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Review Article

REVIEW ARTICLE

Pesticide-induced disturbances of bee gut microbiotas

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One sentence summary: A synthesis of studies investigating interactions between pesticides, bee gut microbial communities and their hosts.

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ABSTRACT

Social bee gut microbiotas play key roles in host health and performance. Worryingly, a growing body of literature shows that pesticide exposure can disturb these microbiotas. Most studies examine changes in taxonomic composition in Western honey bee (*Apis mellifera*) gut microbiotas caused by insecticide exposure. Core bee gut microbiota taxa shift in abundance after exposure but are rarely eliminated, with declines in Bifidobacteriales and *Lactobacillus* near *melliventris* abundance being the most common shifts. Pesticide concentration, exposure duration, season and concurrent stressors all influence whether and how bee gut microbiotas are disturbed. Also, the mechanism of disturbance—i.e. whether a pesticide directly affects microbial growth or indirectly affects the microbiota by altering host health—likely affects disturbance consistency. Despite growing interest in this topic, important questions remain unanswered. Specifically, metabolic shifts in bee gut microbiotas remain largely uninvestigated, as do effects of pesticide-disturbed gut microbiotas on bee host performance. Furthermore, few bee species have been studied other than *A. mellifera*, and few herbicides and fungicides have been examined. We call for these knowledge gaps to be addressed so that we may obtain a comprehensive picture of how pesticides alter bee gut microbiotas, and of the functional consequences of these changes.

Keywords: social bees; gut microbiomes; pesticide exposure; symbiosis; host performance

INTRODUCTION

Bees provide critical pollination services for natural and agricultural plant communities. Despite their ecological and economic importance, populations of some bee species have experienced dramatic declines in recent decades (Goulson *et al.* 2015; Zattara and Aizen 2021). These declines are thought to be driven by a combination of factors, including land-use change, climate change, increases in pathogen and parasite loads, and pesticide use (Goulson *et al.* 2015).

Bees are exposed to a variety of pesticides—including herbicides, fungicides and insecticides—that have a suite of lethal and sublethal effects on their health. Bees encounter pesticides while foraging for nectar and pollen (their primary sources of sugar and protein, respectively) and transport these residues back to their nests; residues of multiple pesticides have been found in pollen and wax samples in bee hives and in bees themselves (Mullin *et al.* 2010; Traynor *et al.* 2016; Botías *et al.* 2017). In addition, bee species that nest in the ground, such as some bumble bees, stingless bees and solitary bees, may also be exposed to pesticide residues in soil while constructing and provisioning their nests (Kopit and Pitts-Singer 2018; Willis Chan *et al.* 2019). These pesticides are often lethal to bees in sufficiently high doses (Sanchez-Bayo and Goka 2014), but may also affect bee health at sublethal doses by impairing foraging, altering gene expression and caste differentiation, and decreasing weight gain, colony growth and reproductive output (Boncristiani *et al.* 2012; Garrido *et al.* 2013; Sandrock *et al.* 2014; Bernauer, Gaines-Day and Steffan 2015; Dos Santos *et al.* 2016; Lima *et al.* 2016; Chmiel *et al.* 2020).

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While a vast amount of research has been dedicated to understanding how pesticides affect bees themselves, we are just beginning to grasp how pesticides affect a key component of bee biology—their gut microbiota. Larval bees have gut microbiotas that are inconsistent in presence and composition both within individuals through time and among individuals within species (Martinson, Moy and Moran 2012; Vojvodic, Rehan and Anderson 2013; Hroncova *et al.* 2015), though the presence of some microbes has been shown to increase survivorship (Corby-Harris *et al.* 2014). Larval gut microbiotas are lost when the fore- and hindgut lining is shed during metamorphosis (Engel and Moran 2013). Adult bees therefore emerge essentially gut microbe-free, and their guts are subsequently recolonized through contact with environmental microbes and/or social interactions with conspecifics (Powell *et al.* 2014; Voulgari-Kokota *et al.* 2019). However, the associations of adult bees with their gut microbes vary widely among species. At one extreme are solitary bees, representing over 75% of the roughly 20 000 known bee species (Danforth *et al.* 2019), whose gut microbiotas are transient, compositionally inconsistent, and are composed primarily of environmental microbes (Martinson *et al.* 2011; McFrederick *et al.* 2014; Voulgari-Kokota *et al.* 2019; Cohen, McFrederick and Philpott 2020; Voulgari-Kokota, Steffan-Dewenter and Keller 2020). These microbes are not thought to be vital to their hosts' health, although much is unknown about their ecology. At the other end of this spectrum are the far less speciose eusocial corbiculate bees (748 known species; Danforth *et al.* 2019). This clade of bees contains three major lineages: honey bees (tribe Apini), bumble bees (tribe Bombini) and stingless bees (tribe Meliponini). The gut microbiotas of these bees are highly intra-specifically conserved, with specialized, host-adapted microbes transmitted among adults through social interaction (Kwong and Moran 2016; Hammer *et al.* 2021). These microbiotas are also characterized by their simple composition, consisting of five core anaerobic or microaerophilic bacterial phylotypes: *Gilliamella* spp. (Gammaproteobacteria; Orbales), *Snodgrassella alvi* (Betaproteobacteria; Neisseriales), *Bifidobacterium* spp. (Actinobacteria; Bifidobacteriales), and *Bombilactobacillus* spp. and *Lactobacillus* near *melliventris* (Firmicutes; Lactobacillales); these last two phylotypes were formerly known as *Lactobacillus* Firm-4 and *Lactobacillus* Firm-5, respectively, and are referred to collectively in this review as the '*Lactobacillus* cluster'. Honey bees tend to have all five core phylotypes present along with *Bartonella apis* and *Frischella perrara*, while many bumble bee species seem to have lost the *Bombilactobacillus* phylotype and gained symbionts from the genera *Bombiscardovia* and *Schmidhempelia* (Kwong *et al.* 2017); stingless bee species vary widely in the core phylotypes they have maintained (Kwong *et al.* 2017). Unlike those of solitary bees, social bee gut microbiotas are known to benefit host health. The best-documented benefits are defense against pathogens and parasites (Koch and Schmid-Hempel 2011; Cariveau *et al.* 2014; Palmer-Young, Raffel and McFrederick 2019; Miller, Smith and Newton 2021) and stimulation of the host immune system (Kwong, Mancenido and Moran 2017; Steele *et al.* 2017; Horak, Leonard and Moran 2020), but these microbial communities also promote host weight gain (Zheng *et al.* 2017), stimulate expression of host detoxification genes (Wu *et al.* 2020a) and may play additional roles in metal detoxification and nutrient processing (Engel, Martinson and Moran 2012; Zheng *et al.* 2017; Lee *et al.* 2018; Rothman *et al.* 2019; Zheng *et al.* 2019; Kešnerová *et al.* 2020), although these final two areas require further investigation.

Social bee microbiotas clearly play a key role in host physiology; thus, it is important to understand how they are affected by pesticide exposure. Beginning in the 1970s and intensifying in the past decade, researchers have conducted studies to determine whether and to what extent pesticides can disturb (i.e. alter the community composition of) bee gut microbiotas and whether these disturbances have negative effects on host health and performance. In this review, we summarize literature investigating the effects of pesticide exposure on bee gut microbial communities, examining researchers' choices of study taxa, pesticides and methods, and synthesizing their results. We also highlight gaps in this area of research that remain to be thoroughly investigated and provide recommendations for future studies.

Gut microbial communities of adult social bees are not only much better characterized than those of solitary and larval bees but they also appear to have a greater influence on host health and performance. As such, it is unsurprising that studies investigating the effects of pesticide exposure on bee gut microbiotas have focused on the adult stage of social corbiculate bees and, therefore, so shall this review.

METHODS FOR INVESTIGATING PESTICIDE-INDUCED DISTURBANCE OF BEE GUT MICROBIOTAS

General experimental design

Experiments that explore the effects of pesticides on bee gut microbiotas are generally designed in the following manner: (i) they are carried out in-lab with groups of adult bees (isolated from the colony) as the experimental replicates, (ii) replicates are grouped into two types of treatments—pesticide-exposed and pesticide-naïve (the exact number of treatment groups depends on the number of pesticides and concentrations tested), (iii) bees in the pesticide-naïve group consume sterile pollen and a sterile sugar solution, while bees in the pesticide-exposed group consume sterile pollen and a sterile sugar solution containing the pesticide for a determined duration, (iv) at some point during or after pesticide exposure, bees are euthanized and (v) changes in microbial presence and/or abundance are examined, usually via 16S rRNA gene amplicon sequencing or quantitative polymerase chain reaction (qPCR) (Table S1, Supporting Information). However, some studies deviate from this general framework. For example, some researchers expose bees to pesticides at the hive level (e.g. Kakumanu *et al.* 2016), some conduct their experiments in the field, allowing bees to freely forage on crops or wildflowers (e.g. Gilliam and Morton 1974; Motta *et al.* 2020), and others expose bee hives to pesticides by placing them next to pesticide-treated crops (e.g. Jones *et al.* 2017; Wintermantel *et al.* 2018).

Host taxa choice

To date, the effects of pesticide exposure on bee gut microbiotas have been examined in only five bee species: *Apis mellifera*, *A. cerana*, *Bombus terrestris*, *B. impatiens* and *Partamona helleri*. Studies on *A. mellifera*, the Western honey bee, vastly outnumber the rest (Fig. 1A), but only eight of 748 social corbiculate bee species are honey bees (genus *Apis*) (Danforth *et al.* 2019). Approximately one-third of the remaining species are bumble bees (*Bombus* spp.) and most are stingless bees (Meliponini, the tribe that includes *Partamona*) (Fig. 1B); both of these taxa are major contributors to pollination of both agricultural and natural plant communities (Ishimatsu *et al.* 1989; Greenleaf and Kremen 2006; Slaa *et al.* 2006; Winfree *et al.* 2007; Garibaldi *et al.*

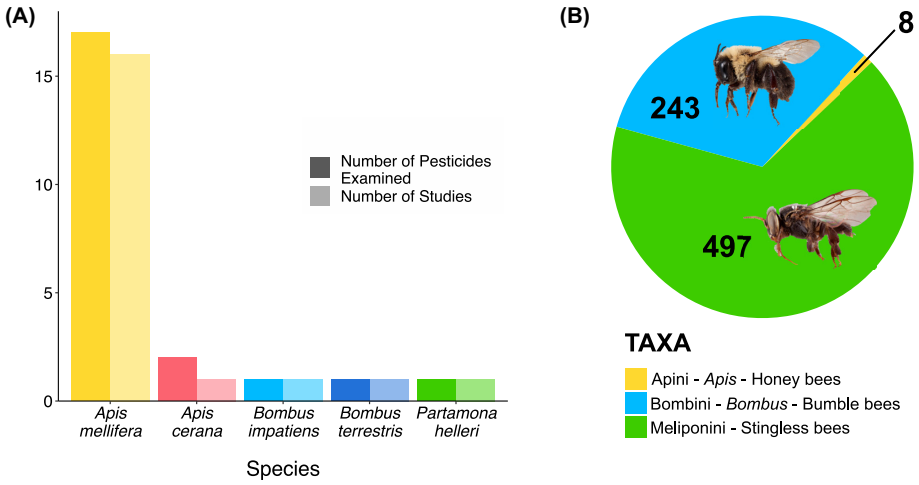

Figure 1. (A) The number of pesticides examined in each host bee species and the number of modern studies (i.e. published 2016 onward) conducted using each host bee species. **(B)** The proportion of social apid bees belonging to each taxon (tribe). Numbers represent the number of species in each taxon based on data from *Discover Life* (Ascher and Pickering 2020). Photos of *Bombus impatiens* and *Partamona helleri* from Dr Laurence Packer (https://www.yorku.ca/bugsrus/resources/resources). Bees are not to scale.

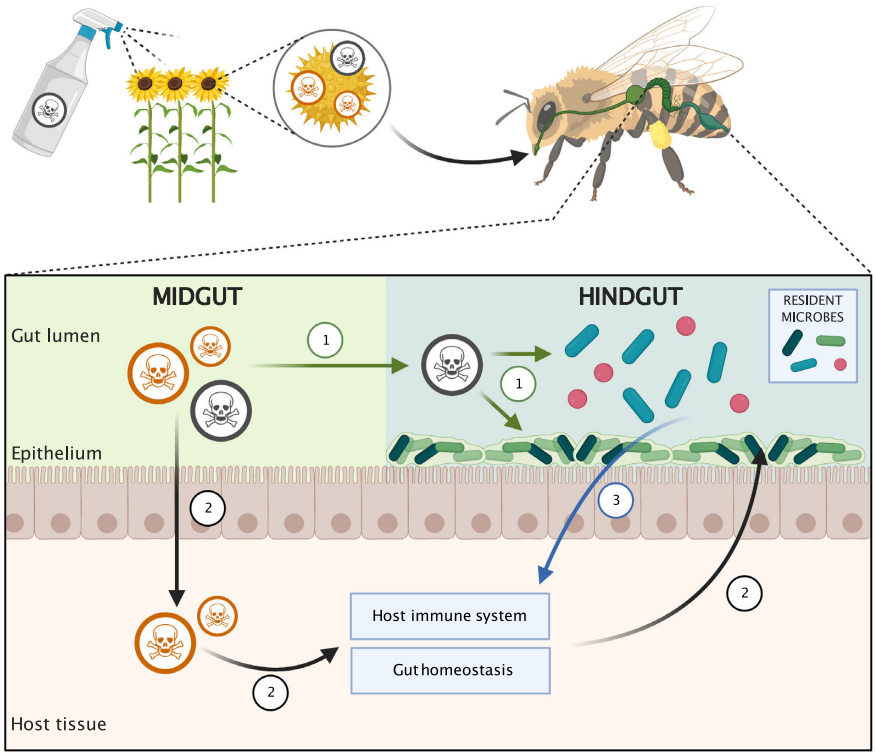

Figure 2. Interactions between pesticides, the bee gut microbiota and the bee host. Bees consume pollen and nectar that may contain pesticide residues. Once pesticide residues have entered the bee host, two main routes of pesticide-induced disturbance exist: (1) Pesticides directly affect the growth of resident microbes, most of which are found in bee hindguts, and (2) Pesticides are absorbed in the host midgut and affect host health, for example by lowering immune defenses or disturbing gut homeostasis; these changes in turn cause the host to lose its capacity to regulate its gut microbiota and the community becomes disturbed. The disturbed gut microbiota then (3) has further impacts on host health, affecting aspects of host performance such as immune system function, pathogen defense, and the gut environment. Created with BioRender.com.

Table 1. Summary of recent studies on pesticide–bee gut microbiota interactions. INSECT = insecticide; FUNG = fungicide; HERB = herbicide. *Lactobacillus* n. *m.* = *Lactobacillus* near *melliventris*. Further details are provided in Table S1 (Supporting Information).

Pesticide	Pesticide type	Host species[a]	Dose ($\mu g\ L^{-1}$)[b]	Exposure duration	Methods[c]	Significantly affected core taxa	Study
Amitraz	INSECT	*Apis mellifera*	1000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
		Apis cerana	1000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
Carbaryl powder	INSECT	*Apis mellifera*	250 g of active ingredient per hectare	2 hours	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	Present only in exposed: *Snodgrassella alvi*	Nogrado *et al.* 2019
Chlorpyrifos	INSECT	*Apis mellifera*	1000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
		Apis cerana	1000	15 and 30 days	16S rRNA gene amp. seq.	None	Yang *et al.* 2019
Clothianidin	INSECT	*Bombus terrestris*	Hives placed along fields with clothianidin-seed-treated crops	21–38 days	qPCR w/ core taxa-specific primers	None	Wintermantel *et al.* 2018
Coumaphos	INSECT	*Apis mellifera*	Two strips in hive	6 weeks	16S rRNA gene amp. seq., PICRUSt	↑ Bifidobacteriales	Kakumanu *et al.* 2016
		Apis mellifera	650 $\mu g\ kg^{-1}$ (pesticide/syrup syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↓ *Lactobacillus* cluster ↓ *Lactobacillus* n. *m.*	Rouzé *et al.* 2019
Fipronil	INSECT	*Apis mellifera*	0.25 $\mu g\ kg^{-1}$ 1.0 $\mu g\ kg^{-1}$ (pesticide/sugar syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↑ *Gilliamella apicola* ↓ *Lactobacillus* cluster ↓ *Lactobacillus* n. *m.* ↑ *Snodgrassella alvi*	Rouzé *et al.* 2019
		Apis mellifera	0.5	17 days	16S rRNA gene amp. seq., active profiling w/ 16S rRNA transcript seq.	When co-exposed to *Nosema ceranae*: ↑ Gammaproteobacteria (Orbales)	Paris *et al.* 2020
Imidacloprid	INSECT	*Apis mellifera*	500	3 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	None	Raymann *et al.* 2018
		In vitro, strains from *Apis mellifera*	1 000 000	72 hours	Single-strain and full gut toxicity assays	None	Raymann *et al.* 2018
		Apis mellifera	0.7	One feeding	Fatty acid profiles	↓ Gram-positive fatty acid biomarkers ↓ Gram-negative fatty acid biomarkers	Diaz *et al.* 2019[d]
		In vitro, strains from *Apis mellifera*	0.7	72 hours	Single-strain toxicity assays	None	Diaz *et al.* 2019[d]
		Apis mellifera	3.5 $\mu g\ kg^{-1}$ (pesticide/sugar syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↓ *Lactobacillus* cluster ↓ *Lactobacillus* n. *m.*	Rouzé *et al.* 2019
		Bombus impatiens	1	4 days	16S rRNA gene amp. seq.	None	Rothman *et al.* 2020
Nitenpyram	INSECT	*Apis mellifera*	300	14 days	16S rRNA gene amp. seq.	↑ Bacilli ↑ Betaproteobacteria ↑ *Bifidobacterium* spp. ↓ Gammaproteobacteria ↓ *Gilliamella* spp. ↓ *Gilliamella apicola* ↑ *Lactobacillus* n. *m.* ↓ Orbales ↓ Orbaceae	Zhu *et al.* 2020

Table 1. Continued

Pesticide	Pesticide type	Host species[a]	Dose ($\mu g\ L^{-1}$)[b]	Exposure duration	Methods[c]	Significantly affected core taxa	Study
Spinosad	INSECT	*Partamona helleri*	810	24 hours	16S rRNA gene amp. seq.	↑ *Gilliamella apicola*	Botina *et al.* 2019
Tau-fluvalinate	INSECT	*Apis mellifera*	Two strips in hive	6 weeks	16S rRNA gene amp. seq., PICRUSt	None	Kakumanu *et al.* 2016
Thiacloprid	INSECT	*Apis mellifera*	200 600 2000	13 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Lactobacillus* n. *m.*	Liu *et al.* 2020
Thiamethoxam	INSECT	*Apis mellifera*	Hives placed adjacent to crops treated with pesticide	Hives placed in April, sampled after peak flowering	16S rRNA gene amp. seq.	↑ Lactobacillales ↓ Proteobacteria	Jones *et al.* 2017
		Apis mellifera	1.7 $\mu g\ kg^{-1}$ (pesticide/sugar syrup)	18 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Bifidobacterium* spp. ↑ *Gilliamella apicola* ↓ *Lactobacillus* cluster ↓ *Lactobacillus* n. *m.*	Rouzé *et al.* 2019
		Apis mellifera	1.5	17 days	16S rRNA gene amp. seq., active profiling w/ 16S rRNA transcript seq.	When co-exposed to *Nosema ceranae*: ↑ Gammaproteobacteria (Orbales)	Paris *et al.* 2020
Boscalid	FUNG	*Apis mellifera*	100	17 days	16S rRNA gene amp. seq., active profiling w/ 16S rRNA transcript seq.	When co-exposed to *Nosema ceranae*: ↑ Gammaproteobacteria (Orbales)	Paris *et al.* 2020
Boscalid/ pyraclostrobin	FUNG	*Apis mellifera*	1990 11410	21 days	16S rRNA gene amp. seq.	↓ *Gilliamella* spp. ↑ *Bombilactobacillus* ↑ *Lactobacillus* n. *m.*	DeGrandi-Hoffman *et al.* 2017
Chlorothalonil	FUNG	*Apis mellifera*	10	6 weeks	16S rRNA gene amp. seq., PICRUSt	↓ Lactobacillales	Kakumanu *et al.* 2016
Glyphosate	HERB	*Apis mellifera* (exposed as larvae)	800 4000 20 000	4 days	16S rRNA gene amp. seq.	↑ Gammaproteobacteria	Dai *et al.* 2018
		Apis mellifera	5000 10000	5 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Bombilactobacillus* ↓ *Lactobacillus* n. *m.* ↓ *Snodgrassella alvi*	Motta *et al.* 2018
		Apis mellifera	5000 10000	5 days	qPCR w/ gen. bacteria primers and core taxa-specific primers	↑ *Lactobacillus* n. *m.* ↓ *Snodgrassella alvi*	Motta *et al.* 2018
		Apis mellifera	169070 (1 mM)	2 days (with 2 days in between)	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↑ *Bombilactobacillus* ↓ *Snodgrassella alvi*	Motta *et al.* 2018
		Apis mellifera	16907 (0.1 mM)	5 days	qPCR w/ gen. bacteria primers and core taxa-specific primers, active profiling via 16S rRNA transcript seq.	↓ *Snodgrassella alvi*	Motta *et al.* 2018
		Apis mellifera	16907 (0.1 mM)	5 days	qPCR w/ *S. alvi*- specific primers	↓ growth of: *Snodgrassella alvi* strains	Motta *et al.* 2018
		In vitro, strains isolated from *Bombus* and *Apis* spp.	1690700 (10 mM)	48 hours	Single-strain toxicity assays	↓ growth of: *Gilliamella apicola* strains *Snodgrassella alvi* strains	Motta *et al.* 2018
		Apis mellifera	253 605 (1.5 mM) 1 268 025 (7.5 mM)	15 days	qPCR w/ gen. bacteria and core taxa-specific primers	↓ *Gilliamella apicola* ↑ *Lactobacillus* cluster ↑ *Lactobacillus* n. *m.* ↓ *Snodgrassella alvi*	Blot *et al.* 2019
		In vitro, strains isolated from *Apis mellifera*	84 535 (0.5 mM) 253 605 (1.5 mM) 845 350 (5 mM) 2 536 050 (15 mM)	48 hours	Single-strain toxicity assays	↓ growth of: *Bifidobacterium* strains *Gilliamella apicola* strains *Lactobacillus* cluster strains *Snodgrassella alvi* strains	Blot *et al.* 2019

Table 1. Continued

Pesticide	Pesticide type	Host species[a]	Dose ($\mu g\ L^{-1}$)[b]	Exposure duration	Methods[c]	Significantly affected core taxa	Study
		Apis mellifera	169 070 (1 mM) 169 070 (1 mM in Roundup)	5 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Gilliamella* spp. ↓ *Snodgrassella* spp.	Motta *et al.* 2020
		Apis mellifera	0.1% Roundup	3 days	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Gilliamella* spp. ↓ *Lactobacillus* n. *m.* ↓ *Snodgrassella* spp.	Motta *et al.* 2020
		Apis mellifera	0.1% Roundup	Four exposures each one week apart	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Bifidobacterium* spp. ↓ *Gilliamella* spp. ↓ *Lactobacillus* n. *m.* ↓ *Snodgrassella* spp.	Motta *et al.* 2020
		Apis mellifera	0.001% Roundup 0.1% Roundup	Four exposures each one week apart	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↓ *Snodgrassella* spp.	Motta *et al.* 2020
		Apis mellifera	0.001% Roundup 0.1% Roundup	One or two exposures	16S rRNA gene amp. seq., qPCR w/ gen. bacteria primers	↑ *Bombilactobacillus* ↓ *Snodgrassella* spp.	Motta *et al.* 2020
		Apis mellifera	Topical exposure: 0.05% Roundup 0.1% Roundup 0.5% Roundup 1.0% Roundup 3.0% Roundup	One spray of ~1.2mL	qPCR w/ *S. alvi*-specific primers	↓ *Snodgrassella* spp. (but only in one of two experiments)	Motta *et al.* 2020
		Apis mellifera	1 690.7 (0.01 mM) 6762.8 (0.04 mM) 11 834.9 (0.07 mM) 16 907 (0.1 mM) 169 070 (1 mM)	15 and 20 days	Active profiling w/ 16S rRNA transcript seq., qPCR w/cDNA	↓ *Snodgrassella alvi*	Motta and Moran 2020
Amino-methylphos-phonic acid (AMPA) (Glyphosate metabolite)	HERB	*Apis mellifera*	166 560 (1.5 mM) 832 800 (7.5 mM)	15 days	qPCR w/ gen. bacteria and core taxa-specific primers	None	Blot *et al.* 2019
		In vitro, strains isolated from *Apis mellifera*	55 520 (0.5 mM) 166 560 (1.5 mM) 555 200 (5 mM) 1 665 600 (15 mM)	48 hours	Single-strain toxicity assays	↓ *Gilliamella apicola*	Blot *et al.* 2019

[a]Workers were used unless otherwise indicated.

[b]Dose is the concentration of the pesticide in sugar syrup or pollen provided to bees unless otherwise indicated; units are $\mu g\ L^{-1}$ unless otherwise indicated.

[c]Amp. = amplicon; seq. = sequencing; gen. = general.

[d]Menthol, oxalic acid and thymol were not included from Diaz *et al.* (2019) as they are primarily marketed as disinfectants, not pesticides.

2013; Pérez-Méndez *et al.* 2020). *Apis* bees thus represent <1% of known social corbiculate bee species, yet one species from this genus is the sole chosen host for 15 of the 19 modern studies on pesticide–bee gut microbiota interactions (Table 1).

Reasons for this large imbalance in host species choice likely include that (i) *A. mellifera* is the most widely managed crop pollinator globally (because of our great dependence on this species for agricultural pollination and honey production, there is great interest in its health); (ii) the species is amenable to research (methods and tools for colony maintenance in both field and lab settings are well developed and colonies are enormous, with workers numbering in the tens of thousands); (iii) the gut microbiota of *A. mellifera* workers is the best-characterized of any bee species (Kwong and Moran 2016), providing a strong foundation for studies on pesticide-induced disturbance; and (iv) *A. mellifera* is widely recognized as a model organism for gut microbiota research in general due to the low diversity of its gut microbial community (Zheng *et al.* 2018).

While the rationale for choosing *A. mellifera* as the host species for studies on pesticide-induced disturbance of bee gut microbiotas is sound, there are differences among the gut microbiotas and ecologies of social corbiculate bee species that may cause variation in how their microbiotas respond to pesticide exposure. To begin, when comparing core phylotypes across social bee gut microbiotas, overall strain diversity is higher in *A. mellifera* than in other species of honey or bumble bees (Powell, Ratnayeke and Moran 2016; Ellegaard *et al.* 2020). In accordance with the species-area relationship in ecology (Connor and McCoy 1979), strain diversity in social bee gut microbiotas is thought to be positively associated with colony size (Powell, Ratnayeke and Moran 2016; Kwong *et al.* 2017; Ellegaard *et al.* 2020). As *A. mellifera* colonies are orders of magnitude larger than those of other bee species, even those within the same genus (*A. mellifera*: 50 000+ individuals; *A. cerana*: 5000+ individuals; *Bombus* spp.: 200+ individuals; Meliponini: 100–8000+ individuals; Wille 1983), their gut microbiotas are expected to be more diverse. Additionally, all *Apis* species form new colonies via colony fission in which a queen takes about half of the workers (and all of their resident gut microbes) from her natal colony with her when she leaves to establish a new colony. In contrast, new queens of other social bee species, like bumble bees, establish new colonies alone, creating a genetic bottleneck for their resident microbes. Colony establishment in bumble bees also occurs after hibernation, a period during which body temperature drastically lowers and food consumption ceases, greatly disturbing core gut microbial taxa (Bosmans *et al.* 2018). The higher gut microbial diversity maintained by *A. mellifera* may be beneficial if different strains of the same phylotype are functionally redundant but vary in their resistance to different environmental stressors. For example, Motta *et al.* (2018) demonstrated that the growth of some *S. alvi* strains—but not others—is inhibited by glyphosate exposure. As stressors change over the lifetime of an individual bee or a whole colony, different strains may become temporarily dominant, yet the overall functioning of the microbial community could be maintained.

Honey bees also differ from other social corbiculate bees in sensitivity to pesticide exposure. The median lethal doses of pesticides for honey bees are generally lower than those for bumble bees and higher than those for stingless bees (perhaps owing to the size differences among these taxa, since doses are often measured in μg bee^{-1}), although stingless bees show an immense range of interspecific variation (Arena and Sgolastra 2014; Sanchez-Bayo and Goka 2014). One of the ways that pesticides disturb gut microbiotas is by impairing host health to the point that the host is unable to properly regulate its gut microbiota, leading to dysbiosis (Box 1; Fig. 2) (Yuan *et al.* 2019). If social corbiculate bee species differ in their pesticide sensitivities, the doses of pesticides and durations of exposure that cause gut microbiota dysbiosis via a decline in host health likely vary as well.

BOX 1:
MECHANISMS OF PESTICIDE-INDUCED DISTURBANCE

There are two principal ways that pesticides can disturb gut microbiotas: (i) by directly affecting the growth of the microbes themselves, and (ii) by affecting the host's ability to regulate its gut microbial community, for example by impairing host health or altering the gut environment (Daisley *et al.* 2020) (Fig. 2).

Pesticides can both promote and inhibit microbial growth. Some microbes, including those found in the guts of insects routinely exposed to pesticides, are able to use certain pesticides as carbon and energy sources to support growth (Russell *et al.* 2011). However, pesticides can also be toxic to microbes—for example, by damaging genetic material, causing structural damage or inhibiting metabolic pathways (De Flora *et al.* 1984; Staley, Harwood and Rohr 2015; Muturi *et al.* 2017; Shahid *et al.* 2019). Glyphosate is one such pesticide that negatively affects the growth of the core bee gut microbe *S. alvi* through inhibition of the shikimate pathway and thereby synthesis of aromatic amino acids (Motta, Raymann and Moran 2018). As demonstrated by Motta *et al.* (2018), testing the growth of microbes in the presence of pesticides *in vitro* can help confirm whether pesticides exert direct effects on gut microbes.

In addition to direct effects on gut microbiotas, pesticides may also impair host health in ways that lead to perturbations in the gut microbiota. The immune system has been shown to play important roles in regulating both vertebrate and invertebrate gut microbiotas (Hooper, Littman and Macpherson 2012; Nyholm and Graf 2012; Engel and Moran 2013). Exposure to a wide array of pesticides alters immune system function in insects (James and Xu 2012), including bees (Boncristiani *et al.* 2012; Di Prisco *et al.* 2013; Aufauvre *et al.* 2014; Brandt *et al.* 2016, 2017), which may impair host regulation of the gut microbiota and consequently alter community structure. Pesticide exposure can also alter physical and physiochemical conditions in the midgut of bees (Catae *et al.* 2018; Araujo *et al.* 2019; Carneiro *et al.* 2020); if similar changes occur in the hindgut, the principal location of bee gut microbes, they may further contribute to shifts in gut microbiota structure. This indirect route of gut microbiota disturbance has been hypothesized to be responsible for disturbances caused by some insecticides. Rouzé *et al.* (2019) found that *in vivo* exposure to four different neurotoxic insecticides decreased the abundance of both *Lactobacillus* cluster and *Bifidobacterium* bacteria in honey bee guts. One proposed explanation for these similarities was that the pesticides disturb bee gut microbiotas through aspecific changes in gut homeostasis rather than direct effects on microbial growth.

It is important to note that these two mechanisms are not mutually exclusive: a pesticide may directly affect the growth of some microbial taxa while also impairing host health, which in turn further disturbs the gut microbiota.

Ultimately, *A. mellifera* is but one of hundreds of social corbiculate bee species whose ecologies and physiologies are sufficiently varied that results from studies investigating pesticide-induced gut microbiota disruption in one species are unlikely to apply to all others. Future research on pesticide–bee gut microbiota interactions should endeavor to broaden the choice of host taxa to adequately reflect the diversity of social bees, though studying species that are not managed for agricultural pollination or honey production will present a significant challenge.

Pesticide choice

In total, 29 pesticides, pesticide formulations, and pesticide metabolites have been examined for their effects on bee gut microbiotas. Eighteen of these compounds have been the focus of recent studies (Fig. 3), and 14 have been shown to affect bee gut microbiota structure *in vivo* (Table 1). The most-studied of these compounds, by far, is the herbicide glyphosate: experiments have examined the effects of the pure compound, a major metabolite, and a commercial formulation on honey bee gut microbiotas in lab and field settings (Blot *et al.* 2019; Motta and Moran 2020; Motta *et al.* 2020), as well as the mechanism of disturbance (Motta, Raymann and Moran 2018; Motta *et al.* 2020), and the disturbance's effect on host performance (Motta, Raymann and Moran 2018). No insecticidal or fungicidal compounds have been investigated in comparable depth, even those that have been examined in multiple studies such as imidacloprid and thiamethoxam. To date, few commercial insecticidal formulations have been tested, some insecticides and fungicides have only been examined in-lab, and the mechanism of disturbance has not been definitively elucidated for any insecticide or fungicide.

While insecticides are currently the most common choice of compound for pesticide–bee gut microbiota studies, increasing the diversity of fungicides and herbicides studied should be a priority. From a conservation standpoint, it is less critical to study the effects of insecticides on bee gut microbiotas as their adverse effects on bee health are already well established (though studies on insecticidal compounds can still provide valuable insights into how bee gut microbiotas are disturbed by pesticide exposure). Herbicides and fungicides, on the other hand, are considered relatively safe for bees, as these compounds tend to have higher lethal doses than bees are likely to encounter while foraging (Mullin *et al.* 2010; Sánchez-Bayo and Goka 2016). However, these pesticides still have a wide range of sublethal effects on bee health (Chmiel *et al.* 2020) and may also directly affect bees' resident microbes. Fungicides and herbicides disrupt molecular pathways found in fungi and plants, respectively, but some of these pathways are also found in bacteria, such as the shikimate pathway targeted by glyphosate (Motta, Raymann and Moran 2018). Additionally, fungicides and herbicides may be used as a source of nutrients by bacteria (Van Eerd *et al.* 2003). Therefore, though a pesticide may have low toxicity to the bee host, it may still affect host health and performance by disturbing the gut microbiota. Herbicide and fungicide residues are commonly found in bee colony material (Mullin *et al.* 2010), so research on pesticide-induced disturbance of bee gut microbiotas would benefit from in-depth investigation on a greater variety of compounds from these groups, particularly those that are widely used. Even in the case of herbicides, where the focus on glyphosate reflects its status as the most-used herbicide globally, current motions to limit and ban glyphosate use make it crucial that we test potential replacements for their effects on bee gut microbiotas (Székács and Darvas 2018; Beckie, Flower and Ashworth 2020; Cruz *et al.* 2021).

Finally, pesticides are often not applied as pure compounds to crops but in formulations (e.g. Roundup® (Monsanto, St. Louis, Missouri) for glyphosate). These formulations contain additional solvents that may themselves affect the bee gut microbiota. For example, exposure to Roundup, but not glyphosate, decreased the abundance of *Gilliamella* and *Bifidobacterium* bacteria in honey bees (Motta *et al.* 2020), suggesting that other components of the Roundup formulation can disturb bee gut microbes. Further applied research should examine the effects of commercial pesticide formulations on bee gut microbiotas so the effects of solvents, on their own or in interaction with the active ingredient, can be better understood.

Measuring responses in the microbiota: early studies

The first attempts to characterize the effects of pesticides on bee gut microbiotas were a series of studies on Western honey bees (*A. mellifera*) led by Martha Gilliam in the 1970s (Gilliam and Morton 1974, 1978; Gilliam *et al.* 1974, 1977; Gilliam, Prest and Morton 1974), followed by a study by Drobníková and Bacílek (1982). All early studies used aerobic culturing techniques to either compare microbes isolated from pesticide-exposed honey bees with those from pesticide-naïve bees (Gilliam and Morton 1974, 1978; Gilliam *et al.* 1974, 1977; Gilliam, Prest and Morton 1974; Gilliam and Morton 1978), or investigate the effects of pesticides on the *in vitro* growth of bacteria isolated from honey bee guts (Drobníková and Bacílek 1982). While some core bee gut microbes can grow aerobically (i.e. *Lactobacillus* cluster phylotypes), many cannot, as they are adapted to an anoxic gut environment (Engel *et al.* 2013). It is therefore likely that the culturing techniques of these early studies failed to capture the true microbial diversity of the guts they examined. However, their results still provide valuable insights into how certain constituents of bee gut microbiotas respond to pesticide exposure (see the 'Community profiles of pesticide-disturbed bee gut microbiotas: Early studies' section).

Measuring responses in the microbiota: current methods

Modern studies (i.e. 2016 onward) primarily use non-culture-based techniques to examine changes in bee gut microbiotas following pesticide exposure (though some studies supplement these approaches with anaerobic and/or microaerophilic culturing (e.g. Motta, Raymann and Moran 2018; Blot *et al.* 2019)). Most studies track changes in taxonomic composition using 16S rRNA gene amplicon or transcript sequencing (e.g. Kakumanu *et al.* 2016; Dai *et al.* 2018; Botina *et al.* 2019; Motta and Moran 2020; Paris *et al.* 2020), often coupled with qPCR-based methods in order to obtain data on gene or transcript copy counts (Motta, Raymann and Moran 2018; Raymann *et al.* 2018; Blot *et al.* 2019; Rouzé *et al.* 2019). While social bee gut microbiotas have previously been examined using metagenomic methods (e.g. Engel, Martinson and Moran 2012; Zheng *et al.* 2019; Ellegaard *et al.* 2020), these methods have not yet been used when investigating bee gut microbiota–pesticide interactions.

qPCR approaches may involve using phylotype-specific primers, and primer choice in this case appears to be relatively important: while some taxa show identical shifts in abundance regardless of the primer pair used, shifts in other taxa appear to be primer-dependent. For example, Rouzé *et al.* (2019) found

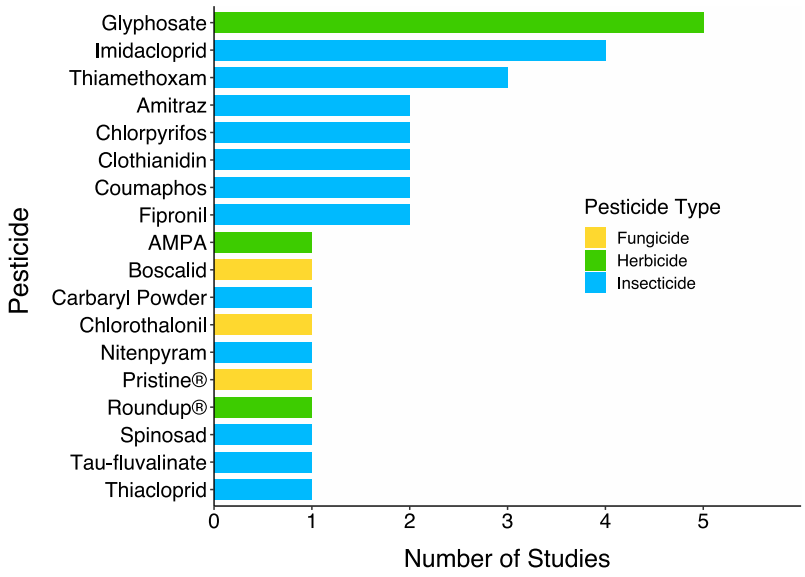

Figure 3. Number of studies that have examined each pesticide. AMPA = amino-methylphosphonic acid.

an increase in *Snodgrassella alvi* abundance when using *S. alvi*-specific primers but not Neisseriaceae-specific primers, and Blot *et al.* (2019) found conflicting results after examining *Gilliamella apicola* abundance using two different primer sets. One explanation for why we observe primer-dependent qPCR results is that different primer pairs target different clades within a given phylotype (Blot *et al.* 2019). For core phylotypes with high strain-level diversity, such as *G. apicola* and *Lactobacillus* cluster spp. (Ellegaard and Engel 2019), a greater diversity of primers may be required to fully capture the responses of all clades to pesticide exposure.

Measuring changes in functional capabilities

Though taxonomic changes in bee gut microbiota community structure after pesticide exposure have been well documented using techniques such as 16S rRNA gene amplicon sequencing (Kakumanu *et al.* 2016; DeGrandi-Hoffman *et al.* 2017; Jones *et al.* 2017), qPCR with taxon-specific primers (Blot *et al.* 2019; Rouzé *et al.* 2019), active profiling via sequencing of 16S and 18S rRNA transcripts (Motta and Moran 2020; Paris *et al.* 2020), and community-level fatty acid profiling (Diaz *et al.* 2019), functional changes have yet to be studied in depth. Understanding how pesticides affect microbial gene expression and metabolism is as important to our understanding of how pesticide exposure affects bee gut microbiotas as knowledge of taxonomic shifts, if not more so. While resident microbes may confer resistance against pathogen and parasite infection physically through exploitative competition for space and nutrients (McLaren and Callahan 2020) and by stimulating the host immune system (Kwong, Mancenido and Moran 2017; Steele *et al.* 2017; Horak, Leonard and Moran 2020), core gut microbe metabolites, such as toxins and short-chain fatty acids, may also protect against infection through direct bacterial antagonism (Steele *et al.* 2017) and by modifying physiochemical conditions in the host gut (Palmer-Young, Raffel and McFrederick 2019). Gut microbiota metabolism also maintains low oxygen levels in bee guts, which affects the products of microbial fermentation (Zheng *et al.* 2017), and there is evidence of some cross-feeding between core bee gut phylotypes (Kwong *et al.* 2014; Kešnerová *et al.* 2017), indicating that microbial metabolism may be important to the proper functioning of a healthy bee gut microbiota. Microbial metabolites may also be absorbed and used by bee hosts, but this remains mostly speculative (Bonilla-Rosso and Engel 2018; Hammer *et al.* 2021). Given the many pathways through which microbial metabolism appears to affect the health of the host and the microbial community, it is important to examine whether and how this metabolism changes after pesticide exposure.

It is also important to examine functional changes because changes in the structure of microbial communities do not always reflect changes in community function. On one hand, gut microbiotas often display a high degree of functional redundancy, possibly due to a high frequency of horizontal gene transfer (Smillie *et al.* 2011; Moya and Ferrer 2016). This is also true of bee gut microbiotas—four out of five core phylotypes (all except *S. alvi*) are sugar fermenters whose primary metabolic products are organic acids, though they also display some phylotype-specific metabolic pathways (Kešnerová *et al.* 2017; Zheng *et al.* 2017), and some toxin-producing genes are found in both *S. alvi* and *G. apicola* (Steele *et al.* 2017). It is possible that despite shifts in community structure caused by pesticide exposure, the overall function of the bee gut microbiota remains consistent through compensation by functionally similar phylotypes. On the other hand, exposure of human gut microbiotas to various xenobiotics produces significant changes in microbial gene expression even when taxonomic shifts are subtle (Maurice, Haiser and Turnbaugh 2013). Core bee gut microbiota phylotypes have a large number of accessory genes (genes that are not found in every strain) (Engel, Stepanauskas and Moran 2014; Ellegaard *et al.* 2015); therefore, even small shifts in bee gut microbiota community structure after pesticide exposure may alter community gene expression or metabolite production in a biologically significant way.

A study by Kakumanu *et al.* (2016) is the only one so far to examine changes in the functional profiles of bee gut microbiotas before and after pesticide exposure. Using PICRUSt (Langille *et al.* 2013) to compare the predicted functional composition of pesticide-exposed and pesticide-naïve honey bee gut microbial communities, they found that exposure to the fungicide chlorothalonil increased predicted gene counts in gene fam-

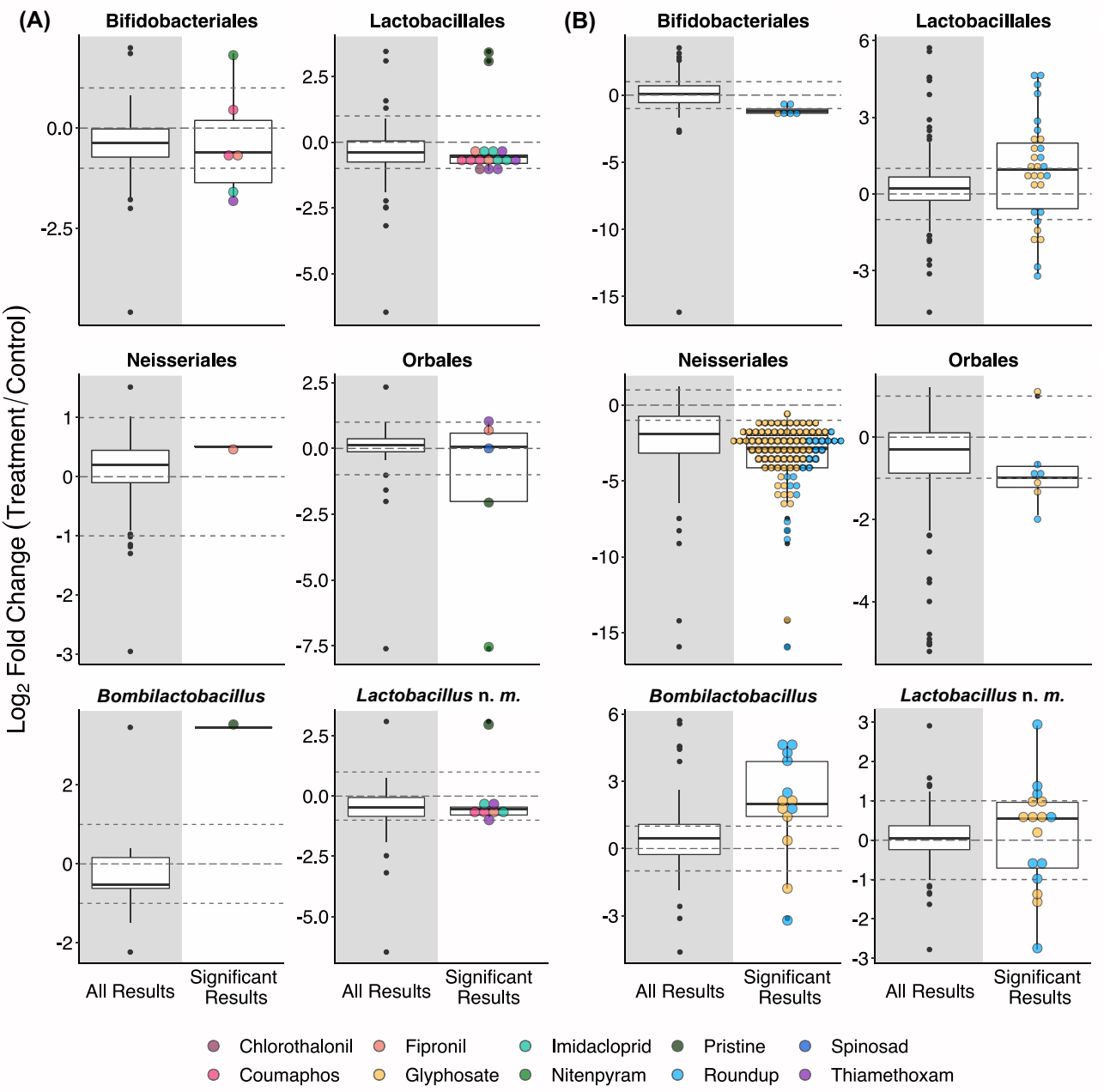

Figure 4. Log_2-fold changes in abundance of core bee gut microbiota taxa in response to exposure from **(A)** non-glyphosate-based pesticides and **(B)** glyphosate-based pesticides. Log_2-fold changes from both relative abundance and gene/transcript copy count data are displayed. Boxes represent medians and interquartile ranges; the whiskers extend to 1.5× the interquartile range. Gray panels show box plots for all results regardless of significance (all results: $n = 17$–272); white panels show box plots for significant results only (significant results: $n = 1$–92). Dot histograms are shown for significant results with dots colored by pesticide. Dashed lines represent log_2-fold changes of $+1$, 0 and -1. *Lactobacillus* n. *m.* = *Lactobacillus* near *melliventris*.

ilies related to oxidative phosphorylation and decreased predicted gene counts in gene families related to sugar metabolism and protease activity (Kakumanu *et al.* 2016). However, it is important to note that microbial functions inferred from 16S rRNA gene sequences, like those in PICRUSt, are rarely accurate due to the prevalence of horizontal gene transfer in microbial communities. A more appropriate alternative to these inferential methods is to track actual community-level changes in either mRNA transcripts or metabolites in response to pesticide exposure using untargeted or targeted metatranscriptomics or metametabolomics. However, such studies have yet to be conducted, likely because, at the time of writing this review, these methods are typically more expensive than standard gene amplicon sequencing. A further constraint is that these methods require large amounts of genetic or metabolomic material, and so may only be feasible for bee species with large colony sizes.

Investigating the transcriptome and metabolome of bee gut microbiotas before and after pesticide exposure could also provide insights into whether and how bee gut microbes metabolize pesticides. Certain microbes are able to detoxify xenobiotics, including pesticides (van den Bosch and Welte 2017; Gressel 2018), while others can transform xenobiotics into more toxic metabolites (Claus, Guillou and Ellero-Simatos 2016;

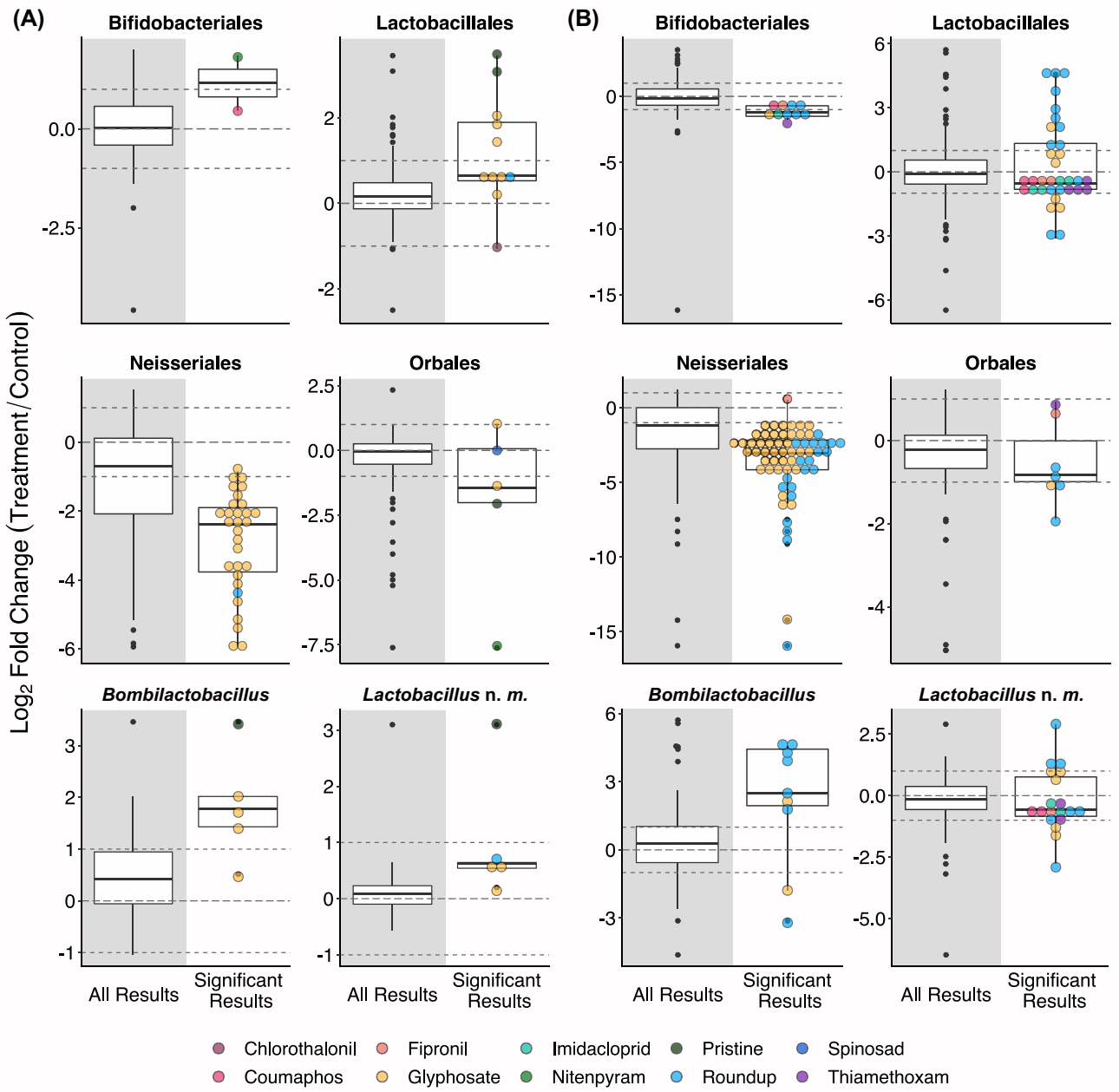

Figure 5. Log_2-fold changes in abundance of core bee gut microbiota taxa in response to pesticide exposure separated by data type. Panel **(A)** shows results from relative abundance data and panel **(B)** shows results from gene/transcript copy count data. Boxes represent medians and interquartile ranges; the whiskers extend to 1.5× the interquartile range. Gray panels show box plots for all results regardless of significance (all results: $n = 48–238$); white panels show box plots for significant results only (significant results: $n = 2–63$). Dot histograms are shown for significant results with dots colored by pesticide. Dashed lines represent log_2-fold changes of +1, 0 and −1. *Lactobacillus* n. *m.* = *Lactobacillus* near *melliventris*.

Collins and Patterson 2020). These processes may also occur in bee gut microbes, though there is currently little evidence to support this; Raymann *et al.* (2018) examined the production of imidacloprid metabolites by bee gut microbes *in vitro* using targeted metabolomics and found only weak evidence for imidacloprid degradation. Furthermore, because absorption—and therefore potential toxicity—of many pesticides occurs in the midgut (Engel and Moran 2013), it is likely that (i) many pesticides are absorbed by the bee host before reaching the gut microbiota, which is primarily present in the hindgut, and (ii) even if these compounds do reach the hindgut and undergo microbial transformation, many metabolites will not be absorbed by the host. Therefore, with our current knowledge of bee host physiology, it seems premature to conclude that microbial transformation of pesticides affects bee host health.

COMMUNITY PROFILES OF PESTICIDE-DISTURBED BEE GUT MICROBIOTAS

Early studies

Studies on pesticide-induced disturbance of bee gut microbiotas published before DNA sequencing was widely available provided early evidence that a variety of pesticides can affect the presence of microbes in honey bees and the growth of microbes isolated from honey bees *in vitro*, laying the foundation for more in-depth future experiments. Exposure to the herbicide 2,4-D had little effect on the presence of Enterobacteriaceae bacteria or non-yeast fungi in honey bee gut microbiotas (Gilliam and Morton 1974; Gilliam, Prest and Morton 1974). However, it did decrease

the presence and diversity of *Bacillus* bacteria (Gilliam and Morton 1978). Both 2,4-D and 2,4,5-T, another herbicide, increased the presence and diversity of yeasts (Gilliam *et al.* 1974, 1977). Drobníková and Bacílek (1982) also demonstrated that yeasts had greater tolerance than bacteria to pesticide exposure. Only one fungicide, folpet, was able to inhibit the growth of yeast isolates; conversely, two fungicides and three insecticides (fenitrothion, pirimiphos-methyl and pirimicarb) inhibited bacterial growth. Together, these early studies suggest that yeasts in bee guts may be better able than other fungi and bacteria to withstand pesticide exposure.

Recent studies

Recent studies have provided a plethora of information on the taxonomic shifts in bee gut microbiotas after pesticide exposure, mostly based on 16S rRNA gene amplicon sequencing data. We conducted a systematic review of *in vivo* taxonomic shifts recorded in recent studies to (i) determine whether microbial abundance tends to increase or decrease after pesticide exposure, (ii) quantify the magnitudes of these increases and decreases and (iii) identify which microbial taxa are most often disturbed by pesticide exposure.

Analysis methods

To synthesize microbial taxonomic shifts recorded in recent studies, we used key word searches on Web of Science and Scopus as well as Google Scholar alerts to find articles in which researchers exposed bees to a pesticide and tracked subsequent changes in their gut microbiotas (Table S2, Supporting Information). We also looked through the references of those articles and relevant reviews for additional papers. From these studies, we extracted the effect sizes of all *in vivo* changes in bacterial abundance between pesticide-exposed and pesticide-naïve bees that were determined by 16S rRNA gene amplicon sequencing, 16S rRNA transcript sequencing or qPCR with taxon-specific primers (Table S3, Supporting Information). Where possible, we took effect sizes from tables and text; however, most values were extracted from figures using the online software WebPlotDigitizer version 4.3 (Rohatgi 2020). Effect sizes for taxonomic groups categorized as 'Other' were excluded. If multiple figures of the same results at different taxonomic resolutions were provided, effect sizes were extracted from the figure with the highest taxonomic resolution. If data were presented for each replicate in a treatment, effect sizes were extracted for each replicate and subsequently averaged across each treatment (Table S3, Supporting Information). We categorized effect sizes by significance, whether the data were relative abundances or gene/transcript copy counts (Box 2), and whether the microbial taxon was part of the core bee gut microbiota. Taxa were only categorized as 'noncore' if there was no possibility of those taxa containing any core phylotypes. We then converted effect sizes to $\log_2$-fold changes in abundance (treatment/control); taxon gains and losses (i.e. where control or treatment abundance of a taxon was zero) were coded as $\log_2$-fold changes of 50 and −50, respectively. We generated two sets of figures using the ggplot2 package in R (Wickham 2016; R Core Team 2017). For the first set (Fig. 4), we included relative abundance data and gene/transcript copy count data for core taxa in the same figure and compared effect sizes for glyphosate separately from all other pesticides; as most effect sizes are from experiments on glyphosate (71% of all effect sizes, 81% of significant effect sizes), when plotted together overall trends were driven primarily by how taxa responded to glyphosate. For the second set of figures (Fig. 5), we separated effect sizes from relative abundance data and gene/transcript copy count data but did not separate effect sizes based on pesticide identity. For the sake of clarity, taxon gains and losses were omitted from both sets of figures. All taxon gains and losses were insignificant.

BOX 2:
TYPES OF DATA USED TO EVALUATE TAXONOMIC SHIFTS

Studies investigating taxonomic shifts in bee gut microbiotas in response to pesticide exposure typically generate two types of abundance data: relative abundances and/or gene/transcript copy counts. Abundance data can be affected at the DNA extraction step (e.g. with preferential extraction of DNA from taxa having cells that are more easily lysed), but also by the sequencing technology. Relative abundances are generated from read count data obtained via high-throughput sequencing. Some sequencing platforms have a set number of reads they can generate, and so the data they create consist of a set of proportions and are known as 'compositional data'; converting read counts to relative abundances reflects this compositional nature. Importantly, relative abundances cannot fluctuate independently, making data difficult to properly analyze (Gloor and Reid 2016; Tsilimigras and Fodor 2016), and they do not account for changes in the total absolute abundance of the microbial community. Consequently, changes in relative abundances may not fully reflect real taxonomic shifts (Gloor *et al.* 2017).

Gene and transcript copy counts, on the other hand, are not compositional data and therefore do not have the same constraints. In the set of studies examined in this review, these copy counts are generated in two ways: (i) by using qPCR with taxon-specific primers (e.g. Blot *et al.* 2019; Rouzé *et al.* 2019; Motta *et al.* 2020), or (ii) by multiplying relative abundance data by total bacterial 16S rRNA gene amplicon counts generated by qPCR (e.g. Liu *et al.* 2020; Motta and Moran 2020; Motta *et al.* 2020). Both methods allow the measured abundances of different taxa to fluctuate independently of one another and attempt to account for changes in absolute microbial abundance, which has been shown to fluctuate significantly in bee gut microbiotas post-pesticide exposure, with most studies observing decreases (Motta, Raymann and Moran 2018; Liu *et al.* 2020; Nogrado *et al.* 2019). While some studies find no significant changes in total bacterial abundance (Motta, Raymann and Moran 2018), it is unlikely that microbial abundances remain perfectly constant across all replicates and treatments in an experiment, and methodologies should be able to account for these fluctuations.

Absolute microbial abundances generated via qPCR may not always be accurate, primarily due to variation in the number of copies of a gene in a microbial genome, as occurs with the 16S rRNA gene. Nevertheless, gene/transcript copy count data represent an essential complement to compositional data to identify which microbial taxa actually increase or decrease in response to a treatment. The development of digital droplet PCR technology, allowing for absolute quantification of amplicons, and the development of increasingly sophisticated algorithms applied to metagenomic datasets may further improve the ability of researchers to obtain estimates of total microbial abundance.

There are three key components to these results: the direction of the average change in abundance, the magnitude of this change and the number of pesticides that cause a disturbance. While it would be useful to have a threshold for magnitude above which changes are biologically significant to host performance, we know too little about the functional consequences of shifts in bee gut microbiotas for bee hosts to be able to define such a threshold (see the 'Performance: the missing piece' section).

Taxonomic shifts in community composition

Examining relative abundance and gene/transcript copy count data together, pesticides generally cause subtle disturbances in core bee gut microbiotas; core taxa shift in abundance, but they are rarely eliminated. Most shifts in abundance are small (i.e. within a $\log_2$-fold change of 1 or −1) (Fig. 4) and are not statistically significant (Table S3, Supporting Information).

Bifidobacteriales and Lactobacillales bacteria both decline in abundance by similar magnitudes after exposure to pesticides (Fig. 4). These taxa are also similarly susceptible to pesticide-induced disturbance: Lactobacillales abundance is significantly disturbed by eight pesticides, while Bifidobacteriales abundance is significantly disturbed by seven. Interestingly, the average decline in Lactobacillales abundance and its susceptibility are driven by only one phylotype: *Lactobacillus* near *melliventris*. This phylotype decreases after exposure to most pesticides except for Pristine® (BASF Crop Protection, Calgary, Canada), a boscalid/pyraclostrobin mixture, which causes increases in abundance, and glyphosate, which can cause changes in both directions. *Bombilactobacillus* spp., on the other hand, increase in abundance by a relatively large magnitude post-pesticide exposure (when considering significant results only) and are significantly disturbed by only three pesticides, with glyphosate-based pesticides in particular having a consistently strong effect.

Neisseriales is similarly resistant to disturbance from pesticides, though glyphosate is a notable exception (Fig. 4). The large decline in Neisseriales abundance (specifically, *Snodgrassella alvi* abundance) after glyphosate exposure is the best-documented pesticide-induced disturbance in bee gut microbiotas—the exact mechanism of disturbance has even been determined for some *S. alvi* strains (Motta, Raymann and Moran 2018). Besides glyphosate, fipronil is the only other pesticide known to significantly disturb Neisseriales, causing a relatively small increase in abundance (Fig. 4).

Finally, the average change in abundance of Orbales after exposure to most pesticides is close to zero: pesticides seem equally likely to cause an increase or decrease in abundance (Fig. 4). Orbales is also relatively susceptible to pesticide-induced disturbance, with six pesticides causing significant shifts in abundance.

In addition to examining the effects of pesticide exposure on core bee gut microbiota taxa, we also examined the average responses of noncore bacteria, not separated by data type (i.e. relative abundances or gene/transcript copy counts) or by pesticide identity (Fig. S1, Supporting Information). Most changes in the abundances of noncore taxa are small and insignificant. However, when changes are significant, abundances are more likely to increase than decrease and the changes tend to be large. Whether this signal captured from noncore taxa is noise or is ecologically relevant remains to be determined.

Though taxonomic composition often changes in response to pesticide exposure, alpha diversity is generally consistent between pesticide-exposed and pesticide-naïve bees regardless of the diversity metric used (Kakumanu *et al.* 2016; Motta, Raymann and Moran 2018; Paris *et al.* 2020), though some studies report a decrease in alpha diversity in pesticide-exposed groups (DeGrandi-Hoffman *et al.* 2017; Liu *et al.* 2020). Effects of pesticides on intra-treatment beta diversity are more varied. When pesticides do cause significant taxonomic shifts in bee gut microbiotas, beta diversity in pesticide-exposed gut microbiotas may increase (Motta, Raymann and Moran 2018; Motta *et al.* 2020), decrease (Dai *et al.* 2018; Motta, Raymann and Moran 2018; Liu *et al.* 2020) or remain the same (Motta, Raymann and Moran 2018; Blot *et al.* 2019; Botina *et al.* 2019) when compared with the control group.

Relative abundance vs gene/transcript copy count data

When examining statistically significant shifts in microbial abundance, average responses to pesticide exposure for relative abundance-based and gene/transcript copy count-based data are notably dissimilar (Fig. 5). Bifidobacteriales, Lactobacillales and *Lactobacillus* near *melliventris* abundances significantly increased on average when analyzed using relative abundance data but decreased on average with gene/transcript copy count data. This indicates that while these taxa may have increased in abundance relative to other taxa in the community, in some cases they may have decreased in absolute abundance. Similar results were observed in a glyphosate study where the relative abundances of some microbes increased post-pesticide exposure, but their gene copy counts decreased (Motta, Raymann and Moran 2018). Additionally, some pesticides, such as fipronil and thiamethoxam, show significant effects on bee gut microbiotas only when using gene/transcript copy counts and not with relative abundance data (Jones *et al.* 2017; Rouzé *et al.* 2019; Paris *et al.* 2020). In fact, it seems that significant declines in abundance, and indeed significant shifts in general, are more commonly observed using gene/transcript copy count data (Fig. 5). Based our analysis, and the fact that gene and transcript copy counts more accurately reflect taxonomic shifts in microbial communities (Box 2), researchers may want to prioritize investigating pesticide-induced disturbances in bee gut microbiotas using gene/transcript copy count methods.

Intra-phylotype variation

While we have synthesized results at the order and phylotype levels, it is worth noting that recent studies have provided evidence that different strains of the same bacterial phylotype can differ in their tolerance of pesticide exposure. For example, the *in vitro* growth of some *S. alvi* strains is more inhibited by glyphosate exposure than the growth of others (Motta, Raymann and Moran 2018). When examining phylotypes with high strain diversity, using a variety of strains (and, if performing *in vitro* work alongside *in vivo* work, strains isolated directly from the bees used in the study) will help to capture the full variation in a given phylotype's response to pesticide exposure.

Hallmarks of bee gut microbiota dysbiosis

In recent years, some hallmarks of bee gut microbiota dysbiosis have been identified, including decreases in *Bifidobacterium* and *Lactobacillus* cluster abundance, increases in the abundance of Gammaproteobacteria, such as Orbales, and proliferation of noncore bacterial phylotypes (Horton, Oliver and Newton 2015; Li *et al.* 2015; Anderson and Ricigliano 2017; Daisley *et al.* 2020). Our synthesis shows that the effects of pesticides on bee gut

microbiotas are consistent with only some of these indicators. We found that, indeed, Bifidobacteriales and *Lactobacillus* near *melliventris* abundances tend to decline after pesticide exposure while noncore bacteria proliferate (Figs 4 and 5; Fig. S1, Supporting Information). However, we also found that *Bombilactobacillus* abundance does not typically change significantly after pesticide exposure (though if it does, it tends to increase) and that Orbales abundance is equally likely to increase or decrease. The common characteristics of bee gut microbiota dysbiosis were primarily established by studies that investigated disturbances caused by antibiotics (Raymann, Shaffer and Moran 2017), suboptimal diets (Maes *et al.* 2016) or abnormal initial gut colonizers (Schwarz, Moran and Evans 2016), not those caused by pesticides whose intended targets are nonbacterial organisms (i.e. insecticides, herbicides and fungicides). It is possible that disturbances caused by these types of pesticides are not severe enough, particularly compared with antibiotic-induced disturbances, to cause all changes commonly observed in bee gut microbiota dysbiosis. Additionally, the dysbiosis profile is likely highly dependent on the pesticide causing the disturbance. For example, significant changes in abundances of core taxa caused by thiamethoxam exposure follow all hallmarks of bee gut microbiota dysbiosis, but significant changes caused by glyphosate do not (Fig. 4).

Duration of and variability in disturbances

An important but little-studied dimension of pesticide-induced bee gut microbiota disturbance is how long the disturbance persists after pesticide exposure ceases. Diaz *et al.* (2019) found that fatty acid profiles of honey bee gut microbiotas can remain disturbed for at least 7 days post-exposure to imidacloprid. Furthermore, Motta *et al.* (2020) demonstrated that honey bee gut microbiotas can remain disturbed for up to four weeks after Roundup® exposure, though it is possible that bees were still being exposed during this time due to pesticide accumulation in hive materials. As adult worker bees generally live around 30–40 days (though longevity varies widely between species and individuals) (Free and Spencer-Booth 1959; Fukuda and Sekiguchi 1966; Goldblatt and Fell 1986; Giannini 1997; Smeets *et al.* 2003; Gomes, Menezes and Contrera 2015), these disturbance durations can represent a significant portion of an adult bee's life span. On the other hand, some evidence suggests that bee gut microbiotas can recover quickly from disturbance. Liu *et al.* (2020) demonstrated that the gut microbiotas of *A. mellifera* workers were disturbed on the 7th day of thiacloprid exposure but had recovered by the 13th day of exposure, possibly due to a decrease in pesticide consumption or rapid adaptation of resident microbes. These results suggest that, in some cases, bee gut microbiotas may be able to recover from pesticide-induced disturbance even during exposure.

Another important question is: does exposure to a given pesticide consistently disturb the same bee gut microbial taxa? Across recent studies, we observe that the consistency of disturbances varies among pesticides. Glyphosate, for example, consistently reduces *S. alvi* abundance across multiple concentrations, formulations, seasons and studies, but its effects on other core taxa members are much more variable (Motta, Raymann and Moran 2018; Blot *et al.* 2019; Motta and Moran 2020; Motta *et al.* 2020). Thiacloprid was also found to consistently disturb *Lactobacillus* near *melliventris* regardless of concentration (Liu *et al.* 2020). However, in other cases, different microbial taxa are disturbed after exposure to the same pesticide. Rouzé *et al.* (2019) found that exposure to 0.25 μg kg^{-1} of the insecticide fipronil reduced the abundance of *Lactobacillus* cluster spp. and *Bifidobacterium* spp. in honey bee guts but that exposure to 1 μg kg^{-1} increased the relative abundance of *G. apicola* and *S. alvi*, and Paris *et al.* (2020) found that fipronil had no effect on the relative abundance of bee gut microbes on its own. Differences in disturbance profiles were also found for low and high concentrations of the fungicide Pristine® (boscalid/pyraclostrobin mixture) (DeGrandi-Hoffman *et al.* 2017), and for almost identical concentrations and exposure durations of thiamethoxam (Rouzé *et al.* 2019; Paris *et al.* 2020).

One possible explanation for why some pesticides produce consistent disturbances and others do not is the mode of action (Box 1). Glyphosate, for example, directly inhibits *S. alvi* growth by blocking an amino acid synthesis pathway (Motta, Raymann and Moran 2018). As this pathway is consistently present in *S. alvi* strains, the effect of glyphosate on *S. alvi* is consistent as well (though some strains are glyphosate-resistant; Motta, Raymann and Moran 2018). Consistent disturbances may be likely when pesticides directly inhibit microbial growth via pathways that are functionally consistent and invariably present in the affected microbes. On the other hand, many insecticides, such as imidacloprid, thiamethoxam and fipronil, are thought to disturb bee gut microbial taxa indirectly through a decline in host health (Rouzé *et al.* 2019). The exact nature of the decline and how it affects the host's ability to regulate its gut microbiota likely depends on a variety of factors such as the host's health before pesticide exposure, host genetics and immune system characteristics. Variation in these factors could cause variation in how host health is altered post-pesticide exposure, which in turn could cause variation in the gut microbiota's disturbance profile even within the same host species.

ADDITIONAL FACTORS THAT INFLUENCE PESTICIDE-INDUCED BEE GUT MICROBIOTA DISTURBANCES

Beyond the host species and the identity of the pesticide being examined, numerous factors can affect pesticide-induced bee gut microbiota disturbances. For this review, we focus on four: pesticide concentrations, exposure duration, seasonality and concurrent stressors.

Pesticide concentration

While it may seem logical that higher concentrations of pesticides would lead to more severe effects, the relationship between concentration and disturbance in bee gut microbiotas appears more complicated. Some studies, such as those of Liu *et al.* (2020) on thiacloprid and Motta and Moran (2020) on glyphosate, have found that disturbances in bee gut microbiotas increase in severity as pesticide concentrations increase. However, other studies have observed disturbances in bee gut microbiotas at low concentrations of a pesticide, but no disturbances at higher concentrations. A pair of studies that exposed honey bees to different concentrations of imidacloprid in sugar syrup for varying lengths of time showed that a concentration of 3.5 μg kg^{-1} could disturb gut microbiotas but a concentration of 500 μg L^{-1} did not (Raymann *et al.* 2018; Rouzé *et al.* 2019). All in all, increasing a pesticide's concentration does not consistently increase the magnitude of its effects on bee gut microbial taxa.

It is also important to consider that the concentration of a pesticide delivered in sugar syrup may not reflect the amount that is consumed or absorbed by bee hosts. Most studies track

consumption of sugar syrup and are therefore able to estimate the amount of pesticide compound consumed per bee. However, few studies have calculated host body burden, or what concentration of the pesticide accumulates in bee host tissues (though see Liu *et al.* 2020 and Raymann *et al.* 2018). Calculating body burden is a staple of toxicological studies (e.g. Rappaport and Smith 2010; Van Meter *et al.* 2014; Inostroza *et al.* 2016) and so analyzing pesticide residues in bee bodies should be considered a priority for future studies on pesticide-induced disturbance of bee gut microbiotas, especially if the pesticide in question is thought to affect bee gut microbial taxa through its effect on host health. Because gathering such data involves destructive sampling, a larger number of bees would be required for a given experiment; consequently, such studies might be more readily accomplished in bee species with larger colonies.

Exposure duration

The length of pesticide exposure is another factor that plays an important role in pesticide-induced bee gut microbiota disturbances. Most studies on insecticides and fungicides use exposure periods longer than 10 days, and, in general, longer periods of exposure appear more likely to disturb bee gut microbial community structure (Table 1). For example, 3 or 4 days of exposure to imidacloprid did not affect *A. mellifera* or *B. impatiens* gut microbiotas (Raymann *et al.* 2018; Rothman *et al.* 2020), but 18 days of imidacloprid exposure disturbed *A. mellifera* gut microbial communities, despite the pesticide's concentration being lower (Rouzé *et al.* 2019). These results imply that the duration of pesticide exposure may matter more than the concentration when determining whether a pesticide disturbs bee gut microbial taxa. However, a long exposure period for a pesticide does not guarantee gut microbiota disturbance (Wintermantel *et al.* 2018; Yang *et al.* 2019), and some studies with short pesticide exposure durations still show perturbations in bee gut microbiotas (Motta, Raymann and Moran 2018; Motta *et al.* 2020) (Box 2).

Pesticides' modes of action may explain some of the variation in the exposure duration required to induce disturbance (Box 1). Longer periods of exposure may be necessary for pesticides that disturb bee gut microbiotas indirectly through impairment of host health as pesticides need time to accumulate in host tissues and affect host physiology. Imidacloprid, which appears to disturb bee gut microbiotas only after longer exposure durations, is hypothesized to be one such pesticide (Raymann, Shaffer and Moran 2017; Rouzé *et al.* 2019). Conversely, pesticides that directly inhibit microbial growth only need to be taken up by gut microbial cells to cause a disturbance, a process that likely takes significantly less time than affecting the health of an entire host organism. Glyphosate is one pesticide that directly inhibits bee gut microbes, and it can consistently disturb honey bee gut microbiotas after exposure periods of 5 days or less (Dai *et al.* 2018; Motta, Raymann and Moran 2018; Motta *et al.* 2020). Therefore, if a pesticide has strong direct effects on bee gut microbes it may be able to cause disturbances after a shorter exposure duration than pesticides that primarily affect gut microbiotas indirectly through host health.

While longer exposure durations appear more likely to produce observable effects of pesticides on bee gut microbiotas, they may not always be field-realistic. Pesticides that are applied as seed coatings—such as neonicotinoids and some fungicides—may be consistently present in agricultural environments over spring and summer months (Krupke *et al.* 2012; Lu *et al.* 2016). However, residues of pesticides applied via spray application may show temporal variation. Generally, there is a period of at least two weeks between pesticide sprays during which time pesticide residues in the environment will degrade and floral turnover may eliminate residue-laden pollen and nectar from the bees' food supply. Therefore, exposure to certain pesticides may be more realistically experienced in pulses and, depending on degradation and floral turnover rates, exposure periods lasting weeks may be unrealistic. Though bee gut microbiotas appear more likely to be disturbed after chronic pesticide exposure, research that aims to be applicable to pollinator management and policy decisions should ensure that the exposure regimes used in experiments accurately reflect field conditions.

Seasonality

The gut microbiotas of many animals vary seasonally (Carey, Walters and Knight 2013; Maurice *et al.* 2015; Ferguson *et al.* 2018) and those of bees are no exception (Bosmans *et al.* 2018; Bleau, Bouslama and Giovenazzo 2020; Kešnerová *et al.* 2020). In social corbiculate bees, this variation occurs in tandem with changes in host diet, behavior and physiology (Fluri *et al.* 1982; Steinmann *et al.* 2015; Orčić *et al.* 2017), including sensitivity to pesticides (Baines *et al.* 2017; Saleem, Huang and Milbrath 2020). The presence of these seasonal differences raises the possibility that interactions between pesticides and bee gut microbiotas may depend on season.

Studies on pesticide–bee gut microbiota interactions that incorporate season into their experimental design demonstrate that which taxa are disturbed by a given pesticide and the extent of the disturbances can vary seasonally. Rouzé *et al.* (2019) compared the abundances of core bee gut microbes after exposure to four insecticides (coumaphos, imidacloprid, thiamethoxam and fipronil) in honey bees in both summer and winter. Though the direction of changes (i.e. whether taxa increased or decreased in abundance) were consistent between summer and winter, the magnitude of many changes depended on season (Rouzé *et al.* 2019). Conversely, Blot *et al.* (2019) found that season had a limited effect on glyphosate-induced disturbances in honey bee gut microbiotas. One possible reason for this discrepancy is a difference in the mode of action of the pesticide (Box 1). Glyphosate directly affects microbial growth through interference with amino acid synthesis (Motta, Raymann and Moran 2018), whereas the four insecticides studied by Rouzé *et al.* (2019) were proposed to perturb the gut microbiota through aspecific changes in the host's gut environment. While the mode of action of glyphosate toxicity would not change seasonally, pesticide-induced changes in the host gut environment may differ based on seasonal changes in host physiology, particularly pesticide sensitivity, which in turn may affect which, and to what degree, taxa are disturbed post-pesticide exposure.

It is also important to consider the ecological reality that seasonal studies on pesticide–gut microbiota interactions are meant to reflect. Many bumble bee species hibernate through the winter, consuming no food for months, which eliminates the opportunity for oral exposure to pesticides. Honey bees, on the other hand, may be chronically exposed to pesticides during the winter via stored food, though little is known about actual pesticide concentrations in overwintering hives. Consequently, it is impossible at the moment to determine ecologically realistic pesticide concentrations for studies examining pesticide–bee gut microbiota interactions in the winter. Until more information on this topic is gathered, it will remain unclear how well results from such studies reflect what is occurring in the field.

Concurrent stressors

In the wild, organisms are rarely exposed to individual stressors. Instead, they often encounter multiple stressors concurrently, and these may act synergistically (Goulson *et al.* 2015). Paris *et al.* (2020) examined the effects of concurrent exposure to pesticides and the microsporidian parasite *Nosema ceranae* on the gut microbiotas of honey bees. They found that co-exposure to *N. ceranae* and various pesticides had primarily synergistic effects on some constituents of the gut microbiota (i.e. Gammaproteobacteria). In a separate study, *N. ceranae* infection was able to cause similar changes in abundance on its own, though a higher number of spores was used (Rouzé *et al.* 2019). Still, the study by Paris *et al.* (2020) demonstrates that under certain conditions, although pesticide exposure may not disturb bee gut microbial communities on its own, it may exacerbate disturbances caused by other stressors.

While studies on exposure to single pesticides are important for establishing which pesticides are capable of disturbing bee gut microbiotas, studies that expose bees to multiple concurrent stressors are more realistic. In addition to concurrent pathogen exposure, future studies should consider concurrent exposure to multiple pesticides, as such exposure is the norm for wild and agricultural bee populations (Mullin *et al.* 2010; Long and Krupke 2016; Traynor *et al.* 2016; Manning, Ramanaidu and Cutler 2017) and pesticides can sometimes interact synergistically (Pillings and Jepson 1993; Schmuck, Stadler and Schmidt 2003; Wade *et al.* 2019).

PERFORMANCE: THE MISSING PIECE

One of the most important questions concerning pesticide-induced disturbance of bee gut microbiotas is what effect, if any, these disturbances have on host performance. Here we use the term 'performance' to encompass the fitness, behavior, physiology and development of hosts at both the individual and colony levels. Previous studies investigating the roles of bee gut microbiotas in host performance have largely used comparisons between bees with conventional microbiotas and those that are gut microbiota-free or deficient (Koch and Schmid-Hempel 2011; Zheng *et al.* 2017; Rothman *et al.* 2019; Wu *et al.* 2020b). However, it is unclear if the subtler changes caused by pesticide exposure affect host performance in a biologically significant manner.

The most often-tracked performance metric is survival in pesticide-exposed vs pesticide-naïve bees (Dai *et al.* 2018; Motta, Raymann and Moran 2018; Raymann *et al.* 2018; Rouzé *et al.* 2019; Yang *et al.* 2019; Liu *et al.* 2020; Motta and Moran 2020; Motta *et al.* 2020; Paris *et al.* 2020; Rothman *et al.* 2020; Zhu *et al.* 2020), but studies have also examined sublethal effects on host behavior (Botina *et al.* 2019), individual and colony development (Dai *et al.* 2018; Wintermantel *et al.* 2018); host weight gain (Dai *et al.* 2018; Wintermantel *et al.* 2018; Liu *et al.* 2020); host gene expression (Zhu *et al.* 2020); and susceptibility to parasite and pathogen infection (Motta, Raymann and Moran 2018; Blot *et al.* 2019; Rouzé *et al.* 2019; Paris *et al.* 2020). The vast majority of these studies examined effects of pesticide-disturbed gut microbiotas on host performance by comparing performance metrics and gut microbiota community structure in pesticide-exposed vs pesticide-naïve bees (e.g. Wintermantel *et al.* 2018; Botina *et al.* 2019; Zhu *et al.* 2020). This experimental design permits researchers to determine that gut microbiota disturbance is not responsible for performance effects if a difference in performance is observed in the absence of changes in gut microbiota structure (e.g. Raymann *et al.* 2018). However, if changes in both gut microbial community structure and performance are observed, it is impossible to determine if performance has been altered due to a pesticide-disturbed gut microbiota or due to direct effects of the pesticide on the host organism; one can conclude that changes in performance are *correlated* with a disturbed gut microbiota, but not that they are *caused* by a disturbed gut microbiota.

There are ways to overcome this hurdle through clever experimental design. Motta *et al.* (2018) conducted an experiment in which groups of honey bees were either positive or negative for (i) presence of a gut microbiota, (ii) glyphosate exposure and (iii) *Serratia marcescens* (an opportunistic bacterial pathogen) infection (Fig. 6). Through comparisons of survival between these groups, this study showed that the presence of a gut microbiota increases the survival of bees infected with *Serratia* and that exposure to glyphosate eliminates this benefit but does not affect host survival on its own, providing strong evidence that glyphosate-induced disturbance of gut microbiotas increases the lethality of *S. marcescens* in honey bees (Motta, Raymann and Moran 2018). This study provides one framework for establishing that a change in performance is due to pesticide-induced disruption of the gut microbiota and not direct effects of the pesticide on the host.

Another possible methodology that has yet to be tested is inoculating microbe-free bees with either a pesticide-disturbed or pesticide-naïve gut microbiota and comparing performance between the two groups. When adult bees emerge from pupation, they are essentially microbe-free and can therefore be experimentally inoculated with a desired gut microbiota. This approach has been used to compare the impact of host genotype vs gut microbiota on pathogen infection (Koch and Schmid-Hempel 2012), determine the metabolic contributions of individual phylotypes in honey bee gut microbiotas (Kešnerová *et al.* 2017) and confirm effects of pesticides on the growth of individual phylotype strains (Motta, Raymann and Moran 2018), among other experiments. Hypothetically, this approach could be extended to studies on pesticide–gut microbiota–host interactions. Feces or gut homogenates from pesticide-exposed bees could be used to inoculate one group of bees with a pesticide-exposed gut microbiota, and feces or gut homogenates from bees that remained pesticide-naïve used to inoculate another group with a pesticide-naïve gut microbiota. The performance of the two inoculated groups could then be compared and, as neither group has been directly exposed to the pesticide, any performance differences would be due to differences in gut microbiota structure. One potential drawback is that the method assumes that a pesticide-disturbed gut microbiota will not recover to a 'normal' community structure in the absence of pesticide exposure, which may not be the case (see the 'Duration of and variability in disturbances' section). It is also important to note that regardless of experimental design, some metrics of performance remain logistically difficult to measure, such as foraging behavior and colony-level traits.

An excellent next step in examining causal relationships between pesticide-disturbed gut microbiotas and bee host performance would be to examine a pesticide with a more typical disturbance profile than glyphosate. The most consistent taxonomic changes post-pesticide disturbance are declines in Bifidobacteriales and *Lactobacillus* near *melliventris* abundance. Glyphosate does not consistently cause either of these taxa to decline, instead causing a severe decrease in Neisseriales abundance, the only pesticide to do so. There are vast differences between the Neisseriales core phylotype, *S. alvi*, and the Bifidobacteriales and *Lactobacillus* near *melliventris* phylotypes,

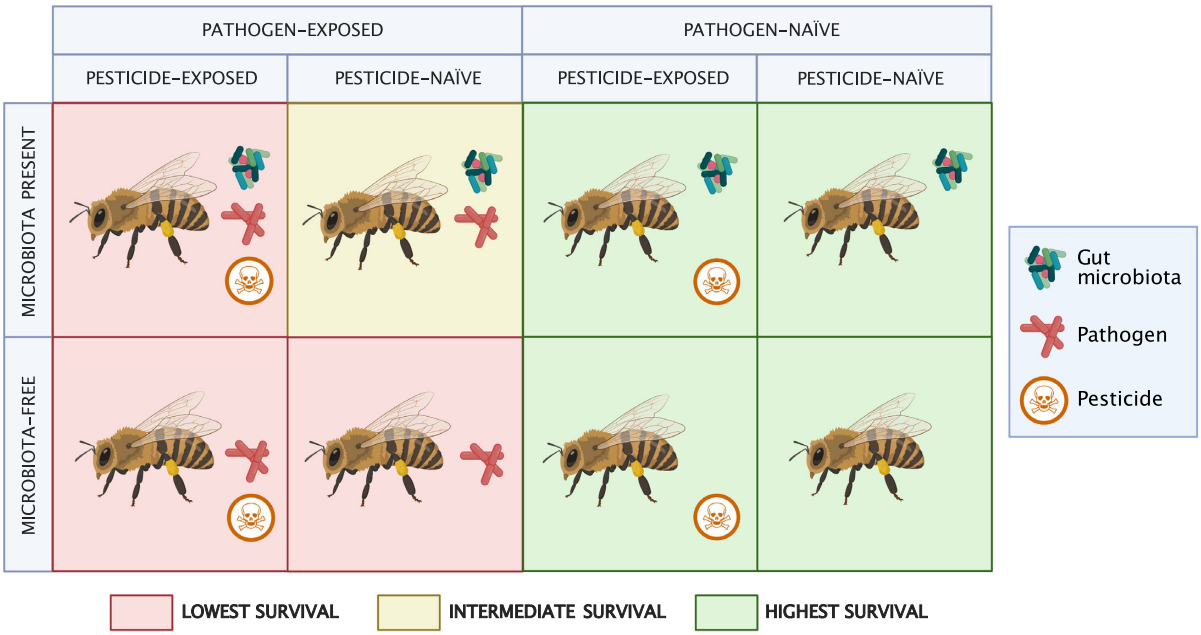

Figure 6. Methodology used in Motta *et al.* (2018), with results. Groups of bees were exposed to combinations of three treatments: inoculation (or not) with a gut microbiota, exposure (or not) to a pesticide and exposure (or not) to a stressor (i.e. pathogen). Bee survival was compared among groups. Groups with same-colored backgrounds showed no significant differences in survival. Created with BioRender.com.

including where they are physically located in the gut (Kwong and Moran 2016), their associations with other core microbes (Bonilla-Rosso and Engel 2018) and their metabolism (Ellegaard *et al.* 2015; Kešnerová *et al.* 2017; Steele *et al.* 2017; Ellegaard and Engel 2019). Consequently, knowing that a glyphosate-disturbed gut microbiota affects bee host performance does not provide much information on whether a similar effect will be observed after a decline in *Lactobacillus* near *melliventris* and/or Bifidobacteriales abundance. Future studies should prioritize examining how any of the pesticides that reduce the abundance of these two phylotypes interact with gut microbiotas to influence bee host performance. Not only would this line of investigation provide information for the chosen pesticide, it would also allow inferences about likely impacts of other pesticides that disturb bee gut microbiotas in a similar manner.

In addition to investigating differences in host performance between bees in pesticide-naïve and pesticide-exposed treatments, investigating within-treatment differences in performance can also provide valuable information about the role of bee gut microbes in host performance. A study by Wintermantel *et al.* (2018) found that *G. apicola* abundance was positively correlated with worker weight and male production in bumble bee colonies foraging on clothianidin-exposed fields, but that this correlation was absent from colonies foraging on clothianidin-free fields. This result highlights how, even in the absence of between-treatment differences in gut microbiotas (Wintermantel *et al.* 2018), bee gut microbes may interact with pesticides to influence host performance.

CONCLUSION

Early culture-based studies in the 1970s provided the first evidence that pesticide exposure could disturb bee gut microbiotas, and these findings have since been confirmed by multiple studies conducted within the past decade. Five bee species have been used as hosts for studying pesticide-induced disturbances of bee gut microbiotas, but studies using *A. mellifera* vastly outweigh the rest. The types of pesticides studied are equally unbalanced, with a greater variety of insecticides having been studied than fungicides or herbicides combined—though glyphosate, an herbicide, is the most thoroughly examined individual pesticide by a wide margin. Most modern studies rely on 16S rRNA gene amplicon sequencing to determine differences in taxonomic profiles between pesticide-naïve and pesticide-exposed bee gut microbiotas. Consequently, taxonomic changes in bee gut microbiotas after pesticide exposure are well documented, but data on functional changes are sparse.

Results from modern studies on bee gut microbiota–pesticide interactions reveal that taxonomic shifts post-pesticide exposure are often subtle, with taxa increasing or decreasing in abundance but rarely being eliminated from the community. Members of Lactobacillales (particularly the *Lactobacillus* near *melliventris* phylotype) and Bifidobacteriales are particularly susceptible to pesticide-induced disturbance and generally decline in abundance post-pesticide exposure, while Neisseriales bacteria are far less sensitive (except to glyphosate). Pesticide-induced disturbances may last for days or even weeks after pesticide exposure has ended (Diaz *et al.* 2019; Motta *et al.* 2020), potentially encompassing a bee's entire adult life span, though recovery may also be possible (Liu *et al.* 2020). Interestingly, pesticide concentration does not appear to have a large effect on the magnitude or severity of disturbance. Instead, exposure duration appears to be more important, with longer durations of pesticide exposure more likely to cause significant disturbances, though these durations may not be field realistic for all pesticides. The time required for a pesticide to disturb bee gut microbiotas may depend on the pesticide's mode of action, with pesticides capable of directly affecting gut microbes able to cause disturbances more quickly. The mode of action may also explain seasonal variation in disturbances: the effects of pesticides that are

hypothesized to disturb bee gut microbiotas via declines in host health have been shown to vary seasonally (Rouzé *et al.* 2019), in tandem with seasonal variation in host ecology, physiology and behavior, while the effects of pesticides that directly affect gut microbes are more seasonally consistent (Blot *et al.* 2019). In fact, pesticides that directly affect gut microbes may cause more consistent disturbances in general, across not only seasons but also concentrations (Motta *et al.* 2020), studies (Motta, Raymann and Moran 2018; Blot *et al.* 2019) and possibly even species, than pesticides that are hypothesized to indirectly affect gut microbiotas through host health (DeGrandi-Hoffman *et al.* 2017; Rouzé *et al.* 2019; Paris *et al.* 2020). Future work determining the precise mode of action for pesticide-induced disturbances in bee gut microbiotas will clarify these trends and may help to predict the consistency of disturbances caused by unstudied pesticides with similar mechanisms; *in vitro* experiments and examining changes in physiochemical gut conditions, host immune gene expression, and host and microbial metabolism after exposure will be useful in this endeavor (Box 1).

Understanding the effects of pesticide-induced bee gut microbiota disturbances on host performance may be the most important question in this field. If pesticide-disturbed gut microbiotas cause bees to perform suboptimally, this would negatively impact not only the bee hosts and their colonies but also the pollination services that these bees provide. While many metrics of performance have been measured, most experiments are designed in such a way that it is impossible to tell whether the pesticide-disturbed gut microbiota is causing the difference in performance or whether the pesticide is acting on the host itself. To date, the only exception is a study by Motta *et al.* (2018) that provides evidence that a pesticide-disturbed bee gut microbiota, specifically a glyphosate-disturbed gut microbiota, impairs bee host performance. To fully understand the biological consequences of pesticide-disturbed gut microbiotas on bee host performance, future studies should be designed to test causal hypotheses, not simply to establish correlation. These experimental designs will be particularly beneficial in investigating how declines in *Lactobacillus* near *melliventris* and/or Bifidobacteriales abundance affect host performance, as these are the most common changes caused by pesticide exposure in bee gut microbiotas.

Moving beyond conventional methodologies, future studies should also attempt to examine metabolic shifts in the microbial community. Even when taxonomic shifts in gut microbiotas are subtle, such as those observed in bees after exposure to some pesticides, large changes in gene expression can occur (Maurice, Haiser and Turnbaugh 2013). This, coupled with the high degree of functional redundancy exhibited by core bee gut microbes (Kešnerová *et al.* 2017; Steele *et al.* 2017; Zheng *et al.* 2017), makes functional changes in bee gut microbiotas worth investigating. Examining these changes may also provide information on the mechanisms through which pesticide-induced disturbances in gut microbiotas affect bee host performance, which may be useful for determining treatments or interventions that can mitigate negative effects.

In addition to more thorough investigations into functional changes, there are other knowledge gaps in this field that future studies can address at the design stage. Specifically, future studies should seek to expand host selection beyond *Apis mellifera* to include social bee species representing different tribes and ecologies, though investigating unmanaged species will be challenging. The strong bias in pesticide choice toward insecticides should also be addressed. Future studies that wish to examine new pesticides should aim to examine the effects of more herbicides and fungicides on bee gut microbiotas, as bees are routinely exposed to pesticides of these types (Mullin *et al.* 2010); the plant and fungal pathways they disrupt may also be present in resident bee gut microbes (e.g. Motta, Raymann and Moran 2018), and knowledge of the effects of more herbicides will be useful when determining safe alternatives for glyphosate (Beckie, Flower and Ashworth 2020; Cruz *et al.* 2021). More precise measurements are also needed of the amount of pesticides absorbed by bee host tissue (i.e. body burden), especially if the pesticides are thought to disturb bee gut microbiotas by impairing host health. Finally, we recommend that future studies move beyond using only relative abundance data to examine taxonomic shifts and instead incorporate methods that account for changes in total microbial abundance, such as gene or transcript copy counts.

Bees are vital pollinators, both for agricultural crops and natural ecosystems, whose population declines are hypothesized to be, in part, due to pesticide exposure. Pesticides affect not only bees themselves but also their resident gut microbial communities, to which they are linked in a fundamental symbiosis. Studies that have investigated pesticide-induced disturbances of bee gut microbiotas have provided critical information for understanding the consequences of pesticide exposure for the taxonomic structure of these microbial communities and how this may impact bee performance. However, this interdisciplinary field is still in its infancy, and there are many facets that remain to be investigated in depth. Expanding our knowledge on the interactions between pesticides, bee hosts and their gut microbiotas will help us generate a more complete picture of not only how pesticides affect bee health and performance but also how toxicants affect host–microbiota symbioses in general.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSRE online.

DATA AVAILABILITY

The data underlying this article are available in GitHub at github.com/michellehotch/FEMS-2021.

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