EFFECTS OF CHRONIC NEONICOTINOID EXPOSURE ON SASKATCHEWAN HONEY BEES

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ABSTRACT

Saskatchewan leads Canada in field crop production, growing approximately fifty percent of Canada's canola and wheat crop each year [1]. As well, Saskatchewan is home to a vibrant beekeeping industry which supports Saskatchewan agriculture through pollination services. Each summer, Saskatchewan beekeepers produce much of their honey crop during the canola bloom, which provides an excellent source of nectar and pollen for honey bees. However, the majority of Saskatchewan canola is grown from seed treated with a neonicotinoid insecticide, most commonly thiamethoxam [2], resulting in chronic neonicotinoid exposure of honey bees foraging on canola. Saskatchewan honey has been shown to contain some of the highest residues of thiamethoxam reported worldwide, at mean concentrations in honey of 17.2 ng/g [3]. Discrepancy in the scientific literature regarding the effects of neonicotinoid residues on non-target insects, such as honey bees, has fostered government re-evaluation of neonicotinoid use in Canada. There is a lack of field and laboratory data regarding the effects of chronic exposure of Saskatchewan honey bees to the high levels of neonicotinoid residues found in this province. To address this gap, the objectives of this thesis were to investigate the effects of chronic neonicotinoid exposure on the health and survival of Saskatchewan honey bees, including nucleus colonies, overwintering colonies, adult honey bee workers and worker honey bee brood. We found that chronic exposure of nucleus colonies to 20 ng/g neonicotinoids significantly decreased colony weight gain (honey production) by 30% and decreased adult bee cluster size by 21%. Chronic exposure to 100 ng/g of the neonicotinoid thiamethoxam significantly decreased overwinter survival of strong fall colonies by 55%, with 20 ng/g thiamethoxam resulting in a statistically nonsignificant, 15% decrease in overwinter survival. Chronic exposure to 40 ng/g thiamethoxam significantly decreased survival of summer adult workers in the laboratory but had no effect on their hyopharyngeal gland development. Compared to adult workers, worker brood required thiamethoxam concentrations which were 23 times higher before significant decreases in survival were observed. Co-exposure of worker adults and brood to neonicotinoids and fungicides was not shown to have additive negative effects on mortality; however, co-exposure of worker brood to fungicides and field-unrealistic doses of thiamethoxam was shown to predispose worker larvae to the bacterial brood disease European foulbrood. In light of these findings, chronic environmental exposure of honey bees to neonicotinoids should be maintained at levels below 20 ng/g to ensure Saskatchewan honey bees and Saskatchewan agriculture continue to thrive.

DEDICATION

This work is dedicated to the past, present, and future students of the WCVM honey bee lab.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AFB	American foulbrood
BOS	boscalid
C1	cage trial 1
C2	cage trial 2
CC	clonal complex
CCD	colony collapse disorder
CCE	carboxylesterase
CFU	colony forming units
CLO	clothianidin
CNS	central nervous system
COLOSS	Prevention of honey bee Colony LOSSes
D	day
DIM	dimethoate
DWV	Deformed Wing Virus
EFB	European foulbrood
F1	field trial 1
F2	field trial 2
GC-MS/MS	gas chromatography coupled with tandem mass spectrometry
GEE	generalized estimating equation
GST	glutathione-S-transferase
HPG	hypopharyngeal gland
HQ	hazard quotient
IMD	imidacloprid
LC-MS/MS	liquid chromatography coupled with tandem mass spectrometry
LC50	lethal concentration 50
LD50	lethal dose 50
MLST	multi-locus sequence typing
N	sample size
nAChR	nicotinic acetylcholine receptor
nAChRD	desensitizing nicotinic acetylcholine receptor
nAChRN	non-desensitizing nicotinic acetylcholine receptor
NOAED	no observable adverse effect dose
NOEC	no observed effect concentration
OD_{600}	optical density at 600 nm
OECD	Organization for Economic Co-operation and Development
P450	cytochrome P450 monooxygenase
PBS	phosphate buffered saline
PMRA	Pest Management Regulatory Agency
PRO	prothioconazole

PROP	propiconazole
PYR	pyrimethanil
RQ	risk quotient
SBI	sterol biosynthesis inhibitor
THI	thiamethoxam
USEPA	United States Environmental Protection Agency
WCVM	Western College of Veterinary Medicine
α-BGT	α-bungarotoxin

Chapter 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Pollinators are essential to agriculture, contributing to 30% of human food production [4]. The western honey bee, *Apis mellifera*, is the most commonly managed pollinator in the world, and is responsible for pollinating over 100 crops in North America [5]. Considering the importance of pollinators to human welfare, it is not surprising that recent declines in honey bee populations [6] have attracted widespread public concern [7].

The causes of honey bee declines are commonly summarized as the four 'P's': parasites, pathogens, pesticides, and poor nutrition [8]. While not an exhaustive list, the four P's are examples of the multiple biotic and abiotic stressors which interact to compromise the immunity and fitness of honey bee colonies, precipitating their collapse [9].

In particular, pesticides belonging to the neonicotinoid class of insecticides have stimulated much scientific and societal debate regarding their potential threat to honey bees [10]. Neonicotinoids contaminate the pollen and nectar of treated crops [11], as well as non-target plant species [12], presenting a source of chronic, sublethal exposure for foraging honey bees [13].

Neonicotinoids cause irreversible, excitatory stimulation of the insect central nervous system [14], altering the behaviour [15] and cognition [16] of individual honey bees exposed to sublethal doses of these insecticides. At the colony-level, effects of chronic, environmental neonicotinoid exposure are less clear, with reports of both decreased [17] and unaffected [18] colony performance, sometimes within the same study [19].

The scientific uncertainty surrounding neonicotinoids and honey bees has resulted in governments adopting often contradictory policies regarding regulation of these pesticides. For example, citing concerns to pollinator health, the European Union has banned the three most commonly used neonicotinoids since 2013 [20], while in Canada, the Pest Management Regulatory Agency deemed the same neonicotinoids a low risk to pollinator health [21–23].

Neonicotinoids are the most common insecticidal seed treatment for crops grown in the agriculturally-dominant province of Saskatchewan [2]. Not surprisingly, Saskatchewan honey bees produce honey with some of the world's highest levels of neonicotinoid contamination [3,24]. Despite the chronic, high environmental exposure of honey bees to neonicotinoids in Saskatchewan, there has been no specific risk assessment of the effects of neonicotinoids on honey bees in this province.

In response to this knowledge gap, this thesis presents field and laboratory data which describe the chronic effects of neonicotinoid exposure on Saskatchewan honey bees in the context of other, concurrent stressors encountered by honey bees in Saskatchewan, including weak colony strength, harsh wintering conditions, fungicide co-exposure and bacterial disease. In this thesis, the experimental exposure of honey bees to neonicotinoids is expressed in terms of the nominal (calculated) concentrations of neonicotinoids in the experimental diets, considering that analytical LC-MS/MS (liquid chromatography coupled with tandem mass spectrometry) measurement of neonicotinoid concentrations in the experimental diets was not performed, with the exception of chapter 2. This thesis models a comprehensive approach to pesticide risk assessment for honey bees by examining the effects of neonicotinoids at the colony and the individual bee level, as well as comparing neonicotinoid effects on worker honey bee adults and brood. The overarching goal of this thesis was to determine the safe, sublethal dose range for honey bees and neonicotinoids in Saskatchewan.

1.2 Pesticides and honey bees – historic review

Beginning in the 1950s, and gaining greater momentum from the late 1960s to the early 1980s, investment in agriculture research and application of technology to farming practices resulted in a dramatic worldwide increase in food production and efficiency; this so called ‘green revolution’was necessary to feed the growing human population [25]. The green revolution saw a shift toward agricultural monocultures and increased application of fertilizer, herbicides and pesticides [25]. Not surprisingly, from 1971 to 1991, farmers on the Canadian prairies increased their pesticide use by 500% [26]. Monoculture farming practices have since been shown to enhance insect pest pressure, and thus encourage use of agricultural insecticides [27].While increased revenue from additional crop acreage compensates farmers for the direct costs of insect damage and insecticide-use, the indirect costs of agriculture intensification, including decreased pollinator habitat and increased pesticide exposure of pollinators, are often not accounted for [27].

In the simplified landscapes created by industrial agriculture [27], farmers frequently rely on commercial honey bee pollination to increase yields [28], and during pollination, honey bees commonly encounter pesticide residues [28]. From 1966-1979, acute honey bee toxicity events were typically associated with exposure to organochlorines, carbamates, organophosphates, or pyrethroids [29]. Signs of pesticide toxicity in honey bees include dead adult bees in or around the hive; abnormal behavior, movement, and waggle dances; inability to fly; dead brood; poor brood

pattern; increased queen supersedure and queenlessness; elevated colony overwinter mortality and increased colony aggression [28]. In 1967, the United States reported a loss of 500 000 honey bee colonies from pesticide poisoning, with 103 000 of these colonies lost to acute poisoning from the carbamate insecticide Carbaryl [28]. Additionally, beekeepers themselves have been responsible for increased exposure of their colonies to pesticides through in-hive application of pyrethroids, organophosphates, formamidine pesticides, organic acids, and monoterpenoids for management of *Varroa destructor*, an ectoparasitic mite of honey bees [29].

To mitigate the risk of pesticides to honey bees, farmers limited pesticide spraying to night or early morning, reduced the quantity of pesticides applied, demanded pesticides with greater selectivity for target pests, and diversified their pest control strategies as part of an integrated pest management plan [28,30]. As well, government bodies, including the Pest Management Regulatory Agency (PMRA) of Canada were established to oversee pesticide regulation and ensure pollinator safety [31].

In Saskatchewan, Canada, there is an interconnected relationship between farmers and beekeepers. Saskatchewan has 41.7% of Canada's cropland [1] and is home to 1,101 beekeepers with 115 000 honey bee colonies [32] which produce approximately 25% of Canada's honey [33]. Saskatchewan provides 50.5% of Canada's canola (*Brassica napus*) production, and from 2006-2011, the area of canola planted in Saskatchewan increased to 63.8% of field crop acres, making canola the top crop in Saskatchewan [1]. Canola is an extremely bee attractive crop; however, it is also a source of pesticide exposure for honey bees as it is largely grown from seed treated with neonicotinoid insecticides [2,34].

1.3 Neonicotinoids

First introduced in 1991, neonicotinoids are systemic insecticides which were quickly adopted by farmers around the world, growing their pesticide market share to 24% by 2008 [35]. Neonicotinoids are widely used in the Canadian prairies and were applied on approximately 44% of crops (10.9 million hectares) in 2012 [2]. Imidacloprid (IMD) was the first neonicotinoid discovered [36] and it remains the top-selling neonicotinoid worldwide [35]; the neonicotinoids thiamethoxam (THI) and clothiandin (CLO) are second and third, respectively, in terms of worldwide sales [35]. THI is the most widely used neonicotinoid on the prairies, followed by CLO and IMD [2].

The widespread, global use of neonicotinoids over the past 30 years is largely due to their excellent chemical and insecticidal properties, including water solubility which allows neonicotinoids to be applied as a seed treatment and translocated systemically into the growing tissues of the plant, where they are toxic to a broad range of insects which feed on the plant [14,35]. Plants grown from neonicotinoid-treated seed also translocate neonicotinoids to their pollen, nectar, and guttation fluid providing a source of neonicotinoid exposure for pollinating honey bees [37]. The versatility of neonicotinoid seed treatments resulted in over six-fold growth of the seed-treatment market from 1990-2008, with neonicotinoids accounting for 80% of this market in 2008 [35]. In 2012, canola was the most common crop on the prairies to be grown from neonicotinoid-treated seed, and intensive canola-growing regions had the highest levels of neonicotinoid use, including our local area of central Saskatchewan [2].

1.3.1 Mechanism of action

Another favorable property of neonicotinoids is their high selectivity for the insect nicotinic acetylcholine receptor (nAChR) and corresponding low selectivity for vertebrate nAChRs, resulting in a high margin of safety for vertebrates [14]. nAChR are post-synaptic, transmembrane ion channels involved in fast cholinergic neurotransmission and are composed of five protein subunits which vary among different subtypes of nAChRs [38,39]. Analysis of the honey bee genome revealed that honey bees have genes for 11 different nAChR subunits [40] and the nAChR subunits of honey bees show high similarity in amino acid sequence to nAChR subunits in other insect species [41].

During nerve impulse transmission, acetylcholine is released from the pre-synaptic axon terminal and binds reversibly to nAChRs on the post-synaptic neuronal membrane, leading to opening of the nAChR ion channels and depolarization of the post-synaptic neuron [39]. Neonicotinoid insecticides interfere with neurotransmission by binding irreversibly and agonistically to nAChRs, leading to prolonged excitation of the post-synaptic neuron [14].

The selectivity of neonicotinoids is mediated by their negatively-charged nitro- or cyano-group which preferentially binds to positively-charged amino acids of the insect nAChR over the negatively-charged amino acids comprising the binding site of the mammalian nAChR [14,42]. The nitro-substituted, *N*-nitroguanidine neonicotinoids, including IMD, THI, and CLO, have higher toxicity to honey bees compared to the cyano-substituted, *N*-cyanoamidine neonicotinoids,

including thiacloprid and acetamiprid [43] (Table 1.3, Table 1.4), likely due to differences in metabolism of these compounds [44].

In mammals, neonicotinoids bind mainly to the α4β2 subtype of nAChR within the brain [14]. In insects, nAChR are primarily found in the central nervous system [14] and are divided into two categories based on their sensitivity to the snake venom α-bungarotoxin (α-BGT): 1) α-BGT sensitive nAChR and 2) α-BGT insensitive nAChR [45,46]. Insect α-BGT sensitive nAChRs are further subcategorized into a desensitizing (nAChRD) subtype and a non-desensitizing (nAChRN) subtype [47], while insect α-BGT insensitive nAChRs are subcategorized into nAChR1 and nAChR2 [48]. Different neonicotinoids bind to different nAChRs in the CNS of insects, which may explain their differences in toxicity and sublethal effects. IMD has been shown to bind to nAChRD, nAChRN, and nAChR1 [47,48]; CLO binds nAChRD, nAChRN, nAChR1, nAChR2 [47,49]; and THI binds nAChRN, nAChR1, and nAChR2, and may have effects on acetylcholine receptors which have both nicotinic and muscarinic properties [46,50,51]. THI is known to be metabolized into CLO within insects and plants, which also contributes to its insecticidal activity [50]. Honey bees vary their expression of nAChRs based on anatomical location within the central nervous system (CNS) [52], life stage [53], and previous neonicotinoid exposure [54], contributing to variation in susceptibility and clinical signs of neonicotinoid toxicity in honey bees.

1.3.2 Exposure of honey bees

Sensitive analytical tools, including gas and liquid chromatography coupled with tandem mass spectrometry (GC-MS/MS and LC-MS/MS, respectively) allow for precise detection and quantification of neonicotinoids and other pesticide residues within nectar, pollen, and guttation fluid of treated plants, as well as within hive matrices, including honey, royal jelly, beebread (stored pollen), and wax [55–57]. Furthermore, as part of tier 1 risk assessment for neonicotinoids and honey bees in Canada [58], neonicotinoid residues in the environment are compared to established Lethal Dose 50 (LD_{50}) or Lethal Concentration 50 (LC_{50}) values which reflect the dose or concentration, respectively, of neonicotinoid which kills 50% of honey bees which are exposed by the oral or contact route [43,59].

During pesticide risk assessment for honey bees, the LD_{50} for a given pesticide is used to calculate a Hazard Quotient (HQ) or Risk Quotient (RQ) which reflects the environmental risk to honey bees posed by that pesticide [58]. A HQ is calculated by dividing the field application rate for a pesticide by the LD_{50}. A RQ can be calculated by dividing the estimate of environmental

pesticide exposure by either the LD_{50} [58] or the 'no observable adverse effect dose' (NOAED) [60]. Traynor *et al.* (2016) calculated modified HQs for pollen and wax by summing pesticide residues in each matrix and dividing by the LD_{50}s for each pesticide to demonstrate that honey bee colonies involved in commercial pollination had beebread with significantly elevated HQs and queen loss was correlated with an increased HQ of wax [61].

1.3.2.1 Environmental residues

Honey bees foraging on crops grown from neonicotinoid-treated seed is one of the most common routes of direct neonicotinoid exposure in Saskatchewan. Table 1.1 shows some of the residues present in canola/oilseed rape grown from seed treated with THI and CLO at label recommendations. Non-target flowering plants adjacent to crops grown from neonicotinoid treated-seed are also a common source of neonicotinoids for honey bees [12,13], likely due to leaching of neonicotinoids from treated seed into the surrounding soil and groundwater [12,62]. Mean residues of THI, CLO, and IMD in soil from oilseed rape fields and field margins have been documented at 0.72 ng/g to 13.28 ng/g [12] and neonicotinoids are considered persistent in soil with reported half-lives in soil ranging from 100 days -3.4 years for IMD, 148 days -19.2 years for CLO, and 7-335 days for THI [62,63].

Table 1.1 Neonicotinoid residues in pollen, nectar, and guttation fluid. Reported mean, median, or maximum residues of CLO, and THI within nectar, pollen (ng/g) or guttation fluid (µg/L) in plants grown from seed treated at label recommendations or non-target plants adjacent to plants grown from treated seed.

Crop	Location	Nectar	Pollen	Guttation fluid	Reference
Canola	Ontario, Canada	2.24 CLO**	2.59 CLO**	-	[18]
Canola	Ontario, Canada	No detection	0.5 – 1.9 CLO	-	[64]
Oilseed rape	Sweden	10.3 CLO	13.9 CLO	-	[65]
Oilseed rape	France	0.65-2.4 THI*	<1 – 3.5 THI*	-	[66]
Oilseed rape	Germany	-	-	130 CLO**	[67]
Oilseed rape	England	3.2 THI	3.26 THI		[12]
Wildflowers from oilseed rape field margins	England	0.1 THI	14.81 THI		[12]

*Denotes median residues; **Denotes maximum residues

1.3.2.2 In-hive residues

Within-hive contamination with neonicotinoids results from the return of foragers who carry contaminated pollen and nectar back to the colony. Table 1.2 summarizes the neonicotinoid residues reported in hive products.

Table 1.2 Neonicotinoid residues in hive products. Reported mean residues of IMD, CLO, and THI within hive products in ng/g.

Wax	Pollen/Beebread	Honey	Location	Reference
		8.2 CLO 17.2 THI	Saskatoon, SK	[3]
		0.32 CLO 0.29 THI 0.35 IMD	Worldwide	[24]
35 CLO 38 THI 26.5 IMD	9.4 CLO 28.9 THI 19.7 IMD	1.9 CLO 6.4 THI 6.0 IMD	Europe, Asia, and North and South America	[68]

1.3.2.3 Acute exposure

Acute LD_{50} values for neonicotinoids and honey bees have been determined empirically (Table 1.3, Table 1.4) with contact LD_{50} values higher than oral LD_{50} values for CLO and THI, likely due to decreased absorption through the adult bee cuticle [69].

Table 1.3 Acute 24-hour contact and oral toxicity of neonicotinoids for adult workers. LD_{50} at 24 hours is expressed in ng/bee.

Neonicotinoid	Contact LD_{50} [43]	Oral LD_{50} [59]
IMD	18	118.74
CLO	22	3.53
THI	30	4.4
Acetamiprid	7100	-
Thiacloprid	14600	-

Table 1.4 Acute 48-hour contact and oral toxicity of neonicotinoids for adult workers. LD_{50} at 48 hours is expressed in ng/bee.

Neonicotinoid	Contact LD_{50} [69,70]	Oral LD_{50} [69,70]
IMD	49 to 102	41 to >81
CLO	44	3
THI	24	5
Acetamiprid	8090	14530
Thiacloprid	38820	17320

During foraging on a crop grown from neonicotinoid-treated seed, honey bee workers would be unlikely to encounter a dose of neonicotinoids which would result in acute toxicity. For example, based on their daily nectar consumption, worker honey bees consuming nectar exclusively from oilseed rape grown from THI-treated seed would receive a dose of 0.553-2.32 ng THI per bee per day for a forager or 0.2714-1.139 ng THI per bee per day for a nurse bee [71]. Comparing to the acute oral LD_{50} of THI for workers (Table 1.3, Table 1.4), these doses of THI

would be considered sublethal. Similarly, honey bees consume 1.1 (worker larva) to 6.5 mg (nurse bee) pollen per day [68], resulting in a maximum dose of approximately 0.02 ng THI per day (Table 1.1) for a nurse bee feeding exclusively on oilseed rape pollen grown from THI-treated seed, a dose which would be 220 times below the acute oral LD_{50} for THI (Table 1.3).

However, neonicotinoid exposure through dust during planting of treated seed has been shown to cause acute toxicity in honey bees. In 2012-2013 the PMRA observed a marked increase in incident reports of acute honey bee mortality in Ontario and Quebec associated with pneumatic planting of corn and soybeans treated with neonicotinoids [72,73]. The PMRA recommended changes to seeding practices to minimize dust and pollinator incident reports declined by 70-80% or more from 2014-2019 [72].

1.3.2.4 Chronic exposure

Due to the systemic nature of neonicotinoids, honey bees are exposed chronically to sublethal doses of neonicotinoids in the environment. Honey bee colonies foraging near corn grown from neonicotinoid-treated seed in Ontario and Quebec were observed to be exposed to neonicotinoids continuously for 12 weeks from May until September, primarily through pollen [13]. Interestingly, much of the neonicotinoid-contaminated pollen collected by honey bee colonies foraging near corn fields was shown to originate from non-target plants such as willow, clover, and buckthorn, which may have absorbed neonicotinoids from contaminated soil and water [13].

Considering that neonicotinoids bind irreversibly to nAChRs, the toxicity of neonicotinoids is not only dose-dependent, but also time-dependent [74,75]. Thus, under conditions of chronic neonicotinoid exposure, the dose of neonicotinoids required to cause toxic effects may decrease with time, although the 24- and 48-hour LD_{50} values shown in Table 1.3 and Table 1.4 demonstrate an increasing trend with time. Time-dependent toxicity of neonicotinoids to honey bees should be further evaluated with chronic neonicotinoid exposure (10 days or more) studies to accurately assess risk [74,75].

1.3.2.5 Concurrent fungicide exposure

Exposure of honey bees to multiple pesticides concurrently is common. In honey bee colonies foraging near neonicotinoid-treated corn fields, 79% of THI detections within a colony co-occurred with a fungicide detection, of which the fungicide boscalid (BOS) was the most common [13]. In a survey of 887 samples of bees, pollen and wax, Mullin *et al.* [55] found an

average of 6.2 pesticides per sample. Of the 52.5% of these samples containing a fungicide, 97.7% also contained an insecticide or acaricide [55]. Considering the multitude of matrices (honey, beebread, pollen, wax, brood food) and individuals in a hive, the concept of a pesticide 'exposome' has been applied to describe pesticide exposure of the colony as a whole [61].

Concurrent fungicide exposure may change the toxicity of neonicotinoids to honey bees due to interference with neonicotinoid metabolism (see 1.4.6). In laboratory trials, co-exposure to the fungicide BOS was found to decrease the acute oral LD_{50} of both THI and CLO for adult workers by almost two-fold [13]. Laboratory co-exposure of worker honey bees to the fungicide propiconazole (PROP) increased the contact toxicity of the *N*-cyanoamidine neonicotinoids acetamiprid and thiacloprid by 105 and 559 fold, respectively, but only increased toxicity of IMD by 1.52 fold, suggesting that pesticide synergism is variable depending on the pesticide and method of metabolism [43].

1.3.2.6 PMRA Risk Assessment

In April 2019, Health Canada's PMRA re-evaluated Canada's neonicotinoid use and deemed pollinator exposure to IMD, THI, and CLO to be acceptable in Canada [21–23]. For each neonicotinoid evaluated, the PMRA must assess the risk of aggregate effects when pollinators are exposed to the same neonicotinoid through different routes of exposure [76]. Based on this re-evaluation, the PMRA instituted improvements to labeling of neonicotinoid products and cancelled or modified some foliar and soil applications of neonicotinoids to turf, fruit, vegetable, and ornamental crops which are attractive to bees [21–23]. The PMRA did not change the regulation or availability of neonicotinoids as a seed treatment [21–23]. The PMRA is presently conducting a Special Review of the risk of IMD, CLO, and THI to aquatic invertebrates with a report expected in fall 2020 [72].

Considering that different neonicotinoids share a similar mechanism of action, the PMRA considers the risk of cumulative effects when pollinators are exposed to mixtures of different neonicotinoids in the environment [76]. Typically, the PMRA calculates the risk of neonicotinoid mixtures to pollinators using the concentration addition model which assumes that the overall effect of a mixture can be predicted based on simple addition of the doses of each component neonicotinoid [77]. However, studies in aquatic invertebrates have shown that the cumulative toxic effects of neonicotinoid mixtures may be greater-than-additive [78,79], or no different from

exposure to single neonicotinoid compounds [80], suggesting that more empirical data on the effects of neonicotinoid mixtures on pollinators is necessary to fully evaluate risk.

1.4 Honey bees

Pollination is essential for sexual reproduction of flowering plants [81]. Global pollination services have increased in value from $200 to $300 billion from 1993 to 2009 [82], reflecting the increased demand for animal-pollinated crops which provide humans with diverse commodities such as fruits, vegetables, livestock forage, fibre, nutraceuticals and fuel [81,82]. Honey bees are the most valuable insect pollinator in the United States, providing $17 billion worth of crop pollination in 2009, in part due to the ease of colony transport and management; the large number of pollinators per colony; and the ability of honey bees to pollinate a wide variety of crops and forage over long distances [81]. Pollination by Canadian honey bee colonies contributed $2.57 billion to Canadian agriculture in 2016, with an additional $1.4-4.6 billion contribution to hybrid canola seed production [33].

Aside from commercial pollination, honey bee colonies are primarily managed for honey production. In 2019, Canadian beekeepers produced over 80 million pounds of honey which was valued at over $173 million [32]. The additional contribution of hive products such as beeswax brings the value of the Canadian honey industry closer to $200 million per year [33].

1.4.1 Definition of eusocial organism

Honey bees live in colonies and have the evolutionarily rare distinction of being a ‘eusocial’ species which is defined by three main traits: (1) colony members belong to groups or ‘castes’ which are either reproductive (few members) or sterile (majority of members), (2) colony members cooperate to raise the colony’s brood, and (3) the colony contains both parent(s) and their offspring [83]. According to these eusocial principles, a honey bee colony is considered a ‘superorganism’ and toxicological studies of honey bees must be specially designed to account for their distinct biology [84].

1.4.2 Biology and worker honey bee development

Within each honey bee colony there are three castes of bees: (1) a single queen, who is responsible for laying eggs and producing pheromones which regulate colony activity; (2) approximately 10 000 to 50 000 female worker honey bees who support the colony through age-based tasks, including cleaning, nursing, building comb, storing and processing food, ventilating, defense and foraging, but do not reproduce themselves; and (3) hundreds of drones, who are

haploid males who contribute genetic diversity to subsequent generations by mating with virgin queens [84,85]. Once a queen lays a fertilized egg within a brood cell, there is a 21-day period of development before the emergence of an adult worker bee [85,86]. This period of development is comprised of 3 days as an egg; 4 days as an unsealed larva which moults through larval instars 1 to 4; 2 days as a fifth instar larva which feeds and spins a cocoon while its brood cell is capped with wax; 2 days as a pre-pupa which terminates with the fifth moult; and 9 days as a pupa until eclosion on day 21, when the newly-formed adult worker bee chews through its wax capping [85,86]. Adult worker bees have an average lifespan of 6 weeks in summer; however, in winter, adult workers can extend their lifespan to 6 months or more through hormonal and metabolic mechanisms [85].

1.4.2.1 Hypopharyngeal glands

Young nursing workers feed the colony's unsealed larvae by producing royal jelly from their hypopharyngeal glands (HPGs) [85]. Located within the head, these glands are best developed in workers at 6 days of age [87]. Nurses consume honey and pollen which is transformed into protein-rich royal jelly within the acini of these glands [88]. As nurses mature into foragers, the HPGs atrophy [87]. Considering their importance in brood nutrition, HPGs have been studied as indicator of worker honey bee stress and both neonicotinoid exposure and low dietary protein have been shown to negatively impact gland development [89].

1.4.3 Beekeeping in Saskatchewan

Although short, the hot, dry summers in Saskatchewan and the abundance of flowering crops enable Saskatchewan beekeepers to produce some of the highest honey yields per colony in Canada [90], with an average of 80 kg of honey harvested per colony in 2019 [91]. Saskatchewan is also known for its harsh, long winters which can present challenges for beekeepers overwintering their colonies [90]. Since 2007, Canadian beekeepers have struggled with overwinter losses in excess of the maximum 15% overwinter loss which is considered sustainable [92]. In winter 2018-2019, Saskatchewan beekeepers reported 21.4% overwinter colony loss which was just below the Canadian average of 25.7% [92]. Lack of colony food stores, queen failure, poor weather, and weak fall colony strength were the biggest challenges for Saskatchewan beekeepers in winter 2018-2019 [92].

In 2019, approximately one third of the colonies that Saskatchewan beekeepers put into winter were small, nucleus colonies which will be used as replacements for full size colonies which

are lost overwinter [91]. Each spring, replacement 'packages' of bees are also imported to Saskatchewan from New Zealand, Australia, and Chile with a 1 kg package consisting of approximately 10,000 workers and a mated queen [90]. Canadian beekeepers import 40,000 to 60,000 packages each year to compensate for overwinter mortality [93].

Colony health is also an important contributing factor to winter stress, and an effective health management plan is often one of the best defenses against overwinter loss.

1.4.4 Common infectious diseases

Beekeepers must protect their colonies' health by prophylaxis and metaphylaxis for a number of bacterial, parasitic, fungal, and viral diseases of honey bees.

1.4.4.1 American foulbrood

American foulbrood (AFB), caused by midgut infection with the spore-forming bacterium *Paenibacillus larvae*, is an endemic disease in Canada which causes death of honey bee brood [94,95]. Control measures for this disease include burning of diseased colonies, gamma or electron-beam irradiation of contaminated equipment, and, in Canada and the United States, antimicrobial metaphylaxis of colonies in spring and fall [95,96]. In 2018-2019, 60% of Saskatchewan beekeepers surveyed used the antibiotic oxytetracycline for AFB metaphylaxis in the spring, and 62% used oxytetracycline metaphylaxis in the fall [92]. Some laboratory studies indicate that neonicotinoids may predispose honey bees to AFB disease. For example, honey bee larvae infected with *P. larvae* spores and exposed to sublethal CLO *in vitro* were shown to have increased mortality from AFB, likely through immunosuppressive effects of CLO on cellular immunity [97].

1.4.4.2 *Nosema*

*Nosem*a disease is caused by infection of the midgut epithelium of adult honey bees with a microsporidian parasite [98] resulting in subclinical infection, dysentery (*Nosema apis*) [95] or colony loss (*Nosema ceranae*) [99]. Since its detection in Canada in 2007 [100], *N. ceranae* is the most common species isolated from Canadian colonies [92]. *Nosema* infection has been correlated with overwinter colony mortality [99] and slow spring build-up of colonies in Canada [101]. In 2018-2019 30% of surveyed Saskatchewan beekeepers treated their colonies with the antibiotic Fumagilin–B® for *Nosema* management in spring and fall [92]. The combined stress of *Nosema* disease and neonicotinoid exposure on honey bees has been explored [102]. In the laboratory, worker honey bees infected with *Nosema* and concurrently exposed to IMD were shown to have

higher mortality, and decreased glucose oxidase activity, an enzyme responsible for production of hydrogen peroxide to maintain colony hygiene [102]. Tesovnik *et al.* [103] found honey bee workers exposed to THI as larvae and adults and infected with *Nosema ceranae* as adults had significant elevations in mortality relative to controls, possibly through THI-induced dysregulated expression of immune and detoxification genes leading to more severe infections with *Nosema ceranae* in these workers.

1.4.4.3 *Varroa* and viruses

Varroa destructor is an ectoparasitic mite of honey bees which weakens and immunocompromises honey bees by feeding on the fat body of honey bee pupae and adults and vectoring viruses as it feeds [104]. Varroa-associated viruses, including Deformed Wing Virus (DWV), can have debilitating effects on honey bees, including hypoplasia and abnormal development of the wings, abdomen, and HPGs [105]. Considering that *Varroa* infestation is one of the leading infectious causes of overwinter colony loss in Canada [101], miticides are commonly applied to colonies for *Varroa* control [92]. 100% of Saskatchewan beekeepers surveyed used chemical control methods for *Varroa* mites in spring 2018 with the most common treatment being Apivar® (amitraz) and 87% of Saskatchewan beekeepers treated for *Varroa* mites in the fall using oxalic acid or Apivar® [92]. The combined stress of neonicotinoid exposure and *Varroa* infestation has been shown to negatively impact honey bee colonies. Exposure of newly-emerged honey bee workers to low, field-relevant doses of CLO in the laboratory was shown in increase replication of DWV vectored by *Varroa* [106]. Furthermore, combined sublethal THI and CLO exposure and *Varroa* infestation of colonies acted synergistically to decrease mass at emergence and longevity of adult workers from these colonies relative to controls [107].

1.4.4.4 European foulbrood

European foulbrood (EFB) is a bacterial disease of honey bee larvae caused by the gram-positive bacterium *Melissococcus plutonius* [108,109]. Clinical signs of EFB may include beige, twisted larvae with a foul odor; a ‘spotty’ brood pattern (frequent empty brood cells indicative of brood mortality); and rubbery scale at the bottom of brood cells [108]. Typically EFB is diagnosed in early spring when colonies may have inadequate nutritional resources and too few nursing bees to care for their rapidly expanding population of brood [108,109]. Larvae are infected with *M. plutonius* from the brood food provided by nursing bees who are asymptomatic-carriers [110,111]. The bacteria proliferates within the midgut of infected larvae leading to starvation and death of the

larva when brood care is inadequate [109]. However, as the spring nectar flow continues, nursing bees become more abundant, and brood care improves, larvae are able to survive *M. plutonius* infection and clinical symptoms of EFB resolve spontaneously [108]. Importantly, *M. plutonius* is able to persist within brood frames from asymptomatic colonies [108] and PCR of worker honey bees from the brood nest could be used to predict risk of EFB the following spring [110,111].

M. plutonius has been categorized into 'typical' and 'atypical' strains [112] and clonal complexes (CC) based on multi-locus sequence typing [113]. In the laboratory, atypical strains belonging to CC12 are most virulent, followed by typical strains belonging to CC3 and CC13, respectively [114,115]. The correlation between laboratory virulence of *M. plutonius* strains and virulence in the field remains unclear. In one study, colonies infected with CC3 strains had a significantly increased proportion of diseased brood and were significantly more likely to be destroyed [116]. Whole genome sequencing identified different virulence factors between different CCs [117]. Recently, the virulence plasmid pMP19 was shown to be critical for the larvicidal activity of CC3 strains [118]. Atypical strains may have more genes for metabolism which may enable more rapid growth within the larval midgut, allowing the bacteria to outcompete the larva for nutrients [117]. To date, investigation of neonicotinoids and susceptibility of honey bees to EFB has not been performed.

1.4.5 Immunity of honey bees

Honey bees have evolved both individual and social immunity to combat the various pathogens described above. As a eusocial superorgansim, there is potential for rapid disease transmission within a colony due to close contact of genetically similar individuals, thus making immunocompetence an important evolutionary investment [119,120].

1.4.5.1 Individual immunity

Honey bees defend themselves from infection using their innate immune system which has both cellular and humoral components [121,122]. Cellular immunity is provided by hemocytes, which are capable of phagocytosing pathogens, as well as surrounding pathogens, with a nodule or capsule of hemocytes [123]. Humoral immunity is provided through antimicrobial peptides secreted from the fat body [124], melanization reactions catalyzed by phenoloxidase which produce melanin which is toxic to bacteria and fungi [125,126], and production of reactive oxygen and nitrogen-containing molecules which are toxic to microbes [127].

The immunocompetence of individual worker honey bees varies with age [128]; brood have higher total hemocyte counts and lower phenoloxidase activity compared to adult workers, while nurse bees have increased fat body mass compared to foragers [119]. Some studies show a negative correlation between age and hemocyte count in adult workers, possibly due to the high energetic cost of cellular immunity [128]. Honey bees have approximately one third of the immune genes present in other insect species; however, decreased immunocompetence of individual bees may be compensated for by their use of social immune mechanisms [120].

1.4.5.2 Social immunity

Honey bees use a diverse array of group behaviours to protect their colony from disease. For example, honey bees generate heat or a 'social fever' [129] to defend against hornets [130] and protect their brood from infection with the cold-adapted fungus *Ascosphaera apis* [129]. Hygienic behaviour is a genetically controlled trait of honey bees by which workers detect and eject diseased brood from the colony [131]. Hygienic colonies have been shown to be more resistant than colonies that lack the trait to pathogens such as *Paenibacillus larvae*, *Ascosphaera apis* [132] and *Varroa* mites [133]. Co-grooming behaviour of adult bees is another social defense strategy against phoretic *Varroa* [133]. Honey bees collect and store antimicrobial plant resins, termed 'propolis', which decrease the requirement for bees within the colony to mount an individual immune response[134].

1.4.5.3 Immunosuppression and neonicotinoids

Neonicotinoid exposure has been shown to negatively impact both individual and social immunity. In individual adult workers, topical exposure to CLO was shown to inhibit NF-κB transcription factors which induce cellular and humoral immunity, leading to increased viral titers of DWV [135]. Additionally, adult workers orally exposed to field-relevant doses of CLO for 48 hours had reduced gene expression of major royal jelly proteins which contribute to humoral immunity as antimicrobial peptides [136]. At the colony level, social immunity was shown to be compromised by chronic exposure to field realistic doses of CLO in pollen over 12 weeks, resulting in a decline in hygienic behaviour over time which was significantly different from controls by the end of the exposure period [13].

Neonicotinoids have also been shown to impair immune function in other bee species. In the solitary bee, *Osmia bicornis*, 3-day laboratory exposure to field-realistic concentrations of thiacloprid in sugar syrup and pollen patty was shown to impair the immune function of males (but

not females), by significantly decreasing hemocyte counts and antimicrobial activity of hemolymph [137].

1.4.6 Honey bee metabolism and detoxification of neonicotinoids and fungicides

In addition to coping with pathogens, honey bees have also developed individual and social mechanisms for processing xenobiotics, including pesticides. Honey bees, use a four-step process for metabolism and detoxification of fungicides and neonicotinoids, including (1) nutritional and social detoxification, (2) phase I functionalization reactions, (3) phase II conjugation reactions, and (4) phase III transport reactions.

1.4.6.1 Social and nutritional detoxification

The complex eusocial behavior of honey bees facilitates avoidance, dilution and processing of pesticides present in pollen and nectar prior to ingestion. For example, foragers may learn to recognize and avoid crops with high pesticide residues, and communicate this information to the rest of the colony [138]. During the production of honey and beebread, pesticide residues are diluted and some pesticide residues may be metabolised by fungal organisms present in beebread. Pollen containing high fungicide residues may be isolated in 'tombs' within the comb if the colony deems it unfit for consumption [139].

Nutritional detoxification refers to consumption of substances which provide honey bees resilience to withstand pesticide stress [138]. Adequate pollen and protein consumption in early adulthood has been linked to enhanced pesticide tolerance [140]. Also, naturally occurring chemicals in pollen and propolis, including quercetin and *p*-coumaric acid, upregulate Phase I detoxification enzymes (see 1.4.6.2) such as cytochrome P450 monooxygenases (P450s), leading to increased survival in honey bees exposed to the neonicotinoid imidacloprid [141]. Nutritional detoxification may be compromised due to decreased pollen diversity available to colonies foraging in landscapes with intensive monoculture farming practices [138].

1.4.6.2 Phase I functionalization reactions

Enzyme-catalyzed Phase I reactions detoxify pesticides into hydrophilic metabolites which can no longer bind to their intended receptors [138]. The workhorse of Phase I pesticide detoxification in honey bees is the P450 superfamily of enzymes which detoxifies pesticides through oxidation. Specifically, the CYP9Q P450 subfamily of enzymes in honey bees, expressed in the brain and the Malpighian tubules, among other tissues, has been shown to detoxify cyano-

substituted neonicotinoids, such as thiacloprid and acetamiprid [43,44]. P450s appear to be less important for detoxification of nitro-substituted neonicotinoids such as IMD [43,44]; however, acute co-exposure of adult workers to imidacloprid and the P450 inhibitor piperonyl butoxide significantly increased mortality by over fivefold relative to IMD alone [142].

Carboxylesterases (CCEs) are another class of Phase I detoxification enzymes which have been shown to participate in neonicotinoid detoxification through hydrolysis of ester bonds [143,144]. Empirically, P450s are more important than CCEs in Phase I metabolism of neonicotinoids. For example, co-exposure of adult workers to IMD and the esterase inhibitor triphenyl phosphate did not significantly increase worker mortality relative to IMD alone [142]. Additionally, IMD exposure did not significantly affect esterase activity in adult workers, unlike organophosphate exposure which was associated with a significant decrease in esterase activity [142]. However, suppression of esterase activity (as well as acetylcholinesterase activity) by organophosphates may partially explain the additive toxicity of organophosphates and neonicotinoids [142].

Phase I detoxification in honey bees is limited by the comparatively small number of genes encoding P450s and CCEs relative to other insects [138,145]. With 46 P450 genes, honey bees have approximately 50% fewer P450 genes compared to fruit flies and mosquitoes, and honey bees are particularly deficient in P450s belonging to the CYP4 clade [145]. The CYP3 clade, containing the CYP9 P450s involved in neonicotinoid metabolism, is comparatively more abundant in the honey bee [145].

Fungicides known as sterol biosynthesis inhibitors (SBIs) are frequent contaminants of pollen [55] and they may compromise Phase I P450-mediated detoxification in honey bees, facilitating additive or synergistic effects on pesticide toxicity [146–148]. SBI fungicides, including the triazole fungicides such as PROP, inhibit fungal growth by interfering with synthesis of fungal ergosterols, compounds which are important for membrane integrity and hormonal communication in fungi [149,150]. During ergosterol biosynthesis, fungal P450s catalyze the removal of an α14-methyl group [149,150]. SBI fungicides bind to the P450s, leading to accumulation of α14-methylsterols which increase fungal membrane permeability and inhibit fungal membrane enzymes [149]. *In vitro* studies of metabolism in honey bee midguts demonstrated that SBI fungicides also inhibit P450 metabolism in honey bees [151]. This finding is consistent with other laboratory studies [146,147] which have demonstrated that co-exposure to

the SBI fungicide PROP enhances the toxicity of the neonicotinoids THI or CLO by up to threefold in adult workers, and increases the toxicity of the diamide insecticide chlorantraniliprole by over sevenfold in adult workers and by 53% in worker brood [148]. Furthermore, co-exposure of adult workers to IMD and the SBI fungicide tetraconzole increased adult mortality by 20% relative to IMD alone [142]. Alternatively, low doses of SBIs (0.03 µg/bee) have been shown to reduce the toxicity of pyrethroids to honey bees, possibly through P450 induction in adult workers [152].

1.4.6.3 Phase II conjugation reactions

Phase II reactions involve enzymatic linkage of Phase I pesticide metabolites to molecules such as glutathione, enhancing solubility of the metabolite and preparing the metabolite for excretion out of the cell [138]. In the honey bee, glutathione-S-transferases (GSTs) are the primary category of Phase II enzymes, and GST upregulation has been observed with sublethal THI exposure in honey bees [143]. Insects have two unique classes of GSTs, the Delta and Epsilon classes, which are critical for pesticide detoxification [145]. Similar to their low genomic inventory of P450 genes, honey bees are also deficient in GST genes compared to other insects, potentially increasing their vulnerability to pesticide toxicity [145]. Mosquitoes and fruit flies have at least eight genes for Delta and Epsilon GSTs, whereas honey bees have no genes for Epsilon GSTs and only one gene for a Delta GST [145]. Compared to P450s, GSTs do not appear to be highly involved in neonicotinoid metabolism [142]. Inhibition of GSTs by diethyl maleate did not significantly increase IMD toxicity to adult workers, and IMD exposure did not significantly alter GST activity in adult workers [142]. Acute co-exposure to the neonicotinoid acetamiprid and the fungicide PROP in *Apis cerana cerana* foragers was observed to induce GST activity, while chronic acetamiprid exposure inhibited GST activity in the midgut [153]. Similar effects were not observed in newly emerged *A. cerana cerana* workers indicating age-dependent differences in detoxification pathways [153].

1.4.6.4 Phase III transport reactions

ATP-binding cassette (ABC) proteins use energy of ATP to excrete Phase II conjugated metabolites out of the cell [138]. Once again, honey bees are deficient in ABC transporter genes; however, these transporters are expressed in a wide variety of honey bee tissues, including the Malpighian tubules, midgut, cuticle, and brain [154]. If ABC transporters are inhibited, the oral toxicity of neonicotinoids, including acetamiprid, thiacloprid, and IMD, was shown to be increased in adult workers, confirming that ABC transporters function in neonicotinoid detoxification [154].

Alarmingly, oxytetracycline has been implicated as a potential ABC inhibitor which can synergize with acaricides and possibly other in-hive pesticides [154].

1.4.6.5 Summary of pesticide detoxification in honey bees

Along the four-step pathway for pesticide detoxification, there is potential for competitive interactions when honey bees are exposed simultaneously to both neonicotinoids and fungicides. Honey bees are deficient in genes for Phase I, II, and III detoxification enzymes and proteins which may enhance their susceptibility to synergistic effects of exposure to multiple pesticides [138]. On the other hand, the efficient behavioural detoxification methods adopted by these eusocial organisms may compensate for their decreased diversity of detoxification pathways [145]. Additionally, some pesticides are known to induce and upregulate detoxification enzymes in honey bees [142], potentially enhancing the detoxification or elimination of neonicotinoids and fungicides, and reducing their toxicity to honey bees.

Pesticides may also behave synergistically with pathogens to impact the immune system of honey bees; accordingly, co-exposure of honey bee colonies to agrochemicals and infectious disease is a hypothesized driver of recent elevations in honey bee colony mortality worldwide [155–157].

1.4.7 Colony collapse disorder

Colony collapse disorder (CCD) was described by vanEngelsdorp et al. [155] after two winters of record levels (31.8% and 35.9%) of colony mortality in the United States, beginning in 2006-2007 [155,158]. CCD is defined as colonies which experience (1) a sudden disappearance of their adult worker bee population with (2) an absence of dead worker bees near the colony and (3) a noticeable time lag in invasion of pests or 'robber' bees to steal food resources from the collapsed colony [155]. Elevations in colony mortality have been reported worldwide, including Canada [92] and Europe [159], stimulating establishment of the Prevention of honey bee COlony LOSSes (COLOSS) network [157], a group of international scientists, beekeepers, and industry representative who conduct collaborative research to understand, monitor, and ultimately prevent colony loss globally [157].

While a precise cause of CCD has not been identified, the general consensus is that CCD results when colonies are exposed to multiple, interacting stressors which immunosuppress colonies and lead to increased infection with pathogens [155]. Mass mortality of honey bee colonies is not a new phenomenon, with at least 18 occurrences reported since 1869 [160];

however, the modern paradigms of commercial beekeeping and agriculture, as well as international trade in honey bees and hive products, have resulted in unprecedented levels of biotic and abiotic stressors which are likely responsible for the continued high levels of overwinter colony loss experienced today [156].

In winter 2018-2019, beekeepers in the United States experienced their highest average overwinter losses (37.7%) since records were kept beginning in 2006-2007 [161]. Over the past 13 years, the average loss of colonies over winter in the United States has been 28.8% [161]. Compared to the United States, Canada has had slightly lower winter loss over the past 13 years, with an average of 25.7 % and a range of 15.3-35% [92]. On average, over the past 11 years, the United States considered 17% overwinter loss to be acceptable; however, there has been a trend toward accepting higher overwinter losses, with as high as 22.2% considered acceptable in 2018-2019 [161].

Considering that the widespread use of neonicotinoids in agriculture preceded the emergence of CCD in 2006, numerous field and laboratory studies have been conducted to better understand the toxicological effects of neonicotinoids on honey bees.

1.5 Neonicotinoid toxicology studies in honey bees

Researchers and regulators have developed a unique suite of toxicology assays, in both the field and the laboratory, to assess the risk to honey bees of environmental exposure to neonicotinoids. These studies must be specially designed to account for the distinct eusocial biology of honey bees [84], as well as to measure sublethal outcomes, rather than acute mortality, as most environmental concentrations of neonicotinoids are considered sublethal for individual honey bees [11].

1.5.1 Colony-level effects of neonicotinoid exposure

Effects of sublethal neonicotinoid exposure on honey bee colonies have been investigated using field studies where colonies forage on crops grown from neonicotinoid-treated seed (Table 1.5), and semi-field studies where colonies are provided artificial diets containing environmental concentrations of neonicotinoids (Table 1.6). The outcome measures of these studies include colony-level inspection parameters such as brood area, foraging activity, adult bee population, mortality, colony weight, disease infestation, pollen and honey stores, and overwinter survival (Table 1.5, Table 1.6). Field studies typically find no significant, negative colony-level effects of chronic neonicotinoid exposure (Table 1.5), or negative effects which are limited to particular

geographic regions [19]. Frequently, the absence of negative effects of neonicotinoids in field studies of honey bee colonies have been attributed to contamination of control colonies with neonicotinoids [18,64], poor study design and replication resulting in inadequate statistical power [65,162,163], and the resilience of honey bee colonies as a 'superorganism' which can compensate for stress by increasing colony investment in reproduction [164]. Results of artificial feeding studies have been mixed (Table 1.6) with some authors observing high levels of overwinter mortality [165], others finding milder effects such as increased queen supersedure [17,166]; and some studies finding no effect [167].

Table 1.5 Field studies examining effects of neonicotinoids on honey bee colonies

Crop	Location	Colonies per treatment group	Neonicotinoid	Outcome measures	Contamination of controls	Significant treatment effect?	Reference
Canola	Ontario, Canada	16	CLO	Honey production, adult worker mortality and longevity, brood area, overwintering success	Yes	No	[18]
Canola	Ontario, Canada	20	CLO	Honey production, disease, number of adult workers and mortality, brood area, overwintering success	Yes	No	[64]
Oilseed rape	Sweden	8	CLO	Number of adult bees	Yes	No	[65]
Oilseed rape	France	12	THI	Overwintering, worker mortality, foraging activity, number of workers, colony weight, brood area, food area	No	No	[66]
Oilseed rape	Hungary, United Kingdom, Germany	66	THI, CLO	Worker numbers, brood cells, storage cells, overwinter survival	No	Significant negative effects in Hungary and UK and positive effects in Germany	[19]

Table 1.6 Artificial feeding studies examining effects of chronic neonicotinoid exposure on honey bee colonies.

Route of exposure and duration	Colonies per treatment group	Neonicotinoid and dose	Outcome measures	Results	Reference
Sucrose syrup for 4 weeks (July-August)	8-9	IMD at 0.0005 ng/µl or 0.005 ng/µl	adult bee activity and population capped brood area before winter disease incidence adult bee mortality number of frames with brood after wintering health and strength of colonies after wintering colony weight	IMD exposure associated with a significant increase in capped brood before winter, increased frames of brood after winter and increased frequency of bees carrying pollen	[167]
High fructose corn syrup for 13 weeks (July - September)	4	IMD at 0.1, 1.1, 5.3, or 10.5 ng/g for 4 weeks and then 20,40,200, or 400 ng/g for 9 weeks	quantity of capped brood colony mortality after winter	94% of IMD treated colonies were dead after winter and only 25% of control colonies were lost	[168]
Sucrose water or high fructose corn syrup for 13 weeks (July-September)	6	IMD or CLO at 0.74 ng/bee/day	quantity of capped brood *Varroa* infestation number of frames containing adult bees (cluster size) colony mortality after winter	IMD or CLO exposure was significantly associated with decreased cluster size and colony collapse after winter	[165]

Table 1.6. continued

Route of Exposure and duration	Colonies per treatment group	Neonicotinoid and dose	Outcome measures	Results	Reference
Pollen patties for 46 days (May-June)	12	combination of 5 ng/g THI and 2 ng/g CLO	number of adult bees amount of brood honey and beebread stores queen presence overwinter survival incidence of swarming	THI and CLO treatment was significantly associated with decreased brood, adult bees, and stored honey and beebread; increased queen supersedure; and decreased swarming	[17]
Pollen patties for 12 weeks (May-August)	7-10	IMD at 5,20,100 ng/g	foraging activity queen presence area of drawn wax, adult bees, brood, honey and beebread stores overwinter survival disease	20 and 100 ng/g IMD treatment was significantly associated with increased queen loss and overwinter mortality and 100 ng/g IMD was significantly associated with increased *Varroa*	[166]

1.5.2 Laboratory studies on individual honey bees

Investigation of the sublethal effects of neonicotinoids on honey bees is most commonly conducted using laboratory experiments [162], which offer a higher degree of control compared to field experiments, but sacrifice the eusocial structure present in a colony, which may buffer pesticide-stress [164], and, more often, examine acute, rather than chronic effects of neonicotinoid exposure [164]. Laboratory experiments have also been criticised for testing concentrations of neonicotinoids which are in excess of environmental concentrations [169].

Sublethal endpoints for individual bees in the laboratory may include measurement of associative learning, lifespan, locomotion, foraging ability, and intraspecific interactions and behaviour [170]. For example, acute, field-realistic, laboratory THI exposure was shown to alter the ability of foragers to associate smells with a stimulus [171], their locomotion [172], their ability to make decisions in a color-based maze [16], and increase their grooming [173]. Acute laboratory neonicotinoid exposure has also been associated with difficulty of foragers to right themselves after falling on their back [173]. In some studies, foragers exposed to sublethal doses of neonicotinoids in the laboratory are subsequently returned to the colony for behavioural evaluation. For example, after return to the colony, neonicotinoid-exposed foragers were shown to perform fewer waggle dance circuits [174] and have lower rates of return to the colony after foraging [175], compared to controls.

In many cases, the negative effects of neonicotinoid exposure described in laboratory experiments are not corroborated by field studies [170]. Further studies linking field and laboratory effects of neonicotinoids are required to explain this discrepancy. As well, most laboratory studies utilize adult workers as test subjects, with more laboratory studies necessary to examine the effects of neonicotinoids on developing worker brood.

1.5.3 Significance of *in vitro* larval rearing as an advancement in honey bee toxicology

Standardized *in vitro* techniques for rearing workers from first instar larvae into adults were developed by Aupinel [176–178] and refined by Schmehl [179]. These *in vitro* techniques have proved useful for testing toxic effects of neonicotinoids on brood by incorporating these pesticides into the artificial larval diet to establish LD_{50}, LC_{50}, or NOAED values (Table 1.7) which can be used in risk assessment.

Table 1.7 Toxicity of neonicotinoids to honey bee worker brood reared *in vitro*.

Neonicotinoid	Duration of exposure	LD_{50} (ng/larva)	LC_{50} (ng/µl)	NOAED (ng/larva)	Reference
IMD	Acute 24 hour exposure	4170	138.84	-	[180]
IMD	Chronic 4 day exposure	-	-	1400	[181]
CLO	Chronic 4 day exposure	-	-	14	[181]
THI	Chronic 5 day exposure	229	1.53	-	[182]

Chronic, *in vitro* exposure of worker larvae to IMD and CLO generated RQs of 0.01 and 0.14, respectively [181], which were less than the level of concern of 1.0 established by the United States Environmental Protection Agency (USEPA) [60] , indicating that the IMD and CLO pose little risk to developing worker brood exposed to environmental concentrations of these neonicotinoids [181].

Despite the relative apparent safety of neonicotinoids to brood, *in vitro* exposure of worker brood to mixtures of pesticides, including fungicides with miticides [183] or insecticides [148], has been shown to synergistically decrease brood survival, suggesting that there is a need for further *in vitro* investigation of worker brood susceptibility to the effects of neonicotinoids in combination with other pesticides.

1.6 Conclusion and objectives

Since 2013, the European Union has banned outdoor agricultural use of IMD, CLO, and THI in Europe citing concerns about the toxicity of these pesticides to pollinators [20,184]. This controversial ban has resulted in challenges for European farmers who must diversify their pest management strategies to protect the health of their crops without access to neonicotinoids [20]. Considering the often conflicting results of field and laboratory studies investigating the effects of neonicotinoids on honey bees, there is need for better risk assessment tools for policy-makers, especially as the PMRA continues to re-evaluate the registration of neonicotinoids in Canada [72].

Due to their distinct eusocial biology, honey bees are not adequately served by traditional pesticide risk assessment, and instead, require specialized risk assessment tools to adequately

capture risk within the complex social environment of a honey bee colony [84]. The field of veterinary toxicological pathology has the potential to offer guidance on risk assessment of neonicotinoids and honey bees [84, 185]. Traditionally, veterinary toxicological pathologists assess the safety of pharmaceuticals in laboratory animals by examining the effects of these drugs at the gross and microscopic level in exposed, individual animals, and extrapolating these findings to the herd or population level.

With this in mind, we suggest that veterinary toxicological pathologists may bring a novel approach to understanding the impact of neonicotinoids on honey bees for determination of safe dose ranges of neonicotinoids for honey bees [185]. In particular, veterinary toxicological pathologists have the skills and expertise necessary to interpret the significance of pesticide-associated effects for both individual worker honey bees as well as for the eusocial 'superorganism' to which those workers belong.

To balance the competing interests of pollinator and crop health, our research aim was to establish an acceptable, safe, sublethal dose range for exposure of honey bees to neonicotinoids in Saskatchewan. Recognizing that the honey bee colony is a 'superorganism', in which direct toxic effects on individual bees, castes, or life-stages may have indirect toxic effects on the overall social structure and function of a colony [84], we examined and correlated the effects of neonicotinoids at both the colony-level and the individual-level, as well as described the effects of neonicotinoids on worker adults and brood. Specifically, the objectives of this thesis were to:

1. Investigate effects of chronic neonicotinoid exposure on nucleus honey bee colonies in spring and summer
2. Investigate effects of chronic overwinter neonicotinoid exposure on honey bee colonies in the field and winter adult honey bee workers in the laboratory
3. Investigate effects of chronic neonicotinoid and fungicide exposure on adult honey bee workers and worker brood in the laboratory
4. Investigate *in vitro* effects of neonicotinoid and fungicide exposure on development of European foulbrood in honey bee worker larvae

Taken together, the combination of field and laboratory experiments in thesis were designed to provide data which may augment the risk assessment procedure for neonicotinoids and honey bees in Saskatchewan.

PREFACE TO CHAPTER 2

Each spring, packages of honey bees are imported from New Zealand by Saskatchewan beekeepers to replace colonies lost overwinter [93]. These packaged nucleus colonies are commonly installed in used brood chambers which contain previously stored Saskatchewan honey and beebread, known to have high concentrations and prevalence of neonicotinoid residues [3]. As summer progresses, these nucleus colonies are exposed to additional neonicotinoid residues during foraging on canola, one of the predominant crops in the Saskatchewan landscape, of which 95% is grown from neonicotinoid-treated seed [2].

To experimentally reproduce this exposure scenario, in Chapter 2, we performed a colony-level, artificial feeding study using New Zealand packaged bees which compared the effects of chronic exposure to three neonicotinoids: THI, CLO, and IMD. The experimental colonies consisted of 68 New Zealand packages which were installed in April and continuously exposed to 0, 20 nM (5 ng/g) or 80 nM (20 ng/g) of THI, CLO or IMD through pollen patties and sugar syrup over 12 weeks during spring/summer in Saskatchewan. During the study, we monitored colony weight gain, adult bee population, and brood area. Results from this chapter demonstrated that 9 and 12 weeks of 80 nM (20 ng/g) neonicotinoid exposure results in significant decreases in colony weight gain, reflecting decreased honey production of the colonies. Significant decreases in adult bee cluster size were also observed after 12 weeks of exposure to 20 ng/g neonicotinoids. This chapter highlights that nucleus colonies in Saskatchewan may experience decreases in performance and strength due to chronic exposure to high-environmental concentrations of neonicotinoids.

Chapter 2: **Comparative chronic toxicity of three neonicotinoids on New Zealand packaged honey bees**

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2.1 Abstract

THI, CLO, and IMD are the most commonly used neonicotinoid insecticides on the Canadian prairies [2]. There is widespread contamination of nectar and pollen with neonicotinoids, at concentrations which are sublethal for honey bees. We compared the effects of chronic, sublethal exposure to the three most commonly used neonicotinoids on honey bee colonies established from New Zealand packaged bees using colony weight gain, brood area, and population size as measures of colony performance. From May 7 to July 29, 2016 (12 weeks), sixty-eight colonies received weekly feedings of sugar syrup and pollen patties containing 0 nM, 20 nM (median environmental dose), or 80 nM (high environmental dose) of one of three neonicotinoids (THI, CLO, or IMD). Colonies were weighed at three-week intervals. Brood area and population size were determined from digital images of colonies at week 12. Statistical analyses were performed by ANOVA and mixed models.

We found a significant negative effect (-30%, $p<0.01$) on colony weight gain (honey production) after 9 and 12 weeks of exposure to 80 nM of THI, CLO, or IMD and on bee cluster size (-21%, $p<0.05$) after 12 weeks. Analysis of brood area and number of adult bees lacked adequate (>80%) statistical power to detect an effect. This study shows that chronic exposure of honey bees to high environmental doses of neonicotinoids has negative effects on honey production. Brood area appears to be less sensitive to detect sublethal effects of neonicotinoids.

2.2 Introduction

Neonicotinoids are the youngest, and arguably the most safe, effective, and widely used class of neuroactive insecticides worldwide [14,186]. THI, CLO, and IMD are the most commonly used neonicotinoid insecticides on the Canadian prairies where they are applied as a seed treatment to a variety of crops, including greater than 95% of canola [2,34]. When used as a prophylactic seed treatment, the neonicotinoid is taken up into the growing plant tissues where it has broad, long-lasting toxicity to a variety of insect pests which feed on these plants [14,186]. Neonicotinoids bind irreversibly, cumulatively [75] and with high affinity and specificity to the post-synaptic nAChRs within the central nervous system of insects, resulting in uncontrolled, excitatory depolarization of post-synaptic neurons [14,187]. Due to the presence of a negatively charged nitro or cyano group, neonicotinoids have a much lower affinity for mammalian nAChRs and corresponding low toxicity to humans [14,186]. Given their systemic nature, low doses of neonicotinoids are present in the nectar and pollen of crops grown from treated seed at mean

maximum concentrations of 1.9 ng/g (~7.6 nM) in nectar and 6.1 ng/g (~24.4 nM) in pollen [188], which are sublethal for honey bees. On a typical foraging trip to a neonicotinoid-treated field, the quantity of neonicotinoids in pollen or nectar gathered by a worker honey bee would represent only 1-3% of its acute oral LD_{50} [188]. Cucurbit crops, exposed to neonicotinoids through a foliar spray or water treatment, may have higher neonicotinoid residues up to 122 ng/g (~488 nM) in pollen and 17.6 ng/g (~70 nM) in nectar [189]. Reported neonicotinoid residues in honey and pollen vary with geographic region; within our local area of Saskatoon, Saskatchewan, mean CLO and THI residues in honey were 8.2 ng/g (~33 nM) and 17.2 ng/g (~69 nM), respectively, based on 26 samples of honey and 19 samples of bee bread from 7 independent apiaries [3]. Saskatoon apiaries have 5-10 times higher neonicotinoid contamination of honey compared to the worldwide average of 1.8 ng/g (7.2 nM) neonicotinoids in honey [24]. Neonicotinoid contamination of honey and bee bread from apiaries in the Saskatoon area is common; greater than 50% of honey and bee bread contains CLO, while THI is detectable in 75% of honey and 21% of bee bread [3]. Within North America, CLO, THI, and IMD were detected concurrently in at least 50% of honey samples [24]. Considering the widespread neonicotinoid contamination of nectar, pollen, and agricultural wetlands [2], as well as the extended half-lives of neonicotinoids in soil [62,63], it is incumbent upon society to use this class of insecticides prudently to minimize pest resistance and non-target effects on pollinators and aquatic invertebrates [14,190,191].

The need for neonicotinoids to safeguard the crop health must be balanced with our reliance on pollinators for 35% of food production worldwide [192]. Managed European honey bee colonies, while contributing the greatest economic value in terms of pollination services [192], suffer from a variety of stressors, including *Varroa destructor* mites; viral, fungal, bacterial, microsporidial, and protozoan pathogens [95]; lack of genetic variability; declining and less diverse bee forage; climate change; and increases in trade and migratory beekeeping [193]. The role of sublethal neonicotinoid exposure in honey bee colony dysfunction is controversial. Laboratory experiments frequently report negative effects of field-realistic neonicotinoid concentrations on individual honey bees, ranging from impaired olfactory learning and long-term memory of honey bee foragers [194] to reduced sperm counts in honey bee queens [195]. In contrast, field studies often conclude that honey bee colonies foraging on neonicotinoid-treated crops have no difference in performance or vitality from colonies in untreated control fields [64]. A recent multi-country field study of honey bee colonies foraging on oilseed rape grown from

neonicotinoid-treated seed found negative effects of CLO exposure on colony size and overwintering in some countries but not others [19]. Artificial feeding studies via sugar syrup and/or pollen patties are a method of controlled, colony-level exposure to neonicotinoids in the field. Outcome measures of these studies usually include colony-level inspection parameters such as brood area, adult bee population, colony weight, disease infestation, hygienic behaviour, pollen and honey stores, internal hive temperature and overwinter survival [13,17,165–168,196]. Results of artificial feeding studies have been mixed, with some authors observing high levels of overwinter mortality [165], others observing queen loss [13,17,166] and decreased social immunity [13]; and some studies finding minimal effects [167].

Despite conflicting scientific literature regarding neonicotinoids, some governments have gone ahead with policy decisions restricting their use. A ban on THI, CLO, and IMD has been in effect in the European Union since December 2013 for all agricultural crops which present a neonicotinoid-exposure risk for bees [20,184]. Similarly, the provincial government in Ontario, Canada encourages farmers to adopt pest management alternatives to prophylactic neonicotinoid treatment of corn and soybean seed by requiring farmers to complete training in integrated pest management and perform a pest risk assessment prior to purchase of neonicotinoid-treated seed [197]. Citing safety concerns for aquatic invertebrates, the PMRA of the Canadian federal government continues to reassess the regulation of THI, CLO, and IMD in Canada [72].

Assessment of the impact these neonicotinoid regulations have had on farmers is ongoing, with an estimated income loss of 880 million EUR per year for the oilseed rape industry due to decreased yields and increased production costs [198], while a more recent, three-year study found that the absence of neonicotinoid seed-treatments had negligible effects on yield and profit of oilseed rape in two out of three years [199]. Considering the potential economic impact of neonicotinoid-use restrictions, there is a need for more reliable scientific evidence regarding the effects of neonicotinoids on honey bee colonies to justify these government policies.

The artificial feeding study presented here aims to address some of the deficiencies identified in the literature on sublethal neonicotinoid exposure of honey bees, including a lack of chronic, colony-level studies which utilize rapidly growing spring nucleus colonies and compare the effects of multiple neonicotinoids [162]. Chronic neonicotinoid toxicity studies are important to detect delayed sublethal effects on honey bee colonies because as length of neonicotinoid exposure time increases, the dose required to induce toxic effects decreases, in part due to the

irreversible binding of neonicotinoids to nAChRs [75]. Surprisingly, there is a lack of protocols for chronic, sublethal neonicotinoid testing in honey bees to guide pesticide risk assessment by regulatory agencies and policy-makers [200,201]. Existing regulatory guidelines for examining the chronic effects of pesticides on honey bee colonies specify a maximum 7 day pesticide exposure followed by a minimum colony observation period of 19-21 days [202,203]. However, a Canadian study of honey bee colonies foraging near corn fields demonstrated that bees may experience continuous exposure to neonicotinoids through pollen for up to 4 months [13], suggesting that the current exposure times used in pesticide risk assessment may be insufficient. There is also a paucity of data on the sublethal effects of neonicotinoids on nucleus colonies, such as packaged bees, which are commonly used for hive replacement. These nucleus colonies may be more susceptible to the chronic, sublethal effects of neonicotinoids, as larger colonies are better able to detoxify and dilute pesticides within the hive [204].

The aim of our study was to compare the colony-level effects of chronic, sublethal exposure to the three most commonly used neonicotinoids (THI, CLO, IMD) on New Zealand packaged honey bees during spring and summer by measuring colony weight gain, capped brood area, and population size.

2.3 Materials and Methods

2.3.1 Experimental colonies

Sixty-eight, one kg packages of honey bees, each with a queen of unknown genetic lineage, were obtained from Kintail Honey (Takapau, New Zealand) in association with Apiflora NZ Ltd (Tauranga, New Zealand) and were installed on April 25, 2016 at the Western College of Veterinary Medicine (WCVM) Goodale Research and Teaching Farm (52°01'50.6"N 106°32'26.6"W) within an approximately 0.2 km^2 area within an alfalfa field surrounded by pasture and fields of canola and cereals. Permission was obtained from the WCVM Associate Dean's Office for utilization of this study site. The colonies were installed in Langstroth standard (full depth) supers containing 10 Langstroth frames with plastic foundation and covered with a wooden lid with a central hole for feeding. The colonies were placed on leveled wooden pallets in groups of 2-4 with the pallets arranged several meters apart.

2.3.2 Preparation

One and a half weeks prior to the start of the trial, the colonies were treated with oxytetracycline (Oxytet-25, Medivet Pharmaceuticals Ltd., High River, Alberta, Canada) and

fumagillin (Fumagilin-B, Medivet Pharmaceuticals Ltd., High River, Alberta, Canada) in accordance with package instructions. All colonies were checked for the presence of a laying queen and the queen was marked. The colonies were provided with *ad libitum* 1:1 (w:w) sugar syrup and pollen patty (Ultra Bee Patties, Mann Lake Ltd., Hackensack, MN, USA) until the initiation of the trial. All colonies had 1.5-2 frames of brood at the start of the experiment and were thus considered uniform in colony strength. A plastic, front-mounted pollen trap (BeeMaid Honey, Winnipeg, MB, Canada) was placed on all colonies on May 13, 2016 (second week of the study) to coincide with the initiation of experimental pollen patty feeding .

2.3.3 Experimental diet

100 ng/µl stock solutions of THI (99.6% purity; 37924, PESTANAL®, Sigma-Aldrich, Oakville, Ontario), IMD (99.9% purity; 37894, PESTANAL®, Sigma-Aldrich, Oakville, Ontario), and CLO (99.9% purity; 33589, PESTANAL®, Sigma-Aldrich, Oakville, Ontario) were prepared in distilled water. Pollen patties were prepared from a mixture of 1 kg soybean flour and brewer's yeast (3:2 ratio), 1.3-2 liters 2:1 (w:w) sucrose syrup and 0.5-2 kg (15.8-39.5% final patty weight) pollen obtained from pollen traps placed on colonies in 2015 and 2016. The quantity of pollen in the patties was increased over the course of the trial to satisfy the nutritional requirements of growing colonies. The neonicotinoid stock solutions were diluted in sucrose syrup (1:1 w:w) to a concentration of either 20 nmol/L (~5 ng/g) or 80 nmol/L (~20 ng/g). Neonicotinoid concentrations were calculated in molarity to obtain an equal number of molecules of each neonicotinoid in the experimental diet. Aliquots of the pollen patties and sugar solutions from treatment and control groups were submitted for measurement of neonicotinoid concentration by LC-MS/MS (Government of Alberta Agriculture and Forestry Chemistry Laboratory, Edmonton, Alberta).

2.3.4 Study design

The colonies were randomized into two treatment groups (20 nM, and 80 nM) for each of the three neonicotinoids (IMD, CLO, and THI) and a control group, with nine colonies per treatment group and fourteen control colonies. The results of a 2015 pilot study of colony weight gain in response to sublethal THI exposure were used to calculate the sample size for this study to achieve a power of 80% with a 95% confidence interval (OpenEpi, Version 3, open source calculator—SSMean).

Beginning May 7, 2016 until July 29, 2016 (12 weeks), 2.3 kg of experimental syrup was top fed to each colony per week using glass mason jars wrapped in tinfoil and covered by plastic pail (to protect from UV light) with three holes in each lid. Experimental pollen patty feeding began a week later, on May 13, 2016 until July 29, 2016. Pollen patties were changed weekly until the week of July 11, 2016 when biweekly patty feeding commenced to keep pace with colony consumption. The weight of pollen patties fed to the colonies was adjusted based on consumption of patties each week. Initially 300 g patties were provided, followed by a decrease to 180 g patties the week of May 20, a subsequent increase to 260 g patties the week of June 17, and a final increase to 500 g patties the week of July 1. The unconsumed syrup and patties were weighed at the end of each week to calculate total feed consumption per colony and total exposure to neonicotinoids expressed in micromoles.

The outcome measures for the trial included colony weight (as an estimate of honey production), brood area (as an estimate of reproduction), and number of adult bees and cluster size of adult bees (as an estimate of population size).

2.3.5 Methods of Measurement

The initial weight of the experimental colonies was measured on May 6, 2016 prior to exposure to neonicotinoids. The colonies were subsequently weighed at three-week intervals throughout the trial (weeks of May 23 [week 3], June 13 [week 6], July 4 [week 9], and July 25 [week 12]). The colonies were weighed in the early morning to maximize the number of bees within the hive, using a mechanical hanging scale (Salter Model 235, Brecknell Scales, Fairmont, MN, USA) with an accuracy to the nearest 0.5 kg. The final colony weights were corrected for any additional supers and frames used for colony expansion

At week 12, digital images of both sides of all drawn frames in each colony were obtained using a 16.2 megapixel Nikon D7000 digital camera (Minato, Tokyo, Japan) mounted on a tripod with a Nikon 18-105 mm lens, a Nikon SU-800 wireless speedlight commander, Nikon SB-R200 wireless remote speedlights and a covered photo box made of white corrugated plastic (0.6 meters in length and slightly wider and higher than a single Langstroth frame). Each frame was placed in the photo box prior to image capture to standardize lighting conditions independently of the ambient lighting. The photos were taken in the morning to maximize the number of bees present inside each colony. The total area of capped brood for each frame was calculated from the photos based on the capped brood area detected by the HoneyBee Complete software (version 4.2, WSC

Scientific GmbH, Heidelberg, Germany) using the auto-recognition function for capped brood and summing the result for all frames in each colony. Adult bees were not brushed from the frames prior to taking photos for brood recognition; however, the brood area was outlined manually for each frame, prior to auto-recognition of capped cells with the HoneyBee Complete software. For photo analysis of number of adult bees, the number of adult bees auto-recognized by the HoneyBee Complete software (version 5.4, WSC Scientific GmbH, Heidelberg, Germany) per frame was summed for each colony. Non-drawn frames were excluded from the analysis. For partially drawn frames, auto-recognition was limited to the areas of the frame with drawn wax. Percent recognition (number of auto-recognized bees/actual number of bees x 100) of the software calculated for four different types of frames (uncapped honey, capped honey, open brood, capped brood) was 63, 64, 82, and 69 percent, respectively. The software underestimation was assumed to be the same across all colonies as photo conditions were consistent (same photo box and camera settings) for all colonies.

Cluster size as a measure of population size [205] was visually assessed at week 6 and week 12. Early in the morning, when bees were still clustered due to lower night temperatures in the prairies, a photo was taken of the top (week 6) or top and bottom (week 12) of each super. The number of interframe spaces (maximum of 11 for a 10 frame super) occupied by adult bees was determined visually to the nearest 0.25 for each super and summed for each colony at week 6. At week 12 the number of interframe spaces occupied on the top and bottom of each super was averaged and the averaged values summed for each colony. Repeatability of the cluster size measurements was assessed for a random sample of 25% of the colonies at week 12 and the Pearson Correlation Coefficient identified a high correlation between the repeated cluster size assessments ($r(14) = 0.9947$, $p<0.001$).

2.3.6 Analysis of Data

Five colonies were excluded from analysis due to queen failure, including one colony from each of the 80 nM THI, 20 nM THI, and 20 nM CLO groups and two colonies from the control group, resulting in a sample size of 63. The twelve remaining control colonies were randomly assigned into the CLO, IMD, and THI groups for all factorial ANOVA analyses. All statistical analyses were performed using Stata/SE 14.2 or 15(College Station, TX). Normality was assessed using Shapiro-Wilk W test. Equality of variances was assessed using Bartlett's test for equal variances or a two-sample variance comparison test. Data were presented as mean ± standard

deviation (SD). Colony weight gain data were interpreted in two ways: (1) cumulative colony weight gain, defined as the difference between the colony weight at a given time point and the initial colony weight prior to the trial, and (2) three-week weight gain, defined as the difference between the colony weight at the indicated week and the colony weight three weeks prior. Similarly, consumption of syrup or pollen patty was analyzed as (1) cumulative consumption per colony over twelve weeks, and (2) weekly consumption per colony. Cumulative colony weight gain, capped brood area, number of adult bees, and cluster size data were analyzed with a 3x3 (dose x neonicotinoid) factorial ANOVA and a Bonferroni multiple comparison test with a p value <0.05 considered significant. Additional analysis of cumulative colony weight gain, as well as analysis of cumulative syrup and patty consumption were performed using a one-way ANOVA with a p value <0.05 considered significant. Specific post-hoc comparisons of the six neonicotinoid-dose treatment groups to the control were performed using a two-sample t-test with equal variances and a p value <0.01 considered significant, based on a Bonferroni correction for six comparisons [206].

Three-week colony weight gain and weekly syrup and patty consumption were analyzed with repeated measures mixed models with restricted maximum likelihood (reml) regression and a p value <0.05 considered significant. The model for three-week weight gain used an unstructured covariance matrix while the model for weekly consumption used an ar1 covariance matrix. The model for three-week weight gain was limited to weeks 6-12 and population size was included in the model by using the cluster size at week 6 as an estimate of the population at weeks 6 and 9 and the cluster size at week 12 as an estimate of the population at week 12. Specific post-hoc comparisons to the control group were performed using pairwise comparisons of predictive margins at chosen time intervals with a p value <0.01 considered significant.

Where no significant difference was observed, statistical power for comparing two means was calculated by the normal approximation method with a two-sided 95% confidence interval (OpenEpi, Version 3, open source calculator—PowerMean). A minimum of 80% statistical power to detect a difference was considered adequate.

2.4 Results

2.4.1 Colony Weight Gain

Colony weight gain data were analyzed by three different, although corroborating, methods including factorial and one-way ANOVA of cumulative weight gain data, and analysis of three-

week weight gain data using a mixed model which accounted for differences in population size of the colonies. As described below, all analyses confirmed a significant negative effect of 80 nM doses of neonicotinoids on colony weight gain at weeks 9 and 12 with the 80 nM CLO group most frequently identified as the group with the lowest weight gain compared to the controls. Factorial ANOVA identified a significant effect of dose on cumulative weight gain at week 9 ($F_{2,54}$=7.59, p=0.0012) and week 12 ($F_{2,54}$=5.79, p=0.0053) with a nonsignificant interaction between dose and neonicotinoid treatment ($F_{4,54}$=0.41, p=0.8021 at week 9 and $F_{4,54}$=0.74, p=0.5711 at week 12), indicating that the effect of dose on weight gain was the same for all neonicotinoids tested. Colonies exposed to an 80 nM dose of IMD, THI, or CLO had a 30.2% and 31.8% decrease in cumulative weight gain compared to the controls at weeks 9 and 12 respectively (p=0.002 at week 9 and p=0.005 at week 12) (Figure 2.1A). Alternatively, analyzing cumulative weight gain data with a one-way ANOVA which compared individual neonicotinoid-dose treatments, significant differences in cumulative weight gain were identified at week 9 ($F_{6,56}$=3.03, p=0.0124) and week 12 ($F_{6,56}$=2.82, p=0.018). Compared to controls, cumulative colony weight gain was 31.9% and 37.8% lower after 9 and 12 weeks of exposure to 80 nM CLO, respectively, (Figure 2.2A [t_{19}= -3.3056, p=0.0037 at week 9] [t_{19} = -3.7956, p=0.0012 at week 12]) and 28.9% lower after 9 weeks of exposure to 80 nM IMD (Figure 2.2B [t_{19} = -3.0597, p=0.0064]). Although statistically nonsignificant, colonies exposed to 80 nM IMD, 20 nM THI, and 80 nM THI for twelve weeks gained 28.3% (Figure 2.2B[t_{19}=-2.7982, p=0.0115]), 31.2% (Fig 2C[t_{18}=-2.4830, p=0.0231]), and 28.9% (Figure 2.2C[t_{18}=-2.2069, p=0.0405]) less weight, respectively, compared to controls. At week 12, there was inadequate statistical power to detect a statistical difference in cumulative weight gain from control for the experimental groups treated with either 20 nM CLO, 20 nM IMD, 20 nM THI, or 80 nM THI. Mixed model analysis of three-week weight gain identified a significant interaction between neonicotinoid-dose treatment and time (χ^2_{12}=22.24, p=0.0349), indicating that the effect of neonicotinoid-dose treatment on colony weight gain changed over time. Post-hoc analysis identified a significant difference of predicted three-week weight gain between the control and 80 nM CLO group from weeks 7-9 and weeks 10-12 (p=0.002, p=0.005).

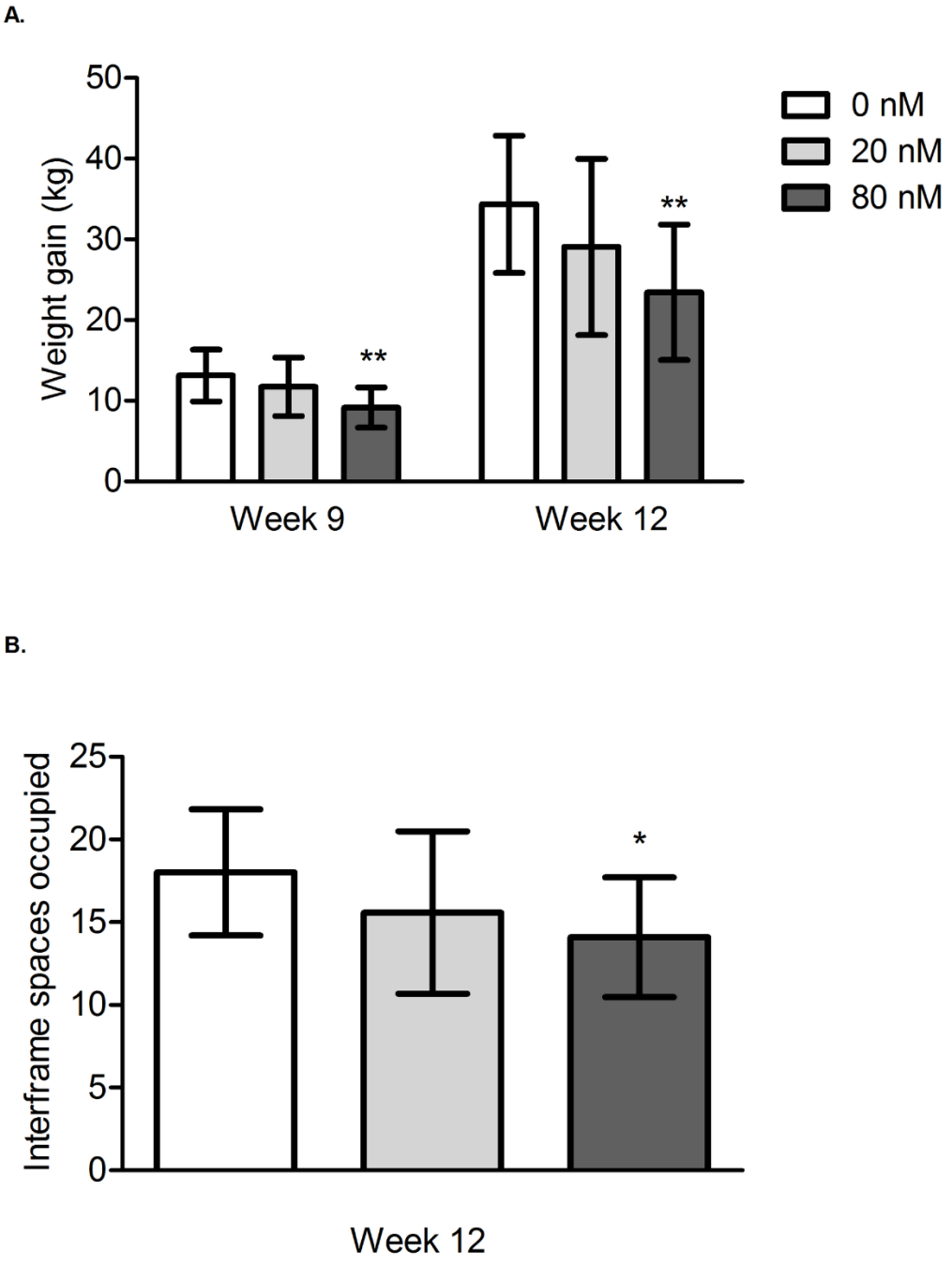

Figure 2.1 Chronic, sublethal neonicotinoid exposure decreases cumulative weight gain and cluster size of honey bee colonies. (A) Decrease in colony weight gain after exposure to 80 nM neonicotinoids for nine and twelve weeks and (B) decrease in colony cluster size after exposure to 80 nM neonicotinoids for twelve weeks. Treatment colonies were exposed to CLO, IMD, or THI, at 20 or 80 nanomolar concentrations. The bars show mean cumulative colony weight gain (A) or cluster size (B) ± SD for each neonicotinoid dose group, which includes all three neonicotinoids tested. **significantly different from control, $P<0.01$. * significantly different from control, $P<0.05$.

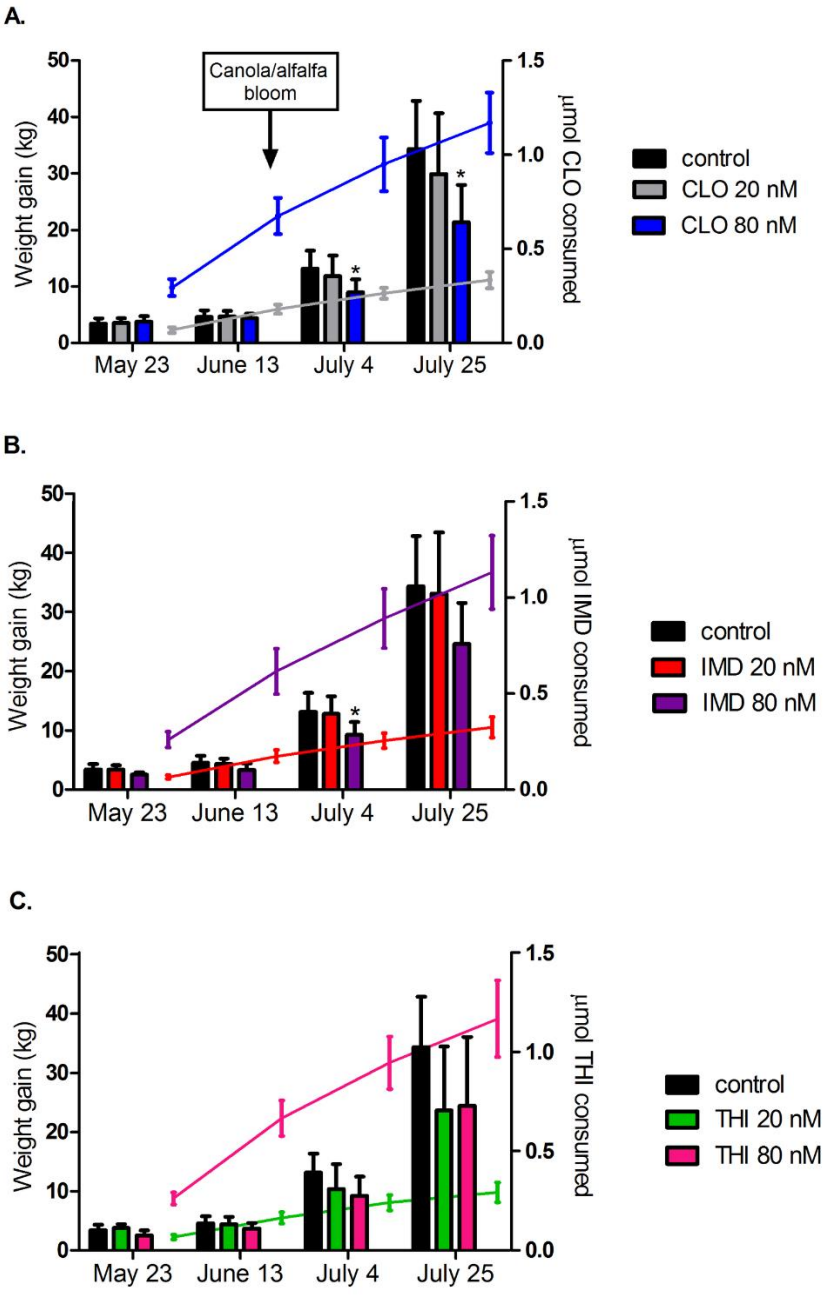

Figure 2.2 Cumulative weight gain of colonies exposed to sublethal doses of individual neonicotinoids for twelve weeks. Treatment colonies were exposed to CLO (A), IMD (B), or THI (C) at 20 or 80 nanomolar concentrations. Colonies exposed to 80 nM CLO (A) and 80 nM IMD (B) demonstrated significant decreases in weight gain from controls at weeks 9 and 12 and week 9, respectively. The bars show mean cumulative colony weight gain ± SD for each group (left y-axis). The curves show mean cumulative consumption of neonicotinoid per colony ± SD in micromoles for the treatment groups (right y-axis). * significantly different from control, $P<0.01$. The timing of the canola and alfalfa bloom surrounding the study site is indicated (A).

2.4.2 Population size and brood area

Twelve-week exposure to 80 nM neonicotinoids had a significant negative effect on cluster size of the colonies (Figure 2.1B), although these differences were not observed in the total adult bee counts or capped brood area. There was a significant effect of dose on cluster size at week 12 ($F_{2,54}$=3.62, p=0.0336) with a nonsignificant interaction between dose and neonicotinoid treatment ($F_{4,54}$=0.31, p=0.8705), indicating that the effect of dose on cluster size was the same for all neonicotinoids tested. Colonies exposed to an 80 nM dose had a 21.7% reduction in cluster size (p=0.03) compared to the controls at week 12 (Figure 2.1B). There was no significant effect of dose ($F_{2,54}$=1.78, p=0.1781) or neonicotinoid treatment ($F_{2,54}$=1.39, p=0.2573) on the number of adult bees at week 12. Although statistically nonsignificant, colonies exposed to an 80 nM dose had 16.7% fewer adult bees compared to the controls at week 12. Similarly, sublethal exposure to neonicotinoids did not have a significant effect on capped brood area after twelve weeks with no significant effect of dose ($F_{2,54}$=0.61, p=0.5497) or neonicotinoid treatment ($F_{2,54}$=0.96, p=0.3898) (Figure 2.3). However, analysis of both number of adult bees and capped brood area at week 12 lacked adequate (>80%) statistical power to detect an effect.

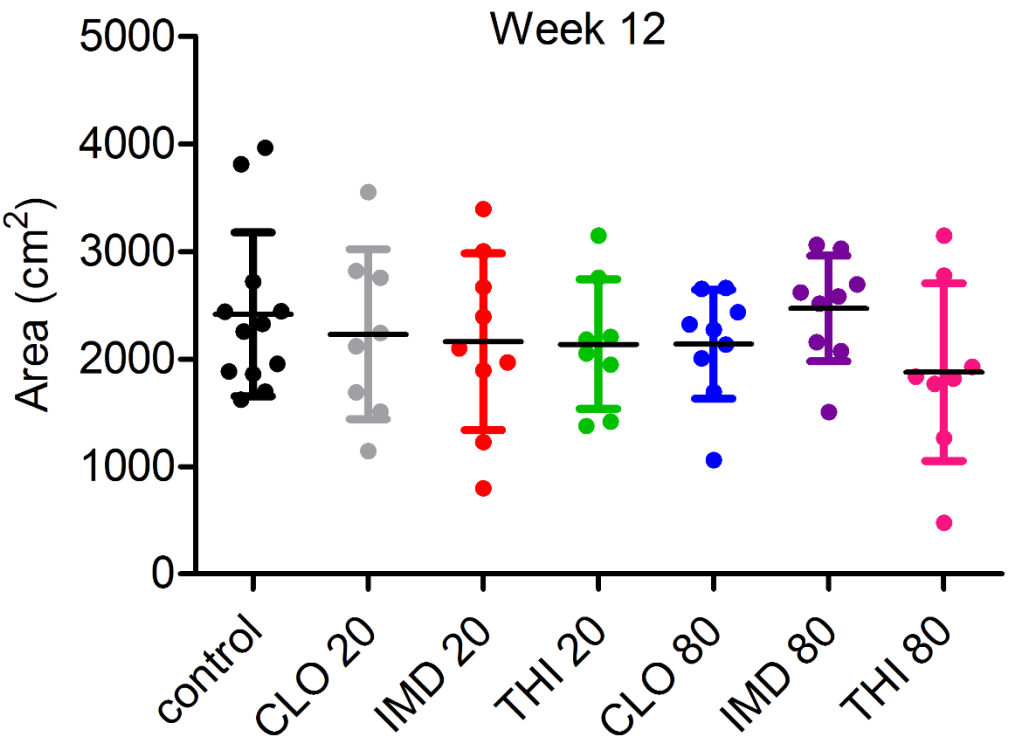

Figure 2.3 Capped brood area of colonies exposed to sublethal doses of neonicotinoid for twelve weeks. Treatment colonies were exposed for twelve weeks to CLO, IMD, or THI at 20 or 80 nanomolar concentrations. Brood area was quantified by analysis of digital images of brood frames with brood recognition software. There was no statistical difference among experimental groups but analyses lacked adequate (>80%) statistical power due to high variability. Mean ± SD are indicated for each group.

2.4.3 Neonicotinoid consumption

The LC-MS/MS measured concentrations of neonicotinoids in experimental pollen patties and sugar syrup were on average 30% (SD 20.4 %) below calculated concentrations in the experimental pollen patties and 0.075% (SD 18.6 %) below calculated concentrations in the sugar syrup (Table A2.1, Table A2.2). A low level of THI contamination (~1 ng/g) was detected in one of each of the 80 nM CLO and 20 nM IMD pollen patty samples likely due to the addition of natural pollen in the experimental patties (Table A2.1). Colony consumption of pollen patties and sugar syrup was analyzed by two complementary methods: (1) one-way ANOVA of cumulative consumption over the entire twelve week exposure period, and (2) mixed model analysis of weekly consumption over twelve weeks. Cumulative consumption of syrup (Figure 2.4B) and pollen patty (Figure 2.4D [$F_{6,56}$=1.27, p=0.2859]) was comparable for all experimental groups with the

exception of colonies exposed to 20 nM THI consuming 18.2% (2.98 kg) less syrup compared to controls ($F_{6,56}$=2.5, p=0.0325; t_{18}=2.9046, p=0.0095). There was no significant difference in weekly consumption of sugar syrup among the 20 nM, 80 nM and control groups (Figure 2.4A [χ^2_2=4.81, p=0.0901]); however, analysis of weekly patty consumption revealed a significant interaction between neonicotinoid dose (0, 20 or 80 nM) and week (Figure 2.4C [χ^2_{20}=61.74, p<0.001]), indicating that the effect of neonicotinoid dose on patty consumption was not constant over time. Colonies exposed to 80 nM neonicotinoids consumed 20.5% (154.2 g), 17.2% (130.9 g), and 14.5% (108.5 g) less patty than control colonies at weeks 10, 11, and 12 respectively (Figure 2.4C [p<0.001 at week 10, p<0.001 at week 11, and p=0.002 at week 12]). There was a marked decline in syrup consumption at week eight coinciding with the bloom of canola and alfalfa in the surrounding environment and widespread availability of nectar (Figure 2.4A). At the same time, consumption of pollen patties increased rapidly in all colonies due to colony growth (Figure 2.4C). The installation of pollen traps on each colony promoted consumption of the experimental patties instead of pollen from the environment during colony expansion.

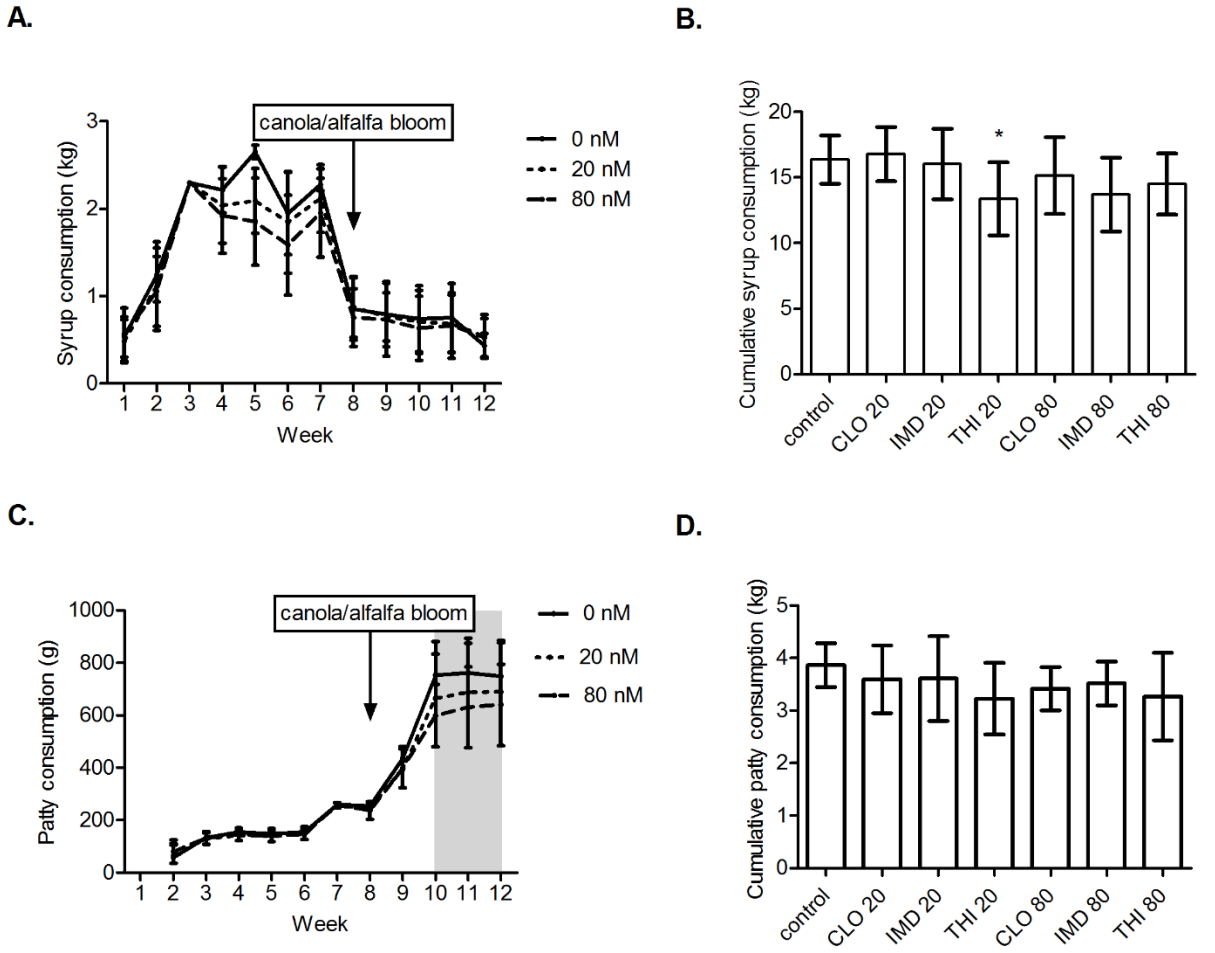

Figure 2.4 Weekly and cumulative feed consumption per colony over 12 weeks. Over twelve weeks, cumulative consumption of syrup (B) and pollen patty (D) was comparable for all experimental groups with the exception of colonies exposed to 20 nM THI consuming 18.2% (2.98 kg) less syrup compared to controls. Shaded area indicates significant differences ($P<0.01$) in weekly pollen patty (C) consumption between control colonies and colonies exposed to 80 nM neonicotinoids. Treatment colonies were exposed to CLO, IMD, or THI at 20 or 80 nanomolar concentrations. Mean weekly (A, C) or cumulative (B, D) consumption per colony ± SD is indicated for each group. * significantly different from control, $P<0.01$. The timing of the canola and alfalfa bloom surrounding the study site is indicated (A, C).

2.5 Discussion

To our knowledge, this is the first colony-level study comparing sublethal effects of three neonicotinoids on honey bees. Similar to the colony-level artificial feeding study by Dively *et al*. [166] and Tsvetkov *et al.* [13] we chose a chronic, twelve-week exposure period, with sublethal neonicotinoid doses of 20 nM (~5 ng/g) and 80 nM (~20 ng/g), representing mid- and high-range, environmentally realistic concentrations, respectively [166,196]. The chosen neonicotinoid doses are similar to the calculated minimum and maximum residues of CLO [5.95 ng/g (~23.8 nM) and 19.04 ng/g (76.2 nM)] and THI [4.592 ng/g (~18.4 nM) and 19.29 ng/g (~77.2 nM)] in the pollen of oilseed rape grown from treated seed [71,207]. Our major finding was that chronic exposure of honey bee colonies to high environmental doses of neonicotinoids decreased colony weight gain by 30% compared to controls, which reflects predominantly honey production of the colonies (Figure 2.1, Figure 2.2). Similarly, Sandrock et al. [17] found that colonies chronically exposed to 2 ng/g (~8 nM) and 5 ng/g (~20 nM) CLO and THI in pollen patties collected significantly less pollen and produced 29% less honey; however, Faucon *et al.* [167] found no significant difference in weight gain of colonies fed syrup with 0, 0.5 ng/ml (~2 nM), and 5 ng/ml (~20 nM) IMD.

Decreased foraging activity, navigational ability, and longevity of worker honey bees [13,175,208,209] of treatment colonies due to neurotoxic effects of neonicotinoids could explain the decreased honey production by the nucleus colonies exposed to 80 nM (equivalent to ~ 20 ng/g) neonicotinoids in our study. Correspondingly, Wu-Smart and Spivak [204] found that small colonies of 3000-7000 bees exposed to 10, 20, 50, and 100 ng/g (~40, 80, 200, and 400 nM) IMD foraged less, although no difference in honey and nectar stores was observed, possibly due to the smaller size of the colonies, and the shorter experimental duration (3 weeks) compared to our study. At concentrations of 10 nM, IMD and CLO have been shown to inhibit the activity of Kenyon cells, neurons of the honey bee brain which are important in sensory processing for effective pollen and nectar collection [210]. Thus, the workers exposed to 80 nM neonicotinoids in our study may have had difficulty distinguishing and remembering floral odors, reducing their foraging success [194]. We cannot rule out that the presence of pollen traps may have weakened our experimental colonies; however, both treatment and control colonies experienced the same pollen restrictions to decrease potential dilution effects in the experimental diets. The 1 kg New Zealand packages of bees used in our study may have been more susceptible to negative effects of neonicotinoid exposure initially due to their small size. Had stronger colonies been used, a negative

effect of neonicotinoid exposure on colony weight gain may not have been observed, as larger colonies may be better able to compensate for colony stress. Furthermore, unknown intake of neonicotinoids in nectar from surrounding fields is a limitation of this, and many other field studies. However, both treatment and control colonies had similar access to surrounding fields, and thus, treatment differences were attributed to the experimental diets, rather than neonicotinoid contamination from natural nectar sources. The use of natural pollen in our experimental pollen patties may also have been a source of neonicotinoid contamination (Table A2.1); however, both treatment and control patties were prepared from the same source of natural pollen.

Nine to twelve weeks of sublethal exposure to neonicotinoids was required before a significant difference in colony weight gain was observed (Figure 2.1, Figure 2.2). This effect was only observed at the higher end of environmentally realistic dosages (80 nM) for IMD at week 9 (Figure 2.2B) and for CLO at weeks 9 and 12 (Figure 2.2A). At week 12, the colonies exposed to the mid- and high-range sublethal concentrations of THI experienced a statistically nonsignificant, approximately 30% lower cumulative weight gain (Figure 2.2C) compared to the control group; however, there was inadequate statistical power to detect a difference from the control. Future studies with larger sample sizes or longer exposure duration would be desirable to confirm whether or not THI significantly impacts colony weight gain and honey production. Significant differences in colony weight gain among treatment and control colonies coincided with the bloom of alfalfa and canola in the surrounding environment. Rapid growth and increased foraging by the colonies in response to widespread availability of nectar may have allowed treatment differences to become more apparent. Thus, timing (for example, during nectar flow), rather than duration of exposure to neonicotinoids may be more important when designing colony-level exposure trials. Consumption of experimental syrup decreased during nectar flow (~week 8), resulting in decreased neonicotinoid exposure of treatment colonies despite an increase in pollen patty consumption (Figure 2.4). On average, colonies experienced a 25% decrease in total micromoles of neonicotinoid consumed per three-week interval from weeks 4-6 to weeks 7-9 (Figure 2.2).

Of the six neonicotinoid-dose combinations tested, colonies treated with 80 nM CLO experienced the greatest decrease in colony weight gain compared to controls, demonstrating 32% and 38% lower cumulative colony weight gain compared to controls after 9 and 12 weeks of exposure (Figure 2.2A). CLO has been shown experimentally to cause greater stimulation of the insect nAChR than IMD and cause larger neuronal depolarizations [210,211], possibly explaining

it's more profound colony-level effects on weight gain. CLO also has the lowest acute 24-hour oral toxicity dose (3.35 ng/honey bee), followed by THI (4.4 ng/bee), and IMD (118.74 ng/bee) [59]. THI might be expected to have similar colony level effects as CLO, considering that THI is metabolized to CLO in insect and plant tissues [50]. In our study, the THI treated colonies, unlike the CLO treatment groups, did not demonstrate significant differences in colony weight gain compared to controls; however, our analysis lacked adequate statistical power to detect a difference. Although statistically nonsignificant, colonies exposed to 20 nM and 80 nM THI had 31% and 29% lower cumulative weight gain, respectively, compared to controls at week 12 (Figure 2.2C).

After twelve weeks of sublethal exposure to 80 nM of THI, CLO, or IMD, the adult bee cluster occupied 3.91 fewer interframe spaces in exposed colonies compared to controls (Figure 2.1B); however, unequivocal effects of neonicotinoid exposure on capped brood area were not demonstrated (Figure 2.3). Although the 80 nM-treated colonies exhibited decreases in both cluster size and adult bee numbers compared to controls; only the decrease in cluster size was statistically significant ($p=0.03$). Inaccuracy of the software used for adult bee detection likely confounded analysis. Lack of statistical power further hindered characterization of population size and brood area in treatment groups compared to controls. Decreases in cluster size associated with exposure to 80 nM neonicotinoids could be explained by shortened lifespan of adult workers secondary to sublethal pesticide exposure during development in the brood comb [13,209]. Decreased life expectancy and higher rates of forager loss as a result of compromised navigational ability [175,208] could have a compounding negative effect on colony population size due to disruption of colony polyethism and resultant reduction in nurse bees available for brood care [13,17,209]. At peak consumption of the experimental diet, colonies exposed to 80 nM neonicotinoids consumed significantly less pollen patty compared to control colonies (131.2 g less pollen patty on average from weeks 10-12 [Figure 2.4C]). This could be partially explained by the decreased population size (as estimated by cluster size) of the 80 nM exposed colonies. Decreased consumption of the experimental patties by treatment colonies is unlikely to be the result of an 'antifeedant effect' of sublethal neonicotinoid doses based on published field and laboratory studies [167,196,212]. Similarly, we could not demonstrate differences in syrup consumption by colonies simultaneously offered four doses (0, 4, 40, and 400 nM) of one of three neonicotinoids (IMD, CLO, or THI) in syrup (unpublished data). Differences in mean cumulative syrup

consumption per colony between treatment group and control groups (maximum difference of 2.99 kg between controls and colonies exposed to 20 nM THI [Figure 2.4B]) were not large enough to explain the differences in cumulative weight gain observed in the treatment colonies compared to controls (Figure 2.1, Figure 2.2).

The significant decrease in cluster size after 12 weeks of 80 nM neonicotinoid exposure in our study contrasts with the absence of an observable effect of sublethal neonicotinoid exposure on brood area, and further contributes to the often conflicting results of previously published artificial feeding studies of IMD to honey bee colonies. Dively *et al.* [166] exposed colonies to 5, 20, and 100 ng/g (~20, 80, and 400 nM) IMD in pollen patties for 12 weeks and found no difference in capped brood or population size associated with treatment. Similarly, Meikle *et al.* [196] demonstrated no difference in capped brood area among colonies exposed to 5 and 20 ppb (~20 and 80 nM) IMD for 6 weeks in sugar syrup, although colonies exposed to the environmentally unrealistic dose of 100 ppb (~400 nM) IMD had a significant decrease in brood area. Faucon *et al.* [167] exposed strong colonies to 0.5 ng/ml and 5 ng/ml (~2 and 20 nM) IMD in sugar syrup for 34 days and found no difference in cluster size or capped brood during the experiment. In contrast, Wu-Smart and Spivak [204] found that exposure of small colonies (<10 000 bees) to 20, 50 or 100 ppb (~80, 200, and 400 nM) of IMD in sugar syrup for three weeks had a negative impact on brood quantity and pattern. Sandrock *et al.* [17] found that strong colonies exposed to a combination of 5 ppb (~20 nM) THI and 2 ppb (~8 nM) CLO in pollen patties for 46 days had 13% less brood and 28% fewer adult bees compared to controls at the end of the exposure period. The social organization of honey bee colonies allows them to be resilient to stress [166]. The addition of multiple, concurrent stressors, such as cold temperature or disease, may exacerbate sublethal effects of neonicotinoids on brood area or population size, explaining the often incongruent findings reported in the literature [19,188]. There may have been a qualitative effect on brood area in the neonicotinoid-exposed colonies in our study; however, this sublethal reproductive effect may have been obscured by increased investment in brood production by exposed colonies [162,164,188]. Furthermore, high variation in the brood area data led to a lack of statistical power and higher than accepted probability of type II error. Prior to the start of our study, both treatment and control colonies received oxytetracycline treatment in accordance with recommended beekeeping practice for installation of New Zealand packaged bees in Canada. Oxytetracycline has been shown to cause significant elevations in brood mortality [213] as well as

decreased diversity of the honey bee gut microbiome leading to increased susceptibility to opportunistic pathogens [214]. In our study, any negative effects of oxytetracycline treatment on colony weight gain, brood area or population size and health should have been experienced equally by control and treatment colonies. Considering that the majority of honey bee colonies in North America receive antibiotic metaphylaxis for AFB, the oxytetracycline treatment of our study colonies is representative of the iatrogenic stress experienced by most North American honey bees. It is important to note that administration of antibiotics to honey bees is not permitted in some jurisdictions outside of North America (e.g. European Union).

One of the major observations in our study was the large amount of variation in our experimental colonies which undermined the statistical power of the colony weight gain, brood area and population size analyses. New Zealand packages were chosen as experimental colonies to standardize colony strength at the beginning of the study; however, the colonies did not have sister queens, introducing genetic variation among colonies. Sandrock *et al.* [17] found that colonies from different genetic lineages of *A. mellifera* differed in their susceptibility to chronic exposure of THI and CLO. Variability in colony genetics could influence the ability of individual bees to detoxify neonicotinoids [167]. Our initial sample size of nine colonies per treatment group was chosen based on results of a previous pilot study of THI exposure on colony weight gain and was similar to the sample size of other artificial feeding studies [166,167]; however, some authors have recommended three to nine times greater samples sizes to compensate for the inherent variability of honey bee colonies [215–217]. Lack of statistical power is not a problem unique to our study and is present in many other studies of sublethal effects of neonicotinoids [162,216], emphasizing the need for more sensitive and specific tests [216,217].

2.6 Conclusion

Similar to other studies [166], we found largely no effect on colony performance at the mid-range doses (20 nM or ~5 ng/g) of neonicotinoids present in the environment. Negative effects of sublethal exposure to neonicotinoids on honey production and cluster size were observed only after 9-12 weeks of exposure to the higher-end of environmentally realistic concentrations (80 nM or ~20 ng/g). Although concentrations of 80 nM neonicotinoids have been documented in honey samples from our local area [3], this concentration is 10 times higher than the average maximum neonicotinoid concentrations in nectar based on a review by Godfray *et al.* [188]. Production of a commodity, in this case honey, is a common method to assess toxicity in food

producing species, such as monitoring milk production to understand ergot toxicity in dairy cattle [218]. Honey production is also an economically relevant parameter for beekeepers and for farmers who rely on the foraging activity of honey bees for crop pollination [162]. The significant differences in weight gain observed in the colonies exposed to 80 nM neonicotinoids in our study suggest that honey production is a useful colony-level parameter to estimate sublethal neonicotinoid exposure in honey bees.

PREFACE TO CHAPTER 3

In Chapter 2 we showed that chronic, 20 ng/g neonicotinoid exposure during spring and summer decreased weight gain and cluster size of nucleus colonies in Saskatchewan, with no significant effect of chronic exposure to 5 ng/g neonicotinoids. Chapter 3 focuses on the effects of chronic 5, 10, 20, and 100 ng/g neonicotinoid exposure on overwinter survival of honey bee colonies and adult, winter honey bee workers. Colonies overwintering in Saskatchewan experience 6 months or more of harsh climactic conditions, during which time they subsist exclusively on honey and beebread stored in the brood chamber, which is known to be contaminated with neonicotinoids [3]. To experimentally reproduce this exposure scenario, we administered THI-contaminated sucrose syrup to honey bee colonies for 5 weeks in fall, and evaluated their survival and strength the following spring. In parallel , we chronically exposed caged winter workers in the laboratory to the same doses of neonicotinoids in sucrose syrup and monitored their survival. Results from this chapter demonstrate that chronic, colony exposure to 100 ng/g THI significantly decreases colony overwinter survival and cluster size, while laboratory survival of winter workers is significantly decreased at 100 ng/g THI or CLO as well as at lower doses of neonicotinoids, from 5-20 ng/g. This chapter highlights that both season (summer vs. winter) and environment (field vs. laboratory) can impact the results of chronic neonicotinoid exposure studies, contextualizing our understanding of the risk of neonicotinoids to honey bees in Saskatchewan.

Chapter 3: **Chronic high dose neonicotinoid exposure decreases overwinter survival of *Apis mellifera* L.**

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3.1 Abstract

Overwinter colony mortality is an ongoing challenge for North American beekeepers. During winter, honey bee colonies rely on stored honey and beebread which is frequently contaminated with the neonicotinoid insecticides CLO and THI. To determine whether neonicotinoid exposure affects overwinter survival of honey bees, we chronically exposed overwintering field colonies and winter workers in the laboratory to THI or CLO at different concentrations and monitored survival and feed consumption. We also investigated the sublethal effects of chronic THI exposure on colony pathogen load, queen quality and colony temperature regulation. Under field conditions, high doses of THI significantly increased overwinter mortality compared to controls, with field-realistic doses of THI showing no significant effect on colony overwinter survival. Under laboratory conditions, chronic neonicotinoid exposure significantly decreased survival of winter workers relative to negative control at all doses tested. Chronic high-dose THI exposure was not shown to impact pathogen load or queen quality, and field-realistic concentrations of THI did not affect colony temperature homeostasis. Taken together, these results demonstrate that chronic environmental neonicotinoid exposure significantly decreases survival of winter workers in the laboratory, but only chronic high dose THI significantly decreases overwinter survival of colonies in the field.

3.2 Introduction

Honey bee colony mortality is most prevalent during the winter months in temperate climates, and since 2007, Canadian beekeepers have experienced average overwinter losses in excess of the 15% economically sustainable threshold [92]. In winter 2019, Canadian beekeepers experienced 25.7% overwinter colony loss on average, with beekeepers attributing their losses to weather, poor queen quality, weak fall colonies, *Varroa* infestation, and starvation [92].

Pesticide exposure through stored honey and pollen is another potential stressor contributing to overwinter colony loss. Canola, also known as oilseed rape, is one of the most common bee-attractive crops grown in Canada, and most of this canola is grown from neonicotinoid-treated seed [2]. The neonicotinoid insecticides CLO and THI, are commonly detected in pollen, nectar, and honey at mean concentrations from 1.9 - 9.4 ng/g CLO and 6.4 - 28.9 ng/g THI [68]. In Saskatchewan, Canada, CLO was detected in 68% of honey samples at mean doses of 8.2 ng/g and THI was detected in 75% of honey samples at a mean of 17.2 ng/g [3].

Previous chronic colony-feeding studies [219], laboratory studies [220,221], and field trials [66] have examined the effects of THI or CLO exposure on overwinter survival of honey bees. A chronic, summer colony-feeding study [219] found significant decreases in overwinter survival with 100 ng/g THI exposure, with no significant effect of 12.5-50 ng/g THI on overwintering. Interestingly, winter workers were more sensitive to chronic neonicotinoid exposure under laboratory conditions, with doses of 20 and 50 ng/g CLO significantly reducing survival of winter adult workers and no effect of 1-10 ng/g CLO on laboratory survival [220]. Similar to the laboratory results, field trials demonstrated that colonies exposed to <1-7 ng/g THI [66] or 0.5-2 ng/g CLO [64] during foraging show no significant difference in overwinter survival relative to controls [64,66]. Although a link between field-realistic THI and CLO exposure and overwinter colony mortality has not been established thus far, the effects of neonicotinoids on overwintering honey bees warrant further study.

Winter worker honey bees have important endocrine and metabolic differences from summer adult workers, which increase their lifespan by 6-8 fold, but may also alter their susceptibility to pesticides. For example, compared to summer foragers, winter workers have low levels of juvenile hormone and high levels of vitellogenin and other proteins in the hemolymph [222–226]; decreased protein synthesis, transport across the midgut, and catabolism [227–229]; and lower activity of monooxygenase enzymes important for pesticide detoxification [230]. It remains unclear how the physiologic differences between summer and winter workers alter pesticide sensitivity, with one study demonstrating increased susceptibility of winter workers to acute THI and CLO exposure compared to summer workers [221], and another study showing decreased sensitivity of winter workers to acute synergistic effects of a pyrethroid insecticide and an imidazole fungicide [231].

Few studies have examined the correlation between field and laboratory effects [232] of chronic neonicotinoid exposure on honey bee workers, particularly in Saskatchewan, Canada where outdoor colonies must endure severe cold during the winter months, and during summer, colonies commonly forage on THI or CLO-treated canola. We hypothesize that chronic dietary neonicotinoid exposure will decrease overwinter survival of (1) honey bee colonies in the field and (2) adult winter honey bee workers in the laboratory during Saskatchewan winter. In the field, we hypothesize that chronic THI exposure will decrease colony size, queen quality, and colony temperature regulation.

We performed both field and laboratory experiments to examine the effects of chronic overwinter neonicotinoid exposure on overwinter survival at field-realistic and high dose neonicotinoid concentrations during the harsh climactic conditions of Saskatchewan winter. In the field experiments, we also investigated the sublethal effects of chronic THI exposure on colony pathogen load, queen quality and colony temperature regulation.

3.3 Materials and Methods

3.3.1 Experimental design

Two field trials (F1 and F2) and two laboratory cage trials (C1 and C2) were conducted (Figure 3.1). The overall objective was to examine the effect of neonicotinoid exposure on colony overwintering. F1 was designed to test 100 ng/g THI as a positive control, as well as 20 ng/g THI which is a high, environmentally realistic dose for Saskatchewan [3]. C1 was designed as a laboratory model of F1 to test the same concentrations of THI and its metabolite CLO, with the addition of a 5 ng/g dose group. Based on the overwinter survival results of F1, we designed F2 to examine whether weak fall colony strength, in combination with field-realistic, 5 and 10 ng/g THI exposure, would predispose colonies to overwinter mortality. We designed C2 as a laboratory model of both F1 and F2 by including all of the doses tested in the field for both THI and its metabolite CLO. C1 and C2 were conducted from March-May when winter worker honey bees are approaching the end of their natural lifespan; thus, negative control survival of approximately 30 days or less was expected. To understand sublethal effects of neonicotinoid exposure on overwintering, we evaluated pathogen levels and queen quality in F1. Based on the absence of sublethal effects observed in F1, we chose a different sublethal outcome (temperature) for F2. Thus, the field and laboratory trials should not be considered replicates, and each trial had an appropriate negative control group for comparison.

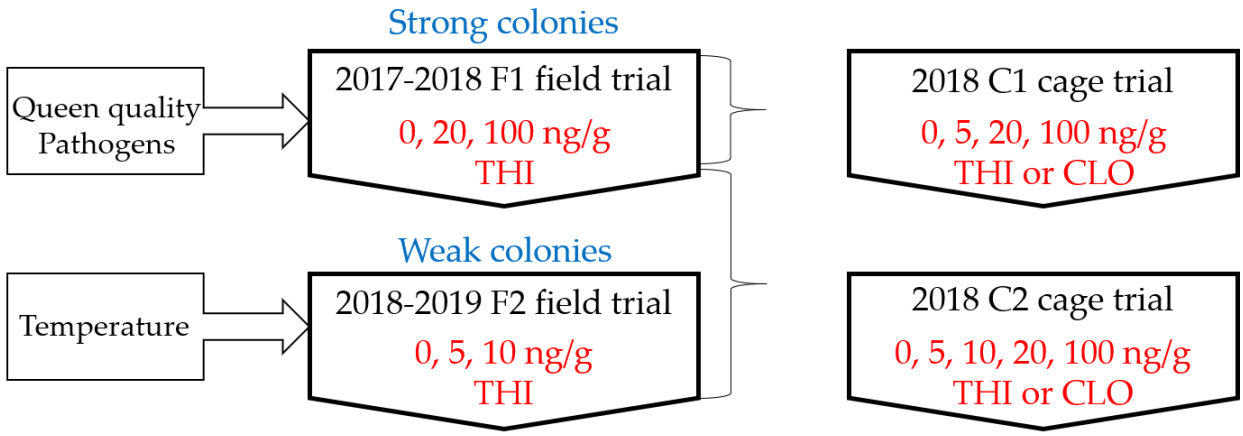

Figure 3.1 Experimental design of field and laboratory cage trials. Field trials examined the effects of chronic overwinter exposure to THI on survival of strong colonies (F1) or weak colonies (F2), as well as sublethal effects on queen quality and pathogen load (F1) and colony temperature regulation (F2). Cage trials examined the effects of chronic exposure to THI or its metabolite CLO on laboratory survival of winter adult workers.

3.3.2 Field trials

To compare the effects of THI exposure on overwinter colony survival, field colonies received *ad libitum* control or THI-contaminated 2:1 (w:v) sucrose syrup over five weeks in fall and overwinter survival was assessed the following spring (Table 3.1). F1 consisted of 60 strong colonies, each equalized to two brood chambers and randomized into treatment groups, with newly-mated sister queens from one genetic lineage. F2 contained 64 weak colonies with newly-mated sister queens from two genetic lineages which were unrelated to the F1 queens. The F2 colonies occupied one to two brood chambers and were stratified across treatment groups. The F1 colonies were not-reused in F2. Both F1 and F2 occurred on land rented or owned by the University of Saskatchewan Goodale Research and Teaching Farm (Clavet, Saskatchewan, Canada) at two different yards which were 2.3 km apart (52°01'50.6"N 106°32'26.6"W in F1 and 52°02'34"N 106°30'45"W in F2).

Table 3.1 Experimental design and sample size (N) of field trials examining survival of outdoor overwintered colonies chronically exposed to THI.

	Field trial 1 (F1)	Field trial 2 (F2)
Trial dates (September - May)	2017-2018	2018-2019
THI doses (ng/g)	0, 20, 100	0, 5, 10
N (colonies) per dose	20, 20, 20	22, 21,21
N for queen quality analysis	6,6,6	-
N for temperature analysis	-	14,11,10
Varroa and *Nosema* monitoring	Yes	No

To prepare the THI treated syrup, a stock of THI in water was prepared at a concentration of 100 µg/ml (F1) or a 10 µg/ml (F2), and the appropriate volume of stock was added to 2:1 sucrose solution to achieve the desired concentration in ng THI per g of syrup. Stock was prepared using analytical standard THI (product number 37924; batch number BCBT3749; purity 99.7%; expiry November 2021; obtained from MilliporeSigma Canada Co., Oakville, Ontario, Canada). Syrup was mixed thoroughly prior to feeding. In F1, colonies were administered sugar syrup through 4 liter top-fed jars initially, followed by 4 liter frame feeders. In F2, colonies received syrup exclusively through frame feeders. Any syrup remaining in the frame feeder in the spring was accounted for when calculating total syrup consumption. In early October the colonies were administered one final feeding of experimental syrup and the colonies were wrapped in groups of four for winter using standard side wraps (R4 thermal rating) and insulating top pillows (R8 thermal rating) with a plywood top cover. Preliminary overwinter colony survival was assessed in April (F1) or March (F2) and final overwinter survival was determined at unwrapping in May.

All study colonies were weighed at three time points: (1) prior to syrup-feeding, (2) prior to winter wrapping, and (3) after spring unwrapping using a mechanical hanging scale (Salter Model 235, Brecknell Scales, Fairmont, MN, USA) with an accuracy to the nearest 0.5 kg.

To estimate population size, the study colonies were 'cluster sized' at four time points: (1) prior to syrup-feeding (F2 only), (2) prior to winter wrapping, (3) during the initial spring survival assessment, and (4) after spring unwrapping. Briefly, using a 16.2 megapixel Nikon D7000 digital camera (Minato, Tokyo, Japan) with a Nikon 18±105 mm lens, a photo was taken of the adult bee cluster on the tops of each occupied super early in the morning prior to bees flying out to forage. The cluster size for each super was estimated by counting the number of interframe spaces to the nearest 0.25 occupied by adult bees in the photos. The overall cluster size for each colony was obtained by summing the cluster size for each super in the colony [233]. At time point 3 in F1 and

F2, the cluster size was based on a photo of the top super only because the second super could not be accessed due to the winter wrap.

3.3.2.1 Pathogen monitoring in F1

In F1, all colonies were sampled for phoretic *Varroa* mites before (August, 2017) and after (October, 2017) treatment with amitraz as well as at the beginning and the end of May, 2018 using an alcohol wash [234] of approximately 300 workers from a brood frame. Briefly, workers were sampled in 200 ml windshield washer fluid (Turbo Power®, All Season Windshield Washer; Recochem Inc., Edmonton, AB, Canada) or methyl hydrate (Turbo Power® Heavy Duty 99.9% Pure; Recochem Inc., Edmonton, AB, Canada) and shaken for 30 minutes on a rotary shaker at 200 rpm [235]. Bees were strained from the wash fluid and the mites were counted to obtain a percent infestation (mites/bees sampled x 100%). In June, 2018 a 17 x 6 cm area of capped brood was uncapped to examine for the presence of *Varroa* in a subset of the surviving colonies (11 colonies from control group; 10 colonies from 20 ng/g group, and 7 colonies from 100 ng/g group). Additionally, all F1 colonies were sampled for *Nosema* spore counts in September, 2017 and May, 2018. *Nosema* spore counts per bee were generated by macerating 60 workers sampled from a honey frame (fall 2017) or the entrance (spring 2018) of each colony in 60 ml phosphate buffered saline for 1 minute using a Stomacher®80 Biomaster (Seward, Davie, FL, USA); and counting spores in 0.02 mm^3 using a haemocytometer (Hausser Scientific, Horsham, PA, USA) and a phase contrast microscope (Olympus IX51, Tokyo, Japan) [236]. Two samples of macerate were counted per colony and the results averaged to obtain a spores per bee count for each colony.

3.3.2.2 Queen quality in F1

Quality of the queens in F1 was assessed by sacrificing and weighing six queens from each control and treatment group in August, 2018. Each queen's spermatheca was diluted in 1 ml Kiev buffer (sodium citrate dihydrate 24.3 g/L, $NaHCO_3$ 2.1 g/L, KCl 0.4 g/L, sulphanilamide 0.3 g/L, D-(+) glucose 3.0 g/L in double distilled water all from MilliporeSigma Canada Co., Oakville, Ontario, Canada) [237] and total spermatozoa counts were performed using a 1:16 dilution of spermatozoa with a haemocytometer (Hausser Scientific, Horsham, PA, USA) and a light microscope (Olympus CX22, Tokyo, Japan) [236]. Sperm viability was assessed by staining 50 µl of spermatozoa in Kiev buffer with SYBR®14 and propidium iodide (LIVE/DEAD™ Sperm Viability Kit, ThermoFisher Scientific, Waltham, MA, USA) and counting live and dead sperm in

a minimum of 10 and maximum of 20, 20X fields to reach a minimum count of 200 sperm per sample using a fluorescent compound microscope (Olympus BX51, Tokyo, Japan) [195].

3.3.2.3 Temperature monitoring in F2

In F2, a Thermochron iButton (DS1921G-F5#, Embedded Data Systems, Lawrenceburg, KY, USA), vacuum packed in a plastic strip, was inserted through the top entrance of each colony on February 19, 2019 to monitor hourly, within-colony temperature until May 8, 2019 (78 days).

3.3.2.4 Analysis of data

All statistical analyses were performed using Stata/SE 15.1 (College Station, TX, USA) with $P < 0.05$ considered significant. Data are presented as medians or means ± standard deviation (SD). Overwinter survival was analyzed by Chi-square and a z-test. Syrup consumption, colony weight gain, queen weight, sperm viability and sperm counts were analyzed using a one-way ANOVA with a Bonferroni multiple comparison test. Cluster size at each time point was analyzed using a one-way ANOVA with a Bonferroni multiple comparison test or a Kruskal-Wallis equality of populations rank test with a Dunn's pairwise comparison test. *Nosema* and *Varroa* data was analyzed using a Kruskal-Wallis equality of populations rank test.

Temperature data was analyzed from surviving colonies in spring 2019 which retained the sensor within the colony throughout the temperature monitoring period (14 colonies in control group, 10 colonies in 10 ng/g group and 11 colonies in 5 ng/g group), excluding days where colonies were opened for cluster sizing or spring treatments. In accordance with Meikle *et al.* [238], a running average temperature was calculated for the 12 hours before and after each temperature measurement, as well as a 'detrended' temperature, calculated by subtracting the running average from each temperature measurement. For each day, the minimum and maximum running average and detrended temperatures were determined for each colony and compared across treatment groups using a linear mixed model. The assumptions of the model were met.

3.3.3 Laboratory cage trials

To evaluate the effects of neonicotinoids on laboratory survival of winter adult workers, winter workers received *ad libitum* 1:1 (w:v) sucrose syrup containing THI or CLO over 30 days and survival was monitored daily (Table 3.2). Negative controls received untreated syrup and positive controls received syrup containing dimethoate (DIM) [239].

Table 3.2 Experimental design of laboratory cage trials examining survival of winter honey bee workers chronically exposed to THI or CLO for 30 days

Trial dates	Cage trial 1 (C1) March 13 - April 11, 2018		Cage trial 2 (C2) April 16 - May 15, 2018	
Negative control	1:1 (w/v) sucrose solution		1:1 (w/v) sucrose solution	
# negative control cages	13		8	
Positive control	1000 ng/g DIM		1000 ng/g DIM	
# positive control cages	3		3	
Mean bees per cage (SD)	10.1 (0.97)		9.9 (1.1)	
# diet evaporation cages	3		3	
Neonicotinoids tested	THI	CLO	THI	CLO
Doses (ng/g)	4.9, 19.5, 97.3	4.2, 16.7, 83.2	4.9, 9.7, 19.5, 97.3	4.2, 8.3, 16.7, 83.2
# cages per dose	5 ,5, 3	5, 5, 3	5 ,5 ,5, 3	5, 5, 5, 3

Adult workers were sampled from a single outdoor, overwintering, queenright colony which was derived from three colonies of different genetic lineages which were merged one week prior to the beginning of C1. The colony was placed indoors at 15 degrees Celsius, 12 hours prior to sampling. The colony did not have brood at the time of sampling for C1 or C2, likely due to unusually cold spring weather. In late March, prior to sampling for C2, the colony was treated for *Varroa* mites with amitraz-impregnated strips (Apivar®, Veto-pharma, France) in accordance with label instructions.

In each trial, stainless steel insect cages (measuring 7.5 x 4 x 5.5 cm; Small Life Supplies, Cambridgeshire, Great Britain) were filled with adult worker honey bees each by gently vacuuming bees directly from the frame into a cage. After collection, prior to the start of each trial, the bees were given a 24-hour acclimatization period where they received untreated 1:1 (w/v) sucrose solution. The cages were kept in darkness within an incubator at 29ºC and 60% relative humidity. In each trial, the cages were randomly assigned to treatment and control groups (Table 2). Each day, the number of dead bees in each cage was recorded and the dead bees were removed. All procedures were performed under red light.

To ensure equal numbers of active molecules of THI and CLO at each concentration tested, concentrations (100, 20, 10, and 5 ng/g) were converted to nanomolar (400, 80, 40, 20 nM). Thus, the actual ng/g doses of THI and CLO tested are presented in Table 3.2; however, for simplicity we will refer to the test doses as 100, 20, 10, and 5 ng/g in the text and figures. A 10 ng/µl stock of THI or CLO in water was prepared, and the appropriate volume of stock added to sucrose solution to achieve the desired concentration in nM. Similarly, for the positive control, a 100 ng/µl

stock of DIM in water was prepared and diluted in sucrose solution to a concentration of 1000 ng/g. Stocks were prepared using analytical standard pesticides from MilliporeSigma Canada Co., Oakville, Ontario, Canada. For each pesticide, the name, product number, batch number, purity, and expiration date is listed: (i) THI, 37924, BCBT3749, 99.7%, November 2021 (ii) CLO, 33589, BCBS3968V, 99.9%, June 2020 (iii) DIM, 45449, BCBS9338V, 99.8%, August 2021.

Fresh treatment solution was provided every third day and the feeding syringes were weighed pre-insertion and post-removal to monitor diet consumption. Three cages without bees were used to monitor diet evaporation in each trial.

3.3.3.1 Analysis of data

All statistical analyses were performed using Stata/SE 15.1 (College Station, TX, USA) with $P < 0.05$ considered significant. Data are presented as means ± standard deviation (SD). For each trial, syrup consumption (mean grams per bee per 3 days) corrected for evaporation was analyzed using a generalized estimating equation (GEE) population averaged model with an exchangeable correlation structure and time and treatment as independent variables. Syrup consumption of the positive control was not included in the analysis.

Survival over 30 days for each trial was modelled using a Weibull hazard function with an accelerated failure time model. The survival data was clustered by cage, with bee survival considered non-independent between bees in the same cage and bee survival considered independent between bees in different cages. Cox-Snell residuals, Martingale residuals, deviance residuals, and proportionality of hazards were assessed graphically to evaluate the goodness-of-fit of the model, the functional form of the model, the presence of outliers, and the model assumptions, respectively.

3.4 Results

3.4.1 Effects of THI on overwintering honey bee colonies in F1

Strong fall colonies exposed to high dose, field unrealistic concentrations of 100 ng/g THI experienced significant ($z = 3.6$, $P < 0.001$), 55% greater overwinter mortality relative to control colonies (Figure 3.2a) and significant ($F_{2,37} = 4.59$, $P = 0.045$), two interframe space decreases in early spring adult bee cluster compared to the control (Figure 3.3a). A dose response was observed for overwinter colony survival (Figure 3.2a), with 10% overwinter loss of control colonies (2/20), 25% overwinter loss of medium dose colonies (5/20), and 65% overwinter loss of high dose colonies (13/20). Strong fall colonies chronically exposed to high environmental doses of 20 ng/g

THI overwinter did not experience significant increases in overwinter mortality relative to the control ($z = 1.2$, $P = 0.2119$).

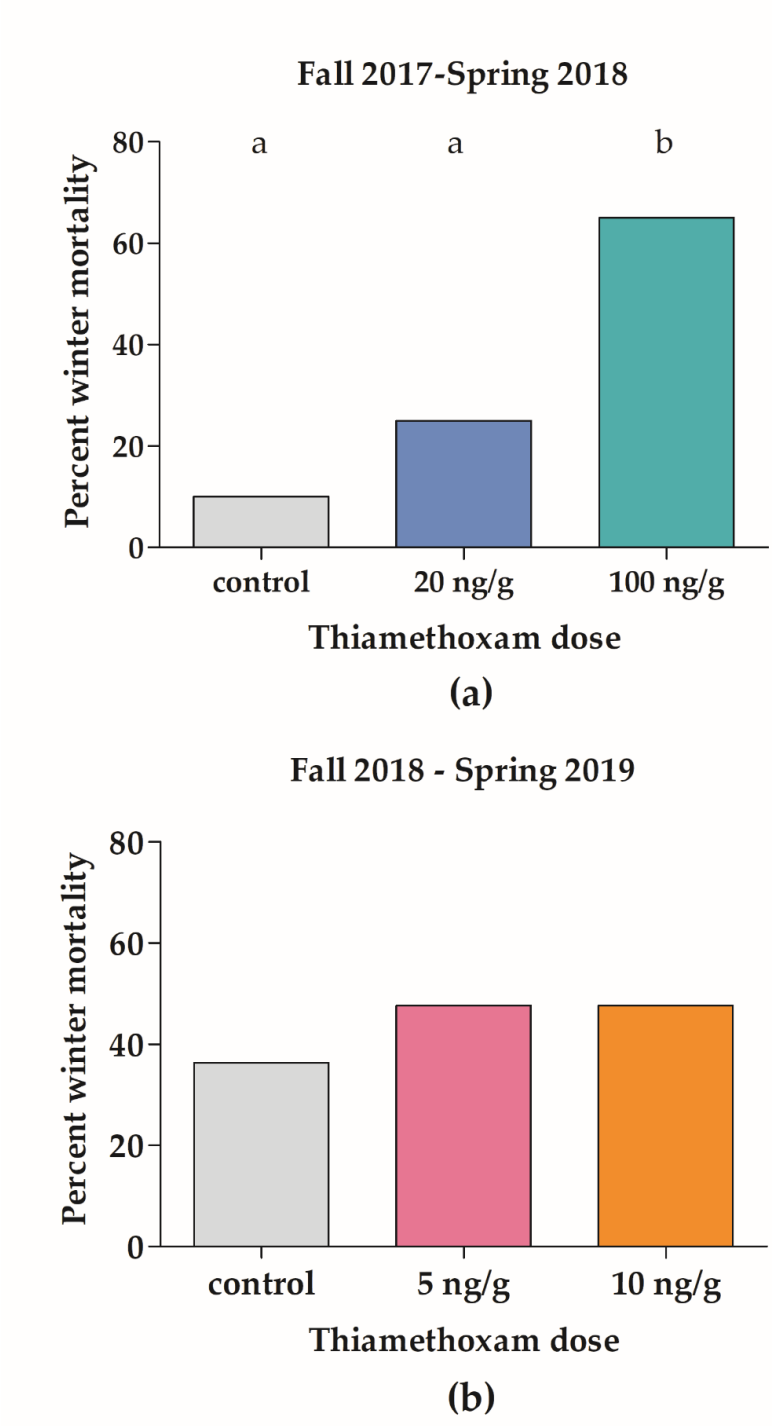

Figure 3.2 Chronic high dose (100 ng/g) thiamethoxam significantly increases overwinter mortality of *Apis mellifera* colonies in the field. Honey bee colonies were chronically exposed to THI over winter in F1 (2017-2018) (a) or F2 (2018-2019) (b) and survival of the colonies was evaluated the following spring. Bars indicate percent overwinter colony loss for twenty to twenty-two colonies per group. Different letters indicate significant differences by a z-test, $P < 0.05$.

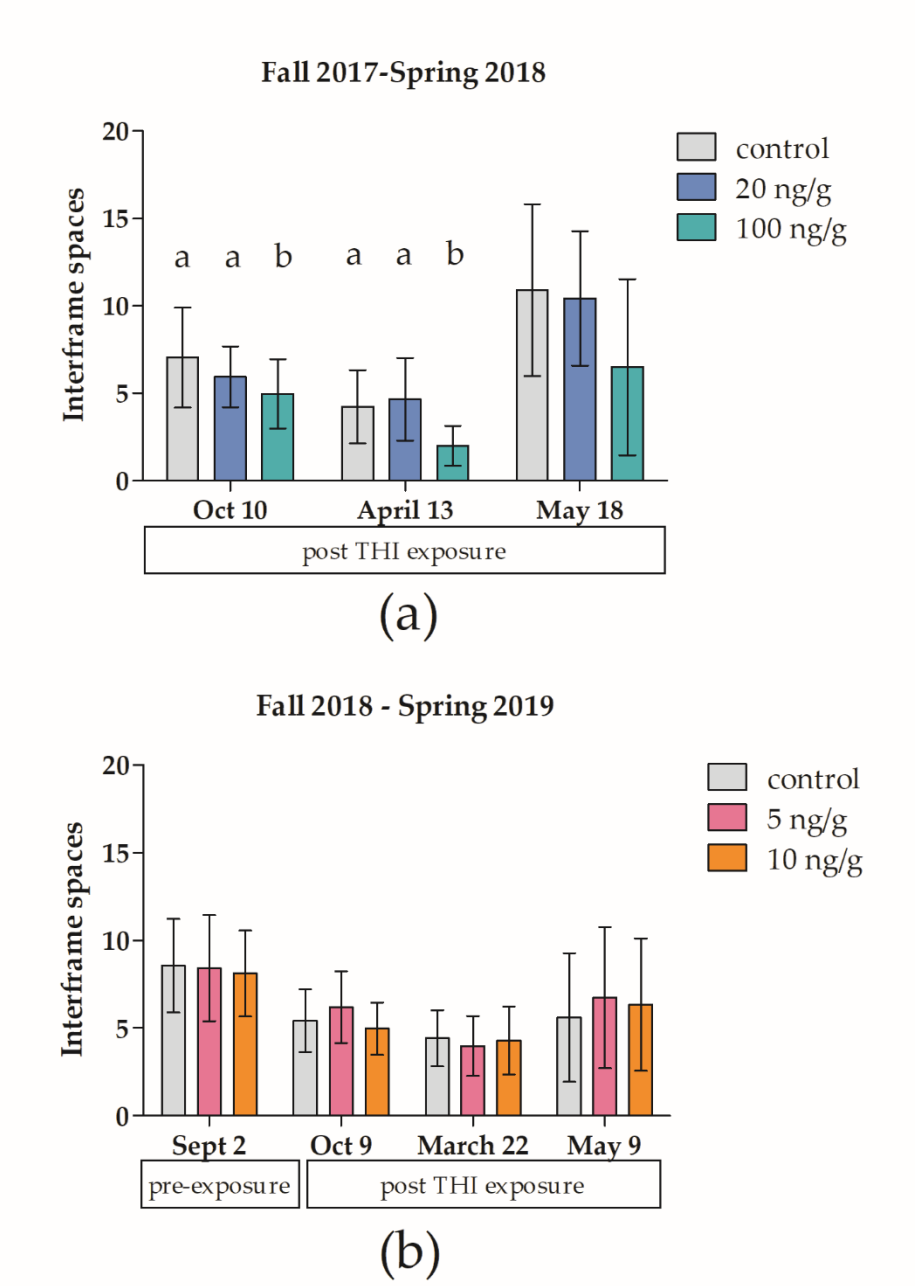

Figure 3.3 Chronic high dose (100 ng/g) thiamethoxam significantly decreases cluster size of *Apis mellifera* colonies in the field. Honey bee colonies were exposed to THI over winter in F1 (2017-2018) (a) or F2 (2018-2019) (b) and adult bee cluster size of the colonies was monitored in the fall and the following spring. Bars indicate mean ± SD interframe spaces occupied by the adult bee cluster for twenty to twenty-two colonies per group. Different letters indicate significant differences at each time point, $P < 0.05$ by ANOVA or Kruskal-Wallis rank test.

During fall feeding, the twenty 100 ng/g THI-treated colonies consumed significantly less syrup (14.25 L, SD = 4.11 L, Figure 3.4a) compared to the twenty control (24.97 L, SD = 4.04 L, $F_{2,57}$ = 37.65, P < 0.001) and twenty 20 ng/g THI-treated colonies (21.89 L, SD = 3.92 L, P < 0.001). Furthermore, the twenty 100 ng/g THI-treated colonies lost significantly less weight (15.85 kg, SD = 4.65 kg) from October 2017 to May 2018 compared to the twenty control colonies (19.3 kg, SD = 4.19 kg, $F_{2,57}$ = 3.34, P = 0.044, Figure 3.5a).

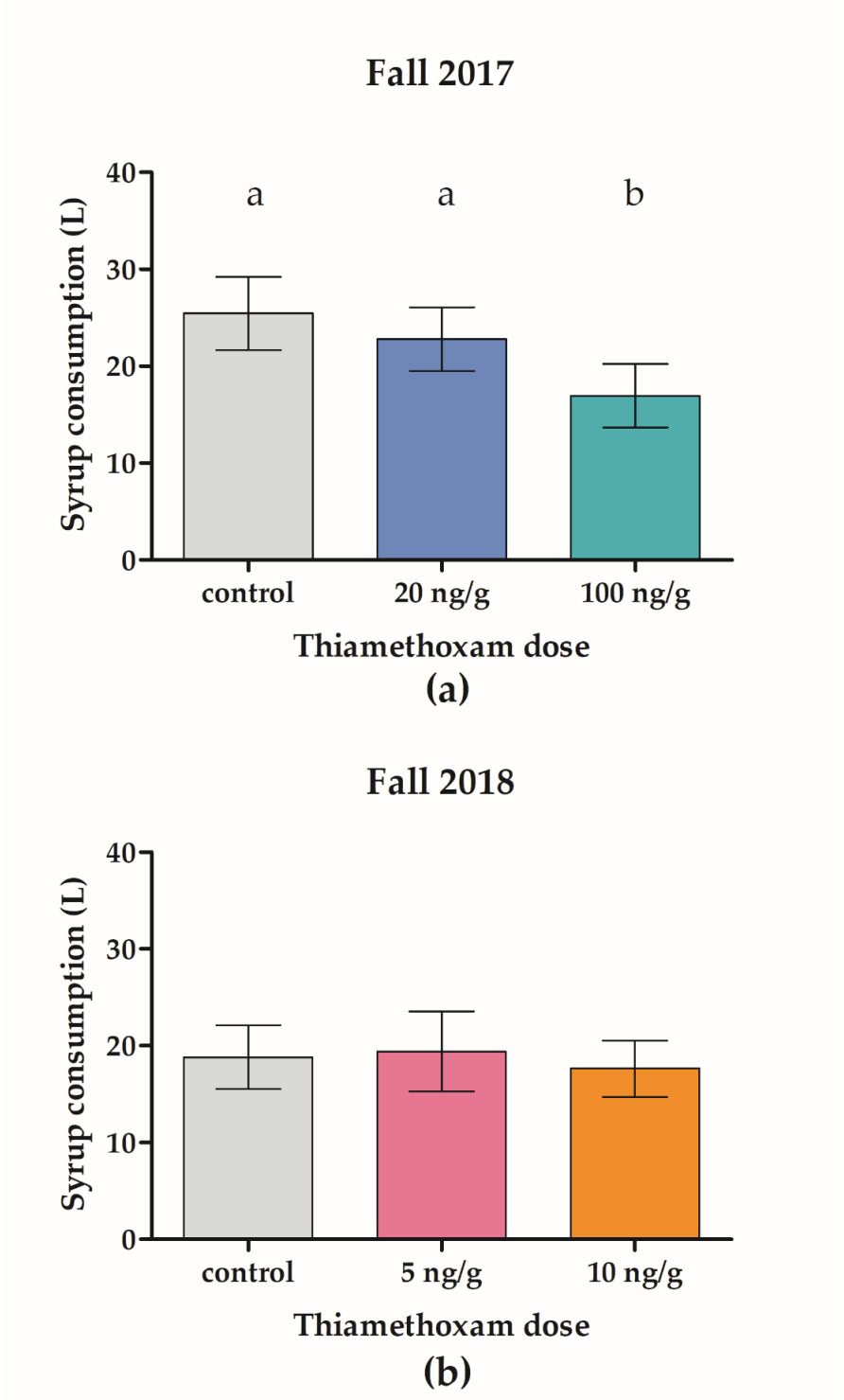

Figure 3.4 Colonies exposed to chronic high dose (100 ng/g) thiamethoxam consumed significantly less syrup. Honey bee colonies were fed control or THI-contaminated sugar syrup *ad libitum* over five weeks during fall 2017 in F1 (a) or during fall 2018 in F2 (b). Bars indicate mean ± SD litres of sugar syrup consumed for twenty to twenty-two colonies. Different letters indicate a significant difference, $P < 0.001$ by ANOVA.

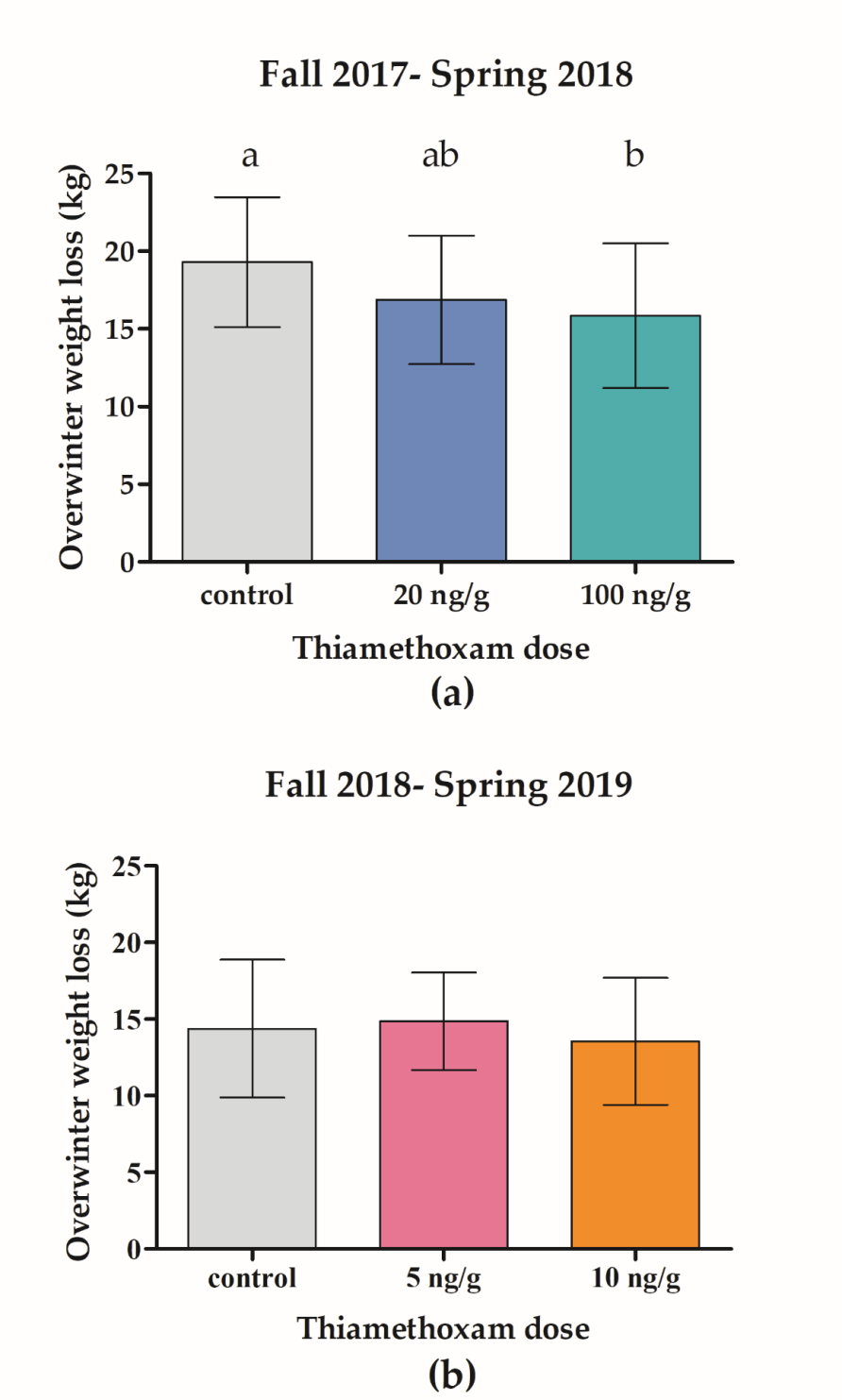

Figure 3.5 Colonies exposed to chronic high dose (100 ng/g) thiamethoxam lost significantly less weight during winter compared to controls. Honey bee colonies were exposed to THI during winter 2017-2018 in field trial 1 (a) or winter 2018-2019 in field trial 2 (b). Bars indicate mean ± SD overwinter (October to May) weight change in kg for twenty to twenty-two colonies. Different letters indicate significant differences, $P < 0.05$ by ANOVA.

Prior to THI exposure, all colonies had low levels of *Varroa* (0.36% infestation, SD = 0.38) and *Nosema* (5.21 x 10^4 spores/bee, SD = 1.55 x 10^5). In October 2017, mean percent *Varroa* infestation declined to 0.02% (SD = 0.082) after amitraz treatment, with no significant difference in infestation across treatment groups ($X^2(2) = 0.295$, P = 0.8628). In early spring 2018, there were no significant THI-treatment effects for *Nosema* infection (6.4 x10^5 spores/bee, SD = 1.42 x 10^6; $X^2(2) = 4.098$, P = 0.1289) or *Varroa* infestation (phoretic *Varroa* not detected in any colony). In late spring 2018, after amitraz treatment, mean percent *Varroa* infestation was 0.014% (SD = 0.063) with no treatment effect on infestation ($X^2(2) = 0.358$, P = 0.8362). *Varroa* was not observed in any of the capped brood examined in the control and THI-treated colonies.

Queen quality was not significantly affected by chronic overwinter THI exposure in F1 (Figure 3.6). Six control queens, six queens exposed to 20 ng/g THI and six queens exposed to 100 ng/g THI did not differ significantly in sperm viability (mean live = 69.4%, SD = 13.69, $F_{2,15}$ = 3.23, P = 0.0681, Figure 3.6a), total sperm count (2.91 x 10^6, SD = 1.35 x 10^6, $F_{2,15}$ = 1.98, P = 0.1729, Figure 3.6b), or queen weight (242 mg, SD = 26.62, $F_{2,15}$ = 3.15, P = 0.0722, Figure 3.6c).

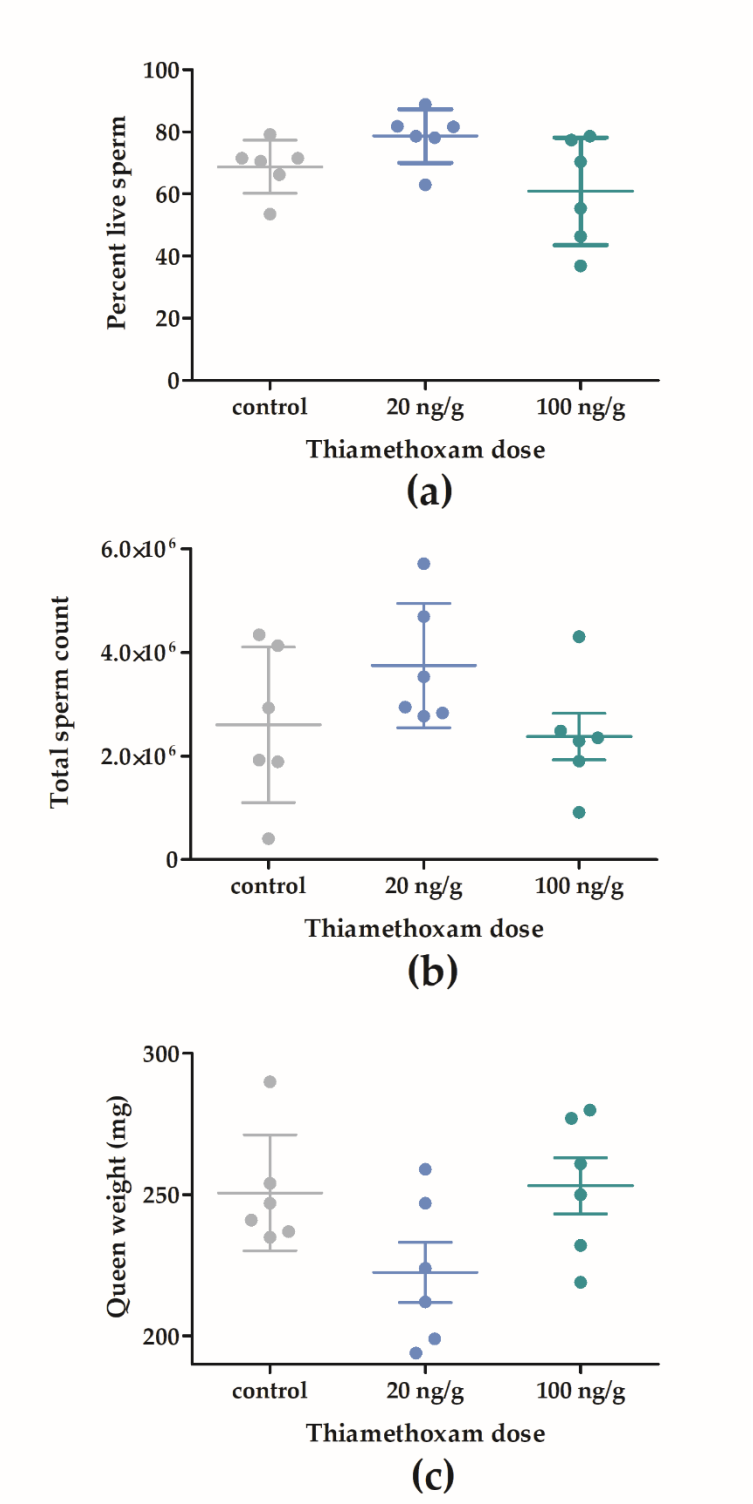

Figure 3.6 Chronic overwinter thiamethoxam (20 ng/g and 100 ng/g) exposure does not impact *Apis mellifera* queen quality. Honey bee colonies were exposed to THI during winter 2017-2018 in F1 and queens were sacrificed in August 2018 for determination of percent sperm viability (a), total sperm counts (b), and queen weight (c). Plots indicate mean ± SD.

3.4.2 Effects of THI on overwintering honey bee colonies in F2

Weak fall colonies chronically exposed to environmental (5 or 10 ng/g) doses of THI did not experience significant decreases in overwinter colony survival ($X^2(2) = 0.743$, $P = 0.699$; Figure 3.2) or colony cluster size relative to control (Figure 3.3). Prior to THI exposure, the colonies in F2 were weaker than the colonies in F1. The mean cluster size in September 2018 in F2 was 8.37 interframe spaces (SD = 2.69, Figure 3.3b), while the mean frames of bees in September 2017 for F1 was 13.04 frames (SD = 3.10). Not surprisingly, the overwinter mortality of controls in F2 (36%, Figure 3.2b) was over four times greater than the overwinter mortality of 112 non-study colonies in our research apiary in winter 2018-2019 (8.9%).

During fall feeding, there was no significant difference ($F_{2,61} = 1.42$, $P = 0.2501$) in syrup consumption (18.6 L, SD = 3.50 L) of the 64 control and treatment colonies (Figure 3.4b). Additionally, there was no significant difference ($F_{2,61} = 0.58$, $P = 0.5616$) in overwinter weight loss from October 2018 to May 2019 (14.27 kg, SD = 3.96 kg) of the 64 control and treatment colonies (Figure 3.5b).

The in-hive temperature for colonies exposed to 5 or 10 ng/g THI was not significantly different from controls (Figure 3.7). The maximum and minimum running average daily temperatures (29.06 °C, SD = 4.49 and 13.44°C, SD = 10.14, respectively; Figure 3.7d) did not differ significantly across treatment groups ($X^2(2) = 0.27$, $P = 0.873$ for maximums and $X^2(2) = 0.53$, $P = 0.7677$ for minimums). Similarly, there was no significant effect of THI treatment on the maximum and minimum detrended daily temperature amplitudes (3.54°C, SD = 2.12 and -4.47°C, SD = 3.23, respectively; Figure 3.7b; $X^2(2) = 3.06$, $P = 0.2163$ for maximums and $X^2(2) = 1.36$, $P = 0.5077$ for minimums).

The in-hive maximum and minimum running average and detrended temperature amplitudes varied significantly over time during F2 ($P < 0.001$; Figure 3.7), with no significant interaction between THI treatment and time ($X^2(154) = 89.14$, $P = 1.0$ for maximum and $X^2(154) = 77.35$, $P = 1.0$ for minimum running average temperature; $X^2(154) = 82.87$, $P = 1.0$ for maximum and $X^2(154) = 169.38$, $P = 0.1877$ for minimum detrended temperature amplitude).

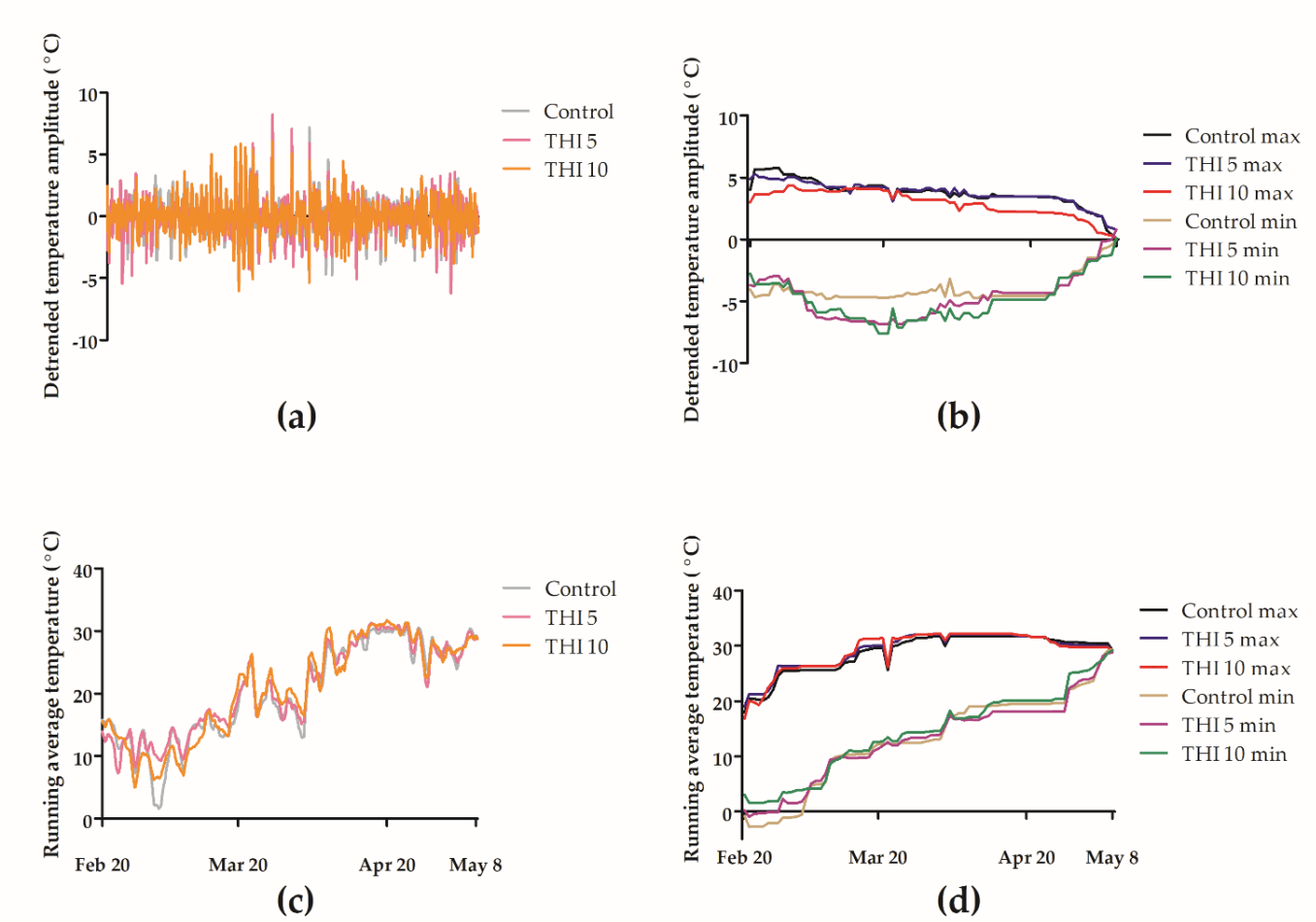

Figure 3.7 Environmental doses of (5 and 10 ng/g) thiamethoxam do not significantly affect within hive temperature during winter and early spring compared to controls. Honey bee colonies were exposed to THI during winter 2018-2019 in F2. Lines indicate mean detrended temperature amplitude (a), maximum and minimum detrended temperature amplitude (b), mean running average temperature (c), and maximum and minimum running average temperature (d) in degrees Celsius for 10-14 colonies for 78 days from February to May, 2019

3.4.3 Effects of chronic THI or CLO exposure of winter adult workers during C1

We found that chronic laboratory neonicotinoid exposure significantly ($X^2(6) = 124.73$, $P < 0.001$) decreased survival time of winter workers relative to control in a dose-dependent manner (Figure 3.8a,c, Table A3.1). The negative control had a median survival of 16.48 days (Table A3.1). As a positive control, 30 workers were treated with 1000 ng/g DIM resulting in a median survival of 2 days.

For the same dose, there was no significant difference ($P > 0.05$) in survival between THI or CLO treated workers (Figure 3.8a,c). Workers treated with 100 ng/g neonicotinoids survived a 67-77% shorter time compared to negative controls; workers treated with 20 ng/g neonicotinoids survived a 38% shorter time relative to negative controls; and workers exposed to 5 ng/g neonicotinoids survived a 17-20% shorter time compared to negative controls (Figure 3.8a,c, Table A3.

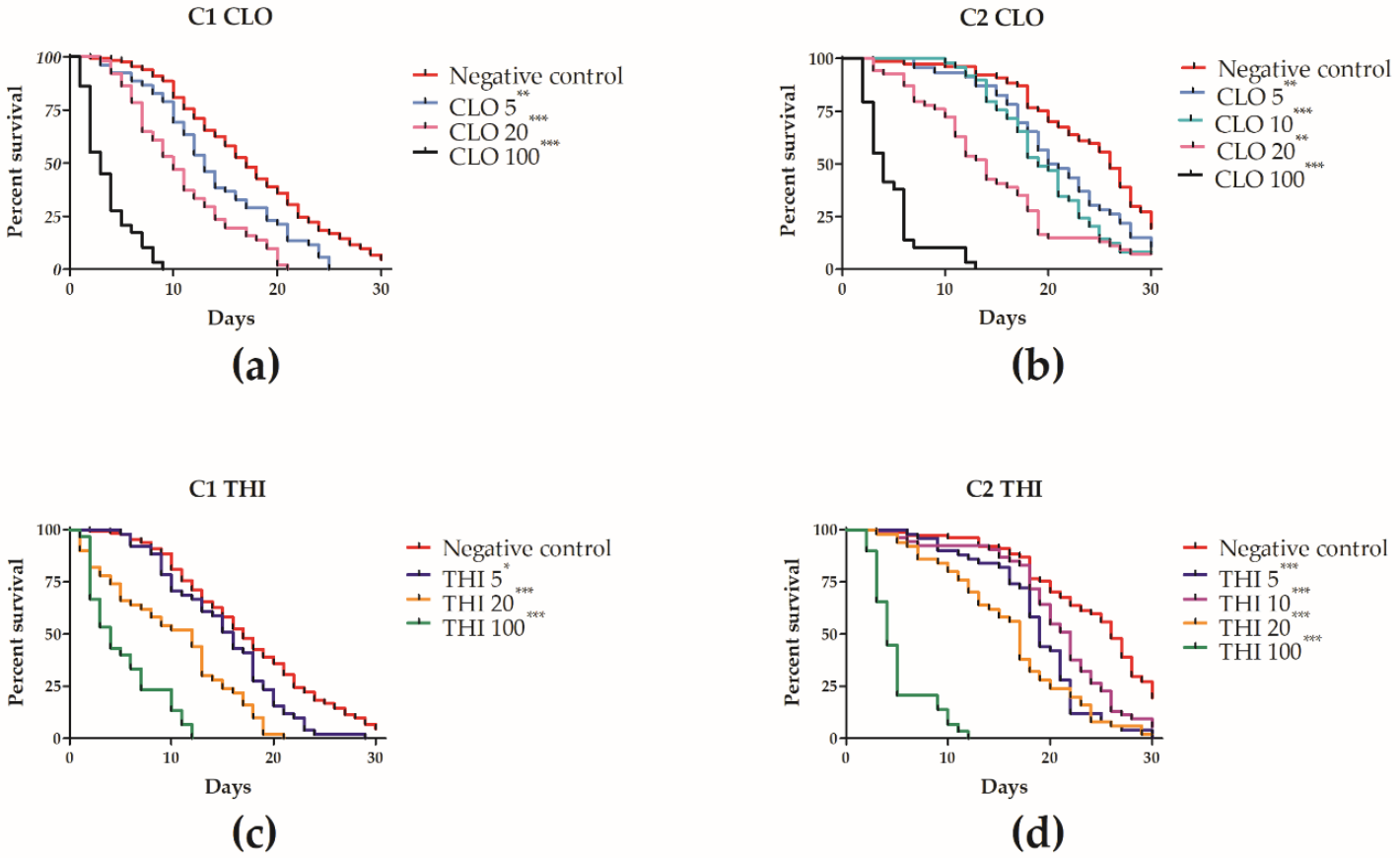

Figure 3.8 Chronic laboratory thiamethoxam or clothianidin exposure significantly decreases winter adult worker *Apis mellifera* survival. Winter workers were exposed to THI or CLO (doses in ng/g) for 30 days through syrup and mortality was monitored daily. Two laboratory cage trials were conducted: C1 in March-April (a,c) and C2 in April-May (b,d). Only C2 included 10 ng/g dose groups. Lines indicate percent daily survival for 131 (a,c) to 77 (b,d) bees in the negative control groups; 29-30 bees in the 100 ng/g groups; and 46-54 bees in the other treatment groups. *,**,*** survival significantly different from control, $P < 0.05$, 0.01, 0.001, by a Weibull accelerated failure time mode

Mean syrup consumption was 0.30 g per bee per three days (SD = 0.19 g); thus consumption was calculated to be 83 µl per bee per day (Figure 3.9). There was no significant difference in syrup consumption of treatment groups relative to control ($X^2(6) = 11.64$, $P = 0.0706$, Figure 3.9a). Syrup consumption varied significantly over time ($X^2(8) = 26.88$, $P < 0.001$), but there was no interaction between time and treatment ($X^2(27) = 29.85$, $P = 0.321$).

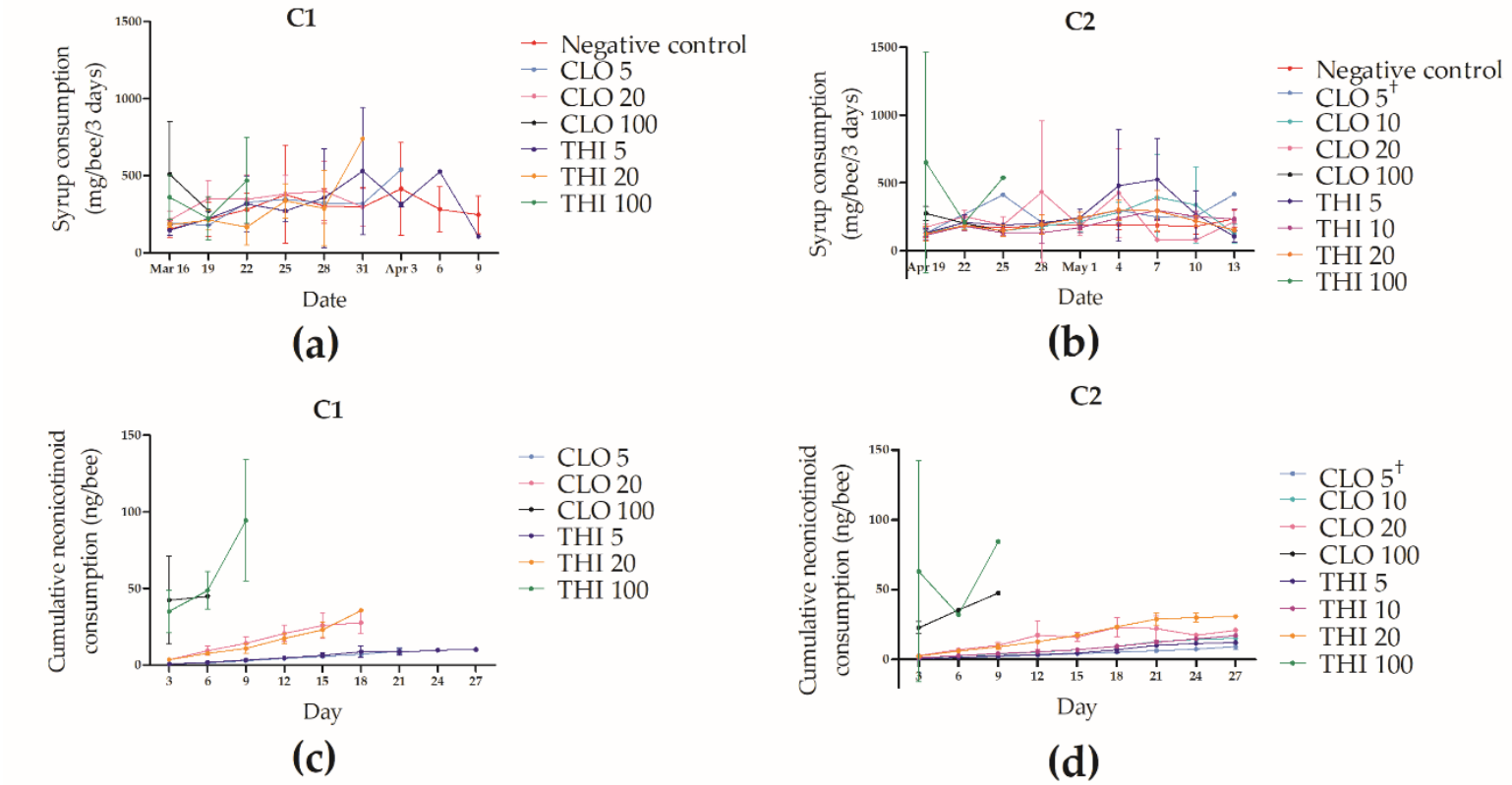

Figure 3.9 Laboratory syrup consumption and cumulative neonicotinoid consumption of winter adult *Apis mellifera* workers. Winter workers were exposed to THI or CLO (doses in ng/g) for 30 days through syrup and mortality was monitored daily. Two laboratory cage trials were conducted: C1 in March-April, 2018 (a,c) and C2 in April-May, 2018 (b,d). Only C2 included 10 ng/g dose groups. Lines indicate mean syrup consumption in mg per bee per 3 days (a,b) and mean cumulative neonicotinoid consumption in ng per bee (c,d). †consumption data for day 6 was missing for the CLO 5 group in C2

3.4.4 Effects of chronic THI or CLO exposure of winter adult workers during C2

We found that chronic laboratory neonicotinoid exposure significantly ($X^2(8) = 167.57$, $P < 0.001$) decreased survival time of winter workers relative to control in a dose-dependent manner (Figure 3.8b,d, Table A3.2). The negative control had a median survival of 23.89 days (Table A3.2). As a positive control, 30 workers were treated with 1000 ng/g DIM resulting in a median survival of 3 days.

For the same dose, from 10-100 ng/g, there was no significant difference ($P > 0.05$) in survival between THI or CLO treated workers. Workers treated with 100 ng/g neonicotinoids survived a 77% shorter time compared to negative controls; workers treated with 20 ng/g neonicotinoids survived a 33-34% shorter time relative to negative controls; and workers exposed to 10 ng/g neonicotinoids survived a 17-21% shorter time compared to negative controls (Figure 3.8b,d, Table A3.2). Workers exposed to 5 ng/g THI survived 15% shorter time than workers exposed to 5 ng/g CLO ($P = 0.003$) and a 27% shorter time than negative controls ($P < 0.001$). Workers exposed to 5 ng/g CLO survived a 13.9% shorter time than negative controls ($P = 0.003$).

Mean syrup consumption was 0.23 g per bee per three days (SD = 0.16 g) and thus, consumption was calculated to be 64 µl per bee per day (Figure 3.9). There was a significant interaction between time and treatment for syrup consumption ($X^2(51) = 94.55$, $P = 0.0002$, Figure 3.9b), indicating that syrup consumption was different over time depending on neonicotinoid treatment. Since consumption per bee per three days was calculated using the final number of living workers in a cage [240], cages which experienced high mortality over the preceding three days (for example, THI 100 in Figure 3.9b,d) had an elevated consumption per bee value, contributing to the interaction between treatment and time.

3.5 Discussion

In this chapter we demonstrated that chronic experimental neonicotinoid exposure during Saskatchewan winter significantly decreased overwinter survival of (1) honey bee colonies in the field at high doses and (2) adult winter worker honey bees in the laboratory at field-realistic and high doses. Our study shows that colonies overwintering in Saskatchewan on canola honey and beebread are at low risk of mortality from chronic neonicotinoid exposure.

3.5.1 Effects of THI on overwintering honey bee colonies in F1

At the colony level, a dose response in overwinter survival was observed for colonies chronically exposed to THI overwinter, with no observed effect of chronic overwinter THI exposure on pathogen load or queen quality.

Colony overwinter survival and cluster size was significantly decreased by exposure to 100 ng/g THI (Figure 3.2a, Figure 3.3a). Our results agree with the colony-level feeding studies of Thompson *et al.* [219] and Overmeyer *et al.* [232] who found that colonies fed 100 ng/g THI during 6 weeks in summer had significant, 50% reductions in number of adult bees relative to controls prior to overwintering and a significant, two times increase in overwinter mortality, but colonies fed lower doses of THI (12.5-50 ng/g THI) had no long-lasting colony effects.

Similar to our study, Overmeyer *et al.* [232] observed that high dose, 100 ng/g THI-exposed colonies consumed less syrup compared to lower dose THI treatments and controls. Decreased colony strength and population size may explain the decrease in syrup consumption and overwinter weight loss (reflecting consumption of overwinter food stores) of the 100 ng/g THI-treated colonies in our study (Figure 3.4a, Figure 3.5a).

As is typical of most colony-level studies, sample size is the greatest weakness of our field trial. While the number of colonies per treatment in F1 (twenty colonies) was larger than some overwinter studies [219], still we had inadequate statistical power to detect an effect at 20 ng/g THI. A strength of our study design is that neonicotinoid exposure in our study occurred immediately prior to overwintering rather than during summer as in other studies [219,232].

Despite an apparent dose response in colony survival (Figure 3.2a), pathogen load and queen quality were not significantly impacted by overwinter exposure to THI at high (20-100 ng/g) doses (Figure 3.6). Considering the overall low levels of *Varroa* and *Nosema* in our study colonies throughout F1, it is not surprising that a treatment effect of THI exposure was not observed; however, we cannot rule out synergy or additive effects of neonicotinoids in colonies with higher disease pressure.

The absence of a treatment effect on queen quality in our study is in contrast to other studies [17,195] which have shown that queens are negatively impacted by chronic, colony-level, field-realistic 4-5 ng/g THI and 1-2 ng/g CLO exposure, demonstrating 60% increases in queen supersedure [17], and significant 20% and 9% decreases in total number and viability of spermatozoa, respectively, in queen spermathecae [195]. The discrepancy in queen quality results

of our study and others may be explained by differences in timing of colony neonicotinoid exposure. In our study, THI exposure took place in the fall when the queen is much less reproductively active, in contrast to other studies in which queens were exposed during development [195] or during summer colony build-up [17]. Considering that queen quality is an oft-cited reason for reduced overwintering success [92], our data would suggest that chronic overwinter neonicotinoid exposure is not responsible for declines in queen reproductive health. Furthermore, our low sample size (6 queens) may have been inadequate to detect treatment effects on queen quality.

3.5.2 Effects of THI on overwintering honey bee colonies in F2

The combination of weak colony strength in fall and chronic, environmentally realistic, 5 or 10 ng/g THI exposure did not significantly increase overwinter mortality or affect temperature homeostasis relative to control colonies (Figure 3.2b, Figure 3.7), suggesting that colonies are resilient in the face of combined stressors. Similar to our findings, Sandrock *et al.* [17] showed that colony overwintering success was not affected by chronic 5.3 ng/g THI and 2.05 ng/g CLO exposure. Furthermore, our findings in F2 support the results of a four-year colony monitoring study which found no correlation between overwinter colony mortality and environmental pesticide residues in bee bread, although the beebread did not contain THI or CLO residues [241]. As in F1 above, despite a sample size per group of twenty-one to twenty-two colonies, we lacked adequate statistical power to detect a treatment effect on survival in F2.

Sublethal effects of field-realistic THI exposure on colony temperature homeostasis were not observed. Laboratory studies have shown that individual worker bees exposed to sublethal doses CLO have decreased ability to detect and respond to environmental stimuli, suggesting a potential mechanism for decreased temperature control within an overwintering colony chronically exposed to neonicotinoids [242]. However, our F2 temperature results suggest that effects of neonicotinoids on individual bees may not scale up to cause colony-level dysfunction. The lack of a treatment effect on temperature regulation in our study contrasts with Colin *et al.* [243] who demonstrated that colonies chronically exposed to 5 ng/g of IMD through sugar syrup had higher average overwinter in-hive temperatures and decreased colony temperature variability, and Meikle *et al.* [244] who observed that a history of commercial pollination activity and agrochemical exposure was associated with lower overwinter internal colony temperatures and increased colony temperature variability. These inconsistent results regarding the effect of pesticide exposure on

colony temperature regulation may be explained by variation in overwintering climate and beekeeping practices. Our study colonies wintered outdoors in Saskatchewan, experiencing ambient temperatures of -20°C and below, while Meikle *et al.* [244] wintered their study colonies indoors at 7°C, and Colin *et al.* [243] wintered their study colonies outdoors in the desert environment of Tuscon, Arizona. Notably, Colin *et al.* [243] were unable to repeat the results of their Tuscon study in an identical trial conducted in Sydney, Australia, underscoring the geographic variation in colony temperature control in response to pesticide exposure.

Considering the absence of lethal or sublethal effects of overwinter, environmental THI exposure demonstrated in our study, parameters other than pesticide exposure should be examined to predict colony overwinter success, including levels of *Varroa*, fall colony strength, deformed wing virus and acute bee paralysis virus titers, queen age, and beekeeper knowledge and experience [241,245].

3.5.3 Effects of chronic THI or CLO exposure of winter adult workers in C1

In accordance with F1, exposure to high dose, 100 ng/g THI or CLO in the laboratory resulted in the greatest decrease in median survival time relative to negative controls ($P < 0.001$, Figure 3.8a,c, Table A3.1). However, in contrast to F1, we found significant effects of chronic THI or CLO exposure on adult winter worker laboratory survival at field realistic (5 ng/g) and high environmental (20 ng/g) doses. Thus, our study demonstrates that winter workers in the laboratory are more sensitive to chronic neonicotinoid exposure compared to colonies overwintering in the field. One explanation for this observation could be that the eusocial structure of a colony buffers pesticide stress while individual workers in the laboratory are rendered more vulnerable to neonicotinoid toxicity due to a lack of eusocial support and the stress of the artificial cage environment [240,246]. Alternatively, in the field, there was likely dilution of the THI administered during fall feeding due to colony consumption of existing brood honey stores overwinter, resulting in decreased THI exposure of workers in the field colonies compared to workers in the laboratory.

3.5.4 Effects of chronic THI or CLO exposure of winter adult workers in C2

In contrast to F2, significant ($P < 0.05$) decreases in winter worker survival were observed after chronic exposure to 5 and 10 ng/g THI or CLO in the laboratory (Table A3.2). While the combined stress of weak colony strength and neonicotinoid exposure did not predispose THI-treated colonies to overwinter loss in the field, the addition of cage-associated stress may have

predisposed winter workers to neonicotinoid-related mortality in the laboratory. For example, the cage volume to bee ratio used in our laboratory trials (17:1) was higher than the 3:1 ratio recommended by others [240] which may have increased the social stress for the workers in our study.

Similar to our findings, Baines *et al.* [221] demonstrated significant negative effects of environmental concentrations of THI or CLO on winter adult worker survival in the laboratory. In contrast to our study, Alkassab and Kirchner [220] found that chronic exposure to 10 ng/g CLO did not significantly decrease winter worker survival in the laboratory, while we found that winter workers exposed to 10 ng/g CLO had a significant, 21% decrease in survival time ($P < 0.001$, Table A3.2) compared to negative controls. A higher daily syrup consumption in our study (77 mg/bee/day vs. 60 mg/bee/day) and a longer exposure time in our study (30 days compared to 12 days) could explain the differences in survival in our study [220].

Compared to the literature on chronic laboratory neonicotinoid exposure of summer workers, our study demonstrates that winter workers are more sensitive to THI under conditions of chronic exposure in the laboratory. In our study, significant decreases in winter worker survival were observed after chronic exposure to 5, 10, and 20 ng/g THI while in chapter 4 [247] we show no significant effect of chronic 10 and 20 ng/g THI exposure on summer worker survival. Similarly, Overmyer *et al.* [232] found no effect of chronic, 117 ng/g THI exposure on summer adult worker survival, while in our study, chronic, 100 ng/g THI exposure resulted in over 70% decreases in survival time of winter workers compared to controls (Table A3.2).

3.6 Conclusion

In summary, chronic, high dose, environmentally unrealistic exposure to 100 ng/g THI was necessary before a significant decrease in overwinter survival of strong fall colonies was observed. This study demonstrated no effect of environmental doses of THI on overwinter survival of weak fall colonies. Considering that the same environmental doses of THI resulted in significant overwinter mortality of winter worker bees in the laboratory, this study highlights the importance of field studies to validate laboratory data.

PREFACE TO CHAPTER 4

Chapter 4 focuses on the effects of chronic, laboratory neonicotinoid and/or fungicide exposure of summer adult honey bee workers and worker honey bee brood. Co-exposure of honey bees to both neonicotinoids and fungicides is common during foraging on canola grown in Saskatchewan. While 95% of Saskatchewan canola is grown from neonicotinoid-treated seed, canola is also commonly sprayed with the foliar, SBI fungicide prothioconazole (PRO) at bloom for control of the fungal pathogen *Sclerotinia sclerotiorum* [248]. Of concern, there is laboratory evidence that SBI fungicides may potentiate negative effects of neonicotinoids on honey bees by inhibiting neonicotinoid metabolism in honey bees co-exposed to neonicotinoids and SBI fungicides [142,146,147]. Thus, in Chapter 4, we mimicked chronic, natural exposure of honey bee worker adults and brood to THI and/or PRO in the laboratory and examined the effects on survival as well as HPG development. Chapter 4 provides a seasonal comparison for the effects of chronic neonicotinoid exposure on winter adult workers discussed previously in Chapter 3. Chapter 4 also provides a laboratory correlate for the colony-level field study discussed in Chapter 2. Results from Chapter 4 demonstrate that adult honey bee workers are more sensitive to negative effects of chronic THI exposure on survival compared to worker honey bee brood. Concurrent, chronic exposure of worker adults or brood to THI in combination with an SBI fungicide did not have additive negative effects on survival. Furthermore, chronic THI and/or PRO exposure was not shown to negatively affect HPG development of adult workers. These results emphasize the importance of conducting risk assessment for neonicotinoids and honey bees utilizing multiple life stages of worker honey bees. Chapter 4 also highlights that environmental (10-20 ng/g) concentrations of THI, alone or in the presence of a fungicide, do not pose a threat to survival of summer adult honey bee workers or honey bee brood in Saskatchewan.

Chapter 4: **Effects of chronic dietary thiamethoxam and prothioconazole exposure on *Apis mellifera* worker adults and brood**[¶]

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4.1 Abstract

Chronic exposure of honey bees to the neonicotinoid THI and the fungicide PRO is common during foraging in agricultural landscapes. We evaluated the survival and HPG development of adult worker honey bees, and the survival of worker brood when chronically exposed to THI or THI and PRO in combination.

We found that thirty days of exposure to 40 ng/g of THI significantly ($P < 0.001$) increased the frequency of death in worker adults by four times relative to solvent control. Worker brood required 23 times higher doses of THI (1 ng/µl or 909 ng/g), before a significant ($P = 0.04$), 3.9 times increase in frequency of death was observed relative to solvent control. No additive effects of simultaneous exposure of worker adults or brood to THI and PRO were observed. At day 8 and day 12, HPG acinar diameter was not significantly different ($P > 0.05$) between controls and adult workers exposed to THI and/or PRO.

These results indicate that chronic exposure to field-realistic doses of THI and/or PRO are unlikely to affect the survival of adult workers and brood.

4.2 Introduction

Honey bees are an ecologically and economically important species due to their pollination activities and production of honey and other hive products. Unfortunately, beekeepers have experienced increased colony losses in recent years which have been attributed to a variety of interacting stressors, including pathogens, malnutrition, poor management, and exposure to pesticides [156]. Of the numerous pesticides detected in North American honey bee colonies [55], the neonicotinoid class of neurotoxicant insecticides [249] has been broadly studied for its negative effects on honey bees at sublethal doses [11].

THI is a widely prevalent neonicotinoid in nectar, honey and pollen from both agricultural crops as well as non-target species [13,250], at median doses in seed-treated oilseed rape nectar from 0.65 ng/g to 2.4 ng/g [66]; at mean doses in honey from 0.29 ng/g [24] to 6.4 ng/g [68], to as high as 17.2 ng/g [3]; and at mean doses in pollen from 0.15 ng/g [250] to 28.9 ng/g [68]. In semi-field studies, chronic, exposure to environmental doses of THI, as well as its metabolite CLO, another neonicotinoid insecticide, have been linked to deleterious, sublethal effects on honey bees, including decreased queen fecundity (at doses of 4 ng/g THI and 1 ng/g CLO) [195], and decreased colony honey production (at doses of 20 ng/g CLO) [233]; however, field studies often find no effect of environmental THI or CLO exposure on honey bee colonies [64,66].

THI is commonly found in association with fungicides, especially in pollen [13,250], including the systemic fungicide PRO [251]. Mean PRO residues in bee pollen were reported at 182.9 ng/g [252]. Fungicide residues within the hive have been correlated to colony dysfunction [253]. For example, adult workers were twice as sensitive to the insecticidal effects of THI when exposed in combination with the fungicide BOS; however, the mechanism of this interaction is unknown [13]. Similarly, SBI fungicides, such as PRO, have been shown to enhance insecticidal properties of neonicotinoids and acaricides in honey bees, likely through inhibition of P450s [138,152]. P450s are an important part of the detoxification and defense system of insects; however, this family of monooxygenases is especially limited in honey bees, which may make them more susceptible to negative effects when exposed to combinations of pesticides [138,145]. The CYP9Q P450 subfamily, expressed in the brain and the Malpighian tubules, among other tissues, has been shown to detoxify the neonicotinoids thiacloprid and acetamiprid in honey bees [44].

Recognizing that foraging bees deliver pesticide-contaminated pollen and nectar to the colony, honey bees are likely exposed to pesticides at the brood stage [11]. Accordingly, the Organization for Economic Co-operation and Development (OECD) has established guidance documents for chronic testing of xenobiotics on both adults [239] and brood [254]. During early larval development, each worker larva receives approximately 30 mg worker jelly [60]. The percent pesticide transfer from contaminated pollen to royal jelly has been demonstrated experimentally to be 0.001-0.016 %, [255] corresponding to a total dose of 1.4×10^{-5} ng THI in 30 mg worker jelly, assuming pollen is contaminated with 28.9 ng/g THI [68]. Later in larval development, each worker larva is fed approximately 180 mg nectar and 5.4 mg pollen [60], corresponding to a total dose of 0.588 ng THI, assuming nectar contains 2.4 ng/g THI [66] and pollen contains 28.9 ng/g THI [68].

HPG development in worker bees is another commonly used functional marker for pesticide exposure, linking pesticide effects in adults and brood. HPG acini reach maximal size in nursing bees of approximately 6 days old with subsequent gland atrophy during the transition to foraging duties [87]. Premature atrophy of these brood food-producing glands has been associated with exposure to insecticides and fungicides, possibly resulting in suboptimal feeding of brood [256].

Considering the high frequency of exposure and sensitivity of honey bees to mixtures of pesticides, there is a need for more studies which evaluate combined effects of neonicotinoids and fungicides, particularly chronic studies beyond standard 10-day feeding trials [239] to allow for development of cumulative toxic effects [68,257]. As such, a comprehensive risk assessment should include an evaluation of cumulative effects on both adults and brood. Accordingly, we hypothesize that (1) chronic exposure to the neonicotinoid THI, alone and in combination with the fungicide PRO, will decrease adult worker survival and HPG development, and (2) THI, alone and in combination with PRO, will decrease survival of worker brood. To test these hypotheses, we reared worker larvae and newly emerged adult workers in the laboratory and exposed them chronically to pesticides through the diet while monitoring survival as well as HPG development in adult workers.

4.3 Materials and Methods

4.3.1 Materials

All pesticides and solvents were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). Pesticide details, including name, product number, lot number, purity, and expiration date were as follows: (i) THI: 37924-100 mg, BCBT8326, ≥98% purity, expires March 2022; (ii) PRO: 34232-100 mg, SZBE225XV, 99.9% purity, expires August 2019; (iii) DIM: 45449-100 mg, BCBS9338V, ≥98% purity, expires August 2021. DIM and THI stocks were prepared in water.[22, 28] PRO was dissolved in 5 mM Tris.

Table 4.1 Concentration of pesticides and solvents in diet administered to adult workers

Test substance	Concentration (ng/g)
THI	10, 20[†], 40, 100[‡]
PRO	360
DIM[§]	1000
THI + PRO	10, 20, 40 THI + 360 PRO

[†]20 ng/g concentration was only tested in survival experiment
[‡]100 ng/g concentration was only tested in the HPG experiment
[§]DIM was only tested in survival experiment

4.3.2 Animals

From July-August 2018, synchronized frames of *A. mellifera* worker brood were obtained by caging the queens in separate colonies for 24 hours with a frame of empty foundation drawn with wax. Frames were marked with the date of egg-laying so that all experimental bees were of known age. All colonies were maintained at a research apiary located at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada) in accordance with the Saskatchewan Apiaries Act [258].

4.3.3 Study design

To compare the effects of chronic exposure of adult workers to THI, alone and in combination with PRO, we monitored the daily mortality over 30 days of groups of 48 newly emerged workers fed THI and/or PRO *ad libitum* in sugar syrup and pollen paste. DIM was used as a positive control for survival. At days 8 and 12, 21 bees per treatment per day were sacrificed for HPG dissection and measurement. Considering that adult worker honey bees have an average lifespan of 42 days during summer [85], decreased survival of negative control workers was expected after day 20 of the experiment; however, a chronic, 30-day exposure period was considered necessary to reflect the reality that worker honey bees transition to foraging duties later in life, and thus are likely to encounter pesticide residues in the environment as they senesce.

For each treatment group, both the sugar syrup and the pollen paste contained the same concentration of pesticides in ng/g (Table 4.1). The concentrations tested included 10 ng/g THI to reflect mean to maximum concentrations of THI in honey; 20 ng/g THI to reflect mean concentrations of THI in pollen [68]; as well as two high doses of THI (40 and 100 ng/g) which

would be sporadically encountered in pollen under environmental conditions [68]. The dose of PRO (360 ng/g) was chosen based on maximum residues reported in pollen [252].

To compare the effects of chronic exposure of worker brood to THI, alone and in combination with PRO, worker larvae were reared *in vitro* according to the protocol of Schmehl *et al*. [179] and fed pesticide or control diet (Table 4.2) from days 3-6. Mortality was monitored daily until eclosion. Two to five replicates (24-60 larvae) were performed of each pesticide-dose combination (Table 4.2). The six concentrations of THI tested ranged from 10 ng/µl (9091 ng/g) to 0.1 ng/µl (91 ng/g), representing a total dose of 1400 ng to 14 ng THI. The lowest experimental dose (14 ng THI) is 24 times greater than estimated environmental exposure of worker brood to THI (0.588 ng THI)[60]. The dose of PRO tested (0.36 ng/µl or 327 ng/g) was lower in the worker brood study compared to the adult worker study (360 ng/g).

Table 4.2 Concentration and dose of pesticides in diet administered from days 3-6 to worker *Apis mellifera* larvae reared *in vitro*

Pesticide(s) (ng/µl)	Pesticide concentration (ng/g) †	Total dose THI (ng/larva)	Total dose PRO (ng/larva)‡	Total dose DIM (ng/larva)
THI 10	9091	1400	-	-
THI 5	4545	700	-	-
THI 2	1818	280	-	-
THI 1	909	140	-	-
THI 0.5	454	70	-	-
THI 0.1	91	14	-	-
PRO 0.36	327	-	50.4	-
THI 10 + PRO 0.36	9091 THI; 327 PRO	1400	50.4	-
THI 5 + PRO 0.36	4545 THI; 327 PRO	700	50.4	-
THI 2 + PRO 0.36	1818 THI; 327 PRO	280	50.4	-
THI 1 + PRO 0.36	909 THI; 327 PRO	140	50.4	-
THI 0.5 + PRO 0.36	454 THI; 327 PRO	70	50.4	-
THI 0.1 + PRO 0.36	91 THI; 327 PRO	14	50.4	-
DIM 43-45§	39000-41000	-	-	6200

†Concentration in µg/kg calculated based on 1 ml diet weighing 1.1 g
‡Total dose calculated based on four days of feeding
§Concentration varied from day 3-6 of feeding

4.3.4 Effects on adult *Apis mellifera* workers

4.3.4.1 Preparation

To obtain newly emerged worker bees from three genetic lineages, synchronized frames of capped brood at day 19 post-oviposition were transferred from the field to an incubator at 33°C and 60% humidity. On day 21 post-oviposition (considered day 0 of the experiment) the newly emerged worker honey bees were allocated to stainless steel insect cages (Small Life Supplies, Cambridgeshire, Great Britain). Bees of different genetic lineages were distinguished with a non-toxic colored paint mark on the thorax (Mitsubishi Pencil Co. Ltd., Downers Grove, IL, USA). For survival analysis, 40 small cages (measuring 7.5 x 4 x 5.5 cm) were filled with 12 bees each, with four bees from each genetic lineage. Four cages were assigned to each treatment group (48 bees per treatment). For HPG analysis, 16 large cages (measuring 8.5 x 5 x 9 cm) were filled with 48

bees each, with 16 bees from each genetic lineage. Two cages of 48 bees were assigned to each treatment group, totaling 96 bees per treatment. Three additional small cages which did not contain bees were used for monitoring diet evaporation.

4.3.4.2 Interventions

Ad libitum 50% (w/v) sucrose syrup and pollen paste, both containing pesticides (Table 4.1), were provided to the caged bees, with the diets changed every three days. The solvent for the treatment groups and solvent control was 5 mM Tris. The solvent for the positive control DIM group and the negative control was water. The syrup was prepared fresh every three days. Pollen paste was prepared using pollen collected from colonies in spring 2018, during bloom of willows and prior to bloom of local agricultural crops. To prepare the paste, a calculated volume of pesticide stock solution was added to sucrose syrup (1:1 w:v); mixed by vortexing; added to pollen in a ratio of 30 g syrup to 100 g pollen; and mixed thoroughly into a paste that contained the desired concentration of pesticides. The pollen paste was divided into aliquots and stored at -20°C in between feedings.

To evaluate HPG development, on days 8 and 12 of the experiment, 21 bees per treatment per day (7 bees x 3 genetic lineages) were sacrificed for HPG dissection, according to previously published protocols [259–261]. Briefly, the bees were anesthetized with CO_2, decapitated, and the heads were embedded in a beeswax dissection plate. Next, the heads were dissected in insect saline (1 L distilled water with 7.5 g NaCl, 2.38 g Na_2HPO_4 and 2.72 g KH_2PO_4) [261] using an Olympus SZ61 stereomicroscope (Olympus, Tokyo, Japan). The isolated glands were stained with ~250 µl of 0.25% (w/v) Coomassie Brilliant Blue R-250 [262] for ~3 seconds and then stored in insect saline at 4ºC for 24-48 hours until digital image capture was performed.

4.3.4.3 Methods of measurement

Adult bee mortality and syrup consumption were monitored daily. Syrup consumption was determined by recording the volume of syrup remaining in the gradated feeding syringe each day. To determine pollen paste consumption, pollen feeders were weighed prior to insertion and upon removal from the cage every three days.

To measure HPG acinar diameter, a wet mount of each HPG sample was prepared in insect saline without a coverslip and 20 unique acini per bee (420 acini per treatment) were photographed using a 10X objective on an Olympus BX51/BX41 microscope and an Olympus DP71 microscope

digital camera. The diameter of each acinus was measured perpendicular to its point of attachment using Image-Pro Premier 9.2 (Media Cybernetics, Rockville, MD, USA).

4.3.4.4 Analysis of data

All statistical analyses were performed using Stata/SE 15.1 (College Station, TX, USA). Data are presented as means ± standard deviation (SD) unless indicated. Mean pollen consumption over three days per bee per cage and mean daily syrup consumption per bee per cage for the treatment groups and negative controls were analyzed using a mixed model with an ar1 covariance matrix. Pollen and syrup consumption were corrected for evaporation. The assumptions of the model were met. Survival analyses over 30 days were performed using Cox proportional hazards regression with cage as a gamma shared frailty group variable. Although 48 bees were initially assigned to each treatment group, final data analysis revealed 47-50 bees per treatment. Proportionality of hazards was confirmed using a post hoc global test. HPG acinar diameter was analyzed using a Kruskal-Wallis equality of populations rank test with a post hoc Dunn's pairwise comparison test.

4.3.5 Effects on worker *Apis mellifera* brood

4.3.5.1 Preparation

Larvae were reared *in vitro* in accordance with the protocol of Schmehl *et al*. [179] and OECD guidelines [254]. Larval diets A, B, and C were prepared in advance as per the recipe of Schmehl *et al*. [179] from royal jelly (Stakich, Troy, MI, USA), D-glucose (Fisher Scientific, Toronto, ON, Canada, #D16-500), fructose (Fisher Scientific, #L95-500), Bacto yeast extract (Fisher Scientific, #B212750), and distilled water. Diet aliquots were frozen at -20ºC and thawed immediately before use. On the fourth day after oviposition, considered day 1 (D1) of the experiment [254], three frames of first instar larvae were transferred from the field to the laboratory in a portable incubator at 35ºC. Within a sterile biosafety cabinet, the larvae were grafted into sterilized queen cell cups (Apihex, Calgary, AB, Canada) containing 20 µl pre-warmed, uncontaminated diet A within a 48-well sterile tissue culture plate (STCP) (Fisher Scientific) kept on a warming plate at 35ºC. Larvae were incubated within a desiccator containing supersaturated K_2SO_4 solution [179] at a mean relative humidity of 83.0% (SD 8.3) in an incubator at a mean temperature of 34.5 ºC (SD 0.19). The larvae were not fed on D2.

4.3.5.2 Interventions

Larvae were exposed to pesticides in the diet from D3-6. On D3, each STCP containing 48 healthy larvae (16 larvae x 3 genetic lineages) was divided into one to three treatment groups and one control group (12-24 larvae per group). From D3-6, each group was fed either a solvent control diet or diet containing test substance (Table 4.2). The solvent was 0.25 mM Tris or water for all THI treatments and controls; 0.25 mM Tris for all THI with PRO treatments and controls; and the solvent was water for the DIM positive controls. The diets were contaminated with test substance and vortexed immediately prior to feeding each day. The volume of the test substance was ≤ 10% of the diet volume.

4.3.5.3 Methods of measurement

Brood mortality was checked once daily throughout development using an Olympus SZ61 stereomicroscope for larvae or unaided visual examination for pupae, in accordance with criteria of Dai *et al.* [181]. To facilitate pupation [179], on D7, or once the larvae had completely consumed their diet, larvae were transferred to a new STCP containing absorbent Kimwipe (Kimberly-Clark, Irving, TX, USA) at the bottom of each cell and incubated in a desiccator containing supersaturated NaCl solution [179] at a mean temperature of 34.6ºC (SD 0.24) and a mean humidity of 67% (SD 7.81). To facilitate eclosion, on D15, the plates were transferred to ventilated plastic boxes (18 x 11 x 9 cm) and incubated in an environmental chamber at 33ºC and 60% humidity until eclosion [254]. Newly emerged bees were provided with *ad libitum* access to non-contaminated pollen paste and sucrose solution (1:1 w/v) and live bees were removed and counted daily.

4.3.5.4 Calculations

Percent larval, pupal, and total survival were calculated as follows for each treatment group [181]:

Larval survival = (# larvae D7/# larvae D3) x 100

Pupal survival = (# adults/# larvae D7) x 100

Total survival = (#adults/# larvae D3) x 100

4.3.5.5 Analysis of data

Data are presented as means ± SD. Larval, pupal, and total survival were compared using a Kruskal-Wallis equality of populations rank test or a one-way analysis of variance (ANOVA). Post hoc comparisons were performed using a Dunn's pairwise comparison test or a Bonferroni multiple comparison test. Overall survival was analyzed with Cox proportional hazards regression

with stratification of the data by plate. Proportionality of hazards was confirmed using a post hoc global test.

4.4 Results

4.4.1 Effects on adult *Apis mellifera* workers

We found that chronic exposure of adult workers to 40 ng/g THI significantly reduced survival ($P < 0.001$) and PRO did not enhance insecticidal activity of THI under laboratory conditions.

Compared to the solvent control, 47-50 adult bees exposed to 10-40 ng/g THI had a significantly increased frequency of death (Wald $X^2(9)=183.51$, $P < 0.001$, Figure 4.1, Table A4.1) after 30 days of exposure; however, compared to the negative control, only the bees exposed to 40 ng/g THI had a significant ($P = 0.002$), 2.8 times increased frequency of death (Table A4.2) after 30 days. The survival of the negative control was not significantly different ($P = 0.363$) from the solvent control. Twenty days of exposure to 40 ng/g THI and 25-30 days of exposure to 10-20 ng/g THI were required before significant differences ($P < 0.05$) in survival from the solvent control were observed (Figure 4.1). On its own, PRO did not significantly increase the frequency of death of adult bees relative to the solvent control (Table A4.1, $P = 0.059$), and the combination of THI with PRO did not alter the frequency of death compared to THI alone (THI 10 vs. THI 10 PRO $P = 0.658$; THI 20 vs. THI 20 PRO $P = 0.532$; THI 40 vs. THI 40 PRO $P = 0.683$). The effect of genetic lineage on survival was non-significant ($P = 0.153$), with a non-significant interaction between treatment and genetic lineage ($P = 0.0661$).

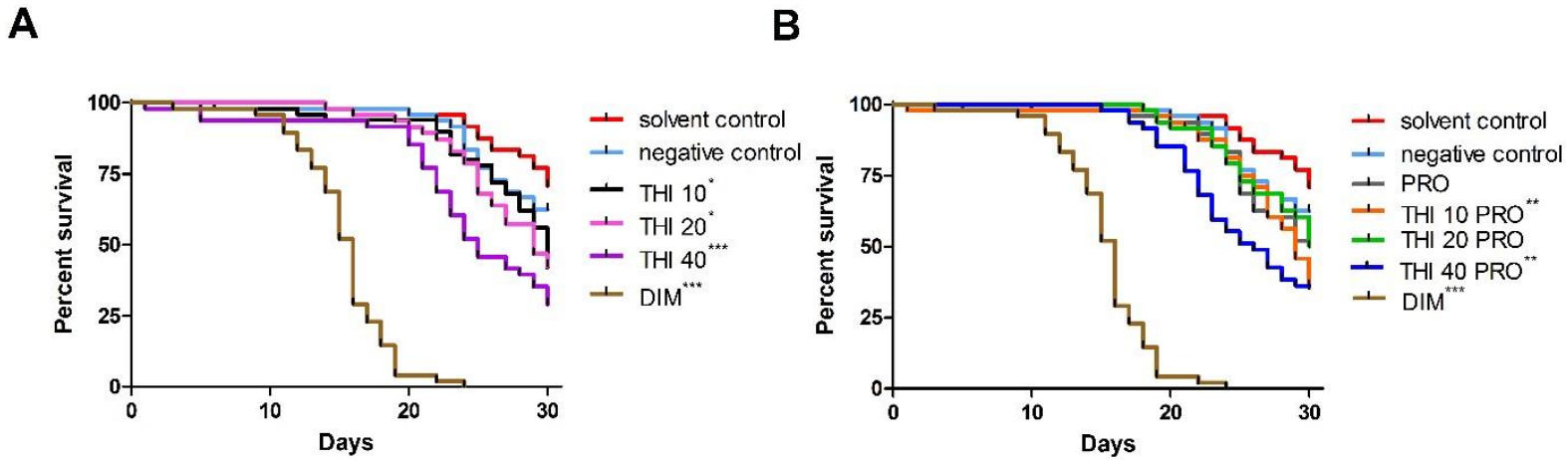

Figure 4.1 Chronic thiamethoxam (A) and thiamethoxam with prothioconazole (B) exposure significantly decreases adult worker *Apis mellifera* survival. Newly emerged workers were exposed to THI (dose specified in ng/g), and/or PRO (360 ng/g) for 30 days through syrup and pollen paste and mortality was monitored daily. The positive control was 1000 ng/g DIM. Lines indicate percent daily survival for 47-50 bees. *,**,***significantly different from solvent control, *P<0.05, ** P<0.01, ***P<0.001, by Cox proportional hazards regression.

Chronic exposure of adult workers to THI and/or PRO did not significantly impact HPG development under laboratory conditions relative to controls. We found no significant difference in acinar diameter of the 21 bees in each treatment group relative to the solvent control or the negative control at day 8 ($X^2(6) = 8.90$, $P = 0.1794$; mean = 102.78, SD = 12.47) and day 12 ($X^2(6) = 14.00$, $P = 0.0298$; mean = 101.34, SD = 12.59) (Figure 4.2, Table A4.3). Despite the overall significant ($P < 0.05$) P value at day 12, post hoc pairwise comparisons did not yield significant differences among treatment groups. When the positive control was included in the analysis, the 100 ng/g THI treated bees demonstrated significant, 11-15 % decreased acinar diameter relative to the 10 ng/g THI PRO treated bees at day 8 ($X^2(7) = 14.321$, $P = 0.0157$) and 12 ($X^2(7) = 19.28$, $P = 0.0190$) and the PRO treated bees at day 12 ($P = 0.0229$).

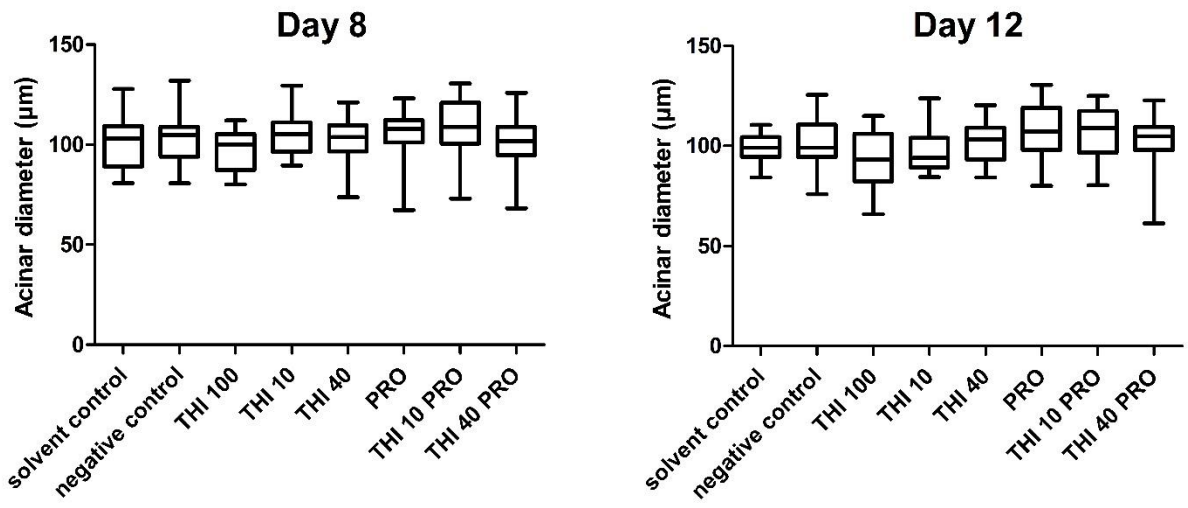

Figure 4.2 Thiamethoxam and prothioconazole exposure does not significantly affect adult worker *Apis mellifera* hypopharyngeal gland development in the laboratory. Newly emerged workers were exposed to THI, and/or PRO for 8 or 12 days through syrup and pollen paste, followed by dissection and measurement of HPG acini. Box and whisker plots indicate median, 25th and 75th percentiles, minimum and maximum for 21 bees.

Syrup consumption was not affected by THI or PRO contamination (Figure 4.3A); however, there was treatment-associated variation in pollen paste consumption (Figure 4.3B). Mean consumption of pollen paste was 8.62 mg per bee per three days (SD = 7.69) and the mean consumption of syrup per bee was 0.023 ml per day (SD = 0.016). Treatment had a significant effect ($X^2(8) = 25.71$, $P = 0.0012$) on pollen paste consumption, with the 40 ng/g THI PRO group consuming 19% ($P = 0.004$) less pollen paste on average compared to the solvent control and the 20 ng/g THI group consuming 15% ($P = 0.006$) more pollen paste on average compared to the solvent control. Statistically significant differences in pollen paste consumption relative to the solvent control were not observed for the other treatment groups. There was no significant effect of treatment on syrup consumption ($X^2(8) = 11.2$, $P = 0.1905$). Consumption of the experimental diet varied significantly over time (pollen $X^2(9) = 1062.87$, $P < 0.001$; syrup $X^2(29) = 1225.68$, $P < 0.001$) and the interaction between treatment and time was non-significant (pollen $X^2(72) = 89.11$, $P = 0.0837$; syrup $X^2(232) = 246.41$, $P = 0.2462$). Peak pollen consumption occurred during the first three days of exposure (mean = 25.6 mg/bee/3 days) and declined, on average, by over 70% during the following six days (Figure 4.3B), after which pollen consumption remained relatively constant at a mean of 5.71 mg/bee per three days (SD = 4.03). Mean cumulative

consumption of THI after 30 days in bees exposed to 10, 20, and 40 ng/g THI was 9.42 (SD = 0.47) ng/bee, 17.60 (SD = 2.49) ng/bee, and 38.27 (SD = 1.64) ng/bee, respectively (Figure 4.3C). Mean cumulative consumption of PRO after 30 days was 320.55 (SD = 39.88) ng/bee (Figure 4.3D).

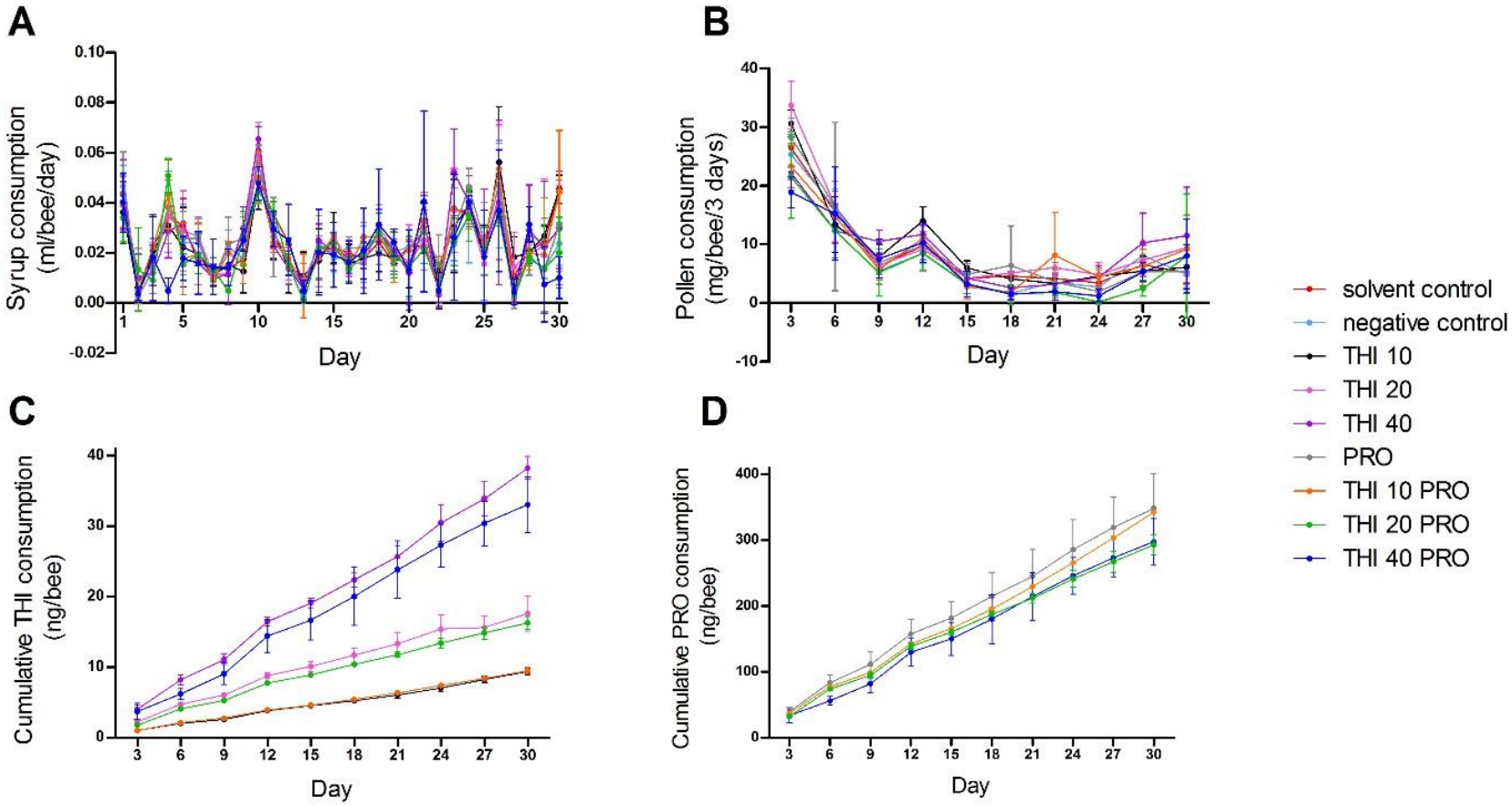

Figure 4.3 Experimental diet consumption of adult worker *Apis mellifera*. Newly emerged workers were exposed to THI (dose specified in ng/g), and/or PRO (360 ng/g) for 30 days through syrup and pollen paste. Lines represent consumption (mean ± SD) over time in (A) ml syrup per bee per day, (B) mg pollen paste per bee per three days, (C) cumulative ng THI per bee in syrup and pollen paste, and (D) cumulative ng prothioconazole per bee in syrup and pollen paste

4.4.2 Effects on worker *Apis mellifera* brood

In contrast to adult workers, at least 23 times higher doses of THI (1 ng/µl or 909 ng/g for brood vs. 40 ng/g for adults) were required before significant brood mortality was observed. When we analyzed survival of individual larvae using Cox proportional hazards regression (Log Rank $X^2(13) = 134.2$, $P < 0.001$, Figure 4.5), significant ($P < 0.05$) decreases in survival to eclosion were observed for 36 to 60 larvae fed 1 to 10 ng/µl THI (140 to 1400 total ng per larvae; Figure 4.5A,C). Larvae fed diet containing lower doses of THI, from 0.1 to 0.5 ng/µl, THI did not experience significant ($P > 0.05$) decreases survival (Figure 4.4, Figure 4.5). Solvent in the diet (water or 0.25 mM Tris) had a non-significant effect ($P = 0.076$) on survival, and there was a non-significant interaction between solvent and treatment ($P = 0.235$).

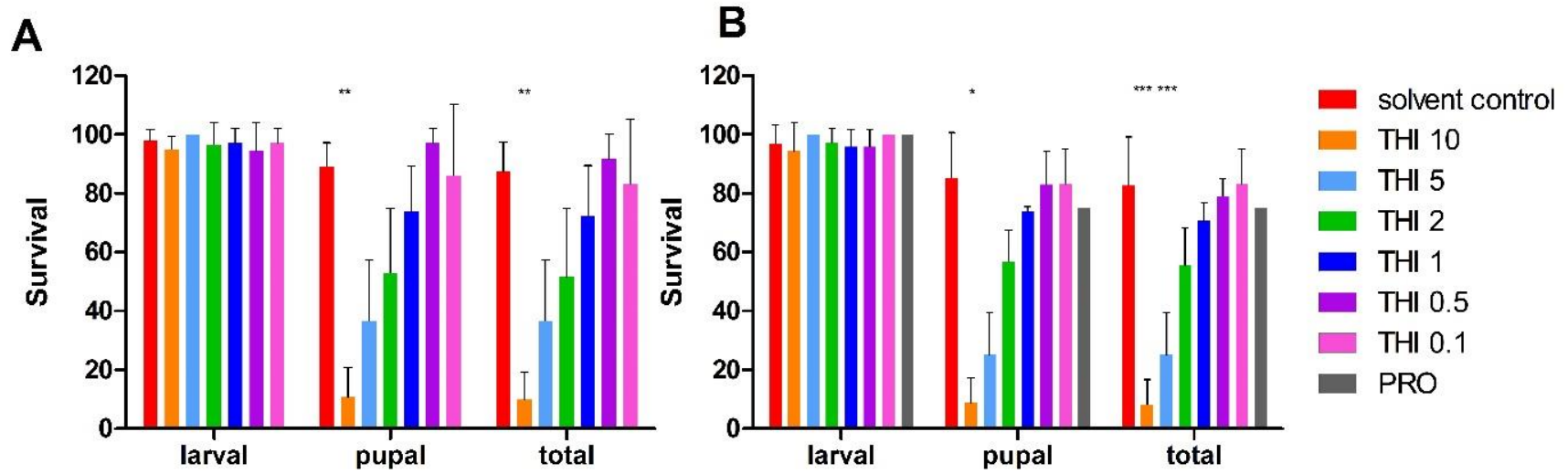

Figure 4.4 Dietary thiamethoxam (A) and thiamethoxam with prothioconazole (B) decreases pupal and total survival, but not larval survival in worker *Apis mellifera* brood reared *in vitro*. Bars indicate percent survival of 24-60 larvae (mean ± SD). From days 3-6, larvae were fed either control diet or experimental diet containing THI and/or PRO. Mortality was monitored daily until eclosion. THI doses are in ng/µl. *,**<*** significantly different from solvent control, *P<0.05,** P<0.01, ***P<0.001, by Kruskal-Wallis rank test or ANOVA.

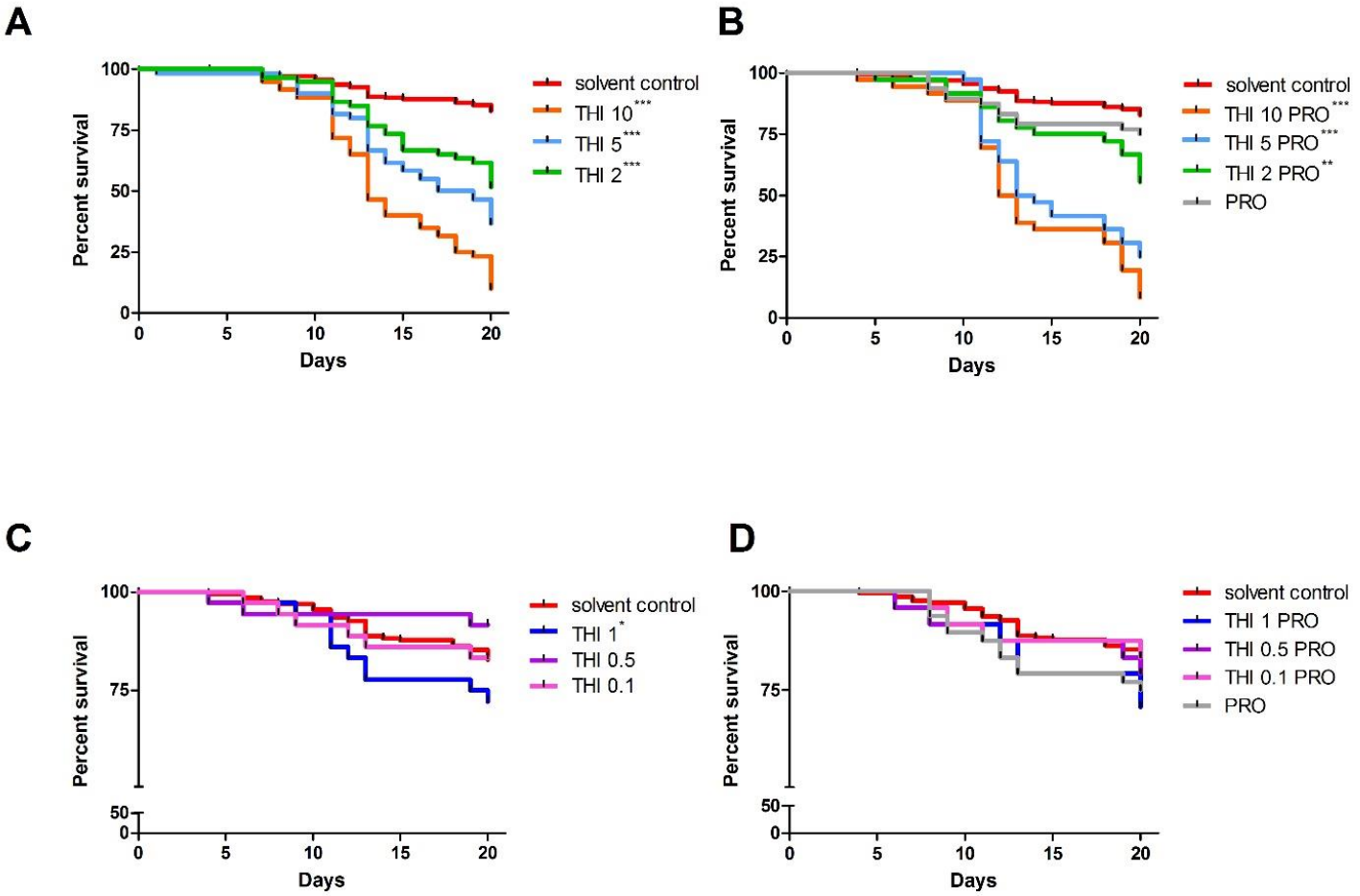

Figure 4.5 Dietary thiamethoxam (A, C) and thiamethoxam with prothioconazole (B, D) decreases survival of worker *Apis mellifera* brood reared *in vitro*. Lines indicate percent daily survival of 24-60 larvae. From days 3-6, larvae were fed either control diet or experimental diet containing THI and/or PRO. Mortality was monitored daily until eclosion. THI doses are in ng/µl. *,**,*** significantly different from solvent control, *P<0.05, ** P<0.01, ***P<0.001, by Cox proportional hazards regression analysis.

Similar to adult workers, dietary PRO did not significantly enhance the insecticidal activity of THI to worker brood *in vitro*. Significant ($P < 0.001$), 70% to 90% decreases in total survival compared to control were observed for 36 larvae fed THI at 5 and 10 ng/µl in combination with PRO ($F(7,16) = 15.11$, $P < 0.001$, Figure 4.4B); however, these decreases in survival were not significantly different from the 58% ($P = 0.0579$) to 89% ($P = 0.001$) decreases in total survival observed for 60 larvae fed 5 and 10 ng/µl THI ($X^2(6) = 23.35$, $P < 0.001$, Figure 4.4A). On its own, PRO did not significantly increase the frequency of death of brood (Figure 4.5; Table A4.4; $P = 0.317$), and the combination of THI with PRO did not alter the frequency of death compared to THI alone (Figure 4.5; THI 10 vs. THI 10 PRO $P = 0.355$; THI 5 vs. THI 5 PRO $P = 0.208$; THI 2 vs. THI 2 PRO $P = 0.549$; THI 1 vs THI 1 PRO $P = 0.624$).

In our experiments, worker brood was most sensitive to pesticides during the pupal stage, with no effect of THI or PRO on larval survival. Mean larval survival for 24-60 larvae was greater than 94% for all treatment and control groups with no significant differences in larval survival among treatment groups (Figure 4.4; $X^2(6) = 2.526$, $P = 0.8655$ for THI; $X^2(7) = 2.091$, $P = 0.9546$ for THI with PRO). Despite high survival during the larval stage, only 11% (SD = 9.99; $P = 0.0022$) and 37% (SD = 14.43; $P = 0.0825$) of the larvae fed 10 and 5 ng/µl THI (Figure 4.4A), respectively, survived the pupal stage to eclosion ($X^2(6) = 24.586$, $P < 0.001$). Similarly, only 9% (SD = 8.39; $P = 0.0136$) and 25% (SD = 14.43; $P = 0.0680$) of the larvae fed 10 ng/µl and 5 ng/µl THI with PRO (Figure 4.4B), respectively, survived the pupal stage to eclosion ($X^2(7) = 17.726$, $P = 0.0133$).

As a positive control, 72 larvae were fed DIM at 43-45 ng/µl (Table 4.2) resulting in a median survival of 10 days and a 8.8 times increased frequency of death relative to control (95% CI = 4.8-16.2, $P < 0.001$). Sporadically, in both treatment and control groups, adult bees and pupae with deformed wings and shortened abdomens were observed.

4.5 Discussion

4.5.1 Effects on adult *Apis mellifera* workers

Chronic dietary exposure of adult workers to 40 ng/g THI, alone, or in combination with PRO, significantly decreased survival under laboratory conditions. Exposure times of at least 20 days were required before significant reductions in survival of the 40 ng/g THI-treated bees were observed (Figure 4.1). This exposure scenario would be unlikely to occur in a field-realistic setting. While doses in excess of 40 ng/g THI are reported in pollen [55,68,263], foraging bees are more

commonly exposed to THI residues in the range of <1 to 3.5 ng/g in pollen and 0.65 to 2.4 ng/g in nectar during flowering of oilseed rape (considered peak exposure in our geographic area) [66]. Furthermore, workers consume greater amounts of nectar (which typically has lower pesticide residues[11,263]) than pollen during their lifetime (e.g. a nurse bee consumes 140 mg nectar per day vs. 9.6 mg pollen per day[60]), thus, concentrations in nectar would be more representative of chronic environmental exposure in adult workers. Importantly, chronic exposure periods of 20 days or more are field-realistic considering that honey bee colonies near neonicotinoid-treated corn fields in eastern Canada were observed to receive continuous neonicotinoid exposure for up to four months (May-August)[13]. In our study, the absence of differential survival prior to twenty days may suggest the need for chronic laboratory testing of pesticides, beyond current 10-day trials[239] to fully characterize risk.

High environmental doses [1,68] of 10-20 ng/g THI, alone, or in combination with PRO, did not significantly reduce survival of adult workers relative to the negative control group (Table A4.2), although significant differences in survival relative to the solvent control group were observed after 25 days of exposure (Table A4.1). Considering that there was no significant difference in survival of the negative control and the solvent control ($P = 0.363$), our results suggest that chronic 10-20 ng/g THI does not significantly impact adult worker survival, which is in agreement with the USEPA colony-level No Observed Effect Concentration (NOEC) of 19 ng/g for CLO[263].

At day 30, the mean cumulative consumption of THI (Figure 4.3C) of the 10, 20, and 40 ng/g THI-treated bees was 2.26, 4.23, and 9.20 times greater than the reported 72 hour acute oral LD_{50} for THI (4.16 ng/bee)[59]. One reason for the observed tolerance of adult workers to doses of THI in excess of the LD_{50} may have been that during chronic exposure, workers were able to upregulate metabolic pathways for excretion and detoxification of dietary THI. Furthermore, the adult workers were provided with natural pollen in the diet which may have contained natural phenolic acid and flavonol compounds shown to protect honey bees against neonicotinoid exposure due to induction of P450s [141].

The addition of the fungicide PRO did not enhance the risk of death from THI exposure (Figure 4.1). This finding is in opposition to other studies demonstrating up to threefold synergistic effects of acute oral exposure to THI or CLO and the SBI fungicide PROP on worker mortality [146,147]. Furthermore, Wade *et al.* [148] demonstrated over 7 fold increased toxicity of the

diamide insecticide chlorantraniliprole in the presence of PROP under conditions of acute topical exposure to adult workers. While PRO belongs to the same class of fungicides as PROP, differences in the chemistries of these two fungicides may explain the inconsistency in synergistic effects with other pesticides.

Differential survival of THI and/or PRO exposed adult workers was not observed during the first two weeks of the experiment; similarly, treatment effects of THI and/or PRO exposure were not observed on HPG development during this time (Figure 4.2). This finding is in contrast to other research groups who found a significant decrease in HPG acinar diameter in workers exposed to similar doses of neonicotinoids on similar days of development [89,259]. Similar to our study, Zaluski *et al*. [256] found that co-exposure of honey bees to fungicides and neurotoxic insecticides did not cause synergistic or additive decreases in HPG acinar area; however, Zaluski *et al*. [256] showed that pesticide exposure had significant negative effects on HPG area, with pesticide-exposed bees having ~94% more HPG acini with reduced cross-sectional area compared to controls. Our hypothesis for the lack of treatment effect on HPG development in this study is that the stress of the laboratory environment, lack of brood [264], and/or inadequate protein in the experimental diet resulted in hypoplasia of the glands, regardless of treatment, preventing demonstration of differential development. The evidence for gland hypoplasia in our experiment includes the 40% reduction in mean acinar diameter of our controls compared to controls in other laboratory studies at similar time points, and the absence of a declining trend in mean acinar diameter of controls from days 8-12 as expected (Figure 4.2)[259,260]. Furthermore, pollen consumption and nutritional value is positively correlated with HPG development [264,265], and it is possible that our experimental pollen paste did not have an optimal nutritional profile [265]; however, Renzi *et al.* [89] demonstrated a significant treatment effect of 0.04 ng/µl THI on HPG development even with a low protein diet. Lack of experimental diet consumption does not appear to explain the results of our HPG investigation. Although mean pollen paste consumption (8.6 mg/bee per 3 days SD = 7.69) was lower in our study compared to some *in vitro* studies [266], it was within the same range as Hatjina *et al.* [260] who found negative effects of pesticide exposure on HPG development. Similar to Hatjina *et al.* [260], mean pollen paste consumption at the start of our experiment, coinciding with HPG development, was significantly ($P < 0.001$) elevated, up to threefold greater than overall mean consumption. Syrup consumption in our study (mean 0.023 ml/bee/day SD = 0.016) was within the range reported for other chronic cage studies [267].

Considering that HPG development may be influenced by the laboratory environment [259]; the results of our study suggest that semi-field exposure [256,259] may be the best experimental context for evaluation of HPGs.

4.5.2 Effects on worker *Apis mellifera* brood

In our study, worker brood was 23 times less sensitive to THI compared to worker adults. Decreased brood susceptibility to neonicotinoids compared to adult bees has been shown previously by other researchers for THI [268], CLO [181] and IMD [181,269]. Some hypotheses for the apparent decreased sensitivity of worker brood to THI compared to adult workers could be a lack of nicotinic acetylcholine receptors in the developing central nervous system of brood [269], or increased availability of detoxification enzymes within the larval fat body [270].

An important difference of our larval THI feeding study from some other larval pesticide feeding trials [181,183] was that we only observed significant mortality in the pupal stage, rather than the larval stage. This observation is consistent with other acute [270] and chronic [182] larval THI feeding studies; however, our findings contrast with the results of Dai *et al.* [181] who found that chronic larval exposure to 10 ng/µl CLO decreased larval survival, but not pupal survival. One possible explanation for this difference is that larvae require time to process THI into active metabolites, such as CLO, and thus, insecticidal effects are delayed until sufficient quantities of metabolites have accumulated in the body [183,270].

Similar to adult workers, PRO did not demonstrate additive effects with THI on brood survival (Figure 4.4, Figure 4.5). This observation is in contrast to other studies which demonstrated that fungicides increase the insecticidal activity of miticides [183] and insecticides [148] to worker larvae reared *in vitro*. For example, in the *in vitro* larval rearing experiments of Zhu *et al.* [183], there was fivefold synergism of 34 ng/µL of the fungicide chlorothalonil with 3 ng/µL fluvalinate and fourfold synergism of 34 ng/µL chlorothalonil with 8 ng/µL coumaphos. Wade *et al.* [148] found 2.25 µg of the fungicide PROP or 5.05 µg of the fungicide iprodione, in combination with 1 µg of the insecticide chlorantraniliprole, significantly decreased adult emergence relative to chlorantraniliprole alone by 53 and 23 percent, respectively. Reasons for the absence of synergism or additive effects observed in our study compared to the work of Zhu *et al.* [183] and Wade *et al.* [148] could be that we tested different pesticide-fungicide combinations at different doses (Table 4.2) resulting in different properties of bioaccumulation and metabolism in worker larvae.

Other laboratory studies [43,44] have shown that P450 inhibitors are synergistic with the cyano-substituted neonicotinoids in exposed honey bees, but have limited interaction with nitro-substituted neonicotinoids such as IMD. Thus, oxidation by P450s may not be as an important method of detoxification of nitro-substituted neonicotinoids such as THI, thus explaining the limited effects of PRO co-exposure on THI activity in this study.

While adult workers experienced decreased survival in response to chronic exposure to 40 ng/g of THI, worker brood did not demonstrate decreased survival to adulthood unless exposed to environmentally unrealistic doses of THI of 1-10 ng/µL (909 to 9091 ng/g). Our findings contrast with Grillone *et al.* [182] who found significant, dose-responsive decreases in survival of larvae fed diets containing THI from 2 ng/µL to 0.2 ng/µL THI. Significantly decreased Africanized *A. mellifera* adult emergence has been reported in larvae acutely exposed to 0.001 mg/L THI [270]; however, other studies refuted this finding [271]. One reason for the disagreement between our work and that of Grillone *et al.* [182] could be that Grillone *et al.* [182] used the *in vitro* larval rearing methods of Aupinel *et al.* [176,178] with five days of THI exposure (corresponding to days 2-6), whereas we used the Schmehl *et al.* [179] protocol with four days of THI exposure (days 3-6), resulting in a 6.6 percent lower total dose per larva in our study for the same test concentration of THI. Nevertheless, our lack of effect at 0.1 ng/µl THI on larval survival is consistent with the no adverse effect concentration established by Dai *et al.* [181] of 0.1 ng/µl for worker larvae fed CLO, a metabolite of THI. Considering that THI residues of 0.1 ng/µl (91 ng/g) represent 3 times mean THI residues in pollen (28.9 ng/g) [68], 38 times median THI residues in nectar (2.4 ng/g) [66] and 24 times estimated dietary exposure[60] of worker brood to THI under field conditions (0.588 ng), we conclude that environmental doses of THI would not impact worker brood survival.

The main weakness of our *in vitro* brood survival data is the lack of statistical power (<80%) for doses of THI from 0.1-1 ng/µl, alone or in combination with PRO. Nonetheless, we feel that the demonstration of a dose-response in our data (Figure 4.4), lends support to our conclusions. Our larval sample size was limited by our adherence to stringent requirements [254] for high control survival. Decreased control survival was observed during early and late season grafting, and may be correlated to colony strength and reproductive activity. Furthermore the occurrence of deformed wings and abdomens in our *in vitro*-reared adults and pupae suggests that the vertical transmission of DWV may have been a confounding factor in our study. The colonies

used for grafting were equally represented in all treatment and control groups; therefore, the risk of DWV infection should have been equal across experimental groups.

4.6 Conclusion

In summary, our laboratory results support that chronic dietary exposure to THI and/or PRO at environmentally relevant doses does not (1) decrease survival or HPG development of adult workers, nor (2) decrease survival of worker brood. Neither adult workers nor brood experienced additive negative effects of THI and PRO co-exposure. Studies such as ours may serve as a model for a comprehensive approach to pesticide risk assessment which encompasses chronic effects of a test substance on multiple life stages, and in combination with other co-occurring pesticides in the environment.

PREFACE TO CHAPTER 5

In Chapter 4 we demonstrated that chronic, *in vitro* THI and fungicide exposure does not pose a significant threat to worker brood mortality at field-realistic concentrations. However, we did not investigate whether chronic, sublethal THI and fungicide exposure would predispose worker brood to disease, such as EFB. Fungicides and/or insecticides are widely used in bee-attractive crops such as blueberries and canola. Interestingly, commercial pollination of blueberries is a reported risk factor for EFB development in honey bee colonies [272–275]. EFB has also become increasingly common among honey-producing colonies in Saskatchewan, which commonly forage on canola (Geoff Wilson, Provincial Specialist, Apiculture, Government of Saskatchewan, personal communication). In Chapter 5 we examined whether chronic THI and fungicide exposure would increase mortality of worker honey bee larvae infected with the pathogenic bacterium *Melissococcus plutonius*, the etiologic agent of EFB. We infected larvae with *M. plutonius* on the day of grafting *in vitro*, chronically exposed the larvae to THI and/or fungicides in the diet, and monitored their survival over six days. We showed that THI in combination with the fungicide PROP, significantly increased larval mortality from EFB *in vitro*, but none of the other THI and fungicide combinations tested were shown to significantly affect larval survival after *M. plutonius* infection. The results from this chapter highlight the importance of examining other sublethal outcome measures, such as susceptibility to disease, to facilitate comprehensive understanding of the effects of neonicotinoids and fungicides on honey bee brood.

Chapter 5: *In vitro* effects of pesticides on European Foulbrood in honeybee larvae

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5.1 Abstract

Neonicotinoid and fungicide exposure has been linked to immunosuppression and increased susceptibility to disease in honey bees. EFB, caused by the bacterium *M. plutonius*, is a disease of honey bee larvae which causes economic hardship for commercial beekeepers, in particular those whose colonies pollinate blueberries. We report for the first time in Canada, an atypical variant of *M. plutonius* isolated from a blueberry-pollinating colony. With this isolate, we used an *in vitro* larval infection system to study the effects of pesticide exposure on development of EFB disease. Pesticide doses tested were excessive (THI and pyrimethanil (PYR)) or maximal field-relevant (PROP and BOS). We found that chronic exposure to the combination of THI and PROP significantly decreased survival of larvae infected with *M. plutonius*, while larvae chronically exposed to THI and/or BOS or PYR did not experience significant increases in mortality from *M. plutonius* infection *in vitro*. Based on these results, individual, calculated field-realistic residues of THI and/or BOS or PYR are unlikely to increase mortality from EFB disease in honey bee worker brood, while the effects of field-relevant exposure to THI and PROP on larval mortality from EFB warrant further study.

5.2 Introduction

EFB, caused by the bacterium *Melissococcus plutonius*, is an enteric disease of honey bee larvae [108]. *M. plutonius* is transmitted to developing larvae through contaminated brood food and proliferates within the larval midgut, leading to larval death, especially under conditions of colony stress [108]. Honey bee larvae respond to bacterial infection through both cellular [97] and humoral immunity [276] provided by hemocytes and antimicrobial peptides, respectively.

Strains of *M. plutonius* have been categorized as 'atypical' variants and 'typical' variants which have genetic differences [112], including variation in cell-adhesion proteins and carbohydrate metabolism [117]; different virulence factors [118]; biochemical differences, including variation in β-glucosidase activity, esculin hydrolysis, and carbohydrate fermentation [114]; as well as variable fastidiousness in their requirements for successful growth in culture media [114]. *In vitro* infection of honey bee worker larvae with *M. plutonius* has been successfully performed [114,115,277], with atypical strains of *M. plutonius* showing higher incidence of larval mortality in comparison with typical strains [114,115,277–279].

There is widespread, chronic, in-hive exposure of honey bees to complex mixtures of agricultural and apicultural fungicides and insecticides through nectar, honeydew, honey, wax, pollen and pollen stored as beebread [55,61,280]. In a survey of 350 pollen samples and 259 wax samples from North American honey bee colonies, 98.4% of pollen and wax was found to have two or more pesticide residues and 61.7% of pollen or wax containing a fungicide also contained insecticide or miticide residues [55]. Increased numbers of pesticide residues in wax, particularly fungicides which inhibit sterol biosynthesis [149], have been significantly associated with colony mortality [61]. There is also concern for negative effects of chronic, in-hive pesticide exposure on developing worker brood [247], although transfer of pesticides from pollen and honey to royal jelly is considered to be low, ranging from 0.001-0.016% [255].

Concentrations of insecticides and fungicides within hive matrices are generally considered to be sublethal for honey bees [55]. In-hive pesticide surveillance in Europe, Asia, and North and South America [68] detected the fungicide BOS in 12.6% of wax and 4.3% of pollen at means of 72.4 ng/g and 22.5 ng/g respectively; the fungicide pyrimethanil (PYR) in 1.4% of wax and 3.5% of pollen at means of 14.3 ng/g and 14.2 ng/g, respectively; the fungicide PROP in 1% of wax and 1.8% of pollen at means of 196.5 ng/g and 5.5 ng/g, respectively; and the neonicotinoid insecticide THI in 7.7% of wax, 12.8% of pollen, and 65% of honey at means of 38 ng/g, 28.9 ng/g, and 6.4

ng/g, respectively. The mean concentration of THI in honey has been reported to be as high as 17.2 ng/g in Saskatchewan, Canada [3] but globally, the average THI concentration in honey has been calculated to be 0.29 ng/g [24]. Environmental concentrations of THI in pollen and nectar have been reported as high as 86 ng/g in pollen from wildflowers adjacent to oilseed rape grown from THI-treated seed [12] and as high as 13.3 ng/g in nectar of oilseed rape grown from THI-treated seed [12]. By comparison, adverse effects of THI exposure on honey bee colonies are not observed until THI concentrations reach 20 to 100 ng/g [219,232,233,281].

Chronic co-exposure of honey bees to fungicides and insecticides within a colony has potential for synergistic negative effects on honey bee health. Compared to other insects, honey bees have fewer genes encoding P450 enzymes used in pesticide detoxification [145], and some fungicides, such as PROP, are inhibitors of insect P450s [149]. Not surprisingly, laboratory co-exposure of honey bees to PROP and insecticides has been shown to synergistically increase toxicity and decrease survival of honey bee adult workers [43,146,147] and worker larvae [148].

Neonicotinoid and fungicide exposure may also alter honey bee susceptibility to pathogens through changes in innate immune function [103,282] and social immunity [9]. Neonicotinoids are hypothesized to immunosuppress honey bees by downregulation of immune genes and pathways [136], including transcription factor NF-κB [135]. For example, the neonicotinoid CLO was shown to decrease cellular immunity and increase mortality of larvae infected with bacterial spores of *Paenibacillus larvae*, the etiologic agent of AFB [97]. Furthermore, fungicide exposure in pollen increased risk of laboratory infection of adult honey bee workers with the microsporidian parasite *Nosema ceranae*; however, neonicotinoid exposure was associated with decreased *Nosema* infection prevalence in honey bee workers [283].

Beekeepers who provide commercial pollination services to blueberry crops in Canada and the United States have reported an increased incidence of EFB in their colonies, both during and after blueberry pollination [272–275]. Elevated levels of fungicides in beebread from blueberry pollination were significantly correlated with colony loss [61]. However, the relationship between fungicide exposure during pollination and EFB is unknown.

To date, no one has investigated whether pesticide exposure alters susceptibility of honey bee worker larvae to EFB. Thus, we used an *in vitro* model to test the hypothesis that pesticide exposure increases mortality of worker honey bee larvae from EFB. Specifically, we determined if honey bee worker larvae are more susceptible to EFB-associated mortality when exposed to (i) the insecticide

THI, (ii) the fungicides BOS, PROP or PYR, or (iii) the combination of THI and BOS, PROP or PYR.

5.3 Materials and Methods

To investigate the effects of pesticide exposure on honey bee larval mortality from EFB *in vitro*, newly-hatched worker larvae were infected with a pure culture of *M. plutonius* on the day of grafting (day 0 (D0)) and exposed to pesticides in the diet from D0 to D5. Survival of the larvae from D0 to D6 was compared between pesticide-exposed larvae and controls (Table 5.1).

Table 5.1 Experimental design of *in vitro* model for testing effects of pesticides on larval mortality from European foulbrood. On D0 of the experiment, larvae received 0.5 µl PBS or 0.5 µl of a pure culture of *M. plutonius* diluted in PBS to contain 500, 250, or 50 CFU. From D0 to D5, larvae were administered control diet or diet contaminated with the pesticides THI and/or BOS, PYR, or PROP. Larval survival was monitored daily until D6.

Experimental group	Inoculation with *M. plutonius* D0	Pesticide administration D0 to D5
Pesticide and *M. plutonius*	0.5 µl *M. plutonius* with 500, 250, or 50 CFU	THI and/or BOS, PYR, or PROP
Pesticide only	0.5 µl PBS	THI and/or BOS, PYR, or PROP
Survival control	0.5 µl PBS	none
Infected control	0.5 µl *M. plutonius* with 500, 250, or 50 CFU	none
Positive control	0.5 µl PBS	THI and BOS

5.3.1 Isolation of an atypical variant of *M. plutonius*

M. plutonius was isolated from a diseased larva from a honey bee colony in blueberry pollination in the Fraser Valley of British Columbia, Canada. Briefly, the macerated larva was streaked on KSBHI agar [brain heart infusion (Difco; Becton, Dickinson and Co., Sparks, MD, USA) media with 0.15 M KH_2PO_4 (Millipore Sigma, Oakville, ON, Canada), 1% soluble starch (Difco; Becton, Dickinson and Co.), 1.5% agar (Difco; Becton, Dickinson and Co.) and 3 µg/ml filter-sterilized nalidixic acid (Millipore Sigma)] [114,284] and incubated at 37°C for 3 days under microaerophilic conditions (Pack-MicroAero, Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan). Colonies resembling *M. plutonius* [284] were subcultured on KSBHI agar and *M. plutonius* identity was confirmed using Gram stain (Figure A5.1) and PCR (Figure A5.2) [285]. Duplex PCR (Figure A5.3) [112] identified the *M. plutonius* isolate as an atypical variant which was further characterized using multi-locus sequence typing (MLST) [113]. Based on comparison to the *M. plutonius* MLST databases (https://pubmlst.org/mplutonius/) [116], the isolate belonged to

sequence type (ST) 19 of Clonal Complex (CC) 12, which was previously identified in the Netherlands [113]. The GenBank accession numbers for the MLST loci sequenced for our *M. plutonius* isolate are as follows: MT127566, MT127567, MT127568, MT127569, MT127570, MT127571 and MT127572.

The *M. plutonius* isolate was subcultured in liquid KSBHI media [114] and incubated at 37°C under microaerophilic conditions and shaking at 100 rpm to an optical density at 600 nm (OD_{600}) of 1.6. The culture was mixed with 20% glycerol and stored in 150 µl aliquots at -80°C which served as the stock culture for all experiments.

5.3.2 Experimental animals

From mid-June through mid-August, 2019, synchronized frames of worker larvae were continuously generated from six caged queens within experimental colonies of *A. mellifera* located at the University of Saskatchewan (Saskatoon, Saskatchewan, Canada). Every 24 hours, a frame of freshly-laid eggs was removed from the cage in each colony and replaced with an empty frame of foundation drawn with wax. Frames of eggs were incubated within the colony for three days until hatching, after which the frames were transported to the laboratory in a portable incubator at 35ºC.

5.3.3 *In vitro* larval rearing

A. mellifera worker larvae were reared *in vitro* [179] for six days (Table 5.1). Larval diets 'A', 'B', and 'C' [179] were prepared from royal jelly (Stakich, Troy, MI, USA), D-glucose (Fisher Scientific, Toronto, ON, Canada), fructose (Fisher Scientific), yeast extract (Fisher Scientific), and distilled water. The larvae were fed diets 'A', 'B', and 'C' in sequence [179] and the three diets differed in the proportion of ingredients they contained to reflect the changes in worker diet composition (gradual increase in sugar and protein with increasing age of larva) fed to worker larvae in a colony [179]. Diet aliquots were frozen at -20ºC and warmed to 35°C prior to feeding. On D0, within a sterilized biosafety cabinet, newly-hatched, first instar larvae were grafted from their frame into sterilized queen cell cups (Apihex, Calgary, AB, Canada) primed with 10 µl of control diet 'A', within a 48-well sterile tissue culture plate (STCP) (Fisher Scientific) kept on a warming pad at 35ºC [179]. Each STCP received larvae from two to three different genetic lineages (16-24 larvae per lineage). After grafting, each larva was fed an additional 9.5 µl of control or treatment diet 'A' mixed with 0.5 µl of sterile phosphate buffered saline (PBS) or 0.5 µl of *M. plutonius* culture diluted in sterile PBS (see section 2.5 below and Table 1). After feeding, the

STCPs were incubated in one of two desiccators containing supersaturated K_2SO_4 solution (Fisher Scientific) [179] within an incubator set at 35°C. From D2 to D5, larvae received daily feedings of control or treatment diet 'B' or 'C' according to the feeding schedule of Schmehl *et al.* (2016) [179]. From D1 to D6, larvae were examined daily and dead larvae were identified based on their discolored, deflated appearance and lack of moving spiracles [181] using unaided visual examination and/or a stereomicroscope (Figure 5.1). Dead larvae were removed each day and recorded.

We had two types of negative controls: a 'survival' control (no pesticide and no *M. plutonius*), which was required to have ≥ 75 % survival at D6 for the data from the corresponding STCP to be included in the study; and an 'infected' control (no pesticide and infected with 500, 250, or 50 CFU *M. plutonius*), for comparison to pesticide-exposed larvae which were infected with the same number of CFU of *M. plutonius* (Table 5.1). Each STCP was divided into four groups including a survival control group on every plate (Table 5.1), with 10-12 larvae per group (mean = 11.94, SD = 0.26). Pesticide-treated larvae received pesticides only or pesticides in combination with *M. plutonius*. 4-7 replicates (mean = 53.05 larvae, SD = 9.02) of each treatment and control group were performed, with the exception of the survival control which had 16-23 replicates (mean = 236.5 larvae, SD = 46.65). Replicates of each group were performed on a minimum of two different, time-staggered STCPs.

Temperature and relative humidity in each of the two desiccators was logged hourly using a HOBO MX Temp/RH Data Logger MX1101 (Onset Computer Corp., Bourne, MA, USA) and found to be, on average, 34.69°C (SD = 0.26) and 93.11% (SD = 10) and 34.71°C (SD = 0.18) and 96.76% (SD = 6.76), respectively, for the duration the experiment.

5.3.4 Larval pesticide exposure

Pesticide stock solutions were prepared in water and/or acetone from analytical standard chemicals (Millipore Sigma). THI (Product 37924, Lot BCBT8326, expiry March 2022) was prepared as a 100 ng/µl stock in distilled water. PROP (Product 45642, Lot BCBW6694, expiry February 2023) was prepared as a 65 ng/µl stock in distilled water and 0.0065% acetone. PYR (Product 31577, Lot BCBW1407, expiry November 2022) was prepared as a 65 ng/µl stock solution in distilled water and 0.5% acetone. Two stock solutions of BOS were prepared. BOS (Product 33875, Lot BCB58868V, expiry Aug 2021) was prepared as a 1170 ng/µl stock in 100%

acetone and BOS (Product 33875, Lot SZBF099XV, expiry April 2020) was prepared as a 1600 ng/µl stock in 100% acetone.

The pesticide concentration remained constant throughout the experiment. Since larvae were grafted into queen cell cups primed with non-contaminated diet 'A' on D0, the pesticide concentration in diet 'A' was adjusted accordingly to account for this dilution. For the larvae treated with THI and/or PROP or PYR, part of the water in the diet was replaced with pesticide stock solution to achieve the desired pesticide concentration in the larval diet (Table 5.2). Due to its poor water solubility, BOS was added by pipetting BOS stock solution (representing 2.5% of diet volume) into the diet adjacent to each larva after feeding. Survival controls for the BOS-treated larvae received an equal volume (2.5%) of acetone. As a positive control (Table 5.1), to confirm BOS activity and exposure, 4 replicates (48 larvae) of 40 ng/µl BOS with 10 ng/µl THI (2.5% acetone) and 4 replicates (48 larvae) of 80 ng/µl BOS with 10 ng/µl THI (5% acetone) were performed and survival was compared to survival control larvae treated with 2.5% acetone (47 larvae) or 5% acetone (48 larvae), respectively.

The THI concentrations selected (Table 5.2) were based on previously tested THI concentrations which did not significantly impact larval survival [247], and the total dose of THI provided in the diet represented 84.2-842 times the calculated, worst-case, field-relevant exposure of a worker larva to THI (1.9 ng), which was calculated based on estimated worker larval consumption of 5.4 mg pollen and 180 mg nectar during development [60] and reported maximal environmental concentrations of THI in pollen (86 ng/g) and nectar (13.3 ng/g) [12]. Thus, the concentrations of THI tested were not intended to be field-realistic; but instead, high concentrations with potential for observable, sublethal effects. The total doses of PROP and BOS administered in the diet (Table 5.2) were based on previously tested [148], field-relevant doses of PROP and BOS calculated based on maximum application rates of these fungicides to almond crops [148]. We used the same total dose of PYR as for PROP (Table 5.2). Based on maximum residues of PYR reported in pollen (83 ng/g) and nectar (4 ng/g) [68], the total dose of PYR tested in our experiment represents 2876 times the calculated, worst-case, field-relevant exposure of a worker larva to PYR (0.779 ng) [60].

Table 5.2 Pesticides, mode of action, concentration and total dose in 160 µl larval diet provided from D0 to D5 to honey bee worker larvae reared *in vitro*.

Pesticide	Mode of action	Diet concentration (ng/µl)	Total dose (ng)
THI	Neonicotinoid insecticide which is a nAChR agonist [14]	1, 10	160, 1600
BOS†	Carboxamide fungicide which inhibits cellular respiration [286]	29	4680
PYR	Anilinopyrimidine fungicide which inhibits protein synthesis [287]	14	2240
PROP	Triazole fungicide which inhibits sterol biosynthesis and cytochrome P450s [149]	14	2240

†Due to its poor water solubility, BOS was pipetted into the larval diet immediately after feeding, unlike the other pesticides which were dissolved directly within the diet.

5.3.5 Larval infection with *M. plutonius*

For experimental larval infection, fresh cultures of *M. plutonius* were prepared daily by thawing an aliquot of stock culture (see 5.3.1 above), diluting it 1/1000 in liquid KSBHI media, and growing the culture for 29 hours at 37°C under microaerophilic conditions and shaking at 100 rpm. To determine the bacterial load of the stock culture after 29 hours, the OD_{600} of the culture was measured and serial dilutions were prepared in sterile PBS. Serial dilutions were plated on KSBHI agar and incubated at 37°C for 3 days under microaerophilic conditions to determine colony forming units (CFU) per ml.

The mean OD_{600} of the cultures used for experimental infection was 0.849 (SD = 0.206) and the mean CFU/ml based on plating of serial dilutions was 9.58×10^7 CFU/ml (SD = 2.85×10^7). On the day of grafting, each larva was administered 0.5 µl of a 1/100, 1/200, or 1/1000 dilution of the stock culture; thus, each larva received 479 CFU (SD = 142.46), 240 CFU (SD = 71.23), or 47.9 (SD = 14.25) CFU, respectively, based on the mean CFU/ml of the stock culture. For simplicity, the bacterial inocula will be referred to as 500, 250, or 50 CFU. Larvae in the survival control group received 0.5 µl of sterile PBS (Table 5.1).

To verify fulfillment of Koch's postulates, control and *M. plutonius*-infected larvae were preserved in 10% neutral phosphate buffered formalin, processed for histopathology using standard automatic tissue processing, embedded in paraffin (Paraplast Plus, Leica Biosystems, Richmond, IL, USA), sectioned into 5 µm sections, and stained with Hematoxylin and Eosin and/ or Gram stain [288,289]. Control and *M. plutonius*-infected larvae were also homogenized in

sterile PBS, streaked on KSBHI agar and incubated at 37°C for 3 days under microaerophilic conditions.

5.3.6 Statistical analysis

Statistical analysis was conducted using Stata/SE 16 (College Station, TX, USA). For each dose of *M. plutonius* (0, 50, 250, 500 CFU) larval survival at D6 was compared between pesticide-treated and control groups using a Pearson Chi-squared test. For the dose of *M. plutonius* resulting in approximately 50% survival of infected controls, additional survival analysis was performed using Cox proportional hazards regression with a post hoc global test to confirm proportionality of hazards.

5.4 Results

We successfully isolated an atypical strain of *M. plutonius* and reproduced EFB disease *in vitro* (Figure 5.1). We also fulfilled Koch's postulates by demonstrating gram positive bacteria within the midgut of infected larvae on histopathology (Figure 5.1d) and culturing back *M. plutonius* from infected larvae. Control larvae did not contain bacteria within the midgut on histopathology (Figure 5.1c), and culture of control larvae did not yield colonies of *M. plutonius*.

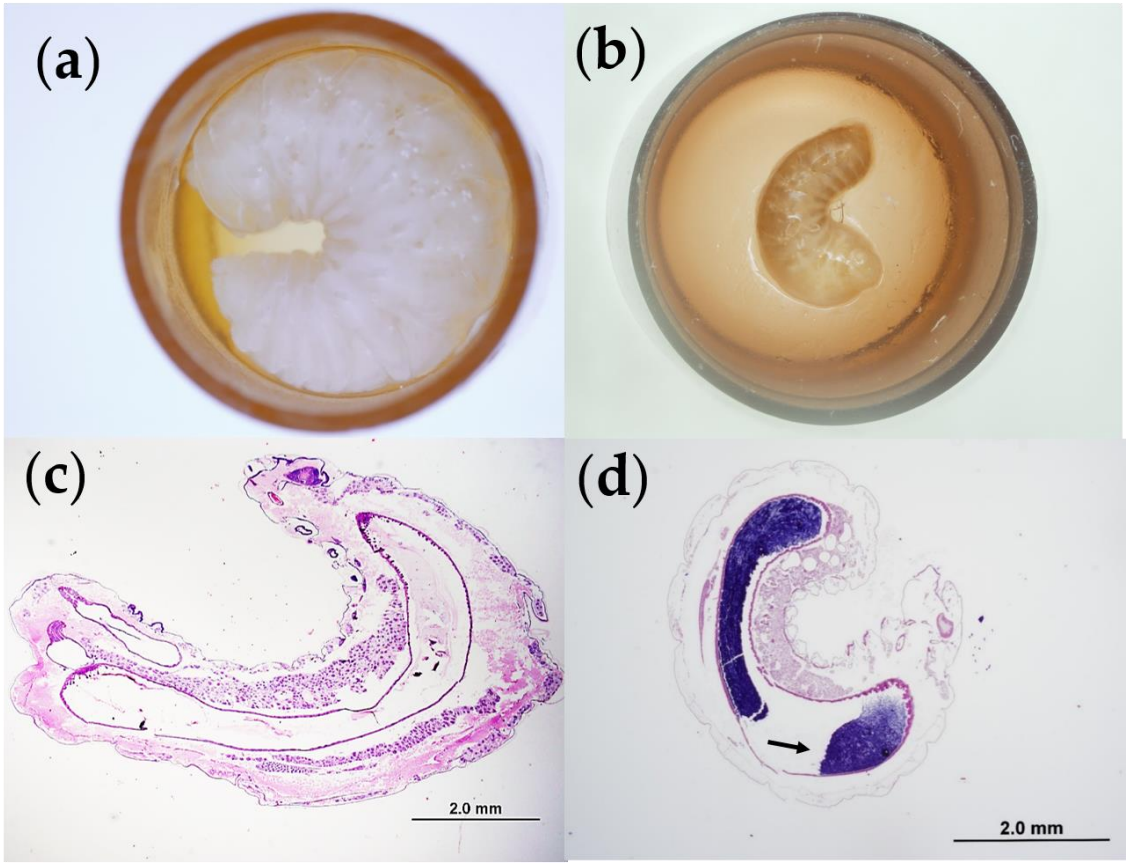

Figure 5.1 Multiplication of *Melissococcus plutonius* in honey bee worker larvae reared *in vitro*. Gross (a), (b) and histologic sections (c), (d) after 6 days of *in vitro* rearing of control larvae (a), (c) and larvae infected with *M. plutonius* (b), (d). The healthy control larvae (a) is white and plump compared to the larvae infected with *M. plutonius* (b) which is decreased in mass, brown and deflated, with prominent tracheae. The Gram-stained section of an infected larva (d), demonstrates a mass of gram-positive bacteria (arrow) within the midgut, which is absent in the section of a control larva stained with Hematoxylin and Eosin (c).

Chronic exposure to THI in combination with PROP significantly decreased survival of larvae infected with 50 CFU *M. plutonius in vitro* (Figure 5.2d, Figure 5.3d). Compared to infected control larvae which received 50 CFU *M. plutonius*, larvae exposed to 1 ng/µl THI and 14 ng/µl PROP and infected with 50 CFU *M. plutonius* had a significantly lower (by 25%) survival over 6 days (Figure 5.2d, $X^2(1) = 3.9625$, P = 0.047). Similarly, when larval survival after infection with 50 CFU *M. plutonius* was analyzed using Cox proportional hazard regression (Figure 5.3), we observed a marginally significant decrease in survival of larvae exposed to THI and PROP compared to infected controls (Figure 5.3d; P = 0.048, Hazard Ratio = 1.85, 95% Confidence Interval (CI) = 1.00 to 3.42; Table A5.1).

By comparison, chronic larval exposure to THI, BOS, PYR, PROP, or THI in combination with the fungicides BOS or PYR, was not shown to significantly affect larval survival over 6 days after infection with 50 CFU *M. plutonius* (Figure 5.2, Figure 5.3; Table A5.1). Similarly, at higher doses (250 and 500 CFU) of *M. plutonius*, there was no significant effect of THI and/or fungicide treatment on survival relative to infected controls (Figure 5.2).

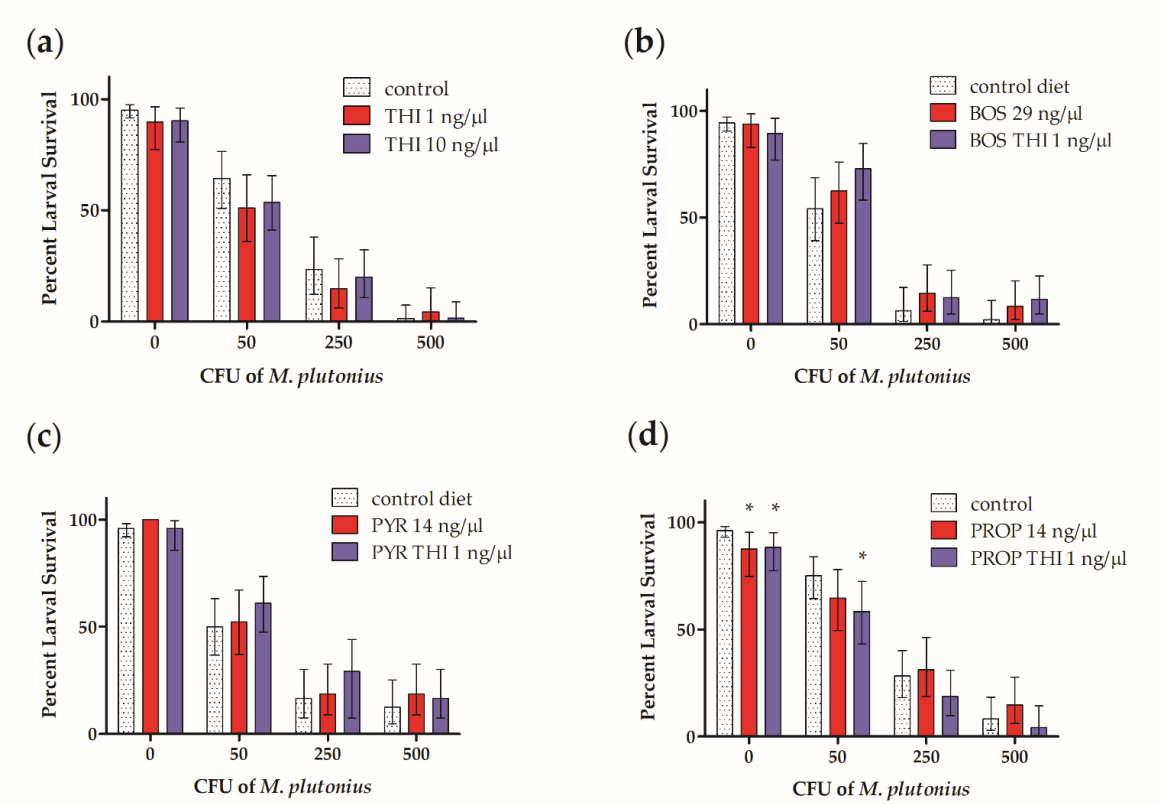

Figure 5.2 Effects of chronic pesticide exposure on larval survival from European foulbrood. (a) Percent survival of larvae fed control diet or diet with THI (1 or 10 ng/µl); (b) Percent survival of larvae fed control diet, diet with BOS (29 ng/µl), or diet with BOS and THI (1 ng/µl); (c) Percent survival of larvae fed control diet, diet with PYR (14 ng/µl), or diet with PYR and THI (1 ng/µl); (d) Percent survival of larvae fed control diet, diet with PROP (14 ng/µl), or diet with PROP and THI (1 ng/µl). Bars show percent larval survival at day 6 with 95% confidence interval for 45-84 worker honey bee larvae reared *in vitro* and infected with 0, 50, 250, or 500 CFU of *M. plutonius* and 191-300 survival control larvae which were unexposed to pesticides and not infected with *M. plutonius*. Percent larval survival was analyzed with a Chi-squared test. * indicates significant difference ($P < 0.05$) relative to control for each inoculum (CFU) of *M. plutonius*. Larval survival from EFB was significantly decreased by co-exposure to the insecticide THI with the fungicide PROP.

In the absence of *M. plutonius* infection, we observed a significant, 9% and 8%, respectively, lower survival of larvae exposed to PROP (Figure 5.2d, $X^2(1) = 6.095$, $P = 0.014$) and THI with PROP ($X^2(1) = 5.88$, $P = 0.015$) compared to controls. Uninfected larvae which were chronically exposed to THI, BOS, PYR, or THI in combination with BOS or PYR, did not experience a significant ($P > 0.05$) decrease in larval survival relative to survival controls (Figure 5.2a-c).

Positive control, high concentrations of 40 and 80 ng/µl BOS with 10 ng/µl THI, significantly decreased larval survival relative to survival controls by 32.19 % ($X^2(1) = 8.1934$, $P = 0.004$) and 96.28 % ($X^2(1) = 28.6138$, $P < 0.001$), respectively, in the absence of *M. plutonius*, confirming the efficacy of the pesticide exposure model used for BOS.

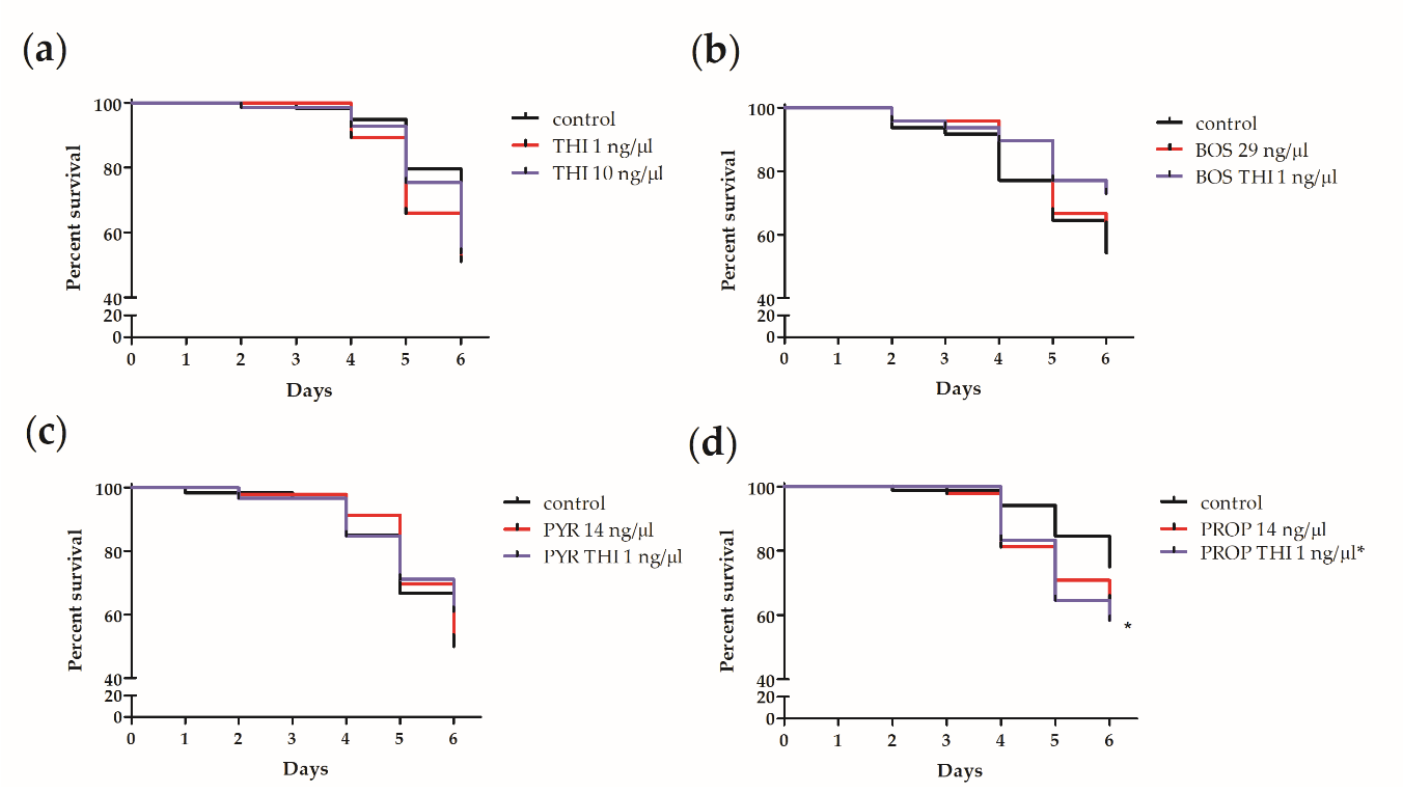

Figure 5.3 Effects of chronic pesticide exposure on survival of larvae infected with 50 CFU of *Melissococcus plutonius*. (a) Percent survival of larvae fed control diet or diet with THI (1 or 10 ng/µl); (b) Percent survival of larvae fed control diet, diet with BOS (29 ng/µl), or diet with BOS and THI (1 ng/µl); (c) Percent survival of larvae fed control diet, diet with PYR (14 ng/µl), or diet with PYR and THI (1 ng/µl); (d) Percent survival of larvae fed control diet, diet with PROP (14 ng/µl), or diet with PROP and THI (1 ng/µl). From D0 to D5 larvae were fed control or pesticide-contaminated diet and mortality was recorded daily for 6 days after grafting on D0. Lines indicate percent daily survival for 46-84 larvae administered 50 CFU on day 0 of *in vitro* rearing. * indicates significant ($P < 0.05$) difference relative to control by Cox proportional hazards regression. THI and PROP exposure increased susceptibility of honey bee worker larvae to mortality from EFB *in vitro* after infection with 50 CFU of *M. plutonius*

5.5 Discussion

We report an atypical isolate of *M. plutonius* for the first time in Canada. The distribution of atypical isolates in Canada is currently unknown. With this isolate, we successfully reproduced EFB disease *in vitro* (Figure 5.1) and using this *in vitro* model, we demonstrated that chronic exposure to a neonicotinoid (THI) or one of three fungicides (BOS, PYR, or PROP) on its own does not increase honey bee worker larvae death from EFB (Figure 5.2, Figure 5.3). However, chronic co-exposure of worker larvae to THI and PROP was shown to significantly decrease survival of larvae infected with *M. plutonius* relative to infected controls (Figure 5.2d, Figure 5.3d). We reiterate that only one of four combinations of THI with a fungicide tested was correlated with a significant increase in mortality from EFB, and the dose of THI used was 84.2 times greater than environmentally relevant exposure; thus, our study does not show that pesticide exposure would predispose honey bees to EFB-associated mortality in the field.

After infection with 50 CFU *M. plutonius*, survival of THI and PROP-exposed larvae over 6 days was significantly lower than infected controls (Figure 5.2d), with chronic THI and PROP exposure significantly increasing the risk of larval mortality by 1.85 times ($P = 0.048$, Figure 5.3d, Table A5.1) relative to infected controls receiving 50 CFU of *M. plutonius*. This finding suggests that THI and PROP co-exposure may have potentiated development of EFB disease, possibly through PROP-mediated inhibition of larval P450s leading to decreased THI detoxification and subsequent THI-mediated impairment of larval antibacterial defenses. The hazard of 1 ng/µl THI with PROP exposure after infection with 50 CFU *M. plutonius* (Figure 5.3d, Table A5.1) was greater than the hazard of exposure to 1 ng/µl THI on its own (Figure 5.3a), which resulted in a non-significant, 1.52 times increase ($P = 0.17$, Table A5.1) in larval mortality relative to infected controls administered 50 CFU of *M. plutonius*. There is previous evidence for PROP-mediated inhibition of honey bee P450s leading to increases in toxicity of THI to adult workers [147] and decreases in survival of worker larvae exposed to the diamide insecticide chlorantraniliprole [148]. The absence of significant differences in survival relative to infected controls of larvae infected with 250 and 500 CFU of *M. plutonius* and exposed 1 ng/µl THI with PROP (Figure 5.2d) could be due to these higher doses of *M. plutonius* overwhelming the larval immune system regardless of its immunocompetence. As well, we emphasize that larval survival was only monitored over 6 days in our experiment, and we cannot rule out a possible time lag in mortality of our infected control group.

Furthermore, consistent with our results, other studies [97,290] have demonstrated increased susceptibility of honey bee larvae to disease when exposed to agrochemicals. For example, the neonicotinoid CLO and the bacterium *Paenibacillus larvae* were shown to act synergistically to decrease survival and total hemocyte count of exposed larvae [2]. Similarly, larvae infected with four RNA viruses and chronically exposed to an organosilicone surfactant adjuvant used in tank mixes of pesticides had significant increases in mortality and viral replication and decreased expression of Toll 7-like receptor which mediates viral immunity [290]. One criticism of these studies [97,290], as well as the present study, is that all experiments lacked a control group of agrochemical-exposed larvae which were infected with a non-pathogenic organism which was similar to the pathogen under study.

Alternatively, the significant decrease in survival of the larvae co-exposed to THI and PROP and infected with 50 CFU of *M. plutonius* (Figure 5.2d) could be explained by direct toxic effects of PROP, rather than increased susceptibility to *M. plutonius*, considering that chronic larval exposure to PROP, or THI with PROP, in the absence of *M. plutonius*, resulted in significant 9% ($P = 0.014$) and 8% ($P = 0.015$), respectively, decreases in larval survival relative to survival controls, although no significant effect of PROP exposure on its own was observed in the presence of *M. plutonius* (Figure 5.2d). The total PROP dose (2.24 µg) administered in our study was based on maximum field application rates to almonds [148]. Wade *et al.* [148] found no significant effect of 2.25 µg PROP on survival of larvae exposed on day 4 of development, but perhaps the chronic exposure scenario in our study provided more time for negative effects of PROP on larval survival to occur. Considering that the total THI dose (160 ng) tested alone and in combination with PROP was 84.2 times the calculated, maximum environmental exposure of worker larvae [60], and on its own THI did not show significant effects on larval survival, further studies are needed to confirm field-relevant doses and to examine the effect of these doses of THI in combination with PROP on larval mortality from *M. plutonius*.

In contrast to studies such as ours which demonstrate negative effects, or no effect of pesticides on susceptibility of larval honey bees to infectious disease, there is some evidence to suggest that pesticides may have a positive, immunostimulatory effect on honey bees. For example, *in vitro* fungicide exposure of honey bee larvae was found to increase gene expression of an immune enzyme in pupae involved in melanization [291]. Additionally, Dickel *et al.* [292] observed a possible hormetic effect of the neonicotinoid thiacloprid on survival of adult workers co-exposed to

the bacterium *Enterococcus faecalis*, suggesting that concurrent bacterial infection and sublethal pesticide exposure may increase longevity of adult honey bees. Future studies with our *in vitro* model of EFB should examine the effect of pesticides on worker mortality over the entire developmental period to eclosion, as well as sublethal parameters such as bacterial load or immune gene expression.

The relevance of the results reported herein is limited to the single atypical *M. plutonius* isolate we tested, and cannot be generalized to other isolates of *M. plutonius* without additional *in vitro* testing. Of note, it is interesting that we reliably reproduced EFB disease *in vitro* with only 50 CFU of *M. plutonius*, while other authors required 56 [115] to 1000 [114] times greater infectious doses of atypical *M. plutonius* to trigger EFB *in vitro*. Differences in strain virulence may explain the discrepancy in these infectious doses.

5.6 Conclusion

An *in vitro* model for testing effects of pesticide exposure on development of EFB disease in honey bee larvae was successfully implemented with an atypical isolate of *M. plutonius* from a blueberry-pollinating colony. Using this model, we demonstrated that a neonicotinoid insecticide (THI) and/or the fungicides BOS or PYR do not increase susceptibility of worker honey bee larvae to mortality from EFB. However, chronic exposure to greater than field-realistic concentrations of THI with the fungicide PROP were shown to significantly increase larval mortality from EFB at low infectious doses *in vitro*, suggesting that further testing of field-relevant THI concentrations in combination with PROP is required. Our established experimental model will enable future testing of additional pesticide combinations to better understand the interaction between pesticides and larval susceptibility to EFB. Studies such as this are important to strike a balance between the farmers' need to control crop pests with agrochemicals and the beekeepers' need for healthy colonies with which to provide pollination services and produce honey.

Chapter 6: General discussion: establishment of a safe, sublethal dose range for neonicotinoids and honey bees in Saskatchewan

6.1 Introduction

Neonicotinoids are widely used by Saskatchewan farmers due to their convenient seed-treatment application, high selectivity for target pests, and high safety for humans [35]. However, ongoing scientific and government debate regarding the safety of these pesticides for honey bees and other non-target invertebrates threatens farmers' access to these pesticides [20,72]. Saskatchewan has been shown to have high levels of neonicotinoid contamination in honey, including a mean THI concentration in honey (17.2 ng/g) [293] which is 59 times the global average (0.29 ng/g) [24]. In addition to chronic neonicotinoid exposure, Saskatchewan honey bee colonies face additional challenges such as harsh, long winters; co-exposure to fungicides and other pesticides; and a variety of infectious diseases, including the re-emerging bacterial disease EFB. Despite chronic exposure to high environmental concentrations of neonicotinoids, in combination with other abiotic and biotic stressors, Saskatchewan honey bees continue to produce high quality honey and provide essential pollination services which enhance the yields of Saskatchewan crops [33]. The objective of this thesis was to evaluate the effects of chronic neonicotinoid exposure on honey bee colonies, adult honey bee workers, and honey bee worker brood to provide clarity regarding the safe, acceptable concentration for neonicotinoids in the Saskatchewan environment which poses no threat to honey bee health and colony performance.

6.2 Major findings, strengths, and limitations

6.2.1 Discussion of Chapter 2

Due to the harsh winters in Saskatchewan, packaged bees from New Zealand are commonly imported to replace overwinter losses in early spring [93]. These nucleus colonies are often installed in used brood chambers which provide an immediate source of neonicotinoid exposure through stored honey and beebread [293]. Neonicotinoid exposure increases during the summer as colonies forage on surrounding, subsequent blooms of canola, which is mostly grown from neonicotinoid-treated seed [2] There is a lack of information on the effects of neonicotinoid exposure on weak, nucleus colonies such as packaged bees [166,204], with most field studies exposing strong colonies to neonicotinoids during summer to mimic neonicotinoid exposure during crop bloom [17]. Chronic exposure of honey bees to neonicotinoids for up to 12 weeks has been shown to occur in Canadian agricultural landscapes [13], in part due to neonicotinoid

contamination of non-target wildflowers [12,13]. Unfortunately, chronic, colony-level neonicotinoid exposure studies are not commonly performed [166], and few colony-level studies investigate the comparative toxicity of sublethal exposure to different neonicotinoids.

In Chapter 2, we performed a chronic, twelve-week, colony-level, neonicotinoid-feeding study to compare the effects of IMD, THI, and CLO on small nucleus colonies during spring and summer in Saskatchewan. We tested mid-range (5 ng/g) and high environmental (20 ng/g) concentrations of neonicotinoids reported in Saskatchewan honey [293] by exposing colonies to neonicotinoids through sugar syrup and pollen patties. After 9 and 12 weeks of exposure, colonies receiving 20 ng/g of IMD, THI, or CLO gained significantly less weight compared to control colonies (-30%, $P < 0.01$), reflecting decreased honey production. Of the neonicotinoids tested, CLO had the greatest negative impact on colony weight gain relative to controls. After 12 weeks of exposure to 20 ng/g of IMD, THI, or CLO, colonies had a significantly reduced cluster size (-21%, $P < 0.05$) relative to controls, reflecting a decrease in adult bee population. Computer-assisted visual analysis of number of adult bees and capped brood area per colony at week 12 did not demonstrate a significant effect of neonicotinoid treatment, although this analysis lacked adequate statistical power to detect an effect.

Some of the strengths of this study include the chronic, field-realistic [10] exposure time as well as concurrent, comparative testing of several neonicotinoids. Some of the limitations of this study include a lack of genetic homogeneity of the study colonies (which did not have sister queens), leading to increased heterogeneity in colony performance and inadequate sample size (nine colonies per treatment group) and inadequate statistical power to detect treatment effects. Similar to many field studies, there was potential neonicotinoid contamination of treatment and control colonies due to colonies foraging for nectar from surrounding crops. While we mitigated exposure of study colonies to contaminated pollen from surrounding crops by installing pollen collectors on all study colonies, we failed to include a control group without pollen collectors to investigate potential effects of suboptimal nutrition due to exclusion of natural pollen. Furthermore, we did not have a low-dose group (for example, 1 ng/g) which may have been more broadly relevant for other parts of the world which have lower levels of environmental neonicotinoid contamination [24].

6.2.2 Discussion of Chapter 3

In Chapter 2, we demonstrated negative effects of chronic exposure to 20 ng/g neonicotinoids on honey production and population size of small colonies during spring and summer in Saskatchewan; these results prompted further investigation into the effects of chronic neonicotinoid exposure on overwintering colonies in Saskatchewan. Overwinter colony losses have been elevated in Canada since 2007 [92]. Overwintering colonies are vulnerable due to limited food stores, the severe climactic conditions during Saskatchewan winter and their potential chronic exposure to agrochemicals through honey and beebread collected and stored during the preceding summer. In Chapter 3, we examined the effects of chronic THI exposure on overwinter survival of colonies in Saskatchewan, and correlated colony-level findings to laboratory effects of chronic THI and CLO exposure on winter adult worker honey bees. We chronically exposed strong colonies to 20 and 100 ng/g THI prior to overwintering and evaluated survival, colony strength, queen quality and pathogen load the following spring. This experiment was repeated the following year, where we tested the effects of chronic exposure to 5 and 10 ng/g THI, in combination with weak colony strength, on overwinter survival, colony strength and temperature homeostasis. Using the same concentrations tested in the field experiments, we also performed laboratory trials to understand the effects of chronic THI or CLO exposure on survival of individual winter adult workers housed in cages.

In the field experiments described in Chapter 3, we found that chronic THI exposure had dose-responsive effects on overwinter survival of strong colonies with 0, 20 and 100 ng/g THI resulting in 10%, 25% and 65% overwinter mortality. Relative to controls, only the colonies exposed to 100 ng/g THI had a statistically significant increase in overwinter mortality (55% greater, $P < 0.001$) and a significant decrease in early spring cluster size (-2 interframe spaces, $P = 0.045$). We found no significant effect of chronic THI exposure on queen quality or severity of colony infection with *Varroa* or *Nosema* . Furthermore, combined stress of weak fall colony strength and chronic exposure to 5 and 10 ng/g concentrations of THI did not impact colony overwinter survival, cluster size, or temperature homeostasis relative to controls. However, in the laboratory experiments described in Chapter 3, combined stress of the artificial cage environment and chronic exposure to 5 or 10 ng/g THI or CLO resulted in significant ($P < 0.05$) negative effects on survival of winter adult workers relative to controls, suggesting that stress associated with an artificial environment may be a significant confounding factor of laboratory studies.

Some of the strengths of the field studies conducted in Chapter 3 include a larger sample size (20-22 colonies per treatment group) compared to the field study in Chapter 2, as well the use of sister queens to decrease genetic variability. Furthermore, neonicotinoid exposure in Chapter 3 occurred immediately prior to overwintering, instead of during the preceding summer [219], facilitating a more direct temporal relationship between neonicotinoid treatment and the outcome measures of the study.

Some of the limitations of the studies in Chapter 3 included the use of a different geographic location in each of the two field trials, limiting comparisons between the two trials. It would have been preferable to randomize the study colonies in each trial to three different locations, with the same locations used in each trial, to account for microclimate effects on overwinter survival in different geographic locations. Another weakness of the field studies in Chapter 3 was potential dilution and contamination of the THI treatment due to existing stores of honey and beebread within the brood chamber of each study colony. Furthermore, the experimental diets in this chapter were not submitted for analytical LC-MS/MS verification of neonicotinoid concentrations, thus the true neonicotinoid exposure of the honey bees in these studies is unknown. Sample size continued to be a problem for the field studies in Chapter 3 with inadequate statistical power to detect treatment effects for concentrations of THI less than 100 ng/g. Although we found no effects of THI exposure on pathogen load, the study colonies had low levels of *Varroa* and *Nosema* at the start of the study, and thus, we cannot rule out effects of chronic neonicotinoid exposure when colonies are experiencing higher disease pressure. As well, we did not monitor colony temperature when colonies were exposed to higher (20 ng/g and 100 ng/g) concentrations of THI; thus, we do not know if these neonicotinoid exposure levels may have triggered dysregulation of temperature homeostasis. A weakness of the laboratory experiments described in Chapter 3 is that we introduced social and nutritional stressors, including a queenless cage environment which lacked eusocial order and pollen supplementation and had a lower than recommended bee density within each cage [240]. These laboratory stressors may have confounded the effects of neonicotinoid exposure on adult winter worker survival.

6.2.3 Discussion of Chapter 4

We complemented the overwinter field study in Chapter 3 by performing laboratory experiments to evaluate effects of chronic neonicotinoid exposure on survival of winter adult workers. Similarly, to complement the spring-summer field study in Chapter 2, further laboratory

experiments were needed to examine the effects of chronic neonicotinoid exposure on summer worker adults and brood. Toxic effects of neonicotinoids on adult workers may have indirect negative effects on brood survival due to decreased brood care by affected workers [84]. Previous studies have shown that THI exposure inhibits development of the HPGs in exposed adult workers [89], thus potentially compromising nutrition of the colony's larvae.

Furthermore, we were interested to explore the effects of chronic exposure of honey bees to both a neonicotinoid and a fungicide, as honey bees are frequently co-exposed to multiple agrochemicals in the environment [13] and the hive [55,61]. SBI fungicides have been previously shown to potentiate neonicotinoid toxicity [142,146,147], possibly through inhibition of P450s, which honey bees use to detoxify some neonicotinoids [43].

One of the main findings of the laboratory experiments in Chapter 4 was that newly emerged adult workers required 20 days of exposure to 40 ng/g THI before significant decrease in survival was observed. Chronic exposure to 10 and 20 ng/g THI did not significantly decrease survival of summer adult workers relative to controls. These findings contrast with the laboratory experiments on winter adult workers in Chapter 3 which found that chronic exposure to 5, 10, and 20 ng/g THI significantly decreased adult worker survival. As well, the absence of significant decreases in laboratory survival of summer adult workers chronically exposed to 20 ng/g THI in Chapter 4 suggests that the negative colony-level effects of 20 ng/g neonicotinoids on honey production and cluster size in Chapter 2 cannot be explained by decreased adult worker survival.

In Chapter 4 we also exposed worker brood chronically to neonicotinoids *in vitro* and we showed that, compared to adult workers, worker brood required exposure to 23 times higher concentrations of THI (909 ng/g) before significant effects on mortality were observed. In contrast to the *in vitro* studies of Dai *et al.* [181], who found that chronic CLO exposure primarily affected larval survival of worker brood, we showed that chronic THI exposure primarily affected pupal survival, rather than larval survival, of exposed worker brood. However, similar to Dai *et al.* [181] who established a NOAEC of 0.1 ng/µl CLO for worker brood, we found no significant decreases in survival of worker larvae exposed to 0.1 ng/µl THI. This data would have been stronger had the neonicotinoid concentrations within the larval diet been verified using LC-MS/MS.

We observed that neither worker adults, nor brood, experienced additive decreases in survival when co-exposed to THI and the SBI fungicide PRO. This finding contrasted with other studies which found that SBI fungicides act synergistically with insecticides to decrease survival

of worker adults [146] and brood [148]. Additionally, in Chapter 4, we showed that HPG development of newly emerged worker adults was not adversely affected by chronic, laboratory exposure to 10, 40, and 100 ng/g THI and/or 360 ng/g PRO. This finding was in opposition to the work of Zaluski *et al.* [256], who showed that exposure of adult nursing bees to an insecticide (fipronil) and a fungicide (pyraclostrobin) decreased HPG development.

A strength of Chapter 4 was that we examined the effects of a neonicotinoid in combination with a fungicide, and we evaluated effects of this exposure on multiple life stages of worker honey bees. A limitation of this study was the use of a single dose of PRO, which was based on empirical residues in pollen [252], but may not reflect the range of application rates of this fungicide in the field. As well, some of the *in vitro*-reared workers in our study had clinical signs of DWV infection at emergence which may have confounded our results. Furthermore, lack of statistical power limited our analysis of brood survival at the lower end of the range of THI concentrations tested. One weakness of our investigation of HPG development in adult workers is that the stressful laboratory cage environment may have contributed to hypoplasia of the workers' HPGs, regardless of treatment group, thus preventing detection of a treatment effect.

6.2.4 Discussion of Chapter 5

The purpose of Chapter 5 was to investigate whether THI and/or fungicide exposure predisposes honey bee larvae to develop EFB *in vitro*. Continuing the theme of Chapter 4, we co-exposed worker brood to neonicotinoid and fungicide mixtures, considering that, compared to other insects, honey bees may be more vulnerable to negative effects of multiple pesticides due to their genomic shortage of P450 enzymes [145]. While neonicotinoid exposure has been shown to compromise individual [135,136] and social immunity of honey bees to infectious disease [13], the effects of neonicotinoids on susceptibility of honey bee larvae to *M. plutonius* infection had not been previously explored.

We found that chronic exposure to THI, in combination with the SBI fungicide PROP, significantly decreased survival, by 25%, of honey bee larvae infected with *M. plutonius*, relative to infected controls. In contrast, exposure to THI, or the fungicides BOS or PYR, alone or in combination, did not increase larval mortality from EFB *in vitro*. The mechanism for the THI and PROP-mediated increase in EFB-associated mortality could be explained by inhibition of larval P450s by PROP, leading to decreased THI detoxification and impairment of larval immunity by THI [138].

A limitation of this study is that the test doses of THI were in excess of the calculated environmental exposure of honey bee larva to THI. Thus, we do not know whether THI and PROP co-exposure would increase mortality from EFB in a field-realistic setting. Furthermore, pesticide concentrations in the experimental larval diet were not verified by LC-MS/MS measurement, contributing to uncertainty regarding pesticide exposure of the larvae in these experiments. Also, our experimental design would have been stronger had we included a control group which tested pesticide exposure in combination with a non-pathogenic strain of *M. plutonius*. Considering that we only tested a single strain of *M. plutonius in vitro*, we cannot generalize our results to other strains of *M. plutonius* encountered by colonies in the field. Furthermore, our study was limited to evaluation of larval survival; a more comprehensive approach would be to monitor brood survival through the entire developmental period to eclosion.

6.3 Importance of research

6.3.1 Discussion of Chapter 2

The main finding of Chapter 2 was that 20 ng/g was the threshold level of exposure for negative, colony-level effects of neonicotinoids. As supported by these results, we propose that environmental neonicotinoid contamination in Saskatchewan should be maintained below 20 ng/g, which would agree with the colony-level, NOEC of 19 ng/g established for CLO by the USEPA [263]. In Chapter 2, we demonstrated that honey production is a valuable sublethal endpoint for colony-level, neonicotinoid toxicology studies. We also emphasized the importance of using nucleus colonies in pesticide-safety testing, as these small colonies may be less able to compensate for pesticide stress. We showed that brood area was not a sensitive measure of colony performance in response to neonicotinoid exposure, indicating a role for *in vitro* toxicological brood assays. Also, Chapter 2 provided a seasonal comparison for Chapter 3 which examined the colony-level effects of chronic neonicotinoid exposure over winter.

6.3.2 Discussion of Chapter 3

The overall conclusion of Chapter 3 was that 100 ng/g was the threshold level of exposure for negative effects of THI on colony overwintering, similar to the findings of Thompson *et al.* [219] who showed that 100 ng/g THI was the lowest observed effect concentration associated with decreases in colony overwinter survival. Importantly, in Chapter 3, we demonstrated that environmental concentrations of THI do not significantly decrease overwinter survival of field colonies. However, in line with the results of Chapter 2, we observed a 15% decrease in overwinter

survival of colonies exposed to 20 ng/g THI relative to controls, which although not statistically significant, may have economic implications for a commercial beekeeper. Taken together, these findings suggest that Saskatchewan honey bee colonies which overwinter on honey collected during the canola bloom in the preceding summer are not at increased risk of overwinter mortality. However, the findings of Chapter 3 lend support for our proposed toxic threshold of 20 ng/g environmental neonicotinoid contamination to minimize overwinter colony loss in Saskatchewan.

Interestingly, in Chapter 3 we showed that the combined stressors of weak colony strength, 5 or 10 ng/g neonicotinoid exposure, and a cold Saskatchewan winter did not predispose colonies to overwinter mortality. This finding contrasts with the widely held view that multiple stressors predispose colonies to collapse, and reinforces the concept that eusociality makes honey bee colonies resilient to stress.

Chapter 3 also revealed that negative effects of neonicotinoid exposure on adult winter worker survival in the laboratory do not correlate with decreased overwinter survival of colonies exposed to the same concentrations of neonicotinoids in the field. This finding reinforces the importance of repetition of laboratory experiments at the colony level.

6.3.3 Discussion of Chapter 4

In Chapter 4, we transitioned from exposing honey bees to neonicotinoids alone, to co-exposure of honey bees to a neonicotinoid and a fungicide, which more accurately reflects pesticide exposure in the environment. We observed that co-exposure to the SBI fungicide PRO did not enhance the insecticidal activity of the neonicotinoid THI to worker adults or brood. This finding is relevant for honey bees in Saskatchewan, as canola grown from seed treated with THI is often sprayed with the fungicide PRO at bloom, and the interaction between these two pesticides in exposed honey bees had not been previously studied. We showed that chronic co-exposure of summer worker adults and brood to THI and PRO did not decrease survival at field-realistic concentrations, nor impact HPG development of nurse bees, alleviating concerns about exposure of Saskatchewan honey bees to these pesticides during foraging on canola.

Furthermore, we demonstrated that worker brood were less sensitive to the effects of chronic THI exposure compared to worker adults. This observation emphasized the importance of pesticide safety testing on multiple honey bee life stages as part of a comprehensive pesticide risk assessment strategy.

6.3.4 Discussion of Chapter 5

In Chapter 5, we used a combined biotic and abiotic stressor approach to examine the effects of neonicotinoid and fungicide exposure on susceptibility of honey bees to disease. Our observation that fungicide exposure did not increase larval mortality from EFB is important for Canadian beekeepers whose colonies pollinate blueberries, considering that blueberry crops are frequently sprayed with fungicides, and beekeepers have reported an increased incidence of EFB in their colonies after blueberry pollination [272–275]. Our finding that THI exposure did not increase mortality from EFB, and that only unrealistically high concentrations of THI, in combination with a fungicide, increase larval susceptibility to EFB, suggests that Saskatchewan honey bee colonies foraging on canola would not be at increased risk of brood disease when exposed to field-realistic concentrations of neonicotinoids.

We demonstrated, for the first time, the presence of an atypical strain of *M. plutonius* in Canada. This is an important first step to further characterizing the strain distribution of *M. plutonius* in Canada. Furthermore, the *in vitro* model of EFB we implemented will be useful for future testing of other pesticides and *M. plutonius* strains.

6.4 Future directions

To improve future toxicological studies of Saskatchewan honey bees and neonicotinoids, there is a need for better surveillance of the environmental neonicotinoid concentrations in Saskatchewan hive products. Our understanding of the environmental concentrations of neonicotinoids in Saskatchewan largely stems from the work of Codling *et al.* [3], who determined the neonicotinoid concentrations in 26 samples of honey and 19 samples of beebread from seven apiaries surrounding Saskatoon, Saskatchewan. Considering the limited sample size and sampling area of Codling *et al.* [3], the long half-lives of neonicotinoids in the soil [62,63], and the variable intensity of agriculture and beekeeping in different regions of Saskatchewan, a broader, more representative study of neonicotinoid concentrations in Saskatchewan hive matrices is necessary to ensure that test concentrations of neonicotinoids used in honey bee toxicology studies such as ours are field-relevant for Saskatchewan honey bees.

Once a more accurate understanding of field-relevant concentrations of neonicotinoids in Saskatchewan is obtained, additional colony-level and laboratory studies of honey bees and neonicotinoids are required to fully understand the impact of these pesticides on honey bee health.

At the colony-level, future studies should investigate the effects of neonicotinoids combined with other stressors. For example, an artificial feeding study could be designed to test the effects of combined, chronic neonicotinoid and fungicide exposure on colony honey production, cluster size, and overwintering.

Additionally, to improve the colony-level overwintering study performed in Chapter 3, a field experiment, using a randomized complete block design, should be performed to test the combined effects of chronic neonicotinoid exposure and high disease pressure on colony overwinter survival, utilizing study colonies which have high levels of *Varroa* infestation and *Nosema* infection in the fall. These studies should take advantage of remote colony sensor technologies which allow continuous monitoring of variables including colony weight, temperature, and humidity [294].

To improve on the laboratory study in Chapter 4, the effects of neonicotinoid and fungicide co-exposure on HPG development of nurse bees should be examined in field colonies to avoid confounding effects of laboratory stress on HPG development.

To augment the laboratory study in Chapter 5, a colony-level model of EFB should also be developed which would allow testing of multiple, concurrent predisposing factors to EFB, including pesticide exposure, weak colony strength and compromised colony nutrition.

Future laboratory studies of neonicotinoids and honey bees should explore the mechanistic underpinning of why worker brood are more resistant to negative effects of chronic neonicotinoid exposure compared to worker adults. For example, P450 functionality and nAChR expression could be compared at different worker life stages. Detoxification enzyme assays could also be utilized to enhance understanding of the combined effects of neonicotinoid and fungicide exposure on worker adults and brood, possibly providing an explanation for the negative effects of THI and PROP co-exposure on worker larvae discussed in Chapter 5. As well, laboratory assays for larval cellular and humoral immunity in response to concurrent pesticide exposure and EFB infection should be performed.

Continued field and laboratory investigation of the effects of neonicotinoids and honey bees will assist with ongoing risk assessment of these pesticides for pollinators in Saskatchewan. While honey bees are an excellent model species to understand the effects of neonicotinoids on pollinators, Saskatchewan is home to over 200 species of wild bees [295], as well as the managed

alfalfa leafcutting bee (*Megachile rotundata* F.), suggesting that future risk assessment should incorporate research on the effects of neonicotinoids on other pollinator species in Saskatchewan.

6.5 Conclusion

A synthesis of Chapters 2-5 suggests that chronic exposure to neonicotinoid concentrations less than 20 ng/g will not have negative consequences for colony performance and survival, nor negative effects on the health and survival of individual worker adults and brood. When chronic neonicotinoid exposure was combined with fungicide exposure, we did not observe additive negative effects on the survival and development of worker adults and brood. Only at neonicotinoid concentrations 84 times greater than environmental were neonicotinoids and fungicides observed to enhance susceptibility of worker larvae to infectious disease. Thus, based on our data, we suggest that Saskatchewan monitor and maintain environmental concentrations of neonicotinoids below 20 ng/g to protect the health of Saskatchewan honey bees.

At present, the concentration of neonicotinoids in Saskatchewan honey is approaching the 20 ng/g threshold for negative effects on Saskatchewan honey bees. The mean concentration of THI in honey from Saskatoon, SK was reported at 17.2 ng/g [293], which is 59 times greater than the global average of 0.29 ng/g THI in honey [24]. This is not surprising considering the estimated 216 000 kg of neonicotinoids applied each year to 11 million hectares of crops in the Canadian prairies [2], and reported half-lives of neonicotinoids in soil of up to 19 years [62,63]. Accordingly, we recommend that Saskatchewan re-evaluate the sustainability of its agricultural neonicotinoid use to ensure that the average neonicotinoid concentration in Saskatchewan honey remains below 20 ng/g.

Neonicotinoids are one of the most versatile and effective classes of insecticides developed to date [35]. These insecticides are a critical tool for Saskatchewan farmers who must protect their canola crops from herbivorous insects [296], including the flea beetle (*Phyllotreta* sp.), which causes over $300 million worth of damage to Canadian canola each year [297]. At the same time, Canadian farmers benefit from the pollination that honey bees provide, including an annual contribution of up to $4.6 billion in pollination services for the production of Canadian hybrid canola seed [33]. The objective of this thesis was to provide empirical data with which to balance farmers' reliance on neonicotinoids for crop protection, and beekeepers' dependence on healthy honey bee colonies for pollination and honey production. Communication of the findings of this

thesis, as well as continued research and collaboration with other scientists, beekeepers, farmers, and policy-makers, will be essential to maintain the sustainability of Saskatchewan agriculture and Saskatchewan beekeeping into the future.

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APPENDIX

Supplemental material and information for this thesis are provided in this Appendix (10 tables and 3 figures total). Data include neonicotinoid concentrations in experimental diet (Chapter 2); survival analysis of winter workers chronically exposed to neonicotinoids (Chapter 3); survival analysis and HPG diameter of summer adult workers chronically exposed to a neonicotinoid and/or a fungicide and survival analysis of worker brood chronically exposed to a neonicotinoids and/or a fungicide (Chapter 4); characterization of an atypical *M. plutonius* strain and survival analysis of worker brood infected with *M. plutonius* and chronically exposed to neonicotinoids and/or a fungicide (Chapter 5).

Table A2.1. Neonicotinoid concentrations within experimental pollen patties. Neonicotinoid concentrations were measured using LC-MS/MS at the Government of Alberta Agriculture and Forestry Chemistry Laboratory in Edmonton, Alberta.

Experimental diet (nM)	Sample size (n)	Calculated concentration (ng/g)	Mean measured concentration ± SD (ng/g)	Mean percent difference from expected ± SD (%)	THI contamination (ng/g)
control	3	0	0	0 ± 0	
CLO 20	4	5	3.7 ± 0.36	-26 ± 7.12	
CLO 80	4	20	13.9 ± 0.70	-30.5 ± 3.49	1.2, n=1
IMD 20	4	5	2.6 ± 0.95	-47.5 ± 19.07	1.1, n=1
IMD 80	4	20	9.9 ± 3.32	-50.5 ± 16.60	
THI 20	3	5	4.6 ± 0.47	-7.33 ± 9.45	
THI 80	3	20	18.1 ± 3.75	-9.5 ± 18.76	

Percent difference from expected was calculated using: $\frac{([measured]-[calculated])}{[calculated]} \times 100\%$.

Table A2.2 Neonicotinoid concentrations within experimental syrup. Neonicotinoid concentrations were measured using LC-MS/MS at the Government of Alberta Agriculture and Forestry Chemistry Laboratory in Edmonton, Alberta.

Experimental diet (nM)	Sample size (n)	Calculated concentration (ng/g)	Mean measured concentration ± SD (ng/g)	Mean percent difference from expected ± SD (%)
control	3	0	0	0 ± 0
CLO 20	3	5	4.4 ± 0.56	-12 ± 11.14
CLO 80	3	20	18.6 ± 0.95	-6.83 ± 4.75
IMD 20	4	5	5.5 ± 1.36	9 ± 27.25
IMD 80	3	20	18.7 ± 1.88	-6.67 ± 9.39
THI 20	3	5	5 ± 0.53	-5.92×10^{-15} ± 10.58
THI 80	4	20	22 ± 5.50	9.75 ± 27.49

Percent difference from expected was calculated using: $\frac{([measured]-[calculated])}{[calculated]} \times 100$

Table A3.1. Survival analysis of winter *Apis mellifera* workers chronically exposed to THI or CLO through syrup in laboratory cage trial C1.

Treatment (ng/g)	Median survival time (days)	Time ratio†	Standard error	P	95% Confidence Interval	
					lower	upper
Negative control	16.48	-	-	-	-	-
CLO 100	3.74	0.227	0.041	<0.001	0.159	0.324
CLO 20	10.27	0.623	0.077	<0.001	0.489	0.794
CLO 5	13.11	0.796	0.059	0.002	0.688	0.920
THI 100	5.48	0.332	0.045	<0.001	0.255	0.433
THI 20	10.22	0.620	0.081	<0.001	0.480	0.802
THI 5	13.73	0.833	0.066	0.021	0.714	0.973

†The time ratio indicates the change in expected survival time for each treatment relative to the negative control by a Weibull accelerated failure time model.

Table A3.2. Survival analysis of winter *Apis mellifera* workers chronically exposed to THI or CLO through syrup in laboratory cage trial C2.

Treatment (ng/g)	Median survival time (days)	Time ratio†	Standard error	P	95% Confidence Interval	
					lower	upper
Negative control	23.89	-	-	-	-	-
CLO 100	5.53	0.231	0.037	<0.001	0.169	0.317
CLO 20	15.75	0.660	0.097	0.005	0.494	0.881
CLO 10	18.92	0.792	0.036	<0.001	0.724	0.866
CLO 5	20.57	0.861	0.043	0.003	0.781	0.950
THI 100	5.53	0.232	0.047	<0.001	0.156	0.343
THI 20	16.10	0.674	0.054	<0.001	0.576	0.789
THI 10	19.80	0.829	0.041	<0.001	0.752	0.914
THI 5	17.39	0.728	0.040	<0.001	0.653	0.812

†The time ratio indicates the change in expected survival time for each treatment relative to the negative control by a Weibull accelerated failure time model

Table A4.1. Cox proportional hazards regression survival analysis of newly emerged adult worker honey bees chronically exposed to dietary THI and/or PRO in the laboratory for 30 days.

				95% Confidence Interval	
Treatment (ng/g)	**Hazard ratio**	**Standard error**	**P**	**lower**	**upper**
Negative control	1.43	0.56	0.363	0.66	3.08
DIM 1000	71.21	27.74	<0.001	33.18	152.81
PRO	2.02	0.76	0.059	0.97	4.21
THI 10	2.30	0.84	0.022	1.13	4.70
THI 10 PRO	2.64	0.95	0.007	1.30	5.35
THI 20	2.43	0.89	0.016	1.18	5.00
THI 20 PRO	1.98	0.74	0.067	0.95	4.13
THI 40	4.00	1.43	<0.001	1.98	8.07
THI 40 PRO	3.54	1.29	0.001	1.73	7.21

The hazard ratio is the effect of a unit change in treatment on the frequency of death relative to **solvent control** for 47-50 bees. Mortality was monitored daily. PRO was administered at 360 ng/g.

Table A4.2. Cox proportional hazards regression survival analysis of newly emerged adult worker honey bees chronically exposed to dietary THI and/or PRO in the laboratory for 30 days.

				95% Confidence Interval	
Treatment (ng/g)	**Hazard ratio**	**Standard error**	**P**	**lower**	**upper**
Solvent control	0.70	0.27	0.363	0.33	1.51
DIM 1000	49.86	18.32	<0.001	24.26	102.44
PRO	1.42	0.50	0.321	0.71	2.82
THI 10	1.61	0.55	0.162	0.83	3.15
THI 10 PRO	1.85	0.62	0.07	0.95	3.58
THI 20	1.70	0.59	0.123	0.87	3.35
THI 20 PRO	1.39	0.49	0.350	0.70	2.77
THI 40	2.80	0.94	0.002	1.45	5.40
THI 40 PRO	2.48	0.84	0.008	1.27	4.83

The hazard ratio is the effect of a unit change in treatment on the frequency of death relative to **negative control** for 47-50 bees. Mortality was monitored daily. PRO was administered at 360 ng/g.

Table A4.3. Hypopharyngeal gland acinar diameter in adult worker honey bees fed diet containing THI and/or PRO for 8 and 12 days in the laboratory.

	Acinar diameter (µm)	
Treatment (ng/g)	**Day 8**	**Day 12**
solvent control	101.04 ± 12.45	99.12 ± 6.34
negative control	102.61 ± 12.34	101.27 ± 12.36
THI 100	97.41 ± 10.03	93.04 ± 15.35
THI 10	104.39 ± 10.55	98.13 ± 11.16
THI 40	102.92 ± 10.69	101.96 ± 10.06
PRO	105.26 ± 11.79	107.73 ± 13.52
THI 10 PRO	109.15 ± 15.42	106.88 ± 11.93
THI 40 PRO	99.44 ± 13.86	102.63 ± 13.37

Values are means ± SD for 21 bees. PRO was administered at 360 ng/g.

Table A4.4. Cox proportional hazards regression survival analysis of worker honey bee brood reared *in vitro* and fed larval diet containing THI and/or PRO for four days.

				95% Confidence Interval	
Treatment (ng/µl)	**Hazard ratio**	**Standard error**	**P**	**lower**	**upper**
THI 0.1	2.31	1.64	0.236	0.58	9.27
THI 0.5	1.08	0.88	0.923	0.218	5.36
THI 1	3.87	2.55	0.04	1.06	14.06
THI 2	4.03	1.54	<0.001	1.91	8.53
THI 5	5.86	2.18	<0.001	2.83	12.15
THI 10	10.70	3.89	<0.001	5.25	21.84
PRO	0.69	0.26	0.317	0.33	1.44
THI 0.1 PRO	1.34	1.02	0.701	0.30	5.99
THI 0.5 PRO	1.72	1.25	0.459	0.41	7.19
THI 1 PRO	2.42	1.67	0.200	0.63	9.38
THI 2 PRO	6.27	3.95	0.004	1.83	21.53
THI 5 PRO	14.43	8.82	<0.001	4.35	47.80
THI 10 PRO	20.60	12.50	<0.001	6.27	67.68

The hazard ratio is the effect of a unit change in treatment on the frequency of death relative to **solvent control** for 24-60 larvae in each treatment group and 204 larvae in the solvent control group. Mortality was monitored daily until eclosion. PRO was administered at 360 ng/g.

Table A4.5. Cox proportional hazards regression survival analysis of worker honey bee brood reared in vitro and fed larval diet containing THI and/or PRO for four days.

Treatment (ng/µl)	Hazard ratio	Standard error	P	95% Confidence Interval	
				lower	upper
THI 0.1	3.38	2.71	0.129	0.70	16.27
THI 0.5	1.58	1.42	0.611	0.27	9.21
THI 1	5.64	4.28	0.023	1.27	24.99
THI 2	5.88	3.16	0.001	2.05	16.87
THI 5	8.55	4.53	<0.001	3.03	24.17
THI 10	15.62	8.19	<0.001	5.59	43.64
THI 0.1 PRO	1.96	1.67	0.431	0.37	10.40
THI 0.5 PRO	2.51	2.06	0.264	0.50	12.56
THI 1 PRO	3.53	2.78	0.109	0.76	16.53
THI 2 PRO	9.15	6.72	0.003	2.17	38.56
THI 5 PRO	21.05	15.12	<0.001	5.15	86.06
THI 10 PRO	30.07	21.49	<0.001	7.41	122.01
Solvent control	1.46	0.55	0.317	0.70	3.06

The hazard ratio is the effect of a unit change in treatment on the frequency of death relative to the **PRO** treated group for 24-60 larvae in each treatment group and 204 larvae in the solvent control group. Mortality was monitored daily until eclosion. PRO was administered at 360 ng/g..

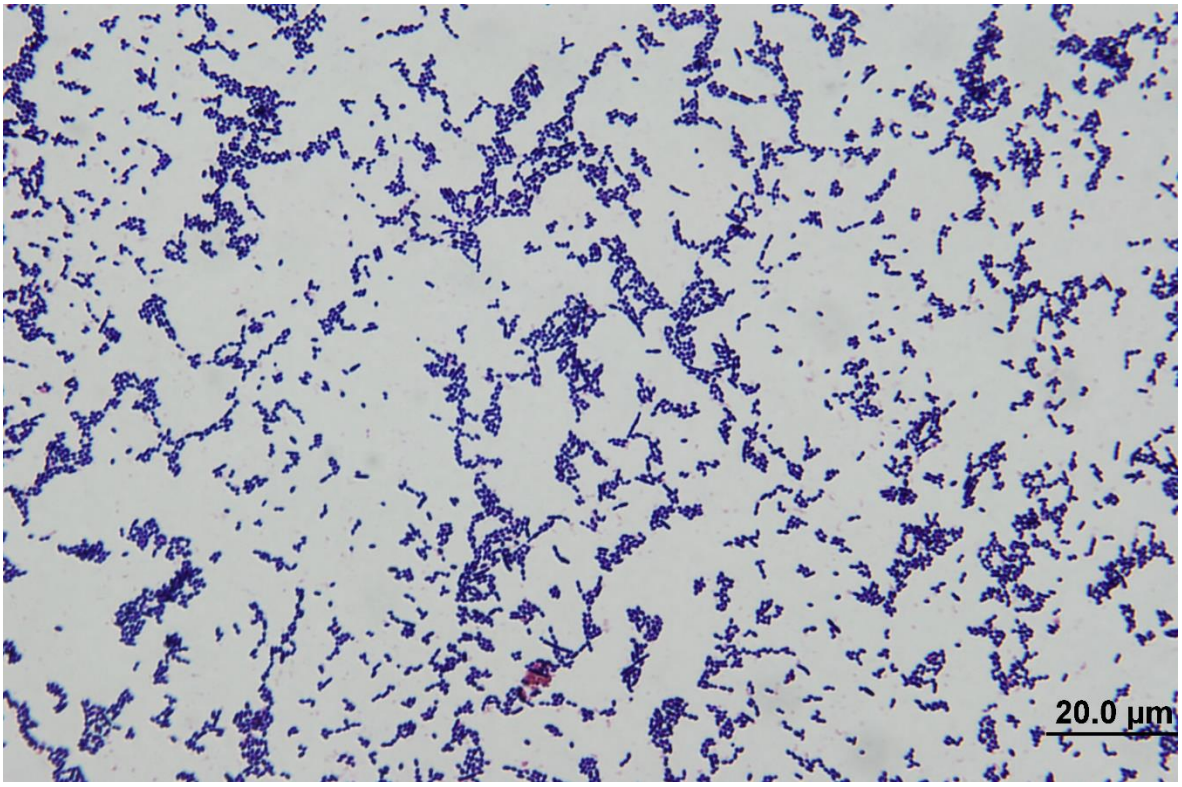

Figure A5.1. **Gram stain of *Melissococcus plutonius* isolate from a honey bee colony pollinating blueberries in the Fraser Valley of British Columbia.** The bacteria forms characteristic short chains of gram-positive lanceolate cocci.

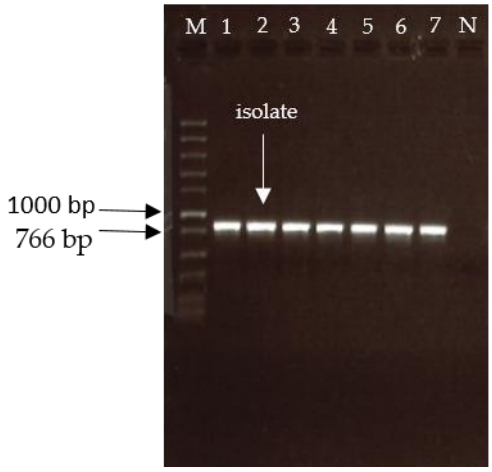

Figure A5.2. **Agarose gel (1%) of PCR product from genomic DNA of *Melissococcus plutonius* isolates.** The white arrow indicates an 812 base pair band which confirms the *M. plutonius* identity of the bacterial isolate utilized in the present study. Lanes 1, 3, and 4-7 represent other *M. plutonius* isolates; N, no template control; M, molecular size marker (Fast DNA Ladder, New England BioLabs, Whitby, ON, Canada).

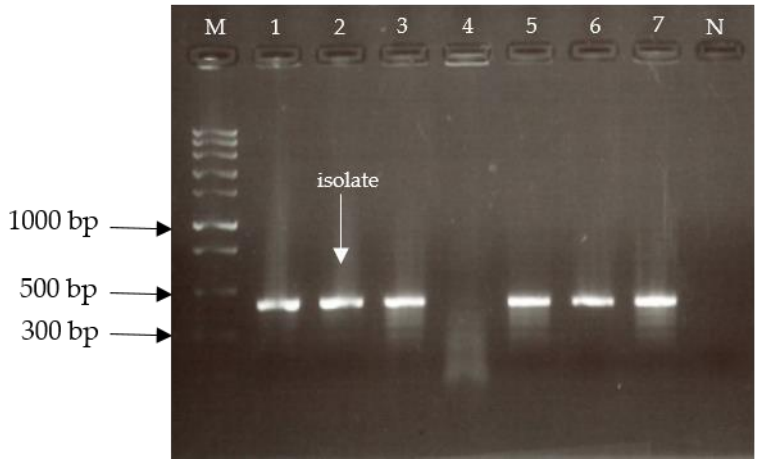

Figure A5.3. **Agarose gel (1%) of duplex PCR product from genomic DNA of *Melissococcus plutonius* isolates.** The white arrow in lane 2 indicates a 424 base pair band which identifies the bacterial isolate used in the present study as an atypical strain of *M. plutonius*. Lanes 1, 3, and 5-7 represent other atypical *M. plutonius* isolates; lane 4 is a PCR reaction failure; N, no template control; M, molecular size marker (Fast DNA Ladder, New England BioLabs, Whitby, ON, Canada).

Table A5.1. Cox proportional hazards regression survival analysis of 46-84 *Apis mellifera* larvae infected with 50 CFU of *M. plutonius* and chronically exposed dietary THI and/or fungicides from D0 to D5 *in vitro*. Mortality was recorded daily for 6 days.

				95% Confidence Interval	
Treatment (ng/µl)	**Hazard ratio[1]**	**Standard error**	**P**	**lower**	**upper**
THI 1	1.52	0.46	0.17	0.84	2.74
THI 10	1.35	0.38	0.29	0.78	2.33
BOS 29	1.16	0.25	0.48	0.77	1.76
THI 1 BOS 29	0.97	0.19	0.88	0.66	1.44
PYR 14	0.91	0.26	0.75	0.53	1.59
THI 1 PYR 14	0.76	0.21	0.33	0.44	1.32
PROP 14	1.57	0.51	0.17	0.83	2.98
THI 1 PROP 14	1.85	0.58	0.048	1.00	3.42

[1] The hazard ratio indicates the effect of a unit change in pesticide treatment on frequency of death relative to the control group which was infected with 50 CFU *M. plutonius* and received uncontaminated diet from D0 to D5.