PROTECTING POLLINATION IN A CHANGING WORLD: METHOD DEVELOPMENT FOR COMMERCIAL POLLINATOR PROVISION AND ASSESSING THE IMPACTS OF CLIMATE CHANGE AND NEONICOTINOIDS ON POLLINATORS

by

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Summary

Pollinators are essential to food security but are under threat because of multiple factors including climate change, habitat loss, emerging infectious diseases and pesticide use. Commercial pollinator provision can support threatened pollination services, however, only a few species are currently available. This study assessed the viability of mass-rearing red mason bees (*Osmia bicornis*) within a lab and greenhouse setting, as well as methods to manipulate the diapausing stage, and thus extend when this species is available to growers. Reproductive rates were too low to be commercially viable, but diapause was successfully extended using fluctuating thermal regimes and could be terminated early using chemical treatment. Climate change was shown to potentially have significant effects on red mason bees, with spring warming likely to advance adult emergence, and delayed winter cooling decreasing survival. Neonicotinoid pesticides impact was then assessed in three species: red mason bees, pollinating flies (*Calliphora vicina*) and buff-tailed bumblebees (*Bombus terrestris*). Sub-lethal, field-relevant, doses had a significant impact on thermal activity thresholds of all species, with buff-tailed bumblebees the most vulnerable. Indeed, RNAseq data in this species highlighted disruption of several core processes linked to thermal adaptation. The implications on pollinator phenology, survival, as well as food security are discussed.

Dedication

Maki,

Ništa od ovoga ne bi bilo moguće bez tebe. Bez tebe, nikada se ne bih ni usudio na to do ostavim sve, preselim se u London, i probam da završim svoj doktorat. Nikada ne bih imao dovoljno samopouzdanja i svakako ne bih uspeo. Ti si bila moja stena i moj oslonac kroz sve ovo. Kroz sve moje suze kasne večeri. Čak i kroz – da budemo iskreni – sve moje ispade. Bez tebe, sve bih to odavno napustio i verovatno počeo da živim na drvetu. Ali ti si uvek bila tu da me čvrsto držiš za ruku, uliješ mi samopouzdanje i rešenost da nastavim, i da učiniš da siđem sa svog drveta.

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List of abbreviations used in this thesis

ACE	Acetamiprid
AChRs	Neuronal acetylcholine receptors
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATP	Adenosine-triphosphate
Atp2a1	calcium transporting ATPase sarcoplasmic/endoplasmic reticulum type
BBF	Blue blowfly
BH	Benjamini–Hochberg
BLAST	Basic Local Alignment Search Tool
BTB	Buff-tailed bumblebee
BWARS	Bees, Wasps and Ants Recording Society
CLO	Clothianidin
CNS	Central Nervous System
CT_{max}	Critical thermal maximum
CT_{min}	Critical thermal minimum
cyp6a2	cytochrome P450 6a2
cyp6k1	cytochrome P450 6k1
cyp9e2	cytochrome P450 9e2
DEFRA	Department for Environment Food and Rural Affairs
DEG	Differentially expressed gene
DF	Degrees of Freedom
EFSA	European Food Safety Authority

EPA	United States Environmental Protection Agency
EPRS	European Parliament Research Service
EU	European Union
FTR	Fluctuating Thermal Regime
h	hour
HSF	Heat shock factor
Hsp	Heat shock protein
IMI	Imidacloprid
InsP3R	inositol 1,4,5-trisphosphate receptor
IPCC	Intergovernmental Panel on Climate Change
IUCN	International Union for the Conservation of Nature
LD	Light:Dark
LD_{50}	Lethal Dose, 50%
LED	Light emitting diode
MACF1	Microtubule-actin cross-linking factor 1
MIDAS	Met Office Integrated Data Archive System
nAChR	Nicotinic acetylcholine receptor
NP	Neonicotinoid Pesticide
NRC	National Research Council of the National Academies
PAR	Photosynthetically active radiation
PC	Principal component
PCA	Principal component analysis
PHGPx	phospholipid-hydroperoxide glutathione peroxidase

RH	Relative Humidity
RMB	Red mason bee
RNA	Ribonucleic acid
RNAi	RNA interference
RNAseq	RNA-Sequencing
scbp1	sarcoplasmic calcium binding protein 1
sHsp	small heat shock protein
STAR	Spliced Transcripts Alignment to a Reference
THIC	Thiacloprid
THIM	Thiamethoxam
UK	United Kingdom
UN	United Nations
UNEP	United Nations Environment Programme
USA	United States of America
UV	Ultraviolet

Chapter 1: Introduction

1.1 The importance of pollination for food security

Pollination is the transfer of pollen from the male to the female reproductive organs of a flower, and for many plants, it is fundamental to their reproductive success (Potts et al., 2016). Many plants are wind pollinated, but 87.5 % of all flowering plant species (Angiosperms) are estimated to rely on animal pollination (Ollerton et al., 2011; Potts et al., 2016). Whilst there are some examples of vertebrate pollinators, the vast majority of pollination is performed by insects (Klein et al., 2007; Allsopp et al., 2008), which are therefore essential for ecosystem functioning (Thomann et al., 2013).

The reliance of many plants on insect pollinators means that an important component of food humans consume is also dependent on those same insects. Indeed, approximately 35% of global crop production (principally fruit and vegetables) is at least partially dependent on pollinators (Klein et al., 2007; Aizen et al., 2008). As a result, in the United States of America (USA) alone, pollination services have been valued at between £2.3 billion and £12.4 billion annually (Hanley et al., 2015), with worldwide estimates ranging between £121.8 – £285.4 billion (Pimentel et al., 1997; Gallai et al., 2009; Lautenbach et al., 2012). With the area of land dedicated to pollinator-dependent crops increasing, especially for oil crops and fruit trees (Aizen et al., 2008; Garibaldi et al., 2011), the need for pollination services is also increasing. However, despite their importance and

value to agriculture, pollinating insects are declining worldwide (Vanbergen et al., 2013; Potts et al., 2016).

The importance of insect pollinators is further emphasised by the pressing need to sustainably increase the amount of food produced worldwide and to ensure food security, which is defined by the United Nations (UN) as existing when “all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food to meet dietary needs for a productive and healthy life” (Shaw, 2007). It is estimated by 2050 there will be around 9.7 billion humans on the planet (UN, 2019) and so to provide all of these people with sufficient food, crop yields will likely have to be increased (Tilman et al., 2011). Past yield increases have relied upon chemical fertilisers and increased use of pesticides, which has caused environmental degradation (Tilman et al., 2002; Springmann et al., 2018). Increasing yields through achieving maximal pollination, on the other hand, may represent a way to partially meet food demands, in a more sustainable way (Garratt et al., 2018).

The challenging goal to ensure food security may be further complicated by the recent global pandemic caused by the severe acute respiratory syndrome coronavirus 2. Previous disease outbreaks, such as the 2014 Ebola epidemic, have disrupted access to food for many communities, due to slowing of trade and migration of people away from food growing areas (World Bank, 2014). Disease outbreaks can also cause economic shocks leading to food price volatility, which may reduce certain communities’ access to food (Kalkuhl et al., 2016). Such

concerns have led the chief economist of the Food and Agriculture Organization to warn of a global food crisis due to the current global pandemic (Torero, 2020).

For the United Kingdom (UK), its withdrawal from the European Union (EU) has been predicted to cause increases in the prices of many foods, which could lead to households in the UK experiencing food insecurity (Lang and McKee, 2018; Barons and Aspinall, 2020). Such predictions are complicated by the changing nature of the UK with the EU (Barons and Aspinall, 2020). How the withdrawal of the UK from the EU will interact with the current global pandemic remains to be seen, but due to the country's reliance on seasonal workers from the EU and the increase in food prices coupled with the economic and trade disruptions resulting from the pandemic, it's likely the UK will become even more food insecure (Petetin, 2020). Altogether, this emphasises the importance of pollination as a method to increase food yield and achieve food security in the UK.

1.2 Threats to pollination

The most important agricultural pollinators are bees (Anthophila) (Klein et al., 2007). However, according to the International Union for the Conservation of Nature's (IUCN) Red List in some EU countries approximately half of the bee species are threatened (Nieto et al., 2014). In particular, highly industrialised countries have suffered large losses. For example, in the Netherlands and the UK, many regions have experienced > 60 % decrease in bee species richness since 1980 (Biesmeijer et al., 2006). Additionally, in Great Britain, Belgium and the Netherlands, there has been a 20-40% reduction in bumblebee (*Bombus*

spp.) species richness between 1950-1989, and other bees' species richness have also declined across this period in Belgium and the Netherlands (Carvalheiro et al., 2013). Powney et al. (2019) also showed that 25% of 139 UK wild bee species have declined in distribution by ~ 20 % since 1980.

In the USA, research has indicated that there have been reductions in bee abundance by 23% in US land area between 2008 and 2013, concentrated most in areas of intensive agriculture (Koh et al., 2016). Declines of bees in the USA have been evidenced by Cameron et al. (2011) who showed that four species of bumblebee (*Bombus occidentalis*, *B. pensylvanicus*, *B. affinis*, and *B. terricola)* have experienced range contractions of between 23-87%, with a resultant loss of population abundance of up to 96% across the 20th century. Further analysis of 150 years of observational records in New Hampshire, showed declines in bumblebee (*B. affinis, B. fervidus, B. vagans and B. terricola*) abundance, with a 96.5 % reduction in the relative abundance of *B. affinis*, and with *B. terricola* range contractions restricting this species to high elevation ranges (Jacobson et al., 2018).

Evidence of declines in pollinating insects outside of North America and Europe is limited (Jamieson et al., 2019), but what information does exist suggests that losses are global (Potts et al., 2016). Moreover, analysis of pollinator-plant mutualisms has suggested that declines in pollinator species can reach a critical point, where they will quickly go extinct (Lever et al., 2014). Rhodes (2018) has argued that current knowledge of pollinator ecology and populations is sufficient for remedial action. Indeed, comprehensive reviews have identified five key

drivers of pollinator declines: land-use change and management, climate change, pesticides, spread of disease, and invasive alien species (Vanbergen et al., 2013; Goulson et al., 2015; Potts et al., 2016).

Of these factors, land-use change has perhaps had the largest impact on pollinator declines. Intensification of agricultural land in industrialised countries over the past 100 years has reduced the amount of forage available for bees (Goulson et al., 2015). For example, in the UK, as a result of the agricultural act of 1947, much of the partially managed and florally diverse hay meadows were transformed into monoculture silage production, the conversion of which resulted in a loss of forage for many bee species (Goulson et al., 2008). Whilst land-use change has undoubtedly caused the loss of pollinator species (Potts et al., 2016), it is likely that the bee species which responded negatively to changes in land-use are not primarily those that are contributing to crop pollination; species that tend to pollinate crops also tend to use these crops as forage and are common in these landscapes (Kleijn et al., 2015). Indeed, Powney et al. (2019) found that, at the same time as declines were observed in many wild bee species, numbers of several key bee species responsible for pollinating flowering crops, such as oil-seed rape (*Brassica napus*), have increased in the UK since 1980. Therefore, to understand how losses of pollinating insects affects crop pollination specifically, research should also target drivers of decline other than land-use change, such as climate change and pesticides (see sections 1.8-1.16). In addition, the role of commercial operations that provide on-demand pollinators, and thus bolster pollination services, should not be overlooked.

1.2 Commercialisation of pollinators

Evidently, there are many challenges to insect pollinators and the ecosystem services they provide. To ensure pollination services to crops and to promote food security, the provision of managed pollinators may be essential. Western honeybees (*Apis mellifera*) have been associated with humans for thousands of years, primarily for honey and beeswax production (Roffet-Salque et al., 2015), and have also been managed to pollinate crops for hundreds of years (Rucker et al., 2012). Whilst it is not clear to what extent agricultural yields are currently reliant on honeybee pollination (Hanley et al., 2015), they are used extensively to fulfil demands worldwide (Klein et al., 2007; Allsopp et al., 2008; Breeze et al., 2011). Indeed, the yields of some fruit and nut crops in the USA have been shown to decrease by > 90% without managed honeybees (Southwick and Southwick, 1992).

Bumblebees are now also produced commercially, primarily for greenhouse pollination (Velthuis and Van Doorn, 2006). Since the 1980's, three main companies dominate the commercial production of bumblebees: Biobest®, Koppert Biological Systems, and Bunting Brinkman Bees (Velthuis and Van Doorn, 2006). Two bumblebee species are chiefly produced, the buff-tailed bumblebee (BTB; *B. terrestris*) for pollination in Europe, and the common eastern bumblebee (*B. impatiens*) for North America (Owen, 2016). Whilst there are 20 commercially important crops pollinated by bumblebees (Delaplane et al., 2000), the tomato (*Solanum lycopersicum*) industry in particular benefits from their commercial provision (Delaplane et al., 2000; Velthuis and Van Doorn, 2006).

Tomato flowers must be shaken to release their pollen, and bumblebees are able to achieve this via buzz pollination, a trait unique to them and several species of solitary bee (Morgan et al., 2016). Previously, pollination of tomatoes was conducted by labourers using vibrating wands; replacement of this process with bumblebees is estimated to save tomato growers approximately £9,000 per hectare every year (Velthuis and Van Doorn, 2006). As of 2015, over a million colonies of bumblebees are produced annually for pollination (Goulson and Hughes, 2015).

There has been extensive work to commercially produce certain species of solitary bees. For example, after being accidentally introduced to North American in the 1940s the alfalfa leafcutting bee (*Megachile rotundata*) was found to greatly increase yields of alfalfa (*Medicago sativa*) and so the bees became increasingly used and commercialised for its pollination (Pitts-Singer and Cane, 2011). A group of solitary bees, the mason bees (*Osmia* spp.), have also shown promise as potential commercial pollinators (Bosch and Kemp, 2002). For instance, the blue orchard bee (*Osmia lignaria*) is currently being investigated as a commercial pollinator for orchards (Bosch and Kemp, 2000; Kraemer et al., 2014).

As a strategy to safeguard pollination services in the face of pollinator declines, the commercial production of other pollinators has not garnered much discussion (the only example to my knowledge is by the United States National Research Council (2007)), and, while conservation should of course remain a priority, a considerate approach to commercialisation of additional species could mitigate losses of pollination services. In addition, given the extent to which food

production is reliant on pollination (Klein et al., 2007), this strategy should not be ignored. With the number of pollination-dependant crops worldwide increasing (Garibaldi et al., 2011), whilst wild pollinator species decline (Potts et al., 2016), commercialised species could provide a cost-effective solution for greenhouse pollination demands and beyond (Delaplane et al., 2000).

The commercial production of pollinators has some additional advantages over relying on wild pollination. Wild populations are susceptible to stochastic changes in the environment, disease and parasitism, and their populations may vary greatly year to year (Bosch and Kemp, 2002). Furthermore, crop production within greenhouses is mostly inaccessible to wild pollinators, and it is currently serviced mostly by commercial species, such as BTBs (Velthuis and van Doorn, 2006). However, there are also problems with current commercial provision of pollinators, particularly with the most widely used bumblebees and honeybees, that must be addressed.

1.3 Issues with current commercial provision of pollinations

1.3.1 Inadequate pollination

Honeybees are utilised in a variety of agricultural systems worldwide (Klein et al., 2007), being also used for certain crops despite there being little evidence that they increase yields or quality (Garibaldi et al., 2013). Indeed, in the UK honeybees appear to have declining importance even in crops dependent on animal pollination (Breeze et al., 2011). For example, in fruit trees honeybees are known to ‘side collect’, meaning that they draw only on the nectar whilst avoiding

taking the pollen (Free, 1960; Bosch and Kemp, 2002; Finta, 2004), thus avoiding pollinating the tree. Moreover, in orchards they have been shown to rarely switch rows, which is essential for pollination of many fruit trees where the same variety is incompatible with itself (DeGrandi-Hoffman et al., 1984; Finta, 2004; Monzon et al., 2004).

Honeybees also meticulously collect pollen, tightly packing pollen away into their corbiculae (pollen baskets), which leaves few grains loose to contact the stigma of the flower for the plant to be pollinated (Westerkamp, 1991). Additionally, honeybees have the ability to communicate with one another about available forage, which means that they can abandon crop plants in preference of other more favourable forage (van Heemert et al., 1990).

As for bumblebees, one potential limitation is that they are known to forage outside of glasshouses (Morandin et al., 2001a; Otterstatter and Thomson, 2008; Dafni et al., 2010), suggesting that they could favour forage other than the crops they are bought to pollinate, or that they may not be providing maximal pollination to the crops. Indeed, pollen sampled from bumblebees (*B. occidentalis* and *B. impatiens*) in some glasshouses suggested that only around 5% of their pollen was from the crop species (Whittington et al., 2004).

1.3.2 Spread of disease

Commercial use, and the trading of pollinators, has been associated with spread of disease. A comprehensive review by Graystock et al. (2016) on this topic contended that the spread of the Varroa mite (*Varroa destructor*), microsporidians

(such as *Nosema cerenae* and *Nosema bombi*), a parasitic mite (*Locustacarus buchneri*) and deformed wing virus can all be associated with commercialisation of honeybees and bumblebees. The high density of hosts and their usage in permeable ecosystems can promote pathogen proliferation (Otterstatter and Thomson, 2008; Murray et al., 2013). Moreover, these diseases spread not only between commercial pollinators but also closely related wild pollinators, i.e. the spread can be bidirectional, moving from commercial to wild populations and vice versa (Graystock et al., 2016).

The diseases spread by commercial pollinators can have substantial consequences. *Nosema bombi* is an obligate intracellular parasite of bumblebees that negatively affects individuals and colony fitness (Otti and Schmid-Hempel, 2007, 2008). By using museum specimens of bumblebees, it was shown that *N. bombi* had much higher infection levels in species of bumblebees that were declining in North America (Cameron et al., 2011) and the spread has been linked to commercial rearing of bumblebees within the USA (Szabo et al., 2012; Cameron et al., 2016).

Similarly, well-documented declines of honeybees have been in part associated with the prevalence of diseases. Two key causative agents of the decline are the Varroa mite (*Varroa destructor*) and *Nosema cerenae* (Neumann and Carreck, 2010; Potts et al., 2010; Goulson et al., 2015). Originally present in the eastern honeybee (*A. cerenae*), the varroa mite has spread to western honeybees (*A. mellifera*) across the globe, facilitated by the global honeybee trade (Rosenkranz et al., 2010; Goulson et al., 2015; Graystock et al., 2016). Similarly, the

microsporidian *Nosema cerenae* (which causes digestive problems, shortening of lifespan, immune suppression, and ultimately death (Higes et al., 2006, 2008; Antúnez et al., 2009)) was originally present in eastern honeybees, spreading to western honeybees and then being transported worldwide through the commercial honeybee trade (Graystock et al., 2016).

1.3.3 Commercial pollinators becoming invasive

Trading of bumblebee and honeybee species across the world also has the potential to cause their unintended introduction into non-native habitats, which can cause negative impacts on the native flora, as well as put these 'alien' commercial species in direct competition with native pollinators (Morales et al., 2017).

Honeybees have long been traded worldwide and now have a global presence (Paini, 2004; Stout and Morales, 2009). Much of the trading occurred long before any consideration was given to its ability to become invasive (Kearns et al., 1998). Now, however, honeybees' invasive potential has been increasingly recognised. For example, feral honeybees (which originated from commercial hives) are now designated as an invasive species by the Australian government (Australian Government: Department of Agriculture, Water and the Environment, 2021). As introduced honeybees have been progressively understood to be invasive, their negative effect on the native flora and fauna has been demonstrated. For example, in Australia, honeybees were found to actively rob pollen from the stigma of a native plant (*Melastoma affine*), decreasing its reproductive capacity,

whilst also disrupting the legitimate pollination activities of native pollinators (Gross and Mackay, 1998). Similarly, in Mexico, honeybees were shown to decrease the number of wild pollinators in coffee (*Coffea arabica*) plantations, as well as decrease crop yields at higher abundances (Badano and Vergara, 2011).

The invasive potential of commercially produced BTBs has also been established. It can withstand a range of climatic conditions, has good dispersal abilities, is very generalist, has a facultative dormancy period, and can thermoregulate (Dafni et al., 2010). Furthermore, many areas of the world are climatically suitable and have appropriate forage for this bee (Acosta et al., 2016). These invasive attributes have meant that the USA, Canada and Australia have banned its importation, recognising the potential downsides of its introduction, such as disease spread, competition with native species and loss of genetic integrity in native species (Velthuis and Van Doorn, 2006; Winter et al., 2006; Dafni et al., 2010). Moreover, certain sub-species of BTBs, if exported, can out-compete native sub-species (Ings et al., 2005).

Of bumblebees, BTBs are also the most widely commercialised and have been for the longest period of time (Velthuis and Van Doorn, 2006), so they have had the most opportunity to invade, often before proper biosecurity protocols have been put in place (Dafni et al., 2010). Indeed, this bumblebee has been characterised as invasive in Japan (Matsumura et al., 2004), Israel (Dafni et al., 2010), Argentina (Torretta et al., 2006; Schmid-Hempel et al., 2014) and Chile (Ruz, 2002; Schmid-Hempel et al., 2014).

1.4 Navigating the disadvantages to commercial pollinator use

Evidently, there are downsides to the mass-rearing and importation of commercial pollinator species, but, at the same time, there are increasing demands for pollination services. In essence, despite their flaws, commercial species may be indispensable, especially with the pressing need to increase the amount of food we produce sustainably (Tilman et al., 2011). Commercial pollinators can provide services that could increase yield and quality of crops with minimal environmental impact, especially if they are used in a way that attempts to combat some of the disadvantages discussed above. Increasing yields by using pollinators rather than, say, increasing the amount of nitrogen fertiliser used, will likely have a smaller impact on the environment (Tilman et al., 2002, 2011; Isaacs et al., 2017). Many crops are pollination-limited as well (Garibaldi et al., 2011). For example, it was shown that increasing pollination in almond (*Prunus dulcis*) always led to a yield increase regardless of nutrient or water limitation (Klein et al., 2015).

To continue using managed pollinators, whilst trying to mitigate the impacts discussed above, a practical strategy could be to increase the diversity of pollinators that we currently commercially produce. As discussed, honeybees (and perhaps bumblebees) are not the best pollinators for every crop, which opens up a commercial opportunity to fund research to find the optimal pollinator for a specific crop. For example, the suitability of mason bees (*Osmia* spp.) to pollinate orchards has been investigated and this group of bees are now used on a small commercial scale (Bosch and Kemp, 2002). Furthermore, focus should

be shifted to commercialising native species, rather than relying on western honeybees and BTBs. This approach has been taken up in North America, where commercial bumblebee production now focuses on native common eastern bumblebees (*B. impatiens*), to avoid the unintended introduction of BTBs (Winter et al., 2006). In the UK, imports are now regulated to prevent the introduction of non-native BTB subspecies*, B. terrestris dalmatinus,* which has invasive potential (Ings et al., 2005; Owen et al., 2015), and instead use the native subspecies *B. t. audax* (Goulson and Hughes, 2015).

1.5 Commercialisation of the red mason bee

The red mason bee (RMB; *Osmia bicornis*) has been identified as a suitable candidate for commercialisation (Bosch and Kemp, 2002) and has also been shown to be an effective pollinator for many important crops. For example, it has been shown that, when RMBs are placed near rapeseed, up to 45% of their collected pollen will be from that plant, indicating that the bees are most often visiting and pollinating this particular crop (Teper and Biliński, 2009). In strawberries (*Fragaria* × *ananassa*), RMBs have been shown to limit the proportion of deformed fruits, and increase the total mass of fruits (Wilkaniec and Radajewska, 1997). Additionally, it has been suggested that RMBs can be effective pollinators of blackcurrant (*Ribes nigrum*; Fliszkiewicz et al., 2011), apples (*Malus domestica*; Gruber et al., 2011), and trees of the genus *Prunus* (which include fruits such as cherries and peaches; Fliszkiewicz et al., 2011; Gruber et al., 2011; Ryder et al., 2020). In fact, RMBs are polylectic (forages on a wide variety of plant species for pollen) and have been shown to use pollen

from at least 19 plant families (Haider et al., 2014). Moreover, RMBs may be better placed than honeybees as pollinators in greenhouses. Honeybees can become disorientated and aggressive in greenhouses, whereas RMBs do not (Holm, 1974; Van der Steen and De Ruijter, 1991; Sedivy and Dorn, 2014).

The area of orchard cultivation continues to rise in the UK (DEFRA, 2015b) and it has been suggested that some orchard crop yields may be limited by pollination (Garratt et al., 2014a). This is where RMBs could become economically important as pollinators. As an example, apple crops alone are currently estimated to be worth over £117 million annually in the UK (DEFRA, 2015). Garratt et al. (2014a) anticipated that yields of apples could be 8% higher with maximal pollination, meaning that if RMBs were able to maximise pollination service provision, only to apple, the yield increase could be worth approximately £9 million annually.

Such advantages have led to several companies being established to provide RMBs to growers (Appendix 1.1), and they are increasingly managed in orchards (Ivanov, 2006; Gruber et al., 2011). However, current commercial practice still relies on collecting wild populations from 'bee hotels' provided to growers (example shown in Figure 1.1). Numbers of bees collected from these wild populations can fluctuate due to weather conditions as well as parasitism etc. (Bosch and Kemp, 2002), so bee availability can vary a great deal between years (R. Dean, personal communication). Adult females visit these nests and use them to build brood cells and oviposit, and the offspring is collected around October-November when the bees have developed into adults and spun their cocoons (Figure 1.2).

Figure 1.1 Example of a trap nest used to collect red mason bees

Commercially available plastic trap nest with cardboard tubes, which can be used to collect red mason bees. Picture taken by N. Howe

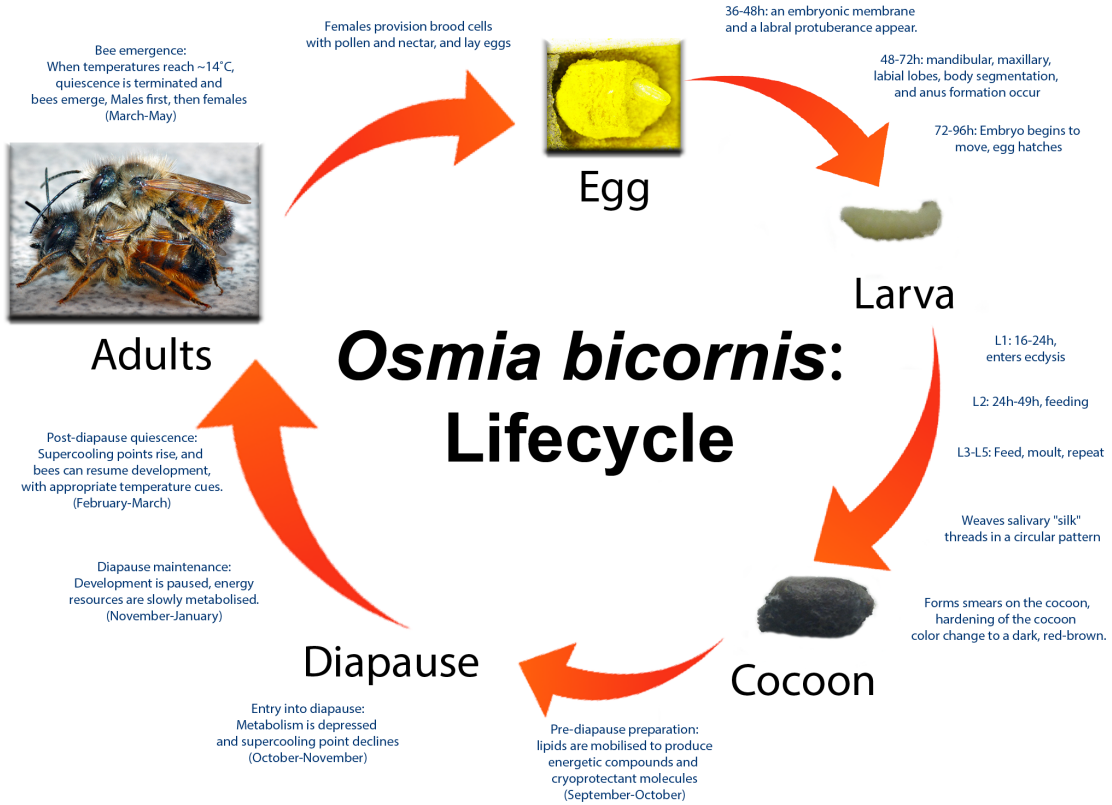


Figure 1.2 Red mason bee lifecycle

The lifecycle of red mason bees as described by Raw (1972), Wasielewski (2011a) and Keller et al. (2013). The black bold text indicates the major lifecycle stages of the bee, whilst the blue text refers to steps involved in the progression to each stage. Pictures of red mason bee stages are original, with the exception of the adults and the egg which came from Wikimedia commons (https://commons.wikimedia.org/wiki/Osmia_bicornis).

To reduce population variability and to improve the stability of RMB production, rearing systems conducted in artificial and controlled conditions (similar to commercial bumblebee production (Velthuis and Van Doorn, 2006)) should be established. RMBs are well-placed for the development of such a rearing system, as they are already managed in many orchards, and their biology is well-understood (Bosch and Kemp, 2002; Gruber et al., 2011). Moreover, Sandrock et al. (2014) have reared RMBs in a sunlight simulation chamber. By building upon this background, a commercial rearing system for RMBs may be possible. Moreover, a key part of the RMB lifecycle is diapause (Figure 1.2) which may offer opportunities, and challenges, to commercial provision and so is explored in detail in the next section.

1.6 RMB diapause

Diapause can be defined as a pre-determined arrest in development that enables insects to avoid environmentally unfavourable conditions (Denlinger, 2008). In temperate and colder regions almost all insects undergo a diapause period to circumvent winter conditions (Denlinger, 2009), whereas in the tropics diapause may allow insects to evade intervals of insufficient food resources or hot, dry conditions (Denlinger, 1986). For RMBs, diapause is an obligate part of their lifecycle (Figure 1.2), meaning they are genetically pre-determined to enter it, regardless of environmental conditions (Denlinger, 2009). This is distinguished from facultative diapause employed by most insects, which occurs in response to environmental cues (Denlinger, 2009).

Diapause is split into several phases: pre-diapause, diapause and post-diapause (Figure 1.3). During post-diapause, many insects, including the RMBs, enter quiescence, which is differentiated from diapause by being a developmental arrest that allows an immediate resumption of development when conditions allow, rather than a fixed arrest period (Hayward et al., 2005; Koštal, 2006). The timing of the different stages of diapause in RMBs is not well-understood, but generally pre-diapause occurs September–October, diapause November–January, and post-diapause quiescence February–March (Wasielewski et al., 2013). In terms of their lifecycle, this means that RMB diapause occurs after adults have developed and spun cocoons (Wasielewski et al., 2011a), thereafter, they will remain in post-diapause quiescence until receiving a temperature cue (around 14˚C), which signals that spring has arrived and conditions are favourable to resume development (Raw, 1972; O'Toole, 2000; Wasielewski et al., 2011a; Figure 1.2).

This developmental arrest presents a challenge for commercial rearing systems. Firstly, there can be no provision of adult bees for pollination services during this diapause period, meaning that all-year-round access to RMBs for growers is currently unfeasible. Secondly, termination of diapause, and thus bee emergence, will not necessarily be timed with the target crop bloom. Instead, emergence will be aligned with the conditions upon which the bees were reared (Bosch and Kemp, 2000, 2002).

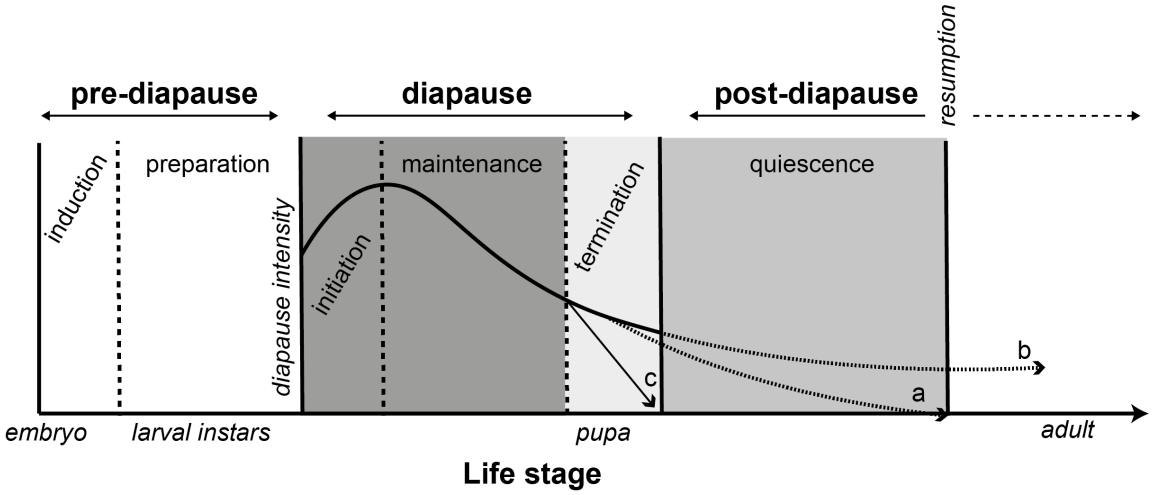


Figure 1.3 Stages of diapause

Figure adapted from Koštál (2006). The line along the x axis indicates developmental progression of one hypothetical insect, with the approximate life stages (embryo, larval instars, etc.) at different times shown (although it should be noted this is not true for every insect, e.g. RMBs enter diapause as adults). Across the top the major stages of diapause as defined by Koštál are shown in bold and are delineated by solid lines, with the sub-stages (induction, preparation, etc.) below in regular text and separated by dotted lines. The curved line shows the diapause intensity of a hypothetical insect, and the three branches (a, b, and c) showing different possible diapause trajectories, where **a** demonstrates resumption of development after an exogenous cue to terminate quiescence, **b** resumption of development after quiescence without an exogenous cue, and **c** when specific environmental conditions are encountered to promote the termination of diapause.

In current commercial operations these challenges are tackled by keeping RMBs in cold storage, to maintain post-diapause quiescence, and then transferring the bees to temperatures of 20-30˚C to promote emergence when they are required (Bosch and Kemp, 2000; Biliński and Teper, 2004; Pitts-Singer et al., 2008; Dmochowska et al., 2013). However, prolonged maintenance of diapause/quiescence has been shown to deplete RMB energy stores

(Dmochowska et al., 2013), which may reduce their survival and longevity (Bosch and Kemp, 2000, 2004; Sgolastra et al., 2011, 2016). To ensure the production of healthy bees, alternative strategies to promote emergence, or maintain diapause/quiescence, should be investigated. Moreover, the aforementioned method does not ensure that RMB emergence will be timed with crop bloom, instead, the rate of emergence is contingent on the length, and temperature, of the wintering period (Bosch and Kemp, 2000; Dmochowska et al., 2013; Giejdasz and Wasielewski, 2017a). Therefore, it would be useful to develop methods to promote RMB emergence quickly, so bees can be delivered to growers, ready to pollinate, on demand.

1.7 Methods to manipulate diapause

Techniques to terminate diapause have been utilised in many other insect species (Appendix 1.2), such as the use of hexane to terminate diapause in *Sarcophaga crassipalpis* (Denlinger et al., 1980). The juvenile hormone analogue, methoprene, has also been shown to accelerate processes involved in termination of diapause, increase in ovary size and changes in protein levels, in RMB females (Wasielewski et al., 2011b), so could potentially be used to promote bee emergence. Whilst diapause programmes diverge between species, the mechanisms of terminating compounds may be somewhat similar, for example, hexane (an alkane) mediates termination of diapause by causing an increase in juvenile hormone and ecdysteroid production (Fujiwara and Denlinger, 2007). This in turn may be similar to how methoprene functions to terminate diapause in RMBs, as methoprene is a juvenile hormone analogue

(Wasielewski et al., 2011b). Therefore, some of the available compounds may influence RMB diapause termination. However, it will be critical to assess the health of the bees after treatment, as it is possible that the compounds could have negative effects on insects, reduce insect longevity for example, which may affect their usage as managed pollinators.

Diapause may also represent an opportunity for commercial operations. If diapause, or quiescence, were to be extended, it would allow RMBs to be stockpiled, building resilience into their provision. Such extensions have been shown, for instance, in the closely related alfalfa leafcutting bee (*Megachile rotundata*), where fluctuating thermal regimes (FTRs) were employed to significantly extend diapause duration (Rinehart et al., 2013). The leafcutting bees were kept at 6˚C with a daily temperature spike of 20˚C and this allowed the bees to be stored for 19 months with no consequences on the survival of bees (Rinehart et al., 2013). A similar strategy may effectively allow RMBs to be stockpiled. Moreover, wild pollinators may be vulnerable to the effects of warming winters due to climate change (Fliszkiewicz et al., 2012), and so FTRs could be a potential strategy to mitigate such effects.

1.8 Climate change

With current emission pledges the climate is set to warm by around 3.2˚C above pre-industrial levels (Rogelj et al., 2016; Allen, 2019), and projections indicate that, regardless of any actions taken, the Earth will warm by 1.5˚C by 2052 (UNEP, 2019). This will be a key challenge for insect pollinators. Insects are

generally incapable of maintaining their body temperatures (key exceptions include honeybees and bumblebees, which have some ability to generate heat (Heinrich, 1974b)); therefore, their development is closely associated with environmental temperature (Bale and Hayward, 2010; Forrest, 2016), and the geographical ranges they can inhabit are dictated by the climate (Deutsch et al., 2008). In a warming world this could cause severe insect losses. Indeed, a recent study by Warren et al. (2018) suggested that a 3.2˚C increase in temperature would cause 49 % of invertebrates, chiefly insects, to lose 50 % of their ranges, with a consequent increase in extinction risk. For pollinating insects, climate change is likely to exacerbate other drivers of their declines (Settele et al., 2016).

For RMBs, a warming world could be particularly challenging. Unlike many insects which have facultative diapause, meaning they can avert entry into diapause if conditions are favourable (Koštal, 2006; Denlinger, 2009), the obligate diapause of RMBs means it is a fixed part of their lifecycle (Wasielewski et al., 2013; Dmochowska-Slezak et al., 2015). Whilst the primary cue for many insects with facultative diapause to enter diapause is daylength (Denlinger, 2009), in some cases, temperature can override this cue and cause insects to avert diapause (Bale and Hayward, 2010). For example, in the blue blowfly (*Calliphora vicina*) temperatures of 20˚C experienced by the adults will cause their offspring to avert diapause (Coleman et al., 2015), thus overriding the primary cue of daylength and allowing them to increase their voltinism (number of broods or generations produced per year). Such changes in voltinism can allow insect pollinators to take advantage of longer growing seasons (Forrest, 2016).

Indeed, Forrest et al. (2019) found that a mason bee (*O. iridis*) is shifting its generation times from two years to one in response to warming temperatures, to take advantage of warmer summers and autumns. However, RMBs will enter diapause regardless of environmental temperature (Wasielewski et al., 2013; Dmochowska-Slezak et al., 2015), meaning they are unable to adjust this part of their lifecycle in response to climate change.

To survive the long overwintering period and promote fitness upon emergence, RMBs, like many insects during diapause preparation (Figure 1.3), accumulate substantial lipid and protein resources before entry into diapause (Dmochowska et al., 2013). However, unlike many other insects, larvae are not able to take advantage of favourable conditions to accumulate extra resources during this period, instead they are entirely dependent on the food resources allocated by their mother within each egg chamber (Wasielewski et al., 2013). Due to climate change, winters are warming particularly rapidly (Williams et al., 2015), and these increased temperatures often boost the metabolism of insects. This raised metabolism, in turn, may lead to the consumption of precious (and limited) energy stores that are crucial for survival and reproduction post-emergence (Hahn and Denlinger, 2007, 2011; Williams et al., 2015).

Reductions in RMB survival due to warmer winters have been demonstrated experimentally. Fliszkiewicz et al. (2012) simulated the effects of warmer winters (one week at 15˚C) in RMB females, versus bees kept outside in natural winter conditions in Poland (control). They showed that RMBs at 15˚C lost body mass and lipid reserves more rapidly than the control bees, from December onwards

(Fliszkiewicz et al., 2012). These results have been substantiated by Schenk et al. (2018b) who showed that increasing temperatures by 3˚C above the monthly average during overwintering led to a reduction in body mass in two species of mason bee (*O. bicornis* and *O. cornuta*). Similarly, increased body mass and lipid store loss has been demonstrated in another species of mason bee (*O. ribifloris*), under temperature conditions projected to occur in 2040-2099 (CaraDonna et al., 2018).

Additionally, warmer temperatures may not only affect maintenance of obligate diapause, they may also affect RMBs during pre-diapause phases. For example, in the blue orchard bee (*O. lignaria*), which also has an obligate diapause, warmer temperatures or delays to the onset of winter have been shown to cause greater energy expenditure during overwintering, leading to lower body mass and increased lipid loss (Sgolastra et al., 2011, 2016). Whether such effects are present in RMBs is not has not yet been investigated.

1.9 Phenological mismatch

Another key issue may be that early termination of diapause and quiescence by increased temperatures can cause RMBs to shift their phenology. This shift could mean they would lose synchrony with their environment, including the plants they pollinate (Bale and Hayward, 2010). Like insects, many plants rely on temperature cues for different parts of their lifecycle, to ensure that the timing of developmental transitions is synchronised with favourable conditions (Blackman, 2017). For example, spring flowering may be a response to warmer temperatures

during or after winter (Amasino and Michaels, 2010). Insect pollinators also commonly use temperatures preceding, during, or after winter as a cue to emerge and to determine their flight period (Memmott et al., 2007; Hegland et al., 2009). With warming temperatures, and climate instability associated with climate change, there are concerns that, despite using similar cues, pollinators and plants in the future will respond dissimilarly, and thus their synchrony will be lost, i.e. there will be phenological mismatches (Memmott et al., 2007; Hegland et al., 2009; Forrest, 2016).

Phenological mismatches between plants and pollinators are well evidenced at high latitudes and elevations (Forrest, 2016). For example, Kudo and Cooper (2019) showed that a bumblebee (*B. hypocrita*) and a spring flowering plant (*Corydalis ambigua*) may lose synchrony as a result of early snowmelt in northern Japan. Where present, the snow insulated the soil, keeping it at 0-2˚C; the advanced snowmelt meant that the surface temperature, which the plant relies on to determine its flowering, increased quickly, whereas the soil temperature, which the bumblebee uses to determine its emergence, increased more slowly (Kudo and Cooper, 2019). These differences in the rate of temperature change caused flowering to occur up to ten days before bumblebee emergence (Kudo and Cooper, 2019). Such a disruption was shown to lead to the lower seed production in *C. ambigua* (Kudo and Ida, 2013). For solitary bees in the Rocky Mountains, a similar mismatch has been demonstrated (Forrest and James, 2011). Here, it was suggested that higher temperatures are required for solitary

bees to terminate diapause and emerge than are required for plants to flower (Forrest and James, 2011).

For temperate regions, there are few detailed examples of mismatches between plants and pollinators. Pollinator phenology is certainly changing, for example Schenk et al. (2018b) showed that warmer temperatures during winter caused mason bees (RMBs and *O. cornuta*) to emerge earlier on average. However, currently it appears that, in general, shifts in pollinator phenology are similar to those occurring in plants (Bartomeus et al., 2011; Forrest, 2016; Morton and Rafferty, 2017; Renner and Zohner, 2018). Nevertheless, as the climate continues to warm, it is unlikely that the trajectories of plant and pollinator phenologies will remain aligned (Hegland et al., 2009; Forrest, 2015). For example, Petanidou et al. (2014) assessed the interactions between 132 plant and 665 pollinating insect species in the Mediterranean and found that, during hotter years, plants that flowered earlier were generally visited by fewer pollinators. This suggests that as the climate warms there will be a loss of pollination services, which could impact food security, as crops may be inadequately pollinated (Memmott et al., 2007; Hegland et al., 2009; Forrest, 2016), and further declines in pollinators could also occur due to lack of forage availability (Kaiser-Bunbury et al., 2010).

For RMBs, to date, there is little evidence of phenological changes due to climate change in wild populations. However, RMBs are likely sensitive to warming as their rate of development and termination of post-diapause quiescence, i.e. emergence, is controlled by temperature (Giejdasz and Wilkaniec, 2002;

Wasielewski et al., 2011b), and other cavity nesting bees have been shown to use temperature as the primary cue for emergence (Bennett et al., 2018). Indeed, Kehrberger and Holzschuh (2019), show that RMB males (but not females) advance emergence in response to warming, but at a slower rate than the spring-flowering pasque flower (*Pulsatilla* vulgaris). Additionally, RMBs may require a period of cold temperatures as a cue to complete diapause (Wasielewski et al., 2011b), as is the case in blue orchard bees (Kraemer and Favi, 2010; Bosch et al., 2010), which, with increasing occurrence of warmer winters, is likely to also be disrupted (Williams et al., 2015). Therefore, it is highly likely that RMBs phenology will shift in response to climate change.

Due to the polylectic nature of RMBs, they may be able to adapt to phenological shifts by exploiting whatever pollen and nectar resources are most available (Radmacher and Strohm, 2010; Eckhardt et al., 2014; Haider et al., 2014), such adaptation has already been evidenced in blue orchard bees (Kraemer and Favi, 2005), but the extent to which this may mitigate the impacts of climate change is unclear. Moreover, even if RMBs can utilise alternative forage, there may be a reduction in the pollination services they provide to crops due to phenological mismatches. The potential loss of crop pollination services from wild bees, and the likely shifting of flowering time of many commercial crops, emphasises the importance of establishing a rearing system for RMBs, as this would allow bees to be provided to crops whenever necessary.

1.10 Climate change and thermal challenges

As previously discussed, insect pollinators are adjusting their life histories in response to climate change. Shifts in voltinism have been evidenced, and whilst in many cases such shifts are adaptive (e.g the production of more offspring and thus increase in fitness demonstrated by many butterflies and moths (Altermatt, 2010)), they can also be maladaptive (Forrest, 2016). After all, organisms alter their developmental trajectory, for instance by averting diapause, with no knowledge of future conditions (Forrest et al., 2019). In the UK, the typically univoltine BTB has been shown to be founding colonies during winter (Stelzer et al., 2010), likely in response to warmer autumns. This shift to a bivoltine (two generation) lifecycle may be damaging to the bee, as Owen et al. (2013) showed that whilst these bumblebees can survive acute cold, they are vulnerable to chronic cold that is common in UK winters. In fact, BTB colonies have been shown to be currently unable to persist throughout a typical winter in the UK (Owen, 2015, PhD thesis). With future climate change, important pollinator species may therefore be exposed to novel thermal challenges.

For RMBs, the unpredictability associated with climate change may mean that bees would emerge early to a ‘false’ spring in response to warmer temperatures, only for temperature to then fall again (Williams et al., 2015). For example, in February 2018 the UK was affected by temperatures 8-10˚C colder than the seasonal average after a mild winter (Greening and Hodgson, 2019), the sudden drop in temperature may have severely affected early emerging insects. Moreover, such extreme events are predicated to become more likely with

climate change (Williams et al., 2015). Whilst there is some evidence of enhanced cold tolerance during diapause (Krunic and Stanisavljevic, 2006), it is unclear how active RMBs would respond to cold conditions. Therefore, in order to fully understand the impacts of a warming climate on key pollinators such as RMBs and BTBs, it is critical to understand their thermal tolerance. Furthermore, insect pollinators will not be exposed to cold in isolation, instead there will be many other stresses that will simultaneously affect them (Sinclair et al., 2013; Kaunisto and Ferguson, 2016). One well-described stress for pollinators, particularly in agricultural ecosystems, is that of pesticides (Sponsler et al., 2019). Of the pesticides that are suggested to impact upon insect pollinators, perhaps the most damaging are the neuroactive insecticides, the neonicotinoids (Goulson et al., 2015).

1.11 Neonicotinoids

The neonicotinoid pesticides (NPs) were developed in the late 1980s and early 1990s as an alternative to organophosphates, carbamates and pyrethroids – to which resistance was prevalent (Simon-Delso et al., 2015). Since the introduction of the first commercially available NP compound, imidacloprid (IMI), NPs have become the most widely used insecticides in the world (Jeschke et al., 2011; Goulson, 2013). There are seven commercially available compounds but the five most commonly used are: IMI, clothianidin (CLO), thiamethoxam (THIM), thiacloprid (THIC) and acetamiprid (ACE) (Jeschke et al., 2011; Goulson, 2013).

1.12 Neonicotinoid structure and mechanism of action

Broadly, the NPs can be split into two groups based on their chemical structure: those possessing a cyanoimino group and those with a nitroimino group (Figure 1.4). The structures of NPs are similar to that of nicotine (Figure 1.4; Millar and Denholm, 2007), and it allows them to bind to nicotinic acetylcholine receptors (nAChRs) (Millar and Denholm, 2007). Unlike nicotine, NPs are selectively more toxic to insects than they are to mammals (Tomizawa and Casida, 2005; Goulson, 2013), as they exhibit a strong binding affinity specifically to insect nAChRs (Tomizawa and Casida, 2005). The effect is magnified as insects also possess more nAChRs that have a high affinity for neonicotinoids (Simon-Delso et al., 2015). Even at low doses, NPs bind to the nAChRs, which causes the firing of action potentials and nervous stimulation, while at higher doses they block nAChRs and cause paralysis and death within minutes (Goulson, 2013; Van Der Sluijs et al., 2015; Simon-Delso et al., 2015).

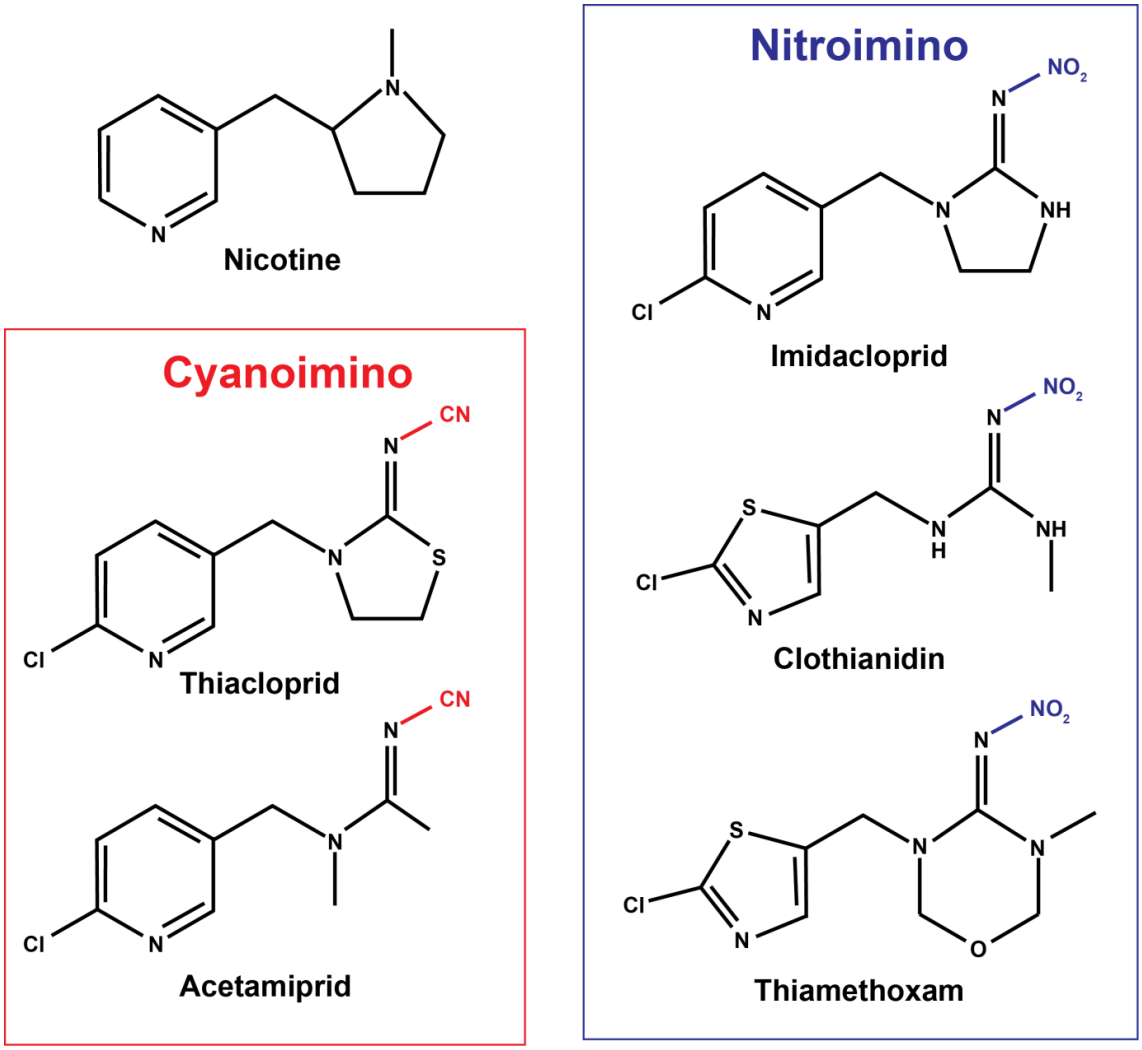


Figure 1.4 Structure of nicotine and the five most widely used neonicotinoids

Chemical structures of nicotine and the five neonicotinoids that Jeschke (2011) describes as the most widely used. The neonicotinoids are split into two groups based on their chemical structures. In the red box, the neonicotinoids that possess a cyanoimino group (highlighted in red), and in the blue box the neonicotinoids that possess a nitroimino group (highlighted in blue).

NPs are small molecules and have physicochemical properties that make them highly water soluble (Bonmatin et al., 2015). This means that, when applied, NPs

are taken-up by the target crop and translocated to all of the plant tissues, offering systemic protection (Bonmatin et al., 2015), which allows for versatility in their application. They can be applied to the seeds prior to planting and still offer protection over a period of weeks, or applied as a foliar spray (Simon-Delso et al., 2015). This flexibility, along with low mammalian toxicity, has promoted their widespread adoption, although they are usually applied as seed treatments (Jeschke et al., 2011; Goulson, 2013). Despite these advantages, however, evidence has emerged in recent years that they may cause detrimental impacts on non-target animals (Goulson, 2013), most notably pollinators (Wood and Goulson, 2017).

1.13 The impacts of neonicotinoids on insect pollinators

The systemic protection offered by NPs means that these insecticides can enter the pollen and nectar in plants (David et al., 2016) (although it not clear how much neonicotinoid is deposited (Gierer et al., 2019)). NPs within nectar and pollen means that adult pollinators will be directly exposed to them, as well as collecting contaminated forage which is then given to developing offspring (Goulson, 2013). In addition, the water-solubility of these insecticides allows them to make their way into the environment and contaminate wildflowers, soil and waterways, providing another route of exposure for pollinators (Bonmatin et al., 2015; Wood and Goulson, 2017). Indeed, wildflowers in the margins of crop fields were shown to be contaminated with NPs even several years after the last application in the UK (Botías et al., 2015; David et al., 2016). The literature on NPs impact on

pollinators indicates that they cause few direct effects on mortality, but lead to a range of sub-lethal effects (Alkassab and Kirchner, 2017).

Sub-lethal effects of NPs on the western honeybee and bumblebees (*Bombus spp*.), notably the BTB, have been particularly well-evidenced. One such effect is a negative impact on learning ability. For example, IMI reduces the ability of honeybees to be trained to respond to a nectar reward at chronic (Decourtye et al., 2003) as well as short 30-minute exposures (Decourtye et al., 2004). Similarly, Stanley et al. (2015b) found that a chronic (24-day) exposure of BTBs to 2.4 µg/l THIM made it more difficult to train the bees to respond to a nectar reward (Stanley et al., 2015b).

NPs may also have direct impacts on motor function. For example, Williamson et al. (2014) found that after a dose of 2-3 µg/l of the NPs IMI, THIM or CLO, honeybees spent more time laying on their backs, unable to right themselves and had reduced postural control (Williamson et al., 2014). Such impacts on motor function may disrupt bee foraging. Yang et al. (2008) measured time intervals for honeybees to travel between a feeding station and the hive, after exposure to 100 µg/l IMI and above, and found that the time intervals required increased in a concentration dependent manner (Yang et al., 2008). These longer trips may indicate that the bees were becoming disorientated after IMI exposure. This hypothesis is supported by Henry et al. (2012) who tracked honeybees after they were released up to 1 km away (a realistic foraging distance; Goulson, 2013) and showed that after being fed 67 µg/l THIM, honeybees returned to their colony approximately 10-30% less often (Henry et al., 2012). Whilst these studies may

not directly show that foraging has been negatively affected, when combined with experiments showing that NP exposed bees are less able to distinguish between flowers (Karahan et al., 2015; Jiang et al., 2018), they strongly suggest that honeybee foraging is disrupted by neonicotinoid exposure (Goulson, 2013; Vanbergen et al., 2013; Hladik et al., 2018).

Bumblebee foraging has also been shown to be affected by NPs. For example, BTBs foraging in a field exposed to 10 µg/l IMI, collected 8% less pollen, despite the foraging time being increased by 18% (Gill et al., 2012). Similarly, BTB workers foraged less often and brought back 31% less pollen per hour, after feeding on 0.7 µg/l IMI in nectar, and 6 µg/l in pollen (Feltham et al., 2014). Such impacts on foraging ability could have devastating consequences on these bees as, without adequate resources, honeybees will have difficulties surviving winter and providing for their offpsring (Goulson et al., 2015), and for bumblebee colonies, adequate nectar and pollen resources are essential to brood rearing and colony growth (Goulson, 2010).

Such impacts have been shown experimentally. Whitehorn et al. (2012) exposed BTB colonies to 6 µg/l IMI in pollen and 0.7 µg/l in nectar over a fortnight, after which the bees could forage in a field for 6 weeks. After this period, the IMI treated colonies were found to be 8 % smaller than unexposed colonies. In addition, the unexposed colonies produced 13.72 queens on average, whereas the treated colonies only produced 2.00 (Whitehorn et al., 2012). This reduced reproduction may have been caused by NPs disrupting foraging (Whitehorn et al., 2012). Additionally, a field study showed that BTB colonies exposed to CLO seed-

treated rapeseed also produced fewer queens (Rundlöf et al., 2015). This effect of NPs is especially problematic for bumblebee populations, as the queens are the sole overwintering stage and founders of future colonies (Goulson, 2010).

1.14 Neonicotinoid usage

In response to the evidenced detrimental effects of NPs on beneficial pollinators, a review by the European Food Safety Authority (EFSA) concluded that NPs represent a risk to wild bees and honeybees and thus a pre-existing moratorium on three neonicotinoids (IMI, CLO, THIM) was extended to a full outdoor ban on their use within the EU (Jactel et al., 2019). Despite this political response, NPs are still widely used across the world, potentially putting many pollinators at risk (Goulson et al., 2018). In the USA, NPs are the preferred and most readily available insecticides, with approximately 300,000 acres treated with them annually (DiBartolomeis et al., 2019). Moreover, NPs are used widely in Canada, and their application is only set to increase as they replace older insecticides such as organophosphates (Anderson et al., 2015). In addition, Denmark has openly defied the EU ban (Sonne and Alstrup, 2019), potentially opening the door for other governments to do the same. NPs therefore continue to be a likely threat to pollinating insects, and there is much that is still not understood about their effects.

1.15 Effects of neonicotinoids on red mason bees

Whilst the consequences of neonicotinoid exposure have been well-studied in bumblebees and honeybees, it is less well known how NPs affect other insect

pollinators, such as solitary bees like RMBs (Blacquiere et al., 2012; Pisa et al., 2014; Van Der Sluijs et al., 2015). When pollinator risk assessments are conducted for pesticides, they are conducted only on the western honeybee with the presumption that the doses which cause the worst-case scenarios for this species will also do the same in other pollinators (Dicks et al., 2016; Sgolastra et al., 2019). This may not be the case, however, and this gap in knowledge needs to be addressed as the majority of the world's bee species are in fact solitary (Michener, 2007) and many of them have been shown to be major contributors to crop pollination (Garibaldi et al., 2013; Woodcock et al., 2013). To better understand the effects of NPs on solitary bees, RMBs may be a good model species, as there is some understanding of the impacts of NPs on these bees (Sandrock et al., 2014), and they are common in agricultural landscapes (Rundlöf et al., 2015).

It has been shown that NPs may reduce the reproductive success of RMBs. Sandrock et al. (2014) assessed the impact of chronic exposure to a mixture of 2.87µg/l and 0.45 µg/l THIM and CLO, respectively, and found that the number of offspring produced was 47.7% lower than in unexposed bees and was significantly male-biased (Sandrock et al., 2014). This study was extended by Rundlöf et al.'s (2015) field study, where it was shown that wild RMBs, exposed to CLO seed-treated rapeseed, did not engage in nesting activity at all. Another field study demonstrated that residues of neonicotinoids in RMB nests reduced the number of brood cells (where eggs are laid) in a concentration dependent manner (Woodcock et al., 2017). It is possible that NPs effects on reproduction

could be due to the effects on ovary development, as a study showed that RMB females exposed to 10 µg/l CLO had demonstrated slowed ovary growth (Sgolastra et al., 2018). This differs from the findings of Peters et al. (2016) who showed that exposure to seed-treated CLO had no detrimental impacts on ovary growth.

The differences in field studies (Rundlöf et al. and Woodcock et al vs. Peters et al.) may be due to environmental contamination. Woodcock et al.'s (2017) study showed geographical variation in the number of reproductive cells produced by RMBs, despite being exposed to identical CLO or THIM seed-treated rapeseed. The authors tested the pollen collected by the bees at different locations and found that other neonicotinoids, which were not part of the experiment, like IMI, were present, which may have driven the greater negative effects found in some of the regions (Woodcock et al., 2017). Alternatively, the dissimilarities could be due to differences in dosage, as it is difficult to determine in field studies how much NPs bees are exposed to (Wood and Goulson, 2017). For example, in Peters et al.'s (2016) study in most cases residues of CLO on crops were below quantifiable levels (i.e. below 1 µg/l), whereas in other studies the field residues were far higher (Alkassab and Kirchner, 2017).

The immune system of RMBs may also be affected by NP exposure. Brandt et al. (2020) showed that high doses of 200-555 µg/l THIC reduced the density of immune cells in RMBs, suggesting that NPs can leave RMBs more vulnerable to disease. Although, future work would need to test lower more field-relevant doses and assess if immunosuppression is a consistent effect between NPs.

While exposure of adult RMBs to neonicotinoids may be damaging, larval exposure may not be. Contaminated larval pollen provisions of RMBs with 0, 1, 3 and 10 µg/l clothianidin, showed no impacts on development time, overwintering survival or resulting adult weight (Nicholls et al., 2017), although the study may have overlooked subsequent sub-lethal effects on adult foraging or longevity.

These studies demonstrate that NPs may be negatively affecting RMBs. However, there is little understanding of sub-lethal effects beyond reproductive output. To better understand the impacts of NPs on RMBs, and solitary bees more broadly, this knowledge gap must be addressed.

1.16 The impacts of neonicotinoids on flies

Bees are often the main focus of insect pollinator research, with other insect orders less well represented (Blacquiere et al., 2012; Pisa et al., 2014; Van Der Sluijs et al., 2015). However, the true flies (Diptera) are also known to be important pollinators (Larson et al., 2001; Ssymank et al., 2008; Orford et al., 2015), as they are well known flower visitors and have been shown to transfer pollen between plants (Orford et al., 2015). In particular, the hoverflies (Diptera: Syrphidae) have been identified as pollinators of crops such as rapeseed (Jauker and Wolters, 2008), whilst also providing pest control through the consumption of aphids (Aphidoidea) (Haenke et al., 2009). Additionally, the blue blowfly (BBF) has been used as an effective pollinator of carrot (*Daucus carota*), onion (*Allium cepa*) and field mustard (*Brassica rapa*) (Schittenhelm et al., 1997; Howlett,

2012). In addition, they are often the dominant pollinator Order within alpine and high latitude terrestrial ecosystems, as they can fly at lower temperatures than many bee species (Totland, 1993; Orford et al., 2015). Despite this, almost nothing is known about the organism or population-level impacts of neonicotinoids on this diverse group.

Many NP studies on Diptera have focused on them as pests to be controlled (e.g. *Drosophila suzukii* (Beers et al., 2011) and *Aedes aegypti* (Paul et al., 2006)). Unsurprisingly, these studies have used very high doses and showed large effects on mortality and oviposition, as was the aim. To date, there are just two studies that have considered the impacts of neonicotinoids on flies as beneficial insects. In one study, hoverfly (*Eristalis tenax*) larvae were exposed to concentrations of THIM up to 500 µg/l, which considerably reduced adult longevity (Basley et al., 2018). However, no effects on survival, development, or on adult energy budgets were found below 500 µg/l (Basley et al., 2018). It is possible though that there are sub-lethal effects of NPs that may compromise the flies' pollinating ability that were not considered by these authors. The other study suggests that 0.01-1 µg/l IMI can deter pollinating Diptera from contaminated pan traps (Easton and Goulson, 2013). As flies are important pollinators, a lack of understanding of the impacts of NPs on them could undermine efforts to protect pollination services, stressing the importance of research into this topic.

In contrast, a large part of our understanding of NP impacts at the molecular level, comes from studies of Diptera, namely *Drosophila melanogaster*. It has been shown that, in this species, resistance to IMI is mediated by a group of enzymes

that metabolise xenobiotics, the cytochrome P450s (Daborn et al., 2001, 2007; Denecke et al., 2017). Additionally, genes involved in neuronal development and function are induced by IMI in *D. melanogaster*, re-affirming that the central nervous system (CNS) is a prime target for these pesticides (Denecke et al., 2017). This observation tallies with NPs' activation of nAChRs in the CNS (Brown et al., 2006). Beyond *D. melanogaster*, a fruit fly not renowned for its pollinating ability, there is little available information about the molecular impact of NPs on Diptera.

1.17 Thermal Biology

Little is known about the impacts of neonicotinoids on the thermal biology of insects. Insects are poikilotherms and are as such largely unable to regulate their body temperature, meaning they are vulnerable to changes in environmental temperature such as cold (Bale and Hayward, 2010; Forrest, 2016). Insects have a variety of strategies to cope with low temperatures (Bale and Hayward, 2010; Overgaard and MacMillan, 2017), but it is possible that these could be disrupted by exposure to neonicotinoids. For example, Potts et al. (2018) showed that THIM and IMI affect the ability of BTBs to recover from cold torpor. They found that dietary exposure to 0.1-1 µg/l of IMI increased the rate at which bees recovered from cold torpor, whilst higher doses (1-125 µg/l) decreased it. In contrast, THIM decreased this rate in a concentration dependent manner (Potts et al., 2018). Tosi et al. (2016) also showed possible effects on thermal biology of pollinators, by demonstrating that 20 µg/l THIM reduced the thorax temperature of African honeybees (*A. mellifera scutellata*), potentially showing that bee

thermoregulation was disrupted. Other than these two studies, there is limited research on the impacts of NPs on pollinator thermal biology. As cold is a common stress that pollinators will encounter (Sinclair et al., 2013; Kaunisto and Ferguson, 2016), it is vital that this area is better understood.

One way to better understand the dual impacts of cold and NPs is to consider activity thresholds. These provide an insight into the limits of insect movement at different temperatures and can be experimentally determined by observing insect behaviour during temperature change. For cold, two specific insect behaviours can be observed: the critical thermal minimum (CT_{min}), which is the temperature at which coordinated movement is lost (Figure 1.5); and chill coma, which is the temperature at which the ability to move an appendage is lost (Figure 1.5). Determining activity thresholds after exposure to NPs would allow an insight into how their effects combine with cold and how they may impact on important activities such as mating, foraging, and predator avoidance (Everatt et al., 2013).

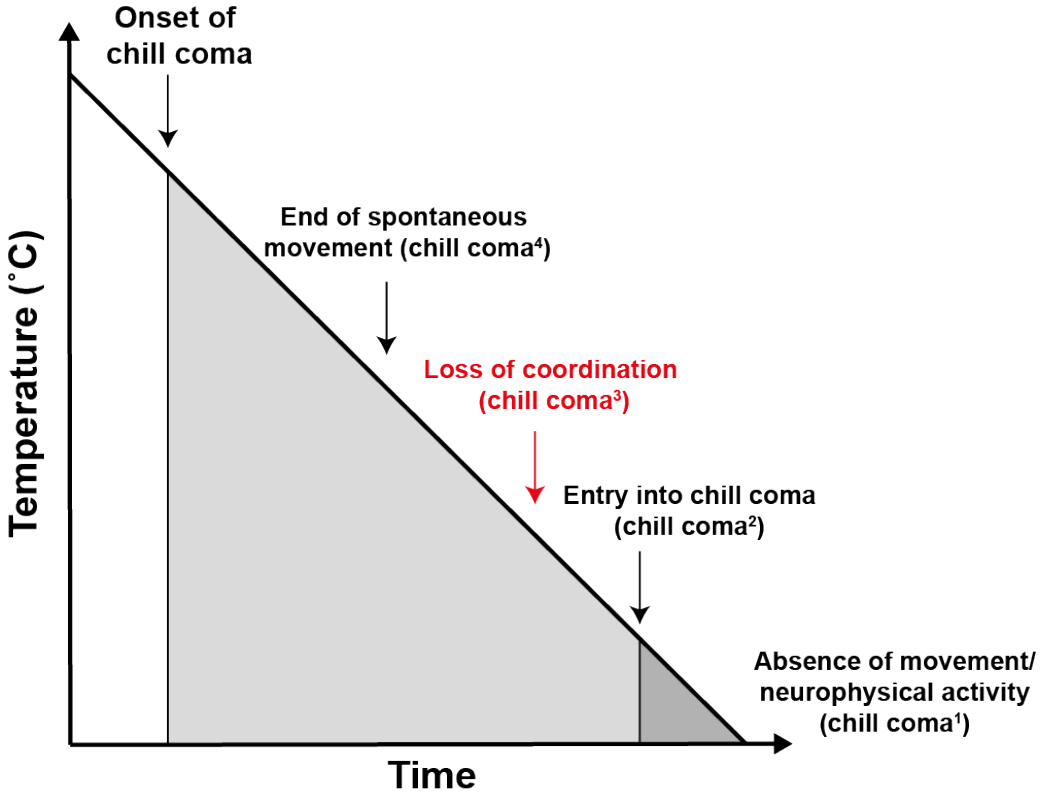


Figure 1.5 Common definitions of critical thermal minimum and chill coma

Figure and legend adapted from Hazell and Bale (2011). In studies of critical thermal minimum and chill coma different behavioural and physiological thresholds are often used interchangeably, so this diagram illustrates the sequence of these thresholds as temperature decreases. The optimum temperature range for normal activity is in white. The section in light grey shows where locomotor efficiency is first compromised, and ends where chill coma is entered (chill coma^2) i.e. where there is an absence of movement/neurophysical activity (chill coma^1), which is designated by a final muscular twitch after which ability to move appendages is lost. Chill coma^4 shows where spontaneous movements end and chill coma^3 shows where coordination is lost. Chill coma the physiological state (chill coma^1) is highlighted in dark grey. In this thesis loss of coordination is used as the threshold to indicate the critical thermal minimum (chill coma^3), and is highlighted in red.

1.18 Molecular responses to neonicotinoids and temperature

As set out above, another area where information is lacking is the molecular mechanisms of the negative impacts of neonicotinoids on pollinators. As with most NP studies, research has again predominantly focused on honeybees and bumblebees, with several studies identifying cytochrome P450s as a key part of the response to NPs (Manjon et al., 2018; Feyereisen, 2018), similarly to the aforementioned Drosopholid studies. In fact, it has been postulated that honeybees and bumblebees particularly sensitivity to NPs are due to there being relatively few of these cytochrome P450s present in the genome, compared to more NP resistant insects such as *D. melanogaster* (Claudianos et al., 2006; Berenbaum and Johnson, 2015).

There are, however, relatively few studies on more global gene expression, such as ribonucleic acid sequencing (RNA-seq). Some studies on honeybees have examined how the transcriptome changes upon neonicotinoid exposure, showing that a broad range of genes associated with immunity, lifespan and development are altered (Wu et al., 2017; Shi et al., 2017). However, for other important pollinators, like bumblebees, transcriptome data are lacking. So far, only two studies have investigated the impacts of NPs on the transcriptome of bumblebees (Mobley and Gegear, 2018; Colgan et al., 2019). In these, as well as that cytochrome P450s, genes involved in muscular regulation and locomotion were also upregulated (Mobley and Gegear, 2018; Colgan et al., 2019). Given the range of impacts of neonicotinoids on bumblebees and their importance to promote crop yields and maintain food security, further investigation is warranted.

Studies of NP exposure in combination with other stressors, such as temperature, are almost entirely lacking from the pollinator literature, and while insect cold tolerance studies abound (see Hayward, 2014a), our understanding of temperature responses in many key pollinators remains poor. So far, no study has assessed this in combination with neonicotinoid exposure.

1.19 Thesis structure

As a BBSRC Industry partnership with the company Biobest®, the thesis begins with the primary aim to investigate methods for commercially mass-rearing RMBs. To that end, the requirements for RMBs to produce offspring in an enclosed environment were explored (Chapter 2). Through these investigations it was found that the seasonal timing of rearing affected the mortality and reproductive outputs of bees. As RMBs only are able to reproduce after a period of overwintering, different methods to prolong or shorten this overwintering period without the negative consequences on survival and reproduction were examined (Chapter 3). It is possible that manipulation of overwintering could also facilitate year-round stockpiling of bees, which would aid their commercialisation, and so this too was investigated (Chapter 3). As these methods involve the control of temperature, the possible impacts of climate change during the overwintering period were also considered (Chapter 3). Here, it was found that warming temperatures may well have negative impact on the survival of RMBs (Chapter 3). Climate change is only one of many possible threats to RMBs though, and so the impacts of neonicotinoid pesticides were investigated (Chapter 4). Here, the joint stress of cold and imidacloprid was probed, as this particular challenge may

occur in a warming world; for example, increasing temperature may advance emergence of RMBs, they may then be exposed to neonicotinoid pesticides, only for temperatures to fall again. As it has previously been well established that imidacloprid has pronounced effects on bumblebees, this study was broadened to include BTBs (Chapter 4). Conversely, as very little is known about the effects of neonicotinoids on fly species, this study was also broadened out to include BBFs (Chapter 4). As it was established that there were substantial effects of cold and imidacloprid on BTBs, and their transcriptome is relatively well characterised, a RNA sequencing study was performed to better understand the changes to the transcripts that underpin the noted impacts of the joint stress (Chapter 5). The overall structure of the thesis is illustrated in figure 1.6. Altogether these investigations sought to determine how stresses, such as temperature and pesticides, may impact pollinator survival, pollination service provision, and ultimately food security, and what strategies are at our disposal to mitigate these effects.

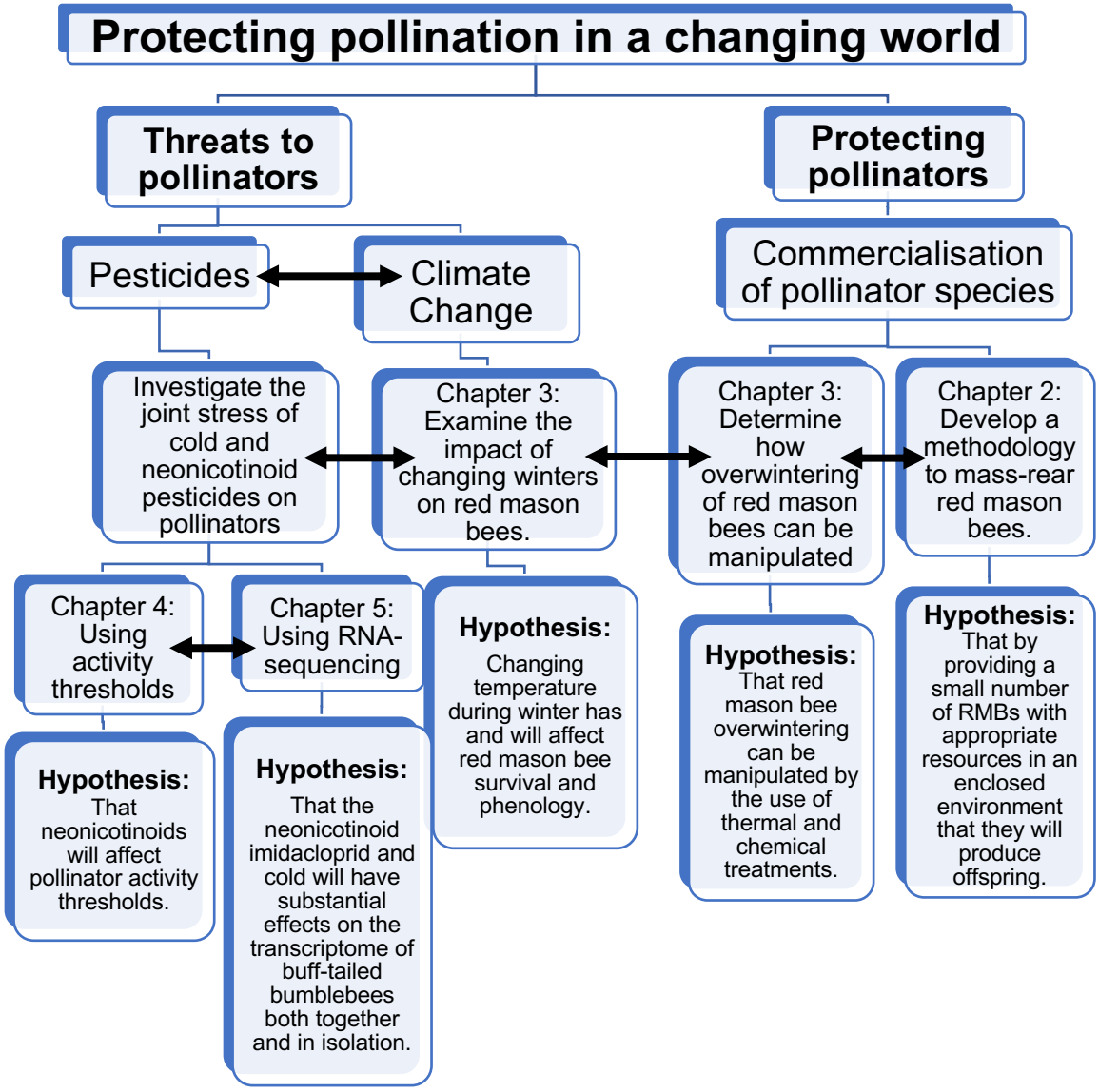


Figure 1.6 Schematic of the thesis chapters and main hypotheses

Here, the main aims and hypotheses of the thesis are illustrated. Double-headed arrows indicate where there are explicit links between the chapters, topics, or aims.

Chapter 2: Commercial viability and mass-rearing of the red mason bee

2.1 Abstract

Pollination is a vital ecosystem service ensuring food security. There are however many challenges to the pollination service provided by natural populations of pollinators and some crop yields may already be limited by insufficient pollination. To combat this, growers regularly use commercially mass-reared bees to enhance fruit set. Mason bees (*Osmia* spp.) are not yet the target of concerted commercial rearing efforts, yet these species have been shown to be effective pollinators of orchards in particular. This chapter introduces the outcomes of experiments aimed to investigate mass-rearing of the red mason bee (*Osmia bicornis*) under laboratory conditions. Light intensity was identified as important in influencing mating and oviposition and had a positive correlation with bee activity and may be a key requirement for rearing. It was also found that large rearing enclosures, and perhaps a high number of nests, may be necessary to prevent cannibalism of eggs. However, very low numbers of adult offspring produced throughout the experiments, indicate that laboratory rearing might not be a viable proposition at the current time. To create a commercial product there are several further challenges that need to be overcome, but the methodology outlined here will be a crucial starting point for any rearing operations.

2.2 Introduction

Pollinator declines have been well-documented. Indeed, in Europe around 9% of bees and 9% of butterflies are endangered (Potts et al., 2016) and in some European Union (EU) countries approximately 50% of known bee species are threatened (Nieto et al., 2014). Such declines have raised concerns that the vital pollination services provided by pollinators are under threat (Potts et al., 2016). In fact, many crops are already limited in their yields by lack of pollination (Garibaldi et al., 2011; Garratt et al., 2014b; Klein et al., 2015). In this context, commercial operations providing on-demand pollinators could deliver pollination services to partly fulfil a potential pollination shortfall.

Currently, managed western honeybees (*Apis mellifera*) and commercially grown bumblebees (*Bombus* spp.) are being used extensively to fulfil pollination demands from growers (Klein et al., 2007; Goulson and Hughes, 2015). For example, mass rearing of bumblebees (e.g. buff-tailed bumblebees [BTBs] and the common eastern bumblebee) has been successfully performed for decades (Velthuis and Van Doorn, 2006). How exactly these bees are reared is not clear as the methodology used by different commercial suppliers is a trade secret (Velthuis and Van Doorn, 2006), but, in general, rearing is performed using mated queens that have just emerged from diapause. The queens are housed in small boxes at 28˚C and 60 % relative humidity (RH), provided with workers (bumblebee or honeybee workers are sufficient), nectar and pollen, to support the queen to produce her own progeny. Once the colony reaches a sufficient size (around 40-100 workers) it is transferred to a larger container and shipped to

growers (Velthuis and Van Doorn, 2006). However, they are perhaps not best suited as pollinators for every situation (Chapter 1, Section 1.3). As a result, there have been recent efforts to promote solitary bees as viable commercial products. For example, the alfalfa leafcutting bee (*Megachile rotundata*) has become intensively managed in the United States of America (USA) and Canada for alfalfa (*Medicago sativa*) pollination (Pitts-Singer and Cane, 2011), and the blue orchard bee (*Osmia lignaria*) is being investigated as a commercial pollinator for orchards (Bosch and Kemp, 2000; Kraemer et al., 2014).

Orchard crops are heavily pollination dependent (Klein et al., 2007; Garratt et al., 2014b). Pollination was shown to be the most important factor for almond yields, above even nutrients and water (Klein et al., 2015), so they are a potentially lucrative market for commercial pollinators. It is estimated that fruit set in apple orchards in the United Kingdom (UK) could be increased by 8% by optimised pollination (Garratt et al., 2014b). In 2017, the apple industry in the UK was worth £128.7 million and is growing annually (DEFRA, 2019), so enhanced pollination could mean an annual estimated £10 million increase in revenue.

Mason bees (*Osmia* spp.) are suitable pollinators for orchard crops (Bosch and Kemp, 2002), due to their high stigma contact and visitation rate, and their emergence is concurrent with orchard blooms (Bosch, 1992; Vicens and Bosch, 2000; Maccagnani et al., 2003; Monzon et al., 2004). They may even be more suited to orchard pollination than western honeybees (*Apis mellifera*), as honeybees tend to ‘side-collect’ from fruit trees, biting into the side of the flower to access only nectar, thus preventing high rates of pollination (Free, 1960; Bosch

and Kemp, 2002; Finta, 2004). Red mason bees (RMBs) are considered particularly desirable species for commercialisation as they share the discussed advantages of other mason bees (Gruber et al., 2011; Schindler and Peters, 2011), as well as pollinating a wider variety of crops (Haider et al., 2014). However, the RMB lifecycle includes obligate diapause rendering them unavailable as pollinators for large parts of the year (Chapter 1, Figure 1.2; Chapter 3) whilst also making it challenging to time their emergence with target crop bloom (Chapter 3). Moreover, there is limited information available about their rearing, as for the most part they are cultivated in orchards under essentially natural conditions (Gruber et al., 2011; Schindler and Peters, 2011).

The management of RMBs was examined in a limited number of pioneering studies. Holm (1974) assessed RMBs as greenhouse pollinators and determined them to be suitable pollinators of *Brassica* crops, as males and females readily visited flowers upon emergence (Holm, 1974). In 1989, research commenced to formalise a greenhouses management system for RMBs (Van der Steen and De Ruijter, 1991). The study focused on the overwintering period and showed that the optimal incubation time was between 120-170 days, with bees stored for longer or shorter durations being less active and unable to forage (Van der Steen and De Ruijter, 1991). There have been relatively few studies since, and focus appears to have shifted to cultivation of these bees in field conditions, typically orchards.

Only one example exists where RMBs were successfully reared under laboratory conditions, although the experiment was designed to ascertain the impact of the

pesticide clothianidin on their reproduction (Sandrock et al., 2014). The results showed that 125 female and 75 male bees produced 808 larval offspring, approximately six per female, indicating that the conditions used in this study could be a promising strategy for mass-rearing. However, the bees were reared in a large (4.3 x 2.4 x 1.8m) climate-controlled room with a sunlight simulation chamber, where temperature, relative humidity and light were controlled to imitate natural conditions (Sandrock et al., 2014). The importance of these variables may be key to develop a rearing protocol for RMBs.

Light intensity is important for RMB activity levels; they are more active on bright sunny days (Menzel et al., 1988; Seidelmann et al., 2010) and studies have used natural light from a window augmented by artificial light in their experimental designs to promote mating (Fliszkiewicz and Wilkaniec, 2009; Conrad et al., 2010; Seidelmann, 2015). In the Sandrock et al. (2014) study the maximum light intensity was 74,000 lux (1,000 µmol photons/m^2/s, approximating a typical sunny day). Additionally, *O. cornuta* has been shown to be active at around 200w/m^2 (~25,000 lux, approximating shade on a sunny day; Vicens and Bosch, 2000). Temperature is also correlated with successful outcomes of RMBs lifecycles: Holm (1974) found that the minimum temperature for activity was 15-18˚C, while Raw (1972) observed that activity in RMBs ceased below 12˚C. Strohm et al. (2002) showed nesting activity was considerably reduced for *O. bicornis* at temperatures under 20˚C, a finding replicated by Fliszkiewicz et al. (2015). It is likely then that a minimum light intensity of 25,000 lux and a temperature of 20˚C or higher may be required to rear RMBs successfully.

Bosch and Kemp (2002) used mason bees as a case study on how to develop a bee from a wild species to a managed crop pollinator, highlighting the key steps required to do this. Since then several more studies have explicitly set out management protocols for RMBs in orchards, examining how to increase population sizes and promote nesting by wild bees by using nesting aids (Ivanov, 2006; Gruber et al., 2011). Indeed, several companies exist that capture wild RMBs using trap nests, maintain them in controlled environments over winter, and then sell RMB cocoons (pre-emerged adults) to growers the following season (Appendix 1.1). However, the number and quality of bees obtained using these management methods can be highly variable due to unpredictable weather conditions and/or high levels of parasitism etc. (Bosch and Kemp, 2002). There is some doubt therefore that such an approach can generate the quantities of bees required to sustain food security. Indeed, within this project, adequate numbers of the UK sub-species of RMB (*O. bicornis rufa*) were difficult to obtain; the UK supplier was unable to meet the modest demands of a scientific experiment, citing poor weather conditions as a reason for low bee numbers (R. Dean, personal communication). This necessitated the use of the European sub-species (*O. b. cornigera*) provided by a German supplier (WAB-Mauerbienenzucht, Konstanz, Germany) for some experiments.

Both RMB subspecies occur naturally in Europe and are potentially suitable for mass-rearing in any commercial operation. Each sub-species could be reared separately for export to their native regions, therefore covering most of Europe (Appendix 2.1). Thus, mass-rearing of RMBs under controlled conditions could

provide bees across Europe to growers and ensure numbers of bees regardless of climatic conditions. Moreover, for commercial pollination provision bees may be required to pollinate many crops in diverse locations with distinct flowering periods. Many crop species are not synchronised with the natural active period of RMBs (spring and early-late summer), but this may be overcome with commercial rearing. There is a possibility that RMB emergence can be chemically forced to time with crop blooms (Chapter 3) and combined with a mass-rearing system year-round provision of RMBs to growers is possible.

In the UK, RMBs are naturally active from around April until July only (Chapter 1, Figure 1.2). In order to supply RMBs year-round commercial rearing may have to shift the natural active period of the bees, which is currently performed by prolonging their dormancy using low temperatures (Bosch and Kemp, 2000; Biliński and Teper, 2004; Pitts-Singer et al., 2008). However, there is evidence that keeping RMBs in cold storage may increase bee mortality (Giejdasz and Wielkaniec, 1998), perhaps due to increased usage of energy resources (Dmochowska et al., 2013). Moreover, a shortening of the dormancy may also have negative effects on the survival of the bees, as this has been demonstrated in other species of mason bee (Sgolastra et al., 2010). It is possible that that different treatments can be used to manipulate RMB diapause and dormancy, which is assessed later in this thesis (Chapter 3), but without such manipulations, the timing of mass-rearing may be somewhat fixed, as prolonged or interrupted storage could cause negative effects on the survival and longevity of the bees.

This chapter outlines the testing of different methods to mass-rear RMBs for a commercial partner, Biobest® Group N.V., in order to see if commercial provision of this species is a viable proposition. Initially, experiments were performed to identify appropriate light intensity, minimum spatial scale of enclosures, suitable food sources, temperature, humidity and material for nest construction. The results from these experiments were then used to inform further experiments, which aimed to identify whether increasing the spatial scale or rearing arenas and light intensity, as well as whether the inclusion of flowering plants as a food source could increase the number of offspring produced. In addition, these experiments sought to explicitly link the activity (walking, flying, and mating) of bees to different factors tested, as activity has been associated with reproductive output (Fliszkiewicz et al., 2015).

Based on the literature discussed here, I hypothesise that by providing a small number of RMBs with appropriate resources (outlined in studies such as Sandrock et al., 2014) in an enclosed environment that they will produce offspring, as has previously been recorded. Additionally, I hypothesise that the number of offspring can be increased by improving the environmental conditions for the RMBs.

2.3 Methods

2.3.1 Study organism

The RMB is a strictly univoltine bee, active from around April to July in the UK (Chapter 1, Figure 1.2). Offspring develop from around July to October, and, upon

reaching adulthood, they wrap themselves in cocoons; the bees then overwinter as pre-emerged adults in an obligatory diapause (Dmochowska et al., 2013), until warmer temperatures cue their emergence in spring (Chapter 1, Figure 1.2).

Reproductive success is mostly dependent on the females, as they exclusively rear the brood: provisioning the brood cells with pollen and nectar, upon which each egg is laid, and constructing separate brood cells with mud partitions (Seidelmann et al., 2010). Due to their role in rearing, females are larger and longer-lived than the males, averaging 10-12 mm in size and living around six-eight weeks, compared to 8-10 mm and two-four weeks in males (Steffan-Dewenter and Schiele, 2004; Gruber et al., 2011).

Two subspecies of RMBs were investigated in this study: *Osmia bicornis rufa* and *Osmia b. cornigera*. *O. b. rufa* were purchased from the Red Beehive Company (formally Red Beehive Ltd.; Southampton, Hampshire, UK) and *O. b. cornigera* were purchased from WAB-Mauerbienenzucht (Konstanz, Baden-Württemberg, Germany). The purchased bees were overwintering as pre-emerged adults in cocoons at the start of each year. To prevent emergence of bees until experiments commenced (see table 2.1), they were stored at 2˚C upon receipt using a Sanyo MIR-254 incubator (Sanyo Electric Co. Ltd., Moriguchi, Osaka, Japan).

Overall, the rearing experiments were divided into two parts: 1. A set of mating and nest establishment trials (hereafter – preliminary experiments), where the design of the rearing environment was iterated upon to determine the minimum

requirements for mass rearing of RMBs and 2. An experiment designed to ascertain whether additional lighting or plants would increase the number of progeny produced by the bees (hereafter – secondary experiment), and also to improve culturing methods within a glasshouse setting.

2.3.2 Enclosure design

2.3.2.1 Preliminary experiments – mating and nest establishment

Six preliminary experiments were conducted at the University of Birmingham (Birmingham, UK), inside Bug-Dorms (MegaView Science Co., Talchung, Taiwan). Each Bug-Dorm (hereafter enclosure; Figure 2.1B) was placed in a climate-controlled room at 20˚C. The temperature was chosen as it is thought to be the minimum suitable temperature for RMBs to fly, mate and provision brood (Raw, 1972; Holm, 1974; Strohm et al., 2002; Fliszkiewicz et al., 2015), and at higher temperatures (> 25˚C) larval development can be negatively affected (Rust et al., 1989; Radmacher and Strohm, 2010), additionally a climate controlled room at 20˚C was available for the rearing experiments. High light intensity conditions (~60,000 lux) were provided using 6 light emitting diode (LED) panels which surrounded the enclosure, set to a 16:8 light:dark (LD) cycle (Figure 2.1A). These LED panels used wavelengths of light around 420-470 nm, which is at the lower end of the visible light spectrum of sunlight (Iqbal, 1983), but is similar to the wavelengths of light used by Sandrock et al. (2014) in their sunlight simulation chamber. Cocooned (pre-emerged adult) bees were introduced to each enclosure at the start of each experiment and allowed to eclose (the number

of bees varied between trials, see Table 2.1). To meet the desired sex ratios cocooned bees were selected based on size (females are generally larger than males), once bees had eclosed the sexes were confirmed based on the colour of clypeal hairs and the size of antenna (Raw, 1972). In addition, cocoons were inspected for signs as parasitism, by rolling them between fingers to ascertain if they were full of parasitic wasp (*Monodontomerus obscurus*) larva (infested cocoons feel rigid as they expanded to their limits with wasp larva), parasitized cocoons were discarded.

Nesting compartments were holes 8 mm wide and 120 mm deep, drilled within unseasoned pine wooden blocks (Figure 2.1D). Wooden nests were used as these have found to generate the most brood cells compared to other materials (Wilkaniec and Giejdasz, 2003). Each drilled hole is hereafter referred as a single 'nest'. RMBs also require a mud-like material to build partitions between brood cells in their nests (Raw, 1972; Ivanov, 2006), so a plastic container with cell construction material was provided (Figures 2.1C and D). This material was made up of a mixture of modelling clay (Scola Creative Arts Products Ltd., Middlewich, UK), Fontainebleau sand (VWR International, Lutterworth, UK) and water in a 2:2:1 ratio, was attached to an additional plastic container filled with water (Figure 2.1C). Water was allowed to diffuse into the mud through one mm perforations of the divider located between the two containers, allowing for a moisture gradient to form in the mud (Figure 2.1C; Sandrock et al., 2014). Water was topped up as necessary.

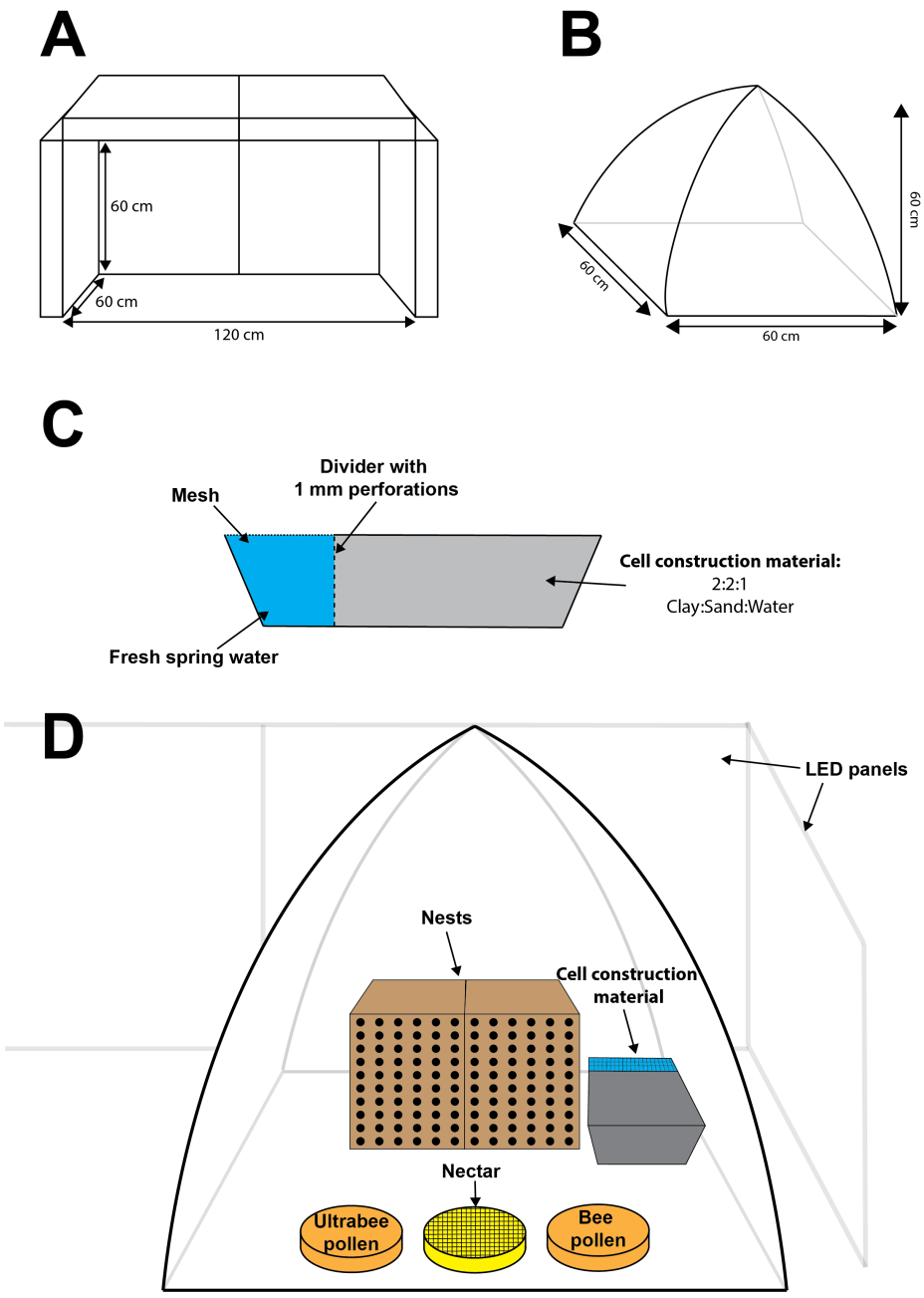


Figure 2.1 Schematic of bee enclosures used in preliminary experiments

A Dimensions of LED panels surrounding enclosures. **B** Dimensions of enclosure. **C** Diagram of container with cell construction material and attached water, used to create a moisture gradient, based on descriptions by Sandrock et al. (2014). **D** Set-up inside enclosure, to scale. Components as indicated in the diagram, detailed in the text.

Pro-Sweet Liquid Feed (Solid sugar 76.5 - 77.5%, Moisture 22.5 – 23.5%; Carbohydrates: Fructose 22%, Dextrose 27%, Sucrose 50%, Maltose .5%, Higher saccharides .5%; Mann Lake Ltd., Hackensack, Minnesota, USA), and was made available to bees, as a nectar resource, in petri dishes covered with mesh (to prevent drowning). Additionally, 25 g of two types of pollen, ground up honeybee pollen (consisting of pollen from > 15 plants; Nutriseed, London, UK) and artificial honeybee pollen, Ultrabee (Mann Lake Ltd.), were added to petri dishes. Pollen and nectar were replaced weekly, and were stored in at 20˚C.

Before anything was introduced to the enclosures, it was irradiated with ultraviolet (UV) light for a minimum of two minutes using a UV cross linker (UVItec, Cambridge, UK), to remove contaminants or pathogens. This is per the standard protocol used in industrial bee rearing, for example at Biobest®.

2.3.2.2 Secondary experiment – effect of lighting and plants in a greenhouse environment

A secondary experiment with five enclosures was conducted in a temperature-controlled greenhouse at the Biobest® facility (Westerlo, Belgium). Key differences to the preliminary experiments are set out in Figure 2.2.

As this was a glasshouse environment light and temperature varied throughout the experiment, but were monitored as described below, In each of the five enclosures, 100 cocoons (size sorted for approximately 60♂♂ and 40♀♀) were introduced at the start of the experiment. An additional 100 cocoons (size sorted

for approximately 60♂♂ and 40♀♀) were added 17 days later. Bees were provided

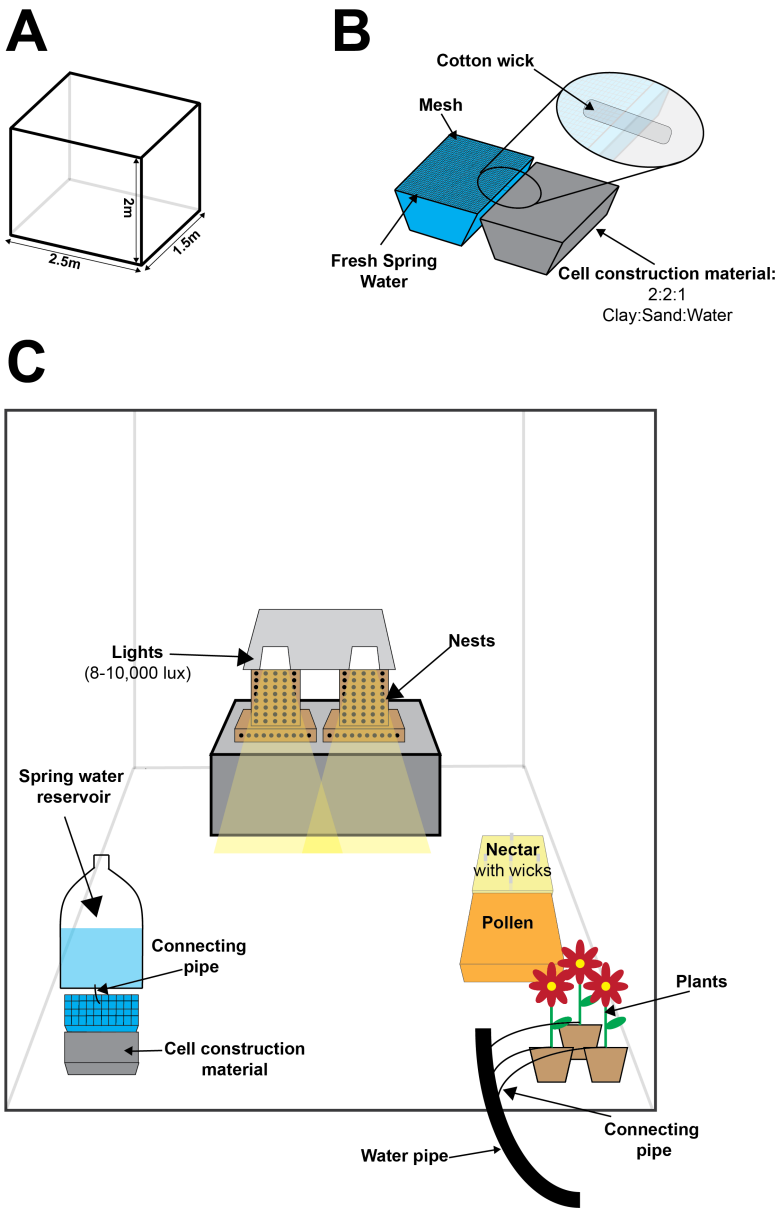


Figure 2.2 Schematic of bee enclosures used in the secondary experiment

A Dimensions of enclosure. **B** Diagram of container with cell construction material and attached water, used to create a moisture gradient, based on descriptions by Sandrock et al. (2014). **C** Set-up inside enclosure, to scale. Components as indicated in the diagram, detailed in the text.

with 100 wooden nests, as described previously. Wooden nesting blocks were placed on a plastic box to elevate them (Figure 2.2C).

In each cage, a mixture of 75% Nutrigluc® (Biobest Group NV, Westerlo, Belgium), a sucrose dominated nectar solution, and 25% water was made available to bees as a nectar resource (Figure 2.2C). Nectar was placed in a small plastic container where cotton wicks allowed its diffusion, so the bees could feed without drowning.

Also, 500 g of ground up honeybee pollen (Biobest Group NV, Westerlo, Belgium) was provided in a small plastic container (Figure 2.2C). Cell-construction material was made available to bees, as described previously (Figure 2.2B). The water was fed from a large spring water container, to ensure it was always topped up (Figure 2.2C).

2.3.3 Design of trials to ascertain impacts of different environment variables on bee reproductive output

Different spatial scales were used, along with different numbers of bees, to determine whether bee density and space influenced reproductive output (Table 2.1). Plants were also added at the start of the experiment for trial 3 (Table 2.1), to identify if their addition would affect the reproductive output of the bees, as RMBs have been noted to mate in and around plants (Seidelmann, 1999). These plants were purchased from Winterbourne House and Garden (Birmingham, UK).

Table 2.1 Summary of differences between mass rearing trials to discern impacts of different environment variables on red mason bee reproductive output

	Trial	Commenced	Subspecies	Enclosure size (m^3)	Light intensity (lux)	Temperature (˚C)	Nests available	Male bees	Female bees	Plants	Rationale
Preliminary experiments	**1**	February 2016	*rufa*	0.06	60,000	20	120	65	44	None	Based on literature synthesis to discern minimum viable rearing conditions
	2	April 2016	*rufa*	0.03	60,000	20	50	7	28	None	To determine if a higher ratio of females:males would increase reproductive output
	3	July 2016	*rufa*	0.11	60,000	20	50	15	15	*Buddleja* sp., *Geranium* sp. *Mentha* sp.	To determine if introduction of plants would increase reproductive output
	4	August 2016	*rufa*	0.03	60,000	20	50	9	25	None	A repeat of the most successful trial (trial 2)
	5	March 2017	*cornigera*	0.65	60,000	20	120	30	30	None	To determine if a larger enclosure and lower number of bees would reduce cannibalism
	6	May 2017	*cornigera*	1.03	3,000	20	120	30	30	None	To determine effect of light intensity
Secondary Experiment	**A**	March 2018	*cornigera*	7.5	11,040* + 8,500	26.6*	100	60†	40†	None	1) To see if large enclosures would reduce cannibalism 2) To monitor whether fluctuations in temperature and light affect activity 3) To test if additional lighting or plants had an effect on reproductive output
	B	March 2018	*cornigera*	7.5	11,040* + 8,500	26.6*	100	60†	40†	*Erica* sp. *Ranunculus* sp.	
	C	March 2018	*cornigera*	7.5	11,040*	26.6*	100	60†	40†	*Erica* sp. *Ranunculus* sp.	
	D	March 2018	*cornigera*	7.5	11,040* + 8,500	26.6*	100	60†	40†	*Erica* sp. *Ranunculus* sp.	
	E	March 2018	*cornigera*	7.5	11,040*	26.6*	100	60†	40†	None	

A description of each trial conducted and the differences therein. In the secondary experiment certain trials (A, B and D) included LED lamps, this is indicated by + 8,500 (how much light intensity was increased at 10 cm distance).
*Average over the course of the experiment
†An additional 100 bees (60♂♂ and 40♀♀) were added 17 days into the experiment

The plants were placed inside the enclosure and were watered every other day. These plants were chosen based on their suitability as pollen sources for bees (Haider et al. 2014), and on what was available.

Light intensity was considered to be very important to rearing of RMBs, so in trials 1-5 high light intensities of 60,000 lux were provided (Table 2.1). However, to ensure that light was as important as hypothesised, in trial 6 RMBs were reared at a light intensity of 3,000 lux. This lower light intensity was provided by a rack of fluorescent strip lighting (although this likely changed the spectral quality of the light as well).

For the secondary experiment conducted at Biobest®, to prevent cannibalism (as observed in the preliminary experiments) the largest enclosures and number of nests possible were used in each experiment, 7.5 m^3 (Figure 2.2A). Preliminary experiments had emphasised the importance of light, so additional lighting was added to some enclosures to determine its effect. Also, whilst plants were added to one of the preliminary experiments it was unclear what their effect was, so the addition of plants was also assessed.

To that end five enclosures were constructed (Figure 2.2) with the only difference being whether or not LED lamps or plants were included (Table 2.1; again, these lights produced wavelengths of light around 420-470 nm). When used, plants were purchased from Aveve NV (Geel, Belgium). Three heath plants (*Erica* spp.) and three buttercup plants (*Ranunculus* spp.) were used, based on descriptions of what plants the bees commonly use as pollen sources (Haider et al. 2014), and what was available. Additional lighting (~8,500 lux) was supplied via two LED

lamps placed on top of nests. As these experiments took place within a greenhouse, light did also fluctuate with weather conditions, so it was monitored throughout, as described below.

2.3.4 Monitoring of mating, reproductive output, mortality, temperature and light

During preliminary experiments, enclosures were observed for 10 mins each day (at 10 am every day) and assessed for mating events, recorded when a male was performing pre-copulatory courtship (as described in Conrad et al., 2010). Based on observations this interval was considered to be a sufficient period of time to sample mating, as pre-copulatory courtship only lasts anything from a few seconds to a few minutes (Conrad et al., 2010) – whether the attempt was successful was not recorded as it is impossible to determine without removal of the bees from the rearing environment. For the secondary experiment, this monitoring occurred twice daily (at 10am and 3pm), and the activity of bees was also recorded. Bees were defined as active when walking, flying, mating, feeding, constructing nests, or provisioning brood cells (modified from Schenk et al. [2018a]). Every time a bee was active this was counted; bees were counted again if they ceased activity for more than five seconds and then recommenced activity. During this observation period, the enclosures were also monitored for mortality.

In the secondary experiment, enclosures were placed within a greenhouse. This meant that light intensity, relative humidity (RH) and temperature naturally varied with weather conditions. Due to this, light intensity was monitored using the Lux Light Meter Pro app (developed by Elena Polyanskaya) on an iPhone SE, which

was validated using a photosynthetically active radiation (PAR) sensor (Quantum sensor, Apogee instruments, Logan, Utah, USA; Appendix 2.2), where three readings were taken for one minute each and averaged together. Temperature and RH were monitored using TinyTag Plus Data Loggers (Gemini, Chichester, UK). As light, RH and temperature varied throughout the experiment and activity was monitored, it allowed assessment of how these factors may impact activity.

At the end of all the experiments, when all the original adult bees had died, the enclosures were assessed for: the individual number of brood cells provisioned, defined as when the females had partitioned a section with mud and laid down pollen and nectar inside; and the number of eggs laid. If eggs were laid, they were monitored to assess the final life stage reached. Altogether, these observations gave the number of offspring produced by the original RMBs and their final life stage i.e., their reproductive output.

The amount of cannibalism was also monitored. Cannibalism was defined as where an egg was missing from a brood chamber but with evidence it had been broken into and there were indentations on the pollen ball where an egg would have originally been laid.

2.3.5 Statistical analyses

An alpha threshold of .05 was used (i.e., results were considered 'significant' if *p*-values were below .05), except where otherwise stated. No data were transformed, unless otherwise stated. Statistical outputs, including test-outputs, degrees of freedom (DF) and p-values are described in the text.

For mating events in the preliminary experiments, a per female ratio were calculated by dividing the total number of observed mating events by the maximum number of female RMBs present to allow comparability between experiments where the number of bees differed. For the secondary experiments, the total number of mating events per condition (Table 2.1) were recorded and as two experiments were identical (+Lights/+Plants) in this case a mean was calculated along with standard error.

Mortality of original adult bees was monitored, but the total number of bees differed between trials (Table 2.1). Therefore, to allow comparability between trials a mortality rate was calculated. This was done by working out the average percentage of total bees that died each day. As the data were not normally distributed (as determined by a Shapiro-Wilk test for normality [$W = .80$; $p = .05$] and due to the bounded nature of percentage data) a binomial generalised linear model was used. Here, the difference between the null and residual deviance indicates if there is an association between the variables. In this case, a *p*-value, as determined by Hosmer-Lemeshow goodness of fit test, is used to identify how well the model fits the data, where a *p*-value > .05 shows no significant difference between the model and the observed data i.e., a good fit.

To find associations between light intensity and bee activity (as defined above) exploratory scatter plots were used to interpret patterns in the data. To allow comparisons between the enclosures, which had different numbers of bees at different times, total activity count was divided by the total number of bees present to give an activity index. As the data were not normally distributed (as determined

by a Shapiro-Wilk test for normality [W = .95; p = .02] and due to the bounded nature of the bee activity data) a binomial generalised linear model was used, as described above.

For activity measurements in the secondary experiment, the four conditions were compared using a Kruskal-Wallis test, as data were not normally distributed (as determined by a Shapiro-Wilk test for normality [W = .95; p = .02], and due to the bounded nature of the bee activity data). To discern differences between the individual groups, for post-hoc analysis, pairwise Mann-Whitney U tests were used, with a Bonferroni correction.

For data on reproductive output of the bees, total counts at the end of the experiment were conducted. Due to the limited amount of data, statistical analyses were not performed.

2.4 Results

2.4.1 Mating

Mating was observed in all preliminary experiments, with the exception of trial 6 (Table 2.2). As this was an iterative process, each rearing design was changed to attempt to improve upon the last, therefore the number of variables changing across these preliminary experiments precludes any analysis of relationships between mating and environmental variables. Two trials were comparable however, trials 2 and 4 (Table 2.1), as these were almost identical except for being conducted in different months (Table 2.2), and having a slightly different male:female ratio (Table 2.2). In this case there were more than five times the

number of mating events per female in trial 2 (April) compared to trial 4 (August) (Table 2.2).

Table 2.2 Observed mating events per female in each of the preliminary experiments and how they compare to the number of males

Trial	Month	Mating events per female	Male:Female Ratio
1	February	1.7	1.5:1
2	April	2.1	0.25:1
3	July	0.7	1:1
4	August	0.4	0.36:1
5	March	0.8	1:1
6	April	0	1:1

Mating events per female were calculated by dividing the total number of observed mating events by the maximum number of female RMBs present. Enclosures were monitored for 10 minutes each day for mating events, as described in the methods.

In the secondary experiments, the incidence of mating was highest where additional lighting was added, while the inclusion of plants appeared to reduce the incidence of mating (Figure 2.3).

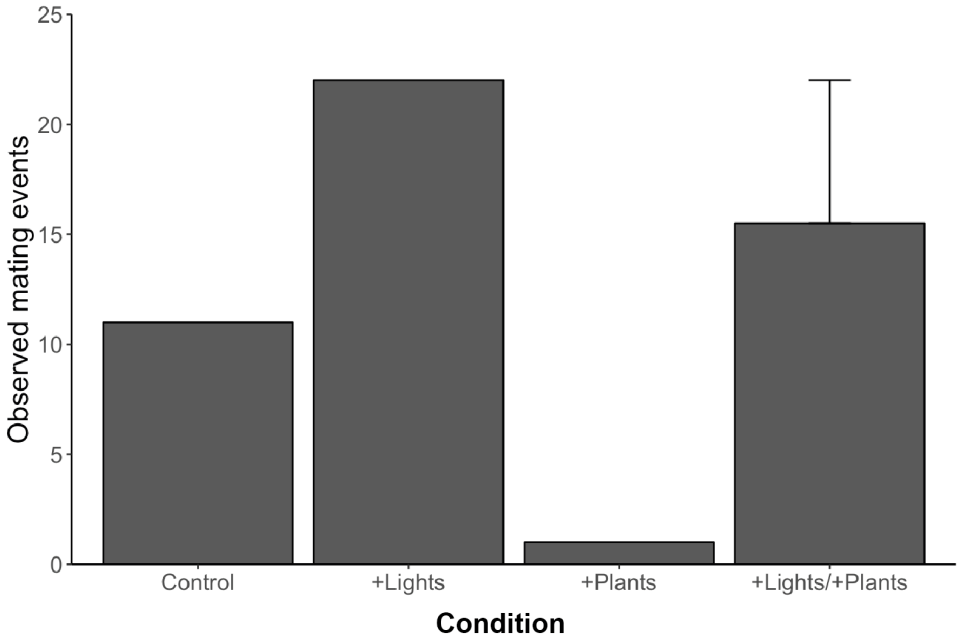


Figure 2.3 Total number of mating events observed in the secondary experiment in each of the different conditions

The x-axis descriptions refer the different conditions in each enclosure (Table 2.1), i.e., whether additional lighting or plants were added. As conditions were identical in trials B and D the total mating events were averaged together in the group '+Lights/+Plants' and an error bar was calculated for this group, with one standard error.

2.4.2 Mortality of original adult bees

For the preliminary experiments a significant trend was found for the mortality rate across the different trials that was associated with the timing of the trial (binomial generalised linear model; y = 1 ÷ (1+ exp-[-5.81 + 0.41x]); null deviance = .11; Residual deviance = .00; DF = 5; p = .99; Figure 2.4), with the lowest mortality rate observed in the trial conducted in February and increasing exponentially in each trial from May towards August (Figure 2.4).

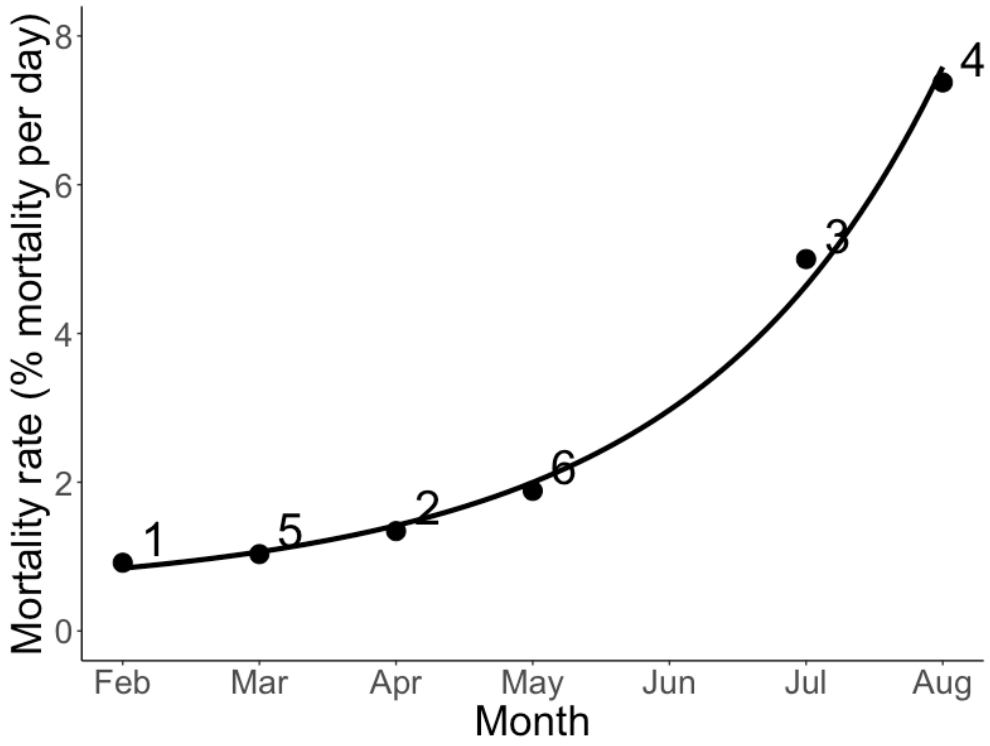


Figure 2.4 Mortality rate of bees as the year advances

Mortality rate was calculated by determining the percentage of bees that died daily. The points show calculated mortality rate in each trial and the line shows a binomial generalised linear model. Different trials (Table 2.1) are denoted by labels next to the points.

2.4.3 Activity of bees during the secondary experiment

During diurnal fluctuations within the greenhouse, temperature and RH showed no association with activity (binomial generalised linear model; null deviance = 15.26; Residual deviances = 15.25 and 15.23 respectively; DF = 59; p = .99 and .99 respectively), but light intensity did (binomial generalised linear model; y = 1 ÷ (1+ exp-[-1.07 + .0001x]); null deviance = 15.26; Residual deviance = 13.37; DF = 59; p = .99; Figure 2.5).

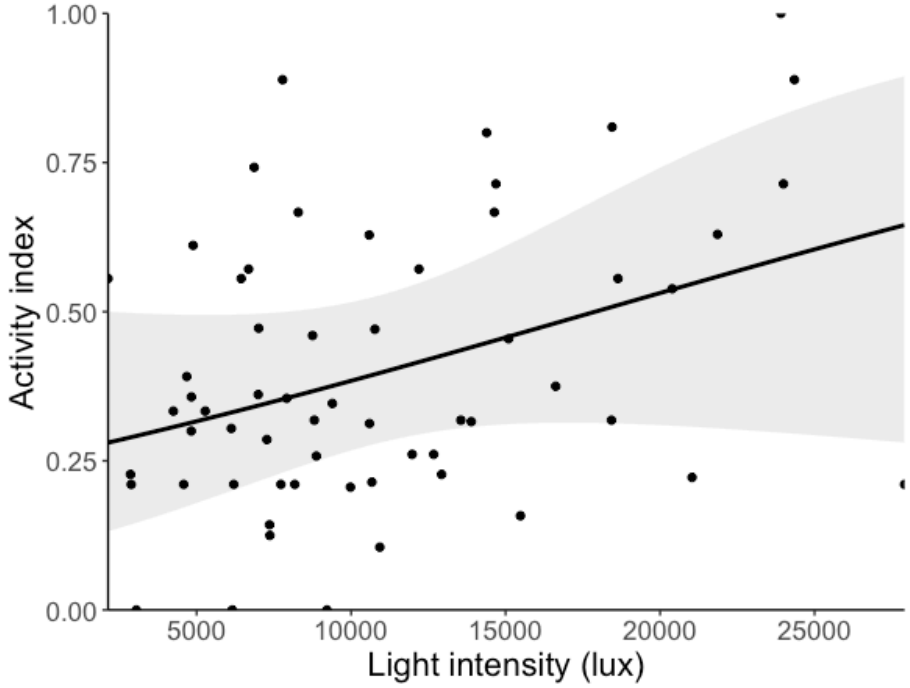


Figure 2.5 The association between light intensity and a calculated bee activity index

The activity index was a measure of bee activity that was calculated by monitoring activity and then dividing it by the number of bees present. To avoid the possible influence of additional lighting and plants on activity only data from trial E was shown (Table 2.1). The line shows a binomial generalised linear model, and the shaded error shows 95% confidence intervals.

Activity of RMBs also differed between the different conditions (Figure 2.6). Where lights were present (and no plants) activity was the highest and was significantly different from the condition where neither plants nor lights were added (Kruskal-Wallis; $\chi^2 = 20.51$; DF = 2; $p < .001$; Figure 2.6).

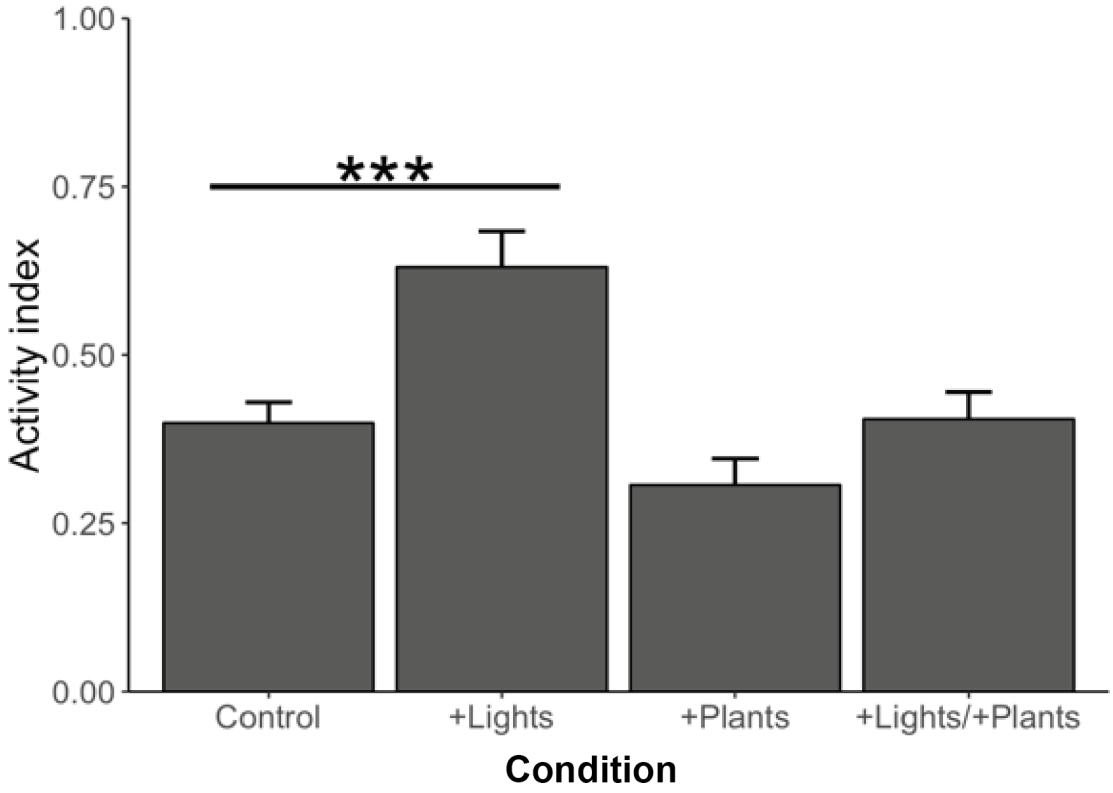


Figure 2.6 Activity observed in the secondary experiment in each of the different enclosures A-E

The x-axis descriptions refer the different conditions in each enclosure, whether additional lighting or plants were added (Table 2.1). The activity index was a measure of bee activity that was calculated by monitoring activity and then dividing it by the number of bees present. The bars show the average activity index in each different condition. The error bars show one standard error. *** indicates a $p < 0.01$ compared to the control condition, as determined by pairwise Mann-Whitney U tests, with a Bonferroni correction.

2.4.4 Reproductive output

For the preliminary experiments, reproductive output was only observed in trials 1, 2 and 5 (Figure 2.7). Only in trial 5 did this reproductive activity lead to a successful production of adult offspring, and in this case one adult was produced (Figure 2.7). This represented only a small fraction of the brood cells that were provisioned (Figure 2.7). In the secondary experiment two trials (control and +Lights) showed reproductive output (Figure 2.7), but only in the control did any progeny survive to adult eclosion (1 individual). In none of the conditions where plants were present was there any reproductive output (Figure 2.7).

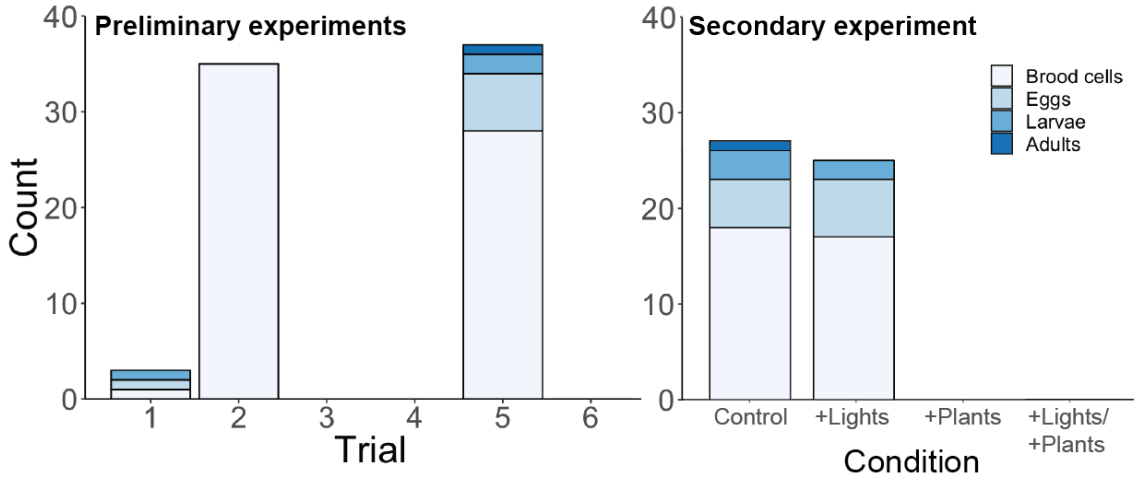


Figure 2.7 Reproductive output of red mason bees in each of the different environmental conditions tested

Bars show total counts of the reproductive outputs of the bees in each of the trials. 'Nests' and 'Brood cells' are defined in the methods section. Brood cells were monitored to assess the final life stage that would be reached in each of the conditions: egg, larvae or adult. For the secondary experiment the x-axis descriptions refer the different conditions in each enclosure, whether additional lighting (+Lights) or plants (+Plants) were added.

Cannibalism of eggs was recorded in two preliminary experiments, but not within the larger enclosures tested in the secondary experiment (Table 2.3).

Table 2.3 Percent cannibalism in each trial where brood cells were constructed

	Trial	Enclosure size (m^3)	Number of nests available	Number of brood cells constructed	Brood cells cannibalised (%)
Preliminary	1	0.06	120	1	0
	2	0.03	50	35	100
	5	0.65	50	28	79
Secondary	A	7.5	100	18	0
	E	7.5	100	17	0

Cannibalism is defined in the methods and was measured in each of the trials where brood cells were constructed. For full details of the trials see Table 2.1.

2.5 Discussion

In this chapter, different methods to mass-rear RMBs under controlled conditions were assessed, in an attempt to circumvent the unpredictability of current field-based commercial provision (Bosch and Kemp, 2002). Appropriate temperature, food, mud resources and nests to rear RMBs were found. However, the maximum number of offspring that reached adulthood in any trial was one (Figure 2.7), highlighting the significant challenge of developing a rearing methodology. Given that a minimum of 60 bees served as input to produce a single adult (Figure 2.7), a viable method to mass-rear RMBs has yet to be determined. However, based

on the experiments, potential pitfalls and promising avenues for future developments in mass-rearing have been identified and are discussed below.

One of the key problems identified in the preliminary experiments was that the bees readily cannibalised eggs (Table 2.3). RMBs have a tendency to be highly competitive, disrupting the offspring provisioning of other females by breaking into and appropriating their nests (Strohm et al., 2002; Cerna et al., 2013). Therefore, after the preliminary experiments were concluded, it was hypothesised that the small cages in which RMBs were being reared enhanced the propensity to be competitive, which led to cannibalisation of eggs. As such, larger enclosures were used in later experiments in which cannibalism was no longer observed (Table 2.3). It also possible that an increased number of nests also lessened this cannibalism (Table 2.3). This highlights that there is a minimum size of rearing environment, and perhaps number of nests, required for successful RMB rearing.

Another key issue highlighted in the preliminary experiments was that the seasonal timing of the experiment seemed to greatly affect the bee mortality rates (Figure 2.4) and to a lesser extent mating (Table 2.2). The lowest mortality rates were observed in February-May (Figure 2.4), which coincides with RMBs' natural active period (Chapter 1, Figure 1.2). The number of observed mating events was perhaps also associated with RMBs natural active period (Table 2.2). This could suggest that RMBs have an endogenous seasonal clock that influences their performance. This is perhaps substantiated by the finding that despite identical conditions in two experiments that took place at different times of year, only the bees reared in April produced offspring, whereas those in August did not (Figure

2.7). Alternatively, the difference in mortality and reproductive output could be due to the depletion of bee lipid stores whilst RMBs are kept in cold storage (Dmochowska et al., 2013). Indeed, postponing RMB emergence has been shown to increase bee mortality (Giejdasz and Wielkaniec, 1998), with similar effects also found in the blue orchard bee, *Osmia lignaria* (Bosch and Kemp, 2003), although see Chapter 3. It is possible too that the prolonged storage, which may have increased mortality (Chapter 3), could have also prevented production of offspring. Indeed, production of offspring was only ever observed between February and April (Figure 2.7).

Light intensity clearly impacts RMB rearing. When reared under low light intensity (~3000 lux) bees did not engage with mating and there was no reproductive output. Anecdotally, under this low light regime bees were observed to cluster near the light source and remain inactive. There was also an association between the activity of bees and with light intensity (Figure 2.5). This correlation was substantiated by the finding that the activity of RMBs was enhanced by the inclusion of LED lights (Figure 2.6). RMBs have been shown to be more active during bright sunny days (Menzel et al., 1988; Seidelmann et al., 2010) and light intensity has also been shown to be important for the rearing of leafcutter bees, (*Megachile rotundata*; Szabo and Smith, 1972; Lerer et al., 1982; J. Rinehart, personal communication). Therefore, a minimum level of light intensity may be required to ensure bee activity. This threshold appeared to be reached by rearing in a greenhouse environment (Figures 2.5 and 2.6), and so using natural light, perhaps augmented by artificial lighting (Figure 2.6), may be a reasonable strategy.

It is unclear whether bee activity is associated with an increased reproductive output (e.g. Figure 2.7); however, it is reasonable to assume that a baseline level of activity is required for reproduction to occur. Flight is necessary for female bees to forage nectar and pollen, and thus to provision nests. Flight activity has been positively linked to light intensity in honeybees and leafcutter bees (Kefuss and Nye, 1970; Szabo and Smith, 1972), which suggests that a reduction in light intensity may lead to a lower flight activity, consequently causing lower reproductive output. Also, in the hairy-footed flower bee (*Anthophora plumipes*), the quantity of pollen foraged appears to be associated with activity of the bees (Stone, 1994). Moreover, increased activity in RMBs has been positively related to reproductive output (Fliszkiewicz et al., 2015). Furthermore, pollination and foraging seem to be positively associated with the activity of bumblebees (Morandin et al., 2001b). In the present study, it is unclear what the association is between activity and reproductive output (Figure 2.6 and 2.7), but altogether activity may be a predictor of RMB reproductive output.

Surprisingly, the presence of plants seemed to slightly reduce the activity of bees (Figure 2.6) and no reproductive output was observed where plants were present (Figure 2.7). It was unclear why this was the case, since the proximity of flowers is typically positively related to the reproductive output of RMBs (Radmacher and Strohm, 2010; Everaars et al., 2011; Coudrain et al., 2016). To speculate, the suppression of activity by plants could be due to bees being more attracted to them rather than to nectar and pollen placed in tubs, causing bees to ignore those resources in favour of plants. Indeed, olfactory and visual cues associated with plants have been well-evidenced to attract bees (Raguso, 2004; Burger et al.,

2010; Dobson et al., 2012). For RMBs, they appear to be adapted to exploit the most abundant food source (Coudrain et al., 2016), whatever that might be, therefore, inclusion of plants may be unnecessary if adequate type and amount of pollen is supplied. This was evidenced by Sandrock et al. (2014) who found that RMBs provisioned at high rates using only ground bee pollen supplied in four Petri dishes, refilled every 3-4 hours. Indeed, in Sandrock et al. (2014), on average each female bee produced approximately seven brood cells, whereas in the present experiment, when provisioning was highest (Preliminary trial 2), on average each female produced only approximately 0.8 brood cells (Figure 2.7). Alternatively, RMBs may interact with alternative plant species differently. RMBs are mostly managed on orchard crops (Gruber et al., 2011; Schindler and Peters, 2011), and have been shown to preferentially use pollen-rich trees to provision brood (Haider et al., 2014), therefore the inclusion of such plants should be examined in future work.

The only published study that purports to demonstrate successful mass-rearing of RMBs is that of Sandrock et al. (2014) and the methodology used in the present study was based on their description. However, Sandrock et al. (2014) showed a much higher reproductive output in their RMB mass-rearing experiments. A major difference between Sandrock et al.'s (2014) and this study is that the light intensity used in their experiment was 75,000 lux provided by a sunlight simulation chamber, which is much higher than what was available in my experiment. As light intensity was shown here to positively influence activity and possibly reproductive output, this difference may be crucial in understanding the success of Sandrock et al. However, such a high-light intensity may not be

available or financially feasible for commercial operations, instead using natural light (such as in a greenhouse; Figure 2.5) may be an appropriate strategy.

Altogether this work highlights the substantial challenge of developing a rearing system. Whilst millions of colonies of five species of bumblebee (*B. terrestris, B. lucorum, B. occidentalis, B. ignitus and B. impatiens*) are now produced by commercial operations each year (Goulson and Hughes, 2015), these rearing systems took decades of research to develop, with initial efforts showing low success rates (Velthuis and Van Doorn, 2006). Similarly, the management system for rearing alfalfa leafcutting bees has been developed over decades (Pitts-Singer and Cane, 2011). My work therefore is an important first step in establishing a RMB rearing system, demonstrating the feasibility of RMB rearing, and will provide key information to future studies. With the recognition of the constraints of light and spatial scale, it may be pertinent for future research to implement certain aspects of semi-field rearing, involving part of the lifecycle in the field and part in the laboratory, to strike a balance between the benefits of controlled conditions and the noted constraints on RMB reproductive output.

For the alfalfa leafcutting bee a semi-field approach is used: the adult bees are released into the field to collect pollen and provision brood, and the offspring are subsequently collected, and maintained under controlled conditions (Pitts-Singer and Cane, 2011). However, these originally European bees are managed outside of their natural range (Pitts-Singer and Cane, 2011), in the USA and Canada, and likely suffer from less parasitism and disease than RMBs would if they were reared similarly (Gruber et al., 2011). For RMBs then, rearing inside

greenhouses, where parasites can be better excluded than in the field, may be an appropriate strategy. This could be coupled with the use of X-rays (see Chapter 3) to identify parasitized cocooned adults and remove them, preventing further spread of parasites (Pitts-Singer and Cane, 2011). Moreover, a greenhouse would allow temperature to be controlled, the inclusion of additional lighting to promote bee activity (Figure 2.6), and perhaps the use of orchard crops.

In this proposed system, the target nests for RMBs could be placed to allow observation and simple removal by a researcher (e.g. inside a box made from transparent material (Strohm et al., 2002)), to determine when oviposition occurs. This could allow offspring to be removed before cannibalism or parasitism may occur (Table 2.3), and they could then be moved to laboratory conditions where development could be tightly controlled (Giejdasz and Wilkaniec, 2002). After offspring develop into adults and enter diapause (Chapter 1, Figure 1.2), it is then possible that they could be transferred to specific storage conditions, which could allow their diapause to be manipulated to better time with target crops whilst preventing increased mortality (Figure 2.4) and any potential effects on mating (Table 2.2), which is investigated in Chapter 3. Such manipulations may also allow for some protection from changing phenology that may be occurring due to climate change (Chapter 3). A synergy of these approaches could help realise year-round provision of RMBs, which may be critical in ensuring crop yields in the face of pollinator shortfalls.

2.6 Conclusions

Whilst mass-rearing of RMBs was demonstrated to be feasible, the reproductive output under the conditions assessed was so low as to not be commercially viable. Improvements most likely to improve this outcome are increasing light intensity, which was found to positively affect the activity and may therefore have an influence on RMB mating and reproduction. As such, higher light intensities or the use of greenhouse and semi-field environments should be tested in future RMB mass-rearing experiments. In addition, small spatial scales appeared to increase bee competition and thus cannibalisation of eggs, which was later alleviated by larger rearing environments, suggesting that an enclosure at least 7.5 m^3 in size should be used to rear RMBs. Future work should seek to establish whether semi-field rearing, where light and spatial scale are lesser constraints, would be feasible. The seasonal timing of establishing cultures also affected the mortality and reproductive outputs of bees; hence, different methods to prolong RMB storage, but to prevent its negative consequences on bee survival, and to promote bee emergence after storage are investigated in the next chapter.

Chapter 3: Manipulating overwintering in the red mason bee could partially mitigate against the negative effects of climate change

3.1 Abstract

Climate change is already altering insect pollinator abundance and phenology, with potentially significant long-term effects on food security. Understanding of the role of warming winter conditions, and associated impacts on pollinator diapause, in driving these changes remains poor, yet is critical to predicting where and when shortfalls in pollinator service provision may occur. Commercially produced pollinators could mitigate against species declines, as well as provide a pollination service at times in the season when wild populations are not active in sufficient numbers. However, even within this commercial setting, an understanding of how to manipulate overwintering diapause is essential in order to synchronise adult emergence with pollination-service demand. This study investigates the impact of changing winter conditions on the obligate diapause of the red mason bee (RMB; *Osmia bicornis*) as part of predicting future phenology and survival patterns. I then directly compare these predictions to existing data on the first and last observations of red mason bees, from the Bees, Wasps and Ants Recording Society (BWARS). Different strategies to manipulate obligate diapause in RMBs (early termination using chemical treatments, or prolonging diapause under different cold storage conditions) are subsequently evaluated as a method to mitigate against shortfalls in pollination

service provision by natural populations. I identify clear shifts in RMB phenology since 1977, where the first observation of the bees has on average shifted 8 days earlier per decade. There were also sizeable negative consequences of delayed and shorter winters on overwintering physiology and survival. A juvenile hormone analogue and hexane were each able to promote early diapause termination, although with some impacts on bee survival. While fluctuating thermal regimes represent a promising strategy to prolong diapause in RMBs without negative impacts on survival. The implications of climate change for insect pollinators more generally, and options to sustain food security, are discussed.

3.2 Introduction

Solitary bees are an important group of pollinators (Garibaldi et al., 2013; Woodcock et al., 2013), but many of these species are also strictly univoltine with obligate diapause, meaning that the timing of their lifecycle is largely fixed (Gill et al., 2017). Many species with facultative diapause will avert diapause if they experience warmer conditions, whereas in a species with obligatory diapause there is no such response, they will enter diapause regardless of the environmental conditions at the time (Denlinger, 2002; Bale and Hayward, 2010). This means these solitary bees with obligate diapause are particularly susceptible to climate change, as, whilst they may advance emergence, they are unable to alter or avert their diapause programme (Biesmeijer et al., 2006).

Before entering diapause, temperate insects stockpile nutrients to survive overwintering and initiate processes to mitigate the effects of cold (Bale and Hayward, 2010). During diapause, if such cold never comes or is delayed it

promotes increased metabolisation of energy stores, which can lead to reduced survival post-diapause (Hahn and Denlinger, 2007, 2011). This is concerning as winters are warming particularly rapidly and may be delayed in response to climate change (Williams et al., 2015). Impacts of altered winters have been evidenced in a solitary bee, the blue orchard bee (*Osmia lignaria*), where delayed onset of winter showed increased energy expenditure and reduced post-diapause survival (Sgolastra et al., 2011, 2016). Similarly, in the RMB it has been shown that warming temperatures during diapause can result in increased usage of energy stores (Fliszkiewicz et al., 2012), but the effect on post-diapause survival is unclear.

It has also been postulated that a warming climate may present a key challenge to species' phenologies. Insects may be particularly affected, as their development depends on temperature (Bale and Hayward, 2010; Forrest, 2016). In temperate climates most insects navigate the harsh conditions of winter by arresting their development, an adaptation known as diapause. After traversing winter, the cues to terminate diapause, and for insects to emerge, are normally daylength and temperature (Bale and Hayward, 2010). Because of this, as temperatures increase, many insects may advance their emergence. However, this shift in emergence may be in a different manner to the insects' respective host plants, resulting in phenological mismatch (Forrest, 2016). This is especially true of those insects less sensitive to daylength cues, which includes many important pollinators like the mason bees (Dmochowska et al., 2013).

For pollinators, phenological mismatches could have sizeable impacts on plant reproduction and therefore crop yields (Forrest, 2015; Morton and Rafferty, 2017). Whilst it has been pointed out that the timing of many pollinator-plant interactions is regulated by temperature and therefore shifts in phenology will occur in the same direction (Forrest, 2015; Renner and Zohner, 2018), the relative rate at which species will respond to climate change is largely unknown (Forrest, 2016). Indeed, there is some evidence that phenological mismatches have already occurred. In two species of mason bee, the RMB and the European orchard bee (*Osmia cornuta*), emergence was shown to occur earlier with warmer temperatures, but the flowering time of a pollinator dependent plant (*Pulsatilla vulgaris*) responded more strongly to the warming, thus resulting in a phenological mismatch (Kehrberger and Holzschuh, 2019). Furthermore, using museum specimens, Scheper et al. (2014), suggested that phenology has shifted earlier for many bees, which has caused mismatches with late flowering host plants.

To ensure pollination services, in spite of the challenges of climate change, commercial mass-rearing systems, where the environment is controlled, may be a useful strategy. The RMB has been identified as a good candidate for such a commercial system, however, I tested different methodologies to mass-rear RMBs and concluded that, whilst possible, such mass-rearing is not viable for commercial operations at present (Chapter 2). As such, it is likely that commercial provisions of RMBs will continue to originate from trap nests placed in orchards and agricultural land. In this case, when supplied, the RMBs' emergence and overwintering period may not be synchronised with the target crops, instead their

emergence would be timed with the location from which they were supplied (Bosch and Kemp, 2000, 2002). Moreover, these natural populations will be subject to possible phenological mismatches.

To attempt to circumvent these problems and to synchronise RMB emergence with target plant blooms, the bees are typically stored at low temperatures to prolong their overwintering until such times as they are needed, at which point they are transferred to higher temperatures to promote emergence (Bosch and Kemp, 2000; Biliński and Teper, 2004; Pitts-Singer et al., 2008). There are two major problems with this approach: firstly, advancing RMB emergence using temperature does not guarantee that it will be timed with orchard bloom, instead, time to emergence seems to depend on the length of the wintering period (Bosch and Kemp, 2000; Giejdasz and Wasielewski, 2017a); secondly, keeping RMBs at low temperatures for prolonged periods appears to reduce bee energy stores (Dmochowska et al., 2013), which in turn may have a negative impact on survival and longevity (Bosch and Kemp, 2000, 2004; Sgolastra et al., 2011, 2016).

Bee emergence not matching crop blooms could be problematic, since even a few weeks can make a big difference to both the plants and the pollinators especially when considering short fruit blooms (Matsumoto, 2014; Schenk et al., 2018a). There are several methods that may circumvent this problem; for example, treatment of RMBs with different compounds may induce emergence (Appendix 1.2). Indeed, Wasielewski et al. (2011b) suggested that a juvenile hormone analogue, methoprene, may force the emergence of RMBs. In females, they suggested this is caused by termination of diapause triggered by the juvenile

hormone and by increased ovarian production (Wasielewski et al., 2011b), however, it is unclear if similar effects occur in male RMBs. Consequences of methoprene treatment on health of RMBs were not investigated in Wasielewski et al. (2011b), but artificial shortening of overwintering in the blue orchard bee (*O. lignaria*) has shown reduced survival (Sgolastra et al., 2010). Any further studies assessing the utility of methoprene to promote RMB emergence should therefore take into account effects on survival and longevity of RMBs.

In other species many compounds have been tested that can influence the termination of diapause (Appendix 1.2). For example, in the flesh fly, *Sarcophaga crassipalpis*, and the tobacco hornworm (*Maduca sexta*) hexane has been shown to stimulate the termination of diapause (Denlinger et al., 1980). Whilst these species have a different diapause system from RMBs, hexane mediates termination of diapause by causing an increase in juvenile hormone and ecdysteroid production (Fujiwara and Denlinger, 2007). As methoprene is a juvenile hormone analogue and has been used to terminate diapause in RMBs (Wasielewski et al., 2011b) it is possible that hexane may have a similar effect by stimulating juvenile hormone production.

In addition to compound treatments to promote bee emergence, it may also be possible to prolong the overwintering period to properly time bee emergence with crops. This would also allow bees to be stored for long periods of time, building resilience into RMB provision. However, as previously discussed, such extensions can lead to negative impacts on bee health. Alternative storage conditions to prolong overwinter may offer a solution. For instance, storing alfalfa

leafcutting bees (*Megachile rotundata*) in a fluctuating thermal regime (FTR) of 6˚C with a daily 1h pulse to 20˚C was shown to prevent any negative effects on bee survival for up to 19 months (Rinehart et al., 2013). Bennett et al. (2013) also showed that FTRs prevent weight loss in the leafcutting bees. This lack of weight loss under FTRs may have come as a result of energy stores not being depleted as rapidly (Dmochowska et al., 2013). In fact, Torson et al. (2015) demonstrated that many metabolic pathways are altered by FTRs and they suggest that FTRs may act to promote repair of chilling injuries and thus restore metabolic activity to approximately that of non-injured insects. If this is indeed the case, FTRs may represent a useful tool to allow prolonged storage of RMBs without deleterious effects on their survival.

Another possible method to prolong overwintering may be to lower the temperature at which bees are stored. RMBs have a supercooling point (the point at which insects freeze) of -26˚C to -31˚C during diapause (Krunic and Stanisavljevic, 2006) and therefore are likely tolerant of much lower temperatures than the current storage regimes of 2-4˚C. Reducing temperature could decrease the rate at which energy stores are used up (Fliszkiewicz et al., 2012) and thus prevent negative impacts on survival and longevity.

This chapter aims to determine the impact of warming winter conditions on the survival and phenology of RMBs, and then assess potential methods of diapause manipulation in a commercial setting to help mitigate against these effects. Altogether, I hypothesise that prolonged autumn temperatures and shorter time at winter temperatures will reduce the survival of RMBs, as it has been

demonstrated in blue orchard bees (Sgolastra et al., 2011, 2016). Moreover, I hypothesise that any such reduction of survival will be as a consequence of depleted lipid stores, which has been shown previously in studies where wintering was impacted (Dmochowska et al., 2013; Sgolastra et al., 2011, 2016). Additionally, considering climate change, I further hypothesise that RMBs phenology will have advance over the past few decades as the climate has warmed, which may also have consequences on survival for the same reasons outlined above. This has been suggested by Scheper et al. (2014), among others. Finally, I also hypothesise that RMB overwintering can be artificially manipulated (shortened or lengthened), through chemical, hormonal and thermal means, as has been demonstrated in previous studies (Denlinger et al., 1980; Wasielewski et al., 2011b; Rinehart et al., 2013).

3.3 Methods

3.3.1 Study species

The subspecies of RMB *Osmia bicornis cornigera* was investigated in this study. These were purchased from WAB-Mauerbienenzucht (Konstanz, Baden-Württemberg, Germany), as the United Kingdom (UK) subspecies (*O. b. rufa*) were no longer available. Unless otherwise stated, upon receipt the bees were stored at 2˚C using a Sanyo MIR-254 incubator (Sanyo Electric Co. Ltd., Moriguchi, Osaka, Japan), until the start of experiments.

3.3.2 Delaying onset of winter

The impact of changing the onset of 2˚C storage, i.e. winter temperatures, on bee survival was determined. Upon receipt, the bees were stored at 11˚C for 0 days, 30 days or 60 days after which they were transferred to 2˚C (Figure 3.1). The duration bees were stored at 11˚C is hereafter referred to as their pre-wintering duration, this is then added to the time they spent at 2˚C to give the total storage duration (Figure 3.1). Three replicates of five male bees and three replicates of five female bees (in total 15♀♀ and 15♂♂) per treatment were assessed for their survival after 90-300 days total storage duration. Survival was designated as successful eclosion of the bees from their pupa (after direct placement at 20˚C, as in industrial rearing), and the percentage survival for each group of five was calculated. The percentage survival for all groups was averaged together to give the mean survival. The bees were also weighed throughout the experiment (approximately every 30 days), using a Sartorius M2P microbalance (Göttingen, Lower Saxony, Germany). With these measurements percentage weight lost was calculated.

Four replicates of five bees (10♀♀ and 10♂♂) under the same thermal conditions were also X-rayed to get an indication of the lipid content stored in their fat bodies in after 120-, 150-, 180- and 270-days total storage duration. Fat bodies were assessed as they are where the majority of the lipids are stored as an energy resource for RMBs to overwinter (Bosch et al., 2010; Radmacher and Strohm, 2011). X-rays were performed at the University of Birmingham Dental Hospital using a Siemens Elema AB X-ray machine (Siemens AG, Berlin, Germany) with

65 kV power, imaging onto phosphor plates. As in Bosch et al. (2010), fat bodies were scored on a semi-quantitative scale depending on how much the fat body was depleted. The fat body depletion scores are shown in Figure 3.2.

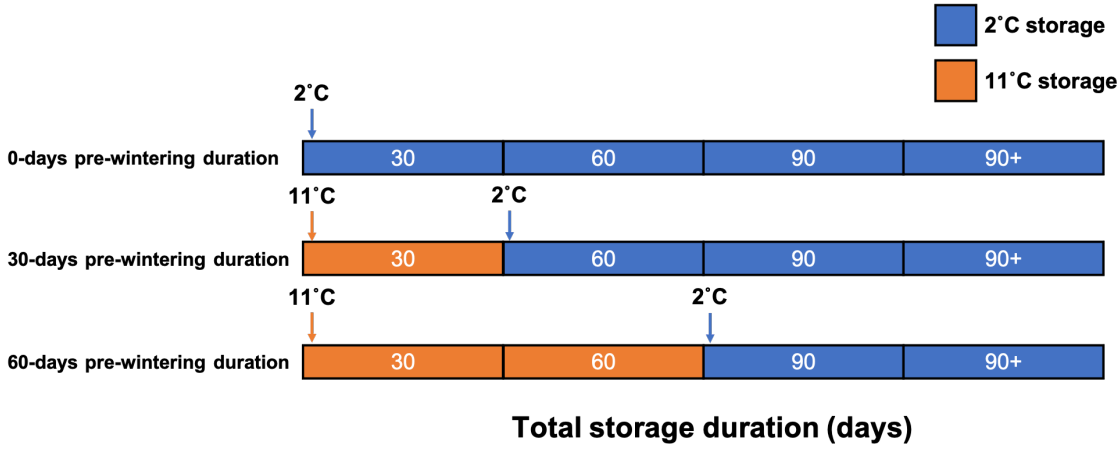


Figure 3.1 Description of experiment to test delaying winter

Each of the three different experiments (0-, 30-, and 60-days pre-wintering) delaying winter are described. The amount of time at 2˚C (blue) and 11˚C (orange) are shown, compared to the total storage duration.

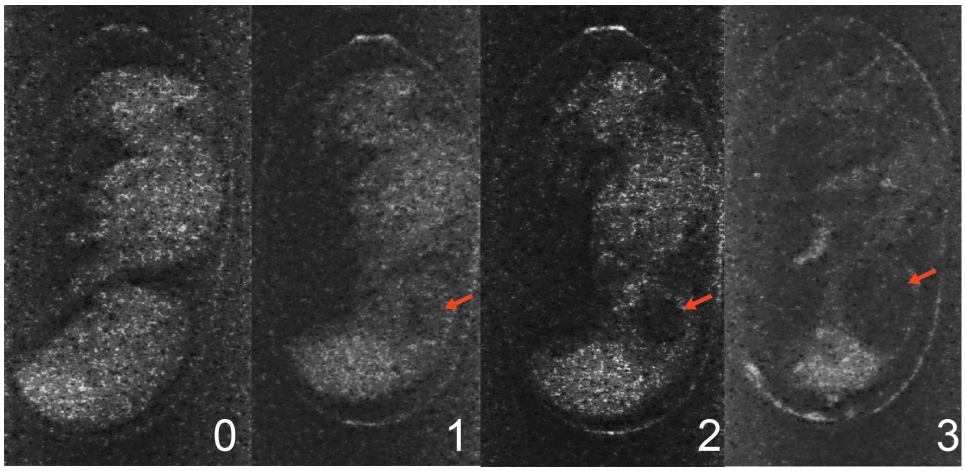


Figure 3.2 Semi-quantitative scale of fat body depletion

X-ray image of four bees, showing differing degrees of fat body depletion based on descriptions in Bosch et al. (2010), where 0 shows no depletion, 1 some depletion, 2 greater depletion and 3 shows fully depleted. Fat bodies in different states of depletion are highlighted with red arrows.

3.3.3 Curtailing length of winter

Restricting the time bees spent at 2˚C, i.e. wintering temperature, was studied for its effects on survival and emergence. RMBs (10♀♀ and 10♂♂) were incubated at 2˚C for one, three, five or seven weeks, after which they were immediatley placed at 20˚C. The rate of emergence (i.e. the number of days since the bees were placed at 20˚C until eclosion) was tracked and percent survival for four sets of five bees in each group was assessed as above.

3.3.4 Determining the active period of RMBs in the UK

Data provided by the Bees Wasps and Ants Recording Society (BWARS), showed every recorded observation of RMBs from 1977-2013. After removing incomplete records these data were used to determine the possible earliest emergence date of RMBs and the possible final active day. In each year of the dataset the earliest dated observation was taken to be the first emergence of the bees, and the latest dated observation was assumed to be the end of the activity period for the bees. Whilst the elevation and latitude may have had an effect on these observations (Hodgson et al., 2011; Forrest, 2016; Appendix 3.1), elevation was not recorded and most (87%) of the records were from below 52 degrees latitude (Appendix 3.1), so any effects of latitude would be small. Indeed, for the first emergence and end of activity period, latitude did not show any significant effect (first emergence: $p = .85$, $R^2 = -.03$; end of activity period: $p = .53$, $R^2 = -.01$) likely because the first and last dated observations were mostly from below 52 degrees latitude.

Regression lines were calculated based on these data, to compare the year with the first emergence, and the end of the activity period. First emergence was assessed using a polynomial 2nd degree regression and the assumptions were validated (Appendix 3.2). Last emergence was assessed using a linear regression.

As there appeared to be a trend in the time of first emergence across the years, it was investigated whether spring temperature was associated with this change in first emergence. Emergence of RMBs has been observed to be sensitive to temperatures above 10˚C (N.H. personal observations; Kehrberger and Holzschuh, 2019). So, to investigate the effect of spring temperature the accumulated number of ˚C per day for the first 100 days of the year was found, using data from the Met Office Integrated Data Archive (MIDAS) Heathrow weather station. In addition, as the number of records submitted to BWARS varied through the years (i.e. the sampling effort) whether this was correlated to the first emergence was also examined.

To determine whether the number of records submitted, or spring temperature was having a greater effect on emergence time, random subsets of data were used to remove the possible effect of number of records. Fifteen records were sampled randomly from each year 100 times, and linear regression was conducted for each of these subsets to see if spring temperatures still significantly explained the variance (excluding 2013, as it only had five values). The *p*- and R^2 values were calculated for each of these 100 regression lines (Appendix 3.3).

3.3.5 Forcing bee emergence

To test different methods to terminate diapause and thus force bee emergence, on the 20th of the month, in December, January, February, March and May, chemical treatments were applied to groups of bees (all bees had begun their overwintering period in the previous October). Two compounds were used: methoprene and hexane. Methoprene was purchased as a powder from Sigma-Aldrich® (St Louis, Missouri, United States of America [USA]) and 600 mg was dissolved in 15 ml acetone to get a concentration of 40 mg/ml. After carefully removing the pupal cap with scissors, 5 µl of 40 mg/ml methoprene was applied topically to the head of each bee, every day for five days (after Wasielewski et al., 2011). A previous study had shown that there was no impact of acetone on RMB emergence (Wasielewski et al., 2011b; Giejdasz and Wasielewski, 2017b), and due to limited numbers of bees available it was not assessed. Hexane (approximately 97% GC) was also purchased from Sigma-Aldrich®. 5 µl of the 97% hexane was applied topically in the same manner as methoprene, however it was only applied once per bee, as per previous hexane forcing experiments in other insects (e.g. Fujiwara and Denlinger, 2007). After application of either methoprene or hexane, bees were placed in an incubator at 20˚C. Two control treatments were performed simultaneously, one where bees were simply placed at 20˚C and another where the pupal cap was carefully cut off before placement at 20˚C. For every month, 80 bees (40♀♀ and 40♂♂) were investigated per treatment.

Survival and rate of emergence were assessed every 24 h, as defined above. For survival, bees were separated into 16 groups of five (a total of 40♀♀ and 40♂♂) and the percentage survival for each group was calculated, which was then averaged to give the mean survival. In the same set of bees, the mean values of emergence for the male and female bees were calculated.

3.3.6 Extension of overwintering

The survival of bees after prolonged periods of storage, seven months and longer, under different thermal regimes was assessed. The different temperature conditions were as follows: 2˚C constant temperature (control); - 5˚C constant temperature; and a FTR of 0˚C with 1 h daily pulse of 5˚C. After seven, 11, 15, 19, and 21 months, the percentage survival (as described above) three replicates of five male bees, and three replicates of five female bees (in total 15♀♀ and 15♂♂) was assessed in each treatment.

Three replicates of five male bees, and three replicates of five female bees (in total 15♀♀ and 15♂♂) under the same thermal conditions were also weighed every one or two months for 14 months. This was performed using a Sartorius M2P microbalance (Göttingen, Lower Saxony, Germany). Using these measurements percentage weight lost was calculated.

3.3.7 Statistical analyses

An alpha threshold of .05 was used (i.e., results were considered ‘significant’ if *p*-values were below .05), except where otherwise stated. No data were

transformed, unless otherwise stated. Statistical outputs, including test-outputs, degrees of freedom (DF) and p-values are described in the text.

For survival data, the raw observations of mortality were transformed into percentage survival by dividing the total number of bees that survived to the end-point of the experiment in each group by the total number of bees in that group (n=30). Binomial 95% confidence intervals were calculated using the Wilson score interval, to account for the bounded nature of the data. Where there appeared to be trends in the data (e.g. Figure 3.6B and Figure 3.10) binomial generalised linear models were used to interpret the data. Here, the difference between the null and residual deviance indicates if there is an association between the variables. In this case, a *p*-value, as determined by Hosmer-Lemeshow goodness of fit test, is used to identify how well the model fits the data, where a *p*-value > .05 shows no significant difference between the model and the observed data i.e., a good fit. For the number of days until emergence when bees experienced shortened winter (Figure 3.6A), the mean number of days until emergence along with standard error were calculated.

For analysis of bee weight loss, mean weights of all bees at each timepoint in each different condition (15♀♀ and 15♂♂) were calculated along with standard error. Linear regression was used to find trends, with polynomial regression used when it increased the R^2 by more than 0.1. Analysis of covariance (ANCOVA) tests were also conducted. And for the x-ray ranking data, a Kruskal-Wallis rank sum test was used to discern overall differences between groups, in this case

binomial 95% confidence intervals were again calculated, along with weighted means.

To determine trends within the phenology data (and for the variables contributing to it), again, linear or polynomial regression were used. Additionally, to control for the effect of sample size subsets (n=15) of the phenology were taken and regression models were used to see if trends remained (Appendix 3.3).

For emergence data, to find differences between groups, measures of significance, and F-statistics, analysis of variance (ANOVA) tests were used. For ANOVA the assumptions of normality were tested using exploratory histograms and homogeneity of variance was tested using Levene's test. Additionally, significant differences between groups were identified used Tukey's honestly significant difference (HSD) test.

3.4 Results

3.4.1 Impacts of shortening winter conditions on red mason bee diapause physiology (lipid storage) and survival

Initially, survival between the different pre-wintering durations was similar, however, survival of the bees pre-wintering for 30 or 60 days declined to 0% after 210 days of storage (Figure 3.3). Comparatively, survival of bees with 0 days of pre-wintering was between 30 and 50% during the same period (Figure 3.3).

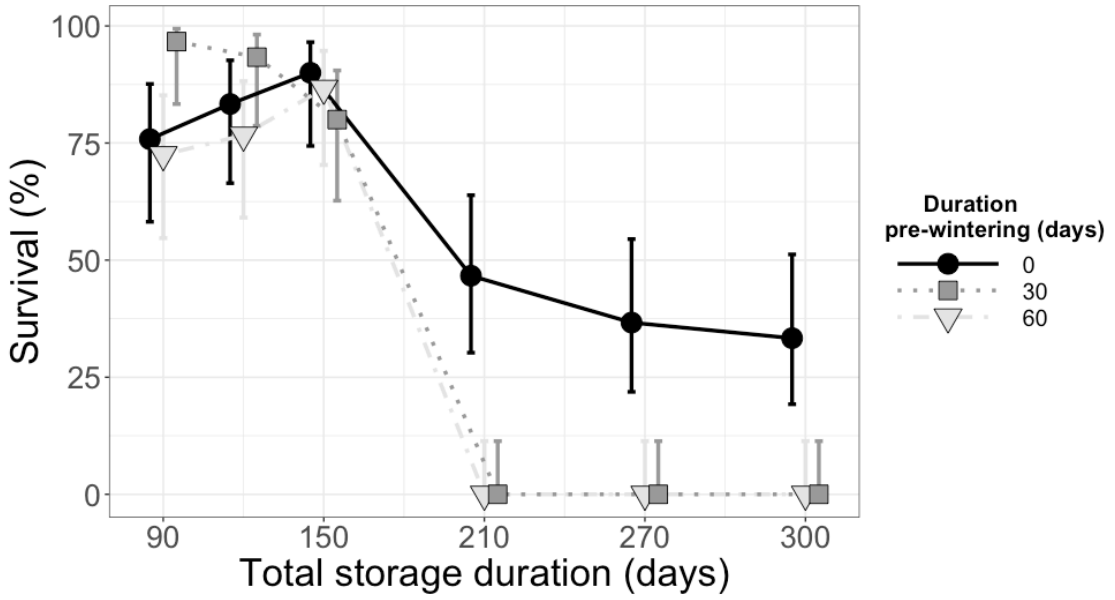


Figure 3.3 Percent survival of bees after long-term storage with differing starts to winter temperatures (2˚C)

Pre-wintering refers to amount of time bees were stored at 11˚C prior to the start of winter (2˚C).Pre-wintering refers to amount of time bees were stored at 11˚C prior to this. Total storage duration refers to pre-wintering time plus time stored at winter temperature. Points show the overall proportion of bees that survived and error bars show 95% binomial proportion confidence intervals.

Longer pre-wintering durations also caused a much more rapid decline in bee weights (Figure 3.4). Indeed, after 210 days of storage, bees that had pre-wintered for 30 (2^{nd} degree polynomial regression; $y = -.21x + .000093x^2 + 89.97$; $R^2 = .14$; F-statistic = 83.67; DF = 957; $p < .001$) or 60 days (2^{nd} degree polynomial regression; $y = -37x + .00076x^2 + 90.34$; $R^2 = .16$; F-statistic = 92.73; DF = 957; $p < .001$) had lost >30 mg, while those with no pre-wintering had only lost around 15 mg (Linear regression; $y = -.065x + 89.24$; $R^2 = .03$; F-statistic = 21.67; DF = 658; $p < .001$; Figure 3.4).

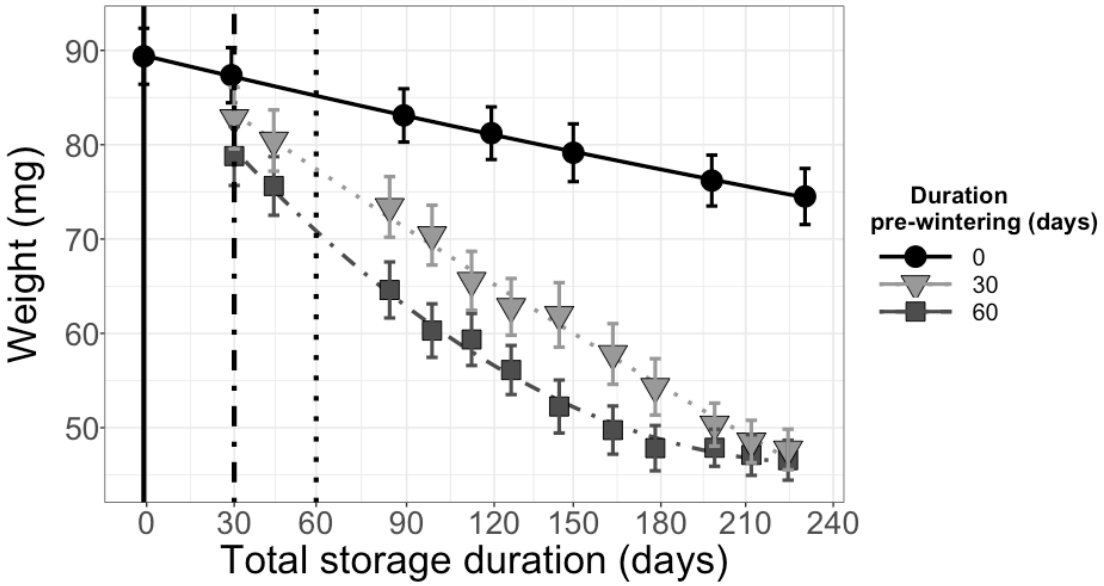


Figure 3.4 Average weight of bees during long term storage with differing starts to winter temperatures (2˚C).

Pre-wintering refers to amount of time bees were stored at 11˚C prior to the start of winter (2˚C). Total storage duration refers to pre-wintering time plus time stored at winter temperature. Trend-lines show linear or 2nd degree polynomial regression. Points show averaged survival of six groups of five bees and error bars show one standard error. The vertical lines show when bees were placed at 2˚C, where the solid line refers to 0-days, the dot-dashed line 30-days, and the dotted line 60-days pre-wintering.

By assessing X-ray images of bees, it was shown that the fat bodies were more depleted in bees subjected to longer pre-wintering durations (Kruskal-Wallis, $\chi^2 = 34.50$, DF = 2; $p < .001$; Figure 3.5). At the time of the first X-ray, in February, the bees with delayed winter onset have already depleted the fat bodies much more than the control (Figure 3.5), implying that much of the depletion may have occurred prior to the experiment, during the typical diapause period (November-January).

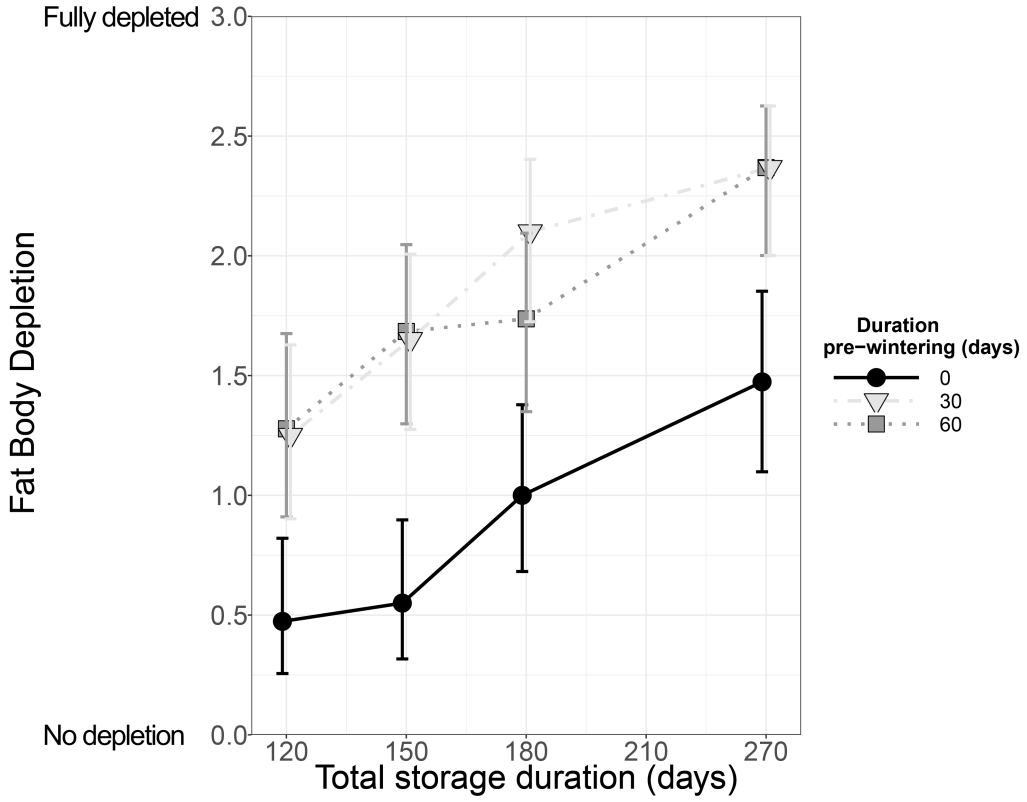


Figure 3.5 Average fat body depletion of bees during long term storage with differing starts to winter temperatures (2˚C)

Pre-wintering refers to amount of time bees were stored at 11˚C prior to this. Total storage duration refers to pre-wintering time plus time stored at winter temperature. Points show weighted averages (n = 20 per group) and error bars show 95% binomial proportion confidence intervals. See methods for full description of how depletion was ranked.

On average, regardless of the amount of time in winter (storage at 2˚C), bees took around 90 days to emerge (Figure 3.6A). Survival of the bees increased exponentially with amount of time stored at 2˚C, with a mean survival of 15% for one week, compared to seven weeks at 2˚C where mean survival observed was 60% (binomial generalised linear model; y = 1 ÷ (1+ exp-[-2.36 + .40x]); null deviance = 5.20; Residual deviance = 2.69; DF = 15; *p* = .99; Figure 3.6B).

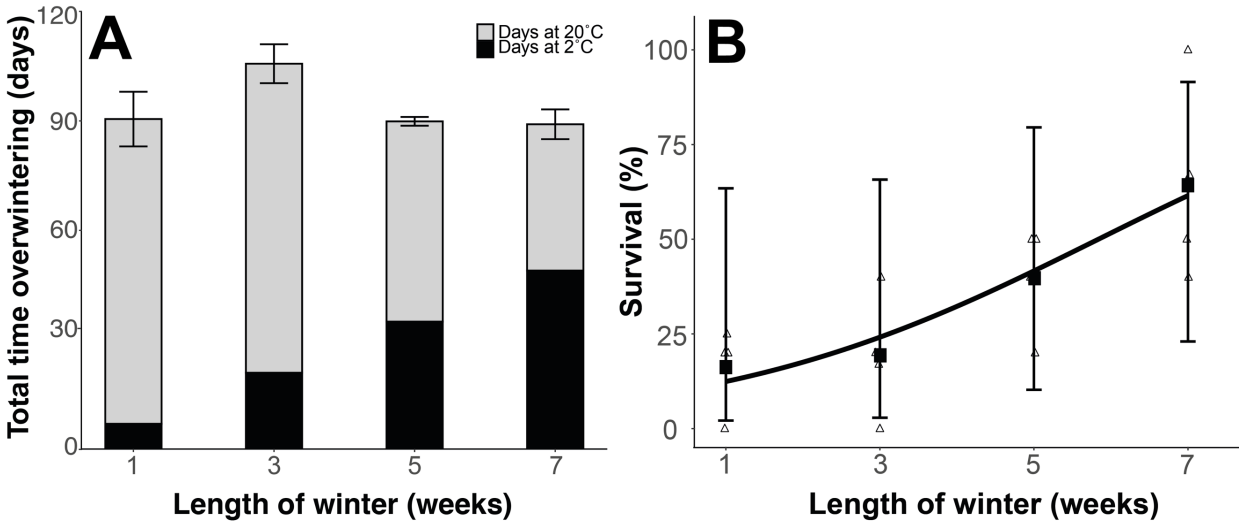


Figure 3.6 Total time bees spent overwintering at 2˚C and 20˚C, and the survival of bees stored for different lengths of time at 2˚C

A Total time bees spent overwintering with the number of days at winter temperature (2˚C), and the mean number of days spent at 20˚C. Error bars represent standard error for the number of days spent at 20˚C. **B** Survival of bees stored for different lengths of time at 2˚C, where the dots represent average survival in individual replicates (n=5) while large points represent overall proportion of bees that survived. The line represents a binomial generalised linear model, and the error bars shows 95% binomial proportion confidence intervals.

3.4.2 Phenology of red mason bees in the UK

The first sighting of bees has been brought forward by ~30 days since 1977 (2nd degree polynomial regression; $y = -44.39x + 25.73 x^2 + 119.60$; $R^2 = .34$; F-statistic = 10.22; DF = 34; $p < .001$; Figure 3.7), the last sighting has progressed by around 5 days (linear regression; $y = .16x - 121.04$; $R^2 = -.02$; F-statistic = .43; DF = 35; $p = .519$; Figure 3.7).

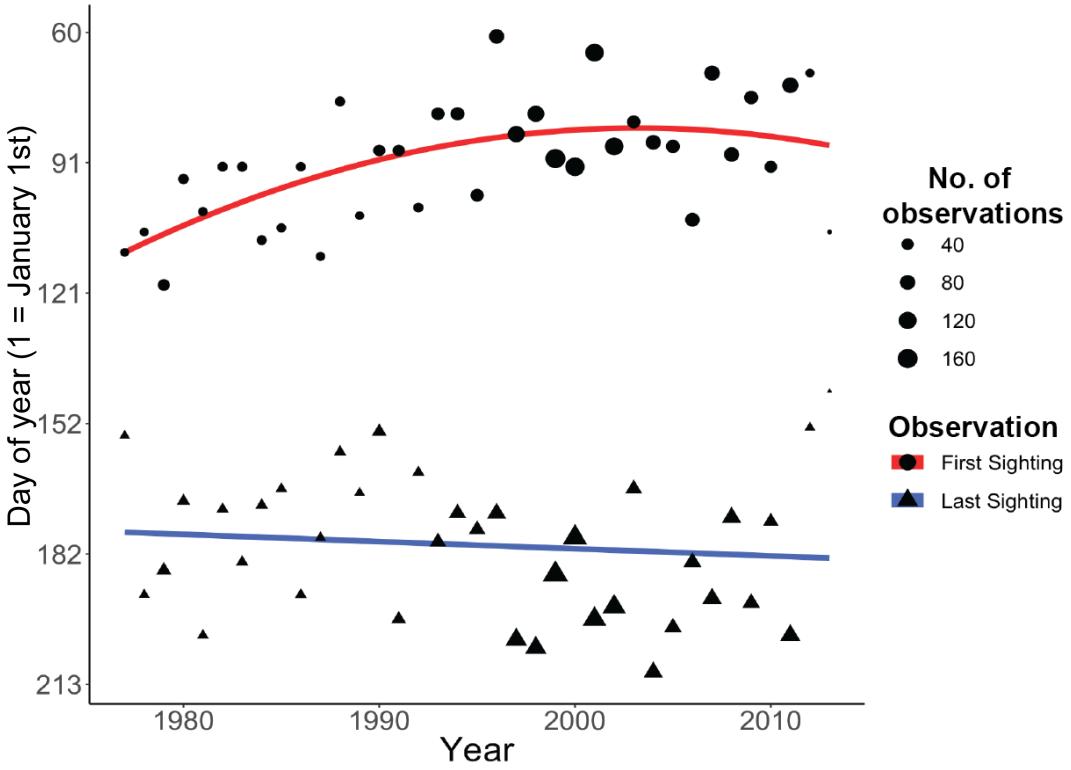


Figure 3.7 The first and last observations of red mason bees in the UK from 1977-2013.

The first and last sighting of red mason bees in the UK 1977-2013, using data provided by the BWARS. The size of the points represents the number of observations. The red line shows 2nd degree polynomial regression of the first sighting of bees and the year (R^2 = .34). The blue shows a linear regression of the last sighting of bees and the year (R^2 = -.02).

When spring temperatures were higher RMBs were observed to emerge earlier (linear regression; y = -.14x + 132.91; R^2 = .22; F-statistic = 11.05; DF = 35; *p* = .002; Figure 3.8A). Similarly, when the number of records were higher, RMBs showed earlier emergence, although this was only up to a point, after 100 records RMBs no longer tended to emerge earlier (2nd degree polynomial regression; y = -39.96x + 30.13x^2 + 119.60; R^2 = .32; F-statistic = 9.44; DF = 34; *p* < .001; Figure 3.8B).

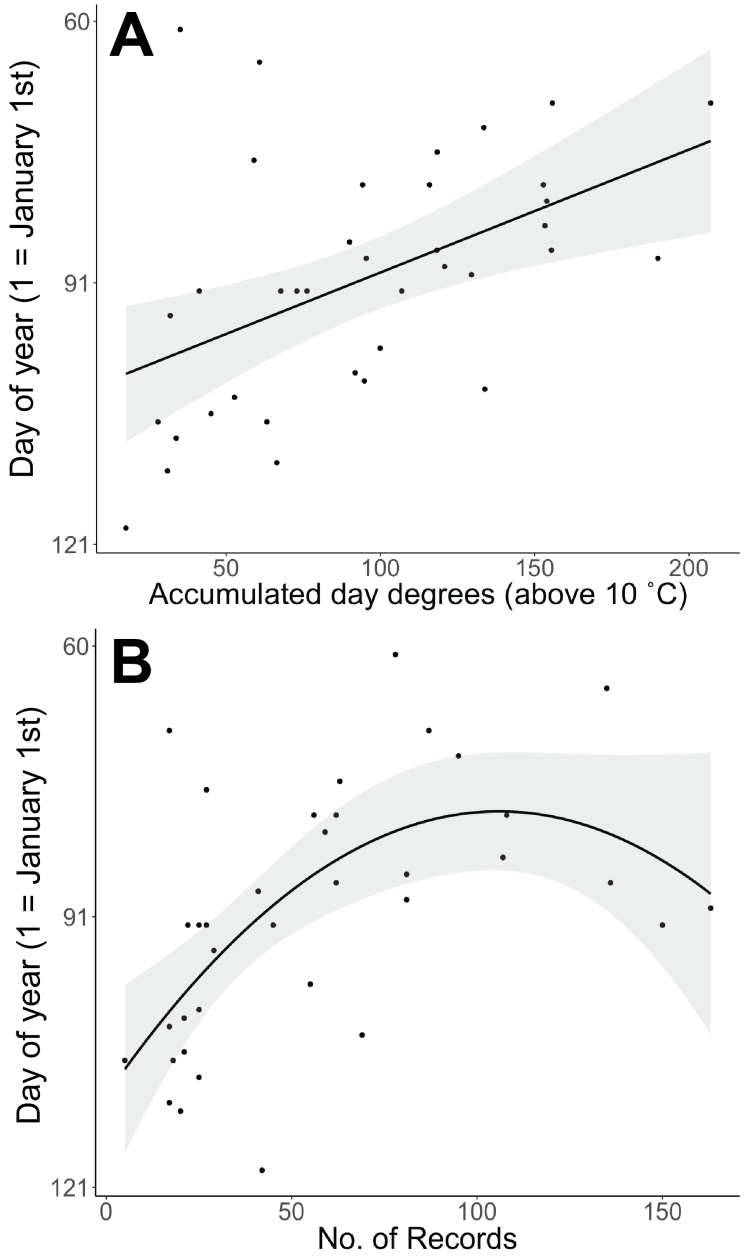


Figure 3.8 The association of spring temperature and number of records with emergence of red mason bees

The first sighting of RMBs in the UK 1977-2013, using data provided by the BWARS, was correlated with different variables. **A** Linear regression of spring temperature (defined in the methods) and the first sighting of RMBs (R^2 = .22, p = .002). **B**. 2nd degree polynomial regression of number of records submitted to BWARS and the first sighting (R^2 = .32, p < .001). The shaded areas show 95% confidence intervals.

When the data were subsetted to eliminate the potential effect of number of records, it was found that most of the time there was still a significant effect of spring temperature (Appendix 3.3). Ninety-five of the 100 regression models showed a significant contribution of spring temperatures ($p < .05$) and 22 of these were highly significant ($p < .001$; Appendix 3.3).

3.4.3 Influence of chemical and hormonal treatments on RMB emergence

In general, the time it took for bees (both male and female) to emerge decreased with calendar month, and the differences between treatments converged (Figure 3.9). Methoprene showed substantial effects on forcing the emergence (ANOVA; F-statistic = 38.94; DF = 3, $p < .001$; Figure 3.9), advancing emergence by 45 days on average in females, compared to the controls (Figure 3.9). There was also a comparable effect on the emergence of males (ANOVA; F-statistic = 27.10; DF = 3; $p < .001$; Figure 3.9). Hexane also showed a large effect on advancing the emergence (ANOVA; F-statistic = 38.94; DF = 3, $p < .001$; Figure 3.9), decreasing the average time to emergence by 20 days in December in females (Figure 3.9). In either case, the effect on emergence decreased as the year advanced (Figure 3.9). Piercing the pupal cap may have had an effect on emergence in some months, but the effect size was small and was only considered significant in February for females (ANOVA with Tukey's HSD; F-statistic = 15.24; DF = 3; $p = .002$; Figure 3.9).

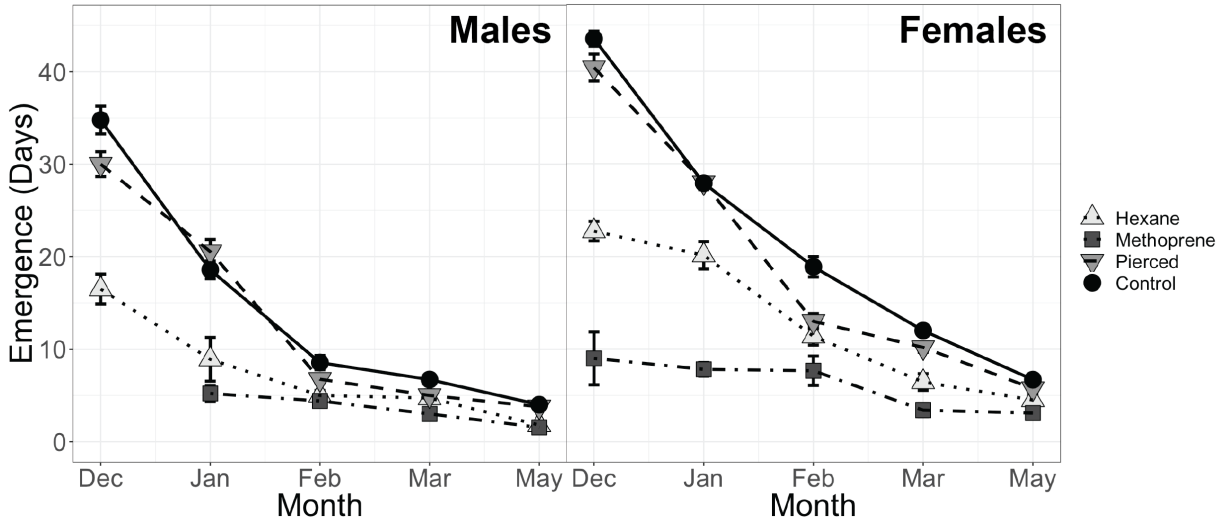


Figure 3.9 Rate of red mason bee emergence with different chemical and hormonal treatments

The number of days taken for bees to emerge after exposure to different chemical treatments and transfer to 20˚C from 2˚C (n = ~40 per group) is plotted relative to the month the experiment has performed. Points show means and error bars show one standard error. The legend refers to the different chemical treatments, where 'Pierced' refers to piercing of the pupal cap of bees. The data point for males in December in methoprene treatment is absent due to no bees surviving this treatment.

The chemical treatments were also shown to have effects on survival (Figure 3.10). Differences between males and females were not considered significant (Kruskal-Wallis; χ^2 = 34.50, DF = 6; p = .13), so for simplicity the results are presented together. Overall, methoprene reduced survival in December and January (binomial generalised linear model; y = 1 ÷ (1+ exp-[-1.74 + .75x]); null deviance = 25.83; Residual deviance = 15.40; DF = 48; p = .97; Figure 3.10), as compared to the controls (binomial generalised linear model; y = 1 ÷ (1+ exp-[1.10 - .019x]); null deviance = 18.41; Residual deviance = 18.40; DF = 48; p = .99; Figure 3.10), with survival being comparable in later months. In contrast,

survival for hexane treated bees declined over time, with the lowest survival in May (binomial generalised linear model; y = 1 ÷ (1+ exp-[.54 - .21x]); null deviance = 19.66; Residual deviance = 18.64; DF = 48; *p* = .99; Figure 3.10). Overall, piercing of the pupal cap appeared to show the highest survival (binomial generalised linear model; y = 1 ÷ (1+ exp-[2.02 - .29x]); null deviance = 17.74; Residual deviance = 16.60; DF = 48; *p* = .97; Figure 3.10). Generally, survival was very variable between months.

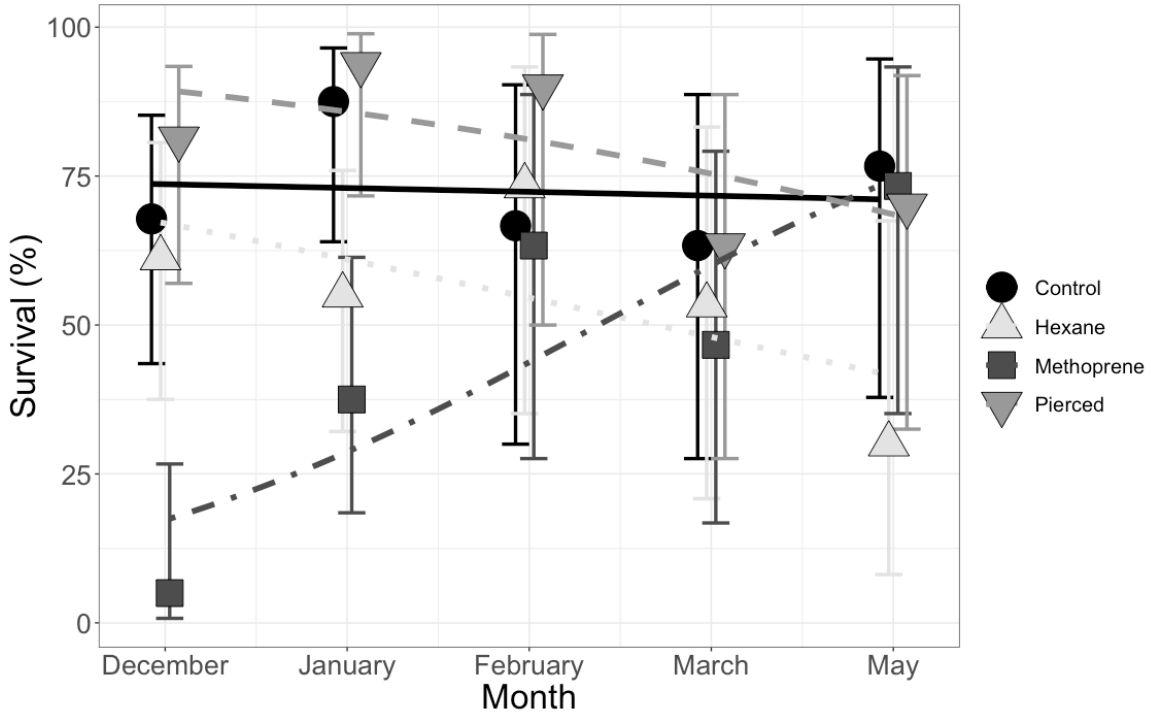


Figure 3.10 Survival of bees treated with hexane and methoprene

Percentage survival of bees after exposure to different chemical treatments and transfer to 20˚C from 2˚C, relative to the month the experiment was performed. The legend refers to the different chemical treatments, where 'Pierced' refers to piercing of the pupal cap of bees. Points show the overall proportion of bees that survived in each treatment and timepoint (n=40), and error bars show 95% binomial proportion confidence intervals. Lines represent binomial generalised linear models.

3.4.4 The effect of different thermal regimes on the survival of RMBs in long-term storage

In the 15-month storage treatment, survival was shown to be much higher in the bees stored in FTR, with survival continuing to be higher for these bees after 19 and 21-months storage (Figure 3.11). Indeed, at the 15-month checkpoint, average survival for the FTR-stored bees was 65% compared to 0% for the control and 5% for bees stored at -5˚C. Survival for the control and bees stored at -5˚C was thereafter 0% (Figure 3.11).

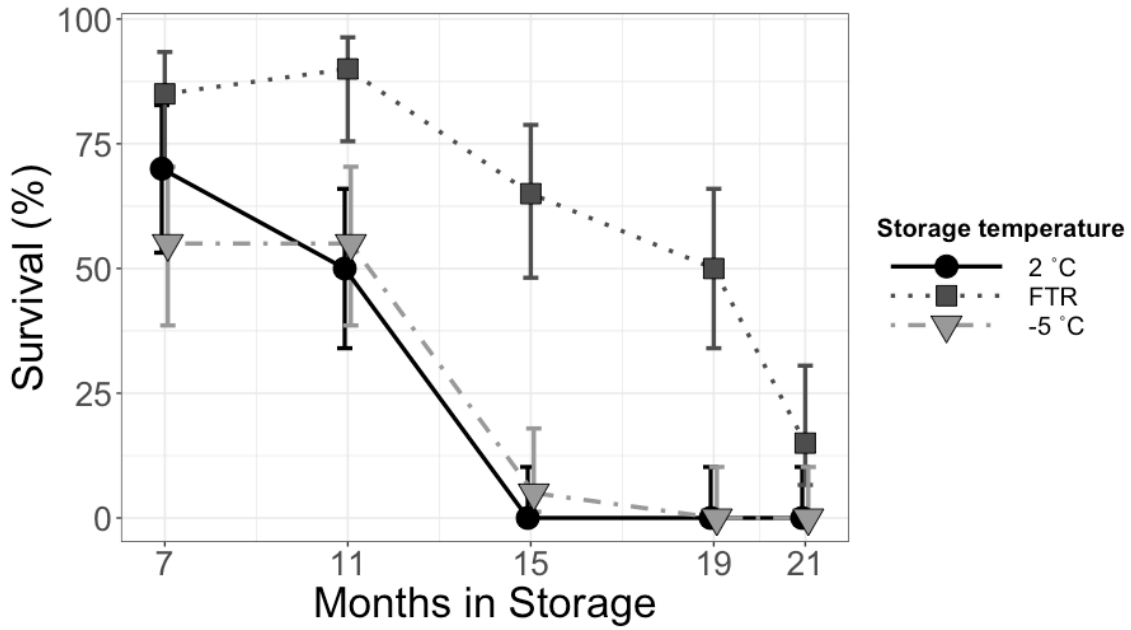


Figure 3.11 The influence of different storage temperatures on the survival of bees in long-term storage

Percent survival of bees after long-term storage of bees in different thermal regimes, where FTR refers to bees stored at 0˚C with 1 h at 5˚C each day. Points show the overall proportion of bees that survived in each treatment and timepoint (n=20), and error bars show 95% binomial proportion confidence intervals.

Bees stored at 2˚C and -5˚C lost weight much more rapidly than those stored in a FTR (ANCOVA with Tukey's HSD; F-value = 32.47; DF = 2; p = .002; Figure 3.12). Indeed, bees stored in FTR had only lost around 13 mg of their starting weight on average after 14 months (2nd degree polynomial regression; y = -127.22x - 5.91x^2 + 85.78; R^2 = .02; F-statistic = 9.44; DF = 947; p < .001), compared to >30 mg for both the 2˚C (2nd degree polynomial regression; y = -271.96x - 25.95x^2 + 77.16; R^2 = .10; F-statistic = 9.44; DF = 947; p < .001), and -5˚C stored bees (2nd degree polynomial regression; y = -260.43x - 52.61x^2 + 80.18; R^2 = .08; F-statistic = 9.44; DF = 947; p < .001; Figure 3.12).

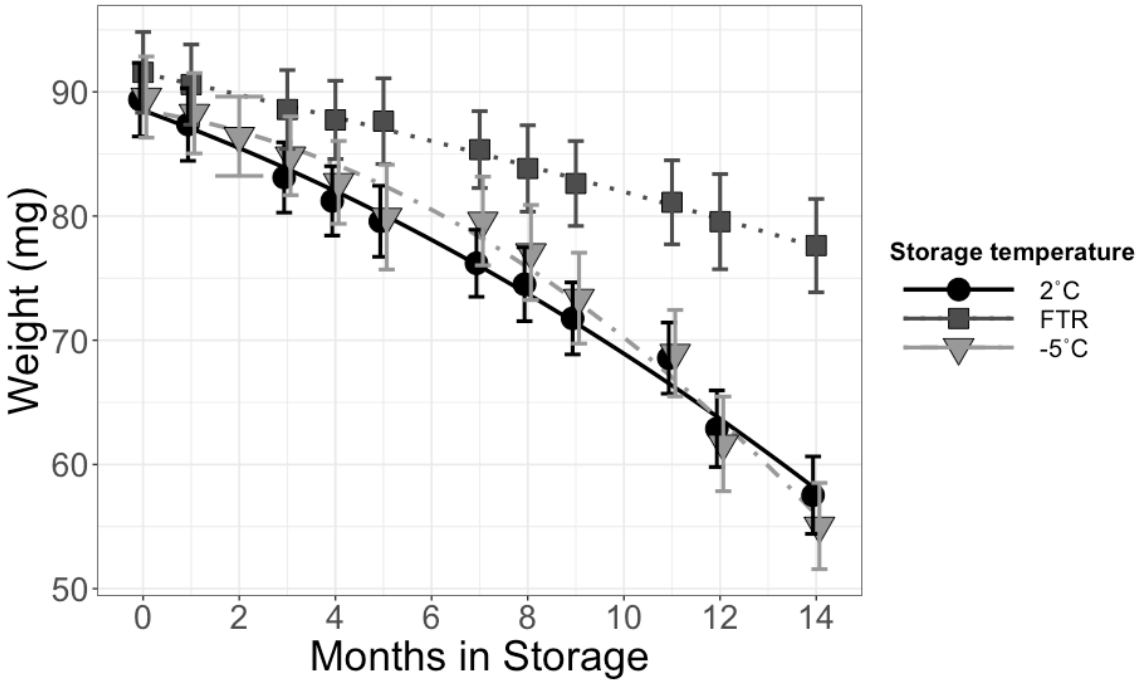


Figure 3.12 Average weight of bees during long-term storage in different thermal regimes

Weight of bees during long-term storage under different thermal regimes, where FTR refers to bees stored at 0˚C with 1 h at 5˚C. Points show mean weight lost (n = 100 per group) and error bars show one standard error. Lines show 2nd degree polynomial regression.

Using X-rays, it was shown that bees stored either at - 5˚C or in a FTR lost less fat body over the period studied, compared to the controls (Kruskal-Wallis, χ^2 = 40.18, DF = 2; p < .001; Figure 3.13). The loss of fat body appeared to be similar between the -5˚C and the FTR (Kruskal-Wallis, χ^2 = 2.52; DF = 1; Figure 3.13; p = .11).

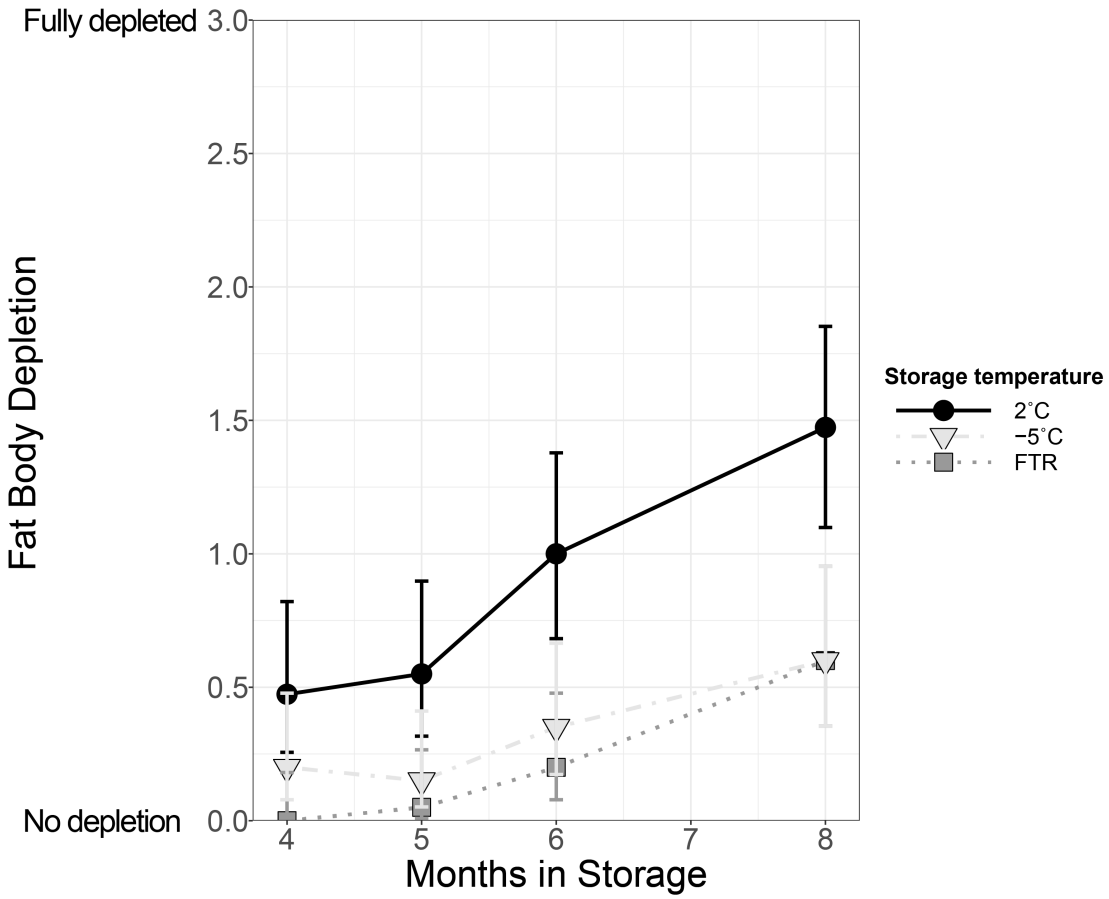


Figure 3.13 Average fat body depletion, as determined by X-rays, of bees during long-term storage in different thermal regimes

Average of the rankings of fat body depletion under different thermal regimes, where FTR refers to bees stored at 0˚C with 1 h at 5˚C. Points show weighted averages (n = 20 per group) and error bars show 95% binomial proportion confidence intervals. See methods for full description of rankings.

3.5 Discussion

In this chapter, the impacts of changing winter conditions and techniques to manipulate the overwintering of RMBs were investigated. Alterations to winter, as may occur with climate change, were also found to have deleterious effects on RMB survival (Figures 3.3 and 3.6), perhaps by increasing lipid usage (Figures 3.4 and 3.5). Furthermore, it was shown that historical climate change may be advancing RMB emergence (Figures 3.7 and 3.8), which may indicate future declines in RMB populations as the climate continues to warm. To mitigate these impacts different methods to manipulate RMB diapause were examined. Hexane and methoprene were shown to advance bee emergence by weeks (Figure 3.9), although methoprene had a negative impact on survival (Figure 3.10). Therefore, hexane may be a useful tool to synchronise RMB with crop blooms. FTRs were also shown to greatly reduce the impacts on survival of long-term storage for nearly two years (Figure 3.11), perhaps by reducing weight loss and lipid consumption (Figures 3.12 and 3.13). This again could be useful for commercial operations, allowing RMBs to be stockpiled and mitigate against pollinator losses that could occur in future climate change scenarios.

Delays to the onset of winter have been shown to reduce survival in blue orchard bees (Sgolastra et al., 2011, 2016); therefore, the impact of delayed onset of winter (storage at 2˚C) on RMB survival was assessed. As in blue orchard bees, delaying winter in RMBs resulted in lower survival (Figure 3.3), but the extent to which the survival was reduced was striking. Whilst it started off similarly to control bees between January and March, survival strongly declined thereafter,

with no bees surviving from May onwards (Figure 3.3). In contrast, when winter was delayed by 60 days in blue orchard bees there was only a 19% decrease in survival after 196 days in storage (Sgolastra et al., 2011), compared to 0% at 210 days here (Figure 3.3). Such a marked reduction in survival may be due to a greatly accelerated loss of body-weight (Figure 3.4), which may be driven by fat body loss, as 30- and 60-day delays to the onset of winter appear to cause depletion of this lipid store much more rapidly (Figure 3.5). This is substantiated by several studies by Sgolastra et al. (2010, 2011, 2016) in blue orchard bees, where delayed winters have been shown to elevate lipid consumption, fat body depletion, and body weight loss. Whilst such effects are well-evidenced in blue orchard bees, this was the first time that delayed onset to winter has been studied in RMBs.

A shortened wintering period on its own did not seem to affect the survival of bees that underwent a delayed onset to winter. RMBs with a pre-wintering duration of 60 days had a survival of 72.5% after only 30 days of winter (Figure 3.3). This is surprising given that when bees had a protracted wintering period of 35 days (with no pre-wintering duration), survival was 40% (Figure 3.6). Shortening of winter alone, therefore, does not explain the pattern of the bee survival. Delays to the onset of winter only showed an impact on survival after several months, which may mean that RMBs have a certain capacity to cope with changes to the timing of winter temperatures; however, they eventually succumb to weight and lipid loss. In the wild, the typical emergence period for the RMBs is mid-March to May (Gruber et al., 2011; Wasielewski et al., 2011b; Radmacher, 2012), which falls within the time period when bee survival was shown to be reduced to zero upon

delayed wintering (Figure 3.3). As RMBs are univoltine and have an obligate diapause from October-January (Raw, 1972; Wasielewski et al., 2013), during which they are unable to obtain more energy resources, they may be particularly susceptible to such delays in the onset of winter, which could severely impact their survival. In the face of climate change such delays may become more commonplace, and indeed already appear to be (Gallinat et al., 2015; Williams et al., 2015), suggesting that RMB populations may decline in a warming world.

To better understand possible impacts of climate change on RMBs, a historical dataset of their observations was assessed. As RMBs overwinter inside cavities, blocked from sunlight, their main emergence cue is likely temperature (Giejdasz and Fliszkiewicz, 2016). Therefore, earlier emergence times could indicate a response to increased temperatures. Looking at the period from 1977 to 2013, first observations of RMBs occur on average 30 days earlier in the past five years compared to 1977 (Figure 3.7). Whilst temperatures have been increasing across this period, the number of records submitted to the dataset examined has also been rising (Figure 3.8). Therefore, it was unclear whether the observed earlier emergence times were caused by the temperatures becoming increased over time or by a growing number of records. However, when subsets of data were tested it was shown that increased spring temperatures were still associated with earlier emergence (Appendix 3.3). Furthermore, it has previously been shown that increases in temperature can promote RMB emergence (Kehrberger and Holzschuh, 2019). Therefore, it is likely that warming is driving the observed change in the first observation of bees.

Climate change has been shown to drive many changes to phenology of insects and plants (Roy and Sparks, 2000; Cleland et al., 2007), although such changes are not always linear (Forrest, 2016). For RMBs, earlier emergence could mean a potential mismatch between them and certain crops they pollinate, such as apples. Apple orchards have been shown to bloom ~2 days earlier per decade due to increasing temperatures (Wolfe et al., 2005; Bartomeus et al., 2013), whereas here I show that RMB emergence advances by ~8 days per decade (Figure 3.6). RMBs are generalist pollinators and therefore may have some capacity to adapt to such a mismatch (Haider et al., 2014), but the crop plants may not.

Based on the results of delaying the onset of winter, earlier RMB emergence times may actually have positive effects on survival. Negative effects of a delay in the onset to winter on survival were only shown after 210 days of total storage, which corresponds to a May emergence. If RMB emergence is advanced by climate change it could prevent such losses. Indeed, with earlier emergence, bees may be able to compensate for lipid stores and weight lost during winter by feeding, if there are suitable plants available (Fliszkiewicz et al., 2012). On the other hand, increased temperatures throughout overwintering or protracted wintering periods may decrease bee survival (Fliszkiewicz et al., 2012), as the shorter the amount of time bees were at winter temperatures, the lower their survival (Figure 3.6). Altogether, the effects of climate change on this important pollinator could be substantial and require more research.

To combat these deleterious effects of climate change, different methodologies to manipulate RMB diapause were examined. Both hexane and methoprene were shown to increase the rate of RMB emergence (Figure 3.9); however, whilst methoprene advanced RMB emergence by as much as 45 days (Figure 3.9), it greatly reduced their survival (Figure 3.10). Methoprene causing impacts on survival is in stark contrast to previous studies on its effects on RMBs, where no changes to survival were reported (Wasielewski et al., 2011b; Giejdasz and Wasielewski, 2017b). Additionally, greater effects on RMB emergence were found here. For example, in Giejdaz and Wasielewski's (2017b) study, the biggest effect of methoprene on bee emergence was advancement by 2-4 days. By contrast, in the present study, at the same time point (December), the emergence was brought forward 34 days in methoprene-treated bees (Figure 3.9). Two differences between experimental conditions were: incubation temperatures, 26˚C compared to 20˚C; and study start date, 9th of December vs the 20th, in Giejdasz and Wasielewski (2017b) vs this study, respectively, all else was the same. These two experimental differences could explain some of the dissimilarities in the results obtained between the studies and may be important to recognise in the development of techniques to manipulate RMB diapause.

Hexane, on the other hand, increased the time to emergence without causing as large effects on survival. Emergence was advanced by hexane by as much as 20 days in December, and by more than a week in January (Figure 3.9). Survival was only significantly different between the hexane-treated and the control bees in January and May, averaging around 60% before May (Figure 3.10). Together, this indicates that hexane may be a useful method to force the emergence of

bees. Furthermore, when Denlinger et al. (1980) used hexane to break diapause in the flesh fly (*S. crassipalpis*) they obtained similar results by application either topically or as a vapour. Vapour exposure could be a useful method in commercial operations, as many bees could be exposed simultaneously; in contrast to topical applications, where each individual bee must be treated, making it very labour intensive. Different methods to apply hexane should be investigated further.

As well as investigating how emergence can be promoted, I also examined whether bees could be stored for prolonged periods in an overwintering state, primarily to be readily available when needed and to ensure supplies of bees are maintained. In the alfalfa leafcutting bee FTRs promoted survival of alfalfa leafcutting bees for more than two years (Rinehart et al., 2013). Similarly, the data obtained in this study suggests that FTRs may represent a promising storage regime to increase bee survival, since, after 15 months of storage, no bees survived when stored at a static temperature of 2˚C, while the survival of bees stored under FTR conditions was 63% (Figure 3.11). In fact, storage using FTRs allowed a small number of bees to survive up to 21 months (Figure 3.11). Practically, these results show that FTRs can allow access to viable bees essentially year-round.

As per the mechanism of FTRs promoting survival and essentially prolonging storage capabilities, this study suggests that a reduction in lipid loss may be important, which aligns with transcriptional changes observed in alfalfa leafcutting bees (Torson et al., 2015). Indeed, RMBs stored in a FTR lost weight much less

rapidly than those stored at 2˚C, and X-ray analysis revealed that they also had reduced depletion of their fat bodies (Figures 3.12 and 3.13). As fat bodies are the location of the majority of the bees' lipid stores (Bosch et al., 2010; Radmacher and Strohm, 2011), this implies that lipid loss was reduced by FTRs. Previous studies have also demonstrated that, when in prolonged storage, RMBs lose lipids, proteins and sugars due to an increased activity of metabolic enzymes (Dmochowska et al., 2013), so FTRs may act by slowing down some of these metabolic processes, thus increasing survival by ensuring adequate energy for emergence. It has been suggested that losses of lipid and other stores during overwintering could impact insects' abilities to survive after the period of overwintering is over (Hahn and Denlinger, 2007, 2011). Indeed, in close relatives of RMBs, blue orchard bees and European orchard bees, weight loss during overwintering appeared to reduce longevity and survival (Bosch and Kemp, 2000, 2004; Sgolastra et al., 2011, 2016). In this study, prolonged storage was also shown to decrease longevity (Figure 3.12), suggesting a similar response of RMBs as other *Osmia* spp..

The increase in survival of FTR-stored bees cannot be accounted for solely by maintaining the bee lipid stores. When bees were stored at -5˚C for prolonged periods, their fat bodies were also maintained (Figure 3.13). After 8 months of storage, there was in fact no difference in fat body depletion between FTR and -5˚C (Figure 3.13); however, bee body weight did differ between the two treatments (Figure 3.12). This implies that weight loss is caused by more than just lipid loss, suggesting that FTRs may also prevent the loss of other important overwintering stores. Indeed, this aligns with the hypothesis of Torson et al.

(2015) who suggest that FTRs act by allowing the repair of chilling injuries and thus restoring normal metabolic function and preventing the build-up of reactive oxygen species. It is likely that bees stored at -5˚C accumulated chilling injuries, which negatively affected their survival after 15 months in storage. FTRs on the other hand, may prevent or minimise such injuries, allowing increased storage times with minimal impacts on survival. Moreover, FTRs are straightforward to implement for commercial providers, as they only require a programmable incubator, so could be rapidly and easily taken up by industry.

3.5 Conclusions

In this study, it was demonstrated that delaying the onset or shortening winter caused drastic declines in the survival of bees, likely propagated by increased weight loss and loss of lipids. In natural populations climate change may have already advanced the earliest emergence of RMBs, as data here indicate that it has shifted earlier by ~30 days from 1977 to 2013, potentially suggesting future phenological mismatches with target crops. Given that shortening the overwintering of bees reduces their survival, this shift in phenology could indicate future declines in RMB populations. To combat these negative impacts of climate change, different techniques to manipulate RMB diapause were investigated. Here, it was demonstrated that the overwintering period of RMBs can be extended and shortened using thermal and chemical techniques. To time RMB emergence with crop blooms application of hexane may be a particularly promising technique, as it was shown to advance emergence without large negative effects on survival and has the potential to be applied in a number of

different ways, reducing labour costs for commercial provision. Furthermore, FTRs are a simple strategy to stockpile RMBs and ensure provision of viable bees, by reducing the impacts of long-term storage on survival, thus mitigating the impacts of climate change. However, there are many other pressures on pollinating insects, including RMBs, that could prove detrimental to their success as a species. One key example is the use of insecticides, which is examined in the next chapter.

Chapter 4: Imidacloprid undermines the thermal biology of three key pollinators

4.1 Abstract

Neonicotinoid pesticides (NPs) have been shown to have a range of sublethal effects on pollinator species, from foraging, to learning, to immobility. However, the impacts of NPs on thermal biology of pollinators are almost completely unknown. Here, I assessed the sub-lethal concentrations of imidacloprid (IMI) and its impact on three important pollinator species – red mason bees (RMBs; *Osmia bicornis cornigera*), blue blowflies (*Calliphora vicina*) and buff-tailed bumblebees (BTBs; *Bombus terrestris audax*) – as temperatures decline, by characterising their activity thresholds. Specifically, I investigated at what temperature they lose the ability to co-ordinate their movement (critical thermal minimum; CT_{min}), and when they enter chill coma. Furthermore, I assessed the impacts of imidacloprid on buff-tailed bumblebee queens' ability to maintain thoracic temperature, i.e. thermoregulate. I showed that buff-tailed bumblebees are particularly sensitive to imidacloprid, as a low field-realistic dose of 9 µg/l increases their critical thermal minimum by 13.9˚C, and to a lesser extent increased the temperature at which they enter chill coma. Similar impacts of imidacloprid are found in red mason bees and blue blowflies, but at much higher concentrations. 9 µg/l imidacloprid also reduced the thermoregulatory ability of buff-tailed bumblebee queens, with bees unable to maintain their thoracic temperature at temperatures on average below 14.2˚C. In contrast, another

neonicotinoid, thiacloprid, was shown to have similar sub-lethal effects on buff-tailed bumblebee critical thermal minimum, but at concentrations 4,000 times higher than imidacloprid. These results suggest that imidacloprid in particular could restrict buff-tailed bumblebee activity throughout the year, which would have sizeable consequences for provision of pollination services and thus for food security.

4.2 Introduction

The neonicotinoid pesticides (NPs) are a group of neuroactive insecticides that in insects preferentially bind to the certain neuroreceptors known as the nicotinic acetylcholine receptors (nAChRs) (Goulson, 2013; Simon-Delso et al., 2015). Due to the specificity for insect nAChRs and systemic protection of target crops, they have been extensively used as insecticides (Jeschke et al., 2011; Simon-Delso et al., 2015). In particular imidacloprid (IMI) has been one of the most widely sold insecticides in the world (Goulson, 2013; Simon-Delso et al., 2015). However NPs have been shown to exhibit various deleterious effects on pollinator species (Godfray et al., 2014; Potts et al., 2016; Alkassab and Kirchner, 2017). Whilst direct lethal effects are rare in field studies, many sub-lethal effects have been identified, including impacts on learning, memory and ability to forage (Alkassab and Kirchner, 2017). The exact doses of NPs to which bees will be exposed to in the environment is unclear (so called 'field-realistic doses'), but in general very low doses, of only 3.5 - 1.1 x 10^2 µg/l are reported (Bonmatin et al., 2005; Blacquiere et al., 2012; Alkassab and Kirchner, 2017). However, many laboratory studies concerning the impacts of NPs on pollinator species have used

much higher doses (Godfray et al., 2014, 2015), instead of outlining what concentrations exactly are sub-lethal for the tested pollinators and how they refer to likely field-relevant concentrations.

Defining a field-relevant concentration of NP is further complicated by different crops showing varying exposure levels to insects. For example, despite similar application rates of clothianidin, maize (*Zea mays*) has been reported to have higher residues of the NP in pollen compared to oilseed rape (Wood and Goulson, 2017). Similarly, maize also shows higher levels of thiamethoxam (THIM) compared to sunflowers (Wood and Goulson, 2017). Throughout the life of the crop as well, the amount of NP present can vary, typically decaying as time passes from the initial pesticide treatment (Bonmatin et al., 2015). Moreover, residues of NPs in pollen, in all plants, seem to generally be higher than those in the nectar (Godfray et al., 2014). As multiple factors can influence the exposure of pollinating insects, including the route of exposure, it is again useful for studies to fully outline how the chosen exposure route and pesticide actually influence mortality in the insect and how they may refer to field-relevant concentrations.

Research on NPs' effects on beneficial insects has focused on bumblebees (*Bombus* spp.) and western honeybees (*Apis mellifera*) as, being dominant pollinators in agricultural systems, they are more likely to be exposed to NPs used to protect crops from pest insects (Woodcock et al., 2016) and are responsible for providing pollination services to many important crops (Klein et al., 2007; Goulson and Hughes, 2015). For example, a field study conducting counts of buff-tailed bumblebee (BTB) colonies exposed to 5 µg/l of the NP clothianidin

showed clear negative effects on reproduction, with approximately 10% fewer workers, 35% fewer queens and 18% fewer drones produced over 5 weeks (Arce et al., 2016). This result is similar to an earlier study, which showed that 6 µg/l of IMI caused an 85% reduction in the production of BTB queens (Whitehorn et al., 2012).

Several studies have indicated that NPs increase neuronal sensitivity to acetylcholine (Manjon et al., 2018). For example, after exposure to NPs less acetylcholine is required to stimulate neuronal acetylcholine receptors (nAChRs), and thus depolarise the mitochondria (Moffat et al., 2015, 2016). These effects eventually lead to nervous overstimulation and thus immobility and death (Goulson, 2013; Van Der Sluijs et al., 2015; Simon-Delso et al., 2015). The associated depolarisation of mitochondria potentially impairs ability to produce adenosine-triphosphate (ATP; Powner et al., 2016), which, as the primary ionic pumps that maintain cellular ionic homeostasis are ATP driven (Treherne and Schofield, 1981), could ultimately result in an impaired ability to maintain ionic balance. Such ionic imbalances could cause depolarisation of the central nervous system, consequently leading to loss of neuromuscular control due to overproduction of action potentials (Koštál et al., 2004; Goulson, 2013; Van Der Sluijs et al., 2015; Simon-Delso et al., 2015). Loss of neuromuscular control might explain why NPs cause instances of immobility as well as reduce overall mobility (Lambin et al., 2001; Medrzycki et al., 2003; Moffat et al., 2016). However, there is no direct evidence of what is causing this immobility in the literature.

Another important area where there is little understanding is the effect of NPs on the thermal biology of insects. For pollinators like the BTB, cold is an unavoidable stress for part of the year (Renault et al., 2002), but there have been few studies on how it may interact with the deleterious effects of NPs. One means of disentangling this is to consider activity thresholds. For cold, two specific measures can be observed: critical thermal minimum (CT_{min}), which is the temperature where neuromuscular signals are disrupted and coordinated movement is lost (Chapter 1; Figure 1.5); and chill coma, which is the temperature at which the ability to move an appendage is lost (Chapter 1; Figure 1.5). As these measures indicate how well insects are able to move under different temperature conditions, investigating these activity thresholds after exposure to NPs could identify how important activities such as mating, foraging, and predator avoidance are affected, as when these actions can occur will be governed by the upper and lower thermal limits of insects, as within this 'window' normal organismal functioning and movement will be able to occur (Everatt et al., 2013).

There is some evidence to suggest that NPs may increase CT_{min}. The onset of CT_{min} has been directly linked with loss of ionic balance (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017), nervous system failure and muscle depolarisation (Robertson et al., 2017). In addition, NPs have been found to cause nervous system dysfunction by their action on nAChRs and it is possible that NPs can cause ionic imbalances (Matsuda et al., 2001; Goulson, 2013; Van Der Sluijs et al., 2015; Simon-Delso et al., 2015). In fact, a previous study from this lab identified that a store-bought, thiacloprid(THIC)-based, pesticide (Provado Ultimate Bug Killer®) caused CT_{min} of BTB workers to increase from

4.2˚C to 17.7˚C at a dose of 4.0 x 10^4 µg/l (Owen, 2015, PhD thesis). However, the concentration used in Owen's (2015) study was, although being sub-lethal, much higher than what has been considered field-relevant (Blacquiere et al., 2012; Alkassab and Kirchner, 2017). As the pesticide used in Owen et al.'s study was for domestic use, and THIC is not as widely used as a pesticide, further study into the effects of NPs on BTBs is warranted. For example, IMI is very widely used (Jeschke et al., 2011), and has been shown to have particularly deleterious effects on bumblebees (Cresswell et al., 2012).

A change in CT_{min} could be detrimental to pollinators and food security. CT_{min} is the point at which organisms lose the ability to co-ordinate their movement (Hazell and Bale, 2011). As such, an increase in CT_{min} would limit the temperatures at which pollinators could be active, which could limit their ability to provision and thereby produce offspring. For example, in Owen's 2015 study, the CT_{min} of 17.7˚C after thiacloprid exposure would be higher than the daily average United Kingdom (UK) temperature for most of the year, suggesting their ability to co-ordinate movement would be limited only to summer months (Owen, 2015, PhD thesis). Moreover, bumblebees are known to be active earlier in spring than many other pollinators (Goulson, 2010), when temperatures are often below 10˚C in the UK (Chapter 3, Figure 3.8), as such, an increase in CT_{min} could substantially limit bumblebees' spring activity and thus their role as crop pollinators.

In the published literature, there are no studies of the effects of NPs on activity thresholds, but there are limited studies on pesticides' influence on critical

thermal maximum (CT_{max}) in aquatic vertebrate species. For example, chlorpyrifos, a pesticide that has a similar mode of action to NPs, was found to decrease the CT_{max} of tadpoles (*Rhinella arenarum*) and reduced locomotor performance (Quiroga et al., 2019). This, together with the findings of Owen's (2015) study, shows that neuroactive pesticides, like NPs, may have pronounced effects on activity thresholds, but there is a dearth of literature on the topic.

The possible disruption of ionic balance and nervous system control caused by NPs could also have consequences beyond CT_{min}. Bumblebees have an ability to increase their body temperature above ambient conditions, i.e. thermoregulate (Heinrich, 1974a, 1975; Goulson, 2010). Without motor control bumblebees may not be able to contract their thorax adequately to generate heat, which could also prevent them flying at lower temperatures (Heinrich, 1975). Indeed, when African honeybees (*Apis mellifera scutellate*) were exposed to THIM their ability to thermoregulate was hindered, especially when exposed to lower (22˚C) temperatures (Tosi et al., 2016). A study of BTBs examined the ability of workers to recover from cold torpor by thermogenesis, and found that IMI increased, while THIM decreased, the rate at which they recovered from cold torpor (Potts et al., 2018). Overall, these studies indicate that NPs may be having an impact on thermoregulation, but the impacts are neither clear nor consistent.

Understanding the effects that NPs have on thermoregulation is crucial, as bumblebee queens in particular are active at cooler periods of the year (Goulson, 2010). Disruption of queens' abilities to function normally in cold could have large impacts on bumblebee numbers since they are the only life stage that remains

active in late autumn and early spring and, as colony founders, they represent a bottleneck in populations (Goulson, 2010). Moreover, if bumblebees were only able to be active at later, warmer, times of the year, they may have to compete with other pollinator species that lack an ability to be active in spring (Free, 1979; Goulson, 2010), i.e. bumblebees would lose a competitive advantage over other pollinators, which could have a negative impact on spring crops that rely on bumblebees.

There is an open question however about the influence of NPs on the activity thresholds of pollinators other than BTBs. For example, whilst Owen et al.'s (2015) study showed an increased CT_{min} after exposure to a NP in BTBs, does the same hold true for other insect pollinators? Previous studies on lethal and sub-lethal effects of NPs on different pollinator species have shown contrasting effects (Blacquiere et al., 2012; Pisa et al., 2014; Van Der Sluijs et al., 2015; Alkassab and Kirchner, 2017). Thus, it is important to extend any assessment of NP impacts on CT_{min} beyond just honeybees and bumblebees. Other important pollinators include solitary bees and flies (Garibaldi et al., 2014; Orford et al., 2015; Potts et al., 2016; Rader et al., 2016), but the impacts of NPs on these groups are not well-understood (Van Der Sluijs et al., 2015; Alkassab and Kirchner, 2017).

Flies can pollinate in colder conditions than honeybees and bumblebees, and are often the dominant pollinator species within alpine and high latitude terrestrial ecosystems (Orford et al., 2015). For example, blue blow-flies (BBFs) have been shown to have similar pollinator efficiency of carrots (*Daucus carota*) compared

to honeybees, in part due to the flies' ability to pollinate at lower temperatures (Howlett, 2012). Even in temperate systems, Diptera can be important pollinators. The role of hoverflies (syrphid Diptera) in pollination is well-evidenced, but there is increasing recognition that non-syrphid Diptera may make valuable contributions to pollination services, as their abundance means that they may contribute more to pollination than syrphids (Orford et al., 2015). Indeed, in a study comparing BBFs to RMBs on pollination of carrots (*Daucus carota*), onions (*Allium cepa*) and field mustard (*Brassica rapa*), the flies were associated with higher germination rates, yield and seed weight than the bees (Schittenhelm et al., 1997).

Despite the evidence that flies can be important pollinators, almost nothing is known about whether NPs can affect their pollinating ability. With the exception of the molecular characterisation of neonicotinoids' effects on *D. melanogaster* (Daborn et al., 2001, 2007; Denecke et al., 2017), most studies concerning Diptera and neonicotinoids have focused on them as pests (e.g. Beers et al., 2011; Paul et al., 2006), and these have considered NPs as a measure of control, trialling high doses (e.g. 84-12,000 µg/l) and showing large effects on mortality and oviposition. There is one study concerning hoverflies (*Eristalis tenax*) as non-target organisms (Basley et al., 2018), and the authors observed no effects on survival, development, or effects on adult energy budgets at doses below 500 µg/l THIM, however, this does not rule out other sub-lethal effects on the organism, as seen in bees.

Solitary bees also pollinate some plants more effectively than honeybees and bumblebees, and are dominant pollination service providers in orchards (Bosch and Kemp, 2002; Gruber et al., 2011; Garibaldi et al., 2013). For example, the RMB has been shown to pollinate a wide range of crops, including rapeseed (Teper and Biliński, 2009), apples (Gruber et al., 2011), and strawberries (Wilkaniec and Radajewska, 1997). However, there are few studies concerning the effects of NPs on solitary bees, and those are conflicting, with some showing negative consequences of NPs (Sandrock et al., 2014; Rundlöf et al., 2015), and others showing only negligible impacts (Abbott et al., 2008; Peters et al., 2016). Furthermore, the impact of NPs on solitary bee activity thresholds is unknown. Given the knowledge gap surrounding NPs effects on solitary bees and flies, it is imperative to elucidate what the effects of NPs are on their activity thresholds. This would help gain a better understanding of how NPs are affecting pollinators in general.

Against this background, the aim of this study was to assess the impact of NPs on the thermal biology of three key pollinator species: BTBs, RMBs and BBFs. I hypothesise that IMI will increase the temperature at which CT_{min} and chill coma are entered in the pollinator species, based on previous assessments from this laboratory (Owen, 2014). In addition, based on the available evidence in honeybees (Tosi et al., 2016), I also hypothesise that the thermoregulation of bumblebee queens will be disrupted by IMI exposure. Finally, I hypothesise that the effects of neonicotinoids will vary between the insects and the neonicotinoid tested, as previous work has indicated that different insects have different

susceptibilities, and the potency of neonicotinoids can differ based on their structures (Cresswell et al., 2012).

4.3 Methods

4.2.1 Insect cultures

Worker BTBs (*B. t. audax*) were purchased from Biobest® and were reared according to the manufacturer's instructions. Queen BTBs were purchased from Biobest® and were immediately transferred to experimental conditions. The bumblebees were not age controlled and instead were selected based on similarity of size. BBFs were caught wild, at the University of Birmingham using an approach described previously (Coleman et al., 2015). Once caught, two generations were reared before experimentation to avoid maternal effects and the influence of acclimatisation from the natural environment. Adult BBFs were reared at 20˚C under 16:8 h (light:dark) LD cycle with sugar and water *ad libitum* until 4-days post-eclosion, at which point they were transferred to experimental conditions. RMB (*Osmia bicornis cornigera*) cocoons (pre-emerged adults) were purchased from WAB-Mauerbienenzucht Ltd. These were maintained at 2˚C before being transferred to 20˚C to eclose. Once bees had eclosed they were sexed based on the colour of clypeal hairs and the size of antenna (Raw, 1972).

4.2.2 Assessing mortality of insects exposed to imidacloprid

All insects were exposed to IMI through oral ingestion. Treatment groups were kept in 20 x 20 x 20 cm cages with mesh windows under 16:8 h LD cycle at 20˚C. IMI was purchased from Sigma-Aldrich® as powder and was dissolved in a 50:50

sucrose:water solution for the BBFs, in Pro-Sweet Liquid Feed (Mann Lake Ltd., Minnesota, United States of America [USA]) for RMBs, and in Biogluc® for the BTBs, to the relevant concentrations. These IMI solutions were then wrapped in foil to prevent degradation (Maienfisch et al., 2001). Small plastic sample pots, with a small 1 mm perforation, containing the 20 ml NP solution were taped to the top of the cage, with a Petri dish lid underneath to catch any drops. All species were exposed *ad libitum* for 24 h. Mortality was then assessed after another 24 h for at least three replicates (n = 5 per replicate for BTB workers; n = 10 for BBFs; n = 5♂♂ & 5 ♀♀ for RMBs). Sub-lethal doses of neonicotinoids were identified as those before mortality increased exponentially in the calculated dose-response relationships.

For queens, mortality was not assessed as too few were available to conduct mortality assessments in addition to testing of CT_{min}, chill coma and thermoregulation. Wu-Smart and Spivak (2018) found that after chronic exposures queens suffered mortality at as low as 1 µg/l IMI. The data from Wu-Smart and Spivak (2018) in addition to mortality data collected from workers in the present study were used to select 9 µg/l as the dose at which CT_{min} and chill coma would be assessed. Queens were exposed to IMI as described above for BTB workers.

4.2.3 Calibrating insect body temperature changes relative to activity threshold arena temperature

The activity threshold arena employed in this experiment is presented in figure 4.1. Before CT_{min} and chill coma experiments were performed, a series of

calibration tests were run, as monitoring the arena temperature alone does not always give an accurate measure of insect body temperature, especially for species, such as BTBs, which can thermoregulate. These calibration tests were performed by attaching a Type K thermocouple to the insect's throrax with fine twine and an adhesive, Oecotak (Oecos Ltd, Kimpton, Hertfordshire, UK). The thermocouples were connected to a datalogger (Pico ® TC-08 Thermocouple Data Logger; Pico® Technology, UK), interfaced with a computer. The arena temperature was meanwhile recorded using a Type K thermocouple, pushed through a hole in the arena wall (Figure 4.1), connected to a thermometer (Tecpel Advanced Digital Thermometer DTM-315; Tecpel Ltd., Taiwan). These data were recorded throughout the temperature ramping protocol, from +20 to -10˚C. The arena temperature and insect body temperature were then plotted, and linear regressions were used to generate equations that could then determine body temperature directly from arena temperature values, and thus thermocouples did not need to be attached to experimental animals.

This same methodology was also used to assess the thermoregulation of queens (i.e. the queens' thoracic temperature was monitored with an attached thermocouple), as thermoregulation was defined as the maintenance of core body temperature as the environment (i.e. the arena) was cooled. By determining when queens ceased to maintain their body temperature (determined as when the thoracic temperature stopped fluctuation and began to decrease towards the arena temperature), a measure of their thermoregulatory ability could be found.

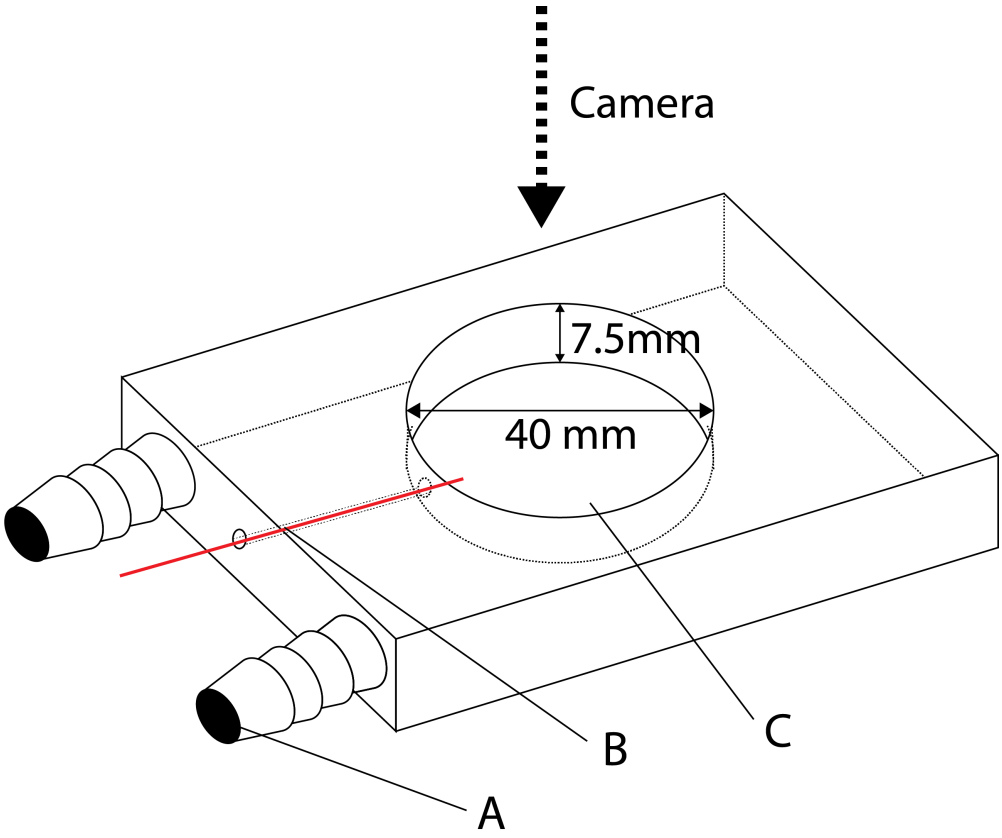


Figure 4.1 Temperature-controlled insect activity arena used to assess CT_{min} and chill coma.

Diagram of aluminium cooling arena, where a camera was positioned above the arena to allow filming of the insects as they were cooled. **A.** Connection to programmable thermal bath, to allow heating or cooling. **B.** Thermocouple to measure temperature. **C.** Arena to place insects, as in Hazell et al. (2008).

4.2.4 Measuring the critical thermal minimum and chill coma of insects

All species' CT_{min} and chill coma were assessed using an adapted version of Hazell et al.'s (2008) methodology. Each species was exposed to its respective sub-lethal IMI dose for 24 h (to ascertain the acute effects, similar to a foraging trip, as above) and were then immediately placed in a central aluminium cooling arena, covered with a thin perspex lid (Figure 4.1).

Insects were cooled using cooled fluids that were controlled via a programmable alcohol bath (Haake Pheonix 11 P2; Thermo Electron Corporation, USA). It has been observed that changes in the rate of cooling can affect at what temperature CT_{min} and chill coma occur (Oyen et al., 2018). Therefore, initially different rates of cooling were assessed: 0.2˚C/min and 0.5˚C/min. When the rate of 0.2˚C/min was tested with BTBs and BBFs and compared to 0.5˚C/min, no differences in CT_{min} or chill coma were found (n=30), thus 0.5˚C/min was used to reduce the timeline of experiments. This was in contrast to RMBs where a rate of 0.5˚C/min gave much lower CT_{min} values than 0.2˚C/min (n=20), as such 0.2˚C was selected to avoid underestimation of activity thresholds.

Once placed in the arena, each species was cooled at their respective rate, as described above. The CT_{min} and chill coma of the insects were recorded using a digital camera (Infinity 1-1- Lemenera Scientific, Ottawa, Canada) with a macro lens (Computar MLH-10X, CBC Corp, USA), with the time and temperature displayed. All videos were blinded, by random number assignment to each before being assessed, to ensure no bias was introduced into the analysis. Videos were played back to determine the temperature at which the last coordinated movement (CT_{min}) and last twitch (chill coma) occurred (Hazell et al., 2008).

4.2.5 Determining impact of altered critical thermal minimum on seasonal activity of buff-tailed bumblebees under field temperatures

To determine how any change in CT_{min} induced by IMI exposure might impact on seasonal activity patterns of BTB workers and queens, their respective CT_{min} values were plotted across average daily UK air temperature profiles over a 10

year period (2007-2017) using data from the Met Office Integrated Data Archive System (MIDAS), Kew Garden Weather Station, London, UK. The mean number of days below the CT_{min} of BTB workers and queens exposed to 9 µg/l was then found for each month and compared to the mean number of days below the CT_{min} of the untreated controls.

4.2.6 Assessing the response of BTB workers to another neonicotinoids: Thiacloprid

As above, THIC was purchased from Sigma-Aldrich® as powder and was dissolved in Biogluc® to the relevant concentrations. Mortality assessments were performed as previously described. Once sub-lethal concentrations had been identified CT_{min} and chill coma were assessed as previously described.

4.2.7 Statistical analyses

An alpha threshold of .05 was used (i.e., results were considered 'significant' if *p*-values were below .05), except where otherwise stated. No data were transformed, unless otherwise stated. Statistical outputs, including test-outputs, degrees of freedom (DF) and p-values are described in the text.

Logit regression was used to identify dose-response relationships in mortality data. Error bars for mortality were calculated as 95% confidence intervals with a Poisson distribution, as these can be constrained between 0 and 100%, making them useful to interpret percentage data, like mortality. A linear or polynomial regression were used for CT_{min} and chill coma data to find relationships between the dose and the response, with polynomial regression used when it increased

the R^2 by more than 0.1. Analysis of covariance (ANCOVA) was used to discern differences between regression lines.

To compare CT_{min} and chill coma data for BTB queens, a Wilcox-Rank Sum Test with a continuity correction was performed, as it is suitable for comparing two groups that are not normally distributed. As only a small number of queens were used in this experiment, a test of normality was not justified, instead data-points were plotted across a histogram and did not appear to be normally distributed.

4.4 Results

4.4.1 Impacts of imidacloprid on pollinator survival

BTB worker mortality increased rapidly at concentrations above 90 µg/l (Figure 4.2). At concentrations above 2.0 x 10^4 µg/l all BTB workers died (Figure 4.2). A line generated by logit regression allowed estimation of the lethal dose for 50% (LD_{50}) of BTBs as 1,000 µg/l.

Overlapping confidence intervals and non-significant differences in mortality of male and female RMBs, according to a Wilcox-Rank Sum Test (W=7920; n=240; p = .23), allowed their responses to be grouped together. According to a logit regression, the mortality began to increase rapidly after 90 µg/l, reaching 88% at a concentration of 5 x 10^5 µg/l, with an LD_{50} of 1.5 x 10^4 µg/l (Figure 4.2). Similarly, for BBFs, mortality rapidly increased at doses above 90 µg/l, with 87% mortality at 1.8 x 10^5 µg/l (Figure 4.2), giving an LD_{50} of 1x10^5 µg/l.

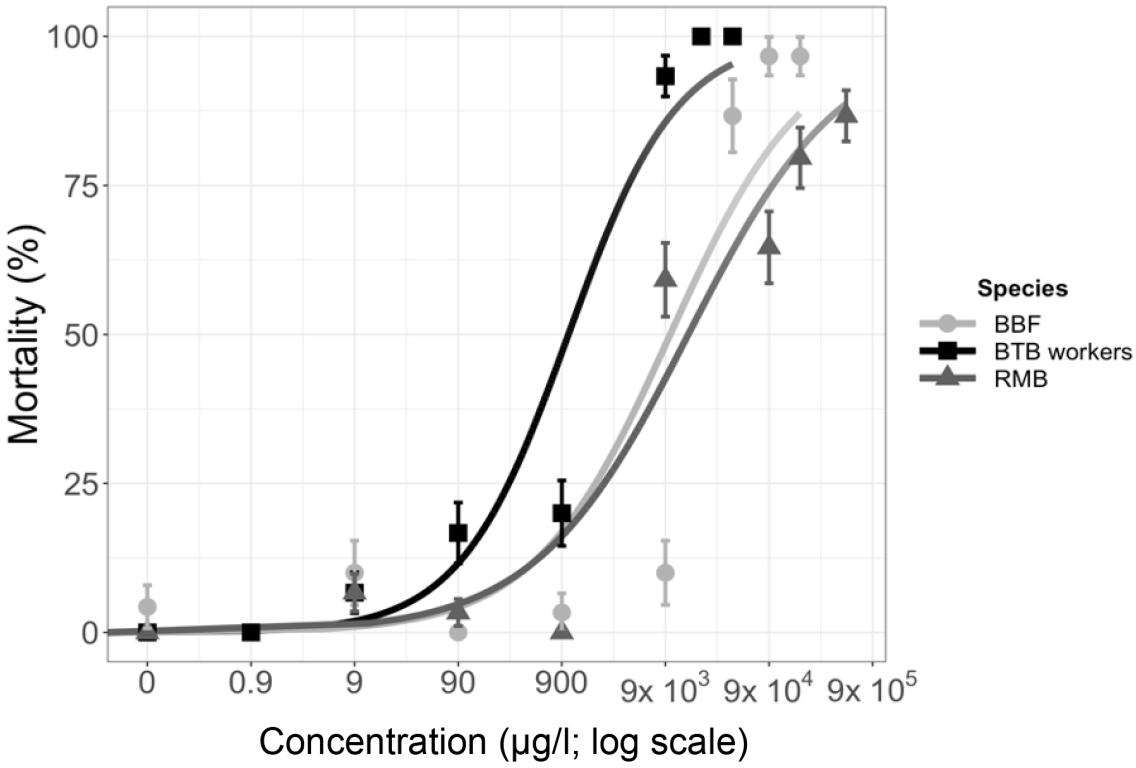


Figure 4.2 Mortality of key pollinator species when exposed to varying doses of imidacloprid

Relationship between the mortality of BTB workers (n = 40 per concentration), RMBs (n=30 per concentration; ♂♂ = 15, ♀♀ = 15), BBFs (n=30 per concentration) and concentration of IMI used, described by logit regression. Points represent means while errors bars show 95% confidence intervals with Poisson distribution.

4.4.2 Influence of imidacloprid on critical thermal minimum and chill coma

Untreated, control, BTB workers had a CT_{min} of 4.7 ± 0.7˚C (mean ± standard error; Figure 4.3A). CT_{min} values increased significantly with IMI concentration (Figure 4.3A), reaching 18.4 ± 0.9˚C at the highest dose of 9 µg/l, (3rd degree polynomial regression; $y = 23.89x + 4.36x^2 + 1.13x^3 – 2.37$; $R^2 = .56$; F-statistic = 60.01; DF = 135; $p < .001$; Figure 4.3A). Chill coma also increased linearly with

IMI dose (Figure 4.3A), changing from -2.6 ± 0.4˚C in controls to 2.8 ± 0.5˚C at the highest dose of 9 µg/l (linear regression; y = .42x - 2.05; R^2 = .25; F-statistic = 45.13; DF = 132; p < .001; Figure 4.3A).

For RMBs, CT_{min} increased with concentration of IMI (linear regression; y = .44x + 1.02; R^2 = .36 F-statistic = 45.06; DF = 79; p < .001; Figure 4.3B). Untreated controls had a CT_{min} of 2.9 ± 0.3˚C compared to 8.7 ± 0.9˚C at the highest dose tested of 900 µg/l (Figure 4.3B). Chill coma also had a positive linear relationship with concentration (linear regression; y = .55x + 4.17; R^2 = .05; F-statistic = 5.05; DF = 79; p = .028; Figure 4.3B), although the size of the effect was small. Chill coma was -1.8 ± 0.3˚C in the controls, but at the highest dose (900 µg/l) it was -1.0 ± 0.2˚C.

Again, CT_{min} increased as the concentration of IMI rose in BBFs (linear regression; y = .48x + 1.09; R^2 = .36; F-statistic = 137.70; DF = 105; p < .001; Figure 4.3C). In the untreated controls CT_{min} was 0.4 ± 0.2˚C, which compares to 9.7 ± 1.1˚C at the highest dose of 9 x 10^3 µg/l (Figure 4.3C). Chill coma also increased with IMI concentration (linear regression; y = .62x + 5.51; R^2 = .09; F-statistic = 18.91; DF = 105; p = .010; Figure 4.3C), however, it appears that this was largely driven by the highest dose (9 x 10^3 µg/l). When the highest dose is removed from the analysis, there was no significant effect of concentration on chill coma (linear regression; y = -.24x + 1.33; R^2 = .00; F-statistic = 0.68; DF = 72; p = .41).

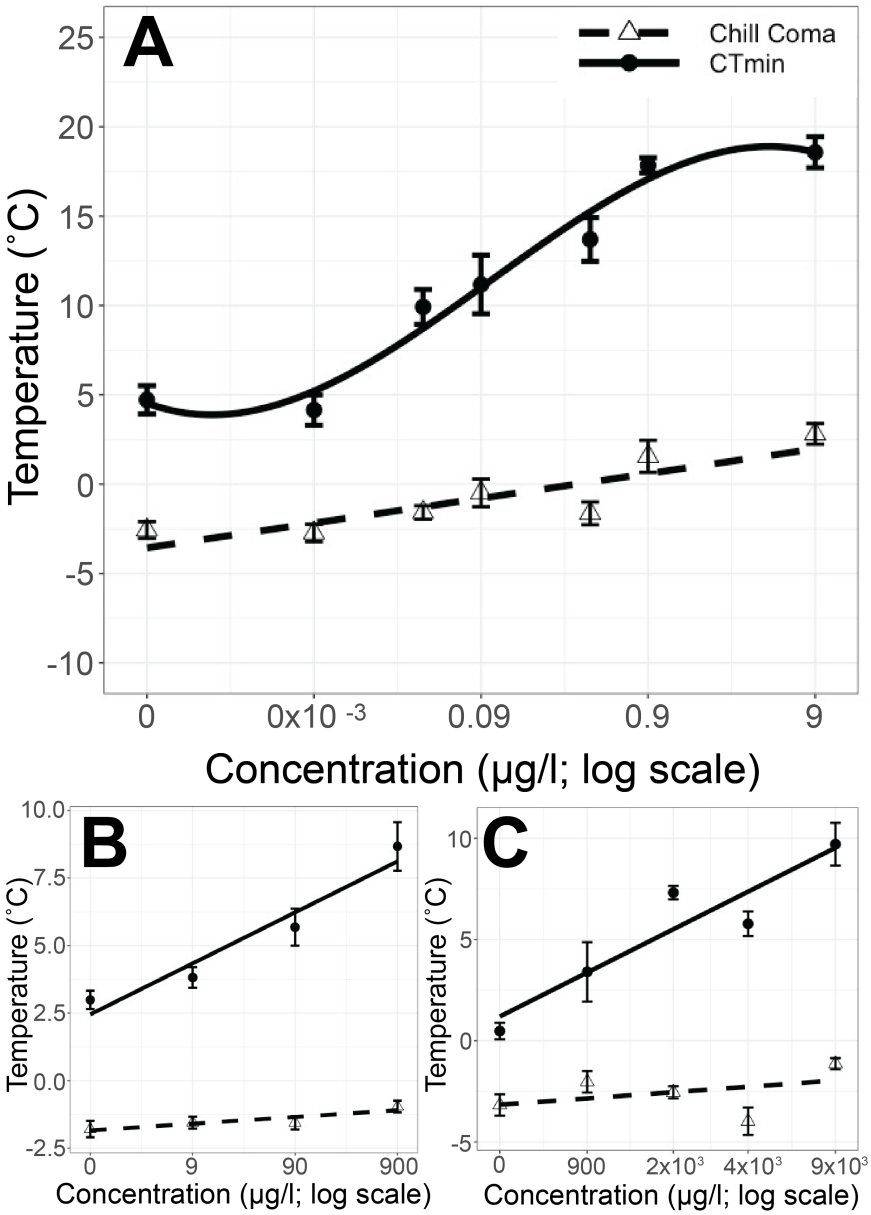


Figure 4.3 Relationship between CT_{min} and chill coma of three pollinators, and concentration of IMI

Points describe means and error bars show one standard error. **A** BTB workers' (n = 25 per concentration) CT_{min} and chill coma, where CT_{min} described is by 3rd degree polynomial regression (R^2 = .56) and chill coma by linear regression (R^2 = .25) respectively. **B** RMB (n = 30 per concentration; ♂♂ = 15, ♀♀ = 15) CT_{min} and chill coma, described by linear regression (R^2: CT_{min} = .36, chill coma=.05). **C** BBF (n=15 per concentration) CT_{min} and chill coma, described by linear regression (R^2: CT_{min} = .36, chill coma=.09).

4.4.3 Imidacloprid impact on buff-tailed bumblebee queen thermoregulation, critical thermal minimum and chill coma

IMI exposure significantly disrupted the ability of queens to thermoregulate (Figures 4.4A and B). Untreated queens were able to maintain a body temperature 7 to 10˚C above the arena temperature until the latter dropped below approximately 9.0˚C (Figures 4.4A and B). In contrast, queens exposed to 9 µg/l IMI lost this ability at an arena temperature of approximately 14.2˚C (Wilcox-Rank sum test; W = 51.5; n = 20; p = .04; Figure 4.4B).

IMI exposure also significantly increased the mean CT_{min} temperature of queens from 3.5 ± 0.6˚C in controls, to 11.3 ± 0.8˚C in bees exposed to 9µg/l (Wilcox-Rank sum test; W = 1; n = 20; p < .001; Figure 4.4C). IMI exposure may have had a small impact on chill coma, as chill coma increased (1.6 ± 0.8˚C) relative to the untreated controls (-1.5 ± 0.9˚C) (Wilcox-Rank sum test; W = 13.5; n =20; p = .06, Figure 4.4C).

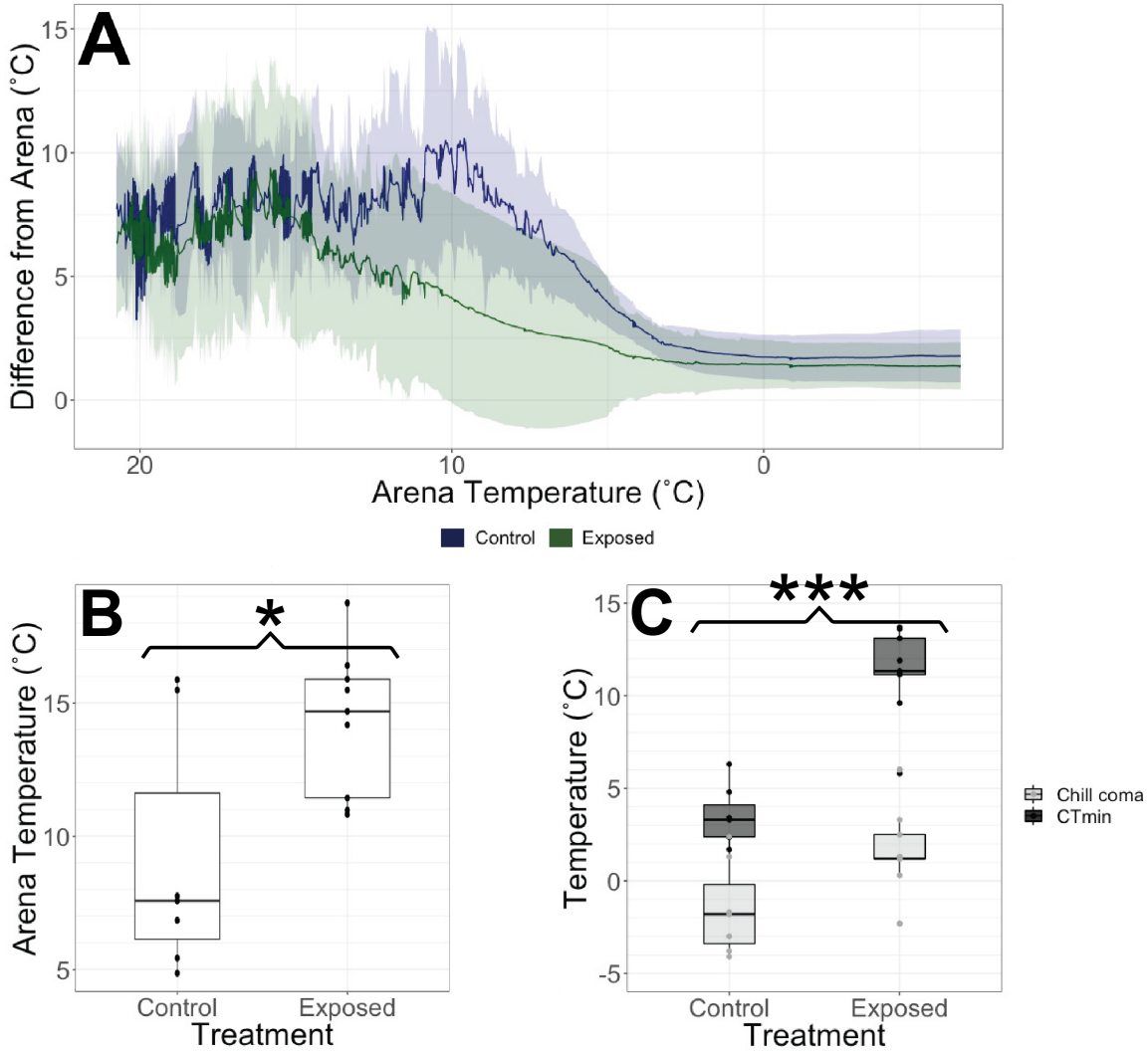


Figure 4.4 The influence of imidacloprid on buff-tailed bumblebee queen thermoregulation, CT_{min} and chill coma

A. Difference between BTB queen body temperature and arena temperature, for bees exposed to imidacloprid (green; n=10) and untreated controls (purple; n=10). Shaded areas show 95% confidence Intervals **B.** Temperature of the cooling arena at the point when queens ceased thermoregulation under each treatment, **C.** CT_{min} and chill coma after exposure to imidacloprid (n=10) versus controls (n=10). Asterisks show significant differences, * = p<0.05, ** = p<0.01, *** = p<0.001.

4.4.3 Implications of increased critical thermal minimum in buff-tailed bumblebees

When CT_{min} values are plotted against UK mean daily temperatures for the period 2007-2017 (Figure 4.5), it is possible to determine the frequency of events when temperatures drop below this value, i.e. where bees are proposed to no longer have coordinated movement (Figure 4.3A). For workers, sub-lethal (9 µg/l) exposures to IMI mean that activity is mostly restricted to the summer months, with only a monthly maximum of around 20 days when temperatures would be suitable in July (Figure 4.5A). BTB queens would be able to be active in every month of the year, but the number of days this would be possible is largely curtailed compared to unexposed bees (Figure 4.5B). On average compared to the controls, exposed queens would only be able to be active on 8.3% as many days in winter (December-February), 57.3% as many days in spring (March-May), and 67.8% as many days in autumn (September-November). In summer (June-August) the number of days above CT_{min} was relatively similar to the controls.

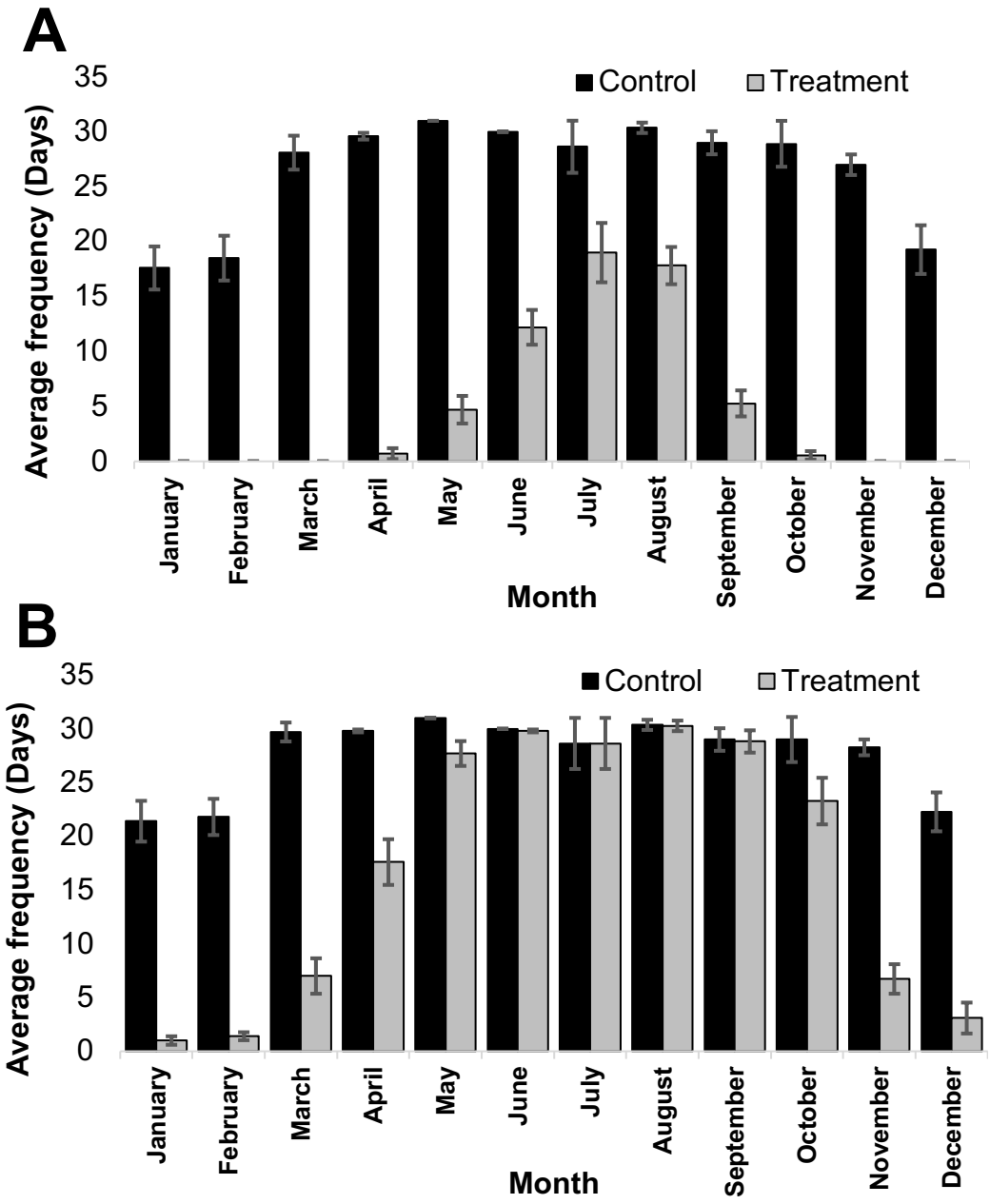


Figure 4.5 Average number of days in the UK above the CT_{min} for buff-tailed bumblebees after 9 µg/l IMI exposure

Average daily air temperature data (2007-2017) were taken from MIDAS, Kew Garden Weather Station. **A** The mean number of days below the CT_{min} of BTB workers exposed to 9 µg/l IMI (Treatment; grey bars), was compared to the untreated controls (Control; black bars). **B** The mean number of days below the CT_{min} of BTB queens exposed to 9 µg/l IMI (Treatment; grey bars), was compared to the untreated controls (Control; black bars). Error bars represent one standard error.

4.4.5 Impacts of thiacloprid on buff-tailed bumblebee mortality and activity thresholds

Another neonicotinoid, THIC, was also tested for impacts on the mortality of BTB. In this case, mortality increased gradually, reaching 20% mortality at the highest dose tested, 5 x 10^5 µg/l (Figure 4.6).

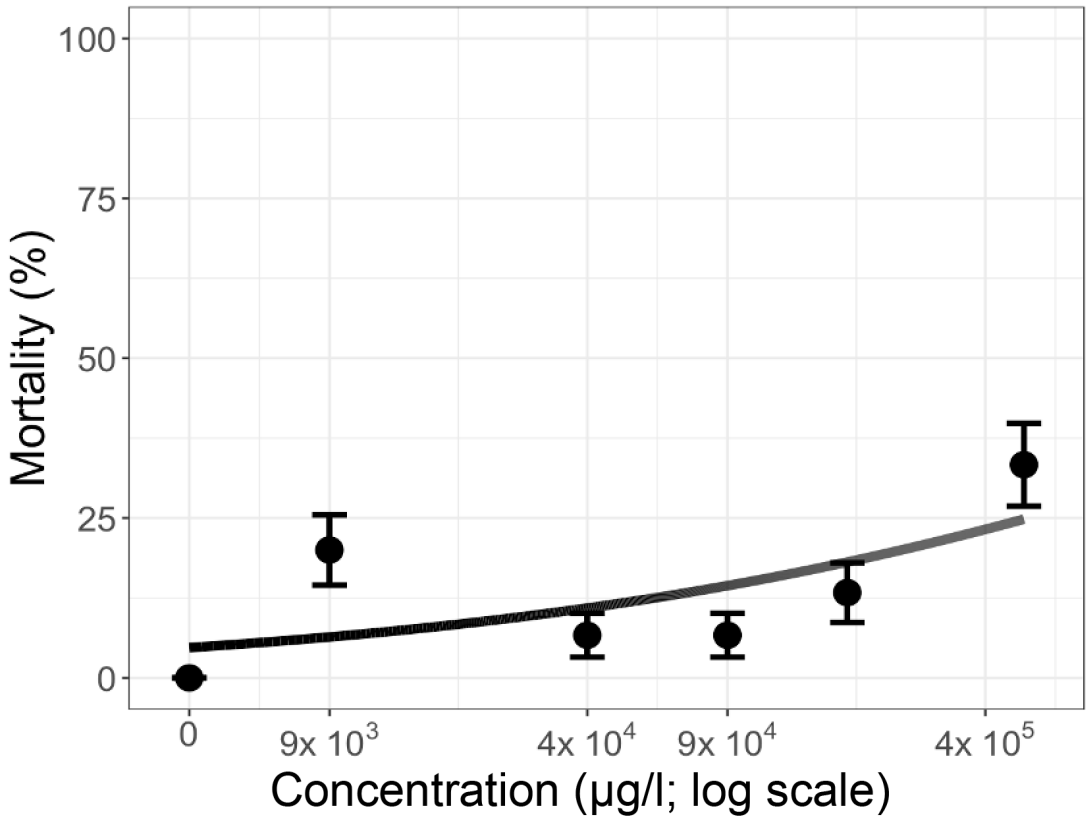


Figure 4.6 Mortality of buff-tailed bumblebee workers when exposed to varying doses of thiacloprid

Relationship between the mortality of BTB workers and concentration of THIC used, described by logit regression. Points represent means while errors bars show 95% confidence intervals with Poisson distribution.

Concentrations of THIC also increased CT_{min} in BTB workers (3rd degree polynomial regression; $y = 6.94x + .34x^2 - 3.10x^3 + 1.58$; $R^2 = .43$; F-statistic =

26.37; DF = 97; $p < .001$; Figure 4.7). Compared to the control CT_{min} (4.7 ± 0.4˚C), BTBs had a much higher CT_{min} at all concentrations tested, within the range of 13.8 – 16.0˚C (Figure 4.7). In addition, chill coma increased with application of THIC, all concentrations tested increased chill coma temperature (3rd degree polynomial regression; $y = 5.79x + .88x^2 - 3.12x^3 + 16.65$; $R^2 = .23$; F-statistic = 30.85; DF = 99; $p < .001$; Figure 4.7).

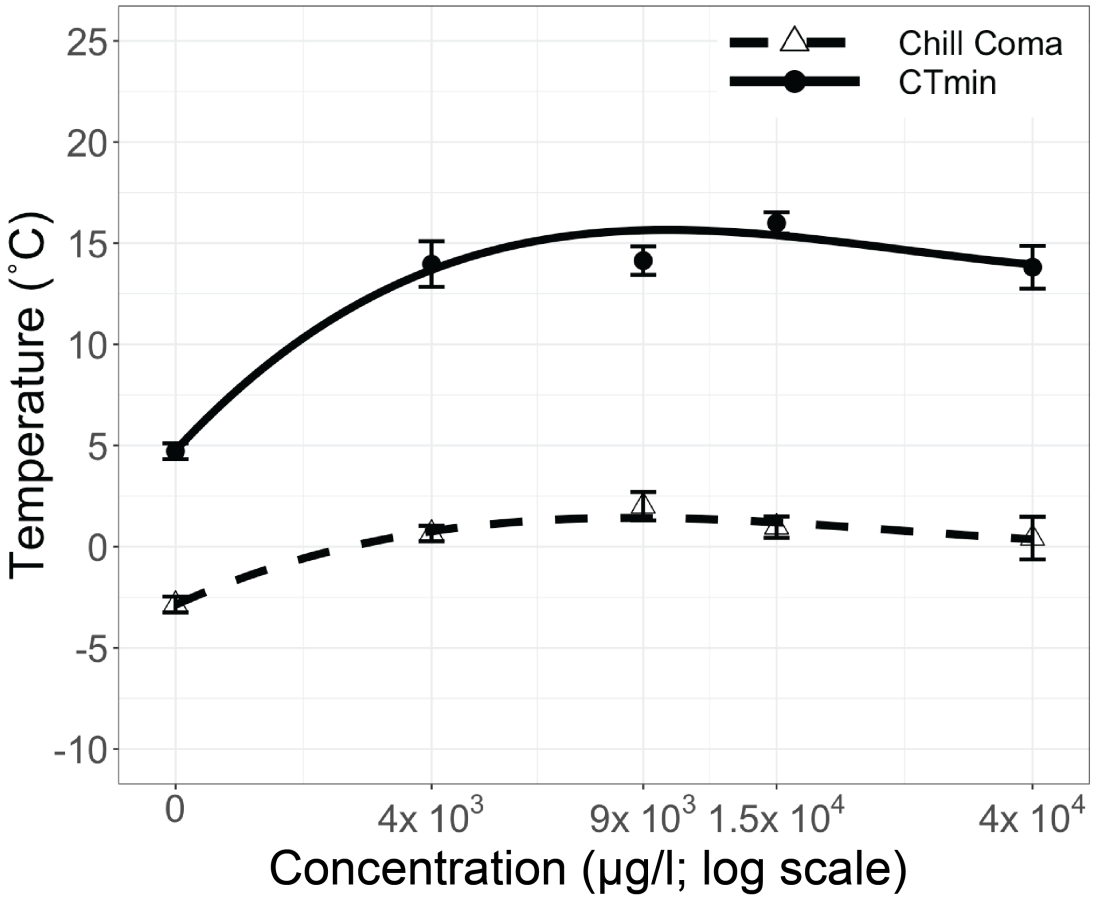


Figure 4.7 The influence of thiacloprid on the CT_{min} and chill coma of buff-tailed bumblebee workers

Relationship between BTB CT_{min} and chill coma, and concentration THIC (n = 18 per concentration), where CT_{min} and chill coma are described by 3rd degree polynomial regression (R^2 for CT_{min} = .43; R^2 for chill coma = .23). Points describe means and error bars describe one standard error.

4.5 Discussion

This study has identified that 9 µg/l IMI represents a sub-lethal dose for BTB workers (Figure 4.2), which is at the lower end of what concentrations of NPs have been recorded as being sub-lethal (Bonmatin et al., 2005; Alkassab and Kirchner, 2017). However, at this dose and even below, significant effects on the thermal biology of BTB workers were found, with CT_{min} increasing by 13.7˚C at 9 µg/l and 12.9˚C at 0.9 µg/l (Figure 4.3A). Moreover, 9 µg/l IMI also increased BTB queen CT_{min} by 7.8˚C (Figure 4.4C) and impeded their ability to thermoregulate (Figure 4.4B). Such impacts could severely restrict BTB activity throughout the year, with workers limited to only 10-20 days in the summer months above their CT_{min} (Figure 4.5A), and only 57.3% as many days available to BTB queens (Figure 4.5B). Interestingly, another NP, THIC, did not have such a strong effect on BTB workers, showing a greatly reduced effect on mortality (Figure 4.6), and only showing effects on CT_{min} at doses almost 1,000x higher than IMI (Figure 4.7). The response to IMI also appeared to be consistent across different pollinator species, albeit at higher doses with RMBs showing increased CT_{min} at doses of 90 µg/l and BBFs at 900 µg/. Overall, this study shows that NPs can have a profound effect on thermal biology phenotypes.

BTBs were shown to be particularly susceptible to IMI, even when exposed to even a very low dose of 9 µg/l (Figure 4.3A). In fact, increased CT_{min} was noted even at doses as low as 0.04 µg/l (Figure 4.3A). BTB queens were less affected, but still demonstrated a significant increase in CT_{min} at a concentration of 9 µg/l IMI (Figure 4.4C). Such concentrations are well within field-relevant

concentrations of IMI (Alkassab and Kirchner, 2017; Wood and Goulson, 2017). This means that after a low field-relevant dose of 9 µg/l BTB worker activity may be restricted to warm days over 18.6˚C. In the UK, that suggests that workers would mainly be able to be active in July and August (Figure 4.5). BTB queens can emerge as early as February to found a colony (Goulson, 2010), during which time if workers were exposed to a low dose of IMI they would be unable to be active (Figures 4.4 and 4.5), and therefore unable to forage to establish a colony. Even in the summer months, activity would be restricted for workers, reducing their foraging rates (Figure 4.5), which would again result in inadequate forage for the colony. Bumblebee workers reaching their CT_{min} at higher temperatures, may also explain the many studies that have described reduced foraging in bumblebees after exposure to IMI (Stanley et al., 2015a; Lämsä et al., 2018; Muth and Leonard, 2019).

Bumblebees have been shown to be active during winter in the UK (Stelzer et al., 2010). During which, the temperatures are almost always below the CT_{min} of BTBs that have been exposed to 9 µg/l (Figure 4.5). Due to climate change associated warming, it has been hypothesized that BTB queens are averting diapause (Bale and Hayward, 2010), resulting in establishing new colonies in autumn, and subsequent winter-active workers. If exposed to IMI during this period they could be extremely vulnerable to winter temperatures. It is important to note that the likelihood of exposure during the winter months is low because few crops are present (DEFRA, 2018), but there is a possibility that ornamental or garden plants may have been treated (Wood and Goulson, 2017), and NPs can persist in the environment for years (Botías et al., 2015; David et al., 2016;

Humann-Guilleminot et al., 2019). During the queens' emergence period in February, if they were exposed to 9 µg/l IMI, then past temperature data suggest only approximately one day would be above the CT_{min} of 11.3˚C (Figures 4.4C and 4.5).

The mechanism by which IMI caused such a substantial increase in CT_{min} may be due to NPs causing misfiring of action potentials and perhaps promoting ion imbalances. NPs appear to stimulate Kenyon cells, causing ion imbalances (Zayas et al., 2002; Moffat et al., 2016), these ion imbalances could lead to depolarisation in the central nervous system causing failure to trigger action potentials. Furthermore, in Moffat et al.'s (2016) study, IMI caused immobility of bees, and in a prior study Moffat et al. (2015), showed that IMI can cause mitochondrial dysfunction. It is possible that this dysfunction led to immobility of bees, perhaps by preventing ionic homeostasis and thus promoting the onset of CT_{min} (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017; see also Chapter 5). Furthermore, by NPs' direct action on the nAChRs they could disrupt action potential signalling (Matsuda et al., 2001). This depolarisation has been explicitly linked to CT_{min} and chill coma (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). Indeed, in the locust (*Locusta migratoria*) depolarisation has been shown to be the mechanism underlying CT_{min}. Altogether it is possible that NP-mediated depolarisation causes the loss of muscular control.

Another neonicotinoid, THIC, also had an impact on BTB workers (Figures 4.6 and 4.7), but at much higher concentrations compared to IMI (Figures 4.2 and 4.3A). Mortality was shown to be only 20% at a concentration of 5×10^5 µg/l THIC

(Figure 4.6), in contrast IMI caused 100% mortality at only 2.0 x 10^4 µg/l (Figure 4.2). Similarly, with sub-lethal effects, a 4 x10^4 µg/l dose of THIC increased the CT_{min} (Figure 4.7), this is compared to only 0.9 µg/l IMI which increased the CT_{min} by several more degrees (Figure 4.3A). Together these results suggest that BTBs are far more sensitive to IMI, compared to THIC. As IMI is used far more widely than THIC (Jeschke et al., 2011), the implies bumblebees are at greater risk, alternatively it could imply that THIC may be more pollinator 'safe' pesticide to use.

The particular susceptibility of bumblebees to IMI may be due to differences in the chemical structure of the different neonicotinoids (Chapter 1, Figure 1.4), which would, therefore, lead to different detoxification responses (Iwasa et al., 2004). Honeybees have been shown to be largely insensitive to THIC as its cyanoimino pharmacore promotes bees' metabolism, which, through the use of carbon esterases and cytochrome P450s, breaks down THIC (Iwasa et al., 2004; Alptekin et al., 2016). Conversely, IMI has a nitriomino group which may not elicit the same metabolic response, so the bees cannot effectively degrade it (Alptekin et al., 2016). Bumblebees display an increased sensitivity to IMI over honeybees (Cresswell et al., 2012) and appear to have a similar overall detoxification response to honeybees (Xu et al., 2013). The lack of bumblebees' ability to degrade IMI may be due then to a lack of a cytochrome P450 mediated response (see Chapter 5), which may cause the increased sensitivity to IMI. This could mean that bumblebees are less able to be competitive with other species that are less affected by IMI.

The data presented here also demonstrate that sub-lethal doses of IMI also significantly reduce the ability of BTB queens to thermoregulate (Figure 4.4A and 4.4B), which would further impinge on their ability to be active in colder months. When queens first emerge, they must forage and provide all of the resources to found a colony on their own (Goulson, 2010). Because of this Thompson and Hunt (1999) have highlighted that queens may be particularly vulnerable as pesticide spraying of crops in spring coincides with their foraging. During this time temperatures can still be very low and they are reliant on their ability to thermoregulate in order to forage effectively (Free, 1979). Moreover, queens thermoregulate in order to maintain brood temperature (Heinrich, 1974a), which is vital to speed up offspring development times, which thus allows new workers to assume foraging responsibilities (Goulson, 2010). If queens are unable to thermoregulate effectively, then the founding of new colonies, and the production of offspring could be curtailed. As queens represent a bottleneck in bumblebee populations (Goulson et al., 2008; Goulson, 2010), an increase in CT_{min} and loss of thermoregulatory ability caused by IMI exposure could have dramatic consequences for populations.

This effect on thermoregulation was partly in agreement with the findings of Potts et al. (2018), who showed that IMI decreased the thoracic temperatures of BTBs and at doses of 100 µg/l bees were slower to warm up after cold torpor. Interestingly, Potts et al. (2018) also showed that low-level doses of IMI (0.1 – 10 µg/l) increased the rate at which bees recovered from cold torpor, which the authors speculate may be due to stimulatory effects of IMI on muscular contraction. It is possible then that at lower doses than those tested here (< 9

µg/l) IMI may cause increased thermoregulation in BTBs, this was the case African honeybees (*A. m. scutellata*) where high doses (2 ng/bee) of thiamethoxam reduced the thorax temperature, whilst low doses (0.2-1 ng/bee) increased it (Tosi et al., 2016). Alternatively, as Potts et al. (2018) were not specifically testing thermoregulation, but rather rate of recovery from cold, bee recovery from CT_{min} and chill coma may also be affected by IMI. Both represent interesting avenues for future research.

Chill coma in BTBs was also affected by IMI, but to a much lesser extent than CT_{min} (Figure 4.3A). This is possibly because chill coma is caused by a different mechanism to CT_{min} (Overgaard and MacMillan, 2017). Chill coma represents the absence of neurophysiological activity altogether, whereas CT_{min} is the loss of muscular coordination (Hazell and Bale, 2011). Chill coma is more likely then caused by a total failure of the central nervous system, CT_{min} rather by the loss of ionic homeostasis and dysregulation of action potentials (Overgaard and MacMillan, 2017), which may be much more readily affected by NPs. With that in mind, chill coma did increase by around 5˚C in BTB workers (Figure 4.3A). At temperatures below the chill coma it is understood that insects acquire injuries and die (MacMillan and Sinclair, 2011), so an increase to this threshold could reduce survival of BTBs, especially if bees are active in winter, where temperatures can often fall below the onset of chill coma of 2.5˚C at 9 µg/l IMI.

IMI was shown to influence CT_{min} and, to a lesser extent, chill coma, in other species too (Figures 4.3B and 4.3C). Sub-lethal concentrations of neonicotinoids increased CT_{min} in BBFs, and RMBs, by approximately 9˚C and 6˚C at the highest

concentrations tested, respectively (Figures 4.3B and 4.3C). Interestingly, the doses at which these effects were observed were very different from that in BTB. For IMI, BTBs showed responses at concentrations as low as 0.04 µg/l (Figure 4.3A), whereas BBFs and RMBs responded at 900 µg/l and 90 µg/l, respectively (Figures 4.3B and 4.3C). Indeed, the concentrations which caused effects on CT_{min} for BBFs and RMBs were far above what is usually considered field-relevant (Bonmatin et al., 2005; Alkassab and Kirchner, 2017). These different responses indicate that the responses of pollinators to NPs cannot be generalised. Moreover, the data presented here are totally novel for Diptera, as sub-lethal effects of NPs have never previously been identified to my knowledge.

Both RMBs and BBFs represent important pollinators. As the results from this study indicate that BTBs are particularly sensitive to IMI (Figure 4.2 and 4.3), the greater tolerance of BBFs and RMBs could indicate that they could be used to supplement more susceptible pollinators where NPs are present. BBFs are also very easy to rear in large numbers (Sherman and Wyle, 1996), and so could be readily produced to provide pollination services. RMBs have been shown to pollinate oilseed rape (Teper and Biliński, 2009), a crop which is currently effectively pollinated by bumblebees and honeybees (Stanley et al., 2013; Bartomeus et al., 2014; Lindström et al., 2016); RMBs could then act as pollinators for oilseed rape if IMI was present. Indeed, RMBs, when pollinating an oilseed rape field with seed-treated clothianidin, were shown to suffer no consequences on development or reproduction (Peters et al., 2016). However, another study looking at oilseed rape field with seed-treated clothianidin showed that RMBs had reduced nesting and reproduction (Rundlöf et al., 2015), therefore

any replacement of honeybees and bumblebees with RMBs should be performed cautiously.

The results presented here could have profound implications for pollinator service provision, which is imperative for food security. Whilst IMI has been banned for outdoor use in the EU and partially banned in parts of the US and Canada (EPA, 2017; Ontario Government, 2017; Bass and Field, 2018; EU, 2018) it is still used in many parts of the world. Furthermore, potential replacements for NPs, for example the sulfoximines, have similar mechanisms of action (Sparks et al., 2013; Simon-Delso et al., 2015). It is therefore important that we understand the impact of NPs on pollinator species, to better tackle any deleterious effects they might have. This study demonstrates that these neuroactive pesticides may have substantial effects on the activity thresholds of important pollinators, so whilst there is a necessity for chemical crop protection to ensure food security (Popp et al., 2013; Wannes, 2019), it must be implemented with caution, lest we impact beneficial insects. The three tested species responded dissimilarly to IMI, with BBFs and RMBs having a much higher tolerance to it, which adds weight to the idea that using honeybees as a proxy for responses of pollinators to pesticides is a flawed approach (Sgolastra et al., 2019). More hopefully, the different responses show that some neonicotinoids may have limited impacts on certain pollinators, which indicates that not all NPs are necessarily bad for all pollinator species. Instead, using a targeted approach, choosing the neonicotinoid based on the pollinators present to protect crops, could prove to be a fruitful approach. RMBs and BBFs, for example, could be reared commercially to fulfil pollination demands (Chapter 2), whilst NPs were still used to protect crops.

4.6 Conclusions

In this chapter the sub-lethal doses of IMI have been found for three key pollinators: BTBs, RMBs and BBFs. In BTBs, sub-lethal concentrations were 9 µg/l and lower. RMBs and BBFs were much more tolerant to IMI, with sub-lethal concentrations found at 90 µg/l and 900 µg/l, respectively. At these sub-lethal concentrations of IMI CT_{min} of BTBs was found to be dramatically increased, by doses as low 0.04 µg/l. Indeed, untreated bees had a CT_{min} of 4.7˚C, which increased to 18.6˚C at 9 µg/l IMI. This could potentially restrict the ability of BTB workers to be active to 10-20 days in the summer months, thus limiting their provision of pollination services. A similar impact on CT_{min} was also found after exposure to THIC, although at much higher doses to IMI, implying that BTBs are particularly sensitive to IMI. A dose of 9µg/l IMI also increased the CT_{min} of BTB queens from 3.5˚C to 11.3˚C and also impacted upon their ability to thermoregulate. Such effects could also have significant knock-on effects on BTB populations, as queens found new bumblebee colonies in the cold spring months, a time when thermoregulation is necessary to allow flight and foraging. Similar effects of IMI on CT_{min} were found in RMBs and BBFs at their own (higher) sub-lethal doses, with 900 µg/l increasing CT_{min} by 5.8˚C in RMBs, and 9,000 µg/l IMI increasing BBF CT_{min} by 9.3˚C. The increased tolerance of these important pollinators could denote that they would be effective pollinators where NPs were present. Altogether in this chapter it is shown that NPs are affecting previously unrecognised thermal biology phenotypes, which curtail pollination services and damage food security. As such, in the next chapter RNA sequencing is used to unpick the molecular mechanism behind this novel response.

Chapter 5: RNA-sequencing suggests imidacloprid combined with chilling disrupts core processes in buff-tailed bumblebees

5.1 Abstract

Cold is an unavoidable stress for any insect at temperate latitudes and the capacity of species to cope with winter cold determines their spatial range, phenology and abundance. Another important stress faced by pollinators is pesticide exposure, with neonicotinoids in particular causing a range of impacts, including negative effects on learning, memory and ability to forage. Understanding the molecular responses to each of these stresses in isolation has advanced considerably in recent years, with the advent of different 'omic' technologies, however, most cold studies to date have focused on responses to sub-zero temperatures and very few have considered temperature and pesticide stress in combination. These combinational stresses can have severe effects, as it has been shown previously that if buff-tailed bumblebees (*Bombus terrestris*) are cooled after exposure to a field-relevant dose (9 µg/l) of the neonicotinoid imidacloprid, they lose the ability to coordinate their movement at 18.6˚C. The present study then represents the first characterisation of the transcriptomic profile of important pollinators, BTBs, as they are exposed to neonicotinoids and chilling. The transcriptional response to imidacloprid agreed with previous studies, and was dominated by genes associated with metabolism, and response to oxidative stress. This study also identified that muscular contraction, ionic

homeostasis, the heat shock response and cytoskeleton remodelling underpin the chilling response in buff-tailed bumblebees, and of these, muscular contraction and the heat shock response appear severely disrupted as a result of combined exposure to imidacloprid.

5.1 Introduction

Cold can be roughly defined as a sub-optimal temperature for the continued development and performance of an organism (Sinclair et al., 2003; Colinet et al., 2015). As most insects are unable to thermoregulate and so are dependent on environmental heat (Bale and Hayward, 2010; Colinet et al., 2015), cold is a particularly pertinent stress. Even for species that can regulate their body temperature to some extent, such as the buff-tailed bumblebee (BTB; Heinrich, 1974, 1975), there are limits on how long this can be sustained, and the process can be disrupted by other factors, such as pesticide exposure (Chapter 4). In temperate regions, cold is unavoidable for part of the year (Renault et al., 2002), and so many studies have attempted to understand the molecular and transcriptional responses of insects to cold (e.g. Yocum et al., 1998; Rinehart et al., 2007; Colinet et al., 2010; Moskalev et al., 2015), however, much of this research has focused on sub-zero temperatures. This is understandable as temperatures below the freezing point of water are important in biological systems (Sinclair et al., 2003), but there are frequent above 0˚C fluctuations in temperature that stress insects (Colinet et al., 2015), and in combination with neonicotinoid exposure BTBs have been shown to be particularly susceptible to

low levels of chilling (Chapter 4). However, little is known about insects' molecular responses in the case of these more subtle chilling stresses.

Core molecular processes known to underpin the cold stress response include: Heat shock proteins (Hsps) (King and Macrae, 2014), ionic homeostasis (MacMillan and Sinclair, 2011; Macmillan et al., 2015) and cytoskeleton reorganisation (Michaud and Denlinger, 2004; Des Marteaux et al., 2018a). Hsps are molecular chaperones that mediate and maintain correct folding of proteins (King and Macrae, 2014). For sub-zero temperatures, there is direct evidence that they are required for cold tolerance and repair of chilling injuries. For example, Rinehart et al. (2007), used RNA interference (RNAi) to show that the suppression of *Hsp23* and *Hsp70* reduced the survival of flesh fly (*Sarcophaga crassipalpis*) pupa exposed to -15˚C. Further, Colinet et al. (2010) used real-time polymerase chain reaction (RT-PCR) to assess change in Hsp expression during and after an 8-hour exposure to 0˚C of fruit flies (*D. melanogaster*). In response, a suite of Hsps were shown to be induced, with *Hsp22*, *Hsp68*, and *Hsp cognate 70* were all upregulated during the first 15 minutes of 0˚C exposure (Colinet et al., 2010). Despite 0˚C being considered a severe stress for many active temperate insects (Bale and Hayward, 2010), as these genes are upregulated so quickly, they may be relevant to understand more subtle cold stresses. For example, *Hsp22* is an example of a small heat shock protein (sHsp), which is a group of low molecular weight (<43kDa) ATP-independent chaperones that are thought to be the first line of defence of the cell against stress (King and Macrae, 2014), and therefore are likely play a role in the response to 'cold' temperatures above 0˚C. Similarly, the role of Heat shock factors (HSFs) may be key, as they

are transcriptional activators of Hsps (Clos et al., 1990; Gomez-Pastor et al., 2017) and so their upregulation could occur at the very inception of the cold stress response cascade (Hayward et al., 2014b).

For many insects, cold stress also involves the loss of ionic balance across cellular membranes, which can lead to loss of water balance, cell death and eventually mortality (MacMillan and Sinclair, 2011; Macmillan et al., 2015). Perhaps in response to this challenge, in the few studies that have considered the transcriptomic response of chilling above 0˚C, there is evidence of a modulation of genes involved in ionic regulation. For example, Torson et al. (2017) assessed the whole body transcriptome of a solitary bee, the alfalfa leafcutting bee (*Megachile rotundata)*, while it was stored at 6˚C for seven days, and found upregulation of *sodium potassium calcium exchanger 6*, which is involved in extruding calcium ions across the mitochondrial membrane (De Marchi et al., 2014). Such a response is likely involved in maintaining ionic homeostasis in the bees, which is in agreement with studies of insect cold acclimatisation (Macmillan et al., 2015; Andersen et al., 2017; Des Marteaux et al., 2017). When the transcriptome of cold-acclimated field crickets (*G. pennsylvanicus*) was assessed, *Ca^{2+} channel protein* was found to be upregulated while *Bumetanide-sensitive Na^{+}-Cl^{+} channel* was shown to be downregulated (Des Marteaux et al., 2017), both of which encode proteins for transmembrane ion transport (Xu et al., 1994; Fleckenstein-Grun, 1996). Further, in fruit flies, several genes involved in sodium-ion transport were also found to be downregulated after five days at 6˚C (MacMillan et al., 2016). These changes in gene expression that protect insects against loss of ionic balance at low

temperatures (Macmillan et al., 2015) therefore may play a role in the response to the above 0˚C temperatures.

This loss of ionic balance during cold exposure may also influence the cell cytoskeleton. In Des Marteaux et al.'s (2017) study it was also found that acclimatisation in crickets caused shifts in the expression of actin and actin-anchoring/stabilizing proteins, tubulin, α-actinin, among other cytoskeletal related genes. As temperatures decline, the ability of cells to export Ca^{2+} is gradually lost (Teets et al., 2013), which can lead to increased intracellular levels of Ca^{2+}, causing a loss of cellular integrity (Michaud and Denlinger, 2004). As the cytoskeleton acts to reinforce the cell (Michaud and Denlinger, 2004; Des Marteaux et al., 2018a), the changes in expression observed by Des Marteaux et al. (2017) may act to prevent the observed loss of cellular integrity when faced with cold stress. Indeed, actin was found to be of an increased density in the rectal tissue of cold-acclimated (12˚C for one week) crickets, and was resistant to depolymerisation (Des Marteaux et al., 2018b).

As set out previously (Chapters 1 and 4), low temperatures also affect insect movement until they reach the critical thermal minimum (CT_{min}; Hazell and Bale, 2011), which is thought to be driven by the loss of ionic balance (Terhzaz et al., 2015; Overgaard and MacMillan, 2017). Cold does not affect insects, or indeed any organism, in isolation, all species encounter many other stresses in their environment (Sinclair et al., 2013; Davies et al., 2014), and it is critical to understand how responses to different stressors interact. For example, sub-lethal doses of pesticides can alter the CT_{min}, with a field realistic dose (9 μg/l) of the neonicotinoid pesticide imidacloprid (IMI) increasing the CT_{min} of BTB workers

from 4.7˚C to 18.6˚C (Chapter 4). As BTBs are highly generalist pollinators that are important for farmland and greenhouse pollination (Delaplane et al., 2000; Goulson, 2010), such an increase in CT_{min} could have significant consequences for food security and crop yields (Chapter 4). It remains unclear, however, what molecular processes underpin this phenotypic change in CT_{min}. Whilst ionic balance is thought to drive onset of CT_{min}, and there is evidence that neonicotinoids can disrupt this balance (Moffat et al., 2015, 2016; Powner et al., 2016), processes involved in muscular control or others may also contribute to CT_{min} onset (Overgaard and MacMillan, 2017). Currently, the interplay of cold and neonicotinoids on molecular processes remains unknown, as they have been studied in isolation thus far, never in combination.

Of the studies that have characterised the molecular impacts of neonicotinoids, only a few published studies have examined bumblebees. This is surprising, as there are many examples of neonicotinoids having a substantial effect on bumblebees (Alkassab and Kirchner, 2017). Mobley and Gegear (2018) used RNAseq to investigate why clothianidin (CLO) impacts the drones and workers of the common eastern bumblebee (*B. impatiens*) differently. After a 5-day exposure of bumblebees to 5 µg/l CLO, the authors found that several cytochrome P450s, genes that are involved in the metabolisation of neonicotinoids (Manjon et al., 2018; Feyereisen, 2018), were differentially expressed in the bee body (wings, legs and proboscis were removed). They also showed altered expression of genes involved in reproduction, immunity and several associated with locomotion, for example *titin* was upregulated (Mobley and Gegear, 2018). The titin family is a group of large proteins that are involved with the passive elasticity

of muscle and muscular contraction (Labeit et al., 1997; Taylor-Burt et al., 2015). Thus, the induction of *titin* expression by CLO suggests that muscular function is affected by neonicotinoids.

Colgan et al. (2019) conducted a transcriptome-wide study assessing how gene expression differed between worker and queen BTBs when exposed to either 6.47 µg/l IMI or CLO for four days. In this study the heads of bees were assessed, as the authors postulate that the main activity of neonicotinoids would be in the brain, and indeed heads did exhibit significant changes in gene expression (Colgan et al., 2019). They found that clothianidin elicited greater gene expression changes than IMI, and that workers showed a greater transcriptomic response than queens (Colgan et al., 2019). In particular, they showed that many metabolism related genes were differentially expressed in response to both neonicotinoids tested (Colgan et al., 2019). In addition, three putative cytochrome P450 genes, and muscle related genes, including *troponin* and *calponin*, were upregulated in response to clothianidin, whilst IMI only induced alternative splicing in one cytochrome P450 (Colgan et al., 2019).

An unpublished PhD thesis study also assessed the transcriptome of BTB guts after 12 h exposure to a high dose of 98 µg/l IMI (Laycock, 2014). In this study, Laycock (2014) showed 26 genes were differentially expressed, including downregulation of a cytochrome P450, probable cytochrome P450 6a13. This may suggest that whilst clothianidin induces expression of these genes (Mobley and Gegear, 2018; Colgan et al., 2019), IMI may not, or at least, to a lesser extent. It is also possible that IMI disrupts the detoxification process carried out by these enzymes. Shi et al. (2017) show that in honeybees exposed to

thiamethoxam cytochrome P450 6AS5 was upregulated, which may indicate that in contrast to IMI, thiamethoxam stimulates the cytochrome P450 detoxification response, or that there is a difference in the response between honeybees and bumblebees. It has been suggested that P450s may only play a role in metabolism of cyano-substituted neonicotinoids, such as THIM (Chapter 1, Figure 1.4), and may not respond to nitro-substituted neonicotinoids like IMI (Iwasa et al., 2004; Alptekin et al., 2016). In support of this claim, a study looking at the transcriptome of honeybee brains found that six cytochrome P450 enzymes were downregulated after exposure to 20 µg/l IMI for 11 days (Li et al., 2019).

Thus, it appears that neonicotinoids may cause changes to the expression of P450 enzymes and may also cause downregulation of expression of muscle related genes, as well as disruptions to the ionic balance of cells. Some evidence also suggests that the Hsps, which appear to show a complex and an important response to cold stress, may be disrupted by IMI. However, no study has assessed the two stresses together and so their combined effects on gene expression are unclear. It was previously postulated that an increase in BTB CT_{min} was derived from IMI causing ionic imbalances (Chapter 4). To assess this hypothesis, along with the molecular underpinnings of other changes, the current chapter uses RNAseq to examine the transcriptomic response of BTBs exposed to IMI as their body temperature declines from 20˚C to 14˚C, as well as the influence of chilling and IMI in isolation. This is the first pollinator study to examine gene expression changes across declining temperatures at the very onset of the chilling response, and well above temperatures expected to cause cold injury. In

combination with exposure to an IMI dose known to alter the CT_{min} (Chapter 4) this chapter aims to identify what molecular processes are being affected.

Given the literature and experimental data presented (Chapter 4), I hypothesise that IMI exposure alone will show similar impacts on gene expression as in previous studies, e.g. genes involved in metabolism, oxidative stress response, and detoxification will be differentially expressed (Mobley and Gegear, 2018; Colgan et al., 2019). When cold exposure is examined in isolation I instead hypothesise that genes involved in muscular contraction, ionic homeostasis, heat shock response, and cytoskeleton regulation will be affected (Colinet et al., 2010; Des Marteaux et al., 2017; Torson et al., 2017). When combined, I hypothesise that IMI and cold exposure will show increased levels of differential expression of genes than either two in isolation, as this will be a more pronounced stress for the insect. Finally, given this experiment will recapitulate aspects of the activity threshold experiment (Chapter 4), I hypothesise that the genes shown to be affected by the combined stress will go some way to explaining an increase to CT_{min} shown by IMI in BTBs. Additionally this chapter serves as an investigation into possible genetic pathways affected by neonicotinoids that will lay the ground-work for future analyses.

5.2 Methods

5.2.1 Experimental conditions

A *Bombus terrestris audax* colony was purchased from a commercial supplier (Biobest®). One colony was used to limit the amount of genetic difference between individuals, as female BTB workers in one colony share 75% of the

genetic information (Goulson, 2010). The colony was provided with *ad libitum* Biogluc® and ground up honeybee pollen (Nutriseed, London, United Kingdom [UK]) every other day (as in Chapter 4).

To replicate conditions of the previous study on CT_{min} (Chapter 4), bees were not age-controlled and instead were selected based on similarity of size. Adult bumblebee workers were exposed to either 0 µg/l (control) or 9 µg/l IMI for 24 h, after which they were immediately placed in a central aluminium cooling arena used to assess CT_{min} (see Chapter 4, figure 4.1). A single bee was held within the arena for each run and cooled at a rate 0.5˚C/min starting from 20˚C, until the desired body temperature was reached. This was determined by attaching a thermocouple to the bee thorax (Chapter 4). Bees were removed once their body temperature had reached the following temperatures during the ramping process: 20˚C (temperature control), 18˚C, 16˚C or 14˚C. These temperatures were specifically selected because they span the CT_{min} observed in IMI-exposed bees (Chapter 4, Figure 4.3A). For each temperature and treatment, the experiment was performed in triplicate (e.g. three bees at 20˚C exposed to 9 µg/l, three bees exposed to 20˚C at 0 µg/l, etc.). Bees were immediately frozen in liquid nitrogen upon removal from the arena.

5.2.2 RNA extraction

As neonicotinoids act on the central nervous system, bees were decapitated in order to localise transcriptional analyses to the brain tissue (Colgan et al., 2019). Using a pestle, heads were ground up in liquid nitrogen until well homogenised, upon which TRIzol (Fisher Scientific, Pittsburgh, USA) was added to extract RNA.

The heads were homogenised further using zircon beads and a Precellys 24 tissue homogeniser (Bertin Technologies, Aix-en-Provence, France). Chloroform was then used for phase separation and isopropanol to precipitate RNA. The precipitated RNA was washed with microelute columns (RNAeasy, Qiagen, Hilden, Germany) to clean and improve the quality of the RNA. Quantity and quality of RNA was assessed using a NanoDrop Microvolume Spectrophotometer (Thermo Fisher, Hemel Hempstead, UK) and Bioanalyzer 2100 technology (Agilent, Santa Clara, CA, USA). Additionally, RNA was run on an electrophoresis gel to ensure that RNA had not been sheared and RNA integrity numbers were calculated.

5.2.3 RNA sequencing

Libraries were constructed from 1 µg of total RNA. All RNA libraries were produced using NEBNext Ultra Directional II RNA Library Prep Kit (New England Biolab E7760L, New England Biolabs, Hitchin, UK), NEBnext Multiplex Oligos for Illumina Dual Index Primers (New England Biolabs E7600S), in the Biomek FxP Laboratory Automation Workstation (Beckman Coulter A31842, Beckman Coulter, High Wycombe, UK), according to provided protocols. Constructed libraries were assessed for quality using the Tapestation 2200 (Agilent G2964AA, Agilent Technologies, Cheadle, UK) with High Sensitivity D1000 DNA screentape (Agilent 5067-5584). This showed that for all samples used for RNAseq, there was no evidence of degradation or genomic contamination.

Multiplex library clustering and sequencing was performed upon the HiSeq4000 by BGI Copenhagen, with data delivered as fastq format files. Prior to assembly,

raw reads were filtered using Trimmomatic (version 0.33) in order to remove duplicated sequences, reads with a quality score below Phred 33 and adapters. High quality reads were mapped to the *Bombus terrestris* transcriptome version 2.0 (Blaxter et al., 2011, available here: http://www.nematodes.org/downloads/databases/Bombus_terrestris/) using Salmon (version 0.8.2; Patro et al. 2017). Salmon was run with default parameters to give Transcripts Per Kilobase Million (TPM) values. Approximately 11 million reads were mapped to 36,354 transcripts of the transcriptome. Mapping rate varied slightly between the 24 samples, within the range of 82-88% (Appendix 5.1).

Transcripts were also mapped to the *Bombus terrestris* genome version 1.0 (Sadd et al., 2015, available here: ftp://ftp.ensemblgenomes.org/pub/metazoa/release41/fasta/bombus_terrestris/dna/) using Spliced Transcripts Alignment to a Reference (STAR; version 2.5.4b; Dobin et al. 2013). however, this had a lower mapping rate than the transcriptome (~76%; Appendix 5.1), therefore, all further analyses were conducted with the transcriptome.

5.2.4 Quality checking

Principal component analysis (PCA) was used to allow patterns in the dataset to be more easily identified; the greater the distance between two groups on a PCA plot, the greater the difference between them. Using PCA and generating a heatmap of the top 300 differentially expressed genes (DEGs), two outliers were identified: one sample treated with 9 µg/l IMI at 20˚C and one sample treated with 0 µg/l IMI at 18˚C. These outliers were removed from further analyses.

5.2.5 Transcript annotation

Transcripts were annotated using MagicBlast (v. 1.3.0; https://ncbi.github.io/magicblast/) against the blast database downloaded on the 4th June 2018 (ftp://ftp.ncbi.nlm.nih.gov/blast/db/) using the default settings. This gave Basic Local Alignment Search Tool (BLAST) accession numbers for transcripts that had a minimum of 86% identity, which was approximately 66% of the 36,354 total transcripts. If further analyses considered transcripts that had not been annotated by MagicBlast, these transcripts were assessed by the BLAST web tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). In this case, transcripts were considered to have a BLAST match if the query cover was a minimum of 60%.

5.2.6 Differential expression analysis

Differential expression analysis was conducted using the DESeq2 package (Love et al., 2014) version 1.22.1 in R (R Core Team, version 3.5.1, 2018). Before analysis, transcripts with read numbers of less than 10 were removed. Transcript abundance, as calculated by Salmon, was used to assess differential expression by estimating variance-mean dependence using the DESeq2 package, which was then converted to $\log_2$ fold change. Benjamini–Hochberg (BH) adjusted p-values were calculated and transcripts with adjusted p-values of less than 0.05 were considered for further analysis. Furthermore, samples with a fold change of between 1 and -1 were removed. For simplicity, if no BLAST match could be found for a transcript, it was not assessed.

Initially, differential expression was assessed just comparing the bees exposed to 9 µg/l IMI to those that were unexposed (i.e. the bees at 20˚C). Differential

expression was calculated as above. Then, just the effects of temperature were assessed by looking for differential expression in the unexposed bees between the different temperature treatments (20˚C, 18˚C, 16˚C and 14˚C). Once specific DEGs related to this were found, a heatmap was constructed using the pheatmap package (Kolde, 2019). The genes that were identified as being differential expressed between temperature treatments in the unexposed bees were then directly compared to DEGs from IMI-exposed bees. Genes that had similar differential expression were clustered together, using Pearson correlation. Transcripts of interest were highlighted in bold.

Genes that were differentially expressed during the combined stress of both temperature change and IMI exposure were then identified. To discern what was driving differences between samples, a PCA plot was constructed from the top 300 DEGs. When separation between samples in the PCA plot were found, the top ten genes most responsible for this (the top and bottom loading genes) were identified using the pcaExplorer package, as determined by their eigenvalues (Marini and Binder, 2019). PCA analysis was also conducted between samples at 14˚C, as this appeared to be where the biggest differences between unexposed and IMI-treated bees was observed.

Lastly, a manually curated group of DEGs involved in the heat shock response, muscular control and cytoskeletal responses was selected where expression changed significantly with temperature and IMI exposure (adjusted p-value < .05). Much of the expression change for this group was more subtle and therefore transcripts with an overall fold change of between 1 and -1 were included.

5.3 Results

5.3.1 The transcriptional response of buff-tailed bumblebees to 9 µg/l Imidacloprid

In total 46 DEGs were found after exposure of BTBs to 9 µg/l IMI, of which 42 returned known identities (Table 5.1). Most of the DEGs are involved with metabolism, for example *Eufriesea mexicana glycogenin-1*, *sorbitol dehydrogenase* and *NADH dehydrogenase* (Table 5.1). There are also several transcripts associated with protection from oxidative stress that were upregulated, e.g. *probable phospholipid-hydroperoxide glutathione peroxidase (PHGPx)*, *catalase and peroxidase* (Table 5.1). Also, one enzyme involved in neonicotinoid metabolisation, *cytochrome P450 9e2* (*cyp9e2)*, was shown to be highly upregulated (Table 5.1).

Table 5.1 Differentially expressed genes in response to 9 µg/l imidacloprid

	Closest BLAST match	Relative log_2 fold change	Standard error	Adjusted p-value
Downregulated vs control	Eufriesea mexicana glycogenin-1	-23.3	4.3	2.29E-04
	translation initiation factor eIF-2B subunit gamma	-23.2	4.3	2.29E-04
	NECAP-like protein CG9132	-9.5	1.4	3.46E-08
	fibroin heavy chain	-8.6	1.3	1.05E-06
	FGGY carbohydrate kinase domain-containing protein	-7.6	1.3	2.91E-05
	receptor expression-enhancing protein 5	-7.6	1.4	3.08E-04
	transient receptor potential cation channel protein painless	-6.9	1.6	1.23E-02
	T-complex protein 1 subunit beta	-3.6	0.9	2.07E-02
	tctex1 domain-containing protein 2	-2.6	0.5	1.79E-04
	ADP-ribosylation factor GTPase-activating protein 2	-2.5	0.5	2.72E-03
	transmembrane and coiled-coil domains protein 2	-1.7	0.4	2.27E-02
Upregulated vs control	sodium-coupled neutral amino acid transporter 9 homolog	22.0	4.4	1.10E-03
	probable phospholipid hydroperoxide glutathione peroxidase	21.6	4.4	1.57E-03

cytochrome b5-related protein	21.5	4.4	1.66E-03
open rectifier potassium channel protein 1	21.3	4.4	1.91E-03
reticulocyte-binding protein PFD0110w	21.2	4.4	1.91E-03
serine-rich adhesin for platelets	21.2	4.4	1.91E-03
cytochrome P450 9e2	21.1	4.4	1.95E-03
nesprin-1	20.4	4.4	3.80E-03
dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial	8.9	1.6	1.90E-04
NADH dehydrogenase [ubiquinone] 1 subunit C2	8.7	1.7	6.97E-04
zinc finger protein 468	8.6	1.5	1.08E-04
B-cell receptor-associated protein 31	8.3	1.7	1.91E-03
GTP-binding protein 10 homolog	8.2	1.7	1.75E-03
Down syndrome critical region protein 3 homolog	6.9	1.7	2.55E-02
sorbitol dehydrogenase_1	3.5	0.7	3.85E-04
sorbitol dehydrogenase_2	3.5	0.9	4.94E-02
Ceratina calcarata spermine synthase-like	2.9	0.7	3.58E-02
catalase	2.8	0.3	4.96E-16
Apis mellifera large subunit ribosomal RNA_1	2.6	0.7	4.88E-02
proline-rich receptor-like protein kinase PERK9	2.6	0.6	2.12E-02
Apis mellifera large subunit ribosomal RNA_2	2.4	0.6	3.26E-02
ADP-ribosylation factor GTPase-activating protein 2	2.3	0.6	2.12E-02
3-hydroxy-3-methylglutaryl-coenzyme A reductase	2.3	0.6	4.72E-02
Apis mellifera large subunit ribosomal RNA_3	2.2	0.5	2.85E-02
fructose-bisphosphate aldolase	2.2	0.5	2.07E-02
Apis mellifera large subunit ribosomal RNA_4	2.0	0.5	1.23E-02
complex III assembly factor LYRM7	1.9	0.5	2.85E-02
Hymenoptera sp. Hym_H1 18S ribosomal RNA gene, partial sequence	1.8	0.4	1.23E-02
Apis mellifera gene for 18S ribosomal RNA, partial sequence	1.8	0.4	4.81E-02
nose resistant to fluoxetine protein 6	1.4	0.3	5.71E-03
aldose reductase	1.0	0.2	3.80E-03

Transcripts were split up depending on whether they were up or downregulated in the imidacloprid-exposed bees. The closest BLAST match was found for each transcript; where an underscore and a number follow a name, it indicates a different transcript had the same blast match as one found previously. Where a species' name is present there was no match found in *Bombus terrestris* and instead shows the closest identity in the named species.

5.3.2 The transcriptional response of buff-tailed bumblebees to declining temperatures, and how this is affected by imidacloprid

In response to a body temperature change from 20 to 14˚C, 98 genes were found to be differentially expressed, of which 62 had BLAST matches. Overall, this set

of DEGs can be characterised as the 'typical' response of BTBs to minor temperature stress (Figure 5.1 left). The influence of IMI on these same genes is then shown (Figure 5.1 right).

Largely, the expression patterns can be split into four clusters based on different expression profiles. Cluster 1 represents genes that were upregulated early in response to temperature (a decrease of 2˚C); on the other hand, in IMI-exposed bees this response appeared to be blocked, or to some extent downregulated (Figure 5.1). This included genes involved in ionic regulation, muscular contraction, and cytoskeleton, e.g. *calcium transporting ATPase sarcoplasmic/endoplasmic reticulum type (Atp2a1),* several variants of *actin clone 205_like*, and *titin*.

Cluster 2 is represented by genes that were downregulated early in response to temperature and become strongly downregulated at 14˚C; whereas in IMI-exposed bees, these genes are mostly upregulated (Figure 5.1). DEGs in Cluster 2 included muscle related genes such as: four variants of *troponin C*, and *sarcoplasmic calcium binding protein 1* (*scbp1*).

Cluster 3 is composed of transcripts with unaltered expression until being downregulated at 14˚C in the control bees, whilst in the IMI exposed bees there is no discernible change in expression (Figure 5.1). This cluster shows another muscular contraction related gene: *troponin I*.

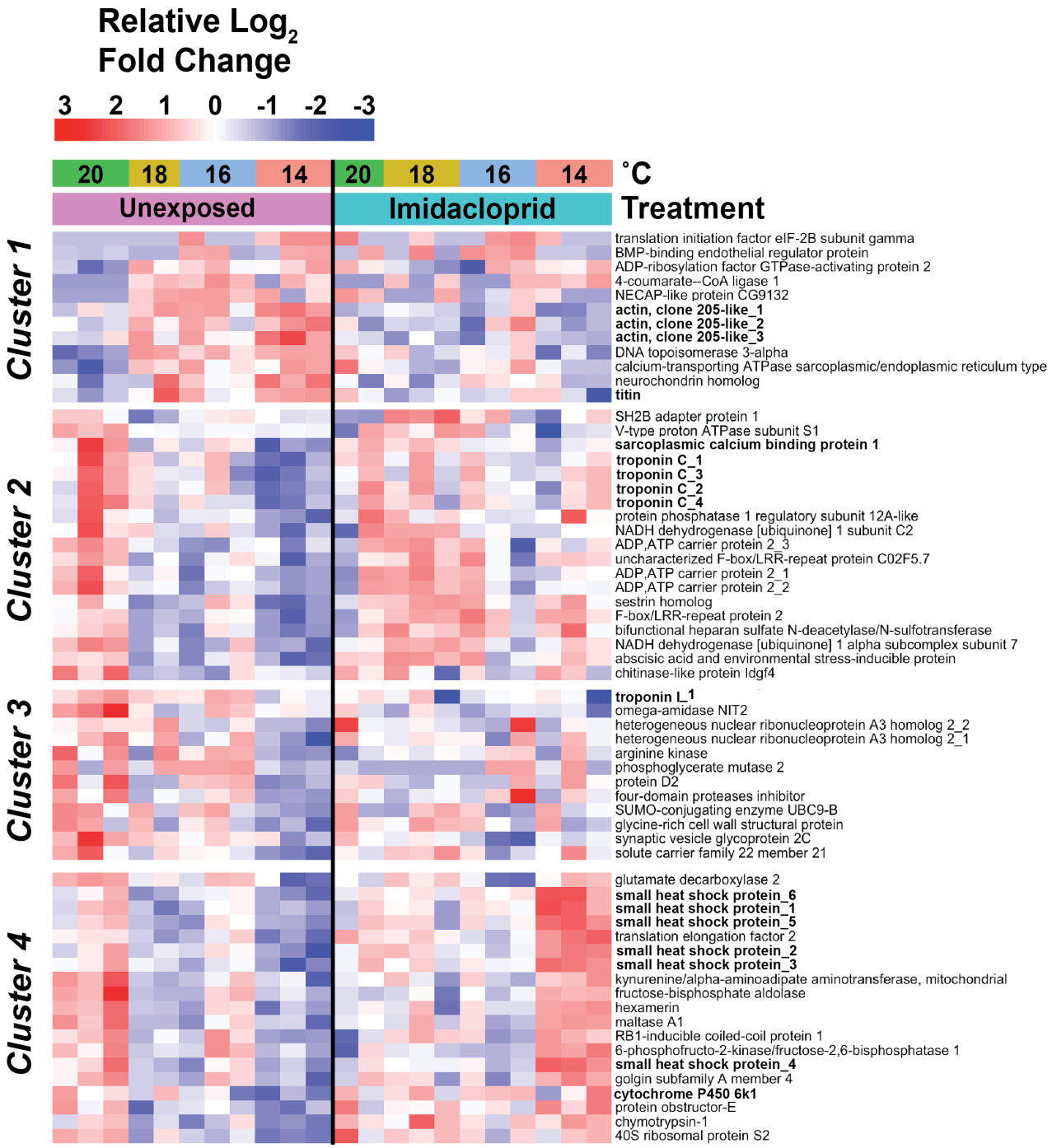

Relative Log2 Fold Change
3 2 1 0 -1 -2 -3
20 18 16 14 20 18 16 14 °C
Unexposed
Imidacloprid
Treatment
Cluster 1
translation initiation factor eIF-2B subunit gamma
BMP-binding endothelial regulator protein
ADP-ribosylation factor GTPase-activating protein 2
4-coumarate--CoA ligase 1
NECAP-like protein CG9132
actin, clone 205-like_1
actin, clone 205-like_2
actin, clone 205-like_3
DNA topoisomerase 3-alpha
calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type
neurochondrin homolog
titin
Cluster 2
SH2B adapter protein 1
V-type proton ATPase subunit S1
sarcoplasmic calcium binding protein 1
troponin C_1
troponin C_3
troponin C_2
troponin C_4
protein phosphatase 1 regulatory subunit 12A-like
NADH dehydrogenase [ubiquinone] 1 subunit C2
ADP,ATP carrier protein 2_3
uncharacterized F-box/LRR-repeat protein C02F5.7
ADP,ATP carrier protein 2_1
ADP,ATP carrier protein 2_2
sestrin homolog
F-box/LRR-repeat protein 2
bifunctional heparan sulfate N-deacetylase/N-sulfotransferase
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7
abscisic acid and environmental stress-inducible protein
chitinase-like protein Idgf4
Cluster 3
troponin I_1
omega-amidase NIT2
heterogeneous nuclear ribonucleoprotein A3 homolog 2_2
heterogeneous nuclear ribonucleoprotein A3 homolog 2_1
arginine kinase
phosphoglycerate mutase 2
protein D2
four-domain proteases inhibitor
SUMO-conjugating enzyme UBC9-B
glycine-rich cell wall structural protein
synaptic vesicle glycoprotein 2C
solute carrier family 22 member 21
Cluster 4
glutamate decarboxylase 2
small heat shock protein_6
small heat shock protein_1
small heat shock protein_5
translation elongation factor 2
small heat shock protein_2
small heat shock protein_3
kynurenine/alpha-aminoadipate aminotransferase, mitochondrial
fructose-bisphosphate aldolase
hexamerin
maltase A1
RB1-inducible coiled-coil protein 1
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1
small heat shock protein_4
golgin subfamily A member 4
cytochrome P450 6k1
protein obstructor-E
chymotrypsin-1
40S ribosomal protein S2

Figure 5.1 The influence of imidacloprid exposure changes on the expression of genes in buff-tailed bumblebees

Heatmap of the relative log_2 fold change of the DEGs in bees as temperature declines (left) and the log_2 fold change of the same temperature decline after exposure to 9 µg/l IMI (right). Each column represents a different biological replicate. Putative transcript names are shown next to each row and were identified using the closest BLAST match. Where an underscore and a number follow a name, it indicates a different transcript had the same blast match as one found previously. Transcripts that had similar differential expression were clustered together, where red represents relative upregulation and blue represents relative downregulation. Transcripts were highlighted in bold if they were part of processes considered important for responding to temperature (e.g. the heat shock response).

Finally, Cluster 4 has a similar expression profile to cluster 2 for control bees, but in IMI exposed bees all these genes are strongly upregulated at 14˚C (Figure 5.1). In this final cluster there are several variants of *small heat shock protein* (*sHsp*), which are involved in the heat shock response, and the putative neonicotinoid detoxifying enzyme *cytochrome P450 6k1* (*cyp6k1*).

5.3.3 The combined effect of imidacloprid and chilling on gene expression

In bees exposed to both temperature decline and IMI, 358 genes were significantly downregulated, while 128 were found to be upregulated. When the top 300 DEGs were assessed by PCA, principal components (PC) 1, 2, and 3 explained 17%, 14% and 11% of the variance, respectively (Figure 5.2). PC1 vs. PC2 showed that there was overlap between the samples of the IMI exposed and the unexposed bees, although the unexposed samples at 14˚C and 18˚C were found to be distinct from the exposed (Figure 5.2A). There was greater separation

between temperature response alone vs. combined temperature and IMI treatment when PC2 and PC3 were compared (Figure 5.2B), where only three unexposed samples, two at 20˚C and one at 14˚C, overlap with the exposed samples.

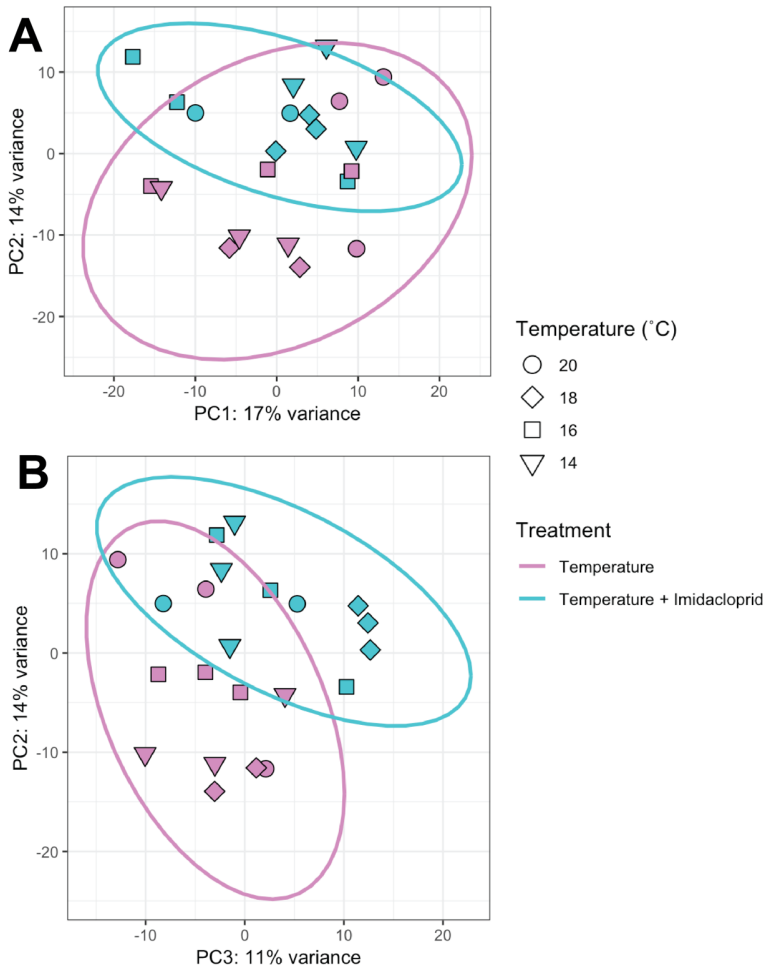


Figure 5.2 Principal Component Analysis plots of top 300 differentially expressed genes

The top 300 DEGs were assessed by PCA to identify differences between IMI- and temperature-exposed (blue) and temperature treatment alone (purple) samples, in addition to differences between temperatures. Different shapes indicate samples from different temperatures. Ovals show 95% confidence intervals. **A** PC1 versus PC2 **B** PC2 versus PC3.

Based on the PCA analysis, PC2 was found to show the largest separation between the unexposed and IMI exposed bees' gene expression. Therefore, the top 20 genes most responsible for this separation, based on their eigenvalues, were identified. Of these genes 14 had BLAST matches (Table 5.2), which included transcripts associated with muscular regulation (variants of *titin* and *paramyosin, long form*), and a putative neonicotinoid metabolising enzyme, *cytochrome P450 6a2* (*cyp6a2*).

Table 5.2 Top 14 annotated transcripts responsible for the separation shown by PC2 in Figure 5.2

	Closest BLAST match	Relative log_2 fold change
Downregulated vs control	titin_2	-8.3
	cytochrome P450 6a2	-3.3
	general odorant-binding protein 56d	-2.8
	general odorant-binding protein 19a	-2.6
	general odorant-binding protein 19a	-2.6
	esterase FE4	-2.1
	zinc carboxypeptidase	-1.8
Upregulated vs control	ATP-dependent RNA helicase p62	7.0
	titin_3	3.2
	hymenoptaecin mRNA	2.7
	paramyosin, long form_2	2.6
	paramyosin, long form_4	2.0
	paramyosin, long form_1	1.9

Transcripts were split up depending on whether they were up- or downregulated in the neonicotinoid exposed bees. The closest BLAST match was found for each transcript; where an underscore and a number follow a name, it indicates a different transcript had the same blast match as one found previously.

In both cases of the PCA (Figures 5.2A and B), most of the difference between the treatment and control appears along PC2. Differences between the temperature groups was harder to discern. Samples do not separate out into clear clusters based on exposure temperature. However, 14˚C samples

demonstrate the greatest separation (Figures 5.2A and B). As such, samples at 14˚C were further assessed. In this case, 1,018 genes were found to be differentially expressed. By performing PCA on the top 300 DEGs clear separation was shown between genes from the unexposed compared to the IMI exposed bees (Figure 5.3). PC1 accounted for 42.7% of the variance, whilst PC2 explained 23.3% of the variance.

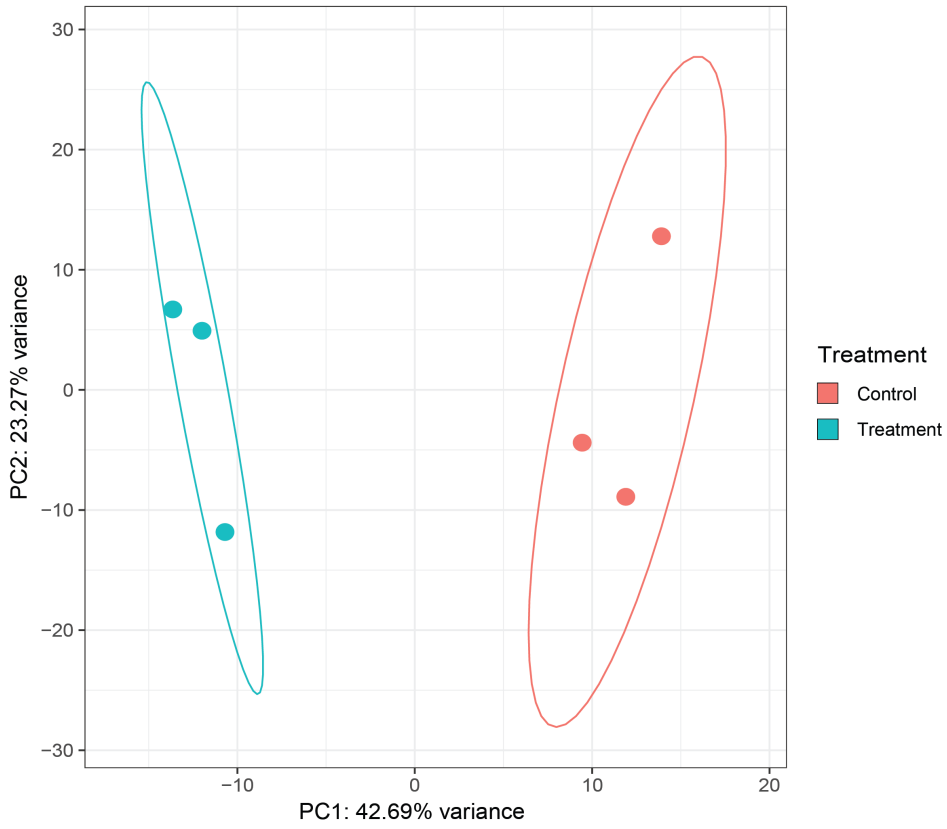


Figure 5.3 Principal Component Analysis plots of top 300 DEGs at 14˚C

The top 300 DEGs at 14˚C were assessed by PCA to identify differences between IMI exposed (Treatment, blue) and unexposed (Control, orange) samples. Ovals show 95% confidence intervals.

The top 20 most influential (top and bottom loadings) genes in the PCA were identified (Figure 5.3) and were checked for BLAST matches, of which 14 returned known identities (Table 5.3). Again, this showed muscle-related genes (variants of *titin*, *myosin heavy chain*, *muscle*, and *paramyosin, long form*).

Table 5.3 Top fourteen annotated transcripts responsible for the separation shown by PC1 at 14˚C in Figure 5.3

	Closest BLAST match	Relative log_2 fold change
Downregulated vs. control	titin_2	-5.26
	myosin heavy chain, muscle_2	-1.71
	myosin heavy chain, muscle_1	-1.28
	FGGY carbohydrate kinase domain-containing protein_1	-3.20
	translation initiation factor eIF-2B subunit gamma	-3.55
	guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	-2.43
	probable E3 ubiquitin-protein ligase HERC4	-3.19
	FGGY carbohydrate kinase domain-containing protein_2	-1.91
	3-hydroxy-3-methylglutaryl-coenzyme A reductase	-1.41
Upregulated vs. control	ATP-dependent RNA helicase p62	6.70
	paramyosin, long form_2	5.04
	paramyosin, long form_1	8.58
	ornithine aminotransferase, mitochondrial	1.33
	Bombus ignitus clone DS_044 18S ribosomal RNA gene	1.18

Transcripts were split up depending on whether they were up or downregulated in the neonicotinoid exposed bees. The closest BLAST match was found for each transcript. Where an underscore and a number follow a name, it indicates a different transcript had the same blast match as one found previously.

5.3.4 Specific processes likely underpinning activity thresholds affected by imidacloprid and chilling

31 transcripts that could be involved in processes concerning activity thresholds (e.g. heat shock response, muscular activity and cytoskeletal processes) were

found to be differentially expressed across temperature and between IMI exposed and unexposed bees (Figure 5.4). Broadly these are separated into two clusters: Cluster 1 is represented by DEGs that were upregulated in response to IMI. These included several variants of the *small heat shock protein*, two additional variants of *titin, inositol 1,4,5-trisphosphat receptor, scbp1, troponin C,* a variant of *twitchin, inositol 1,4,5-trisphosphate receptor* (*InsP3R*), and *microtubule-actin crosslinking factor 1 (MACF1)*. Cluster 2, on the other hand, showed DEGs that are downregulated in response to IMI (Figure 5.4). This cluster included: *heat shock factor protein* (HSF), a variant of *twitchin*, two variants of *actin clone 205_like* and another variant of *titin*.

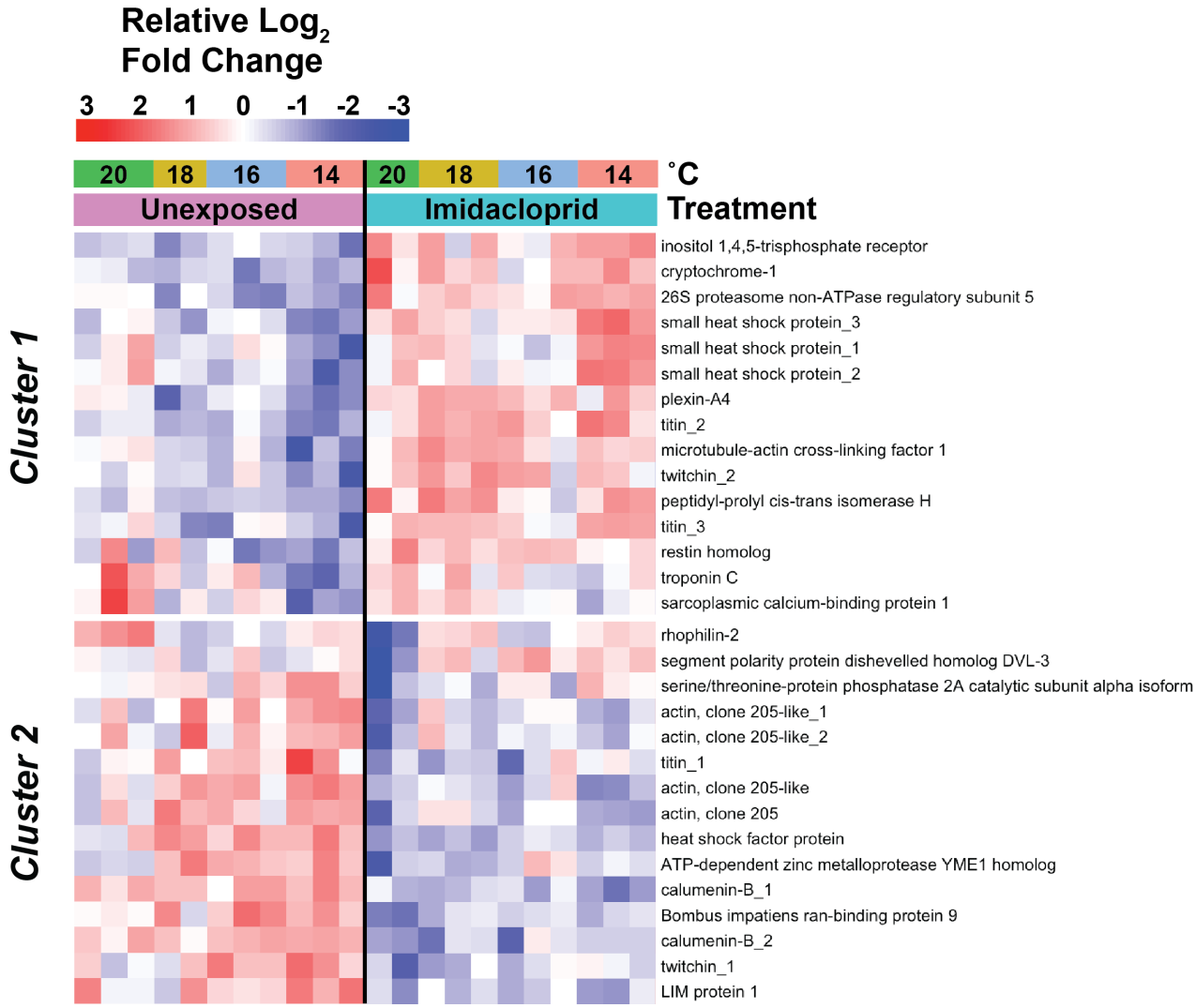


Figure 5.4 Differentially expressed genes of imidacloprid treated over control bees that are involved in muscular control and response to temperature

Heatmap of the relative log_2 fold change of the DEGs that were associated with muscle and response to temperature. Log_2 fold change unexposed bees (left) compared to the log2 fold change of bees exposed to 9 µg/l IMI (right). Each column represents a different biological replicate. Putative transcript names are shown next to each row and were identified using the closest BLAST match; where an underscore and a number follow a name, it indicates a different transcript had the same blast match as one found previously. Transcripts that had similar differential expression were clustered together, where red represents relative upregulation and blue represents relative downregulation.

5.4 Discussion

This study represents the first comprehensive transcriptomic assessment of any pollinator in response to mild chilling. It is worth highlighting that the temperatures tested (20 to 14˚C) represent the actual insect body temperature, and thus are likely to constitute a mild stress for worker bees which typically sustain body temperatures well above ambient when foraging (Goulson, 2010; Stelzer et al., 2010). Furthermore, Chapter 4 has already indicated that this temperature range spans when most worker bees hit their CT_{min} when also exposed to sub-lethal IMI doses. That said, several of the gene expression changes observed were subtle, and the log_2 change in expression used as a threshold in this study was therefore slightly lower than in previous RNAseq studies of pesticide responses in bees (e.g. Mobley and Gegear, 2018). Even these small changes in gene expression, however, clearly have substantial effects on the thermal biology of BTBs (see Chapter 4), so it is imperative they are better understood.

In agreement with previous studies, IMI exposure alone was shown to impact several key processes. For example, a cytochrome P450, *cyp9e2*, was highly upregulated (Table 5.1). The cytochrome P450s are a family of enzymes that are involved in the metabolisation of neonicotinoids (Manjon et al., 2018; Feyereisen, 2018). Previous transcriptomic studies on bumblebees have shown induction of cytochrome P450s after exposure to neonicotinoids (Mobley and Gegear, 2018; Colgan et al., 2019), suggesting that in BTBs these genes will be promoted to degrade neonicotinoids. However, there has been little evidence for their involvement in the response to IMI. As *cyp9e2* showed such high upregulation

(22.1-fold increase) it may be key to BTB response to IMI. In fact, enzymes belonging to this subfamily of cytochrome P450s (CYP9Q) have been shown to be key to determining the sensitivity of bumblebees to neonicotinoids (Manjon et al., 2018; Feyereisen, 2018), so *cyp9e2* is well-placed for further study into the BTB IMI response. As only one cytochrome P450 was found in response to IMI, in comparison to the greater number shown in response to other neonicotinoids (e.g. Mobley and Gegear, 2018; Colgan et al., 2019), it also supports the hypothesis that bumblebees are particularly sensitive to IMI as it does not induce an equivalent detoxification response as shown for other NPs (Chapter 4).

Some other key processes were also shown to be affected by IMI exposure alone. For example, metabolism and oxidative stress. The majority of the DEGs associated with IMI are involved in metabolism (e.g. *Eufriesea mexicana glycogenin-1*, *sorbitol dehydrogenase*, *NADH dehydrogenase*), with some up and some downregulated (Table 5.1), suggesting that metabolism is reorganised in response to IMI. Such a response has been previously shown in BTBs (Colgan et al., 2019), and may suggest that IMI perturbs BTB energetic balance, as in honeybees (Cook, 2019). *PHGPx*, *catalase and peroxidase* were shown to be upregulated after IMI treatment (Table 5.1), and encode key enzymes involved in protection from oxidative stress (Felton and Summers, 1995; Hu et al., 2010), therefore they are indicative of IMI promoting oxidative stress (Dussaubat et al., 2016). This is in line with many previous studies on both bumblebees and honeybees that have shown upregulation of several processes involved in oxidative stress in response to neonicotinoids (Badiou-Bénéteau et al., 2012;

Christen et al., 2016; Dussaubat et al., 2016; Mobley and Gegear, 2018; Colgan et al., 2019).

The present study also identifies the normal expression of genes responsive to declining temperature and shows that field-relevant IMI exposure perturbs this expression, with several core processes emerging as being affected: muscular contraction, ionic homeostasis, the heat shock response, cytoskeleton remodelling, and detoxification of neonicotinoids.

5.4.1 Muscular contraction

As temperature declined several muscle related genes were affected, including Troponin I and four variants of Troponin C (Figure 5.1 left). Both Troponin C and I are part of the troponin complex, which is integral to calcium-mediated muscular contraction and regulation (Bhagavan et al., 2015). Similarly, *scbp1* was downregulated (Figure 5.1), and also plays a role in the cellular Ca^{2+} homeostasis, acting to sequester these ions. This gene is also closely associated with the sarcoplasmic reticulum (Hermann and Cox, 1995) and, as such, may play a role in regulation of calcium excitation in muscles mediated by the troponin complex (Gao et al., 2006; Iwamoto, 2011; Rohrback et al., 2015). Together, the expression of these transcripts could indicate that, as temperature declines, genes involved in muscular contraction are downregulated, which aligns with the observation that as ambient temperatures decrease insects generally slow their movement and eventually reach their CT_{min} (Hazell and Bale, 2011).

At the same time these transcripts were downregulated, a variant of *Titin* was upregulated at temperatures below 20˚C in unexposed bees (Figure 5.1 left). Titin

proteins are important for muscular contraction, anchoring the sarcomere and contractile machinery, and are also essential for passive tension in muscle (Labeit et al., 1997; Taylor-Burt et al., 2015). It is possible, therefore, that this upregulation in titin helps sustain passive tension in muscles and reinforce actin-myosin cross bridges that may be affected by cold stress (Lee and Herzog, 2008; Ranatunga, 2018) - in effect stabilising the muscle against the effects of cold. Interestingly, however, other variants of the gene were downregulated (Figure 5.4), suggesting that the response of the overall titin machinery to temperature change may be complex.

When BTBs were exposed to IMI in combination with declining temperature, all the previously discussed muscle-associated responses were altered, along with some others (Figures 5.1 and 5.4). Moreover, some of genes showing the biggest changes between IMI-exposed and unexposed bees were muscle-related (Figure 5.2 and Table 5.2), especially at 14˚C (Figure 5.3 and Table 5.3). Notably, troponin associated transcripts and *scbp1* were downregulated to a lesser extent and *titin* was upregulated in IMI exposed bees as temperatures fell (Figure 5.1). In fact, during chilling variants of *titin* were strongly affected by IMI exposure, having some of the biggest fold differences between unexposed and exposed bees (Table 5.2). A change in *titin* expression has previously been shown in CLO-exposed bumblebees (Mobley and Gegear, 2018), suggesting that *titin* is neonicotinoid sensitive. Moreover, during temperature decline three variants of *paramyosin, long form* were strongly upregulated in IMI-dosed BTBs (Table 5.2), especially when compared at 14˚C (Table 5.3). Paramyosin forms part of the myosin filaments in invertebrates (Sonobe et al., 2016), a core component of the

muscle contractile system. Another core component of this system is *myosin heavy chain*, which was, in contrast to paramyosin, found to be strongly downregulated in IMI exposed bees at 14˚C (Table 5.3).

In combination the responses of *titin*, *paramyosin long form,* and *myosin heavy chain* could indicate that IMI is stimulating the restructuring of contractile tissue during chilling. The relative amounts of paramyosin are associated with the thickness of invertebrate muscle fibres, generally with more paramyosin leading to thicker and longer fibres (Hooper et al., 2008). Myosin heavy chain is the motor protein of muscle thick filaments, and the relative amounts of it and paramyosin are implicated in the strength of muscular contraction (Wells et al., 1996; Hooper et al., 2008). Titin is also associated with the structure of the muscle filaments and sarcomere (Labeit et al., 1997). A decreased amount of *myosin* and *titin*, with an increased amount of *paramyosin* (as in exposed bees at 14˚C) could indicate that muscle filaments are longer, which could in turn increase contractile function. This would indicate that IMI stimulates the function of muscles under chilling.

As a neonicotinoid, IMI binds to the nicotinic acetylcholine receptor (nAChR), which in turn causes the firing of action potentials (Goulson, 2013). The firing of these action potentials could act to promote muscle contraction. Indeed, in several studies the application of neonicotinoids has been shown to cause muscular twitching (Baines et al., 2017) or immobility (Lambin et al., 2001; Moffat et al., 2016), which could indicate insects are unable to control muscular activity. This may also be further substantiated by the expression of *twitchin,* which was found to be downregulated in IMI exposed bees (Figure 5.4). *Twitchin* (synonym

unc-22) is a member of the titin-like family, as such it is thought to be a key regulator of muscular contraction (Mayans et al., 2013; Matsunaga et al., 2015). This is evidenced by *twitchin* knock-out mutants in *Caenorhabditis elegans* showing uncontrollable twitching (Moerman et al., 1988; Matsunaga et al., 2015). Also, in *D. melanogaster* an ortholog of *twitchin*, *projectin*, is thought to regulate flight muscle contraction (Ayme-Southgate et al., 2008). Indeed, it appears that twitchin inhibits the rate of muscle relaxation and thus has an impact on the overall contraction/relaxation cycle and therefore locomotion (Funabara et al., 2007; Matsunaga et al., 2015). Thus, the expression of *twitchin* seen in this study may cause IMI treated bees to make uncontrollable movements and twitch, something that has been observed in previous studies with bees exposed to neonicotinoids (Milatovic, 2014; Baines et al., 2017; Chapter 4). Overall, IMI appears to disrupt several processes relating to normal muscular contraction as BTBs are chilled, and as well as a possible stimulatory effect which may result in BTBs being less able to coordinate their movement. Further studies should use other parts of the bee for transcriptional analysis, such as leg muscles, as it is possible that simply looking at heads may underestimate the impacts of IMI on muscles.

5.4.2 Ionic homeostasis

It is well-understood that as temperatures decline ionic homeostasis becomes hard to maintain for insects, eventually resulting in a loss of coordinated movement (i.e. they reach their CT_{min}) and the accumulation of chilling injuries (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). Moreover,

recovery from CT_{min} is associated with a restoration of ionic balance (MacMillan et al., 2012). Additionally, it has been shown that when insects are held at low, above zero, temperatures gene expression is altered to promote the maintenance of ionic balance (Des Marteaux et al., 2017; Torson et al., 2017), which will likely buffer against cold-induced injuries (Macmillan et al., 2015). Influx of Ca^{2+} into cells specifically has been found to be responsible for such injuries (Bayley et al., 2018), and therefore buffering against overloading of intracellular Ca^{2+} may be crucial to protect against damage. As these studies focus on prolonged cold exposure, or lower temperatures, than the present study it could be predicted that there is little change in gene expression with regard to ionic homeostasis, or it will be associated with small initial shifts to maintain ionic homeostasis. As BTBs experience chilling it was shown that two genes that may be involved with ionic homeostasis had altered expression: *scbp1* and *Atp2a1* (Figure 5.1).

Both *scbp1* and *Atp2a1* are involved in cellular Ca^{2+} homeostasis but showed divergent expression profiles (Figure 5.1), which may be explained by their complementary action on Ca^{2+}. For instance, *scbp1* acts as Ca^{2+} sink and has been associated with the excitation of muscle (Gao et al., 2006; Iwamoto, 2011; Rohrback et al., 2015), so its downregulation may suggest a reduction of muscular activity, as proposed above. Conversely, *Atp2a1* acts to transport intracellular Ca^{2+} across the sarcoplasm or endoplasmic reticulum (depending on the cell type) to be stored, and is associated with relaxation of muscles (Toyoshima et al., 2000). Therefore, its upregulation as temperatures decline may also be associated with a reduction of muscular activity. As no other DEGs were associated with ionic homeostasis, the data here suggest that this low-level

chilling does not substantially disrupt ionic maintenance, rather muscular activity is reduced. Such a loss of ionic homeostasis may occur at lower temperatures, or when bees are exposed to additional stresses.

Chapter 4 hypothesised that IMI exposure causes a loss of ionic balance across cellular membranes in BTBs, thus increasing their CT_{min} substantially. This is further supported by the RNAseq data presented here, as whilst there were no genes involved in ionic regulation found to be significantly differentially expressed after exposure to 9 µg/l IMI alone (Table 5.1), in combination with chilling several DEGs were found. In particular, *scbp1* (Cluster 3, Figure 5.1), was downregulated in response to declining temperature, but its expression was sustained when IMI and low temperatures were experienced in combination (Cluster 3, Figure 5.1). Similarly, *Atp2a1* was downregulated under the combined stress (Cluster 1, Figure 5.1). These expression profiles could identify processes to maintain ionic homeostasis in response to a greater amount of Ca^{2+} being present after IMI exposure, as there is some evidence that IMI may cause dysfunction of the mitochondria which could ultimately result in ionic imbalances (Zayas et al., 2002; Moffat et al., 2016).

A possible increased presence of Ca^{2+} in the cell is also supported by the upregulation of *InsP3R* in IMI exposed bees (Figure 5.4). InsP3R is a calcium-sensitive protein channel that essentially converts external stimuli to intracellular Ca^{2+} signals (Yoshida and Imai, 1997; Foskett et al., 2007). IMI is well-understood to cause the misfiring of action potentials and overstimulation of nerve cells (Matsuda et al., 2001; Goulson, 2013; Van Der Sluijs et al., 2015;

Simon-Delso et al., 2015), which results from Ca^{2+} influx (Moffat et al., 2016). Therefore, the upregulation of *InsP3R* may be a result of IMI's stimulation of nerve tissue promoting action potentials via Ca^{2+}. An increased amount of Ca^{2+} could also precipitate an increase in BTB CT_{min} (MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017).

5.4.3 The heat shock response

It was shown in unexposed BTB that six variants of *sHsp* were downregulated as temperature declined (Figure 5.1). In contrast, it appears that *HSF* was upregulated at temperatures lower than 20˚C (Figure 5.4). While sHsps are often involved in the response to temperature stress, acting as molecular chaperones to ensure proteins are correctly folded during stress (Jakob et al., 1993; Bakthisaran et al., 2015), their exact role in the response to cold is yet to be fully characterised, and they also perform many other non-stress functions (Carra et al., 2017). The sHsps have been shown to be upregulated during the recovery of insects from cold (Colinet et al., 2010; Rinehart et al., 2007), which may suggest that in general sHsps are more involved in recovery from cold, rather than responding to cold itself; something that has also been proposed in larvae of the gall fly (*Eurosta solidaginis*; Zhang et al., 2011). In BTB queens the sHsp (of which the transcripts discussed here are transcriptional variants) is downregulated in the brain during diapause, in response to one month at 4˚C, implying that sHsp may not contribute to cold tolerance in this tissue at least (Kim et al., 2008). On the other hand, *HSF* may be responding to promote the activation of other Hsps, as cold declines (Clos et al., 1990; Gomez-Pastor et al.,

2017). Together, the expression of *sHsps* and *HSF* may indicate that as temperatures decline, the ATP-independent sHsps play less of a role, instead larger ATP-dependent Hsps facilitate correct protein folding in BTBs. Further study of the expression of Hsps at temperatures below 14˚C are required to clarify their role, as well as examining their expression in other tissues, such as the muscles.

When BTBs were exposed to 9 µg/l IMI, it appears the 'normal' expression patterns of both *sHsp* and *HSF* during cooling were disrupted (Figures 5.1 and 5.4). Instead of *sHsps* being downregulated as temperature declines, overall, they appear to maintain their expression in IMI-exposed bees, until they are strongly upregulated at 14˚C (Figure 5.1). Similarly, instead of being upregulated, *HSF* expression remains unaltered across all temperatures (Figure 5.4). These expression profiles could mean that the heat shock response is derailed by IMI exposure. HSF functions to promote Hsps (Clos et al., 1990; Gomez-Pastor et al., 2017), so its lack of upregulation indicates that IMI may be preventing the activation of Hsps that would typically be involved in the response to cold stress, leaving BTBs vulnerable to protein misfolding and cellular damage (King and Macrae, 2014).

Other studies also suggest that IMI may disrupt the heat shock response. For example, when IMI-treated (13 µg/l) Colorado potato beetles (*Leptinotarsa decemlineata*) were exposed to 43˚C for five hours, Hsp70 was not upregulated as much as is typical in response to the heat shock; at the same time mortality increased compared to untreated beetles (Chen et al., 2015). As Hsp70 is

transcriptionally activated by HSF (Nielsen et al., 2005), the lack of upregulation of Hsp70 noted by Chen et al. (2015) could be due to a similar repression of *HSF* upregulation found in the present study. In honeybees (*Apis mellifera*) IMI has been shown to reduce the expression levels of *Hsp70* and *Hsp90* in dose-dependent manner (Koo et al., 2015), which may also be mediated by lower expression of HSF. Further, Laycock (2014) also showed that *Hsp83*, was also repressed in response to a high dose of IMI. Altogether, the present study and others suggest that IMI may render BTBs less able to respond to cold (and possibly other) stresses.

5.4.4 Cytoskeleton

Three variants of *actin, clone 205_like* were upregulated as temperatures declined in unexposed bees (Figure 5.1). Actin, clone 205_like shares close homology with actin_87E in *D. melanogaster*, which is involved in cytoskeleton structure and muscle contraction (Röper et al., 2005). An upregulation of cytoskeletal-associated genes would be consistent with work by Des Marteaux *et al.* (2017), that found that actin may be upregulated to prevent cytoskeletal depolymerisation in response to cold, to prevent loss of cellular integrity during cold stress (Des Marteaux et al., 2018a, 2018b). However, this process appears to be disrupted by IMI exposure, as here the actin genes were downregulated as temperatures declined (Figure 5.1). This could mean that bees exposed to IMI are at risk of cytoskeletal depolymerisation.

Several other cytoskeleton associated genes also appeared to be affected by IMI exposure and cold in combination. *MACF1* was upregulated in the IMI-exposed

bees, whereas in unexposed bees it was downregulated at all temperatures below 20˚C (Figure 5.4). MACF1 may play a key role in cytoskeletal dynamics, particularly in the integration of actin and microtubules (Kodama et al., 2003). Furthermore, *restin homolog* was also upregulated under the same conditions (Figure 5.4), and may play a similar role to MACF1 (Lantz and Miller, 1998). These expression profiles could show a disruption of the cytoskeletal changes that occur in response to temperature decline. Instead of stabilisation of actin, microtubules may be bundled through the activity of *MACF1* and *restin* (Huber et al., 2015; Voelzmann et al., 2017), perhaps to increase resistance to IMI, as MACF1 has been shown to contribute to cytoskeleton-mediated pyrethroid resistance in the soybean aphid (*Aphis glycines*; Bi et al., 2016). Alternatively, *MACF1*'s upregulation could be associated with IMI-mediated stimulation of muscular activity, as it is associated with movement; for example, knocking out *MACF1* in *C. elegans* causes movement defects (Jørgensen et al., 2014).

5.4.5 Detoxification of neonicotinoids

Whilst *cyp9e2* was shown to be stimulated by IMI alone (Table 5.1), in contrast, it was shown that as temperatures decreased in unexposed BTBs, *cyp6k1* was downregulated (Figure 5.1), which is similar to the findings of Des Marteaux et al. (2017). Cyp6k1 has been shown to be associated with neonicotinoid resistance in the soybean aphid (Kim et al., 2015), and detoxification of IMI in the Colorado potato beetle (Clements et al., 2018). As such, this change in expression could indicate that as temperatures decline, certain metabolic processes, like metabolisation of xenobiotics, are repressed in favour of activities to help cope with cold stress. During the joint stress, when BTBs were exposed to IMI and low

temperatures, *cyp6k1* was instead upregulated (Figure 5.1). This gene showed the strongest upregulation at 14˚C, which may show the combination of chilling and IMI is causing the greatest stress on BTBs. An increase in *cyp6k1* expression has also been shown in bumblebee transcriptomic studies after exposure to either IMI or clothianidin (Mobley and Gegear, 2018; Colgan et al., 2019). Why increased expression of *cyp6k1* was only found under the joint stress in the present study is unclear, but it may suggest that exposure to low temperatures and neonicotinoids may promote further detoxification mechanisms.

Interestingly, another cytochrome P450, *cyp6a2*, was strongly downregulated in response to IMI and temperature (Table 5.2). This is similar to the findings of Li et al. (2019) who found that in the brains of honeybees exposed to 20 µg/l IMI *cyp6a2*, along with some other cytochrome P450s, is downregulated. This could suggest, contrary to the response of *cyp6k1,* that IMI actually impairs detoxification processes. However, in *D. melanogaster* overexpression of *cyp6a2* imparted no additional resistance to neonicotinoids (Daborn et al., 2007). Indeed, there appears to be little evidence of *cyp6a2* contributing to IMI detoxification or tolerance, instead it appears to be involved in the metabolisation of several other xenobiotics (Kim et al., 2015; Pan et al., 2015; Pang et al., 2016). It is unclear what the exact role of *cyp6a2* is in BTBs, but it is possible that it does not respond to IMI and so its activity may be repressed in favour of other processes that allow detoxification of neonicotinoids.

5.4.6 Future directions

The genes discussed are well-placed for further analysis: confirmation of their expression by RT-PCR, assessment of whether protein levels correspond to the transcript levels observed, and perhaps observing the effect of silencing these genes using RNAi. It would be particularly informative to see if RNAi silencing of certain genes identified here (e.g. *Twitchin*, *Troponin, scbp1*) can recreate the observed effects on insect activity thresholds (Chapter 4). However, the implementation of RNAi can be challenging, as the efficiency of silencing is highly variable between species, and it is not fully understood how the RNAi signal is amplified and spread throughout the insect (Scott et al., 2013; Zotti and Smagghe, 2015; Vogel et al., 2019). Indeed, to my knowledge there is only one published example of RNAi being implemented in BTBs (Deshwal and Mallon, 2014), but the efficiency of the technique was unclear.

Another important next step would be to assess whether the predicted protein products of the genes found to be important are indeed changed after IMI exposure. Changes in gene transcript levels are important to understand, but they do not necessarily correlate with the abundance of proteins they encode (Schwanhüusser et al., 2011). Moreover, the characterisation of these proteins could help determine what different splice variants of genes are being produced. For example, there were several different *titin* variants, all with different levels of expression, so the overall *titin* response is difficult to predict at present.

5.5 Conclusions

This study provides the only characterisation to date of the transcriptomic response of an important pollinator, the BTB, to the combined effects of IMI and chilling. Typically, as temperatures decline, BTBs reduce the expression of many muscle related genes, increase the expression of cytoskeleton related genes, and the heat shock response is enacted. Together, this suggests that muscle activity is slowed, cells are reinforced, and heat-shock processes are initiated to combat the effects of cold. Upon exposure to IMI, all these processes are disrupted. Additionally, cytoskeleton-associated genes, such as *actin clone 205-like*, were downregulated in response to cold and IMI exposure. It also appears that the heat shock response is altered, illustrated by the lack of induction of *HSF* expression and the changed expression pattern of several *sHsps*. Ionic homeostasis response pathways also appeared to be disrupted by IMI exposure, since *scbp1* and *InsP3R* were found to be upregulated under the joint stress of chilling and IMI. Loss of coordination by bumblebees at temperatures above 15˚C when exposed to sub-lethal doses of IMI (Chapter 4), could be partly due to a direct impact of IMI on muscular control and partly due to loss of ionic homeostasis. Overall, the data shown here suggest that IMI may be causing previously unrecognised disruption of core molecular processes in BTBs. As bumblebees are widely used as effective crop pollinators, this could have profound effects on pollination provision and thus food security, and therefore warrants further study.

Chapter 6: General Discussion

6.1 Overview

Around 35% of worldwide food production is at least partly dependent on animal pollination, chiefly performed by insect pollinators (Klein et al., 2007). This dependence is emphasised by the rising amount of crop production that requires pollination (Aizen et al., 2008; Garibaldi et al., 2011) and the increasing need to produce more food sustainably, for a growing human population (Tilman et al., 2002; Springmann et al., 2018). However, declines in pollinator species have been well-documented (Biesmeijer et al., 2006; Carvalheiro et al., 2013; Nieto et al., 2014; Koh et al., 2016; Jacobson et al., 2018), and have been associated with five key drivers: land-use change and management, climate change, pesticides, spread of disease, and invasive alien species (Vanbergen et al., 2013; Goulson et al., 2015; Potts et al., 2016). Whilst land-use change is thought to be the primary cause of declines (Potts et al., 2016), climate change and pesticide use may be particularly relevant to those pollinators that pollinate agricultural crops (Goulson et al., 2015; Kleijn et al., 2015; Powney et al., 2019). Until recently these threats have been examined in isolation, very few studies have considered their impacts on pollinators in combination, i.e. multi-stressor environments.

Understanding these drivers of pollinator declines is essential to ensuring their continued provision of pollination services. In addition to conservation strategies, alternative solutions to safeguarding pollinator service provision should be investigated. One possible example is to develop commercial rearing techniques (NRC, 2007), which have been already been successful for bumblebees and

honeybees in supplying pollination services (Velthuis and Van Doorn, 2006; Allsopp et al., 2008; Breeze et al., 2011).

Here, mass-rearing protocols for red mason bees (RMBs; *Osmia bicornis*) were examined (Chapter 2), in addition to different techniques to manipulate the overwintering of RMBs to facilitate commercial rearing (Chapter 3). Moreover, the effects of climate change on the obligate diapause of the RMB, along with the phenology of RMBs were examined (Chapter 3). Then, the impacts of imidacloprid (IMI) and cold were assessed on RMBs and this was widened out to also include buff-tailed bumblebees (BTBs; *Bombus terrestris*) and blue blow flies (BBFs; *Calliphora vicina*) (Chapter 4). Finally, the transcriptomic response of IMI and a cold stress were examined in BTBs (Chapter 5), as in this species was where the most substantial effect was found (Chapter 4) and because BTBs have relatively well-characterised genomes and transcriptomes. These studies and key results are summarised in Figure 6.1.

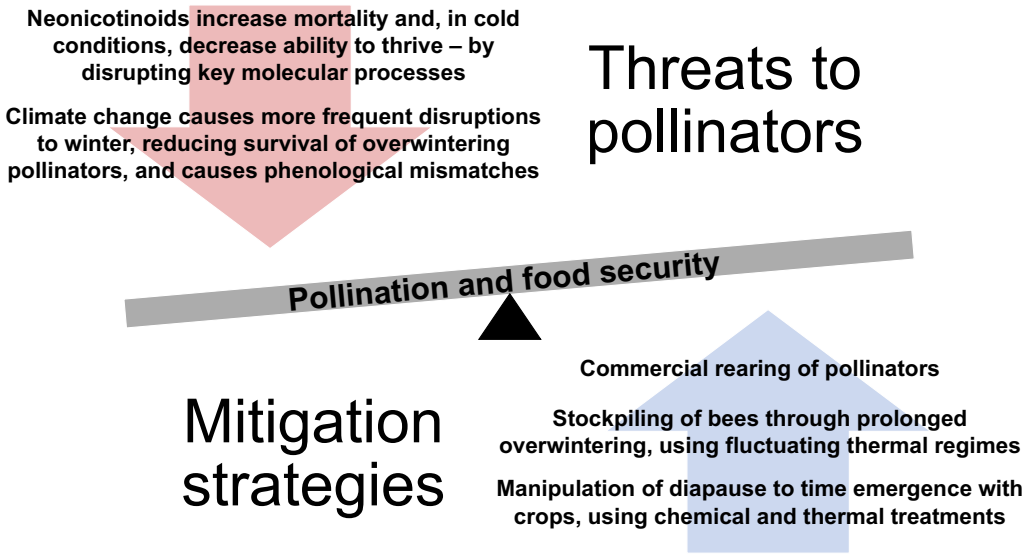


Figure 6.1 Summary of the investigations in this thesis

6.2 Feasibility of the commercial rearing of red mason bees

RMBs may be particularly desirable as commercial pollinators as they are the most polylectic of the mason bees (Haider et al., 2014) and have been shown to be effective pollinators of several important crop species (Biliński and Teper, 2004; Teper and Biliński, 2009; Fliszkiewicz et al., 2011). Although, rather than managing RMBs in orchards which can leave them susceptible to parasitism and adverse weather conditions (Bosch and Kemp, 2002), it may be prudent to rear these bees under more controlled conditions.

6.2.1 Mass-rearing methodologies for red mason bees

The results of this thesis suggest that the mass-rearing of RMBs under controlled conditions is possible, but at present it is not commercially viable (Chapter 2). With the methodologies trialled, RMBs successfully mated and provisioned offspring, but the maximum number of adult offspring produced was only one per rearing attempt (Chapter 2, Figure 2.8). The amount of offspring required for a commercial operation to reach viability is unclear but based on my own experiments I conservatively estimate that the minimum costs to provide RMBs all the resources they require to produce offspring were £289 (Table 6.1). However, this calculation did not include labour costs or any additional resources that may be required to increase RMB reproductive output. Therefore, the production of a single adult bee at an estimated sale price of £0.70 (based on the retail price of RMBs from other providers (Appendix 1.1)), would not allow an operation to break even, let alone make any profit.

Table 6.1 Estimated minimum costs for laboratory rearing of red mason bees

Resource	Estimated minimum cost (£)
Enclosure (0.65 m^3 bug-dorm)	75.00
Starting bee population (100 individuals)	70.00
Nests (24 wooden nest blocks)	120.00
Cell-construction material	12.00
Pollen resources	2.00
Nectar	10.00
Grand total	**289.00**

The costs here are estimates based on the minimum amount of resources that were required for RMBs to provision brood (Chapter 2).

Based on the number of oocytes available, RMB females appear to have a maximum fecundity of 42 offspring (Maeta and Kurihara, 1971; Giejdasz et al., 2016). However, in the wild the number of offspring reared can range considerably, with observations showing that a single female can produce from two to 34 offspring (Raw, 1972; Strohm et al., 2002; Giejdasz et al., 2016). If a level of reproduction similar to the medium possible (i.e. 17-20 offspring produced per female) could be attained in commercial ventures, then a starting population of 100 RMBs (50 ♂♂ and 50 ♀♀) could produce around 1,700-2,000 offspring, allowing an estimated sale revenue of £1,190-1,400. Such numbers may then facilitate commercial viability.

For future studies, I would propose employing semi-field rearing of RMBs. Low light intensity and small enclosure sizes, in particular, were shown to negatively impact RMB rearing (Chapter 2), both of which could be alleviated in a semi-field system. For example, by releasing the adult females into a field or greenhouse environment to collect pollen and provision brood and then collecting the offspring

to rear under controlled conditions (although care would need to be taken to prevent damaging developing brood (Giejdasz and Wilkaniec, 2002)). In bumblebee rearing systems labour is one of the biggest outlays (Velthuis and Van Doorn, 2006), and the same is likely true for RMB rearing. A semi-field system may allow a reduction in these labour costs, as it would allow RMBs to be left unattended whilst they provision brood. Additionally, RMBs are docile and rarely sting (Raw, 1972; O'Toole, 2000), so they could be reared in pre-existing plant production greenhouses (e.g. an orchard nursery) to utilise the available forage whilst minimising the need for additional space, providing an economic way to cultivate RMBs.

If such a system were pursued it could increase the risk of parasites as, if nests were placed outside of an enclosed system, they may be more accessible to parasites (Strohm et al., 2002). However, in this thesis I demonstrated using X-rays to examine RMBs (Chapter 3, Figure 3.2), which also allows detection of parasites within cocoons (pre-emerged adults) in a non-invasive manner (Pitts-Singer and Cane, 2011). The system I employed used only simple X-ray plates (at an approximate cost of £70 each), and a dental X-ray device (approximately £2,000 in value). Whilst expensive initially, such a system could allow the assurance of parasite-free bees to growers, which may be an extremely valuable commercial advantage. Moreover, there are also handheld dental x-ray machines that could provide a cheaper alternative. However, this technique would not allow detection of parasites that target RMBs before they wrap themselves in cocoons (Chapter 1, Figure 1.2), but by combining it with other techniques to reduce parasitism (e.g. use of wood or bamboo nests (Wilkaniec and Giejdasz, 2003;

Horth and Campbell, 2018) and regular nest cleaning (Pitts-Singer and Cane, 2011)) the parasite exposure risk in a semi-field system could be minimised.

In this proposed system the offspring of RMBs would be reared in controlled laboratory conditions (as in Giejdasz and Wilkaniec (2002)), which provides opportunity to manipulate the lifecycle of RMBs. Offspring development could be adjusted using different temperature regimes (Giejdasz and Wilkaniec, 2002), and once RMBs reached diapause (Chapter 1, Figure 1.2) this again could be manipulated to allow the development of a stocking system of RMBs: extension of diapause to allow storage, then quick resumption of development and emergence when bees are required. Such manipulations were investigated in Chapter 3.

6.2.2 Manipulations of red mason bee diapause

RMBs are dormant for around 6-7 months per year (Chapter 1, Figure 1.2) due to diapause and a post-diapause quiescent period, which currently constrains when bees are available to growers. To allow RMBs to be transported to different growers in different regions, and to synchronise emergence with the staggered timing of target plant blooms across these regions, methods to force or delay RMB emergence were assessed (Chapter 3). Here, it was found that methoprene and hexane are potential compounds that can promote RMB emergence (Chapter 3, Figure 3.9). However, methoprene may reduce RMB survival (Chapter 3, Figure 3.10). Hexane, on the other hand, may increase the emergence rate of RMBs by up to 18 days in males and 22 days in females (Chapter 3, Figure 3.9), without as severe effects on survival as methoprene

(Chapter 3, Figures 3.10). Hexane may also have the advantage of being flexible in its mode of application; it can be applied either topically or as a vapour to terminate diapause in other insect species (Denlinger et al., 1980). A vapour application would be far less labour intensive than topical treatments, so may be advantageous for commercial operations. Whether hexane as a vapour would promote RMB emergence as efficiently as a topical application is a key area of future investigation. Either way hexane is a useful compound for commercial operations to synchronise RMB emergence more closely with early flowering target crops.

The diapause of RMBs also offers an advantage. It has been shown that the diapause period of the alfalfa leafcutting bee (*Megachile rotundata*) can be safely extended for up to a year and a half without negative effects, by the use of a fluctuating thermal regime (FTR; Rinehart et al., 2013). Similarly, here it was shown that RMB diapause can be extended for up to 11 months without any adverse effects on survival using this approach. In fact, survival under FTR was consistently higher than at an industry-standard constant temperature of 2˚C for up to 21 months (Chapter 3, Figure 3.11). This may have been due to a decreased use of energy stores (Chapter 3, Figures 3.12 and 3.13). Such storage regimes are simple to implement and could be readily used by current commercial operations to stockpile RMBs for potentially more than one season, as well as staggering their emergence times. RMB numbers are likely to fluctuate year on year based on climatic conditions (Bosch and Kemp, 2002) and wild populations of bees have largely been found to be experiencing declines (Potts et al., 2016; Powney et al., 2019). Therefore, FTRs could represent an important

technique to maintain pollination services. However, it remains to be seen whether bees stored in this manner are as effective at producing offspring, and so this is a crucial target of future work.

6.2.3 The future of commercial red mason bee provision

Data from this thesis suggest that existing methods for establishing RMB rearing systems are far from being commercially viable. However, some of the next steps to improve these methods have been identified. Manipulations of diapause in this species is feasible and could supplement current RMB management practices to lengthen the period this species is available to growers.

Another key area of investigation should be determining the economic advantage of rearing RMBs relative to other species. Whilst RMBs are effective orchard pollinators (Fliszkiewicz et al., 2011; Gruber et al., 2011) and pollinate several other important crops, such as rapeseed (Teper and Biliński, 2009), growers may be content to rely on 'natural' pollination, rather than purchasing additional pollinators. Key to the commercial success of bumblebee production was the huge economic advantage of their use for greenhouse tomato pollination, which was previously costing growers approximately £9,000 per hectare annually in manual labour (Velthuis and Van Doorn, 2006). This allowed bumblebee production to quickly reach commercial viability, despite relatively high production costs in the early stages (Velthuis and Van Doorn, 2006).

It has previously been suggested that pollination is limiting yields in United Kingdom (UK) orchards (Garratt et al., 2014a, 2014b), so further investigation into this is warranted to promote a greater recognition of the problem amongst

growers. Orchard crops are also likely to be further limited under future climate change, due to loss of pollination services and direct effects of warming on fruiting (Polce et al., 2014; Fraga et al., 2019). Therefore, this should be further investigated and emphasised to growers. Indeed, in this thesis there is data to suggest that the phenologies of apples and RMBs are diverging (Chapter 3, Figure 3.7), and thus apples may see pollination deficits in the future unless mitigating strategies can be developed. Internationally, there may be more scope to develop commercial provision of orchard specialist pollinations. For example, apples have been exported to several regions (e.g. Sichuan, China) that lack appropriate pollinators, resulting in growers eventually removing apple trees for less pollination-dependent crops, or even resorting to hand-pollination (Partap and Ya, 2012).

As an alternative strategy, there are other pollinator species that could be suitable targets for commercialisation. For example, the blue blowfly (BBF; *Calliphora vicina*) has been shown to be a pollinator of several vegetables (Schittenhelm et al., 1997; Howlett, 2012), and due to a long history of use in medical entomology (Donovan et al., 2006), its biology is well-understood. Furthermore, vast numbers of BBFs can be reared in the laboratory using very straightforward methodology (Coleman et al., 2015; Chapter 4). Several hoverfly species (Diptera: Syrphidae) are already an attractive commercial target for biocontrol (Haenke et al., 2009), and as effective pollinators of a wide range of crops (Biesmeijer et al., 2006; Jauker and Wolters, 2008), they represent an ideal commercially produced insect system to enhance food security. A greater range of commercially available pollinators could also allow the most appropriate pollinator to be used for a target

crop and may bolster provision of pollination services in the face of potential pollinator shortfalls.

As RMBs are not currently commercially viable, their rearing does not represent a good strategy to mitigate pollinator losses at present. Until such a system is developed, conservation of RMBs, and the pollination services they provide, is essential. As previously discussed, there are five key factors that are implicated in pollinator declines: land-use change and management, climate change, pesticides, spread of disease, and invasive alien species (Vanbergen et al., 2013; Goulson et al., 2015; Potts et al., 2016), and in agricultural settings RMBs may be particularly vulnerable to pesticides applied to protect crops. Additionally, RMBs may be especially susceptible to climate change. Therefore, these challenges to RMBs were investigated.

6.3 Climate change and red mason bees

With an obligate diapause, RMBs will prepare for and enter diapause regardless of environmental conditions (Wasielewski et al., 2011a; Dmochowska et al., 2012). This could leave them vulnerable to increases in temperature that can significantly disrupt their phenology and winter survival. For example, it has been shown that increases in winter temperatures during RMB diapause deplete lipid stores (Fliszkiewicz et al., 2012), which in turn reduce survival post-diapause. While warming in late winter and early spring can result in early emergence (Wasielewski et al., 2011a), with subsequent impacts on survival if cold conditions return.

6.3.1 Red mason bee overwintering and climate change

Here, it was shown that RMB overwintering may be negatively affected by increasing temperatures (Chapter 3). Extended autumn temperatures caused a dramatic decline in survival of RMBs, with no bees subjected to such conditions surviving after 210 days total storage – which is within their normal emergence period (Chapter 3, Figure 3.3). This was linked to a loss of bee mass over this period, likely as a result of lipid loss and thus depleted energy reserves (Chapter 3, Figures 3.4 and 3.5). Interestingly, the weight loss and depletion of lipids continued long after the initial increase of temperature (November-December), continuing until June the following year (Chapter 3, Figures 3.4, 3.5), implying that a short delay to the onset of winter temperatures could cause long-term impacts on the diapause programme, reducing post-winter survival. Altogether, this could mean that RMB populations will decline in future because delays to winter, or warmer autumns, are likely to become more common under future climate change (Gallinat et al., 2015; Williams et al., 2015).

These results also emphasise the utility of different RMB storage regimes. Whilst a commercial rearing system is not currently viable (Chapter 2), growers and producers of RMBs could use the FTR system discussed in this thesis to promote survival of overwintering bees (Chapter 3). As temperatures continue to increase, and the length and onset of winter changes, leaving RMBs in the field to overwinter may become unworkable, so storing bees in FTRs is a straightforward solution. Moreover, by extending the overwintering period FTRs could allow a portion of RMBs to be ‘stocked’ and left in reserve to bolster pollinator provision,

if there are shortfalls in a particular year, which may become more likely due to climate change (Chapter 3).

6.3.2 Red mason bee phenology and climate change

A historical dataset of RMB observations was used to discern how RMB phenology has changed over time. From 1977 to 2013 it was shown that the first observation of RMBs has advanced by approximately 30 days (Chapter 3, Figure 3.7). The first observation was taken as a proxy for RMB emergence, and it was concluded that spring temperatures were the predominant driver of this earlier emergence (although increasing sampling effort, and thus more sightings overall, may partly explain why RMBs in certain locations are being seen earlier than previous years; Chapter 3, Figure 3.8). Therefore, it is likely that past climate change has promoted the earlier emergence of RMBs. This is corroborated by accounts of RMBs. In 1972, Raw described RMBs as being active “in May and July” (Raw, 1972), whereas in 2000 O’Toole described them as a “spring bee” active from “March” to “end of June” (O’Toole, 2000). Additionally, as temperature is the cue for RMBs to emerge in spring (Wasielewski et al., 2011a), it is highly likely that their emergence is advancing with increasing temperatures.

Earlier spring emergence could cause RMB phenology to mismatch with that of the plants they pollinate. The results in this thesis suggest that their mean emergence date is advancing by approximately 8 days per decade since 1977, whereas a potential pollination target for RMBs, apple, has been shown to be shifting its phenology forward by only 3 days per decade (Wolfe et al., 2005; Bartomeus et al., 2013). However, the results here are only a proxy for

emergence. Furthermore, phenological mismatches would need to be shown empirically, by direct observations of RMBs and the associated plant forage.

In temperate regions there are few detailed examples of phenological mismatches in solitary bees (although see Kehrberger and Holzschuh, 2019). These results then show that this is knowledge gap that urgently needs to be filled, and that long-term datasets (such as that provided by the Bees Wasps and Ants Recording Society) can identify in what species there are potential phenological mismatches. There are many important pollinating bees that have a similar lifecycle to RMBs and also use temperature as the main cue to for emergence (e.g. other mason bees (Schenk et al., 2018b) and alfalfa leafcutting bees (Bennett et al., 2018)). Therefore, these pollinators should be the target of future research, as it is possible they are at risk of becoming phenologically mismatched with target crops (Schenk et al., 2018a). Additionally, as shortened overwintering periods were shown to reduce RMB survival (Chapter 3, Figure 3.6), changing phenology may precipitate further bee declines (Schenk et al., 2018b).

Another consequence of the advancing phenology of RMBs may be that they are exposed to colder conditions than those they are adapted to. Whilst temperature under climate change will be on average warmer, it is also likely that the climate will be more unpredictable and so there may be episodes of cold occurring after bee emergence (Williams et al., 2015). For example, in the UK in February 2018 there was an period of intense cold following a relatively mild winter (Greening and Hodgson, 2019), which may have impacted upon many emerged insects.

Besides the direct effects of cold, it is also possible that low temperatures could combine with other stresses, like pesticides, which can have significant effects on pollinators (Chapters 4 and 5).

6.4 Impacts of neonicotinoids and cold on pollinators

Whilst neonicotinoids have been restricted in their use in the European Union (Jactel et al., 2019), they are still widely used elsewhere (Anderson et al., 2015; Goulson et al., 2018; DiBartolomeis et al., 2019). This is concerning as neonicotinoids have been shown to cause a range of sub-lethal effects on bumblebees and honeybees (Alkassab and Kirchner, 2017). However, there is little known about their effects on solitary bees, and almost nothing known about their effects on flies (Blacquiere et al., 2012; Pisa et al., 2014; Van Der Sluijs et al., 2015). Moreover, there is limited understanding of neonicotinoids' effects on the thermal biology of pollinators.

Here, the lack of understanding of the thermal of biology of pollinators was addressed by investigating the critical thermal minimum (CT_{min}) of three pollinators (RMBs, BTBs and BBFs) after exposure to IMI (Chapter 4). IMI exposure substantially increased the CT_{min} of all species (Figure 4.3), which is suggestive of a general mechanism, perhaps disrupting the signalling of action potentials (Matsuda et al., 2001; MacMillan and Sinclair, 2011; Overgaard and MacMillan, 2017). The transcriptomic analysis of BTBs showed that after IMI exposure many transcripts associated with the response to temperature were dysregulated (Chapter 5), which may suggest that exposure to IMI makes BTBs less able to adapt to low temperatures (Chapter 5, Figures 5.1 and 5.4).

Additionally, genes associated with muscular control were dysregulated (Chapter 5, Figure 5.1 and 5.4), which may also result in the observed increase in CT_{min} (Chapter 4). Whether these responses are generalisable to other pollinators, like RMBs and BBFs, remains unclear, and should be a focus of future studies. With the recent sequencing of the RMB genome (Beadle et al., 2019), the gene expression of RMBs exposed to NPs should be a key research outlook.

These results also clearly illustrate a previously unrecognised example of a substantial impact on pollinators occurring due to combined stresses, which individually may be sub-lethal, but in combination have significant impacts on survival and/or fitness. After exposure to a very low field-relevant dose of IMI, BTBs showed a loss of coordinated movement at only 18.6˚C body temperature (Chapter 4, Figure 4.3). Additionally, 9 µg/l IMI and subtle chilling of BTBs body temperature caused substantial changes in gene expression (Chapter 5), much more than was shown when each stress was examined individually (Chapter 5). This was especially in genes relating to muscular contraction and ionic homeostasis (Chapter 5, Figures 5.1 and 5.4), which could prevent BTBs regulating their body temperature and moving to escape cold (Chapter 4, Figures 4.3 and 4.4). As pollinator phenology may be shifting (e.g. Chapter 3, Figure 3.7), it is possible that they will experience more cold spells after their emergence (Bale and Hayward, 2010), therefore these combinational stresses may be increasingly important as the climate changes. It has been proposed that combined stresses are driving bee declines (Goulson et al., 2015), which is supported by the data shown here. Therefore, future studies should urgently investigate other combined

stresses that are experienced by pollinators in the field, even beyond pesticides and cold, as it is possible there are many other unidentified impacts.

6.5 Study limitations

There were a few limitations to the work presented here. It is understood that the thermal experiences of mason bees (including RMBs) throughout their development and wintering period can affect their later survival and longevity (Bosch and Kemp, 2004; Kemp and Bosch, 2005; Pitts-Singer et al., 2014; Giejdasz and Fliszkiewicz, 2016). Whilst total control of thermal history is unworkable without creation of a laboratory rearing system (Chapter 2), it is possible that the prior thermal experience of the diapausing RMBs purchased for this study influenced the survival experiments conducted in different years during this work. As RMB survival was variable it is unclear whether that was the case in this study (Chapter 3), but experiments only drew comparisons with bees in the same cohort (i.e. ones that were purchased at the same time from the same place and so presumably had the same thermal history) so it is likely this effect was minimal.

There is also the question of how well commercially produced BTBs represent the responses of wild BTBs. The rearing methods for these bees are, understandably, protected as intellectual property by Biobest® and other commercial produces, so it is unknown exactly how they were reared, and also to what extent they may be inbred. This is important as introgression can adversely affect fitness (Whitehorn et al., 2009). Whilst previous experiments in this lab have suggested that BTBs are representative of wild bees, at least in

terms of their use in activity threshold experiments (Owen, 2015, PhD Thesis), a more thorough analysis may be desirable.

For the transcriptomic work, there were also some limitations. BTBs' genes are bioinformatically inferred because for many of them their roles have not been demonstrated experimentally (Colgan et al., 2019). The results shown here therefore should only suggest possible processes that have been affected. The function of individual genes will need to be confirmed in future studies. Inter-individual responses were also variable (Chapter 5, Figure 5.1), showing that whilst BTB workers share 75% of their genetic information, there is a still variability between individuals. Future work should also consider age-controlling the bees, as it is possible this has an effect on the vulnerability of BTBs to NPs (Blacquiere et al., 2012).

6.6 Conclusions

With the many threats facing pollination services, commercial provision of pollinators may be a key tool to promote yields and achieve food security. This mitigation strategy was investigated by attempted development of a mass-rearing protocol for RMBs (Chapter 2). Whilst unsuccessful, the data shown here provide an essential first step and identify how moving to a semi-field system may be a prudent strategy to rear RMBs commercially. Before such a system can be developed RMBs remain vulnerable to many challenges in the environment. Indeed, it was shown that climate change may be damaging to RMBs (Chapter 3). Delayed onset and shortening of winter reduced survival in the bees, moreover RMBs are likely changing their phenology in the face of warming

temperatures and so are vulnerable to mismatches with target crops (Chapter 3). Therefore, identifying pollinators with similar lifecycles and the risks they face from climate change should be a priority. Such impacts may also be partially mitigated by the use of FTRs that were shown to increase the shelf-life of RMBs (Chapter 3). NPs were also shown to have damaging effects on pollinators, increasing their CT_{min} (Chapter 4). BTBs were especially susceptible and this work demonstrates a previously unrecognised combinational effect of cold and pesticides on these important pollinators, which suggests that this is an area of study that urgently needs to be addressed. Indeed, a transcriptomic study in BTBs suggested that the gene expression of several core processes was disrupted by the combination of IMI and chilling (Chapter 5). Overall, this work identifies several previously unrecognised effects on pollinators, which may have wide-ranging impacts on food security and suggests possible mitigation strategies.

References

Abbott, V. A., Nadeau, J. L., Higo, H. A. and Winston, M. L. (2008) 'Lethal and sublethal effects of imidacloprid on *Osmia lignaria* and clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae)', *Journal of Economic Entomology,* 101(3), pp. 784-796.

Acosta, A. L., Giannini, T. C., Imperatriz-Fonseca, V. L. and Saraiva, A. M. (2016) 'Worldwide alien invasion: A methodological approach to forecast the potential spread of a highly invasive pollinator', *PLoS ONE,* 11(2), pp. e0148295.

Agui, N., Mihara, M. and Kurahashi, H. (1991) 'Effect of juvenile hormone analogue on ovarian development of the reproductive-diapausing parasitic fly, *Melinda pusilla*', *Medical Entomology and Zoology,* 42(4), pp. 311–317.

Aizen, M. A., Garibaldi, L. A., Cunningham, S. A. and Klein, A. M. (2008) 'Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency', *Current Biology,* 18(20), pp. 1572-1575.

Alkassab, A. T. and Kirchner, W. H. (2017) 'Sublethal exposure to neonicotinoids and related side effects on insect pollinators: honeybees, bumblebees, and solitary bees', *Journal of Plant Diseases and Protection,* 124(1), pp. 1-30.

Allen, M., Antwi-Agyei, P., Aragon-Durand, F., Babiker, M., Bertoldi, P., Bind, M., Brown, S., Buckeridge, M., Camilloni, I. and Cartwright, A. (2019) 'Technical Summary: Global warming of 1.5° C. An IPCC Special Report on the impacts of global warming of 1.5° C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty'. Available at: https://www.ipcc.ch/sr15/ (Accessed: 20 August 2019).

Allsopp, M. H., de Lange, W. J. and Veldtman, R. (2008) 'Valuing insect pollination services with cost of replacement', *PLoS ONE,* 3(9), pp. e3128.

Alptekin, S., Bass, C., Nicholls, C., Paine, M. J., Clark, S. J., Field, L. and Moores, G. D. (2016) 'Induced thiacloprid insensitivity in honeybees (*Apis mellifera* L.) is associated with up-regulation of detoxification genes', *Insect Molecular Biology,* 25(2), pp. 171-180.

Altermatt, F. (2010) 'Climatic warming increases voltinism in European butterflies and moths', *Proceedings of the Royal Society B: Biological Sciences,* 277(1685), pp. 1281-1287.

Amasino, R. M. and Michaels, S. D. (2010) 'The timing of flowering', *Plant Physiology,* 154(2), pp. 516-520.

Amiri, A., Bandani, A. R. and Darvishzadeh, A. (2012) 'Effects of the insect growth regulators methoxyfenozide and pyriproxyfen on adult diapause in sunn pest *Eurygaster integriceps* (Hemiptera: Scutelleridae)', *Journal of Agricultural Science and Technology,* 14(6), pp. 1205-1218.

Amorós-Jiménez, R., Pineda, A., Fereres, A. and Marcos-García, M. Á. (2012) 'Prey availability and abiotic requirements of immature stages of the aphid predator *Sphaerophoria rueppellii*', *Biological Control,* 63(1), pp. 17-24.

Andersen, M. K., Folkersen, R., MacMillan, H. A. and Overgaard, J. (2017) 'Cold acclimation improves chill tolerance in the migratory locust through preservation of ion balance and membrane potential', *Journal of Experimental Biology,* 220(3), pp. 487-496.

Anderson, J. C., Dubetz, C. and Palace, V. P. (2015) 'Neonicotinoids in the Canadian aquatic environment: a literature review on current use products with a focus on fate, exposure, and biological effects', *Science of the Total Environment,* 505, pp. 409-422.

Antúnez, K., Martín-Hernández, R., Prieto, L., Meana, A., Zunino, P. and Higes, M. (2009) 'Immune suppression in the honey bee (*Apis mellifera*) following

infection by *Nosema ceranae* (Microsporidia)', *Environmental Microbiology,* 11(9), pp. 2284-2290.

Arce, A. N., David, T. I., Randall, E. L., Ramos Rodrigues, A., Colgan, T. J., Wurm, Y. and Gill, R. J. (2017) 'Impact of controlled neonicotinoid exposure on bumblebees in a realistic field setting', *Journal of Applied Ecology,* 54(4), pp. 1199-1208.

Arpagaus, M. (1987) 'Vertebrate insulin induces diapause termination in *Pieris brassicae* pupae', *Roux's Archives of Developmental Biology,* 196(8), pp. 527-530.

Arpagaus, M., Beydon, P. and Lafont, R. (1986) 'Effects of ecdysone injection on diapause termination in *Pieris brassicae* pupae. Evidence for a stimulatory action on endogenous ecdysone production', *Reproduction Nutrition Développement,* 26(6), pp. 1306-1317.

Australian Government: Department of Agriculture, Water and the Environment (2021) *Invasive bees*. Available at: https://www.environment.gov.au/biodiversity/invasive-species/insects-and-other-invertebrates/invasive-bees (Accessed: 04 May 2021).

Ayme-Southgate, A. J., Southgate, R. J., Philipp, R. A., Sotka, E. E. and Kramp, C. (2008) 'The myofibrillar protein, projectin, is highly conserved across insect evolution except for its PEVK domain', *Journal of Molecular Evolution,* 67(6), pp. 653-669.

Badano, E. I. and Vergara, C. H. (2011) 'Potential negative effects of exotic honey bees on the diversity of native pollinators and yield of highland coffee plantations', *Agricultural and Forest Entomology,* 13(4), pp. 365-372.

Badiou-Bénéteau, A., Carvalho, S. M., Brunet, J. L., Carvalho, G. A., Buleté, A., Giroud, B. and Belzunces, L. P. (2012) 'Development of biomarkers of exposure to xenobiotics in the honey bee *Apis mellifera*: application to the systemic

insecticide thiamethoxam', *Ecotoxicology and Environmental Safety,* 82, pp. 22-31.

Baines, D., Wilton, E., Pawluk, A., de Gorter, M. and Chomistek, N. (2017) 'Neonicotinoids act like endocrine disrupting chemicals in newly-emerged bees and winter bees', *Scientific Reports,* 7(1), pp. 10979.

Bakthisaran, R., Tangirala, R. and Rao, C. M. (2015) 'Small heat shock proteins: Role in cellular functions and pathology', *Biochimica et Biophysica Acta - Proteins and Proteomics,* 1854(4), pp. 291-319.

Bale, J. S. and Hayward, S. A. (2010) 'Insect overwintering in a changing climate', *Journal of Experimental Biology,* 213(6), pp. 980-94.

The World Bank (2014) The economic impact of the 2014 Ebola epidemic: short- and medium-term estimates for West Africa. The World Bank. Available at: https://elibrary.worldbank.org/doi/abs/10.1596/978-1-4648-0438-0 (Accessed: 17 April 2021).

Barons, M. J. and Aspinall, W. (2020) 'Anticipated impacts of Brexit scenarios on UK food prices and implications for policies on poverty and health: a structured expert judgement approach', *BMJ Open,* 10(3), pp. e032376.

Bartomeus, I., Ascher, J. S., Wagner, D., Danforth, B. N., Colla, S., Kornbluth, S. and Winfree, R. (2011) 'Climate-associated phenological advances in bee pollinators and bee-pollinated plants', *Proceedings of the National Academy of Sciences of the United States of America,* 108(51), pp. 20645-20649.

Bartomeus, I., Park, M. G., Gibbs, J., Danforth, B. N., Lakso, A. N. and Winfree, R. (2013) 'Biodiversity ensures plant-pollinator phenological synchrony against climate change', *Ecology Letters,* 16(11), pp. 1331-1338.

Bartomeus, I., Potts, S. G., Steffan-Dewenter, I., Vaissière, B. E., Woyciechowski, M., Krewenka, K. M., Tscheulin, T., Roberts, S. P., Szentgyörgyi, H., Westphal, C. and Bommarco, R. (2014) 'Contribution of insect pollinators to crop yield and quality varies with agricultural intensification', *PeerJ,* 2, pp. e328.

Basley, K., Davenport, B., Vogiatzis, K. and Goulson, D. (2018) 'Effects of chronic exposure to thiamethoxam on larvae of the hoverfly', *PeerJ,* 6, pp. e4258.

Bass, C. and Field, L. M. (2018) 'Neonicotinoids', *Current Biology,* 28(14), pp. R772-R773.

Bayley, J. S., Winther, C. B., Andersen, M. K., Grønkjær, C., Nielsen, O. B., Pedersen, T. H. and Overgaard, J. (2018) 'Cold exposure causes cell death by depolarization-mediated Ca', *Proceedings of the National Academy of Sciences of the United States of America,* 115(41), pp. E9737-E9744.

Beadle, K., Singh, K. S., Troczka, B. J., Randall, E., Zaworra, M., Zimmer, C. T., Hayward, A., Reid, R., Kor, L., Kohler, M., Buer, B., Nelson, D. R., Williamson, M. S., Davies, T. G. E., Field, L. M., Nauen, R. and Bass, C. (2019) 'Genomic insights into neonicotinoid sensitivity in the solitary bee *Osmia bicornis*', *PLoS Genetics,* 15(2), pp. e1007903.

Beers, E. H., Van Steenwyk, R. A., Shearer, P. W., Coates, W. W. and Grant, J. A. (2011) 'Developing *Drosophila suzukii* management programs for sweet cherry in the western United States', *Pest Management Science,* 67(11), pp. 1386-1395.

Bennett, M. M., Petersen, K., Yocum, G., Rinehart, J., Kemp, W. and Greenlee, K. J. (2013) 'Effects of extended prepupal storage duration on adult flight physiology of the alfalfa leafcutting bee (Hymenoptera: Megachilidae)', *Journal of Economic Entomology,* 106(3), pp. 1089-1097.

Bennett, M. M., Rinehart, J. P., Yocum, G. D., Doetkott, C. and Greenlee, K. J. (2018) 'Cues for cavity nesters: investigating relevant zeitgebers for emerging leafcutting bees,' *Journal of Experimental Biology,* 221(10), jeb175406.

Berenbaum, M. R. and Johnson, R. M. (2015) 'Xenobiotic detoxification pathways in honey bees', *Current Opinion in Insect Science,* 10, pp. 51-58.

Bhagavan, N. V. and Ha, C.-E. (2015) 'Chapter 19 - Contractile Systems', in Bhagavan, N.V. and Ha, C.-E. (eds.) *Essentials of Medical Biochemistry.* 2nd edn. San Diego, California, USA: Academic Press, pp. 339-361.

Bi, R., Pan, Y., Shang, Q., Peng, T., Yang, S., Wang, S., Xin, X., Liu, Y. and Xi, J. (2016) 'Comparative proteomic analysis in *Aphis glycines* Mutsumura under lambda-cyhalothrin insecticide stress', *Comparative Biochemistry and Physiology - Part D: Genomics and Proteomics,* 19, pp. 90-96.

Biesmeijer, J. C., Roberts, S. P., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A. P., Potts, S. G., Kleukers, R., Thomas, C. D., Settele, J. and Kunin, W. E. (2006) 'Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands', *Science,* 313(5785), pp. 351-354.

Biliński, M. and Teper, D. (2004) 'Rearing and utilization of the red mason bee—*Osmia rufa* L.(Hymenoptera, Megachilidae) for orchard pollination', *Journal of Apicultural Science,* 48, pp. 69-74.

Blackman, B. K. (2017) 'Changing Responses to Changing Seasons: Natural Variation in the Plasticity of Flowering Time', *Plant Physiology,* 173(1), pp. 16-26.

Blacquière, T., Smagghe, G., van Gestel, C. A. and Mommaerts, V. (2012) 'Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment', *Ecotoxicology,* 21(4), pp. 973-992.

Bodnaryk, R. P. (1985) 'Ecdysteroid levels during post-diapause development and 20-hydroxyecdysone-induced development in male pupae of *Mamestra configurata* Wlk', *Journal of Insect Physiology,* 31(1), pp. 53-58.

Bonmatin, J. M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E. A., Noome, D. A., Simon-Delso, N. and Tapparo, A. (2015) 'Environmental fate and exposure; neonicotinoids and fipronil', *Environmental Science and Pollution Research,* 22(1), pp. 35-67.

Bonmatin, J. M., Moineau, I., Charvet, R., Colin, M. E., Fleche, C. and Bengsch, E. (2005) 'Behaviour of imidacloprid in fields. Toxicity for honey bees', in Lichtfouse E., Schwarzbauer J., Robert D. (eds) *Environmental Chemistry.* Berlin; Heidelberg, Germany: Springer, pp. 483-494.

Bosch, J. (1992) 'Parasitism in wild and managed populations of the almond pollinator *Osmia cornuta* Latr.(Hymenoptera: Megachilidae)', *Journal of Apicultural Research,* 31(2), pp. 77-82.

Bosch, J. and Kemp, W. (2000) 'Development and emergence of the orchard pollinator *Osmia lignaria* (Hymenoptera: Megachilidae)', *Environmental Entomology,* 29(1), pp. 8-13.

Bosch, J. and Kemp, W. P. (2002) 'Developing and establishing bee species as crop pollinators: the example of *Osmia* spp. (Hymenoptera: Megachilidae) and fruit trees', *Bulletin of Entomological Research,* 92(1), pp. 3-16.

Bosch, J. and Kemp, W. P. (2003) 'Effect of wintering duration and temperature on survival and emergence time in males of the orchard pollinator *Osmia lignaria* (Hymenoptera: Megachilidae)', *Environmental Entomology,* 32(4), pp. 711-716.

Bosch, J. and Kemp, W. P. (2004) 'Effect of pre-wintering and wintering temperature regimes on weight loss, survival, and emergence time in the mason bee *Osmia cornuta* (Hymenoptera: Megachilidae)', *Apidologie,* 35(5), pp. 469-479.

Bosch, J., Sgolastra, F. and Kemp, W. P. (2010) 'Timing of eclosion affects diapause development, fat body consumption and longevity in *Osmia lignaria*, a univoltine, adult-wintering solitary bee', *Journal of Insect Physiology,* 56(12), pp. 1949-1957.

Botías, C., David, A., Horwood, J., Abdul-Sada, A., Nicholls, E., Hill, E. and Goulson, D. (2015) 'Neonicotinoid residues in wildflowers, a potential route of chronic exposure for bees', *Environmental Science & Technology,* 49(21), pp. 12731-12740.

Brandt, A., Hohnheiser, B., Sgolastra, F., Bosch, J., Meixner, M. D. and Büchler, R. (2020) 'Immunosuppression response to the neonicotinoid insecticide thiacloprid in females and males of the red mason bee *Osmia bicornis* L', *Scientific Reports,* 10(1), pp. 4670.

Breeze, T. D., Bailey, A. P., Balcombe, K. G. and Potts, S. G. (2011) 'Pollination services in the UK: How important are honeybees?', *Agriculture, Ecosystems & Environment,* 142(3-4), pp. 137-143.

Brown, L. A., Ihara, M., Buckingham, S. D., Matsuda, K. and Sattelle, D. B. (2006) 'Neonicotinoid insecticides display partial and super agonist actions on native insect nicotinic acetylcholine receptors', *Journal of Neurochemistry,* 99(2), pp. 608-615.

Burger, H., Dötterl, S. and Ayasse, M. (2010) 'Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee', *Functional Ecology,* 24(6), pp. 1234-1240.

Cameron, S. A., Lim, H. C., Lozier, J. D., Duennes, M. A. and Thorp, R. (2016) 'Test of the invasive pathogen hypothesis of bumble bee decline in North America', *Proceedings of the National Academy of Sciences of the United States of America,* 113(16), pp. 4386-4391.

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F. and Griswold, T. L. (2011) 'Patterns of widespread decline in North American bumble bees', *Proceedings of the National Academy of Sciences of the United States of America,* 108(2), pp. 662-7.

CaraDonna, P. J., Cunningham, J. L. and Iler, A. M. (2018) 'Experimental warming in the field delays phenology and reduces body mass, fat content and survival: Implications for the persistence of a pollinator under climate change', *Functional Ecology,* 32(10), pp. 2345-2356.

Carra, S., Alberti, S., Arrigo, P. A., Benesch, J. L., Benjamin, I. J., Boelens, W., Bartelt-Kirbach, B., Brundel, B. J. J. M., Buchner, J., Bukau, B., Carver, J. A., Ecroyd, H., Emanuelsson, C., Finet, S., Golenhofen, N., Goloubinoff, P., Gusev, N., Haslbeck, M., Hightower, L. E., Kampinga, H. H., Klevit, R. E., Liberek, K., Mchaourab, H. S., McMenimen, K. A., Poletti, A., Quinlan, R., Strelkov, S. V., Toth, M. E., Vierling, E. and Tanguay, R. M. (2017) 'The growing world of small

heat shock proteins: from structure to functions', *Cell Stress Chaperones,* 22(4), pp. 601-611.

Carvalheiro, L. G., Kunin, W. E., Keil, P., Aguirre-Gutiérrez, J., Ellis, W. N., Fox, R., Groom, Q., Hennekens, S., Van Landuyt, W., Maes, D., Van de Meutter, F., Michez, D., Rasmont, P., Ode, B., Potts, S. G., Reemer, M., Roberts, S. P., Schaminée, J., WallisDeVries, M. F. and Biesmeijer, J. C. (2013) 'Species richness declines and biotic homogenisation have slowed down for NW-European pollinators and plants', *Ecology Letters,* 16(7), pp. 870-8.

Chen, J., Kitazumi, A., Alpuerto, J., Alyokhin, A. and de Los Reyes, B. (2016) 'Heat-induced mortality and expression of heat shock proteins in Colorado potato beetles treated with imidacloprid', *Insect Science,* 23(4), pp. 548-54.

Christen, V., Mittner, F. and Fent, K. (2016) 'Molecular Effects of Neonicotinoids in Honey Bees (*Apis mellifera*)', *Environmental Science & Technology,* 50(7), pp. 4071-81.

Claudianos, C., Ranson, H., Johnson, R. M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R. and Oakeshott, J. G. (2006) 'A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee', *Insect Molecular Biology,* 15(5), pp. 615-36.

Cleland, E. E., Chuine, I., Menzel, A., Mooney, H. A. and Schwartz, M. D. (2007) 'Shifting plant phenology in response to global change', *Trends in Ecology and Evolution,* 22(7), pp. 357-65.

Clements, J., Sanchez-Sedillo, B., Bradfield, C. A. and Groves, R. L. (2018) 'Transcriptomic analysis reveals similarities in genetic activation of detoxification mechanisms resulting from imidacloprid and chlorothalonil exposure', *PLoS ONE,* 13(10), pp. e0205881.

Clos, J., Westwood, J. T., Becker, P. B., Wilson, S., Lambert, K. and Wu, C. (1990) 'Molecular cloning and expression of a hexameric *Drosophila* heat shock factor subject to negative regulation', *Cell,* 63(5), pp. 1085-1097.

Coleman, P. C., Bale, J. S. and Hayward, S. A. (2015) 'Meat feeding restricts rapid cold hardening response and increases thermal activity thresholds of adult blow flies, *Calliphora vicina* (Diptera: Calliphoridae)', *PLoS ONE,* 10(7), pp. e0131301.

Colgan, T. J., Fletcher, I. K., Arce, A. N., Gill, R. J., Ramos Rodrigues, A., Stolle, E., Chittka, L. and Wurm, Y. (2019) 'Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees', *Molecular Ecology,* 28(8), pp. 1964-1974.

Colin, T., Meikle, W. G., Wu, X. and Barron, A. B. (2019) 'Traces of a neonicotinoid induce precocious foraging and reduce foraging performance in honey bees', *Environmental Science & Technology,* 53(14), pp. 8252-8261.

Colinet, H., Lee, S. F. and Hoffmann, A. (2010) 'Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*', *FEBS Journal,* 277(1), pp. 174-85.

Colinet, H., Sinclair, B. J., Vernon, P. and Renault, D. (2015) 'Insects in fluctuating thermal environments', *Annual Review of Entomology,* 60, pp. 123-40.

Conrad, T., Paxton, R. J., Barth, F. G., Francke, W. and Ayasse, M. (2010) 'Female choice in the red mason bee, *Osmia rufa* (L.) (Megachilidae)', *Journal of Experimental Biology,* 213(Pt 23), pp. 4065-73.

Cook, S. C. (2019) 'Compound and dose-dependent effects of two neonicotinoid pesticides on honey bee (*Apis mellifera*) metabolic physiology', *Insects,* 10(1), pp. 18.

Coudrain, V., Rittiner, S., Herzog, F., Tinner, W. and Entling, M. H. (2016) 'Landscape distribution of food and nesting sites affect larval diet and nest size, but not abundance of *Osmia bicornis*', *Insect Science,* 23(5), pp. 746-753.

Cresswell, J. E., Page, C. J., Uygun, M. B., Holmbergh, M., Li, Y., Wheeler, J. G., Laycock, I., Pook, C. J., de Ibarra, N. H., Smirnoff, N. and Tyler, C. R. (2012)

'Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid)', *Zoology (Jena),* 115(6), pp. 365-71.

Daborn, P., Boundy, S., Yen, J., Pittendrigh, B. and ffrench-Constant, R. (2001) 'DDT resistance in *Drosophila* correlates with Cyp6g1 over-expression and confers cross-resistance to the neonicotinoid imidacloprid', *Molecular Genetics and Genomics,* 266(4), pp. 556-63.

Daborn, P. J., Lumb, C., Boey, A., Wong, W., Ffrench-Constant, R. H. and Batterham, P. (2007) 'Evaluating the insecticide resistance potential of eight *Drosophila melanogaster* cytochrome P450 genes by transgenic over-expression', *Insect Biochemistry and Molecular Biology,* 37(5), pp. 512-9.

Dafni, A., Kevan, P., Gross, C. L. and Goka, K. (2010) '*Bombus terrestris*, pollinator, invasive and pest: An assessment of problems associated with its widespread introductions for commercial purposes', *Applied Entomology and Zoology,* 45(1), pp. 101-113.

David, A., Botías, C., Abdul-Sada, A., Nicholls, E., Rotheray, E. L., Hill, E. M. and Goulson, D. (2016) 'Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops', *Environmental International,* 88, pp. 169-178.

Davies, S. A., Cabrero, P., Overend, G., Aitchison, L., Sebastian, S., Terhzaz, S. and Dow, J. A. (2014) 'Cell signalling mechanisms for insect stress tolerance', *Journal of Experimental Biology,* 217(1), pp. 119-28.

De Marchi, U., Santo-Domingo, J., Castelbou, C., Sekler, I., Wiederkehr, A. and Demaurex, N. (2014) 'NCLX protein, but not LETM1, mediates mitochondrial Ca2+ extrusion, thereby limiting Ca2+-induced NAD(P)H production and modulating matrix redox state', *Journal of Biological Chemistry,* 289(29), pp. 20377-85.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M. and Pham-Delègue, M.-H. (2004) 'Imidacloprid impairs memory and brain

metabolism in the honeybee (*Apis mellifera* L.)', *Pesticide biochemistry and physiology,* 78(2), pp. 83-92.

Decourtye, A., Lacassie, E. and Pham-Delègue, M. H. (2003) 'Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season', *Pest Management Science,* 59(3), pp. 269-78.

DeGrandi-Hoffman, G., Hoopingarner, R. and Baker, K. (1984) 'Pollen transfer in apple orchards: tree-to-tree or bee-to-bee?', *Bee World,* 65(3), pp. 126-133.

Delaplane, K. S. and Mayer, D. F. (2000) *Crop pollination by bees.* Wallingford, England; New York, USA: CABI.

Denecke, S., Fusetto, R., Martelli, F., Giang, A., Battlay, P., Fournier-Level, A., O' Hair, R. A. and Batterham, P. (2017) 'Multiple P450s and variation in neuronal genes underpins the response to the insecticide imidacloprid in a population of *Drosophila melanogaster*', *Scientific Reports,* 7(1), pp. 11338.

Denlinger, D., Campbell, J. and Bradfield, J. (1980) 'Stimulatory effect of organic solvents on initiating development in diapausing pupae of the flesh fly, *Sarcophaga crassipalpis*, and the tobacco hornworm, *Manduca sexta*', *Physiological Entomology,* 5(1), pp. 7-15.

Denlinger, D. L. (1976) 'Preventing insect diapause with hormones and cholera toxin', *Life Sciences,* 19(10), pp. 1485-1489.

Denlinger, D. L. (1986) 'Dormancy in tropical insects', *Annual Review of Entomology,* 31, pp. 239-264.

Denlinger, D. L. (2002) 'Regulation of diapause', *Annual Review of Entomology,* 47, pp. 93-122.

Denlinger, D. L. (2008) 'Why study diapause?', *Entomological Research,* 38(1), pp. 1-9.

Denlinger, D. L. (2009) 'Chapter 72 – Diapause', in Resh, V.H. and Cardé, R.T. (eds.) *Encyclopedia of insects*. 2nd edn. Amsterdam, Netherlands: Elsevier, pp. 267-271.

Department for Environment, Food and Rural Affairs (2015) *Horticultural Statistics Dataset: Table 6 - Value of home production marketed for the calendar year in the UK*: National Statistics. Available at: https://www.gov.uk/government/statistics/horticulture-statistics-2015 (Accessed: 09 September 2018).

Department for Environment, Food and Rural Affairs (2018) *Farming statistics: livestock populations at 1 December 2017 – UK*: National Statistics. Available at: https://www.gov.uk/government/statistics/farming-statistics-livestock-populations-at-1-december-2017-uk (Accessed: 09 September 2018).

Des Marteaux, L. E., McKinnon, A. H., Udaka, H., Toxopeus, J. and Sinclair, B. J. (2017) 'Effects of cold-acclimation on gene expression in Fall field cricket (*Gryllus pennsylvanicus*) ionoregulatory tissues', *BMC Genomics,* 18(1), pp. 357.

Des Marteaux, L. E., Stinziano, J. R. and Sinclair, B. J. (2018b) 'Effects of cold acclimation on rectal macromorphology, ultrastructure, and cytoskeletal stability in *Gryllus pennsylvanicus* crickets', *Journal of Insect Physiology,* 104, pp. 15-24.

Des Marteaux, L. E., Štětina, T. and Koštál, V. (2018a) 'Insect fat body cell morphology and response to cold stress is modulated by acclimation', *Journal of Experimental Biology,* 221(21), pp. jeb189647.

Deshwal, S. and Mallon, E. B. (2014) 'Antimicrobial peptides play a functional role in bumblebee anti-trypanosome defense', *Developmental and Comparative Immunology,* 42(2), pp. 240-3.

Deutsch, C. A., Tewksbury, J. J., Huey, R. B., Sheldon, K. S., Ghalambor, C. K., Haak, D. C. and Martin, P. R. (2008) 'Impacts of climate warming on terrestrial ectotherms across latitude', *Proceedings of the National Academy of Sciences of the United States of America,* 105(18), pp. 6668-72.

DiBartolomeis, M., Kegley, S., Mineau, P., Radford, R. and Klein, K. (2019) 'An assessment of acute insecticide toxicity loading (AITL) of chemical pesticides used on agricultural land in the United States', *PLoS ONE,* 14(8), pp. e0220029.

Dicks, L. V., Viana, B., Bommarco, R., Brosi, B., Arizmendi, M. D., Cunningham, S. A., Galetto, L., Hill, R., Lopes, A. V., Pires, C., Taki, H. and Potts, S. G. (2016) 'Ten policies for pollinators', *Science,* 354(6315), pp. 975-976.

Dinesh, K., Sharan, S., Mishra, P., Ram, K. and Maheshwari, G. (2007) 'Effect of Fenoxycarb on termination of pupal diapause in *Antheraea mylitta* Drury (Lepidoptera: Saturniidae)', *Journal of Experimental Zoology, India,* 10(2), pp. 265-270.

Dmochowska, K., Giejdasz, K., Fliszkiewicz, M. and Zoltowska, K. (2012) 'Changes in the antioxidative system of the red mason bee (*Osmia rufa*)(Hymenoptera: Megachilidae) induced by artificially elongated diapause', *Journal of Apicultural Science,* 56(1), pp. 99.

Dmochowska, K., Giejdasz, K., Fliszkiewicz, M. and Zółtowska, K. (2013) 'Prolonged postdiapause: influence on some indicators of carbohydrate and lipid metabolism of the red mason bee, *Osmia rufa*', *Journal of Insect Science,* 13, pp. 77.

Dmochowska-Ślęzak, K., Fliszkiewicz, M., Giejdasz, K. and Żółtowska, K. (2015) 'The antioxidant system in diapausing and active red mason bee *Osmia bicornis*', *Physiological Entomology,* 40(1), pp. 82-89.

Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M. and Gingeras, T. R. (2013) 'STAR: ultrafast universal RNA-seq aligner', *Bioinformatics,* 29(1), pp. 15-21.

Dobson, H. E., Ayasse, M., O'Neal, K. A. and Jacka, J. A. (2012) 'Is flower selection influenced by chemical imprinting to larval food provisions in the generalist bee *Osmia bicornis* (Megachilidae)?', *Apidologie,* 43(6), pp. 698-714.

Dong, Y. C., Chen, Z. Z., Clarke, A. R. and Niu, C. Y. (2019) 'Changes in Energy Metabolism Trigger Pupal Diapause Transition of', *Frontiers in Physiology,* 10, pp. 1288.

Donovan, S. E., Hall, M. J., Turner, B. D. and Moncrieff, C. B. (2006) 'Larval growth rates of the blowfly, *Calliphora vicina*, over a range of temperatures', *Medical and Veterinary Entomology,* 20(1), pp. 106-14.

Dussaubat, C., Maisonnasse, A., Crauser, D., Tchamitchian, S., Bonnet, M., Cousin, M., Kretzschmar, A., Brunet, J.-L. and Le Conte, Y. (2016) 'Combined neonicotinoid pesticide and parasite stress alter honeybee queens' physiology and survival', *Scientific Reports,* 6(1), pp. 1-7.

Easton, A. H. and Goulson, D. (2013) 'The neonicotinoid insecticide imidacloprid repels pollinating flies and beetles at field-realistic concentrations', *PLoS ONE,* 8(1), pp. e54819.

Eckhardt, M., Haider, M., Dorn, S. and Müller, A. (2014) 'Pollen mixing in pollen generalist solitary bees: a possible strategy to complement or mitigate unfavourable pollen properties?', *Journal of Animal Ecology,* 83(3), pp. 588-597.

Ellis, C., Park, K. J., Whitehorn, P., David, A. and Goulson, D. (2017) 'The neonicotinoid insecticide thiacloprid impacts upon bumblebee colony development under field conditions', *Environmental Science & Technology,* 51(3), pp. 1727-1732.

European Union (2018) *L 132*. European Union: European Union. Available at: https://eur-lex.europa.eu/legalcontent/EN/TXT/PDF/?uri=OJ:L:2018:132:FULL&from=DA (Accessed: 04 February 2019).

Evenden, M., Armitage, G. and Lau, R. (2007) 'Effects of nutrition and methoprene treatment upon reproductive diapause in *Caloptilia fraxinella* (Lepidoptera: Gracillariidae)', *Physiological Entomology,* 32(3), pp. 275-282.

Everaars, J., Strohbach, M. W., Gruber, B. and Dormann, C. F. (2011) 'Microsite conditions dominate habitat selection of the red mason bee (*Osmia bicornis*,

Hymenoptera: Megachilidae) in an urban environment: A case study from Leipzig, Germany', *Landscape and Urban Planning,* 103(1), pp. 15-23.

Everatt, M. J., Bale, J. S., Convey, P., Worland, M. R. and Hayward, S. A. (2013) 'The effect of acclimation temperature on thermal activity thresholds in polar terrestrial invertebrates', *Journal of Insect Physiology,* 59(10), pp. 1057-1064.

Feltham, H., Park, K. and Goulson, D. (2014) 'Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency', *Ecotoxicology,* 23(3), pp. 317-23.

Felton, G. W. and Summers, C. B. (1995) 'Antioxidant systems in insects', *Archives of Insect Biochemistry and Physiology,* 29(2), pp. 187-97.

Feyereisen, R. (2018) 'Toxicology: Bee P450s take the sting out of cyanoamidine neonicotinoids', *Current Biology,* 28(9), pp. R560-R562.

Finta, K. (2004) *Insect Pollination of Apple Orchards.* PhD Thesis, University of West Hungary, Hungary.

Fleckenstein-Grün, G. (1996) 'Calcium antagonism in vascular smooth muscle cells', *Pflugers Archiv European Journal of Physiology,* 432(3), pp. R53-60.

Fliszkiewicz, M., Giejdasz, K., Wasielewski, O. and Krishnan, N. (2012) 'Influence of winter temperature and simulated climate change on body mass and fat body depletion during diapause in adults of the solitary bee, *Osmia rufa* (Hymenoptera: Megachilidae)', *Environmental Entomology,* 41(6), pp. 1621-30.

Fliszkiewicz, M., Giejdasz, K. and Wilkaniec, Z. a. (2011) 'The importance of male red mason bee (*Osmia rufa* L.) and male bufftailed bumblebee (*Bombus terrestris* L.) pollination in blackcurrant (*Ribes nigrum* L.)', *The Journal of Horticultural Science and Biotechnology,* 86(5), pp. 457-460.

Fliszkiewicz, M., KuśnierczaK, A. and Szymaś, B. (2015) 'Reproduction of the red mason solitary bee *Osmia rufa* (syn. *Osmia bicornis*)(Hymenoptera:

Megachilidae) in various habitats', *European Journal of Entomology,* 112(1), pp. 100-105.

Fliszkiewicz, M. and Wilkaniec, Z. (2009) 'Potential possibilities of insemination of mason bee (*Osmia rufa* L.) females by a single male in laboratory conditions', *Annals of Warsaw University of Life Sciences - SGGW, Animal Science,* 46, pp. 51-58.

Forrest, J. R. (2015) 'Plant–pollinator interactions and phenological change: what can we learn about climate impacts from experiments and observations?', *Oikos,* 124(1), pp. 4-13.

Forrest, J. R. (2016) 'Complex responses of insect phenology to climate change', *Current Opinion in Insect Science,* 17, pp. 49-54.

Forrest, J. R., Cross, R. and CaraDonna, P. J. (2019) 'Two-year bee, or not two-year bee? How voltinism is affected by temperature and season length in a high-elevation solitary bee', *The American Naturalist,* 193(4), pp. 560-574.

Forrest, J. R. and Thomson, J. D. (2011) 'An examination of synchrony between insect emergence and flowering in Rocky Mountain meadows', *Ecological Monographs,* 81(3), pp. 469-491.

Foskett, J. K., White, C., Cheung, K. H. and Mak, D. O. (2007) 'Inositol trisphosphate receptor Ca2+ release channels', *Physiological Reviews,* 87(2), pp. 593-658.

Fraga, H., Pinto, J. G. and Santos, J. A. (2019) 'Climate change projections for chilling and heat forcing conditions in European vineyards and olive orchards: a multi-model assessment', *Climatic Change,* 152(1), pp. 179-193.

Free, J. (1960) 'The behaviour of honeybees visiting flowers of fruit trees', *Journal of Animal Ecology*, 29(2), pp. 385-395.

Free, J. B. (1979) *Bumblebee economics.* Cambridge, Massachusetts, USA: Harvard University Press.

Fujiwara, Y. and Denlinger, D. L. (2007) 'High temperature and hexane break pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, by activating ERK/MAPK', *Journal of Insect Physiology,* 53(12), pp. 1276-82.

Funabara, D., Hamamoto, C., Yamamoto, K., Inoue, A., Ueda, M., Osawa, R., Kanoh, S., Hartshorne, D. J., Suzuki, S. and Watabe, S. (2007) 'Unphosphorylated twitchin forms a complex with actin and myosin that may contribute to tension maintenance in catch', *Journal of Experimental Biology,* 210(24), pp. 4399-410.

Gadenne, C., Varjas, L. and Mauchamp, B. (1990) 'Effects of the non-steroidal ecdysone mimic, RH-5849, on diapause and non-diapause larvae of the European corn borer, *Ostrinia nubilalis* Hbn', *Journal of Insect Physiology,* 36(8), pp. 555-559.

Gallai, N., Salles, J.-M., Settele, J. and Vaissière, B. E. (2009) 'Economic valuation of the vulnerability of world agriculture confronted with pollinator decline', *Ecological economics,* 68(3), pp. 810-821.

Gallinat, A. S., Primack, R. B. and Wagner, D. L. (2015) 'Autumn, the neglected season in climate change research', *Trends in Ecology and Evolution,* 30(3), pp. 169-76.

Gao, Y., Gillen, C. M. and Wheatly, M. G. (2006) 'Molecular characterization of the sarcoplasmic calcium-binding protein (SCP) from crayfish *Procambarus clarkii*', *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* 144(4), pp. 478-87.

Garibaldi, L. A., Aizen, M. A., Klein, A. M., Cunningham, S. A. and Harder, L. D. (2011) 'Global growth and stability of agricultural yield decrease with pollinator dependence', *Proceedings of the National Academy of Sciences of the United States of America,* 108(14), pp. 5909-5914.

Garibaldi, L. A., Carvalheiro, L. G., Leonhardt, S. D., Aizen, M. A., Blaauw, B. R., Isaacs, R., Kuhlmann, M., Kleijn, D., Klein, A. M. and Kremen, C. (2014) 'From

research to action: enhancing crop yield through wild pollinators', *Frontiers in Ecology and the Environment,* 12(8), pp. 439-447.

Garibaldi, L. A., Steffan-Dewenter, I., Winfree, R., Aizen, M. A., Bommarco, R., Cunningham, S. A., Kremen, C., Carvalheiro, L. G., Harder, L. D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N. P., Dudenhöffer, J. H., Freitas, B. M., Ghazoul, J., Greenleaf, S., Hipólito, J., Holzschuh, A., Howlett, B., Isaacs, R., Javorek, S. K., Kennedy, C. M., Krewenka, K. M., Krishnan, S., Mandelik, Y., Mayfield, M. M., Motzke, I., Munyuli, T., Nault, B. A., Otieno, M., Petersen, J., Pisanty, G., Potts, S. G., Rader, R., Ricketts, T. H., Rundlöf, M., Seymour, C. L., Schüepp, C., Szentgyörgyi, H., Taki, H., Tscharntke, T., Vergara, C. H., Viana, B. F., Wanger, T. C., Westphal, C., Williams, N. and Klein, A. M. (2013) 'Wild pollinators enhance fruit set of crops regardless of honey bee abundance', *Science,* 339(6127), pp. 1608-1611.

Garratt, M. P., Breeze, T. D., Jenner, N., Polce, C., Biesmeijer, J. C. and Potts, S. G. (2014a) 'Avoiding a bad apple: Insect pollination enhances fruit quality and economic value', *Agriculture, Ecosystems & Environment,* 184(100), pp. 34-40.

Garratt, M. P., Truslove, C., Coston, D., Evans, R., Moss, E., Dodson, C., Jenner, N., Biesmeijer, J. and Potts, S. (2014b) 'Pollination deficits in UK apple orchards', *Journal of Pollination Ecology,* 12(2), pp. 9-14.

Giejdasz, K. and Fliszkiewicz, M. (2016) 'Effect of Temperature Treatment during Development of *Osmia rufa* L., on Mortality, Emergence and Longevity of Adults', *Journal of Apicultural Science,* 60(2), pp. 221-232.

Giejdasz, K., Fliszkiewicz, M., Bednárová, A. and Krishnan, N. (2016) 'Reproductive potential and nesting effects of *Osmia rufa* (syn. *bicornis*) female (Hymenoptera: Megachilidae)', *Journal of Apicultural Science,* 60(1), pp. 75-85.

Giejdasz, K. and Wasielewski, O. (2017a) 'Effect of Artificial Prolonged Wintering on Emergence and Survival of *Osmia rufa* Adults', *Journal of Apicultural Science,* 61(1), pp. 127-137.

Giejdasz, K. and Wasielewski, O. (2017b) 'Methoprene and temperature stimulation of emergence and longevity in adults of *Osmia rufa* L.(Megachilidae; Apoidea) during wintering period', *Journal of Apicultural Science,* 61(1), pp. 139-147.

Giejdasz, K. and Wielkaniec, Z. (1998) 'Effect of the activation of the bee *Osmia rufa* L., Megachilidae on the emerging dynamics of imagines and their survival rate', *Pszczelnicze Zeszyty Naukowe,* 42(1), pp. 265-271.

Giejdasz, K. and Wilkaniec, Z. (2002) 'Individual development of the red mason bee (*Osmia rufa* L., Megachilidae) under natural and laboratory conditions.', *Journal of Apicultural Science,* 46(1), pp. 51-57.

Gierer, F., Vaughan, S., Slater, M., Thompson, H. M., Elmore, J. S. and Girling, R. D. (2019) 'A review of the factors that influence pesticide residues in pollen and nectar: Future research requirements for optimising the estimation of pollinator exposure', *Environmental Pollution,* 249, pp. 236-247.

Gill, H. K., Goyal, G. and Chahil, G. (2017) 'Insect diapause: a review', *Journal of Agricultural Science and Technology A,* 7, pp. 454-473.

Gill, R. J., Ramos-Rodriguez, O. and Raine, N. E. (2012) 'Combined pesticide exposure severely affects individual- and colony-level traits in bees', *Nature,* 491(7422), pp. 105-8.

Godfray, H. C., Blacquière, T., Field, L. M., Hails, R. S., Petrokofsky, G., Potts, S. G., Raine, N. E., Vanbergen, A. J. and McLean, A. R. (2014) 'A restatement of the natural science evidence base concerning neonicotinoid insecticides and insect pollinators', *Proceedings of the Royal Society B: Biological Sciences,* 281(1786), pp. 20140558.

Godfray, H. C., Blacquière, T., Field, L. M., Hails, R. S., Potts, S. G., Raine, N. E., Vanbergen, A. J. and McLean, A. R. (2015) 'A restatement of recent advances in the natural science evidence base concerning neonicotinoid insecticides and

insect pollinators', *Proceedings of the Royal Society B: Biological Sciences,* 282(1818), pp. 20151821.

Gomez-Pastor, R., Burchfiel, E. T. and Thiele, D. J. (2018) 'Regulation of heat shock transcription factors and their roles in physiology and disease', *Nature Reviews Molecular Cell Biology,* 19(1), pp. 4-19.

Goulson, D. (2010) *Bumblebees: behaviour, ecology, and conservation.* Oxford, UK: Oxford University Press.

Goulson, D. (2013) 'REVIEW: An overview of the environmental risks posed by neonicotinoid insecticides', *Journal of Applied Ecology,* 50(4), pp. 977-987.

Goulson, D. and Hughes, W. O. (2015) 'Mitigating the anthropogenic spread of bee parasites to protect wild pollinators', *Biological Conservation,* 191, pp. 10-19.

Goulson, D., Lye, G. C. and Darvill, B. (2008) 'Decline and conservation of bumble bees', *Annual Review of Entomology,* 53, pp. 191-208.

Goulson, D., Nicholls, E., Botías, C. and Rotheray, E. L. (2015) 'Bee declines driven by combined stress from parasites, pesticides, and lack of flowers', *Science,* 347(6229), pp. 1255957.

Goulson, D. and signatories (2018) 'Call to restrict neonicotinoids', *Science,* 360(6392), pp. 973.

Ontario Government (2017) *Neonicotinoid Regulations*: Ontario Government. Available at: https://www.ontario.ca/page/neonicotinoid-regulations (Accessed: 07 August 2019).

Graystock, P., Blane, E. J., McFrederick, Q. S., Goulson, D. and Hughes, W. O. H. (2016) 'Do managed bees drive parasite spread and emergence in wild bees?', *International Journal for Parasitology: Parasites and Wildlife,* 5(1), pp. 64-75.

Greening, K. and Hodgson, A. (2019) 'Atmospheric analysis of the cold late February and early March 2018 over the UK', *Weather,* 74(3), pp. 79-85.

Gross, C. and Mackay, D. (1998) 'Honeybees reduce fitness in the pioneer shrub *Melastoma affine* (Melastomataceae)', *Biological Conservation,* 86(2), pp. 169-178.

Gruber, B., Eckel, K., Everaars, J. and Dormann, C. F. (2011) 'On managing the red mason bee (*Osmia bicornis*) in apple orchards', *Apidologie,* 42(5), pp. 564-576.

Haenke, S., Scheid, B., Schaefer, M., Tscharntke, T. and Thies, C. (2009) 'Increasing syrphid fly diversity and density in sown flower strips within simple vs. complex landscapes', *Journal of Applied Ecology,* 46(5), pp. 1106-1114.

Hahn, D. A. and Denlinger, D. L. (2007) 'Meeting the energetic demands of insect diapause: nutrient storage and utilization', *Journal of Insect Physiology,* 53(8), pp. 760-73.

Hahn, D. A. and Denlinger, D. L. (2011) 'Energetics of insect diapause', *Annual Review of Entomology,* 56, pp. 103-21.

Haider, M., Dorn, S., Sedivy, C. and Müller, A. (2014) 'Phylogeny and floral hosts of a predominantly pollen generalist group of mason bees (Megachilidae: Osmiini)', *Biological Journal of the Linnean Society,* 111(1), pp. 78-91.

Hallmann, C. A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D. and de Kroon, H. (2017) 'More than 75 percent decline over 27 years in total flying insect biomass in protected areas', *PLoS ONE,* 12(10), pp. e0185809.

Hamel, M., Géri, C. and Auger-Rozenberg, M. A. (1998) 'The effects of 20-hydroxyecdysone on breaking diapause of *Diprion pini* L.(Hym., Diprionidae)', *Physiological Entomology,* 23(4), pp. 337-346.

Hanley, N., Breeze, T. D., Ellis, C. and Goulson, D. (2015) 'Measuring the economic value of pollination services: Principles, evidence and knowledge gaps', *Ecosystem Services,* 14, pp. 124-132.

Hayward, S. A. L. (2014) 'Application of functional 'Omics' in environmental stress physiology: insights, limitations, and future challenges', *Current Opinion in Insect Science,* 4, pp. 35-41.

Hayward, S. A. L., Manso, B. and Cossins, A. R. (2014) 'Molecular basis of chill resistance adaptations in poikilothermic animals', *Journal of Experimental Biology,* 217(1), pp. 6-15.

Hayward, S. A. L., Pavlides, S. C., Tammariello, S. P., Rinehart, J. P. and Denlinger, D. L. (2005) 'Temporal expression patterns of diapause-associated genes in flesh fly pupae from the onset of diapause through post-diapause quiescence', *Journal of Insect Physiology,* 51(6), pp. 631-40.

Hazell, S. P. and Bale, J. S. (2011) 'Low temperature thresholds: are chill coma and CTmin synonymous?', *Journal of Insect Physiology,* 57(8), pp. 1085-1089.

Hazell, S. P., Pedersen, B. P., Worland, M. R., Blackburn, T. M. and Bale, J. S. (2008) 'A method for the rapid measurement of thermal tolerance traits in studies of small insects', *Physiological Entomology,* 33(4), pp. 389-394.

Hegland, S. J., Nielsen, A., Lázaro, A., Bjerknes, A. L. and Totland, Ø. (2009) 'How does climate warming affect plant-pollinator interactions?', *Ecology Letters,* 12(2), pp. 184-195.

Heinrich, B. (1974a) 'Thermoregulation in bumblebees - I. Brood incubation by *Bombus vosnesenskii* queens', *Journal of Comparative Physiology,* 88(2), pp. 129–140.

Heinrich, B. (1974b) 'Thermoregulation in endothermic insects', *Science,* 185(4153), pp. 747-56.

Heinrich, B. (1975) 'Thermoregulation in bumblebees - II. Energetics of warm-up and free flight', *Journal of Comparative Physiology B*.

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J. F., Aupinel, P., Aptel, J., Tchamitchian, S. and Decourtye, A. (2012) 'A common pesticide decreases foraging success and survival in honey bees', *Science,* 336(6079), pp. 348-350.

Hermann, A. and Cox, J. A. (1995) 'Sarcoplasmic calcium-binding protein', *Comparative Biochemistry and Physiology -- Part B: Biochemistry and Molecular Biology,* 111(3), pp. 337-345.

Higes, M., Martín, R. and Meana, A. (2006) '*Nosema ceranae*, a new microsporidian parasite in honeybees in Europe', *Journal of Invertebrate Pathology,* 92(2), pp. 93-95.

Higes, M., Martín-Hernández, R., Botías, C., Bailón, E. G., González-Porto, A. V., Barrios, L., Del Nozal, M. J., Bernal, J. L., Jiménez, J. J., Palencia, P. G. and Meana, A. (2008) 'How natural infection by *Nosema ceranae* causes honeybee colony collapse', *Environmental Microbiology,* 10(10), pp. 2659-2669.

Hladik, M. L., Main, A. R. and Goulson, D. (2018) 'Environmental risks and challenges associated with neonicotinoid insecticides', *Environmental Science & Technology,* 52(6), pp. 3329-3335.

Hodgson, J. A., Thomas, C. D., Oliver, T. H., Anderson, B. J., Brereton, T. and Crone, E. (2011) 'Predicting insect phenology across space and time', *Global Change Biology,* 17(3), pp. 1289-1300.

Holm, S. N. (1974) *'Osmia rufa* L.(Hym. Megachilidae) as a Pollinator of Plants in Greenhouses', *Insect Systematics & Evolution,* 4(3), pp. 217-224.

Hooper, S. L., Hobbs, K. H. and Thuma, J. B. (2008) 'Invertebrate muscles: thin and thick filament structure; molecular basis of contraction and its regulation, catch and asynchronous muscle', *Progress in Neurobiology,* 86(2), pp. 72-127.

Horth, L. and Campbell, L. A. (2018) 'Supplementing small farms with native mason bees increases strawberry size and growth rate', *Journal of Applied Ecology,* 55(2), pp. 591-599.

Howlett, B. (2012) 'Hybrid carrot seed crop pollination by the fly *Calliphora vicina* (Diptera: Calliphoridae)', *Journal of Applied Entomology,* 136(6), pp. 421-430.

Hu, Z., Lee, K. S., Choo, Y. M., Yoon, H. J., Kim, I., Wei, Y. D., Gui, Z. Z., Zhang, G. Z., Sohn, H. D. and Jin, B. R. (2010) 'Molecular characterization of a phospholipid-hydroperoxide glutathione peroxidase from the bumblebee *Bombus ignitus*', *Comparative Biochemistry and Physiology - B Biochemistry and Molecular Biology,* 155(1), pp. 54-61.

Huber, F., Boire, A., López, M. P. and Koenderink, G. H. (2015) 'Cytoskeletal crosstalk: when three different personalities team up', *Current Opinion in Cell Biology,* 32, pp. 39-47.

Humann-Guilleminot, S., Binkowski, Ł. J., Jenni, L., Hilke, G., Glauser, G. and Helfenstein, F. (2019) 'A nation-wide survey of neonicotinoid insecticides in agricultural land with implications for agri-environment schemes', *Journal of Applied Ecology,* 56(7), pp. 1502-1514.

Ings, T. C., Schikora, J. and Chittka, L. (2005) 'Bumblebees, humble pollinators or assiduous invaders? A population comparison of foraging performance in *Bombus terrestris*', *Oecologia,* 144(3), pp. 508-516.

Iqbal, M. (1983) *An introduction to solar radiation.* Toronto, Canada; New York, USA: Academic Press.

Isaacs, R., Williams, N., Ellis, J., Pitts-Singer, T. L., Bommarco, R. and Vaughan, M. (2017) 'Integrated crop pollination: combining strategies to ensure stable and sustainable yields of pollination-dependent crops', *Basic and Applied Ecology,* 22, pp. 44-60.

Ivanov, S. (2006) 'The nesting of *Osmia rufa* (L.)(Hymenoptera, Megachilidae) in the Crimea: Structure and composition of nests', *Entomological Review,* 86(5), pp. 524-533.

Iwamoto, H. (2011) 'Structure, function and evolution of insect flight muscle', *Biophysics (Nagoya-shi),* 7, pp. 21-28.

Iwasa, T., Motoyama, N., Ambrose, J. T. and Roe, R. M. (2004) 'Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*', *Crop protection,* 23(5), pp. 371-378.

Jacobson, M. M., Tucker, E. M., Mathiasson, M. E. and Rehan, S. M. (2018) 'Decline of bumble bees in northeastern North America, with special focus on *Bombus terricola*', *Biological Conservation,* 217, pp. 437-445.

Jactel, H., Verheggen, F., Thiéry, D., Escobar-Gutiérrez, A. J., Gachet, E., Desneux, N. and Neonicotinoids Working Group (2019) 'Alternatives to neonicotinoids', *Environment International,* 129, pp. 423-429.

Jakob, U., Gaestel, M., Engel, K. and Buchner, J. (1993) 'Small heat shock proteins are molecular chaperones', *The Journal of Biological Chemistry,* 268(3), pp. 1517-20.

Jamieson, M. A., Carper, A. L., Wilson, C. J., Scott, V. L. and Gibbs, J. (2019) 'Geographic biases in bee research limits understanding of species distribution and response to anthropogenic disturbance', *Frontiers in Ecology and Evolution,* 7, pp. 194.

Jauker, F. and Wolters, V. (2008) 'Hover flies are efficient pollinators of oilseed rape', *Oecologia,* 156(4), pp. 819-823.

Jeschke, P., Nauen, R., Schindler, M. and Elbert, A. (2011) 'Overview of the status and global strategy for neonicotinoids', *Journal of Agricultural and Food Chemistry,* 59(7), pp. 2897-908.

Jiang, X., Wang, Z., He, Q., Liu, Q., Li, X., Yu, L. and Cao, H. (2018) 'The effect of neonicotinoid insecticide and fungicide on sugar responsiveness and orientation behavior of honey bee (*Apis mellifera*) in semi-field conditions', *Insects,* 9(4), pp. 130.

Jørgensen, L. H., Mosbech, M. B., Færgeman, N. J., Graakjaer, J., Jacobsen, S. V. and Schrøder, H. D. (2014) 'Duplication in the microtubule-actin cross-linking factor 1 gene causes a novel neuromuscular condition', *Scientific Reports,* 4, pp. 5180.

Kaiser-Bunbury, C. N., Muff, S., Memmott, J., Müller, C. B. and Caflisch, A. (2010) 'The robustness of pollination networks to the loss of species and interactions: a quantitative approach incorporating pollinator behaviour', *Ecology Letters,* 13(4), pp. 442-52.

Kalkuhl, M., von Braun, J. and Torero, M. (2016) *Food Price Volatility and Its Implications for Food Security and Policy* Cham. Heidelberg, Germany: Springer International Publishing.

Karahan, A., Çakmak, I., Hranitz, J. M., Karaca, I. and Wells, H. (2015) 'Sublethal imidacloprid effects on honey bee flower choices when foraging', *Ecotoxicology,* 24(9), pp. 2017-25.

Kaunisto, S., Ferguson, L. V. and Sinclair, B. J. (2016) 'Can we predict the effects of multiple stressors on insects in a changing climate?', *Current Opinion in Insect Science,* 17, pp. 55-61.

Kearns, C. A., Inouye, D. W. and Waser, N. M. (1998) 'Endangered mutualisms: the conservation of plant-pollinator interactions', *Annual Review of Ecology and Systematics,* 29(1), pp. 83-112.

Kefuss, J. and Nye, W. P. (1970) 'The influence of photoperiod on the flight activity of honeybees', *Journal of Apicultural Research,* 9(3), pp. 133-139.

Kehrberger, S. and Holzschuh, A. (2019) 'Warmer temperatures advance flowering in a spring plant more strongly than emergence of two solitary spring bee species', *PLoS ONE,* 14(6), pp. e0218824.

Keller, A., Grimmer, G. and Steffan-Dewenter, I. (2013) 'Diverse microbiota identified in whole intact nest chambers of the red mason bee *Osmia bicornis* (Linnaeus 1758)', *PLoS ONE,* 8(10), pp. e78296.

Kemp, W. and Bosch, J. (2005) 'Effect of temperature on *Osmia lignaria* (Hymenoptera: Megachilidae) prepupa–adult development, survival, and emergence', *Journal of Economic Entomology,* 98(6), pp. 1917-1923.

Keulemans, W., Bylemans, D. and de Coninck, B. (2019) *Farming Without Plant Protection Products: Can We Grow Without Using Herbicides, Fungicides and Insecticides?*: Brussels, Belgium: European Parliament, European Union.

Kidokoro, K., Iwata, K., Fujiwara, Y. and Takeda, M. (2006) 'Effects of juvenile hormone analogs and 20-hydroxyecdysone on diapause termination in eggs of *Locusta migratoria* and *Oxya yezoensis*', *Journal of Insect Physiology,* 52(5), pp. 473-479.

Kim, B. G., Shim, J. K., Kim, D. W., Kwon, Y. J. and Lee, K. Y. (2008) 'Tissue-specific variation of heat shock protein gene expression in relation to diapause in the bumblebee *Bombus terrestris*', *Entomological Research,* 38(1), pp. 10-16.

Kim, J. I., Kwon, M., Kim, G.-H., Kim, S. Y. and Lee, S. H. (2015) 'Two mutations in nAChR beta subunit is associated with imidacloprid resistance in the *Aphis gossypii*', *Journal of Asia-Pacific Entomology,* 18(2), pp. 291-296.

King, A. M. and MacRae, T. H. (2015) 'Insect heat shock proteins during stress and diapause', *Annual Review of Entomology,* 60, pp. 59-75.

Kleijn, D., Winfree, R., Bartomeus, I., Carvalheiro, L. G., Henry, M., Isaacs, R., Klein, A. M., Kremen, C., M'Gonigle, L. K., Rader, R., Ricketts, T. H., Williams, N. M., Lee Adamson, N., Ascher, J. S., Báldi, A., Batáry, P., Benjamin, F., Biesmeijer, J. C., Blitzer, E. J., Bommarco, R., Brand, M. R., Bretagnolle, V.,

Button, L., Cariveau, D. P., Chifflet, R., Colville, J. F., Danforth, B. N., Elle, E., Garratt, M. P. D., Herzog, F., Holzschuh, A., Howlett, B. G., Jauker, F., Jha, S., Knop, E., Krewenka, K. M., Le Féon, V., Mandelik, Y., May, E. A., Park, M. G., Pisanty, G., Reemer, M., Riedinger, V., Rollin, O., Rundlöf, M., Sardiñas, H. S., Scheper, J., Sciligo, A. R., Smith, H. G., Steffan-Dewenter, I., Thorp, R., Tscharntke, T., Verhulst, J., Viana, B. F., Vaissière, B. E., Veldtman, R., Ward, K. L., Westphal, C. and Potts, S. G. (2015) 'Delivery of crop pollination services is an insufficient argument for wild pollinator conservation', *Nature Communications,* 6, pp. 7414.

Klein, A. M., Hendrix, S., Clough, Y., Scofield, A. and Kremen, C. (2015) 'Interacting effects of pollination, water and nutrients on fruit tree performance', *Plant Biology,* 17(1), pp. 201-208.

Klein, A. M., Vaissière, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C. and Tscharntke, T. (2007) 'Importance of pollinators in changing landscapes for world crops', *Proceedings of the Royal Society of London B: Biological Sciences,* 274(1608), pp. 303-313.

Kodama, A., Karakesisoglou, I., Wong, E., Vaezi, A. and Fuchs, E. (2003) 'ACF7: an essential integrator of microtubule dynamics', *Cell,* 115(3), pp. 343-354.

Koh, I., Lonsdorf, E. V., Williams, N. M., Brittain, C., Isaacs, R., Gibbs, J. and Ricketts, T. H. (2016) 'Modeling the status, trends, and impacts of wild bee abundance in the United States', *Proceedings of the National Academy of Sciences of the United States of America,* 113(1), pp. 140-145.

Kolde, R. (2019) pheatmap: Pretty Heatmaps. R package version 1.0.12.

Koo, J., Son, T.-G., Kim, S.-Y. and Lee, K.-Y. (2015) 'Differential responses of *Apis mellifera* heat shock protein genes to heat shock, flower-thinning formulations, and imidacloprid', *Journal of Asia-Pacific Entomology,* 18(3), pp. 583-589.

Kostál, V. (2006) 'Eco-physiological phases of insect diapause', *Journal of Insect Physiology,* 52(2), pp. 113-27.

Kostál, V., Vambera, J. and Bastl, J. (2004) 'On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*', *Journal of Experimental Biology,* 207(9), pp. 1509-21.

Kraemer, M. E. and Favi, F. D. (2005) 'Flower phenology and pollen choice of *Osmia lignaria* (Hymenoptera: Megachilidae) in central Virginia', *Environmental Entomology,* 34(6), pp. 1593-1605.

Kraemer, M. E. and Favi, F. D. (2010) 'Emergence phenology of *Osmia lignaria* subsp. *lignaria* (Hymenoptera: Megachilidae), its parasitoid *Chrysura kyrae* (Hymenoptera: Chrysididae), and bloom of *Cercis canadensis*', *Environmental Entomology,* 39(2), pp. 351-358.

Kraemer, M. E., Favi, F. D. and Niedziela, C. E. (2014) 'Nesting and pollen preference of *Osmia lignaria lignaria* (Hymenoptera: Megachilidae) in Virginia and North Carolina orchards', *Environmental Entomology,* 43(4), pp. 932-941.

Krunić, M. D. and Stanisavljević, L. Z. (2006) 'Supercooling points and diapause termination in overwintering adults of orchard bees *Osmia cornuta* and *O. rufa* (Hymenoptera: Megachilidae)', *Bulletin of Entomological Research,* 96(3), pp. 323-326.

Kudo, G. and Cooper, E. J. (2019) 'When spring ephemerals fail to meet pollinators: mechanism of phenological mismatch and its impact on plant reproduction', *Proceedings of the Royal Society B: Biological Sciences,* 286(1904), pp. 20190573.

Kudo, G. and Ida, T. Y. (2013) 'Early onset of spring increases the phenological mismatch between plants and pollinators', *Ecology,* 94(10), pp. 2311-20.

Kuwano, E., Fujisawa, T., Suzuki, K. and Eto, M. (1991) 'Termination of Egg Diapause by Imidazoles in the Silkmoth, *Antheraea yamamain*', *Agricultural and Biological Chemistry,* 55(4), pp. 1185-1186.

Labeit, S., Kolmerer, B. and Linke, W. A. (1997) 'The giant protein titin. Emerging roles in physiology and pathophysiology', *Circulation Research,* 80(2), pp. 290-294.

Lambin, M., Armengaud, C., Raymond, S. and Gauthier, M. (2001) 'Imidacloprid-induced facilitation of the proboscis extension reflex habituation in the honeybee', *Archives of Insect Biochemistry and Physiology,* 48(3), pp. 129-134.

Lang, T. and McKee, M. (2018) 'Brexit poses serious threats to the availability and affordability of food in the United Kingdom', *Journal of Public Health,* 40(4), pp. e608-e610.

Lantz, V. A. and Miller, K. G. (1998) 'A class VI unconventional myosin is associated with a homologue of a microtubule-binding protein, cytoplasmic linker protein–170, in neurons and at the posterior pole of *Drosophila* embryos', *The Journal of Cell Biology,* 140(4), pp. 897-910.

Larrere, M., Lavenseau, L., Tasei, J. N. and Couillaud, F. (1993) 'Juvenile hormone biosynthesis and diapause termination in *Bombus terrestris*', *Invertebrate Reproduction & Development,* 23(1), pp. 7-14.

Larson, B., Kevan, P. and Inouye, D. W. (2001) 'Flies and flowers: taxonomic diversity of anthophiles and pollinators', *The Canadian Entomologist,* 133(4), pp. 439-465.

Lautenbach, S., Seppelt, R., Liebscher, J. and Dormann, C. F. (2012) 'Spatial and temporal trends of global pollination benefit', *PLoS ONE,* 7(4), pp. e35954.

Laycock, I. (2014) *Biological effects and effect mechanisms of neonicotinoid pesticides in the bumble bee Bombus terrestris.* PhD Thesis, University of Exeter, Exeter, UK.

Lee, E. J. and Herzog, W. (2008) 'Effect of temperature on residual force enhancement in single skeletal muscle fibers', *Journal of Biomechanics,* 41(12), pp. 2703-2707.

Lemmen, J. K. and Evenden, M. L. (2016) 'The roles of juvenile hormone and biogenic amines on pheromone response plasticity and diapause termination in male *Caloptilia fraxinella*', *Entomologia Experimentalis et Applicata,* 158(2), pp. 184-201.

Lerer, H., Bailey, W., Mills, P. and Pankiw, P. (1982) 'Pollination activity of *Megachile rotundata* (Hymenoptera: Apoidae)', *Environmental Entomology,* 11(5), pp. 997-1000.

Lever, J. J., van Nes, E. H., Scheffer, M. and Bascompte, J. (2014) 'The sudden collapse of pollinator communities', *Ecology Letters,* 17(3), pp. 350-359.

Li, Z., Yu, T., Chen, Y., Heerman, M., He, J., Huang, J., Nie, H. and Su, S. (2019) 'Brain transcriptome of honey bees (*Apis mellifera*) exhibiting impaired olfactory learning induced by a sublethal dose of imidacloprid', *Pesticide Biochemistry and Physiology,* 156, pp. 36-43.

Lindström, S. A., Herbertsson, L., Rundlöf, M., Smith, H. G. and Bommarco, R. (2016) 'Large-scale pollination experiment demonstrates the importance of insect pollination in winter oilseed rape', *Oecologia,* 180(3), pp. 759-69.

Lister, B. C. and Garcia, A. (2018) 'Climate-driven declines in arthropod abundance restructure a rainforest food web', *Proceedings of the National Academy of Sciences of the United States of America,* 115(44), pp. E10397-E10406.

Lämsä, J., Kuusela, E., Tuomi, J., Juntunen, S. and Watts, P. C. (2018) 'Low dose of neonicotinoid insecticide reduces foraging motivation of bumblebees', *Proceedings of the Royal Society B: Biological Sciences,* 285(1883).

Maccagnani, B., Ladurner, E., Santi, F. and Burgio, G. (2003) *'Osmia cornuta* (Hymenoptera, Megachilidae) as a pollinator of pear (*Pyrus communis*): fruit-and seed-set', *Apidologie,* 34(3), pp. 207-216.

MacMillan, H. A., Andersen, J. L., Loeschcke, V. and Overgaard, J. (2015) 'Sodium distribution predicts the chill tolerance of Drosophila melanogaster raised in different thermal conditions', *American Journal of Physiology - Regulatory Integrative and Comparative Physiology,* 308(10), pp. R823-R831.

MacMillan, H. A., Knee, J. M., Dennis, A. B., Udaka, H., Marshall, K. E., Merritt, T. J. and Sinclair, B. J. (2016) 'Cold acclimation wholly reorganizes the Drosophila melanogaster transcriptome and metabolome', *Scientific Reports,* 6, pp. 28999.

Macmillan, H. A. and Sinclair, B. J. (2011) 'Mechanisms underlying insect chill-coma', *Journal of Insect Physiology,* 57(1), pp. 12-20.

MacMillan, H. A., Williams, C. M., Staples, J. F. and Sinclair, B. J. (2012) 'Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*', *Proceedings of the National Academy of Sciences of the United States of America,* 109(50), pp. 20750-5.

Maes, D. and Van Dyck, H. (2001) 'Butterfly diversity loss in Flanders (north Belgium): Europe's worst case scenario?', *Biological conservation,* 99(3), pp. 263-276.

Maeta, Y. and Kurihara, M. (1971) 'Anatomical and histological studies on the oogenesis and oosorption of terminal oocytes within the genus Osmia', *The Entomological Society of Japan,* 39(2), pp. 138-158.

Maienfisch, P., Angst, M., Brandl, F., Fischer, W., Hofer, D., Kayser, H., Kobel, W., Rindlisbacher, A., Senn, R., Steinemann, A. and Widmer, H. (2001) 'Chemistry and biology of thiamethoxam: a second generation neonicotinoid', *Pesticide Management Science,* 57(10), pp. 906-13.

Manjon, C., Troczka, B. J., Zaworra, M., Beadle, K., Randall, E., Hertlein, G., Singh, K. S., Zimmer, C. T., Homem, R. A., Lueke, B., Reid, R., Kor, L., Kohler, M., Benting, J., Williamson, M. S., Davies, T. G. E., Field, L. M., Bass, C. and

Nauen, R. (2018) 'Unravelling the Molecular Determinants of Bee Sensitivity to Neonicotinoid Insecticides', *Current Biology,* 28(7), pp. 1137-1143.e5.

Marini, F. and Binder, H. (2019) 'pcaExplorer: an R/Bioconductor package for interacting with RNA-seq principal components', *BMC Bioinformatics,* 20(1), pp. 331.

Matsuda, K., Buckingham, S. D., Kleier, D., Rauh, J. J., Grauso, M. and Sattelle, D. B. (2001) 'Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors', *Trends in Pharmacological Science,* 22(11), pp. 573-80.

Matsumoto, S. (2014) 'Apple pollination biology for stable and novel fruit production: search system for apple cultivar combination showing incompatibility, semicompatibility, and full-compatibility based on the S-RNase allele database', *International Journal of Agronomy,* 2014, pp. 1-9.

Matsumura, C., Yokoyama, J. and Washitani, I. (2004) 'Invasion status and potential ecological impacts of an invasive alien bumblebee, *Bombus terrestris* L.(Hymenoptera: Apidae) naturalized in Southern Hokkaido, Japan', *Global Environmental Research English Edition,* 8(1), pp. 51-66.

Matsunaga, Y., Qadota, H., Furukawa, M., Choe, H. H. and Benian, G. M. (2015) 'Twitchin kinase interacts with MAPKAP kinase 2 in *Caenorhabditis elegans* striated muscle', *Molecular Biology of the Cell,* 26(11), pp. 2096-111.

Mayans, O., Benian, G. M., Simkovic, F. and Rigden, D. J. (2013) 'Mechanistic and functional diversity in the mechanosensory kinases of the titin-like family', *Biochemical Society Transactions,* 41(4), pp. 1066-71.

Medrzycki, P., Montanari, R., Bortolotti, L., Sabatini, A. G., Maini, S. and Porrini, C. (2003) 'Effects of imidacloprid administered in sub-lethal doses on honey bee behaviour. Laboratory tests', *Bulletin of Insectology,* 56, pp. 59-62.

Memmott, J., Craze, P. G., Waser, N. M. and Price, M. V. (2007) 'Global warming and the disruption of plant-pollinator interactions', *Ecology Letters,* 10(8), pp. 710-7.

Menzel, R., Steinmann, E., De Souza, J. and Backhaus, W. (1988) 'Spectral sensitivity of photoreceptors and colour vision in the solitary bee, *Osmia rufa*', *Journal of Experimental Biology,* 136(1), pp. 35-52.

Michaud, M. R. and Denlinger, D. 'Molecular modalities of insect cold survival: current understanding and future trends'. *International congress series*: Elsevier, 32-46.

Michener, C. D. (2007) *The bees of the world.* 2nd edn. Baltimore, Maryland, USA: Johns Hopkins University Press.

Milatović, D. (2014) 'Chapter 23 – Insecticides', in Gupta, R. (ed.) *Biomarkers in Toxicology*. 1st edn. Cambridge, Massachusetts, USA: Academic Press, pp. 389-407.

Millar, N. S. and Denholm, I. (2007) 'Nicotinic acetylcholine receptors: targets for commercially important insecticides', *Invertebrate Neuroscience,* 7(1), pp. 53-66.

Mishra, P., Sharan, S., Kumar, D., Singh, B., Subrahmanyam, B. and Suryanarayana, N. (2008) 'Effect of ecdysone on termination of pupal diapause and egg production in *Antheraea mylita* Drury', *Journal of Advanced Zoology,* 29, pp. 128.

Mobley, M. W. and Gegear, R. J. (2018) 'One size does not fit all: Caste and sex differences in the response of bumblebees (*Bombus impatiens*) to chronic oral neonicotinoid exposure', *PLoS ONE,* 13(10), pp. e0200041.

Moerman, D. G., Benian, G. M., Barstead, R. J., Schriefer, L. A. and Waterston, R. H. (1988) 'Identification and intracellular localization of the unc-22 gene product of *Caenorhabditis elegans*', *Genes and Development,* 2(1), pp. 93-105.

Moffat, C., Buckland, S. T., Samson, A. J., McArthur, R., Chamosa Pino, V., Bollan, K. A., Huang, J. T. and Connolly, C. N. (2016) 'Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees', *Scientific Reports,* 6, pp. 24764.

Moffat, C., Pacheco, J. G., Sharp, S., Samson, A. J., Bollan, K. A., Huang, J., Buckland, S. T. and Connolly, C. N. (2015) 'Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*)', *FASEB Journal,* 29(5), pp. 2112-9.

Monzón, V. H., Bosch, J. and Retana, J. (2004) 'Foraging behavior and pollinating effectiveness of *Osmia cornuta* (Hymenoptera: Megachilidae) and *Apis mellifera* (Hymenoptera: Apidae) on “Comice” pear', *Apidologie,* 35(6), pp. 575-585.

Morales, C. L., Sáez, A., Garibaldi, L. A. and Aizen, M. A. (2017) 'Disruption of pollination services by invasive pollinator species', in Vilà, M., Hulme, P. (eds.) *Impact of Biological Invasions on Ecosystem Services*. Heidelberg, Germany: Springer International Publishing, pp. 203-220.

Morandin, L., Laverty, T., Kevan, P., Khosla, S. and Shipp, L. (2001a) 'Bumble bee (Hymenoptera: Apidae) activity and loss in commercial tomato greenhouses', *The Canadian Entomologist,* 133(6), pp. 883-893.

Morandin, L. A., Laverty, T. M. and Kevan, P. G. (2001b) 'Bumble bee (Hymenoptera: Apidae) activity and pollination levels in commercial tomato greenhouses', *Journal of Economic Entomology,* 94(2), pp. 462-467.

Morgan, T., Whitehorn, P., Lye, G. C. and Vallejo-Marín, M. (2016) 'Floral sonication is an innate behaviour in bumblebees that can be fine-tuned with experience in manipulating flowers', *Journal of Insect Behaviour,* 29, pp. 233-241.

Morton, E. M. and Rafferty, N. E. (2017) 'Plant–pollinator interactions under climate change: The use of spatial and temporal transplants', *Applications in Plant Sciences,* 5(6), pp. 1600133.

Moskalev, A., Zhikrivetskaya, S., Krasnov, G., Shaposhnikov, M., Proshkina, E., Borisoglebsky, D., Danilov, A., Peregudova, D., Sharapova, I., Dobrovolskaya, E., Solovev, I., Zemskaya, N., Shilova, L., Snezhkina, A. and Kudryavtseva, A. (2015) 'A comparison of the transcriptome of *Drosophila melanogaster* in

response to entomopathogenic fungus, ionizing radiation, starvation and cold shock', *BMC Genomics,* 16(13), pp. 1-18.

Murray, T. E., Coffey, M. F., Kehoe, E. and Horgan, F. G. (2013) 'Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations', *Biological Conservation,* 159, pp. 269-276.

Muth, F. and Leonard, A. (2019) 'A neonicotinoid pesticide impairs foraging, but not learning, in free-flying bumblebees', *Scientific reports,* 9(1), pp. 1-13.

National Research Council of the National Academies (2007) *Status of pollinators in North America.* Washington, D.C., USA: National Academies Press.

Neumann, P. and Carreck, N. L. (2010) 'Honey bee colony losses', *Journal of Apicultural Research,* 49(1), pp. 1-6.

Nicholls, E., Fowler, R., Niven, J. E., Gilbert, J. D. and Goulson, D. (2017) 'Larval exposure to field-realistic concentrations of clothianidin has no effect on development rate, over-winter survival or adult metabolic rate in a solitary bee, *Osmia bicornis*', *PeerJ,* 5, pp. e3417.

Nielsen, M. M., Overgaard, J., Sørensen, J. G., Holmstrup, M., Justesen, J. and Loeschcke, V. (2005) 'Role of HSF activation for resistance to heat, cold and high-temperature knock-down', *Journal of Insect Physiology,* 51(12), pp. 1320-1329.

Nieto, A., Roberts, S. P. M., Kemp, J., Rasmont, P., Kuhlmann, M., García Criado, M., Biesmeijer, J. C., Bogusch, P., Dathe, H. H., De la Rúa, P., De Meulemeester, T., Dehon, M., Dewulf, A., Ortiz-Sánchez, F. J., Lhomme, P., Pauly, A., Potts, S. G., Praz, C., Quaranta, M., Radchenko, V. G., Scheuchl, E., Smit, J., Straka, J., Terzo, M., Tomozii, B., Window, J. and and Michez, (2014) *European Red List of bees*. Luxembourg: European Union. Available at: https://ec.europa.eu/environment/nature/conservation/species/redlist/downloads/European_bees.pdf (Accessed: 23 March 2016).

Nishitsutsuji-Uwo, J. and Nishimura, M. S. (1975) 'Further studies on non-hormonal agents which mimic insect brain hormone', *Applied Entomology and Zoology,* 10(1), pp. 52-57.

Numata, H. and Hidaka, T. (1984) 'Termination of Adult Diapause by a Juvenile Hormone Analogue in the Bean Bug, *Riptortus clavatus*', *Zoological Science,* 1(5), pp. 751-754.

O'Toole, C. (2000) *The red mason bee: taking the sting out of bee-keeping: a practical guide to managing Osmia rufa as a pollinator in gardens, allotments, and orchards.* London, UK: Osmia Publications, Natural History Book Service.

Ollerton, J., Winfree, R. and Tarrant, S. (2011) 'How many flowering plants are pollinated by animals?', *Oikos,* 120(3), pp. 321-326.

Orford, K. A., Vaughan, I. P. and Memmott, J. (2015) 'The forgotten flies: the importance of non-syrphid Diptera as pollinators', *Proceedings of the Royal Society B: Biological Sciences,* 282(1805), pp. 20142934.

Otterstatter, M. C. and Thomson, J. D. (2008) 'Does pathogen spillover from commercially reared bumble bees threaten wild pollinators?', *PLoS ONE,* 3(7), pp. e2771.

Otti, O. and Schmid-Hempel, P. (2007) '*Nosema bombi*: A pollinator parasite with detrimental fitness effects', *Journal of Invertebrate Pathology,* 96(2), pp. 118-24.

Otti, O. and Schmid-Hempel, P. (2008) 'A field experiment on the effect of *Nosema bombi* in colonies of the bumblebee *Bombus terrestris*', *Ecological Entomology,* 33(5), pp. 577-582.

Overgaard, J. and MacMillan, H. A. (2017) 'The integrative physiology of insect chill tolerance', *Annual Review of Physiology,* 79, pp. 187-208.

Owen, E. L. (2015) *Bumblebee ecophysiology: assessing the impacts of climate change and pesticide use on Bombus terrestrial audax and B. t. dalmatinus.* PhD Thesis, University of Birmingham, Birmingham, UK.

Owen, E. L., Bale, J. S. and Hayward, S. A. (2013) 'Can winter-active bumblebees survive the cold? Assessing the cold tolerance of *Bombus terrestris audax* and the effects of pollen feeding', *PLoS ONE,* 8(11), pp. e80061.

Owen, E. L., Bale, J. S. and Hayward, S. A. (2016) 'Establishment risk of the commercially imported bumblebee *Bombus terrestris dalmatinus*—can they survive UK winters?', *Apidologie,* 47(1), pp. 66-75.

Owen, R. E. (2016) 'Chapter 9 - Rearing Bumble Bees for Research and Profit: Practical and Ethical Considerations', in Chambó E. D. (ed.) *Beekeeping and Bee Conservation-Advances in Research*. Online: IntechOpen, pp. 225-241. Available at: https://www.intechopen.com/books/beekeeping-and-bee-conservation-advances-in-research/rearing-bumble-bees-for-research-and-profit-practical-and-ethical-considerations (Accessed 19 April 2018).

Oyen, K. J. and Dillon, M. E. (2018) 'Critical thermal limits of bumblebees (*Bombus impatiens*) are marked by stereotypical behaviors and are unchanged by acclimation, age or feeding status', *Journal of Experimental Biology,* 221(8), pp. jeb165589.

Paini, D. (2004) 'Impact of the introduced honey bee (*Apis mellifera*)(Hymenoptera: Apidae) on native bees: a review', *Austral Ecology,* 29(4), pp. 399-407.

Pan, Y., Yang, C., Gao, X., Peng, T., Bi, R., Xi, J., Xin, X., Zhu, E., Wu, Y. and Shang, Q. (2015) 'Spirotetramat resistance adaption analysis of *Aphis gossypii* Glover by transcriptomic survey', *Pesticide Biochemistry and Physiology,* 124, pp. 73-80.

Pang, R., Chen, M., Liang, Z., Yue, X., Ge, H. and Zhang, W. (2016) 'Functional analysis of CYP6ER1, a P450 gene associated with imidacloprid resistance in *Nilaparvata lugens*', *Sci Rep,* 6, pp. 34992.

Park, K.D. and Kim, H.-R. (1989) 'Hormonal Control of Induction and Termination of Diapause in *Pieris rapae* L', *Korean Journal of Zoology,* 32(1), pp. 1-8.

Partap, U. and Ya, T. (2012) 'The human pollinators of fruit crops in Maoxian County, Sichuan, China', *Mountain Research and Development,* 32(2), pp. 176-186.

Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. and Kingsford, C. (2017) 'Salmon provides fast and bias-aware quantification of transcript expression', *Nature Methods,* 14(4), pp. 417-419.

Paul, A., Harrington, L. C. and Scott, J. G. (2006) 'Evaluation of novel insecticides for control of dengue vector *Aedes aegypti* (Diptera: Culicidae)', *Journal of Medical Entomology,* 43(1), pp. 55-60.

Petanidou, T., Kallimanis, A. S., Sgardelis, S. P., Mazaris, A. D., Pantis, J. D. and Waser, N. M. (2014) 'Variable flowering phenology and pollinator use in a community suggest future phenological mismatch', *Acta Oecologica,* 59, pp. 104-111.

Peters, B., Gao, Z. and Zumkier, U. (2016) 'Large-scale monitoring of effects of clothianidin-dressed oilseed rape seeds on pollinating insects in Northern Germany: effects on red mason bees (*Osmia bicornis*)', *Ecotoxicology,* 25(9), pp. 1679-1690.

Peters, D. S. (1978) 'Systematik und Zoogeographie der west-paläarktischen Arten von *Osmia* sstr, *Monosmia* und *Orientosmia*', *Senckenbergiana Biologica,* 58, pp. 287-346.

Petetin, L. (2020) 'The COVID-19 crisis: an opportunity to integrate food democracy into post-pandemic food systems', *European Journal of Risk Regulation,* 11(2), pp. 326-336.

Pimentel, D., Wilson, C., McCullum, C., Huang, R., Dwen, P., Flack, J., Tran, Q., Saltman, T. and Cliff, B. (1997) 'Economic and environmental benefits of biodiversity', *BioScience,* 47(11), pp. 747-757.

Pisa, L. W., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Downs, C. A., Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M., McField, M., Morrissey,

C. A., Noome, D. A., Settele, J., Simon-Delso, N., Stark, J. D., Van der Sluijs, J. P., Van Dyck, H. and Wiemers, M. (2015) 'Effects of neonicotinoids and fipronil on non-target invertebrates', *Environmental Science and Pollution Research,* 22(1), pp. 68-102.

Pitts-Singer, T. L., Bosch, J., Kemp, W. P. and Trostle, G. E. (2008) 'Field use of an incubation box for improved emergence timing of *Osmia lignaria* populations used for orchard pollination', *Apidologie,* 39(2), pp. 235-246.

Pitts-Singer, T. L. and Cane, J. H. (2011) 'The alfalfa leafcutting bee, *Megachile rotundata*: the world's most intensively managed solitary bee', *Annual Review of Entomology,* 56, pp. 221-237.

Pitts-Singer, T. L., Cane, J. H. and Trostle, G. (2014) 'Progeny of *Osmia lignaria* from distinct regions differ in developmental phenology and survival under a common thermal regime', *Journal of Insect Physiology,* 67, pp. 9-19.

Polce, C., Garratt, M. P., Termansen, M., Ramirez-Villegas, J., Challinor, A. J., Lappage, M. G., Boatman, N. D., Crowe, A., Endalew, A. M., Potts, S. G., Somerwill, K. E. and Biesmeijer, J. C. (2014) 'Climate-driven spatial mismatches between British orchards and their pollinators: increased risks of pollination deficits', *Global Change Biology,* 20(9), pp. 2815-28.

Popp, J., Pető, K. and Nagy, J. (2013) 'Pesticide productivity and food security. A review', *Agronomy for sustainable development,* 33(1), pp. 243-255.

Potts, R., Clarke, R. M., Oldfield, S. E., Wood, L. K., Hempel de Ibarra, N. and Cresswell, J. E. (2018) 'The effect of dietary neonicotinoid pesticides on non-flight thermogenesis in worker bumble bees (*Bombus terrestris*)', *Journal of Insect Physiology,* 104, pp. 33-39.

Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O. and Kunin, W. E. (2010) 'Global pollinator declines: trends, impacts and drivers', *Trends in Ecology and Evolution,* 25(6), pp. 345-53.

Potts, S. G., Imperatriz-Fonseca, V., Ngo, H. T., Aizen, M. A., Biesmeijer, J. C., Breeze, T. D., Dicks, L. V., Garibaldi, L. A., Hill, R., Settele, J. and Vanbergen, A. J. (2016) 'Safeguarding pollinators and their values to human well-being', *Nature,* 540(7632), pp. 220-229.

Powner, M. B., Salt, T. E., Hogg, C. and Jeffery, G. (2016) 'Improving mitochondrial function protects bumblebees from neonicotinoid pesticides', *PLoS ONE,* 11(11), pp. e0166531.

Powney, G. D., Carvell, C., Edwards, M., Morris, R. K. A., Roy, H. E., Woodcock, B. A. and Isaac, N. J. B. (2019) 'Widespread losses of pollinating insects in Britain', *Nature Communications,* 10(1), pp. 1018.

United Nations Environment Programme (2019) *Emissions Gap Report 2019*. Nairobi, Kenya: United Nations. Available at: https://www.unep.org/resources/emissions-gap-report-2019 (Accessed: 15 March 2020).

Pullin, A. and Bale, J. (1989) 'Effects of ecdysone, juvenile hormone and haemolymph transfer on cryoprotectant metabolism in diapausing and non-diapausing pupae of *Pieris brassicae*', *Journal of Insect Physiology,* 35(12), pp. 911-918.

Quiroga, L. B., Sanabria, E. A., Fornés, M. W., Bustos, D. A. and Tejedo, M. (2019) 'Sublethal concentrations of chlorpyrifos induce changes in the thermal sensitivity and tolerance of anuran tadpoles in the toad *Rhinella arenarum*?', *Chemosphere,* 219, pp. 671-677.

Rader, R., Bartomeus, I., Garibaldi, L. A., Garratt, M. P., Howlett, B. G., Winfree, R., Cunningham, S. A., Mayfield, M. M., Arthur, A. D., Andersson, G. K., Bommarco, R., Brittain, C., Carvalheiro, L. G., Chacoff, N. P., Entling, M. H., Foully, B., Freitas, B. M., Gemmill-Herren, B., Ghazoul, J., Griffin, S. R., Gross, C. L., Herbertsson, L., Herzog, F., Hipólito, J., Jaggar, S., Jauker, F., Klein, A. M., Kleijn, D., Krishnan, S., Lemos, C. Q., Lindström, S. A., Mandelik, Y., Monteiro, V. M., Nelson, W., Nilsson, L., Pattemore, D. E., Pereira, N. e. O., Pisanty, G., Potts, S. G., Reemer, M., Rundlöf, M., Sheffield, C. S., Scheper, J.,

Schüepp, C., Smith, H. G., Stanley, D. A., Stout, J. C., Szentgyörgyi, H., Taki, H., Vergara, C. H., Viana, B. F. and Woyciechowski, M. (2016) 'Non-bee insects are important contributors to global crop pollination', *Proceedings of the National Academy of Sciences of the United States of America,* 113(1), pp. 146-151.

Radmacher, S. (2012) *Temperature effects on the development in the solitary bee Osmia bicornis (Hymenoptera, Megachilidae)-Development time, brain microstructure, and cognitive abilities.* PhD Thesis, Universität Regensburg, Regensburg, Germany.

Radmacher, S. and Strohm, E. (2010) 'Factors affecting offspring body size in the solitary bee *Osmia bicornis* (Hymenoptera, Megachilidae)', *Apidologie,* 41(2), pp. 169-177.

Radmacher, S. and Strohm, E. (2011) 'Effects of constant and fluctuating temperatures on the development of the solitary bee *Osmia bicornis* (Hymenoptera: Megachilidae)', *Apidologie,* 42(6), pp. 711-720.

Raguso, R. A. (2004) 'Flowers as sensory billboards: progress towards an integrated understanding of floral advertisement', *Current Opinion in Plant Biology,* 7(4), pp. 434-40.

Ranatunga, K. W. (2018) 'Temperature Effects on Force and Actin-Myosin Interaction in Muscle: A Look Back on Some Experimental Findings', *International Journal of Molecular Sciences,* 19(5), pp. 1538.

Raw, A. (1972) 'The biology of the solitary bee *Osmia rufa* (L.)(Megachilidae)', *Transactions of the Royal Entomological Society of London,* 124(3), pp. 213-229.

Renault, D., Salin, C., Vannier, G. and Vernon, P. (2002) 'Survival at low temperatures in insects: what is the ecological significance of the supercooling point?', *Cryo-Letters,* 23(4), pp. 217-28.

Renner, S. S. and Zohner, C. M. (2018) 'Climate change and phenological mismatch in trophic interactions among plants, insects, and vertebrates', *Annual Review of Ecology, Evolution, and Systematics,* 49, pp. 165-182.

Reynolds, J. A., Nachman, R. J. and Denlinger, D. L. (2019) 'Distinct microRNA and mRNA responses elicited by ecdysone, diapause hormone and a diapause hormone analog at diapause termination in pupae of the corn earworm, *Helicoverpa zea*', *General and Comparative Endocrinology,* 278, pp. 68-78.

Rhodes, C. J. (2018) 'Pollinator decline–an ecological calamity in the making?', *Science Progress,* 101(2), pp. 121-160.

Rinehart, J. P., Li, A., Yocum, G. D., Robich, R. M., Hayward, S. A. and Denlinger, D. L. (2007) 'Up-regulation of heat shock proteins is essential for cold survival during insect diapause', *Proceedings of the National Academy of Sciences of the United States of America,* 104(27), pp. 11130-11137.

Rinehart, J. P., Yocum, G. D., Kemp, W. P. and Greenlee, K. J. (2013) 'A fluctuating thermal regime improves long-term survival of quiescent prepupal *Megachile rotundata* (Hymenoptera: Megachilidae)', *Journal of Economic Entomology,* 106(3), pp. 1081-1088.

Robertson, R. M., Spong, K. E. and Srithiphaphirom, P. (2017) 'Chill coma in the locust, *Locusta migratoria*, is initiated by spreading depolarization in the central nervous system', *Scientific Reports,* 7(1), pp. 10297.

Roffet-Salque, M., Regert, M., Evershed, R. P., Outram, A. K., Cramp, L. J., Decavallas, O., Dunne, J., Gerbault, P., Mileto, S., Mirabaud, S., Pääkkönen, M., Smyth, J., Šoberl, L., Whelton, H. L., Alday-Ruiz, A., Asplund, H., Bartkowiak, M., Bayer-Niemeier, E., Belhouchet, L., Bernardini, F., Budja, M., Cooney, G., Cubas, M., Danaher, E. M., Diniz, M., Domboróczki, L., Fabbri, C., González-Urquijo, J. E., Guilaine, J., Hachi, S., Hartwell, B. N., Hofmann, D., Hohle, I., Ibáñez, J. J., Karul, N., Kherbouche, F., Kiely, J., Kotsakis, K., Lueth, F., Mallory, J. P., Manen, C., Marciniak, A., Maurice-Chabard, B., Mc Gonigle, M. A., Mulazzani, S., Özdoğan, M., Perić, O. S., Perić, S. R., Petrasch, J., Pétrequin, A. M., Pétrequin,

P., Poensgen, U., Pollard, C. J., Poplin, F., Radi, G., Stadler, P., Stäuble, H., Tasić, N., Urem-Kotsou, D., Vuković, J. B., Walsh, F., Whittle, A., Wolfram, S., Zapata-Peña, L. and Zoughlami, J. (2015) 'Widespread exploitation of the honeybee by early Neolithic farmers', *Nature,* 527(7577), pp. 226-230.

Rogelj, J., Den Elzen, M., Höhne, N., Fransen, T., Fekete, H., Winkler, H., Schaeffer, R., Sha, F., Riahi, K. and Meinshausen, M. (2016) 'Paris Agreement climate proposals need a boost to keep warming well below 2˚C', *Nature,* 534(7609), pp. 631-639.

Rohrback, S. E., Wheatly, M. G. and Gillen, C. M. (2015) 'Calcium binding to *Procambarus clarkii* sarcoplasmic calcium binding protein splice variants', *Comparative Biochemistry and Physiology Part - B: Biochemistry and Molecular Biology,* 179, pp. 57-63.

Rosenkranz, P., Aumeier, P. and Ziegelmann, B. (2010) 'Biology and control of *Varroa destructor*', *Journal of Invertebrate Pathology,* 103(1), pp. S96-119.

Rotheray, G. E. and Gilbert, F. (2011) *The natural history of hoverflies.* London, UK: Forrest text, Natural History Book Service.

Roy, D. and Sparks, T. (2000) 'Phenology of British butterflies and climate change', *Global Change Biology,* 6(4), pp. 407-416.

Rucker, R. R., Thurman, W. N. and Burgett, M. (2012) 'Honey bee pollination markets and the internalization of reciprocal benefits', *American Journal of Agricultural Economics,* 94(4), pp. 956-977.

Rundlöf, M., Andersson, G. K., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., Jonsson, O., Klatt, B. K., Pedersen, T. R., Yourstone, J. and Smith, H. G. (2015) 'Seed coating with a neonicotinoid insecticide negatively affects wild bees', *Nature,* 521(7550), pp. 77-80.

Rust, R., Torchio, P. and Trostle, G. (1989) 'Late embryogenesis and immature development of *Osmia rufa cornigera* (Rossi) (Hymenoptera: Megachilidae)', *Apidologie,* 20(4), pp. 359-367.

Ruz, L. (2002) 'Bee pollinators introduced to Chile: a review', in Kevan, P. G. and Imperatriz-Fonseca, V. L. (eds.) *Pollinating bees*, *The conservation link between Agriculture and Nature*. Brasilia, Brazil: Ministério do Meio Ambiente, pp. 155-167.

Ryder, J. T., Cherrill, A., Prew, R., Shaw, J., Thorbek, P. and Walters, K. F. (2020) 'Impact of enhanced *Osmia bicornis* (Hymenoptera: Megachilidae) populations on pollination and fruit quality in commercial sweet cherry (*Prunus avium* L.) orchards', *Journal of Apicultural Research,* 59(1), pp. 77-87.

Röper, K., Mao, Y. and Brown, N. H. (2005) 'Contribution of sequence variation in *Drosophila* actins to their incorporation into actin-based structures in vivo', *Journal of Cell Science,* 118(17), pp. 3937-3948.

Sandrock, C., Tanadini, L. G., Pettis, J. S., Biesmeijer, J. C., Potts, S. G. and Neumann, P. (2014) 'Sublethal neonicotinoid insecticide exposure reduces solitary bee reproductive success', *Agricultural and Forest Entomology,* 16(2), pp. 119-128.

Schenk, M., Krauss, J. and Holzschuh, A. (2018a) 'Desynchronizations in bee–plant interactions cause severe fitness losses in solitary bees', *Journal of Animal Ecology,* 87(1), pp. 139-149.

Schenk, M., Mitesser, O., Hovestadt, T. and Holzschuh, A. (2018b) 'Overwintering temperature and body condition shift emergence dates of spring-emerging solitary bees', *PeerJ,* 6, pp. e4721.

Scheper, J., Reemer, M., van Kats, R., Ozinga, W. A., van der Linden, G. T., Schaminée, J. H., Siepel, H. and Kleijn, D. (2014) 'Museum specimens reveal loss of pollen host plants as key factor driving wild bee decline in The Netherlands', *Proceedings of the National Academy of Sciences of the United States of America,* 111(49), pp. 17552-7.

Schindler, M. and Peters, B. (2011) 'Mason bees *Osmia bicornis* and *Osmia cornuta* as suitable orchard pollinators?', *Erwerbs-Obstbau,* 52(3/4), pp. 111-116.

Schittenhelm, S., Giadis, T. and Rao, V. (1997) 'Efficiency of various insects in germplasm regeneration of carrot, onion and turnip rape accessions', *Plant Breeding,* 116(4), pp. 369-375.

Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., Escudero, L. R., Salathé, R., Scriven, J. J. and Schmid-Hempel, P. (2014) 'The invasion of southern South America by imported bumblebees and associated parasites', *Journal of Animal Ecology,* 83(4), pp. 823-37.

Schwanhäusser, B., Busse, D., Li, N., Dittmar, G., Schuchhardt, J., Wolf, J., Chen, W. and Selbach, M. (2011) 'Global quantification of mammalian gene expression control', *Nature,* 473(7347), pp. 337-42.

Scott, J. G., Michel, K., Bartholomay, L. C., Siegfried, B. D., Hunter, W. B., Smagghe, G., Zhu, K. Y. and Douglas, A. E. (2013) 'Towards the elements of successful insect RNAi', *Journal of Insect Physiology,* 59(12), pp. 1212-21.

Sedivy, C. and Dorn, S. (2014) 'Towards a sustainable management of bees of the subgenus *Osmia* (Megachilidae; Osmia) as fruit tree pollinators', *Apidologie,* 45(1), pp. 88-105.

Seidelmann, K. (1999) 'The race for females: the mating system of the red mason bee, *Osmia rufa* (L.)(Hymenoptera: Megachilidae)', *Journal of Insect Behavior,* 12(1), pp. 13-25.

Seidelmann, K. (2015) 'Double insurance of paternity by a novel type of mating plug in a monandrous solitary mason bee *Osmia bicornis* (Hymenoptera: Megachilidae)', *Biological Journal of the Linnean Society,* 115(1), pp. 28-37.

Seidelmann, K., Ulbrich, K. and Mielenz, N. (2010) 'Conditional sex allocation in the Red Mason bee, *Osmia rufa*', *Behavioral Ecology and Sociobiology,* 64(3), pp. 337-347.

Settele, J., Bishop, J. and Potts, S. G. (2016) 'Climate change impacts on pollination', *Nature Plants,* 2(7), pp. 16092.

Sgolastra, F., Arnan, X., Cabbri, R., Isani, G., Medrzycki, P., Teper, D. and Bosch, J. (2018) 'Combined exposure to sublethal concentrations of an insecticide and a fungicide affect feeding, ovary development and longevity in a solitary bee', *Proceedings of the Royal Society B: Biological Sciences,* 285(1885), pp. 20180887.

Sgolastra, F., Arnan, X., Pitts-Singer, T. L., Maini, S., Kemp, W. P. and Bosch, J. (2016) 'Pre-wintering conditions and post-winter performance in a solitary bee: does diapause impose an energetic cost on reproductive success?', *Ecological Entomology,* 41(2), pp. 201-210.

Sgolastra, F., Bosch, J., Molowny-Horas, R., Maini, S. and Kemp, W. P. (2010) 'Effect of temperature regime on diapause intensity in an adult-wintering Hymenopteran with obligate diapause', *Journal of Insect Physiology,* 56(2), pp. 185-194.

Sgolastra, F., Hinarejos, S., Pitts-Singer, T. L., Boyle, N. K., Joseph, T., Luckmann, J., Raine, N. E., Singh, R., Williams, N. M. and Bosch, J. (2019) 'Pesticide exposure assessment paradigm for solitary bees', *Environmental Entomology,* 48(1), pp. 22-35.

Sgolastra, F., Kemp, W. P., Buckner, J. S., Pitts-Singer, T. L., Maini, S. and Bosch, J. (2011) 'The long summer: pre-wintering temperatures affect metabolic expenditure and winter survival in a solitary bee', *Journal of Insect Physiology,* 57(12), pp. 1651-1659.

Shaw, D. J. (2007) 'World Food Summit, 1996', *World Food Security*: London, UK: Palgrave Macmillan, pp. 347-360.

Sherman, R. A. and Wyle, F. A. (1996) 'Low-cost, low-maintenance rearing of maggots in hospitals, clinics, and schools', *American Journal of Tropical Medicine and Hygiene,* 54(1), pp. 38-41.

Shi, T. F., Wang, Y. F., Liu, F., Qi, L. and Yu, L. S. (2017) 'Sublethal effects of the neonicotinoid insecticide thiamethoxam on the transcriptome of the honey bees (Hymenoptera: Apidae)', *Journal of Economic Entomology,* 110(6), pp. 2283-2289.

Shiomi, K., Ishida, Y., Ikeda, M., Sato, Y., Saito, H., Imai, K., Isobe, M. and Yamashita, O. (1994) 'Induction of non-diapause eggs by injection of anti-diapause hormone rabbit serum into the diapause type of the silkworm, *Bombyx mori*', *Journal of Insect Physiology,* 40(8), pp. 693-699.

Sielezniew, M. and Cymborowski, B. (1997) 'Effects of ecdysteroid agonist RH-5849 on pupal diapause of the tobacco hornworm (*Manduca sexta*)', *Archives of Insect Biochemistry and Physiology: Published in Collaboration with the Entomological Society of America,* 35(1-2), pp. 191-197.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D. W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C. H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E. A., Morrissey, C. A., Noome, D. A., Pisa, L., Settele, J., Stark, J. D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van der Sluijs, J. P., Whitehorn, P. R. and Wiemers, M. (2015) 'Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites', *Environmental Science and Pollution Research,* 22(1), pp. 5-34.

Sinclair, B. J., Ferguson, L. V., Salehipour-shirazi, G. and MacMillan, H. A. (2013) 'Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects', *Integrative and Comparative Biology,* 53(4), pp. 545-556.

Sinclair, B. J., Vernon, P., Klok, C. J. and Chown, S. L. (2003) 'Insects at low temperatures: an ecological perspective', *Trends in Ecology & Evolution,* 18(5), pp. 257-262.

Singtripop, T., Wanichacheewa, S. and Sakurai, S. (2000) 'Juvenile hormone-mediated termination of larval diapause in the bamboo borer, *Omphisa fuscidentalis*', *Insect Biochemistry and Molecular Biology,* 30(8-9), pp. 847-54.

Sonne, C. and Alstrup, A. K. O. (2019) 'Denmark defies EU neonicotinoid ban', *Science,* 363(6430), pp. 938.

Sonobe, H., Obinata, T., Minokawa, T., Haruta, T., Kawamura, Y., Wakatsuki, S. and Sato, N. (2016) 'Characterization of paramyosin and thin filaments in the smooth muscle of acorn worm, a member of hemichordates', *Journal of Biochemistry,* 160(6), pp. 369-379.

Southwick, E. E. and Southwick Jr, L. (1992) 'Estimating the economic value of honey bees (Hymenoptera: Apidae) as agricultural pollinators in the United States', *Journal of Economic Entomology,* 85(3), pp. 621-633.

Sparks, T. C., Watson, G. B., Loso, M. R., Geng, C., Babcock, J. M. and Thomas, J. D. (2013) 'Sulfoxaflor and the sulfoximine insecticides: chemistry, mode of action and basis for efficacy on resistant insects', *Pesticide Biochemistry and Physiology,* 107(1), pp. 1-7.

Sponsler, D. B., Grozinger, C. M., Hitaj, C., Rundlöf, M., Botías, C., Code, A., Lonsdorf, E. V., Melathopoulos, A. P., Smith, D. J., Suryanarayanan, S., Thogmartin, W. E., Williams, N. M., Zhang, M. and Douglas, M. R. (2019) 'Pesticides and pollinators: A socioecological synthesis', *Science of the Total Environment,* 662, pp. 1012-1027.

Springmann, M., Clark, M., Mason-D'Croz, D., Wiebe, K., Bodirsky, B. L., Lassaletta, L., de Vries, W., Vermeulen, S. J., Herrero, M., Carlson, K. M., Jonell, M., Troell, M., DeClerck, F., Gordon, L. J., Zurayk, R., Scarborough, P., Rayner, M., Loken, B., Fanzo, J., Godfray, H. C. J., Tilman, D., Rockström, J. and Willett, W. (2018) 'Options for keeping the food system within environmental limits', *Nature,* 562(7728), pp. 519-525.

Ssymank, A., Kearns, C., Pape, T. and Thompson, F. C. (2008) 'Pollinating flies (Diptera): a major contribution to plant diversity and agricultural production', *Biodiversity,* 9(1-2), pp. 86-89.

Stanley, D. A., Garratt, M. P., Wickens, J. B., Wickens, V. J., Potts, S. G. and Raine, N. E. (2015a) 'Neonicotinoid pesticide exposure impairs crop pollination services provided by bumblebees', *Nature,* 528(7583), pp. 548-550.

Stanley, D. A., Gunning, D. and Stout, J. C. (2013) 'Pollinators and pollination of oilseed rape crops (*Brassica napus* L.) in Ireland: ecological and economic incentives for pollinator conservation', *Journal of Insect Conservation,* 17(6), pp. 1181-1189.

Stanley, D. A., Smith, K. E. and Raine, N. E. (2015b) 'Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide', *Scientific Reports,* 5, pp. 16508.

Steffan-Dewenter, I. and Schiele, S. (2004) 'Nest-site fidelity, body weight and population size of the red mason bee, *Osmia rufa* (Hymenoptera: Megachilidae), evaluated by mark-recapture experiments', *Entomologia Generalis,* 27(2), pp. 123-131.

Stelzer, R. J., Chittka, L., Carlton, M. and Ings, T. C. (2010) 'Winter active bumblebees (*Bombus terrestris*) achieve high foraging rates in urban Britain', *PLoS ONE,* 5(3), pp. e9559.

Stone, G. N. (1994) 'Activity patterns of females of the solitary bee *Anthophora plumipes* in relation to temperature, nectar supplies and body size', *Ecological Entomology,* 19(2), pp. 177-189.

Stout, J. C. and Morales, C. L. (2009) 'Ecological impacts of invasive alien species on bees', *Apidologie,* 40(3), pp. 388-409.

Strohm, E., Daniels, H., Warmers, C. and Stoll, C. (2002) 'Nest provisioning and a possible cost of reproduction in the megachilid bee *Osmia rufa* studied by a new observation method', *Ethology Ecology & Evolution,* 14(3), pp. 255-268.

Suman, D. S., Wang, Y. and Gaugler, R. (2015) 'The insect growth regulator pyriproxyfen terminates egg diapause in the asian tiger mosquito, *Aedes albopictus*', *PLoS ONE,* 10(6), pp. e0130499.

Szabo, N. D., Colla, S. R., Wagner, D. L., Gall, L. F. and Kerr, J. T. (2012) 'Do pathogen spillover, pesticide use, or habitat loss explain recent North American bumblebee declines?', *Conservation Letters,* 5(3), pp. 232-239.

Szabo, T. and Smith, M. (1972) 'The influence of light intensity and temperature on the activity of the alfalfa leaf-cutter bee *Megachile rotundata* under field conditions', *Journal of Apicultural Research,* 11(3), pp. 157-165.

Takeda, S. and Hasegawa, K. (1975) 'Alteration of egg diapause in *Bombyx mori* by ouabain injected into diapause egg producers', *Journal of Insect Physiology,* 21(12), pp. 1995-2003.

Taylor-Burt, K. R., Monroy, J., Pace, C., Lindstedt, S. and Nishikawa, K. C. (2015) 'Shiver me titin! Elucidating titin's role in shivering thermogenesis', *Journal of Experimental Biology,* 218(5), pp. 694-702.

Teets, N. M., Yi, S. X., Lee, R. E. and Denlinger, D. L. (2013) 'Calcium signaling mediates cold sensing in insect tissues', *Proceedings of the National Academy of Sciences of the United States of America,* 110(22), pp. 9154-9159.

Teper, D. and Bilinski, M. (2009) 'Red mason bee (*Osmia rufa* L.) as a pollinator of rape plantations', *Journal of Apicultural Science,* 53(2), pp. 115-120.

Terhzaz, S., Teets, N. M., Cabrero, P., Henderson, L., Ritchie, M. G., Nachman, R. J., Dow, J. A., Denlinger, D. L. and Davies, S. A. (2015) 'Insect capa neuropeptides impact desiccation and cold tolerance', *Proceedings of the National Academy of Sciences of the United States of America,* 112(9), pp. 2882-2887.

Thomann, M., Imbert, E., Devaux, C. and Cheptou, P. O. (2013) 'Flowering plants under global pollinator decline', *Trends in Plant Science,* 18(7), pp. 353-359.

Thompson, H. M. and Hunt, L. V. (1999) 'Extrapolating from honeybees to bumblebees in pesticide risk assessment', *Ecotoxicology,* 8(3), pp. 147-166.

Tilman, D., Balzer, C., Hill, J. and Befort, B. L. (2011) 'Global food demand and the sustainable intensification of agriculture', *Proceedings of the National Academy of Sciences of the United States of America,* 108(50), pp. 20260-20264.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R. and Polasky, S. (2002) 'Agricultural sustainability and intensive production practices', *Nature,* 418(6898), pp. 671-7.

Tomizawa, M. and Casida, J. E. (2005) 'Neonicotinoid insecticide toxicology: mechanisms of selective action', *Annual Reviews of Pharmacology and Toxicology,* 45, pp. 247-68.

Torero, M. (2020) 'Without food, there can be no exit from the pandemic', *Nature,* 580(7805), pp. 588-589.

Torretta, J. P., Medan, D. and Abrahamovich, A. H. (2006) 'First record of the invasive bumblebee *Bombus terrestris* (L.)(Hymenoptera, Apidae) in Argentina', *Transactions of the American Entomological Society,* 132(3), pp. 285-289.

Torson, A. S., Yocum, G. D., Rinehart, J. P., Kemp, W. P. and Bowsher, J. H. (2015) 'Transcriptional responses to fluctuating thermal regimes underpinning differences in survival in the solitary bee *Megachile rotundata*', *Journal of Experimental Biology,* 218(7), pp. 1060-1068.

Torson, A. S., Yocum, G. D., Rinehart, J. P., Nash, S. A., Kvidera, K. M. and Bowsher, J. H. (2017) 'Physiological responses to fluctuating temperatures are characterized by distinct transcriptional profiles in a solitary bee', *Journal of Experimental Biology,* 220(18), pp. 3372-3380.

Tosi, S., Démares, F. J., Nicolson, S. W., Medrzycki, P., Pirk, C. W. and Human, H. (2016) 'Effects of a neonicotinoid pesticide on thermoregulation of African

honey bees (*Apis mellifera scutellata*)', *Journal Insect Physiology,* 93-94, pp. 56-63.

Totland, Ø. (1993) 'Pollination in alpine Norway: flowering phenology, insect visitors, and visitation rates in two plant communities', *Canadian Journal of Botany,* 71(8), pp. 1072-1079.

Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) 'Crystal structure of the calcium pump of sarcoplasmic reticulum at 2.6 A resolution', *Nature,* 405(6787), pp. 647-55.

Treherne, J. E. and Schofield, P. K. (1981) 'Mechanisms of ionic homeostasis in the central nervous system of an insect', *Journal of Experimental Biology,* 95, pp. 61-73.

Tsurumaru, S., Kawamori, A., Mitsumasu, K., Niimi, T., Imai, K., Yamashita, O. and Yaginuma, T. (2010) 'Disappearance of chorion proteins from *Bombyx mori* eggs treated with HCl solution to prevent diapause', *Journal of Insect Physiology,* 56(12), pp. 1721-1727.

United States Environmental Protection Agency (2017) *U.S. Environmental Protection Agency's Policy to Mitigate the Acute Risk to Bees from Pesticide Products*: Office of Pesticide Programs. Available at: https://www.regulations.gov/document/EPA-HQ-OPP-2014-0818-0477 (Accessed: 12 May 2019).

van der Sluijs, J. P., Amaral-Rogers, V., Belzunces, L. P., Bijleveld van Lexmond, M. F., Bonmatin, J. M., Chagnon, M., Downs, C. A., Furlan, L., Gibbons, D. W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E. A., Morrissey, C. A., Noome, D. A., Pisa, L., Settele, J., Simon-Delso, N., Stark, J. D., Tapparo, A., Van Dyck, H., van Praagh, J., Whitehorn, P. R. and Wiemers, M. (2015) 'Conclusions of the Worldwide Integrated Assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning', *Environmental Science and Pollution Research,* 22(1), pp. 148-54.

van der Steen, J. and de Ruijter, A. 'The management of *Osmia rufa* L. for pollination of seed crops in greenhouses'. *Proceedings of Experimental and Applied Entomology*, 2, pp. 137-141.

van Heemert, C., De Ruijter, A., van den Eijnde, J. and van der Steen, J. (1990) 'Year-round production of bumble bee colonies for crop pollination', *Bee World,* 71(2), pp. 54-56.

Vanbergen, A. J. and the Insect Pollinators Initiative (2013) 'Threats to an ecosystem service: pressures on pollinators', *Frontiers in Ecology and the Environment,* 11(5), pp. 251-259.

Velthuis, H. H. and Van Doorn, A. (2006) 'A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination', *Apidologie,* 37(4), pp. 421-451.

Vicens, N. and Bosch, J. (2000) 'Pollinating efficacy of *Osmia cornuta* and *Apis mellifera* (Hymenoptera: Megachilidae, Apidae) on 'Red Delicious' apple', *Environmental Entomology,* 29(2), pp. 235-240.

Voelzmann, A., Liew, Y. T., Qu, Y., Hahn, I., Melero, C., Sánchez-Soriano, N. and Prokop, A. (2017) '*Drosophila* Short stop as a paradigm for the role and regulation of spectraplakins', *Seminars in Cell and Developmental Biology,* 69, pp. 40-57.

Vogel, E., Santos, D., Mingels, L., Verdonckt, T. W. and Broeck, J. V. (2018) 'RNA interference in insects: protecting beneficials and controlling pests', *Frontiers in Physiology,* 9, pp. 1912.

Vuillaume, M. and Berkaloff, A. (1974) 'LSD treatment of *Pieris brassicae* and consequences on the progeny', *Nature,* 251(5473), pp. 314-315.

Wang, J., Zhou, H. Y., Zhao, Z. M. and Liu, Y. H. (2014) 'Effects of juvenile hormone analogue and ecdysteroid on adult eclosion of the fruit fly *Bactrocera minax* (Diptera: Tephritidae)', *Journal of Economic Entomology,* 107(4), pp. 1519-1525.

Warren, R., Price, J., Graham, E., Forstenhaeusler, N. and VanDerWal, J. (2018) 'The projected effect on insects, vertebrates, and plants of limiting global warming to 1.5˚C rather than 2˚C', *Science,* 360(6390), pp. 791-795.

Wasielewski, O., Giejdasz, K., Wojciechowicz, T. and Skrzypski, M. (2011a) 'Ovary growth and protein levels in ovary and fat body during adult-wintering period in the red mason bee, *Osmia rufa*', *Apidologie,* 42(6), pp. 749-758.

Wasielewski, O., Wojciechowicz, T., Giejdasz, K. and Krishnan, N. (2011b) 'Influence of methoprene and temperature on diapause termination in adult females of the over-wintering solitary bee, *Osmia rufa* L', *Journal of Insect Physiology,* 57(12), pp. 1682-1688.

Wasielewski, O., Wojciechowicz, T., Giejdasz, K. and Krishnan, N. (2013) 'Overwintering strategies in the red mason solitary bee—physiological correlates of midgut metabolic activity and turnover of nutrient reserves in females of *Osmia bicornis*', *Apidologie,* 44(6), pp. 642-656.

Wells, L., Edwards, K. A. and Bernstein, S. I. (1996) 'Myosin heavy chain isoforms regulate muscle function but not myofibril assembly', *The EMBO Journal,* 15(17), pp. 4454-4459.

Westerkamp, C. (1991) 'Honeybees are poor pollinators—why?', *Plant systematics and Evolution,* 177(1), pp. 71-75.

Whitehorn, P. R., O'Connor, S., Wackers, F. L. and Goulson, D. (2012) 'Neonicotinoid pesticide reduces bumble bee colony growth and queen production', *Science,* 336(6079), pp. 351-2.

Whitehorn, P. R., Tinsley, M. C., Brown, M. J., Darvill, B. and Goulson, D. (2009) 'Impacts of inbreeding on bumblebee colony fitness under field conditions', *BMC Evolutionary Biology,* 9, pp. 152.

Whittington, R., Winston, M. L., Tucker, C. and Parachnowitsch, A. L. (2004) 'Plant-species identity of pollen collected by bumblebees placed in greenhouses for tomato pollination', *Canadian Journal of Plant Science,* 84(2), pp. 599-602.

Wilkaniec, Z. and Giejdasz, K. (2003) 'Suitability of nesting substrates for the cavity-nesting bee *Osmia rufa*', *Journal of Apicultural Research,* 42(3), pp. 29-31.

Wilkaniec, Z. and Radajewska, B. 'Solitary bee *Osmia rufa* L.(Apoidea, Megachilidae) as pollinator of strawberry cultivated in an unheated plastic tunnel'. *III International Strawberry Symposium 439*, pp. 489-494.

Williams, C. M., Henry, H. A. and Sinclair, B. J. (2015) 'Cold truths: how winter drives responses of terrestrial organisms to climate change', *Biological Reviews,* 90(1), pp. 214-235.

Williamson, S. M., Willis, S. J. and Wright, G. A. (2014) 'Exposure to neonicotinoids influences the motor function of adult worker honeybees', *Ecotoxicology,* 23(8), pp. 1409-18.

Winter, K., Adams, L., Thorp, R., Inouye, D., Day, L., Ascher, J. and Buchmann, S. (2006) *Importation of non-native bumble bees into North America: potential consequences of using Bombus terrestris and other non-native bumble bees for greenhouse crop pollination in Canada, Mexico, and the United States*: North American Pollinator Protection Campaign. White Paper. Available at: https://www.researchgate.net/profile/Oliver-Williamson/publication/31227280_Introduction/links/5655fd8c08aefe619b1cfc39/Introduction.pdf (Accessed: 07 August 2019).

Wolfe, D. W., Schwartz, M. D., Lakso, A. N., Otsuki, Y., Pool, R. M. and Shaulis, N. J. (2005) 'Climate change and shifts in spring phenology of three horticultural woody perennials in northeastern USA', *International Journal of Biometeorology,* 49(5), pp. 303-309.

Wood, T. J. and Goulson, D. (2017) 'The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013', *Environmental Science and Pollution Research,* 24(21), pp. 17285-17325.

Woodcock, B., Edwards, M., Redhead, J., Meek, W., Nuttall, P., Falk, S., Nowakowski, M. and Pywell, R. (2013) 'Crop flower visitation by honeybees,

bumblebees and solitary bees: Behavioural differences and diversity responses to landscape', *Agriculture, Ecosystems & Environment,* 171, pp. 1-8.

Woodcock, B. A., Bullock, J. M., Shore, R. F., Heard, M. S., Pereira, M. G., Redhead, J., Ridding, L., Dean, H., Sleep, D., Henrys, P., Peyton, J., Hulmes, S., Hulmes, L., Sárospataki, M., Saure, C., Edwards, M., Genersch, E., Knäbe, S. and Pywell, R. F. (2017) 'Country-specific effects of neonicotinoid pesticides on honey bees and wild bees', *Science,* 356(6345), pp. 1393-1395.

Woodcock, B. A., Isaac, N. J., Bullock, J. M., Roy, D. B., Garthwaite, D. G., Crowe, A. and Pywell, R. F. (2016) 'Impacts of neonicotinoid use on long-term population changes in wild bees in England', *Nature Communications,* 7, pp. 12459.

Wu, M. C., Chang, Y. W., Lu, K. H. and Yang, E. C. (2017) 'Gene expression changes in honey bees induced by sublethal imidacloprid exposure during the larval stage', *Insect Biochemistry and Molecular Biology,* 88, pp. 12-20.

Wu-Smart, J. and Spivak, M. (2018) 'Effects of neonicotinoid imidacloprid exposure on bumble bee (Hymenoptera: Apidae) queen survival and nest initiation', *Environmental Entomology,* 47(1), pp. 55-62.

Xu, J., Strange, J. P., Welker, D. L. and James, R. R. (2013) 'Detoxification and stress response genes expressed in a western North American bumble bee, *Bombus huntii* (Hymenoptera: Apidae)', *BMC Genomics,* 14, pp. 874.

Xu, J. C., Lytle, C., Zhu, T. T., Payne, J. A., Benz, E. and Forbush, B. (1994) 'Molecular cloning and functional expression of the bumetanide-sensitive Na-K-Cl cotransporter', *Proceedings of the National Academy of Sciences of the United States of America,* 91(6), pp. 2201-2205.

Xu, K., Niu, Q., Zhao, H., Du, Y. and Jiang, Y. (2017) 'Transcriptomic analysis to uncover genes affecting cold resistance in the Chinese honey bee (*Apis cerana cerana*)', *PLoS ONE,* 12(6), pp. e0179922.

Xu, W. H. and Denlinger, D. L. (2003) 'Molecular characterization of prothoracicotropic hormone and diapause hormone in *Heliothis virescens* during diapause, and a new role for diapause hormone', *Insect Molecular Biology,* 12(5), pp. 509-16.

Yang, E. C., Chuang, Y. C., Chen, Y. L. and Chang, L. H. (2008) 'Abnormal foraging behavior induced by sublethal dosage of imidacloprid in the honey bee (Hymenoptera: Apidae)', *Journal of Economic Entomology,* 101(6), pp. 1743-1748.

Yocum, G. D., Joplin, K. H. and Denlinger, D. L. (1998) 'Upregulation of a 23 kDa small heat shock protein transcript during pupal diapause in the flesh fly, *Sarcophaga, crassipalpis*', *Insect Biochemistry and Molecular Biology,* 28(9), pp. 677-682.

Yoshida, Y. and Imai, S. (1997) 'Structure and function of inositol 1, 4, 5-trisphosphate receptor', *The Japanese Journal of Pharmacology,* 74(2), pp. 125-137.

Zayas, R. M., Qazi, S., Morton, D. B. and Trimmer, B. A. (2002) 'Nicotinic-acetylcholine receptors are functionally coupled to the nitric oxide/cGMP-pathway in insect neurons', *Journal of Neurochemistry,* 83(2), pp. 421-431.

Zhang, Q., Zdarek, J., Nachman, R. J. and Denlinger, D. L. (2008) 'Diapause hormone in the corn earworm, *Helicoverpa zea*: Optimum temperature for activity, structure–activity relationships, and efficacy in accelerating flesh fly pupariation', *Peptides,* 29(2), pp. 196-205.

Zhang, W., Ricketts, T. H., Kremen, C., Carney, K. and Swinton, S. M. (2007) 'Ecosystem services and dis-services to agriculture', *Ecological Economics,* 64(2), pp. 253-260.

Zotti, M. J. and Smagghe, G. (2015) 'RNAi technology for insect management and protection of beneficial insects from diseases: Lessons, challenges and risk assessments', *Neotropical Entomology,* 44(3), pp. 197-213.

Černá, K., Zemenová, M., Macháčková, L., Kolínová, Z. and Straka, J. (2013) 'Neighbourhood society: nesting dynamics, usurpations and social behaviour in solitary bees', *PLoS ONE,* 8(9), pp. e73806.

Appendices

Appendix 1.1 List of commercial suppliers, and sale price, of red mason bees

Name of company	Website	Email	Sale price (per cocoon)	Country of operation
MasonBees UK	http://www.masonbees.co.uk/	contact@masonbees.co.uk	N/A	UK
Dragonfli	www.dragonfli.co.uk	sales@dragonfli.co.uk, julianives@dragonfli.co.uk	£1.50	UK
WAB - Mauerbienenzucht	http://mauerbienen.com/	info@Mauerbienen.com	£0.69*	Germany
Bienenhotel.de	http://bienenhotel.de	mail@bienenhotel.de	£0.57*	Germany
Mauerbienen.eu	http://mauerbienen.eu	bioresearch.schubert@t-online.de	£0.60	Germany

*Lower prices are available when bulk purchasing

Appendix 1.2 Table of hormonal and chemical treatments to terminate diapause in insects

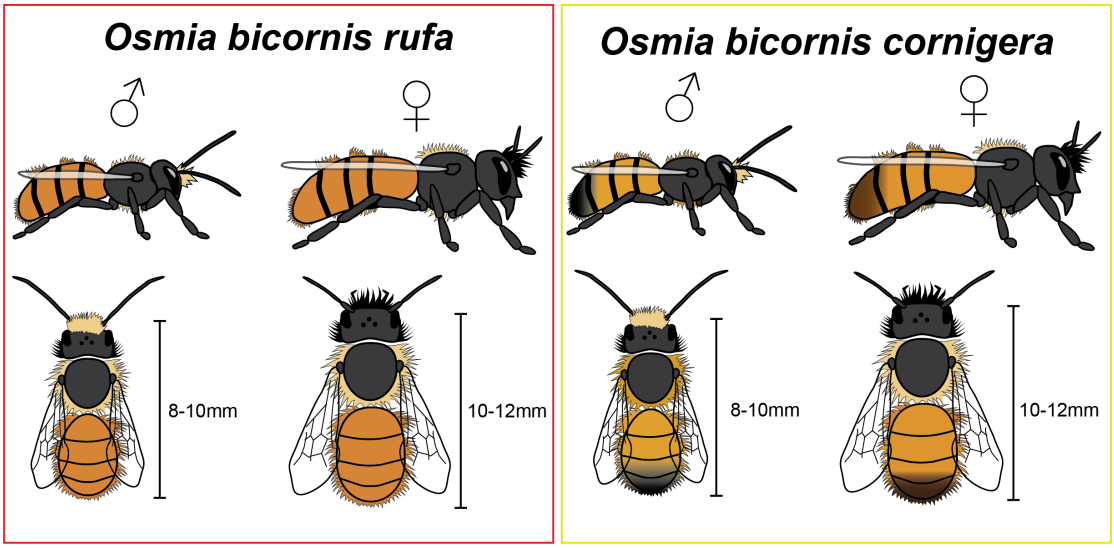
Termination Treatment		Dose	Method of application	Species	Diapause life-stage	Reference
Juvenile Hormone, its analogs and mimics	Methoprene	0.1 µg	Topical	*Caloptilia fraxinella*	Reproductive diapause	Evenden et al., 2007
		1 µg	Topical	*Caloptilia fraxinella*	Reproductive diapause	Lemmen and Evenden, 2016
		200 µg	Topical	*Locusta migratoria*	Egg diapause	Kidokoro et al., 2006
		5 x 1 µg	Topical	*Melinda pusilla*	Reproductive diapause, adults	Agui et al., 1991
		200 µg	Topical	*Omphisa fuscidentalis*	Diapausing larvae	Singtripop et al., 2000
		5 x 200 µg	Topical	*Osmia bicornis*	Adults, Post-diapause quiescence	Wasielewski et al., 2011b
		200 µg	Topical	*Oxya yezoensis*	Egg diapause	Kidokoro et al., 2006
		0.6 µg	Topical	*Riptortus clavatus*	Diapausing adults	Numata and Hidaka, 1984
	Ecdysterone	2-6 µg	Topical	*Antheraea mylitta*	Diapausing pupae	Mishra et al., 2008
		500 ng	Injection	*Pieris brassicae*	Diapausing pupae	Pullin and Bale, 1989
		500 ng	Injection	*Pieris brassicae*	Diapausing pupae	Arpagaus et al., 1986
		10 µg	Topical	*Sarcophaga crassipalpis*	3rd instar larvae – pre-diapause induction	Denlinger, 1976
		0.01 µg	Injection	*Sarcophaga crassipalpis*	Pupa – immediately prior to diapause	Denlinger, 1976

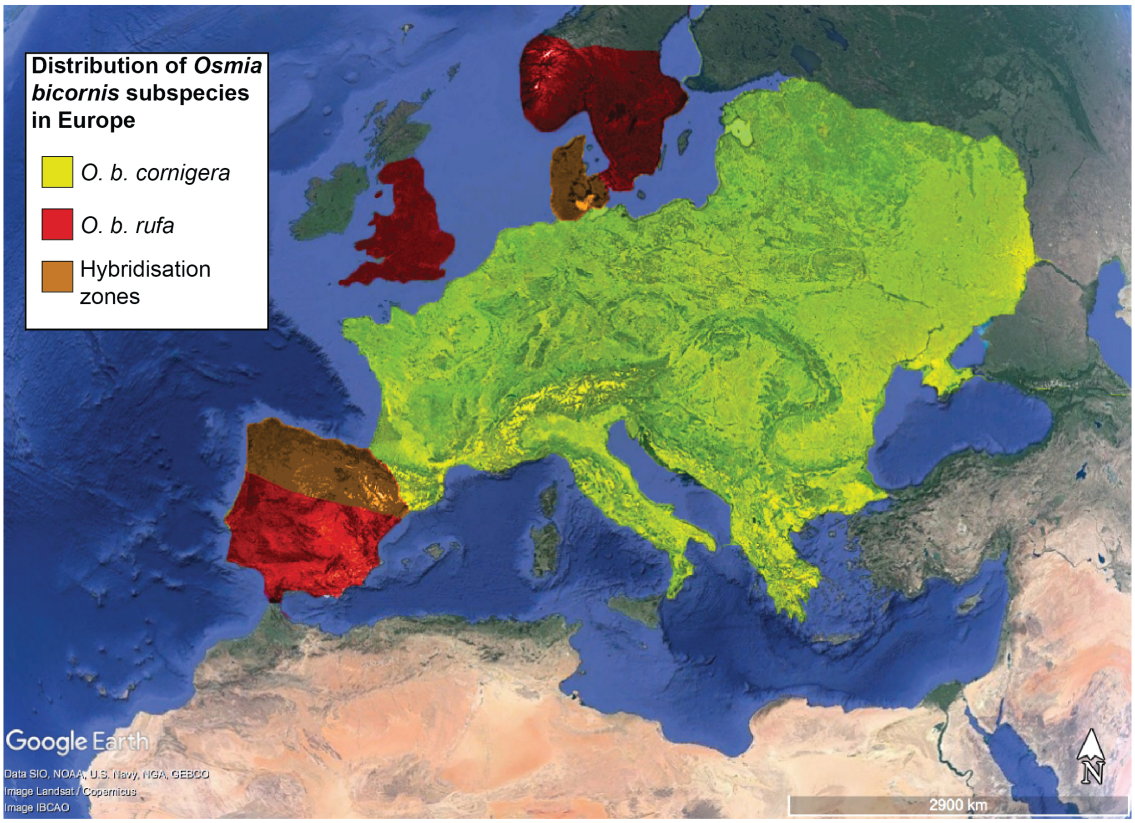
	1 µg	Injection	*Sarcophaga crassipalpis*	3rd instar larvae – pre-diapause induction	Denlinger, 1976
ß-ecdysone	20 µg	Topical (in combination with low temperature, 5ºC)	*Antheraea yamamai*	Egg diapause	Kuwano et al., 1991
20-hydroxyecdysone	0.05-1.5 µg	Topical	*Bactrocera minax*	Diapausing pupae	Wang et al., 2014
	0.2 µg	Injection	*Bactrocera minax*	Diapausing pupae	Dong et al., 2019
	2-10 µg per gramme insect	Injection	*Diprion pini*	Prepupal diapause	Hamel et al., 1998
	0.5 g	Injection	*Helicoverpa zea*	Diapausing pupae	Reynolds et al., 2019
	4.8g	Soaked for 24h	*Locusta migratoria*	Egg diapause	Kidokoro et al., 2006
	7.5 µg per gramme insect	Injection	*Mamestra configurata*	Pupal diapause	Bodnaryk, 1985
	0.1-0.5 µg	Injection	*Pieris rapae*	Diapausing pupae	Park and Kim, 1989
RH-5849	1 µl	Topical	*Ostrinia nubilalis*	Diapausing larvae	Gadenne et al., 1990
RH-5949	0.15–10 µg per gram insect	Injection	*Maduca sexta*	Diapausing pupae	Sielezniew and Cymborowski, 1997
Hydroprene	200 µg	Topical	*Locusta migratoria*	Egg diapause	Kidokoro et al., 2006
	200 µg	Topical	*Oxya yezoensis*	Egg diapause	Kidokoro et al., 2006
Pyriproxyfen	0.25-1 µg	Soaked in treated water	*Aedes albopictus*	Egg Diapause	Suman et al., 2015
	10 µg	Topical	*Eurygaster integriceps*	Reproductive diapause	Amiri et al., 2012

	Fenoxycarb	1 µg per gramme insect	Topical	*Antheraea mylitta*	Diapausing pupae	Dinesh et al., 2007
Diapause Hormone and its analogs	Diapause hormone	0.1 µg	Injection	*Helicoverpa zea*	Diapausing pupae	Zhang et al., 2008
		30 µg	Injection	*Helicoverpa zea*	Diapausing pupae	Reynolds et al., 2019
		0.1-0.2 µg	Injection	*Heliothis virescens*	Diapausing pupae	Xu and Denlinger, 2003
	'1963'	0.5 nmole	Injection	*Helicoverpa zea*	Diapausing pupae	Reynolds et al., 2019
Alkanes	Hexane	2 µl	Injection	*Manduca sexta*	Diapausing pupae	Denlinger et al., 1980
		2 µl	Topical	*Sarcophaga crassipalpis*	Diapausing pupae	Denlinger et al., 1980
		-	Vapour exposure (1-2h)	*Sarcophaga crassipalpis*	Diapausing pupae	Denlinger et al., 1980
	Acetone	2 µl	Topical	*Manduca sexta*	3 days prior to onset of diapause	Denlinger et al., 1980
		2 µl	Topical	*Sarcophaga crassipalpis*	3 days prior to onset of diapause	Denlinger et al., 1980
Miscellaneous	Lysergic acid diethylamide	20 µg	Topical	*Pieris brassicae*	Pre-diapause induction	Vuillaume and Berkaloff, 1974
	Ouabain	6 µg	Topical	*Bombyx mori*	Laying of non-diapausing eggs	Takeda and Hasegawa, 1975
	Cholera toxin	1 µg	Injection	*Sarcophaga crassipalpis*	Pre-diapause induction	Denlinger, 1976
	Antiserum of diapause hormone	0.1 µl	Injection	*Bombyx mori*	Laying of non-diapause eggs	Shiomi et al., 1994
	Iron (II) Chloride	0.4-6 g	Injection	*Bombyx* sp.	Diapausing pupae	Nishitsutsuji-Uwo and

Iron (III) Chloride	0.5-8.1 g	Injection	*Bombyx sp.*	Diapausing pupae	Nishimura, 1975 Nishitsutsuji-Uwo and Nishimura, 1975
Carbon dioxide	-	Narcosis (coupled with 20ºC and 13L/11D light conditions)	*Bombus terrestris*	Diapausing adults	Larrere et al., 1993
Hydrogen chloride	Specific gravity: 1.075-1.110	Vapour exposure (5 mins)	*Bombyx mori (Silkmoth)*	Pre-diapause induction, eggs	Tsurumaru et al., 2010
Precocene	20 µg	Topical (in combination with low temperature, 5ºC)	*Antheraea yamamai*	Egg diapause	Kuwano et al., 1991
Imidazole	20 µg	Topical (in combination with low temperature, 5ºC)	*Antheraea yamamai*	Egg diapause	Kuwano et al., 1991
Bovine insulin	5 µg	Injection	*Pieris brassicae*	Diapausing pupae	Arpagaus, 1987

A non-exhaustive list of different treatments to terminate diapause, or post-diapause quiescence, available in the published literature. Where possible, doses were converted to µg to aid comparison.





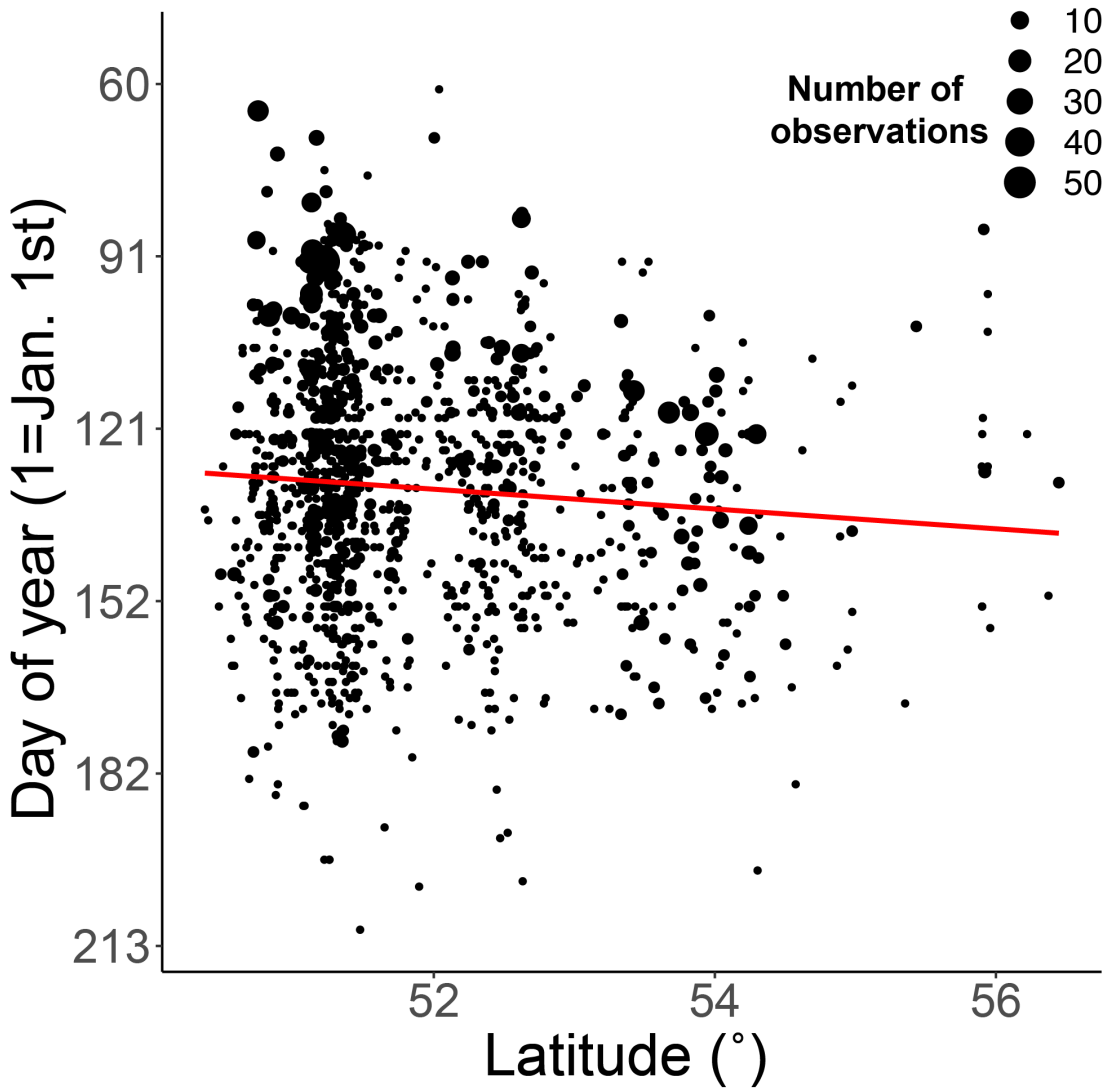
Appendix 2.1 Description of Osmia bicornis subspecies and their distribution in Europe

The illustrations of *O. b. rufa* and *O. b. cornigera* were drawn by me based on descriptions by Peters (1978). The distribution map was adapted from Conrad and Ayasse (2016).

Appendix 2.2 Comparison light readings with a PAR sensor and the Lux Light Meter Pro app

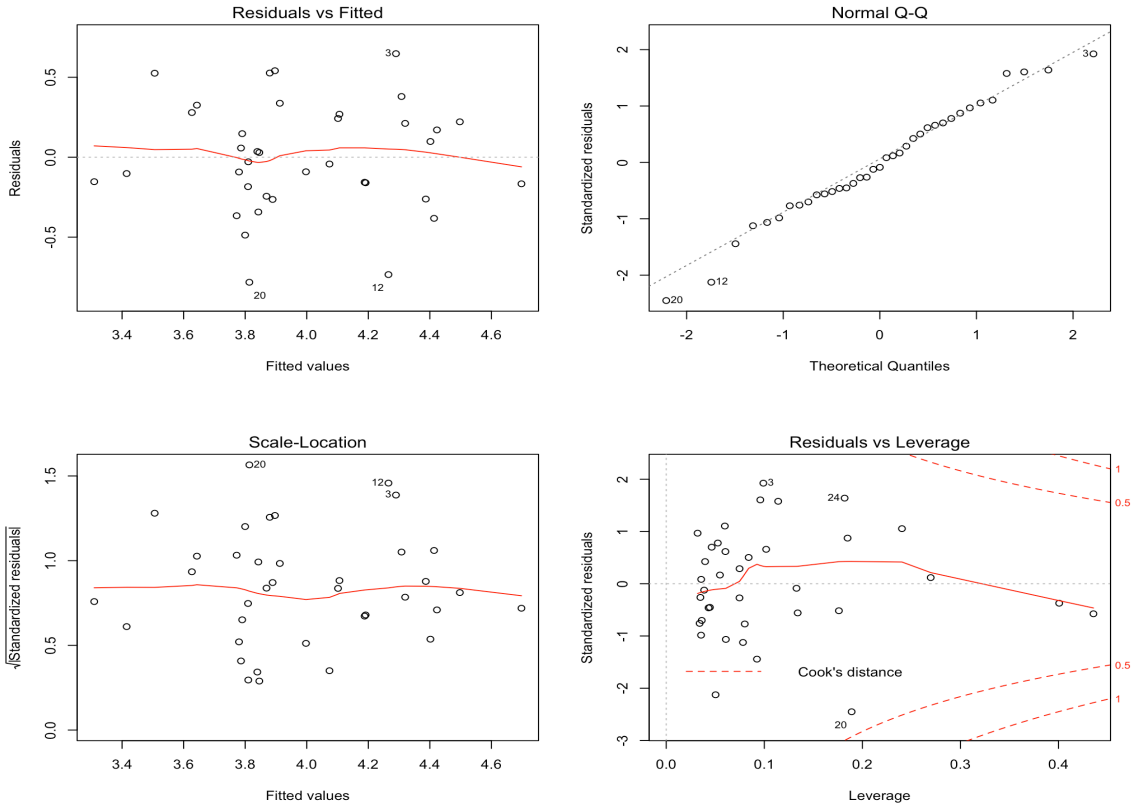
Lux Light Meter Pro app (lux)	PAR sensor (µmol/m^2/s)	PAR readings converted to lux
2,986 (±123.1)	56 (±1.4)	3,024 (±75.6)
4,467 (±87.2)	88 (±2.0)	4,752 (±108.0)
12,674 (±89.3)	230 (±1.3)	12,420 (±70.2)
15,382 (±102.9)	285 (±1.6)	15,390 (±86.4)
26,589 (±67.3)	488 (±2.2)	26,352 (±118.8)
34,864 (±156.3)	691 (±4.6)	37,314 (±248.4)
59,876 (±56.5)	1,100 (±0.3)	59,400 (±16.2)

Readings show the average of three measurements each taken over the course of a minute and are shown with the standard error. Lux Light Meter Pro readings were taken on an iPhone SE, while PAR readings were collected using a Quantum sensor from Apogee instruments. PAR readings were converted to lux by multiplying them by 54 - as suggested by Apogee instruments.



Appendix 3.1 Date of all observations from BWARS RMB dataset compared to latitude

Each point refers to a single observation and the size of the point shows if multiple observations overlap. The red line shows a linear regression (y = 1.53x + 63.05; F-score = 8.61; DF = 1246; R^2 = .04; p < .01).



Appendix 3.2 A linear model to assess first emergence of RMBs appears valid based on diagnostic plots

Residuals vs. Fitted shows the predictive pattern between the predictor variables and the outcome. To meet the assumptions of a linear model residuals should be spread relatively evenly around a horizontal line. The Q-Q plot shows how normally distributed the data were, if there is little deviation from the diagonal line then the data is considered normally distributed. Scale-Location checks if variance is homoscedastic (equal) by seeing if points are distributed randomly around a horizontal line. Residuals vs. Leverage shows if any particular subject in the model is influential (i.e. its exclusion would alter the model) by assessing their score for Cook's distance. If a point overlaps with the Cook's distance line, then it is influential.

Appendix 3.3 List p and R^2 values determined from linear regression of 100 subsets of the BWARS data.

Model	p-value	R^2
1	0.00119	0.2475
2	0.025	0.1139
3	0.0105	0.1532
4	0.122	0.04153
5	0.000102	0.344
6	0.000675	0.2708
7	0.18	0.02448
8	0.00208	0.2241
9	0.115	0.04421
10	0.00459	0.19
11	0.00162	0.2346
12	0.0538	0.07874
13	0.0138	0.1409
14	0.000989	0.2552
15	5.25E-05	0.368
16	0.000409	0.2909
17	2.68E-06	0.4664
18	0.00141	0.2404
19	2.11E-04	0.3166
20	0.00691	0.172
21	4.42E-02	0.08771
22	0.00112	0.25
23	1.19E-03	0.2475
24	0.000472	0.2852
25	2.53E-03	0.2157
26	3.93E-05	0.3783
27	1.98E-02	0.1247
28	3.26E-02	0.1017
29	1.75E-02	0.1302
30	1.51E-05	0.411
31	2.22E-03	0.2214
32	7.51E-05	0.3551
33	3.03E-02	0.105
34	1.78E-04	0.3231
35	6.36E-04	0.2732
36	8.34E-03	0.1636
37	2.60E-04	0.3085
38	3.20E-04	0.3005
39	2.69E-02	0.1106
40	1.28E-03	0.2445
41	3.22E-04	0.3002
42	3.54E-03	0.2013
43	4.59E-03	0.19

44	6.13E-02	0.07273
45	1.41E-04	0.3318
46	6.26E-05	0.3617
47	5.05E-05	0.3694
48	6.31E-04	0.2735
49	1.85E-04	0.3216
50	6.60E-06	0.4382
51	2.45E-02	0.1149
52	3.94E-04	0.2923
53	3.90E-05	0.3785
54	2.14E-03	0.223
55	6.09E-04	0.275
56	1.45E-02	0.1389
57	1.79E-02	0.1292
58	1.21E-04	0.3374
59	2.19E-02	0.1199
60	3.91E-04	0.2926
61	2.65E-06	0.4667
62	6.08E-05	0.3628
63	1.14E-02	0.1495
64	1.02E-04	0.344
65	3.23E-02	0.1021
66	4.72E-03	0.1888
67	3.30E-04	0.2993
68	2.25E-05	0.3976
69	2.78E-03	0.2118
70	7.93E-03	0.1659
71	1.66E-05	0.4079
72	4.75E-05	0.3716
73	2.33E-04	0.3128
74	7.29E-05	0.3562
75	1.14E-03	0.2492
76	1.44E-05	0.4127
77	1.81E-03	0.2301
78	1.88E-03	0.2283
79	0.000116	0.3392
80	0.000709	0.2688
81	4.50E-05	0.3735
82	0.0171	0.1311
83	0.00429	0.193
84	0.0013	0.244
85	0.00361	0.2005
86	9.08E-05	0.3482
87	0.00218	0.2221
88	7.71E-06	0.4332
89	0.00127	0.2449
90	0.000697	0.2695
91	6.50E-05	0.3603

92	0.00275	0.2123
93	7.92E-05	0.3532
94	0.000505	0.2824
95	0.0413	0.09082
96	0.0198	0.1245
97	0.0148	0.1377
98	0.00255	0.2154
99	0.0182	0.1285
100	7.10E-05	0.3572

Complete list *p* and R^2 values determined by linear regression models generated to test the relationship between spring temperature and the first sighting of RMBs when BWARS data was subsette

Appendix 5.1 Mapping rate of buff-tailed bumblebee sequencing samples on the transcriptome and genome

Sample	Transcriptome mapping rate (%)	Genome mapping rate (%)
1	85.33	79.27
2	82.39	78.55
3	84.73	82.26
4	83.29	78.71
5	84.33	74.66
6	82.42	81.45
7	83.61	74.82
8	82.44	81.21
9	83.17	81.17
10	85.05	65.98
11	84.68	70.69
12	85.36	69.44
13	85.78	70.89
14	84.94	75.55
15	87.96	59.05
16	86.45	71.91
17	83.81	80.80
18	85.06	81.93
19	83.70	83.79
20	84.58	74.14
21	85.22	75.41
22	83.91	81.23
23	85.20	78.10
24	83.67	83.78

Read mapping rate of each of the 24 BTB total RNA samples when sequenced and then mapped to either the transcriptome or genome. Reads were mapped to the transcriptome using Salmon (Patro et al., 2017), and to the genome using STAR (Dobin et al., 2013).