Assessing Different Insecticides for Potential Use as a Toxic Standard in Pesticide Risk Assessment Protocols with *Bombus impatiens* (Cresson)

by

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in
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ABSTRACT

ASSESSING DIFFERENT INSECTICIDES FOR POTENTIAL USE AS A TOXIC STANDARD IN PESTICIDE RISK ASSESSMENT PROTOCOLS WITH BOMBUS IMPATIENS (CRESSON)

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Professor C. Scott-Dupree
Professor G. C. Cutler

Bumble bees (Bombus spp.) are important pollinators in natural and agricultural landscapes. Bumble bees can be exposed to pesticides via spray deposition, contact with plant surfaces, or ingestion of contaminated nectar. Therefore, there is a need to develop pesticide risk assessment protocols for bumble bees. Recently, laboratory protocols were established using Bombus impatiens (Cresson) (the North American surrogate bumble bee), but tier II semi-field methods still need to be refined and validated. In this thesis, I determined the contact and oral median lethal dose (LD₅₀) for dimethoate on B. impatiens which has not been previously reported. Secondly, I investigated the effects of dimethoate and three insect growth regulators (IGRs) to determine their suitability as toxic standards for use in semi-field pesticide risk assessment. I found that none of the IGRs caused adverse effects on B. impatiens reproduction, but dimethoate was highly toxic to B. impatiens under semi-field and laboratory conditions.
ACKNOWLEDGEMENTS

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I’d like to thank our funding body, Bayer Crop Science. Without your support, this research would not have been possible.

Thank you to the Scott-Dupree lab (past and present members) for all your assistance in field set-up, data collection and so much more. A big thank you to Dr. Andrew Frewin and Graham Ansell for all your help both in the field and in the lab. Andrew, thank you for all the stats help. I would also like to extend my deepest gratitude to Dr. Angela Gradish. Your support and advice throughout this project has been invaluable. Learning from you has made me a better scientist and I truly appreciate all the help you’ve provided to me over the past few years.

Lastly, I’d like to thank my family. To my parents, Mike and Dee, your love and support saw me through until the end. To my sister, Neesha, thank you for listening, making me laugh and for alleviating my stress. I’d like to thank my partner, Josh, thank you for making the move to Guelph and for sticking it out the past three years. I know it hasn’t always been easy, but I truly appreciate it. Thank you for always listening, for always loving me (even when I was a mess) and for always being in my corner. To whatever comes next.
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D80: colonies placed in buckwheat plots treated with dimethoate at 80 g a.i./ha

D80-2: extra colonies placed in D80 plots 24 h after dimethoate application when D80 colonies were found dead

D200: colonies placed in buckwheat plots treated with dimethoate at 200 g a.i./ha

EFSA: European Food Inspection Agency

HBRC: University of Guelph Honey Bee Research Centre

ICPPR: International Commission for Plant-Pollinator Relationships

IGR: Insect growth regulator

LD$_{50}$: Median lethal dose

OECD: Organisation for Economic Co-operation and Development

USEPA: United States Environmental Protection Agency
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Chapter 1

Literature Review

1.1 Value of Bumble Bee Pollination

Approximately 35% of global food production relies, to some extent, on pollination by insects (Klein et al. 2007). The value of insect pollination services is estimated to be $220 billion USD per year (Gallai et al. 2009). Most pollination can be attributed to unmanaged animal pollinators, with bees being the largest contributors (Garibaldi et al. 2013).

In North America and Europe, bumble bees (Bombus spp.) visit over 29 different agricultural crops (McGregor 1976; Thompson and Hunt 1999) and are recognized as important agricultural pollinators (Mader et. 2010; Button and Elle 2014). Their importance as pollinators can be attributed to both their ability to forage in inclement weather as well as their ability to sonicate, or ‘buzz’ pollinate. Buzz pollination occurs when a bumble bee contacts the anthers of a flowers and rapidly vibrate its flight muscles on the thorax resulting in the release of large quantities of pollen (Heinrich 1979; Williams et al. 2014). In contrast, honey bees are incapable of buzz pollination and must forage under warm and sunny conditions (Carron 1999) (Table 1.2).

Several important crops require buzz pollination (e.g. Solanum spp) or have greater yields when they are buzz pollinated (e.g. Vaccinium spp) (Table 1.1) (Buchmann 1983). Currently, most Canadian greenhouses use the commercially available common eastern bumble bee, Bombus impatiens (Cresson) to pollinate tomatoes and peppers (Banda and Paxton 1991; van Ravestijn and van der Sande
1991) and blueberry growers often rely on the pollination services of wild bumble bees to improve yields (Plowright et al. 1978; Button and Elle 2014).

Table 1.1. Comparing the benefits of bumble bee pollination services to honey bee pollination services in some agricultural crops.

<table>
<thead>
<tr>
<th>Plant Genus</th>
<th>Benefits of bumble bee species pollination</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vaccinium</em> spp.</td>
<td>Increased • amount of pollen deposited on stigma • pollen deposition per stigmatic visit • overall fruit set</td>
<td>Button and Elle 2014 Javorek et al. 2002 Dogterom 1999 Ratti et al. 2008</td>
</tr>
<tr>
<td><em>Solanum</em> spp.</td>
<td>Increased • overall fruit set • pollen deposition • fruit size • fruit weight</td>
<td>Banda and Paxton 1991 van Ravestijn and van der Sande 1991 Morandin et al. 2001</td>
</tr>
<tr>
<td><em>Citrullus</em> spp. <em>Cucumis</em> spp.</td>
<td>• Increased seed set in cucumbers and watermelon • Lowered fruit abortion rates</td>
<td>Stanghellini et al. 1998a, b</td>
</tr>
<tr>
<td><em>Brassica</em> spp.</td>
<td>Increased • pollen deposition • stigmal contact</td>
<td>Woodcock et al. 2013</td>
</tr>
</tbody>
</table>

1.1.1 Bumble bee Life History

Bumble bees (*Bombus* spp.) are native to Europe, Asia and North America, and are found in the Arctic tundra, deserts and subtropical forests, with the highest diversity around the Tibetan plateau (Heinrich 1979; Williams et al. 2014). In the southern hemisphere, bumble bees are native to parts of South America but have been introduced to Australia, New Zealand and Tasmania (Heinrich 1979; Williams et al.)
Bumble bees are eusocial insects, in which related individuals cooperate in foraging for food, raising brood and protecting the nest (Heinrich 1979; Williams et al. 2014).

The bumble bee colony cycle begins in spring when a queen emerges from diapause (Williams et al. 2014). After feeding, she searches for an appropriate nesting site, such as a grass tussock, an abandoned rodent hole, a hollow log or an aboveground manmade structure (Williams et al. 2014). Once a suitable nesting site is located, the queen creates a pollen patty and lays her first batch of eggs (Heinrich 1979; Plowright and Laverty 1984). Eggs that are fertilized will develop into diploid females (workers or queens), whereas unfertilized eggs will develop into haploid males (Oster and Wilson 1978). Generally, the bumble bee colony cycle has an initial ergonomic phase when only workers are produced, followed by a terminal phase when new queens and males are produced (Oster and Wilson 1978; Plowright and Laverty 1984).

Once the first group of adult worker bees eclose, the queen ceases foraging and remains inside the colony for the remainder of her life (Heinrich 1979; Plowright and Laverty 1984; Williams et al. 2014). Workers are responsible for foraging, defending the nest and caring for brood (Heinrich 1979; Williams et al. 2014). Towards the end of the colony cycle, males and new queens emerge (Oster and Wilson 1978). Generally, males leave the nest shortly after emerging, but new queens tend to remain within the colony to build fat reserves (Heinrich 1979; Williams et al. 2014). When new queens have sufficient fat reserves, they leave the colony to mate (Heinrich 1979; Williams et al. 2014). As the autumn and colder weather approaches, the old queen, workers and
males die, and the newly mated queens find suitable overwintering sites (e.g. grass tussocks, decomposing logs, loose soil) (William et al. 2014).

1.2 Bumble Bees and Pesticides

In temperate regions, most bumble bee species have a single generation, usually in the summer, making them vulnerable to disturbances (Plowright and Laverty 1984; Thompson and Hunt 1999; Thompson 2001). Some bumble bee species are in decline in North America (Potts et al. 2010; Cameron et al. 2011) and Europe (Williams 1982; Biesmeijer et al. 2006; Kosior et al. 2007; La Féon et al. 2010). Suspected drivers of these declines include habitat loss and fragmentation (Ricketts et al. 2008; Le Féon et al. 2010; Vanbergen et al. 2013); climate change (William and Osbourne 2009; Vanbergen et al. 2013; Kerr et al. 2015); parasites and disease (Meus et al. 2011; Szabo et al. 2012; Vanbergen et al. 2013); and exposure to pesticides (Brittain et al. 2010; Szabo et al. 2012; Vanbergen et al. 2013).

Pesticides have received a great deal of attention both in academia and mainstream media as a potential cause of bee decline. Major routes of insecticide exposure for foliar applied insecticides to bumble bees include spray deposition directly on the bumble bees, contact with plant surfaces (leaf, flower, pollen and nectar) on which a pesticide has been deposited, and ingestion of contaminated nectar or pollen (USEPA 2014). The major route of exposure for seed-treated systemic insecticides is contact or ingestion of pesticide dislodged off the seed during planting, or ingestion of residues found in nectar or pollen following translocation of the pesticide through the plant (Brittain and Potts 2011; USEPA 2014). When exposure occurs, effects can be lethal or sublethal, the latter of which may result in lower reproductive outputs through
physiological and hormonal changes or through changes in locomotive functions that can disrupt foraging and feeding behaviours, all of which can negatively impact colony survival (Thompson 2001).

1.3 Pesticide Risk Assessment

Pesticides are regulated by federal agencies around the world to ensure they pose minimal risk to the environment and human health. An important step in this regulation involves assessing toxic effects of the pesticide to surrogate organisms under various levels of exposure. To assess the effects of pesticides on non-target arthropods, pesticide data have traditionally only been required for the honey bee. Due to growing concerns over global bumble bee declines and given that there are pronounced behavioural, physiological, and ecological differences between honey bees and bumble bees (Table 1.2), it is the honey bee may not be a suitable surrogate for bumble bees when assessing pesticide toxicity and exposure. Since bumble bees are widely distributed across North America and are prevalent in agricultural landscapes, regulators are interested in incorporating pesticide toxicity data for bumble bees for pesticide registration and re-registration. This will require the development of standardized test protocols to assess toxicity of pesticides on bumble bees.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Honey bees</th>
<th>Bumble bees</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tongue Length Classification</strong></td>
<td>Short</td>
<td>Short, medium, long (vary by species, varies by individual)*****</td>
</tr>
<tr>
<td><strong>Sociality (population size)</strong></td>
<td>Social (10,000 – 50,000)</td>
<td>Social (50 – 300)</td>
</tr>
<tr>
<td><strong>Days for Development</strong></td>
<td>Worker: 21</td>
<td>Worker: 25</td>
</tr>
<tr>
<td></td>
<td>Drone: 24</td>
<td>Male: 24</td>
</tr>
<tr>
<td></td>
<td>Queen: 16</td>
<td>Queen: 36</td>
</tr>
<tr>
<td><strong>Strength of Age Related Division of Labour in Workers</strong></td>
<td>Strong 1-20 days old: Hive Duties - Nursing 21+: Foragers</td>
<td>Weak†</td>
</tr>
<tr>
<td><strong>Forager Type</strong></td>
<td>Generalist</td>
<td>Generalist</td>
</tr>
<tr>
<td><strong>Nesting Location</strong></td>
<td>Above ground</td>
<td>At ground level</td>
</tr>
<tr>
<td><strong>Peak Foraging Period</strong></td>
<td>Mid-Day</td>
<td>Early Morning – Late Afternoon</td>
</tr>
<tr>
<td><strong>Degree of Floral Constancy</strong></td>
<td>High</td>
<td>Low-Medium</td>
</tr>
<tr>
<td><strong>Degree of Communication</strong></td>
<td>High</td>
<td>Low (no dancing structure recorded)</td>
</tr>
<tr>
<td></td>
<td>(waggle dance to inform sisters of food sources)</td>
<td></td>
</tr>
<tr>
<td><strong>Colony Life Cycle</strong></td>
<td>Perennial</td>
<td>Annual</td>
</tr>
<tr>
<td><strong>Queen Lifespan</strong></td>
<td>Multiple years (2-4)</td>
<td>One Year</td>
</tr>
<tr>
<td><strong>Reproductive Success</strong></td>
<td>Colony overwintering success Colony swarming</td>
<td>Overwintered mated queens</td>
</tr>
</tbody>
</table>

*Caron 1999  
**Williams et al. 2014  
***Plowright and Laverty 1984  
†Jandt et al. 2009
1.3.1 Tiered Level Testing

A tiered approach is currently used to estimate the risk of pesticides to honey bees. Tier I studies are done in the laboratory to estimate acute (e.g., contact and oral LD$_{50}$, or the pesticide dose that causes 50% mortality of a test population) and chronic toxicity of pesticides to individual bees, including measures of effects on behaviour and brood development (Lee-Steere and Steeger 2014). Results from tier I tests can be compared to conservative (worst case) estimates of exposure to generate risk quotients, that are then compared to predetermined acute or chronic toxicity “trigger values” or “level of concern (LOC)” (Lee-Steere and Steeger 2014). Trigger values and LOCs for honey bees are calculated by using hazard quotients (HQ) (in Europe) or risk quotients (RQs) (in North America) (USEPA 2012; EFSA 2013). Both HQ and RQ estimate pesticide exposure to the bees by setting the oral or acute median lethal dose (LD$_{50}$) ($\mu$g a.i./honey bee) as the denominator while either the pesticide application rate (g a.i./ha) (for HQ) (EFSA 2013) or the contact or dietary exposure values (USEPA 2012) serve as the numerator. These values can then be multiplied by an additional assessment factor of 10 that is supposed to account for differences between bee species (USEPA 2012; EFSA 2013); however, this approach does not account for the size or weight of the bee nor does it account for the foraging behaviour differences between bee species (Table 1.2), all of which can affect susceptibility (van der Steen 1999; Thompson and Hunt 1999).

Risk quotients that exceed the trigger value indicate that higher tier (more realistic assessments of hazard or risks) are required (EFSA 2013). For honey bees, tier II testing involves exposing colonies to pesticides under “semi-field” conditions or
placing colonies in an enclosure (around 40 m²) containing bee-attractive flowering plants that have been treated with either the pesticide, a toxic standard, or a negative control (OECD 2007; EFSA 2013; USEPA 2014; Lee-Steere and Steeger 2014). A toxic standard serves as proof of exposure to the pesticide and provides information on the sensitivity of the bees to the test conditions. Semi-field tests offer a more realistic exposure scenario than laboratory testing; however, being confined can stress honey bee colonies (Table 1.3) (USEPA 2014). If adverse effects are seen tier II, then tier III field scale testing is done. In tier III testing, colonies forage freely in an open field containing bee-attractive flowering plants that have been treated with the pesticide in question. For logistical purposes, full field studies often only compare the pesticide being tested against a negative control. Off-site foraging can occur, which can confound results. It is difficult to find suitable sites that are within the same geographical region but are also far enough apart to minimize honey bee workers from foraging between treatments (Table 1.3). Because of behavioural and life-history differences, the tiered methods for honey bees need to be modified for bumble bees. Therefore, incorporation of bumble bees into the regulatory risk assessment process will require the development and validation of methods at all three tiers for use with bumble bees.

**Tier I Tests**

Laboratory (acute contact and acute oral) protocols (tier I) have currently been accepted for use with the common eastern bumble bee (*Bombus impatiens* (Cresson)) and the buff tailed bumble bee (*B. terrestris* L.) (OECD 2017; Cabrera et al. 2015). In these new tier I protocols, dimethoate is recommended as the toxic standard for acute testing with *B. terrestris* and *B. impatiens*. Dimethoate is an acetylcholinesterase
inhibiting insecticide that affects all stages of development and is also the current recommended toxic standard in honey bee laboratory pesticide risk assessments (OECD 1998a, b). The median lethal dose (LD$_{50}$) of dimethoate for $B.\ terrestris$ (Schaefer et al. 1996; van der Steen 1999; Marletto et al. 2003; Heard et al. 2017) and for honey bees (Gough et al. 1994; OECD 1998a, b) is established but the LD$_{50}$ for $B.\ impatiens$ is unknown. Therefore, if dimethoate is recommended a toxic standard in acute laboratory testing with $B.\ impatiens$, LD$_{50}$ values specific to $B.\ impatiens$ that can be recommended in the tier I protocols must be generated.
Table 1.3. Important endpoints and considerations of the strengths and limitations of the three tiers of toxicity studies for pollinators*

<table>
<thead>
<tr>
<th>Tier Level</th>
<th>Key Endpoint Assessed</th>
<th>Strength</th>
<th>Weakness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tier I – Acute</td>
<td>Mortality</td>
<td>• Quantifiable test doses</td>
<td>• Acute exposure only</td>
</tr>
<tr>
<td>Oral</td>
<td>Oral LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>• Dose-response curve generated</td>
<td>• Only one type of exposure</td>
</tr>
<tr>
<td>Tier I – Acute</td>
<td>Mortality</td>
<td>• Some sub-lethal effects can be measured</td>
<td>• Effects assessed at the individual level</td>
</tr>
<tr>
<td>Contact</td>
<td>Contact LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tier II – Semi-Field</td>
<td>Colony strength</td>
<td>• Multiple exposure routes (contact and oral)</td>
<td>• Short-term exposure (7-10 days)</td>
</tr>
<tr>
<td></td>
<td>Brood pattern and development</td>
<td>• Controlled setting (minimize external pesticide exposure)</td>
<td>• Potential stress on colonies from confinement</td>
</tr>
<tr>
<td></td>
<td>Foraging activity</td>
<td>• Standard test protocol (OECD)</td>
<td>• Often low statistical power due to lack of replication</td>
</tr>
<tr>
<td></td>
<td>Worker mortality</td>
<td>• Colony-level effects</td>
<td>• Use of surrogate crop</td>
</tr>
<tr>
<td></td>
<td>Queen health</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tier III – Field</td>
<td>Colony strength</td>
<td>• Most realistic exposure scenario</td>
<td>• Logistical constraints</td>
</tr>
<tr>
<td></td>
<td>Brood pattern and development</td>
<td>• Gives insight in lower tier uncertainties</td>
<td>• Confounding impact of off-site exposure</td>
</tr>
<tr>
<td></td>
<td>Foraging activity</td>
<td></td>
<td>• Costly</td>
</tr>
<tr>
<td></td>
<td>Worker mortality</td>
<td></td>
<td>• Often low statistical power due to lack of replication</td>
</tr>
<tr>
<td></td>
<td>Queen health</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table adapted from USEPA 2014

1.3.2 Tier II Tests

The purpose of semi-field (tier II) studies is to look at colony level effects of pesticides (vs. individual bee effects in tier I) under more realistic “worst-case” scenarios (Cabrera et al. 2015). To do this, bees are placed in an enclosure and forage on a pesticide-treated, flowering attractive plant. Semi-field pesticide toxicity studies must contain a minimum of three treatment groups: the test pesticide, the negative control,
and the toxic standard positive control. Although some recommendations have been made, tier II methods for bumble bees still need to be refined and validated.

**Proposed Tier II Methods**

Proposed tier II testing for bumble bees involves similar protocols to the tunnel studies used for honey bees. Cabrera et al. (2015) suggested that the tunnel design would be easily transferable to bumble bees. To date, different sized enclosures have been used in semi-field tests with bumble bees, for example 6 m² (Gretenkord and Drescher 1996) to 450 m² (Sechser and Freuler 2003), but a recommended tunnel area for *B. terrestris* is at least 60 m² (ICPPR unpublished data, 2017). Bumble bee colonies contain fewer individuals (50-200) than honey bee colonies (10,000-60,000), which makes it possible to use smaller enclosures. Using smaller enclosures allows for increased replication of experimental units, which increases statistical power (Bowley 2015). Increasing the number of experimental units is preferred because there can be large variability in the size and development of bumble bee colonies (Cabrera et al. 2015).

Further recommendations include placing commercial colonies at the centre of the enclosure and allowing the bees to forage on a treated flowering plant species for a minimum of 2 weeks (Cabrera et al. 2015). In Europe, the recommended surrogate flower plant is purple tansy (*Phacelia tanacetifolia*) or oil see rape (*Brassica napus*) (ICPPR unpublished data, 2017). Recently, in Canada, the suitability of common buckwheat (*Fagopyrum esculentum*), red clover (*Trifolium pratense*) and purple tansy (*P. tanacetifolia*) as surrogate flowering plants was assessed for commercial *B. impatiens* colonies (Gradish et al. 2016). Colony development (i.e. change in colony
weight, number of adults (queens, workers and males) produced) did not significantly
differ between the three plant types, but workers from colonies placed in tents with
buckwheat foraged significantly more than those placed in tents with red clover or
purple tansy (Gradish et al. 2016). The higher foraging activity, combined with its rapid
growth and establishment, is why buckwheat was recommended as the surrogate
flowering plant to use in future tier II testing for *B. impatiens* (Gradish et al. 2016).
The pesticides being tested should be sprayed at the maximum recommended label
rate on the product label (Cabrera et al. 2015; ICPPR unpublished data, 2017). Both a
toxic standard, a pesticide known to cause adverse effects in the test species, and, a
negative control, water or solvent used to dissolve the formulated pesticide, should also
be used in the test (Cabrera et al. 2015; ICPPR unpublished data, 2017). In Europe,
dimethoate applied at a rate of 800 g a.i./ha is the recommended toxic standard for use
in semi-field pesticide toxicity studies with *B. terrestris* (ICPPR unpublished data 2017).
However, in North America, a suitable toxic standard and rate of application has yet to
be identified and validated for use with *B. impatiens* semi-field pesticide toxicity studies.
Dimethoate is the current toxic standard in honey bee pesticide risk assessments and in
tier I bumble bee testing (OECD 2017a, b) and is recommended for use in tier II testing
with *B. terrestris* (ICPPR unpublished data 2017). Novaluron, diflubenzuron and
tebufenozide are insect growth regulators (IGRs). IGRs disrupt larval development of
several Lepidopteran pests and can, theoretically, disrupt bumble bee larval
development (Mommaerts et al. 2006 a, b; Cutler and Scott-Dupree, 2007). Therefore,
both dimethoate and IGRs have the potential to used as toxic standards in semi-field
studies with *B. impatiens*. 
Differences in ecology between *B. impatiens* and *B. terrestris* should be considered when making regulatory guideline recommendations for *B. impatiens*. First, *B. terrestris* colonies produce more queens (21-56); males (25-164) and workers (136-284) compared to *B. impatiens* (queens 9-11; males 8-41; workers 28-160) colonies (Duchateau and Velthius 1988; Goulson et al. 2001; Cnaani et al. 2002; Gradish et al. 2016). On average, development time from egg until eclosed adult for *B. impatiens* queens is 37 d, which is longer than the average total development time for *B. terrestris* queens (33 d) (Cnaani et al. 2002; van der Steen 2008). Interestingly, *B. impatiens* workers take less time to eclose (24 d) than *B. terrestris* workers (29 d) (Ribeiro et al. 1999; Cnaani et al. 2002). Lastly, *B. terrestris* colonies go through a competition phase where callow workers lay their own eggs, destroy the queen’s eggs and exhibit aggression towards the queen (Duchateau 1989; Bloch and Hefetz 1999). However, these behaviours are rarely observed in *B. impatiens* colonies (Cnaani et al. 2002).

Another consideration is determining the bumble bee colony development stage, either early or late, that is most susceptible to pesticide exposure. Using the stage at which the bumble bee colony is most susceptible to pesticide exposure provides a “worst-case” scenario (Cabrera et al. 2015). The ICPPR non-*Apis* working group (unpublished data 2017) recommends ordering colonies with approximately 10 workers and allowing the colonies to develop for 2 weeks before the colonies are placed in the field. Other recommendations include recording the colony weight twice weekly, counting number of foraging bees on the crop and determining worker mortality (Cabrera et al. 2015). Recommended endpoint measures include the number of queens produced per colony, mean weight of queens produced per colony (EFSA 2013;
Cabrera et al. 2015; Sterk et al. 2016), the mean number of males produced (EFSA 2013, ICPPR unpublished data, 2017), number of dead or living immature stages (eggs, larvae, pupae), mean number of workers, and weight of alive workers (ICPPR unpublished data, 2017).

1.4 Summary

Developing standardized and repeatable protocols for assessing effects of pesticides is an important step in helping characterize potential risks to pollinators. There are pronounced biological and ecological differences between honey bees and bumble bees, and as such, current methods used to characterize risks of pesticides to honey bees may not be suitable for bumble bees. By generating data in the development of standardized methodology for assessing pesticide risk to *B. impatiens*, my research can help clarify species differences in response to pesticides. Furthermore, by contributing to the development of standardized test protocols for assessing pesticide risk to *B. impatiens*, pesticide manufacturers will be able to use these protocols to submit reliable and accurate information on the risk their pesticides pose to bumble bees. Therefore, through understanding differences in pesticide susceptibility between bee species and developing standardized and validated bumble bee risk assessment protocols, regulatory agencies will be able to make informed decisions on pesticide use.
1.5 Objectives

Data on assessing pesticide toxicity to honey bees are plentiful but, comparatively, similar pesticide toxicity data for bumble bees are scarce. This is especially true for *B. impatiens*, which is recommended as the North American surrogate bumble bee species in pesticide risk assessments. Dimethoate has been recommended as the toxic standard in the acute contact and acute oral laboratory protocols but the acute oral and contact LD$_{50}$ for *B. impatiens* are unknown. Furthermore, in North America, tier II, semi-field pesticide risk assessment protocols still need to be established and validated for use with *B. impatiens*. Development of semi-field protocols requires identifying an appropriate toxic standard that can be used in future semi-field pesticide risk assessment protocols. Therefore, the purpose of my research is to:

1) Generate dimethoate contact and oral LD$_{50}$ estimates for *B. impatiens* and *A. mellifera* to compare the sensitivity of these two species; and second, to compare the contact and oral LD$_{50}$ values of dimethoate for *B. impatiens* to reported LD$_{50}$ values for *B. terrestris*.

2) Determine the suitability of dimethoate, diflubenzuron, novaluron and tebufenozide as toxic standards for use in tier II hazard assessments with bumble bees by characterizing their effects on *B. impatiens* under lab and semi-field conditions at different application rates.
2.1 Abstract

The purpose of this study was to compare susceptibility of the common eastern bumble bee (*Bombus impatiens* (Cresson)) and the European honey bee (*Apis mellifera* L.) to dimethoate, the recommended toxic standard for use in tier I and tier II pesticide risk assessments with honey bees. I determined that the oral and contact median lethal doses (LD$_{50}$) for *B. impatiens* were 0.41 µg a.i./bee and 0.51 µg a.i./bee, respectively, which was 2- to 5-fold greater than the acute oral (0.18 µg a.i./bee) and contact (0.1 µg a.i./bee) LD$_{50}$ for *A. mellifera*. The LD$_{50}$ values for *B. impatiens* were 10- to 20-fold lower than those previously reported for *B. terrestris*, suggesting *B. impatiens* is far more susceptible to dimethoate than *B. terrestris*. Therefore, different recommended acute contact and oral doses of dimethoate may be required in the North American guidelines for future tier I pesticide testing with *B. impatiens*.

2.2 Introduction

Bumble bees are important pollinators for both agricultural and natural landscapes; however, declines in some species of bumble bees across North America (Potts et al. 2010; Cameron et al. 2011) and Europe (Williams 1982; Biesmeijer et al. 2006; Kosior et al. 2007; La Féon et al. 2010) have been reported. Bumble bee declines can be attributed to several factors, including exposure to pesticides (Brittain et al. 2010; Szabo et al. 2012; Vanbergen et al. 2013). Like honey bees, bumble bees can be exposed to pesticides while foraging via bodily contact with spray droplets or residues on plants, or
through the consumption of contaminated nectar and pollen from a treated crop.

Currently, honey bees are used to estimate the risk of pesticides to all bees when generating data required for pesticide registration, but, because of their differences in life history and behaviour, honey bees and bumble bees may differ in their susceptibility to pesticides. Because of this potential difference, regulatory agencies in Europe and North America may, in the near future, require pesticide risk assessments for bumble bees for pesticide registration and reregistration.

Standardized and validated tier I (laboratory) methods to evaluate the acute toxicity of pesticides to bumble bees were recently released (OECD 2017a, b) based on ring-testing completed using *B. impatiens* (the proposed North American surrogate bumble bee) and *B. terrestris* (the European surrogate bumble bee). In these new OECD guidelines, dimethoate is the recommended toxic standard. A toxic standard serves to confirm exposure and provides information on the sensitivity of the bees to the test conditions (Cabrera et al. 2015). Dimethoate is an organophosphate insecticide that is frequently used as toxic standard in assessing pesticide toxicity with honey bees in laboratory testing (Gough et al. 1994; OECD 1998a, b). The median lethal dose (LD$_{50}$) of dimethoate for honey bees (both through acute contact and acute oral exposure) is established, (Gough et al. 1994) but the LD$_{50}$ for *B. impatiens* is unknown. If dimethoate is recommended as a toxic standard in acute oral and contact tier I testing with *B. impatiens*, we need to generate LD$_{50}$ values specific to *B. impatiens* that can be recommended in the OECD protocols. Currently, *B. terrestris* has acute oral and contact LD$_{50}$ estimates for dimethoate but it is unclear whether *B. impatiens* has a similar sensitivity to dimethoate.
Therefore, the purpose of my study was to: 1) generate an acute contact and acute oral LD$_{50}$ of dimethoate for *B. impatiens* and *A. mellifera* and compare the sensitivity of these two bee species to dimethoate; and 2) compare the acute contact and oral LD$_{50}$ values for dimethoate on *B. impatiens* to LD$_{50}$ values for *B. terrestris*.

2.3 Methods & Materials

*Insecticide*

Formulated dimethoate (Lagon® 480 E, Loveland Products Canada Inc, Dorchester, Ontario, Canada) was used in all experiments.

*Bumble Bee Dimethoate Application Procedure*

For acute oral experiments, dimethoate was dissolved in 50% w/v sucrose solution at 6.25, 12.5, 25, 50 and 100 ppm. Bees received 40 µl of treated solution, resulting in doses of 0.25, 0.5, 1, 2, and 4 µg a.i./bee.

For acute contact experiments, dimethoate was dissolved in acetone and 2 µL of solution was applied with a micropipette applicator at 0.16, 0.31, 0.70, 0.75, 1.1, 1.18, 1.25, 2.5, 5, 10 and 20 µg a.i./bee (78, 156, 350, 375, 550, 588, 625, 1250, 2500, 5000 and 10 000 ppb solutions, respectively). Initially, I used the same dose-range as the ICPPR ring-testing group at 1.25, 2.5, 5 and 10 µg a.i./bee, except formulated dimethoate was dissolved in a 1% Tween® solution instead of Triton™-X; however, beading occurred when the dimethoate-1% Tween solution was applied to the bumble bee thorax. To prevent beading, new solutions were made where formulated dimethoate was instead dissolved in acetone. Because 76% mortality occurred at the lowest dose after 48 h of exposure, I performed another experiment using the following doses: 0.16,
0.31, 0.70, 0.75, 1.1 and 1.18 µg a.i./bee.

**Honey Bee Dimethoate Application Procedure**

For acute oral experiments, dimethoate was dissolved in 50% w/v sucrose solution at 0, 2.5, 5, 10 and 20 ppm. Bees fed *ad libitum* for 4 h. The dose received was calculated after the exposure period.

For acute contact experiments, dimethoate was dissolved in acetone and 1 µL was applied with a micropipette applicator at 0 (control), 0.05, 0.1, 0.2 or 0.3 µg a.i./bee (50, 100, 200 and 300 ppb).

2.3.1 Bumble Bees

**Test Insects & General Set-Up**

Three *B. impatiens* colonies containing ~60-80 workers were obtained from Biobest Biological Systems® (Leamington, ON) and were used within 1 week of the delivery date (OECD 2017a, b). Groups of 20 workers per colony were randomly collected under red light using forceps and placed into a 1 L glass mason jar with a mesh screen ventilated lid. Bees were then anesthetized with CO₂ for 10-12 s and placed individually into plastic Nicot® honey bee queen cages. A 1 mL syringe with the tip cut off and containing 50% w/v sucrose was inserted inside a hole cut in the plastic bottom of each cage and secured in place with masking tape. Cages were placed on a slight incline, so the sucrose solution flowed toward the tip of the syringe (Figure 2.1). Bees were then placed in a growth chamber (40 ± 10% relative humidity (RH) and 24 ± 1°C) and kept in the dark for 12 h to acclimate to the test conditions. After this acclimation period, any moribund or dead bees were removed.
Figure 2.1. Nicot® queen cage (A), with plastic bottom and tape (B) to secure the 1 mL syringe filled with 50% w/v sucrose solution (C) housing an individual *Bombus impatiens* worker used for acute oral and acute contact testing.

2.3.2 Bumble Bee Acute Exposure

**Acute Oral LD$_{50}$**

After the 12 h acclimation period, bees were individually anaesthetized again with CO$_2$ and weighed. While each bee was being weighed, the syringe of untreated sucrose was removed from the Nicot cage and the hole was covered with tape. Once weighed, the workers were placed back inside the sealed Nicot cage and, after all weights were taken, bees were then placed back in the growth chamber in complete darkness for 3 h without food. A syringe containing 40 µL of treated solution was weighed and then inserted into the corresponding Nicot cage of each bee. Control bees received 50% w/v sucrose solution only. The bees were given 3 h to consume the treated solution. Then the syringes were removed and replaced with clean, 1 mL syringes filled with untreated 50% w/v sucrose solution for *ab libitum* feeding. Syringes containing treated solution were then weighed again, and the change in weight of each syringe was used to determine the dose received by each bee. Mortality was assessed at 4, 24, and 48 h after exposure. Bees that did not move when poked with a dissection probe were
considered dead. Each bumble bee acute oral bioassay was performed three times (10 bees/treatment per bioassay) for a total of 30 bees/treatment.

**Acute Contact LD\(_{50}\)**

Bees were acclimated and anaesthetized as described above. While anaesthetized, bees were weighed and then 2 µL of dimethoate dissolved in acetone was applied to the dorsal thorax using a micropipette. Controls were treated with acetone only. The syringes were refilled with 50% w/v sucrose solution before being placed back in the Nicot cages for the bees to feed *ad libitum*. Mortality was assessed at 4, 24, and 48 h after exposure. Bees that did not move when poked with a dissection probe were considered dead. Each bumble bee acute contact bioassay was performed three times (10 bees/treatment), for a total of 30 bees/treatment.

**2.3.5 Honey Bee**

**Test Insects**

In June of 2017, newly emerged (<24 h old) *A. mellifera* workers were obtained from the Honey Bee Research Centre (HBRC) (University of Guelph, Guelph, ON). Workers were acclimatized in a growth chamber (40% RH and 24 ± 1°C) for 24 h before testing.
2.3.6 Honey Bee Acute Exposure

**Acute Oral**

Two experiments were performed to determine the oral LD\textsubscript{50} of dimethoate for *A. mellifera*. The first experiment was set-up using the OECD protocols where the bees were housed in wooden cages (14.5 × 13.0 × 9 cm, height, length and width) with a mesh wire floor and a glass front and provided sucrose through an inverted scintillation vial with two small holes (1 mm diameter) cut in the lid (Figure 2.2) and kept in a growth chamber at 24 ± 1°C with 40 ± 10% RH (OECD 1998a). Treated sucrose was supplied via a 1 mL syringe with the tip removed but under these conditions, *A. mellifera* workers did not feed on the treated sucrose solution after the starvation period. When there was no feeding in my first experiment, the 1 mL treated syringe was switched for a 30 mL syringe since Huang et al. (2014) reported that their largest syringe tested (20 mL) provided the best steady food supply compared to other feeder designs. However, the 30 mL syringe did not increase feeding in my study.

The second experiment involved placing ten workers from the same colony in a 500 mL clear plastic deli container (11.6 × 7.6 cm Pro-Kal®, Shortreed Paper Inc., Guelph, Ontario, Canada) with the bottom removed and replaced with a 30 x 30 cm piece of craft netting, secured with a rubber band. The plastic containers were then placed into 946 mL waxed paper cups (13.5 cm × 11.7 cm Solo®, Toronto, Ontario, Canada). The feeder was made up of a cotton dental wick (6-7 cm long) placed inside a 25 mL scintillation vial containing 10 mL of 50% sugar solution. The feeders were placed beneath the mesh flooring and the wicks were cut slightly shorter than the top of the vial to reduce contact between the bee’s tarsi and the wick yet allow each bee to
access to the solution with their proboscis. The bees were acclimatized for 12 h in a growth at 50 ± 10% RH and 29 ± 1°C and provided with untreated 50% w/v sucrose solution *ad libitum*. Prior to dimethoate exposure, honey bees were starved for 3 h. During the starvation period, four dimethoate treated sucrose solutions (2.5, 5, 10, and 20 ppm) were prepared. After the starvation period, each group was randomly given one of the four dimethoate treated feeders or a control feeder (untreated 50% sucrose solution). Each treatment group was replicated four times, for a total of 40 bees/treatment. After 4 h, the treated feeder was replaced with untreated sucrose solution, so the bees could feed *ad libitum* (OECD 1998a). Feeders were weighed before they were placed inside the containers and again after they were removed to determine the amount of sucrose solution that was consumed. Three extra containers were set-up with no bees and randomly placed among the treated containers to measure evaporation of the sucrose solution, which allowed for a correction factor when determining consumption. Mortality was assessed 4, 24, and 48 h after exposure. Bees that did not move when poked with a dissection probe were considered dead. Bees were kept in 24 h darkness (except when being treated and during mortality assessments) at 50 ± 10% RH and 29 ± 1°C.

**Acute Contact**

Honey bees were acclimated as described above and were housed in a wooden cage (14.5 × 13.0 × 9 cm, height, length and width) and provided sucrose through an inverted scintillation vial with two small holes (1 mm diameter) cut in the lid (Figure 2.2) and kept in a growth chamber at 24 ± 1°C with 40 ± 10% RH (OECD 1998a). Honey bees were obtained as described above and acclimatized for 12 h before beginning the
experiment and provided with untreated 50% w/v sucrose solution *ad libitum*.

Thereafter, ten workers were anaesthetized using CO₂ and individually-treated on the dorsal side of the thorax with 1 µL of untreated acetone (control) or dimethoate at 0.05, 0.1, 0.2 or 0.3 µg a.i./bee dissolved in acetone. There were five replicate cages per treatment group, for a total of 50 sister worker honey bees per treatment. After being treated, each group was placed back in their wooden cage and supplied with untreated 50% w/v sucrose solution (OECD 1998b) (Figure 2.2). Mortality was assessed as described above 4, 24 and 48 h after exposure. Bees were kept in 24 h darkness (except when being treated and during mortality assessments) at 40 ± 10% RH and 24 ± 1°C.

### 2.3.7 Data Analysis

Statistical analyses were performed using the University Edition of SAS statistical software (SAS Statistical Analysis Systems 2017, Cary, NC, USA). For honey bees, the *Probit* procedure with a lograte model was used to determine the median lethal dose (LD₅₀) with 95% fiducial limits (FL), the chi-square values and the slope. For bumble bees, the *Probit* procedure with a lograte model using bumble bee weight as a covariate was used to determine the median lethal dose (LD₅₀) with 95% fiducial limits (FL), the chi-square values and the slope. Weight was used as a covariate in with *B. impatiens* because, comparatively, weights are highly variable (ranging between 0.0958-0.401 g) between *B. impatiens* workers. No control mortality occurred in both acute contact and oral studies with *A. mellifera* and *B. impatiens*. 
Figure 2.2. Cage used for housing 10 *Apis mellifera* workers for acute contact exposure. The wooden cage has a glass pane in the front, wire mesh flooring and an inverted scintillation vial with two small holes (~1 mm) cut into the lid to allow *ad libitum* feeding of 50% w/v sucrose solution.

### 2.4 Results

The oral LD$_{50}$ of dimethoate for *B. impatiens* workers was 0.41 µg a.i./bee (Table 2.1). I was unable to determine the oral LD$_{50}$ of dimethoate for *A. mellifera* because no measurable feeding took place. The contact LD$_{50}$ for *B. impatiens* workers was 0.51 µg a.i./bee, which was 5-fold higher than the contact LD$_{50}$ for *A. mellifera* of 0.10 µg a.i./bee (Table 2.1).
Table 2.1. Acute contact (C) and oral (O)* toxicity of dimethoate after to *Apis mellifera and Bombus impatiens after a 48 h exposure period.

<table>
<thead>
<tr>
<th>Species</th>
<th>Exposure Route</th>
<th>n</th>
<th>Slope ± SE</th>
<th>LD$_{50}$ (µg a.i./bee)</th>
<th>95% Fiducial Limits</th>
<th>$\chi^2$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. mellifera</td>
<td>C</td>
<td>200</td>
<td>13.2 ± 2.33</td>
<td>0.10</td>
<td>0.09 – 0.11</td>
<td>0.86</td>
<td>0.4214</td>
</tr>
<tr>
<td>B. impatiens</td>
<td>C</td>
<td>240</td>
<td>5.1 ± 0.75</td>
<td>0.51</td>
<td>0.40 – 0.62</td>
<td>1.63</td>
<td>0.1487</td>
</tr>
<tr>
<td>B. impatiens</td>
<td>O</td>
<td>180</td>
<td>5.4 ± 0.88</td>
<td>0.41</td>
<td>0.32 – 0.50</td>
<td>0.81</td>
<td>0.7463</td>
</tr>
</tbody>
</table>

*A. mellifera acute oral experiment did not yield usable data to be analyzed

2.5 Discussion

Based on my results, B. impatiens is less susceptible than A. mellifera to dimethoate when exposed via direct contact. My findings are consistent with the trend of bumble bee species being less susceptible than honey bees to a range of insecticides, including some pyrethroids, organophosphates, carbamates, and neonicotinoids (Bailey et al. 2005; Scott-Dupree et al. 2009; Arena and Sgolastra 2014; Heard et al. 2017).

Generally, pesticide susceptibility in bees is thought to be inversely proportional to body size (Devillers et al. 2003; Arena and Sgolastra 2014; Uhl et al. 2016), which my findings support since B. impatiens workers weighed, on average 0.211 g, making them 2 x heavier than the average A. mellifera worker (0.100-0.120 g) (Devillers et al. 2003; Arena and Sgolastra 2014; Heard et al. 2017). In addition, because bumble bees and honey bees possess a similar number of xenobiotic detoxifying enzymes including cytochrome P450s, alcohol dehydrogenases, hydroxylases and peroxidases (Xu et al. 2013), interspecific differences in pesticide susceptibility may be related to other factors, such as bee age. For instance, newly emerged honey bees possess fewer detoxifying enzymes than older, foraging workers (Smirle and Winston 1988; Słowińska et al. 2015). In addition, cuticle permeability decreases as honey bees age, therefore,
pesticide uptake may be greater in younger worker bees (Falcón et al. 2014; Uhl et al. 2016). Thus, because the honey bees in my study were 1 d old, it is possible they were more sensitive to dimethoate exposure than the older bumble bees; but, more research on the effect of age on detoxifying enzymes and cuticular penetration in bumble bee workers is needed to explore this hypothesis.

Most of the acute pesticide exposure data for bumble bees has been generated with B. terrestris and relatively few data for B. impatiens exist. Interestingly, B. impatiens may be more sensitive to dimethoate than B. terrestris. In my study, when B. impatiens workers were exposed to the acute contact dose range in the OECD ring-tested protocols (unpublished data, 2015), 76% mortality occurred at the lowest dose after 48 h. Interestingly, my results differ from other labs that tested this acute contact dose range with B. impatiens. Using the same acute contact and oral dose range, these North American labs did not report high acute contact mortality, but high acute mortality following oral exposure (Cutler personal communication). It is possible that because I used acetone in my acute contact study as opposed to Triton X-100 (surfactant), more of the dimethoate was absorbed through the bee cuticle. For example, technical dimethoate dissolved in acetone was absorbed significantly faster through the Periplaneta americana cuticle compared to dimethoate dissolved in water (Olson and O’Brien 1963). Therefore, it is possible the bees in my study absorbed more dimethoate than those exposed to dimethoate dissolved in water with Triton X-100. Previous methods for development of pesticide acute toxicity test for bumble bees, including

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Dalhousie University
dose-range selection, have been done with *B. terrestris* (Schaefer et al. 1996; van der Steen 1999; Marletto et al. 2003; Heard et al. 2017). Because *B. impatiens* mortality was high at the lowest recommended OECD dose, I used a lower dose range (0.16-1.18 µg a.i./bee). My results, when compared to those from the open literature for *B. terrestris*, suggests that *B. impatiens* is 2- to 17-fold more susceptible to dimethoate by contact than *B. terrestris* (van der Steen 1999; Marletto et al. 2003). Conversely, when exposed to wet and dry residues, *B. terrestris* may be 16-fold and 19-fold more susceptible to spinosad and spinetoram than *B. impatiens* (Gradish et al. 2012). It is possible that differences in direct contact pesticide susceptibility may be the result of inherent species differences in susceptibility to different insecticides. For example, Wu et al. (2010) found that *B. hypocrita* was 2x less susceptible to acetamiprid via direct contact compared to *B. patagia* and *B. ignitus*. However, when consumed, Wu et al. (2010) found no interspecific differences in susceptibility to acetamiprid. My findings seem to suggest that *B. impatiens* has a similar susceptibility to dimethoate (LD$_{50}$ 0.41 µg a.i./bee) as *B. terrestris* (0.38-1.7 µg a.i./bee) when consumed (Schaefer et al. 1996; van der Steen 1999; Marletto et al. 2003; Heard et al. 2017). Along with dimethoate, *B. impatiens* and *B. terrestris* may also have similar oral susceptibilities to deltamethrin and spinetoram (Tasei et al. 1994; Besard et al. 2011; Gradish et al. 2012). Contact susceptibility between bee species may be more variable compared to oral susceptibility. More acute oral and contact toxicity data are needed to confirm differences in pesticide susceptibility between bumble bee species.

Two experiments were performed to generate an oral LD$_{50}$ for dimethoate on *A. mellifera* and neither were successful. In my first attempt, I used the same wooden cage
set-up that was used in the acute contact experiment. Workers did not seem to be feeding on the untreated sucrose during the acclimation period. It is possible that honey bee workers did not feed because of their age. Inside the hive, newly eclosed and 1 d old honey bee workers tend to receive more food from older donor bees and exhibit low trophallactic activity (Free 1957; Crailsheim 1998; Moritz and Hallmen 1986; Brodschneider et al. 2017). In addition, honey bee workers that emerge without the presence of nurse bees tend to have reduced levels of amino acids in their haemolymph which can affect their feeding abilities (Crailsheim 1986; Crailsheim and Leonhard 1997). Therefore, it is possible that the young age of the honey bee workers in my study resulted in reduced feeding. In addition to honey bee age, the temperature in my first experiment may have been too low (24°C) which could have impacted feeding behaviour since other studies using the wooden cage set-up had feeding success between 29-35°C (Kovac et al. 2007; Kovac et al. 2014; Huang et al. 2014). After the starvation period, when the untreated sucrose was substituted for the treated dimethoate inside a 1 mL syringe with the tip cut off, the bees continued not feeding. When no feeding occurred, the 1 mL syringe was replaced with a larger 30 mL syringe since, previously, the use of a 20 mL syringe (the largest tested) as a feeder was reported to provide the steadiest supply of food to workers, which increased survivorship when compared to other feeder designs (Huang et al. 2014). However, the 30 mL syringe did not increase feeding in my study. The wooden cages may have contributed to reduced feeding. The effect of cage design on honey bee feeding has not been directly tested, although one study reported that groups of 10-15 bees housed in the wooden cages had the shortest survivorship over time compared to groups of bees
housed in other cage designs (Huang et al. 2014). However, the changes in bee survivorship between cages did not significantly differ until after day 5 (Huang et al. 2014). After I increased the temperature, some feeding did occur in my second experiment. The bees were the same age in both my first and second experiment, which suggests that temperature played a larger role in feeding behaviour than age. However, feeding in my second experiment was inconsistent across replicates and no measurable feeding occurred to determine the dose received by the workers. Interestingly, Brodschneider et al. (2017) found that food sharing was better distributed among 30 bees caged together than 10 bees caged together. In addition, 10 caged bees did not better distribute radioactive $^{14}$C-labelled sugar solution when offered 100 µL or 25 µL.

Currently, no LD$_{50}$ of dimethoate for _B. impatiens_ exists in the published literature, making my study the first to generate an acute contact and oral LD$_{50}$ for dimethoate. Overall, _B. impatiens_ is less susceptible to dimethoate via direct contact than honey bees. Interestingly, because of the potential difference in susceptibility to dimethoate between _B. impatiens_ and _B. terrestris_ when topically applied, a separate OECD (2017a) acute contact toxic standard dose recommendation may be needed to achieve $\geq 50\%$ mortality for _B. impatiens_. Hopefully, more acute contact and oral data on _B. impatiens_ pesticide susceptibility will be generated to increase our understanding on differences in pesticide susceptibility. The new acute oral and contact guidelines for bumble bees will facilitate future research on pesticide susceptibility of _B. impatiens_ in tier I studies.
Chapter 3
Evaluating different insecticides as potential toxic standards for use in pesticide risk assessment with Bombus impatiens (Cresson)


3.1 Abstract

The decline of some bumble bee species across North America and Europe is a cause for concern. Pesticides are a suspected driver of bumble bee declines. The development of North American bumble bee pesticide risk assessment protocols requires the identification and validation of a toxic standard for use with Bombus impatiens (Cresson), the proposed surrogate bumble bee for these studies in North America. Potential toxic standards were evaluated in both micro-colonies and in tier II (semi-field) studies. In a 2016 tier II study, 100% of queenright B. impatiens colonies died within 24 h of dimethoate exposure at 400 g a.i./ha (1/3 of the max label rate). In contrast, diflubenzuron applied at its max label rate (257 g a.i./ha) had no effect on colony weight, foraging activity or the number of new queens, males or workers produced. Thereafter, a micro-colony screen was done with dimethoate, novaluron and tebufenozide to determine their suitability as toxic standards. As in the 2016 field season, dimethoate in honey-deionized water solutions at 129.5 mg a.i./L or 12.9 mg a.i/L caused 100% mortality of B. impatiens in micro-colonies. Conversely, no adverse
effects on days to oviposition, number of males produced, or male weights were observed in micro-colonies fed novaluron (35.6 mg a.i./L or 3.6 mg a.i./L) or tebufenozide (14.1 mg a.i./L or 1.4 mg a.i./L) treated honey-deionized water solution. Based on the 2016 semi-field and micro-colony results, dimethoate was the only insecticide used in my 2017 semi-field study. Queenright \textit{B. impatiens} colonies were exposed to dimethoate at 200, 80, 40 g a.i./h or water (controls). Within 72 h of exposure all colonies exposed to dimethoate died. If dimethoate is to be used as the toxic standard with \textit{B. impatiens}, in micro-colonies or in semi-field studies, a rate that elicits observable adverse effects, but not death, must be identified.

\textbf{3.2 Introduction}

Exposure to pesticides may be a factor in bumble bee declines (Brittain et al. 2010; Szabo et al. 2012; Vanbergen et al. 2013). Bumble bees can be exposed to pesticides while foraging via bodily contact with spray droplets or residues on plants, or through the consumption of contaminated nectar and pollen from a treated crop. Since pesticide exposure is a concern, regulatory agencies in Europe and North America may soon require pesticide risk assessments for bumble bees for pesticide registrations. Tier I (laboratory) methods to assess acute toxicity of pesticides on bumble bees were recently established (OECD 2017a, b) and, although some recommendations have been made, tier II (semi-field) methods still need to be refined and validated. The current recommendation is to use \textit{Bombus terrestris} as the European surrogate and \textit{B. impatiens} as the North American surrogate bumble bee, as both species are
commercially available and native to their respective continents (Cabrera et al. 2015; OECD 2017).

Semi-field studies look at colony level effects of pesticides under realistic “worst-case” scenarios (Cabrera et al. 2015). To do this, bees are placed in an enclosure and forage on a pesticide-treated, flowering attractive plant. Semi-field pesticide toxicity studies must contain a minimum of three treatment groups including the: 1) test pesticide; 2) negative control; and, 3) toxic standard. A toxic standard serves as proof of exposure to the pesticide and provides information on the sensitivity of the bees to the test conditions and is required in acute lab testing and in semi-field pesticide toxicity studies (Cabrera et al. 2015). In Europe, dimethoate applied at a rate of 800 g a.i./ha is the recommended toxic standard for use in semi-field pesticide toxicity studies with *B. terrestris* (ICPPR unpublished data 2017). In North America, a toxic standard has yet to identified and validated for use in *B. impatiens* semi-field pesticide toxicity studies.

The purpose of this experiment is to find a suitable toxic standard for use in semi-field pesticide toxicity assessments with *B. impatiens*. The effects of dimethoate (an organophosphate), and three insect growth regulators (IGRs), diflubenzuron, novaluron and tebufenozide were evaluated for their potential use as toxic standards in semi-field pesticide toxicity testing with *B. impatiens*. Dimethoate is an acetylcholinesterase inhibitor that affects all stages of development and is the current toxic standard in honey bee pesticide risk assessments and in tier I bumble bee testing (OECD 2017a, b) and is recommended for use in tier II testing with *B. terrestris* (ICPPR unpublished data 2017). Chitin synthesis inhibitors (e.g., novaluron and diflubenzuron) and ecdysone agonists (e.g., tebufenozide) are used to control the larval stages of several Lepidopteran pests
by interfering with larval molting, and theoretically can interfere with bumble bee larval development (Mommaerts et al. 2006 a, b; Cutler and Scott-Dupree, 2007). Because IGRs only affect larval stages and not the adults, they have the potential to disrupt bumble bee colony growth and development. Therefore, IGRs have the potential to be used as toxic standards under semi-field conditions.

The effects of diflubenzuron and dimethoate were evaluated to determine their suitability as a toxic standard for tier II toxicity tests with *B. impatiens*. Prior to the 2017 field season, the suitability of novaluron and tebufenozide as potential toxic standards were screened using *B. impatiens* micro-colony bioassays. Because neither elicited adverse effects on the micro-colonies, they were not used in my 2017 tier II study.

### 3.3 Material and Methods

#### 3.3.1 2016 Semi-Field Experiment

**Test Insects**

Thirty-six queenright *Bombus impatiens* (Cresson) colonies with approximately 40-50 workers were obtained from Biobest Biological Systems® (Leamington, ON). Each colony arrived with a sealed bottle of sugar solution (Biogluc®, Biobest Biological Systems®) as a nectar substitute. Colonies arrived four days prior to placement in the field. Upon arrival, colonies were supplemented with approximately 5 g of ground pollen obtained from the University of Guelph Honey Bee Research Facility (HBRF) and lids on the sugar solution bottles were removed, allowing bees to feed *ad libitum*. The day colonies went into tents, (hereafter Day 0), initial colony weights were recorded. In addition, three photographs of each colony were taken under red light to determine the
number of workers. The number of workers per colony was counted from each photograph and a mean number of workers in each colony was calculated. During the experiments, colonies were weighed twice weekly, with at least 72 h between weight recordings (Appendix A).

**Insecticides**

Insecticide formulations used included dimethoate (Lagon® 480 E, Loveland Products Canada Inc, Dorchester, Ontario, Canada) and diflubenzuron (Dimilin® 25%WP, Chemtura Canada Co, Elmira, Ontario, Canada). Dimethoate was applied to buckwheat plots at 400 g a.i./ha (1/3 of max label rate) and diflubenzuron was applied at its maximum label rate of 257 g a.i./ha.

**Planting and Field Set-Up**

In both 2016 and 2017, a 6 ha field of buckwheat was planted 2 km southwest of Eden, Ontario (42° 48' 38.2608” N, 80° 44' 5.2944” W) (Figure 3.1A). Once buckwheat plants reached the second true leaf stage, 3.5 m² plots were marked with staking flags. Plots were hand weeded throughout the duration of experiment. A total of 36 plots (12 plots/treatment), with a minimum distance of 4 m between each plot were set up. When the buckwheat was 20-25% flowering, a wooden stand (5 cm²) with a plywood base (30 x 35 cm) was placed in the corner of each plot. Following this, tents (3.35 x 3.35 x 2.29 m, Instant Screen House®, Coleman Canada Inc.) were placed over the plots such that the wooden stands were approximately 0.5 m into the plot, away from the sides of the tent. Once the plots were equipped with a tent and a stand, and colonies had been
weighed and photographed, one *B. impatiens* colony was placed on each stand and the entrance opened.

**Foraging Activity Assessments**

Foraging activity assessments involved an observer standing outside the tent, with a clear view of the colony entrance. Bees were allowed to acclimate to the presence of observers for two minutes. Following the acclimation period, the number of active foragers visibly visiting flowers. The observer then counted the number of workers entering or exiting the colony over a 10 min period. Foraging activity assessments were repeated 1-2 h later on each observation day. The first observation day was day 1, and the last was on day 16, there were a total of 7 observation days staggered over the duration of the experiment (Appendix A).

**Insecticide Application**

When 90-95% of the buckwheat was flowering on Day 6, colony entrances were closed at 5:00 AM the morning of spray application. Insecticides were mixed with water in 2 L plastic bottles 24 h before spray application. The bottles were stored in the dark until the morning of application. Colonies and tents were moved off the plots prior to treatment application. Plots were sprayed with either dimethoate at 400 g a.i./ha, diflubenzuron at 257 g a.i./ha or water (controls). The insecticides were applied using a 28 mm inverted spray header at a pressure of 60 psi using a 4 nozzle (TeeJet® VisiFlo Flat Spray 800 2VS) and a 2 m handheld boom. The 2 L insecticide bottles and CO₂ cylinder were both carried in a heavy-duty back frame (W 315-107 bracket from
Bellspray Inc). After insecticides were sprayed, the colonies were placed back on their stands with the entrances closed. Tents were placed back on plots once the residue had dried, approximately 1-2 h after application. Once tents were placed back over plots, colonies were opened and left to forage. On the evening of Day 16, colony entrances were closed, and the colonies returned to the University of Guelph. Three pictures of the inside of each colony were taken under red light and the number of workers counted (Table 3.1). Biogluc feeders were opened to allow ad libitum feeding and colonies were kept in a dark room. The following morning, the Biogluc feeders were closed and colonies were moved to an area of natural forage, described below.

**Residue Analyses**

Of the 36 plots, six were set aside for residue analysis (2/treatment). Samples were taken twice. Early samples were taken 24 h after spray application, while late samples were taken on the final day of the colonies being on the plots (Day 16). During sampling, approximately 7-8 actively foraging workers (bees on flowers collecting nectar or pollen) were caught in each residue tent. This was done using a net, catching one bee at a time, placing her in a labelled and sealed cup that was placed in a cooler with ice packs. Dimethoate residue tents had no active foragers so no bees were collected. The bees from the diflubenzuron and control residue tents were brought back to the University and stored in a -20°C freezer.

Worker bees that were collected were dissected and had their crops removed and placed inside 1 mL microcentrifuge tubes for eventual residue analysis. Crops that could not be kept intact had the nectar squeezed out into the tube. Crops from different
treatments and timing of sampling (early vs. late) were dissected separately to avoid cross-contamination of samples. Nectar and crop samples were spun in a microcentrifuge at 6600 rcf for 30 s so that nectar on the sides of the microcentrifuge tubes pooled in the bottom. In 2016, a total of 4 control residue samples (2 early and 2 late) and 4 diflubenzuron samples (2 early and 2 late) were sent for analyses at Activation Labs Ltd (Ancaster, Ontario). A control nectar sample was spiked at three levels – 1 x limit of quantification (LOQ), 10 x LOQ, and 20 x LOQ. Samples were then shaken vigorously in a mixture of high purity water and acetonitrile. Magnesium sulphate and sodium chloride were added to separate the aqueous water layer from the organic acetonitrile layer. Samples were centrifuged and run through a primary secondary amine dispersive clean-up. Thereafter, residue samples were filtered through a 0.22 um polyvinylidene difluoride syringe filter and analyzed for diflubenzuron and dimethoate by liquid chromatography–mass spectrometry coupled with tandem mass spectrometry.

**Post Exposure**

On the morning of Day 17 (7 July 2016), live colonies were moved to the 4th Canadian Division Training Centre (Meaford, Ontario) and placed in a meadow behind the ammunition storage building. All 20 (10 control and 10 diflubenzuron) colonies were placed inside a single plywood shelter and affixed with a black paper geometric shape to reduce workers from one colony entering a different colony, also known as drifting. The base of the shelter was 72 × 147 cm; the back of the shelter was 133 × 112 cm; and the angled roof was 77 × 152 cm (Figure 3.1B). Three cinder blocks were placed
across the base to keep the structure secured to the ground. Every Monday and Thursday for 4 weeks, each colony was closed and removed from the wooden shelter to record the number of queen pupae, newly emerged queens and colony weight. To perform the assessments, colony entrances were closed and colonies were then gently removed from the wooden shelter, one at a time. Once the assessments were recorded, the colony was placed back in the shelter and entrance was re-opened. On 5 August 2016 an invasion of ants caused the sudden death of 8 colonies, so the remaining colonies were removed from the area of natural forage and brought to the University of Guelph. Colonies were kept in a growth room with a photoperiod of 24 h darkness at 24 ± 1°C and 30 ± 10% RH (except when recording number of queen pupae, new queens and colony weights). Each colony received 5 g of honey-bee collected pollen from the HBRC Monday, Wednesday and Friday. Colonies were weighed every Monday and Thursday and fed Biogluc ad libitum. Thereafter, new queen emergence was monitored daily, and colonies were frozen two weeks after the first queen emergence. If no new queens emerged, then colonies were frozen 8 weeks after the removal from the buckwheat plots (August 30, 2016).

**Colony Dissection**

After being frozen, each colony was dissected, and number of eggs, larvae, pre-pupae, and pupae were counted and categorized as either dead (having a brown or black colour) or alive (maintaining a creamy white colour). Number of adult workers, males and queens were counted and if that number exceeded 20, then 20 bees were randomly selected from each group (workers, males and queens) to be weighed.
Figure 3.1. A 2016 semi-field study setup for *Bombus impatiens*. Screened enclosures in 6 ha field of buckwheat near Tillsonburg, Ontario (A), and a post-insecticide exposure plywood protective structure for colonies in an area of natural forage at the 4th Canadian Division Training Centre near Meaford, Ontario (B).

**Data Analysis**

Data (initial colony size, final colony size, foraging activity (the number of foragers entering or exiting the colony and the number of active foragers), colony weights, number of immature stages (eggs, larvae and pupae) and number of adults (queens, workers and males)) were analysed with an ANOVA in the University Edition of SAS statistical software (SAS Statistical Analysis Systems 2016, Cary, NC, USA). The type I error rate was set at 0.05 for all statistical comparisons. Foraging activity, colony weights, number of immature stages and number of adults between treatments, did not meet the assumptions of the error of the variance analyses (random, homogeneous, and normally distributed).

A repeated measure ANOVA using the *Glimmix* procedure was used to assess foraging activity and colony weights. Variance was partitioned into the fixed effect of treatment and day, the interaction of day × treatment and the random effect of
replication × treatment. Foraging activity was analysed using a negative binomial distribution and colony weights were analysed using a gamma distribution, a log link was applied to both variables. Means were compared using Tukey’s multiple means comparison adjustment.

Data on number of eggs, larvae, pupae and adults per colony were analyzed with the Glimmix procedure using a negative binomial distribution with a log link. Variance was partitioned into the fixed effect of treatment and the random effect of replication × treatment. Means were compared using Tukey’s multiple means comparison adjustment.

### 3.3.2 Micro-colony Experiment

Based on the results of the 2016 field season, I used queenless micro-colonies to screen two IGRs, novaluron and tebufenozide, to determine their suitability as toxic standards for use in semi-field toxicity studies with *B. impatiens*.

**Test Insects**

Eight colonies were delivered from Biobest Biological Systems® (Leamington, ON). Each colony arrived with a sealed bottle of sugar solution (Biogluc®, Biobest Biological Systems®) as a nectar substitute. Upon arrival, the colonies were supplemented with approximately 5 g of ground pollen obtained from the University of Guelph Honey Bee Research Facility (HBRF) and lids on the Biogluc bottles were removed, allowing the bees to feed *ad libitum.*
**Insecticides and Treatments**

Insecticide formulations used included dimethoate (Lagon® 480 E, Loveland Products Canada Inc, Dorchester, Ontario, Canada) and two IGRs: novaluron (Rimon®10 EC, MacDermid Agricultural Solutions Canada Company, Guelph, Ontario, Canada) and tebufenozide (Confirm®240F, Nippon Soda Co., Ltd, Tokyo, Japan).

Rates for treatments were determined based on USEPA assumption that: 110 ppm pesticide residue = 1 lb/acre (or 1.121 kg/ha) of active ingredient applied via foliar spray (USEPA 2014). This assumption is based on the terrestrial residue exposure (T-REX) model used as a surrogate for estimating pesticide concentrations in nectar of flowers that are sprayed directly with pesticides (EFSA 2014). The maximum label rate and a tenth of maximum label rate were used to calculate the approximate residue level for each insecticide. Estimated pesticide residue levels were calculated using the following equation:

\[
\text{estimated pesticide residue (in ppm) = } \left[ \frac{110 \text{ ppm}}{1.121 \text{ kg ha}^{-1}} \right] \times \text{maximum label rate of pesticide}
\]

Micro-colonies were randomly assigned to one of seven treatments: dimethoate (129.5 mg a.i./L or 12.9 mg a.i/L), novaluron (35.6 mg a.i/L or 3.6 mg a.i/L), tebufenozide (14.1 mg a.i/L or 1.4 mg a.i/L) or a control consisting of untreated 50% v/v honey-deionized water solution. Formulated insecticides were dissolved in 50% v/v honey-deionized water solution (hereafter honey-water solution).

**Micro-colony Set-Up**

Micro-colonies were assembled 24 h after the eight colonies were received from Biobest. A group of five workers from one of the eight parent colonies were randomly
selected for each of the 70 micro-colonies (7 treatments x 10 micro-colonies/treatment) in this study. Five workers were placed in a 500 mL clear plastic deli container (11.6 × 7.6 cm Pro-Kal®, Shortreed Paper Inc, Guelph, Ontario, Canada) with the bottom removed and replaced with a 30 cm² piece of craft netting secured to the plastic container with a rubber band. The plastic containers were then placed into 946 mL waxed paper cups (13.5 × 11.7 cm, Solo®, Toronto, Ontario, Canada). The feeder was a 25 mL scintillation vial filled with 10 mL of treatment solution. Thereafter, a cotton dental wick soaked in one of the six treatments and subsequently placed inside a corresponding feeder (Figure 3.2). To determine the amount of honey-water solution consumed, weights of the feeders were taken before they were placed in the micro-colonies, and once again when the feeders were removed. Feeders were secured to the base of the waxed paper cups with a twist tie beneath the micro-colonies (Figure 3.2).

To account for evaporation of the honey-water solution, two micro-colony containers without bees were set up. Evaporation feeders were weighed and changed on same schedule as above. Micro-colonies were kept in 24 h darkness (unless making observations or changing feeders) at 24 ± 1 °C and 33 ± 10% RH in a growth chamber and arranged as a completely randomized design.

Micro-colony feeders were changed every Monday, Wednesday and Friday for 45 d; treated honey-water solution was provided for 14 d before all micro-colonies were switched to untreated 50% v/v honey-deionized water solution. Each micro-colony also received pollen balls made from a paste consisting of ground honey bee-collected mixed floral pollen from the HBRC, deionized-water and honey (from HBCR) in a 6:1:1 ratio, respectively. Using a toothpick, pollen balls were coated in melted bee’s wax (from
HBCR) and placed in the freezer until use. At study initiation, one 2 g pollen ball treated with 1 mL of the insecticide solution to obtain the desired concentration, was placed inside each micro-colony and left there for the duration of the experiment. On Day 10, to supplement for pollen, untreated 1 g pollen balls were placed in each micro-colony and were changed twice weekly. Micro-colonies received a total of ten pollen balls for the remaining duration of the experiment.

Figure 3.2. *Bombus impatiens* queen-less micro-colony set up. A 50% v/v honey-deionized water feeder (A) sits beneath the netting, allowing workers to feed *ad libitum*. Workers rear and initiate brood on initial wax coated 2g pollen ball (B) and are supplemented with 1g wax coated pollen balls twice weekly (C).

**Data Analysis**

Micro-colonies were monitored daily for the duration of the experiment (45 d). Initially, 70 micro-colonies were established but 24 h after set-up, all workers died in
micro-colonies exposed to 12.95 mg a.i./L and 129.5 mg a.i./L dimethoate treated honey-water solutions. These colonies were excluded from analysis (n=20). Throughout the course of the experiment micro-colonies were terminated if 3 or more of the 5 workers died (n=16). These micro-colonies were excluded from further analysis, except when comparing the number of bee deaths that occurred per treatment group.

Date to oviposition, number of males emerged and average male weights, number of worker deaths, amount of honey-deionized water solution consumed and number of eggs, larvae and pupae between treatments were analyzed in the University Edition of SAS statistical software (SAS Statistical Analysis Systems 2017, Cary, NC, USA). An analysis of variance (ANOVA) was performed for each variable using the Glimmix procedure. Data on number of males emerged, number of immature stages, number of worker deaths and days to oviposition did not meet the assumptions of the error of the variance analyses (random, homogeneous and normally distributed). Variance was partitioned into the fixed effect of treatment and the random effect of replication × treatment. Number of emerged males, number of immature stages and number of worker deaths were analysed using a negative binomial distribution with a log link. Days to oviposition were analysed using a gamma distribution with a log link. Means were compared using Tukey’s multiple means comparison adjustment. The type I error rate was set at 0.05 for all statistical comparisons.

3.3.3 2017 Semi-Field Experiment

Test Insects

Fifty-two queenright colonies each containing 20-30 workers were received from Biobest Biological Systems® (Leamington, ON) 48 h before they were placed in the
Each colony arrived with pollen and a sealed bottle of sugar solution (Biogluc®, Biobest Biological Systems®) as a nectar substitute. On Day 0 (10 July 2017), the 48 colonies containing approximately 20 workers and one queen and no males or evidence of bumble bee wax moth (Aphomia sociella L.) infestation were weighed. The Biogluc containers were closed prior to placing the colonies in the field.

**Insecticide**

Formulated dimethoate (Lagon® 480 E, Loveland Products Canada Inc, Dorchester, Ontario, Canada) were used in this study. Dimethoate was applied to buckwheat plots at a rate of 40, 80 or 200 g a.i/h and water was applied to control plots.

**Planting and Field Set-Up**

The same field site, set-up and planting from 2016, with only minor changes, was used in 2017. The minor changes involved setting up a total of 48 plots (4 treatments and 12 plots/treatment). Because diflubenzuron and dimethoate residues were found in control plots in 2016 (Table 3.2), the minimum distance between each plot was increased to 10 m to eliminate potential contamination due to drift. To reduce contact between the tent and the stands, stands in 2017 were placed along the front middle edge of the tent instead of the back corner. In 2017, all tent and *B. impatiens* colony entrances faced east.
**Foraging Activity Assessments**

Foraging activity assessments were executed as described in 2016 for a total of 6 observation days (Appendix A).

**Insecticide Application**

When 90-95% of the buckwheat was flowering (Day 6; 16 July 2017) colony entrances were closed between 8:40-9:15 PM the evening before spray application. Insecticides were prepared and applied as in 2016. On the morning of day 7 (17 July 2017) at 5:45 AM colonies and tents were moved off the plots prior to treatment application. Plots were sprayed with dimethoate either at 40, 80 or 200 g a.i/h (hereafter D40, D80 and D200) or water (controls). As in 2016, after insecticides were sprayed, the colonies were placed back on their stands. Tents were placed back on plots once the residue had dried, approximately 1-2 h after application. Once tents were placed back over the plots, the colony entrance was opened, and bees were left to forage.

**Post-Dimethoate Exposure**

Twenty-four h after dimethoate was applied, 9 x D80 and 9 x D200 colonies were dead. As a result, a decision was made to place the 4 unallocated colonies inside D80 plots for observational purposes. These colonies were referred to as “D80-2”.

On Day 14 (25 July 2017), colonies were moved from buckwheat plots and brought to the University of Guelph and placed in a growth chamber and maintained under the same conditions as the previous year. Each colony received 5 g of honey-bee collected pollen from the HBRC Monday, Wednesday and Friday. They were weighed
every Monday and Thursday and fed on Biogluc *ad libitum*. New queen emergence was monitored daily, and colonies were frozen two weeks after the first queen emergence. If no new queens emerged, then colonies were frozen 8 weeks after the removal off the buckwheat plots (18 September 2017). Colonies were frozen and excluded from analyses if the foundress queen died before the end of the experiment.

*Colony Dissection*

While frozen, each colony was dissected, and the number of eggs, larvae, pre-pupae, and pupae were counted and categorized as either dead (having a brown or black colour) or alive (maintaining a creamy white colour). The number of adult workers, males and queens were counted if that number exceeded 20, then 20 individuals were randomly selected from each group (workers, males and queens) and weighed.

*Data Analysis*

Colony weights before dimethoate exposure met the assumptions of the error of the variance analyses (random, homogeneous and normally distributed) and were analysed using a repeated measure ANOVA in the *Glimmix* procedure using the University Edition of SAS statistical software (SAS Statistical Analysis Systems 2017, Cary, NC, USA). Variance was partitioned into the fixed effect of treatment and day, the interaction of day × treatment and the random effect of replication × treatment. Means were compared using Tukey’s multiple means comparison adjustment. The type I error rate was set at 0.05 for all statistical comparisons.
Within 72 h, all 30 colonies exposed to dimethoate treated buckwheat died. Means and standard errors for colony weights after exposure, number of foragers entering or exiting the colony, number of active foragers and the dissection endpoints (number of immature stages, number of adults and adult weights) are reported, but no analyses were performed.

3.4 Results
3.4.1 2016 Field Results

Colony Size, Colony Weights, and Foraging Assessments

Treatment had no effect on colony weights while on buckwheat plots ($F = 1.12; df = 1, 18; P=0.3039$) (Figure 3.3). The effect of day was significant ($F = 51.96; df = 5, 90; P=<0.0001$); but, the interaction of treatment × day was not ($F = 1.04; df = 5, 90; P=0.3966$). This indicates that the effect of day did not vary by treatment (Figure 3.3). Colony weights initially declined when on the buckwheat plots, but all colonies gained weight when they were moved to the area of natural forage (Figure 3.5).

Treatment had no effect on the number of active foragers ($F =2.12; df = 1, 264; P = 0.1467$) (Figure 3.4A) or on the number of foragers entering or exiting colonies ($F =1.04; df = 1, 264; P = 0.3020$) (Figure 3.4B). Foraging activity increased significantly between days for both number of foragers entering or exiting the hive ($F =59.04; df = 6, 264; P = <0.0001$) and number of active foragers ($F =40.54; df = 6, 264; P =<0.0001$). The interaction of treatment × day was not significant for the number of foragers entering or exiting the colony ($F =0.96; df = 6, 264; P = 0.4563$) or for the number of active foragers ($F =0.59; df = 6, 264; P = 0.7419$).
Table 3.1. Mean (± SE) initial number of *Bombus impatiens* workers in colonies on Day 0 (23 June 2016) and mean final number of workers on day 16 (6 July 2016).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Initial Mean ± SE Number of Workers</th>
<th>Final Mean ± SE Number of Workers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.6 ± 2.69</td>
<td>87.1 ± 5.39</td>
</tr>
<tr>
<td>Diflubenzuron</td>
<td>47.9 ± 2.69</td>
<td>82.1 ± 5.39</td>
</tr>
<tr>
<td>Dimethoate*</td>
<td>49.7 ± 2.69</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*All colonies treated with dimethoate died 48 hr after spray application

Figure 3.3. Mean (± SE) weight of control (n=10), diflubenzuron (n=10) and dimethoate (n=10) *Bombus impatiens* colonies while inside buckwheat plots during the summer of 2016 for 16 d. The solid vertical line depicts when plots were treated with either 400 g a.i./ha of dimethoate, 257 g a.i./ha of diflubenzuron or water on Day 6. After Day 6, all colonies exposed to dimethoate treated buckwheat plots died (n=10).
Figure 3.4 Mean (± SE) (A) number of active *Bombus impatiens* foragers or (B) number of foragers entering and exiting the colony entrance in buckwheat plots treated with either 400 g a.i./ha of dimethoate (n=10), 257 g a.i./ha of diflubenzuron (n=10) or water (n=10) per observation period per day (n=7) during the summer of 2016 for 16 d. The solid vertical line depicts when insecticide treatments were applied on day 6. After day 6, all colonies exposed to dimethoate treated buckwheat plots died (n=10).
Post Treatment Exposure Observations

When *B. impatiens* colonies were in the post-treatment exposure site near Meaford foundress queens died in seven diflubenzuron colonies and five control colonies. Wild *B. impatiens* queens were found in nine diflubenzuron colonies and eight control colonies. Out of the seven diflubenzuron colonies that died prematurely, six contained at least one wild *B. impatiens* queen, and one of those six also contained a dead *B. citrinus* female. Similarly, four of the five control colonies that died early contained at least one wild *B. impatiens* queen and one of those four also contained a dead *B. vagans* worker. On 4 August 2016, ants (*Crematogaster cerasi* Fitch as identified by Dr. Gary Umphrey\(^2\)) were found eating the nest structure (honey and pupae) in six diflubenzuron colonies and seven control colonies. The foundress queen in five controls and three diflubenzuron ant-infested colonies died. To prevent further queen loss, the colonies were brought back to the University of Guelph on 5 August 2016. At the end of the experiment (8 weeks after the colonies were moved off the buckwheat plots) bumble bee wax moth (*A. sociella*) larvae were found in three diflubenzuron colonies and three of the five control colonies.

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\(^2\) Dr. Gary Umphrey, umphrey@uoguelph.ca  
Associate Professor, Statistics  
Mathematics and Statistics  
University of Guelph
Figure 3.5. Change in mean (± SE) colony weights while foraging in buckwheat over the course of 16 d in the summer of 2016 for control colonies (n=10) and diflubenzuron colonies (n=10). The narrow black vertical line depicts when insecticide treatments were applied on day 6. Colonies were moved to an area of natural forage on day 17 (vertical dashed line) where control (n=5) and diflubenzuron (n=3) colonies remained until they were brought back to the university (thick black vertical line) on Day 46.

**Residue Analyses**

Dimethoate and diflubenzuron residues were detected in early control residue samples (CE1 and CE2) and late control residue samples (CL1 and CL2) (Table 3.2). Similarly, dimethoate residues were detected in the early (DE1 and DE2) and late (DL1 and DL2) diflubenzuron residue samples (Table 3.2).
Table 3.2. Summary of nectar residue sample analysis results from 2016, where early collection period was 24 h after insecticide application (E), and late collection was one week thereafter (L). All colonies that were exposed to dimethoate died 24 h after insecticide application, as a result no dimethoate residue samples were collected.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Collection ID*</th>
<th>Residue Level (ppb)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Diflubenzuron**</td>
<td>Dimethoate***</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>CE1</td>
<td>0.153</td>
<td>9.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CE2</td>
<td>0.185</td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL1</td>
<td>0.159</td>
<td>0.547</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CL2</td>
<td>0.799</td>
<td>0.339</td>
<td></td>
</tr>
<tr>
<td>Diflubenzuron</td>
<td>DE1</td>
<td>27.7</td>
<td>52.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DE2</td>
<td>23.8</td>
<td>5.84</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DL1</td>
<td>7.79</td>
<td>2.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DL2</td>
<td>0.751</td>
<td>0.301</td>
<td></td>
</tr>
</tbody>
</table>

*aResidue samples from replicates (1 & 2) were kept separate for analysis

**Diflubenzuron: LOD = 0.035 ppb and LOQ = 1.00 ppb

***Dimethoate: LOD = 0.114 ppb and LOQ = 0.80 ppb

Colony Dissection

Treatment had no effect on the mean number of adult queens ($F = 0.44; df = 1, 6; P = 0.5323$), workers ($F = 0.13; df = 1, 6; P = 0.7271$), or males ($F = 0.1.19; df = 1, 6; P = 0.3164$) per colony (Table 3.3). Queen ($F=1.90; df = 1, 51; P = 0.1738$) and worker ($F=0.72; df = 1, 165; P=0.3988$) weight did not differ between treatments, but, males in diflubenzuron-treated colonies weighed significantly more than males in control colonies ($F =8.09; df = 1, 111; P = 0.0053$) (Table 3.3). Treatment also had no effect on the number of eggs ($F = 0.00; df = 1, 6; P = 0.9604$), larvae ($F = 0.10; df = 1, 6; P = 0.7662$) or pupae ($F =0.27; df = 1, 6; P = 0.6195$) (Table 3.3).
Table 3.3. Mean (± SE) number of eggs, larvae, pupae and adults (queens, workers and males) and mean weight of queens, workers and males in *Bombus impatiens* colonies after exposure to buckwheat plots treated with either 257 g a.i./ha of diflubenzuron (n=3) or water (n=5) for 7 d. After exposure, colonies were moved to an area of natural forage to continue their development for 29 d then in a growth chamber for 23 d and frozen and dissected.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment (mg a.i./L, n)</th>
<th>Control (0, n=5)</th>
<th>Diflubenzuron (257, n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. eggs</td>
<td></td>
<td>10.6 ± 7.95</td>
<td>0 ± 0.00</td>
</tr>
<tr>
<td>No. larvae</td>
<td></td>
<td>81.4 ± 20.40</td>
<td>44.0 ± 14.47</td>
</tr>
<tr>
<td>No. worker or male pupae</td>
<td></td>
<td>35.8 ± 8.05</td>
<td>43.3 ± 12.46</td>
</tr>
<tr>
<td>No. queen pupae</td>
<td></td>
<td>2.8 ± 2.77</td>
<td>0.67 ± 0.95</td>
</tr>
<tr>
<td>No. queens</td>
<td></td>
<td>6.4 ± 4.24</td>
<td>13.0 ± 10.91</td>
</tr>
<tr>
<td>No. males</td>
<td></td>
<td>30.4 ± 16.69</td>
<td>80.7 ± 26.80</td>
</tr>
<tr>
<td>No. workers</td>
<td></td>
<td>62.4 ± 16.42</td>
<td>73.0 ± 24.72</td>
</tr>
<tr>
<td>Queen weight (g)</td>
<td></td>
<td>0.5181 ± 0.01631</td>
<td>0.5484 ± 0.01793</td>
</tr>
<tr>
<td>Male weight* (g)</td>
<td></td>
<td>0.1521 ± 0.00469</td>
<td>0.1724 ± 0.00536</td>
</tr>
<tr>
<td>Worker weight (g)</td>
<td></td>
<td>0.1665 ± 0.00537</td>
<td>0.1741 ± 0.00717</td>
</tr>
</tbody>
</table>

*Males in diflubenzuron colonies weighed significantly more than control males (α=0.05)*
3.4.2 Micro-colony Experiment

There was no significant difference in number of days to oviposition \( (F = 1.93; \, df = 4, \, 44; \, P = 0.1224) \), number of males produced \( (F = 1.32; \, df = 4, \, 44; \, P = 0.2782) \) or male weight \( (F = 0.81; \, df = 4, \, 11; \, P = 0.5439) \) between treatments (Table 3.5). Additionally, treatment had no effect on the number of workers that died \( (F = 0.50; \, df = 4, \, 45; \, P = 0.7328) \) (Table 3.4). Treatment also had no effect on number of eggs \( (F = 1.40; \, df = 4, \, 29; \, P = 0.2602) \), larvae \( (F = 1.02; \, df = 4, \, 29; \, P = 0.4116) \) or pupae \( (F = 0.57; \, df = 4, \, 29; \, P = 0.6865) \) produced (Table 3.4). However, treatment did result in significantly greater honey-water solution consumption per day \( (F = 14.67; \, df = 4, \, 641; \, P < 0.0001) \). Micro-colonies treated with tebufenozide at 14.1 ppm and novaluron at 3.6 ppm consumed significantly more honey-water solution per day than the control, tebufenozide at 1.1 ppm and novaluron at 35.6 ppm (Table 3.4).
Table 3.4. Mean (± SE) number of days to oviposition, males, worker deaths, eggs, larvae, pupae and mean male weights and mean amount of nectar consumption per day and number of worker deaths of two insect growth regulators on *Bombus impatiens* queenless micro-colonies. Micro-colonies were fed 50% v/v honey-water for 14 d that were treated with either novaluron (at 3.6 or 35.6 mg a.i./L) or tebufenozide (at 1.1 or 14.1 mg a.i./L). Control colonies were fed untreated honey-water solution.

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Control (0, n=6)</th>
<th>Novaluron (3.6, n=7)</th>
<th>Novaluron (35.6, n=7)</th>
<th>Tebufenozide (1.1, n=7)</th>
<th>Tebufenozide (14.1, n=7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days to first oviposition</td>
<td>5.8 ± 0.38</td>
<td>5.3 ± 0.35</td>
<td>5.3 ± 0.37</td>
<td>4.7 ± 0.31</td>
<td>5.9 ± 0.38</td>
</tr>
<tr>
<td>No. males</td>
<td>1.7 ± 0.97</td>
<td>2.7 ± 1.38</td>
<td>0.1 ± 0.16</td>
<td>1.9 ± 0.98</td>
<td>1.7 ± 0.91</td>
</tr>
<tr>
<td>No. worker deaths</td>
<td>1.8 ± 0.55</td>
<td>1.0 ± 0.37</td>
<td>1.4 ± 0.46</td>
<td>1.8 ± 0.55</td>
<td>1.4 ± 0.46</td>
</tr>
<tr>
<td>No. eggs</td>
<td>0.3 ± 0.31</td>
<td>1.3 ± 0.84</td>
<td>3.6 ± 2.12</td>
<td>3.0 ± 1.80</td>
<td>1.9 ± 1.16</td>
</tr>
<tr>
<td>No. larvae</td>
<td>2.3 ± 1.47</td>
<td>5.9 ± 3.23</td>
<td>1.6 ± 0.96</td>
<td>2.1 ± 1.26</td>
<td>5.3 ± 2.93</td>
</tr>
<tr>
<td>No. pupae</td>
<td>0.8 ± 0.55</td>
<td>1.0 ± 0.59</td>
<td>0.3 ± 0.24</td>
<td>0.9 ± 0.53</td>
<td>1.3 ± 0.73</td>
</tr>
<tr>
<td>Male weight (g)</td>
<td>0.141 ± 0.0090</td>
<td>0.123 ± 0.0078</td>
<td>0.131 ± 0.0156</td>
<td>0.141 ± 0.0078</td>
<td>0.134 ± 0.0078</td>
</tr>
<tr>
<td>Nectar consumed/day (g)*</td>
<td>2.09 ± 0.063</td>
<td>2.33 ± 0.058</td>
<td>1.99 ± 0.058</td>
<td>1.87 ± 0.058</td>
<td>2.39 ± 0.058</td>
</tr>
</tbody>
</table>

*Micro-colonies exposed to tebufenozide at 14.1 mg a.i./L or novaluron at 3.6 mg a.i./L consumed significantly more honey-deionized water solution per day than all other treatments.*
3.4.3 2017 Field Results

Treatment had no effect on colony weight prior to insecticide treatment ($F = 0.21; df = 3, 72; P = 0.8833$) (Table 3.5). The effect of day was significant ($F = 119.68; df = 1, 72; P < 0.0001$); however, the interaction of treatment × day was not significant ($F = 0.02; df = 3, 72; P = 0.9969$) indicating that mean treatment weights on the same day were not significantly different from each other (Table 3.5).

Prior to dimethoate application, foraging activity was consistent across treatments (Figure 3.7). However, 24 h after insecticide application, no foragers were observed in D40, D80 or D200 plots. The lack of foraging resulted in inspection of all colonies treated with dimethoate. Upon inspection, it was determined that 1 of the D40 colonies was dead (queen dead or absconded, workers dead or absent), and the other 9 were severely affected (queen and workers erratically walking around and very few workers present within colony). Additionally, 9 colonies from D80 plots and 9 from D200 plots were dead. After 48 h, 6 more colonies in D40 plots had died, and 72 h after dimethoate application, all colonies treated with dimethoate died (Figure 3.6). The four D80-2 colonies had low foraging activity compared to the controls (Figure 3.7) and upon inspection of the D80-2 colonies, there appeared to be relatively few workers present inside the colonies 7 days after the D80-2 colonies went into the D80 plots. Colony weights initially declined when on the buckwheat plots, but all colonies gained weight when they were returned to the lab (Figure 3.8).
Table 3.5. Mean (± SE) weight of *Bombus impatiens* colonies on the first day out in the buckwheat plots on Day 0 (10 July 2017) and on Day 4 (14 July 2017) prior to insecticide treatment application.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean (± SE) Colony Weight (g)</th>
<th>Day 0</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>579.2 ± 2.58</td>
<td>558.7 ± 2.58</td>
<td></td>
</tr>
<tr>
<td>Dimethoate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>579.5 ± 2.58</td>
<td>559.8 ± 2.58</td>
<td></td>
</tr>
<tr>
<td>80</td>
<td>577.8 ± 2.58</td>
<td>557.7 ± 2.58</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>577.9 ± 2.58</td>
<td>558.5 ± 2.58</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.6. Total number of *Bombus impatiens* colonies that were living (queen and workers alive) 1, 2 and 3 d after dimethoate treatments (40, 80 and 200 g a.i./h) were applied to flowering buckwheat plots (n=10).
Figure 3.7. Mean (± SE) number of active *Bombus impatiens* foragers (A) and mean number of foragers entering and exiting the colony entrance (B) in buckwheat plots treated with dimethoate applied at 40 g a.i./ha (D40, n=10); applied at 80 g a.i./ha (D80, n=10); applied at 200 g a.i./ha (D200, n=10) and control buckwheat plots (n=10) per observation period per day (n=2) prior to dimethoate application. The solid vertical line depicts when plots were sprayed with insecticide on day 7. On day 8, all colonies exposed to plots treated with dimethoate at 80 g a.i./ha (n=10) and 200 g a.i./ha (n=10) were dead and 4 unallocated colonies were placed inside D80 plots (D80-2).
Figure 3.8. Mean (± SE) colony weights while foraging in the summer of 2017 over the course of the 70 d development season for control colonies (n=10) and dimethoate treated colonies at 40 g a.i./h (n=10), 80 g a.i./h (n=10) and 200 g a.i./h (n=10). The solid vertical line at day 7 depicts when plots were sprayed with insecticide treatments. On day 8, all colonies exposed to buckwheat plots treated with dimethoate at 80 g a.i./h (D80; n=10) or 200 g a.i./h (D200; n=10) were dead and 4 unallocated colonies were placed inside D80 plots where colonies had died (D80-2). By day 10 all colonies exposed to buckwheat plots treated with dimethoate at 40 g a.i./h (D40) died. Colonies were brought back to the University of Guelph on day 18 (vertical dashed line).

Dissection

Mean number of immature stages (eggs, larvae and pupae) and adults (queens, workers and males) (Table 3.6) and mean weights of adult stages are reported for the control colonies and the D80-2 colonies (Table 3.6). No statistical analyses were performed because the D80-2 colonies were put into the plots one week after the
control colonies so variation between endpoints are confounded by time of placement and cannot be attributed to differences in treatment.

Table 3.6. Mean (± SE) number of eggs, larvae, pupae, queen, males and workers and mean weight of queens, workers and males for control (n=10) and dimethoate 80-2 (n=4) *Bombus impatiens* colonies. Twenty-four h after dimethoate treatments were applied to buckwheat plots, 9 colonies in buckwheat plots treated with 80 g a.i./ha (D80) of dimethoate died. Unallocated colonies were placed inside D80 buckwheat plots (dimethoate 80-2).

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Treatment (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n=10)</td>
</tr>
<tr>
<td>No. eggs</td>
<td>22.3 ± 3.31</td>
</tr>
<tr>
<td>No. larvae</td>
<td>87.0 ± 10.62</td>
</tr>
<tr>
<td>No. worker or male pupae</td>
<td>61.9 ± 4.39</td>
</tr>
<tr>
<td>No. queen pupae</td>
<td>3.0 ±1.62</td>
</tr>
<tr>
<td>No. queens</td>
<td>9.1 ± 3.82</td>
</tr>
<tr>
<td>No. males</td>
<td>50.0 ± 11.21</td>
</tr>
<tr>
<td>No. workers</td>
<td>131.7 ± 13.97</td>
</tr>
<tr>
<td>Queen weight (g)</td>
<td>0.6108 ± 0.01477</td>
</tr>
<tr>
<td>Male weight (g)</td>
<td>0.1374 ± 0.00261</td>
</tr>
<tr>
<td>Worker weight (g)</td>
<td>0.1792 ± 0.00341</td>
</tr>
</tbody>
</table>
3.5 Discussion

Finding a suitable toxic standard for use in semi-field pesticide toxicity studies involving *Bombus impatiens* has proved to be challenging. In 2016, I applied dimethoate at 400 g a.i./ha, a rate that was used in Europe with *B. terrestris* in a semi-field study. In this study, dimethoate was applied while *B. terrestris* workers were actively foraging and exposed colonies experienced sublethal effects, including a higher number of dead larvae and fewer workers compared to the control colonies (Almanza et al. 2015). Conversely, in my study, *B. impatiens* colonies exposed to dried residues of dimethoate applied at the same rate, died within 24 h after application. The rate of 400 g a.i./ha was acutely toxic to the bees. My findings suggest that *B. impatiens* is more susceptible to dimethoate than *B. terrestris* under semi-field conditions.

Diflubenzuron, applied at 257 g a.i./ha, had no effect on foraging activity for *B. impatiens* which was also true for *B. terrestris* foraging activity when exposed to diflubenzuron at 80 g a.i./ha (Almanza et al. 2015). Although diflubenzuron did not adversely affect the number of immature stages, males, workers and queens produced in *B. impatiens* colonies, these colonies did produce significantly larger males (Table 3.4). It could be that diflubenzuron elicits a hormetic effect, resulting in increased male weights. For instance, some insecticides can increase the number of males produced by *B. impatiens* (Ramanaidu and Cutler 2012; Cutler 2013; Cutler and Rix 2015). It is also possible that this increase in male weight is the result of random chance. Random chance seems more plausible than hormesis since brood development in *B. terrestris* colonies exposed to diflubenzuron treated *Phacelia tanacetifolia* was severely affected, and in some cases resulted in complete brood loss (Gretenkord and Drescher 1996;
Almanza et al. 2015). However, because *B. impatiens* may differ from *B. terrestris* in susceptibility to diflubenzuron, more research is needed to elucidate my findings.

During my IGR screening using *B. impatiens* micro-colonies, I found that both novaluron and tebufenozide had no effect on the number of males produced. Similar to my results, tebufenozide also does not appear to affect the number of males produced in *B. terrestris* micro-colonies (Mommaerts et al. 2006b) or *A. mellifera* queen development (Thompson et al. 2005). Because tebufenozide specifically binds to target ecdysone receptors found in Lepidopteran pests, it does not appear to interfere in the brood development of non-targeted insects, such as bumble bees (Mommaerts et al. 2006b). I found novaluron to have no effect on male reproduction in *B. impatiens* queenless micro-colonies. Similarly, Malone et al. (2007) reported that *B. terrestris* micro-colonies were unaffected when exposed to novaluron. Conversely, other studies have found that novaluron can adversely affect brood development in honey bees (Fine et al. 2017), in alfalfa leafcutter bees (*Megachile rotundata*) (Hodgson et al. 2010; Pitts-Singer and Barbour 2017) and in *B. terrestris* (Mommaerts et al. 2006b). Mommaerts et al. (2006a) found *B. terrestris* micro-colonies exposed to novaluron (40 mg a.i./L) produced significantly fewer males than control colonies. Interestingly, in my study, the *B. impatiens* micro-colonies treated with novaluron at 35.6 mg a.i./L produced the fewest number of males, but the effect was not significant. It is possible that the loss of 3-4 replicates per treatment because 2 or more workers died, may have lowered the statistical power in my study. Therefore, novaluron may adversely affect *B. impatiens*; however, further research with novaluron is required to clarify my findings. Although novaluron and tebufenozide had no effect on number of males produced, those micro-
colonies exposed to novaluron at 3.6 mg a.i./L and tebufenozide at 14.1 mg a.i./L consumed significantly more honey-water solution suggesting a potential hormetic effect. Hormesis is a biphasic dose-response that occurs when low doses of pesticides trigger a biological response (Cutler and Rix 2015). For example, increased frequency in *B. terrestris* foraging was observed when exposed to low doses of the neonicotinoid, thiamethoxam (Stanley and Raine 2016). Similarly, *B. impatiens* micro-colonies produced significantly more males when workers were topically treated with low concentrations of *Bacillus subtilis* (Ramanaidu and Cutler 2012). It is plausible that some characteristic of both novaluron and tebufenozide is causing the bees to consume more honey-water solution, but more research is recommended to confirm these findings.

Colony weight was unaffected by treatment, but in both years, all colonies lost weight while in the screened enclosures in the buckwheat plots. The loss of weight was not indicative of declining colony health, as there was no evidence of dead workers or brood inside the colonies prior to insecticide treatment. It is possible that colony weight loss resulted from the consumption of stored resources and increased foraging activity. Prior to placement in the field, colonies were provided with pollen and able to feed on the Bioglucc solution *ad libitum*. However, once in the field, these unlimited food resources were removed to encourage foraging. Therefore, it is likely that colonies consumed their stored resources while acclimating to their new surroundings, which would contribute to colony weight decline. In addition, as time in the buckwheat plots progressed, foraging activity inside the screened enclosures increased, and, because colonies were weighed while foragers were active, it is possible that fewer workers were
present inside the colonies at the time of weighing, contributing to the colony weight decrease. Similarly, in a study by Gradish et al. (2016), they determined that *B. impatiens* colonies restricted to foraging in screened enclosures (the same enclosure I used for my studies) containing buckwheat, purple tansy or red clover, initially lost weight. Conversely, in other semi-field studies with *B. impatiens*, colonies did not lose weight likely because they were allowed to feed *ad libitum* on sucrose solution (Larson et al. 2013) or were supplemented with pollen (Gels et al. 2002). In my study *B. impatiens* colonies did not have *ad libitum* access to their Biogluc feeders which may have account for the difference in colony weights between my study and others.

Initial recommendations for semi-field method development with bumble bees made by Cabrera et al. (2015) suggest terminating colonies after two weekly reductions in weight. In my study, when in the area of natural forage in 2016, colonies from diflubenzuron and in control colonies unexpectedly lost and gained weight (Figure 3.5) making it unreliable to use two weekly reductions in colony weight as a termination point. Perhaps a more reliable termination point is the production of new queens (Gradish et al. 2016). Colonies in my study were terminated 2 weeks after the emergence of the first queen, which was 8 weeks after the colonies were moved off the buckwheat plots. Similarly, in Europe, the ICPPR suggested removing new queens twice weekly and freezing colonies 21 d after the first queen pupae are detected (ICPPR unpublished data, 2017). In contrast to my study and Gradish et al. (2016), the ICPPR recommend freezing colonies that do not produce new queens when the last control hive is frozen. This termination point fails to consider the scenario where a control colony produces no queens, which can occur in *B. impatiens* colonies.
Since there are differences between \textit{B. impatiens} and \textit{B. terrestris} in reproductive outputs and pesticide sensitivity, separate North American semi-field regulatory guidelines may be needed for use with \textit{B. impatiens}.

After I moved the colonies to the area of natural forage, several concerns arose, including the presence of other bumble bee species and the presence of ants inside colonies. Also concerning was the presence of wild \textit{B. impatiens} queens found inside 10 of 12 colonies that had died early. Invading \textit{B. impatiens} queens were identified because before their appearance, none of the colonies contained queen pupae. This intraspecific usurping behaviour can occur because \textit{B. impatiens} queens vary in their emergence from hibernation, and those queens that emerge later may find suitable nesting sites already occupied and take up residency over an existing nest by usurping the foundress queen (Kearns and Thomson 2001; Velthuis and van Doorn 2006; Goulson 2010). Other bumble bee species (\textit{B. citrinus} and \textit{B. vagans}) were also found inside some of the \textit{B. impatiens} colonies. This interspecific usurping behaviour is a form of social parasitism (Goulson 2010). Dead female lemon cuckoo bumble bees, \textit{B. citrinus}, were found inside 2 diflubenzuron colonies, presumably in their attempt to usurp the queen. This obligate social parasite has various \textit{Bombus} host species, including \textit{B. impatiens}, and will try to kill the foundress queen and some workers (Goulson 2010; Williams et al. 2014). If successful, the \textit{B. citrinus} female will then lay her own eggs after destroying all the foundress queen’s eggs and will use the \textit{B. impatiens} workers to rear her brood (Goulson 2010; Williams et al. 2014). The presence of the \textit{B. vagans} female can be explained through social parasitism or through
accidental drift into the nest (Goulson 2010). To date, there is no evidence that *B. vagans* workers enter other bumble bee species nests to lay eggs; however, this behaviour has been seen with *B. terrestris*. Aggressive *B. terrestris* workers are known to enter unrelated *B. terrestris* nests to lay eggs and will occasionally try and usurp the colony of *B. lucorum* (Goulson 2010). It is more likely *B. vagans* accidentally drifted into the *B. impatiens* colony but more research on this matter is needed to confirm.

The presence of intruding bumble bee species was not the only biological stressor on the *B. impatiens* colonies. Ants (*Crematogaster cerasi* Fitch) caused the death of 8 colonies. Interestingly, bumble bee colonies have previously been kept in the same area of natural forage in Meaford without ant infestation (Cutler and Scott-Dupree 2014). In 2016, *B. impatiens* colonies likely happened to be placed near an ant nest (Umphrey, personal comm. August 8, 2016). Since many ant species, including *C. cerasi*, are opportunistic foragers, bumble bee nests full of protein rich bee larvae and eggs, make for easy prey (Young 1979; Ramirez and Cameron 2003). Future studies with commercial bumble bee species should mitigate ant invasions by creating a barrier around the housing structure.

My goal in 2017 was to find a rate to apply dimethoate that would elicit an observable adverse effect on *B. impatiens* colonies without causing total colony death. Unfortunately, even when I applied dimethoate at 1/2, 1/5 and 1/10 the rate used in 2016, all colonies exposed to dimethoate died within 72 h. For anecdotal purposes, I placed four extra colonies inside D80 plots but observed that, even after 7 d, these D80-

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2 colonies had low foraging activity and found these D80-2 colonies had relatively few workers present inside the colonies. Originally, these extra colonies were excluded from use in my experiment because they contained adult bumble bee wax moths (A. sociella). When bumble bee wax moth larvae emerge, they chew through wax, food reserves and bumble bee brood, which weakens colony health (Goulson 2010). It is possible the D80-2 colonies were already weakened by the bumble bee wax moth larvae making them more sensitive to dimethoate residues. It is also possible that dimethoate residues on the buckwheat were still toxic 7 d after application, regardless of initial colony condition.

Dimethoate resulted in the loss of entire treatment groups in both micro-colonies and semi-field experiments indicating that B. impatiens is highly sensitive to dimethoate. In contrast, all other insecticides that were tested – novaluron and tebufenozide in micro-colonies and diflubenzuron in 2016 semi-field study – did not cause observable adverse effects with B. impatiens. If dimethoate is to be the toxic standard for use in pesticide risk assessments with B. impatiens, an appropriate, lower rate must be found before it can be recommended. In summary, the effects of these four insecticides on B. impatiens micro-colonies and queenright colonies has created a path that future research can follow which will help clarify the findings of my study.
Chapter 4

General Discussion and Conclusions

Over the past 10 years, some North American species of bumble bee have declined (Potts et al. 2010; Cameron et al. 2011). Several factors, including exposure to pesticides, may contribute to bumble bee declines (Brittain et al. 2010; Szabo et al. 2012; Vanbergen et al. 2013). Bumble bees, like honey bees, can be exposed to pesticides by contact with spray droplets while foraging, through contact with residues on plants, or through the consumption of contaminated nectar and pollen from a treated crop.

Unlike honey bee colonies that last for multiple years and contain 10,000 – 50,000 individuals, bumble bee colonies are annual and only produce between 50 – 200 workers (Caron 1999; Thompson and Hunt 1999; Williams et al. 2014; Stoner 2016). Therefore, the loss of a few workers from pesticide exposure is likely to have a larger impact on a bumble bee colony. Additionally, future bumble bee colonies depend on the number of mated queens that overwinter and successfully establish colonies the following spring, whereas honey bee colonies reproduce through swarming (Caron 1999; Thompson and Hunt 1999; Williams et al. 2014). Thus, the death of a bumble bee queen that emerges in the spring is essentially the loss of a potential colony. Finally, those queens that do establish colonies depend on the first group of workers that emerge to bring back nectar and pollen until more bumble bee brood emerge. Consequently, young bumble bee colonies are likely to be more vulnerable to pesticide exposure. To date, honey bees are used to estimate the risk of pesticides to all bees for pesticide registration and re-registration, but, because of differences in life history and...
behaviour, honey bees and bumble bees may differ in their susceptibility to pesticides. Because of this potential difference, regulatory agencies in Europe and North America will soon require pesticide risk assessments for bumble bees for pesticide registration and reregistration.

To incorporate bumble bees into the regulatory process, standardized and validated methods must be developed for them. Recently, acute contact and acute oral laboratory protocols (tier I) were released for use with the proposed North American surrogate bumble bee, *B. impatiens*, and the proposed European surrogate bumble bee, *B. terrestris* (OECD 2017a, b; Cabrera et al. 2015), and semi-field (tier II) methods are being developed for use in Europe with *B. terrestris*. Currently, dimethoate is the recommended toxic standard in the OECD laboratory guidelines (2017) and in the semi-field protocols being drafted for *B. terrestris* (ICPPR unpublished data, 2017). To use dimethoate as a toxic standard with *B. impatiens* in laboratory studies, we must determine the acute contact and oral median lethal dose (LD$_{50}$), and, similarly, in semi-field studies, we need to find a rate of dimethoate that elicits observable adverse effects in *B. impatiens* colonies.

When comparing the acute contact susceptibility of dimethoate of *A. mellifera* and *B. impatiens*, I found *B. impatiens* to be less susceptible (LD$_{50}$ 0.51 µg a.i./bee) than *A. mellifera* (LD$_{50}$ 0.10 µg a.i./bee). Although I was unable to generate an oral LD$_{50}$ for *A. mellifera*, the oral LD$_{50}$ of dimethoate for *B. impatiens* was 0.40 µg a.i./bee. However, based on dimethoate LD$_{50}$ values for *A. mellifera* in the literature (0.10-0.35 µg a.i/bee) (Gough et al. 1994), *B. impatiens* may be less susceptible to acute oral exposure to dimethoate than *A. mellifera*.
To find a suitable toxic standard for use in semi-field pesticide toxicity studies with *B. impatiens*, I first evaluated the effects of diflubenzuron and dimethoate when applied to buckwheat plots. Diflubenzuron was applied at its maximum label rate of 257 g a.i./ha, and dimethoate was applied at a rate that was successfully used with *B. terrestris* in Germany, 400 g a.i./ha. I found diflubenzuron-treated colonies did not differ from control colonies in foraging activity, weight, or number of queens, workers or males produced. In contrast, all colonies from dimethoate-treated buckwheat plots were dead within 24 h of application. Based on the 2016 results, I wanted to see if two other IGRs, novaluron and tebufenozide, and lower doses of dimethoate, could be used as potential toxic standards for the 2017 field season. To screen for their potential suitability as toxic standards, I set up queenless *B. impatiens* micro-colonies and exposed them to dimethoate at 129.5 or 12.9 mg a.i./L, tebufenozide at 14.1 or 1.4 mg a.i./L and novaluron 35.6 or 3.6 mg a.i./L. All micro-colonies treated with dimethoate died within 24 h of exposure, but novaluron and tebufenozide had no effect on worker survival or the number of males produced. Interestingly, micro-colonies treated with 1.4 mg a.i./L of tebufenozide or 3.6 mg a.i./L of novaluron consumed significantly more honey-water solution per day, suggesting a potential hormetic effect. Based on my 2016 semi-field results and my micro-colony results, I decided to focus on dimethoate in my 2017 field season. I applied three different rates of dimethoate, 200, 80 and 40 g a.i./ha, to buckwheat plots but within 72 h after dimethoate application, all *B. impatiens* colonies, except the control colonies, had died.

To generate an acute contact and oral LD\(_{50}\) of dimethoate for *B. impatiens*, I used ring testing protocols that were also used with *B. terrestris* (OECD unpublished
The oral LD$_{50}$ of dimethoate for *B. impatiens* generated in my study (0.41 µg a.i./bee) was within the range of the oral LD$_{50}$ for *B. terrestris* (0.38 – 1.7 µg a.i./bee) (Schaefer et al. 1996; van der Steen 1999; Marletto et al. 2003; Heard et al. 2017), suggesting a similar susceptibility of both species to dimethoate when consumed. In addition to dimethoate, *B. terrestris* and *B. impatiens* may also have a similar oral susceptibility to deltamethrin (*B. impatiens*: LD$_{50}$ 0.825 µg a.i./bee after 48 h vs. *B. terrestris*: LD$_{50}$ 0.6 µg a.i./bee after 24 h) and spinoteram (*B. impatiens*: LD$_{50}$ 17.7 mg a.i./L after 48 h vs. *B. terrestris*: LD$_{50}$ 20.8 mg a.i./L after 72 h), suggesting that these species are similar in their susceptibility to some pesticides if consumed (Tsai et al. 1994; Besard et al. 2011; Gradish et al. 2012). However, the acute contact dose range from the ring-test protocols were too high to generate a LD$_{50}$ for dimethoate on *B. impatiens*, as the lowest dose of 1.25 µg a.i./bee caused 76% mortality. I found the acute contact LD$_{50}$ for *B. impatiens* to be 0.51 µg a.i./bee, lower than the acute contact LD$_{50}$ range for *B. terrestris* of 1.25 - 10 µg a.i./bee (van der Steen 1999; Marletto et al. 2003). This difference suggests that *B. impatiens* may be more susceptible to dimethoate following acute contact exposure than *B. terrestris*. There is other evidence to suggest that differences in pesticide susceptibility between bumble bee species exist. For example, *B. hypocrita*, *B. ignitus* and *B. patagiatus* differed in their susceptibility to acetamiprid (an insecticide) and copper abietate (a fungicide): *B. hypocrita* was more susceptible to both pesticides following contact exposure than both *B. ignitus* and *B. patagiatus* suggesting that pesticides can differ in their effects on different bumble bee species (Wu et al. 2011).
The notion that pesticides may differ in their effects on various bumble bee species has implications for developing guidelines in assessing pesticide risk to *B. impatiens*. For example, recently released OECD acute contact protocols (2017a) suggest applying dimethoate at 10 µg a.i./bee to achieve a mortality of ≥ 50% (OECD 2017a), but, based on the acute contact results from my study, this dose would likely result in 100% mortality if applied to *B. impatiens* workers. Therefore, a lower recommended dose of dimethoate may be required for use with *B. impatiens* in acute topical exposure tests. However, because there is no other data on the LD$_{50}$ of dimethoate for *B. impatiens*, more research is needed to verify my findings.

My research from 2016 and 2017 provides further evidence that, as with acute contact exposure, *B. impatiens* may also be more susceptible to dimethoate under semi-field conditions than *B. terrestris*. For instance, the rate of dimethoate I used in 2016 was based on results obtained from a study in Monheim, Germany with *B. terrestris*. When dimethoate was applied at 400 g a.i./ha to purple tansy in semi-field tunnels containing actively foraging *B. terrestris* on, colonies produced fewer workers and larvae, but all colonies survived (Almanza et al. 2014). Furthermore, the ICPPR (unpublished data, 2017) tier II recommendation is to apply dimethoate at 800 g a.i./ha as the toxic standard in semi-field pesticide risk assessments with *B. terrestris* in Europe, again using purple tansy as the surrogate plant. Since 100% of the *B. impatiens* colonies exposed to 40 g a.i./ha died in my study, it is unlikely that the recommended rate of 800 g a.i./ha of dimethoate will be suitable for use with *B. impatiens* in North America. Therefore, if dimethoate is to be used as the toxic standard to assess pesticide risk to *B. impatiens* under semi-field conditions, a lower rate must be
found. In my study, buckwheat was selected as the surrogate flowering plant instead of purple tansy (recommended for use with *B. terrestris*) (ICPPR unpublished data, 2017). Although purple tansy tends to produce pollen of comparatively higher nutritional quality (Pernal and Currie 2000) than buckwheat (Sommerville 2001), Gradish et al. (2016) found no difference in *B. impatiens* colony development. Importantly, Gradish et al. (2016) found buckwheat to elicit significantly higher foraging activity in *B. impatiens* colonies when compared to purple tansy. Thus, I used buckwheat in both years to ensure adequate exposure to diflubenzuron and dimethoate.

It is possible that after further research, dimethoate may not be a suitable toxic standard for use with *B. impatiens* under semi-field conditions and another toxic standard must be identified. One way to identify a different toxic standard involves generating a list of acute oral and contact LD$_{50}$s of pesticides for *B. impatiens*. Because there are many acute pesticide toxicity studies with *B. terrestris*, future studies should use pesticides that have previously been tested with *B. terrestris* to start generating LD$_{50}$ data with *B. impatiens*. Having a comprehensive list of the acute oral and contact LD$_{50}$ data for pesticides on both *B. terrestris* and *B. impatiens* will further clarify any species differences in their pesticide susceptibility and provide much needed information on the sensitivity of *B. impatiens* to pesticides. Based on the LD$_{50}$ results with *B. impatiens*, those insecticides that have a high acute toxicity could then be further screened using queenless micro-colonies. Micro-colonies should be exposed to concentrations they might encounter in the field, which can be estimated using the EPA residue calculation (110ppm pesticide residue = 1 lb/acre of active ingredient applied) (USEPA 2014). Using a combination of acute contact and oral tests followed by micro-
colony bioassays should hopefully identify a few insecticides that have the potential to be used as toxic standards under semi-field conditions. Another way to identify a potential toxic standard for use in semi-field toxicity studies with *B. impatiens*, would be to investigate those pesticides that have reportedly caused adverse affects at the colony level (i.e. reduction in colony weight, reduced number of new queens or males, death of the foundress queen, reduced foraging) with either *B. terrestris* or *B. impatiens*. To identify any potential differences in sensitivity between species, I recommend those pesticides that have reportedly caused adverse affects at the colony level with *B. terrestris*, be screened with *B. impatiens* micro-colonies, prior to use in the field. For example, *B. terrestris* colonies that consumed λ-cyhalothrin treated sucrose solution (3.75 ppb) for two weeks had increased worker mortality and resulted in foundress queen death (Ceuppens et al. 2015), suggesting λ-cyhalothrin may be highly toxic to *B. terrestris* colonies when consumed. However, because there are potential differences in pesticide susceptibility between *B. terrestris* and *B. impatiens*, the sensitivity of *B. impatiens* colonies to λ-cyhalothrin should be screened using micro-colony bioassays. In contrast, if there is clear evidence of a pesticide causing adverse effects on *B. impatiens* colonies, it may be possible to forgo the micro-colony screening process. For example, *B. impatiens* colonies placed in enclosures containing white clover treated with either chlorpyrifos applied at 1.12 kg a.i./ha, carbaryl applied at 6.10 kg a.i./ha or cyfluthrin applied at 0.77 kg a.i./ha weighed less and had fewer workers, fewer number of honey pots and fewer brood chambers than the controls (Gels et al. 2002). Since chlorpyrifos, carbaryl and cyfluthrin caused adverse affects on *B. impatiens* colonies under semi-field conditions without killing the foundress queens, workers and brood, it is
possible that any of these three insecticides have the potential to be used as a toxic standard in semi-field pesticide toxicity testing with *B. impatiens*. Evidently, identifying a toxic standard for use in semi-field toxicity studies with *B. impatiens* is an ongoing process that will require further investigation.

In contrast to the tunnel design (around 40 m²) recommended for use with *B. terrestris* (ICPPR unpublished data, 2017) and currently used for *A. mellifera* (EPPO 2010), I used a smaller tent design (3.5 m²) in my semi-field pesticide toxicity studies with *B. impatiens*. Using smaller tents as opposed to larger, more permanent tunnels, equates to greater mobility in experimental set-up, as the tents can be easily collapsed and moved to various locations. Additionally, using smaller tents allows for a larger number of replicates per treatment, which strengthens the statistical power of the experiment (Bowley 2015). Although the small size of the tents can be beneficial, the tent design could stress colonies if there is reduced resources available upon which to forage. However, over both years, there was no evidence of declining colony health while in the buckwheat plots. In contrast to the tents, using larger tunnels has the benefit of increased forage, effectively reducing potential colony stress; but, the use of tunnels often results in a lower number of replicates which can reduce statistical power. Overall, tunnels and tents have been shown to support bumble bee colony development and growth in pesticide toxicity semi-field studies (Larson et al. 2013; Almanza et al. 2015; Gradish et al. 2016). Moving forward, future studies may want to directly compare the tent design to the tunnel design for both *B. impatiens* and *B. terrestris* to determine if one design promotes better colony development and growth.
To find a toxic standard for use in semi-field studies with *B. impatiens*, future studies can continue to build off my findings by investigating lower rates of dimethoate (e.g. 1/2, 1/4 and 1/10 of 40 g a.i./ha). If dimethoate is deemed unsuitable as a toxic standard, other pesticides need to be investigated (e.g. chlorpyrifos, carbaryl, cyfluthrin and λ-cyhalothrin) to verify their potential suitability as toxic standards. The results from this study provide strong evidence that *B. impatiens* is more susceptible to dimethoate than *B. terrestris*. Understanding the difference in pesticide susceptibility between *B. impatiens* and *B. terrestris* will help regulators in North America and Europe make accurate and informed decisions on pesticide use and policy development which may mitigate further bumble bee loss.
References


**Dogterom, M.H. 1999.** *Pollination by Four Species of Bees on Highbush Blueberry.* PhD thesis, Simon Fraser University, Vancouver, BC.


Appendix A: Significant dates during *Bombus impatiens* semi-field studies in 2016 and 2017


<table>
<thead>
<tr>
<th>Event</th>
<th>2016</th>
<th>2017</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start Date (Bees out in Field)</td>
<td>June 21</td>
<td>July 10</td>
</tr>
<tr>
<td>End Date (Bees removed from Field)</td>
<td>July 6</td>
<td>July 25</td>
</tr>
<tr>
<td>Dates of Colony Weights</td>
<td>June 21, 23, 29</td>
<td>July 10, 13, 19, 21, 25</td>
</tr>
<tr>
<td></td>
<td>July 1, 4, 6</td>
<td></td>
</tr>
<tr>
<td>Forager Activity Observation Dates</td>
<td>June 22, 24, 28, 29</td>
<td>July 11, 14, 18, 19, 21, 24</td>
</tr>
<tr>
<td></td>
<td>July 1, 4, 6</td>
<td></td>
</tr>
<tr>
<td>Spray Application Date</td>
<td>June 27</td>
<td>July 17</td>
</tr>
<tr>
<td>Treatments</td>
<td>Diflubenzuron at 257 g a.i./ha</td>
<td>Dimethoate: 40, 80 and 200 g a.i./ha</td>
</tr>
<tr>
<td></td>
<td>Dimethoate at 400 g a.i./ha</td>
<td></td>
</tr>
<tr>
<td>Residue Sample Collection Dates</td>
<td>June 28 and July 6</td>
<td>July 18 and 24</td>
</tr>
<tr>
<td>Moved to Area of Natural Forage</td>
<td>July 7</td>
<td>N/A</td>
</tr>
<tr>
<td>Moved to University of Guelph</td>
<td>August 5</td>
<td>July 25</td>
</tr>
<tr>
<td>End Date (8 weeks after colonies</td>
<td>August 30</td>
<td>September 18</td>
</tr>
<tr>
<td>removed from buckwheat plots)</td>
<td></td>
<td></td>
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