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The Behavioral Toxicity of Insect Growth Disruptors on *Apis mellifera* Queen Care

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As social insects, honey bees (*Apis mellifera*) rely on the coordinated performance of various behaviors to ensure that the needs of the colony are met. One of the most critical of these behaviors is the feeding and care of egg laying honey bee queens by non-fecund female worker attendants. These behaviors are crucial to honey bee reproduction and are known to be elicited by the queen's pheromone blend. The degree to which workers respond to this blend can vary depending on their physiological status, but little is known regarding the impacts of developmental exposure to agrochemicals on this behavior. This work investigated how exposing workers during larval development to chronic sublethal doses of insect growth disruptors affected their development time, weight, longevity, and queen pheromone responsiveness as adult worker honey bees. Exposure to the juvenile hormone analog pyriproxyfen consistently shortened the duration of pupation, and pyriproxyfen and diflubenzuron inconsistently reduced the survivorship of adult bees. Finally, pyriproxyfen and methoxyfenozide treated bees were found to be less responsive to queen pheromone relative to other treatment groups. Here, we describe these results and discuss their possible physiological underpinnings as well as their potential impacts on honey bee reproduction and colony performance.

Keywords: reproduction, pesticide, sublethal effects, pollinator, honey bee, insect growth regulator, insect hormone mimic

INTRODUCTION

Managed honey bees (*Apis mellifera*) are crucial agricultural pollinators that improve food security for growing global populations (Southwick and Southwick, 1992; Aizen et al., 2008; Calderone, 2012), and the transportation of large numbers of pollinators into agricultural fields is simplified by the social nature of honey bees, where thousands of worker bees live together in a self-contained unit (Winston, 1987). This communal living arrangement relies upon a complex social structure wherein tasks such as reproduction, rearing offspring, and gathering food are delegated by caste and by age (Hölldobler and Wilson, 2009). Like most social insects, honey bee workers exhibit temporal polyethism, or age related division of labor (Johnson, 2010). In a productive colony, older

workers leave the hive to forage, while younger workers remain in the hive and perform nursing and housekeeping tasks (Seeley, 1982). Of the many tasks typically performed by younger workers, one of the most critical is tending to the queen (Allen, 1960).

Inside a functional colony, young worker bees feed and care for a single egg-laying honey bee queen, enabling her to produce fertilized eggs that develop into the next generation of workers (Oster and Wilson, 1978). The behavior of worker bees attending to the queen, commonly referred to as queen retinue behavior, is reliant on the responsiveness of workers to the queen's nine-component pheromone blend (Robinson, 1985; Slessor et al., 1988; Kaminski et al., 1990; Keeling et al., 2003). Attraction to queen pheromone (QP) can be influenced by numerous parameters including the worker's physical health (Walton et al., 2018), queen health and physiology (Kocher et al., 2009; Rangel et al., 2016; Walsh et al., 2020), worker reproductive potential (Galbraith et al., 2015), and worker age (Allen, 1960; Pham-Delègue et al., 1993). Workers that are less responsive to QP may perform other tasks such as foraging (Pham-Delègue et al., 1993). These divisions of labor create a strong codependence between hive members, and the continued functioning of a colony is reliant on the balanced performance of these behaviors (Oster and Wilson, 1978). However, various stressors can shift the optimal balance of these divisions, resulting in a disruption to the normal processes required to sustain colony activities like reproduction (Perry et al., 2015; Booton et al., 2017; Bordier et al., 2017), which may eventually lead to colony loss.

Since 2007, beekeepers have been reporting high annual colony losses (vanEngelsdorp et al., 2007, 2008; Kulhanek et al., 2017) caused primarily by stress due to parasitic infestations (Neumann and Carreck, 2010; Steinhauer et al., 2021), pathogenic infections (Berthoud et al., 2010), poor nutrition (Leonhardt and Blüthgen, 2012; Donkersley et al., 2014), and exposure to pesticides (Sanchez-Bayo and Goka, 2014; Dively et al., 2015). These ubiquitous and interacting stressors have been shown to negatively affect various aspects of honey bee health including larval and pupal development (Wu et al., 2011; Chen et al., 2016), longevity (Wu et al., 2011; DeGrandi-Hoffman and Chen, 2015), immune function (Nazzi and Pennacchio, 2018; Harwood and Dolezal, 2020), and memory (van Dame et al., 1995; Siviter et al., 2018). Perhaps one of the more troubling and cryptic effects of these stressors relate to disruptions in the performance of important honey bee social behaviors and alterations to the expected pattern of temporal polyethism (Tasei, 2001; Thompson et al., 2007; Fine and Corby-Harris, 2021).

Changes in honey bee worker behavior and colony dynamics, which can be difficult to diagnose in field colonies (Henry et al., 2015), are known to occur in response to both abiotic and biotic stressors such as parasites (Downey et al., 2000), pathogens (Goblirsch et al., 2013; Natsopoulou et al., 2016), pesticides (Robinson, 1985; Tasei, 2001; Thompson et al., 2007), and poor nutrition (Free, 1961; Schulz et al., 1998; Mattila and Otis, 2006). Larval nutrition, which directly affects caste determination (Haydak, 1970; Leimar et al., 2012; Slater et al., 2020), and worker responsiveness to QP (Walton et al., 2018) is of particular importance in regulating temporal polyethism, and agrochemical exposure is well known to affect honey bee behavior (Robinson, 1985; Ciarlo et al., 2012; Liao et al., 2017; Colin et al., 2019). As managed pollinators, honey bees frequently encounter agrochemicals when they are applied in their foraging range or administered directly into hives to control parasite infestations (Mullin et al., 2010; Traynor et al., 2016, 2021a). Inside the colony, honey bee larvae may be exposed to agrochemical residues through the wax of their cell (Mullin et al., 2010) and potentially through diet, though the extent to which the latter occurs is debated (Böhme et al., 2018). Therefore, it is important to investigate and understand the potential effects of developmental exposure to agrochemicals on adult bees.

One of the most ubiquitous classes of agrochemicals honey bees can encounter are insect growth disruptors (IGDs) (Traynor et al., 2016; Fine and Corby-Harris, 2021), which are commonly applied in blooming almond orchards (CalDPR, 2019; Wade et al., 2019). IGDs are agrochemical pesticides that target pathways associated with insect growth and development (Pener and Dhadialla, 2012), though they are also known to impact numerous aspects of adult insect behavior including oviposition (Smagghe and Degheele, 1994; Hamaidia and Soltani, 2021), mating (Thompson et al., 2005), memory and learning (Abramson et al., 2004), and behavioral maturation (Jaycox et al., 1974; Robinson, 1985). Because IGDs exhibit low lethality to adult honey bees (Tasei, 2001), they are often applied to blooming crops where bees forage, such as almonds (Mullin et al., 2016; Wade et al., 2019; CalPIP Home - California Pesticide Information Portal). Foragers that encounter IGDs and other pesticides can return to the colony with contaminated resources (Mullin et al., 2010; Traynor et al., 2016, 2021a), resulting in a systemic exposure scenario in which all colony members including the queen and developing larvae may be at risk (Traynor et al., 2021a,b). Because of the known effects of IGDs on insect development, much consideration is given to the lethal effects of IGDs on immature pollinators (Tasei, 2001; Chen et al., 2016; Milchreit et al., 2016), and growers may attempt to select IGDs that exhibit less toxicity to honey bee brood when making an application while bees are foraging. There has been significant progress made in understanding how IGD exposure affects adult worker health and behavior (Robinson, 1985; Abramson et al., 2004; Fisher et al., 2018), however, the sublethal effects of developmental exposure to IGDs are seldom explored. Given the importance of the pathways targeted by IGDs, it is possible that adult bees exposed during development to IGDs may exhibit altered behavioral patterns as adults (Fourrier et al., 2015).

In this work, we explored the effects of sublethal doses of IGDs during honey bee development as described by Moriarty (1969). Metrics examined included the survival of immature bees, the longevity of adult bees, and their queen pheromone responsiveness. We focus on three IGDs known to be applied in flowering almond orchards while bees are foraging: the juvenile hormone (JH) analog pyriproxyfen, the ecdysone agonist methoxyfenozide, and the chitin synthesis inhibitor diflubenzuron (CalPIP Home - California Pesticide Information Portal). Here, we examine how larval exposure to these chemicals affects adult bee responsiveness to queen pheromone and discuss

implications for the continued health and performance of an affected colony.

MATERIALS AND METHODS

Honey Bees

First instar honey bee larvae were obtained from *Apis mellifera* colonies maintained according to standard commercial methods at the Harry H. Laidlaw Jr. Honey Bee Research Facility at the University of California at Davis. To best account for differences in susceptibility to IGDs due to genetic background (Crailsheim et al., 2013), three colonies were used per experimental replicate with 1 colony replaced between replicates 1 and 2 due to an accidental queen death. In total, 4 colonies, herein referred to as Colonies 1–4, with queens of Caucasian descent were used. All colonies were apparently healthy with no obvious evidence of disease visible upon inspection.

Larval Rearing

Larvae were reared according to an established protocol (Schmehl et al., 2016) following first instar eclosion and fed an artificial, royal jelly based diet. Briefly, queens from 3 colonies were caged for 24 h for each experimental replicate, and first instar larvae were grafted into queen cups (day 0) set into 48 well plates approximately 72–84 h later. Subsequently, larvae were housed inside a modified desiccator within a Heratherm^{TM} incubator (Thermo Fisher Scientific, Waltham, MA, United States) maintained at 34.5°C. Relative humidity inside the desiccator was maintained at 95% using a saturated aqueous solution of potassium sulfate. Larvae were reared on artificial diet composed of royal jelly, sugars, yeast extract, and water at different concentrations depending on larval age for 5 days after grafting and transferred to new 48 well plates lined in Kim wipes prior to pupation only after all treatment diet had been consumed. While pupating, larvae were maintained in a separate desiccator within the same incubator with relative humidity maintained at 75% using a saturated aqueous solution of sodium chloride. Because contact with the meconium is suspected to cause mortality in developing larvae (Crailsheim et al., 2013), the larvae were removed from the experiment if they were observed to have defecated prior to the transfer.

For each colony, 144 larvae were grafted onto four 48 well plates. Each of the 4 plates were randomly assigned to receive 1 of 4 dietary treatments on days 2–5 of larval development for a total of 3 plates per treatment for each biological replicate. The experiment was repeated twice (Replicates 1 and 2) during the month of June. In the second replicate, a single colony used in Replicate 1 was replaced with a new colony due to an accidental queen death.

Diet Preparation and IGD Exposure

All treatments were administered through 140 μL royal jelly based diet at chronic dosages scaled to generate sublethal effects. Methoxyfenozide was administered at 16.1 μg/mL of diet (Wade et al., 2019), diflubenzuron at 0.1674 μg/mL (Dai et al., 2018), and pyriproxyfen at 0.164 μg/mL (Fourrier et al., 2015). To prepare the diet, stock solutions were prepared by dissolving IGDs in a 1:1 acetone/methanol solution to accommodate solubility differences between diflubenzuron and methoxyfenozide and stored at −20°C when not in use. See Schmehl et al. (2016) for the recommended volumes of diet used per day. For each treatment, 0.05 mL of stock solution was added to 9.95 mL of diet and vortexed for 30 s for a final solvent concentration of 0.5%. An equivalent volume of pure solvent was added to the control diet. All dietary treatments were prepared fresh daily and warmed in an incubator set to 34.5°C for 30 min prior to their administration. The treatment period began on the second day after grafting and concluded after the final day of feeding, prior to the transfer of larvae to the pupation plates.

Mortality and Eclosion Monitoring

On day 2, prior to the treatment period, larvae that did not survive the grafting procedure were removed from the experiment. From this point until transfer to pupation plates, larval mortality was monitored daily. Dead larvae were identified by black coloration, a deflated appearance, or lack of spiracle movement (Crailsheim et al., 2013). Mortality was recorded and dead larvae were removed.

Because of previously observed mortality caused by excessive handling during pupation (Fine, unpublished), larvae were left undisturbed after transfer to pupation plates until day 9, when pupation status and survivorship were noted for each larva. Thereafter, immature bees were monitored daily for pupation, and mortality checks resumed. Dead pupae were identified by black or brown coloration or obvious lack of development. Upon discovery, failed pupations, dead pupae, and dead pre-pupa were noted and removed from their wells, which were cleaned with 10% bleach solution on a cotton swab.

Date of pupation was recorded for each bee, and beginning on day 15, each plate of pupae was checked twice daily for adults.

Adult Care

Pharate Bee Caging

Eclosing adults were weighed to the nearest tenth of a milligram using a Mettler Toledo ML104T scale (Columbus, OH, United States) and transferred to modified cup cages (Evans et al., 2009) assembled from 16oz clear plastic cups glued onto egg laying plates (ELP), keeping bees from different colonies and treatments separated. ELPs, which are described by Fine et al. (2018), consist of artificial plastic comb designed for the collection of fertilized eggs from a mated honey bee queen. In a colony, pharate adults do not exit their cells until their cuticle has sufficiently hardened (Elias-Neto et al., 2009). Bees reared using standard *in vitro* methods typically eclose in 48 or 24 well plates (Crailsheim et al., 2013; Schmehl et al., 2016), and in this work, they were transferred to cup cages immediately after they were observed to have eclosed as pharate adults. This novel *in vitro* handling practice presents pharate bees with an opportunity to move into ELP cells and complete their sclerotization process in a more natural, physically protected place.

Each cage had a 2 cm diameter hole covered with mesh on the side of the cup for ventilation and another 1 cm hole on the top through which eclosing bees and diet were added. While in use,

the top hole was covered with a piece of laboratory tape. Prior to use, 2 mL of 30% (w/w) aqueous sucrose solution and 1 mL deionized water were added to the cells of the ELP and diet was replenished daily. Because an adult bee's behavioral development and responsiveness to queen pheromone is influenced by early exposure to queen pheromone (Robinson et al., 1998; Hoover et al., 2003; Grozinger and Robinson, 2007; Vergoz et al., 2009), we added 1 queen equivalent (Qeq) of artificial 9 component QP blend, administered as 1/10th of a TempQueen (INTKO Supply, Vancouver, Canada) lure fixed to a safety pin, to all cup cages.

Pharate Bee Microbial Inoculation

Newly eclosed adult bees in a colony acquire their gut microbiome from natural comb and interactions with nurse bees, but *in vitro* reared bees do not have an opportunity to be exposed to the microbial communities that typically inhabit their digestive tract (Zheng et al., 2018). In this work, newly eclosed, *in vitro* reared bees were given a microbial inoculum prepared from the digestive tracts of nurse bees as described by Powell et al. (2014) while in cup cages. Nurse bees were collected from the callow bees' hives of origin and anesthetized by chilling at 4°C in a refrigerator for 15 min. The nurses were then dissected alive on a petri dish floating in an ice bath to reduce movement while preserving gut flora. Immediately after dissection, the entire gut of a single nurse from crop to rectum was homogenized with a pestle and added to 250 μL of 50% sucrose solution (Kwong et al., 2017). The microbial solutions of four nurse bees from a single hive were mixed and placed into one cell of each of the cup cages corresponding to the nurse bees' hive of origin in addition to the 30% sucrose solution prior to adding newly eclosed bees. The microbes were ingested and assumed to spread through the population through trophallaxis (Powell et al., 2014).

Cup cages were placed in an incubator maintained at 34 ± 0.5°C. Humidity was maintained at 75 ± 10% RH using a saturated aqueous sodium chloride solution placed on the bottom shelf of the incubator. These conditions were selected to mimic those of a healthy honey bee colony (Winston, 1987). Bees were maintained in cup cages for 12–72 h depending on the timing of eclosion, and mortality was noted daily.

Callow Bee Caging

On day 19 after larval grafting, approximately 1–3 days after adult eclosion, bees were transferred from cup cages to petri dish cages derived from Shpigler and Robinson (2015). Alterations to this design included two 1.3 cm diameter holes in the edge of the petri dish for 2 mL feeder tubes, with an additional 0.6 cm diameter hole centered between them. Petri dish cages were assembled with a wax foundation base and stood on edge with feeder holes facing up using wooden stands (see **Figure 1**). A ball of pollen supplement made with 1:1 w/w 50% sucrose solution and MegaBee pollen supplement powder weighing roughly 1.5 g was added to the bottom of each dish, and a maximum of 10 bees were added from the cup cages to each dish. Bees were kept in separate cages according to treatment and colony of origin. One Qeq of artificial QP secured onto a safety pin and tied to a length of fishing line was dangled into the cage through a feeder hole and taped so that the safety pin rested in the center of the dish. Two feeders made from 2 mL microcentrifuge tubes, one containing a 50% sucrose solution and one deionized water, were added to the dish and the smaller hole between them was taped closed.

Petri dish cages were kept inside an incubator maintained at 34 ± 0.5°C and 75 ± 10% RH. Bees remained in this incubator until they were approximately 7–9 days old, which corresponds to the period when worker bees are most responsive to queen pheromone (Kaminski et al., 1990). During this time, mortality was recorded daily and bees were fed *ad libitum*. Details on the minimum number of bees used for behavioral assessments are given in the "Statistical Analysis" section.

Queen Pheromone Response Behavior

Synthetic QP is widely used as a substitute for a mated queen in packages and temporarily queenless colonies prior to the introduction of a new queen (Naumann et al., 1990). For this reason, a nine component synthetic QP blend, which is

FIGURE 1 | Petri dish cages with *Apis mellifera* workers approximately 8 days after adult eclosion inside heated PCR cabinet. Workers were exposed during larval development to IGDs. This image was taken prior to recording worker responses to queen pheromone. Coded labels correspond to treatment and hive.

comprised of the same components as natural QP (Keeling et al., 2003), was used in this experiment in leu of QP extracted from a sacrificed queen. QP lures for observations were made by fixing 1 Qeq of a TempQueen plastic strip to a 25 cm long wooden dowel and wrapping a piece of parafilm around the dowel 5.5 cm from the QP. The parafilm skirt ensured the QP would sit in the center of the petri dish cage during observations (**Figure 1**). To increase the novelty of the stimulus and encourage bees to respond to QP, safety pins with QP were removed from the petri dish cages 24 h before beginning observations.

On day 25 after grafting, when bees were between 7 and 9 days old, petri dish cages on stands were placed in a PCR workstation (AirClean 600 PCR Workstation, AirClean Systems) with a space heater (Fan-Forced Utility Heater, Soleil, Geneva Industrial Group) to maintain a temperature of roughly 32°C. The bees were permitted to acclimate to the new environment for 30 min before observations began.

Before each recording, a QP lure was inserted through the small center hole of each petri dish cage. The lure was positioned in the cage with the TempQueen strip facing toward the lid, visible to the camera, with the back of the dowel pressed into the wax foundation for stability. Once the lures were in place, 5 min video recordings were taken for 3 petri dish cages at a time. After the recording, the lures were removed and placed in the next set of 3 cages before beginning a new recording. This process was repeated until the bees in all petri dish cages had been recorded, rotating the placement of the cages in the PCR Workstation. The cycle was then repeated two more times for a total of three 5 min videos for each petri dish cage.

The queen pheromone responsiveness of workers was assessed using a modified point sampling approach described by Rangel et al. (2016). This assessment involved counting the number of bees antennating, licking, or otherwise contacting the QP strip during 10 s intervals with 20 s between observations. For each video recording, the number of contacts observed during the entire 5 min period was summed and divided by the number of bees present in the dish.

Statistical Analysis

Kaplan Meier Survival Analysis was used to evaluate differences in immature and adult survivorship among treatment groups. Although adult bees were maintained for 6–9 days prior to the behavioral assay, the majority were 8 days old at the conclusion of the experiment. Because bees eclosing at different time points were mixed as adults, all bees were assigned an age of 2 days at the time of transfer to petri dish cages.

Separate linear mixed effects models (LMM) were constructed to assess treatment dependent differences in pupal development time and weight at adult eclosion with replicate and source colony treated as random effects. Significance of predictors was evaluated using Wald's tests. *Post hoc* comparisons between treatments were made using Tukey's HSD tests. Generalized linear models (GLM) with a binomial distribution were used for each replicate to evaluate the proportion of adult bees eclosing from each treatment group with obvious deformities for each replicate. Random effects were not incorporated in this analysis due to issues with model singularity relating to the homogeneity of the data.

Spearman's rank correlation coefficient was used to assess the relationship between the number of bees in a petri dish cage and worker QP response. A significant correlation between the number of bees in a dish and the number of QP responses per bee was observed when petri dish cages containing less than 3 bees were included in the analysis (Replicate 1: $R_s = 0.0554$, $p = 0.0440$; Replicate 2: $R_s = 0.0805$, $p = 0.0059$), therefore, only cages containing 3–10 bees were used to assess worker QP response (Replicate 1: $R_s = 0.0118$, $p = 0.6773$; Replicate 2: $R_s = -0.0308$, $p = 0.3186$). Using the censored worker QP response data, a generalized linear mixed effects model (GLMM) was constructed with QP response per bee as the response variable and treatment group as a predictor. Replicate and source colony were treated as random effects and a Poisson distribution was specified. Prior to analysis, QP response data were transformed to integers using the following equation and rounding to the nearest whole number: $(x + 1) \times 10^2$. Significance of predictors was evaluated using Wald's tests. *Post hoc* comparisons between treatments were made using Tukey's HSD tests.

For this work, all statistical analysis was performed in R Studio 1.2.5003 (Boston, MA, United States). Figures were prepared using R Studio, JMP Pro 15 and Photoshop CC 2019 (Adobe Inc., San Jose, CA, United States).

RESULTS

Development: First Instar to Adult Eclosion

Survivorship to Adult Eclosion

There was no difference in larval survivorship among treatment groups in Replicates 1 or 2 (Replicate 1: $\chi^2 = 0.4$, df = 3, $p = 1.00$; Replicate 2: $\chi^2 = 3.5$, df = 3, $p = 0.3$). Overall, average survivorship was 76.30 ± 0.51% for Replicate 1 and 73.43 ± 2.25% for Replicate 2.

Development Time

The duration of pupation was significantly affected by developmental exposure to pyriproxyfen (T = −8.986, df = 751.3, $p \leq 0.0001$) and diflubenzuron (T = 2.047, df = 752.7, $p = 0.0410$; **Figure 2**), though *post hoc* pairwise comparisons between diflubenzuron and all treatment groups indicated no significant differences. Conversely, *post hoc* pairwise comparisons revealed that pyriproxyfen treated bees experienced significantly shortened pupation periods relative to all other treatments. See **Table 1** for a summary of *post hoc* Tukey HSD tests.

External Morphology

No effect of treatment on the rate of deformities was observed (see **Table 1**). Wing deformation was the only deformity observed in eclosing bees in this study, and overall, rates of this deformity remained low among all replicates (Replicate 1 = 3.39 ± 1.27%, Replicate 2 = 0.52 ± 0.52%).

Treatment had no effect on the weight of eclosing bees (Diflubenzuron: T = 1.777, df = 745.9, $p = 0.0760$;

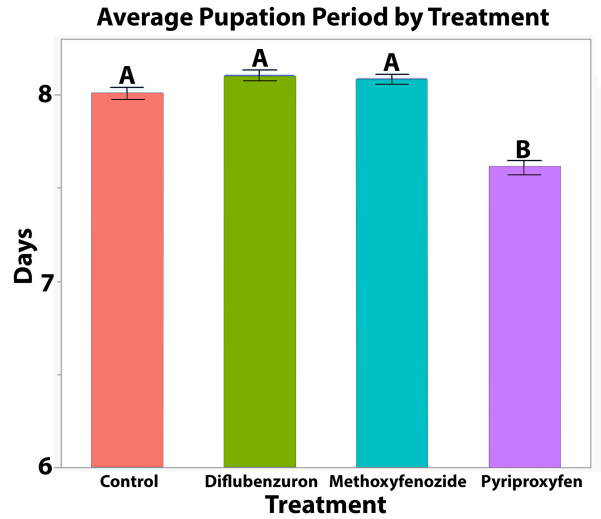

FIGURE 2 | Average ± S.E. time from first observation of successful pupal eclosion to successful adult eclosion of *Apis mellifera* workers following developmental exposure to IGDs. Significance indicated by letters (LMM, Tukey HSD, See **Table 1**).

Methoxyfenozide: T = −0.747, df = 744.3, p = 0.4551, Pyriproxyfen: T = 0.553, df = 744.6, p = 0.5807).

Adult Survivorship and Behavior

Adult Survival

Over the course of the 8 days following the final day of adult eclosion, the survivorship of adult bees in Replicate 1 was negatively affected by developmental exposure to IGD laced diet (χ^2 = 38.5, df = 3, $p \leq 0.0001$; **Figure 3**). Bees that had been developmentally exposed to pyriproxyfen exhibited the lowest rates of survival relative to all treatments (Control: $p \leq 0.0001$; Methoxyfenozide: $p \leq 0.0001$; Diflubenzuron: $p = 0.0054$), and bees developmentally exposed to diflubenzuron had lower survival relative to control ($p = 0.0054$). In Replicate 2, there was no effect of treatment on adult survivorship observed (χ^2 = 3.9, df = 3, $p = 0.3$).

Queen Pheromone Response

Developmental exposure to methoxyfenozide and pyriproxyfen had significant effects on the QP responses of worker bees (Pyriproxyfen: Z-value = −5.342, $p \leq 0.0001$; Methoxyfenozide: Z-value = −3.325, $p = 0.0009$; **Figure 4**). *Post hoc* pairwise testing between treatments showed that pyriproxyfen and methoxyfenozide treated bees responded significantly less to queen pheromone relative to all other treatments except for each other. See **Table 1** for *post hoc* test statistics and p-values.

DISCUSSION

Exposure to agrochemicals has been identified as a major contributing factor in honey bee colony losses (Goulson et al., 2015), yet the effects of sublethal agrochemical exposure during honey bee development are still not fully understood. For social insects, the performance of altruistic behaviors that contribute to the overall productivity of the colony rather than the individual is necessary to sustain the structure of the superorganism (Hamilton, 1963; Oster and Wilson, 1978; Ratnieks and Wenseleers, 2008; Ratnieks and Helanterä, 2009;

TABLE 1 | Results of statistical analysis of pupation time, QP response per bee and rate of deformities in eclosing *Apis mellifera* adults following developmental exposure to IGDs.

Measurement	Model	Treatments	T/Z Value	*P*-Value
Pupation Time	**LMM;** treatment = fixed factor, hive and replicate = random effects	Control-Methoxyfenozide	1.850	0.250
		Control-Pyriproxyfen	**−8.986**	**<0.001**
		Control-Diflubenzuron	2.047	0.171
		Methoxyfenozide-Pyriproxyfen	**−10.709**	**<0.001**
		Methoxyfenozide-Diflubenzuron	−0.218	0.996
		Pyriproxyfen-Diflubenzuron	**−10.810**	**<0.001**
Queen Pheromone Response 3–10 bees per dish	**GLMM** treatment = fixed factor, hive and replicate = random effects	**Control-Methoxyfenozide**	**−3.325**	**0.005**
		Control-Pyriproxyfen	**−5.342**	**<0.001**
		Control-Diflubenzuron	0.379	0.981
		Methoxyfenozide-Pyriproxyfen	−2.073	0.162
		Methoxyfenozide-Diflubenzuron	**−3.634**	**0.002**
		Pyriproxyfen-Diflubenzuron	**−5.580**	**<0.001**
Deformities	**GLM:** Replicate 1	Control-Methoxyfenozide	0.015	0.988
		Control-Pyriproxyfen	0.016	0.988
		Control-Diflubenzuron	0.015	0.988
	GLM: Replicate 2	Control-Methoxyfenozide	0	1
		Control-Pyriproxyfen	0.004	0.997
		Control-Diflubenzuron	0	1

Significant results indicated in bold.

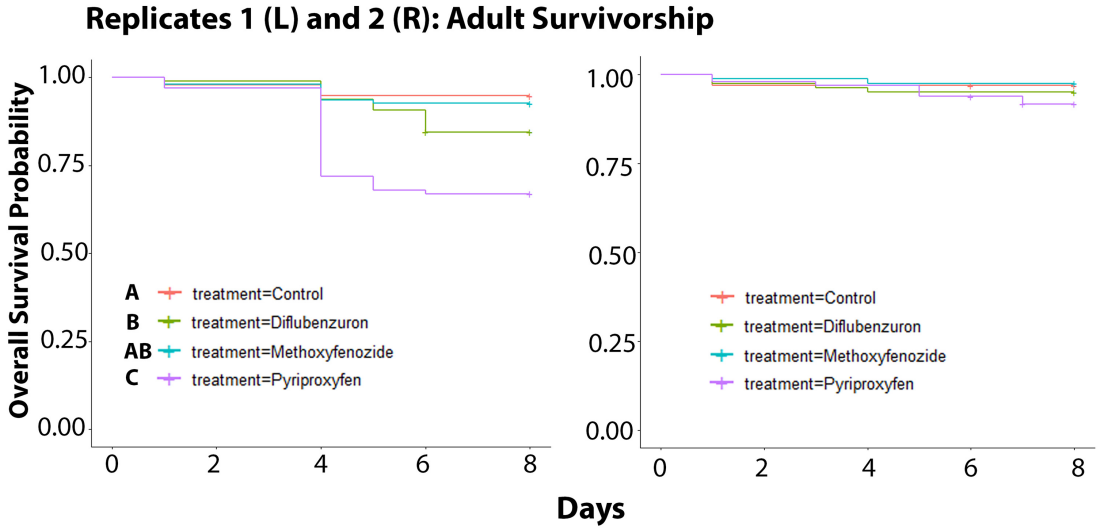

FIGURE 3 | Survivorship of adult *Apis mellifera* workers following developmental exposure to IGDs for Replicates 1 (Kaplan Meier Survival Analysis, $\chi^2 = 38.5$, df = 3, $p \leq 0.0001$) and 2 ($\chi^2 = 3.9$, df = 3, $p = 0.3$). Significance indicated by letters.

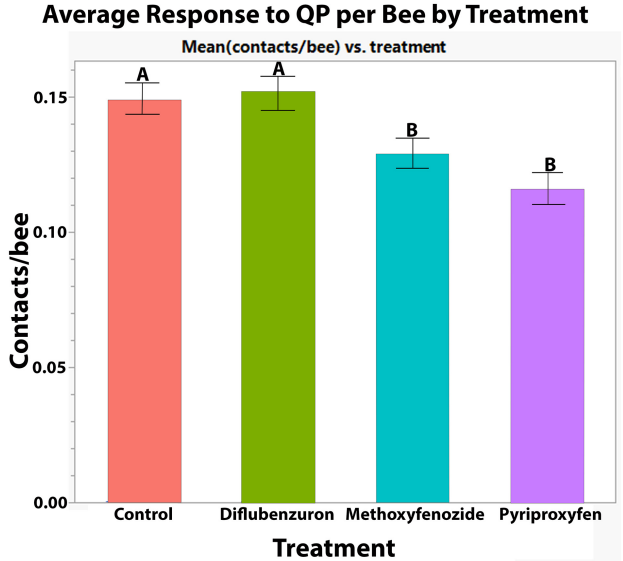

FIGURE 4 | Average ± S.E. number of contacts with QP lure per bee made by *Apis mellifera* workers in petri dish cages following developmental exposure to IGDs. Significance indicated by letters (GLMM, Tukey HSD).

Shorter and Rueppell, 2012), and any shifts in these behaviors may be deleterious to the colony unit (Perry et al., 2015). The results of this work demonstrate that developmental exposure to IGDs can influence adult survival and the performance of a social behavior that is necessary to sustain queen productivity and colony expansion (Allen, 1960).

Residues of methoxyfenozide have been reported in the pollen stores of colonies at concentrations ranging from 5.7–1820.0 ppb in 2.2% of samples (Rennich et al., 2013, 2014), and diflubenzuron has been measured at 84.3–252.0 ppb in 0.6% of samples (Rennich et al., 2013, 2014). Pyriproxyfen has been found at 1.5–277.0 ppb in 0.9% of samples (Rennich et al., 2013, 2014), though a more recent survey reported a lower range of 1–13.6 ppb in 0.76% of samples (Traynor et al., 2021a). In comparison, the doses used in our study correspond to 16100 ppb (g/mL) methoxyfenozide, 167.4 ppb diflubenzuron, and 164 ppb pyriproxyfen. Therefore, the concentrations used here are high, but such concentrations of pyriproxyfen and diflubenzuron can be found within honey bee colonies. The highest reported concentrations of methoxyfenozide, however, are nearly ten-fold lower than the concentration used here, making it unlikely that larvae would be directly exposed to the levels used in this work.

For many compounds, much lowered concentrations have been observed in larval queen diet following colony exposure (Böhme et al., 2018), and developing workers only receive a small amount of pollen in their diet in the latter days of larval development (Haydak, 1970), suggesting that larvae are at much lower exposure risk through diet relative to adults. It is not known how prevalent IGD residues are in royal jelly, but numerous studies have shown profound impacts on larvae following colony exposure, suggesting that IGDs do translocate to larval diet at appreciable concentrations, albeit when colonies are directly exposed to heavily contaminated food sources [as reviewed in Fine and Corby-Harris (2021)]. Furthermore, beekeepers frequently report heavy brood loss following almond pollination, where IGDs, along with other agrochemicals, are often applied directly to blooming crops (Pollinator Stewardship Council, 2014). Another source of IGD exposure is contaminated wax (Mullin et al., 2010), though this exposure scenario may affect larvae differently than through direct ingestion. Ultimately, more work is needed to evaluate the concentrations of IGDs in larval diet, but this

work demonstrates that IGDs, particularly those that mimic insect hormones, can affect the behavior of adult bees exposed during development.

In this work, exposure to the JH analog, pyriproxyfen, and the ecdysteroid agonist, methoxyfenozide, resulted in significantly fewer observed responses to artificial QP. Unlike diflubenzuron, which acts to inhibit chitin synthesis in developing bees (Grosscurt, 1978), hormone mimics act on the insect endocrine system to interfere with developmental processes (Williams, 1967; Hoffmann and Lorenz, 1998; Jindra and Bittova, 2020). In adult honey bees, rising JH triggers the transition from in hive tasks like nursing and queen care to riskier tasks like foraging and guarding (Robinson, 1985). Ecdysteroids are involved in oocyte maturation and embryogenesis (Bloch et al., 2002), and caged feeding studies have demonstrated that they can affect hypopharyngeal gland development (Corby-Harris et al., 2016, 2019). However, in developing bees, JH and ecdysteroids have different functions. Primarily, JH and the major ecdysteroid in honey bees, makisterone A (Feldlaufer et al., 1986) act in concert to regulate the timing of molting and development (Weir, 1970). Dramatic changes in the titers of these hormones can disrupt this process completely by delaying or accelerating molting too drastically for the insect to recover (Dhadialla et al., 1998), but natural variation in these titers, particularly in JH, can affect caste determination.

During larval development, increases in the hemolymph titers of JH cause female larvae to develop as queens rather than workers (Bloch et al., 2002). Relative to workers, queens experience shortened pupation periods and greatly increased reproductive potential as adults (Winston, 1987). In this work, pyriproxyfen treated bees consistently experienced shorter pupation periods compared to other treatments, which is suggestive of a queen intermediate state. Queen intermediates or intercaste honey bees, are understood to be physiological and behavioral hybrids between queens and workers (Beetsma, 1979) and are less likely to participate in typical worker bee tasks (Hillesheim et al., 1989; Mattila et al., 2012). Shortened development time in response to pyriproxyfen has been previously demonstrated (Bitondi et al., 1998; Elekonich et al., 2003; Fourrier et al., 2015), and Fourrier et al. (2015) found that developmental pyriproxyfen exposure resulted in decreased performance of social behaviors in adult bees. Reproductive potential is known to negatively influence QP responsiveness (Galbraith et al., 2015), which may be the cause of the reduced QP response observed after exposure to pyriproxyfen.

While the effects of sublethal methoxyfenozide exposure during development on adult bees are less well established, it has been shown that colony level exposure can result in decreased thermoregulation (Meikle et al., 2019). Here, we demonstrated that the underpinning of queen retinue behavior, QP responsiveness, can also be affected by methoxyfenozide exposure. More work is needed to explore why methoxyfenozide treatment resulted in lower responsiveness to QP, but these results suggest that, like JH, ecdysteroids and their agonists may affect the physiology and brain development of honey bees during larval development.

Beyond the reduced QP responsiveness, reductions in adult longevity were particularly pronounced for pyriproxyfen treated bees in Replicate 1, which experienced more than 25% higher mortality relative to the control group prior to the behavioral assay. Similarly, diflubenzuron treated bees experienced reduced adult survival relative to control bees. Although the exact cause of the mortality reported in Replicate 1 cannot be determined from this work, it is possible that pyriproxyfen and diflubenzuron treated bees were more vulnerable to a stressor introduced through an uncontrolled variable in this experiment. Due to a queen loss event, a new colony was introduced in the second experimental replicate. Response to stressors like pesticide exposure is known to have a heritable component (Rinkevich et al., 2015; Milone and Tarpy, 2021), though striking differences in sensitivity within the same stock are unlikely. Another possibility is that the variation was related to differences in the adult microbiome of the inoculated worker bees. The honey bee microbiome is known to significantly impact honey bee health (Zheng et al., 2017; Raymann and Moran, 2018; Vernier et al., 2020; Retschnig et al., 2021). The microbial inoculations in this work were performed to more realistically mimic colony conditions, though this practice may have exposed our bees to pathogens which are known to affect honey bee health and behaviors in unpredictable ways (Goblirsch et al., 2013; Gómez-Moracho et al., 2017; Geffre et al., 2020). Furthermore, combined stressors like pathogens and agrochemicals are known to synergize (Doublet et al., 2015; Fine et al., 2017; O'Neal et al., 2018). Therefore, while any negative effects of the microbial inoculations used in this work are purely speculative, we suggest that it may benefit future work to use a standardized microbial inoculum.

This work demonstrates that pyriproxyfen and methoxyfenozide affect the performance of a social behavior intrinsic to colony reproduction and longevity while inducing no obvious abnormalities in brood or newly eclosed adults. Stress induced changes in honey bee behavior are known to negatively affect colony dynamics and accelerate colony failure (Thompson et al., 2007; Perry et al., 2015). Until recently, the majority of studies have focused on behavioral effects of stress during insect adulthood, though developmental conditions are also known to influence physiological and behavioral characteristics of adult insects (Mousseau and Dingle, 1991; Rossiter, 1991; Fox, 1993). Like all other insects, honey bee development is hormonally regulated, and changes to larval and pupal hormone balance can result in mortality or altered physiological and behavioral phenotypes (Tunaz and Uygun, 2004; Fourrier et al., 2015; Jindra and Bittova, 2020).

This work did not investigate the reproductive potential or physiology of pyriproxyfen and methoxyfenozide treated bees, and thus, the underlying cause of the decreased responsiveness to QP cannot be definitively determined. However, it can be inferred from models examining the influence of precocious foraging on colony reproduction that if a high enough proportion of bees are unresponsive to a true, fertilized queen, colony populations would quickly dwindle due to the low number of new workers produced (Thompson et al., 2007; Perry et al., 2015). Given the importance of honey bee pollinators to global food production,

it is imperative to understand how IGDs and other stressors may influence the development of immature bees and how these changes may contribute to the success or failure of colony units.

DATA AVAILABILITY STATEMENT

Upon request, the raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

EL contributed to the experimental design, implementation, and reporting of the results with guidance from JF. JF performed statistical analysis. SC assisted in performing relevant research and in the implementation of the experiment. EL and JF wrote the final manuscript. All authors edited the manuscript.

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