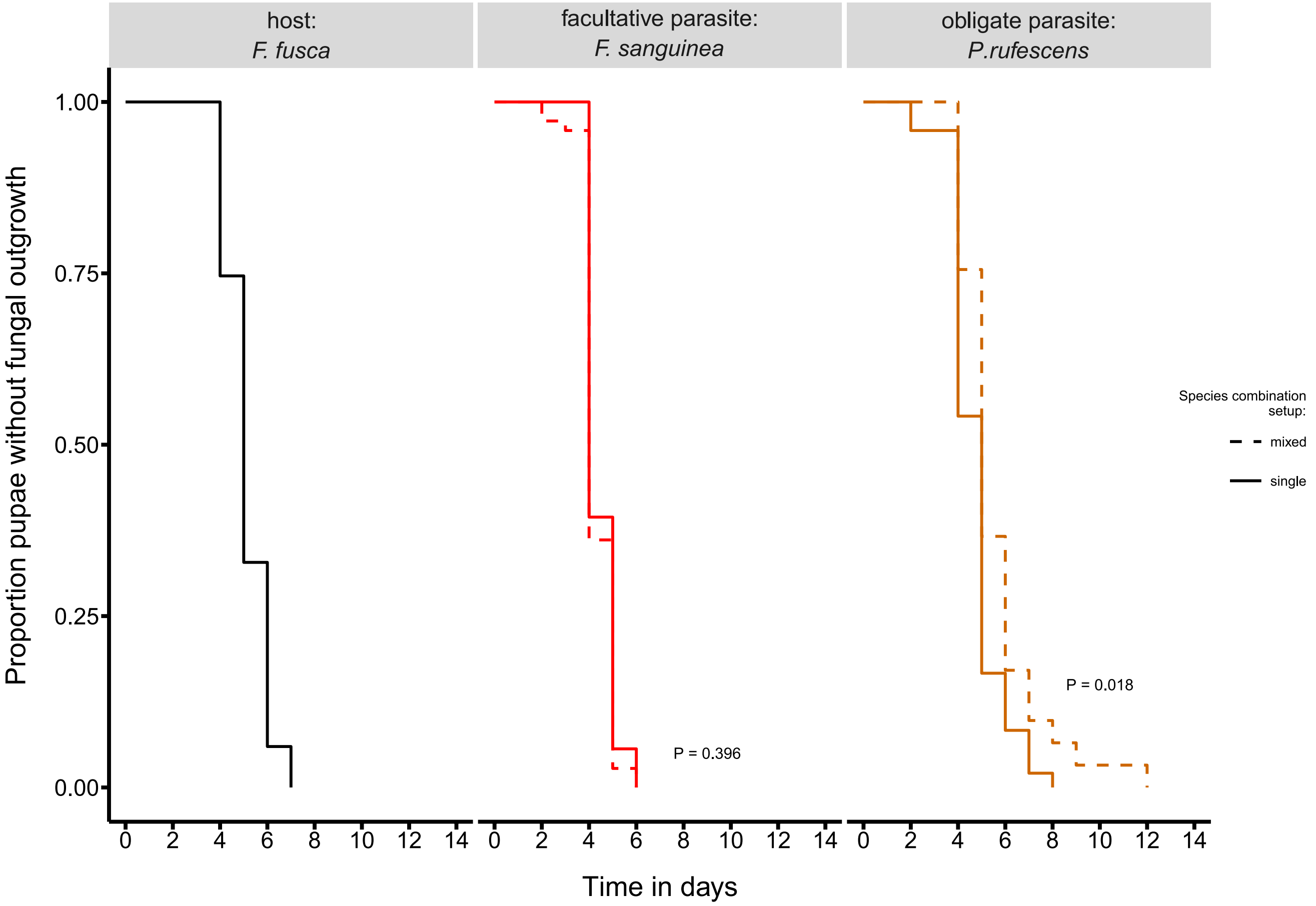

Fig. 4 Proportion of pupae without fungal outgrowth shown as Kaplan–Meier curves over the course of the experiment (14 days) in single species setups (solid line) involving the host species *F. fusca* (black) on the left, in single (solid line) and mixed (dashed line) setups involving the facultative social parasite *F. sanguinea* (red) in the middle and in single and mixed setups involving the obligate social parasite *P. rufescens* (orange) on the right. The letter P denotes the p-value in Cox mixed-effects models comparing fungal outgrowth in single vs. mixed species setups

LR-Test, χ2 = 0.720, df = 1, p = 0.396). In contrast, but in line with the result of *P. rufescens* workers never performing pupae grooming, fungal outgrowth probability on pupae of *P. rufescens* was almost twice as high in single species setups when cared for by two *P. rufescens* workers than in mixed species setups when cared for by one *P. rufescens* and one *F. fusca* worker (Fig. 4; COXME, Hazard Ratio: 1.83, LR-Test, χ2 = 5.563, df = 1, p = 0.018).

After contact with fungus exposed pupae for 24 h, workers of all three species experienced some degree of mortality for the following 13 days. In 93% of the cases (91 out of 98), mortality was followed by visible fungal outgrowth of the entomopathogenic fungus from dead bodies, indicating that direct or indirect contact to fungus exposed pupae was a major driver of worker mortality. Pupae contact occurence number and duration were not different between *F. sanguinea* workers from mixed and single species setups but contact duration was significantly lower in *F. sanguinea* compared to *F. fusca* (Figs. 2B and 3B; Supplementary Material 1, Table S2, Table S3). Despite this lower pupae contact duration, *F. sanguinea* workers in mixed species setups had, like *F. fusca* workers, an almost three times higher probability of dying over the course of the experiment (Fig. 5; *F. sanguinea* hazard ratio HR: 2.77, 95% CI: 0.95 – 8.09; *F. fusca* HR: 2.67, 95% CI: 0.88 – 8.06) compared to *F. sanguinea* workers in single species setups, a difference which was however not statistically significant (COXME, LR-test, χ2 = 3.999, df = 2, p = 0.136). This difference in mortality between *F. sanguinea* workers in mixed versus single species setups, was not related to a difference in between worker allo-grooming or self-grooming behaviour, as allo-grooming did not differ between species and experimental setups (Figs. 2C and 3C; Supplementary Material 1, Table S2, Table S3) and self-grooming, although significantly elevated in duration but not occurence number in *F. sanguinea* compared to *F. fusca*, did not differ between *F. sanguinea* workers in mixed and single species setups (Figs. 2D and 3D, Supplementary Material 1, Table S2, Table S3).

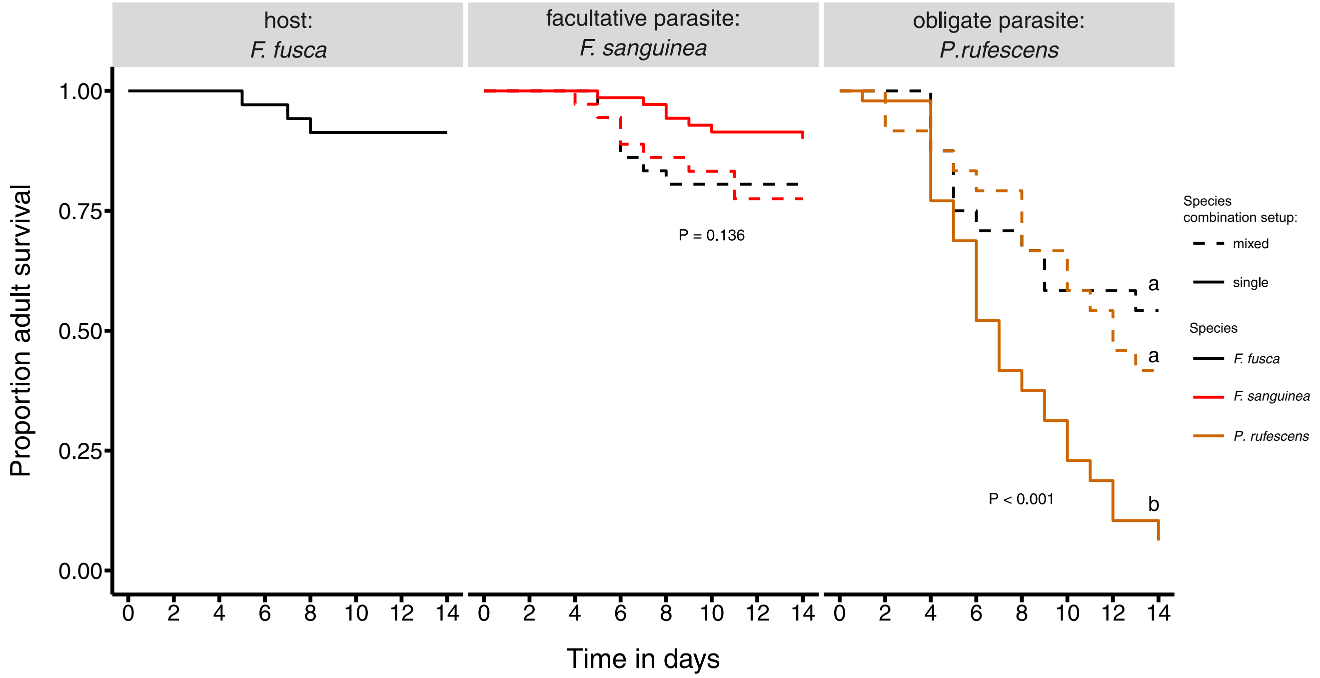

Fig. 5 Worker mortality shown as Kaplan–Meier curves over the course of the experiment (14 days) in single species setups (solid line) involving the host species *F. fusca* (black) on the left, in single (solid line) and mixed (dashed lines) setups involving workers of the facultative social parasite *F. sanguinea* (red) and the host species *F. fusca* (black) in the middle and in single and mixed setups involving workers of the obligate social parasite *P. rufescens* (orange) and the host species *F. fusca* (black) on the right. Note that the mixed species setups always involved workers of *F. fusca*, The letter P denotes the p-value in Cox mixed-effects models comparing worker survival in single vs. mixed species setups. Lower case letters indicate statistically significant differences at α = 0.05

The lower worker mortality of *F. sanguinea* workers in single species setups compared to *F. sanguinea* and *F. fusca* workers in mixed species setups did however correspond to a significantly upregulated behaviour of acidopore grooming (Figs. 2E and 3E, Supplementary Material 1, Table S2, Table S3).

Although *P. rufescens* workers were never observed to perform pupae grooming, the probability of dying was significantly higher for *P. rufescens* workers in single species setups compared to both *P. rufescens* and *F. fusca* workers in mixed species setups, the latter two not differing in mortality (Fig. 5; HR: 3.05, 95% CI: 1.52 – 6.13 and HR: 3.76, 95% CI: 1.77 – 7.99, respectively; COXME, LR-test, $\chi2 = 14.925$, df = 2, $p < 0.001$; post-hoc Tukey comparisons: *P. rufescens* in single species setups vs. *P. rufescens* and *F. fusca* in mixed species setups: $p = 0.002$ for both, *P. rufescens* vs *F. fusca* in mixed species setups: $p = 0.614$). In contrast to worker mortality patterns of *F. sanguinea*, the elevated mortality of *P. rufescens* workers in single species setups corresponded to significantly higher pupae contact occurence number of *P. rufescens* workers in single species setups (Fig. 2B; Supplementary Material 1, Table S4), which occurred however for a much lower duration compared to pupae contacts of both *F. fusca* and *P. rufescens* workers in mixed species setups (Fig. 3B; Supplementary Material 1, Table S5). Mortality differences involving *P. rufescens* were not related to differences in between worker allo-grooming, as worker allo-grooming was never observed for *P. rufescens* (Figs. 2C and 3C; Supplementary Material 1, Table S1) and occurred only rarely for a limited duration from *F. fusca* towards *P. rufescens* in mixed species setups (mean allo-grooming occurence number ± s.e.: 0.08 ± 0.06, mean allo-grooming duration ± s.e.: 3.42 ± 2.60; see also Supplementary Material 1, Table S1). Worker self-grooming differences were also unrelated to this mortality differences. Albeit worker self-grooming events occurred at significantly lower numbers in *P. rufescens* workers from mixed species setups compared to both *F. fusca* workers in mixed species setups and *P. rufescens* workers in single species setups, the latter two not differing from each other, worker self-grooming duration was not different between *F. fusca* and *P. rufescens* workers in mixed setups or *P. rufescens* workers in single setups (Figs. 2D and 3D; Supplementary Material 1, Table S4, Table S5). Finally, acidopore grooming events occurred at similar numbers and durations in *P. rufescens* and *F. fusca* workers in mixed as well as *P. rufescens* workers in single species setups (Figs. 2E and 3E; Supplementary Material 1, Table S4, Table S5).

Discussion

In this study we set out to investigate consequences of a transition to a social parasitic lifestyle on immune defence traits of the facultative social parasitic ant *Formica sanguinea*, the obligate social parasitic ant *Polyergus rufescens* and their common social host species *Formica fusca* during sanitary brood care. We found that upon exposure of social parasitic pupae to a fungal entomopathogen, *F. fusca* performs most sanitary pupae grooming behaviour when housed together with *F. sanguinea* or *P. rufescens*, more so than when housed alone toward their own pupae. This suggests that social parasitic ants may exploit immune services of their social host. We also found that *F. sanguinea* workers engage in sanitary brood grooming and worker allogrooming behaviour irrespective of the presence of *F. fusca*, while *P. rufescens* workers were never observed to engage in these sanitary behaviours, suggesting that the facultative social parasite has retained immune defence traits of its free-living, social ancestor while the obligate social parasite has lost essential immune defence traits. Finally, we found that in the absence of a host species, *F. sanguinea* significantly increases the sanitary behaviour of acidopore grooming, indicating a conditional expression of venom uptake for sanitary purposes according to the presence of a social host in this species.

The result of a higher amount of pupa grooming by *F. fusca* towards heterospecific pupae of *F. sanguinea* and *P. rufescens* than towards conspecific pupae agrees with the hypothesis that social parasitic ants exploit immune services of their hosts. Moreover, the higher pupa grooming by *F. fusca* towards heterospecific pupae of *P. rufescens* than *F. sanguinea* indicates that host immune defence exploitation is in proportion to the degree of social parasite specialization on the host. The role of social parasite specialization in our experiment is in line with results obtained in other studies. In an experiment by Mori et al. (1996) workers of two free-living host species, *Formica cunicularia* and *Formica rufibarbis*, cared for pupae of *P. rufescens* and for conspecific pupae at comparable rates, while pupae of *F. sanguinea* were not accepted by the two species although both can serve as hosts in colonies of *F. sanguinea*. Similarly, the host species *Formica occulta* has been found not to discriminate against pupae of the obligate slave maker *Polyergus breviceps* but to discriminate against pupae of the facultative slave maker *Formica wheeleri* (Zimmerli and Mori 1993). Caution is however warranted in interpreting the results of immune service exploitation and the role of social parasite specialization in our study, as our experimental paradigm did not involve setups with uninfected pupae and/or the choice between heterospecific pupae of the social parasites and conspecific pupae of the host.

Discrimination as well as acceptance of heterospecific brood of ant social parasites is influenced by many different factors (Schultner and Pulliainen 2020). For example, *F. fusca* can discriminate against non-nestmate conspecific and heterospecific eggs of a social parasite in populations with high social parasite pressure (Chernenko et al. 2011, 2013), while *F. fusca* discrimination abilities are strongly reduced in populations without parasites (Martin et al. 2011). On the other hand, the two host species *Formica gnava* and *Formica occulta* do not generally accept eggs of the obligate slave maker ant *P. breviceps* (Johnson et al. 2005) while acceptance of *P. rufescens* pupae by *Formica* host species is dependent on host species identity and in the case of *F. cunicularia* also on worker ontogeny, i.e. whether workers originated from parasitized or unparasitized colonies (D’Ettorre et al. 2002). It has also been suggested that discrimination against heterospecific brood mirrors phylogeny to a certain extent, so that more closely related species are more likely to rear each other’s brood (Schultner and Pulliainen 2020). This is however unlikely to explain the higher amount of pupa grooming by *F. fusca* in our study, as *P. rufescens* is more distantly related to *F. fusca* than *F. fusca* to *F. sanguinea* (Borowiec et al. 2020).

Failure to discriminate against parasite brood may be linked to chemical cues that are frequently used by social insect societies to distinguish foreign from colony own (Lenoir et al. 2000; D’Ettorre and Heinze 2001; Johnson et al. 2005). For example, adult individuals of obligate slave making species such as *P. rufescens* are known to mimic their hosts in terms of their cuticular hydrocarbon mixture (Yamaoka n.d.; Kaib et al. 1993; Bonavita-Cougourdan et al. 1997; D’Ettorre et al. 2002; Guillem et al. 2014; Kleeberg and Foitzik 2016) while *F. sanguinea* and other related facultative slave making species, *Formica asevra*, have been documented to promote their own recognition cues in their slaves, rather than employing a strategy of chemical mimicry (Włodarczyk 2016; Włodarczyk and Szczepaniak 2017; Scheckel 2022). However, the role of chemical cues in relation to acceptance and care of developmental stages of social parasitic ants has so far not been investigated and it might be that developmental stages of social parasitic ants emit chemical or other signals, which make them more attractive targets of brood care behaviour (Brian 1975; Creemers et al. 2003; Casacci et al. 2013; Pull et al. 2018).

Finally, acceptance, discrimination and care of heterospecific social parasitic brood might also be dependent on the social context, i.e. the presence of host brood (Sumner et al. 2003b). The general exploitation of care behaviours by social parasites comes with fitness costs to the hosts, resulting in selection acting on defensive traits (Grüter et al. 2018). Therefore, for enslaved host workers a line of defence might be to either destroy parasite brood or to reproduce within parasitic nests, a defensive line that has also been termed slave-rebellion (Achenbach and Foitzik 2009; Achenbach et al. 2010; Czechowski and Godzińska 2015). An experimental paradigm involving a choice between heterospecific pupae of social parasitic species and conspecific pupae of the host would shed light on this possibility.

In addition to the higher amount of pupa grooming by *F. fusca* towards social parasitic pupae our study also found that workers of the obligate social parasite *P. rufescens* never engaged in social immune services such as sanitary brood grooming or worker allo-grooming behaviour. Consequently, fungal outgrowth on *P. rufescence* pupae was expedited in the absence of host *F. fusca* workers. Previous studies investigating the participation of social parasites in colony tasks including food gathering, nest maintenance as well as general brood care also found that social parasites contributed little or nothing to these tasks but that some can expand their behavioural repertoire in the absence of a social host species (Sakagami and Hayashida 1962; Kutter 1968; Wilson 1975; Dobrzańska 1978a, b; Stuart and Alloway 1985; Topoff et al. 1985; Mori and Le Moli 1988; Czechowski 1989; Hunter and Mathis 2020). In line with this, we also found that *P. rufescens* increases pupae contact frequency but decreases pupae contact duration in the absence of *F. fusca*. Such a context dependent expression of vestigial brood care behaviours has already been suggested by Dobrzanska (1978a, 1978b) for *P. rufescens* and is known for anti-parasite behaviours in other animals (Rayner et al. 2022).

Moreover, we found that while acidopore grooming was still performed by *P. rufescens* and unaffected by the presence of *F. fusca*, *P. rufescens* showed a significantly increased self-grooming rate but not duration in the absence of *F. fusca*. Despite this, *P. rufescens* workers experienced an almost ten-times higher mortality followed by fungal entomopathogen outgrowth in absence of *F. fusca* compared to its presence. This might suggest an impaired ability of *P. rufescens* to prevent fungal infection. To behaviourally defend against fungal infections, formicine ants do not only mechanically remove spores during grooming but also use their venom after acidopore grooming as a chemical disinfectant (Tragust et al. 2013, reviewed in Tragust 2016). Interestingly, *P. rufescens* has only a very low percentage of formic acid in its venom (Stumper 1952, reviewed in Koch et al. 2025), the main active venom component against fungal entomopathogens. This might partly explain an impaired ability of *P. rufescens* to defend against fungal infections. However, the high mortality of *P. rufescens* in the absence of *F. fusca* might also have been indirectly caused. Although in our study we could observe the occurrence of stomodeal, i.e. mouth to mouth, trophallaxis between workers of *P. rufescens*, feeding in obligate slave-makers in general (Buschinger 2009; Rabeling 2020) and in *P. rufescens* specifically (Beck 1961) is often assumed to be absent or impaired and dependent on the presence of a host ant. Thus, whether the lower survival of *P. rufescens* in the absence of *F. fusca* is a direct consequence of an impaired ability to defend itself against fungal pathogens, indirectly caused by their inability to appropriately feed themselves or a combination of both will need to be established in future experiments.

In contrast to *P. rufescens*, we found that workers of the facultative social parasite *F. sanguinea* groomed their fungal entomopathogen exposed pupae at similar rates in single and mixed species setups and that fungal outgrowth of *F. sanguinea* pupae was unaffected by the presence of the host. The latter result is somewhat surprising as we found that the presence of *F. sanguinea* and *F. fusca* workers in mixed species setups resulted in a higher combined pupae grooming effort than the presence of only *F. sanguinea* workers in single species setups. Consequently, control of the fungus should have been better in the presence of *F. fusca* workers. Moreover, we found that *F. sanguinea* workers suffered almost three times less mortality in the absence of *F. fusca* host workers, although their contact with fungal exposed pupae in the form of pupa grooming and general pupae contact was similar in mixed and single species setups as was the level of personal care in the form of self-grooming and social care in the form of allo-grooming. How can we explain these results? A possible answer lies in the fact that in the absence of *F. fusca* we observed a significantly increased occurrence and duration of acidopore grooming in *F. sanguinea* workers. Assuming an equal and proportional uptake of venom during the occurrence and duration of acidopore grooming, this may indicate an increased use of venom for sanitary purposes on behalf of *F. sanguinea* in the absence of a host species. The venom gland reservoir of *F. sanguinea* is bigger than that of *P. rufescens* (Beck 1972) and while the formic acid content of the venom of *P. rufescens* in relation to its body weight is the lowest of all so far measured formicine ant species, *F. sanguinea* has a formic acid content between 3.7–6.4%, similar to that of *F. fusca* (3.3–3.6%) and other Serviformica species (Stumper 1952; Koch et al. 2025). An increased use of venom for sanitary care of pupae as well as personal care of workers in the absence of a host can both explain why fungal outgrowth on *F. sanguinea* pupae was not influenced by the presence of *F. fusca* and why *F. sanguinea* workers survived better in the absence of *F. fusca*. This interpretation of our findings warrants further investigation in experiments specifically targeted at testing whether venom use for sanitary purposes is indeed condition dependent in *F. sanguinea*, unlike other social immune defence traits in superorganisms (Cremer and Pull 2024). Further experiments will also need to investigate whether venom unrelated factors, unbeknown and uncontrolled for, might have contributed to and/or produced the mortality patterns observed in *F. sanguinea*.

Overall, our results indicate that the social parasitic ants *F. sanguinea* and *P. rufescens* exploit immune services of their social host ant *F. fusca* and that while the facultative social parasite *F. sanguinea* has retained essential immune defence traits to have the option as either free-living (social) or parasitic species, the obligate social parasite *P. rufescens* relies on services provided by its social host and has lost essential immune defence traits, like many other ancestral traits associated with a free-living cooperative life style. Moreover, our study found evidence suggesting that formicine ant social parasites are able to invest less into the maintenance, in the case of *P. rufescens*, and use, in the case of *F. sanguinea,* of the venom gland and its antimicrobial secretion likely as a consequence of their exploitation of the disease defence system of their host and to reduce the cost associated with venom as an immune defence trait. Further experiments will be needed to validate these results. In addition to experiments outlined in the discussion above, a potential fungal-dose dependency of our experimental outcomes and the generality of a reduced maintenance and use of immune defence traits in proportion to the degree of social parasite specialization on the host, warrant further investigation. With respect to the latter, future investigations should include several different facultative and obligate dulotic ant species together with their hosts, preferably not only including dulotic species in the ant genera *Formica* and *Polyergus*, but also *Rossomyrmex* (Sanllorente et al. 2018; Borowiec et al. 2020). Despite these limitations, our study supports the view that morphological, physiological and behavioural adaptations of social parasites with respect to immune defence offer interesting systems to understand gains and losses in evolution (Cini et al. 2015, 2019) that can be scrutinized to study costs imposed at maintaining and using immune defence traits or to study evolutionary pressures that shape investment in these traits (Tragust 2016).

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00265-025-03639-8.

Acknowledgements The authors would like to thank Christian Jünemann for help with the data collection. S.T. is supported by the German Science Foundation (DFG TR 1972/1-1, 520989725), P. B. was funded through an iDiv MLU Support Fund for Integrative Biodiversity Research in Saxony-Anhalt (MLU|BioDivFund; Project-No.: R02020830–16).

Author's contribution S.T. designed the study. T.W. collected the ants and S.T. and P.B. performed the experiment. S.T. analysed the data and wrote the first draft of the manuscript. All authors read, revised and commented on subsequent versions of the manuscript and approved the final manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. Not applicable.

Data availability Raw data generated in this study with a corresponding input file for R as well as a code and script file for R to reproduce figures, tables and analyses can be found in this article and its supplementary material (Supplementary Material 2 and Supplementary Material 3 for data and script file, respectively).

Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

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