CORRECTION

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In the final sentence of paragraph 4 of 'Effects on SR' in the Results section, the total number of proboscis extensions is given, rather than percentages. The correct sentence is published below.

On average, control nectar foragers had a lower mean total PER bee^{-1} (4.1±2.3 responses) than control pollen foragers (4.8±2.1 responses).

The authors apologise for any confusion this may have caused.

RESEARCH ARTICLE

A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing

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SUMMARY

A nicotinic acetylcholine receptor agonist, imidacloprid, impairs memory formation in honey bees and has general effects on foraging. However, little is known about how this agonist affects two specific aspects of foraging: sucrose responsiveness (SR) and waggle dancing (which recruits nestmates). Using lab and field experiments, we tested the effect of sublethal doses of imidacloprid on (1) bee SR with the proboscis extension response assay, and (2) free-flying foragers visiting and dancing for a sucrose feeder. Bees that ingested imidacloprid (0.21 or 2.16 ng bee^{-1}) had higher sucrose response thresholds 1 h after treatment. Foragers that ingested imidacloprid also produced significantly fewer waggle dance circuits (10.5- and 4.5-fold fewer for 50% and 30% sucrose solutions, respectively) 24 h after treatment as compared with controls. However, there was no significant effect of imidacloprid on the sucrose concentrations that foragers collected at a feeder 24 h after treatment. Thus, imidacloprid temporarily increased the minimum sucrose concentration that foragers would accept (short time scale, 1 h after treatment) and reduced waggle dancing (longer time scale, 24 h after treatment). The effect of time suggests different neurological effects of imidacloprid resulting from the parent compound and its metabolites. Waggle dancing can significantly increase colony food intake, and thus a sublethal dose (0.21 ng bee^{-1}, 24 p.p.b.) of this commonly used pesticide may impair colony fitness.

Key words: imidacloprid, sucrose response threshold, waggle dance communication, foraging, pesticide.

INTRODUCTION

Acetylcholine (ACh) plays a major role in insect synaptic neurotransmission (Breer and Sattelle, 1987; Tomizawa and Casida, 2003). One ACh receptor, the nicotinic acetylcholine receptor (nAChR), belongs to the superfamily of Cys-loop ligand-gated ion channels and mediates signal transmission at cholinergic synapses (Jones et al., 2006). In honey bees, nAChRs are expressed in brain areas associated with mechanosensory antennal information, visual and olfactory processing, learning and memory (Gauthier, 2010). Neonicotinoids target nAChRs (Matsuda et al., 2001) and thus provide a tool to explore bee neurobiology. In honey bees, the neonicotinoid imidacloprid inhibits receptors for γ-aminobutyric acid (GABA), a major neurotransmitter in the central nervous system (Thany, 2010). Imidacloprid is a partial agonist of nAChRs in brain areas associated with olfaction, learning and memory: cultured antennal lobe neurons (Barbara et al., 2005; Barbara et al., 2008) and cultured Kenyon cells from mushroom bodies (Déglise et al., 2002). Suchail et al. found that imidacloprid is metabolized relatively quickly and has a half-life of 4–5 h (Suchail et al., 2004a). Imidacloprid's metabolites also interfere with habituation and memory formation in honey bees (Guez, 2001; Decourtye et al., 2004b).

Researchers have also examined the effects of imidacloprid on honey bee behavior, particularly its effects on foraging, a key aspect of colony fitness (Sherman and Visscher, 2002). Imidacloprid reduces foraging rates (Decourtye et al., 2004a; Ramirez-Romero et al., 2005), delays a forager's return visit to food (Yang et al., 2008) and impairs olfactory associative learning (Decourtye et al., 2003; Decourtye et al., 2004b; Decourtye et al., 2004a), which plays a significant role in the learning of rewarding food sources (Riffell, 2011). Other studies have reported that imidacloprid reduces feeder visitation and feeding (Kirchner, 1999; Decourtye and Devillers, 2010). This reduction in feeder visits may be due to decreased recruitment and persisted for days, even after the feeder was switched from imidacloprid to uncontaminated sucrose (Decourtye et al., 2004a).

Studies have reported preliminary observations that bees trained to a sucrose feeder laced with imidacloprid (20–100 p.p.b.) begin trembling or may decrease the frequency of waggle dancing upon their return to the nest (Kirchner, 1999; Dechaume-Moncharmont, 2003). A reduction in waggle dancing can significantly reduce colony weight gain and fitness (Sherman and Visscher, 2002; Dornhaus and Chittka, 2004). However, it is unclear whether such behaviors are due to the perception of the food being contaminated, or to the ingestion of imidacloprid, which subsequently alters recruitment even to uncontaminated food.

Foraging behavior is also influenced by sucrose responsiveness (SR), whether a bee finds a nectar source sweet enough to feed upon. Bees that consume only nectar with a high sugar concentration (high response threshold bees) respond to fewer sucrose concentrations in a test series. Bees that consume even low concentration nectar are called low response threshold bees and will accept low, medium and high sugar concentrations. Sucrose responsiveness is influenced by multiple factors, including the environment (recent feeding experiences) (Pankiw et al., 2001) and genetics. Page and Fondrk identified quantitative trait loci that are

linked to SR and the probability of individuals foraging for pollen (Page and Fondrk, 1998). Pollen and water foraging are correlated with a low sucrose response threshold, and nectar foraging is associated with a higher sucrose response threshold (Page and Fondrk, 1995; Pankiw and Page, 2000). Empty foragers, bees that return without pollen or nectar, have high sucrose response thresholds (Drezner-Levy et al., 2009). Pankiw and Page reported such 'finicky foragers' had even lower SR than bees bringing back nectar (Pankiw and Page, 2000). Thus, an increase in the number of 'finicky foragers' should change the willingness of foragers to collect nectars with lower sucrose concentrations. This could reduce food flow to the nest, because bees normally collect nectar over a wide range of concentrations (Seeley, 1995).

Aliouane et al. examined the effects of the neonicotinoid thiamethroxam on SR, and found that a bee fed 1 ng of thiamethroxam shows a significant reduction in its willingness to extend its proboscis (proboscis extension response, PER) and feed on 3% or 10% (w/w) sucrose solutions (Aliouane et al., 2009). Lambin et al. topically applied imidacloprid on the thorax and found that it decreases SR at high doses of 5, 10 and 20 ng bee^{-1} (Lambin et al., 2001). However, no honey bee studies have examined the effect of ingested imidacloprid on SR, or tested the effect of imidacloprid at lower doses that correspond to more realistic field exposure levels [1.1–4.3 ng bee^{-1} (Rortais et al., 2005)].

These field exposure levels are an important consideration, because imidacloprid is a pesticide widely used to control plant-sucking agricultural pests (Tomizawa and Casida, 2003) such as aphids and leafhoppers (Elbert et al., 2008). Concern has therefore grown about the effects of neonicotinoids on non-target insects such as honey bees (Desneux et al., 2007). Imidacloprid is commonly applied as a seed dressing. It is translocated primarily within the xylem and is absorbed by different plant tissues (Sur and Stork, 2003). Nectar and pollen can consequently contain imidacloprid at concentrations that negatively affect honey bees (Bonmatin et al., 2003; Bonmatin et al., 2005). In addition, the guttation drops (xylem sap) that exude on the tips and edges of corn leaves grown from pesticide-treated seeds can contain high levels of pesticide (Girolami et al., 2009). Bees collect these guttation drops (Shawki et al., 2006), which can contain lethal imidacloprid concentrations [47,000±9960 p.p.b., mean ± s.d. (Girolami et al., 2009)].

Thus, the goals of our study were to determine how sublethal doses of the nAChR agonist imidacloprid affect honey bee preference for nectar sweetness and honey bee foraging behavior. We measured (1) the short-term effects of imidacloprid on honey bee sucrose response threshold (SR) and (2) the longer term effects of imidacloprid metabolites on honey bee foraging preferences and waggle dancing. To the best of our knowledge, the results from this study provide the first detailed data on how imidacloprid alters forager SR and waggle dancing, effects that may contribute to decreased colony fitness.

MATERIALS AND METHODS
This study was conducted at the UC San Diego Biological Field Station (La Jolla, CA, USA) between February and October 2010, and February and August 2011. We performed two experiments with five healthy colonies of *Apis mellifera ligustica* Spinola 1806 used consecutively. In the first experiment (short-term effect of imidacloprid), foragers from three colonies were placed in modified PER harnesses, restraining movement to only their mouthparts and antennae (Takeda, 1961), and their SR was measured after 1 h. In the second experiment (longer term effect of imidacloprid metabolites), we measured the responses of free-flying foragers from

the two remaining colonies visiting a feeder 24 h after treatment. We chose this short time interval for our SR assay to facilitate comparisons with the results of other investigators (Aliouane et al., 2009). We did not measure the longer term effect of imidacloprid on SR after 24 h. Prolonged captivity can result in a lower survival rate and deteriorated PERs unless bees are fed a significant quantity of sucrose solution, a manipulation known to affect their SR (Mujagic and Erber, 2009). In the foraging experiment, free-flying foragers would not return to the feeder 1 h after they had been treated, but would return the next day (24 h later). Thus, the foraging experiment examined the longer-term effects of imidacloprid and its metabolites on foraging and recruitment behavior.

Imidacloprid dose and concentration
Rortais et al. estimated that bees collecting nectar could receive imidacloprid doses of 1.1–4.3 ng bee^{-1} (Rortais et al., 2005). Because the effects of imidacloprid are dose dependent (Decourtye and Devillers, 2010), we tested two different doses within this range, covering a 10-fold span. We used a micropipette to feed bees 7 µl of imidacloprid (Sigma-Aldrich PS2086 analytical standard) suspended in 2.0 mol l^{-1} unscented pure sucrose solution: 0.21 ng, at 24 p.p.b. or 2.16 ng at 241 p.p.b. To calculate p.p.b., we used 1263.36 kg m^{-3} as the density of 2.0 mol l^{-1} sucrose solution (56% sucrose w/w) at room temperature [table 322/1 in Bubnik et al. (Bubnik et al., 1995)]. The lowest concentration (24 p.p.b.) that we used is similar that used in previous studies (Kirchner, 1999; Decourtye et al., 2003) and the higher concentration (241 p.p.b.) is similar to the highest concentration (240 p.p.b.) used in another study (Decourtye et al., 2003).

Effects on SR
The SR bioassay is based upon the PER and assesses a bee's perception of sugar (Page et al., 1998). The response threshold is measured by stimulating a bee's antennae with an ascending sucrose concentration gradient. The lowest sucrose concentration that will elicit proboscis extension is the response threshold (Marshall, 1935; Scheiner et al., 2004).

Honey bees were trained 1.5 m from the colony entrance to a nectar (2.0 mol l^{-1} unscented sucrose, 56% w/w) or pollen (freshly ground, collected from honey bees) feeder. We defined nectar and pollen foragers as bees that foraged only on nectar or pollen, respectively, at these feeders and thus would bring back only a nectar or a pollen load to the colony (Pankiw and Page, 2000).

Three standard apiary colonies were used for this experiment. At the feeders, bees were individually captured inside plastic vials within 1 s of landing to ensure consistent responsiveness (Mujagic and Erber, 2009). Holes were made in the cap of each vial to allow ventilation and permit feeding with a micropipette (7 µl of 2.0 mol l^{-1} sucrose solution containing imidacloprid at 0, 0.21 or 2.16 ng bee^{-1}). We then harnessed bees inside stainless steel tubes, and placed them in an incubator (30°C, 70% humidity) for 1 h to allow full absorption of imidacloprid from the gut (see El Hassani et al., 2008). No further imidacloprid was provided.

The SR for each individual was measured by stimulating the two antennae simultaneously for 3 s with an ascending sucrose concentration series of 0%, 0.1%, 0.3%, 1%, 3%, 10%, 30% and 50% (w/w) (Mujagic and Erber, 2009). Unlike a previous method (Page et al., 1998), water was not used between each sucrose concentration. These test sucrose solutions contained no imidacloprid and were prepared with analytical grade sucrose and double-distilled water. All bees were tested with all sucrose concentrations, using an inter-test interval of 2 min (Page et al.,

1998). For each trial, 7–15 bees were tested. Half of the group was treated with the control solution and half with one of the imidacloprid doses. Only a proboscis extension that was complete (extending through the mouthparts) was recorded as a response.

We gauged SR in two different ways. We measured the sucrose response threshold (SRT), the lowest sucrose concentration in a series that elicited proboscis extension (see Page et al., 1998). We also measured the total PER bee^{-1}, the total number of proboscis extensions elicited by the sucrose series [the gustatory response score (Mujagic and Erber, 2009)]. A high response threshold results in a low total PER score because this corresponds to a bee only extending its proboscis for higher concentration sucrose solutions. After testing, control bees were permanently marked on their thorax with enamel paint (so that they would not be mistakenly reused) and released back to the their colonies. Bees treated with imidacloprid were not released, and were killed by freezing.

Effects on foraging and dancing
To test the effect of imidacloprid on foragers, we consecutively used two observation colonies and trained bees to a feeder (50% w/w sucrose solution, with no treatment) located 1.5 m from each colony entrance. We used this short feeder distance to encourage dancing and to limit the costs of food collection, thereby presenting a highly desirable food source, even at low sucrose concentrations. At this distance, recruiting bees perform what is classically called the 'round dance' (von Frisch, 1967). However, recent analyses suggest that the term 'waggle dance' should be applied to a continuum of behaviors encompassing both the round dance and waggle dance (Gardner et al., 2008). We follow this terminology and focus on the number of dance circuit repetitions, which are positively correlated with recruitment for both round and waggle dance variants (von Frisch, 1967).

Each colony was housed in a standard Langstroth three-frame observation hive placed inside a temperature-controlled room (30°C). Bees entered and exited each colony through a vinyl tube piercing the wall. To facilitate observations, each colony had an adjustable entrance slide that allowed bees to enter and exit from only one side, where they consequently performed all recruitment dances. We uniquely marked foragers with different combinations of enamel paint on the thorax.

We conducted one trial per day from 09:00h to 12:00h during May–August 2011 and used two colonies consecutively. We only used bees that consistently visited the 50% sucrose feeder before the treatment. We captured each bee in a separate vial, and fed it inside the vial with 7 μl of the control (pure 2.0 mol l^{-1} sucrose) or imidacloprid (0.21 ng bee^{-1}, 24 p.p.b. in 2.0 mol l^{-1} sucrose) solution. Bees were incubated for 1 h to allow full absorption of the imidacloprid and then released back to the hive. The next day, we measured the number of visits made by each bee to a series of sucrose concentrations (50%, 30%, 10% and 3% w/w, presented in this order). This series of sucrose concentrations contained no imidacloprid. Each concentration was presented for 25 min. For every forager returning from these sucrose concentrations, we measured the unloading wait time (a measure that influences the probability of a forager dancing) (Seeley, 1992) and counted the number of dance circuits made by the forager.

Statistics and analysis
We measured SR in two ways and corrected for multiple tests ($k=2$, $\alpha=0.05$) on the same data by applying the sequential Bonferroni correction (Sokal and Rohlf, 1995). We used ANOVA (JMP v9.0 statistical software) to test the effects of colony (random effect, REML algorithm), forager type (pollen or nectar), treatment (imidacloprid at 0, 0.21 or 2.16 ng bee^{-1}), and the interaction of forager type × treatment on the two different measures of SR. Both SR measures were square-root transformed to normalize the data. Significant effects were further analyzed with Tukey honestly significant difference (HSD) *post hoc* tests.

To analyze the effect of imidacloprid on foraging, we used a repeated-measures nominal logistic model to determine the effect of treatment (sucrose alone or with imidacloprid at 0.21 ng bee^{-1}), individual (bee), colony and phase (ingestion of 50% sucrose before and ingestion of 50%, 30%, 10% and 3% sucrose after treatment) on whether the individual accepted or rejected the sucrose concentration (yes or no). Separately, the number of times a bee fed at each sucrose concentration was analyzed using repeated-measures ANOVA (EMS algorithm). As before, we applied the sequential Bonferroni correction. We tested the effects of treatment (imidacloprid at 0 or 0.21 ng bee^{-1}), individual, colony (random effect) and concentration (50%, 30%, 10% and 3% sucrose solution).

To analyze the effects of imidacloprid on recruitment behavior (dancing), we also used repeated-measures ANOVA (EMS algorithm). Bees only danced for 50% and 30% sucrose and thus we used data just from these concentrations in our dance analysis. We tested the effects of treatment (imidacloprid at 0 or 0.21 ng bee^{-1}), individual (bee), colony (random effect) and phase (before or after treatment) on the number of dance circuits and unloading wait time. We applied the square-root transformation to the number of dance circuits and a log transformation to the unloading wait time to normalize these data distributions. Throughout this paper, we report data as means ± s.d.

RESULTS
Effects on SR
In total, we tested 314 nectar and 209 pollen foragers from three colonies. Fig. 1A,B shows the mean responses of these bees to the sucrose concentration series. In nectar foragers, there were fewer PER responses at both imidacloprid doses as compared with the controls. In pollen foragers, the lower imidacloprid dose (0.21 ng bee^{-1}) resulted in behaviors similar to those of controls, but this was not the case for bees given the higher dose (2.16 ng bee^{-1}).

We first examined the effects of imidacloprid on SRT. In the overall model, treatment had a significant effect on SRT ($F_{2,516}=16.96$, $P<0.0001$) and colony accounted for 7.5% of model variance. There was no significant overall effect of forager type (pollen or nectar forager, Fig. 1; $F_{1,1}=1.17$, $P=0.4763$). However, the interaction between treatment and forager type was significant ($F_{2,516}=3.82$, $P<0.0226$). On average, control nectar foragers had a higher SRT than control pollen foragers (10.6±15.2% and 5.9±13.2% sucrose, respectively).

Nectar foragers treated with imidacloprid increased their SRT in comparison to controls. The mean response thresholds for nectar foragers were 10.6%, 18.9% and 19.2% sucrose for treatment with 0, 0.12 and 2.16 ng imidacloprid bee^{-1}, respectively (significant differences between control and both imidacloprid doses, Fig. 1C, Tukey HSD$_{REML}$, $P<0.05$). Pollen foragers treated with imidacloprid also increased their SRT in comparison to controls. The mean response thresholds for pollen foragers were 5.9%, 5.7% and 18.1% for 0, 0.12 and 2.16 ng imidacloprid bee^{-1}, respectively (significant differences between 0 and 2.16 ng doses, Fig. 1D, Tukey HSD$_{REML}$, $P<0.05$).

We next examined the effects of imidacloprid on total PER bee^{-1}. The results were consistent. Treatment had a significant effect on
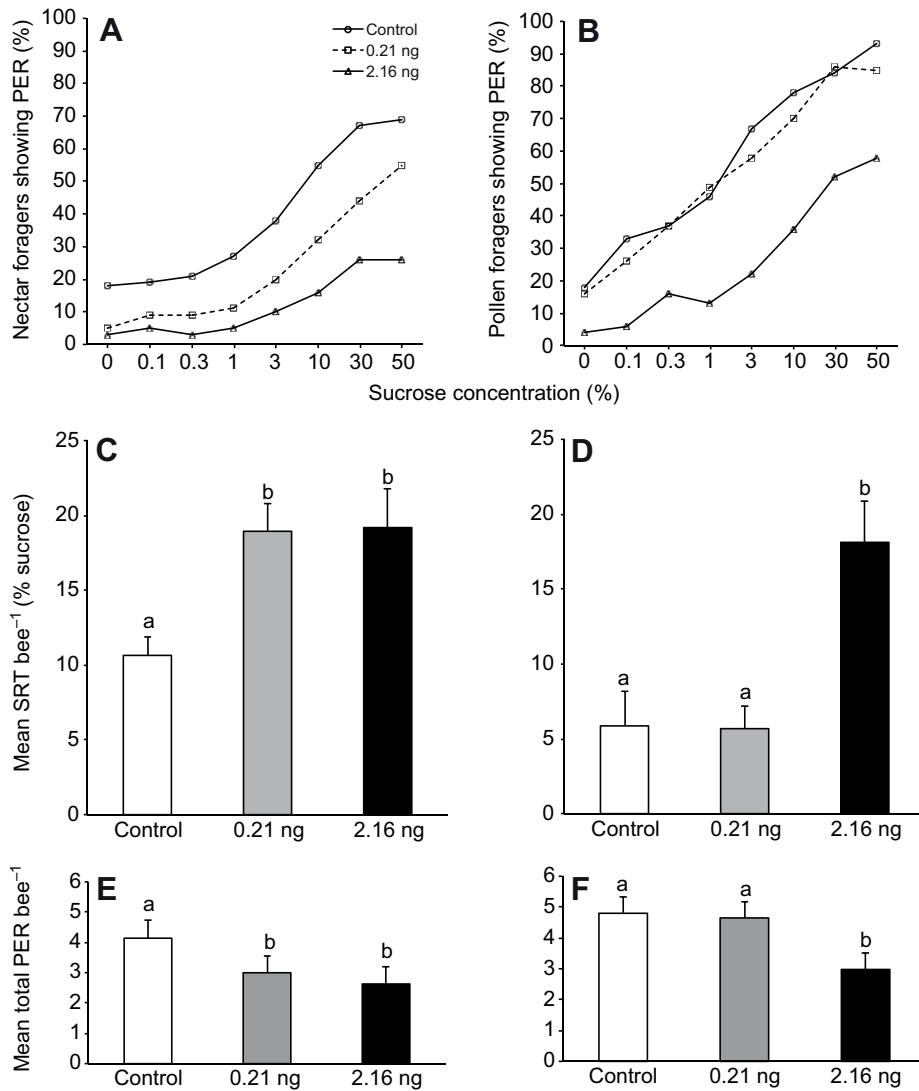
Fig. 1. The effect of imidacloprid (0, 0.21 and 2.16 ng bee^{-1}) on the sucrose response threshold (SRT) of nectar ($N_{control}$=151, $N_{0.21 ng}$=111, $N_{2.16 ng}$=52 bees) and pollen ($N_{control}$=95, $N_{0.21 ng}$=67, $N_{2.16 ng}$=47 bees) foragers. (A,B) The percentage of total proboscis extension responses (PER) to each sucrose concentration for (A) nectar and (B) pollen foragers. We measured the SR in two different ways and therefore show the (C) mean SRT for nectar foragers, (D) mean SRT for pollen foragers, (E) mean total PER bee^{-1} for nectar foragers and (F) mean total PER bee^{-1} for pollen foragers. Different letters indicate significant differences. Error bars are s.e.m. Nectar and pollen foragers were analyzed together in the full model. In these plots, we divide them into nectar and pollen forager groups because of the significant interaction between forager type and treatment (see Results).

total PER bee^{-1} ($F_{2,516}$=24.31, P<0.0001), and colony accounted for 6.2% of model variance. There was no significant effect of forager type ($F_{1,1}$=1.98, P=0.3952). However, there was a significant treatment × forager interaction ($F_{2,516}$=3.2, P=0.0400; Tukey HSD$_{REML}$, P<0.05). On average, control nectar foragers had a lower mean total PER bee^{-1} than control pollen foragers (4.1±2.3% and 4.8±2.1% sucrose, respectively).

Nectar foragers treated with imidacloprid had fewer total PER bee^{-1} in comparison to controls. The mean total PER bee^{-1} for nectar foragers was 4.1, 3.0 and 2.6 responses for treatment with 0, 0.12 and 2.16 ng imidacloprid bee^{-1}, respectively (significant differences between control and both imidacloprid doses, Fig. 1D, Tukey HSD$_{REML}$, P<0.05). Pollen foragers treated with imidacloprid also had fewer total PER bee^{-1} in comparison to controls. The mean total PER bee^{-1} for pollen foragers was 4.8, 4.7 and 3.0 responses for 0, 0.12 and 2.16 ng imidacloprid bee^{-1}, respectively (significant differences between control and the 2.16 ng dose, Fig. 1E, Tukey HSD$_{REML}$, P<0.05).

Effects on foraging bees

In this experiment, we used 65 bees from two additional colonies. We first examined acceptance (yes or no) of different sucrose concentrations at a feeder 24 h after treatment. In the full model, there was no significant effect of the interaction treatment × phase (χ^2_4=5.77, P=0.21). We therefore used the reduced model without interaction, in which there were no significant effects of colony (χ^2_1=1.83, P=0.18) or treatment (χ^2_1=0.32, P=0.57, Fig. 2A). Individuals exhibited significant variation in the sucrose concentrations that they accepted (χ^2_{61}=88.82, P=0.0115). Respectively, 100% and 93.8±0.2% of individuals accepted 50% sucrose solution in the before and after phases. However, as expected, fewer individuals accepted lower concentration sucrose solutions (Fig. 2A). Thus, there was a significant effect of phase (χ^2_4=337.03, P<0.0001).

We also analyzed the number of times each bee fed at each sucrose solution after treatment with imidacloprid. The results were consistent. In the full model, there was no significant effect of the interaction treatment × concentration ($F_{1,194}$=0.0006, P=0.98). In the reduced model with no interaction, there were no significant effects of treatment ($F_{1,195}$=0.20, P=0.65), colony ($F_{1,195}$=0.33, P=0.56) or individual ($F_{61,195}$=1.28, P=0.11). As expected, the number of times a bee fed at each sucrose concentration increased with increasing sucrose concentration ($F_{1,195}$=352.23, P<0.0001).
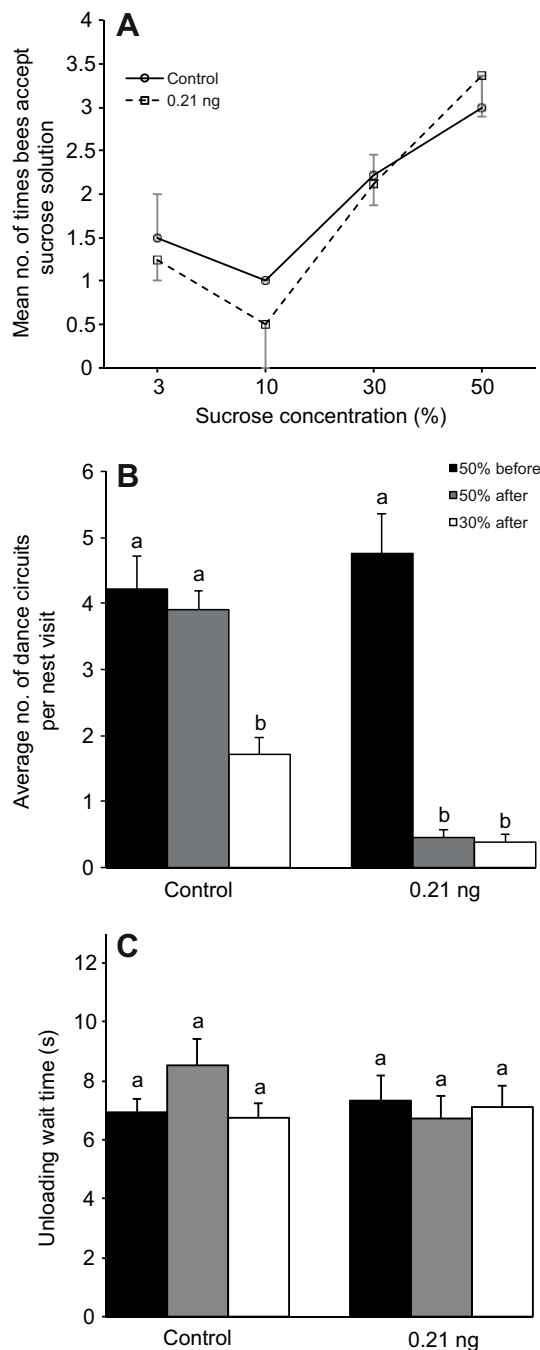
Fig. 2. The effects of imidacloprid on foraging and recruitment behavior. Foragers were trained to a feeder outside the colony. (A) The number of times that foragers accepted (landed on and collected at least once) each different sucrose concentration provided in a descending series ($N_{control}$=32, $N_{0.21 ng}$=33). (B,C) The resulting behavior inside the nest for 50% and 30% sucrose solutions: (B) the number of waggle dance circuits performed and (C) the unloading wait time. Different letters indicate significant differences. Error bars are s.e.m.

We then examined the effects of imidacloprid (24 h after treatment) on waggle dancing. The number of dance circuits was significantly affected by the imidacloprid treatment ($F_{1,117}$=9.02, P=0.0033). Only colony was not a significant factor ($F_{1,117}$=0.28, P=0.60). There was a significant effect of phase ($F_{2,117}$=124.46, P<0.0001) and a significant interaction of treatment $\times$ phase

($F_{2,117}$=56.09, P<0.0001) because control bees responded differently from imidacloprid-treated bees. Tukey HSD *post hoc* tests (Q=2.898, P<0.05) revealed no significant difference in the number of dance circuits performed by control bees *versus* imidacloprid-treated bees before treatment and control bees after treatment (Fig. 2B). Thus, before the treatment, bees in the control and imidacloprid groups performed the same number of dance circuits, and control bees did not change their dance behavior after being captured and given the sham treatment.

However, imidacloprid-treated bees performed significantly fewer dance circuits after imidacloprid treatment (the fewest of any category, Q=2.898, P<0.05). As anticipated, control and imidacloprid bees produced fewer dance circuits for 30% than for 50% sucrose solution (Fig. 2B). For 30% sucrose solution, imidacloprid-treated bees produced significantly fewer dance circuits than control bees (Q=2.898, P<0.05, Fig. 2B). Finally, there were significant individual differences in the degree to which imidacloprid reduced dancing ($F_{60,117}$=2.39, P<0.001).

The likelihood of a bee dancing is influenced by the unloading wait time (Seeley, 1992). However, we did not find any significant effect of treatment, phase or sucrose concentration on unloading wait time. In the full model, the interaction treatment $\times$ phase did not account for significant variation in unloading wait time ($F_{2,117}$=1.91, P=0.15). In the reduced model without this interaction, there were no significant effects of treatment, colony or phase ($F_{1,117}$≤0.29, P≥0.60). There was significant individual variation between bees ($F_{60,117}$=1.87, P=0.002). Thus, the sucrose concentration and imidacloprid treatment, not the time that foragers waited to be unloaded in the nest, affected the number of dance circuits.

DISCUSSION

Nectar and pollen foragers treated with 0.21 or 2.16 ng of the nAChR agonist imidacloprid showed a significant decrease in their SR 1 h after treatment. They extended their proboscises only for higher concentration sucrose solutions as compared with control bees. Analyses of two measures of SR yielded the same results. Treated bees had elevated SRT and lower total PER bee^{-1} (lower gustatory response scores) compared with controls. In addition, there were dose-dependent differences in the effects of imidacloprid on nectar and pollen foragers. These foragers were defined by what they were collecting immediately before testing. Both doses of imidacloprid significantly reduced SR in nectar foragers. Pollen foragers only reduced their SR in response to the higher dose. We also provide the first detailed results showing that imidacloprid metabolites can affect honey bee recruitment dances. Imidacloprid has a metabolic half-life of 4–5 h (Suchail et al., 2004a). When tested 24 h after imidacloprid ingestion, foragers treated with 0.21 ng (24 p.p.b.) imidacloprid performed 10.5- and 4.5-fold fewer dance circuits, respectively, for 50% and 30% sucrose solutions at a feeder as compared with controls. Honey bee waggle dancing can significantly enhance colony fitness (Sherman and Visscher, 2002; Dornhaus and Chittka, 2004). Thus, decreased waggle dancing for relatively high quality nectar should negatively affect colony fitness.

The concentration of imidacloprid used is relevant to our results because pesticides are applied at specific concentrations (reviewed by Decourtye and Devillers, 2010). However, there is variation in the size of the liquid load that each bee collects (Pankiw et al., 2004). Thus, knowing the actual dose that an individual bee receives is important for determining the precise effect of imidacloprid. Rortais et al. estimated that bees collecting nectar could be exposed to

imidacloprid doses of 1.1–4.3 ng bee^{-1} (Rortais et al., 2005). Acute oral LD$_{50}$ (the dose at which 50% of individuals die) for imidacloprid ranges from 3 ng bee^{-1} to >80 ng bee^{-1}, and contact LD$_{50}$ ranges from 7 to 81 ng bee^{-1} (Decourtye and Devillers, 2010). At sublethal doses of 1.25–20 ng bee^{-1}, imidacloprid can reduce mobility or induce tremors (Decourtye and Devillers, 2010). Honey bee larvae consuming an artificial diet with 400 p.p.m. of imidacloprid have significantly increased apoptotic cell death in their midguts (Gregorc and Ellis, 2011). However, other studies report no adverse effects of imidacloprid on colony health (Schmuck et al., 2001; Nguyen et al., 2009). These conflicting results may be due to several factors (Decourtye and Devillers, 2010), including different imidacloprid doses, colony variation in sensitivity to imidacloprid (Suchail et al., 2001), and disease or malnutrition (Cresswell, 2011). Some studies report synergistic effects. Imidacloprid at concentrations of 0.7, 7 and 70 p.p.b., coupled with the parasite *Nosema*, increased mortality as compared to imidacloprid or *Nosema* alone (Alaux et al., 2010). Honey bee larvae that are indirectly exposed to brood food with 5 or 20 p.p.b. of imidacloprid emerge as adults that are more susceptible to *Nosema* infection (Pettis et al., 2012).

In our study, bees were fed imidacloprid at doses of 0.21 and 2.16 ng bee^{-1} (corresponding to concentrations of 24 and 241 p.p.b., respectively, in 7 μl of 2.0 mol l^{-1} sucrose solution) to explore the effects of imidacloprid over a 10-fold range. The lower concentration (24 p.p.b.) is similar to that previously used in sublethal studies on honey bees (Kirchner, 1999; Decourtye et al., 2003; Yang et al., 2008). The higher concentration (241 p.p.b.) is close to the highest concentration (240 p.p.b.) used in another study (Decourtye et al., 2003) to study the effect of imidacloprid on honey bee PER. Bees are not typically exposed to such high concentrations in nectar (Decourtye and Devillers, 2010). However, exposure to even higher concentrations (47,000±9960 p.p.b.) is possible as bees can collect the guttation droplets exuded from the leaves of corn plants whose seeds were treated with imidacloprid (Girolami et al., 2009). Nevertheless, the lower dose and concentration (0.21 ng bee^{-1} at 24 p.p.b.) corresponds to what bees would more likely encounter. This lower dose was sufficient to decrease SR (Fig. 1A,C,E) and the number of dance circuits (Fig. 2B) in nectar foragers. The higher dose and concentration decreased SR in pollen foragers (Fig. 1B,D,F), which typically have lower SR than nectar foragers (Pankiw and Page, 2000).

We conducted an acute oral toxicity study, administering only one dose per bee, instead of several doses over a period of time (a chronic toxicity study), because we wished to determine how a bee responds to a single exposure to imidacloprid. A limitation of this approach is that it only provides information on the effects of a single dose, not the gradual accumulation of multiple doses. However, a strength of this approach is that it demonstrates a clearly defined outcome at one point in time. A single exposure to imidacloprid can have a strong and significant effect on SR and recruitment dancing. It would be useful for future studies to examine the effects of long-term, multiple exposures.

Our results on SR are in line with those reported in other studies. Harnessed bees given only pure sucrose solution (control bees, Fig. 1) had response thresholds similar to those measured in 1 week old bees (Pankiw and Page, 2000; Scheiner et al., 2004). Lambin et al. found that imidacloprid applied topically on the thorax at 1.25 or 2.5 ng bee^{-1} does not significantly alter SR, although higher doses (5, 10 and 20 ng) significantly reduce SR, particularly after 60 min (Lambin et al., 2001). Bees are more sensitive to neonicotinoids given orally than applied topically (Decourtye and Devillers, 2010). Thiamethoxam, another neonicotinoid, reduces SR when bees are

fed approximately 1 ng day^{-1} (Aliouane et al., 2009). Thus, nAChR agonists may generally impair SR in honey bees, perhaps by decreasing their appetitive motivation and resulting in responses only to very sweet food.

Another study (Kirchner, 1999) noted that imidacloprid at concentrations of 20–100 p.p.b. causes foragers to tremble dance and reduces the precision of distance and direction information in the waggle dance, presumably by increasing variation in the duration and angle of the waggle phase. Interestingly, honey bees perform tremble dances after consuming different poisons added to sugar solution (Schneider, 1949; Schick, 1953). Dechaume-Moncharmont suggested that a feeder with 20 p.p.b. imidacloprid may reduce the frequency with which foragers waggle dance (Dechaume-Moncharmont, 2003). Here, we provide the first clear evidence that nectar foragers significantly reduce waggle dancing by 10.5 and 4.5-fold for 50% and 30% sucrose, respectively (Fig. 2B), 24 h after ingesting a single dose of imidacloprid (0.21 ng bee^{-1} at 24 p.p.b.).

This reduction in dancing was not due to foragers rejecting contaminated sucrose solution or to the food source containing undesirable trace components (Afik et al., 2008). We tested foragers with uncontaminated solutions of pure sucrose. In addition, this decrease in waggle dancing did not arise from increased unloading wait times, because these were not significantly different between control and imidacloprid-treated bees (Fig. 2C). The decrease in waggle dancing was also not due to transfer of imidacloprid to nestmates. Foragers were released back to the hive 1 h after the control or imidacloprid dose was given, sufficient time for the 7 μl to be fully absorbed into the hemolymph (El Hassani et al., 2008) and thus reducing the possibility of imidacloprid being transferred to nestmates. However, even if a small amount of imidacloprid was transmitted to nestmates, control and imidacloprid-treated bees spent the same time period inside the nest (24 h) and had contact with nestmates. Nonetheless, there was a strong and significant reduction in waggle dancing by imidacloprid-treated bees compared with control bees (Fig. 2B).

Other studies have demonstrated a generalized reduction in movement and overall activity in honey bees treated with imidacloprid (Teeters et al., 2012; Suchail et al., 2001). We found highly specific effects. Imidacloprid-treated bees reduced the number of dance circuits that they performed, but did not change their foraging and flight activity for the feeder. They continued to visit and collect nectar ranging in concentration from 3% to 50% sucrose (w/w) at a rate that was not significantly different from that of controls (Fig. 2A). This behavior was surprising because we expected imidacloprid to reduce SR in foraging bees, as it did in our harnessed bees. Mujagic and Erber note that it is not possible to infer from laboratory PER measurements whether the same bees will accept similar sucrose concentrations in the field (Mujagic and Erber, 2009). However, based upon our PER results, we expected a general foraging decrease in imidacloprid-treated bees.

A difference in the effect of imidacloprid *versus* the effect of its metabolites may account for our results. The harnessed bees were tested 1 h after treatment whereas foraging bees were tested 24 h after they were treated. We did this because control and treatment bees would not forage 1 h after treatment. However, imidacloprid has a metabolic half-life of 4–5 h (Suchail et al., 2004a), and thus imidacloprid's metabolites (Suchail et al., 2000; Suchail et al., 2004b) may not affect SR, whereas the parent compound does. Imidacloprid's metabolites can persist for more than 48 h (Suchail et al., 2004b) and may cause longer term changes in dance behavior, particularly if bees are chronically exposed.

In summary, our results provide further insight into how imidacloprid affects honey bee foraging behavior. These effects are time dependent: SR decreased 1h after treatment, but foragers showed no change in the sucrose concentrations that they would collect when tested 24h after treatment. Thus, foraging efficiency may be temporarily reduced if foragers have higher response thresholds (in the short term) and accept fewer available nectar sources. Over the long term, reductions in waggle dancing should affect colony fitness by reducing honey weight gain in situations where recruitment is important (Sherman and Visscher, 2002; Dornhaus and Chittka, 2004). The links between the molecular disruptions caused by this nAChR agonist and these complex behaviors may be intricate. However, our results suggest a new tool for investigating the neural basis of SR and a forager's motivation to waggle dance.

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