



The survival consequences of grooming in the honey bee *Apis mellifera*

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Abstract

In social animals, disease management behaviors such as grooming occur in response to diverse stimuli, making it difficult to interpret the evolution and function of these phenotypes. The honey bee has a grooming repertoire that includes self-directed behaviors and allogrooming from nestmates. Many stimuli provoke these behaviors, and their impacts on individual and colony survivorship are unclear. We evaluated the effects of two different stressors on grooming frequencies and survivorship. We found that self-grooming frequency is activated in distinct ways in response to pathogen infection, pesticide treatment, and social context. Moreover, self-grooming frequency predicts individual survival. Allogrooming interactions were less common and did not predict individual survival. The honey bee highlights the difficulty inherent in interpreting the evolution and function of grooming interactions in highly social species.

Keywords Social immunity · *Varroa* mite · Allogrooming

Introduction

Disease management is an integral component of social evolution, because proximity to conspecifics increases risk of pathogen spread. In many social species, individuals perform behaviors that provide their groupmates disease or parasite protection ("social immunity" behaviors; Silk et al. 2003; Cremer et al. 2007, 2018; Smolinsky et al. 2009; Evans and Spivak 2010; Zhukovskaya et al. 2013; Stroeymeyt et al. 2014; Pritchard 2016). Grooming is one such phenotype; in group living animals, it has both self-directed (self-grooming) and social (allogrooming) forms, each with

distinct implications for disease transmission to the group, and for individual and group survivorship (Fefferman et al. 2006; Boroczky et al. 2013; Zhukovskaya et al. 2013; Kaluff et al. 2016).

Despite the ubiquity of grooming, the connections between grooming and disease resistance at the individual or group level remain poorly understood. This is because a variety of stimuli induce self- and allogrooming, and only some of these stimuli are relevant to disease spread. For example, in insects, self-grooming is stimulated by foreign objects such as parasites and pollen (Pettis and Pankiw 1998; Land and Seeley 2004), but also chemical irritants (Cox and Wilson 1984; Golenda and Forgash 1986), subtle changes in cuticular chemical profiles (Kovac and Maschwitz 1990), and microorganisms (Yanagawa et al. 2012). Furthermore, the impacts of grooming activities on survival are sometimes indirect (Zhukovskaya et al. 2013); in mammals, allogrooming results in parasite removal, but it is also linked to bonding, reciprocity, nurturing, social status, and immune function (DeVries et al. 2003; Silk et al. 2003; Li et al. 2016, 2019; Eads et al. 2017; Schweinfurth et al. 2017; Chen and Hong 2018). Finally, in both vertebrate and invertebrate species, grooming is a common displacement behavior, often expressed in response to stress (Troisi 2002; Root-Bernstein 2010); its frequency could indicate stress levels

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or immune system status while having no clear immediate benefit. Thus, grooming is broadly linked to health, but its impacts on individual survivorship and/or disease spread among members of a social group may depend on the stimulus that elicits the behavior.

Grooming and other social immunity behaviors are common in the honey bee (*Apis mellifera*). Colonies are composed of a single queen and 20,000–40,000 highly physically interactive female workers (Winston 1987). Presumably as a result of strong selection for disease and parasite management, the honey bee has evolved a grooming repertoire (Walker and Hughes 2009; Evans and Spivak 2010; Hamiduzzaman et al. 2017; Cremer et al. 2018). Workers self-groom and allogroom diseased nestmates (spontaneously, but also in response to a dance signal; Land and Seeley 2004). Both behaviors are elicited by ectoparasites, but also infections with various pathogens (Waddington and Rothenbuhler 1975; Land and Seeley 2004; Evans and Spivak 2010; Richard et al. 2012; Carr et al. 2020; Russo et al. 2020), suggesting a variety of potential functional outcomes for grooming, and perhaps a general connection between grooming and immune system activation in the recipient (Boroczky et al. 2013).

The consequences of honey bee grooming behaviors remain unclear, and have not been thoroughly investigated across the variety of contexts that stimulate the behavior. For example, in studies focused on ectoparasites, there is relatively weak evidence that elevated allogrooming impacts mortality or parasite infestation at the colony level (at least for *A. mellifera*; Pettis and Pankiw 1998; Boecking and Spivak 1999; Evans and Spivak 2010; Pritchard 2016). Moreover, to our knowledge, no study has assessed mortality impacts of allogrooming or self-grooming for other contexts that elicit the behavior, e.g., pathogen infection (see Waddington and Rothenbuhler 1975). In the current study, we use a lab-based approach to assess whether allogrooming or self-grooming are associated with increased individual worker bee survival in the face of two different types of stressors. The first is a yeast (*Saccharomyces cerevisiae*) injection, which broadly and rapidly activates the immune system, stimulates self- and allogrooming, and results in behavioral and mortality effects over 24 h (Carr et al. 2020). The second is topical treatment with the metabolic pesticide tetradifon, which alters behavior and causes mortality in a stress-dependent manner (Li-Byarlay et al. 2014). We chose these stressors, because there are established methods to apply them without 100% mortality, as well as known relationships to behavior. They are also distinct in terms of their application method (Barron et al. 2007) and mode of action, allowing us to investigate whether there are relationships between grooming and survivorship that can be generalized across contexts.

Materials and methods

We performed experiments on August 5–8, 2020 at the University of Kentucky Research Farm (Lexington, Kentucky, USA), using full-sized colonies advertised as Italian, Carniolan, and Russian hybrids (Schoolhouse Bees, Covington, KY, USA). No colonies were undergoing *Varroa destructor* mite treatment at the time of the experiment.

On the morning of the experiments, we collected focal bees at the entrance of the colonies using a vacuum. Focal bees are likely a combination of foragers, guards, and other middle-aged bees (Winston 1987). We targeted these individuals, because they could be collected easily without tracking individual behavior. Notably, these individuals are older than the bees that typically specialize on allogrooming (Cini et al. 2020). Prior to treatment (described below), focal bees were kept in cages (~40 bees per colony) in a 34 °C incubator and fed ad libitum 50% sucrose.

Individual bees were assigned haphazardly to one of four treatment groups ($N=10$ /group) representing all combinations of yeast treatment (yes/no) and pesticide treatment (yes/no, Table 1). Bees with no yeast or pesticide treatment were handled for a timeframe resembling other treatment combinations. We duplicated this set up across two social treatments, one where focal bees were placed in a social group after treatment (allowing allogrooming interactions), and one where treated focal bees were kept in isolation (preventing such interactions). Thus overall, this experiment included 80 focal bees ($N=10$ for each of four treatments across two social conditions). Each source colony ($N=10$) was represented by one individual per treatment combination per social condition ($N=8$ focal bees per colony). All treatment combinations are outlined in Table 1.

Due to the large number of treatment combinations in this study, we did not evaluate the impacts of our pesticide vehicle alone, or the effects of a yeast-free sham injection.

Table 1 Focal bee treatment combinations

| Number of focal bees | Yeast treatment | Pesticide treatment | Social treatment |
|----------------------|-----------------|---------------------|------------------|
| 10 | No | No | Isolated |
| 10 | No | No | Group |
| 10 | Yes | No | Isolated |
| 10 | Yes | No | Group |
| 10 | No | Yes | Isolated |
| 10 | No | Yes | Group |
| 10 | Yes | Yes | Isolated |
| 10 | Yes | Yes | Group |

Each of 10 colonies was represented by one focal bee per treatment combination. We collected self-grooming data for all focal individuals, and allogrooming data for group social treatments

Though we cannot rule out impacts of the vehicle (dimethyl formamide, DMF; Hewlett et al. 2018) and sham injection, in previous work, we found that saline injection did not alter self-grooming or allogrooming compared to a no-stab control (Carr et al. 2020). Similarly, DMF has been shown in other studies to have minimal impacts on behavior compared to a sham control (Sovik et al. 2013).

Treatments

All bees were anesthetized in a 4 °C refrigerator. Bees were removed from the refrigerator, treated (or handled), and marked on the thorax with paint (Testors, Rockford, IL, USA). The paint allowed observers to set up the experiment while remaining blind to treatment during the assays. Bees treated with both yeast and pesticide received the yeast treatment first and the pesticide immediately after. Following treatment or handling, we placed focal bees in 100 mm by 20 mm plastic dishes provisioned with 50% sucrose. Focal bees assigned to the isolated social treatment were placed in a dish alone, while bees from the group treatment were placed in a dish with three untreated nestmates from the same colony (Rittschof et al. 2018; Carr et al. 2020). Two observers treated and then observed 10 dishes at a time each, performing the set up and observations twice a day for 2 days (resulting in 80 total focal bees observed).

Yeast-treated bees were injected under the third abdominal tergite with 2 µL of a solution of *Saccharomyces cerevisiae*. Dehydrated *S. cerevisiae* (Dadant & Sons, Frankfort, KY, USA) was cultured in 10% glucose and diluted to an OD600 of ~0.56 using bee saline (Yang and Cox-Foster 2007; 0.156 M NaCl, 0.003 M KCl, 0.002 M CaCl₂, pH 7.0). Injections were performed using a 30-gauge 50 µL hand-injector syringe (Hamilton Company, Reno, NV, USA; Kucharski and Maleszka 2003; Carr et al. 2020). In a previous study, this dosage resulted in 2% mortality after 1 h, and 90% mortality after 24 h (Carr et al. 2020).

Pesticide-treated bees received a 1 µL thorax topical application of tetradifon (Sigma-Aldrich, St. Louis, MO, USA) dissolved in DMF (Sigma-Aldrich). Tetradifon inhibits Complex V of mitochondrial oxidative phosphorylation, which means that, much like neonicotinoids, it modulates immune system function by altering reactive oxygen species production (James and Xu 2012). Because of its metabolic effects, this pesticide has social-context-dependent impacts on aggression, a behavior that is correlated with allogrooming activity and immune activation (Rittschof et al. 2019; Carr et al. 2020). We reasoned that this pesticide may exacerbate the mortality impacts of yeast treatment, especially in combination with variation in grooming activity (Huang and Robinson 1992). We applied a concentration of 21.1 µg/µL, which typically results > 95% survivorship (Li-Byarlay

et al. 2014). Thus, we did not expect the pesticide alone to impact mortality.

Behavioral observations

All bees were given 1 h to acclimate after treatment. At this point, all bees were standing and moving, allowing us to start the 1 h observation period. We watched each dish for 1 min at a time, tallying grooming behaviors (described below). Observers rotated through each dish throughout the hour and collected data for a total of 6, 1 min observation periods per focal bee. We assessed survivorship following the 1 h observation period (2 h total after treatment) and 24 h after treatment.

A self-grooming event occurred when the focal bee pulled on her antennae, rubbed her head with her forelegs, or rubbed her thorax or abdomen with her middle or hind legs. An allogrooming event occurred when a groupmate stroked the focal bee with its antennae or legs, licked her, or ran her antennae through her mandibles (Carr et al. 2020). Compared to other behavioral contexts, such as aggression, where interactions are sudden and rapid, allogrooming behaviors are slow and the receiving bee typically remains stationary. We did not observe any allogrooming solicitation dances, nor did we keep a record of the dances. We also recorded allogrooming behaviors directed towards groupmates by the focal bee. However, because our goal in this experiment was to assess whether experiencing allogrooming improved survivorship, we did not keep track of groupmates individually, nor did we assess whether allogrooming in these cases impacted mortality.

Statistics

Self-grooming and allogrooming behaviors were summed separately across the 6 observation periods. Source hive is a blocking factor and, therefore, is included as a random effect in all analyses. We performed simple regressions and linear mixed models (LMMs) in JMP Pro 13.2.0 and generalized linear mixed models (GLMMs) in R 4.0.3 using the glmer function (lme4 package; significant main effects in GLMMs were assessed using the anova function). We examined the distributions of model residuals for normality to confirm quality of model fits. We used LMMs to assess the impacts of pesticide treatment, yeast treatment, and their interaction on self-grooming behavior. For allogrooming, because events were rarer, we used separate LMMs to individually assess the impacts of each treatment on allogrooming behavior. We used binomial GLMMs for all analyses of survivorship. The tests used are described in the appropriate location in the Results. Due to the multiple statistical tests performed on our data set, we performed a Benjamini–Hochberg correction to check which *P* values less than 0.05 (four

P values, please see below) remained below the threshold for significance with a false discovery rate of 5%.

Results

No focal bees died 2 h after yeast and pesticide treatment (similar to previous studies), and only 18% died after 24 h, which is far fewer than expected based on a previous study using the same yeast treatment (Carr et al. 2020). This discrepancy could reflect the time of year during which the experiments were performed, or other unexplained variation in stress susceptibility (Corby-Harris et al. 2018; Carr et al. 2020). Models with single fixed effects showed that pesticide, yeast, and social treatments failed to significantly impact mortality (binomial GLMMs; yeast: $X^2_1 = 3.2$, $P = 0.07$, pesticide: $X^2_1 = 1.4$, $P = 0.24$, social: $X^2_1 = 1.4$, $P = 0.24$). More complex models containing combinations of these factors did not have improved Akaike Information Criterion (AIC) values nor did they impact the significance of treatment main effects (data not shown).

All treatments impact self-grooming frequency, which is correlated with survivorship

Self-grooming was common and uncorrelated with allogrooming events in the group social treatment ($R^2 = 0.002$, $P = 0.77$). All but two focal bees self-groomed (0–17 events, mean = 6.2). Because we wanted to test the hypothesis that yeast, pesticide, and/or social treatment have cumulative impacts on grooming and mortality, we built an LMM including yeast, pesticide, social treatment and all two- and three-way interaction terms. We found that pesticide treatment ($F_{1,63} = 16.4$, $P = 0.0001$), yeast treatment ($F_{1,63} = 6.7$, $P = 0.01$), and the interaction of yeast and social treatment ($F_{1,63} = 5.0$, $P = 0.03$) significantly predicted the total number of self-grooming events (Fig. 1). The significant yeast and social treatment interaction suggests the main effect of yeast cannot be separated from the social effects (notably, this *P*-value did not survive the Benjamini–Hochberg correction, see Methods). There was no main effect of social treatment ($F_{1,63} = 0.0$, $P = 1.0$), and no interaction between yeast and pesticide treatment ($F_{1,63} = 2.4$, $P = 0.13$), pesticide and

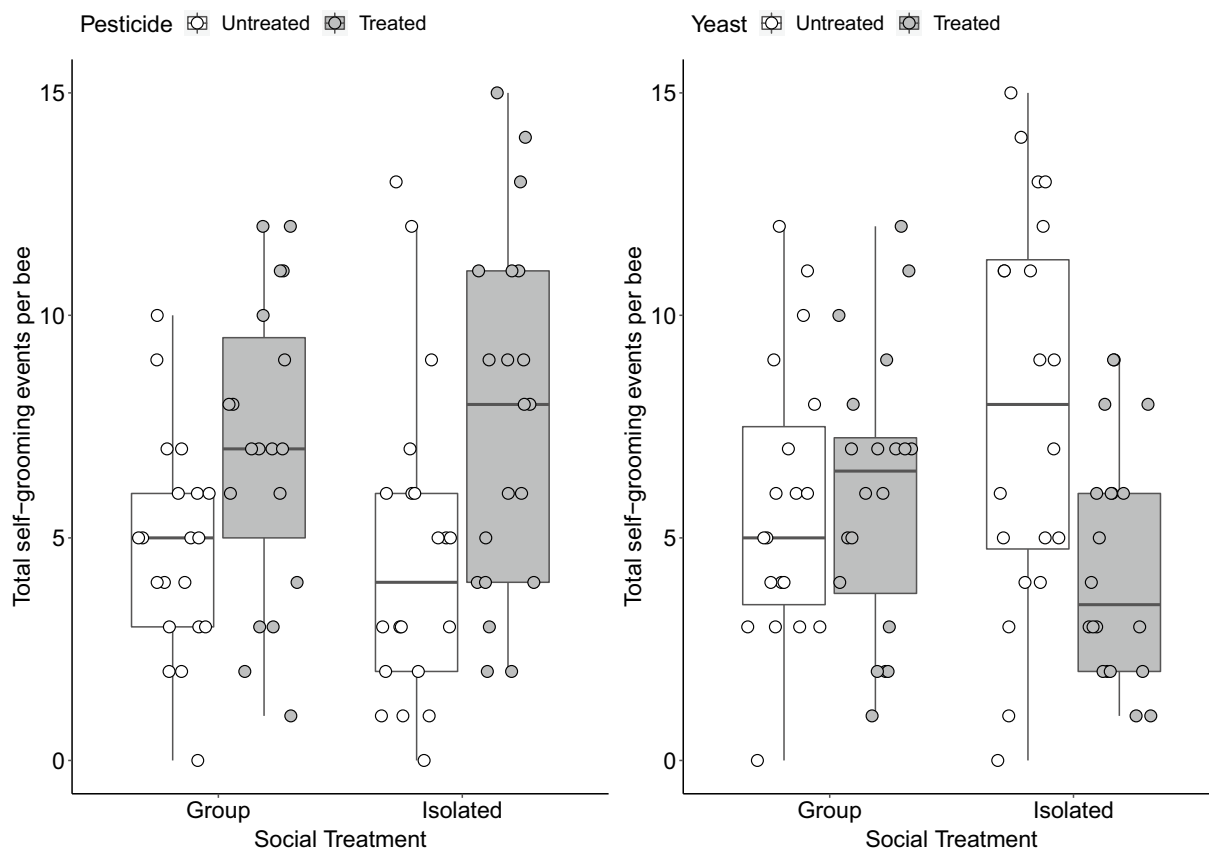


Fig. 1 Yeast, pesticide, and social treatments impact self-grooming events. Topical pesticide treatment significantly impacted self-grooming (left). These effects were similar across social treatments. Yeast

injection significantly impacted self-grooming, but with a significant interaction with social context (right)

social treatment ($F_{1,63}=0.04$, $P=0.83$), or the three-way interaction ($F_{1,63}=0.1$, $P=0.73$). A comparison of other models containing all combinations of main effects and interaction terms gave three models with similarly low AIC values. Like our hypothesis-based model, each contained all main effects and the yeast and social treatment interaction effect, but with different combinations of the other two-way interaction effects. Analyses of these simpler models did not impact the significance of any factors (data not shown).

Pesticide treatment increased self-grooming by 60%. Yeast treatment decreased self-grooming by about 25% in the isolated social treatment only. These results demonstrate that multiple types of factors (e.g., chemical exposure, immune system stimulation from injection or activation of the olfactory system by yeast presence) induce a grooming response in the honey bee. In addition, despite low overall mortality, self-grooming was a significant predictor of mortality at 24 h (Fig. 2, binomial GLMM, $X^2_1=6.8$, $P=0.009$), suggesting this behavior either enhances survival or serves as an indicator of a resilient physiology among those who express it.

Allogrooming did not respond to treatment and did not predict survivorship

Rates of allogrooming were similar to our previous study using yeast treatment (Carr et al. 2020). 48% of focal bees were allogroomed at least once by a groupmate. The total number of allogrooming events directed at the focal bee ranged from 0 to 5. Because of the low occurrence of allogrooming, we performed simple analyses with single main effects to determine whether either pesticide or yeast treatment alone impacted the occurrence of these behaviors (social treatment is not relevant for allogrooming as all bees were in groups). The number of allogrooming events did not differ as a function of yeast (LMM, $F_{1,29}=0.42$, $P=0.52$) or pesticide treatment ($F_{1,29}=0.02$, $P=0.90$). Analyses of allogrooming activity directed by the focal bee towards group members yielded similar non-significant results (data not shown).

Unlike self-grooming, allogrooming was not predictive of focal bee mortality (Fig. 2, binomial GLMM, $X^2_1=0.04$, $P=0.84$). Results were the same when allogrooming was treated as a yes/no binomial variable (data not shown).

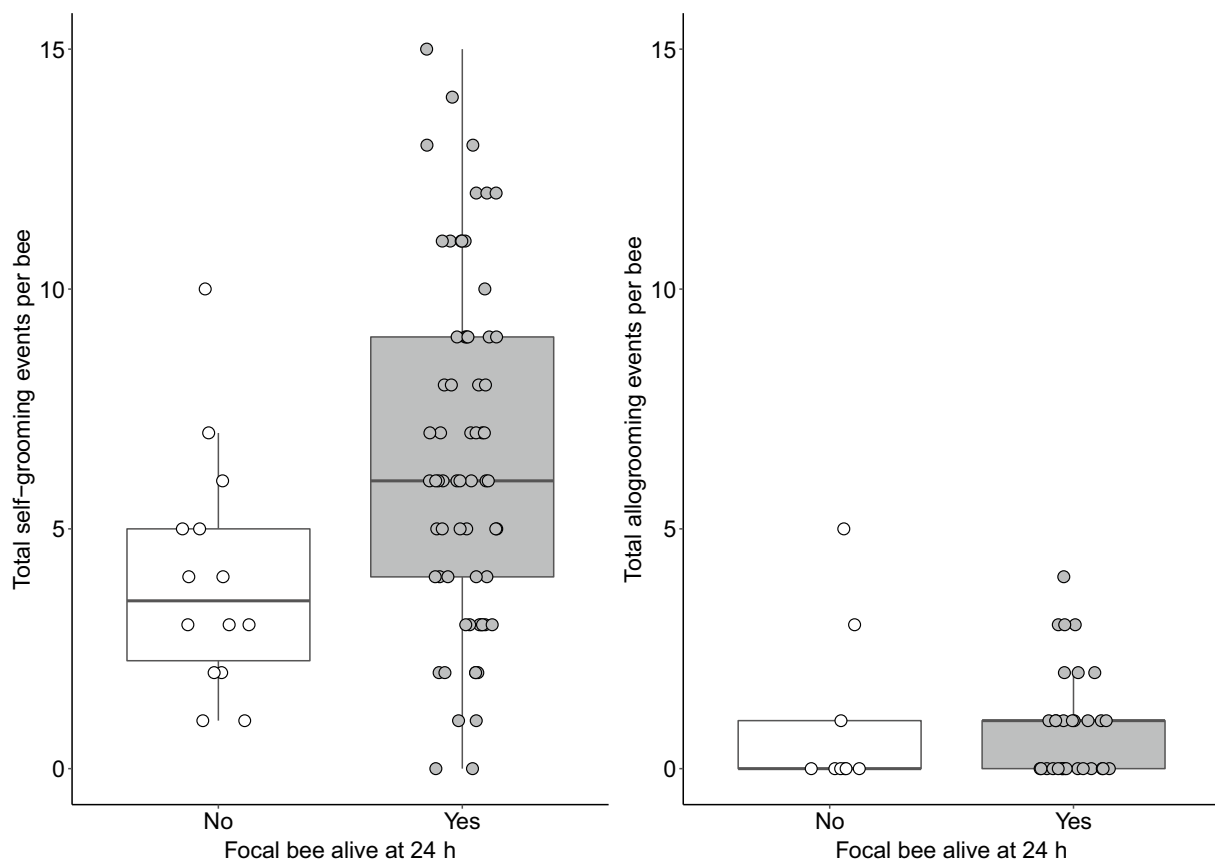


Fig. 2 Relationship between grooming and 24 h survivorship. Self-grooming (left) was significantly higher in focal bees that survived 24 h after treatment. There was no difference in allogrooming activity

(right) for focal bees that died or survived after 24 h. Note that allogrooming could only take place in the social group treatment ($N=40$ focal bees)

Discussion

In the current lab-based study, an immune-stimulating yeast treatment and topical pesticide treatment both modulated self-grooming behaviors, though impacts of yeast were dependent on social context. Individuals displaying more self-grooming had higher survivorship after 24 h even though the yeast and pesticide treatments had no significant impacts on mortality. Neither yeast infection nor topical pesticide application impacted allogrooming, despite its occurrence in about 50% of focal bee observations. Similarly, allogrooming did not predict focal bee survivorship.

Our results support other studies showing that grooming behaviors are triggered by a variety of stimuli, including foreign body and microorganism presence, immune activation, and chemical contact. It is thus reasonable to hypothesize that the function of these behaviors extends beyond foreign particle removal (Zhukovskaya et al. 2013). Better knowledge of the mechanisms that regulate grooming could provide new insights into its function. Possible regulatory mechanisms include immune system activation (Carr et al. 2020), a change in an individual's perception of her own odor profile (Richard et al. 2012), or stimulation of mechanosensory hairs (Land and Seeley 2004; Nicholls et al. 2016).

Variation in self-grooming could reflect differences in individual perceptual abilities, sensory thresholds, or activity levels. The association between self-grooming and survival supports the hypothesis that self-grooming is correlated with internal immune state and health resilience. It is important to note that we assessed grooming behaviors shortly after infection, but immune system dynamics can result in mortality and behavioral consequences over a longer timeframe (e.g., 24 h). In future studies, it would be interesting to examine the temporal dynamics of the immune system response and grooming behaviors, especially in the context of a replicating virus or other transmissible pathogen. This would help clarify the connection between immune activation, grooming activity, and the functional consequences at the group level.

Our results showing a correlation between positive health outcomes and self-grooming activity parallel a recent study showing that allogrooming specialists have elevated immune system activity, presumably as protection against potential infection (Cini et al. 2020). It is interesting to note that while pesticide and yeast treatments induced self-grooming, and self-grooming was associated with increased survival, neither yeast nor pesticide treatment impacted mortality. This could suggest that some aspect of the focal bee physiology predisposed it to groom or not, but that our treatments were not the main cause of

death. Another possibility is that our low mortality rate, though sufficient to detect highly significant effects of self-grooming, masked mortality impacts of either treatment.

In our study, we focused our behavioral analyses on a mixed group of middle-aged and older worker bees, and we did not control for variation in individual age or task specialization. This choice could impact the observed rate of allogrooming, as it is unlikely we gathered allogrooming specialists (Pettis and Pankiw 1998; Cini et al. 2020). Our approach may have also added behavioral variation within and among groups in our study (Cini et al. 2020). Though death rates in our experiment were extremely low compared to treatments provoking altruistic suicide, it is important to consider that the adaptive value of social immunity behaviors could change depending on the cost of losing specific individuals relative to the cost of infection spread; the cost of individual mortality decreases with age as productive working time decreases (Kuszevska and Woyciechowski 2014). This could result in age-dependent infection responses. For example, there is evidence that foragers stressed with CO₂ exposure and growth inhibitors spontaneously leave the colony prior to their own deaths, evidence of “altruistic suicide” (Rueppell et al. 2010). Future studies should investigate the mechanisms that link physical grooming processes to internal immune function or other measures of stress or disease, including the ways in which these relationships change with age and task. A better understanding of these mechanisms could help explain why the levels of some other behaviors, e.g., nest defense behaviors (performed by a similar cohort of bees to those used in our study), are correlated with immune system function and grooming activity (Rittschof et al. 2019; Carr et al. 2020).

Despite its self-directed nature, our results suggest a social component to self-grooming: yeast-injected individuals showed decreased self-grooming activity when kept in isolation. Isolated individuals may lack a motivation to groom, e.g., if grooming restores perceptual abilities (Boroczky et al. 2013) or socially relevant odor profiles (Richard et al. 2012). Alternatively, social isolation is a form of stress that impacts self-directed grooming behaviors in other species (Cinini et al. 2014). Isolation alters individual physiology in honey bees, but over a much longer timescale than that encompassed by the current study (Huang and Robinson 1992). We did not find evidence that social isolation alone induced differential mortality, suggesting that it does not cause severe acute stress. Future studies could explore how the presence of nestmates, or the frequency of interaction with nestmates, alters grooming behaviors.

Self-grooming is a critical component of the honey bee grooming solicitation dance (Bozic and Valentincic 1995). We did not notice or record dances in the current study, and the lack of correlation between self-grooming and allogrooming suggests very few occurred (Pettis and Pankiw

1998) despite a substantial overall rate of allogrooming. Though the dance is highly effective at inducing allogrooming (Bozic and Valentincic 1995), past studies suggest that the self-grooming component may play a larger role in parasite and pathogen resistance than the allogrooming response from nestmates (Pettis and Pankiw 1998). Our results support this finding, though it is important to note that our sample size, coupled with the low frequency of allogrooming, may have limited our ability to detect the relationships between health challenges, allogrooming, and survivorship.

Particularly for allogrooming, its function, triggers, and impacts at the colony level remain unclear (Aumeier 2001; Evans and Spivak 2010). Allogrooming is a widespread trait that appears very early in social insect evolution (Fefferman et al. 2006). However, modeling studies suggest allogrooming only increases colony survival when disease exposure is periodic, not constant (Fefferman et al. 2006). Allogrooming may have evolved under very different disease and parasite conditions than present day, and as a result, the behavior could persist without a clear survival benefit. Alternatively, it is possible that the benefits of allogrooming emerge only under very specific contexts, e.g., when an individual is sick with a pathogen that is capable of spreading amongst individuals, or in cases where honey bees couple grooming with other parasite defense capabilities, for example the ability to injure ectoparasites once removed (Guzman-Novoa et al. 2012; Smith et al. 2021). Exposure route (e.g., injection or parasite-mediated versus feeding) is known to impact viral pathogenicity and immune function (Yang and Cox-Foster 2007; Grozinger and Flenniken 2019; Al Naggar and Paxton 2020), and thus may be tied to the efficacy of allogrooming. Future studies could evaluate these possibilities by monitoring the extent to which, e.g., virus-infected bees direct behaviors towards healthy individuals, and the consequences for these interactions. Studies could also evaluate grooming interactions and their consequences across genotypes that differ in, for example, mite-biting behaviors. The honey bee highlights the complexity of interpreting the evolution and function of grooming interactions in highly social species.

The weak positive impacts of allogrooming could suggest that self-grooming (a key feature of the grooming repertoire that is also part of the allogrooming dance signal) may be the more critical disease management or survival component of the grooming repertoire (Pettis and Pankiw 1998; Aumeier 2001; Bąk and Wilde 2016). Such a pattern would be significant, because self-grooming does not put nestmates at risk of contracting a disease or becoming parasitized through contact (Bozic and Valentincic 1995; Richard et al. 2012). Though some studies show self-grooming is an important component of parasite defense (Aumeier 2001; Guzman-Novoa et al. 2012), to our knowledge, no study has assessed whether self-grooming modulates individual survivorship in other contexts that elicit the behavior. Here we demonstrate

that self-directed behaviors may serve important individual and social functions, and perhaps should be considered alongside allogrooming in studies of the evolution of social immunity behaviors.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00040-022-00868-2>.

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Author contributions AMF collected data and wrote the manuscript, RRW designed experiment, collected data, and edited the manuscript, MV designed figures and wrote the manuscript, CCR designed the experiment, analyzed data, and wrote the manuscript.

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Declarations

Conflict of interest Authors declare no competing interests.

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