1 **Primer Effects of the Honey Bee Queen Pheromone 9-ODA on Drones (*Apis***
2 ***mellifera*)**

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14 **ABSTRACT**

15 In many social insect species, pheromones coordinate defining features of social life.
16 Queen-produced pheromones mediate many of these processes, and thus there is
17 substantial interest in understanding both the mechanisms by which queen
18 pheromones organize behavior and how these chemical communication systems
19 evolved. It is hypothesized that queen social pheromones evolved from sex
20 pheromones found in their solitary ancestors. Here we begin to test this theory in
21 the honey bee, where the queen-produced pheromone 9-ODA (9-oxo-2-decenoic
22 acid) serves as both a social pheromone (priming physiological processes mediating
23 worker behavioral maturation) and sex pheromone (attracting males during mating
24 flights). While we expected the primer effects of 9-ODA on workers to represent a
25 derived worker-specific function, we surprisingly found similar effects in drones.
26 Exposure to 9-ODA resulted in a significant increase in expression levels of
27 *vitellogenin* in drones. Since previous studies in workers found *vitellogenin* levels
28 regulate behavioral maturation, we investigated 9-ODA's effects on sexual
29 maturation in drones. 9-ODA-exposed drones initiated mating flights later and took
30 fewer flights than control drones. Our results demonstrate that honey bee queen

pheromone has primer effects on drone bees, and thus chemical communication systems involving honey bee drones are more complex than previously appreciated.

KEYWORDS: Social organization, primer pheromone, drone, queen pheromone, sex pheromone, honey bee

INTRODUCTION

Social insects use chemical communication systems to organize nearly every aspect of colony life (reviewed in Czaczkes et al., 2015; Grozinger, 2015; Le Conte & Hefetz, 2008; Matsuura 2012; Slessor et al., 2005). Pheromones can regulate a myriad of social behaviors including brood care, defense, resource acquisition, reproductive division of labor between reproductive queens and sterile workers, and task differentiation among workers. Queen-produced pheromones are particularly important in regulating these social behaviors, and thus there has been long-standing interest in elucidating the origin and evolution of queen pheromones. It has been hypothesized that queen pheromones originally served as sex pheromones to attract males, and later evolved to regulate social behavior among the females in the colony (Kocher & Grozinger, 2011; Oi et al., 2015). Indeed, there is mounting evidence that cuticular hydrocarbons, which vary significantly according to physiological state (Amsalem et al., 2015; Liebig et al., 2000; Sledge et al., 2001), can

serve as fertility signals in ants and bees (reviewed in Howard & Blomquist, 2005), and thus these could represent chemical signals in solitary ancestors that are now being used to mediate social interactions in social species. However, we have little understanding of the sensory and neurophysiological mechanisms underpinning this hypothesized transition. The honey bee (*Apis mellifera*) is an ideal model system to investigate these questions, since the same queen pheromone (9-oxo-2-decenoic acid, hereafter 9-ODA) that attracts male drones to virgin queens during mating flights also mediates social interactions between the queen and workers in a colony (reviewed in Grozinger, 2015; Le Conte & Hefetz, 2008; Slessor et al., 2005).

In honey bee workers, the function of 9-ODA has been studied primarily in the context of a five-component blend produced by the mandibular glands of honey bee queens called 'queen mandibular pheromone' or QMP. The primary component of QMP is 9-ODA, and the remaining components include (R)-and (S)-9-hydroxy-(E)-2-decenoic acid (9-HDA), methyl *p*-hydrobenzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) (Slessor et al., 1988). QMP acts as a releaser pheromone, triggering short-range attraction of workers to the queen or a pheromone lure (Slessor et al., 1988) causing the workers to pick up the pheromone and spread it throughout the hive (Naumann et al., 1993). QMP also acts as a primer pheromone, slowing the behavioral maturation of worker bees from the nursing (brood care) behavioral and physiological state to the foraging state (and thus keeping workers in the nursing state longer), as well as reducing the numbers of foraging bees in a colony (Pankiw et al., 1998).

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83 QMP has many specific physiological effects on worker bees that underpin this
84 delayed maturation. For example, exposure to QMP reduces hemolymph titers of
85 juvenile hormone (JH) (Pankiw et al., 1998): reducing JH titers delays behavioral
86 maturation, while increasing titers accelerates behavioral maturation (Schulz et al.,
87 2002; Sullivan et al., 2000) Exposure to QMP increases worker expression levels of
88 the nutrient storage and egg-yolk precursor protein Vitellogenin (Vg) (Fischer &
89 Grozinger, 2008): Vg acts as a negative regulator of JH (Guidugli et al., 2005) and
90 higher levels of *vg* are associated with slower behavioral maturation (Antonio et al.,
91 2008). Furthermore, exposure to QMP reduces expression levels of the
92 transcription factor *Kr-h1* in the brains of worker bees: lower levels of *Kr-h1*
93 expression are associated with nursing versus foraging behavior (Grozinger &
94 Robinson, 2007; Grozinger et al., 2003). The effects of 9-ODA alone have not been
95 investigated as thoroughly, but exposure to 9-ODA also reduces levels of JH (Kaatz
96 et al., 1992) and *Kr-h1* (Grozinger et al., 2007). Note that 9-ODA alone (in the
97 absence of the other QMP components) does not significantly elicit short-range
98 attraction of workers (Grozinger et al., 2007; Slessor et al., 1988).

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100 In drones, 9-ODA stimulates releaser effects, eliciting long-range attraction to
101 queens during mating flights (Boch et al., 1975; Brockmann et al., 2006; Gary, 1962).
102 Possible primer effects of 9-ODA on drones have not been investigated. However,
103 many of the same physiological processes that regulate behavioral maturation in
104 workers regulate sexual maturation in drones. Drones initiate mating flights when

they are approximately 6-10 days old (Howell & Usinger, 1956; Fukuda & Ohtani, 1977; Rowell et al., 1986; Rueppell et al., 2006). JH titers in drones rise significantly when drones are 5 days old, corresponding with the onset of mating flights (Giray & Robinson, 1996). Treatment with a JH analog causes an early onset of mating flights (Giray & Robinson, 1996). Furthermore, based on comparisons on different genotypic strains of bees and transcriptomic analysis of worker behavioral maturation and drone sexual maturation, similar suites of genes regulate these two processes (Giray & Robinson, 1996; Zayed et al., 2012). Additionally, protein synthesis and protein titers of *vg* in drones rise rapidly in the days following eclosion as adults, peaking close to the time when drones initiate flights (~5 days old) and dropping thereafter, in a pattern similar to honey bee workers (Trenczek et al., 1989). However, it should be noted that in workers, JH levels rise in aging nurses, stay high after they transition to foraging and they exhibit a negative correlation with *vg* levels, while in drones, both *vg* and JH levels peak as mating flights are initiated, and then both decline steadily as drones age. Thus, though similar processes are involved in behavioral and sexual maturation, there may be differences in how these are regulated.

Here we test the hypothesis that 9-ODA originated as a sex pheromone/attractant in honey bees, and only later evolved to regulate social behavior in workers, likely through the emergence of novel and worker-specific sensory and neurophysiological pathways. Under this scenario, 9-ODA would serve as a social pheromone in workers, but would not elicit similar responses in drones. Since 9-

ODA alone and in the context of QMP regulates physiological processes associated with worker behavioral maturation, and since sexual maturation in drones appears to be regulated by similar mechanisms, we examined the effect of 9-ODA exposure on drone sexual maturation (the time to initiate mating flights and numbers of mating flights attempted) and associated physiological processes (expression levels of *vg*).

METHODS

General Honey Bee Rearing

Honey bee colonies were maintained according to standard apicultural practices at apiaries at The Pennsylvania State University, University Park, PA. To minimize genetic diversity among the workers used in the cage studies, we used workers produced by single-drone inseminated (SDI) queens (obtained from Glenn Apiaries, Fallbrook, CA). For the field trials with single cohort colonies, bees were derived from naturally mated (multiple-drone inseminated) queens. Note that since drones are produced from unfertilized eggs, brother drones are equally related regardless of the mating status of the queen. Replication of experiments used different colony sources, and replicates are referred to as “trials.”

For experiments requiring newly emerged workers (callow), honeycomb frames of emerging brood were collected and stored in a dark incubator at 34°C and 50% relative humidity. Emerging bees (<24 hours old) were collected and placed in

cages/colonies. For experiments requiring mature nurses, individuals were identified and collected as they inspected and fed larvae in comb cells within the brood nest of their colony.

Drone brood was obtained by caging a queen overnight on a honeycomb frame of drone comb cells (which are larger than the cells used to rear female worker brood), facilitating the laying of unfertilized, drone-destined eggs by the queen. These frames were maintained in the colony until drone emergence was imminent, at which point the frame was removed from the colony, placed in an incubator, and monitored for emergence. For the cage experiments requiring both callow workers and drones, worker emergence was synchronized to occur with drone emergence.

In the cage studies, 20 callow or mature nurse (henceforth “nurse”) workers and 10 callow drones from a single colony source were placed in individual Plexiglas cages (10x7x7cm). Workers were included because young drones require feeding by workers to survive (Free, 1957). Cages were provided with 50% sucrose and crushed pollen *ad libitum*. Cages were treated once daily with 20 µg of synthetic 9-ODA (Contech International, Victoria, BC), dissolved in 1% water/ isopropanol or a solvent-only control on a glass slide, as in (Grozingier et al., 2007). This amount of 9-ODA corresponds to 0.1 queen equivalents of the pheromone (Slessor et al., 1990) and has previously been shown to result in nurse-like levels of JH and *vg* (Fischer & Grozingier, 2008; Grozingier et al., 2003; Kaatz et al., 1992). Cages were maintained in a dark incubator at 34°C and 50% relative humidity throughout the duration of

the experiments. The experiments conducted herein on honey bees did not require any IACUC or related approvals and are in accordance with international animal care and use ethical standards.

Effect of 9-ODA Exposure on vg Expression

In the first set of cage studies, 10 one-day old drones were caged with 20 callow workers. We set up 6 replicates of untreated and 9-ODA treated cages. After 48 hours, the bees from all the cages were collected on dry ice and stored at -80°C. This was replicated across two colony sources, for a total of two trials.

In the second set of cage studies, 10 one-day old drones were caged with either 20 callow workers or 20 nurses. Cage exposure to 9-ODA or a solvent control was increased to 72 hours, for a total of 4 different treatments, with 6 cages per treatment. Bees were collected on dry ice after 72 hours and stored at -80°C. These studies were replicated across three colony sources, for a total of three trials.

To characterize *Vg* expression, 2-3 frozen individuals per cage were collected, using at least four of the six cages/treatment (see figures for the exact number of replicates in each trial). The digestive system was completely removed from the abdomen, leaving the abdominal cuticle and attached fat body, the primary site of vitellogenin synthesis (Amdam et al., 2009).

Individual abdominal fat bodies from collected drones were homogenized and RNA extracted using an RNeasy RNA isolation kit (Qiagen, Valencia CA). cDNA was synthesized from 150 µg of extracted RNA using Superscript II reverse transcriptase (Invitrogen, Carlsbad CA). Expression levels were quantified using an ABI PRISM 7900 sequence detector using the SYBR Green detector method (Applied Biosystems, Foster City CA). Samples were tested in triplicate and results averaged. Genomic DNA dilutions were used to construct a standard curve and relative quantities of RNA in each sample were calculated. Primer specificity and lack of genomic DNA contamination was confirmed using a dissociation curve and negative controls. Expression levels reported for *vg* (primer sequences, 5' to 3', F: AGTTCCGACCGACGACG, R: TTCCCTCCCACGGAGTCC) were normalized to the geometric mean of two housekeeping genes, *actin* (F: CCTAGCACCATCCACCATGAA, R: GAAGCAAGAATTGACCCACCAA) and *GAPDH* (F: GCTGGTTTCATCGATGGTTT, R: ACGATTTCGACCACCGTAAC); primers were successfully used in previous studies (Corona et al., 2007; Grozinger et al., 2003; Wang et al., 2014). The Grubbs test for statistical outliers was used to identify outliers and only one sample was removed from the analysis. The data was log transformed and confirmed to be normally distributed using a goodness of fit test. Significant differences in relative expression levels were assessed using a two-way ANOVA with trial, pheromone treatment, and rearing conditions as factors for multiple trials or a Student's *t* test for single trials (when full model interaction effects prohibited the use of ANOVA, but after test assumptions were confirmed to be satisfied). JMP 9.0.2 (SAS Institute Inc., Cary NC) was the statistical software used for all analyses.

Effect of 9-ODA Exposure on Trophallaxis Rates and Hypopharyngeal Gland Development

To assess the impacts of 9-ODA exposure on worker's likelihood to feed drones, we measured trophallaxis rates in each of the three trials of the second set of cage studies. Behavior was monitored under red light, two hours after the pheromone or solvent control treatment had been administered. Worker-drone trophallaxis events were recorded within each cage every five minutes for 40 minutes, repeated daily over the 72 h duration of the experiment. Event totals per cage per day served as the raw data for statistical analysis. Daily trophallaxis event means were compared within worker rearing groups using a repeated measures ANOVA using trial and treatment as factors with day as the repeated measure.

To assess the impacts of 9-ODA exposure on the worker's ability to feed drones, we measured hypopharyngeal gland size in callow or nurse bees in Trials 1 and 2 of the second cage study, using a protocol described in (Crailsheim & Stolberg, 1989). Six workers were collected across at least four cages per treatment after 72 hours of treatment. For each individual, the left hypopharyngeal gland was dissected in molecular grade water and eight random acini were measured lengthwise using an ocular micrometer at high magnification. Acini measurements were averaged per individual and means were compared across treatment groups using an ANOVA with trial and treatment as factors.

Effect of 9-ODA Exposure on Drone Sexual Maturation and Reproductive Behavior

Two single cohort colonies with equivalent amounts of callow workers, two nectar frames, one pollen frame and two empty frames were established in tandem from a single colony source. 100 newly emerged drones were individually number-tagged (Betterbee, Greenwich, NY) on their thorax and placed within each colony, marking the start of the experiment. One colony received a daily application of 200µg, or one queen equivalent (Pankiw et al., 1996), of synthetic 9-ODA while the other colony received a solvent control. The pheromone treatment was hung in a central location within the colony and allowed to diffuse throughout. The experiment was replicated in a second trial with a new colony source.

Each colony was outfitted with long runways to facilitate the observation of drone flights. Colonies were observed daily and continuously during typical drone flight times, with observations ceasing two days after the initiation of drone flights, since all the drones had taken flights at this point. Individual drone flights from and returning to the colony were documented, allowing us to discern the age of initial flight and flight rates for all individuals. Flight onset and frequency was used as a measure of behavioral and reproductive maturation. Drones will take short orientation flights followed by longer mating flights, usually in the same day (Howell & Usinger, 1956). We monitored the day that drones initiated flight activity, as well as the frequency with which individuals took flights. Though we may have achieved greater resolution of the reproductive status of the drones by assessing flight length (longer flights are typically assumed to be mating flights) (Howell & Usinger, 1956),

we propose that capturing age of flight onset and frequency are reasonable measures to assess drone reproductive state. Indeed, studies on drone mating flights have consistently found mating flights to begin seven to eight days into adulthood, sometimes earlier, and that the length of a flight is not necessarily indicative of reproductive status, as flight lengths of young adult drones (7-8 days old) can resemble those of older drones (15-20 days old) (Howell & Usinger, 1956; Rowell et al., 1986; Rueppell et al., 2006). The cumulative proportion of drones flying across treatments was compared using Kaplan-Meier survival analysis across both trials. The average number of daily flights taken per flying individual was compared using a Kruskal-Wallis Rank Sums tests due to data violations of normality assumptions.

RESULTS

Effect of 9-ODA Exposure on vg Expression

The first set of cage studies showed no significant effect of two days of 9-ODA exposure on *vg* expression levels in drones in Trial 1 ($t(13) = -0.193$, $p = 0.425$) and a significant effect of exposure in Trial 2 ($t(13) = -2.99$, $p = 0.0052$), with 9-ODA exposure resulting in significantly higher levels of *vg* expression (Figure 1).

To better resolve the outcomes from the first study, we repeated the cage experiment with three additional colony sources and increased 9-ODA exposure from 48 to 72 hours for our treatment groups. Additionally, we reared drones with

one-day old (callow) bees as well as mature nurse bees. Since 9-ODA exposure can slow behavioral maturation of young bees (Kaatz et al., 1992; Pankiw et al., 1998), it is possible that the 9-ODA altered the physiology of the young (callow) bees, thereby altering their ability to feed the drones, and thus altering drone *vg* levels. Thus, we included mature nurse bees, assuming they are less likely to be impacted by exposure to 9-ODA.

A significant effect of pheromone treatment ($p=0.014$), trial ($p=0.001$), pheromone x rearing ($p=0.0051$) and pheromone x trial x rearing ($p=0.015$) was found in our initial analysis (ANOVA, $F_{11,103}=8.132$, $p=0.0001$) and so the trials were analyzed separately and by rearing regime. Of the callow reared replicates (Figure 2A), Trial 1 and Trial 3 showed an effect of 9-ODA exposure on drone physiology, resulting in significantly higher levels of *vg* expression than the solvent controls (T1, $t(19)=-1.94$, $p=0.0336$; T3, $t(21)=-1.994$, $p=0.0296$). Trial 2 showed no statistical difference between treatments ($t(14)=-0.049$, $p=0.4809$).

Of the mature nurse-reared replicates (Figure 2B), Trial 2 and Trial 3 showed a significant effect of 9-ODA exposure on drone *vg* expression, with higher levels expressed in the pheromone exposed groups (T2, $t(13)=-1.921$, $p=0.0385$; T3, $t(22)=-1.766$, $p=0.0456$). No statistically significant differences were found in Trial 1 ($t(14)=0.5405$, $p=0.7013$).

Effect of 9-ODA Exposure on Trophallaxis Rates and Hypopharyngeal Gland Development

To determine if 9-ODA exposure altered drone-worker interactions or the ability of workers to feed drones, we evaluated trophallaxis events and the size of worker hypopharyngeal glands (which produce food for both developing brood and adult workers and drones) (Crailsheim, 1991). There was no significant effects of pheromone treatment ($p=0.4853$) on callow-reared drone trophallaxis rates, though there was a significant trial effect ($p=0.0005$) and no trial x treatment interaction ($p=0.1607$; Figure 3A, Repeated measures ANOVA $F_{5,27} = 1.021$, $p=0.0013$). No significant effects of pheromone treatment ($p=0.3855$), trial ($p=0.1384$), or trial x treatment interactions ($p=0.2735$) on nurse-reared drone trophallaxis rates were found (Figure 3B, Repeated measures ANOVA $F_{5,31} = 0.2271$, $p=0.2488$).

There were no significant effects of pheromone treatment ($p=0.2619$), trial ($p=0.7584$), or interactions ($p=0.1229$) on hypopharyngeal gland size in callow workers (Supplementary Figure 1, ANOVA $F_{3,20}=1.3417$, $p=0.2891$). A trial x treatment ($p= 0.06$) interaction effect in the nurse gland size analysis was found (ANOVA $F_{3,20}= 7.3323$, $p= 0.0017$) and trials were analyzed separately. In the first trial, nurse hypopharyngeal glands were significantly smaller in the pheromone treated group (Supplementary Figure 2, $t(10)= 3.53$, $p=0.0055$). In the second nurse trial, gland sizes across treatments were not significantly different ($t(10)= -1.005$, $p=0.339$).

Effect of 9-ODA Exposure on Drone Sexual Maturation and Reproductive Behavior

Drones were reared in colonies treated with 9-ODA or a solvent control. A significant effect of 9-ODA exposure on the onset of flight behavior was found, with significantly less pheromone-exposed drones flying on day 7 than those in the control group across the two trials (Figure 4A, Kaplan Meir Survival, Wilcoxon $X^2(1) = 8.959$, $p = 0.0028$). Overall, 31% of control and 19% of 9-ODA exposed drones took at least one flight on day 7, representing a difference of ~61% in flight activity between treatments. By day 8, 95% of control and 93% of 9-ODA exposed drones had taken at least one flight. Overall, 10 control and 15 9-ODA drones did not take a single flight through the course of the study.

For each trial, we assessed the mean number of flights taken by all flying drones within each treatment and compared these means across treatments. In both trials, there was no significant effect of treatment on the average number of flights individuals took on day 7 (Figure 4B, T1 Wilcoxon Rank Sums $X^2(1) = 0$; T2 Wilcoxon Rank Sums $X^2(1) = 0.7833$, $p = 0.3761$). On day 8, control drones took a significantly greater average number of flights in both trials (T1 Wilcoxon Rank Sums $X^2(1) = 12.24$, $p = 0.0005$; T2 Wilcoxon Rank Sums $X^2(1) = 7.638$, $p = 0.0057$).

DISCUSSION

Here we describe, for the first time, a pheromone that elicits primer effects on adult male honey bees (*Apis mellifera*). Exposure to 9-ODA reduces expression levels of *vitellogenin*, a protein that has been shown to regulate honey bee worker behavioral

maturation (reviewed in Amdam et al., 2009) and whose protein synthesis rates and hemolymph titers are strongly correlated with the onset of mating flights in drones (Trenczek et al., 1989). Furthermore, exposure to 9-ODA delayed the timing of mating flight initiation and reduced the number of flights individual drones take. Overall, these results are similar to the effects of 9-ODA exposure – in the context of the full QMP blend- on worker bees, where exposure increases *vg* expression levels in caged worker bees (Fischer & Grozinger, 2008) , delays workers' transition to foraging and reduces the number of foraging flights observed for a colony (Pankiw et al., 1998).

Expression levels of *vg* were significantly increased in drones exposed to 9-ODA in five out of eight cage trials and across multiple rearing regimes. Variation across trials may be a result of innate differences in physiological sensitivity to the effects of 9-ODA across the eight distinct male genotypes we used, a phenomenon previously described in workers (Galbraith et al., 2015; Kocher et al., 2010; Pankiw et al., 1994, 1995). It is interesting to note that there was substantial variation in *vg* levels in 9-ODA exposed groups, which may have reduced our power to observe significant impacts on *vg* levels. If 9-ODA is spread to drones primarily through trophallaxis, as it is with workers (Naumann et al., 1991), then this mode of exposure may result in inter-individual variation in dose as a function of trophallaxis events experienced by each drone and the feeding worker having picked up 9-ODA prior to feeding males. Indeed the largest variances in *vg* expression occurred exclusively in the 9-ODA exposed groups (Figure 2).

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388 Our results indicate that the impacts of 9-ODA on drone physiology are direct
389 effects, and not due to changes in the interactions of drones and workers or worker
390 physiology. Previous studies have found *vitellogenin* synthesis and gene expression
391 levels are sensitive to nutrition in workers (Bitondi & Simões, 1996; Nilsen et al.,
392 2011), and thus we were concerned that the effects we observed were due to
393 altered feeding of the drones by the workers in the 9-ODA versus solvent
394 treatments. To address potential effects that poor nutritional quality or inadequate
395 trophallaxis as provided by workers may have had on drones, we included a mature-
396 nurse rearing group. We reasoned that nurses have well-developed hypopharyngeal
397 glands (Crailsheim & Stolberg, 1989) and specialize in rearing/feeding other bees
398 and should therefore provide adequate and consistent nutrition to drones,
399 regardless of exposure to 9-ODA. Pheromone treatment was found to have no effect
400 on trophallaxis rates regardless of rearing regime (Figure 3) or on hypopharyngeal
401 gland size in callow workers (Supplementary Figure 1). In the first nurse-reared
402 trial (Supplementary Figure 2), nurses in the 9-ODA treatment had smaller
403 hypopharyngeal glands than the control nurses. This difference is likely to be a
404 function of random nurse sampling from within a colony at the start of the study.
405 Interestingly, however, drones reared by these nurses may have experienced a
406 blunting of the treatment's effects on their *vg* expression (see Figure 2B, Trial 1).
407 This association is correlative, but highlights a potential avenue through which the
408 social environment might modulate 9-ODA's effects on drones. Nonetheless, in the
409 absence of differences in worker feeding rates and gland size between treatments,

9-ODA exposure was observed to significantly increase *vg* expression in drones, suggesting it is having a direct impact on drone physiology (Figure 2).

The function of *Vg* in drones remains to be elucidated. In honey bee workers, *Vg* has pleiotropic functions, regulating worker behavioral maturation (Nelson et al., 2007), juvenile hormone titers (Guidugli et al., 2005), metabolic pathways (Corona et al., 2007; Nilsen et al., 2011), and immunity (Amdam et al., 2004). In drones, and based on known vitellogenin functions in worker honey bees or other insects, it has been postulated that *Vg* may play a role in metabolism or in regulating aspects of social behavior (Piulachs et al., 2003). Unfortunately, claims suggesting such functions in drones have remained mostly unsupported by experimental evidence (Amdam et al., 2009). As our results demonstrated that 9-ODA exposure delayed the onset of mating flights and reduced the number of mating flights in drone bees, it suggests that *Vg* may play a role in regulating sexual maturation in drones, as it does in regulating behavioral maturation in workers.

The observed impacts on behavior could have been produced as a result of 9-ODA's effects on *vg* expression levels. In workers, *Vg* regulates behavioral maturation (Antonio et al., 2008), in part through its interactions with JH (reviewed in Amdam & Page, 2010). Though the function of *Vg* in drones is unclear, the endocrine mechanisms regulating behavioral transitions in workers and drones are similar in key respects. *Vg* is higher in nurses and immature drones compared to foragers and sexually mature drones. *Vg* synthesis reaches a peak in drones between the fourth

and fifth day of adulthood (Trenczek et al., 1989), after which Vg declines quickly, reaching undetectable levels after two weeks. Soon after Vg begins declining and just before mating flights begin, JH titers in the hemolymph peak (Giray & Robinson, 1996). This pattern of Vg and JH regulation in drones is consistent with Vg and JH's mutually suppressive relationship (Amdam et al., 2007; Guidugli et al., 2005; Nelson et al., 2007), and suggest endocrine pathways through which 9-ODA may impact the timing and frequency (Tozetto et al., 1997) of drone reproductive behavior, though further studies are needed to confirm this.

Our results demonstrated that 9-ODA regulates sexual maturation (in terms of the time to initiate flights) and sexual behavior (in terms of the numbers of flights attempted) in drone honey bees, but it remains to be determined how this impacts the fitness of drones. Sexual maturation may be delayed and mating flights reduced in queenright, normally functioning colonies if there is a trade-off with reproductive success. Precocious behavioral maturation in workers results in poorer foraging success: workers take fewer flights, bring back less food resources, and have shortened lifespans (Perry et al., 2015). Studies in drones have not been as extensive, but immature drones attempting mating flights often fall off the hive entrance and ultimately die, as they are unable to fly and return to the hive (Villar, personal observations). Reducing flight frequency may reduce the risk of exhaustion or predation, or it is possible that the 9-ODA exposed drones took fewer, but longer, flights than the solvent exposed drones. How flight duration versus flight frequency impacts mating success remains to be determined. Interestingly, drones infected

with the gut microsporidian *Nosema* took significantly shorter flights than uninfected drones and there was a trend for these drones to initiate flights earlier, suggesting the pathogen negatively impacted the drones' sexual physiology and behavior (Holt et al., 2017). Furthermore, drones do not produce an optimal seminal fluid mix, consisting of accessory mucus gland proteins and adequately matured and motile spermatozoa, in the early stages of adulthood (reviewed in Page & Peng, 2001) and thus an early-flying drone may not have sufficient semen to effectively inseminate the queen (note that the queen usually mates with an average of 12 drones, and mixes the pool of semen to use for subsequent egg-fertilization) (Tarpy et al., 2004). Drones also produce chemical signals in their mandibular glands that help them form mating aggregations, and this may be reduced in young drones (Lensky et al., 1985). However, it is possible that in a failing colony lacking a queen (note that loss of a queen is strongly correlated with colony loss (vanEngelsdorp et al., 2013), the absence of 9-ODA can stimulate the remaining males in the colony to initiate mating flights earlier and with greater frequency.

In conclusion, this study provides evidence for a newly described primer role of 9-ODA on drones in honey bees. Not only are primer pheromones quite rare in the animal kingdom, but there are few cases of primer pheromones impacting males - the majority impact female fertility and maturation (Wyatt, 2003). Furthermore, while it has been hypothesized that social pheromones regulating female reproductive division of labor in social insect societies could have evolved from solitary ancestors using the same or similar compounds as sex pheromones (Kocher

& Grozinger, 2011; Oi et al., 2015), our results do not provide support for this model for the queen-produced pheromone, 9-ODA, in honey bees. According to our results, 9-ODA appears to regulate common physiological pathways (involving Vg and JH) in both drones and workers, and thus it did not evolve to mediate novel social behaviors in workers. However, it is difficult to determine if 9-ODA actually serves as the sex pheromone in the solitary ancestor of honey bees, as all *Apis* species are social (Michener, 1974), all produce 9-ODA (Blum, 1996; Plettner et al., 1997), and 9-ODA may ultimately be taxon specific (Oystaeyen et al., 2014). Thus, both the sex and social function of 9-ODA may be newly evolved in social *Apis* species.

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