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USING VIDEO-TRACKING TO ASSESS SUBLETHAL EFFECTS OF PESTICIDES ON HONEY BEES (*APIS MELLIFERA* L.)

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Abstract—Concern about the role of pesticides in honey bee decline has highlighted the need to examine the effects of sublethal exposure on bee behaviors. The video-tracking system EthoVisionXT (Noldus Information Technologies) was used to measure the effects of sublethal exposure to tau-fluvalinate and imidacloprid on honey bee locomotion, interactions, and time spent near a food source over a 24-h observation period. Bees were either treated topically with 0.3, 1.5, and 3 μg tau-fluvalinate or exposed to 0.05, 0.5, 5.0, 50, and 500 ppb imidacloprid in a sugar agar cube. Tau-fluvalinate caused a significant reduction in distance moved at all dose levels ($p < 0.05$), as did 50 and 500 ppb imidacloprid ($p < 0.001$). Bees exposed to 50 and 500 ppb spent significantly more time near the food source than control bees ($p < 0.05$). Interaction time decreased as time in the food zone increased for both chemicals. This study documents that video-tracking of bee behavior can enhance current protocols for measuring the effects of pesticides on honey bees at sublethal levels. It may provide a means of identifying problematic compounds for further testing. Environ. Toxicol. Chem. 2012;31:1349–1354. © 2012 SETAC

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INTRODUCTION

Although the honey bee (*Apis mellifera* L.) is indispensible for the pollination of crops in the United States, beekeepers are experiencing serious problems in maintaining the health and number of colonies. Managed honey bee colonies have declined by 45% over the past 60 years [1]. Whereas losses in the 1960s and 1970s were attributed to pesticides such as the organochlorines and organophosphates [2], subsequent declines correspond to the introduction of the *Varroa* mite (*Varroa destructor* Anderson & Trueman [3]). To combat this parasite, beekeepers began using acaricidal chemicals directly in the hive. The transition in U.S. agriculture toward the use of systemic pesticides, such as neonicotinoid insecticides, has generated additional concern about the role of pesticides in honey bee decline.

Although research investigating causes of large-scale honey bee losses is extensive [4], assessment of the risk posed by sublethal exposure to pesticides is limited by the lack of efficient tools to detect and quantify these effects. Specific guidelines for the use and registration of agrichemicals in the United States are mandated by the Federal Insecticide, Fungicide, and Rodenticide Act (http://www.epa.gov/pesticides/regulating/laws.htm), and the U.S. Environmental Protection Agency (U.S. EPA) determines the level of exposure that poses a hazard to honey bees. In the current paradigm, toxicity values are established for individual compounds in a three-tier system that first tests acute contact toxicity [5]. When the median lethal dose (LD50) is less than 11 μg per bee, the toxicity of foliar residues of the toxicant is measured in 24-h intervals in tier two. If prolonged residue activity is detected, toxicity is examined under conditions that resemble actual field use in tier three. Presently, mortality is the only endpoint that is measured, and data on sublethal effects are not required for pesticide registration [1]. Fortunately, the need for improved methods to predict sublethal behavioral risks has gained recognition [6,7].

Recent studies [8,9] have reported a variety of chemicals to which honey bees are exposed on a regular basis due to contamination of the hive environment. Among these, the pyrethroid tau-fluvalinate was nearly ubiquitous in samples of hive products and was detected at concentrations as high as 204 ppm. It is used in the product Apistan to manage *Varroa* populations and is administered on impregnated plastic strips that are hung between brood frames. Although it targets the *Varroa* mite, concern still exists about its safety to honey bees when exposed to sublethal residues in the hive. Most pyrethoids are highly toxic to honey bees although they are able to tolerate high concentrations of tau-fluvalinate, partly due to rapid detoxification by cytochrome P450 monooxygenases [10]. However, this does not imply that exposure is without harm, as honey bees may be especially vulnerable to pesticides because they have fewer genes encoding detoxification enzymes than other insects [11]. Sublethal effects have been documented in reproductive castes [12,13], but little is reported regarding the behavioral effects of this acaricide on honey bees.

Sublethal exposure to systemic pesticides is also a concern, as these chemicals are translocated to nectar and pollen and present a novel means of exposure in the honey bee diet. Of particular concern are the neonicotinoid insecticides. They are used extensively in the United States on turf, as seed treatments for field crops and as foliar treatments of fruits and vegetables, some of which require commercial pollination services [14]. As systemics, they can be detected in nectar and pollen throughout the blooming season [15]. Although exposure may not cause mortality, sublethal effects on behavior, learning, longevity, and development have been reported [6].

The acute toxicity tests used in the current approach to risk assessment provide an incomplete measure of effects on bees because only short-term survival of adults is considered [6]. Decline in colony health has been associated with ppm levels of

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pesticide residues in hive products [16], and systemic neonicotinoids can impair honey bee health at ppb levels [17]. Currently, the proboscis extension response assay (PER) and manual observations of behaviors are used to assess sublethal effects on behaviors and learning processes [18,19]. This study examined the utility of an automated video-tracking system, EthoVisionXT (Ver 7.0; Noldus Information Technologies), to monitor the behavioral effects of sublethal exposure to tau-fluvalinate and imidacloprid. Video-tracking systems have proved useful in investigations of circadian rhythms in the honey bee and other insects [20,21]. We tested the utility of video-tracking in generating the following meaningful parameters that reflect the effects of sublethal pesticide exposure on honey bee activity and behavior: the distance that honey bees traveled in a 24-h period, the amount of time a pair of worker bees spent interacting, and the amount of time spent near a food source.

MATERIALS AND METHODS

Chemicals

Technical-grade chemicals were used for all experiments in this study. Both tau-fluvalinate and imidacloprid were obtained from Chem Services.

Honey bees

The University of Nebraska–Lincoln maintains 14 honey bee colonies on the East Campus, which provided bees for the EthoVision experiments. Italian queens (C.F. Koehnen & Sons) were introduced to these colonies in April 2009 and 2010. The colonies were treated with the antibiotic Terramycin (oxytetracycline) in March 2010 to prevent bacterial brood diseases and Fumidil B (fumagillin) to control *Nosema* spp. infection. Apiguard (Vita [Europe]) and oxalic acid were used for control of *Varroa* mites.

Bees were collected by taking frames of late-stage brood from field colonies and placing them in a dark, humid incubator (model H024; Darwin Chambers) maintained at 34°C. Newly emerged adult bees were brushed daily from these frames into screened wooden boxes (1,620 cm^3), provisioned with a 1:1 (w/w) sugar water solution, and returned to the incubator for 3 to 4 d to allow them to mature before treatment and observation.

Video-tracking

Honey bee activity was monitored using the automated video-tracking software system EthoVisionXT. For each video-tracking experiment, 32 individual bees were randomly selected from a cohort of workers that had been anesthetized with carbon dioxide. Anesthetized bees were distributed into 16 polystyrene Petri dishes (9 cm), two bees per dish. Each dish was bisected with a piece of 3-mm wire mesh to keep the pair separate but allow interaction. Each bee was provisioned with a 0.5×1.0-cm cube of sucrose agar for food and moisture. Agar was composed of 8 g granulated cane sugar, 0.17 g agar powder, and 20 ml distilled water. New batches were made as needed.

The 16 dishes were placed beneath a video surveillance camera on a frosted Plexiglas surface that was illuminated from below with an infrared light encased in a 45.72×53.34-cm plywood box. All external light was eliminated by enclosing the entire unit in black plastic. Humidity was maintained within the enclosure at 80% RH by using a sonic humidifier (model V5100NS; Kaz USA) controlled by an automated humidity gauge.

A 26-h video of bee activity was recorded using the MPEG-recorder in the EthoVision software package. Recordings began in the morning between 9:00 and 10:00 hours to account for circadian rhythms in bee activity. The initial and final hours of video were excluded from the analysis to allow bees to recover from anesthetization and maintain consistent 24-h tracks. Using an image from the video, 32 arenas were defined with the EthoVisionXT software to establish where activity was to be tracked, and zones of interest were highlighted. Each Petri dish consisted of two arenas, one for each bee, and the sucrose agar was identified as the "food zone." The software scanned each arena 15 times per second to determine the positions of all 32 bees simultaneously as time-series coordinates (x, y) within each arena. These coordinates were translated into actual distances by calibrating the program to the actual dimensions of the arena (9-cm diameter of the dish).

A complete track record of the bees' movement patterns for the entire 24-h observation was obtained. The parameters investigated in this study were distance traveled (m) by each pair of bees, amount of time spent in the food zone (min), and interaction time (min) between the bees that shared a dish. Distance traveled was determined for each bee by summing the distance between a bee's coordinates in consecutive samples. The time spent in the food zone was the total time (total number of samples) that each bee was located on or adjacent to the sucrose agar cube. Interaction time was defined as the number of samples in which the two bees in neighboring arenas were located within 1.5 cm of each other, a distance at which bees were observed interacting through the screen divider.

Treatments

Honey bee workers were treated topically with tau-fluvalinate and orally with imidacloprid to mimic the likely route of exposure under field conditions. Imidacloprid was administered orally in sucrose agar containing 0.0, 0.05, 0.5, 5.0, 50, and 500 ppb imidacloprid, which was dissolved in distilled water and incorporated into the agar. The sublethal ranges correspond to LD10 and lethal concentration at 10% (LC10) estimates determined in preliminary bioassays. Bees were treated topically with 0.0, 0.3, 1.5, and 3 μg tau-fluvalinate in an acetone solution using a 50-μl syringe fitted to a repeating dispenser (model PB-600; Hamilton). One microliter of solution was applied to the thoracic notum of each bee while it was anesthetized. Untreated sucrose agar blocks were provided to bees treated with tau-fluvalinate.

Data and statistical analysis

Data on distance traveled, time in the food zone, and time spent interacting were measured for each pair of bees that shared a dish using the statistical approach described by Sams-Dodd [22]. Raw data were exported from EthoVisionXT for statistical analysis in R [23] using a General Linear Model with the multcomp package [24]. Pairwise comparisons were performed between dose level and control using Dunnett's post-hoc test (two-tailed).

RESULTS

Distance traveled

Analysis revealed that EthoVisionXT was capable of detecting differences in honey bee activity between treated and control groups for both tau-fluvalinate and imidacloprid. For distance traveled, bees treated with tau-fluvalinate moved significantly less than control bees at all dose levels ($p < 0.001$,

$F_{4,40} = 95.92$) (Fig. 1a). Those treated with 0.3 and 3 μg tau-fluvalinate traveled an average distance of 66.79 m (± 7.03 m) and 62.28 m (± 9.72 m), respectively, over 24 h, and those treated with 1.5×10^{-3} μg traveled 49.14 m (± 4.70 m). The control bees traveled at least 30% further than treated bees with a mean distance of 95.64 m (± 7.03 m). Bees exposed to 50 and 500 ppb imidacloprid also traveled significantly shorter distances (38.26 ± 1.95 m and 42.72 ± 4.28 m, respectively) than control bees (74.41 ± 4.37 m) ($p < 0.001$, $F_{6,106} = 184.36$) (Fig. 2a). No statistically significant difference in distance traveled was observed between groups exposed to 0.05, 0.5, and 5.0 ppb imidacloprid, which traveled 84.18 m (± 8.08 m), 81.43 m (± 4.70 m), and 70.11 m (± 4.39 m), respectively.

Time in food zone

The effect of exposure on the amount of time treated bees spent in the food zone (i.e., on or adjacent to the sucrose agar cube) was affected by both tau-fluvalinate ($p < 0.01$, $F_{4,40} = 3.848$) and imidacloprid ($p < 0.001$, $F_{6,106} = 18.145$). However, the Dunnett's test revealed that this was not significantly different from the control group for any dose level of tau-fluvalinate (Fig. 1b). Those treated with 0.3, 1.5, and 3 μg tau-fluvalinate spent 55.30 min (± 33.88), 72.17 min (± 23.38), and 83.00 min (± 42.99), respectively, in the food zone. Time in the food zone increased with higher levels of imidacloprid exposure (Fig. 2b). Although the group exposed to 0.05 ppb spent less time in the food zone than the control (78.98 ± 15.89 min vs 114.68 ± 18.96 min), each subsequent increase in exposure was accompanied by an increase in time spent near the sucrose. Groups exposed to 0.5, 5.0, 50, and 500 ppb spent 130.39 min (± 35.49), 245.66 min (± 56.40), 441.09 min (± 170.00), and 587.62 min (± 196.90), respectively, in the food zone. This equates to 30.6% of the observation period spent near the sucrose for bees exposed to 50 ppb imidacloprid, and 40.8% for those exposed to 500 ppb, compared with 8.0% of the observation period spent near the sucrose for the control group.

Time interacting

The level of exposure also had a significant influence on the amount of time a pair of bees spent interacting when exposed to either tau-fluvalinate ($p < 0.001$, $F_{4,40} = 17.903$) or imidacloprid ($p < 0.001$, $F_{6,106} = 10.194$). Again, interaction is defined as neighboring bees within 1.5 cm of each other. Mean interaction times for bees treated with 0.3, 1.5, and 3 μg tau-fluvalinate at 641.57 min (± 116.32), 394.15 min (± 146.07), and 122.61 min (± 58.87), respectively (Fig. 1c). No patterns were identified relative to control bees, which spent 311.83 min (± 51.05) interacting. The imidacloprid trials presented a more consistent trend (Fig. 2c). The control group spent the most time interacting (147.44 ± 32.34 min), followed by the groups exposed to 0.05 ppb (106.29 ± 40.90 min), 0.5 ppb (84.02 ± 22.70 min), 5.0 ppb (82.00 ± 25.72 min), and 500 ppb (69.91 ± 34.34 min). Bees exposed to 50 ppb imidacloprid spent 32% less time interacting than the control group with 47.07 min (± 22.37). However, no significant differences were observed in pairwise comparisons between the control and the various dose levels of tau-fluvalinate and concentration of imidacloprid.

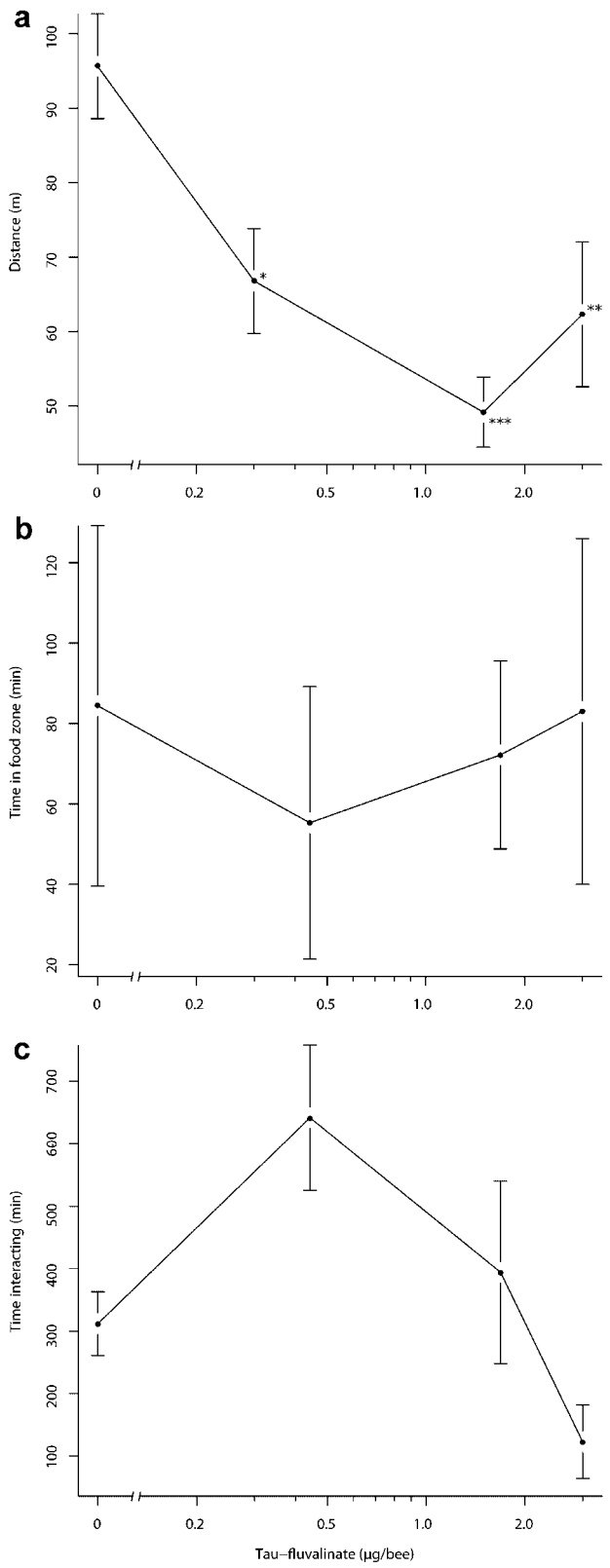

Fig. 1. Effect of a single topical application of tau-fluvalinate on (**a**) distance travelled, (**b**) time spent feeding, and (**c**) time spent interacting for a pair of honey bees over a 24-h period. Doses included: vehicle control ($n = 12$); 0.3 ($n = 12$); 1 ($n = 10$); and 3 μg/bee ($n = 10$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

DISCUSSION

The objective of this study was to examine the utility of the EthoVisionXT video-tracking system to detect sublethal behav-

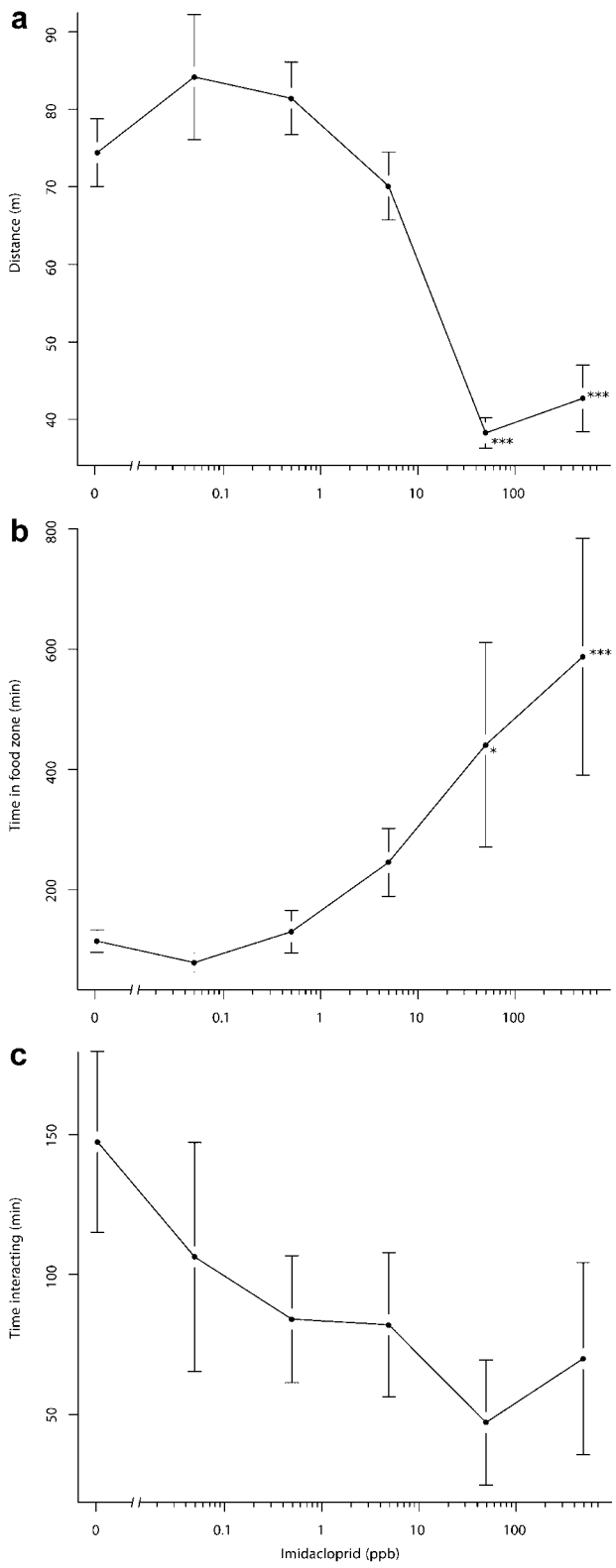

Fig. 2. Effect of orally administered imidacloprid on (**a**) distance travelled, (**b**) time spent feeding, and (**c**) time spent interacting for a pair of honey bees over a 24-h period. Concentrations of imidacloprid in food included: control ($n = 28$); 0.05 ($n = 16$); 0.5 ($n = 16$); 5 ($n = 28$); 50 ($n = 12$); and 500 ppb ($n = 12$). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

ioral effects of tau-fluvalinate and imidacloprid on worker honey bees. This automated method relies on determining the positions of the bees in the arenas, and the resultant x, y coordinates are used for calculating their locomotor activity, how they use the arena, and their relative position to each other. Distance traveled, time interacting, and time in the food zone by a pair of bees provided reliable indicators of tau-fluvalinate and imidacloprid exposure. Although results were more consistent for imidacloprid in this study than tau-fluvalinate, the EthoVisionXT system provided an efficient means of observing honey bee activity under the influence of pesticides for multiple factors on multiple bees simultaneously. This allowed for comparisons between control groups and different levels of exposure while maintaining consistency in time and environmental variables for all treatments. It is worth noting that the control bees included in the tau-fluvalinate and imidacloprid treatment series were not identical in the distance traveled, time in food zone, or time spent interacting. These differences between controls in the two treatment groups may be the result of topical exposure to acetone, which the tau-fluvalinate control group received, but the imidacloprid control group did not.

As a pyrethroid, tau-fluvalinate kills *Varroa* mites by blocking the voltage-gated sodium channels of the nervous system, causing nerve hyperexcitability that paralyzes the mite [25]. In honey bees, Haarmann et al. [12] reported that queens treated with high doses of tau-fluvalinate were smaller than untreated queens. Similarly, Rinderer et al. [13] found that drones exposed to tau-fluvalinate weighed less, produced fewer sperm, and were less likely to survive to sexual maturity. However, relatively little has been documented regarding the sublethal behavioral effects of this acaricide on worker honey bees. Taylor et al. [26] reported impaired response to odor stimuli in foragers exposed to fluvalinate-treated filter paper. In this study, a significant reduction in locomotor activity was observed after exposure to all dose levels of tau-fluvalinate (Fig. 1a), suggesting that this video-tracking system is sufficiently sensitive to measure sublethal behavioral effects of this acaricide.

Neonicotinoid compounds act as agonists of nicotinic acetylcholine receptors (nAChRs) [27]. They cause persistent activation of cholinergic receptors, leading to hyperexcitation and eventual death [28]. As seen in Figure 2a, imidacloprid has a stimulatory effect on locomotor activity at the lowest level of exposure, but an opposite effect at the highest concentrations. Similar results have been documented by Lambin et al. [29], who reported increased motor activity following topical application of imidacloprid at 1.25 ng per bee but a decrease in mobility of bees treated with >5 ng per bee. The stimulatory effect may be indicative of nicotinic activation by low doses of the insecticide, whereas a nonspecific toxic effect is seen at higher doses. Although the oral LD50 of neonicotinoids is much higher than the estimated daily ingestion of a forager [30], if treated crop plants constitute the majority of a colony's nectar and pollen resources while in bloom, multiple exposures to sublethal levels may occur for several weeks [31]. Several studies report on the lethal toxicity of imidacloprid after repeated ingestion [31,32]. However, sublethal effects, particularly impaired learning and orientation, have been induced at levels as low as 0.1 ng per bee [33]. Foragers may be exposed to as much as 0.6 ng imidacloprid per day when Gaucho-treated sunflowers are the primary nectar and pollen source of a colony [30]. Colin et al. [34] reported impaired foraging performance after exposure to imidacloprid at levels as low as 6 ppb. Similarly, Decourtye et al. [35] detected changes in foraging behavior at 4 ppb imidacloprid in sugar water, and both demonstrated reduced visitation to syrup feeders contaminated with this insecticide at 3 ppb and 24 ppb, respectively.

In addition to detecting similar activity patterns under the influence of imidacloprid, the EthoVisionXT system may be used to identify the no observed effect concentration (NOEC). Decourtye et al. [36] determined the NOEC using the conditioned PER assay at 24 ppb for oral treatment with imidacloprid and 60 ppb with its metabolite, hydroxy-imidacloprid. Differences were detected between the control group and bees treated with 50 ppb imidacloprid in distance traveled (Fig. 2a). Future studies should aim at establishing threshold exposure levels to evaluate the sensitivity of the system to different chemicals and define the NOEC.

Comparing the correlation between parameters provides a more complete depiction of bee activity. As time in the food zone increased, interacting time decreased for both tau-fluvalinate- and imidacloprid-treated bees (Figs. 1b,c and 2b,c). This is not surprising because the sucrose agar cube was placed at the far side of the arena, away from the mesh screen through which interactions occurred. For imidacloprid, as distance traveled decreased, time in the food zone increased. It seems that the bees ingested the imidacloprid, became intoxicated, and did not venture far from the cube afterward. Alternatively, a consequence of intoxication may have been increased hunger or thirst. El Hassani et al. [37] reported a dose-dependent increase in responsiveness to water in a water-triggered PER assay after exposure to acetamiprid, a neonicotinoid related to imidacloprid. In this case, intoxicated bees would also be expected to occupy the food zone more frequently than the control group. Future studies could discriminate between these hypotheses by using EthoVisionXT to record the frequency of food visits and interactions rather than the total time bees spend engaged in these behaviors.

CONCLUSIONS

The impact of pesticide exposure at the sublethal level should not be underestimated as these effects may induce physiological impairment that ultimately results in the loss of disoriented foragers and overall decline in colony health. Results indicate that EthoVisionXT is sufficiently sensitive to detect changes in honey bee activity induced by minute, albeit field-relevant pesticide exposure. Significant behavioral effects were noted for both chemicals. This system is capable of continuously monitoring multiple bees simultaneously over an extended period of time to detect changes in activity with sensitivity comparable to that of other methods. Sensitive screening methods are needed to improve the current risk assessment scheme, and video-tracking has the potential to identify problematic compounds for further testing to adequately evaluate the hazard of agrichemicals to honey bees and other pollinators. Implementing such screening systems would improve the efficiency of risk assessment and enhance current protocol by providing a means to more accurately measure impacts at the sublethal level.

REFERENCES

1. U.S. National Academy of Sciences. 2007. *Status of Pollinators in North America*. National Academy Press, Washington, DC.
2. Atkins EL. 1992. Injury to honey bees by poisoning. In Graham JM, ed, *The Hive and the Honey Bee*. Rev. Dadant and Sons, Hamilton, IL, USA.
3. Anderson DL, Trueman JWH. 2000. *Varroa jacobsoni* (Acari: Varroidae) is more than one species. *Exp Appl Acarol* 24:165–189.
4. Williams GR, Tarpy DR, van Engelsdorp D, Chauzat MP, Cox-Foster DL, Delaplane KS, Neumann P, Pettis JS, Rogers REL, Shutler D. 2010. Colony collapse disorder in context. *BioEssays* 32:845–846.
5. van Engelsdorp D, Speybroeck N, Evans JD, Nguyen BK, Mullins C, Frazier M, Frazier J, Cox-Foster D, Chen Y, Tarpy DR, Haubruge E, Pettis JS, Saegerman C. 2010. Weighing risk factors associated with bee colony collapse disorder by classification and regression tree analysis. *J Econ Entomol* 103:1517–1523.
6. Desneux N, Decourtye A, Delpuech J. 2007. The sublethal effects of pesticides on beneficial arthropods. *Annu Rev Entomol* 52:81–106.
7. Thompson HM, Maus C. 2007. The relevance of sublethal effects in honey bee testing for pesticide risk assessment. *Pest Manage Sci* 63:1058–1061.
8. Frazier M, Mullin C, Ashcroft S. 2008. What have pesticides got to do with it? *Am Bee J* 148:521–523.
9. Mullin CA, Frazier M, Frazier JL, Ashcroft S, Simonds R, van Engelsdorp D, Pettis JS. 2010. High levels of miticides and agrochemicals in North American apiaries: Implications for honey bee health. *PLoS One* 5:e9754.
10. Johnson RM, Wen Z, Schuler MA, Berenbaum MR. 2006. Mediation of pyrethroid insecticide toxicity to honey bees (Hymenoptera: Apidae) by cytochrome P450 monooxygenases. *J Econ Entomol* 99:1046–1050.
11. Claudianos C, Ranson H, Johnson RM, Biswas S, Schuler MA, Berenbaum MR, Feyereisen R, Oakeshott JG. 2006. A deficit of detoxification enzymes: Pesticide sensitivity and environmental response in the honey bee. *Insect Mol Biol* 15:615–636.
12. Haarmann T, Spivak M, Weaver D, Weaver B, Glenn T. 2002. Effects of fluvalinate and coumaphos on queen honey bees (Hymenoptera: Apidae) in two commercial queen rearing operations. *J Econ Entomol* 95:28–35.
13. Rinderer TE, De Guzman LI, Lancaster VA, Delatte GT, Stelzer JA. 1999. *Varroa* in the mating yard: I. The effects of *Varroajacobsoni* and Apistan® on drone honey bees. *Am Bee J* 139:134–139.
14. Quarles W. 2008. Pesticides and honey bee colony collapse disorder. *IPM Practitioner* 30:1–10.
15. Cutler GC, Scott-Dupree CD. 2007. Exposure to clothianidin seed-treated canola has no long-term impact on honey bees. *J Econ Entomol* 100:765–772.
16. Johnson RM, Ellis MD, Mullin CA, Frazier M. 2010. Pesticides and honey bee toxicity—USA. *Apidologie* 41:312–331.
17. Decourtye A, Devillers J, Cluzeau S, Charreton M, Pham-Delègue MH. 2004. Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotox Environ Safe* 57:410–419.
18. Pham-Delègue MH, Decourtye A, Kaiser L, Devillers J. 2002. Behavioural methods to assess the effects of pesticides on honey bees. *Apidologie* 33:425–432.
19. Ramirez-Romero R, Chaufaux J, Pham-Delègue MH. 2005. Effects of Cry1Ab protoxin, deltamethrin and imidacloprid on the foraging activity and the learning performances of the honeybee, *Apis mellifera*, a comparative approach. *Apidologie* 36:601–661.
20. Eban-Rothschild AD, Bloch G. 2008. Differences in the sleep architecture of forager and young honeybees (*Apis mellifera*). *J ExpBiol* 211:2408–2416.
21. Rosato E, Kyriacou CP. 2006. Analysis of locomotor activity rhythms in Drosophila. *Nat Protoc* 1:559–568.
22. Sams-Dodd F. 1995. Automation of the social interaction test by a video-tracking system: Behavioural effects of repeated phencyclidine treatment. *J Neurosci Methods* 59:157–167.
23. R Development Core Team. 2008. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria.
24. Hothorn T, Bretz F, Westfall P. 2008. Simultanous interference in general parametric models. *Biometrical J* 50:346–363.
25. Davies TGE, Field LM, Usherwood PNR, Williamson MS. 2007. DDT, pyrethrins, pyrethroids, and insect sodium channels. *IUBMB Life* 59:151–162.
26. Taylor KS, Waller JD, Crowder LA. 1987. Impairment of classical conditioned response of the honey bee (*Apis mellifera* L.) by sublethal doses of synthetic pyrethroid insecticides. *Apidologie* 24:249–266.
27. Tomizawa M, Casida JE. 2003. Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annu Rev Entomol* 48:339–364.
28. Jeschke P, Nauen R. 2008. Neonicotinoids—from zero to hero in insecticide chemistry. *Pest Manage Sci* 64:1084–1098.
29. Lambin M, Armengaud C, Raymond S, Gauthier M. 2001. Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee. *Arch Insect BiochemPhysiol* 48:129–134.
30. Rortais A, Arnold G, Halm M, Touffet-Briens F. 2005. Modes of honeybees exposure to systemic insecticides: Estimated amounts of contaminated pollen and nectar consumed by different categories of bees. *Apidologie* 36:71–83.

31. Ellis MD, Teeters BS. 2011. Assessing the risks of honey bee exposure to pesticides. *Am Bee J* 151:682–683.
32. Suchail S, Guez D, Belzunces L. 2001. Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environ Toxicol Chem* 20:2482–2486.
33. Schmuck R, Schöning R, Stork A, Schramel O. 2001. Risk posed to honeybees (*Apis mellifera* L., Hymenoptera) by an imidacloprid seed dressing of sunflowers. *Pest Manage Sci* 57:225–238.
34. Guez D, Suchail S, Gauthier M, Maleszka R, Belzunces L. 2001. Contrasting effects of imidacloprid on habituation in 7- and 8-day old honeybees (*Apis mellifera*). *Neurobiol Learn Mem* 76: 183–191.
35. Colin ME, Bonmatin JM, Moineau I, Gaimon C, Brun S, Vermandere JP. 2004. A method to quantify and analyze the foraging activity of honey bees: Relevance to the sublethal effects induced by systemic insecticides. *Arch Environ ContamToxicol* 47:387–395.
36. Decourtye A, Pham-Delègue MH. 2002. Assessing the sublethal effects of pesticides on the honey bee. In Devillers J, Pham-Delègue MH, eds, *Honeybees: Estimating the Environmental Impact of Chemicals*. Taylor & Francis, New York, NY, USA, p. 79.
37. El Hassani AK, Dacher M, Gary V, Lambin M, Gauthier M, Armengaud C. 2008. Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). *Arch Environ Contam Toxicol* 54:653–661.