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A Potential Collective Defense of *Drosophila* Larvae Against the Invasion of a Harmful Fungus

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The establishment of a collective defense is an important means of controlling the spread of harmful microbes in group-living animals. Collective defenses are associated with costs resulting from the investment in resources and the risk taking of infections or the exposure to microbial toxins for the performing individual and are often assumed to have evolved in (eu)social insects, like bees and ants, as a result of close contact and pathogen transmission between nestmates. We hypothesize that collective antimicrobial defense mechanisms are potentially also found in insects that exhibit simpler forms of sociality or even mere aggregation behavior. The larvae of the saprophagous fruit fly *Drosophila melanogaster* develop in high-density aggregations on rotting fruits, which are often colonized by insecticidal filamentous fungi. Here we show that fruit fly larvae suppress the invasion of a harmful fungus not only by the summative effect of individuals at high densities but also because larger groups of larvae at the same density can control fungal growth more efficiently. We achieved the necessary manipulation of the group size by increasing the number of larvae in proportion to an increase in habitat size, thereby excluding the effect of density changes on fungal growth as a confounding factor. We found evidence that part of the variation in the ability to suppress the fungus in this group size-dependent manner can be explained by genetic variation at the insects’ foraging \((f-or)\) locus. Group size therefore influences the extent to which the larval aggregates suppress the spread of a harmful fungus. This indicates a potential collective defense against habitat invasion by pathogenic fungi. The selection pressure on the efficiency of this potential defense strategy may contribute to the evolution of aggregation behavior in non-(eu)social insects.

**Keywords:** aggregation, density dependence, *Drosophila*, foraging phenotype, group size, group living, pathogenic fungi, social immunity

**INTRODUCTION**

Allogrooming, application of antimicrobial substances, “weeding,” and removal of diseased broods are traits that enable group-living insects—dependent on their social organization—to defend themselves collectively against the invasion of harmful microbes (Cremer et al., 2007; Meunier, 2015; van Meyel et al., 2018). “Social” or “collective” imply that antimicrobial behavioral and physiological strategies are performed jointly and/or toward each other. Therefore,
collective defense constitutes an additional shield that complements the protection achieved by individual defenses.

The individual-level processes mediating the collective defense are thought to have evolved in response to increased pathogen transmission in eusocial insects—the eusocial framework (Cremer et al., 2007; Cotter and Kilner, 2010; Kappeler et al., 2015; Nuotclà et al., 2019). For this reason, collective antimicrobial defense is often seen in the context of social immunity (Cremer et al., 2007). However, as highlighted by Meunier (2015) and van Meyel et al. (2018), antimicrobial defense strategies, such as release of self-produced antibiotics, hygienic behavior, or allogrooming, are found not only in eusocial but also in non-(eu)social and even solitary insects. If these strategies are performed collectively and result in positive feedback on individual fitness, such a feedback may be a selective force favoring group living and the social complexity of such groups—the group living framework (Meunier, 2015; Biedermann and Rohlf's, 2017; van Meyel et al., 2018; Nuotclà et al., 2019). According to the group living framework, one would expect to observe collective defense strategies in insects that are non-eusocial or in those that form only semi-social aggregations (Nuotclà et al., 2019). In such aggregations, resistance against harmful microbes can be achieved passively due to density-dependent effects (Figure 1). However, this seemingly improved control of microbes is a mere numerical summation of the effects of otherwise competing individuals, i.e., an effect that would not be considered as collective defense or social immunity (Cotter and Kilner, 2010; Meunier, 2015; van Meyel et al., 2018). Thus, if an antimicrobial trait is important for a possible collective defense in a given system, one would expect that the efficiency of the expression of this trait increases with increasing group size, independent of the actual density of individuals (Figure 1). To test this, careful experimental manipulation is required to control for the confounding effect of density.

Within this group living framework, we hypothesize that an ancestral stage in the early evolution of collective antimicrobial defense is due to the advantages of simple semi-social aggregation behavior (Biedermann and Rohlf's, 2017). Semi-social aggregations are widespread in insects that breed in ephemeral resources, e.g., dung, carrion, fruits. On such resources, the developing insect larvae have to cope with numerous saprotrophic and often harmful microorganisms, insecticidal bacteria, and fungi (Janzén, 1977). Aggregative behaviors of non-social insects have frequently been observed in association with such microbes [numerous examples described in Wertheim et al. (2005)]. In the fruit-inhabiting Drosophila model system, the harmful effect of molds on larval development is based on a constitutive and inducible formation of insecticidal secondary metabolites (Caballero Ortiz et al., 2013), which trigger several disease symptoms or even kill the larvae (Wölffe et al., 2009; Trienens et al., 2010). However, fruit fly larvae in high-density aggregations can successfully suppress the spread of insecticidal mold fungi, e.g., Aspergillus sp. and Penicillium sp., in their feeding habitat and thus achieve higher per capita fitness (Rohlf's, 2005). The formation of fungus-controlling aggregations not only seem to matter in Drosophila (Hodge et al., 1999; Wertheim et al., 2002; Rohlf's and Hoffmeister, 2003) but also contribute to the management of detrimental fungi in facultative eusocial ambrosia beetles, for example (Biedermann and Taborsky, 2011; Nuotclà et al., 2019). The insects probably achieve the destruction of hyphal tissue by using their mouth parts, e.g., by chewing the mycelium. In addition, chemical components such as the release of antimicrobial peptides could also be involved in the inhibition of fungal growth.

As outlined above, in such aggregations, group size-specific effects could be masked by insect density-dependent suppression of harmful fungi or not exist at all, i.e., there may be no collective behavior resulting from interactions between group members that contributes to the suppression of harmful microbes. For this reason, we investigate here the hypothesis that larger groups of fruit fly larvae have a higher capacity to suppress the growth of a harmful fungus, which would indicate the involvement of an antimicrobial collective action. To test this, we manipulated the group size of Drosophila melanogaster larvae while not altering their density and quantified the suppression of the insecticidal fungus Aspergillus nidulans by these groups. This fungus species is representative of various taxa of widespread molds that negatively influence insect development in different decomposer systems by the formation of insecticidal secondary metabolites (Janzén, 1977; Hodge et al., 1999; Drott et al., 2017; Küntzer, 2018). Additionally, in order to gain a first insight into how selection might favor different behaviors in the face of microbial invasion, we also investigated the extent to which genetic variation in movement behavior, determined by allelic variation in the foraging locus (Anreiter and Sokolowski, 2019), contributes to the suppression of fungal growth in the breeding substrate of Drosophila.

MATERIALS AND METHODS

Organisms

Experimental D. melanogaster larvae were obtained from an outbred lab population that was established from field-caught flies in 2006 (Wölffe et al., 2009). The population has been kept since with non-overlapping generations, where larvae were reared under moderate densities in several flasks containing breeding substrate. Subsequently, the enclosed flies have been joined as one population in a cage provided with food and water. A flask with breeding substrate was attached to the cage to allow egg laying. This strain was used in Experiment 1 and Experiment 2.

Aspergillus nidulans (strain RDIT2.3) colonies were grown on malt extract agar (30 g malt extract, 5 g peptone, and 20 g agar Kobe I, filled up to 1 L with purified water). Fungal conidiospores for inoculation of experimental setups were harvested from 7-day-old colonies by rinsing the colony with saline solution (8.6 g NaCl, 300 mg KCl, 350 mg CaCl₂ per liter of demineralized water). Conidiospore titer was estimated using a hemocytometer (Neubauer improved). Experiment preparations were conducted in a laminar flow cabinet. We used heat-sterilized tools and substrate. Eggs and plastic frames were treated with sodium hypochlorite to eliminate adherent microbes.
General Experimental Setup

To manipulate insect group size rather than insect density, we designed experimental arenas of different sizes. For this, we created rectangular plastic frames that consisted of plastic strips (polyester strengthened with paper inlay, 15 mm height, 250 µm thickness, the length depends on the arena size), which were pleated at three folding edges and fastened at the fourth to obtain frames of respective sizes (Figure 2A). The plastic frames were filled with fruit agar (50/50 v/v% mashed banana, water, and 36 g agar/L) and placed in 50- and 90-mm Petri dishes, respectively. We created a clearly defined fungal growth zone by forcing the unidirectional growth of the fungi. Filter paper strips (3 mm × arena length) were soaked in a suspension of conidiospores (1 million conidia per microliter); this conidia titer was used to ensure the development of a homogeneous growth front of the fungus. The air-dried filter strips were then placed flush with the frame on the fruit agar (Figure 2B). All setups were pre-incubated at 25°C and constant darkness for 48 h. Then, the larvae were transferred to the arenas and further incubated at 25°C and constant darkness.

To quantify the expansion of the fungi, we photographed the arenas 12, 24, 36, and 48 h after larval transfer and measured the substrate area covered by fungal tissue (“a” in Figure 2C) growing out of the filter paper (“f” in Figure 2C; using ImageJ; //imagej.net for measurement). In the insect treatment, the peripheral growth zone of the fungi was severely damaged by the feeding activity of the larvae. There were still few hyphal fragments visible in this growth zone, but these were not quantifiable, so we did not consider these fragments to be part of an intact colony (Figure 2C). To reduce variation due to observational errors, we performed three independent measurements of each colony and used their means for statistical analysis. The expansion of fungi on the substrate was quantified after 12, 24, 36, and 48 h of confrontation with the larvae as the average expansion per millimeter of growth front (in mm).

Experiment 1: Suppression of the Insecticidal Fungus Aspergillus nidulans by Differently Sized Groups of Drosophila melanogaster Larvae

To test the effect of larval group size difference on the expansion of a harmful fungus, in this first experiment, we used plastic frames of 25 × 25 mm and 50 × 25 mm, i.e., the latter was twice as large as the former. The plastic frames were filled with 4 and 8 ml fruit agar, respectively, inoculated, and incubated as described above. Before the transfer of larvae, the arenas were randomly assigned to the larval treatment. We transferred 10 first-instar larvae into the small arenas and 20 of them into the large arenas, resulting in a larval density of 0.4 larvae per millimeter of fungal growth front (larvae/mm) or 1.6 larvae per cm² arena in both arena types. In total, there were 10 replicates per treatment, with an additional 10 larval-free control colonies per arena type. Due to the aberrant growth of the fungus in one small arena, we were forced to reduce the number of the previously assigned replicates for the larval treatment to nine. The arenas were prepared and incubated, and fungal growth was quantified as described in “General Experimental Setup”. The expansion of the fungi on the substrate was quantified after 12, 24, 36, and 48 h of confrontation with the larvae as average expansion per millimeter of growth front (in mm).

Experiment 2: Suppression of the Insecticidal Fungus Aspergillus nidulans by Differently Sized Groups of Drosophila melanogaster Larvae at Two Levels of Larval Density

The aim of this second experiment was twofold: first, to verify the previous observation made in Experiment 1 by using a wider range of group sizes and, second, to test whether the effect of
group size can be observed at different larval densities. For this purpose, arenas with a size of 25, 50, or 75 × 25 mm were filled with 4, 8, or 12 ml fruit agar, respectively. The filter paper strips inoculated with conidia were adapted to the size of the arena. By reducing and increasing the number of larvae, we changed the differences between the groups compared to Experiment 1. We transferred groups of 5, 10, and 15 larvae, resulting in a constant larval density of 0.2 larvae per millimeter growth front of the fungus, or 20, 40, and 60 larvae, resulting in a constant larval density of 0.8 larvae per millimeter growth front of the fungus (Supplementary Figure S1). We conducted 20 replicates per combination; however, in individual cases, the final replicate number was reduced due to contaminations of the substrate. The arenas were prepared and incubated, and fungal growth was quantified as described in “General Experimental Setup.”

Moreover, to corroborate an overall density-dependent effect on fungal pathogen expansion in the insects breeding sites, we additionally transferred larvae in all group sizes to all arena sizes. This resulted, over all arena types, in a density range of 0.06 to 2.4 (that is, five larvae in 75-mm arenas to 60 larvae in 25-mm arenas; a full list of combinations is provided in Supplementary Tables S1, S2 and Supplementary Figure S1). The results of this density range experiment can be found in the Supplementary Material.

Experiment 3: Effect of Larval Drosophila melanogaster Foraging Phenotype on the Suppression of Insecticidal Aspergillus nidulans

Since the fly larvae actively seek out the fungus, the containment of the fungus is largely based on the behavior of the larvae. Based on this, we have analyzed whether genetic variation in the locomotor activity of D. melanogaster larvae contributes to differences in the suppression of fungal expansion. We used two D. melanogaster strains, “rover” and “sitter,” which differ in allelic variation at the foraging (for) locus and express two different behavioral phenotypes: the larvae of the “rover” phenotype travel longer distances during foraging compared to the “sitter” larvae (Sokolowski, 1985; de Belle et al., 1989). We used arenas of 25 and 50 × 25 mm and transferred groups of 10 and 20 larvae, respectively, with 10 replicates each. Fungal growth was quantified at 12, 24, 36, and 48 h after larval transfer.

To test whether there is indeed a variation in larval behavior that can be correlated with the variation in fungal suppression, we used the images taken after 12, 24, 36, and 48 h to count the number of larvae that were in contact with the fungal colonies. The arenas were prepared and incubated, and fungal growth was quantified as described in “General Experimental Setup”.

Statistics

In Experiment 1, we first tested whether the arena size had an impact on the expansion of unconfronted fungal colonies and, further, the null hypothesis that differences in larval group size do not significantly affect fungal expansion by applying generalized estimating equations (GEE) and fitting marginal generalized linear models (GLM), which take the repeated measures into account. We specified the model with a Gaussian distribution and identity link function, an auto-regressive correlation structure (on the basis of equally spaced cluster), and a fully iterated jackknife estimator. After model selection, fitting was estimated with Q-Q plots.

In concordance, we tested in Experiment 2 whether larval group size and larval density contributed to the variance in the data using the GEE. Different to the above-stated model specification, here we fitted a Gaussian distribution with a log link function (all other settings were the same as above). The effect of
differences in the locomotion behavior of *Drosophila* larvae on fungal expansion (Experiment 3) was likewise analyzed using the GEE procedure with the same model settings as for Experiment 2.

The aggregation of larvae at the rim of the fungal colony was analyzed with a glm specifying a quasibinomial distribution of larval counts in contact with fungal colony to account for overdispersion. In model specifications, larval group size was nested within foraging type, and time was specified as a random factor.

All statistic procedures were performed in R (R Core Team, 2019; "geepack" 1.2-4, Højsgaard et al., 2005; "car" 3.0-3).

**Bioethics Statement**

All aspects of the present research have been conducted in compliance with national and international legislation and fundamental ethical principles. Experimental work with the invertebrate *D. melanogaster* does not require specific measures regarding the Animal Welfare Act.

**RESULTS**

**Experiment 1: Suppression of the Insecticidal Fungus *Aspergillus nidulans* by Differently Sized Groups of *Drosophila melanogaster* Larvae**

In our first experiment, the group size of *D. melanogaster* was 10 or 20 larvae with a constant density of 0.4 larvae per millimeter fungal growth front. Colony expansion was recorded 12 to 48 h post-larval transfer (Figure 3). This early phase of interaction of *Drosophila* larvae with filamentous mold fungi—about 25% of the insects’ development time—determines whether the fly larvae are able to suppress the growth of insecticidal mold fungi sustainably and survive until pupation (Rohlf et al., 2005). In unconfronted colonies, the arena size had no effect on fungal expansion (analysis of Wald statistic $\chi^2 = 0.031$, $p = 0.86$; Figure 3, Table 1, Q-Q plot Supplementary Figure S2A). Compared to these unconfronted controls, the colonies of fungi exposed to fruit fly larvae expanded more slowly. In addition, the spread of the fungus was more suppressed by the larger group, with the effect of group size increasing over time (analysis of Wald statistic, time $\times$ group size: $\chi^2 = 28.94$, $p < 0.001$; Figure 3; Table 1, Q-Q plot Supplementary Figure S2B).

**Experiment 2: Suppression of the Insecticidal Fungus *Aspergillus nidulans* by Differently Sized Groups of *Drosophila melanogaster* Larvae at Two Levels of Larval Density**

To verify the group size effect observed in Experiment 1, we repeated and extended this experiment by increasing the number of arena types and thus the number of possible group sizes. Moreover, group size variation was tested at two different density levels, 0.2 and 0.8 larvae per millimeter growth front of the fungus. As in Experiment 1, the mycelial expansion was significantly reduced, with increasing group size as a function of time (analysis of Wald statistic, time $\times$ group size: $\chi^2 = 36.56$, $p < 0.001$; Figure 4 and Table 2), thus verifying the
Factors influencing the expansion of *Aspergillus nidulans* in Experiment 2. Results of the most parsimonious GEE model (after backward elimination of non-significant factors) are shown.

| Explanatory factor | Model estimate ± SE | Wald | p-value |
|--------------------|----------------------|------|---------|
| Intercept          | 0.8890 ± 0.0278      | 1021.88 | < 0.001 |
| Time               | 0.0167 ± 0.0007      | 571.92  | < 0.001 |
| Density            | −1.2100 ± 0.0893     | 167.91  | < 0.001 |
| Group size         | 0.0073 ± 0.0017      | 18.77   | < 0.001 |
| Time × Density     | 0.0241 ± 0.0022      | 117.39  | < 0.001 |
| Time × Group size  | −0.0002 ± 0.0001     | 31.38   | < 0.001 |
| Alpha              | 0.534 ± 0.076        |        |         |
| $R^2_{marg}$       | 0.852                |        |         |

Previously made observation. Moreover, density also contributed significantly to the reduction of fungal expansion in a time-dependent manner (analysis of Wald statistic, time × density: $\chi^2 = 67.40, p < 0.001$; Figure 4, Table 2, Q-Q plot Supplementary Figure S2C). This means that while the effect of group size is apparent at later points in time, the effect of larval density on fungal expansion is stronger at earlier points in time. However, the absence of a statistically significant three-way interaction between time × density × group size (analysis of Wald statistic $\chi^2 = 1.48, p = 0.22$, Table 2) suggests that the overall effect of larval group size on fungal control is not different at the two densities.

The density–range experiment revealed highly significant differences in fungal growth expansion. With increasing larval densities, fungal growth was increasingly strongly suppressed (analysis of Wald statistic, time × group size difference × density–range: $\chi^2 = 65.20, p < 0.001$; Supplementary Figure S3, Supplementary Table S3, Q-Q plot Supplementary Figure S2D).

**Experiment 3: Effect of Larval *Drosophila melanogaster* Foraging Phenotype on the Suppression of Insecticidal *Aspergillus nidulans***

To explore the possible contribution of genetic variation in larval locomotor activity to the suppression of *A. nidulans*, we quantified the effect of allelic variation at the *rover* locus on foraging phenotype (Supplementary Figure S2E). Generally, larger groups suppressed fungal expansion more effectively than smaller groups in a time dependent manner (analysis of Wald statistic, time × group size: $\chi^2 = 6.0, p = 0.004$; Figure 5A and Table 3, Q-Q plot Supplementary Figure S2D).

Variation in the foraging phenotype possibly leads to differences in larval behavior directed against the fungus, which might provide an explanation for why the genotypes differ in their quantitative effect on the fungus. To test this possibility, we calculated the proportion of larvae found to aggregate at the growth front of the fungus and are in touch with the mycelium. Based on the analysis of the images taken 12 to 48 h post-larvae transfer for fungal growth measurements, we found that a higher proportion of larvae of the *sitter* phenotype in direct contact with the fungal colony than the larvae of the *rover* phenotype (analysis of deviance, type II: *rover* vs. *sitter* $F_{1,155} = 51.65$, $p < 0.001$; time: $F_{1,155} = 126.40, p < 0.001$; time × group difference).
FIGURE 5 | Effect of genetically determined variation in the locomotion behavior of Drosophila melanogaster larvae on the spread of the insecticidal filamentous fungus Aspergillus nidulans. (A) Inhibition of the fungal expansion by rover and sitter Drosophila larvae in different group sizes (constant density = 0.4 larvae/mm). Fungal growth was determined for four time points after larval transfer as the average expansion of the fungus into the substrate surface per millimeter of growth front. Lines represent the fitted model estimates for the four time points (GEE; Table 4). (B) Proportion of larvae in direct contact with the fungal colonies at four different time points after larval transfer (Table 4).

size: $F_{1,155} = 6.449$, $p = 0.0121$; Figure 5B, Table 4, Q-Q plot Supplementary Figure S2F). Further, the decline of larvae in contact with the colony at time point 48 h coincides with the generic behavior of D. melanogaster larvae to leave the substrate surface around the beginning of the third larval stage, where they start digging deeper into the substrate.

DISCUSSION

The existence and effect size of collective actions, such as defense, can be inferred from the extent to which the efficiency of performing a particular fitness-related “task” increases with increasing group size, while the density of individuals involved remains constant (Cotter and Kilner, 2010; Dornhaus et al., 2012). In a series of independent experiments, we tested whether larvae of the fruit fly D. melanogaster can collectively increase the efficiency of such a “task” that is directed against an insecticidal fungus invading the insects’ habitat. While insect density clearly is, as expected, a significant factor in the suppression of the harmful fungus A. nidulans, we repeatedly found that larvae in larger groups, regardless of these density effects, suppressed the invasion of this fungus into their habitat more sustainably. This indicates that the emergence of a collective action in larger larval
Aspergillus nidulans found in contact with fruit fly larvae are attracted (Stötefeld et al., 2015). In because both yeast and mold fungi emit volatile metabolites to weaken, or even kill fly larvae. It therefore remains to be seen to manage the microbial environment and thereby stabilize habitat conditions to the benefit of their fitness (Biedermann and Taborsky, 2011; Hwang and Lin, 2013; Meunier, 2015). On the contrary, it is assumed that collective actions have their evolutionary origin in comparatively simple semi-social aggregation behaviors, such as the Drosophila system, which lack comparatively complex behavioral structuring, nest building, and altruism (Meunier, 2015; Biedermann and Rohlf, 2017). Our finding thus supports the suggestions made by Meunier (2015) or van Meyel et al. (2018) that, in tracing the origin of complex collective defenses, such as social immunity, we need to better understand the extent to which non-(eu)social insects have evolved group-based strategies to manage the microbial environment and thereby stabilize habitat conditions to the benefit of their fitness (Biedermann and Rohlf, 2017). Several recent studies have started revealing the mechanisms underlying social interactions, incl. collective actions, between Drosophila larvae, as related to food choice (Lihoreau et al., 2016), spatial foraging (Dombrovski et al., 2017), microbiome- and pheromone-mediated mutual attraction (Mast et al., 2014; Venu et al., 2014), and physical contact (Otto et al., 2016). Drosophila fruit flies and their interactions with microbes could thus be a suitable model system that would allow us to better understand how social complexity in insects can evolve from behavior in simple social precursor systems.

### DATA AVAILABILITY STATEMENT

Raw data are available as Supplementary Material in Table 1.XLSX and additional information in Data Sheet 1.docx.
AUTHOR CONTRIBUTIONS

MR and MT designed the study, did the statistical analyses, and wrote the manuscript. MT performed the experiments and generated the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00079/full#supplementary-material
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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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