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The possible role of ritualized aggression in the vibration signal of the honeybee, *Apis mellifera*

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Cooperative activities in advanced insect societies are organized by complex systems of communication signals that may have evolved from interactions among nestmates in primitively social ancestors. Primitively social colonies are organized by dominance and aggression, which suggests that some communication signals in advanced societies may have evolved through ritualized aggression. For example, many primitively social insects perform repetitive body movements during aggressive encounters and such movements in ancestral species may have become ritualized into vibratory signals, such as 'abdominal wagging' in *Polistes* wasps, and the 'vibration signal' of the honeybee *Apis mellifera*. We explored this possibility by examining the performance of the vibration signal by queenless honeybee workers. Under typical colony conditions, the vibration signal helps to coordinate cooperative labour; however, the function of the signal under queenless conditions is poorly understood. When a colony is permanently queenless, the ovaries of some workers activate and there is a pronounced increase in overt aggression, expressed as 'mauling', in which workers attack nestmates with greater ovarian activation. Here, we show that workers that performed vibration signals throughout their adult lifetimes had greater ovarian activation and an increased tendency to perform mauling. These results suggest that vibration signal performance is associated with the reproductive competition that characterizes queenless colonies. We next compared brain–gene expression patterns in persistent queenless vibrators, persistent recipients and nonvibrating control bees, for eight candidate genes associated with aggressive nest defence in honeybees and dominance in the primitively social wasp *Polistes metricus*. Five of the eight genes were upregulated in persistent vibrators compared to recipients and controls. Our results are consistent with the idea that the vibration signal of the honeybee evolved from repetitive aggressive movements, perhaps by co-opting components of a conserved genetic module associated with aggression.

In the insect societies, the evolution of social complexity has occurred hand in hand with the evolution of increasingly elaborate systems of chemical and vibroacoustic communication signals (Hölldobler & Wilson, 2009; Hunt & Richard, 2013; Richard & Hunt, 2013). A major goal in the study of social insects is to understand how the communication signals that organize advanced societies may have evolved from pre-existing behaviours in primitively social and solitary ancestors (Block & Grozinger, 2011; Dolezal & Toth, 2014; Liang et al., 2012; Woodard et al., 2011). Colonies of primitively social species are organized through interactions that involve dominance and aggression (De Souza & Prezoto, 2012; Jeanne, 2009; O'Donnell, 2001; 2006; Thompson, Donaldson, Johnstone, Field, & Cant, 2014). This raises the possibility that some communication signals used to orchestrate cooperative activities in advanced insect societies may have evolved through ritualized aggression (Lambda, Chandrasekhar, & Gadagkar, 2008; Molina & O'Donnell, 2009; Penick, Brent, Dolezal, & Liebig, 2014; Powell & Tschinkel, 1999). Comparative sociogenomics provides an array of approaches for exploring the molecular basis and evolution of animal signals (Block & Grozinger, 2011; Toth & Robinson, 2007; Toth et al., 2014). Candidate genes associated with dominance and aggression in primitively social species can be identified and the activity of these genes can be compared between signallers and recipients within an advanced social species, as well as among species showing different levels of social complexity. Such comparisons can elucidate the possible role of aggression in the performance of a particular communication signal, as well as reveal

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possible scenarios for signal evolution (Alaux, Sinha, et al., 2009; Sen Sarma, Rodriques-Zas, Hong, Zhong, & Robinson, 2009; Toth et al., 2014, 2007).

A signal that may contain elements of ritualized aggression is the vibration signal of the highly social honeybee *Apis mellifera*. The vibration signal (sometimes called the shaking signal) consists of a worker rapidly vibrating its own body dorsoventrally for 1–2 s, usually while grasping a recipient with its legs (Milum, 1955). The signal exerts a nonspecific, modulatory influence that enhances a wide variety of cooperative activities, including foraging, brood care, nest maintenance, caste interactions and colony reproduction (Cao, Hyland, Malechuck, Lewis, & Schneider, 2007, 2009; Pierce, Lewis, & Schneider, 2007; Schneider & Lewis, 2004; Slone, Stout, Huang, & Schneider, 2012).

Although the vibration signal shows no signs of aggression under queenright conditions (the typical colony state in which a laying queen is present), three lines of evidence suggest that the signal may have associations with aggressive behaviour. First, genes that are expressed in aggressive contexts in honeybee colonies contribute to vibration signal performance. Vibrating workers in queenright colonies show distinct brain–gene expression patterns that have significant overlap with those of workers exposed to alarm pheromone, which is associated with stinging and aggressive colony defence (Alaux, Duong, et al., 2009; Alaux, Sinha, et al., 2009). Second, the vibration signal may be associated with reproductive competition among workers in queenless colonies. When a honeybee colony becomes permanently queenless, some workers activate their ovaries and there is a dramatic increase in overt aggression expressed as 'mauling', in which workers attack, bite and harass nestmates with greater ovarian development (Sakagami, 1954; Malka, Shnieor, Katzav-Gozansky, & Hefetz, 2008). Under queenless conditions, the tendency to engage in vibration signal activity is associated with both ovarian activation and mauling behaviour (Schneider & McNally, 1991). Third, the vibration signal may share genetic mechanisms in common with vibratory signals associated with dominance and aggression in primitively social species. In the primitively social wasp *Polistes metricus*, dominant females perform a vibration-like display called 'abdominal wagging', which may be associated with reproductive competition and influence the behaviour of subordinates (Jeanne, 2009; Jandt, Tibbetts, & Toth, 2014). Dominant *P. metricus* females show distinct brain–gene expression profiles that have significant overlap with aggression-related genes in *A. mellifera*, the fruit fly *Drosophila melanogaster,* and the mouse *Mus musculus* (Toth et al., 2014, 2007). A preliminary comparison of the brain–gene expression patterns of worker honeybees performing vibration signals under queenright conditions with those of dominant *P. metricus* females showed significant overlap for a series of genes that track dominance in the wasp (Supplementary Material; Table S1). Because bee and wasp sociality evolved independently (Hölldobler & Wilson, 2009), bee–wasp gene expression similarities are not meant to suggest a linear trajectory from primitively to advanced social; instead, these overlapping genes may represent elements of a conserved gene expression module related to aggression in a wide variety of taxa.

Taken together, these observations raise the possibility that the vibration signal of the honeybee may have evolved from vibratory dominance displays in primitively social ancestors through ritualized aggression. Although the vibration signal is used to promote cooperative labour in honeybees under most circumstances, it may still contain elements of aggression that are expressed under queenless conditions and involve gene expression patterns that are also expressed in aggressive contexts. However, the possible role of aggression in the vibration signal is poorly understood. Only a single study has explored the vibration behaviour of queenless workers and it was restricted to 'snapshot' comparisons of the ovarian development of dyadic pairs of individuals immediately engaged in performing and receiving a single signal (Schneider & McNally, 1991). Lifetime tendencies to engage in vibration signal activity under queenless conditions have not been determined, although such studies are more likely to reveal the long-term associations between ovarian development, signalling behaviour and the underlying patterns of gene expression. Also, brain–gene expression patterns of queenless honeybee workers engaging in vibration signal activity have never been compared to those associated with dominance in primitively social species, or those associated with honeybee aggressive behaviour.

The purpose of our study was to examine the behavioural and molecular associations between vibration signal activity and aggression in queenless honeybee colonies. We did this by (1) generating adult lifetime behavioural profiles for vibration signal activity, (2) determining the associations between persistent signalling activity, ovarian development and aggressive mauling, and then (3) comparing brain–gene expression levels among persistent vibrators, persistent recipients and nonvibrating control bees for a series of candidate genes that have a known association with aggression and dominance in honeybees and *Polistes* (Table 1). We predicted that lifetime tendencies to engage in vibration signal performance under queenless conditions would be positively associated with ovarian activation and mauling behaviour. We also predicted that expression levels for aggression-related genes would be higher in queenless vibrators than in vibrated recipients and nonvibrating controls.

METHODS

Establishing Queenless Observation Colonies

Vibration signal performance was examined in three queenless observation colonies (colonies A, B and C) maintained on the campus of the University of North Carolina at Charlotte for a 5-week period from June to July 2011. To establish the observation colonies, we first set up three small queenless colonies in the field by transferring four frames of comb filled with brood and food and 8000–10 000 workers (but not the queen) from each of three large, unrelated field colonies maintained in 10-frame Langstroth hive boxes. The small queenless field colonies were examined every 2–3 days for the next 10 days and all developing queen cells were destroyed. At the end of the 10-day period, there were no young larvae remaining that could be reared into new queens, resulting in permanently queenless conditions. Each queenless field colony was then transferred into a four-frame observation hive equipped with Plexiglas sides containing hinged access ports through which workers could be removed for genetic analysis and determination of ovarian activation. The observation colonies were fed 50% sucrose solution (by volume) as needed to maintain similar levels of food stores among colonies throughout the 5-week study period.

Establishing Populations of Marked Workers

We examined vibration signal behaviour for individually recognizable workers that arose from single-drone inseminated (SDI) queens. Because of the haplodiploid method of sex determination in honeybees, workers arising from the same queen mother and drone father share 75% of their genome in common. Utilizing workers from SDI queens standardized the genetic background of individuals belonging to the same matriline, which facilitated comparisons of gene expression patterns (Alaux, Duong,

Table 1
The eight candidate genes examined in persistent vibrators, persistent recipients and control workers from queenless honeybee colonies that track aggression and dominance in other species

Putative gene			Association with		*Drosophila* orthologue
Name	*Apis* GB	Function	*Polistes* dominance[a]	*A. mellifera* aggression[b,c]	
AmADH	GB12741	Aldehyde dehydrogenase	Differentially expressed in brains of dominant vs subordinate wasps	Greater expression in older vs younger workers (aggressive tendencies increase with age)	FBgn0012036; ethanol response; pyruvate metabolism; oxidation/reduction
AmBlack	GB19363	Cysteine sulfinic acid decarboxylase	Differentially expressed in brains of dominant vs subordinate wasps	Greater expression in African vs European honeybee workers	FBgn0000153; aggressive behaviour; visual behaviour; beta-alanine biosynthesis; pigmentation
AmCG17737-like	GB19193	Unknown	Differentially expressed in brains of dominant vs subordinate wasps	Unknown	FBgn0035423
AmCyp9f2	GB17793	Cytochrome P450 9f2	Differentially expressed in brains of dominant vs subordinate wasps	Unknown	FBgn0038037; aggressive behaviour
AmCG9369-like	GB15506	Dehydrogenase/reductase 11-like	Differentially expressed in brains of dominant vs subordinate wasps	Greater expression in older vs younger workers (aggressive tendencies increase with age)	FBgn0030332
AmDopR1	GB30031	Dopamine receptor 1	Unknown	Soldier behaviour; greater expression in older vs younger workers (aggressive tendencies increase with age)	FBgn0011582; learning and memory; sucrose response
AmJHamt	GB10517	JH acid methyltransferase	Unknown	Soldier behaviour	FBgn0028841
AmOct-TyrR	GB17991	Octopamine and tyramine receptor	Unknown	Soldier behaviour; guarding behaviour	FBgn0004514; olfaction

'*Polistes* dominance' refers to genes that showed differential expression between dominant and subordinate workers, foundresses, or queens. For each gene, the given 'name' was based on homology to annotated genes from *Drosophila* melanogaster.
[a] Toth et al. (2007).
[b] Alaux, Duong, et al. (2009).
[c] Alaux, Sinha, et al. (2009).

et al., 2009). Three commercially produced SDI queens (Glenn Apiaries, Fallbrook, CA, U.S.A.) were established in separate field colonies 6 weeks before the study began, to ensure that by the time workers were gathered for marking all were offspring of the SDI queens. The field colonies used to house the SDI queens were separate from those used to create the queenless observation hives.

To obtain workers for marking, frames of sealed brood were taken from each SDI-queen colony, placed in pre-labelled nylon-mesh cages, and kept in an incubator (33.5 °C; 50% RH). Workers were collected within 24 h of emergence and each was marked by gluing a plastic tag (Opalithplättchen, Graze, Germany) to the thorax with a unique colour/number/paint mark combination that allowed for individual identification, as well as identification of each worker's matriline. In this manner, the larval development of each tagged worker occurred under queenright conditions, whereas its entire adult life occurred under queenless conditions, which simulated the situation in which workers emerge in a colony that has become permanently queenless. Cohorts of 200 tagged workers were added to each observation colony at 5-day intervals until a total of 600 had been introduced into each hive. All tagged workers added to a given observation colony arose from the same SDI queen, such that colony A received workers only from one SDI queen, colony B only from a second SDI queen, and so on.

Generating Lifetime Behavioural Profiles

Each queenless observation colony was monitored by at least two observers continuously during 0700–1800 hours each day, 7 days a week, throughout the study period. Observers rotated among the observation colonies on an hourly basis to minimize observer bias. During each hour, we recorded the identity of every tagged worker that performed the vibration signal, that received the vibration signal, that performed mauling and that received mauling. Mauling was identified based on the descriptions provided by Sakagami (1954) and Malka et al. (2008): the worker performing mauling would bite or rapidly and frantically chew on the abdomen of the recipient, which could adopt a 'flinching' posture and sometimes attempt to escape. Each observation colony was monitored for a total of 385 h over the 5-week study period, which minimized the possibility that we missed tagged workers engaging in the focal activities. We monitored only the behaviour of the tagged workers; we did not record the activity of the unmarked workers in the colonies, nor did we determine colony-wide levels of vibration signal and mauling activity.

Workers could both perform and receive the vibration signal during their lifetimes under queenless conditions. Therefore, at the end of the study period, we determined the following aspects of vibration signal activity for each individual tagged worker monitored: (1) the total number of vibration signals performed and received; (2) the daily rate of signalling activity (signals performed or received/day); and (3) the total number of days on which the bee performed or received vibration signals. Additionally, we classified each tagged worker that engaged in vibration signal activity as either a 'persistent' vibrator or recipient, or a 'nonpersistent' vibrator or recipient. Persistent individuals were those that performed or received vibration signals for 2 or more days during their adult lifetimes; nonpersistent workers were those observed to perform or receive vibration signals during only 1 day of the study period. Vibrating bees often produce a series of signals (range 2–20 or more/min) and can perform several bouts of signalling per day (Schneider & Lewis, 2004). Workers can also receive vibration signals repeatedly during a given period (Cao et al., 2009). Thus, workers classified as persistent individuals typically performed or received far more vibration signals during their lifetimes than did nonpersistent workers. In addition to the aspects of vibration activity determined, we also determined for each tagged individual the total events of mauling performed and received during its adult lifetime.

Collecting Bees for Analysis

At the end of the study period, we collected two groups of tagged workers from each observation colony. First, throughout the final week of the study period, we collected persistent vibrators, persistent recipients and nonvibrating control bees for brain–gene expression analysis. In addition to identifying persistent vibrators and recipients as workers that had previously engaged in signalling activity for at least 2 days during their adult lifetimes, we used the following collection criteria to enhance the detection of gene expression patterns associated with the vibration signal. We required that persistent vibrators collected for analysis could only have produced signals and never received them during their adult lifetimes, and that they also had to be performing the signal at the moment of collection. We required that persistent recipients had only received and never produced signals during their lifetimes, and that they were receiving a vibration signal at the moment of collection. For each persistent vibrator and recipient collected, we also collected a same-age control worker that had never been observed to perform or receive vibration signals during its lifetime. Persistent vibrators, recipients and controls were removed through the hinged access ports of the observation hives using padded forceps and flash-frozen in liquid nitrogen, following the methods of Alaux, Duong, et al. (2009). The head of each bee was immediately removed, placed in a prelabelled microcentrifuge tube and stored at −80 °C for subsequent genetic analysis. The thorax and abdomen of each bee were placed in a prelabelled vial and stored in 70% isopropyl alcohol for ovarian activation analysis. Our stringent collection requirements, coupled with the natural attrition of workers as the study progressed, limited the number of individuals available for collection during the final week of the study, and we collected a total of 15 persistent vibrators, 14 persistent recipients and 18 controls from our three colonies for the examination of brain–gene expression patterns.

Second, on the final day of the study period, we collected all remaining tagged workers for ovarian activation analysis. Each observation colony was opened, every frame was removed, and all remaining tagged workers were plucked off with forceps and killed by submersion in 70% isopropyl alcohol. Each tagged worker was then transferred to a separate microcentrifuge tube and stored in 70% isopropyl alcohol at −20 °C until dissected to determine ovarian development. The typical life span of a worker during spring and summer is 4–6 weeks, and many of the tagged workers that we monitored in the observation colonies were lost through natural attrition before the end of the 5-week study period. Consequently, we were able to collect and dissect a total of 285 bees for which we also had complete lifetime behavioural profiles (including the individuals collected for genetic analysis). These dissected workers provided a large sample for examining the associations between ovarian development and adult lifetime behavioural profiles for vibration signal activity. All workers collected for genetic and ovarian analysis experienced 3–5 weeks of queenless conditions as adults before they were removed from the observation colonies.

Determining Ovarian Activation

Workers were dissected and the level of ovarian activation was assessed following previously described procedures (Dixon, Kuster, & Reuppell, 2014; Schneider & McNally, 1991). We used the presence and maturity of forming oocytes to classify the left and right ovary of each worker as reproductively 'active' (individual oocytes clearly present and deforming the shape of the ovariole) or 'inactive' (oocytes absent or not exceeding the width of the ovariole) (Dixon et al., 2014). If the left and right ovaries of an individual bee differed in development, we classified the worker according to its greatest level of activation.

Analysing the Relationships between Ovarian Activation and Lifetime Behavioural Profiles

We used two approaches to assess the associations between ovarian activation and lifetime vibration signal activity. First, log-linear analysis was used to compare the proportion of workers with active and inactive ovaries that performed vibration signals for 2 or more days during their lifetimes. Second, we use repeated measures ANOVA to compare workers with active and inactive ovaries for the number of vibration signals performed per day. A separate ANOVA was conducted to compare workers with active and inactive ovaries for daily rates of receiving vibration signals. In the ANOVAs, the subject was the individual tagged bee and the between-subjects factors were ovarian activation category (active or inactive) and colony. The analyses that examined the relationships between ovarian activation and vibration signal activity were restricted to the 285 dissected bees.

To examine the relationship between the vibration signal and mauling, we used log-linear models to compare the proportions of vibrator and recipient tagged bees that performed mauling during their lifetimes. A separate log-linear analysis was conducted to compare the proportion of vibrators and recipients that received mauling. These analyses were based on all tagged bees for which we generated behavioural profiles, whether or not they were ultimately collected for genetic and ovarian activation analysis. Because workers could potentially perform and receive vibration signals during their lifetimes, we used the following criteria to ensure that each tagged individual was classified as either a vibrator or a recipient and used only once in each analysis. If an individual bee both performed and received vibration signals during its lifetime, it was classified according to which activity it engaged in most often.

Determining Brain–Gene Expression Patterns

Our goal was to compare queenless persistent vibrators, persistent recipients and nonvibrating control honeybees for the expression levels of eight previously identified candidate genes (Table 1) that are known to be involved in aggressive behaviour in *A. mellifera* and to be associated with differences in dominance status in *Polistes* wasps (both workers and queens), and which show significant overlap with brain–gene expression patterns associated with vibration signal performance by queenright workers (Alaux, Duong, et al., 2009; Alaux, Sinha, et al., 2009; Toth et al., 2014). The head of each collected persistent vibrator, persistent recipient and control bee was freeze-dried at 13.9 Pa (0.105 torr) for 95 min using a 2.5-litre Benchtop Cascade Freeze Dryer to facilitate the removal of cuticle and other extraneous tissues from individual brains. Dissections were conducted on dry ice in the direct presence of molecular biology-grade RNase free ethanol. The hypopharyngeal glands and suboesophageal ganglion were carefully removed during dissection to prevent possible nonbrain tissue contamination (Wagener-Hulme, Kuehn, Schulz, & Robinson, 1999). Dissected brains were placed in the bottom of 1 ml microtubules and stored at −80 °C for RNA extraction.

Total amount of RNA of individual brains was purified using Qiagen RNeasy Mini Kit. The protocol for the purification of total RNA from animal tissues (RNeasy Mini Handbook, 4th ed.) was used with the following modification. Given the size of brain tissue (<20 mg) 350 μl of RLT Buffer (Qiagen) was used during homogenization. Additionally, DNase digestion was performed on all samples to remove potential contamination of genomic DNA. RNA was

eluted with 60 μl of RNase free water. The concentration (ng/μl) and quality assessments, via 260/280 and 260/230 ratios, of RNA extractions were obtained using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Samples with RNA concentrations below 28.8 ng/μl were dried down to ensure that all transcripts were amplified and not just highly expressed mRNAs. To synthesize cDNA from extracted RNA, the protocol issued for Invitrogen SuperScript II Reverse Transcriptase was utilized. In addition, for each qRT-PCR run, two negative controls were routinely run to rule out contamination from water sources and from RNA: a control with only water, and a single, randomly selected sample in which no reverse transcriptase was added to the cDNA synthesis reaction.

Genes of interest (Table 1) were identified from two sources. First, we prioritized genes that showed significant overlap in differential expression between honeybee vibrational signal performers (Alaux, Duong, et al., 2009) and *P. metricus* differing in dominance status (Toth et al., 2014; Supplementary Material, Table S1). Bee and wasp genes were assigned as putative orthologues based on a previous study that used identified conserved *Drosophila* homologues for each gene (Toth et al., 2010). Second, we added three additional genes with associations to different forms of aggression (response to alarm pheromone, subspecies differences in aggression, and age) in honeybees (Alaux, Sinha, et al., 2009). These three additional genes were of special interest because they had functions associated with biogenic amines and juvenile hormone, both of which have established relationships to aggression and dominance in other invertebrates (Kravitz & Huber, 2003). We obtained gene sequences for the genes of interest by retrieving honeybee gene models from BeeBase (designated with GB identifiers) and giving them putative names based on their best hits to *D. melanogaster* annotated genes. These nucleotide sequences were then used to design primers using Primer Quest online from Integrated DNA Technologies (Coralville, IA, U.S.A.). A full list of primer sequences is provided in the Supplementary Material (Table S2). Real-time qPCR was then performed with a Roche 480 Light Cycler using 2× SYBR Green Master Mix (Applied Biosystems, www.lifetechnologies.com). Relative expression of each gene was calculated by comparing to standard curves of genomic DNA generated from whole *A. mellifera* bodies. Each sample was run in triplicate with mean concentration values later used for statistical analysis. Three control genes, *AmActin*, *AmeIF3-S8* and *Amef1-alpha,* were used to normalize expression levels of each candidate gene; the geometric mean of all three control genes taken together was used for normalization. The normalized expression levels were compared among the three groups of worker using one-way ANOVA, followed by post hoc comparisons using Tukey tests. Post hoc comparisons between persistent vibrators and persistent recipients assessed the influence of a bee's role in vibration activity on brain–gene expression patterns. Post hoc comparisons with the nonvibrating controls provided insights into the degree to which levels of gene expression were associated with signalling activity under queenless conditions.

RESULTS

Relationships among Lifetime Vibration Activity, Ovarian Activation and Mauling Behaviour

Of the 1800 total tagged bees added to the three observation hives (600 per colony), 871 engaged in vibration signal activity during their adult lifetimes under queenless conditions. Of these, 248 (mean ± SE = 82.3 ± 10.8/colony) were vibrators, of which 35.9% were persistent vibrators and 64.1% were nonpersistent vibrators. On average, 13.7 ± 1.81% of the tagged bees added to each colony performed vibration signals at some level during the study period. The remaining 623 tagged bees that engaged in vibration activity (208.0 ± 22.03/colony) were classified as vibrated recipients, with 29.9% identified as persistent recipients and 70.1% identified as nonpersistent recipients. Of the 285 dissected bees for which we also had adult lifetime behavioural profiles, 132 had active ovaries and 153 had inactive ovaries.

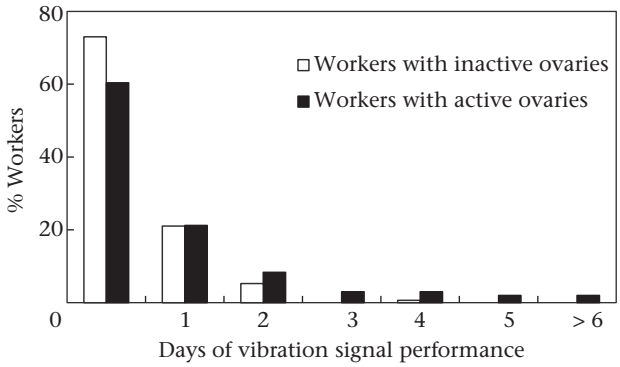

Figure 1. Percentage of queenless workers with active and inactive ovaries that performed vibration signals for the indicated number of days during their lifetimes. Workers performing vibration signals for ≥2 days were classified as persistent vibrators.

Examination of the 285 dissected individuals revealed that vibration signal performance was associated with ovarian activation. Although workers with active and inactive ovaries could perform vibration signals, those with active ovaries were more likely to produce signals for 2 or more days during their lifetimes (log-linear analysis: $\chi^2_1 = 4.54$, $P = 0.033$; Fig. 1). This trend was exhibited similarly among the three colonies (log-linear analysis: $\chi^2_2 = 2.52$, $P = 0.283$), suggesting that the association between ovarian activation and persistent signal performance was consistent among the three worker matrilines examined. Furthermore, workers with active ovaries had higher daily rates of signal performance than workers with inactive ovaries (ANOVA: $F_{1,283} = 6.42$, $P = 0.0118$; Fig. 2) and this trend was exhibited similarly among the three observation colonies (ANOVA: $F_{2,283} = 0.72$, $P = 0.488$). In contrast, workers with active and inactive ovaries did not differ in the number of vibration signals received/day during their lifetimes (ANOVA: $F_{1,283} = 0.16$, $P = 0.693$; Fig. 2).

The lifetime behavioural profiles of the 871 bees that engaged in vibration signal activity revealed that vibrators were more likely to perform mauling than were vibrated recipients. Of the 248

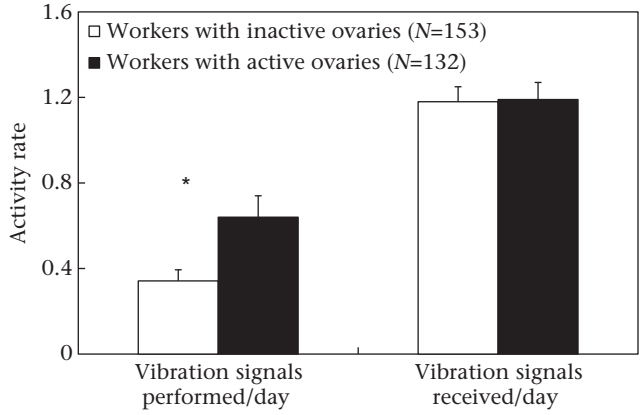

Figure 2. Daily rates (mean ± SE) at which workers with and without ovarian activation performed and received vibration signals during their lifetimes under queenless conditions. *$P < 0.05$.

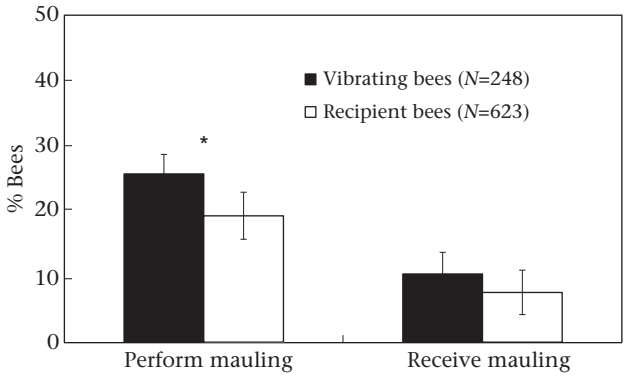

Figure 3. Percentage (mean ±SE) of queenless vibrating bees and vibrated recipients that performed and received mauling during their lifetimes. *$P < 0.05$.

queenless vibrators monitored, 26% performed bouts of mauling during their adult lifetimes, whereas 19% of the 623 vibrated recipients did so (log-linear analysis: $\chi^2_1 = 4.49$, $P = 0.0341$; Fig. 3). This trend was exhibited similarly among colonies (log-linear analysis: $\chi^2_2 = 1.57$, $P = 0.457$). In contrast, queenless vibrators and vibrated recipients did not differ in their lifetime tendencies to receive mauling from nestmates (log-linear analysis: $\chi^2_1 = 1.94$, $P = 0.164$; Fig. 3).

Gene Expression Levels

Some of the bees collected for genetic analysis were lost or damaged during preparation. We determined gene expression levels for a total of 13 persistent vibrators, 10 persistent recipients and 15 control bees that never performed or received vibration signals during the study period. Persistent vibrators tended to have normalized levels of gene expression that were greater than those of persistent recipients and controls (Fig. 4). These differences were significant for five of the genes (*AmADH*: ANOVA: $F_{2,35} = 6.526$, $P = 0.004$; *AmBlack*: $F_{2,35} = 14.893$, $P < 0.0001$; *AmCG9369-like*: $F_{2,35} = 7.2441$, $P = 0.002$; *AmDopR1*: $F_{2,35} = 3.8758$, $P = 0.0302$; *AmOct-TyrR*: $F_{2,35} = 5.8982$, $P = 0.006$). Post hoc comparisons revealed that vibrator expression levels for these five genes were all significantly greater than those of recipients, and exceeded those of the controls with the exception of *AmDopR1* (Fig. 4). In addition, differences in expression levels approached significance for a sixth gene (*AmCyp9f2*: $F_{2,35} = 3.2115$, $P = 0.052$) and post hoc comparisons again revealed that expression levels were higher in vibrators than in recipients (Fig. 4). Indeed, normalized gene expression levels for vibrators exceeded those of recipients for all eight genes examined (Fig. 4), although differences between vibrators and controls were more variable and reached significance for four of the genes. Thus, there was a general trend for vibrators to have higher expression levels than recipients for a series of genes associated with aggressive colony defence in honeybees and/or dominance in *P. metricus*, suggesting these genes may contribute to signal performance under queenless conditions.

DISCUSSION

Approximately 14% of the tagged bees in our queenless colonies performed vibration signals during their adult lifetimes. Similar values have been reported for queenright honeybee colonies (Schneider & Lewis, 2004), although there can be considerable variability among matrilines and patrilines in the tendency to perform vibration signals (Duong & Schneider, 2008). The adult lifetime behavioural profiles we generated for our tagged workers suggested that the vibration signal may be associated with the ovarian activation and overt aggression that occur under permanently queenless conditions. Vibration signal performance was positively associated with ovary activation and the tendency to perform mauling, an aggressive act associated with reproductive competition (Malka et al., 2008; Sakagami, 1954). Our results suggest that the vibration signal may contain elements of aggression, which are normally not expressed behaviourally when the signal is used to promote cooperative activities under queenright conditions, but which may become evident in queenless colonies where overt aggression among workers increases.

In contrast to our results, Schneider and McNally (1991) reported that queenless workers that performed vibration signals had less developed ovaries than recipients. Furthermore, whereas we found that vibrators had greater ovarian activation and a greater tendency to perform mauling than vibrated recipients, previous studies have reported that workers performing mauling tend to have less developed ovaries than recipients of mauling (Malka et al., 2008; Sakagami, 1954). These apparently contradictory results may reflect methodological differences between the different studies. We determined adult lifetime behavioural profiles and ovarian activation for workers throughout a prolonged period of queenlessness, whereas Schneider and McNally (1991) examined dyadic vibrator–recipient pairs that were immediately involved in the performance of a single signal. It is possible that persistent signal production is positively associated with ovarian activation throughout an individual's lifetime under queenless conditions, yet relative ovarian development may influence the immediate selection of a recipient for the performance of a particular vibration signal. Similarly, we determined lifetime tendencies of vibrators and vibrated recipients to perform and receive mauling, whereas previous studies compared the relative ovarian development of dyadic pairs of queenless workers performing and receiving mauling (Malka et al., 2008; Sakagami, 1954). Although our vibrators tended to have greater ovarian activation than vibrated recipients, we did not determine relative ovarian development for dyadic pairs of bees engaged in mauling. The criterion we used to identify workers with active ovaries (individual oocytes clearly present and deforming the shape of the ovariole) included a range of ovarian development. Queenless workers that perform mauling often show a level of ovarian development that would have been classified as 'active' in our study, yet direct their mauling towards more developed nestmates (see Figure 1 in Malka et al., 2008). Thus, it is possible that queenless workers performing vibration signals can have a greater level of ovarian activation than vibrated recipients but a lower level of ovarian development than the specific individual they select for a particular mauling event. Regardless of possible methodological differences between the different studies, our results suggest that under queenless conditions the performance of vibration signals is positively associated with both ovarian activation and the tendency to maul nestmates.

The brain–gene expression patterns revealed that five of the eight candidate genes associated with aggression and dominance in honeybees and *P. metricus* were significantly upregulated in persistent queenless vibrators compared to persistent recipients and nonvibrating controls. Post hoc comparisons revealed that the expression levels of vibrators were consistently higher than those of recipients for all eight genes examined, and exceeded expression levels of controls for four of the eight genes. The comparisons with control bees suggest that differences in expression levels were positively associated with the tendency to engage in vibration signal activity for half of the genes examined. For those workers that engaged in vibration activity, signalling role (vibrator or recipient) strongly influenced brain–gene expression levels for all

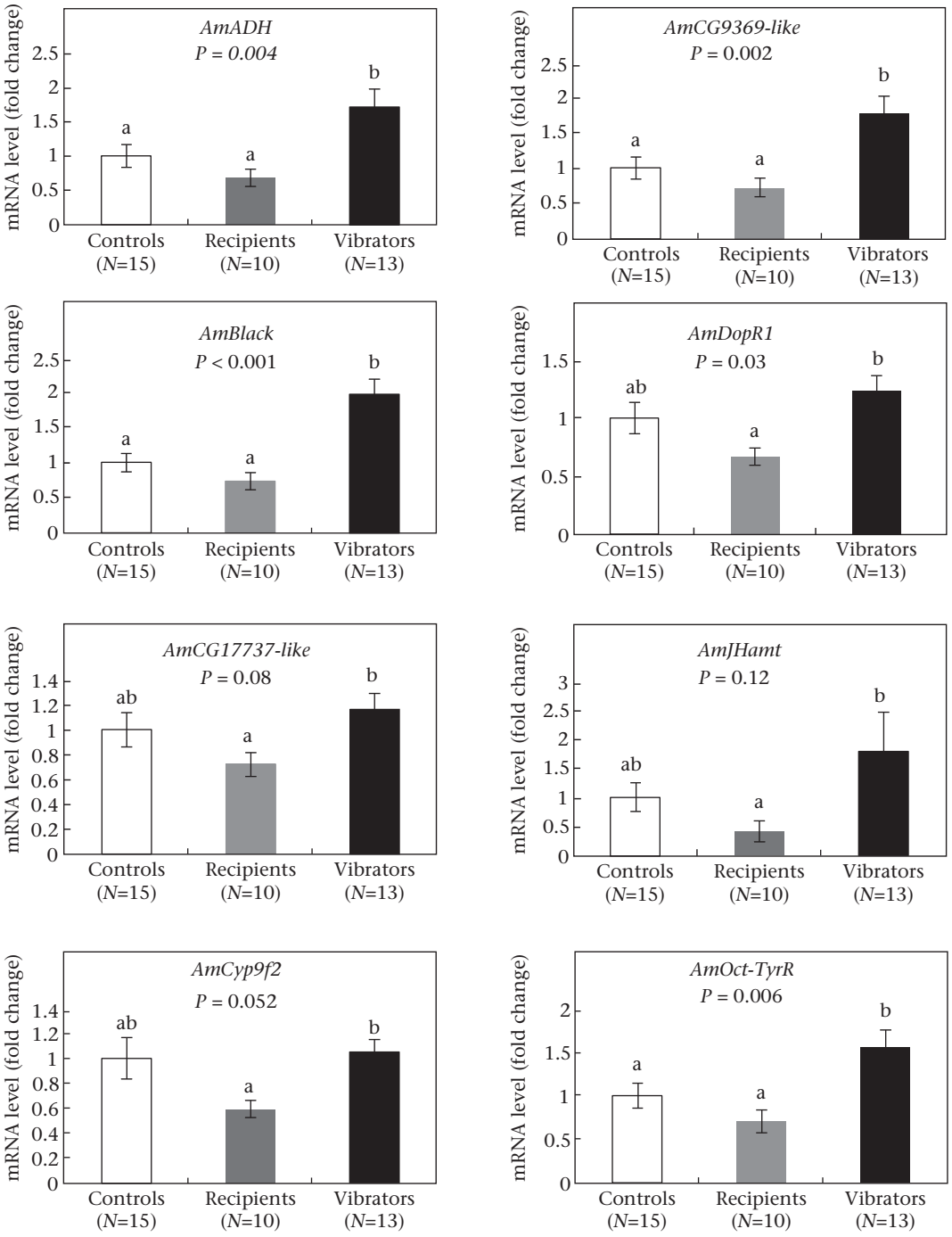

Figure 4. Gene expression levels, shown as mRNA level after normalization to internal control genes, and expressed as fold change relative to controls, for the eight candidate genes compared between queenless persistent vibrators, persistent recipients and nonvibrating control workers. Different letters above the bars denote significant differences based on Tukey post hoc tests.

candidate genes investigated, even if differences with controls were not detected.

Honeybee workers performing vibration signals under queen-right conditions and those engaged in aggression show brain–gene expression patterns associated with heightened arousal and behavioural sensitization (Alaux, Duong, et al., 2009; Alaux, Sinha, et al., 2009). Any commonalities we observed between gene expression profiles for our queenless vibrators and aggressive workers may therefore reflect a basic shared state of arousal rather than direct links with aggression per se. However, several of the candidate genes examined in this study have been implicated in the regulation of aggression in other species. Of particular interest are the genes *AmBlack* and *AmCyp9f2*, both of which are associated with dominance in *P. metricus* and aggression in *Drosophila* (Table 1). Likewise, the gene *AmOct-TyrR* is associated with aggressive nest defence in honeybees and is also involved in the regulation of aggression in other invertebrate as well as vertebrate species (Dierick & Greenspan, 2007; Hunt et al., 2007; Nelson & Chiavegatto, 2001). Although definite conclusions cannot be made at this time, our results suggest that at least some of the

genes examined in this study could be components of a genetic module that contributes to aggressive behaviour in a wide array of social and solitary species (Block & Grozinger, 2011; Toth & Robinson, 2007; Toth et al., 2014). This module may have been co-opted during the evolution of aggressive signals and may now contribute to the performance of vibratory displays in *Polistes,* as well as the performance of the honeybee vibration signal under both queenless and queenright conditions.

Taken together, our results are consistent with the hypothesis that the vibration signal evolved through ritualized aggression. In many primitively social insects, aggressive and dominance interactions involve repetitive movements, such as head butting, biting, lunging, darting and oscillations of the abdomen and other body parts (Jandt et al., 2014; Jeanne, 2009; Rehan & Richards, 2013; Sumana & Starks, 2004; Velthuis & Gerling, 1983; West-Eberhard, 1978). Furthermore, in some species these same aggressive interactions are also used to organize decentralized, cooperative activities such as foraging and resource sharing (Bruyndonckx, Kardile, & Gadagkar, 2006; De Souza & Prezoto, 2012; Lamba, Chandrasekhar, & Gadagkar, 2008; O'Donnell, 2006; Sumana & Starks, 2004). Such repetitive motions of a common ancestor may have become ritualized into vibratory signals, such as abdominal wagging in *Polistes*, which is used in dominance interactions, and the vibration signal of the honeybee, which is used predominantly to organize cooperative labour under typical colony conditions. The molecular mechanisms associated with the performance of aggressive movements may continue to provide the genetic underpinnings for the production of vibratory displays, which may still show elements of aggression under conditions where reproductive competition is expressed. The modulation of cooperative activities by vibratory, tactile signals is widespread in the social insects (Hölldobler & Wilson, 2009; Hunt & Richard, 2013; Schneider & Lewis, 2004; Suryanarayanan, Hermanson, & Jeanne, 2011), which raises the possibility that ritualized aggression is a common feature in the evolution of the complex communication systems that characterize the advanced insect societies.

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Supplementary Material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.anbehav.2014.09.030.

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