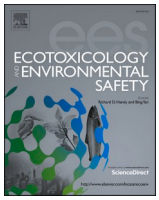
Ecotoxicology and Environmental Safety 204 (2020) 111108

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv



Assessment of lethal and sublethal effects of imidacloprid, ethion, and glyphosate on aversive conditioning, motility, and lifespan in honey bees (*Apis mellifera* L.)

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ARTICLE INFO

Keywords:
Apis mellifera, imidacloprid
Ethion
Glyphosate
Aversive conditioning

ABSTRACT

Honeybees (*Apis mellifera*) play an important role in agriculture worldwide. Several factors including agrochemicals can affect honey bee health including habitat fragmentation, pesticide application, and pests. The growing human population and subsequent increasing crop production have led to widespread use of agrochemicals and there is growing concern that pollinators are being negatively impacted by these pesticides. The present study compares acute exposure to imidacloprid (0.2 and 0.4 mgL^{-1}), ethion (80 and 106.7 mgL^{-1}) or glyphosate (0.12 and 0.24 mgL^{-1}) on aversive learning and movement, to chronic exposure at these and higher concentrations on movement, circadian rhythms, and survival in honey bee foragers. For acute learning studies, a blue/yellow shuttle box experiment was conducted; we observed honey bee choice following aversive and neutral stimuli. In learning studies, control bees spent >50% of the time on yellow which is not consistent with previous color bias literature in the subspecies or region of the study. The learning apparatus was also used to estimate mobility effects within 20 min of exposure. Chronic exposure (up to 2 weeks) with the above metrics was recorded by an automated monitoring system. In chronic exposure experiments, RoundUp®, was also tested to compare to its active ingredient, glyphosate. We found that imidacloprid and ethion have negative impacts on aversive learning and movement following a single-dose and that chronic exposure effects were dose-dependent for these two insecticides. In contrast, glyphosate had no effect on learning and less of an effect on movement; RoundUp® showed dose-dependent results on circadian rhythmicity. Overall, the results suggest that short-term exposure to imidacloprid and ethion adversely affect honey bee foragers and chronic exposure to glyphosate may affect pollination success.

1. Introduction

Agricultural ecosystems play a key role in human food production worldwide (Klein et al., 2006). Agriculture and related industries contributed 5.4% of the United States gross domestic product (GDP) in 2016 (USDA, 2017). Pollination services are important both for agricultural and wild plant reproduction and honey bees make marketable products (Aizen and Harder, 2009; Devillers, 2002; Gallai et al., 2009; Klein et al., 2006; Paudel et al., 2015; Potts et al., 2010a; Rogers et al., 2014). Pollination services of croplands represented a $24 billion industry in the United States in 2013, with honey bees accounting for nearly 63% of that total (White House Office of the Press Secretary, 2014). However, in tandem with increasing need for pollination services, expansion of agricultural land use has led to greater application of chemical pesticides and can be sources of other risks (Schreinemachers and Tipraqsa, 2012). For crops such as almonds, honey bee hives are relocated from their apiaries to the orchards for maximum production, however this increases the likelihood that they will encounter disease during travel and pesticides through nectar and pollen collection (Alger et al., 2018; Calderone, 2012; Pohorecka et al., 2012).

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https://doi.org/10.1016/j.ecoenv.2020.111108
Received 19 February 2020; Received in revised form 16 July 2020; Accepted 30 July 2020

Several factors may decrease honey bee health such as habitat fragmentation, pesticide application, and pests, with reports of decline in Europe and North America (Barbosa et al., 2015; Bortolotti et al., 2003; Bruckner et al., 2020; Goulson et al., 2015; Meixner, 2010; Meixner et al., 2015; Potts et al., 2010b; Smith et al., 2014;, 2013Vanbergen; van Engelsdorp et al., 2007; van Engelsdorp et al., 2009). In the United States, there has been high mortality (>25%) of honey bee colonies since 2006 (Bruckner et al., 2020; Hayes et al., 2008; van Engelsdorp et al., 2007, 2011, 2012). This population decline is attributed to biotic and abiotic agents such as pathogens (*Varroa* spp., *Nosema apis*) (Fries, 2010; van Engelsdorp et al., 2009), parasites (*Varroa destructor*) (Cox-Foster et al., 2007; Le Conte et al., 2010), agrochemicals (Frazier et al., 2008; Godfray et al., 2014), climate change (Spivak et al., 2010), and habitat loss (Potts et al., 2010a). It is unlikely that a single factor is responsible for poor honey bee health; rather, the described factors work in tandem and result in population decline (Johnson et al., 2013; Mullin et al., 2010; van Engelsdorp et al., 2009). These interactions are especially concerning when sublethal effects of one factor increases the lethality of another (Neumann and Carreck, 2010; Potts et al., 2010a). One such example of this type of interaction is increased *Nosema* infection following fungicide exposure in honey bees (Pettis et al., 2012).

Pesticide exposure has various effects on honey bees that make them particularly worth study. Depending on exposure concentration, pesticide ingestion can be acutely lethal, causing near immediate mortality, or sublethal, causing atypical behavior or physiological changes (Di Prisco et al., 2013; Henry et al., 2012). Behavioral effects, such as reduced foraging accuracy and classical conditioning, have been found following pesticide exposure in honey bees (Abramson et al., 1999, 2004, 2012; De Stefano et al., 2014; Henry et al., 2012; Karahan et al., 2015; Muth and Leonard, 2019; Stone et al., 1997). However, how motility, circadian rhythms, or spatial awareness in aversive environments are effected by exposure to various pesticides is not as well understood. Aversive associative conditioning is a potential indicator of lapses in complex bee behaviors that are needed to succeed in foraging for pollen and nectar while avoiding predation (Gauthier, 2010). This can be investigated using laboratory experiments such as those by Abramson et al. (1982) or Dinges et al. (2013).

This study will seek to understand how pesticide exposure to imidacloprid, ethion, or glyphosate, affect honey bee aversive learning of shock-color pairings, motility, circadian rhythms, and survival. Our rationale for using these agrochemicals is that they are used globally in agricultural settings and ingested by honey bees through pollen, nectar, and water collection (Foster et al., 2004; Johnson and Pettis, 2014; Rubio et al., 2014). Glyphosate, RoundUp®, and neonicotinoids such as imidacloprid are differentially regulated by country (see Environmental Protection Agency, 2020, European Commission, 2013, or Wenk et al., 2018 for examples) despite negative impacts on honey bees that have been demonstrated in the literature (Berg et al., 2018; Bortolotti et al., 2003; Dai et al., 2018; Decourtye et al., 2004a, 2004b; Faghani et al., 2018; Farina et al., 2019; Herbert et al., 2014; Jiang et al., 2018; Karahan et al., 2015; Zhang and Nieh, 2015). Ethion has been understudied as the lethal concentrations of this pesticide are higher than the ecologically relevant exposures; however, sublethal outcomes can be equally detrimental and are therefore worthy of study.

This study provides data that can be used to help beekeepers around the world, particularly in regions where beekeeping is expanding, honey bees are native, and where the industry has been suggested as a tool for socio-economic elevation, such as the Middle East and North Africa (Amulen et al., 2017; Komeili, 1990; Schouten and Lloyd, 2019). In Middle Eastern countries, pesticide regulations vary significantly; determining sublethal and lethal exposure concentrations may impact the pursuit or success of beekeeping social programs (Wenk et al., 2018). For example, in Iran there are 2.7 million hives and an average of 10 kg of honey produced annually per hive (Food and Agricultural Organization, 2005). Iran is fourth globally in honey production, and has the fifth largest number of colonies in the world (Bee Culture, 2018). Approximately 1400 tons of pesticides are used in Iran annually and crop pollination relies on honey bees (Bee Culture, 2018; Morteza et al., 2017). In regions such as this, deformed wing virus and other pathogens present both agricultural and biodiversity threats as honey bees are a native and economically valuable species (Haddad et al., 2017). The effects of pesticides on honey bees may increase susceptibility to these threats, therefore understanding their effects is valuable.

Imidacloprid is a crop-systemic neonicotinoid pesticide and is used to control a wide range of insect pests such as aphids and whiteflies (Elbert et al., 1991; Nauen et al., 2001). It is effective for pest management but also affects non-target insect species such as honey bees (Jones and Sattelle, 2010). Imidacloprid is mainly applied as seed coatings or soil applications but can be found in pollen (mean: 2–36 ng g^{-1}), nectar (mean: 0.13–1.9 ng g^{-1}) and leaves (3.25–19.64 ng g^{-1}, Jiang et al., 2018). In insects, imidacloprid has a high agonistic affinity to nicotinic acetylcholine receptors (nAChR) which affects the central nervous system (Elbert et al., 2008; Schmuck et al., 2003). Nicotinic acetylcholine receptors play vital roles in synaptic transmission in the central nervous system and acetylcholine is an excitatory neurotransmitter in the insect brain (Gauthier, 2010).

In addition to lethality (Wu et al., 2011), sublethal effects of imidacloprid on bees are noted including behavioral disorders, difficulty in flight orientation, and impairment of social interactions and can occur across the honey bee lifespan (Colin et al., 2019; Decourtye et al., 2004b; Desneux et al., 2007; Maini et al., 2010). Poor choice-making may be a combination of impaired olfactory and learning retention and neurophysiological changes that affect consumption (Cook, 2019; Decourtye et al., 2003, 2004b; Williamson and Wright, 2013). Additionally, field studies have shown imidacloprid in the pollen of plants like sunflower, maize, and canola, which are often collected by honey bees as protein sources (Arathi et al., 2018; Bonmatin et al., 2005). For this reason, understanding how this pesticide affects learning and color preference may provide vital information toward assessing pollinator risk.

In addition to imidacloprid use, the organophosphate pesticide ethion has been found in beeswax (131 ng g^{-1}), this is likely the result of collecting contaminated floral products (Johnson et al., 2010). Ethion is used to control pests by inhibiting the cholinergic system through degradation of the enzyme acetylcholinesterase. This results in overstimulation of post-synaptic neurons or muscle cells (Pohanka, 2011). Acetylcholinesterase is present throughout the brain and is vital to movement and memory across animals (Gauthier et al., 1992). Studies using the proboscis extension reflex with olfactory conditioning after exposure to organophosphate pesticides have shown effects on olfactory learning and memory (Williamson and Wright, 2013). Although the LD_{50} of ethion is relatively high, 106 mgL^{-1} (Delkash-Roudsari et al., unpublished results), understanding the sublethal effects of chemicals that honey bees may be exposed to is vital to determining potential factors of reduced colony health.

There is not much literature on the impacts of ethion on honey bees because the lethal exposure is considerably higher than other pesticides such as imidacloprid. Ethion has been found in propolis (mean: 40 ± 10 ng g^{-1}, Valdovinos-Flores et al., 2017), honey (median: 18 ng g^{-1}, Pareja et al., 2011), and beeswax (up to 131 ng g^{-1}, Johnson et al., 2010). The presence of ethion in these honey bee products implies that individuals likely ingest the insecticide over the course of their lifespan; therefore, experiments to understand sublethal endpoints and chronic exposure metrics should be conducted.

In addition to insecticides, herbicides may affect honey bees. Glyphosate, the active ingredient in Roundup®, is the most commonly used herbicide in the United States. It is a broad-spectrum herbicide used to control broadleaf weeds and grasses in agricultural and non-agricultural settings (Benbrook, 2016; Lundgren, 2018; Motta et al., 2018; Myers et al., 2016). Bees are exposed to RoundUp® and its active ingredient, glyphosate, when sprayed on blooming flora or following use on weed species, it can be consumed by pollinators collecting pollen,

nectar, or water from contaminated sources (Seide et al., 2018). Glyphosate concentrations in honey (~1.2 × 10^5 ng L^{-1}, Berg et al., 2018) demonstrate that honey bees are collecting and storing contaminated floral products. Glyphosate kills plants and some microorganisms by preventing the biosynthesis of essential aromatic amino acids and other secondary metabolites that are necessary for growth (Shilo et al., 2016). Studies have shown that glyphosate can disturb beneficial gut microbiota in bees, weakening the immune system (Motta et al., 2018), and reduce navigational abilities (Balbuena et al., 2015), sucrose responsiveness, olfactory learning, and food uptake (Goñalons and Farina, 2018; Herbert et al., 2014). Studies have shown mixed mortality effects in brood following glyphosate application and work with zebrafish has shown different outcomes to glyphosate as opposed to the formulation, RoundUp® (Bridi et al., 2017; Gregorc and Ellis, 2010; Thompson et al., 2014). For this reason, both glyphosate and RoundUp® will be used to study chronic toxicity effects.

Disruptions in the ability to learn is a common sublethal indicator for individual bee effects (Decourtye et al., 2003). Although some agrochemicals may not be lethal to honey bees in field conditions, during blooming, foragers may ingest sublethal amounts that affect their learning and decision making (Suchail et al., 2001). Prior studies on honey bees have worked to understand the sublethal and lethal effects of agrochemicals on learning behavior using proboscis extension response learning (Muth and Leonard, 2019), artificial flower patches (Karahan et al., 2015), and pesticide spray studies (McArt et al., 2017), however, there is less information on the effects of agrochemicals on aversive conditioning or chronic individual honey bee exposure outcomes. Zhang and Nieh (2015) used aversive olfactory learning in a sting extension response assay and found that chronic exposure of imidacloprid impaired aversive short-term learning and memory retention. Contrastingly, Colin et al. (2020) found imidacloprid alone did not reduce performance in an aversive conditioning environment. Urlacher et al. (2016) found chlorpyrifos (organophosphate pesticide) has no adverse effect on aversive olfactory conditioning but severely affected appetitive olfactory memories.

Honey bees encounter numerous aversive stimuli including predators, insect repellents, and pesticides, that it would be adaptive to avoid. In prior studies, honey bees stopped flying toward an aversive stimulus and reduced visitation on feeders containing an essential-oil-based pesticide (Abramson et al., 2006). In the present study, we used a color-pairing learning paradigm with electric shock avoidance (ESA). In the ESA conditioning assay, the conditioned stimulus is electric shock (punishment) and the unconditioned stimulus is color (blue or yellow). The learning outcomes following ESA conditioning in prior experiments have been similar to those found when using appetitive stimuli (see Mackintosh, 1974).

The rationale for studying the effect of pesticides on aversive conditioning is twofold. First, the study of agrochemicals on the behavior of honey bees would be incomplete if restricted to the study of appetite learning. Secondly, it has been suggested that the use of the proboscis extension response technique (PER) is fraught with problems of replication, inconsistent PER procedures between laboratories, and seasonal effects (Abramson et al., 2011; Scheiner et al., 2013). These problems with the PER technique have stimulated interest in using aversive techniques.

In addition to aversive conditioning, chronic sublethal and lethal exposure was investigated using a 24-h behavioral monitoring system. This system uses motility, day/night movement, and mortality as endpoints following chronic exposure through water (Chicas-Mosier et al., 2019). This system has previously been used to study aluminum exposure in honey bees and provides unmatched chronic exposure data.

2. Materials and methods

2.1. Aversive conditioning experiment

2.1.1. Honey bee collection

Foragers were collected off of a feeder of 20% (v/v) sucrose solution. Feeders were approximately 15 m from the hives and collection occurred daily between 800 and 1000 h during late summer 2019. Hives (3) were maintained weekly and verified for forager departure, queen health, and eggs. *A priori* testing for hive pesticide exposure was not conducted, however collection of honey bees from the same three hives should account for some preexisting exposure effects (Chicas-Mosier et al., 2019). Only foragers were collected to try to standardize for age as they are typically 21–30 days of age (Huang et al., 1994). Foraging honeybees were caught in glass jars off of the side of the feeder and then transferred to holding cages that contained a Petri dish of 1:2 honey: sucrose mixture for feeding *ad libitum*. Bees were then transported to the Behavioral Biology and Comparative Psychology laboratory at Oklahoma State University, 12.4 km away.

2.1.2. Pesticide Dosing

A preliminary study was conducted to compute the lethal dose of 50% (LC_{50}) and 30% (LC_{30}) of honey bees in Iran following the method of Miranda et al. (2003) and Laurino et al. (2013) with little modification. A bioassay experiment was conducted to determine which concentrations of imidacloprid and ethion caused between 15 and 85% mortality in honey bees. Following this determination, concentrations between them were tested. To test exposure, honey bees were collected and starved for 2 h. Following starvation, bees were allowed to free-feed from intermediate concentrations in 50% w/v sucrose solutions for 1 h. After dosing, honey bees were given no-treatment sucrose solutions *ad libitum* for 24 h. This bioassay was conducted with 25 bees in 3 replicates per concentration in a dark incubator at 35 ± 1 °C and 50 ± 5% relative humidity. Mortality rates after 24 h were used to generate a dose-response curve in Polo Plus software and determine LC_{30} and LC_{50} (Delkash-Roudsari, unpublished data).

Pestanal Analytical Standard grade imidacloprid (CAS: 138,261-41-3, >98%), ethion (CAS: 563-12-2, >95%), and glyphosate (CAS: 1071-83-6, >98%) were purchased from Sigma-Aldrich (St. Louis, MO USA). Each pesticide was dissolved in water at 2x the highest concentration used then diluted by adding the stock to 2 M sucrose solution at the necessary ratio for each needed exposure concentration, rather than serial dilutions. For imidacloprid and ethion, the highest dose tested corresponds to the LD_{50} and the lowest dose tested corresponds to the sublethal dose for 30% of tested bees in the preliminary study (LD_{30}) (Delkash-Roudsari et al., unpublished results). As LD_{50} and LD_{30} for glyphosate was not available, dosages were chosen to be significantly below those previously reported in honey (~1.2 × 10^5 ng L^{-1}, Berg et al., 2018) and water (8 × 10^5-2x10^7 ng L^{-1}, Dai et al., 2018) to determine sublethal effects. Final concentrations were: imidacloprid 0.2 mg L^{-1} (LD_{30}) and 0.4 mg L^{-1} (LD_{50}), ethion 80 mg L^{-1} (LD_{30}) and 106.7 mg L^{-1} (LD_{50}), and glyphosate 0.12 mg L^{-1} and 0.24 mg L^{-1} in 1 M sucrose. Per bee dosages were: imidacloprid LD_{30}: 2 ng $bee^{-1}$and LD_{50}: 4 ng bee^{-1}, ethion LD_{30}: 667 ng $bee^{-1}$and LD_{50}: 889.2 ng bee^{-1}, and glyphosate Low: 1.2 ng $bee^{-1}$and High: 2.4 ng bee^{-1}. All solutions were mixed thoroughly before feeding to maintain solution/suspensions. Solutions with sucrose were stored at 4 °C and used for up to 1 week before remaking to limit bacterial growth, stock pesticide solutions in water were refrigerated and remade as needed. Ten minutes before the aversive conditioning experiment, bees were fed 10 μl of a treatment solution (per bee dose).

2.1.3. Exposure procedure

Following honey bee collection and return to the laboratory, bees were individually selected from a holding cage, a wood framed box with wire-mesh walls (42L × 35.6 W × 41 H cm) with a removable base for

cleaning and a fabric access sleeve. Bees were collected from the top screen of the holding cage; these bees were overall more active than bees that remained on the bottom of the container. Bees were removed via a matchbox and dosed with 10 μL of treatment using a pipette. Following dosing, each bee was held for 10–19 min inside the matchbox before being introduced to the shuttlebox apparatus. This amount of time was selected as it has previously been used to study acute effects on learning (Abramson et al., 2004).

2.1.4. Apparatus

The aim of this experiment was to assess exposure outcomes of sublethal (LD_{30}) and lethal doses (LD_{50}) of imidacloprid, ethion, and glyphosate (no LD values were available so concentrations based on Dai et al. (2018) and Berg et al. (2018)) on honey bee foraging behavior in an aversive learning experiment. The test was run using a shuttle box as described in Dinges et al. (2013, 2017). The apparatus consisted of two separate choice compartments measuring 135 mm × 20 mm × 5 mm. This size allows for continuous contact of the honey bee cuticle to the shock grid. Each choice compartment is atop a continuous shock grid. Following placement in the compartment, bees are secured using a Plexiglas cover for visibility throughout the experiment (Supplemental Image 1). Each compartment was separate to eliminate social cues, and was cleaned prior to introduction of subjects using an ethanol soaked tissue to remove odors.

The shock grid was connected to an external power source that provided a shock of 7 V at 0.05 A (Black et al., 2018; Varnon and Abramson, 2018). Current was administered to the grid after the master bee entered the designated shock portion of the apparatus. Distinct visual stimuli were presented underneath the grid, consisting of two individual paint swatches, one blue and the other yellow. Paint swatches were chosen to most closely match the visual appearance of enamel paint (1632 T and 1208 T, Testors Vernon Hills, IL), which have previously been the standard for honey bee behavioral studies (Chicas-Mosier et al., 2019). Each compartment contained two infrared beams, on either side of the center line. When the subject crossed the centerline, the apparatus, which is connected to a propeller controller (Varnon and Abramson, 2013, 2018) and control panel, recorded the time-point and wrote the data in accordance with the protocol designed by Dinges et al. (2013, 2017) (Supplemental Image 1).

2.1.5. Discrimination task

Prior to initiation of automated data collection, bees were given a 3-min recovery period to allow for habituation to the apparatus. Recovery periods have varied in prior studies; however, given the short experimental period of the present work, 3-min was determined to be sufficient (Black et al., 2018; Dinges et al., 2013). Following the recovery period, the apparatus began collecting data after both subjects were detected by the apparatus and the bee listed as master entered the side of the shuttle box designated as safe; safe and shock were counterbalanced by color (Table 1, Black et al., 2018). Each experimental session consisted of two consecutive 3-min trials, with the second trial starting immediately after the conclusion of the first, consistent with the original protocol (Dinges et al., 2017). Bees were introduced in pairs consisting of a master bee: controlled onset and offset of shock by entering and exiting the designated side, and the yoked bee: shocked whenever the master bee entered the designated shock side, regardless of its location and serving as an unpaired control (Black et al., 2018; Dinges et al., 2013, 2017). Non-experimental pairs consisted of two bees whose behavioral data was recorded, but no shock was administered. These pairs serve as a behavioral control (Black et al., 2018).

2.1.6. Aversive conditioning data analysis

Color bias in baseline bees was measured using a one-way *t*-test against a hypothesized mean of 90 s, this would be equivalent to spending significantly more than 50% of a trial on a single side/color of the shock apparatus. Bees were removed from the dataset if they did not cross the photocells >3x over the course of a trial to eliminate the possibility of no-movement bees unevenly weighing time. Other measured variables, including cumulative time per side and average time spent per colored side, were analyzed via SAS JMP 13 using analysis of variance (ANOVA) with Tukey HSD for all pairs to determine differences between experimental groups and concentrations within pesticide treatments as compared to controls.

2.2. Chronic exposure using the trikinetics monitor system

2.2.1. Honey bee collection

Honey bees were collected in individual 15 mL falcon tubes from the same feeder as described for the aversive conditioning experiment. Each falcon tube was outfitted with a lid filled with a pea-sized dollop of approximately 1:2 honey: sucrose mixture for sustenance (Chicas-Mosier et al., 2019). The food mixture was covered with a piece of cheesecloth, approximately 2 cm × 2 cm, to limit the bees from sticking while allowing nutritive access (Chicas-Mosier et al., 2019).

2.2.2. Monitoring system

The monitor apparatus (Supplemental Image 2) automatically records data for up to 32 bees in individual 15 mL falcon tubes as described in Chicas-Mosier et al. (2019). Each falcon tube contains several aeration holes. On the opposite end of the falcon tube from the lid, an aeration hole was filled with a piece of filter paper approximately 25 mm × 30 mm. The strip of filter paper extended from the inside of the falcon tube down to a 40 cm L x 1 cm inner diameter section of Chlorinated Polyvinyl Chloride (CPVC) pipe attached to the monitor. The CPVC pipe reservoir was filled with up to 40 mL of water before placing the monitor with bees inside an incubator (24 h darkness, 35 ± 2°C, 42% humidity). The water with or without treatment flows up the filter paper to each bee (8 bees/CPVC pipe).

The monitors contain six photocells encircling the center of the falcon tube, these record each time a bee crosses the centerline of the tube. Bees that do not cross the centerline for 24 h are recorded as deceased. Circadian rhythms, activity level, and captive lifespan are recorded via this system. Monitors were kept in darkness for the entirety of the experiment with the exception of water and food replacements during which the bees were exposed to red light (Chicas-Mosier et al., 2019). Bees do not have vision in the red spectrum so the red light should not influence the circadian behaviors (Peitsch et al., 1992). Every subsequent 48 ± 8 h the CPVC pipes were filled with up to 20 mL of water (as needed) by treatment. Every other water refill included a recapping with a fresh food lid for each living bee (Chicas-Mosier et al., 2019). All

Table 1
Description of shuttlebox locations and sides for aversive learning apparatus with sample sizes for each treatment group.

Bee Role	Side Correct	Shock Location	Number of Bees Tested						
			Control	Imida LD_{30}	Imida LD_{50}	Ethion LD_{30}	Ethion LD_{50}	Gly Low	Gly High
Baseline	Blue	None	98	41	20	39	24	40	20
Master Blue	Yellow	Blue	40	36	22	34	38	36	18
Master Yellow	Blue	Yellow	78	40	36	38	38	38	36
Yoked Blue	Yellow	Anywhere, dependent on Master bee	72	38	36	36	38	38	36
Yoked Yellow	Blue	Anywhere, dependent on Master bee	74	38	38	40	38	36	40

monitor experiments ran for up to two weeks or until all bees were recorded as deceased.

2.2.3. Chronic exposure dosing

Automation of the monitor system allows for quick data collection using additional exposure concentrations and pesticides. In contrast to the aversive conditioning experiment, all treatments were in deionized water only (24–32 bees/treatment/concentration) and the pesticide concentrations reflect exposure concentrations rather than dosages. Dose metrics cannot be quantified further than exposure concentration due to the nature of the Trikinetics system: each water reservoir supplies treatment to 8 bees and some leaking is expected so input/output measurements would be inaccurate (Chicas-Mosier et al., 2019). Chronic experiments also included Roundup®, a herbicide formulation that uses glyphosate as the active ingredient. Concentrations (n = 32 bees each) used were Roundup®: 24 mg L^{-1}, 12 mg L^{-1}, 6 mg L^{-1}, 1.2 mg L^{-1}, 0.12 mg L^{-1}, Glyphosate: 24 mg L^{-1}, 12 mg L^{-1}, 6 mg L^{-1}, 1.2 mg L^{-1}, 0.12 mg L^{-1}, Imidacloprid: 0.8 mg L^{-1}, 0.4 mg L^{-1}, 0.2 mg L^{-1}, Ethion: 80 mg L^{-1}, 106 mg L^{-1}, 160 mg L^{-1} and respective deionized water temporal controls for each experiment (n = 120). One-liter stock solutions were made of each high concentration and refrigerated at 4 °C. Dilutions of 120 mL/concentration were made directly from the stock solution (non-serially) every 48hr for water replacement. For each pesticide, the LD_{30} and LD_{50} were used if available (see Pesticide Dosing for details) as well as an additional higher concentration to test an expected lethal dose.

For Roundup® and glyphosate, LD values were not available so values found in the literature were selected (Berg et al., 2018; Dai et al., 2018; Faghani, 2018; Elandalloussi et al., 2008). This was chosen rather than using the application rate for Roundup® to reflect concentrations found in honey and in water. RoundUp® (RoundUp® Ready-to-Use Weed and Grass Killer, Monsanto Company) solutions reflect the amount of active ingredient, glyphosate, additional volume was added to account for other ingredients in the formulation.

2.2.4. Chronic dosing data analysis

Statistical analysis was run in SAS JMP 13. ANOVA with Tukey HSD for all pairs was used to compare control monitors. Multiple controls monitors worth of data (n = 24 bees each) were collected to account for potential temporal differences between monitors experiments. Control monitors showed significant variation ($t = 1.96$, $0.842 > p > 0.0001$). For this reason, baseline values were created by subtracting the appropriate minute-by-minute control from each experimental value. One-way t-tests were then employed to compare values to a hypothesized value of 0. Survival was compared to controls via Log-Rank tests.

3. Results

3.1. Aversive conditioning discrimination results

Regardless of exposure, average time spent on blue during baseline data collection was significantly less than 90 s ($77.32 < \bar{x} < 83.14$, $13.07 < sd < 21.03$), indicating a yellow bias ($-7.19 < t < -3.23$, $p < 0.0008$).

Average duration is a measure of the average amount of time (in seconds) spent by bees on the correct side (Table 1). ANOVA showed significant variation among imidacloprid trials ($F(2, 156) = 3.81$, $p = 0.024$) and post-hoc Tukey HSD showed higher baseline results from LD_{30} and LD_{50} ($p = 0.018$, Fig. 1) with neither significant from controls. The same analysis for ethion and glyphosate showed that master bees had no differences in baseline data.

Both yoked duration values for imidacloprid LD_{30} were significantly higher than baseline values (ANOVA: $F(4, 188) = 4.84$, $p = 0.001$, Tukey: $p_{blue} = 0.0062$, $p_{yellow} = 0.0022$). There were no significant differences between roles for the LD_{50} bees for imidacloprid. Glyphosate did not have significant differences between bee roles in either

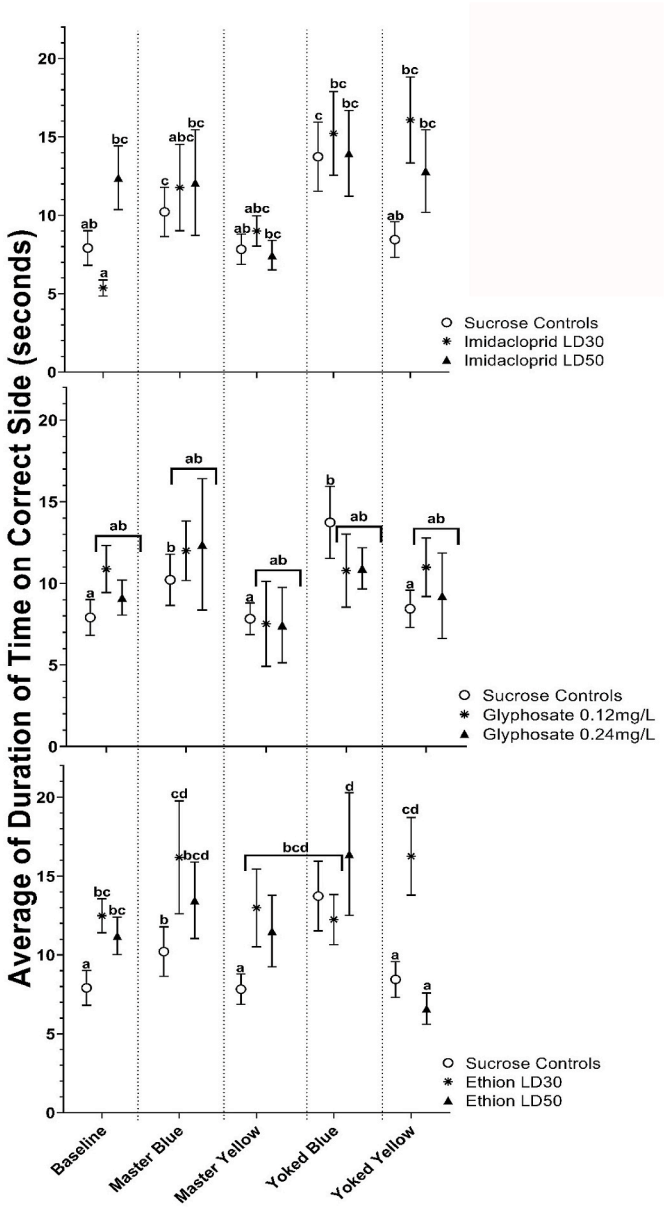

Fig. 1. Average duration of time that bees spent on the correct side after acute oral exposure with imidacloprid (LD_{30}:2 ng $bee^{-1}$and LD_{50}: 4 ng bee^{-1}), glyphosate (Low: 1.2 ng $bee^{-1}$and High: 2.4 ng bee^{-1}) or ethion (LD_{30}: 667 ng $bee^{-1}$and LD_{50}: 889.2 ng bee^{-1}) and experimental condition (±SEM). Letters represent means that are significantly different ($p < 0.05$).

concentration. Ethion LD_{30} was significantly higher than baseline for average duration on the correct side in yoked yellow (ANOVA: $F(4, 182) = 3.87$, $p = 0.0048$, Tukey: $p = 0.006$) and master blue (Tukey: $p = 0.01$), the latter is consistent with the observed yellow bias, however yoked yellow is not consistent with bias and may be a result of reduced activity. For ethion LD_{50}, the yoked blue was higher than baseline (ANOVA: $F(4,171) = 3.57$, $p = 0.0079$, Tukey: $p = 0.037$) and yoked yellow was lower than yoked blue (Tukey: $p = 0.016$), which is consistent with the observed bias.

In addition to average time spent per side, the cumulative time correct was compared between treatments (Fig. 2). For imidacloprid and ethion bees there were no significant differences within baseline metrics.

When bees were compared within treatment by concentration across bee role (master versus yoked) sucrose controls showed significant variation ($F(4,357) = 4.5$, $p = 0.0014$) with an increase in time on the

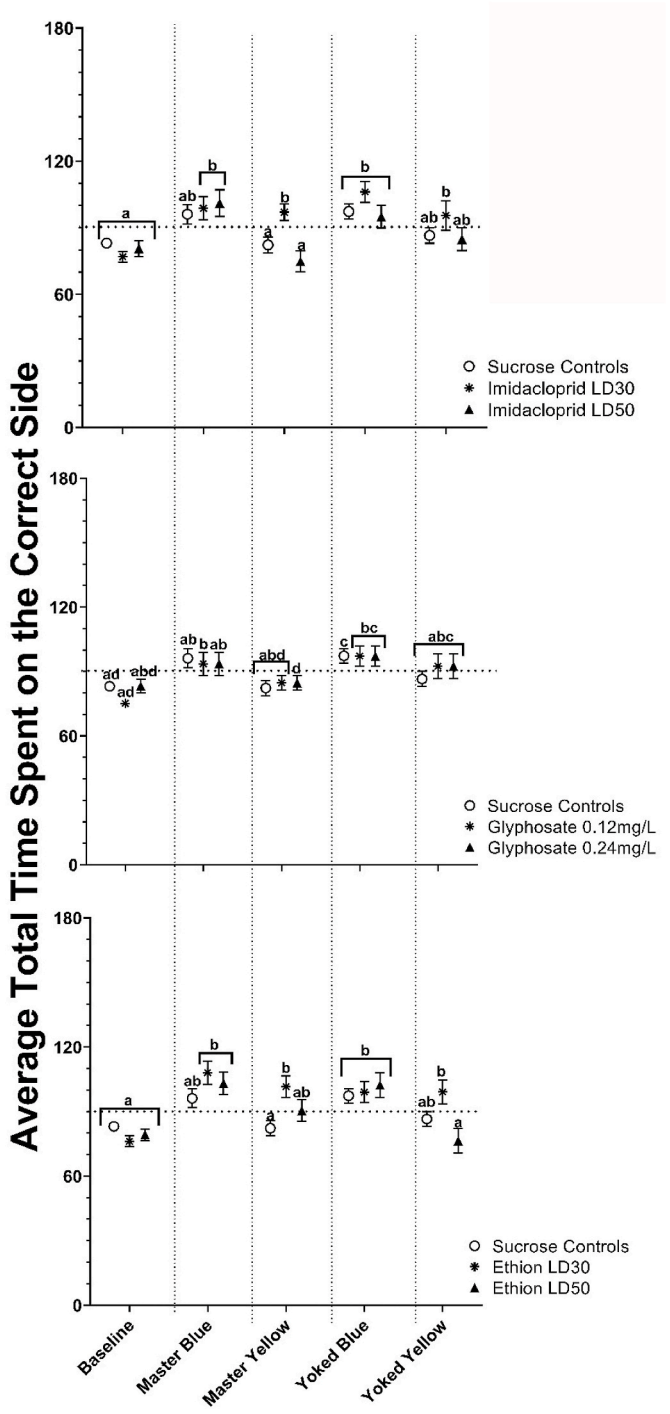

Fig. 2. Average time spent on the correct time after acute oral exposure with imidacloprid (LD_{30}:2 ng $bee^{-1}$and LD_{50}: 4 ng bee^{-1}), glyphosate (Low: 1.2 ng $bee^{-1}$and High: 2.4 ng bee^{-1}) or ethion (LD_{30}: 667 ng $bee^{-1}$and LD_{50}: 889.2 ng bee^{-1}) and experimental condition (±SEM). Letters represent means that are significantly different ($p < 0.05$).

correct side between master yellow and yoked blue ($p = 0.0085$), and yoked blue and baseline values ($p = 0.0092$), both are consistent with the yellow bias observed. Imidacloprid LD_{30} bees ($F(4,188) = 5.55$, $p = 0.0003$) were significantly higher in cumulative time correct in all phases as compared to baseline values ($p < 0.039$) but there were no other significant differences. For LD_{50} imidacloprid bees ($F(4,147) = 4.00$, $p = 0.0041$), there was a significant increase from master yellow to master blue ($p = 0.0073$), and yoked blue ($p = 0.026$) which remain consistent with yellow color bias. Similar to imidacloprid, low concentrations of glyphosate ($F(4,183) = 4.12$, $p = 0.0032$) showed significant differences from baseline with an increase to master blue values ($p = 0.03$) and yoked blue ($p = 0.0038$) and a decrease to yoked yellow ($p = 0.045$), all consistent with bias. Higher concentrations of glyphosate ($F(4,145) = 4.9$, $p = 0.001$) only showed a significant decrease in cumulative time between master blue and master yellow ($p = 0.0137$) and yoked blue and master yellow ($p = 0.0019$), consistent with bias. LD_{30} for ethion ($F(4,182) = 6.5$, $p < 0.0001$) was significantly higher than baseline across all other roles ($p_{master\ blue} < 0.001$, $p_{master\ yellow} = 0.0018$, $p_{yoked\ yellow} = 0.0055$, $p_{yoked\ blue} = 0.0077$). Whereas LD_{50} values were significantly more variable with master blue higher than baseline ($p = 0.03$) and were significant increases in time from yoked yellow to master blue ($p = 0.0029$) and yoked blue ($p = 0.0041$), consistent with bias.

3.2. Chronic exposure using the trikinetics monitors results

Temporal controls showed significant variation, likely as a result of changing season from summer to autumn ($t = 1.96$, $0.842 > p > 0.0001$). To account for changes in controls, treatment data were subtracted from control monitor data that were started within the same 4-day period. This created baseline values that are used as the comparison for each treatment. Motility, day/night activity (circadian rhythmicity), and mortality data are included in the analysis.

3.3. Rhythmicity data

3.3.1. Comparison of daytime and nighttime activity

All exposure concentrations of Roundup®, with the exception of 1.2 mg L^{-1}, reduced daytime (0600–1800h) and nighttime activity (1800-0600 h) as compared to controls (Table 2). Contrastingly, the 1.2 mg L^{-1} concentration had comparatively high daytime activity as compared to controls and drastically higher activity at night (Fig. 3, Table 2). Honey bees exposed to 6 mg L^{-1} and 24 mg L^{-1} of the active ingredient of Roundup®, glyphosate, showed significantly reduced daytime activity. Glyphosate concentrations had strong effects on nighttime activity with 0.12 mg L^{-1}, 12 mg L^{-1}, and 24 mg L^{-1} reducing activity and 1.2 mg L^{-1} increasing nighttime activity.

All exposure concentrations of imidacloprid significantly decreased daytime activity as compared to baseline; this is particularly stark during the late afternoon implying an earlier onset of nighttime reduced activity than controls. Nighttime detrimental effects of imidacloprid were also highly significant for all concentrations. The LD_{50} (106 mgL^{-1}) for ethion did not have an impact on daytime activity, however the LD_{30} (80 mgL^{-1}) significantly decreased activity during the day, as did 160 mgL^{-1}. All ethion concentrations decreased nighttime activity.

3.3.2. Average activity during captive lifespan

Average activity was averaged by individual monitor longevity to remove effects of low activity from deceased bees (Chicas-Mosier et al., 2019). Two concentrations of Roundup® significantly increased average activity, 1.2 mg L^{-1} and 6 mg L^{-1}, whereas 0.12 mg L^{-1} and 12 mg L^{-1} did not affect activity and 24 mg L^{-1} decreased average activity (Fig. 4, Table 2). For glyphosate exposure, 6 mg L^{-1} and 12 mg L^{-1} significantly increased average daily activity. All concentrations of imidacloprid significantly reduced overall daily activity. Ethion did not have an effect on overall activity when averaged by captive lifespan.

3.3.3. Mortality

Of the contaminants used in the monitor apparatus, only imidacloprid significantly reduced captive lifespan. All concentrations of imidacloprid used significantly depressed longevity as compared to controls using Log-Rank tests on survival curves (0.2 mg L^{-1} and 0.4 mg L^{-1}: $\chi^2 = 12.06$, $p = 0.0024$, 0.8 mg L^{-1}: $\chi^2 = 15.24$, $p < 0.0001$).

Table 2
Significance table for monitors activity and circadian rhythmicity data compared to zero (baseline). P-values included if 0.05* > p > 0.0001***.

Pesticide		$\frac{mg}{L}$	$\bar{x}$	Sd	df	t-value	p-value	Effect
Roundup®	**Day**	**0.12**	−403.2	101.5	12	−14.3	***	↓
		1.2	108.5	143.1	12	2.7	*	↑
							0.018	
		6	−299.9	135.4	12	−8.0	***	↓
		12	−332.4	149.6	12	−8.0	***	↓
		24	−595.4	118.9	12	−18.1	***	↓
	Night	**0.12**	−110.6	52.8	10	−7.0	***	↓
		1.2	255.4	133.7	10	8.8	***	↑
		6	−140.4	112.2	10	−4.2	***	↓
		12	−246.5	96.56	10	−8.5	***	↓
		24	−220.0	77.5	10	−9.42	***	↓
	Avg. 24hr Activity	**0.12**	**No Observed Effect**					
		1.2	24897.8	24652.1	14	3.9	**	↑
							0.0008	
		6	23482.3	14892.8	14	6.1	***	↑
		12	**No Observed Effect**					
		24	−11019.4	19427.1	13	2.1	*	↓
							0.03	
Glyphosate	**Day**	**0.12**	**No Observed Effect**					
		1.2	**No Observed Effect**					
		6	−183.7	177.3	12	−3.7	**	↓
							0.0014	
		12	**No Observed Effect**					
		24	−336.5	106.1	12	−11.4	***	↓
	Night	**0.12**	−196.4	90.8	10	−7.2	***	↓
		1.2	71.2	44.9	10	5.2	**	↑
							0.0004	
		6	**No Observed Effect**					
		12	−73.3	69.9	10	−3.5	**	↓
							0.0059	
		24	−54.0	52.8	10	−3.4	**	↓
							0.0069	
	Avg. 24h	**0.12**	**No Observed Effect**					
	Activity	**1.2**	**No Observed Effect**					
		6	11026.0	15870.6	14	2.7	*	↑
							0.018	
		12	14131.7	24080.4	14	2.3	*	↑
							0.039	
		24	**No Observed Effect**					
Imidacloprid	**Day**	**0.2**	−1138.8	351.9	12	−11.7	***	↓
		0.4	−1192.1	380.1	12	−11.3	***	↓
		0.8	−1085.5	323.0	12	−12.1	***	↓
	Night	**0.2**	−662.1	314.2	10	−7.0	***	↓
		0.4	−689.6	314.9	10	−7.3	***	↓
		0.8	−562.9	285.5	10	−6.5	***	↓
	Avg. 24h	**0.2**	−19604.3	17153.7	14	−4.4	**	↓
	Activity						**0.0006**	
		0.4	−21740.7	15465.7	14	5.4	***	↓
		0.8	−47392.2	27107.0	14	6.3	***	↓
Ethion	**Day**	**80**	−760.8	24,701	12	−11.1	***	↓
		106	**No Observed Effect**					
		160	−290.2	127.3	12	−8.2	***	↓
	Night	**80**	−442.5	249.4	10	−6.7	***	↓
		106	−268.6	106.8	10	−8.3	***	↓
		160	−143.5	106.6	10	−4.5	**	↓
							0.0006	
	Avg. 24h	**80**	**No Observed Effect**					
	Activity	**106**	**No Observed Effect**					
		160	**No Observed Effect**					

4. Discussion

There is growing concern that agrochemicals are creating stress and subsequently increasing mortality in honey bee colonies (Bernal et al., 2010). Some variants of agricultural pesticides are taken into the hive by worker bees and distributed to the young while others are used by beekeepers to treat colonies against disease and pests such as *Varroa destructor* (Mullin et al., 2010; van Engelsdorp et al., 2009). Several studies have used conditioning experiments and biochemical analysis of bees to understand toxicity of agrochemicals such as organophosphates, neonicotinoids, and miticides (Abramson et al., 2012; Al Naggar et al., 2015; Badawy et al., 2015; Bernadou et al., 2009; Colin et al., 2020; Decourtye et al., 2005; El Hassani et al., 2005). Studies have shown that there are a wide range of agricultural exposure concentrations for honey bees (Mullin et al., 2010). Therefore, in this study, two concentrations were used to investigate the effect of agricultural chemicals on learning and additional concentrations to measure chronic exposure. The present work adds to the body of literature that demonstrates the sublethal effects of pesticide exposure in honey bees. These effects are particularly relevant as reduced honey bee health is a concern for both food security and economic reliance, especially in developing nations (Fikado, 2019; Food and Agricultural Organization, 2005).

Important behavioral impacts on adult worker honey bees are the changes seen to circadian rhythms (Moore et al., 1998). Forager honey

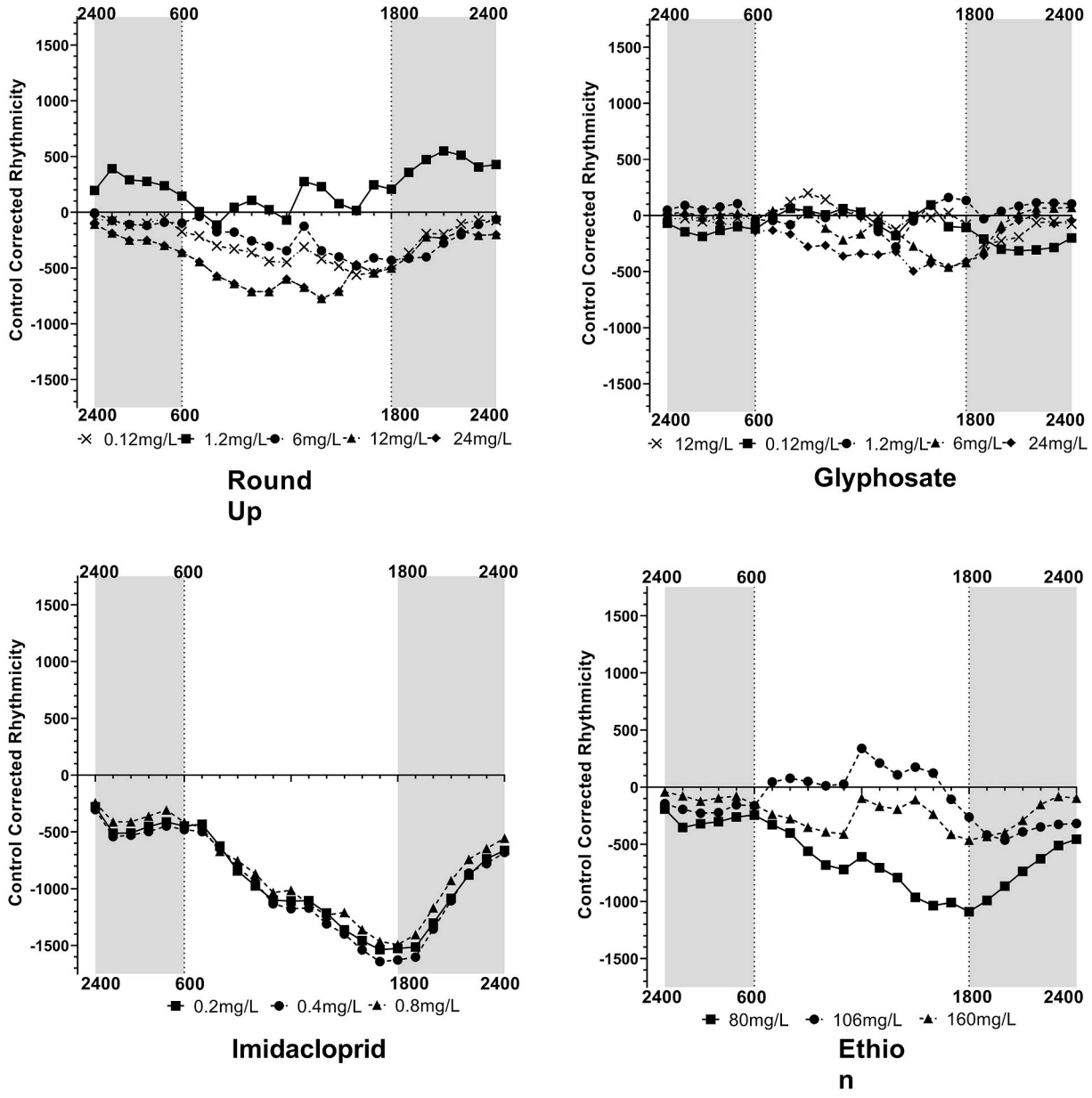

Fig. 3. Average 24-h activity by concentration. Zero line represents baseline control data. Shaded sections represent nighttime hours although bees were kept in 24-h darkness.

bees rely on accurate circadian rhythms to anticipate day–night changes in their environment and maintain foraging schedules. Timing of rest periods is one of the most important functions of the circadian rhythm as deprivation of sleep increases the expression of sleep genes the next day, and can impair learning performance (Eban-Rothschild and Bloch, 2012). Overall, the results of the monitor experiments show that honey bee ingestion of the tested agrochemicals reduced circadian rhythm adherence to a 12-h day-night schedule and that the overall mean activity decreased (except for ethion-treated bees). These results demonstrate that, despite the different modes of action for these compounds, they can have adverse long-term effects on worker bees, which can ultimately disrupt colony performance and endanger the health of the hive. Although the tested subspecies showed altered circadian rhythms, Chicas-Mosier et al. (2019), showed that toxicity may be subspecies specific and should therefore be tested with other honey bee populations. The results of these chronic dosing studies in *Apis mellifera mellifera* suggests that the agrochemicals tested may directly cause individual bee failure from prolonged exposure.

The differences that can be noted between our results and the preliminary experiments used to establish the LD_{30} and LD_{50}, include chemical sources from distribution centers in the United States rather than Iran, and the honeybee subspecies used to determine doses was native to Iran as compared to introduced subspecies in the United States. This may explain why the imidacloprid preliminary data that was used to determine the sublethal exposure concentrations in Iran (Delkash-Roudsari, unpublished results) showed higher lethality than anticipated in the present study. Additionally, previous behavioral tests included a starvation stage but in this shuttle box experiment the honey bees were fed *ad libitum* before starting the experiment. Differences in the data on honey bee motility compared to other studies may also be impacted by the operational definition of chronic toxicity as 2 weeks in the current study compared to 2-days in some prior works (Tosi and Nieh, 2017).

Previous work in aversive conditioning in honey bees has included some pesticide exposure comparisons. Similar to the shuttlebox apparatus developed by Dinges et al. (2013), the APIS-chamber allows for aversive conditioning of honey bee with odor or color stimuli (Colin

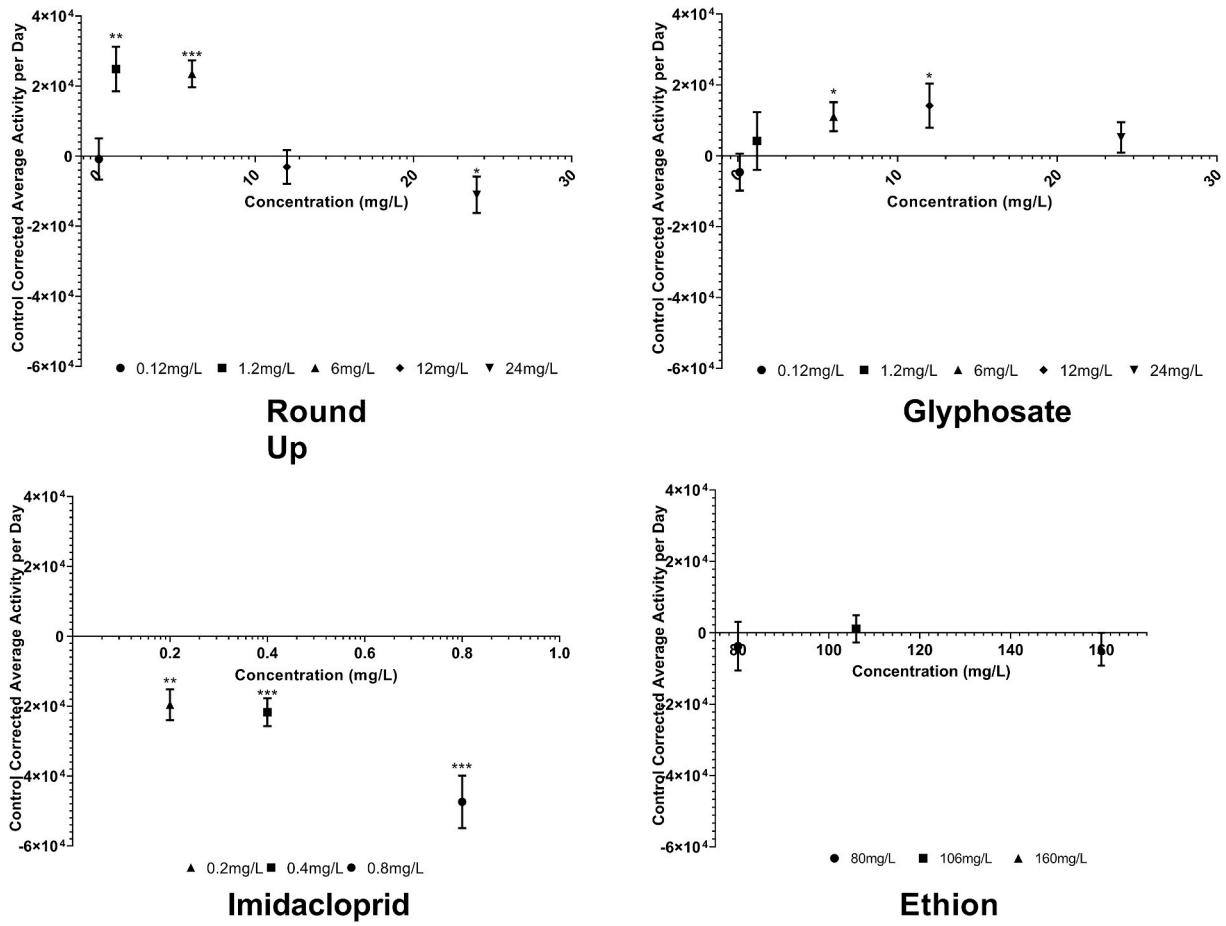

Fig. 4. Average daily activity by pesticide concentration with zero-value baselines. (±SEM). *p < 0.05, **p < 0.01, ***p < 0.001 from baseline of 0.

et al., 2020; Kirkerud et al., 2013). In an APIS experiment, imidacloprid at 5 ppb in sucrose did not reduce performance or speed of honey bees; however, when combined with the miticide thymol, performance was reduced (Colin et al., 2020). In Bartling et al. (2019), the neonicotinoid clothianidin reduced responsiveness to odorant stimuli at 300 pg/bee. In the present study, the neonicotinoid imidacloprid, caused a significant depression in movement in honey bees that likely would impact their ability to effectively forage which has also been noted in prior studies (Bortolotti et al., 2003; Decourtye et al., 2004a, 2004b; Ramirez-Romero et al., 2005). The data presented above further validate the negative impacts on aversive learning in honey bees following neonicotinoid exposure.

In addition to variation in day-night activity following most pesticide exposures, average daily activity across the captive lifespan also showed interesting results. Roundup®, the marketed formulation of glyphosate, showed a similar but markedly higher activity response than glyphosate exposed bees, indicating that the formulation increases cumulative risk to bees that the active ingredient itself does not pose, especially at low exposure concentrations. Similar data has been found in zebrafish but further research with additional species and honey bee subspecies should be conducted to fully understand the risk of formulations compared to active ingredients (Bridi et al., 2017).

In the shuttlebox experiment, a longer average duration of time was used to indicate potential impacts on bee motility and the average cumulative time as an indicator of associative learning. The data from the present experiment demonstrate that imidacloprid and ethion may impair aversive learning and reduce movement in honey bees in a short period (<20 min) with continued effects throughout the captive lifespan. Glyphosate and ethion do not seem to cause immediate learning impairment. The above experiments demonstrate that further analysis should be conducted to fully understand the effects of these pesticides following acute and chronic exposure.

Previous studies have shown that prior color experience effects color biases in bees and parasitoid wasps (Black et al., 2018; Langley et al., 2006). The aversive conditioning experiment described above showed significant yellow color bias. Von Frisch (1967) found that honey bees observe and discriminate colors when searching for food. Honey bees also can modify their flower color fidelity depending on the reward offered in the nectar but this can be limited by toxicant exposure (Amaya-Márquez et al., 2017; Black et al., 2018; Cheng and Wignall, 2006; Chicas-Mosier, 2017, 2019). Hori et al. (2006) trained bees to yellow and attributed it to a high sensitivity to the yellow wavelength, similar to the results demonstrated in the present study. There are differences in color bias between honey bee subspecies and it is possible that some subspecies are more sensitive to color stimuli than others (Abramson et al., 2008; Köppler et al., 2007). The cause of these differences is not well understood, but factors like aggression (Abramson et al., 2008), foraging habits (Cakmak et al., 2010), and available food (Black et al., 2018), may influence these biases.

The results of agrochemical studies on honeybees have been variable with some showing negative impacts on behavior (see Bortolotti et al. (2003), Goñalons and Farina (2018), Tsvetkov et al. (2017). or Yang et al. (2008), for examples), some showing minimal impacts (see Al Naggar et al. (2015) or Pilling et al. (2013) for examples), and others showing mixed results (see Decourtye et al. (2004b), Demares et al. (2016), El Hassani et al. (2005), or Ramirez-Romero et al. (2005)). The present study suggests that mixed results could be the result of impacts on specific systems (e.g. learning but not motility) and that more standardization and comparative study designs are needed to improve understanding. Standardization in exposure concentrations such as

whether they are found in pollen, nectar, or honey may also work to improve the inter-study reliability (Al Naggar et al., 2015; Ramirez-Romero et al., 2005).

In addition to changes in exposure concentration, direct comparisons are limited by whether active ingredients or formulations are used as seen here and in the literature (Bortolotti et al., 2003; Decourtye et al., 2004b; Laurino et al., 2013). In addition to route, the present study, along with others, show that duration of exposure to agrochemicals prior to behavioral testing may influence the outcome of the experiment (Abramson et al., 2004; Williamson and Wright, 2013) as well as whether the bees were fed with honey or sucrose prior to exposure (Laurino et al., 2011; Ramirez-Romero et al., 2005). Considering season, Decourtye et al. (2003) found that honey bees were more susceptible to neonicotinoids in winter than in summer exposure, this may have affected the data presented here as results reflect late season bees.

The results presented in this article suggest that exposure to imidacloprid, ethion, glyphosate, or Roundup® at the described concentrations can adversely affect forager bees, and subsequently affect colony health through contaminated pollen and nectar collection. The present study adds to the literature that demonstrates sublethal exposure effects in honey bees as well as potential variation in toxicity depending on the subspecies. Further studies should work to estimate the risk of sublethal exposures to pesticides to different subspecies of honey bee.

Credit authorship contribution statement:

Sahar Delkash-Roudsari: Conceptualization, data collection, manuscript writing. Ana M. Chicas-Mosier: Conceptualization, Formal analysis, manuscript editing. Charles I. Abramson: Conceptualization, manuscript editing. Seyed Hossein Goldansaz: preliminary study conceptualization, Khalil Telebi-Jahromi: preliminary study conceptualization, Ahmad Ashouri: preliminary study conceptualization

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This project was funded by National Science Foundation grants including the Graduate Research Fellowship Program (grant number: 1144467), Bridge to the Doctorate (grant number: HRD-1612560), and Partnership for International Research and Education (grant number: 2015–1545803). The authors would also like to recognize the Lawrence L. Boger Endowed Professorship for International Studies at Oklahoma State University. The authors would also like to thank Timothy Black for technical assistance with the shuttlebox apparatus.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2020.111108.

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