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An alternative pathway to eusociality: Exploring the molecular and functional basis of fortress defense

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Some animals express a form of eusociality known as "fortress defense," in which defense rather than brood care is the primary social act. Aphids are small plant-feeding insects, but like termites, some species express division of labor and castes of aggressive juvenile "soldiers." What is the functional basis of fortress defense eusociality in aphids? Previous work showed that the acquisition of venoms might be a key innovation in aphid social evolution. We show that the lethality of aphid soldiers derives in part from the induction of exaggerated immune responses in insects they attack. Comparisons between closely related social and nonsocial species identified a number of secreted effector molecules that are candidates for immune modulation, including a convergently recruited protease described in unrelated aphid species with venom-like functions. These results suggest that aphids are capable of antagonizing conserved features of the insect immune response, and provide new insights into the mechanisms underlying the evolution of fortress defense eusociality in aphids.

KEY WORDS: **Aphids, eusociality, fortress defense, immunity, *Pemphigus obesinymphae*.**

Ants, termites, some bees, and wasps express an advanced form of social behavior known as eusociality. They form complex social groups characterized by overlapping generations, division of labor, and brood care. Studies of eusocial insects provide the modern foundations for sociobiology (Hamilton 1964; Wilson 1971; Hölldobler and Wilson 2008), but it has long been recognized that social complexity varies across taxa (Choe and Crespi 1997; Thorne and Traniello 2003; Korb and Heinze 2008). There appears to exist qualitatively distinct forms of social life histories, implying a broad set of selective and ecological conditions operating in social evolution (Alexander et al. 1991; Kapheim et al. 2015). In some species, defense against predation and exploitation are the primary expression of altruistic traits, and brood care—a hallmark of eusociality—is largely absent. These species have been described as "fortress defenders," and include some termites, aphids, thrips, and noninsect species, such as snapping shrimp, all of which lack cooperative brood care and instead produce soldier-like morphs specialized for defense (Crespi 1994; Queller and Strassmann 1998). Defense-based sociality is thought to be favored in conditions in which family groups occur in or on defendable and limited food resources (Alexander 1974; Crespi 1994; Queller and Strassmann 1998). The discovery of such societies was a key advance in the development of a broad ecological theory of social evolution (Costa 2006; Bourke 2011). However, outside of termites (Korb et al. 2015), the functional basis of defense remains to be fully determined for species expressing the "non-canonical" fortress defense eusociality.

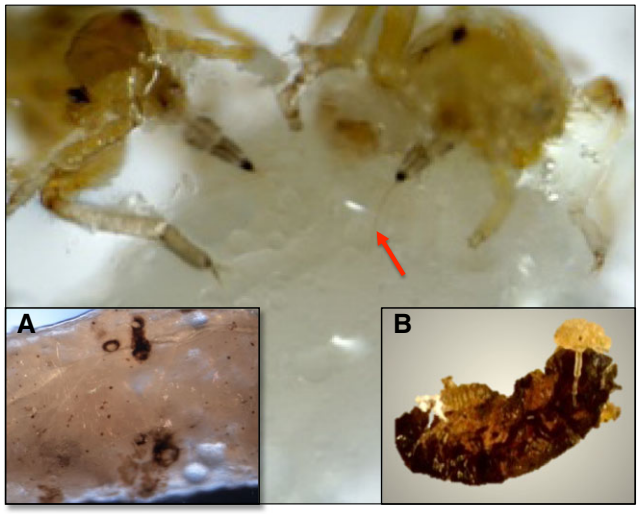


Figure 1. The soldiers of many social aphid species attack other insects with their mouthparts. The picture shows two *Pemphigus obesinymphae* aphid soldiers attacking a fly larva. The red arrow indicates the needle-like stylet of the aphid inserted into through the cuticle of the fly larva. (A) The many darkened wounds on a larva after attack. (B) A heavily melanized fly larva with aphid soldiers still attached.

Pemphigus obesinymphae (Aphididae: Eriosomatinae) is a social aphid that forms relatively simple social groups within tumor-like galls they induce on the leaves of poplars (*Populus* spp.; Salicaceae) in North America (Moran 1993). These groups are composed of a reproducing female and her daughters. The daughters, all of which are first instar nymphs, express aggressive behaviors toward predators, and are called "soldiers." Sociality is rare in aphids, but has evolved independently several times in two subfamilies, the Hormaphidinae and the Eriosomatinae (Stern and Foster 1996). Soldiers are the defining feature of sociality in aphids. They typically swarm and overwhelm other insects, wounding and even killing them by piercing their victims' integuments with their mouthparts or other morphological weapons (Fig. 1; Video S1; Kurosu and Aoki 1988; Moran 1993; Foster 1990; Foster and Rhoden 1998; Shibao et al. 2010; Hattori et al. 2012; Lawson et al. 2014). Species with soldiers are commonly referred to as "social," and those without as "nonsocial," Social and their nonsocial relatives share many ecological traits. For example, all social species form tumor-like galls on their host plants, inside which they feed and reproduce parthenogenetically, but closely related nonsocial species also form galls. In some species, such as the hormaphidine, *Tuberaphis styraci*, an "advanced" form of sociality occurs, and true castes of sterile soldiers are formed that coexist with normal, fertile siblings in colonies of thousands of aphids (Aoki and Kurosu 2010). In others, such as the eriosomatine *P. obesinymphae*, more "primitive" forms of sociality are expressed. All first instar nymphs are aggressive and soldier-like, but are not sterile and may lack obvious morphological specialization, and colony sizes are comparatively small (Moran 1993; Abbot 2009).

From a functional standpoint, it is not entirely clear how social aphids are lethal. Aphids are soft-bodied herbivorous insects, and it is not obvious how they successfully defend themselves against the diverse insects that attack or exploit their colonies. Previous work on a hormaphidine aphid, *T. styraci* (Aphididae: Hormaphidinae), revealed that soldiers can egest a proteolytic insecticidal venom when they attack (Kutsukake et al. 2004; 2008). Whether chemical defenses are general or even required features of sociality in aphids, and how they have evolved and function, are not yet clear (Kutsukake et al. 2008).

As is the case in many eusocial aphids, the nymphal soldiers of the North American eusocial aphid *P. obesinymphae* attack other insects by swarming and piercing them with their needle-like mouthparts (Abbot 2015). In preliminary work, we used *Drosophila* larvae as surrogates for the predatory fly larvae (syrphids and chaemymiids) that specialize on social aphids in the genus *Pemphigus* (Abbot et al. 2001), and observed that when *P. obesinymphae* aphid soldiers attack, melanized injury is clearly evident (Fig. 1; Video S1). Melanization plays many roles in invertebrates, including in pigmentation, wound healing, and innate immunity (Ashida and Brey 1995; Hoffmann 2003; Cerenius and Soderhall 2004; Tang 2009). Because the metabolic process that generates melanin (the prophenoloxidase cascade, or proPO) involves the hydroxylation and oxidation of phenols into quinones (Bolton et al. 2000; Nappi and Christensen 2005) and the generation of various reactive cytotoxic intermediates, the proPO response is tightly regulated and localized to minimize nonspecific damage to vulnerable tissues, a process known as "auto-reactive self harm" (Sadd and Siva-Jothy 2006). We thus hypothesized that an important facet of how aphid soldiers are effective in defense against other insects involves their ability to antagonize and disrupt the insect immune response. We tested the idea that the phenotypic effect of attack by aphid soldiers on other insects is inherently immunological, and therefore is a key to understanding how fortress defense eusociality evolves in this group.

Using the functional advantages provided by Drosophila as models for the larval dipteran predators that commonly prey on gall-forming aphids, we demonstrate that attack by aphid soldiers antagonizes the insect phenoloxidase and wounding response, resulting in melanized wounds and the overexpression of genes in the melanization pathway. We show that the effect is not general to other components of innate immunity, but appears restricted to melanization itself. We demonstrate partial rescue in the pseudopredator with impaired melanization, implicating the melanization response in the lethality of aphid soldiers. Comparative transcriptomic sequencing of a social aphid and a nonsocial congener

identified secretory candidates in aphid soldiers, including various proteolytic enzymes that are upregulated, one of which is homologous to the venomous cysteine protease previously characterized in an unrelated eusocial species (Kutsukake et al., 2004, 2008), and therefore possibly convergently recruited for social functions in *P. obesinymphae*. It remains to be demonstrated if and which secretory candidates antagonize the insect immune response. However, one implication of these results is that in aphids, traits that facilitate life on plants facilitate life in social groups as well. Traits for exploitation of plant tissues and manipulation of plant immune responses (Mather et al., 2017) likely contribute to the effectiveness of their defense against predators. We interpret these results in terms of the adaptations all aphids possess for feeding on plants—in essence, an herbivorous ground plan out of which sociality evolves.

Material and Methods

COLLECTIONS

In the summers of 2012 and 2013, eusocial *P. obesinymphae* were collected from cottonwood trees (*Populus deltoides*) in Dyersburg, Tennessee, and nonsocial *Pemphigus populi-caulis* were collected from *P. deltoides* in the vicinity of Nashville, Tennessee. Wild-type *Drosophila melanogaster* lines were sampled from stocks at Vanderbilt University in Nashville. Drosophila mutant lines were ordered from the Bloomington Drosophila Stock Center (Indiana University) with the following genotypes and stock IDs: ple^4 st^1 e^1/TM3, Sb^1 (tyrosine hydroxylase mutant; BS3279), Df(2R)min, Pu^1/T(2;3)ap^{Xa}, ap^{Xa} (GTP cyclohydrolase mutant; BS173), Dp(1;2;1)AT/+; hk^1 Ddc^7 (Dopa decarboxylase mutant; BS360), and y^1 w^*; P{EP}$Dhpr^{G6439}$ (dihydropteridine reductase mutant; BS27207). Mgat1^1/CyO-GFP Drosophila larvae were used as general controls for using mutant flies in our experimental framework, and for nonspecific effects of aphids on *D. melanogaster* larvae. Mgat1, or mannosyl (α-1,3-)-glycoprotein β-1,2-N-acetylglucosaminyltransferase 1, is involved in N-linked glycosylation, and the Mgat1^1/CyO-GFP heterozygotes exhibit some impairment of the N-linked glycan biosynthesis, but are otherwise phenotypically similar to wild type (Sakar and Schachter 2001). hb^{12} st^1 e^1/TM3, Sb^1 (*hunchback*; BS1755) was used as an additional control for the ple^4 st^1 e^1/TM3, Sb^1 stock. All experimental manipulations used Drosophila third instar larvae (L_3).

DEGREE OF *DROSOPHILA* MELANIZATION

To visually evaluate and quantify melanization in larvae attacked by aphid soldiers, we used double-blind observational assays coupled with quantifications of phenoloxidase activity. We quantified the melanization phenotype before and after attack by aphid soldiers by using a subjective visual assay of melanization. We collected *P. obesinymphae* galls in the vicinity of Nashville, Tennessee in July 2013. To facilitate observation, galls were split in half, and we placed individual Drosophila larvae, a surrogate for common Dipertan predators of galling aphids, in galls with aphids or in galls in which the aphids had been removed. Larvae remained in galls for 1 h. Each *D. melanogaster* larvae was photographed before attack, immediately following attack, after removing the attacking soldiers, and 1 h following attack. Only Drosophila larvae being attacked by at least 10 aphid soldiers were included in the study. Using a double-blind assay, the Drosophila larvae were assigned a score based on the proportion of the body melanized: 1 (no observable melanization) to 4 (surface completely melaninized; Fig. S1). The sample size for each group was greater than or equal to 10.

PHENOLOXIDASE ASSAYS

Phenoloxidase levels in Drosophila tissues were measured using a simple procedure based on the transformation of 3,4-dihydroxyphenyl-alanine (L-dopa) to dopachrome in the presence of phenoloxidase, measured as function of optical density, as described by Lario et al. (1993). Larvae were singly placed in freshly collected *P. obesinymphae* galls for 45 min, and subsequently removed with a small paintbrush. A control group of larvae of similar size was not exposed to the aphids and was labeled the "unattacked" group. To differentiate between phenoloxidase activation during mechanical wounding of the epithelia by aphid stylets, and circulating phenoloxidase activation in the hemolymph, Drosophila hemolymph was extracted from both the attacked larvae and the control group using fine needles. After hemolymph extraction, the larval midguts were removed using a pair of dissecting tweezers. The remaining cuticle was then placed in a 1.5 mL Eppendorf tube and ground by a pestle. The tubes were centrifuged briefly, and the liquid portion was removed by a pipette for use in the phenoloxidase assay, in addition to the extracted hemolymph. Immediately after extraction, the hemolymph was transferred to ice-chilled 96-well plate containing 50 μL of 20 mM Tris buffer, pH 7.0, with 1% sodium citrate. The cuticle fluids were transferred by pipette to wells containing the same mixture. After the hemolymph and cuticle fluids were collected, 50 μL of 3,4L-dopa, 5 mg/mL in Tris buffer, were added to each well. Mixtures were incubated at 32°C for 45 min, and the wells were subsequently read in a spectrophotometer at a wavelength of 490 nm. Blank wells containing 50 μL Tris buffer and 50 μL L-dopa in Tris but no hemolymph or cuticle fluids were used as controls.

SURVIVORSHIP ASSAYS AND RESCUE EXPERIMENTS

To examine if the overactivation of the melanization pathway affected the survivorship of *D. melanogaster* following attack by aphid soldiers, we compared the survivorship of Drosophila

mutants deficient in the melanization response to wild-type Drosophila. To measure survivorship, we introduced larvae to a gall, monitored the larvae every 20 min and noted the time to death, defined as when the larvae stopped moving. Time to death data were log-transformed prior to analysis and tested for normality using the Shapiro-Wilk test (Sokal and Rohlf 1995). Statistical comparisons were performed by way of Student *t* tests on the mean time to death of each mutant line attacked and unattacked by aphid soldiers (Sokal and Rohlf 1995). We then compared mean time to death of each attacked mutant line against the mean time to death of attacked "control" wild-type larvae using a one-way ANOVA, correcting for multiple comparison's using Dunnett's (1955) post hoc test (Dunnett 1955). For each line evaluated, sample sizes were 10 *Drosophila* larvae or greater. All analyses were performed in JMP version 11.0 (SAS Institute Inc., Cary, NC, 1989-2007). Reported *P* values are two-tailed.

ACTIVATION OF MELANIZATION PATHWAY—DROSOPHILA

To examine which genes were differentially regulated following attack by aphid soldiers, we used quantitative polymerase chain reaction (PCR) to assess expression levels of multiple immune genes in two experimental groups of *D. melanogaster* larvae: (1) larvae attacked by *P. obesinymphae* for 1 h; or (2) larvae placed in an empty gall for 1 h. One hour was chosen because it was prior to the average observed time of death caused by aphid soldiers (Lawson et al. 2014), but enough time to observe a melanization response. To provide a standardized reference and to establish whether our assays with aphids were sensitive enough to detect immunological activation, we also measured the expression of each of these genes in Drosophila that were stabbed with sterile needles (Supporting Information Methods and Materials). Total RNA was extracted from whole body individual larvae of each treatment using the RNeasy Mini Kit (Qiagen, Valencia, CA) and eluted in 40 μL of RNase-free water. RNA samples from at least six individuals were obtained for each treatment. Extracted RNA was treated with DNase to eliminate any residual genomic DNA. For first strand cDNA synthesis, 2 μL of RNA was added to 4 μL of 5 × iScript reaction mix, 1 μL of iScript reverse transcriptase and RNase-free water to a total volume of 20 μL (Bio-Rad, Hercules, CA). Samples were incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. All cDNA was stored at −20°C.

Primers for genes in multiple immune pathways were used (Table S1). All had a primer efficiency over 95%. Real-time PCR (qPCR) was carried out on a Bio-Rad Real-Time PCR Detection System in 25 μL reactions and based off from Coggins et al. (2012). Forward and reverse 0.3 μM primers were mixed with 12.5 μL Power SYBR Green Master Mix (Applied Biosystems) and added to 300 ng cDNA and molecular grade water. All qPCR reactions done in triplicate on different plates. Reactions were carried out: 40 cycles at 90°C for 15 sec, 60°C for 1 min, followed by a melt curve up to 95°C. Threshold cycle (CT) values from the Bio-Rad software were used for expression analysis.

Expression levels of mRNA were calculated with the comparative CT method. CT values were normalized to the expression of a nonregulated internal control gene, Actin 5C (Act5c) (Livak and Schmittgen 2001; Ling and Salvaterra 2011) and calibrated to mean expression of larvae from empty galls using the comparative CT method: $\Delta\Delta CT = [\Delta CT \text{ treatment X sample 1}] - [\text{average } (\Delta CT \text{ calibrator sample})]$, where treatment X represents the different manipulations to larvae. Fold change was calculated by $2^{-\Delta\Delta CT}$ (Ponton et al. 2011). Mean fold change from at least six individual samples was charted for each treatment. For validation of primer efficiencies, ΔCT values were calculated with serial dilutions of template cDNA. The data were fit using linear regression. Absolute values of the slope less than 1 were assumed to have similar efficiencies (Ling and Salvaterra 2011).

IDENTIFICATION OF CATHEPSIN B ORTHOLOGS

For the cathepsin B orthologs in *P. obesinymphae*, RNA was isolated from three experimental groups of aphids collected in mid-summer in the vicinity of Nashville, Tennessee and flash frozen immediately after collection: (1) first instar soldiers; (2) apterous third/fourth instar nymphs; (3) winged alates. Groups of aphids totaling 0.05 g were pooled in each group, and 20 samples were prepared per group. RNA extraction and isolation was performed using the total RNA isolation reagent (TRIR; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Previous work had generated partial sequences of three *catB* paralogs (J. N. Mezzanotte and P. Abbot, unpubl. data). cDNA was prepared using a GeneRacer Kit SuperScript III RT Model (Invitrogen Life Technologies) to synthesize RACE-ready cDNA, according to the manufacturer's instructions. From these partial sequences, we designed gene-specific primers (GSPs) for each of the three *catB* paralogs (Table S2). RACE PCR amplifications were carried out at volumes of 10 μL, containing 1X Invitrogen 10X buffer, 0.8 mM of each deoxynucleoside triphosphate and $MgCl_2$, 0.5 μM of each primer, 0.05 U/μL Hot STARTAQ polymerase, sterile PCR-grade water, and approximately 5–10 ng of the cDNA. Reaction conditions were one cycle at 95°C for 2 min 30 sec; 35 cycles of 95°C for 20 sec, 56°C for 20 sec, and 72°C for 45 sec; followed by one cycle at 72°C for 2 min 30 sec. In some cases, nested PCR reactions were performed using the GeneRacer 5′ Nested Primer and or the GeneRacer 3′ Nested Primer. Successful reactions were cloned via a pCR2.1-TOPO vector (Invitrogen Life Technologies) and TOPA TA cloning kit using Top 10 chemically competent cells, according to the manufacturer's instructions. Positive colonies were screened for the insert with the Invitrogen vector primer *IVT7* paired with the oligo dT and

the paralog-specific primers for the SuperScript III RT-generated cDNA or with the GeneRacer primer and GSP used for RACE-generated cDNA. Reaction conditions were 95°C for 3 min; 35 cycles of 94°C for 30 sec, 52°C for 30 sec, and 72°C for 1 min; followed by one cycle at 72°C for 20 min, using the reagent concentrations previously described. The results of each reaction were visualized on 2% agarose gels as described. Colonies that screened positive for the insert were amplified in three 10 μL PCR amplifications using the reagent concentrations and reaction conditions listed above and the Invitrogen vector primers *T7IVG* and *M13RIVG*. Three μL of the products of each reaction were visualized on 2% agarose gels using the methods described, and the products of successful reactions were cleaned enzymatically using shrimp alkaline phosphatase and exonuclease I and were submitted for DNA sequencing at the Vanderbilt University Core sequencing facility.

RNA SEQUENCING

To characterize transcriptional regulation of genes potentially associated with defense, we took advantage of the natural variation in the presence and absence of sociality in closely related North American *Pemphigus* aphids. The experiment was designed to identify genes differentially expressed in soldiers relative to developmentally equivalent, nonaggressive larvae of a nonsocial species (Lawson et al. 2014). In *Pemphigus obesinymphae*, only first instar larvae are soldiers, and there are no subcastes of first instar nonsoldiers. Later instars do not express aggressive behaviors. Thus, genes related to defense should be overexpressed in first instar soldiers relative to later instar larvae. However, some of these genes will be associated with expression differences related to development and age, rather than behavior. To highlight genes with putative social defense functions in soldiers, we first used Illumina sequencing of RNA transcripts to identify a set of genes with age-related expression differences in a nonsocial species (the first and late instar larvae in the nonsocial *P. populi-caulis*). We then removed these genes from our dataset, and after performing the equivalent Illumina study in the social *P. obesinymphae*, analyzed only the set of age-invariant orthologs for expression differences in the social *P. obesinymphae* dataset. In effect, this set is "normalized" for age by the nonsocial *P. populi-caulis*, and those orthologs that are differentially expressed in the social species are candidates for genes related to social traits.

Larval *P. obesinymphae* and *P. populi-caulis* from four clones, respectively, were sorted by instar, and for both, RNA was extracted from pooled samples of 50 first instar nymphs and pooled samples of five fourth instar, resulting in eight pooled samples in total (2 species × 2 samples of first instar larvae × 2 samples of fourth instar larvae = 8). Each sample was extracted using the Qiagen RNeasy Mini Kit® for cells, tissues, and yeast, including the DNase digestion protocol. Purified RNA was quantified with a NanoDrop ND-1000 spectrophotometer. Eight libraries were constructed and sequenced through a single-end read protocol on the Illumina Genome Analyzer at the Vantage core at Vanderbilt University (http://vantage.vanderbilt.edu). Illumina instrument software performed data analysis and base calling.

Low-quality bases and Illumina adapter sequences were trimmed from reads using Trimmomatic (version 0.27; Bolger et al. 2014). Quality-trimmed reads from all *P. obesinymphae* samples and all *P. populi-caulis* samples were pooled and assembled into two de novo transcriptomes using Trinity (Haas et al. 2013). Putative protein coding regions of these de novo transcriptomes were annotated using the Trinity plugin TransDecoder. All putative protein coding regions of these transcriptomes were annotated by aligning to the reference *Acyrthosiphon pisum* transcriptome (International Aphid Genomics Consortium 2010).

Reads from each individual sample of *P. populi-caulis* first instar and late instar were aligned to putative coding sequences if the de novo *P. populi-caulis* transcriptome using Bowtie2 with default parameters (Langmead and Salzberg 2012). Reads from each *P. obesinymphae* soldier and nonsoldier sample were also aligned to the de novo *P. obesinymphae* transcriptome using the same process. Differential gene expression was determined using DESeq2 (Love et al. 2014). Genes were considered differentially expressed if the fold difference between either soldier and nonsoldier samples for *P. obesinymphae* or 1st and late instar samples for *P. populi-caulis* exceeded a twofold change and the adjusted *P*-value as reported by DESeq2 was less than 0.1.

Orthologous genes were identified between *P. populi-caulis* and *P. obesinymphae* using OrthoMCL, which clusters genes into groups of orthologs using BLAST (Li et al. 2003). Orthogroups were identified as being uniquely differentially expressed in *P. obesinymphae* if at least one *P. obesinymphae* gene in the orthogroup was differentially expressed and no *P. populi-caulis* genes in the orthogroup were differentially expressed.

PHYLOGENETIC ANALYSIS

Cathepsin B copies from *P. obesinymphae* and Genbank *(A. pisum* NM001126136; *Nilaparvata lugens* AJ316141, *Toxoptera citricida* AY737543, *A. pisum* NM001126146, *Astegopteryx styracophila* AB371615, *Astegopteryx spinocephala* AB371616, *Tuberaphis takenouchii* AB371614, *T. styraci* AB371608, *Tuberaphis sumatrana* AB371612, *Tuberaphis taiwana* AB371610, *Tuberaphis coreana* AB167466, *T. citricida* AY737533, *A. pisum* NM 001134928, *A. styracophila*, *AB371626*, *Astegopteryx spinocephala* AB371627, *Cerataphis jamuritsu* AB371628, *T. takenouchii* AB371624, *T. taiwana* AB371621, *T. coreana* AB167467, *Tuberaphis sumatrana* AB371622, *T. sumatrana* AB371623) were aligned using the implementation of MAAFT in Geneious version 9.1 (Biomatters; http://www.geneious.com) using default parameters, and then manually checked by eye

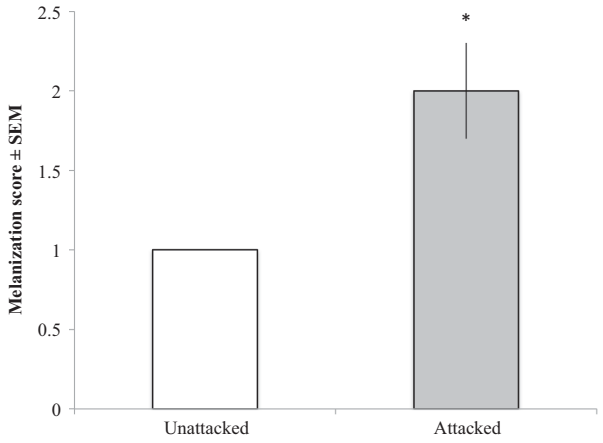


Figure 2. The average degree of melanization score of *Drosophila melanogaster* larvae in an empty gall for 1 h (unattacked control) or attacked by aphid soldiers for 1 h. The average was taken for at least 10 replicates for each group. The bars specify the standard error and the asterisk indicates a significant difference ($P < 0.05$).

(Katoh et al. 2002). A consensus maximum-likelihood bootstrap tree was derived in RAxML 7.0.4 using a general time reversible (GTR) + GAMMA model of sequence evolution, and 1000 bootstrap replicates (Stamatakis 2006). The presence of conserved residues and domains, including signal peptides, was determined by alignment against a *D. melanogaster* cathepsin B (CG10992) and submission of the *catB P. obesinymphae* peptide sequences to copies to the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/; Petersen et al., 2011).

ANALYSIS OF POSITIVE SELECTION

We derived values of the number of nonsynonymous substitutions per nonsynonymous site (Ka) and the number of synonymous substitutions per site (Ks) and tested for evidence of Ka/Ks > 1 using a covarion-based approach implemented in the online software Ka/Ks Calculation tool, using the default settings (Siltberg and Liberies 2002; http://services.cbu.uib.no/tools/kaks).

Results

MELANIZATION AND PHENOLOXIDASE RESPONSE

Relative to unattacked controls, attack by aphid soldiers led to significant increases in the degree of melanization (Fig. 2; $n = 31$; contingency analysis, $\chi^2 = 18.70$, degrees of freedom [df] = 1, $P < 0.0001$). We next assayed phenoloxidase activity in attacked larvae hemolymph. There was a significant elevation of phenoloxidase in the hemolymph of larvae attacked by aphid soldiers compared to unattacked controls (attacked: $n = 16$; average $OD_{attacked} = 0.012 \pm 0.0045$ SEM; unattacked: $n = 15$; average $OD_{unattacked} = -0.005 \pm 0.0046$ SEM; one-way ANOVA, df = 1, $F = 6.68$, $P = 0.02$).

TRANSCRIPTIONAL INDUCTION OF GENES INVOLVED IN IMMUNITY IN RESPONSE TO ATTACK BY APHID SOLDIERS

We quantified the expression of four genes involved in the proPO pathway for melanization, including GTP cyclohydrolase (*Punch*), dihydropteridine reductase (*Dhpr*), dopa decarboxylase (*Ddc*), and tyrosine hydroxylase (*Pale*). Previous microarray studies identified the induction of these genes following septic injury (De Gregorio et al. 2001), and the rapid expression and accumulation of *Pale* and *Ddc* transcripts near wound sites in embryos and early larvae (Ashida and Brey 1995). At 1 h posttreatment, all of the genes quantified in the melanization pathway were expressed by greater than twofold in all treatment groups relative to unattacked controls, likely indicating a biologically significant response (Coggins et al. 2012). *Ddc* and *Dhpr* exhibited about a threefold increase in larvae attacked by aphid soldiers (Fig. 3A and B; *Ddc*: Wilcoxon–Mann–Whitney test, $\chi^2 = 1.20$, df = 1, $P = 0.27$; *Dhpr*: $\chi^2 = 0.83$, df = 1, $P = 0.36$). We found a sixfold increase expression in *Pale* (Fig. 3C; Wilcoxon–Mann–Whitney test, $\chi^2 = 3.84$, df = 1, $P = 0.05$) and a sevenfold increase in *Punch* following aphid attack (Fig. 3D; Wilcoxon–Mann–Whitney test, $\chi^2 = 4.81$, df = 1, $P = 0.03$).

We examined multiple genes involved in the Toll pathway, including Spätzle (*spz*), Spätzle-processing enzyme (*SPE*), Cactus–Dorsal (*cact*), and Defensin (*Def*) (Jang et al. 2006). The Toll signaling pathway forms a central component of innate immunity in insects. At 1 h posttreatment, we found no differences in expression of *cact*, *SPE*, or *Def* (Fig. S2A–C; *cact*: Wilcoxon–Mann–Whitney test, $\chi^2 = 0.00$, df = 1, $P = 1.00$; *SPE*: $\chi^2 = 0.64$, df = 1, $P = 0.42$; *Def*: $\chi^2 = 0.54$, df = 1, $P = 0.46$). However, there was a greater than fivefold increase in the expression of *spz* following attack by *P. obesinymphae* aphids over unmanipulated controls (Fig. S2D**;** Wilcoxon–Mann–Whitney test, $\chi^2 = 8.47$, df = 1, $P = 0.004$).

Next, we quantified expression differences in genes in the Drosophila Imd pathway after attack by aphid soldiers, including peptidoglycan recognition protein, *PGRP-LC* and Cecropin A1 (*CecA1*). The Imd signaling pathway is induced during infection of Gram-negative bacteria (Brennan and Anderson 2004). There was little variation in *PGRP-LC* expression between treatments relative to controls (Fig. S3A**;** Wilcoxon–Mann–Whitney test, $\chi^2 = 0.12$, df = 1, $P = 0.73$). *CecA1* was not significantly upregulated from controls (Fig. S3B**;** Wilcoxon–Mann–Whitney test, $\chi^2 = 0.34$, df = 1, $P = 0.56$). Thioester-containing protein 1 (*Tep1*) is expressed in hemocytes and is involved in immune responses in the epithelial cells in the JAK/STAT pathway (Agaisse and Perrimon 2004), including early melanization responses in the epithelia (Blandin and Levashina 2004). *Tep1* expression was increased by almost fivefold in larvae attacked by aphid soldiers (Fig. S4A; Wilcoxon–Mann–Whitney test, $\chi^2 = 4.33$, df = 1,

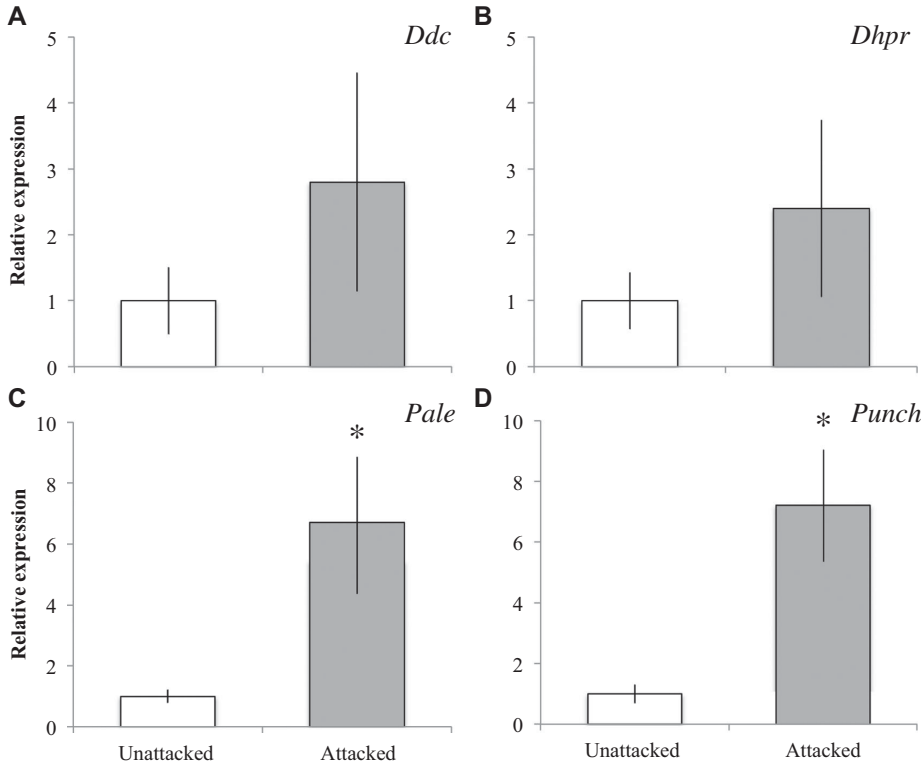


Figure 3. **Mean 2ΔΔ Ct expression (±SEM) relative to an action control of immune gene transcripts involved in the melanization cascade in larvae either in an empty gall (white bar) or attacked by aphid soldiers (gray bar) at 1 h post-treatment (A) Dopa decarboxylase (*Ddc*) was overexpressed by over twofold in the attack by aphid soldier group compared to controls. (B) Dihydropteridine reductase (*Dhpr*) was induced greater than twofold in the group attacked by aphid soldiers relative to controls. (C) Tyrosine hydroxylase (*Pale*) was transcriptionally induced by greater than sixfold in the treatment group attacked by aphid solders relative to controls. (D) GTP cyclohydrolase (*Punch*) was expressed sevenfold higher in larvae attacked by aphid soldiers. An asterisk indicates a significant difference ($P < 0.05$).**

$P = 0.04$). *Tep2* expression did not vary notably in any treatment group (Fig. S4B**;** Wilcoxon–Mann–Whitney test, $\chi^2 = 0.23$, df $=$ 1, $P = 0.63$). Superoxide dismutase (*Sod*) and Catalase (*Cat*) prevent the damaging effects of superoxide and hydrogen peroxide, respectively, by converting these compounds into oxygen and water (Mackay and Bewley 1989). Catalase can induce expression of AMPs and is a scavenger of free radicals that are released in the melanization response (Mackay and Bewley 1989). Although *Sod* was not differentially expressed upon aphid attack (Fig. S5A**;** Wilcoxon-Mann-Whitney test, $\chi^2 = 1.33$, df $= 1$, $P = 0.25$), *Cat* exhibited a 2.5-fold increase in expression in larvae attacked by *P. obesinymphae* soldiers (Fig. S5B; Wilcoxon–Mann–Whitney test, $\chi^2 = 6{,}44$, df $= 1$, $P = 0.01$). Some of the overexpressed genes described above are outside of the proPO pathway, but have been implicated in the melanization response and the response to septic injury (Figs. S2D, S3B, S4A, and S5B, respectively).

PARTIAL RECOVERY OF SURVIVORSHIP IN MUTANTS

To determine if cytotoxic effects of melanization could contribute to the lethality of aphid soldiers, we compared the average time to death of larvae from four different Drosophila mutant lines (*Ddc, Dhpr, Pale*, and *Punch* mutants) with three attacked controls, including a mutant for a nonimmunological process (*N*-linked glycosylation) and wild-type larvae. If the activation of cytotoxic intermediates in the melanization pathway contributes to the lethality of aphid soldiers, we would expect greater survivorship of mutants deficient in melanization over those without impaired melanization. Additional controls included measuring the average larval survival in each line in the experimental arenas in the absence of aphids (unattacked). Across all lines, time to death was significantly faster in attacked than unattacked controls (note that all larvae was ultimately died due to removal from fly media and dessciation). The difference in time to death between the unattacked controls and aphid attacked for each *Drosophila* line is shown (Table 1). When comparing average time to death of attacked larvae in each melanization mutant line with that of the wild type, we found significant variation in mean survival time, but only *Pale* mutants survived significantly longer when attacked by aphid soldiers than similarly attacked wild-type larvae (overall ANOVA: df $= 5$; F ratio $= 8.80$; $P < 0.001$;

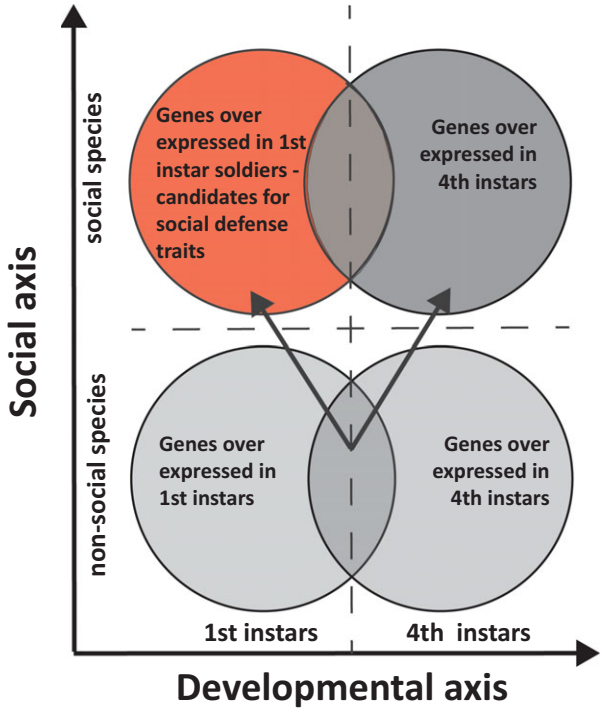
Table 1. **The average time to death in minutes of multiple *Drosophila melanogaster* larval lines defective in various genes involved in the melanization response attacked by aphid soldiers. Table_footnote: Survival of attacked larva in each line was significantly different from unattacked controls (unattacked vs. attacked). Survival of mutant lines attacked by aphid soldiers against a control (attacked wild type; italics) was not significantly different for any mutant line, except *Pale* mutants. Bold indicates a significant difference ($P < 0.05$).**

	Time to death (min)		Unattacked vs. attacked			Attacked wild type vs. mutant line		
Drosophila line	Unattacked	Attacked	X^2	df	p	Δ mean time to death vs. control (min)	LSD	p
Wild type	368.42	86.00	19.42	1	**< .0001**	–	–	–
Multi-stab	368.	134.00		1	**< .0001**			**0.02**
Glycan mutant	447.00	91.00	24.30	1	**< .0001**	−5.00	−0.56	0.97
Punch mutant	406.00	99.31	20.95	1	**< 0.0001**	+13.30	−0.54	0.98
Dhpr mutant	392.00	42.11	40.59	1	**< 0.0001**	−43.90	−0.14	0.13
Pale mutant	362.00	192.00	6.04	1	**0.01**	+106.00	0.07	**0.03**
Ddc mutant	364.44	128.00	12.10	1	**0.0005**	+42.00	−0.13	0.15

Dunnett's method for means comparisons using mean survival time of wild type as a control, with a *P*-value of 0.05: for *Pale* mutants, $|d| = 2.50$, least significant difference (LSD) $= 0.068$, $P = 0.029$; for all other comparisons, LSD < 0, $P > 0.05$).

COMPARATIVE TRANSCRIPTOMICS OF SOCIAL AND NON-SOCIAL APHIDS

To identify potential immune-modulating gene candidates in aphid soldier morphs, we used RNA sequencing to compare soldiers of a eusocial species (*P. obesinymphae*) to age-matched nymphs of a sympatric nonsocial species (*P. populi-caulis*). In *P. obesinymphae*, all first instar nymphs express soldier-like behaviors, meaning that any nonsoldiers in the colony are also developmentally more mature. Comparing soldiers and nonsoldiers within species is equivalent to comparing nymphs that differ in age, potentially confounding behavioral differences with developmental ones. We used the nonsocial *P. populi-caulis* transcriptome to identify genes that do not vary in expression across development, and then asked which of these exhibit differential expression in soldiers of *P. obesinymphae* (Fig. 4). We produced a *P. obesinymphae* transcriptome contained 36,655 transcripts with an N50 of 953; in these transcripts, 15,038 putative unique genes were identified. The assembled *P. populi-caulis* transcriptome contained 46,769 transcripts with an N50 of 1114; 15,089 putative unique genes were identified in these transcripts. A total of 2323 orthogroups were differentially expressed in the social aphids, but only 235 were "developmentally static." Of the 235 developmentally invariant genes in the nonsocial aphid that were over- or underexpressed in *P. obesinymphae* soldiers, 144 encode peptides with functional annotations in the current release of the pea aphid gene set (version 2.1; Fig. S6). Although gene set enrichment of these 144 genes is unlikely to be representative of the whole set, significantly enriched terms included a number of molecular functions related to proteolysis and catalytic activity (Fig. S6), including a member of the CLIP domain serine protease family (ACYPI000458-PA) thought to be among the activators of the proPO cascade in insects (35) (Fig. S6). However, enrichment for proteolytic activity was a general feature of the developmentally invariant genes we evaluated, and many genes related to proteolysis were downregulated in soldiers (Fig. S6), indicating that the differentially expressed genes in *P. obesinymphae* are drawn from a background of proteolytic gene products that *Pemphigus* aphids express ubiquitously and dynamically, whether they are social. Remarkably, however, one gene overexpressed in *P. obesinymphae* soldiers (*catB*) is a homolog of a group of papain proteases identified with venomous properties in a phylogenetically unrelated eusocial species. Kutsukake et al. (2004, 2008) identified two paralogous copies of *catB* in *T. styraci* and related species (Aphididae: Hormaphidinae), one of which (*catB-S*) is fast-evolving, expressed and secreted in a caste-specific manner, and is pharmacologically active against natural enemies. The other (*catB-N*) is not fast evolving, and is ubiquitously expressed in all morphs. BLAST searches against the Genbank nr database indicated that the 522 bp fragment exhibited highest similarity to the *catB-N* orthologs (best hit: putative cathepsin B-N; *Toxoptera citrida,* Aphididae: Aphidinae; AY 737533; *e*-value $= 4.27 \times 10^{-72}$). Based on RPKM values, the *P. obesinymphae* gene was expressed fourfold higher in *P. obesinymphae* soldiers (mean RPKM = 49.2) compared to fourth instar nymph (mean RPKM = 3.1) that do not express soldier behavior, and about 2.5-fold higher than first instar nymph in the nonsocial *P. populi-caulis* (mean RPKM = 16.9). We cloned the *catB* gene in *P. obesinymphae* and identified three paralogs; the expressed fragment was sequence identical to the 3' end of one of the cloned copies (*P. obesinymphae catB 3*). Maximum-likelihood phylogenetic reconstruction placed all of the three *P. obesinymphae* copies in the N-type clade identified in *T. styraci* (Fig. S7). We were unable to successively measure copy-specific expression of catB in *P. obesinymphae*, but in terms



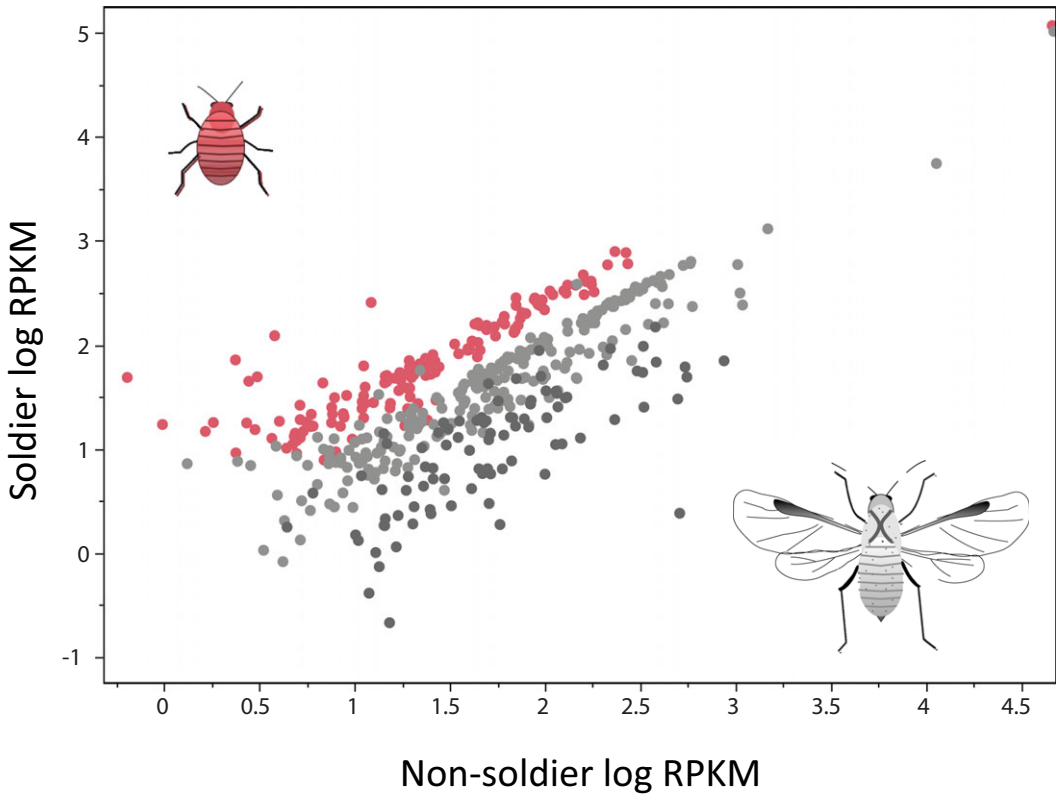


Figure 4. Top: The experimental design to differentiate changes in gene expression over development (developmental axis) and those due to social evolution (sociality axis). The genes that are invariant overdevelop in a nonsocial species (intersection of bottom gray circles) that are differentially expressed in the first instar soldiers of a social species (red circle, top) that are candidates for social traits. Bottom: Plot of RPKM values of soldiers (red) against winged, nonsoldier members of a colony (dark gray; light gray = not differentially expressed).

of the aggregate expression of the catB paralogs via quantitative PCR, expression was higher in *P. obesinymphae* soldiers relative to the more developmentally mature third/fourth instar nymph and winged alates (Fig. S8). Annotation of the *P. obesinymphae catB* indicated the conservation of numerous residues in all three paralogs important for activity, including presence of N-terminal signal peptides in each aphid paralog (Fig. S9). Unlike the evidence from *catB-S* in *Tuberaphis* of positive selection in coding regions of the soldier-specific copies (Kutsukake et al., 2004, 2008), we found no evidence of positive selection on the three *P. obesinymphae catB* paralogs (Fig. S10). Thus, if *catB* has venomous functions in *P. obesinymphae*, the developmental/caste-specific differences appear to be regulatory, and not the result of coding differences. Datasets associated with the results reported above have been deposited in Dryad.

Discussion

The functional basis of fortress defense eusociality lies in how soldiers express aggressive behavioral responses toward other insects, and their lethality. Our results indicate that lethality by aphid soldiers derives in part from how they antagonize immunity in their victims (Figs. 1 and 2). The insect innate immune response is an evolutionary ancient and multifaceted defense against infection, parasitism, and wounds, and involves both constitutive cellular responses (e.g., phagocytosis) and induced humoral responses (e.g., the production of antimicrobial peptides and reactive species; Haine et al. 2008). Response to injury and infection is tightly regulated to minimize self-harm associated with the generation of toxic intermediates (Sadd and Siva-Jothy 2006). We found evidence that initially following attack, aphid soldiers activate components of the melanization and cuticular wounding response (Fig. 3). We did not find evidence of marked induction of Toll or Imd signaling pathways (Fig. S2 and S3), both of which are tuned to respond to fungal and bacterial infection, suggesting that aphid soldiers do not translocate microbial symbionts, plant pathogens, or other microbially derived triggers or virulence factors. However, the short period over which we measure expression may have precluded detection of such factors. Although more work is needed, our results are consistent with the idea that, like at least one other social aphid species (Kutsukake et al., 2004; 2008), *P. obesinymphae* aphid soldiers secrete. Some of these may have immunity-activating properties.

It has long been assumed that lethality by aphid soldiers is the result of the physical wounding and damage they cause with their mouthparts, or simply a direct consequence of the salivary proteolytic activity at wound sites. And it is likely that some of the differences in the transcriptional responses we measured are the result of the general pathology associated simply with dying from attack. However, survivorship can be partially rescued in Drosophila *Pale* mutants relative to wild-type controls, suggesting that epithelial injury alone and general pathology may not entirely explain aphid lethality (Table 1). We were particularly interested in the *Pale* mutant, because tyrosine hydroxylase catalyzes the rate-limited step in melanin biosynthesis (Shi et al. 2014; the conversion of tyrosine to L-dopa), and occurs upstream of most of the biosynthetic steps associated with cytotoxic intermediates and free radical reactions (Nappi and Vass 1998). Possibly then, the general toxicity we observed is associated with more systemic deleterious effects of unchecked melanization (Sadd and Siva-Jothy 2006). That aphid soldiers exploit vulnerabilities in the immune responses of their victims with pharmacologically active secretions (venoms) is consistent with general patterns in venom biology (Fry et al. 2009), as with the venom of the wasp *Nasonia*, which causes significant upregulation of multiple immune genes in its fly host, leading to cell death and developmental arrest (Martinson et al. 2014). However, future work will need to determine more precisely the relative contributions of the various secreted molecules and injuries that aphid soldiers cause. Drosophila are useful in this regard. Interpretation of immunological responses in ecological contexts can be notoriously tricky (Adamo 2004), especially so in this case, in which plant-feeding aphids attack other insects. But Drosophila provide functional tools, and serve as good stand-ins for the fly larvae that commonly prey on gall-forming aphids (Wilch 1999). Although there has been little study of the natural enemies of social aphids, it is tempting to speculate that some may be coevolving with social aphids, underlying the escalation of the chemical weapons soldiers express in defense (Kutsukake et al., 2004). However, many of the insects that attack aphids like *P. obesinymphae* are generalist consumers of insects. Wilch (1999) characterized the predators of a related gall-forming species that co-occurs with *P. obesinymphae* on poplars in the western United States. Among the common species she identified were lepidopterans (Gelechiidae and Pyralidae), lacewings (Chrysopidae and Hemerobiidae), pirate bugs (Anthocoridae), and an aphid fly (Chaemaemyiidae). In some cases, multiple predators were present in the same galls. Assuming this community is similar to those associated with the often syntopic *P. obesinymphae*, specificity between social aphids and their predators is not likely. This is the basis for our assumption that effectiveness of aphid soldiers must derive from more generalized effects, such as that associated with cuticular injury and, as we have hypothesized, because they antagonize conserved features in the immune response shared by most insects.

THE PHARMACOLOGY OF SOCIALITY IN APHIDS

Eusocial Hymenoptera and Isoptera exhibit an array of venoms, toxins, and other biochemical and morphological weaponry for defense with well-characterized biochemical and functional targets (Brand et al. 1972; Sobotnik et al. 2010). This is not the

case for species such as eusocial aphids or thrips, and the chemical ecology of these remains poorly described (Turnbull et al. 2012). We found a number of differentially expressed genes with proteolytic activity in *P. obesinymphae* soldiers that are developmentally invariant in the related *P. populi-caulis*, which is not social. We used this approach (variable in the social species, developmentally invariant in the nonsocial one) to get around the difficulty that in aphids, soldiers are restricted to particular age classes, meaning that development and caste can be easily confounded. But the approach is conservative, and we likely missed differentially expressed genes that relate to caste or behavior in social aphids. However, it is notable that despite the conservative approach, we found that the eriosomatine *P. obesinymphae* soldiers overexpress a cathepsin B protease homologous to the protease expressed by hormaphidine aphids, suggesting the possibility of convergent recruitment of proteases like *catB* in aphid social evolution (Fig. S8; Kutsukake et al. 2004; 2008). These aphids are not close taxonomic relatives, and in both subfamilies, the vast majority of species are not social. Thus, shared social traits between the two subfamilies are candidates for evolutionary convergence. Cathepsin B and related gene families play important roles in host plant colonization in aphids generally (Mathers et al. 2017), and may be readily recruited for venomous activities in social aphids. However, we do not know if the differentially expressed enzymes are secreted and if so whether their effects extend beyond protein degradation at wounds to activation of melanization. Although CLIP domain serine proteases are thought to be the principle agents activating the melanization cascade, various other proteases have been shown to induce melanization (Harrison and Bonning 2010). Thus, there is scope for the *P. obesinymphae catB* to express activity or act as cofactors related to induction of melanization, but this remains to be determined (Fig. S8). We found no evidence of fast evolution in the *catB* peptide, indicating that if *catB* has been co-opted for defense, selection is acting on gene regulation, and not the protein itself (Fig. S10). Possibly, the differences in evolutionary trends in *catB* reflect genome-level effects of differences in the age and complexity of sociality in the two groups, resembling patterns emerging in the eusocial Hymenoptera (Rehan and Toth 2015).

Conclusions

The identification of a distinct mode of social evolution in recent years (fortress defense sociality) begs questions regarding the developmental, molecular, and functional bases of traits that facilitate and distinguish this particular life history and social syndrome (Rehan and Toth 2015). Insect species like aphids and thrips that express fortress defense sociality are gall formers, and the association has long been identified as validation of ecological theories of insect sociality, centered on the idea that living in limiting, defensible resources favor both the evolution of traits for defense as well as extended associations between relatives in multigenerational groups (Crespi 1994; Queller and Strassmann 1998). What these results thus highlight is how traits that evolve and function in antagonistic or competitive interactions can be repurposed in group-living species for social, cooperative functions. In this respect, if the vestiges of a predatory past in social insects can be found in such traits as a wasp's stinger, then the analogs in eusocial aphids are the herbivorous traits that underlie their uniquely successful lifestyles on plants.

AUTHOR CONTRIBUTIONS

SPL and PA designed the experiment; SPL, LTS, AWL, JNM, and HWW conducted the experiment; SPL, ALL, and PA analyzed the data; and SPL, LTS, and PA wrote the manuscript.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Degrees of melanization in *Drosophila melanogaster* larvae.

Figure S2. Relative mean expression (±SEM) of immune gene transcripts involved in the Toll pathway in *Drosophila melanogaster* larvae either in an empty gall unattacked (white bar), stabbed with a sterile needle (gray bar) or attacked by aphid soldiers (black bar), at 1 h posttreatment.

Figure S3. Relative mean expression (±SEM) of immune gene transcripts involved in the Imd pathway in *Drosophila melanogaster* larvae either in an empty gall unattacked (white bar), stabbed with a sterile needle (gray bar), or attacked by aphid soldiers (black bar), at 1 h posttreatment.

Figure S4. Relative mean expression (±SEM) of thioester-containing proteins in *Drosophila melanogaster* larvae either in an empty gall unattacked (white bar), stabbed with a sterile needle (gray bar), or attacked by aphid soldiers (black bar), at 1 h posttreatment.

Figure S5. Relative mean expression (±SEM) of superoxide dismutase (*SOD*) and catalase (*Cat*) in *Drosophila melanogaster* larvae either in an empty gall unattacked (white bar), stabbed with a sterile needle (gray bar), or attacked by aphid soldiers (black bar), at 1 h posttreatment.

Figure S6. The patterns of functional enrichment of differentially expressed genes in soldiers and alates of *Pemphigus obesinymphae* (blue) relative to the pea aphid background (orange).

Figure S7. Maximum-likelihood tree of selected *catB* homologs.

Figure S8. Relative expression of *catB*-like paralogs across developmental stages in *Pemphigus obesinymphae* normalized by *ef1a* (top) and *cyclophilin* (bottom).

Figure S9. Amino acid alignment of selected *catB* homologs.

Figure S10. Ka/Ks ratios for cathepsin B-S/N-like gene copies from various species in the genus *Tuberaphis,* and three paralogous copies from *Pemphigus obesinymphae.*

Figure S11: The average degree (±SEM) of melanization score of *Drosophila melanogaster* larvae in an empty gall for 1 h (unattacked control), stabbed with a sterile needle dipped in PBS, stabbed with a sterile needle >10 times, or attacked by aphid soldiers for 1 h (gray bars).

Figure S12. Relative mean expression (±SEM) of immune gene transcripts involved in the melanization cascade in *Drosophila melanogaster* larvae either in an empty gall unattacked (white bar), stabbed with a sterile needle (gray bar), or attacked by aphid soldiers (black bar), at 1 h posttreatment.

Table S1. Primers used for qPCR of genes in multiple immune pathways.

Table S2. Gene-specific primers for each of the three *catB* paralogs in *Pemphigus obesinymphae*.

Video 1. A *Pemphigus obesinymphae* gall, which has been sectioned in half, revealing the large, nearly immobile foundress in the upper section of the gall, and her many small first instar daughters. A *Drosophila melanogaster* larva has been placed in the gall, and moves rapidly as the nymphal soldiers attack it.