

# The influence of drone physical condition on the likelihood of receiving vibration signals from worker honey bees, *Apis mellifera*

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**Abstract** Honey bee workers will perform vibration signals on adult drones, which respond by increasing the time spent receiving trophallaxis. Because trophallaxis provides the proteins for sexual maturation, workers could direct vibration signals towards drones showing certain physical characteristics, potentially influencing drone development and colony reproductive output. We examined the influence of drone condition on the likelihood of receiving vibration signals by comparing body weight, protein concentrations, and hemolymph juvenile hormone (JH) titers between drones that received the vibration signal and same-age, non-vibrated controls. Vibrated and control drones did not differ in total body weight, abdomen weight, abdomen-to-body weight ratio, total protein concentrations, or hemolymph JH titers. In contrast, vibrated drones had significantly lower thorax weight and smaller thorax-to-body weight ratios compared with controls. Because relative thorax weight may affect flight ability and mating success, workers could use the vibration signal to increase the care received by less developed drones, potentially contributing to the production of greater numbers of competitive males. However, the differences in thorax weights, while significant, were very

small, and it is unknown how such slight differences might be assessed by workers or affect drone performance. Nevertheless, vibration signals performed on drones may provide opportunities for exploring the effect of the quality of reproductive individuals on caste interactions in honey bees.

**Keywords** Drones · Vibration signal · Reproductive potential · Caste interactions · Worker–drone interactions · Communication

## Introduction

Interactions between sterile workers and sexual castes are often major determinants of colony reproductive success in social insects (Hölldobler and Wilson, 1990; Tarpay et al., 2004). Knowledge of caste interactions is therefore necessary for understanding the organization of colony-level reproductive events. Caste interactions have been particularly well studied in the honey bee, *Apis mellifera*, especially worker–queen interactions associated with reproductive swarming and queen replacement (Schneider et al., 2001; Gilley and Tarpay, 2005; Pierce et al., 2007; Schneider and DeGrande-Hoffman 2003, 2008). In contrast, less is known about interactions between workers and drones (males), even though drones are major components of colony reproductive output (Boes, 2010). The sole function of drones is to mate with virgin queens at drone congregation areas (DCAs), where thousands of drones from many different colonies aggregate 15–60 m above ground and pursue and copulate in-flight with virgin queens that fly through the cluster (Koeniger et al., 2005a, b). There is intense competition for queens at a DCA and drones with greater size and flight capabilities may have competitive advantages (Coelho, 1991; Berg et al., 1997; Radloff et al., 2003; Koeniger et al.,

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2005a; Jaffé and Moritz, 2010). Larger, heavier drones also produce more sperm and drones contributing greater volumes of semen are disproportionately represented in a queen's offspring (Duay et al., 2003; Schlüns et al., 2004). A drone can mate only once, because it dies after ejaculation. Thus, a colony can increase its reproductive success through drones primarily by producing and fielding greater numbers of larger, higher-quality males. Any interactions that enable workers to influence these phenomena should convey strong selective advantages.

Honey bee workers build drone comb, raise drone larvae, and adjust total drone production to changes in colony conditions and forage availability (Boes, 2010). After drones emerge as adults, workers interact with them primarily through trophallaxis (mouth-to-mouth feeding) and grooming (Ohtani, 1974). Trophallaxis provides the proteins necessary for the development and sexual maturation of young drones and helps maintain the condition of mature drones (Hrassnigg and Crailsheim, 2005). Grooming may further contribute to the maintenance of drone physical condition.

Workers may also use the vibration signal (sometimes called the shaking signal) to influence the development and behavior of adult drones (Boucher and Schneider, 2009). The signal, which consists of a worker rapidly vibrating its body dorso-ventrally for 1–2 s while grasping a recipient with its forelegs, is performed on drones of all ages, although younger drones tend to be vibrated at higher rates (Boucher and Schneider, 2009). Drones of all ages respond to the signal with increased activity, which contributes to greater proportions of time spent receiving trophallaxis (Boucher and Schneider, 2009). Because trophallaxis is the source of proteins necessary for the development of the flight muscles and sexual organs, workers may use the vibration signal to enhance drone maturation and maintenance. Although drones can engage in trophallaxis without receiving vibration signals, the signal may be used to fine-tune drone care, which could contribute to colony reproductive output in three different ways. First, vibration signals may be directed towards larger, more developed drones, resulting in a subset of heavier, higher-quality males that have increased mating advantages at DCAs. Second, workers may direct signals towards smaller drones to increase the care they receive, resulting in greater total numbers of drones that will be competitive at DCAs. Alternatively, workers may vibrate drones randomly, resulting in a general enhancement of drone care independent of the immediate condition of individual recipients.

The purpose of our study was to examine the influence of drone condition on the likelihood of receiving vibration signals from workers. Our specific objective was to compare vibrated drones and age-matched, non-vibrated controls with respect to three main characteristics that may reflect

physical condition and potentially influence mating performance: body weights, protein concentrations, and hemolymph juvenile hormone (JH) titers. Drone body weight is positively associated with reproductive potential. Heavier drones have greater sperm production, fewer sperm abnormalities, and show increased representation of spermatozoa in a queen's spermatheca (Schlüns et al., 2003; Gencer and Firatli, 2005; Zaitoun et al., 2009). Thorax weight, and particularly the thorax-to-body weight ratio, reflects flight muscle development (Harrison, 1986; Marden, 1989; Coelho, 1991). Drones with higher relative thorax weight may have greater power output and maneuverability during flight, which could enhance mating-flight performance (Coelho, 1996; Kraus et al., 2003). Abdomen weight and the abdomen-to-body weight ratio may reflect maturation of the sexual organs (Hrassnigg and Crailsheim, 2005; Mazed and Mohanny, 2010). Protein concentrations are often used to assess body condition in honey bees (van Engelsdorp et al., 2009; DeGrandi-Hoffman et al., 2010), and the protein content of the thorax and abdomen may reflect development of the flight musculature and sexual organs, respectively (Hrassnigg and Crailsheim, 2005). JH influences male sexual development in many insects and stimulates flight behavior in honey bee drones (Giray and Robinson, 1996; Tozetto et al., 1997). If vibrations signals are directed toward more developed drones, we predicted that vibrated individuals should show greater body weights, protein concentrations, and hemolymph JH titers compared with non-vibrated controls. Conversely, if signals are directed toward less developed drones, vibrated individuals should show lower values for the different characteristics examined. If drones are vibrated randomly with respect to physical condition, we predicted no differences between vibrated and non-vibrated individuals for the variables investigated.

## Materials and methods

### Colony setup

We monitored worker–drone interactions in three four-frame observation colonies maintained on the campus of the University of North Carolina Charlotte for a 5-week period from May through July, 2010. Each colony contained a queen, 8,000–10,000 workers, and four frames of comb filled with food and brood in all stages of development. The observation hives were equipped with plexiglass sides that contained hinged access ports through which drones could be removed for analysis.

We established a population of individually marked drones of known age in each observation colony. To obtain drones for marking, empty frames of drone comb were

placed into four unrelated field colonies 2–4 weeks before the observation hives were established. Frames with emerging drones were placed in separate nylon mesh cage and transferred to an incubator (33.5°C; 50% RH). Adult drones were collected within 24 h of emergence and marked using plastic tags (Opalithplättchen; Graze, Germany) with unique color/number/paint mark combinations glued to the thorax. In this manner, drones could be individually identified and their exact age (in days) was known throughout the study. Tagged drones were added in cohorts of 50–100 individuals every 1–4 days until a total of 775 were introduced into each colony. Throughout the study period, the tagged drone accounted for 5–8% of the total colony population, which is comparable to the abundance of drones in naturally occurring colonies during spring and early summer (Winston, 1987).

### Sample collection

We monitored the observation colonies continuously from 0800–1700 h 5–6 d/wk during the five-week study and recorded the identity of all tagged drones observed to receive vibration signals. Each colony was monitored by two observers simultaneously and observers rotated among colonies on an hourly basis to minimize observer bias. Throughout the study period, we collected matched pairs of vibrated and non-vibrated tagged drones through the hinged access ports of the observation hives. Vibrated tagged drones were removed from the colonies immediately upon receiving vibration signals. For each vibrated drone collected, we collected simultaneously a non-vibrated, control tagged drone that was the same age and located in the same cluster of drones in the nest. The tagged drones in our observation colonies showed typical patterns of clustering based on age, with younger drones forming clusters in areas of brood comb and older drones clustering in the peripheral comb areas where honey was stored (Winston, 1987). Workers that vibrate drones will move back-and-forth through a drone cluster contacting drones repeatedly, but perform signals on only some of them (Stout et al., 2011). By collecting control drones from the same cluster as the vibrated counterparts, we increased the likelihood that a control was contacted, but not vibrated by the same worker that performed signals on the recipient drone. Although we undoubtedly missed some signals on drones, we never observed any of the controls to receive signals from the vibrating worker moving through the drone cluster at the time the vibrated counterpart was collected, nor did any receive signals during the total 504 h that the three colonies were monitored during the study period. In this manner, we were able to carefully match vibrated and control drones that were exactly the same age and had a high probability of

being contacted and potentially assessed by the same signaling worker during the same immediate time period.

Each collected drone was placed in a separate Ziplock<sup>®</sup> bag labeled with the drone's identity, age, and type (recipient or control) and immediately placed in a freezer at –20°C until cold anesthetized. Total body weight was measured by weighing each anesthetized drone to the nearest 0.1 mg using a Metler digital scale. Immediately after weighing, hemolymph was collected using standard protocols (Huang et al., 1994; Schneider et al., 2004), expelled into 500 µl acetonitrile to precipitate blood proteins and JH-degrading enzymes, and stored at –20°C for later JH analysis. The remaining drone carcass was placed in a pre-labeled vial and stored at –80°C for subsequent protein analysis.

### Protein analysis

Protein concentrations were estimated for each drone using BCA Protein Assay kits (Pierce Scientific, Rockford, IL), following a modified procedure developed for honey bee workers (van Engelsdorp et al., 2009). Drones were removed from –80°C storage, separated into head, thorax, and abdomen and each body region was weighed to the nearest 0.1 mg using a Metler digital scale. The wings and legs were removed from the thorax before weighing, because the appendages can become damaged or detached during freezing, and because such an estimate of thorax weight provides a good indication of relative flight muscle mass (Harrison, 1986). The weighed head, thorax, and abdomen were homogenized in 150, 600, and 500 µl, respectively, of extraction buffer (1 × PBS + 0.5% Triton X-100), placed on ice for 30 min, centrifuged at 14,000 rpm for 5 min, and supernatants were transferred into separate 0.5 µl microcentrifuge tubes. BCA tests were then performed on the protein extracts following established protocols (van Engelsdorp et al., 2009). Protein absorbance was read using a Nanodrop<sup>®</sup> spectrophotometer at a wavelength of 562 nm. Final levels of soluble protein were calculated using standard curves generated from nine known concentrations of Bovine Serum Albumen (0–2,000 µg/ml).

### Determining JH titers

JH III in the hemolymph samples was extracted and JH titers were estimated according to well-established procedures used in honey bees (Huang et al., 1994; Jassim et al., 2000). Liquid scintillation counting was performed using a Tricarb 2100TR (Packard), which gave the radioactivity in DPM (disintegrations per minute) for each sample. A standard curve with various amounts (0, 3, 10, 30, 100, 300, 1,000, 3,000, and 10,000 pg) of standard JH-III (Sigma) was obtained each day following the protocol of Huang and

Robinson (1996). The amount of JH in the samples was corrected by dividing by two, because the racemic mixture of JH standard overestimates the 10R JH in biological samples by a factor of two.

### Statistical analysis

We used Repeated Measurers Analysis of Covariance (ANCOVA; SAS 1997) to compare vibrated and control drones with respect to the weight of the total body, thorax, abdomen, thorax-to-body weight ratio, abdomen-to-body weight ratio, and the protein concentrations of the entire body, thorax, and abdomen. In the analyses, the age-matched drone pair was the subject, drone type (recipient or control) was the within-subjects factor, and colony was the between-subjects factor. We used age as a covariate because as our drones aged they showed typical patterns of development, with steady decreases in total body weight and abdomen weight and increases in thorax weight and protein concentrations (Gencer and Firatli, 2005; Hrasnigg and Crailsheim, 2005; Mazeed and Mohanny, 2010). Body weights and protein concentrations were  $\log_{10}$  transformed and proportional data were arcsine transformed before analysis to achieve normality (Sokal and Rohlf, 1995).

We could not use Repeated Measures ANCOVA to compare hemolymph JH titers for our drones, because JH titers are not linearly associated with age: JH levels increase rapidly in young drones, reach peak levels at 6–9 days of age coincident with the onset of flight activity, and then decrease around the age of sexual maturity and remain lower for the rest of a drone's life (Tozetto et al., 1995; Giray and Robinson, 1996). To account for these age-related changes, we classified each drone as sexually immature (<12 days old) or sexually mature ( $\geq$ 12 days old; Winston, 1987) and then compared vibrated and control drones using Repeated Measures ANOVA with colony and maturation status as between subjects factors. JH titers were  $\log_{10}$  transformed before analysis to achieve normality.

Our values for thorax weight, abdomen weight, and all protein concentrations were relative estimates, because we removed the legs and wings and extracted hemolymph before separating the drones into body regions for weighing and protein determinations (total body weight was determined before these manipulations). However, all appendages were completely removed from each drone and the mean volume of hemolymph collected from the vibrated drones ( $5.75 \pm 0.27 \mu\text{l}$ ) and control drones ( $6.08 \pm 0.30 \mu\text{l}$ ) did not differ ( $F_{1,94} = 0.94$ ,  $P = 0.334$ ). Thus, each drone received the same treatments that could have influenced weight and protein concentrations and our relative estimates allowed for meaningful comparisons between the vibrated and control individuals.

For all analyses, degrees of freedom were estimated by the Satterthwaite method (SAS, 1997). The sequential Bonferroni adjustment (Rice, 1989) was used to determine significance levels ( $\alpha = 0.05$ ) for the different comparisons of weights and protein concentrations made between vibrated and controls drones. Mean values are reported as  $\pm 1$  SE.

### Results

We collected 95 pairs of vibrated and controls drones ( $31.7 \pm 2.33/\text{colony}$ ), for a total of 190 individuals examined. The mean age of the collected drones was  $8.4 \pm 0.42$  days (range: 1–24 days). The vibrated drones received  $1.3 \pm 0.05$  signals before collection (range: 1–3 signals). For each of the characteristics examined, we found no drone type-by-colony interaction (for all interactions:  $F_{2,92}$  ranged from 0.02–1.43;  $P$  ranged from 0.268–0.984), suggesting that the differences between vibrated and control drones showed similar trends among colonies. We therefore focused our results on the main effect of drone type.

Vibrated and control drones did not differ for total body weight, abdomen weight, the abdomen-to-body weight ratio, or any of the protein concentrations examined (Table 1). In contrast, drones that received vibration signals had lower thorax weight and smaller thorax-to-body weight ratios compared with non-vibrated controls. Although these differences were slight, they were highly significant (Table 1).

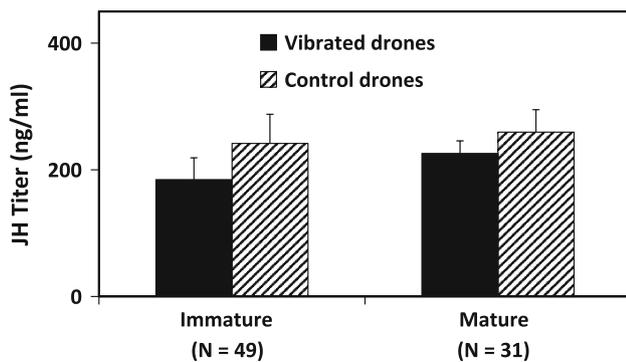
We were unable to obtain hemolymph samples from several drones and some samples were lost during analysis. As a result, JH titers were determined for a total of 80 recipient-control pairs, of which 49 were classified as sexually immature and 31 as mature. Both immature and mature vibrated individuals had slightly lower JH titers than same-age controls (Fig. 1). However, the difference was not significant ( $F_{1,74} = 0.43$ ,  $P = 0.514$ ), but was more pronounced for the immature drones, resulting in a significant drone type-by-maturation status interaction ( $F_{2,74} = 4.51$ ,  $P = 0.014$ ; Fig. 1).

### Discussion

The majority of drone characteristics that we examined did not influence the likelihood of receiving vibration signals from workers. Vibrated and control drones did not differ in most aspects of body weight investigated, protein concentrations, or hemolymph JH titers. Thus, any contribution of the vibration signal to drone performance may occur independently of an assessment of a variety of characteristics that potentially reflect the developmental and maturation status of individual recipients.

**Table 1** Comparisons of the weights and protein concentrations (mean  $\pm$  SE) for the 95 pairs of vibrated and control drones examined

Characteristic	Vibrate drones	Control drones	Statistic	P
Weight (mg)				
Body	235.6 $\pm$ 2.62	239.5 $\pm$ 2.35	$F_{1,94} = 3.37$	0.068
Thorax	89.5 $\pm$ 0.95	92.7 $\pm$ 0.86	$F_{1,94} = 12.59$	0.0006
Abdomen	92.1 $\pm$ 1.67	92.9 $\pm$ 1.54	$F_{1,94} = 0.96$	0.330
Thorax/body weight ratio	0.38 $\pm$ 0.003	0.39 $\pm$ 0.004	$F_{1,94} = 7.09$	0.009
Abdomen/body weight ratio	0.39 $\pm$ 0.004	0.39 $\pm$ 0.004	$F_{1,94} = 0.71$	0.488
Protein (mg/ml)				
Body	39.7 $\pm$ 1.66	39.2 $\pm$ 1.76	$F_{1,94} = 0.41$	0.525
Thorax	14.3 $\pm$ 0.87	15.1 $\pm$ 0.98	$F_{1,94} = 1.77$	0.187
Abdomen	14.1 $\pm$ 0.49	13.1 $\pm$ 0.45	$F_{1,94} = 3.10$	0.082

**Fig. 1** Hemolymph juvenile hormone (JH) titers (mean  $\pm$  SE) for the sexually immature (<12 days old) and sexually mature ( $\geq$ 12 days old) drones examined. Samples sizes indicate the number of recipient/control pairs of drones analyzed

Nevertheless, several lines of evidence suggest that workers do not vibrate drones entirely at random. Vibrating workers perform signals on less than 30% of the drones they contact as they roam through the nest and can return repeatedly to the same individual and vibrate it multiple times (Boucher and Schneider, 2009; Stout et al., 2011). Thus, workers may actively select certain drones as recipients. Furthermore, vibrated drones had significantly lower thorax weight and thorax-to-body mass ratios compared with non-vibrated controls, suggesting that some aspects of drone condition may influence signal performance. Relative thorax weight may indicate power output and maneuverability during flight, which could affect a drone's ability to catch and copulate with queens (Coelho, 1996). Drones respond to the vibration signal with increased time spent in trophallaxis, which supplies the proteins needed for flight muscle development (Hrassnigg and Crailsheim, 2005; Boucher and Schneider, 2009). Workers may therefore use the signal to make fine-tuned adjustments to the care received by slightly less developed drones, which could help to address possible deficiencies in maturation and flight capacity. Vibrated drones also had slightly lower

hemolymph JH titers compared with controls. Although these differences were not significant, they occurred for both sexually immature and mature recipients. JH influences drone sexual development and promotes flight activity (Giray and Robinson, 1996; Tozetto et al., 1997). Although the endocrine responses of vibrated drones are unknown, workers that receive vibration signals show increases in hemolymph JH titers (Schneider et al., 2004). If drones respond similarly, then directing the signal towards individuals with slightly lower JH levels could further contribute to drone maturation and flight ability. Thus, while most of our results revealed no differences between vibrated and control drones, several of our findings occurred in the direction predicted by the hypothesis that workers direct vibration signals towards less developed males, which respond in a manner that potentially contributes to the production of greater total numbers of drones that will be competitive at DCAs.

However, any relationship between the vibration signal and drone condition must remain speculative, because we did not examine other drone characteristics, such as seminal vesicle development or sperm numbers, that may reflect reproductive potential, nor did we determine the actual mating success of vibrated and non-vibrated individuals. Furthermore, although the differences we observed in thorax weights were highly significant, they were very small and we do not know what effect (if any) such slight variations have on drone performance. Nevertheless, these tiny differences may have influenced the tendency of workers to vibrate drones, although it is unknown how such differences might be assessed. Similarly, adult virgin queens reared in the same colony show only slight differences in weight and size, yet these small differences are associated with fighting ability and the number of vibration signals received from workers during the period when virgin queens battle to the death the inherit the natal nest (Schneider and DeGrande-Hoffman 2003; Tarpay et al., 2004). Workers often destroy large numbers of developing queen cells (Hatch et al., 1999; Schneider and DeGrandi-Hoffman, 2002) and will also

destroy larval drones that show developmental abnormalities (Woyke, 1963). Thus, workers may “weed out” lower-quality sexual individuals during rearing, resulting in emerged adults that show only minor differences in many morphological and physiological characteristics (Tarpy et al., 2004). Although the associations between these subtle differences and reproductive capacities are often unclear, they may influence caste interactions in ways that enhance colony reproductive success (Gilley and Tarpy, 2005; Tarpy et al., 2004). Our results suggest that the vibration signal is part of the caste interactions associated with the male component of colony reproductive output in honey bees. Vibratory signals are involved in colony reproduction in a variety of wasps, bees, and ants (Hölldobler et al., 1996; Pierce et al., 2007; Jeanne, 2009; Suryanarayanan et al., 2010) and may provide valuable tools for exploring the influence of reproductive quality on caste interactions in many insect societies.

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