Sublethal effects of pesticide exposure in the buff-tailed bumblebee (*Bombus terrestris*): novel behavioural assay approaches

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Author declaration

I hereby declare that this thesis and the work presented in it are my own. Work which is not my own has been clearly identified and no material within this thesis has been submitted previously for the award of a degree by this, or any other, university.

Laura Elizabeth James

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Abstract

The aim of the work described in this thesis was to assess the potential sublethal effects of pesticide exposure on buff-tailed bumblebee (*Bombus terrestris audax*) mobility, navigation, learning and memory through the development of novel bee behavioural assays. The development of such comparative testing platforms adds core tools to our ability to assess sublethal outcomes across a broad behavioural range and provides a sound basis to compare pesticidal impacts across species. This is currently limited, due to a deficit of studies on non-*Apis* bees. In Chapter 2, core gaps in the existing use of aversive training in bee cognitive studies are highlighted. In Chapter 3, a novel thermal-visual arena was piloted to aversively condition bumblebees to locate a cool reward zone, suggesting ambient temperature as a fruitful avenue for bee aversive learning research. In Chapter 4, this was further confirmed through trials comparing aversive conditioning to other conditioning methodologies, demonstrating that *B. terrestris* foragers responded best to aversive conditioning elements. In Chapter 5, the thermal-visual arena was used to assess the impact, via oral exposure, of sublethal doses of the neonicotinoid insecticides thiacloprid (500 and 5000ppb) and thiamethoxam (10 and 100ppb) and the sulfoximine insecticide sulfoxaflor (5 and 50ppb) on *B. terrestris* navigation and learning, demonstrating that thiamethoxam prevents bees from improving in key training parameters. In Chapter 6, new ways of examining behavioural templates were explored utilising power law analyses, uncovering a speed-curvature power law present in the walking trajectories of bees. In Chapter 7, this speed-curvature power law was found to be disrupted under thiamethoxam (10 and 100ppb) exposure. In Chapter 8, potential genetic determinants of individual differences in *B. terrestris* learning ability were elucidated through RNA-seq analyses of 'good' and 'bad' learners. In Chapter 9, a novel colour learning assay was employed to examine imidacloprid (10ppb) impact on associative and reversal learning in *B. terrestris* foragers, revealing that foraging behaviours, but not learning, are affected by chronic oral exposure. The results presented in Chapters 5, 7 and 9 clearly demonstrate that low doses of pesticides can have important sublethal effects on buff-tailed bumblebees, and can affect previously unstudied parameters (e.g. power laws governing movement (Chapters 6 and 7) and that current standard toxicological assessments often miss such subtle (not immediately lethal) impacts.

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Chapter 1: Introduction

1.1 Bees

1.1.1 The value of animal pollinators

Pollinators are crucial to ecosystem functioning in a wide range of terrestrial environments. This is accentuated in ecosystems dominated by agriculture, where plant productivity is closely linked to plant-pollinator interactions (Kevan, 1999). Estimates of the reliance of the world's 352,000 angiosperm species on animal pollination vary widely. Earlier estimations ranged from 67%-96% (Axelrod, 1960; Renner, 1996; Williams, 1994), but contemporary studies suggest 80-87.5% of angiosperms rely on animal pollination in some form (National Research Council, 2007; Ollerton et al., 2011).

Insects are key pollinators, playing a vital role in global food production and security, ecosystem functioning and health and wild plant reproduction (Gill et al., 2016; Klein et al., 2007; Kwak et al., 1998). It is estimated that 35% of all food crops (Klein et al., 2007) and 84% of European crops rely on insect pollination (Williams, 1994). Bees are considered the most important insect pollinators (Williams, 1994; Gallai et al., 2009), pollinating 90% of the world's top 107 crop types (Klein et al., 2007). An estimated 5-8% of global crop supply could be lost without their pollination services (Aizen et al., 2009).

Assessments of bee importance for global crop pollination are often ambiguous (Ghazoul, 2005) and data sources unclear (Klein et al., 2007). The major 'calorie crops'; wheat, rice and maize, do not require insect pollination. However, 75% of the world's leading food crops show increased seed or fruit set with insect pollination (Klein et al., 2007). On an individual crop basis, bees can even improve yield of self-pollinating species, with honeybee pollination of coffee crops (*Coffea Arabica*) boosting yield by >50% (Roubik, 2002). This suggests that the value of pollination services could be underestimated in some cases. A substantial economic value has been placed on pollination as an ecosystem service to global agriculture, with estimates ranging from $153 billion USD to $577 billion USD (Cairns et al., 2017; Gallai et al., 2009; Lautenbach et al., 2012) worldwide, amounting to circa 9.5% of the world value of agricultural production per annum (Gallai et al., 2009).

1.1.2 Bees: diversity and scope

There are over 25,000 known species of bee (superfamily: Apoidea), however, the sociality of bees varies greatly, from solitary species to colonies with thousands of individuals. The most socially advanced or 'eusocial' species, such as *Apis* (honeybees) and Meliponinae (tropical stingless bees) are all found within the Apidae family. Groups such as the bumblebees (*Bombus* spp.) (also Apidae) are considered 'primitively social', due to their comparatively less complex social organisation. Nonetheless, the social bees are in the minority, with the majority of bee species being solitary.

1.1.3 Apis mellifera: importance for pollination and research

The Western honeybee, *Apis mellifera*, is considered the most economically important crop pollinator globally (Abrol, 2012; Calderone, 2012; Kevan, 1999), playing a role in the pollination of 66% of the world's top 1500 crop species (Roubik, 2002). Human domestication and management of *A. mellifera* has resulted in wide spread global proliferation, with a current distribution across all continents other than Antarctica and some oceanic islands (Hung et al., 2018). The hive-structure and large number of workers within *A. mellifera* colonies makes them adept agricultural pollinators as they can be easily reared in large numbers and transported to key crop pollinations sites e.g. orchards, as and when needed (Abrol, 2012).

Due to its diverse range of behaviours, the honeybee is a useful model organism to explore bee learning and memory, a topic which has been intensively studied for many decades (Frisch, 1967; Erber and Menzel, 1972; Menzel, 2001; Farina, Grüter and Díaz, 2005; Menzel et al., 2005; Menzel and Giurfa, 2006; Giurfa, 2007; Galizia, Eisenhardt and Giurfa, 2012; Giurfa and Sandoz, 2012; Zhang et al., 2012). However, this concentration on honeybees has resulted in a literature database with a distinct bias, overlooking other important pollinator species, particularly wild bees which make up the majority of bee species on earth (Danforth et al., 2019).

1.1.4 Non-Apis bees: importance for pollination and research

The economic value of pollination is often mistakenly attributed entirely to honeybees (Parker et al., 1987). Wild bee species contribute significantly to crop pollination (Garibaldi et al., 2013, 2011; Kremen et al., 2002; Morandin and Winston, 2005), even enhancing honeybee pollination of certain crops (Greenleaf & Kremen, 2006; Garibaldi et al., 2013; Nicholson & Ricketts, 2019) and providing a buffer against the potential loss of honeybees (Winfree et al., 2007).

Wild species (e.g. bumblebees and solitary bees) often provide unique pollination services, pollinating crops inaccessible to honeybees (Buchmann, 1983; Larson and Barrett, 1999). For example, bumblebees are superior pollinators of many fruit crops, including raspberry (*Rubus idaeus*) (Willmer et al., 1994), tomato (*Solanum lycopersicum*) (Koppert B.V., 2017) and blueberry (*Cyanococcus* spp.) (Javorek et al., 2002). An *A. mellifera* worker would have to visit a blueberry flower four times to collect the same amount of pollen as a single visit from a bumblebee (Javorek et al., 2002).

Morphological, physiological and behavioural specialisations of bumblebees facilitate crop pollination advantages. Flower morphology restricts honeybee access to some crop species; however, bumblebee species have highly diverse body sizes and proboscis lengths (Winter et al., 2006), allowing them to access and provide pollination services to a wide range of plant species. The Red clover (*Trifolium pratense*), for example, has deep floral corollas which cannot be accessed by honeybees and therefore the species relies upon long-tongued bumblebees, e.g. *Bombus hortorum*, for pollination (Free, 1993). Bumblebees can also carry more pollen per hair (Willmer et al., 1994), pollinate more flowers per bee (Goulson, 2010) and forage for longer hours (Willmer et al., 1994; Winter et al., 2006) and in less favourable weather conditions (Garibaldi et al., 2013; Winter et al., 2006) than their honeybee counter parts. This is due in part to their larger body size and increased density of body hairs, allowing them to continue foraging in temperatures as low as 2°C and as high as 32°C (Winter et al., 2006), unlike honeybees who become largely inactive below 16°C (Heinrich, 2004). Furthermore, approximately 6-8% of angiosperms (including many of the Solanaceae e.g. tomatoes) have poricidal anthers which dehisce via small pores, limiting pollen removal (Buchmann, 1983). Release of pollen requires vibration of the indirect flight muscles at a high

frequency, an ability to sonicate or 'buzz pollinate' which bumblebees possess (Larson and Barrett, 1999). As well as providing wild pollination services to agriculture, bumblebees have been commercially produced for pollination for several decades now (shipped globally since the 1980's (Goka et al., 2001)), generating an estimated $1.25 billion (USD) in pollination services annually in the USA alone (Ghazoul, 2005).

Solitary bees are also known to be valuable crop pollinators and are increasingly reared commercially (Freitas and Pereira, 2004). Pollination supplementation with the solitary bee O*smia bicornis* (Megachilidae) leads to earlier fruit set in commercial cherry orchards than pollination by wild bees alone (Ryder et al., 2020) and *Megachile rotundata* (the alfalfa leaf cutting bee) is commercially reared for alfalfa pollination in the US and Canada due to its superior pollination abilities (over *A. mellifera*) (Osborne and Free, 2003). The *Medicago sativa* crop (Lucerne seeds) also relies heavily on *M. rotundata* pollination, as these bees are better adapted to reach the flower's pollen and nectaries (Delaplane and Mayer, 2000).

1.2 Bumblebee life histories

1.2.1 Meet the bumblebees

There are approximately 250 bumblebee species (Hymenoptera: Apidae: *Bombus*) worldwide (Michener, 2007). The UK has 24 species, with only eight found commonly (Michener, 2007). *Bombus terrestris* (Linnaeus, 1758), the 'buff-tailed bumblebee', is native to the western Palaearctic region (CABI, 2019). *B. terrestris* is by far the most studied non-*Apis* bee and is an important commercial pollinator. *B. terrestris* is a polylectic species, which visits a great number of plant species (for example, 309 reported plant species in France and Belgium (Rasmont, 1988), 570 in Poland (Ruszkowski, 1971) and 62 in Turkey (Özbek, 1997)), making them good generalist pollinators. Two subspecies of *B. terrestris* are found in the UK; *B. terrestris audax* and *B. terrestris dalmatinus* (Rasmont et al., 2008).

B. terrestris is increasingly used in laboratory and field studies as a model wild pollinator species to investigate topics such as pollination ecology, immunology, social behaviour, learning and memory, parasitism and reproduction (Billiet et al., 2016; Bloch and Hefetz, 1999; Gumbert, 2000; Leadbeater and Chittka, 2007; Müller, 2011; Piiroinen and Goulson, 2016; Wintermantel et al., 2018).

1.2.2 Bumblebee life cycle

Bumblebees have an annual lifecycle (Figure 1.1), in which only newly emerged queens survive into the following year (Alford, 1975; Heinrich, 2004). The new queens mate, usually during a single mating flight (for example, *B. terrestris* and *B. lucorum*) (Baer et al., 2003) and then seek out hibernation sites to over-winter in a state of diapause (Alford, 1975).

Emergence timing of queens varies across species from late winter to early spring (Goulson, 2010). Emerged queens begin searching for appropriate nest sites, often underground in disused rodent burrows or existing holes (Goulson, 2010). Queens forage to collect pollen and nectar which they provision in nectar pots and a pollen clump, into which they lay an initial batch of eggs (approximately eight to sixteen) (Goulson, 2010; Heinrich, 2004). Eggs are incubated at 30-32°C until they hatch into larvae (approximately four days) (Heinrich, 1972a, 1972b). Bumblebee larvae have four instars. It takes 10-14 days for larvae to develop, produce a cocoon and pupate. In a further 14 days pupae hatch, making the total development time from larvae to adult approximately 4-5 weeks (Goulson, 2010). Although, this is dependent on food supply and nest temperature (Alford, 1975). New workers take over the responsibilities of food provision and brood care for the hive. Towards the end of the season (April – August), if colonies attain a sufficient size, new reproductives (queens and males) are produced (Alford, 1975).

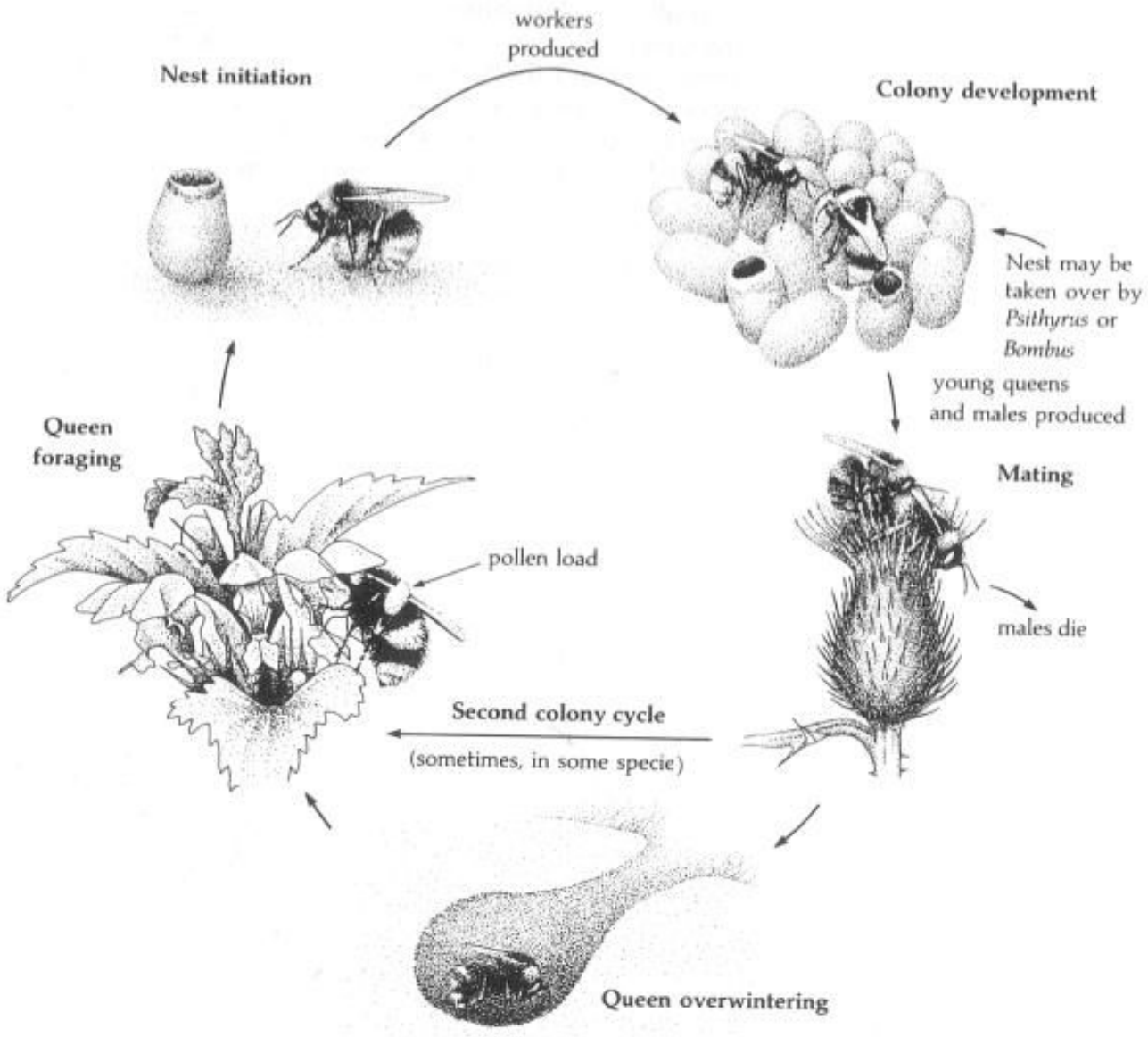

Figure 1.1: Bumblebee lifecycle. Taken from Prys-Jones and Corbet (2011).

1.2.3 Bumblebee colony life & foraging

Bumblebee colony life is markedly less organised than the comparative uniform regimen of the honeybee hive. Bumblebee nests have an almost 'accidental' appearance, with honey pots and pollen pupal cells arranged in clumps across the centre of the nest (Figure 1.2).

Figure 1.2: A mature B. terrestris colony settled in a nest box displaying honey pots, pupal and larval cells and workers © Laura James.

Despite their less advanced sociality, and workers being generalists (no defined task allocation as in honeybees or ants), bumblebees regularly exhibit complex learning and behaviours such as nest homeostasis, between forager communication, and rapid sensory learning.

Temperature control

Nest homeostasis allows hives to thermally regulate the brood to ensure normal development (Alford, 1975; Heinrich, 2004). Nest temperature is tightly regulated, with nests having a remarkably stable temperature of 30 ± 1°C (Goulson, 2010). Temperature fluctuations are controlled through either increased incubation or fanning of the brood (Vogt, 1986). Collective nest homeostasis provides further metabolic benefit to foragers, as it allows flight temperature to be easily attained (bumblebees cannot take off if muscle temperatures are below 30°C (Heinrich, 2004)). Contrastingly, solitary foragers may not be able to forage until later, giving social species a competitive resource gathering advantage (Goulson, 2010).

Efficient foragers

Bumblebees depend exclusively on pollen and nectar for their nutrient intake. Pollen is a rich protein source and nectar provides sugar and water for adult energy expenditure. Acquiring these nutrient rich floral rewards requires efficient foraging behaviours. Foraging can be problematic and unpredictable as floral rewards temporally change, since floral species produce nectar at different times throughout the day (Waser, 1982) and are depleted by rivals and conspecifics (Pleasants and Zimmerman, 1979), meaning that many flowers may actually be empty (Waser and Mitchell, 1990). Bumblebees must also learn how to handle flowers with complex structures to gain hidden floral rewards (Heinrich, 1976). Forager workers must therefore continually make efficient decisions between flowers with a plethora of colours, shapes, abundance and rewards (Goulson, 2010). Flight is particularly costly for bumblebees, which are reported to have one of the highest metabolic rates recorded in any organism (Goulson, 2010), making efficient foraging particularly important.

Bees have been shown to have innate preferences for colours in the wavelengths 400-420nm (near ultraviolet, violet and blue on the human visible light spectrum) and 510-520nm (green – human visible light spectrum) (Lunau, 1990), corresponding to bee trichromatic vision which is centred on green, blue and ultraviolet photoreceptors (Peitsch et al., 1992). However, foragers can readily overcome these innate colour preferences with floral reward experience (Gumbert, 2000). Bees are remarkable learners, utilising shape, colour and scent, both in isolation and combination, to identify previously rewarding flower species and achieve rapid sensory learning (Menzel and Erber, 1978).

1.3 Bee declines

1.3.1 The Insect 'apocalypse'

Insects are the most abundant and speciose animal group, making up approximately two thirds of all land-dwelling species (Sánchez-Bayo and Wyckhuys, 2019; Thomas et al., 2004). Insects are declining on a global level, and at markedly quicker rates than those observed for vertebrates or plants (Dirzo et al., 2014; Sánchez-Bayo and Wyckhuys, 2019; Thomas et al., 2004). A long term population monitoring study in 2017 discovered a 76% decline in flying insects in protected areas in Germany over a 27 year time frame (Hallmann et al., 2017). A further study in Puerto Rico details biomass losses of 78-98% for arthropods during a 36 year period (Lister and Garcia, 2018). A more recent review (73 studies) of the drivers of insect decline concluded that >40% of insect species are at threat of extinction and that the Hymenoptera (bees, wasps, ants and sawflies), the Lepidoptera (moths and butterflies) and the dung beetles (Coleoptera) are most at risk (Sánchez-Bayo and Wyckhuys, 2019).

1.3.2 Bee decline

The decline of insect pollinators has being noted on a global level (Biesmeijer et al., 2006a; Bommarco et al., 2012; Cameron et al., 2011; Potts et al., 2016; Sánchez-Bayo and Wyckhuys, 2019; Vanbergen, 2013) and requires thorough investigations of the underlying drivers of this decline (Gill et al., 2016; Goulson et al., 2015). Both wild and managed bee species are facing global declines (Biesmeijer et al., 2006b; Potts et al., 2010; vanEngelsdorp et al., 2008) and these declines are often mirrored by a decline of the plant species which they pollinate (Biesmeijer et al., 2006a). Of particular concern are wild pollinator species, which are inherently harder to monitor and study than managed species such as the Western honeybee (*A. mellifera*). A multitude of biotic and abiotic stressors in the agricultural environment could be leading to these declines, such as, habitat loss and fragmentation, pesticides, pathogens, parasites and climate change.

1.3.3 Bumblebee decline

Globally, there has been a predicted 30% decline in bumblebee species since the 1870s (Wilson-Rich et al., 2014). Of the 27 bumblebee species originally found in the UK, three, the

Cullum's bumblebee (*Bombus cullumanus*) (1941), the Short-haired bumblebee (*Bombus subterraneus*) (1988) and the Apple bumblebee (*Bombus pomorum*) (1864), have become extinct in the last 150 years (Wilson-Rich et al., 2014) and most remaining species are declining (Biesmeijer, Roberts, Reemer, Ohlemuller, et al., 2006; P. H. Williams, 1982). Similarly, bumblebee declines have been reported across Europe (Kosior et al., 2007; Williams and Osborne, 2009).

In the United States (US), a meta analyses of bee fauna (438 species) over a 140 year period found that bumblebees (Bombus) were the only group to have significant species richness declines (Bartomeus et al., 2013). Additionally, four bumblebee species (*B. occidentalis, B. pensylvanicus, B. affinis, and B. terricola*) have undergone extensive range declines, becoming rare or absent in their historic ranges in the last 20 years (Cameron et al., 2011). In Asia, Japan and China have also documented declines in *Bombus* subspecies (Da-Rong, 1999; Inoue et al., 2008; Matsumura et al., 2004; Xie et al., 2008). However, although the general trend is decline, not all bumblebee species are declining and some are extending their ranges e.g. *B. terrestris* (introduced) and *B. hypnorum* (Downing and Grimwood, 2017; Goulson and Williams, 2000; Schmid-Hempel et al., 2007).

1.4 Factors implicated in the decline

1.4.1 Agricultural intensification as an umbrella term

These biodiversity declines do not appear to be slowing or abating and there is compelling evidence that they are largely anthropological in cause, with habitat loss (destruction or degradation) cited as the largest causal factor (Brown and Paxton, 2009). Other key factors are pesticide usage, climate change, invasive species, parasites and diseases (Downing and Grimwood, 2017; Krupke et al., 2012). The main causes for bumblebee declines can be categorised under the umbrella term of 'agricultural intensification'. The Green Revolution of 1940-1970 was characterised by a shift from low input farming to industrialised, intensive production as well as the introduction of large-scale monocultures, synthetic pesticide regimes and the removal of semi-natural habitats (e.g. hedgerows and woodland) to allow for large scale mechanisation improvements (Sánchez-Bayo and Wyckhuys, 2019), creating a new

suite of challenges for wild pollinators. These factors rarely act in isolation, but often as synergists, making disentangling their effects challenging.

1.4.2 Habitat loss and change

A primary driver of pollinator decline is habitat loss due to intensive agricultural conversion (Brown and Paxton, 2009; Winfree *et al.*, 2009; Goulson, Nicholls, Botías and Rotheray, 2015; Sánchez-Bayo and Wyckhuys, 2019). Globally, intensive farming is synonymous with simplified crop environments, large monocultures and the removal of semi-natural edge habitats, e.g. hedgerows, field boundaries, buffer zones and woodland, to facilitate mechanisation (Tscharntke et al., 2005). Urbanization and agricultural expansion had claimed 30-50% of global land surface by the end of the 20th century (Vitousek et al., 1997) and the UK has lost a predicted 97% of its flower-rich grassland since the 1930s (Howard et al., 2003). Habitat loss and fragmentation results in reduced bee foraging resources and nesting sites and division of local populations, and consequently, a reduction in genetic and demographic mixing (Winfree et al., 2009). Landscape heterogeneity protects wild bees against increasing temperature variability (Papanikolaou et al., 2017) and bumblebee lineage survival increases significantly in areas with high value floral habitats (Carvell et al., 2017). Land use changes such as these appear to be particularly detrimental for bumblebees and wild bee species (Williams and Osborne, 2009), perhaps due to their traditional reliance on semi-natural habitats for nesting and overwintering sites (Bartual et al., 2019).

1.4.3 Parasites and disease

Although bees naturally encounter a range of parasites, parasitoids and diseases (including viruses, bacteria and fungi), human management of bee colonies has led to accidental and increased spread of parasites and disease among bees (Colla et al., 2006; Goulson et al., 2015; McGivney, 2020). For example, the spread of the parasitic mite *Varroa destructor* from *Apis cerana* (Asian honeybee) to *A. mellifera* (European honeybee). The *Varroa* mite is an external parasitic mite which attacks and feeds on the fat bodies of larvae, pupae and adults, laying eggs on larvae as they develop (Ramsey et al., 2019). *Varroa* is also a vector of bee diseases such as deformed wing virus (DWV), playing a role in colony collapse (Nazzi et al., 2012; Rosenkranz et al., 2010). The combined effect of these two stressors has led to major *A.*

mellifera colony losses in Europe and North America (Nazzi et al., 2012; Rosenkranz et al., 2010). Fortunately, *Varroa* does not seem to be able to parasitize bees outside of the *Apis* genus (Goulson, Nicholls, Botías and Rotheray, 2015). Honeybees are also commonly infected by the highly virulent fungal pathogen *Ascosphaera* (causing chalkbrood disease) (Aronstein and Murray, 2009) and the microsporidian *Nosema ceranae* (Morse and Flottum, 1997).

Wild bees have not avoided the spread of *N. cerana*, which has now been reported in wild bumblebee species in China, South America and Europe (Graystock et al., 2013; Li et al., 2012; Plischuk et al., 2009). *N. cerana* is also more virulent in bumblebees than in its original honeybee hosts (Graystock et al., 2013) and there is an absence of knowledge for other wild bee species (Goulson, Nicholls, Botías and Rotheray, 2015). Bumblebees are also affected by their own *Nosema* species; *Nosema bombi*, which is demonstrated to have strong effects on fitness (Meeus et al., 2011; Otti and Schmid-Hempel, 2007). Commercial bumblebee colonies are regularly infected with *N. cerana*, *N. bombi* and DWV, as well as the parasite *Apicystis bombi,* which can then be passed to wild bee species with devastating impacts (Goka et al., 2001; Graystock et al., 2013; Tian et al., 2018). The increasing incidence of disease and parasite spill-over from managed colonies to wild species (Evison et al., 2012; Genersch et al., 2006; Klee et al., 2007; Li et al., 2012; Mcmahon et al., 2015; Plischuk et al., 2009) is highly concerning for wild bee declines and conservation.

1.4.4 Climate change

Climate change is resulting in increased weather extremes, including temperature, drought, early snow melt and precipitation events. A meta analyses of long term data for 66 bumblebee species across Europe and North America found that increasing frequency of hotter temperatures (as caused by climate change) was the core predictor for bumblebee species extinction risk, and that this effect was present irrelevant of land use changes (Soroye et al., 2020). Species generally shift their ranges latitudinally northward in response to climate change (at a median rate of 16.9 km per decade) (Chen et al., 2011), potentially leading to spatial mismatches between pollinators and flowers (Schweiger et al., 2010). However, evidence suggests bumblebees in Europe and North America are not shifting their ranges, resulting in rapid reductions in their populations in southern ranges (Kerr et al., 2015) and placing them at increased risk of rapid species declines. Climate change can also lead to

temporal asynchrony between bees and flowers (Schweiger et al., 2010); this is not only potentially disastrous for bees (by reducing floral resources by 17-50%, less time to forage, early emergence (Memmott et al., 2007)), but can increase the risk of pollination deficits for important crop species (Polce et al., 2014).

1.4.5 Historic pesticide usage

Globally, modern agriculture relies on the systematic and widespread usage of synthetic pesticides to control weeds (herbicides), fungal pathogens (fungicides) and crop pests (insecticides). The biggest worldwide increase in pesticide usage was driven by the synthesis of new synthetic pesticides e.g. nematocides, rodenticides, avicides and herbicides (Mrak, 1984). The organochlorines, e.g. dichloro-diphenyl-trichloroethane (DDT), were introduced post WWII and quickly became popular insecticides. DDT was the most effective insecticide ever synthesized, with reports of low human toxicity, high insect toxicity and persistent action (Davis, 2014). These new synthetics greatly increased agricultural productivity. Although, in laboratory studies, toxicity initially appeared low, DDT and other chlorinated hydrocarbons were found to bioaccumulate in ecosystems, reaching highly toxic levels in top line predators (Carson, 1962). Rachael Carson (1962) presented a strong critique of the indiscriminate use of insecticides (particularly DDT) that resulted in widespread ecological contamination and detrimental effects on invertebrates and vertebrates across the USA. Consequently, the organochlorines were banned for agricultural usage in many countries.

However, this ban did not reduce the use of insecticide products for intensive agricultural production, and the introduction of the organophosphates (1960s), carbamates (1970s) and pyrethroids (1980s) contributed greatly to agricultural pest control and improved crop yields for the remainder of the 20th century (Aktar et al., 2009). The organophosphates garnered interest due to their ability to control aphid pests, which DDT could not (Russell, 2001) and the carbamates due to their low mammalian toxicity (unlike the organophosphates) (Davis, 2014). The emergence of organophosphates and carbamate resistant pests, and cataloguing of negative environmental impacts, vastly reduced the palate of acceptable insect control tools available to agriculture, and the need for new synthetic insecticides continued to grow. Synthetic pyrethroids (e.g. permethrin) have high insecticidal action (permethrin LD_{50} 0.7mg/g for insects), low mammalian toxicity (permethrin LD_{50} exceeds 1000mg/Kg), rapid

soil degradation and lower recommended application rates, suggesting less risk of environmental contamination (Cornell University 1993). The relative safety profile and efficacy of pyrethroids however led to their prophylactic application, and insecticide resistance has emerged to the majority of these compounds.

Newer, targeted, smarter pesticides (e.g. the neonicotinoids, diamides) seemed to herald a new era whereby the ecological tragedies of the likes of the DDT should no longer be repeated. Nonetheless, 50 years since the ban of DDT, ecological evidence is still mounting against insecticides. Reports suggest cases of both acute and chronic pesticide poisonings in humans, ecosystems and wildlife are still common across the world (Davis, 2014).

1.5 Neonicotinoids

1.5.1 Introducing the neonicotinoids

Introduced in the 1980's, the neonicotinoids are a newer class of insecticides which are highly toxic to insects (Tomizawa and Casida, 2005), particularly sucking and chewing herbivorous pests (Jeschke et al., 2011). The group comprises nitenpyram (Sumitomo), acetamiprid (Nippon Soda), thiacloprid (Bayer CropScience), imidacloprid (Bayer CropScience), thiamethoxam (Syngenta), clothianidin (Bayer CropScience and Sumitomo) and dinotefuran (Mitsui Chemicals) (Jeschke et al., 2011). Neonicotinoids are systemic, allowing their translocation into all tissues of treated plants, making them potentially toxic to any insect which feeds upon it (Bonmatin et al., 2015). Molecular structures of the seven neonicotinoids can be found in Figure 1.3.

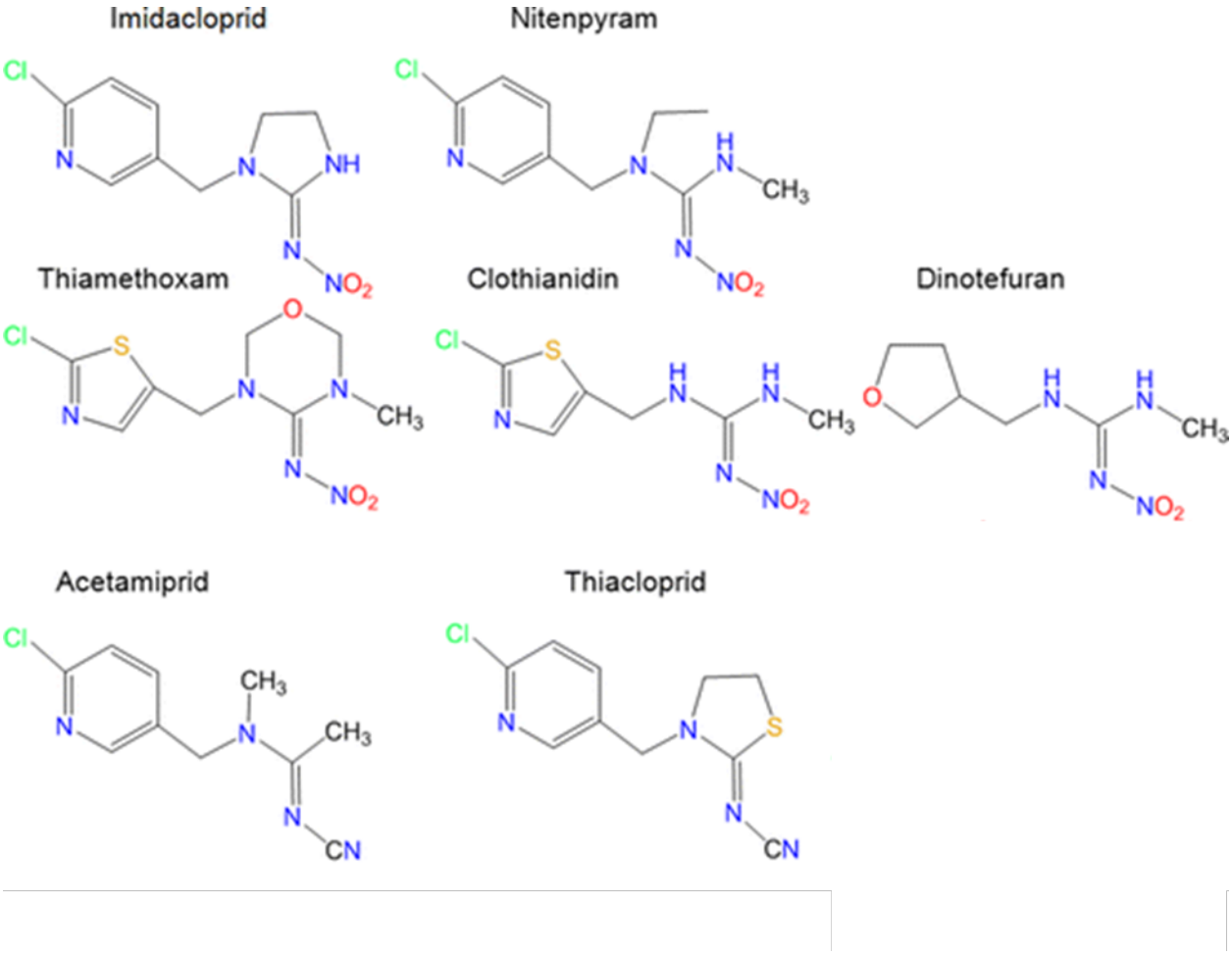

Figure 1.3. Molecular structures of the neonicotinoids. Taken from Simon-Delso et al. (2015).

1.5.3 Molecular targets and mode of action

Neonicotinoids have a similar structure to nicotine, making them potent antagonists to nicotinic acetylcholine receptors (nAChRs) in the central nervous system (Tomizawa and Casida, 2005). Treatment with high dosages of neonicotinoids leads to receptor overstimulation and blockage, and ultimately, paralysis and death (Matsuda et al., 1998). They show low acute and chronic toxicity to birds, fish and mammals (Tomizawa and Casida, 2005). This is due in part to their low affinity for vertebrate nAChRs relative to insects (Tomizawa and Casida, 2009, 2005, 2003) and is responsible for their appealing toxicological profile and their current status as the most widely used insecticides in the world (Goulson, 2013; Jeschke & Nauen, 2008; Simon-Delso et al., 2015). Neonicotinoids target insect nAChRs, known to be localised on the antennal lobes and mushroom bodies in the bee brain (Bicker,

1999; Kreissl and Bicker, 1989), making it possible that neonicotinoids could affect learning and memory.

1.5.4 Application methods

In developed countries the neonicotinoids clothianidin, imidacloprid and thiamethoxam have been most widely used as seed dressing (>91% of usage in agriculture) on cereal, oilseed rape, potatoes and beet crops (DEFRA, 2012). However, neonicotinoids can also be applied as foliar sprays, seedling dips, seed pilling, trunk injections, soil treatments and dredges, granular formulations, chemigation and as bait formulations (Simon-Delso et al., 2015).

1.5.5 Primary routes of exposure to bees

Much of the controversy around the pesticide debate focuses on the potential impacts to bees. Route of exposure may be key to degree of impact and foraging bees may be exposed to pesticide residues simultaneously through a multitude of mechanisms (Krupke et al., 2012). Bees can come into direct contact with pesticide residues through foliar sprays and beehive applications (contact exposure) (Greig-Smith et al., 1994) or indirect contact via feeding on contaminated nectar, pollen or water sources (guttation droplets) (oral exposure) which are collected and returned to the hive (Bonmatin et al., 2015; Girolami et al., 2009). Oral ingestion is the most toxic route of exposure in the honeybee (Mengoni Goñalons et al., 2015) with LD_{50} values for ingestion of clothianidin and imidacloprid recorded at 4-5ng per honeybee, a dosage which is 1/10,000th of the LD_{50} for DDT (Suchail et al., 2000).

Neonicotinoids have been used as seed coats on popular bee forage plants such as oil seed rape, sunflower and maize. The systemic nature of the compounds results in residues in both nectar and pollen of seed-treated crops (Goulson, 2013). Research suggests that systemic compounds are selectively transported into the nectar, reaching much higher concentrations than other plant parts (Davis, Shuel, & Peterson, 1988). Contaminated nectar and pollen are likely to be the most important route of exposure to social bees due to the large volumes collected and consumed by bees and their brood.

Foliar neonicotinoid sprays on fruit crops may result in exposure during foraging (DEFRA, 2012). Management practices have attempted to reduce pollinator exposure by spraying early in the morning or evening to avoid key foraging times. However, these practices largely

avoid honeybee foragers, but not necessarily wild bee species such as bumblebees, which are most active at these times (Goulson, 2010).

Residue reports in pollen and nectar vary widely and should be treated with some caution. Generally, reported residues in nectar (ranging from <1 to 8.6ppb across 20 studies, EFSA 2012) are lower than those in pollen (ranging from <1 to 51 ppb across 20 studies, EFSA 2012) and seem to be highest when crops are treated with foliar sprays (Dively and Kamel, 2012).

Spray drift provides an additional route of potential exposure to foraging bees. Drift of pesticide residues can lead to weed or wild flower contamination in field margins adjacent to treated fields (David et al., 2016; Greig-Smith et al., 1994), resulting in floral resource contamination despite spray management practices e.g. early or late spraying. The sowing of seed involves 'seed drilling', a further potential exposure route. Drilling produces dust from neonicotinoid treated seeds which can drift off-target and contaminate nectar, pollen and water sources, resulting in honeybee mortality (Greig-Smith et al., 1994; Nuyttens and Verboven, 2015; Pistorius et al., 2008).

Cavity and ground-nesting solitary bee species (e.g. *Megachile* and *Osmia* genera) are likely to experience the agricultural environment entirely differently to managed, social species (e.g. *A. mellifera*), and therefore face potentially different exposure routes e.g. through contaminated soils (Anderson and Harmon-Threatt, 2019). Key biological, physiological and ecological differences e.g. habitat location, nesting behaviour, immune response and detoxification mechanisms (Kapheim et al., 2015) can result in differential routes and intensities of exposure of solitary bees to pesticide compounds (Heard et al., 2017; Hooven et al., 2014).

As well as exposure route (oral or contact), temporal exposure period (acute or chronic) must also be considered in pollinator risk assessments. Acute exposure occurs in a single event e.g. forager consumes nectar from a contaminated flower and receives a single pesticide dosage. These effects can be compounded when multiple routine exposures occur over a longer time period (chronic) e.g. when regularly foraging on a contaminated mass flowering crop. Garbuzov *et al.* (2015) suggest that acute exposure events are most likely to occur, as landscapes usually provide a multitude of pollen and nectar foraging options, reducing the likelihood of exclusive foraging on a contaminated crop, a fact which is often overlooked in

exposure studies. Nonetheless, species such as bumblebees demonstrate high patch constancy, repeatedly visiting the same floral cluster (Woodgate et al., 2016) or specialising on a select floral species (Russell et al., 2017). This could potentially lead to chronic field exposure if bees are specialising on treated patches or species.

1.5.6 Impacts on bees: mortality

The evolution of toxicological study was borne from a need to assess the public health risks of chemical compounds prior to their registration. Entomological toxicology aims to measure a compound's effectiveness on target insects and, increasingly, to determine potential impacts to non-target beneficials prior to agrochemical licensing.

Pesticide Environmental Risk Assessments (ERAs) follow a tiered structure in which compounds move from Tier 1 to Tier 3 assessments only if they do not pass the benchmark for environmental safety set in the previous tier (Sánchez-Bayo and Tennekes, 2017). Tier 1 are laboratory-based toxicological assessments which consider acute lethality, utilising LD_{50} or LC_{50} parameters, which allows determination of the pesticide dose/concentration for which 50% of a population die (median lethal dose) (Surber, 1946). A low LD_{50} value indicates a highly toxic compound. These toxicological endpoints signify acute toxicity and mortality over a relatively short time period (usually a maximum of 96 hours) and are often unrepresentative of field exposure scenarios (Sánchez-Bayo and Tennekes, 2017). Experiments are designed to calculate a hazard quotient (HQ) (EPA, 1999), which if above 0.1, compounds progress to Tier 2 testing. Tier 2 assessments involve more field realistic conditions, e.g. semi-field trials and across other agronomically important groups e.g. *Bombus.* Compounds which progress to Tier 3 are tested in full field trials. These tiered testing regimes (particularly Tier 1) inherently assume that the sensitivity of a single model species (usually *A. mellifera*) can be extrapolated to represent cross-species sensitivity (Sánchez-Bayo and Tennekes, 2017, 2015), creating potential oversight of differential effects across bee species which may have very different ecological and physiological profiles e.g. solitary bees. If pesticides pass the tiered regulatory system, they are usually registered for a period of 10 years with restrictions only placed on a single product or crop basis. This approach has been criticised in favour of a systems-wide approach which instead takes into account the

spatiotemporal application of mixtures and sequential treatments of compounds which commonly occurs in the agricultural landscape (Topping et al., 2020).

Chronic exposure studies are usually only conducted in mammal tests (due to their relevance for human health), creating a historic deficit for chronic, field realistic, toxicology data for non-target invertebrates e.g. bees, at the Tier 1 testing level. In the last five years, regulatory bodies appear to be heeding calls to address this deficit, with chronic testing of honeybee foragers now being conducted for periods of 10 days in the laboratory (Hesketh et al., 2016; OECD, 2016).

Meta analyses have reported no significant lethal effects of field-realistic doses of imidacloprid on honeybees across 13 laboratory and semi-field conditions studies (Cresswell, 2011) and in the existing research, there appears little evidence for direct mortality effects on honeybees and bumblebees from neonicotinoids in the field. For example, using the honeybee LD_{50} value for imidacloprid oral toxicity (Suchail et al., 2000), an individual would have to consume approximately 2.6ml of nectar or 1g of pollen to reach this dosage (Goulson, 2013). Given the weight of the average worker (~0.1g) this seems highly unlikely and lethal effects could be easily dismissed.

ERAs have been criticised for being 'out of step' with current scientific knowledge, due to their disregard of factors which have intensified in the agricultural environment in recent years, e.g. habitat loss and homogeneity and climate change (Topping et al., 2020), and their limited study of sublethal effects within Tier 1 assessments (Sánchez-Bayo and Tennekes, 2017). Current regulatory assessments may therefore be unrepresentative of the threats faced by non-target organisms in a modern agricultural environment.

1.5.7 Impacts on bees: sublethal

The study of sublethal effects is fairly new, as traditionally ERAs have focused on mortality measures e.g. LD_{50}s. The number of studies on bees has grown rapidly in the last decade but remains largely focused on *A. mellifera* and imidacloprid (Blacquière et al., 2012; Cresswell, 2011; Laycock, 2014; Lundin et al., 2015; Siviter et al., 2018b; Walters, 2013). A review of 543 studies conducted on sublethal effects of neonicotinoids on bees found that 78% of studies utilised imidacloprid, 34% thiamethoxam, 33% clothianidin, 19% acetamiprid, 18%

thiacloprid, 7% dinotefuran and just 6% nitenpyram, highlighting the literature's weighting towards imidacloprid, thiamethoxam and clothianidin in the study of bee detrimental effects (Lundin et al., 2015). Similarly, a metanalyses of 771 neonicotinoid studies published in the five years prior to 2014 found the majority concerned imidacloprid (Laycock, 2014).

Due to the economic importance of honeybees, non-*Apis* bees, such as bumblebees and solitary bees are largely overlooked in sublethal testing, creating a considerable gap in our knowledge base (Muth and Leonard, 2019; Siviter et al., 2018b). Sublethal effects are inherently harder to observe than direct mortality, making their study more difficult in both the field and the laboratory. Nonetheless, individual sublethal effects can have crucial knock on impacts when scaled up to the hive and landscape scale and their study is pivotal in our understanding of off-target pesticide impacts.

Detrimental sublethal-effects have been reported across a range of bee parameters e.g. on foraging behaviours (Mommaerts et al., 2010; Ramirez-Romero et al., 2005; Yang et al., 2008), feeding (Brown et al., 2017), navigation (Henry et al., 2012) and learning and memory (Mengoni Goñalons et al., 2015). The relevance of these studies to bee and / or colony survival following pesticide exposure are considered below.

Foraging behaviour, navigation and homing success

Feeding and nectar intake are essential to bee foraging activity as flight is energetically costly (Visscher et al., 1996). One study found that field realistic doses (5.32 ppb) of thiamethoxam reduced feeding in two bumblebee species, *Bombus pratorum* and *Bombus pascuorum,* implying potential repellency or toxicity effects (Brown et al., 2017). However, the same study did not observe a feeding reduction in two other species (*B. lucorum* and *B, terrestris*) (Brown et al., 2017), suggesting highly species-specific effects. Dietary ingestion of imidacloprid was shown to reduce feeding rate and locomotor activity in worker bumblebees (*B. terrestris*) (Cresswell et al., 2014b, 2012); however, it should be noted that this was at a high dosage (100ppb), making field effects unlikely. Imidacloprid also reduced motivation to forage, volume of nectar collected and foraging bout initiation in *B. impatiens* (Muth and Leonard, 2019).

Exposure to some neonicotinoids has also been found to reduce homing ability and affect flight and foraging performance in a number of bumblebee studies. Kenna *et al.* (2019)

established that realistic, acute imidacloprid exposure (10 ppb) affected flight performance in *B. terrestris* (Kenna et al., 2019). The study demonstrated initial increases in forager flight velocity over the first 3/4km, followed by marked decreases in flight duration and distance, suggesting potential detrimental consequences on bumblebee foraging range and floral resource access (Kenna et al., 2019).

An early study on bee foraging behaviour demonstrated that sublethal effects can drastically impact forager survival if homing ability is impaired (Henry et al., 2012). When individual honeybees (653 individuals) were treated with 1.34 ng/bee thiamethoxam and released away from their colony (tracked using radio frequency identification (RFID) tags), between 10 to 30% of the foragers failed to return to the colony per day (Henry et al., 2012). Stanley et al. (2016) also employed RFID tags to track foraging bumblebees chronically exposed (5-43 days) to thiamethoxam (2.4ppb). The authors found that bumblebees from the treated colony were more likely to return to the hive when released 1km away, but this observation was lost when bees were released at 2km. They suggest this effect may have resulted from enhanced orientation experience during the longer foraging bouts conducted by treated bees. They also reported that thiamethoxam exposure was detrimental to pollen collection and led to longer foraging bouts (Stanley et al., 2016).

Although less well researched, thiacloprid has also been reported to have detrimental effects on foraging. Tison et al. (2016) chronically exposed (several weeks) honeybee foragers to 5.4ppb thiacloprid, and found that foraging, navigation and homing success were detrimentally affected (Tison et al., 2016). This is concerning given that thiacloprid is widely acknowledged to be less toxic to bees (Iwasa et al., 2004; Pisa et al., 2015).

Experiments have also demonstrated neonicotinoid impact on bee motor function, with differing results. Williamson et al. (2014) found that honeybees which were chronically fed (24 hours) 2–3 ppb doses of imidacloprid, thiamethoxam or clothianidin demonstrated reduced postural control and increased cleaning behaviours. Acute doses of imidacloprid (10ppb and 1.25 ng/bee) resulted in hyperactivity (Kenna et al., 2019; Lambin et al., 2001) and really high chronic doses (100-500ppb) reduced bee activity and increased immobility (Medrzycki et al., 2003; Williamson et al., 2014). Williamson et al. (2014) suggest that the reduced homing and foraging abilities seen in response to neonicotinoid exposure could be as a direct result of compromised motor functions (like those reported above).

Learning and memory

Laboratory trials have been used to examine fine scale effects of the neonicotinoids on bee learning and memory. Chronic exposure to imidacloprid significantly impairs short-term aversive learning by 87% and reduces memory retention by 85% in honeybees (Zhang & Nieh, 2015). Imidacloprid also impairs associative learning (Mengoni Goñalons et al., 2015), visual learning (Han et al., 2010), and olfactory learning and memory (Decourtye et al., 2004; Williamson and Wright, 2013) in honeybees. In bumblebees, olfactory learning speed and short-term memory was impaired by 2.4 ppb thiamethoxam exposure, but ability to learn was not (Stanley et al., 2015).

The majority of sublethal cognition studies are on honeybees, consider a single learning modality (olfactory learning), and utilise a single learning protocol (the proboscis extension response (PER)) (Muth et al., 2019; Muth and Leonard, 2019). However, Muth et al. (2019) demonstrated that imidacloprid impaired scent (olfactory), but not colour (visual) learning in *B. impatiens* (Muth et al., 2019; Muth and Leonard, 2019), highlighting the importance of multi-modality learning studies and a move away from single paradigm, single species (*A. mellifera*) assays.

However, learning and memory effects are not always consistent even within species. A contrary study found that imidacloprid exposure had little effect on *A. mellifera* olfactory learning, and in fact found a modest learning and memory improvement when bees were exposed to combined treatment with coumaphos (organophosphate acaricide) (Williamson et al., 2013). Comparative studies of deltamethrin (pyrethroid), imidacloprid and the cry toxin Cry1Ab, found that honeybee foraging activity was reduced by all three compounds, but that learning capacity was only reduced by deltamethrin (Ramirez-Romero et al., 2005). Furthermore, Schmuck et al. (2001), examining lethal and sublethal impacts of imidacloprid on honeybee feeding, breeding, mortality and colony vitality reported no adverse effects in any of these categories at a concentration of 20 ppb, a higher value than the field realistic level of 1.5 ppb. However, It is important to consider mode of application, as these results (Schmuck et al., 2001) were obtained from imidacloprid seed coatings on sunflower; other crop species and application methods could produce differing results. The effect of imidacloprid on learning and memory does not seem to be permanent and is recoverable if

exposure is ceased and bees fed with clean sucrose (Williamson and Wright, 2013), potentially explaining differing experimental findings if observation periods and exposure regimes differ.

Studies have also demonstrated differential sublethal effects between honeybees and bumblebees. Piiroinen & Goulson (2016) demonstrated that, in isolation, clothianidin impairs olfactory learning in honeybees but not bumblebees (Piiroinen and Goulson, 2016). However, when treated with clothianidin and infected with the parasite *N. ceranea*, learning rate was again impaired in honeybees but bumblebees had a marginally faster rate of learning compared to controls (Piiroinen and Goulson, 2016). The authors were unclear about the potential cause of this increased olfactory learning ability, but clearly there is an interactive effect of multiple stressors, perhaps affecting motivation due to hunger as a result of parasite load. These findings are surprising, as Stanley et al. (2015) found that exposure to thiamethoxam (metabolised to clothianidin) impaired bumblebee olfactory learning (Stanley et al., 2015). Pesticide impacts on native bee pollinators clearly cannot be viewed in singularity.

Forager collection of nectar and pollen resources is essential for the energetic provision of the hive's workers, queen and developing brood, and is critical to colony growth and production of new reproductives (Crone and Williams, 2016). Therefore, it can be seen how sublethal effects on resource collection parameters e.g. foraging ability, feeding rate, motor function, may have knock-on effects on colony fitness.

Reproduction, immunity and colony fitness

Research suggests that chronic exposure to neonicotinoids can impair bee reproduction (Brown et al., 2017), and weaken bee immune systems making them more susceptible to other stressors such as parasites and disease (Di Prisco et al., 2013), contributing to potential reduction in colony fitness. In the laboratory, chronic thiamethoxam exposure results in a length reduction of terminal oocytes in queens of four bumblebee species (*B. terrestris, B. lucorum, B. pratorum*, *B. pascorum*) (Brown et al., 2017), and a reduction in brood production in *B. terrestris* (Laycock et al., 2014), suggesting possible fertility and colony fitness implications. However, the Laycock et al. (2014) study only reported these brood effects at extremely high, non-field realistic thiamethoxam concentrations (39 and 98 ppb), but this

group have previously reported similar declines in *B. terrestris* fecundity (reduction in brood production of one third) at environmentally realistic ranges (1 μg/L-1) (Laycock et al., 2012).

Reproductive and colony fitness effects persist at the landscape level (not just in the laboratory), with field and semi-field studies reporting detrimental effects on colony growth and reproduction. Imidacloprid exposure leads to slower colony growth (10 ppb, 4-week exposure (Gill et al., 2012)) and reduces queen production up to 85% in *B. terrestris* (Whitehorn et al., 2012). Rundlöf et al. (2015) show that, in a field setting, clothianidin coated seed (Elado on oilseed rape) reduced solitary bee (*Osmia bicornis*) nesting, wild bee density (bumblebee and solitary bees), bumblebee colony growth and reproduction (*B. terrestris*) (Rundlöf et al., 2015). Seed coating influenced the weight of developing commercial *B. terrestris* colonies, with markedly smaller weight changes (colony weight expected to increase as colony grows) (Rundlöf et al., 2015). Treated *B. terrestris* colonies (treated in the lab and then placed in the field) also produced significantly less reproductives (males and queens) (Rundlöf et al., 2015), putting this study in line with the findings reported in other studies (Arce et al., 2017; Gill et al., 2012; Whitehorn et al., 2012). Although there is a dearth of studies in solitary bees, a recent study reports that solitary bee exposure to imidacloprid, through field realistic contact with soil residues, affects developmental speed and adult longevity (Anderson & Harmon-Threatt, 2019).

Research suggests that thiacloprid exposure may be less detrimental to bee reproduction and colony fitness parameters. Odemer & Rosenkranz (2018) did not find a negative impact of field thiacloprid exposure (2 ppb) on the population dynamics or overwintering success of honeybee colonies, irrespective of whether applied alone or in combination with tau-fluvalinate (Odemer and Rosenkranz, 2018). However, thiacloprid has been shown to be detrimental to bee immunity, affecting honeybee sensitivity to *N. ceranae* parasitism (Pettis et al., 2012; Vidau et al., 2011).

Neonicotinoid exposure has also been linked to compromised immunity in bumblebees. *B. impatiens* pulsed exposure to 7 ppb imidacloprid led to a reduction in hemolymph antimicrobial activity even 6 days after exposure ceased (Czerwinski and Sadd, 2017). Immunity studies such as these highlight the importance of considering the multi-stressor nature of the agricultural environment in which bees are likely exposed to pesticide compounds, disease, parasites and pathogen loads simultaneously.

Neonicotinoid pesticides & bee decline: a cautionary tale?

Research linking neonicotinoid pesticide use to bee decline has been criticised for its lack of replication of realistic field exposure. Even when field-realistic doses are used, feeding can often be unnatural e.g. treated food is placed in the hive or in a preferential location (Whitehorn et al., 2012). This may be unrepresentative of foraging behaviour, as certain insects have been show to detect and actively avoid neonicotinoid (imidacloprid) residues in food sources (Easton et al., 2013).

Large-scale interspecies studies are rare in the field. A recent study (Woodcock et al., 2017) examined the impact of clothianidin or thiamethoxam seed coatings across different bee species (European honeybee, *A. mellifera*, buff-tailed bumblebee, *B. terrestris* and red mason bee, *O. bicornis*) and different countries (Hungary, Germany and UK). Colony viability (overwintering worker, brood cell and storage cell numbers) during crop flowering period and in the following year, found both negative (United Kingdom, Hungary) and positive (Germany) effects. Reduced colony size (-24%) was also recorded (honeybees in Hungary) in the year following exposure. In the wild bee species neonicotinoid residues were negatively correlated with reproduction (Woodcock et al., 2017). However, it is important to note, that of the 14 parameters studied across 3 countries, 33 of 42 factors showed no significant impact, 3 a positive impact and 6 a negative impact, highlighting the importance of reporting non-effects as well as detrimental impacts.

1.5.8 Bee detoxification mechanisms

The N-nitroguanidine neonicotinoids (imidacloprid and thiamethoxam) are highly toxic to bees (Iwasa et al., 2004; Nauen et al., 2001). Honeybee and bumblebee sensitivity to the N-cyanoamidine thiacloprid is orders of magnitude less than to the N-nitroguanidine compounds (Iwasa et al., 2004), but until recently the mechanisms of this differential sensitivity remained unknown.

Varying recovery rates have been shown in insects which underwent ‘pulsed’ exposure to imidacloprid. The behavioural activity (ventilation and locomotion) of *Chironomas* larvae recovers within 6 days (Azevedo-Pereira et al., 2011), the feeding rates of aphids (*Myzus persicae*) (Nauen, 1995) and coccinellid beetles (*Serangium japonicum*) (He et al., 2012) within

24 hours and the egg production of whitefly (*Bemisia tabaci*) within 48 hours (He et al., 2011). It is clear from these timespans that clearance of pesticide residues is highly organism dependent, but there is also suggestion of large differential toxicity between species groups (Reid et al., 2020). The causes of this differential sensitivity across compounds and between species has been the topic of much debate, posited to be either differences in target site receptor (nAChR) affinity or differential metabolism.

Metabolic studies show that, the honeybee, from a single dietary dose of imidacloprid (50 ppb), can remove the compound and its metabolites from the body within 24 hours (Suchail et al., 2004). This clearance is predominantly due to degradation by metabolism, as opposed to excretion of the compound. The ability to detoxify an ingested compound therefore seems likely to be integral to a species ability to recover from sublethal effects. Bumblebees appear slower to metabolise imidacloprid, with whole body clearance after 48 hours (Cresswell and Robert, 2013). Dietary ingestion of imidacloprid (100 ppb) reduces feeding and locomotor activity in bumblebees, but interestingly, the same effect is not seen in honeybees (Cresswell et al., 2014b, 2012). This is presumably because honeybees can continually metabolise a daily intake of 2ng (approximately half the oral LD_{50} of 4.5ng) (Cresswell, 2011). A large disparity is seen in the bumblebees, which comparably, were only capable of clearing <70% of assimilated imidacloprid each day, resulting in significantly higher whole-body imidacloprid levels (Cresswell, 2011) and perhaps explaining the longer (48 hours) whole body clearance time (Cresswell *et al.*, 2013).

New studies suggest that the cause of differential bee metabolism (and therefore sensitivity) to neonicotinoids, is a result of differences in bee cytochrome P450s of the CYP9Q family (Manjon et al., 2018). Ligand binding and inhibitor studies demonstrate that bee variation in sensitivity to the N-nitroguanidine, versus the N-cyanoamidine, neonicotinoids is not a result of differential receptor affinity, but is due to metabolic differences provided by divergent P450s (Manjon et al., 2018). Honeybees possess a P450 (CYP9Q3) which metabolises thiacloprid, but not imidacloprid, and bumblebees possess functional (slightly less efficient) orthologs (CYP9Q4 & CYP9Q6) (Manjon et al., 2018; Troczka et al., 2019), providing an explanation as to the sensitivity (vulnerability & susceptibility) of these species to the N-nitroguanidines (e.g. imidacloprid).

As well as metabolic ability, anatomical differences may play a role in the differential rates of compound clearance from a bee's body. The relatively large honey stomach of the bumblebee could result in new ingestion of the compound (stored in nectar in the honey stomach) after initial nectar collection (Cresswell *et al.*, 2014).

1.5.9 Current legislation and the need for new approaches

In 2013, due to mounting environmental concerns (such as the above-mentioned experimental evidence), clothianidin, imidacloprid and thiamethoxam, were banned in the EU for use on crops considered attractive to bees (European Commission, 2013). In 2018 this ban was further extended to include all outdoor agricultural usages, with the only remaining usage being indoors in permanent glasshouse structures. Until recently, both thiacloprid and acetamiprid could still be used in agricultural settings. Acetamiprid is approved for use until 2033 (European Commission, 2018a), but thiacloprid's approval was not renewed in April 2020, due to its potential endocrine disrupting properties (European Food Safety Authority; Abdourahime et al., 2019).

As more and more pesticides (especially neonicotinoids) are withdrawn from the European market or are perceived to be 'old' 20th century chemistries which have acknowledged detrimental environmental impacts, we must continually contrast the current pesticide landscape with its alternatives, whether they be more beneficial or more detrimental. The current legislative bans on neonicotinoid compounds results in the need for a radical reassessment of integrated pest management (IPM) practices, and implementation of different agrochemical strategies to protect our crops, that are hopefully more species specific and protect bees and other beneficials.

1.6 Knowledge gaps and thesis objectives

Based on the literature reviewed above several current deficits in the study of pesticide sublethal effects on bees have been identified:

1. Studies examining sublethal effects on non-*Apis* bee species (Siviter et al., 2018b)
2. Studies utilising non-olfactory learning paradigms (particularly non-PER (Proboscis Extension Response) paradigms) to examine cognitive sublethal effects (Muth and Leonard, 2019; Siviter et al., 2018b)

3. Chronic studies for bumblebee adults and larvae (Abdourahime et al., 2019)
4. Comparative testing platforms which facilitate across species and across compound testing (my own conclusion drawn from the current literature)

These major knowledge gaps prevent the full evaluation of pesticide impacts on pollinators. There is an urgent need to rectify this to appropriately revaluate our current and future pesticide usage and ensure both agricultural and biodiversity sustainability long term. The work in this thesis therefore aimed to address these deficits by assessing sublethal effects of chronic pesticide exposure on bumblebee mobility, navigation, learning and memory through the development of novel behavioural assays and approaches which will provide new tools for cross-species and cross-compound comparisons.

1.7. Thesis hypotheses and summary

In **Chapter 2,** the hypothesis that aversive conditioning is currently underutilised as a bee learning paradigm was assessed through a methodological review of bee aversive learning studies to date. The review confirmed previous findings (Muth and Leonard, 2019; Siviter et al., 2018b) that bee learning assays are weighted towards the study of *A. mellifera* and olfactory PER paradigms. However, the review also suggested that aversive stimuli have the potential to be highly effective but are currently underutilised in bee learning and memory studies, providing a potential avenue for new research.

In **Chapter 3, to** address the hypothesis; can ambient temperature be utilised as an effective, ecologically relevant aversive training tool for bumblebees?, a novel *B. terrestris* aversive learning assay (the thermal-visual arena) was developed. Temperature appeared to be strongly perceived by bumblebees, motivating bees to locate and remain in the arena's cool reward zone. This suggests temperature could be a fruitful avenue for new bee aversive learning research.

In **Chapter 4**, the hypothesis that aversive training is the best conditioning paradigm for use in the thermal visual arena was tested by comparison to other training methods (appetitive, combined appetitive and aversive, control). The effectiveness of ambient temperature as an aversive training method was confirmed, as bees in treatments containing aversive stimuli spent significantly more time in the reward zone. The value of the arena's visual pattern

stimuli for bee learning was also assessed and found to facilitate quicker bee learning of the reward zone location (compared to bees without a pattern). However, this effect was short-lived, and benefit disappeared by trial 10. Aversive conditioning was highly effective, even in bees which were not provided with the additional visual pattern, again suggesting its value to bee learning research.

In **Chapter 5**, the hypothesis that low and high dose chronic pesticide exposure to two neonicotinoids (thiamethoxam and thiacloprid), and a sulfoximine insecticide (sulfoxaflor), may impact foragers' ability to complete the aversive learning task developed in Chapter 4 was assessed. Sublethal effects of these pesticides on food consumption were also examined. In the high dose experiments, oral thiacloprid exposure (5000ppb) significantly reduced *B. terrestris* food consumption. High dose thiamethoxam exposure (100ppb) led to increased bee mortality. Low dose thiamethoxam exposure (10ppb) prevented foragers from improving training parameters and caused hyperactivity. The thermal-visual arena was demonstrated to provide a successful assay platform for cross compound comparisons of sublethal effects in free moving *B. terrestris* foragers.

In **Chapter 6**, the hypothesis that power law analyses can be used to develop null behavioural templates for bumblebees was tested by analysing the walking trajectories in all Chapter 4 training treatments. We discovered that walking bumblebees adhere to a speed curvature power law previously observed in humans, other primates and *Drosophila* larval trajectories.

In **Chapter 7**, we tested the hypothesis that the null behavioural templates provided by power laws in Chapter 7 would be modified under the pesticide exposure regimes tested in Chapter 5. Thiamethoxam exposed bees in the low dose experiment (10ppb) had a significantly higher power law exponent, which became even more pronounced in the higher dose experiments (100ppb). These results suggest that power law analyses could provide a novel approach to the study of subtle sublethal effects.

In **Chapter 8**, the hypothesis that observed individual differences in *B. terrestris* aversive learning ability in Chapter 4 could be a result of genetic differences was investigated. RNA-seq and bioinformatic analyses were used to examine underlying genetic determinants of 'good' and 'bad' learners. 83 significant (<0.05) and 35 highly significant (<0.01) unique differentially expressed genes were identified.

In **Chapter 9**, a new colour learning assay was developed to assess the hypothesis that neonicotinoid pesticides could affect both associative and extinction learning in free-flying *B. terrestris* foragers. The latter, extinction learning, has never been studied in regard to pesticide impacts. Initial testing was completed with imidacloprid. Results supported previous findings (Muth and Leonard, 2019) that imidacloprid exposure affects foraging behaviours but not learning, with treated bees taking longer to make correct choices and making more errors.

In **Chapter 10**, the key novel findings of this thesis, potential implications and future research directions are discussed.

Chapter 2

Aversive conditioning in bees: are we missing a trick?

Chapter 2: Aversive conditioning in bees: are we missing a trick?

Statement of contribution

The following Chapter is based on a paper prepared for journal submission. As primary author I was responsible for conceiving the review, designing the data collection, conducting the literature review and statistical analyses and writing the manuscript. My supervisory team, Linda Field, Ian Mellor, and T. G. Emyr Davies reviewed and edited the manuscript. Three anonymous reviewers provided comments on the manuscript during the *Animal Cognition* review process and their comments were used to edit the final thesis manuscript.

2.1 Introduction

Bees rely on learning and memory for predator avoidance, social interaction, sexual behaviours and foraging (Dukas, 2008; Giurfa & Menzel, 2013; Menzel & Benjamin, 2013; Menzel *et al*., 2005). For example, whilst foraging, honeybees must learn and memorize floral cues, such as colour and odour, to recognise floral species with good rewards (Giurfa, 2007; Menzel, 2012). Bees, like all animals, must also learn to adapt their behavioural responses to match potential positive (for instance, food or a mate) or negative (such as a predator or danger) outcomes (Alcock, 1975). This 'associative learning' allows animals to predict the outcomes of their behaviours by forming a memory association between their behavioural response and a consequence (operant (reward/punishment) conditioning) (Skinner, 1936; Skinner, 1938) or between previously neutral stimuli (e.g. odour or colour) and other meaningful stimuli (e.g. food or danger) (classical or Pavlovian conditioning) (Pavlov, 1927).

Associative learning or conditioning can be classified as either 'aversive' or 'appetitive' dependent on its reinforcement outcome. Aversive associative learning results in an unpleasant stimulus reducing the frequency of a behaviour, whereas appetitive associative learning usually results in positive responses to previously neutral signals. This has the effect of driving an animal away from (aversive) or towards (appetitive) reinforcement stimuli (Mackintosh, 1983; Schull, 1979). These two learning forms are mediated by distinct neural circuits which govern reinforcements with opposing valences (Rudy, 2014). Research has demonstrated that insects' have remarkable learning capabilities in response to this type of conditioning (Brembs and Heisenberg, 2001; Giurfa, 2013; Sokolowski et al., 2010) and that

these training mechanisms can be utilised in the laboratory to better understand bee learning, short and long-term memory pathways, and behaviour. Associative learning in the honeybee has been primarily studied in the laboratory using two opposing classical conditioning assays; the appetitive olfactory conditioning of the proboscis extension response (PER) (Bitterman *et al*., 1983; Giurfa & Sandoz, 2012) and the aversive olfactory conditioning of the sting extension response (SER) (Carcaud et al., 2009; Giurfa et al., 2009; Tedjakumala et al., 2014) (aversive learning in light of the SER is reviewed in Tedjakumala & Giurfa (2013)).

The PER is a natural behavioural reflex in which the bee extends the proboscis in response to a food reward. Olfactory conditioning of the PER involves a bee learning to associate an initially neutral odour presented to the antenna (the conditioned stimulus (CS)) with a sucrose reward (the unconditioned stimulus (US)). This then leads to the bee learning to extend its proboscis in response to the given odour alone, without the reward. The PER thereby provides an ecologically relevant tool to study how honeybees perceive and learn floral odours, mimicking foraging behaviour in which a bee experiences a floral odour at the same time as receiving a nectar food reward. In laboratory experiments the PER is often combined with techniques to monitor neural activity, including electrophysiology or bioimaging, or to manipulate target learning pathways (Giurfa and Sandoz, 2012).

Aversive conditioning occurs in the field when worker bees encounter predators, unfavourable climatic conditions, repellents, and agrochemicals, which require adept escape and avoidance responses (Zhang & Nieh, 2015). In field scenarios, it appears that individual honeybee foragers can both assimilate knowledge of aversive conditions and disseminate this knowledge to other individuals via negative feedback signals during forager dancing (Kietzman and Visscher, 2015), this results in reduced visitation rates to the unfavourable site by bees that have not visited the site (Nieh, 2010). Aversive conditioning in the form of punishment has also been demonstrated in honeybees whereby in field experiments bees stop flying to a target once punished (Abramson *et al*., 2006).

The SER is a naturally elicited response of honeybees to sensory stimuli, including alarm pheromone (Balderrama et al., 2002; Breed et al., 2004; Núñez et al., 1997, 1983), dark colours, and human sweat (Free, 1961). Like the PER, the SER can be used in experimental systems, in this case to study aversive learning (Bos et al., 2014; Carcaud et al., 2009; Geddes et al., 2013; Giannoni-Guzmán et al., 2014; Giurfa et al., 2009; Guiraud et al., 2018; McQuillan

et al., 2014; Roussel et al., 2012, 2010; Tedjakumala et al., 2014; Vergoz et al., 2007b; Zhang and Nieh, 2015). In the SER assay, bees learn to associate an odour (Carcaud et al., 2009; Giurfa et al., 2009), visual (Mota et al., 2011), or gustatory (Guiraud et al., 2018) stimulus (CS) with an aversive electric shock stimulus (US) and will then extend their stinger in response to the aversively conditioned odour alone, in the absence of punishment. Temperature, rather than electricity, has also been used as an aversive stimulus in a modification of the SER assay (Cholé et al., 2015; Junca et al., 2019, 2014; Junca and Sandoz, 2015). The development of the SER paradigm (Vergoz et al., 2007a) originated as the result of an impasse between *Drosophila* and *Apis* scientists studying associative learning. Knowledge exchange was hampered by the near exclusive use of olfactory PER conditioning by *Apis* scientists and of the T-maze protocol and electric shock as an unconditioned stimulus by *Drosophila* scientists. Therefore, based on prior work which demonstrated that the SER could be triggered in harnessed bees in the laboratory (Balderrama et al., 2002; Núñez et al., 1997, 1983), the SER paradigm was developed.

Other types of bee behavioural studies have used electric shock as an aversive stimulus combined with mechanical force to simulate a predatory attack (Zhang and Nieh, 2015), or electric grids in which walking bees receive an electric shock when they make an incorrect choice (for example, by entering an area indicated by a specific colour or pattern) (Abramson, 1986; Agarwal, Giannoni Guzmán, *et al.*, 2011; Dinges *et al.*, 2013; Giannoni-Guzmán *et al.*, 2014; Avalos *et al.*, 2017; Kirkerud, Schlegel and Galizia, 2017; Plath *et al.*, 2017; Black *et al.*, 2018; Marchal *et al.*, 2019; Nouvian and Galizia, 2019). Such 'place avoidance' studies are an alternative aversive conditioning method in which a negative stimulus occurs until the required behavioural response is elicited, i.e. the individual "escapes" the aversive stimulus (Mackintosh, 1983). The results of such 'escape conditioning' have been found to be similar to those achieved with food rewards in appetitive conditioning (Mackintosh, 1974). Other studies have utilised further different aversive stimuli, such as an aversive odour (formic acid) (Abramson, 1986) or aversive gustatory compounds (Avarguès-Weber et al., 2010; Ayestaran et al., 2010; de Brito Sanchez et al., 2015; Howard et al., 2019).

Overall, the use of aversive conditioning in bee studies is still comparatively rare (reviewed in Tedjakumala and Giurfa, 2013). Increasingly, authors are highlighting both the lack of aversive learning studies in bees (compared to other insect groups) and to appetitive studies in the

field of bee cognition (see the reflections of Dinges *et al*., 2013; Kirkerud *et al*., 2017; Muth & Leonard, 2019; Siviter *et al*., 2018). Despite these observations and recent efforts to improve aversive conditioning paradigms (through use of other methods than the SER alone) aversive studies on bees remain few and far between.

2.1.1 Aims of this review

Two recent reports have highlighted current deficits in the bee learning and memory literature when it comes to being able to effectively apply this knowledge to assess the detrimental impacts of pesticides:

a) Siviter *et al*. (2018) stated that existing literature on bee learning and memory demonstrates a "heavy focus on *Apis*, with a dearth of studies on bumblebees and other wild bees … research on non- *Apis* species, such as bumblebees (including species other than *B. terrestris*) and solitary bees, is sorely needed, and the development of non PER- based paradigms".

b) Muth and Leonard (2019) stated that "we need to address cognition across more than a single (usually olfactory) modality" and "to address a broader range of cognitive abilities and learning scenarios … (as)…by focusing just on olfactory associative learning we are likely only seeing a part of the picture",

Although these conclusions refer to the study of pesticide impacts on bee learning and memory, it is unlikely that this bias of a dominant conditioning paradigm (olfactory appetitive conditioning via the PER) and a dominant bee group (*Apis*) exists in only this specific field of research; rather that it is symptomatic of a wider descrepancy in bee learning studies as a whole. To effectively assess potential pesticide impacts on bee learning and memory (as in Chapter 5 and 7 of this thesis) it is vital that we are aware of gaps in the methodologies employed in the study of bee learning and memory as a whole, so that newly designed methodologies can address prior pitfalls in this area.

Therefore, this review aims to:

1. Systematically assess the methods ustilised in the current literature on aversive conditioning in bees.

2. Highlight the advantages and pitfalls associated with current aversive training methods.

3. To question why aversive conditioning remains so underutilised as a bee learning paradigm and to highlight future fruitful avenues of research.

2.2 Methods of literature identification and exclusion

In the present study the search terms below were used to retrieve literature concerning any form of aversive conditioning of bees: ("bumblebee*" OR "bumblebee*" OR "honeybee*" OR "honeybee*" OR "bee*" OR "apis" OR "bombus") AND ("aversive" OR "aversive conditioning" OR "aversive learning" OR "punishment" OR "escape" OR "avoidance"). The search used Web of Science (all databases and across all available record years) and Google Scholar as the primary search databases and was conducted in January 2020.

Our search returned a total of 135 papers in Web of Science for “TITLE” specific searches. Of these, 44 had titles that met the inclusion criteria of an aversive conditioning task utilised in a bee species (see the Appendix for Chapter 2 for full list of the 44 screened papers). The abstracts and full manuscripts were then assessed and 14 papers were rejected because they did not directly study an aversive conditioning paradigm or were not a primary research study. One additional paper was identified,utilising the same search terms in Google Scholar. This gave a total of 31 relevant studies which directly employed aversive paradigms to condition bees. These were then allocated into groups according to: the genus studied, the conditioning paradigm used (aversive, both appetitive and aversive), the learning assay used, the type of aversive stimuli used, and whether the bees were harnessed or free moving.

2.3 Results

2.3.1 Genus studied

In support of Siviter *et al.,* (2018) and Muth and Leonard's (2019) assertions, the literature we identified was largely on the genus *Apis*, with 30 of the 31 (97%) studies being on the Western honeybee (*A. mellifera*), and only one study conducted on a non-*Apis* species (Fig. 1). This singular example was *B. terrestris* (the buff-tailed bumblebee), a social bee species. There were no studies on solitary bee species. This bias towards *Apis* does not therefore exist only in the field of pesticide and toxicology studies but also in the wider field of bee conditioning studies.

2.3.2 Conditioning paradigm

Of the 31 studies, 19 (61%) used aversive conditioning alone and 12 (39%) used both aversive and appetitive conditioning (Fig. 1). For the latter only the assay used for aversive conditioning was considered in our further analyses (for example, in studies which used the PER as a solely appetitive paradigm as a comparator to an aversive conditioning paradigm, the PER was not counted as being used as an aversive conditioning paradigm).

2.3.3 Learning assay

Across all 31 papers a total of seven different types of assay were used to assess aversive conditioning on bees. These were categorised into five groups (Figure 2.1):

1) The sting extension response/reflex (SER)
2) The proboscis extension response/reflex (PER)
3) Y-maze
4) Electric shock place avoidance (Shuttlebox OR electric shock avoidance assay (ESA) OR automatic performance index system (APIS) or a Y-maze which utilised an electric shock grid for place avoidance)
5) Flight arena

Shuttlebox, ESA, APIS, and the modified Y-maze assays were grouped together (n = 34 as three studies utilised two assay methodologies) as these assays involve bees in an enclosed walking

area, using electric shock in the form of a grid on the arena floor as an aversive stimulus. The SER was used in 16 (47%) studies, the PER in four (12%), a classical Y-maze in three (9%), a flight arena in one (3%), and an electric shock place avoidance assay in 10 (29%) (Figure 2.1).

Classical Y-maze studies associated with conditioning, using a gustatory reward or punishment (Avarguès-Weber *et al.,* 2010, Howard *et al*., 2019 and De Brito Sanchez *et al*., 2015), were separated from the one example of the Y-maze which utilised an electric shock as a place avoidance assay (Nouvian and Galizia, 2019).

2.3.4 Type of aversive stimuli

Of the 31 total studies (n = 32 in this section as one study utilised two different aversive stimuli; odour and electric shock), 22 of the studies (69%) used electricity as an aversive stimulus, five (16%) used an aversive gustatory compound (usually quinine), four (12%) used temperature in the form of a probe or direct heat application, and one (3%) used an aversive odour (formic acid). The use of electric shock was further broken down into studies making use of a shock in a walking arena through a grid on the floor (n = 10, 43%) and those which delivered the shock through a probe or direct contact electrode (n = 13, 57%). One study used both methods for electric shock delivery.

2.3.5 Harnessing of bees

Bees were harnessed or immobile for at least one experiment in 19 (61%) of the 31 studies when PER or SER tests were being conducted.

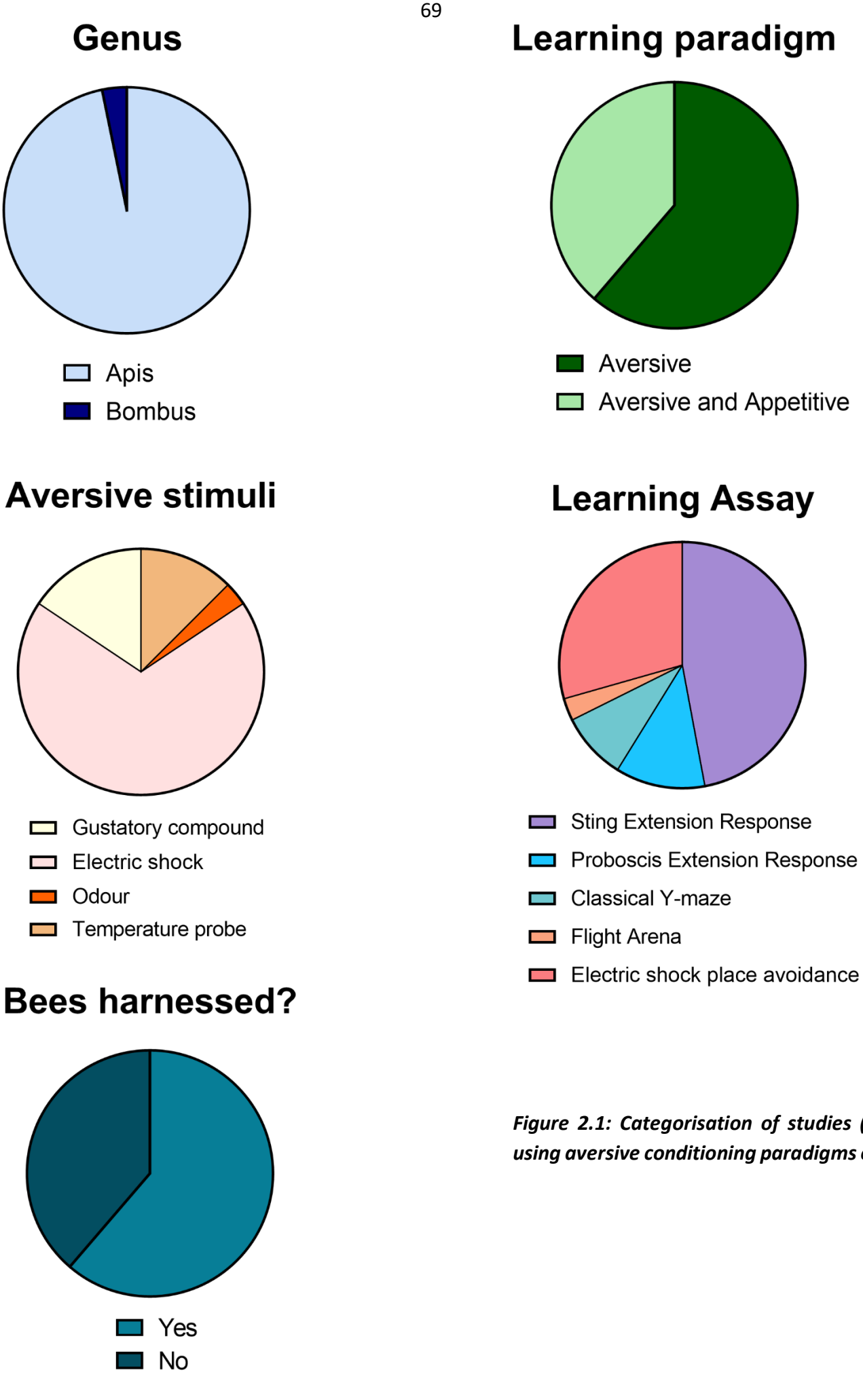

Figure 2.1: Categorisation of studies (n = 31) using aversive conditioning paradigms on bees.

2.4 Discussion

Almost 30 years ago, Smith, Abramson and Tobin (1991) concluded that; as much of what we know about learning has been developed from work with aversive stimuli, and aversive paradigms have been highly successful in other species, their continued development in bees would be of upmost value. In the intervening years, research interest in the aversive conditioning of bees has increased, as is evidenced by the literature search conducted in this review. However, relatively few studies were identified (n = 31) and there is clearly a need to evaluate the aversive methodologies used across these studies to better understand the research gaps which still exist and guide a clear agenda for future research efforts.

2.4.1 The value of aversive stimuli

Differences in the life histories and physiology of different bee species may play a role in their relative cognitive abilities. However, arguably, aversive conditioning remains a relevant cross-species paradigm, as all species face aversive stimuli (such as predators and unfavourable temperatures) in the wild. Aversive conditioning provides a useful tool to study differences in learning ability between species, as it isn't affected by hunger-induced motivation, which can govern the effectiveness of appetitive learning studies (Friedrich et al., 2004; Krashes et al., 2009). For appetitive studies some differences between species can be predicted, for example foragers from social species may have higher foraging motivation than solitary species as they are required to continually collect resources for the whole hive. Nectar carrying capacity is also dependent on honey stomach size which differs both within and between species, potentially further affecting appetitive motivations. In contrast, aversive stimuli do not require individuals to be motivated by hunger and therefore may reduce cross-species variation in stimuli response. Nonetheless, individual variation in stimuli response may persist within species. For example, Roussel *et al.* (2009) demonstrated that honeybee foragers and guards differed in their responsiveness to electric shocks and so in their responsiveness to aversive conditioning via the SER. More recently, Junca *et al*. (2019) also observed that, in honeybees, different hive patrilines differed in their shock responsiveness and learning capabilities, supporting the notion that aversive learning capabilities can differ within a honeybee hive. These considerations highlight the potential limitations of all conditioning

methods (including aversive conditioning) and of utilising methods which minimises intra- and inter-species variation, even if it can never be completely removed.

2.4.2 The need for non-Apis studies

Ongoing research continues to highlight the dearth of learning and memory studies in wild bees (Kirkerud et al., 2017; Muth and Leonard, 2019; Siviter et al., 2018b), as is evidenced from this review where 97% of the studies used *A. mellifera* as the study organism. This bias can be understood, based on the economic importance of *A. mellifera* for commercial crop pollination (Morse and Calderone, 2000; Williams, 1994). However, more research is needed on wild social bee species (bumblebees other than *B. terrestris*), solitary, and stingless bee species, e.g. *Osmia bicornis* and *Tetragonula iridipennis*, which are also proven key crop pollinators (Button and Elle, 2014; Kishan et al., 2017; Ryder et al., 2020; Willmer et al., 1994; Winfree et al., 2007) and for which comparatively little is known about cognition.

2.4.3 Benefits and limitations of tethered laboratory techniques: PER & SER

Although traditionally used for appetitive olfactory conditioning we identified four reports where the PER has been paired with an aversive stimulus, for example, an aversive gustatory compound or an electric shock (Ayestaran et al., 2010; de Brito Sanchez et al., 2015; Smith et al., 1991; Vergoz et al., 2007a). Smith, Abramson and Tobin (1991) first demonstrated the power of aversive stimuli in proboscis extension conditioning by showing that bees could be conditioned to supress their innate PER when an odour was paired with an electric shock. The PER conditioning protocol is approaching 60 years old and, as such, it is a long-established learning assay with an embedded and long-standing prominence in the literature. As discussed in this review, newer aversive assays have been developed, but these are relatively new attempts compared to the PER, and it is important to note the relevance of time in learning assay development and establishment. The established nature of the olfactory PER protocol perhaps explains why other aversive protocols remain scarce and, in many cases, unoptimized. For example, learning efficiency is known to be lower in SER conditioning versus PER conditioning (Vergoz et al., 2007a), again, potentially as a result of the amount of time over which the PER has been optimised in honeybees.

There is no doubt that the use of laboratory techniques with harnessed immobile bees, such as in the PER and the SER (Vergoz et al., 2007a), provide unique opportunities to use physiological and biochemical techniques, including pharmacology, imaging and electrophysiological methods, to explore the neurophysiology of aversive learning (Cholé et al., 2015; Giurfa and Sandoz, 2012; Menzel, 2012; Tedjakumala and Giurfa, 2013). It should be noted that although free-moving paradigms may allow bees more ecologically relevant movements, they are impractical for assessing the neural basis of aversive learning in living animals. This is where the PER and the SER have exceptional benefits through allowing, for example, the direct recording of neural activity (Roussel et al., 2010). The 'best protocol' therefore depends on the research question being asked, which is not always one of 'most ecological relevance'.

However, there remain limitations to these restrictive lab-based methodologies in certain scenarios, as is highlighted by several authors (Abramson, Sokolowski and Wells, 2011; Dinges *et al.,* 2013; Cholé, Junca and Sandoz, 2015; Muth and Leonard, 2019; Frost, Shutler and Hillier, 2012). Cholé*,* Junca and Sandoz (2015) point out that both the SER and PER only produce binary responses in an all or nothing extension of the proboscis (PER) or the sting (SER), while potentially missing finer behavioural gradients (e.g., effects on feeding, foraging and flight parameters) which could be captured in free moving scenarios. The harnessing of bees further limits naturalistic movements and behaviours which bees may display in a more natural (allowing free movement and foraging) learning environment. Dinges *et al.,* (2013) also highlight that the PER has been shown to have methodological inconsistencies across labs (Abramson et al., 2011; Frost et al., 2012). Since the PER usually relies on olfactory conditioning it may also have limitations in its use to test the impacts of stressors, such as pesticides, which have been shown to alter olfactory processing (Andrione et al., 2016; Dinges et al., 2013; Siviter et al., 2018b). The PER may also not be inherent in all species, as has been suggested for stingless bees (Abramson et al., 2011), and the SER is only relevant to bees which have a stinger or that readily sting for defence (e.g. not males, solitary bees, stingless bees), all of which has driven a need for alternative conditioning models.

2.4.4 Place-avoidance/place learning assays

As is clear from this review, alternative methods of conditioning do exist and, in some cases, provide more ecologically relevant training scenarios for bees. Place avoidance/place learning assays can be seen as one such paradigm (Agarwal et al., 2011; Dinges et al., 2013; Kirkerud et al., 2017), in which free-moving subjects learn to avoid an environment which is associated with an aversive stimulus, usually a mild electric shock applied via a grid on the floor. This aversive stimulus is usually paired with a defining property of the environment, such as, a colour or pattern or light or dark. Thus, the subject learns to 'escape' the electric shock by moving to an environment without the associated environmental property. In mice, place avoidance assays which use shocks as a negative reinforcer have been shown to be no more stressful than exploring a familiar environment (Lesburguères et al., 2016). The results of such conditioning are similar to those achievable through appetitive conditioning with food rewards (Mackintosh, 1974), making this a useful tool to study learning and memory. However, it should be noted that the associations created by these protocols may vary from case to case. For example, the APIS (Kirkerud et al., 2017, 2013; Plath et al., 2017) and the Y-maze protocols developed in the same lab (Nouvian and Galizia, 2019), rely on bees learning an association between a set colour, e.g. yellow, and the electric shock (operant learning plus a Pavlovian component). The categorisation of these assays as place avoidance/place learning may therefore be misleading for these assays, as these bees are learning to avoid a colour as opposed to the place itself. Whereas, in the ICARUS protocol (Marchal et al., 2019) bees learn to display a phototactic response (towards or away from light), as opposed to a set colour. These studies demonstrate the importance of considering what bees are learning in each aversive protocol (and its consequences), as well as the overall design.

2.4.5 Electric shock as an aversive stimulus

Although the development of spatial assays, for example place avoidance assays, simulate more-realistic free moving scenarios for bees, these assays have use of electric shocks as the aversive stimulus in common with the SER. It is well evidenced from the studies reviewed here that bees can effectively perceive electric shocks as aversive stimuli, however, the question remains whether there is a natural analogue to such shocks in the 'bee world'. Nonetheless, Zhang & Nieh's (2015) development of an electro-mechanical predator to

simulate predation on honeybee foragers (by applying a pinching bite at a fixed force coupled with an electric shock) is a good example of efforts to develop techniques which provide a more naturalistic delivery of aversive stimuli to study bee learning. Zhang & Nieh (2015) suggest that electric shocks, delivered in this way, can produce sensory neural responses analogous to that of a predatory bite. Electric shocks, whilst not being ecologically realistic, have been used throughout classical experimental psychology studies (for example, in rats; Milad et al., 2006; Pezze and Feldon, 2004; Tarpley et al., 2010) and have revealed fundamental information about how aversive conditioning works. Electric shocks clearly have their place in learning assays, however, a move towards more environmentally realistic aversive stimuli seems appropriate given that laboratory studies are often criticised for their lack of real-word applications (reviewed in Giannelli, 1985).

2.4.6 Temperature as an aversive stimulus

Junca *et al*. (2014) argue that an electric shock of any kind is an unrealistic stimulus for a bee. Instead, these authors demonstrate that temperature can elicit a similar SER response and suggest that it provides a more ecologically relevant stimulus. In a further paper, Junca and Sandoz (2015) demonstrated that bees are able to learn CS-US associations even when a temperature probe is used on a non-sensory area of the bee's body, such as the back of the head or the abdomen. This suggests that bees are highly capable of learning aversive stimuli associations even when that association is in a non-natural setting. However, the use of a probe to administer temperature remains highly unrealistic, irrespective of whether the responses to temperature are similar wherever on the bee's body the heat is administered. A more relevant stimulus might be to increase ambient temperature, as it has been demonstrated that bees locate and remain in zones at their temperature preference (Grodzicki and Caputa, 2005; Ohtani, 1992). This is alongside strictly avoiding temperatures above 44^0C and rejecting food presented to them at above 50^0C (Junca et al., 2014). This aversion to high temperatures is vital to hive survival in social bees as deviations from normal brood temperature can lead to morphological, neurological, and behavioural defects (Koeniger, 1978; Tautz *et al.*, 2003), as well as increased mortality (Jones et al., 2005; Scheiner et al., 2013; Tautz et al., 2003). High temperature is demonstrated to be perceived by bees as a strongly aversive stimulus (Junca et al., 2014; Junca and Sandoz, 2015). However, studies utilising ambient temperature are seemingly non-existent in studies of bee learning and

memory. It is also important to note additional considerations when using temperature as an aversive stimulus, for example, potential damage to individuals caused by heat which may affect their ability to perform in aversive assays and whether this would be consistent across tested species. It may also be prudent to ask whether the use of heat or electric shock (as discussed above) is ethical in insect studies (recently reviewed in Fischer and Larson, 2019).

2.4.7 Bitter tastes as aversive stimuli

A further aversive stimulus used in just five (16%) of the studies reviewed here (Avarguès-Weber et al., 2010; Ayestaran et al., 2010; de Brito Sanchez et al., 2015; Howard et al., 2019; Rodríguez-Gironés et al., 2013) was an aversive gustatory compound. For invetebrates, aversive taste perception is important for the detection of potential toxicity in plant compounds, particularly in pollen and nectar for bees. Insects have been shown to have dedicated bitter gustatory receptors (GRs) (Amrein and Thorne, 2005; Kent et al., 2008; Wanner and Robertson, 2008), however, honeybees have relatively few GRs (Robertson and Wanner, 2006) and so their ability to percieve aversive tastes has been queried (Ayestaran et al., 2010). This research casts some doubt on whether such tastes can act as affective aversive training stimuli in bees.

Previous studies have shown that harnessed honeybees in the lab did not reject potentially aversive compounds such as quinine (Ayestaran et al., 2010; de Brito Sanchez et al., 2005), but free-flying bees were able to learn visual discrimination tasks when a wrong choice was associated with an aversive quinine stimulus (Avarguès-Weber et al., 2010). This difference may be because the harnessed bees had no alternative source of food whereas free-flying bees were given alternative sources. However, there is some suggestion that aversive tastes may stop acting as a negative reinforcer in free flying choice experiments (Avarguès-Weber et al., 2010; Rodríguez-Gironés, Trillo and Corcobado, 2013), where bees may learn to use their antenna to probe a solution prior to consumption. Bee antennae are capable of detecting sucrose, but not quinine, thus bees may simply be learning the absence of sucrose as non-rewarding as opposed to negative reinforcement of the aversive stimulus. Despite the effectiveness of aversive taste stimuli in some studies, the controversy and seemingly context-dependent ability of honeybees to detect bitter substances (de Brito Sanchez, 2011; de Brito Sanchez et al., 2015) and that honeybees have been shown to have a reduced number

of GRs compared to other insect species (Marchal et al., 2019), makes using aversive gustatory compounds to assess aversive learning difficult and somewhat unreliable. Bitter taste perception also remains under studied in other non-*Apis* bee species and there is a need for the development of assays utilising aversive stimuli which are consistently perceived as aversive across species and learning context.

2.4.8 The relevance of aversive stimuli

Aversive conditioning remains a highly ecologically relevant and practical way to study bee learning and memory. Nonetheless, the use of such conditioning remains relatively rare (in bee learning studies), as evidenced by only 31 studies being identified by this review. Furthermore, within the existing studies there is a strong bias towards the use of harnessed-bee laboratory techniques (61%), particularly the use of the SER assay (47%), and the majority of studies use electric shocks as the aversive stimulus (69%). Nonetheless, increased interest in the development of assays which use different aversive stimuli, in more natural scenarios, is evident, if not yet predominant. Furthermore, research must continue to develop in the study of other economically important pollinators, one example being *B. terrestris*.

As Siviter *et al*. (2018) identify, the lack of non-PER tests and gaps in the current use of aversive conditioning are actively preventing our ability to fully evaluate potential stressor impacts on pollinators. Therefore, there is a need to rectify this through the continued development of aversive conditioning paradigms, particularly across non-*Apis* species, to be able to appropriately evaluate current and future environmental stressors on bee learning and memory.

2.4.9 Limitations of the search terms

The search terms utilised in this review were not all encompassing and terms were used in Web of Science for “TITLE” specific searches. It is therefore likely that some relevant studies may have been missed from the review, in spite of an additional Google Scholar search utilising the same search terms. Studies which had more cryptic titles or were not well digitised/accessible online may have been missed, as may new studies published since the literature search was conducted in January 2020. Below, additional studies identified during the peer review process for journal submission, are discussed.

A recent study by Colin *et al.* (2020) utilised an aversive assay to demonstrated that visual learning is negatively impacted by co-exposure to both imidacloprid and thymol (miticide). This study's methodology is concordant with the overall findings of this review, focusing on *A. mellifera* and ustilising an electric shock place avoidance assay (the automatic performance index system (APIS)).

A key study by Giurfa *et al.* (2009) was missed by the search terms in the original literature search. This study provides a full parameterisation of the SER, detailing the effect of trial number and interstimulus/intertrial intervals on memory retention. The study was conducted in harnessed honeybees. Another omitted study, by Carcaud *et al.* (2009), demonstrates the success of this form of aversive learning (SER) in honeybees which, when placed in a maze, avoided the odour which had previously been paired with an electric shock.

2.4.10 Relevance of associative assays for field scenarios

Critics of laboratory studies argue that research needs to shift in focus away from individual performance assessments using associative assays, as these fail to provide a field relevant assessment of the effects of pesticides on pollinators (Suryanarayanan, 2013). Laboratory studies are inherently simplisitic recreations of factors which may be encountered by foraging bees in the field. The use of lab-based studies is usually not designed to be a direct simulation of field conditions, but to allow the discovery and observation of potentially finer sublethal effects which may be missed, or difficult to study, in the field. Associative learning is by no means the only parameter which can be used to study bee performance in the lab (e.g. fitness parameters, movement parameters, flight parameters), but as associative learning is so integral to bee foraging success, it provides a key avenue for the assessment of sublethal stressors (Mengoni Goñalons and Farina, 2018; Mustard et al., 2020; Muth and Leonard, 2019; Raine and Chittka, 2008; Siviter et al., 2018b). In the pesticide testing arena, laboratory studies can allow the tracking of exact levels of pesticide exposure on an individual, providing key information on likelihood of sublethal effects at field realisitc concentrations. As mentioned above, lab based studies such as the PER or SER, provide unique opportunities to use physiological and biochemical techniques to explore the neurophysiology of aversive learning and direct neorological effects. In conjunction with semi-field and field based studies (e.g. RFID/QR code tracking studies which follow foragers throughout their lifetime), I believe

that laboratory studies play a key role in elucidating the underlying mechanisms of sublethal effects (for example, Wu et al., 2017).

2.4.11 Conclusions and implications for future research

The results of this analyses show that aversive conditioning is a highly relevant method for the study of bee cognition and yet it remains underutilized in honeybees, as well as other wild bee species. There remains a need for more aversive training studies to better understand non-*Apis* bee species' learning and cognition, which will further aid in the evaluation of potential detrimental impacts (e.g. stressors such as parasite load, disease or pesticides) on bee learning and memory across species. The development of sound, ecologically relevant, species-encompassing, aversive assays can provide tools to assess negative impacts on our bee pollinators. Such assays will allow a further accumulation of evidence which can reduce negative effects on bees through impacts on policy change.

This literature review highlights existing gaps and flaws in the study of aversive conditioning in bees, and to address these we propose the following avenues for future research:

1. Studies using aversive conditioning on non-*Apis* species (bumblebees and solitary bees) to facilitate the continued accumulation of data on effectiveness of aversive conditioning in these groups.
2. Further development of existing or new aversive conditioning assays which use relevant aversive stimuli other than electric shocks, for example, ambient temperature.
3. Development of aversive assays which can be readily used across species.

Chapter 3

Pilot study: is bumblebee aversive training possible in the thermal-visual arena?

Chapter 3: Pilot study: is bumblebee aversive training possible in the thermal-visual arena?

3.1 Introduction

3.1.1 Bee navigation

Navigation relies on the interplay of a variety of components and processes, requiring integration of spatial information across stimulus modalities, retrieval and formation of memory and selective activation of task-specific memories. The simplest forms of navigation require only the ability to move to or away from a stimulus. However, more complex forms involve the creation of an internal representation of the environment, one's location within this environment, the location of a desired destination and the variety of routes which could be taken from current location to desired goal (Wiener et al., 2011). Directional and non-directional sensory cues provide a platform for navigation. Directional resource cues, such as the chemical gradients of pheromones or food odour (Baker, 1985) can be used to detect and locate resources in a landscape. Topographical (reviewed in Kheradmand & Nieh, 2019) or celestial features (for example, the sun, stars, and moon) (Dovey et al., 2013; Kraft et al., 2011) can also be utilised for directional and orientation information in navigation.

How insects model their external environment to facilitate navigation and spatial learning has fascinated researchers for decades. The notion of the "cognitive map" (Tollman, 1948) is often used when considering spatial navigation across a variety of species, e.g., humans (Arnold et al., 2013), rats (Tollman, 1948) and birds (Kramer, 1952). Cognitive mapping is the ability to flexibly use spatial information to solve a simple problem (Jacobs and Menzel, 2014). Simply, this would be evidenced by a participant's ability to use a novel route to go from current location to desired location formulated from prior spatial knowledge of the environment. In a real-world scenario, cognitive mapping allows a displaced animal to orient from a novel location to its familiar home location.

Harmonic radar have been used to show that honeybees (*A. mellifera*) navigate according to a spatial-map like memory (Menzel et al., 2011, 2005). Honeybees can maintain memories of multiple locations (Menzel *et al.* 2011) and these memories can be a culmination of their own past experiences and information imparted to them by nest mates via waggle dances (Frisch,

1967). Honeybees are able to use this acquired information flexibly to use “short-cuts” to and from goal locations and to continually incorporate new experiences and information (Menzel et al., 2011). Bees are remarkable central place foragers, able to utilize landmarks to locate foraging patches and return to a central hive. Bees regularly forage over large distances, making an ability to relocate the hive key to foragers of social bee species. In bees, such navigational abilities rely heavily on a ‘celestial compass’, governed by polarised light (Frisch, 1967) and using optic flow as an odometer to determine the distance travelled from the hive (Shafir and Barron, 2010). However, bees also utilise visual landmarks to navigate to and from the hive (Cartwright and Collett, 1983, 1982; Collett et al., 2013, 2002; Woodgate et al., 2017), using orientation flights to develop a visual memory of landmarks and goal location (Zeil et al., 1996). When a forager re-encounters these landmarks they inform which action should be performed next, for example, to approach the landmark (Frisch, 1967), turn left or right (Zhang et al., 1996), or in a particular compass direction (Chittka et al., 1995). In this way, visual and spatial cues can be used to teach bees in learning tasks (Collett et al., 2002; Dyer, 1998; Srinivasan, 2010; Zhang et al., 2012).

3.1.2 Appetitive and aversive learning

Although bees have long been used as a model insect species in which to study learning and memory, this study has focused near exclusively on the use of appetitive learning in the Western honeybee (*A. mellifera*). The effects of pesticides on bee spatial learning and memory has been identified as a key research gap, and new assays to assess this called for (Ofstad, 2011). Furthermore, a single Pavlovian learning protocol, the PER, has been used to assess honeybee learning and memory for over 50 years (discussed in Chapter 2). Recently, new aversive conditioning protocols have been developed for the honeybee, e.g. the SER, (Tedjakumala and Giurfa, 2013), but there remains a dearth of aversive methodologies, particularly in respect to non-*Apis* bee species (reviewed in Chapter 2). Appetitive (reward) and aversive (punishment) systems are mediated by differential sets of aminergic neurons in the honeybee brain. Punishment is known to be signalled by dopaminergic neurons, whereas reward is signalled by octopaminergic neurons, allowing the separate learning of appetitive and aversive experiences (Menzel and Benjamin, 2013). The consequence of this is that bees should be able to learn both an appetitive and an aversive learning task, as modulation of the systems is entirely separate.

The way in which bees experience the world is very different to humans. Bees rely on a variety of environmental stimuli to signal both rewards and danger. Within social bee colonies, such as honey and bumblebees, hive temperature is critical to brood development and changes in brood temperature of even 0.5°C have been shown to have a significant influence on bee health, mortality, behavioural and morphological defects (Groh et al., 2004; Jones et al., 2005; Tautz et al., 2003). Interestingly, pupae raised at non-optimal temperatures are also more susceptible to pesticide impacts as adults (Medrzycki et al., 2010) and pupal development temperature can affect task allocation in adult bees (Becher et al., 2009). Detection of temperature fluxes and tight thermoregulation are therefore vital to the health of social bee colonies. Honeybees control the temperature of the hive with utmost precision, maintaining the brood between 32°C and 35°C (Hammer et al., 2009). If hive temperature becomes too high then bees can implement behavioural mechanisms, such as fanning, to remove the hot air or collect water to cool the hive back to the optimum temperature. When the hive temperature drops too low bees can generate metabolic heat through flight muscle vibrations or as bumblebees do; build a waxy covering over the brood to keep the heat in. These behavioural modifications under temperature stress suggest that bees have highly adept temperature detection systems, allowing these behavioural modifications to be implemented.

An ability to detect and respond to temperature is not just applicable to avoidance of over or under heating by bees, as bumblebees, honeybees and solitary bees have been shown to be adept at detecting flowers based on floral differences in temperature patterns, utilising thermal detectors in their tarsi and antenna (Dyer et al., 2006; Hammer et al., 2009; Harrap et al., 2017; Norgate et al., 2010; Whitney et al., 2008). Some floral heat patterns are as much as 11°C warmer than the surrounding flower (Harrap et al., 2017). More importantly, bumblebees have been shown to be able to use these heat patterns to discern which flowers have the highest floral rewards (Harrap et al., 2017), and it is likely that these heat signals work in conjunction with other floral cues (e.g. scent and colour) to attract pollinators to floral nectar rewards. This demonstrates that temperature is an integral and reliable cue to bees; involved in both avoidance (of too high temperatures) and attraction (in terms of floral reward signalling), making it an appropriate environmental stimulus to utilise in bee conditioning.

Specific thermal receptors for peripheral temperature detection have been identified in the honeybee, for example the Hymenoptera specific Transient Receptor Potential Ankyrin (HsTRPA), which has been identified in many bee sensory structures, including the legs, proboscis and antennae (Junca and Sandoz, 2015; Kohno et al., 2010). Although bees clearly have the means to detect and perceive high temperatures as aversive stimuli, as identified in Chapter 2, studies utilising temperature as an aversive stimulus are extremely rare.

3.1.3 Research gaps

As highlighted in Chapter 2, the literature shows a bias towards honeybee studies in both the field of ecotoxicology and learning and memory. Furthermore, a critical gap still exists in the study of alternative (non-appetitive) conditioning paradigms, such as aversive conditioning, in non-*Apis* groups (Muth and Leonard, 2019; Siviter et al., 2018b). Although work in this direction has begun, there is still a vast inequality in terms of our understanding of *Bombus spp*. behaviours and learning and memory impacts in comparison to the Western honeybee (*A. mellifera*). The major knowledge gaps that exist at present also prevent the full evaluation of pesticide impacts on alternative pollinators. The objectives of this study (this Chapter and Chapter 4) were to use a thermal-visual arena platform in a pilot study and ultimately in a full-scale, replicated trial (Chapter 4) to determine whether heat can be effectively used as an aversive training mechanism for bumblebees. This will allow assessment of *B. terrestris audax* foragers' ability to perceive a high ambient temperature (45°C) as an effective aversive stimulus that delivers motivation to complete a learning task (i.e., identify and return to an inconspicuous cool reward zone in an unappealingly hot arena by utilising visual pattern cues).

3.1.4 Aims of the pilot study

1. Develop and optimise a thermal-visual arena for aversive conditioning of the bumblebee *B. terrestris (the buff-tailed bumblebee).*
2. Assess whether temperature can effectively be used as an aversive stimulus for bumblebees
3. Assess whether this form of aversive conditioning can be used to achieve behavioural modification in the form of a learnt task in *B. terrestris* in this novel thermal-visual arena.

To meet these aims, it was necessary to assess whether bumblebees can determine the difference between the heated and cool tiles of the arena floor by simply walking across them and that they can then learn the location of these cool reward zone tiles over a series of learning trials.

3.2 Methods and Results

3.2.1 The conditioning paradigm

In the thermal-visual arena, we exploit bees' exceptional spatial memory abilities, over a series of training trials, to teach foragers to utilise visual cues to locate a reward zone. The arena uses an aversive conditioning paradigm, with environmental temperature as an aversive stimulus. Heat is a natural stimulus for bumblebees and temperature variations play an important role in bees' life. In the arena, foragers are conditioned to locate a cool 'reward' zone (25°C) in an unappealing hot arena (45°C).

3.2.2 Thermal-visual arena experimental design

The thermal-visual arena (Figure 3.1) is a novel aversive place learning assay for bumblebees. The arena was constructed based upon a design used by Ofstad, Zuker, & Reiser (2011) to study walking *Drosophila* trajectories. To facilitate control of the arena's temperature, the arena floor consists of a Peltier array of 64 2.5 x 2.5 cm individually controllable thermoelectric Peltier elements arranged in an 8 x 8 grid. The grid is covered in white masking tape to create a conspicuous, featureless surface which can be easily cleaned and replaced between trials to prevent scent marking by foragers. This surface also facilitates easy tracking of a dark bee silhouette on a light, white background (Figure 3.1.C). A thermal imaging camera (FLIR C2 compact thermal camera, FLIR Systems UK, West Malling, Kent, UK) fixed above the arena (see Figure 3.1 A and D) allows for confirmation that no large-scale thermal gradients exist across the platform during trials, which may have influenced test subjects.

A Perspex tube placed onto the Peltier platform creates the arena walls (Figure 3.1.A). A 'landscape' of visual patterns is adhered to the surface of the tube's circumference to create a visual landscape consisting of repeating patterns of horizontal bars, stars, dots and vertical bars, denoting four quadrants of the circumference (see Figure 3.1.C).

Light-emitting diodes (LEDs) (2100 lumens, colour temperature 6500K) around the top edge of the arena (Figure 3.1.C) are used to light the arena consistently above the bee flicker fusion frequency to prevent potential behavioural disturbances (Inger et al., 2014). The arena was kept in a controlled environment room maintained at 22°C on a day: night cycle of 16:8 hrs.

3.2.3 Arena optimization

As the original arena design (Ofstad et al., 2011), upon which the thermal-visual arena was based, was designed for testing *Drosophila melanogaster* it was necessary to optimise the new arena to allow the study of the much larger, more socially complex *B. terrestris.*

Temperature optimisation

Research has shown that bees are highly capable of finding and remaining in areas of preferred temperature and of avoiding unfavourable temperatures (Grodzicki and Caputa, 2005; Ohtani, 1992; Scheiner et al., 2013). Initially, as used in Ofstad *et al*. (2011), the temperature of the arena's "hot", aversive tiles were set at 35°C. This is a highly aversive temperature for *Drosophila* (Ofstad, 2011), however, *Bombus* spp. have a much higher temperature tolerance and are highly efficient at regulating their body temperature across a large range of ambient temperatures (Oyen and Dillon, 2018). Bumblebees have been shown to be able to fly and forage normally at temperatures between 16°C and 36°C (Couvillon et al., 2010), making it unlikely that 35°C would be perceived as an aversive training condition. The critical thermal maximum (CT_{max}) is a measure of an organism's upper thermal tolerance limit and can thus be defined as a highly aversive stimulus. For three bumblebee species (*Bombus huntii, Bombus bifarius* and *Bombus sylvicola*) CT_{max} was recorded at 44-46°C (Oyen et al., 2016), It was therefore decided that 45°C would be an ecologically relevant temperature as an aversive condition for *B. terrestris*.

Optimizing the reward zone

It was apparent during the pilot trials that one Peltier tile (2.5cm x 2.5cm) was not large enough to be the cool, 'reward' zone for *B. terrestris*. Originally designed for *Drosophila*, a much smaller insect, bumblebee worker size ranges between 11-17 mm in length, meaning that inclusive of leg reach, a bee can easily span more than one of the Peltier tiles when stationary. This made it difficult for individuals to identify the single tile cool zone beneath them when sat upon it. Due to the wiring of Peltier tiles in the maze, there was no immediate way to control four adjacent tiles to increase the cool zone surface area (Figure 3.2.A). It was therefore necessary to rewire the Peltier array to facilitate a larger reward zone (Figure 3.2.B).

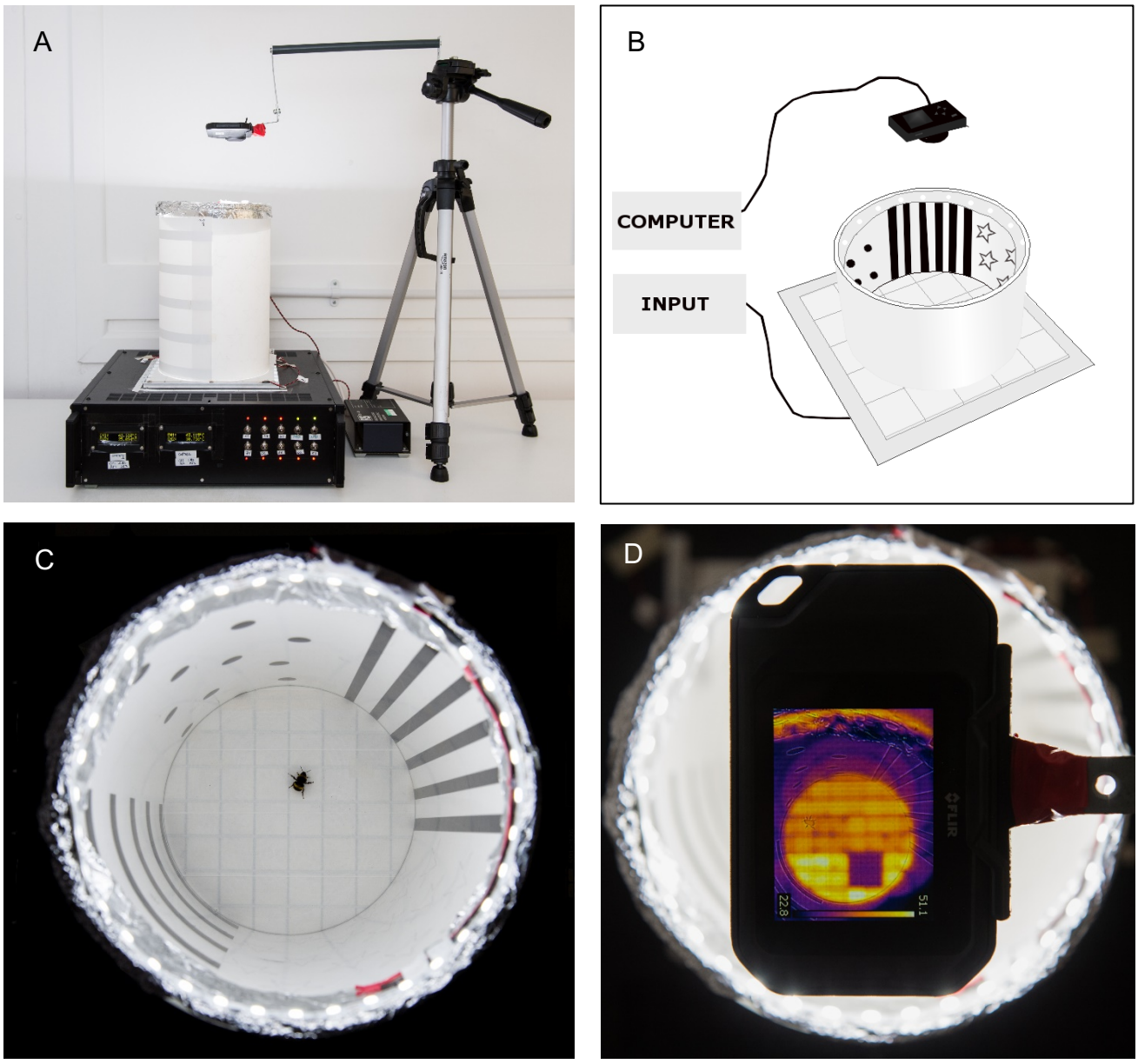

Figure 3.2: The thermal-visual arena. A) Arena set up, with thermal imaging camera placed above the arena. B) Birds-eye view of the arena displaying circumference wall patterns. C) Thermal image of the reward platform, showing the cool reward zone tiles (in purple).

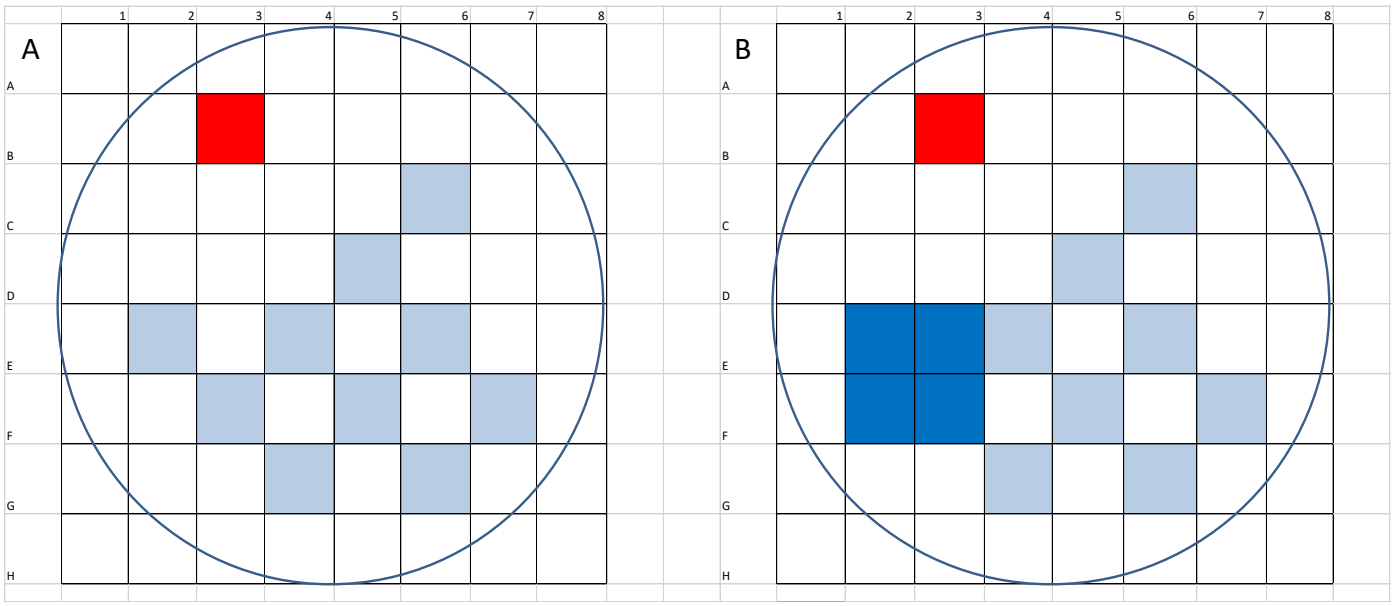

Figure 3.2: Peltier design. A) Original design of Peltier elements on floor of thermal-visual arena. Light blue squares indicate individually controllable tiles which are wired to be switched between hot and cold. Red tile indicates start position for bees for all trials. B) The new design indicates in dark blue the new reward zone of four, adjacent controllable tiles. This facilitates a larger, controllable reward zone appropriate for bumblebees.

To temporarily overcome the reward zone size issue, whilst the arena was being rewired, an alternative scenario was tested, whereby four controllable tiles closest to each other were set to cool to try to generate a larger "cool zone" (Figure 3.3.A). However, in this arrangement, the central tile could not be cooled and was therefore still heated to 45°C (Figure 3.3.A). To temporarily cover this hot tile and extend the cool zone, a corrugated card platform was constructed (Figure 3.3.C) over the central hot tile to act as an insulator. The stepped platform could easily be climbed by the bees, allowing them to escape the heat and test whether the heated floor acted as an aversive training condition. However, it is important to note, that at this stage bees clearly may have been using the platform as a visual cue to the cool zone and not utilising the visual patterns around the arena's edge. This was therefore only a short-term

solution to continue to assess whether aversive training was possible, until the Peltier tiles could be rewired.

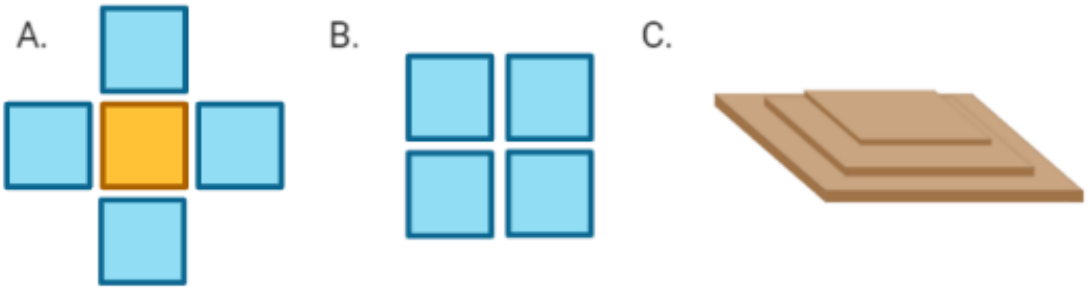

Figure 3.3: Peltier rewiring design. A) Arrangement of cool zone Peltier tiles prior to re-wiring (location E4, F3, F5, G4 (cool tiles) and F4 (hot tile) on Figure 3.2.A). Blue indicates cold tiles during the raised card platform trials, orange indicates the central hot tile which could not be cooled. B) New proposed rearrangement of Peltier tiles to provide a larger cool zone (F2, F3, E2, E3 on Figure 3.2.B). C) Diagram of the corrugated card platform constructed to provide a cool reward zone in early pilot studies prior to Peltier reconfiguration.

3.2.4 Pilot study methodology

Bee test subjects for pilot studies

All subjects were *B. terrestris audax* female workers from standard research hives (without cotton wool, colony size: standard) obtained from Biobest Belgium NV (Westerlo, Belgium). For the pilot study, ten workers were removed at a time from the central hive and kept individually for the duration of the trials. It was initially deemed appropriate to use the standardised procedure of immobilising the hive through sedation with CO_2 (Dietemann et al., 2013). However, in initial trials of this method, when workers recovered from sedation they preceded to attack and kill the queen. This happened even if the queen herself was not sedated (trialled as it was thought perhaps a change in her pheromone production when sedated was triggering the workers' behaviour). The potential behavioural impacts of CO_2 sedation on ovary activation in honeybees (Koywiwattrakul et al., 2005) has been documented, but we could find no recorded behaviour of this type in bumblebees. However,

to avoid future colony losses, hives are no longer sedated in any way, instead, individuals are selected and handled under red light instead.

To study bees' walking trajectories, it was necessary to confine foragers to the test platform. Bees' wings were clipped using dissection scissors and a queen marking cage (EH Thorne Ltd, Market Rasen, UK) and each bee was placed into an individual roller cage (EH Thorne Ltd, Market Rasen, UK) and provided with 50% (w/v) sucrose solution in a 1ml syringe with its tip cut off to allow easy feeding access to bees (Figure 4). Roller cages containing bees were kept in an incubator at 25°C at 55% humidity on a 16:8 hr day: night cycle. Cages were placed adjacent to each other to maintain visual and olfactory communication between hive members. Post wing clipping, bees were left to recover for 24 hours prior to the start of trials.

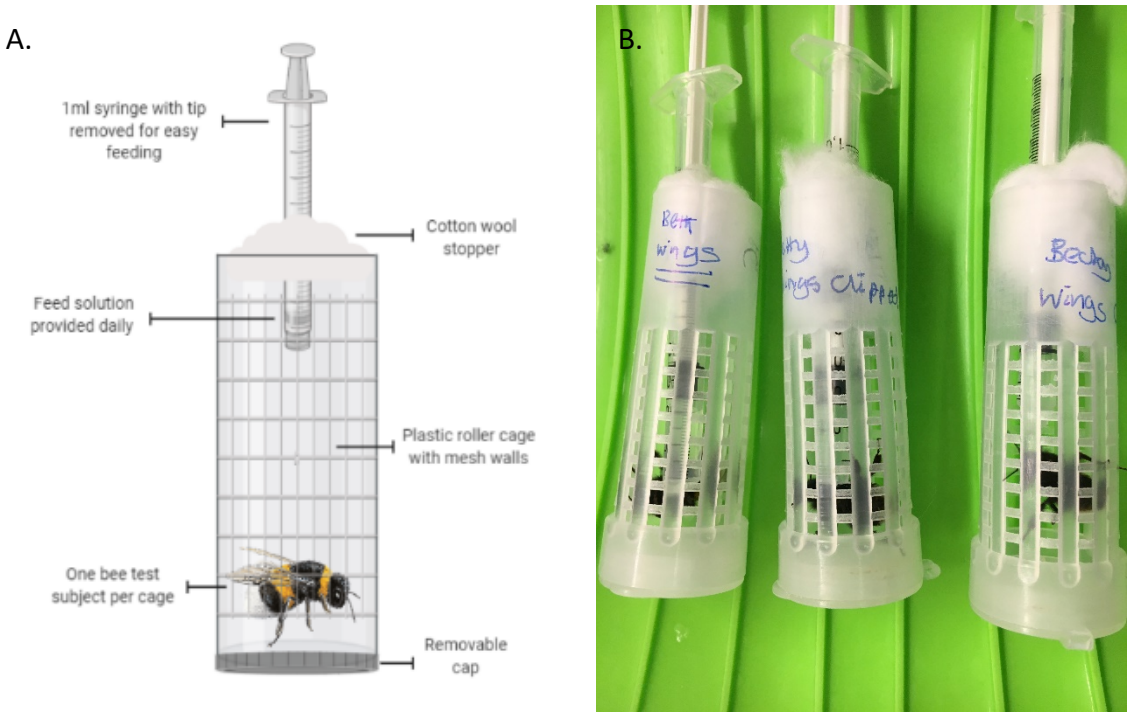

Figure 3.4: Roller cage housing. Diagram of A) and in situ B) modified roller cages used to house bee test subjects. Each cage has a removable cap to facilitate easy removal of bees. The uncapped end was plugged with cotton wool and a 1ml syringe (tip cut off) of 50% sucrose solution provided to allow bees to feed.

Experimental design

A pilot study was designed to test whether it was plausible to train bees with temperature as an aversive stimulus in a novel thermal-visual arena. As, even with arena optimisation for bumblebees (increased arena temperature and reward zone size, see 'Arena optimisation'), it is unclear whether bees would be able to sense and perceive the arena floor temperature as a strongly aversive stimulus.

Initially, it was decided to utilise both appetitive conditioning (sucrose reward) and aversive conditioning (heated floor of the maze) to give bees the highest incentive to find the reward zone. To motivate bees to find the sucrose reward, they were starved prior to trials. Initially this starvation period was trialled at 3 hours, which proved too long as bees became very lethargic and inactive, which would have affected foraging or searching ability. 2, 1 and 0.5-hour starvation periods were trialled as alternatives. 1 hour seemed to be adequate to incentivise foraging efforts without causing detrimental effects.

Tiles 2F, 3F, 3E and 2E of the arena were used as cool reward tiles (25 °C) (Figure 3.2.B) and all other tiles set to 45°C (see 'arena optimization'). Tile 3B was used as the start tile for behavioural trials. 20 µl 50% sucrose solution was positioned at the centre of the cool reward zone. Bees underwent trials in the arena individually to prevent social learning from nest mates (such as is seen in Alem *et al.*, 2016). Bees were positioned and held on the start tile using a clear Perspex tube until the start of the trial. Upon release, each bee was timed for a five-minute trial. The time taken for the bee to locate the cool zone and the time the bee spent within the cool zone (within the five-minute trial) was recorded.

Spaced conditioning, in which temporal spacing exists between successive conditioning trials, has been shown to lead to higher memory consolidation in bees, especially at long intervals (Menzel et al., 2001). How learning trials are spaced is shown to be the dominant factor in both acquisition and retention of memory in bees (Menzel et al., 2001). Thus, instead of conducting all 10 training trials on one day, bees were given 10 x five-minute training trials spaced over three days (Monday: trials 1-4, Tuesday: trials 5-7, Wednesday: trials 8-10 and flash frozen). This was kept consistent across all replicates.

For each bee, initially (trials 1-5) two training stimuli (aversive and appetitive) were used to provide the most incentive for the bee to learn the reward zone location. From trial 6 through

to 10, only one training stimulus was used (aversive) to determine whether aversive conditioning alone was enough to still motivate the bee to locate the reward zone.

Between each trial the arena was wiped down using 70% ethanol and the masking tape floor covering was replaced to prevent individuals from using scent marks from previous trials to solve the task and locate the reward zone. Bees were confined to roller cages in-between trials to prevent further foraging experience whilst not in the arena and standardise the amount of foraging experience in the arena each bee received. Pilot trials were repeated three times, with a total of 28 bees, to allow adjustment of experimental technique.

Video tracking and processing

All trials were recorded using a FLIR C2 compact thermal camera (FLIR Systems UK, West Malling, Kent, UK) attached to a tripod above the arena (Figure 1). Videos were recorded using CamStudio 2.7 video capture software (https://camstudio.org/).

Trial 1 and trial 10 videos were processed for each bee to allow a comparison of pre- and post-training results. Video files were processed in Ctrax: The Caltech Multiple Walking Fly Tracker (Branson et al., 2009) to produce tracking data of x and y coordinates and timestamps from each trial. Ctrax coordinate files were imported into MATLAB (MATLAB and Statistics Toolbox Release 2012b, The MathWorks, Inc., Natick, Massachusetts, United States).

3.2.5 Preliminary results

Trajectory plots of pilot study bees pre- and post- training

Trajectory maps of the routes bees took in trial 1 (pre-training) and trial 10 (post-training) were produced in MATLAB using the raw X and Y coordinates outputted by CTRAX tracking software. These trajectories allow an initial visual assessment of a) whether the aversive stimulus of a heated floor provides enough motivation to encourage bees to locate the reward zone and b) whether there seemed to be any improvement in this ability between pre- and post-training trials. Figure 3.5 gives an example set of pre- and post-training trajectory graphs for four of the bees in the pilot trial. The figure demonstrates that all four of the given bees are seemingly able to locate the cooler reward zone location by trial 10 (post-training), as is evidenced by the centralisation of bee trajectories to the reward zone

location in the bottom left of the arena. This suggests that *B. terrestris* foragers can perceive ambient temperature as an aversive stimulus when walking in the arena and are actively seeking out the cooler zone as a reward.

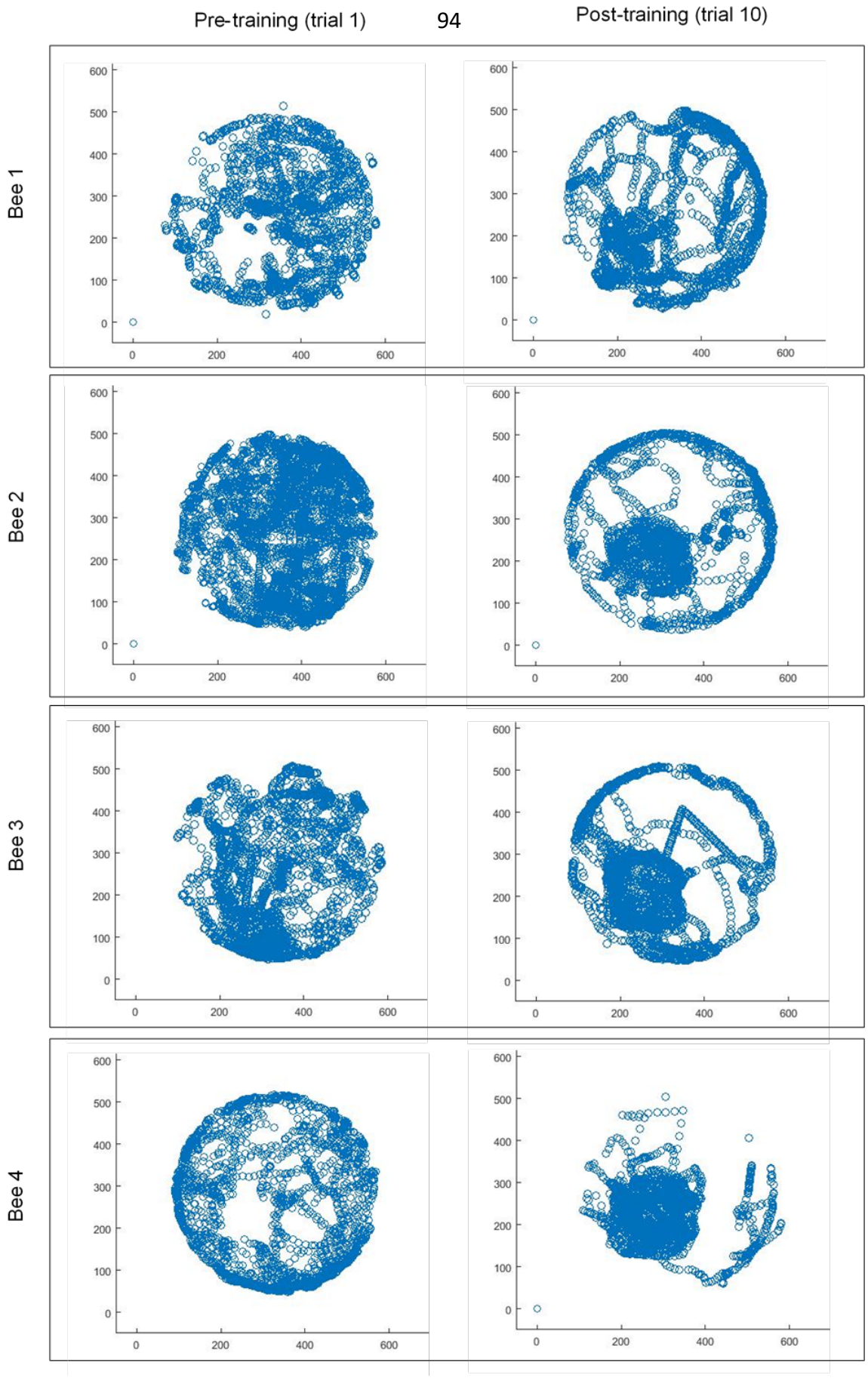

Figure 3.5: Example trajectory plots pre- (trial 1) and post-training (trial 10) for four of the pilot study bees. Marked improvement in ability to locate the reward zone (bottom left of the arena) is seen in trial 10 versus trial 1.

3.4 Discussion

The example pre- training trajectory graphs (Figure 3.5) demonstrate that, when first placed in the novel arena environment, irrespective of the aversive stimulus of the heated floor and the appetitive reward provided in the cool reward zone, individuals trace widely diverse paths, covering all areas of the arena, with some bees (e.g. bees 1 and 4) avoiding the reward zone entirely.

Several explanations could be put forward for these movement patterns. Potentially the first instinct of the bees when placed in an unfavourable temperature environment is to explore all possible options for escape and therefore covering the most possible paths within the arena is a good strategy for locating a possible escape route. Equally, these trajectories could be visual representation of a bee's response to a novel environment, in which the bee is trying to learn as much as possible about the novel space they are in by covering as much ground as possible. Seeming avoidance of the reward zone in trial 1 (e.g. by bees 1 and 4) could again be a response to avoiding the conspicuous card platform used as a reward zone prior to the rewiring of the Peltier tiles (Figure 3.3).

However, by trial 10 (post-training), it appears from the trajectory visuals that individuals have learnt the location of the cool reward zone (located in the bottom left of the arena, Figure 3.5). Individuals appear increasingly motivated to locate and remain in the cool reward zone when compared to the trial 1 trajectories. In comparison to pre-training, the trial 10 bee trajectories, when they do leave the reward zone, show more directed, specific exploratory paths out from this zone to a facet of the arena and back, rather than the dense web of paths seen in trial 1. This implies that individuals are learning and remembering the location of the reward zone to be able to return directly to it. These pilot results are promising, suggesting that bees can learn the reward zone location through aversive conditioning in the thermal-visual arena. Initially, both aversive and appetitive stimuli were used in trials 1-5 to provide the highest motivational push and pull to bees to locate the reward zone. However, due to the joint use of these two stimuli for trials 1-5, it is difficult to disentangle which method of conditioning (aversive or appetitive) is the most effective training approach within the thermal-visual arena. To acertain which conditioning works best in the arena separate, comparative training trials needed to be undertaken.

In future trials, new avenues for improved tracking need to be explored. Here CTRAX was used post-recording to track individual bees across video frames. However, as can be seen from Figure 3.5, occasionally the software picks up non-bee movements within the video frame, for example changes in the wires between the Peltier elements (evidenced by outlier dots, outside of the arena circumference in some of the trajectory graphs in Figure 3.5). In future, new tracking software, e.g. idTracker (Pérez-Escudero et al., 2014), and enhanced parameterisation will be trialled to reduce the number of errors which are picked up outside of the arena circumference.

In the current pilot trials it is difficult to ascertain whether bees are utilising the landscape patterns around the circumference of the arena to locate the reward zone, as for the majority of the pilot study, a card platform (Figure 3.3) was used to create the cool reward zone whilst Peltier rewiring took place. This means that although it appears from the trajectory graphs (Figure 3.5) that bees are learning that the aversive environment is unfavourable and that escape to the cool reward zone is favourable, we cannot be sure which of the visual cues are being used by bees (the landscape patterns or the card platform), and is likely that the conspicuous card platform is playing a large role in visual location. In future trials, the Peltier array will be reconfigured to ensure the reward zone is inconspicuous and cannot be visually discerned from any of the other floor tiles (Figure 3.3). Therefore, if individuals are still able to locate and remain in the reward zone (as suggested in the post-training trajectories in Figure 3.5), then we can have increased confidence that bees are using the landscape patterns on the arena wall as a navigational tool.

These initial data suggest that the heated floor of the thermal-visual arena can indeed be perceived as a strongly aversive stimulus by *B. terrestris*. It is now necessary to ascertain whether, although well perceived, aversive conditioning is the most effective method of training bees to locate the arena's reward zone? If aversive conditioning is not comparably effective to other training methods, then there is little point in its use over other such methods.

Chapter 4

The thermal-visual arena: a meaningful aversive assay for bumblebees

Chapter 4: The thermal-visual arena: a meaningful aversive assay for bumblebees.

4.1 Introduction

Insects rely upon learning and memory for integral parts of their life histories; for predator avoidance, social interaction, foraging, and sexual behaviours (Dukas, 2008; Greggers and Menzel, 1993). In comparison to their often impressive behavioural repertoires insects have relatively simple, modular brains, with separate olfactory, visual and tactile pathways merging in the mushroom bodies (MBs); the primary sites of insect learning and memory (Menzel and Giurfa, 2001). MBs are paired structures found in the insect brain, essential for the storage and retrieval of memories and research has previously associated these structures with olfactory learning and memory (Akalal et al., 2006; Heisenberg, 1998). The role of MBs has been studied widely in the model insect species, *D. melanogaster* (Heisenberg, 2003; Keene and Waddell, 2007) and *A. mellifera* (Giurfa, 2013). In these species MBs were shown to have a key role in the retrieval, storage, and encoding of appetitive and aversive elemental learning (Giurfa, 2013; Heisenberg, 2003; Keene & Waddell, 2007).

Foraging bees are required to use visual information to recall routes to and from the nest and between food sources, as well as for vital pattern recognition (such as the shape of the hive), landmarks, and rewarding floral resources (Pahl et al., 2010). Bees are remarkable visual learners and can learn to distinguish between a variety of pattern orientations including vertical, horizontal, and oblique lines, as well as learning distinct features of these patterns (e.g. black or white, thin or broken lines) (Srinivasan, Zhang, & Witney, 1994; van Hateren, Srinivasan, & Wait, 1990; Wehner, 1972). Honeybees can readily distinguish pattern orientation of unidirectional patterns, even at a distance, and can learn to associate set pattern orientations with rewards, so avoiding unrewarding orientations (Srinivasan et al., 1994). The location of a pattern within the bee visual field is also key to recognition, with visual field position topographically represented in the bee central nervous system and the central lower sector of the visual field appearing most adept at pattern recognition (Wehner, 1972). This ability to discriminate between pattern orientation appears to be derived from green-receptor channel signalling in the bee visual system, making bee orientation assessment similar to that of the mammalian cortex (Srinivasan et al., 1993). Taking into

account these bee visual perception factors, the pattern array chosen for the thermal-visual arena assay is made up of four quadrants of distinct black and white patterns, at least two of which (vertical and horizontal bars) are known to be highly distinguishable by bees (Figure 4.1).

As discussed in Chapters 2 and 3, aversive conditioning studies are strongly ecologically relevant to bee life histories as, in the field, aversive conditions are commonly encountered by bees. Examples of these conditions include; predator encounters, unfavourable climatic conditions, repellents, and agrochemicals (Botías et al., 2017; Colin et al., 2020; Jones and Dornhaus, 2011; Kingsolver et al., 2013; Martinet et al., 2015; Prado et al., 2019; Rodríguez-Gironés, 2012). These encounters require adept escape and avoidance responses with escape behaviours providing a key means of predator avoidance in the wild (Zhang & Nieh, 2015). Escape conditioning is an aversive conditioning training method in which a negative event occurs until a required response is given, upon which the event terminates and the individual "escapes" the aversive condition (Mackintosh, 1983). The results of such escape conditioning are similar to those achieved with food rewards in appetitive conditioning (Mackintosh, 1974), promoting this method for use in future aversive bee learning paradigms, and here in the thermal-visual arena.

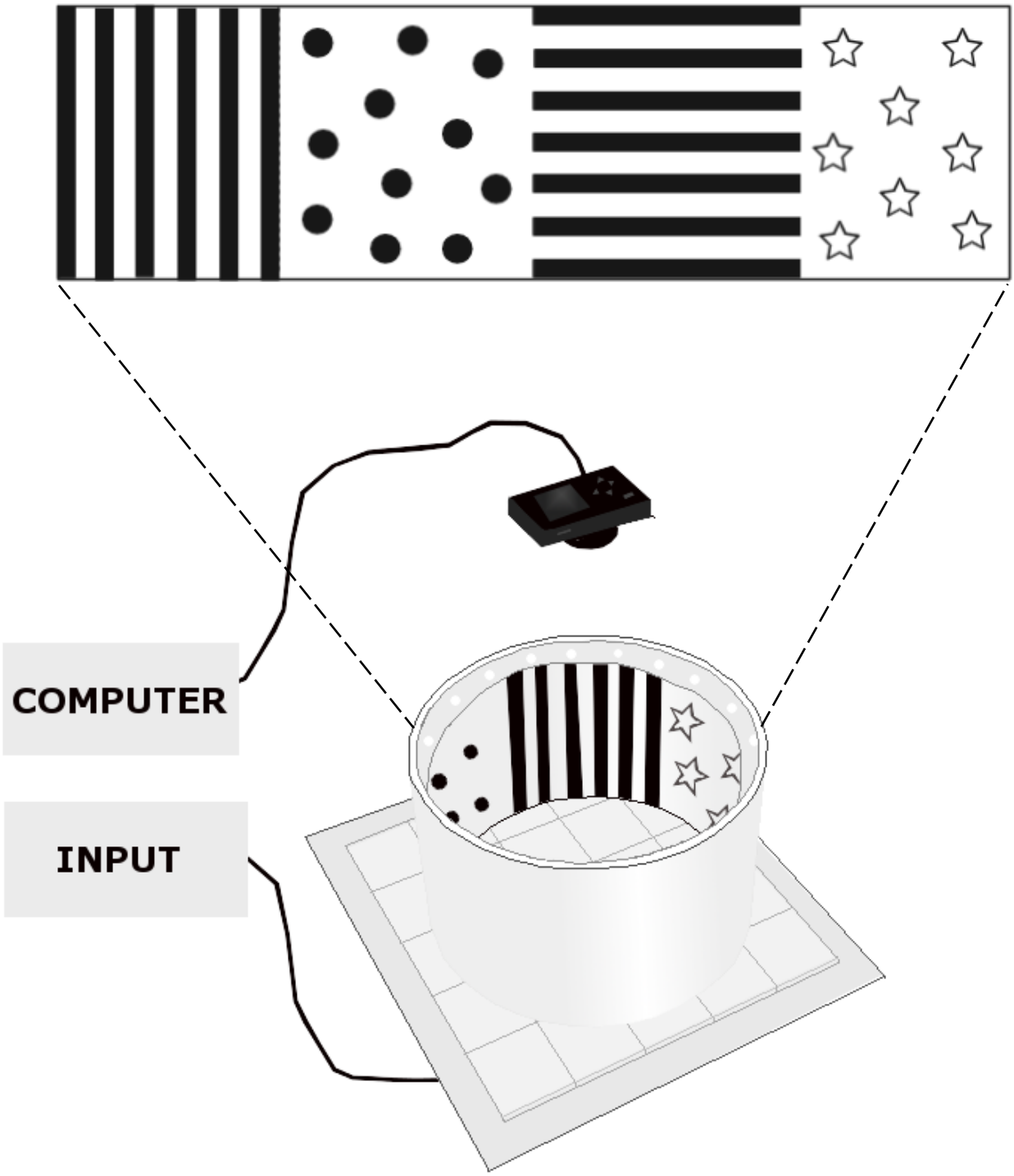

Figure 4.1: Schematic of the thermal-visual arena pattern array. The array features four quadrants, each containing a different pattern of either vertical bars, horizontal bars, solid black circles or black outlined stars.

4.1.1 Aims of this study

The objective of this work was to assess the effectiveness of aversive escape conditioning in the thermal-visual arena (suggested in Chapter 3) in comparison to alternative conditioning paradigms (appetitive, appetitive + aversive, control) to ascertain which method provides the most effective training for *B. terrestris* in the thermal-visual arena. As, regardless of whether aversive conditioning appears to be effective (Chapter 3), if it is not the most effective training tool, then this could explain its underutilisation for bee studies. It was also necessary to assess the degree to which bees utilise the arena's patterns to complete the learning task.

The aims of this study were therefore:

1. To compare aversive (high temperature) conditioning to alternative training methods including appetitive (sucrose reward) conditioning, combined conditioning (aversive & appetitive), and no conditioning (control), to determine the best training method to achieve the task in the thermal-visual arena.
2. To assess whether bees use visual cues around the outside of the arena to complete the learning task through red light trials.
3. To determine which parameters, give the best indication of "training" (that bees have found and remembered the reward zone location) in the thermal-visual arena.

4.2 Methods

As evidenced by the pilot study (Chapter 3), bumblebees can be trained to locate a reward zone within the thermal-visual arena. Nonetheless, it is unclear whether aversive conditioning, for which the arena was designed, is the most effective method of training in the arena. To determine this, three different methods of conditioning were compared. The training methods assessed are listed in Table 4.1.

Table 4.1: The training conditions and control treatment used in the trials and the relevant stimuli provided.

Training condition:	**Stimuli:**
1) Appetitive	20µl 50% sucrose solution reward given each time the bee enters the reward zone.
2) Aversive	Arena floor heated to 45°C, cool reward zone at 25°C.
3) Combined aversive and appetitive	Conditions 1 and 2 combined. A 20µl 50% sucrose solution reward provided in the cool reward zone.
4) Control	No training stimuli presented: a room temperature arena with no reward zone.

4.2.1 Rationale behind training conditions

It is known that appetitive and aversive learning pathways are separated in the bee brain; with aversive learning governed by dopaminergic neurons and appetitive learning governed by octopaminergic neurons (Agarwal et al., 2011; Mizunami et al., 2009; Terao and Mizunami, 2017). This should allow the bee to learn appetitive and aversive tasks independently of one another. The individual training methods employed (Table 4.1, training conditions 1 and 2) facilitate the study of the influence of a single training type on bumblebee behaviour in the arena. In contrast to the pilot study, there are no appetitive responses involved in the aversive alone treatment, thus providing the opportunity to study true aversive (or punishment) learning in *B. terrestri*s. However, the combined aversive/appetitive treatment (Table 4.1, training condition 3) should allow examination of whether combined punishment and reward learning provides higher motivation to complete the task than either of the treatments alone.

4.2.2 Bee test subjects

As in the pilot study (Chapter 3), all bees were *B. terrestris audax* female workers from research hives obtained from Biobest Belgium NV (Westerlo, Belgium). Hives were research hives (*Bombus terrestris audax*), without cotton wool, colony size: standard. Upon arrival of

the new hive the colonies were settled in wooden nest boxes (29 x 21 x 16 cm) (Figure 4.2) by transferring the existing hive comb into the nest box and then transferring all hive members onto the comb one by one using forceps under red light. This approach appears to not alter the behaviour of the workers or queen, in contrast to the CO_2 sedation used previously (see pilot study). Hives were provided with Biogluc (Biobest Belgium NV, Westerlo, Belgium) in gravity feeders in a Perspex foraging tunnel (26×4×4 cm) connected to the nest box. Pollen was also provided in falcon tube caps in the Perspex tunnel. Gravity feeders and pollen were replenished, as necessary, to ensure the colony never ran out of food. The new housing design, using wooden nest boxes and Perspex forager tubes, facilitates age cohorting and selection of only forager bees – factors which were not taken into consideration in the pilot trial but may be important in terms of learning and memory capacity.

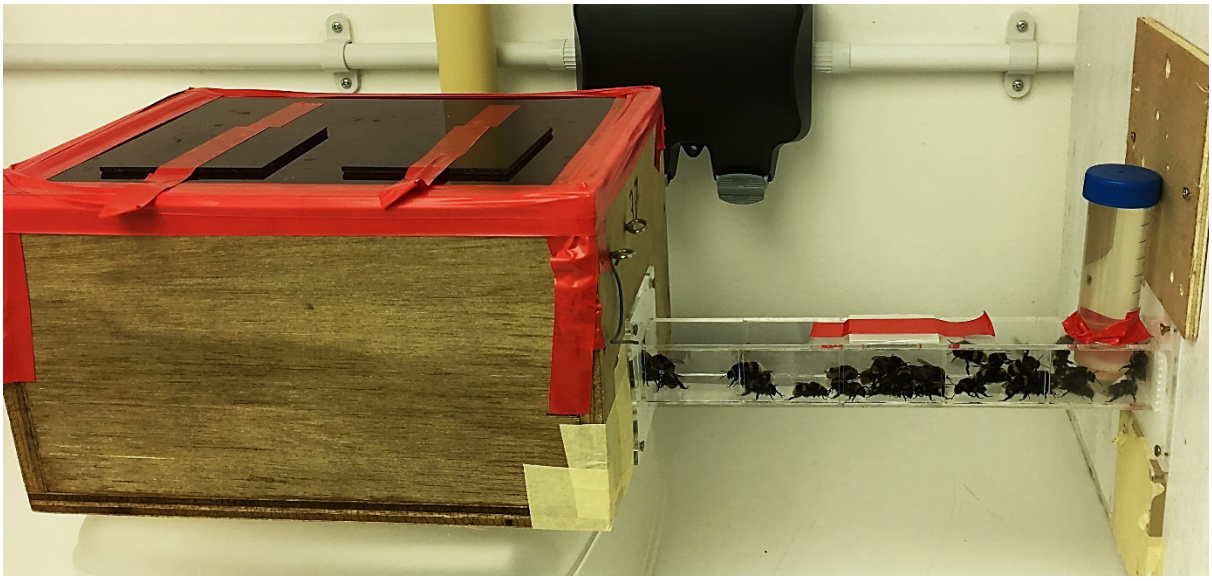

Figure 4.2: Experimental hive setup. B. terrestris colonies settled into wooden nest boxes (29 x 21 x 16 cm) and provided with Biogluc in gravity feeders in a Perspex foraging tunnel (26×4×4 cm).

4.2.3 Bee marking

All original hive members were marked using a queen marking kit (EH Thorne, Market Rasen, UK). Individuals were removed from the hive using forceps, placed in a queen marking cage, and a number tag adhered to the back of the thorax using a small dot of resin glue. Original hive members were all given a white number tag to distinguish them from newly emerged individuals. Newly emerged individuals are marked in colour groups by age cohort. All individuals that emerged within one week are deemed an age cohort and assigned the same colour. Individuals are left to mature for one-week post marking, so that all individuals used in a trial were at a minimum of 1-week post emergence and emerged within the same week of each other.

4.2.4 Forager monitoring

The hive was observed each day and foragers of each age cohort were identified in the foraging tube by their colour and number. From the foragers recorded in each age cohort ten individuals were randomly selected to be tested per trial. The ten selected individuals were then randomly assigned to either the treatment or control group.

4.2.5 Wing clipping

To confine individuals to the test platform surface, bees' wings were clipped using a queen marking cage (EH Thorne Ltd, Market Rasen, UK) under red light to avoid having to sedate individuals using CO_2.

4.2.6 Study period

Although hives are commercially available all year round, it was decided that due to potential variability in the behaviour of colonies produced during winter months (as observed early on in this PhD), trials would only take place during March-October to ensure high reliability and reproducibility of trials.

4.2.7 New bee housing

New, larger rectangular Perspex cages were used to individually house the ten selected bees during trials (Figure 4.3). The roller cages used for the previous pilot study do not allow room for active movement or encourage foraging behaviours, whereas the larger cage design should facilitate more active searching behaviour. Bees in the cages were supplied with cotton wool soaked in 50% (w/v) sucrose solution placed in a falcon tube cap. This method was adopted to allow easier access to the feed than from the previously used syringes (Figure 4.3.A), as bees appeared to be more able to access the sucrose soaked into the cotton wool, rather than the gravity fed sucrose at the tip of the syringe. The gravity fed sucrose syringe method of food provision tended to become inaccessible to bees due to air bubbles developing in the syringe during feeding.

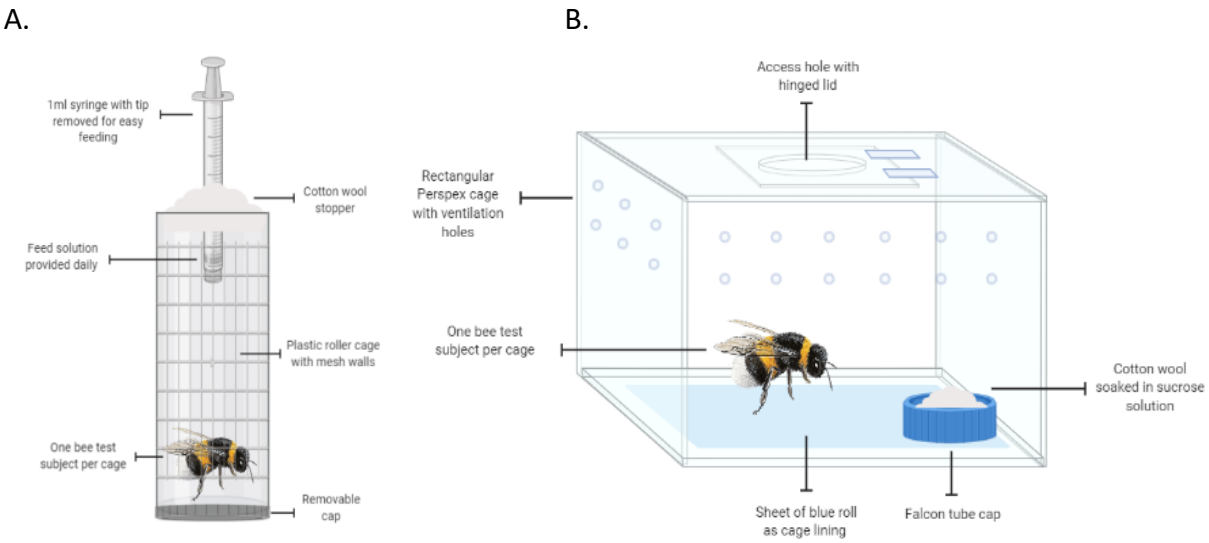

Figure 4.3: New bee housing design. A) Previous roller cage design used in Chapter 3. B) New design utilising rectangular Perspex cages.

4.2.8 New training protocol

The experimental design included 10 training trials for each bee, three minutes each in duration (this was optimised from the five minutes used in the pilot study as three minutes appeared enough for individuals to locate the reward zone in most cases). Bees have several concurrent memory phases; short-term memory (STM), mid-term memory (MTM) and long-term memory (LTM) (Menzel, 2001). STM can be created through a single learning trial and lasts for only a few minutes, with working memory of a visual pattern stimulus decaying exponentially after around eight seconds (Zhang et al., 2005). Multiple conditioning trials can allow the formation of MTM lasting for several hours and LTM occurs 1-2 days post learning (Menzel & Benjamin, 2013). The experiments were therefore conducted as a series of 10 trials, spaced over three days, to give bees the maximum capacity to fix the task into LTM.

All rewards (cool zone or sucrose reward) were inconspicuous, i.e. the cool tiles were not visually distinguishable from any other tiles and the sucrose solution was provided directly on to the masking tape covered tiles of the platform when bees entered the reward zone

location. This meant that the sucrose solution was not provided in a feeder of any kind which bees may have become conditioned to visually recognise through prior feeding. The sucrose reward (20µl) was small enough that it tended to be consumed in one go by the foragers, meaning that it did not remain as a visual cue to the reward zone location upon the next entry.

A replicate was made up of three tests (one per treatment) (A, B and C) (see 4.2.9 below). Each test contained ten bees from the same hive; 5 treatment bees and 5 controls bees. Each replicate (three tests) had a total of 30 bees; 5 appetitive bees, 5 aversive bees, 5 appetitive and aversive bees, and 15 control bees (5 run alongside each treatment). There were three test replicates; all treatments were replicated three times across three different hives (total of 90 bees).

Each bee experienced only one training condition (listed as 1-4 in Table 4.1) for the duration of the 10 trials. Each condition was randomly allocated to five of ten randomly selected foragers from a marked age cohort (random number generator software was used to select ten foragers from the list of all observed foragers in the age cohort, and to allocate treatment condition). These five training treatment bees were tested alongside five control bees of the same hive and age. This ensured bees from each hive and age cohort can be compared to controls from that hive and cohort. These bees may also then be compared to replicates from other hives of the same age cohort.

4.2.9 Order of trials, training method and combination of bee participants

Per replicate (n=3):

Test A: 5 control (1), 5 appetitive (2) for 10 trials

Test B: 5 control (1), 5 aversive (3) for 10 trials

Test C: 5 control (1), 5 combined aversive and appetitive (4) for 10 trials

All individuals were starved for one hour prior to trials. This time frame was determined based upon the pilot studies. This aimed to remove starvation as a confounding variable as if hunger increases searching behaviour, all individuals are pre-exposed to this. If not discussed in the text, all other experimental procedures were identical to the pilot study.

4.2.10 New video tracking technique

Debut video capture software (Debut Video Capture Software Version 5, NCH Software, Inc., 6120 Greenwood Plaza Blvd, Greenwood Village CO, USA) was used to record the thermal-visual arena trials. Video files were then tracked using idTracker, a proprietary software package with proven use in insect research (Pérez-Escudero et al., 2014). idTracker was used here, as in the pilot study Ctrax tracking did not always prove to be reliable, as some of the trajectory plots showed large accelerations and jumps which are unlikely to be the bee's true trajectory. Therefore, to assure reliability of results, all data was reprocessed using idTracker which produced more 'normal', expected walking trajectories.

4.2.11 Data analysis

Training parameters studied

To produce training parameters for each bee, tracking data was exported from idTracker as a .csv file, converted and saved as a .txt file for trajectory analysis (by Jess Evans, Statistics Department, Rothamsted Research) using custom R scripts. The following parameters were calculated for all bees in each treatment (control n = 42, appetitive n = 15, aversive + appetitive n = 15, aversive n = 14 (one aversive bee died prior to completing all 10 trials)) for trial 1 (defined as pre-training) and trial 10 (defined as post-training) videos:

1. Route the bee took within the arena during the trial (also a 'smoothed route' map)
2. Number of times the bee entered each zone within the arena. From this data it was calculated the number of times the bee entered the reward zone.
3. Time the bee spent in the reward zone.
4. The distance the bee was from the reward zone throughout the trial.
5. Average speed of the bee throughout the trial.
6. Total distance travelled by the bee in that trial.
7. The difference in these parameters, for each bee, between pre- (trial 1) and post- (trial 10) training.

Assessing interactions between treatment, training and period

To determine whether training condition affected the percentage of time a bee spent in the reward zone, or the time at which the bee first entered the reward zone, mixed model analyses were undertaken using custom R scripts (by Jess Evans, Statistics Department, Rothamsted Research).

The data were analysed in two steps due to the large proportion of zeros (indicating bees that did not enter the reward zone at all). For the first step, the five bees within each hive-age cohort that received the same treatment were treated as a batch and the proportion of these that entered the reward zone were tested using a binomial general linear mixed model (GLMM). In the second step, the data analysis was restricted to bees that entered the reward zone during the time limit (by the end of the trial). The remaining data were then analysed using a linear mixed model. Both percentage of time spent in reward zone and time first entered reward zone were analysed in this way.

1. *Analysis of controls only:*

The process described above was first carried out only on bees that received the control condition. The models included a single fixed term for period (i.e. pre- vs. post- training). This was to check that the control was suitable in that there was no change between periods when no stimulus was present. The random structure (indicating the structure of the experiment) for the GLMM was Hive/Batch and for the LMMs was Hive/Batch/Bee.

2. *Analysis of all treatments:*

The same process as above was then carried out, accounting for all treatment combinations. Treatments were considered as a 2×2×2 factorial structure, i.e. there were three treatment factors each with two levels:

1. Aversive: yes vs no
2. Appetitive: yes vs no
3. Period: pre-training vs post-training

Therefore, the fixed structure for each model was appetitive* aversive* period. For each model the full factorial structure was considered as a starting point and then backward

selection was used to remove non-significant interaction terms. The random structure was again Hive/Batch for the GLMM and Hive/Batch/Bee for the LMMs.

Treatment differences in training parameters

All of the following training parameter analyses were conducted in GraphPad Prism 8 for Windows (version 8.1.2, GraphPad Software, La Jolla California USA, www.graphpad.com). A suite of normality tests (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov) were run on all datasets, the results of which can be seen in Table 4.2, and determined whether ANOVA (parametric) or Kruskal-Wallis (non-parametric) statistical tests were used for treatment comparisons.

Table 4.2: Parameter datasets taken from bee trajectory data

Dataset	**Data normal?**	**Statistical test run**
'Time spent pre-training'	No	Kruskal-Wallis(non-parametric)
'Time spent post-training'	No	Kruskal-Wallis (non-parametric)
'Times entered pre-training'	No	Kruskal-Wallis (non-parametric)
'Times entered post-training'	No	Kruskal-Wallis (non-parametric)
'Total distance pre-training'	Yes	ANOVA: multiple comparisons
'Total distance post-training'	Yes	ANOVA: multiple comparisons
'Average speed pre-training'	Yes	ANOVA: multiple comparisons
'Average speed post-training'	Yes	ANOVA: multiple comparisons

4.3 Results

It was decided to assess a range of the behavioural parameters recorded during the trials, to ascertain which was the most useful measure of 'training' in the context of this novel arena. These parameters included the trajectory or 'route' a bee took during a trial, the number of times the bee entered the reward zone, the amount of time the bee spent in the reward zone within the trial, the distance the bee was from the reward zone throughout the trial, and the average speed and total distance travelled by the bee across the trial. These parameters were calculated pre- (trial 1) and post- (trial 10) training for each bee.

4.3.1 Trajectories: what can they tell us?

The calculated training parameters can be used to visualise what 'training' may look like for bees in each training environment. Below are some example parameters from one bee in each of the training treatments; control (Figure 4.4), appetitive (Figure 4.5), aversive + appetitive (Figure 4.6), and aversive (Figure 4.7), all pre- and post- training.

If the route maps are considered as visual representations of training, bees appear to implement differing exploratory strategies, dependent on the reward or punishment environment they are in. Pre-training (Figures 4.4A, 4.5A, 4.6A and 4.7A), individuals across all treatments trace similar exploratory trajectories which indicates that training has not yet induced behavioural modification. In the control condition (Figure 4.4), the individual traces concentric paths which delineate the arena boundary, showing little change in trajectory pattern between pre- (Figure 4.4A) and post- (Figure 4.4B) training. However, in the appetitive reward environment post training (Figure 4.5B), some localisation of the bee trajectory to the left-hand side of the arena is apparent. This corresponds to where the reward zone is situated but this is not distinct or specific to the exact reward zone location. This localisation to the reward zone is further pronounced in the two conditions containing an aversive training element (aversive + appetitive (Figure 4.6) and aversive (Figure 4.7)). In the aversive conditions, with a heated floor, individuals were motivated to locate and remain in the cool reward zone. Therefore, post-training trajectories (Figure 4.6B and Figure 4.7B) show directed exploratory paths out from the reward zone to a facet of the arena and return to the reward zone. This is not surprising as this is the condition which should provide foragers with the most motivation to remain in the reward zone, with two rewards (sucrose and cool zone) and

a punishment in the form of the heated arena floor. As no other navigational cues are provided, this provides an initial suggestion that individuals can utilise the arena's landscape patterns to navigate to and from the reward zone.

Control bees

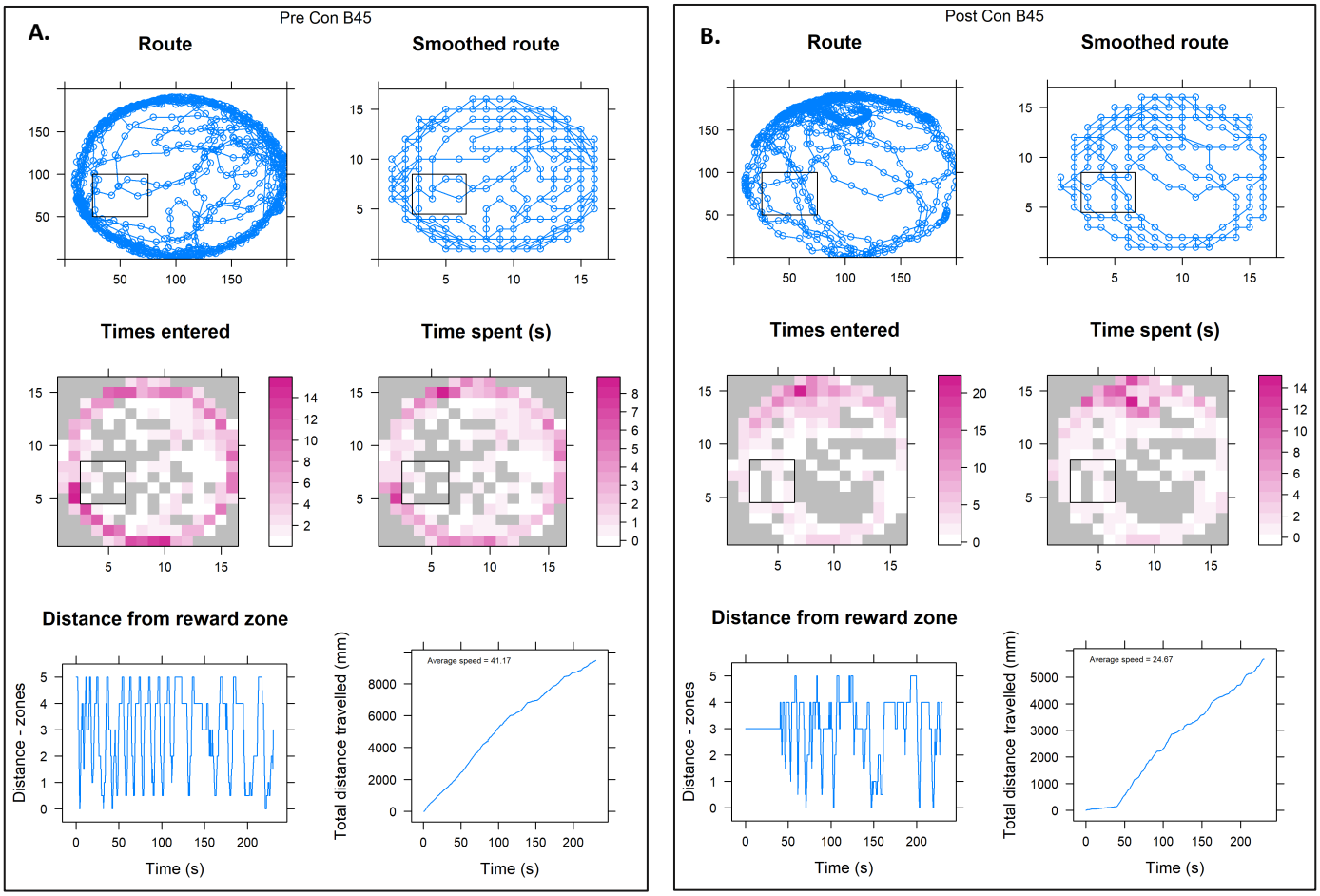

Figure 4.4: Training parameters from a control bee (B45) pre- A) and post- B) training. The black square indicates reward zone location within the arena.

Appetitive bees

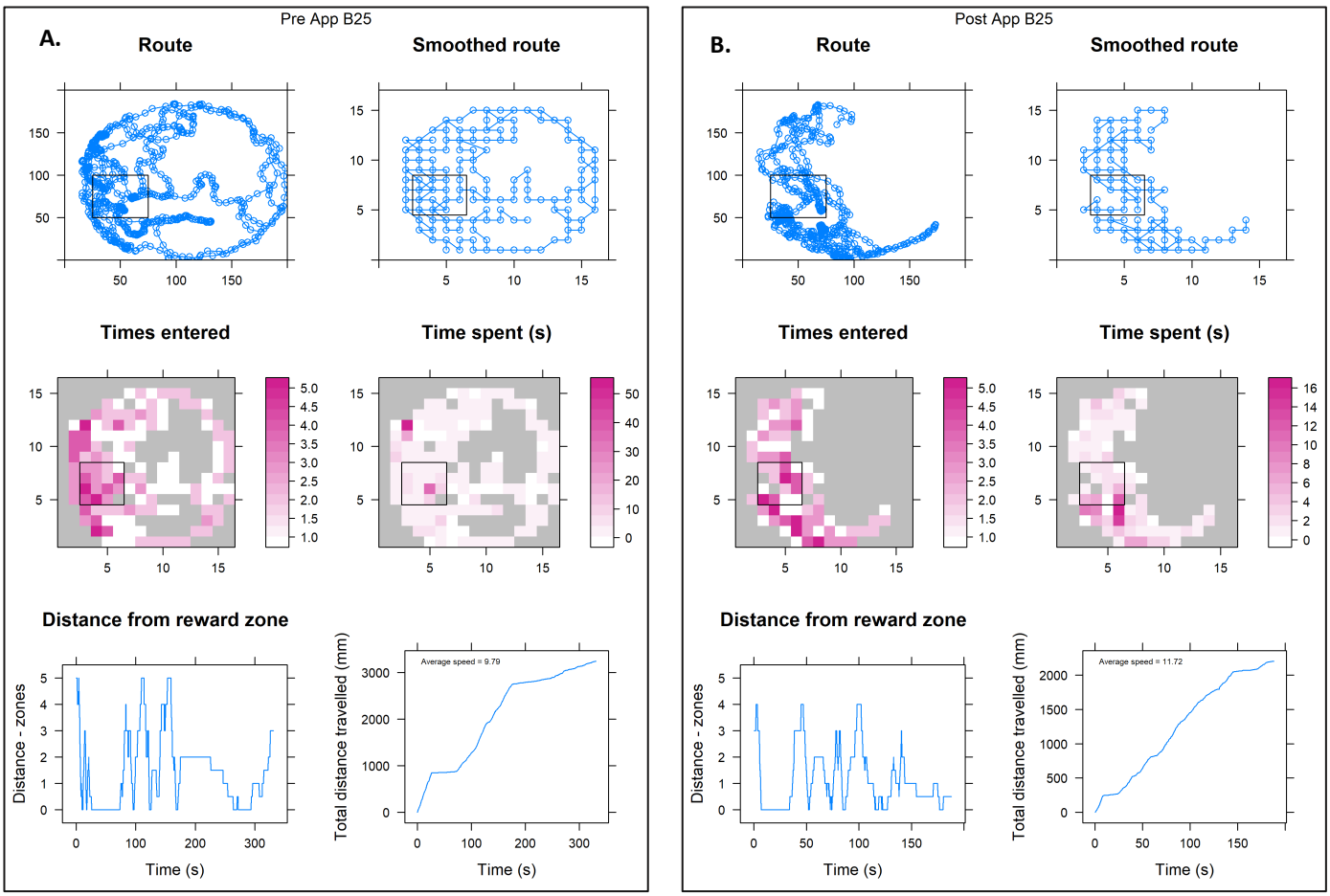

Figure 4.5: Training parameters from an appetitive bee (B25) pre- A) and post- B) training. The black square indicates reward zone location within the arena.

Appetitive + aversive condition

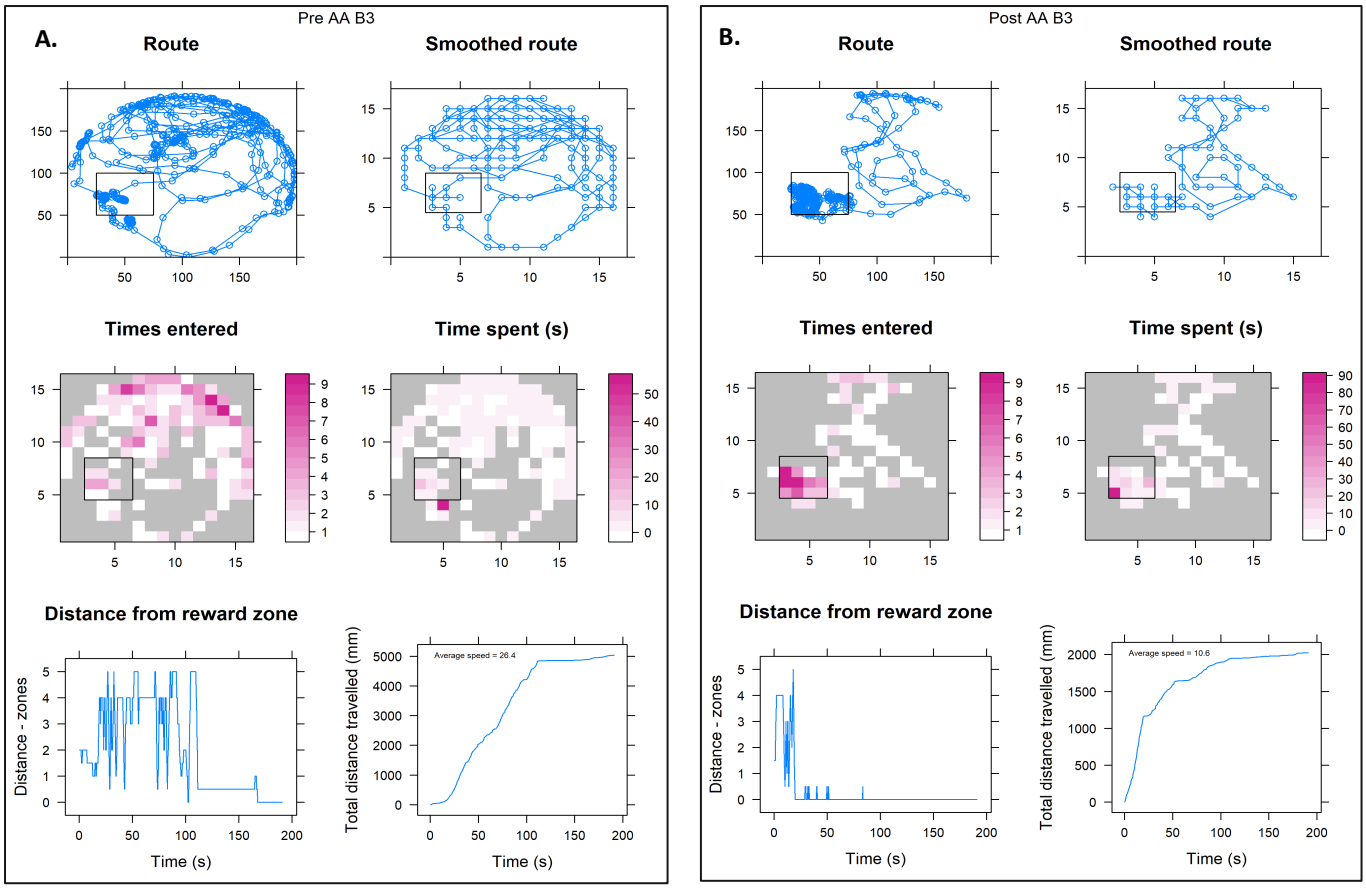

Figure 4.6: Training parameters from an appetitive + aversive bee (B3) pre- A) and post- B) training. The black square indicates reward zone location within the arena.

Aversive condition

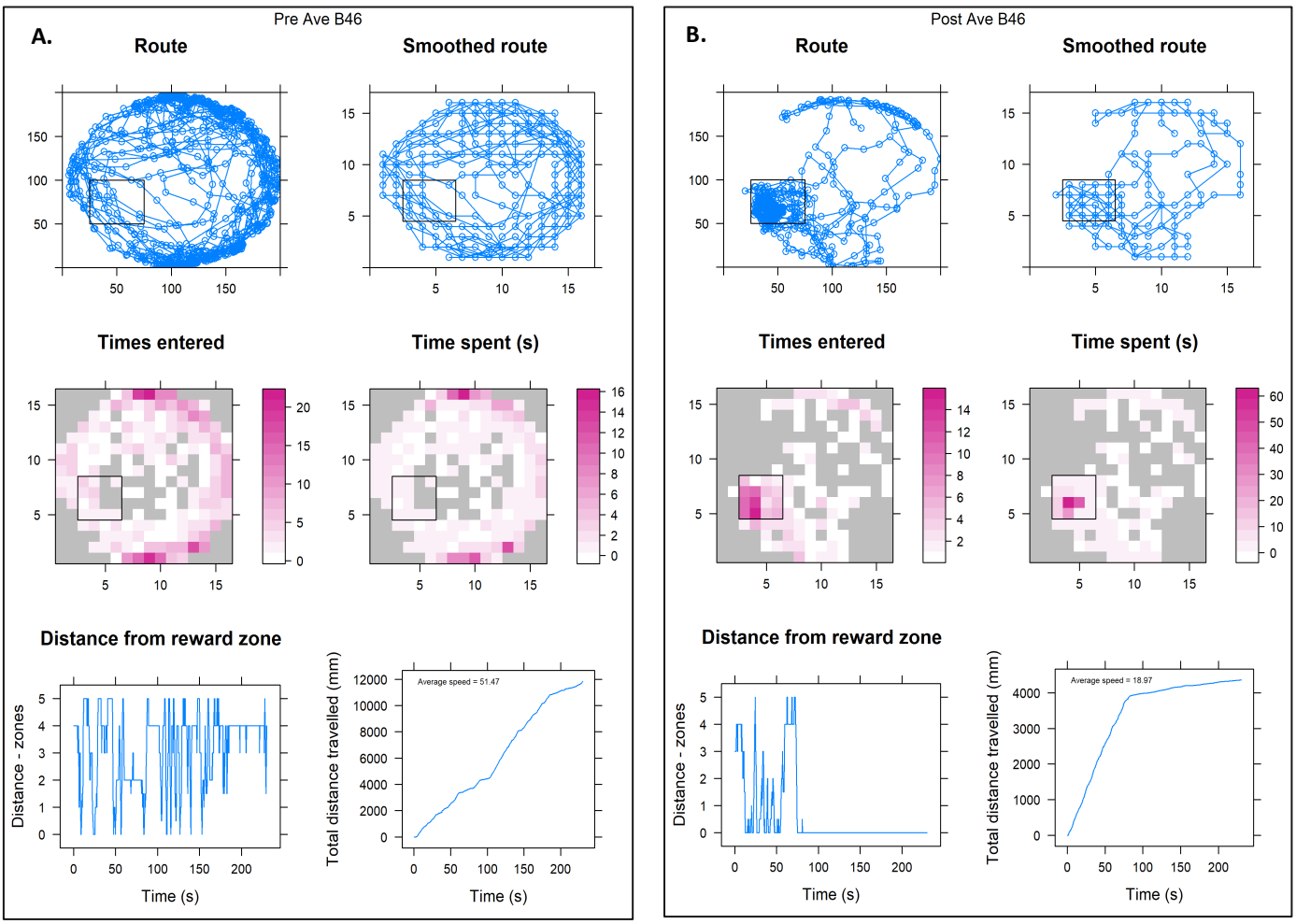

Figure 4.7: Training parameters from an aversive bee (B46) pre- A) and post- B) training. The black square indicates reward zone location within the arena.

4.3.2 GLMM and LMM: interactions between treatment, training and period

Validating the controls

There was no evidence of a difference between pre- and post- training in the controls, in terms of the proportion of bees that entered the reward zone. The mean proportion is approximately 0.5 but the variation is high. There was some evidence to suggest that the percentage of time spent in the reward zone by control bees was different pre- and post-training. However, this is a difference between 0.68% and 1.39% which is an acceptable/negligible difference given the values seen in the other treatments.

Treatment comparisons

A general linear mixed model (GLMM) was first utilised to compare the percentage of time spent in the reward zone and time the bee first entered the reward zone across treatments and pre- and post-training. None of the three main effect terms of the model (whether a treatment contained aversive conditioning, appetitive conditioning or the period – trial 1 or trial 10) was found to be significant. However, there was evidence of an interaction between the aversive training treatment and period; post-training more aversive bees entered the reward zone (67%) than non-aversive (56%).

There was a significant difference in time spent in the reward zone between the bees which received the aversive training condition (2.12%) and those that did not (1.76%). There was also a significant difference in time spent in the reward zone between bees that received appetitive training (3.71%) and those that did not (1%). There is also a significant difference in the time which bees first entered the reward zone between bees that received appetitive (8.55 seconds) and those that didn't (22.01 seconds). This suggests that in the appetitive containing treatments, bees may have also been utilising olfactory cues to locate the sucrose reward.

4.3.3 Treatment differences in training parameters

The behavioural parameter datasets listed in Table 4.2 were compared across treatments. Our null hypotheses were that the training treatment a bee experiences would have no impact on these parameters. For the following analyses the sample sizes for each treatment were: aversive + appetitive n = 15, appetitive n = 15, aversive n = 14 and control n = 44. All box and whisker plots were generated in Prism and the Tukey method was used to calculate whisker parameters (see GraphPad Software, 2020 for details).

Time spent in the reward zone

To assess the time bees spent in the reward zone pre- and post- training, Kruskal Wallis tests were conducted (non-parametric ANOVA equivalent) with a Dunn's correction for multiple comparisons. Time spent in the reward zone has significant treatment comparisons both pre- and post-training (Figure 4.8).

i) Time spent pre-training

Pre-training, the appetitive treatment is the only treatment which is highly significantly different from the control group (P = <0.0001****) (Figure 4.8A). The aversive (P = >0.99) and aversive + appetitive (P = 0.39) treatments were not significantly different from the control group at this pre-training stage. Other significant pre-training comparisons were between the aversive alone and the appetitive alone treatment groups (P = 0.0006***) and the aversive alone and the appetitive + aversive treatment groups (P = 0.03*) (Figure 4.8A).

ii) Time spent post-training

Post-training, both aversive-containing treatments were highly significantly different from the control group (control vs. aversive: P = <0.0001****, control vs. aversive + appetitive: P = <0.0001****), but not from each other (P = >0.99) (Figure 4.8B). The appetitive treatment was not significantly different from control (P = 0.14), aversive (P = 0.33) or aversive + appetitive groups (P = 0.11) (Figure 4.8B).

iii) Difference in time spent in reward zone pre- and post-training

The difference in time spent in the reward zone pre- and post-training was calculated for each bee (post-training – pre-training value). Whereby a positive value indicates a bee improved, spending more time in the reward zone by trial 10 (post-training) and a negative value indicates the bee got worse at the task, decreasing the time spent in the reward zone post-training.

Both the aversive alone (P = 0.001***) and the combined aversive + appetitive (P = 0.0097**) treatments were significantly different from the control group and from the appetitive group (both groups P = <0.0001****), but not from each other (Figure 4.8C). Appetitive conditioning was not significantly different from the control group (p = 0.097) (Figure 4.8C). This further supports the notion that appetitive training is not successful in this arena and that the use of an aversive conditioning element is highly effective.

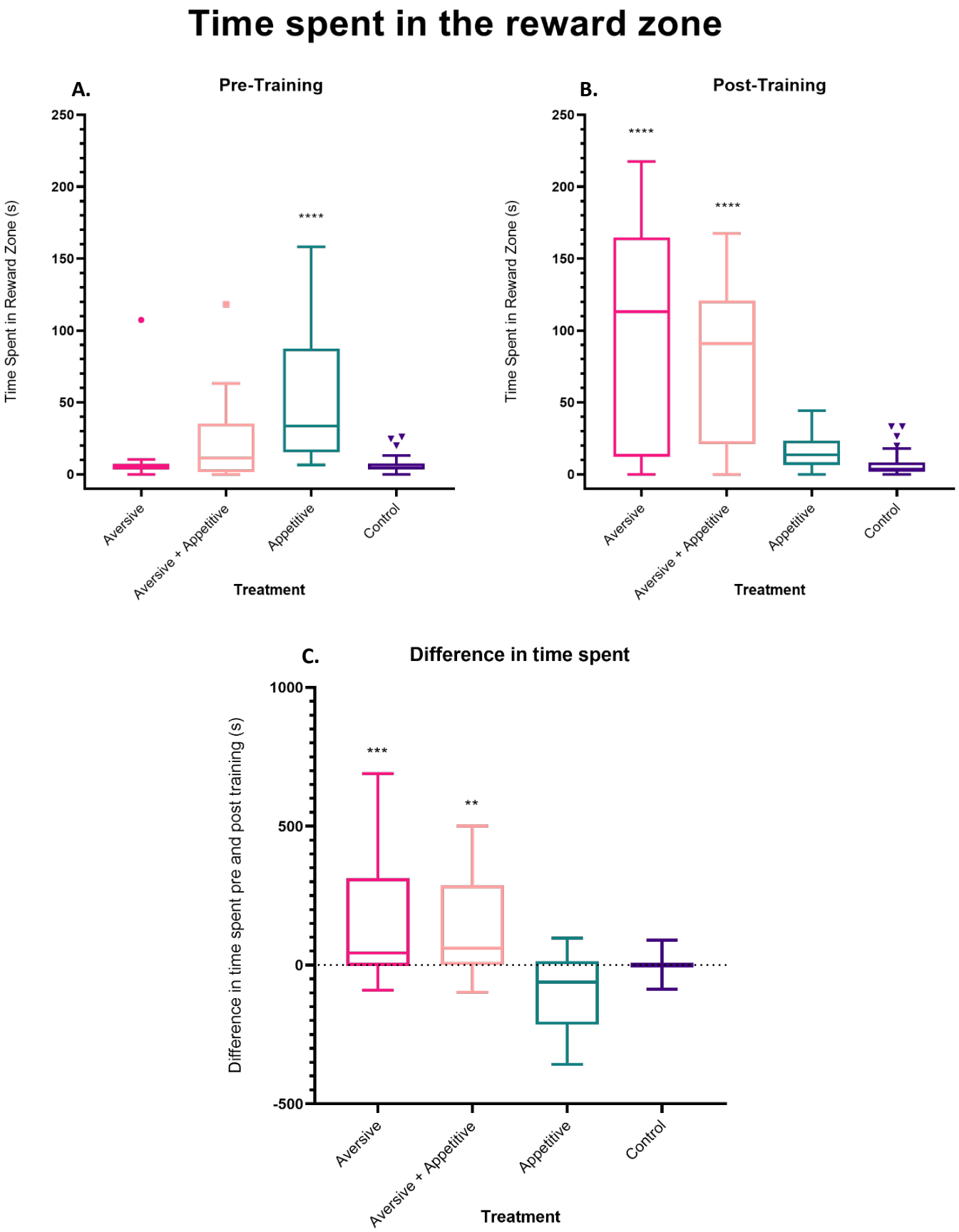

Figure 4.8 (A-C): Time spent in the reward by bees. A. Time spent pre-training. B. Time spent post-training. C. Difference in time spent (post-training – pre-training value). (Aversive + Appetitive n = 15, Appetitive n = 15, Aversive n = 14, Control n = 44).

Number of times entered the reward zone

To assess the number of times bees entered the reward zone pre- and post-training, Kruskal Wallis tests were conducted (non-parametric ANOVA equivalent) with a Dunn's correction for multiple comparisons.

i) Number of times entered pre-training

Pre-training, the only treatment significantly different from the control group was the appetitive group (P = 0.0286*). The only other significant comparison was between the appetitive and the appetitive + aversive treatment (P = 0.006**) (Figure 4.9A).

ii) Number of times entered post-training

Post training, none of the treatment groups were significantly different from the control group. There was also no significant difference in number of times entered between any of the other treatments (Figure 4.9B).

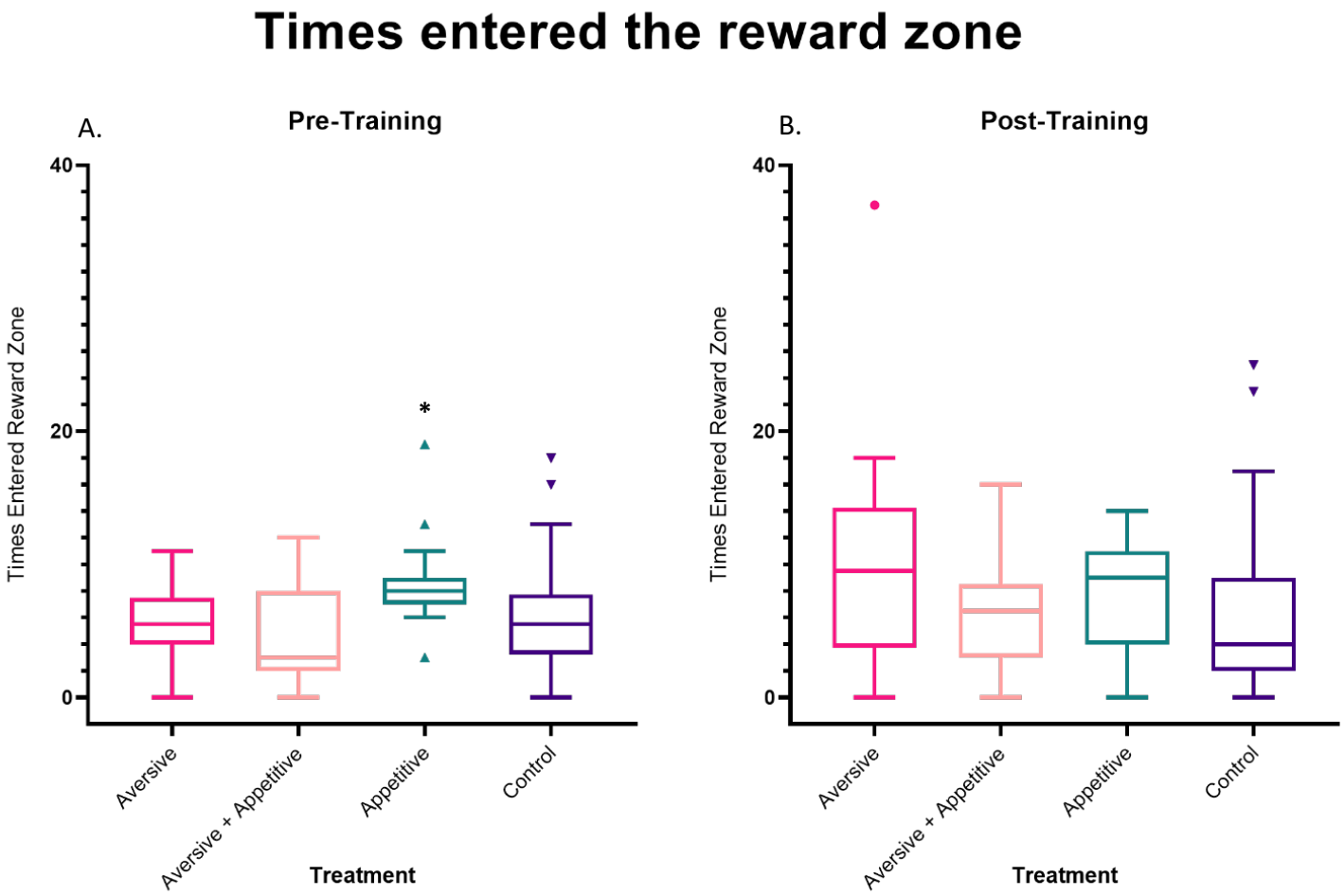

Figure 4.9 (A-B): Number of times bees entered the reward zone. A. pre-training. B. post-training. (Aversive + Appetitive n = 15, Appetitive n = 15, Aversive n = 14, Control n = 44).

Distance travelled

To assess the distance travelled by bees in pre- and post- training trials, ANOVA tests with multiple comparisons were conducted with a Tukey's correction for multiple comparisons.

i) Total distance travelled pre and post training

There was no significant difference in the total distance travelled pre- or post-training by bees in any of the treatments (pre-training; P = >0.5 for all treatments. Post-training; P = >0.7 for all treatments) (Figure 4.10 A and B).

ii) Difference in distance travelled pre- and post-training

The difference in total distance travelled pre- and post-training was calculated for each bee (post-training – pre-training value). Whereby, a positive value indicates a bee travelled further by trial 10 (post-training) and a negative value indicates the travelled less distance post-training (Figure 4.10C).

iii) Comparing distance travelled pre- and post-training

When pre- vs post-training distance travelled data sets (ANOVA with matched pair comparisons of each treatment pre- and post- training) are compared, there are significant differences in the distance travelled of all the treatment groups. All treatments decreased the distance travelled post-training; Aversive (pre vs. post, P = 0.0001***), Aversive + Appetitive (pre vs, post, P = 0.0121*), Appetitive (pre vs. post, P = 0.0068**). and Control (pre vs. post, P = 0.0002***) (Figure 4.10D).

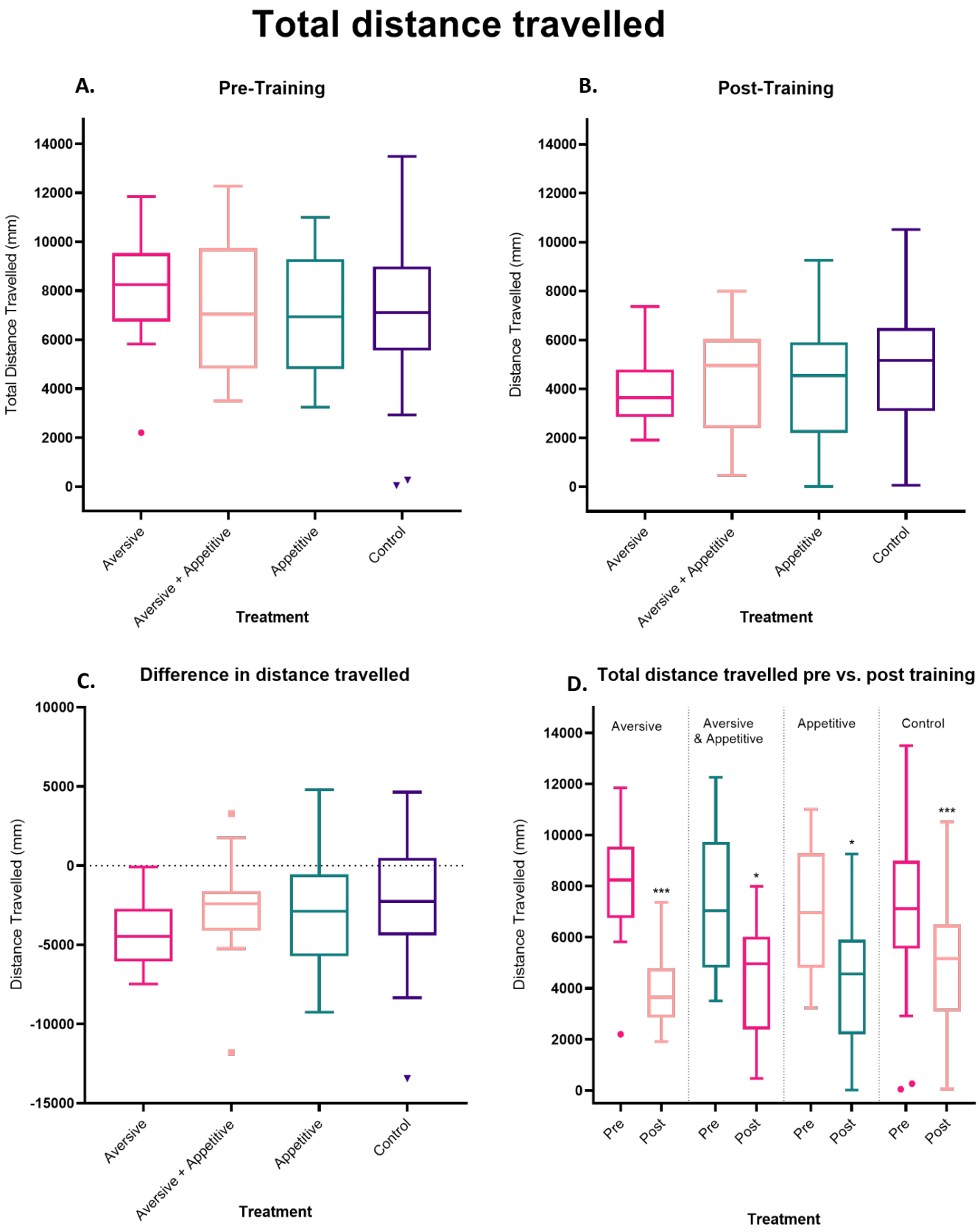

Figure 4.10 (A-D): Total distance travelled by bees. A. Total Distance travelled pre-training. B. Total distance travelled post-training. C. Difference in distance travelled (post-training – pre-training value). D. Total distance travelled pre- versus post-training. (Aversive + Appetitive n = 15, Appetitive n = 15, Aversive n = 14, Control n = 44).

Average speed

i) Average speed pre- and post-training

To assess the average speed of bees in pre- and post- training trials, ANOVA tests with multiple comparisons were conducted with a Tukey's correction for multiple comparisons. There was no significant difference in the average speed of bees in any of the treatments in the pre- or post-training trials (pre-training; P = >0.5 in all treatments. Post-training; P = >0.5 in all treatments) (Figure 4.11 A and B).

ii) Difference in average speed between pre- and post-training

The difference in average speed pre- and post-training was calculated for each bee (post-training – pre-training value). Whereby, a positive value indicates a bee sped up, moving faster by trial 10 (post-training) and a negative value indicates the bee slowed down, decreasing the speed post-training (Figure 4.11C). There were no significant differences between treatments.

iii) Comparing average speed pre- and post-training

When pre- vs post- training average speed data sets (ANOVA with matched pair comparisons of each treatment pre- and post-training) are compared there were significant differences in the average speed of three of the treatment groups; Aversive (pre vs. post, P = 0.006***), Aversive + Appetitive (pre vs, post, P = 0.01*) and Control (pre vs. post, P = 0.007**). The Appetitive treatment did not significantly alter speed pre vs. post-training (P = 0.2) (Figure 4.11D).

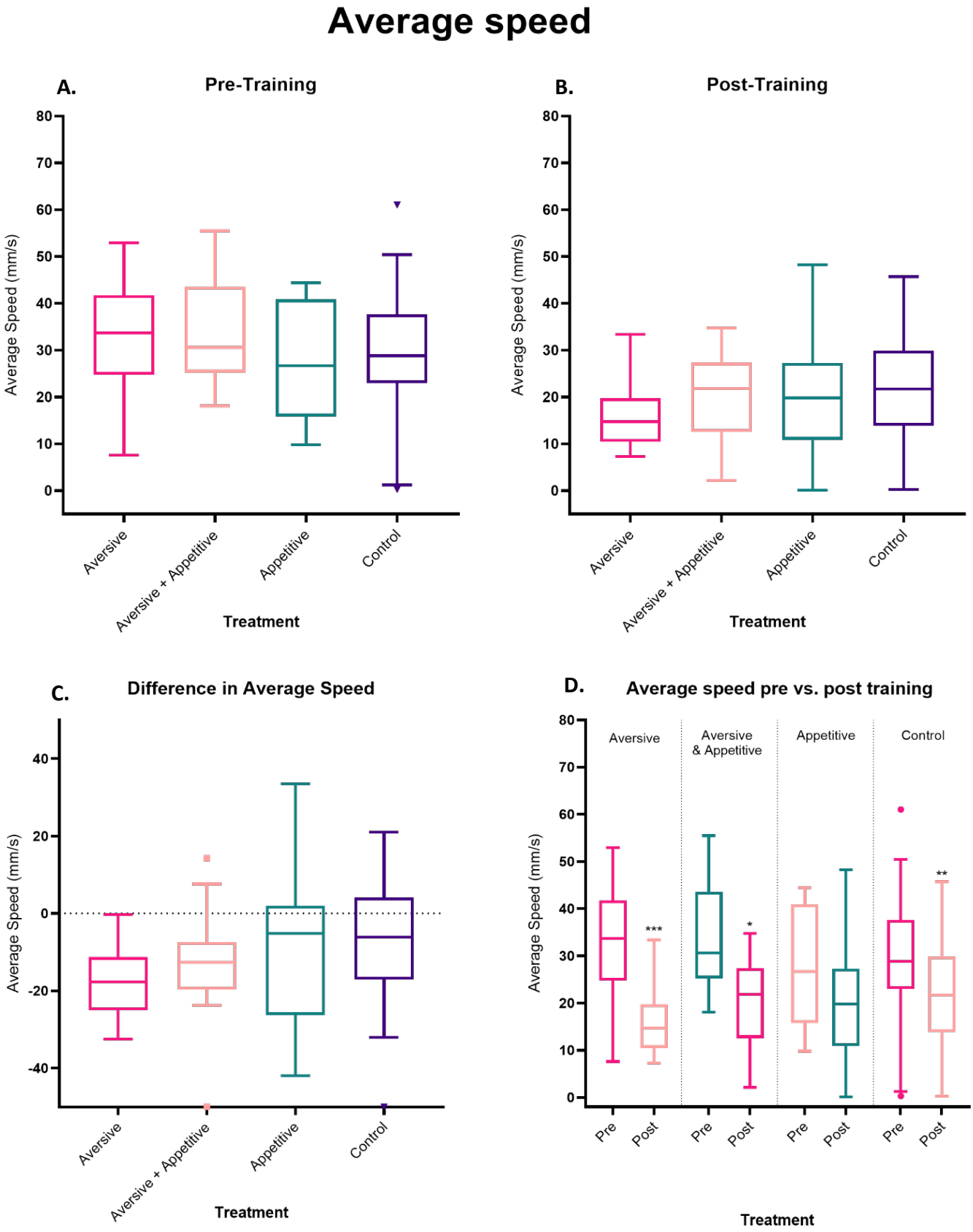

Figure 4.11 (A-D): Average speed of bees in training trials. A. Average speed pre-training. B. Average speed post-training. C. Difference in average speed (post-training – pre-training value). D. Average speed pre- versus post-training. (Aversive + Appetitive n = 15, Appetitive n = 15, Aversive n = 14, Control n = 44).

Impact of hive on training parameters

Inter-hive variation in learning ability could play a large role in overall treatment response variability. It was therefore decided to segregate the data by hive to examine potential differences in hive response to treatments. Some of the datasets were non-normal (aversive treatment and control treatment datasets) and therefore in these cases non-parametric Kruskal-Wallis testing with multiple comparisons was conducted instead of an ANOVA with multiple comparisons.

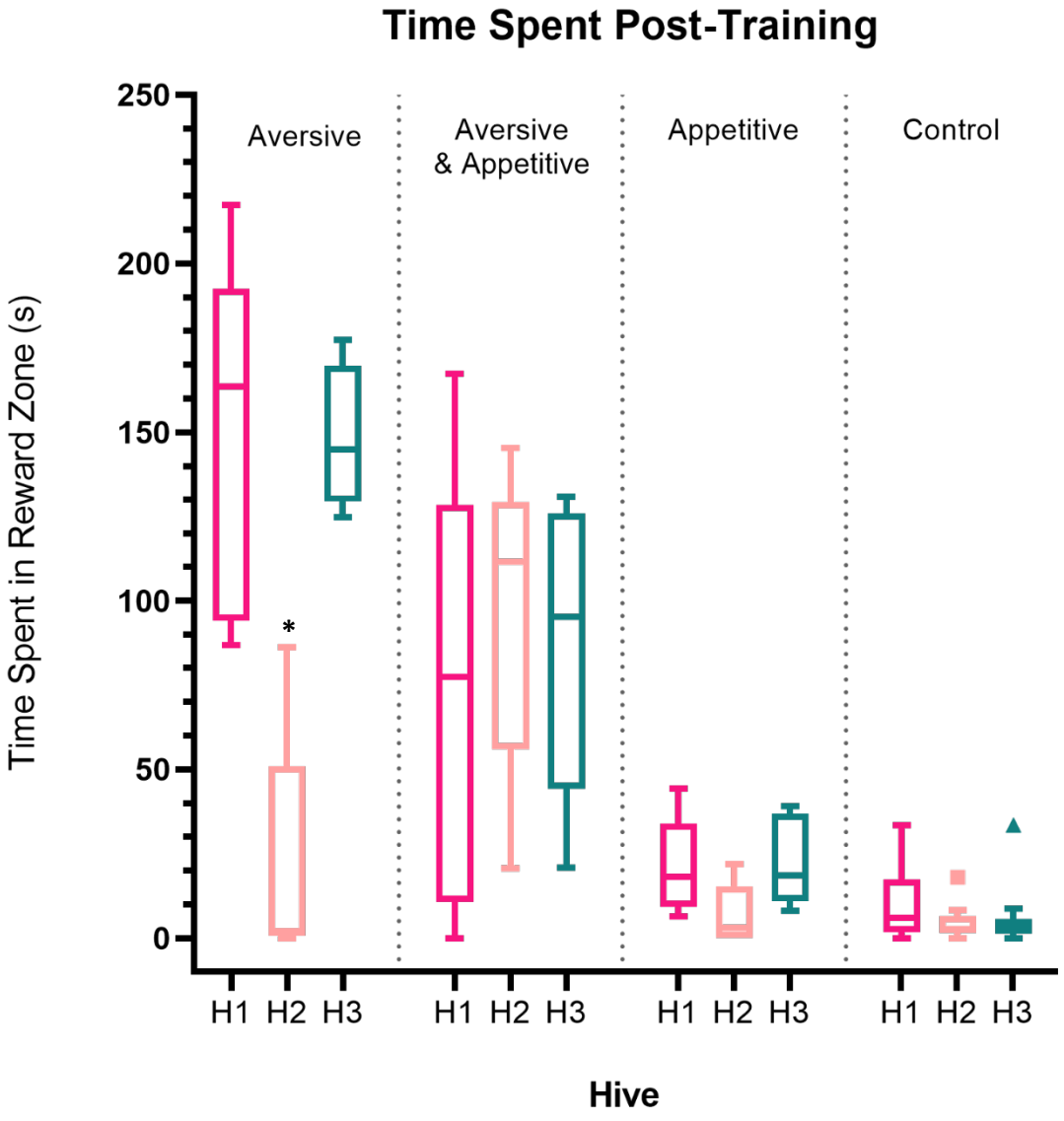

Figure 4.12: Time spent in reward zone post-training by hive. (Aversive + Appetitive n = 15, Appetitive n = 15, Aversive n = 14, Control n = 44).

The results of the analysis clearly show that there is variation in the time spent in the reward zone post-training between hives, even within the same treatment and particularly within the aversive training treatment (Figure 4.12). Within the aversive conditioning group, hive 2 bees spent significantly less time in the reward zone post-training compared to both Hive 1 (P = 0.02*) and Hive 3 (P = 0.04*) bees. There was not a significant difference in time spent post-training between Hive 1 and Hive 3 (P = >0.99). These observations (that bees from Hive 2 are not able to respond as well to the aversive heat stimulus) are the basis for the RNA sequencing experiment in Chapter 8, in which potential genetic determinants of differential learning ability between individuals are investigated. However, it is important to note that this apparent lack of response to the heat stimuli seen in the performance of hive 2 foragers may not be a result of differential learning abilities, but could instead be as a result of individual's abilities to cope with a high temperature environment e.g. hive 2 bees were less heat tolerant. These underlying causations of the observed behaviour cannot be fully elucidated here.

Large inter-hive differences in time spent in the reward zone were not seen in the aversive + appetitive, appetitive or control treatment groups (Figure 4.12). In the appetitive treatment, although there are no significant inter-hive differences (Hive 1 vs. Hive 2 (P = 0.23), Hive 1 vs. Hive 3 (P = 0.97) and Hive 2 vs. Hive 3 (P = 0.15)), it is clear that the comparisons containing Hive 2 are more distinct with lower P values (0.23, 0.15) than the Hive 1 vs. Hive 3 comparison (0.97). In the combined appetitive + aversive treatment group there are no significant inter-hive comparisons; Hive 1 vs. Hive 2 (P = 0.74), Hive 1 vs. Hive 3 (P = 0.88) and Hive 2 vs. Hive 3 (P = 0.96). Similarly, in the control group there are no significant inter-hive comparisons; Hive 1 vs. Hive 2 (P = 0.71), Hive 1 vs. Hive 3 (P = 0.90) and Hive 2 vs. Hive 3 (P = >0.99).

In the case of the combined aversive + appetitive treatment, the lack of a significant difference in time spent is perhaps because there are two training stimuli present (aversive heat, appetitive food reward). In the control group no task is given and therefore we would expect a random, even spread of time spent in the reward zone across treatments. The appetitive treatment group do display some inter-hive differences in time spent in reward zone post training, although this is not as marked for Hive 2 as in the aversive group.

Reanalysis of data, omitting Hive 2 from the analyses

In the analysis below two of the most significant training parameters (time spent in the reward zone and difference in time spent) were reanalysed with Hive 2 bees omitted to assess the variation contributed by Hive 2 inclusion.

i) Time spent in the reward zone

When Hive 2 is omitted from the 'time spent in reward zone' pre- and post-training datasets (Figure 4.12) it can be seen that, pre-training, the previously non-significant Aversive vs. Appetitive treatment comparison becomes significant (P = 0.0165*) and that the appetitive vs. control treatment comparison remains highly significant (P = 0.0006***). Post-training, previously, the appetitive treatment was not significantly different from any other treatment group. With Hive 2 omitted, the aversive vs. appetitive comparison becomes significant (P = 0.04*). The aversive vs. control (P = <0.0001****) and appetitive + aversive vs. control (P = 0.0006***) comparisons remain highly significant. There are no other significant treatment comparisons in time spent in the reward zone pre- or post-training. Figure 4.12 suggests that a large portion of the post-training variation in the aversive treatment can be attributed to Hive 2.

ii) Difference in time spent in the reward zone pre- and-post training

The difference in time spent in the reward zone pre- and post-training was calculated for each bee with Hive 2 data sets removed. Pre-training values were taken away from post training values for each bee in each treatment. A positive value indicates an improvement in the parameter, i.e. the bee spent more time in the reward zone post training. A negative value indicates the contrary, that a bee spent less time in the reward zone post-training.

The Aversive treatment was highly significantly different from the control (P = 0.0009***) and Appetitive groups (P = 0.0003***) but not from the Aversive + Appetitive group (P = >0.99). The Aversive + Appetitive group was also significantly different from the Appetitive alone group (P = 0.0298*). Neither the Aversive + Appetitive (P = 0.14) or the Appetitive alone (P = >0.99) groups were significantly different from the control group (Figure 4.13B). The omission of the Hive 2 dataset further highlights the dominant role of aversive conditioning in this arena.

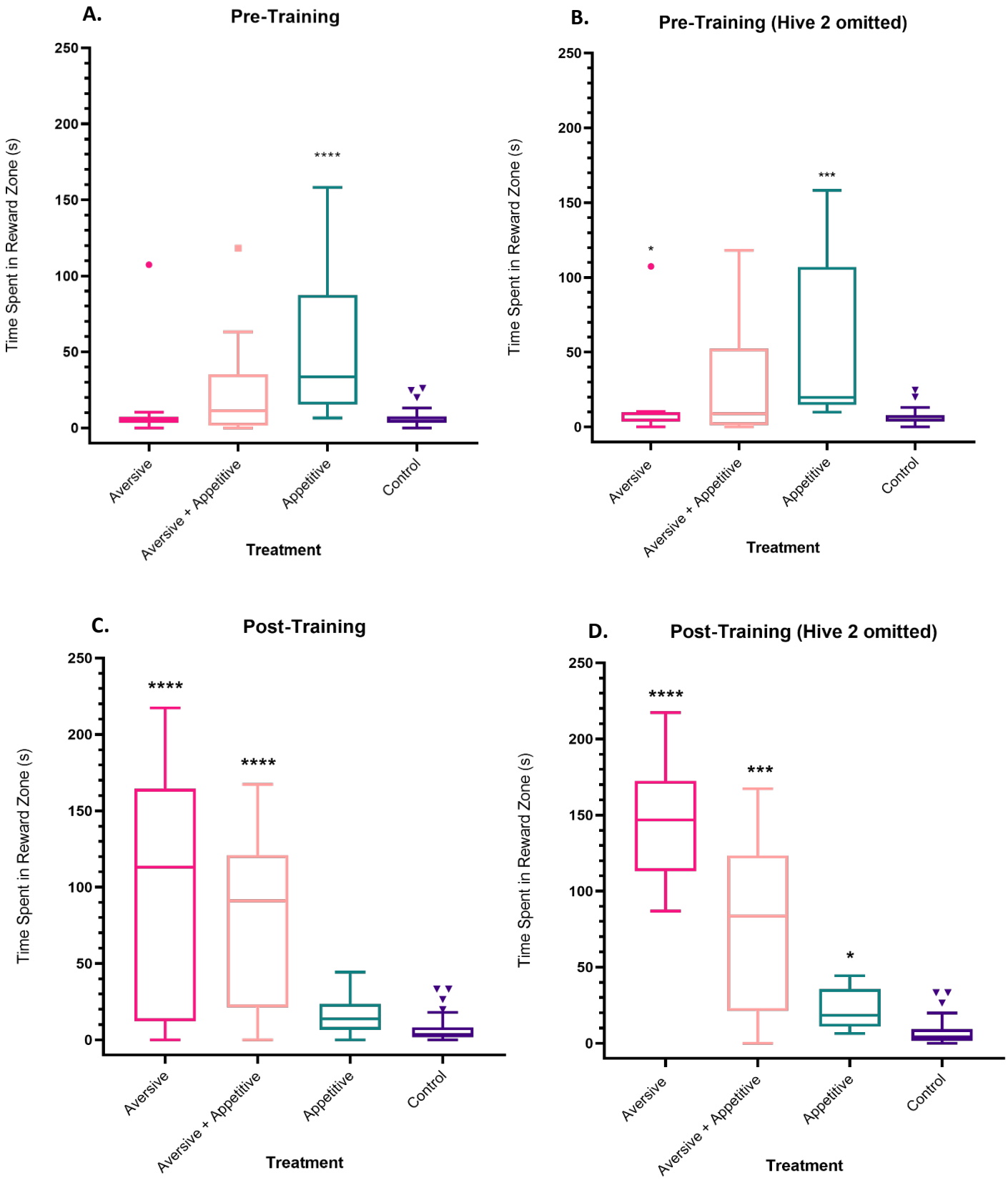

Figure 4.12 (A-D): Time spent in the reward zone. A) and C): Original pre- and post-training dataset. B) and D): modified pre- and post-training dataset with Hive 2 removed.

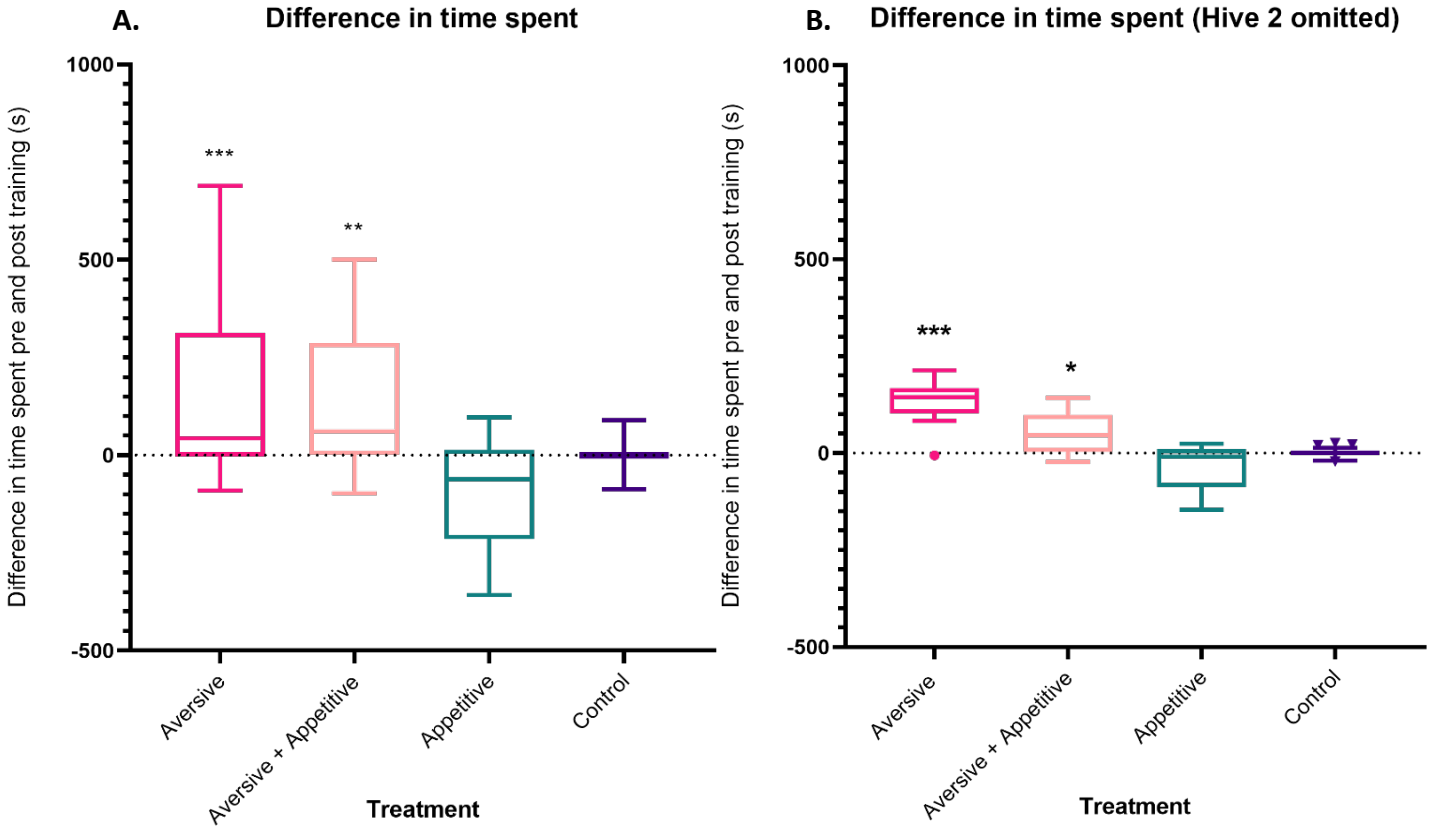

Figure 4.13 (A, B): Difference in time spent in the reward zone pre- and post- training. A) Original dataset. B) Dataset with Hive 2 removed, displaying the variation, particularly in the aversive treatment, attributable to Hive 2.

4.3.4 Red light trials

To determine whether bees utilised the arena's peripheral visual pattern for navigation within trials, it was decided to conduct red light trials. Since bees have photoreceptor cells with spectral sensitivity only to UV, blue and green (Chittka and Wells, 2004), insensitivity of bees to red light can be exploited in an experimental setting. This is because it means that a human experimenter can view and manage experiments, but bees within the arena experience complete darkness.

Bees were placed into the trial arena under red light with either no pattern (white paper) on the arena wall or under LED lighting with the visual pattern adhered to the arena wall (as in the standard trials above). This comparison allowed assessment of if the arena's aversive task could be completed without the visual pattern as a learning cue.

Bees were only given the aversive learning task (no appetitive or combined treatment), as this was deemed the most successful training method from the trials conducted above. Bees which were trialled under red light with no arena pattern are referred to as the "dark" treatment. Bees in the normal, LED lit arena with the arena pattern are referred to as the "light" treatment. The light treatment acted as the visual learning control. As for previous analyses, a range of training parameters were compared between light and dark treatments (Table 4.1). For all statistical analyses sample sizes were light treatment n = 5 and dark treatment n = 10.

Red light trials: time spent in reward zone

i) Pre-training

A two tailed independent t-test was utilized. There was a significant difference in the time bees spent in the reward zone pre-training (P = 0.0096**), with light treatment bees spending significantly more time in the reward zone (light treatment mean = 31.93, dark treatment mean = 6.499) (Figure 4.14A).

ii) Post-training

A non-parametric Mann-Whitney test was utilized. There was no significant difference between treatments (dark vs. light) in time bees spent in the reward zone post-training (P = 0.5941) (Figure 4.14B).

iii) Difference pre- and post-training within treatment

There was a significant difference in the amount of time bees spent pre- vs. post-training in both treatment groups; light (P= 0.0234*) and dark (P = 0.0005***) (Two-tailed paired t-tests between pre- and post-training values for each bee), with bees spending significantly more time in the reward zone post-training compared to pre-training values (Figure 4.14D).

Red light trials: times entered the reward zone

i) Pre-training

A two tailed independent t-test was utilized. Pre-training, there was a significant difference in the number of times bees entered the reward zone between treatments (P = 0.0019**) (light treatment mean = 24, dark treatment mean = 9.7), with bees in the light treatment entering the reward zone significantly more (Figure 4.15A).

ii) Post-training

A non-parametric Mann-Whitney test was utilized. Post-training, there was no significant difference in the number of times bees entered the reward zone between treatments (dark vs. light) (P = 0.8838) (Figure 4.15B).

Difference pre- and post-training

A non-parametric Mann-Whitney test was utilized. There was a significant difference between the treatments (dark vs. light) in the pre- and post-training difference in number of times bees entered the reward zone (P = 0.0453*), with bees in the dark treatment improving significantly more than the bees in the light treatment (pre vs. post training) (Figure 4.15C).

Red light trials: Average speed

i) Pre-training

A two tailed independent t-test was utilized. Pre-training, there was no significant difference in the average speed which bees travelled across treatments (P = 0.1350) (Figure 4.16A).

ii) Post-training

A non-parametric Mann-Whitney test was utilized. Post-training, there was no significant difference in the average speed which bees travelled across treatments (P = 0.2065) (Figure 4.16B).

iii) Difference pre- and post- training

A two tailed independent t-test was utilized. There was no significant difference in the difference in bees' average speed between pre- and post-training trials across treatments (P = 0.0736) (Figure 4.16C).

Red light trials: Total distance travelled

i) Pre-training

A two tailed independent t-test was utilized. There was no significant difference in the total distance travelled by bees pre-training between treatments (P = 0.1350) (Figure 4.17A).

ii) Post-training

A non-parametric Mann-Whitney test was utilized. There is no significant difference in the total distance travelled post-training between treatments (P = 0.2065) (Figure 4.17B).

iii) Difference pre- and post-training

A two tailed independent t-test was utilized. There was no significant difference in the difference in total distance travelled pre- and post-training between treatments (P = 0.0736) (Figure 4.17C).

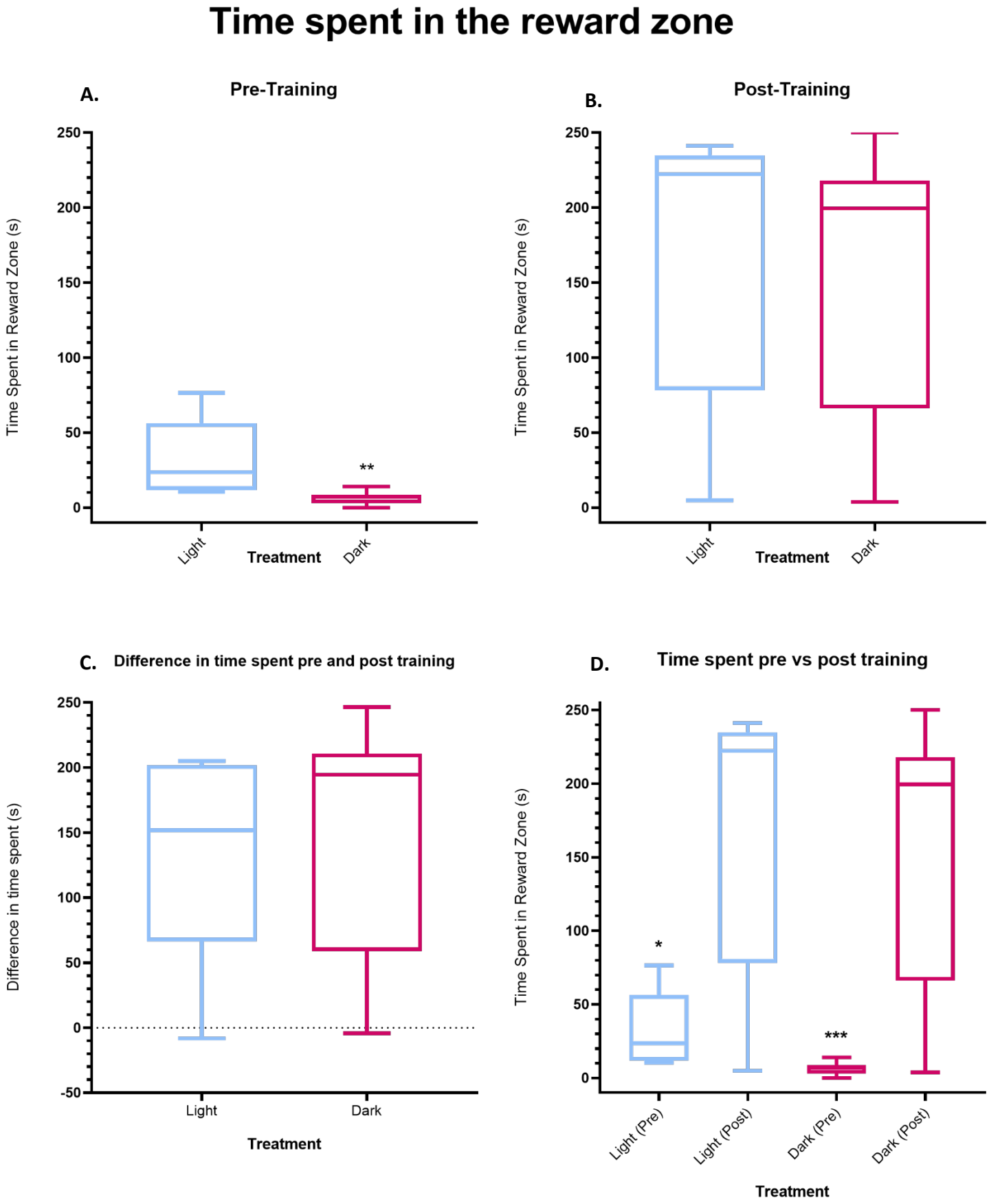

Figure 4.14 (A-D): Time spent in the reward zone by bees in the red-light trials. A. Time spent pre-training. B. Time spent post-training. C. Difference in time spent (post-training – pre-training value). D. Time spent pre- versus post-training. (Light treatment n = 5, dark treatment n = 10).

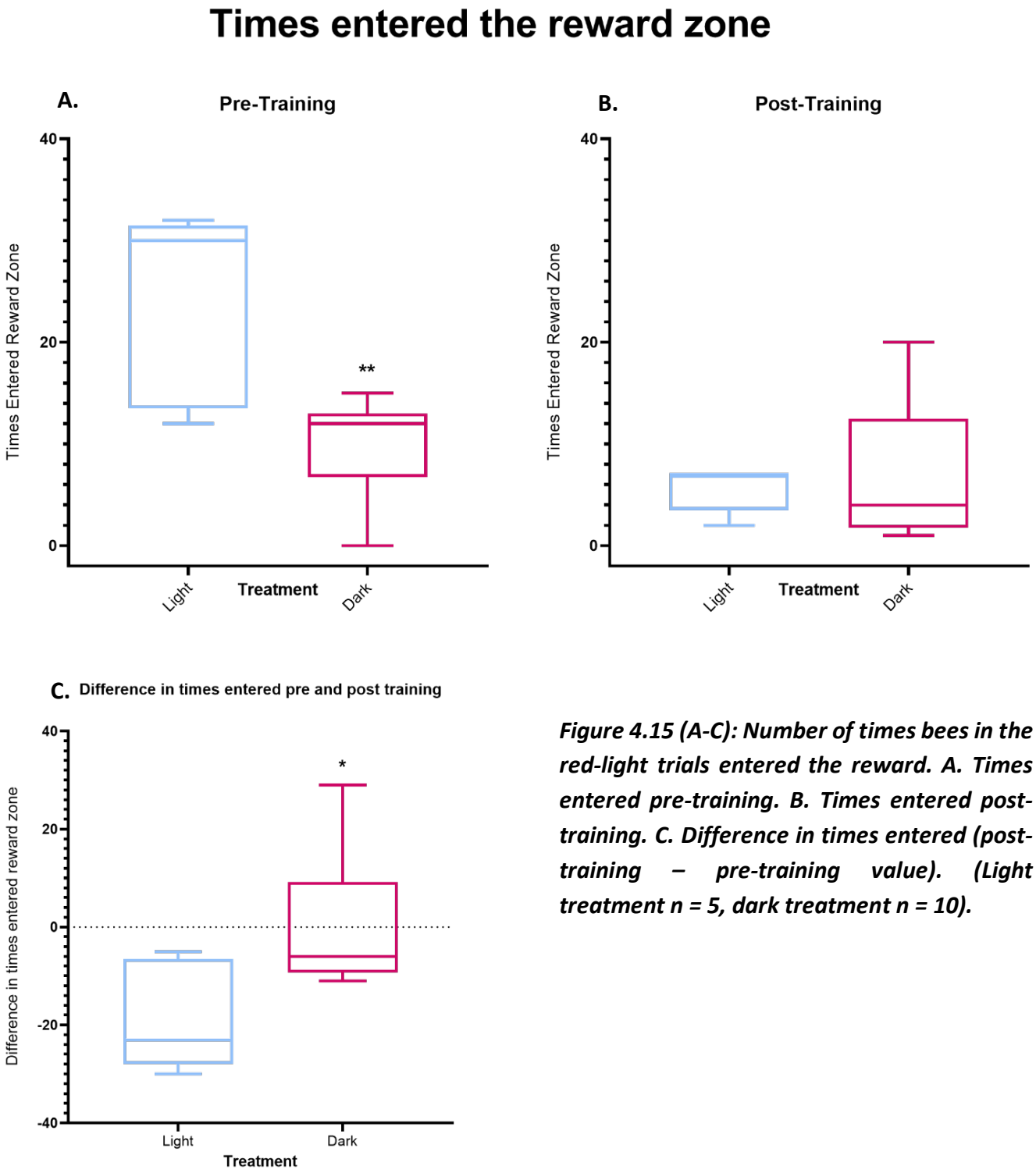

Figure 4.15 (A-C): Number of times bees in the red-light trials entered the reward. A. Times entered pre-training. B. Times entered post-training. C. Difference in times entered (post-training – pre-training value). (Light treatment n = 5, dark treatment n = 10).

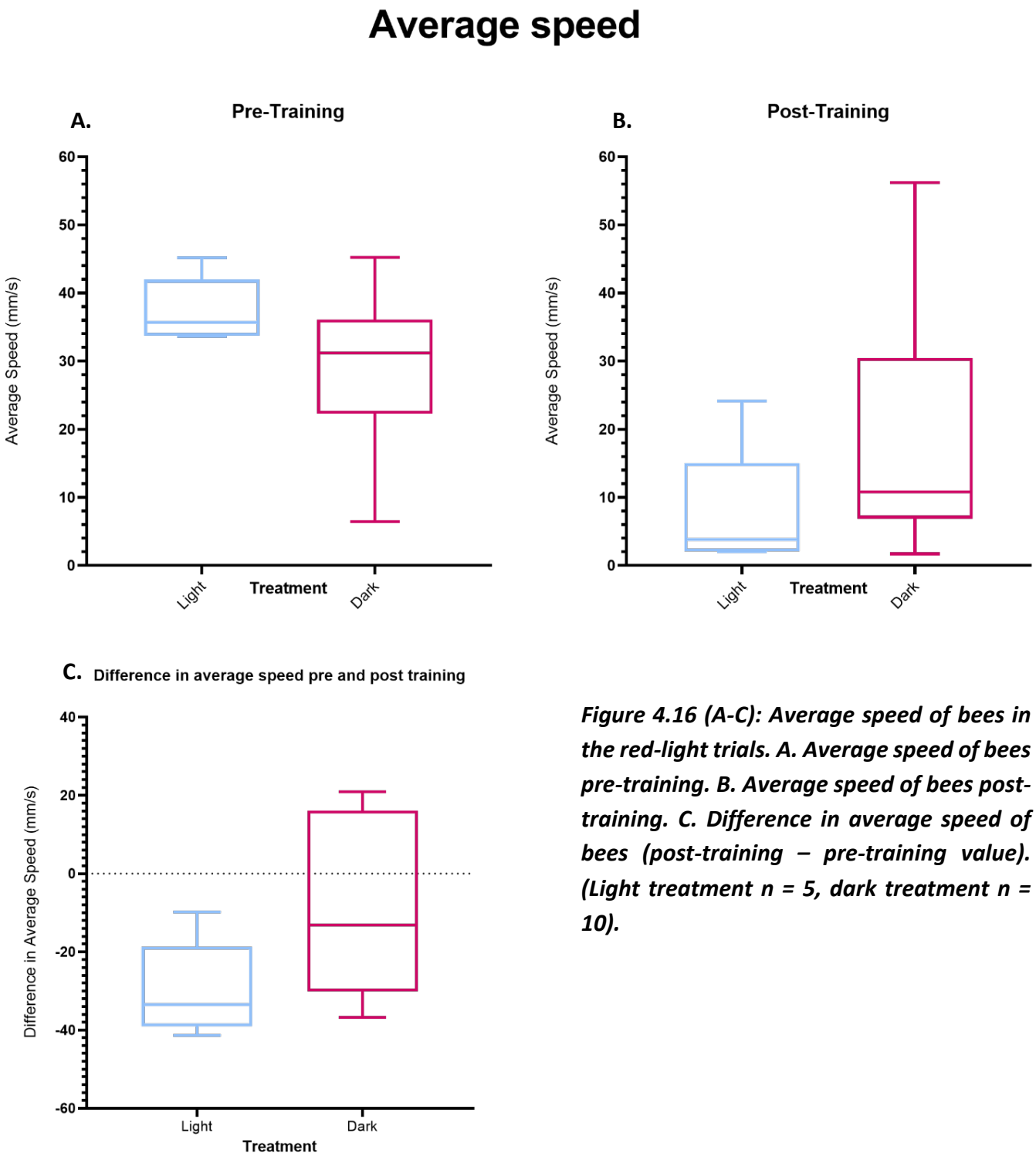

Figure 4.16 (A-C): Average speed of bees in the red-light trials. A. Average speed of bees pre-training. B. Average speed of bees post-training. C. Difference in average speed of bees (post-training – pre-training value). (Light treatment n = 5, dark treatment n = 10).

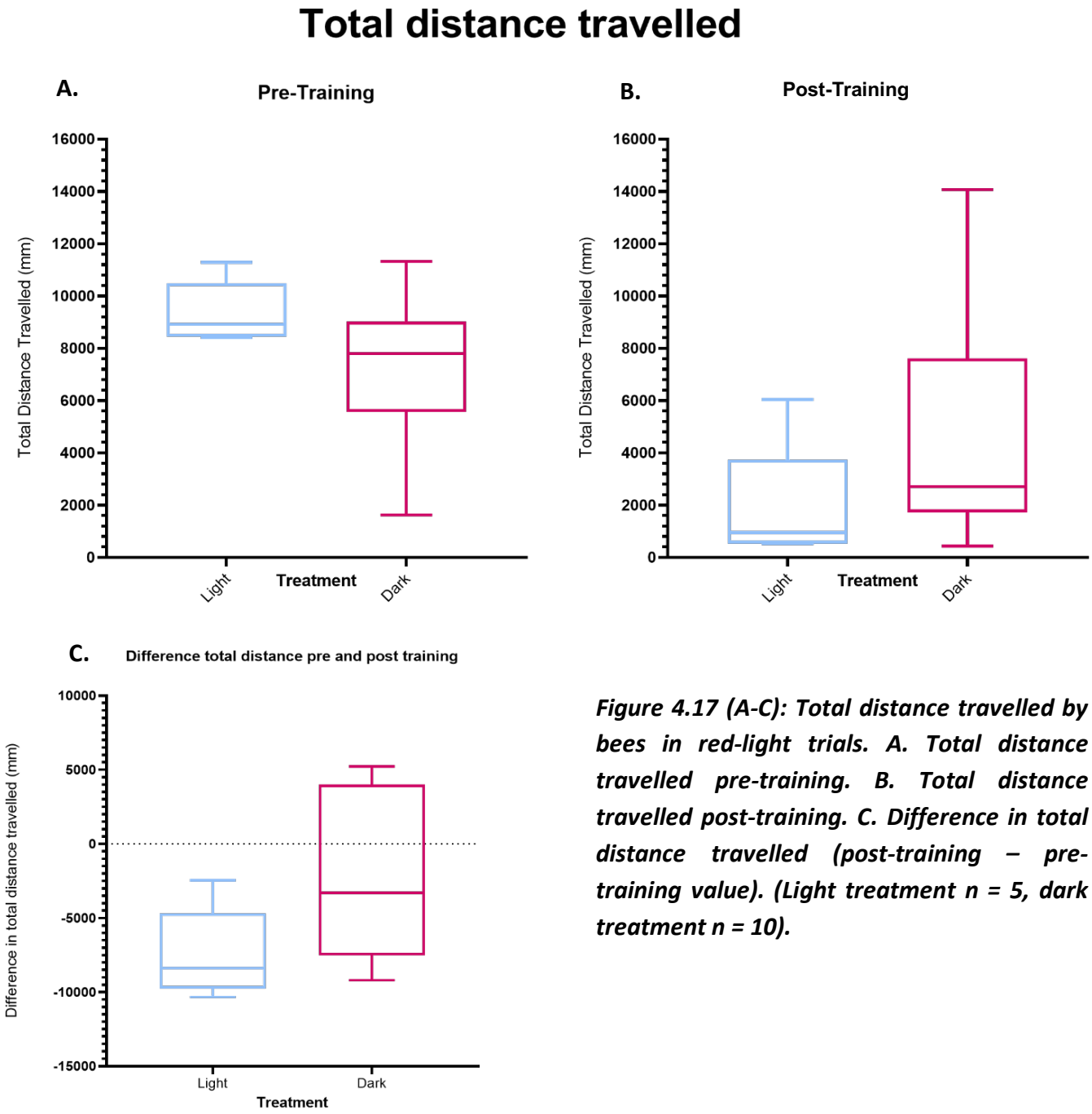

Figure 4.17 (A-C): Total distance travelled by bees in red-light trials. A. Total distance travelled pre-training. B. Total distance travelled post-training. C. Difference in total distance travelled (post-training – pre-training value). (Light treatment n = 5, dark treatment n = 10).

4.4 Discussion

When considering 'training' it is important to consider both qualitative visual training indicators, utilising the trajectory graphs, as well as quantitative indicators of training, such as the amount of time spent in the reward zone, the time the bee first enters the reward zone, and the number of times a bee enters the reward zone. Together these two approaches produce a more comprehensive analysis of what training manifests as in the bumblebee than either parameter singularly.

Our findings show that aversive training is possible in the thermal-visual arena. This is supported by both visual and quantitative training parameters. Visual trajectories of bee routes during trials demonstrate that bees in each treatment trace distinct paths, with aversive conditioning containing treatments leading to trajectories most localised to the reward zone by trial 10. When considering the visual indicators of training, the 'route' graphs (Figures 4.4-4.7) imply that the bees in each training condition implement differing exploratory strategies, dependent on the reward or punishment environment they are in.

In the two treatments which utilise aversive conditioning we see increasingly directed exploratory paths to and from the reward zone. Whereas, although individuals in the appetitive treatment displayed some localisation to the reward half of the arena, individuals seem to track more varied paths, not constrained to set routes, perhaps suggesting that a sucrose reward alone was not enough to motivate an individual to remain specifically in the reward zone. This makes ecological sense, as in the field a floral food source, once depleted, will not replenish immediately and a forager would gain the most benefit by searching for further local food sources rather than remaining on the depleted patch (Pyke, 1980). The more directed trajectories of the bees in the aversive containing conditions imply that the bees were capable of both learning and remembering the location of the inconspicuous reward zone and were able to navigate directly back to it from their position in the arena. Although, the graphical representations of the training parameters suggest that aversive training of *B. terrestris* is possible in the thermal-visual arena, the subjectivity in interpreting what is classified as 'training' when using visual representations such as the trajectory graphs, introduces some variability in interpretation. Therefore, it is important to take these visual

representations in combination with the statistically significant results found by examining the alternative training parameters.

When considering the analysed training parameters pre-training, bees in the appetitive treatment spent significantly more time in the reward zone than those in the control treatment (P = 0.0001) and bees in the aversive treatment spent significantly less time in the reward zone than both the appetitive and the combined appetitive and aversive treatment. This suggests that the appetitive element of the sucrose reward was a strong incentive very early on in training. Post-training, both aversive containing treatments differed significantly from the control group, spending significantly more time in the reward zone (P = 0.001). The aversive containing groups also significantly increased the amount of time spent in the reward zone post- versus pre-training, whereas, appetitive and control groups did not. These results show that bees which experienced aversive conditioning (aversive + appetitive or aversive alone) significantly increased time spent in the reward zone and as the GLMM analyses show, were more likely to enter the reward zone post-training, compared to any other training treatment.

The significant difference in time spent in the reward zone between the aversive + appetitive and the appetitive treatments supports the influential role of aversive conditioning. The only difference between these two treatments is the aversive training element, implying it is the aversive aspect which provides additional training motivation to make this treatment significantly more effective than appetitive alone. The fact that the aversive + appetitive treatment did not significantly increase time spent in the reward zone from the aversive alone treatment further supports the notion the aversive conditioning is the most effective training method. This implies that the appetitive element does not improve training outcomes over aversive training. Interestingly, bees which did not receive appetitive conditioning (those in the aversive alone condition) found and entered the reward zone sooner than bees in other treatments, further supporting aversive conditioning as the most effective training motivator in this context.

Pre-training, the appetitive treatment entered the reward zone significantly more than both the control (P = 0.02) and combined aversive and appetitive (P = 0.006) groups. However, post-training, no significant differences in the number of times the reward zone was entered were observed. This implies that ‘time spent in the reward zone’ may be a better indication

of training for the bumblebee than the 'number of times bee enters the reward zone'. This is supported by the fact that visual measures of training, in the form of the trajectory graphs (Figures 4.4-4.7), more closely support the findings of the 'time spent in reward zone' parameter. This is that aversive conditioning treatments provide the most training motivation, giving increased weight to this parameter. In a similar vein, the 'average speed' and 'total distance' parameters were not significantly different between treatments pre- or post- training and we see a significant difference for all treatments when comparing pre- and post-training values, suggesting these parameters are poor discriminators of training between groups.

These results show that aversive conditioning paradigms can be highly effective in bee training assays, filling an identified gap (Siviter et al., 2018b) in the bee behavioural assay tool kit. The development of novel, ecologically relevant aversive conditioning paradigms for wild pollinator species such as *B. terrestris*, reduces the need to use traditional appetitive paradigms, such as the proboscis extension reflex (PER), where bees are harnessed and unable to move freely, potentially modifying behaviours e.g. accepting different sucrose concentrations, or being more likely to ingest toxic compounds (Ayestaran et al., 2010; Mujagic and Erber, 2009; Muth et al., 2018) and missing finer behavioural scales (Giurfa & Sandoz, 2012). Aversive conditioning removes the need for time delays or starvation periods in appetitive paradigms and eliminates confounding foraging variables such as the size of a forager's honey stomach or variation in foraging ability, providing a high throughput, streamlined training system.

It is important to develop assays which are ecologically relevant to their target species. The aversive assays which exist prior to the thermal-visual arena are based almost exclusively on the managed pollinator; the western honeybee *A. mellifera* (Núñez et al., 1983., Breed, Guzmán-Novoa and Hunt, 2004., Abramson et al., 2006., Dinges et al., 2013., Zhang and Nieh, 2015., Junca et al., 2014 and Junca and Sandoz, 2015.) and have limited ecological relevance to real-world scenarios. The thermal-visual arena bridges these gaps, providing an assay developed specifically for a larger wild insect pollinator, the bumblebee, and utilises environmental temperature as an ecologically relevant aversive stimulus. The development of such assays can provide new routes to study learning and memory in wild pollinator species.

Determining the extent to which bees utilize the visual patterns around the arena's perimeter for navigation is difficult within the current experimental set up. Initially, an attempt was made to develop a memory test in replacement of trial 10. For example, after 9 training trials with the allocated treatment (appetitive, aversive or combined), no stimuli were provided (identical to the control environment) for trial 10 to see whether bees would still travel to the learnt reward zone location. However, this did not appear to act as a memory test for the bees, where their behaviour in the aversive trial conditions seemed to immediately perceive that there was no aversive stimulus to avoid and no need to seek an 'escape'. As well as this finding, in the appetitive trial conditions the behaviour of the bees indicated that there was a rapid discovery that there was no reward at the location and therefore the best strategy was to search the arena for this reward or for a route to escape the arena. This resulted in trajectories which did not have an appearance comparable to the previous 'trained trajectories' and were more like control trajectories. This made interpretation difficult as to whether the bees had or had not learnt the reward zone location.

A second memory test was trialled by rotating the landscape pattern 90° clockwise for trial 10, again to determine whether bees moved to where they had learnt the reward zone should now be, in relation to the new orientation of the landscape pattern. However, this test was flawed as due to the selective wiring of the thermal-visual arena Peltier array, we could not in fact relocate the cool reward zone by 90° to relate to the new pattern. Therefore, it was again difficult to determine whether bees had learnt the location in relation to the pattern or discovered the absence of a reward and began searching the arena instead.

For future studies, a larger fully thermally controllable arena should be used to conduct such tests in a more robust manner. As no navigational cues are present in the arena, other than the circumference landscape patterns, we can infer that the bees are likely to be using these 'landmark' patterns (Cartwright and Collett, 1982, 1983; Collett, Harland and Collett, 2002; Collett, Chittka and Collett, 2013) as spatial navigation tools, but further testing utilising the red light trials was implemented to ascertain this.

During the red light trials it is evident that pre-training, bees in the 'light' condition, with the visual pattern element of the arena present, spent significantly more time ($P = 0.0096$), and entered the reward zone significantly more ($P = 0.0019$), than the 'dark', red light condition bees. Post-training there was no significant difference in either of these parameters between

dark and light groups. This suggests that initially bees in the light group were able to use the pattern's visual cues to complete the task quicker than the dark group, learning where the reward zone was within the first training trial. However, by trial 10, perhaps due to the small size of the thermal-visual arena and the relatively high probability of locating the reward zone by chance, the visual pattern is not necessary for the dark bees to locate the reward zone. Both groups (light (P = 0.02*) and dark (P = 0.005**)) spent significantly more time in the reward zone post-training compared to their pre-training values, showing that both groups improved significantly from the initial parameter values in trial 1. Bees in the 'dark' group were placed in the dark with no pattern stimuli instead of in a light arena no pattern stimuli (arguably a more comparable control to the light condition), as the room in which the arena trials were conducted had large arrays of ceiling lights and panels which I wanted to be certain bees were not using as alternative navigational cues. Jin et *al.* (2014) showed that, in a visual arena, bumblebees (*B. terrestris*) use local cues (e.g. a coloured square at the reward site) over spatial panoramic guides (e.g. maze visual signals). However, bees also utilised panoramas for navigation, helping the bees to localise to the reward quadrant in under a minute of the trial (Jin et al., 2014). This study supports the red-light trial findings here, suggesting a role for panoramic cues (such as the thermal-visual arena's pattern) in spatial guidance (aiding quick localisation to the reward quadrant in the light group), but that local cues are perhaps more salient (e.g. the arena floor's aversive temperature).

The limited size of the thermal-visual arena, and the resulting distance a bee can view the spatial pattern cues from, may be a limiting factor in the bees' accurate usage of the visual patterns in the red-light trials. Bees in the light treatment appear to have been able to more readily locate the reward zone in trial 1 compared to dark bees in the same trial, suggesting usage of the visual cues provided in the arena. However, this ability to better locate the reward zone may have been more distinct if the arena (and visual cues) were on a larger scale. Research has shown that bees' ability to discriminate patterns relies on a memory of the spatial layout they are in and on the flicker generated by the eye during scanning flights (Zhang & Horridge, 1992). In our arena bee subjects were confined to walking on the test platform, perhaps reducing their ability to produce fully formed spatial memories through flicker scanning (honeybees scan scenery sequentially rather than in one 'glance'(Nityananda et al., 2014; Spaethe et al., 2006)). Studies have shown that freely flying bees display higher

visual learning capacities than restricted bees, potentially due to their ability to conduct active scanning whilst learning (Buatois et al., 2017). What is clear from the literature is that black and white stripes of varying orientations are easily learnable patterns for bees (Srinivasan et al., 1993, 1994; van Hateren et al., 1990), meaning that the visual cues we provided for bees in the arena should be highly learnable. However, the delivery of these patterns to test bees' visual systems could be optimised (e.g. larger arena or allowing bees to fly). It is likely that in the small scale of the thermal-visual arena, the bees learnt that one pattern denoted the quadrant in which the reward zone was located but not the exact location of the zone, e.g. vertical bars indicated the area to search within.

A benefit of the experimental design in this assay is that it allowed for forager monitoring and age cohorting within hives. This removed the potentially confounding variable of age which is overlooked in many bee behavioural studies. Although it is not clear how bumblebee castes are established, and role division and foraging behaviours have not been shown to be age related (Lisa J. Evans and Raine, 2014; Tobback et al., 2011), we can assume that, the older the bee, the more external experience of its environment it has had (e.g. prior learning is shown to influence PER conditioning outcomes (reviewed in Frost et al., 2012) and therefore age standardisation should still be considered for learning assays.

Despite the arena utilising an environmentally relevant aversive stimulus (heat), we must acknowledge that the arena is far from a natural setting for a bumblebee. The scale of the arena is not optimal (due to the small size) and bees' wings were clipped to restrict them to the test surface. To facilitate increased 'natural' bumblebee behaviours, future assays should consider a larger scale arena and perhaps no wing clipping, instead utilising a different method to restrict bees to the test surface (e.g. a heated ring as used in Ofstad *et al.* (2011)). This would allow bees to fly and navigate over a larger area, potentially producing clearer foraging decision outputs and making the 'trained bee' phenotype even clearer.

Interspecific (between hive) and intraspecific (within hive) variation in individual bee training parameters makes determining these phenotypes even more difficult. Raine *et al.* (2006) point out that there could be the assumption that bees are under an intense selective pressure to maximise their learning ability. Foraging bees must navigate across highly complex environments, handle widely diverse floral rewards, and monitor these rewards to achieve optimal reward (Knauer & Schiestl, 2015; Menzel et al., 2005; Nicholls & Hempel de

Ibarra, 2017; Núñez, 1970); all these skills are integral to foraging success. It could therefore be predicted that very little variation in learning ability would be observed, both between hives and individuals, as such a strong selective pressure should eliminate such variation. However, even within identical training groups, there appears to be both individuals and hives which do not conform to the identified trend and are unable to learn the task. For example, Hive 2 spent significantly less time in the reward zone post-training than either Hive 1 ($P = 0.02$) or Hive 3 ($P = 0.04$), and experimentally omitting Hive 2 from the dataset introduces new significance between the aversive and appetitive training groups both pre- ($P = 0.0165$) and post- ($P = 0.0006$) training. However, it should be reiterated that the direct source of these individual differences remains unclear. Differential task ability may be as a result of differential learning ability between individuals from different hives, but it may also be a result of differential abilities to e.g. tolerate the heat stress of the thermal-visa arena. Other examples of such individual differences have been recorded in the literature (Chittka and Thomson, 1997; L. Li et al., 2017; Muller and Chittka, 2012) and may play a role in which bees would be the most successful foragers in the wild (discussed in detail in Chapter 8).

In our laboratory studies it was impossible to determine which of our foragers would be good at learning before the commencement of trials and therefore all bees which were marked as active foragers in the Perspex hive tubes had an equal chance of being included in the trials. Theoretically this could skew data across treatment groups, if, by chance good foragers were all allocated to one treatment group. Previous studies have used transcriptomics to examine the genetic basis of differences between groups of bees given a task to learn and those which are in a control group (Li et al., 2018). However, no transcriptomic studies to date have examined the individual differences seen when bees are exposed to the same learning environment, an avenue further investigated in Chapter 8 of this thesis.

We have proven that, in this context of the thermal-visual arena, aversive conditioning is a highly effective training tool to teach bees to locate and remain in a cool reward zone. However, that does not mean that overall, this method of training is always better for bumblebees, as behavioural and motivational contexts will differ, and it is therefore impossible and inappropriate to equate the nature and the strength of the unconditioned stimulus in each learning scenario (appetitive or aversive). As has been noted, honeybees reject sucrose solution presented at above 50°C (Junca et al., 2014), it is therefore possible

that in the combined appetitive + aversive treatment, the sucrose reward was not acting as a salient appetitive stimulus. However, this does not explain why, post-training, appetitive alone bees do not spend any significantly different amount of time in the reward zone versus the control group (P = 0.14). This could, nonetheless, be a factor of satiety, as each time a bee collects a 20µl sucrose reward for entering the reward zone the bee's honey stomach would become increasingly full, with no way of purging until the trial ceased. The reward provided was 20µl and the average bumblebee can carry approximately 0.2 ml (200µl) in their honey stomach meaning that approximately 10+ rewards may result in filling of the honey stomach.

However, if satiety is the primary reason for a lack of time spent in the reward zone in trial 10, we would expect to see the same satiety response in trial 1, which we arguably do not, as initially bees in the appetitive treatment spend significantly more time in the reward zone (P = 0.0001). It may also be the case that the salience of the appetitive stimulus decreases across trials as there is a higher motivation; escape, and no negative association with leaving the reward zone to try to do so. Whereas the aversive stimulus remains consistent in its negative association across all 10 trials. Therefore, these trials demonstrate the effectiveness of aversive conditioning in this arena but not necessarily at the detriment of appetitive conditioning.

The 'training phenotypes' developed from the training parameters studied here, particularly the trajectory graphs and 'time spent in the reward zone' could be used as 'null behavioural templates' for the bumblebee. This will allow us to see how these templates are disrupted under biological stressors, such as parasite load or pesticide exposure. The success of aversive conditioning as a training parameter in the thermal-visual arena provides promise for the further use of aversive assays in bee training studies. The development of this arena as a species-specific assay for the bumblebee adds a tool for the study of wild pollinator species. The assay bridges a gap in the need for techniques to be developed across bee species, outside of the Western honeybee. The ability to understand the null behavioural templates of species across environmental conditions will become increasingly key to understanding the impact of agricultural stressors, such as pesticides, on our pollinators.

Recently, Scheiner et al. (2020) presented their development of 'a novel visual place learning arena for honeybees which relies on high temperatures as aversive stimuli' (Scheiner et al., 2020). Similar to the thermal-visual arena developed in this thesis (Chapters 3 and 4), the

Scheiner et al. assay is a thermal-visual place learning paradigm based on the use of temperature as an aversive stimulus, building upon a previous design used to study *Drosophila* (Ofstad, Zuker, & Reiser, 2011). Work on the thermal-visual arena presented in this thesis began in 2016 and was consequently published as a novel place learning assay for bumblebees in a PLOS ONE paper in January 2020 (James et al., 2020) (Chapter 6), preceding the publication of the Scheiner et al. work in April 2020. That two separate research groups have recognised the deficit in aversive learning assays in this field (reviewed in Chapter 2) and produced similar thermal-visual place learning assays, utilising temperature, stands testament to its effectiveness as a bee training tool and supports the findings reported here in Chapters 3 and 4 of this thesis.

The results reported in the Scheiner et al. (2020) study are discussed and contrasted here. Scheiner et al. (2020) measured bee aversive learning performance as 'time taken to reach the safe spot', finding that honeybees became faster at locating the safe spot with each training trial. The study supports the findings here, that bees spent significantly more time in the reward zone post-training, and that aversive temperature is highly effective at improving bee (bumblebee) training parameters. The group utilised temperatures between 42°C and 50°C to optimise honeybee avoidance, resulting in a similar optimal temperature (46°C) to the one used in this study (45°C), supporting our choice of 45°C as an optimum motivator of bee avoidance. The group also utilised 10 training trials (as in this thesis) as an optimum trial number for assessing learning performance (increased from an initial 6 in their earlier trials which did not prove enough to assess performance). The use of landmarks to aid bee navigation in the arena and allow bees to reach the reward zone more quickly was also supported by the group's honeybee findings. The group utilised technology which was not present in our thermal-visual design, e.g. the use of an LED screen to project patterns and allow easy landmark repositioning. This provides promising avenues for further development of thermal-visual assays utilising enhanced visual aspects. The thermal-visual paradigm is clearly highly effective in the study of bee navigation and visual learning in a controlled laboratory environment and facilitates comparative analyses across species (e.g. Drosophila (Ofstad et al., 2011) and honeybees (Scheiner et al., 2020)).

Chapter 5

Assessing the impacts of two neonicotinoids and a sulfoximine pesticide on *Bombus terrestris* performance in the thermal-visual arena

Chapter 5: Assessing the impacts of two neonicotinoids and a sulfoximine pesticide on *Bombus terrestris* performance in the thermal-visual arena

5.1 Introduction

There is a growing body of evidence pointing to pesticide exposure as a key threat to insect pollinators (reviewed in Chapter 1), particularly for social bees who communally collect and store food resources for their nest. The risk of pesticides to bees is made up of a combination of exposure level and compound toxicity. Effects can be complex and will differ depending on individual species biology e.g. ability to metabolize toxins, the pesticide compound, and whether other interacting stressors are present e.g. pathogen load (Collison et al., 2016). Bees can become exposed to pesticide compounds whilst foraging on treated crop flowers, having a broad range of potential lethal and sublethal effects on bee behavior, physiology, learning and memory at seemingly low exposure levels and with potential knock-on effects to hive functioning and survival (reviewed in Chapter 1).

A wide range of sublethal effects have been recorded in bees as a result of neonicotinoid exposure (reviewed in Blacquière et al., 2012), with compounds affecting learning and memory (Andrione et al., 2016; Mustard et al., 2020; Siviter et al., 2018b; Stanley et al., 2015; Tison et al., 2017a; Williamson et al., 2014; Williamson and Wright, 2013; Zhang and Nieh, 2015), brood care (James D. Crall et al., 2018), larval development (Abbott et al., 2008; Decourtye et al., 2005; Tasei et al., 2000), immunosuppression (Brandt et al., 2017), homing success (Fischer et al., 2014; Henry et al., 2012), reproduction (Whitehorn et al., 2012), thermoregulation (James D. Crall et al., 2018) and foraging and motivation (Arce et al., 2017; Feltham et al., 2014; Gill et al., 2012; Gill and Raine, 2014; Lämsä et al., 2018; Muth et al., 2019; Dara A Stanley et al., 2015). However, it should be noted that traditional toxicology testing has largely focused on honeybees as a model organism for all bee species and only more recently has research taken a wider approach to studying sublethal effects in non-*Apis* bees.

It is has been posited that neonicotinoids with a nitro group (imidacloprid, dinotefuran, clothianidin, and thiamethoxam) are more harmful to bees than the cyano-neonicotinoids (acetamiprid and thiacloprid) (Blacquière et al., 2012; Iwasa et al., 2004; Laurino et al., 2011;

Mommaerts et al., 2010; Wood and Goulson, 2017). However, recent studies have highlighted differential cross-species neonicotinoid sensitivity and suggested a role for genetic determinants (Beadle et al., 2019; Manjon et al., 2018; Reid et al., 2020), highlighting the importance of studying compound effects across species.

Two neonicotinoids were selected for use in this study, thiamethoxam and thiacloprid. Thiamethoxam has been reported to have a range of sublethal effects at field realistic exposure levels on both honeybees; reduced larval and pupal survival and decreased adult emergence and survival (Grillone et al., 2017; Henry et al., 2012; Tavares et al., 2017, 2015; Tesovnik et al., 2017), impaired flight and decreased homing success (Henry et al., 2012; Tosi et al., 2017), impaired locomotion, organ disruptions (Friol et al., 2017), immunosuppression (Tesovnik et al., 2020) decreased motor function and hyper activity (Tosi and Nieh, 2017) and bumblebees; reduced worker survival, brood production and food consumption (Laycock et al., 2014), decreased egg laying (Baron et al., 2017). However, honeybees are still predominantly used as a model organism to study pesticide impacts and there remains a lack of studies on impacts on other taxa such as bumblebees. In this study we wanted to examine whether thiamethoxam, proven to have sublethal effects at field-realistic exposure levels, would influence *B. terrestris* forager's ability to respond to aversive conditioning in the thermal-visual arena. We also wanted to study food consumption, as there are limited reports on the effect of field realistic doses (Laycock et al., 2014).

Thiacloprid has largely been reported as "not harmful to bees", particularly on manufacturer's websites where it is listed as part of an insecticide group which "poses(s) no risk to bees… and can be applied to flowering crops" (Bayer Crop Science UK, 2020a). Differential, lower toxicity has been reported in honeybees in comparison to the other neonicotinoids (Iwasa et al., 2004). Despite this assertion, sublethal effects have been reported in a number of studies on honeybees; olfactory learning and memory (Tison et al., 2017), foraging behavior, immunosuppression (Brandt et al., 2017), homing success, communication and navigation (Tison et al., 2016) and in bumblebees on colony development and reproduction (Ellis et al., 2017; Havstad et al., 2019). There is a need to continue to investigate potential sublethal effects of thiacloprid on bees, particularly non-*Apis* groups, as there remains conflicting reports of its effect at field realistic dosages (Rundlöf and Lundin, 2019).

A relatively new sulfoximine-based insecticide, sulfoxaflor [methyl(oxo){1-[6-(trifluoromethyl)-3-pyridyl]ethyl}-k6-sulfanylidene]cyanamide (Figure 5.1), has been proposed as a potential replacement to the neonicotinoid compounds (thiamethoxam, clothianidin and imidacloprid) now banned for outdoor usage in the EU. The sulfoximines have the same molecular target as the neonicotinoids, as an insect nAChR antagonist. However, sulfoxaflor has a distinct mode of action, with promising effects against neonicotinoid resistant sap-sucking pests including aphids, whiteflies, hoppers, and Lygus (Sparks et al., 2013; Zhu et al., 2011). The presence of sulfoximine in its structure confers a unique set of structure-activity relationships, making its mode of action unique and potentially less damaging to non-target insects (Sparks et al., 2013). Sulfoxaflor is currently registered for use in 81 countries worldwide and has recently been re-registered in the USA, where its registration was initially pulled due to concerns over non-target pollinator effects (United States Environmental Protection Agency (EPA), 2019). Sulfoxaflor was flagged as a potential future threat to bees in a horizon scanning publication in 2016, due to the lack of knowledge of its sublethal effects (Brown et al., 2016) and the Food Safety Authority (2019) have identified chronic studies on adults and larvae and studies on bumblebees as a core data gap in the effective assessment of sulfoxaflor risk to bees. To date there only a handful of (very recent) studies considering potential sublethal effects of sulfoxaflor, which suggest that there may be negative fitness impacts of exposure on bumblebee colonies, with worker production and reproductive output reduced (Siviter et al., 2018) and possible impacts on bee oxidative stress leading to early onset of apoptosis and mortality (Chakrabarti et al., 2020). However, further studies have reported no negative effects on olfactory conditioning or working memory (Siviter et al., 2019) and no direct effect on larval mortality (Siviter et al., 2020). Nevertheless, when *B. terrestris* larvae were treated in combination with the fungal parasite *Nosema bombi,* a significant negative impact on larval mortality was reported (Siviter et al., 2020). The existing research is by no means extensive and has largely looked at acute exposure regimes. Sulfoxaflor's similarity in both its insect target (nAChRs) and systemic mode of action, to the neonicotinoids gives it the potential to have severe detrimental effects on bee learning and memory; however, at present this has barely been studied.

It is vital that alternative replacements to the neonicotinoids are assessed in a timely manner, so that we are not playing catch-up with potentially devastating deleterious effects, as has been the case for several of the neonicotinoids. Compounds such as thiamethoxam, imidacloprid or clothianidin should be tested alongside neonicotinoids thought to be less harmful to bees (e.g. thiacloprid) and potential replacement compounds such as sulfoxaflor. Too often compounds are tested in isolation, making it difficult to determine whether current, or indeed, newer compounds in fact have fewer sublethal effects than the compounds that have gone before them.

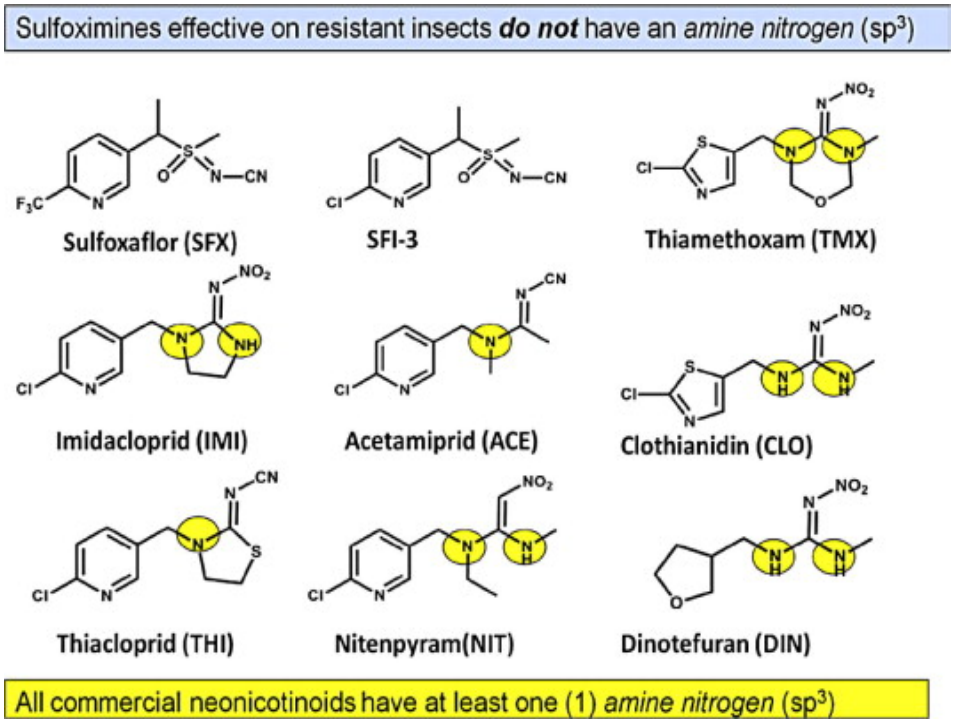

Figure 5.1: The structure of sulfoxaflor in comparison to the neonicotinoids, highlighting the neonicotinoids' presence of one or more sp^3 nitrogens. Taken from Sparks et al. (2013).

A continuing problem in the assessment of pesticide impacts on wild pollinators, such as bumblebees, is that research gaps preclude accurate risk assessment due to a lack of information. This has resulted in the publication of reports stating that it is unclear whether compounds have deleterious effects on wild pollinators (Food Safety Authority, 2019). It is therefore vital that these knowledge gaps are filled as quickly as possible, using sound and field realistic methodologies, so that accurate assessments of pesticide impacts can be collated. Quantifying pesticide impacts on non-target species is vitally important in being able

to identify the potential wider-reaching ecological impacts of compounds (Forbes and Calow, 1999; Walthall and Stark, 1997). Of interest is whether realistic levels of pesticide exposures can illicit negative behavioural impacts on beneficials, which has the potential to have knock-on effects on the wider ecosystem.

5.1.1 Research gaps

When considering reliable risk assessments for pesticide impacts on wild pollinators, the European Food Safety Authority (2019) concluded that several data gaps exist; for chronic data (adult and larvae) on bumblebees, for solitary bees and for acute and chronic risk to non-*Apis* bees (Abdourahime et al., 2019). It is clear that the assessment of pesticide compounds on wild pollinators such as *Bombus* spp. is still incredibly patchy in comparison to the assessments conducted on honeybees and that further assessment is necessary to be able to adequately gage sublethal effects (Mommaerts and Smagghe, 2011; Siviter et al., 2018).

The proportional lack of pesticide impact studies on non-*Apis* bees needs to continue to be addressed. Mommaerts and Smagghe (2011) put forward a tiered approach to improving the gaps in assessment of bumblebee species; tier 1 consisting of laboratory tests on individual insects, tier 2 involving extended laboratory tests which include the evaluation of pesticides impacts on key processes such as worker survival, reproduction and behaviour and tier 3, completing semi-field and/or field tests (Mommaerts and Smagghe, 2011). The studies described here aim to contribute to the tier 1 and tier 2 type studies described and increase the knowledge base of sublethal impacts on bumblebee species.

5.1.2 Approach

Most assessments of pesticide impacts on bee learning and memory in the literature have focused on testing appetitive conditioning paradigms, as highlighted by Siviter *et al.* (2018), who noted that there was simply not enough ‘non-appetitive paradigm’ research to conduct a review of pesticide impacts with anything but appetitive conditioning studies. As was found in Chapters 3 and 4, aversive conditioning has the potential to be a highly effective way of studying bee learning and memory and yet is massively underutilized and basically non-existent in the study of non-*Apis* bee species. The current dearth of studies considering the impact of pesticide exposure on wild bee species’ ability to complete aversive learning tasks

means that we could be missing a wide range of potential sublethal effects which have simply not been studied. Having demonstrated in Chapter 4 that aversive conditioning is an effective training mechanism in *B. terrestris*, it was deemed feasible to proceed to testing pesticide compounds in the thermal-visual arena, to elucidate potential pesticide impacts on aversive learning tasks.

A continuing criticism of the body of research examining sublethal effects on bees, is that studies often utilize unrealistically high pesticide exposure levels, which some argue a foraging bee would never come into contact within the field (Carreck and Ratnieks, 2014; Godfray et al., 2014). It therefore remains uncertain whether certain pesticide compounds could have drastic impacts at the low sublethal levels likely to be encountered by foraging bees.

5.1.3 Aims of this study

The aims of this study were to determine whether environmentally appropriate levels of neonicotinoid pesticides (thiacloprid and thiamethoxam) and a sulfoximine-based pesticide (sulfoxaflor) have significant effects on the bumblebee *B. terrestris*' ability to respond to aversive conditioning and complete the thermal-visual arena's learning task. To do this we used dietary dosages to expose *B. terrestris* foragers to two sublethal concentrations; a low 'field realistic' concentration and a high 'worst-case scenario' concentration of the three pesticide compounds and monitored their feeding behaviour. Bees were then required to complete training trials in the thermal-visual arena to determine how exposure to the pesticide compounds affected the training parameters identified in Chapter 4.

5.2 Methods

5.2.1 Colonies

Two queen-right colonies of *B. terrestris audax* were obtained from Biobest (Biobest, Westerlo, Belgium), each colony contained a queen and approximately 200 workers. Bees were settled in wooden nest boxes (29 x 21 x 16 cm) using the same protocol as described in Chapter 4 (section 4.2.2). Hives were provided with Biogluc (62% sugar concentration consisting of 37.5% fructose, 34.5% glucose, 25% sucrose, 2% maltose, and 1% oligosaccharides) (Biobest, Westerlo, Belgium) in two gravity feeders, modified from laboratory falcon tubes by puncturing small holes to allow feeding, in a Perspex foraging tunnel connected to the hive. Hives were given a regular supply of pollen directly into the hive to allow *ad libitum* feeding. Biogluc is the standard sugar syrup used in bumblebee rearing facilities.

5.2.2 Training protocol

The training protocol for all treatment groups was identical to that used for the 'Aversive' treatment group in Chapter 4, with each bee given individual access to the thermal-visual arena for ten, three-minute, aversive training trials across three days. The Aversive environment is created by heating the Peltier tiles of the thermal-visual arena's floor to 45°C and cooling an area of four Peltier tiles to 25°C to create a cool reward zone (see section 3.2.2). As demonstrated in the previous Chapter, the 45°C arena floor provides strong aversive motivation for bumblebee foragers to locate and remain in the cool reward zone.

5.2.3 Age cohorts and marking

As described in section 4.2.3 of Chapter 4, bees were tagged with coloured bee marking discs (EH Thorne, Market Rasen, UK) within weekly age cohorts, and only bees of the same age cohort were used within each trial. Age cohorts were monitored to record active foragers who regularly left the hive to collect Biogluc from the feeders. Of the active foragers recorded in each age cohort, 12 were randomly selected to be used in each trial. All forager age cohorts were one-week post-emergence when used in trials to standardise forager age.

The 12 selected foragers were then randomly allocated, three bees per treatment, to one of four treatments: 1) control (clean Biogluc - no pesticide dosage given), 2) thiacloprid, 3) thiamethoxam or 4) sulfoxaflor. Foragers' wings were clipped using a queen marking cage and dissection scissors (EH Thorne, Market Rasen, UK) and bees were then housed in individual Perspex cages with access to a modified 1.5ml Eppendorf feeder to facilitate the assessment of individual food consumption (Figure 5.2).

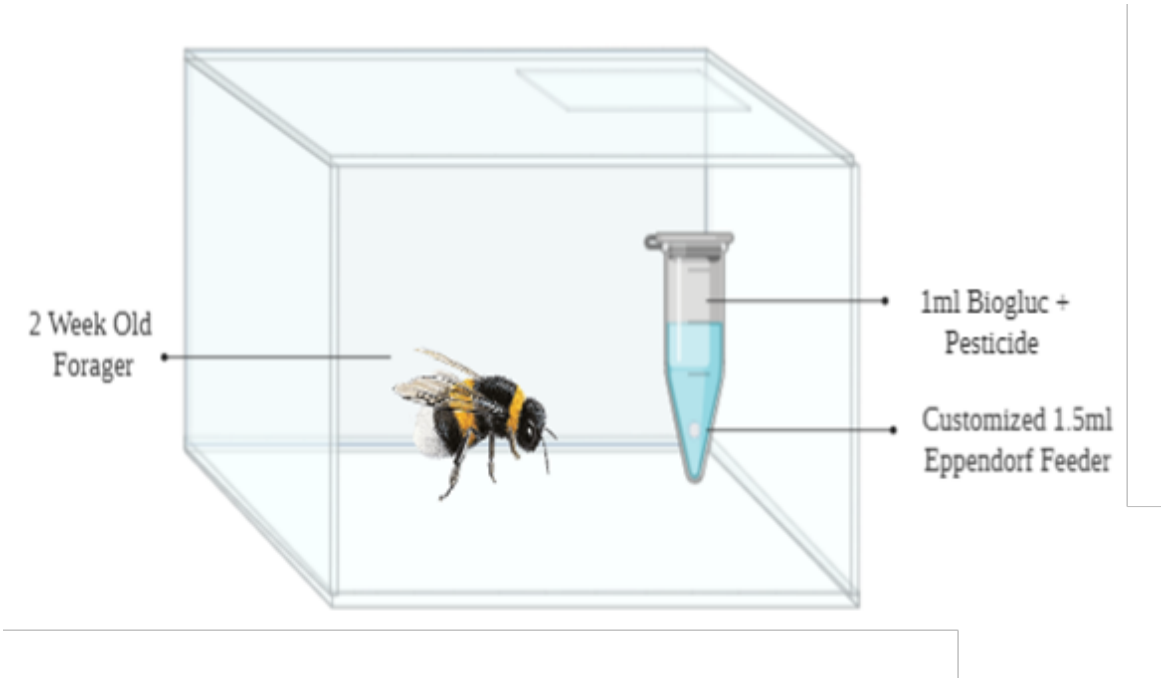

Figure 5.2: New bee housing design utilising individual Perspex cages with new feeders adapted from 1.5ml Eppendorf tubes, to facilitate individual bee food consumption tracking.

5.2.4 Choice of pesticide compounds and exposure

B. terrestris foragers were exposed to one of three pesticide compounds (sulfoxaflor, thiamethoxam or thiacloprid) to assess impacts on the behavioural parameters identified in Chapter 4 in the thermal-visual arena. The neonicotinoids thiamethoxam and thiacloprid were selected for testing as although thiamethoxam is one of the three neonicotinoids now banned for outdoor use in the EU, it is still widely used in the rest of the world. Thiacloprid was selected as it is one of the cyano-substituted neonicotinoids generally considered to have lower bee toxicity and therefore is not part of the EU moratorium (Iwasa et al., 2004).

As well as testing two neonicotinoid pesticides, it was decided to also test a sulfoximine compound; sulfoxaflor. The sulfoximines are an emerging group of insecticides intended to replace neonicotinoids.

Bees were exposed to pesticide compounds orally through feeding on dosed Biogluc sugar syrup. All pesticides were technical grade and not formulation. Pesticide exposed bees were compared against control bees which were given clean Biogluc solution with no pesticide.

Two dosage levels were tested (in separate experiments) for each compound: a 'low' sublethal dose (dosages taken from literature reports of residue levels found in pollen and nectar in the field) and a 'high' sublethal dose (designated as ten times the low sublethal dose).

Due to the nature of the assay, bees are required to walk within the thermal-visual arena to locate the cool reward zone. Therefore, using sublethal pesticide dosages, which would still maintain bee mobility was essential. The full pesticide exposure protocol used for both low and high dose trials can be found in supplementary material 2.

5.2.5 Trial design

A total of 72 *B. terrestris* foragers were tested across the low and high dose pesticide experiments (36 bees in low dose, 36 in high dose). Three replicates were conducted for both low and high dose experiments. All bees were one-week post emergence and of the same age cohort, when used in the trials.

Chronic exposure and temporal spacing of trials

All bees in all treatments were subjected to an aversive conditioning task (as described in Chapter 4); a heated arena floor with an inconspicuous cool reward zone. Bee trials were run sequentially from bee 1 to bee 12 for each set of trials, and treatments were randomised across bees to limit the influence of bee order. The order in which bees were run was also randomized prior to trial 1 and then remained the same throughout the trials (1-10) to ensure relatively equal spacing between trials. Chronic pesticide exposure was conducted over a six-day period; three days prior to the start of training trials and then continued for the three days of the trials (see supplementary material 2 for full protocol). Temporal spacing of trials

was kept the same as Chapter 4 trials: with trials 1 - 4 conducted on one day, trials 5 - 7 on the next and trials 8 -10 on the final day.

Low-sublethal dose pesticide trials

A low dose thiamethoxam concentration of 10 ppb was decided upon, based on studies reporting thiamethoxam residues found in the nectar and pollen of treated plants in the field (Castle et al., 2005; Pohorecka et al., 2013; Rundlöf et al., 2015; Sanchez-Bayo and Goka, 2014; Thompson et al., 2013; Wood and Goulson, 2017) and previous studies which have also used this concentration as a field realistic value (Samuelson et al., 2016; Stanley and Raine, 2016). A low dose thiacloprid concentration of 500 ppb was used, based on field nectar residues reported by Ellis et al. (2017), and a low dose sulfoxaflor concentration of 5 ppb, based on a predicted field-realistic concentration used by Siviter *et al.* in the only sulfoxaflor bumblebee exposure studies to date (Siviter et al., 2019, 2018b, 2018a). Dosages were achieved through serial dilution of pesticides with acetone and water and then final dilutions into Biogluc.

A total of 36 bees were tested at the low dose concentrations. Each replicate represents a different age cohort from the same hive (Hive 1).

- Replicate 1: 3 sulfoxaflor, 3 thiacloprid, 3 thiamethoxam and 3 control bees
- Replicate 2: 3 sulfoxaflor, 3 thiacloprid, 3 thiamethoxam and 3 control bees
- Replicate 3: 3 sulfoxaflor, 3 thiacloprid, 3 thiamethoxam and 3 control bees

High-sublethal dose pesticide trials

The high sublethal dose concentrations were calculated as ten times the low sublethal dose concentrations, equating to 100 ppb for thiamethoxam, 5000 ppb for thiacloprid and 50 ppb for sulfoxaflor. This represents a possible “worst case field scenario” for potential detrimental impacts in extreme exposure cases.

The MFRC (Maximum Field Recommended Concentration) for thiamethoxam is 100 ppm (Mahmoudi-Dehpahni et al., 2020; Mommaerts et al., 2010). This concentration (100ppb) has been reported in guttation droplets (Girolami et al., 2009) and much higher doses of 310ppb in bee tissues (Hladik et al., 2016). Here, 0.1ppm (100ppb) thiamethoxam was used for the high dose trials, just 1/1000 of the MFRC, equivalent to the dosage found in guttation droplets and lower than the in-tissue report. The MFRC for thiacloprid is 120 ppm (Mommaerts et al.,

2010), here, 5ppm (5000ppb) thiacloprid was used for high dose trials, 1/24 of the MFRC. Sulfoxaflor MFRC is 60ppm (Fernández et al., 2017), far higher than the 5ppm (5000ppb) used here, 1/12 of the MFRC.

A total of 36 bees were tested at the high dose concentrations specified above. Each replicate represents a different age cohort from the same hive (Hive 2).

- Replicate 1: 3 sulfoxaflor, 3 thiacloprid, 3 thiamethoxam and 3 control bees
- Replicate 2: 3 sulfoxaflor, 3 thiacloprid, 3 thiamethoxam and 3 control bees
- Replicate 3: 3 sulfoxaflor, 3 thiacloprid, 3 thiamethoxam and 3 control bees

5.2.6 Food consumption recording

On day 1 of the experiments, each bee was individually caged and given access to a single 1.5ml Eppendorf feeder (Figure 5.2). Individual (empty) Eppendorf feeders were weighed, and their weight recorded. Feeders were filled with a standardised amount (1ml) of Biogluc solution and reweighed. Each subsequent day of the experiment (day 2 - day 7), feeders were weighed to assess individual food consumption, emptied, cleaned, refilled and reweighed. Control evaporation feeders were also set up in empty cages to determine potential food loss through evaporation and ensure accuracy of food consumption data. The average of the evaporation from the five evaporation tests per day was calculated as 0.022g. This value was therefore taken away from all bee consumption values prior to data analysis, to take loss through evaporation into account.

5.2.7 Trial recording and video processing

As in Chapter 4, all trials were recorded using a FLIR C2 thermal camera (FLIR Systems UK, West Malling, Kent, UK) situated above the arena. New Debut video capture software (Debut Video Capture Software Version 5.xx, NCH Software, Inc., 6120 Greenwood Plaza Blvd, Greenwood Village CO, USA) was used to capture video recordings. Recorded video files were tracked with idTracker using custom parameters (Pérez-Escudero et al., 2014).

5.2.8 Data analyses

Sample sizes for the low dose trials were n = 36, 9 bees per treatment (control, thiacloprid, thiamethoxam, sulfoxaflor). For the high dose trials: n = 35, as the thiamethoxam treatment only had 8 (rather than 9) bees, as one bee died during the three-day exposure prior to trial 1 on day four, therefore, no trial data was recorded.

Training parameters

As in Chapter 4, training parameter data for each bee was produced from tracking files using custom R scripts (Jess Evans, Statistics Department, Rothamsted). Based upon the analyses and results of Chapter 4, a smaller range of influential parameters for both trial 1 (pre-training) and trial 10 (post-training) were analysed for all bees in each treatment:

1. Bee trajectory maps: the route the bee takes within the arena during the trial
2. Time the bee spent in the reward zone.
3. The distance bees travel throughout the trial.
4. Average speed of the bee throughout the trial.

Seven thiamethoxam bees and one thiacloprid bee in the high dose treatment experiment died prior to trial 10. In such a scenario, the last trial completed prior to death was used as the “post-training” trial for analyses. Two of the six thiamethoxam bees were omitted entirely from analyses (bee IDs 78 and 9, thiamethoxam) as they died during the initial three-day pesticide exposure period prior to commencement of trial 1 on day 4 and therefore no trial data were collected. Of the remaining five bees which were deceased prior to trial 10, trial 4 (bee IDs 80, 19 and 17, thiamethoxam), trial 5 (bee ID 42, thiamethoxam) and trial 7 (bee ID 78, thiacloprid) were used as post-training trials for analyses. All statistical analyses of training parameters were conducted in GraphPad Prism 8.2.1 (GraphPad Software, 2020).

Food consumption

Three new data sets were generated from the pesticide food consumption data recorded during trials (complete food consumption dataset and estimates for per-bee pesticide consumption (ng) can be found in Chapter 5 appendix: Tables A5.1, A5.2 and A5.3). These were:

1) The low dose trials total five-day food consumption for each bee in each treatment.
2) The high dose trials total five-day food consumption for each bee in each treatment.
3) The high dose trials total three-day food consumption (as in the high dose trials seven of the nine thiamethoxam bees died prior to day five food consumption measurements).

5.3 Results

5.3.1 Normality testing

A suite of normality tests was conducted (Anderson-Darling, D'Agostino & Pearson, Shapiro-Wilk and the Kolmogorov-Smirnov test). For the low dose experiments, the 'time spent pre-training' dataset was non-normal, all other data sets were normal. Parametric statistical tests or non-parametric equivalents were used accordingly.

5.3.2 How does chronic pesticide exposure affect food consumption?

Low dose five-day total consumption

Figure 5.3 (A) shows the total amount of food consumed over the five days of the low dose trials by bees in each treatment. All treatments consumed significantly more food than that lost in the evaporation test feeders (One-way ANOVA with Tukey correction for multiple comparisons, P values: evaporation vs. control (0.0049**), evaporation vs. thiacloprid (0.0035**), evaporation vs. sulfoxaflor (0.0275*) and evaporation vs. thiamethoxam (0.0034**), as would be expected. There were no other significant differences in the amount of food consumed between any of the other treatments in the low dose trials.

High dose five-day total consumption

Figure 5.3 (B) shows the total amount of food consumed by bees in the high dose pesticide trials. The thiamethoxam treatment was removed from this analysis as several thiamethoxam bees died by day four, skewing the amount of food being consumed by day five in that treatment group. The other two pesticide compounds (thiacloprid and sulfoxaflor) were compared to the control group (One-way ANOVA with Tukey correction for multiple comparisons). Bees in the thiacloprid treatment consumed significantly less food (P = 0.0008***) compared to the control group. There was no significant difference in food consumption between the sulfoxaflor and control groups (P = 0.1567) or between the thiacloprid and sulfoxaflor groups (P = 0.0603). However, the thiacloprid vs. sulfoxaflor comparison was close to the significance threshold of P = 0.05 (P is 0.06), suggesting that thiacloprid bees ate less than the sulfoxaflor treated bees, although this was not significant.

High dose 3-day total consumption

Figure 5.3 (C) shows the total amount of food consumed across treatments in the initial three-day period of high dose exposure. Three-day analysis was conducted to allow us to compare consumption of thiamethoxam to the other pesticides, prior to the thiamethoxam bee deaths on day four. As in the high dose five-day data, at three days, bees in the high dose thiacloprid treatment consumed significantly less food than bees in the control treatment (One-way ANOVA with Tukey correction for multiple comparisons, P = 0.0112*). There were no other significant comparisons between treatments and thiamethoxam bees did not consume significantly different amounts of food (vs. controls or other treatments) prior to the deaths of some bees on day four.

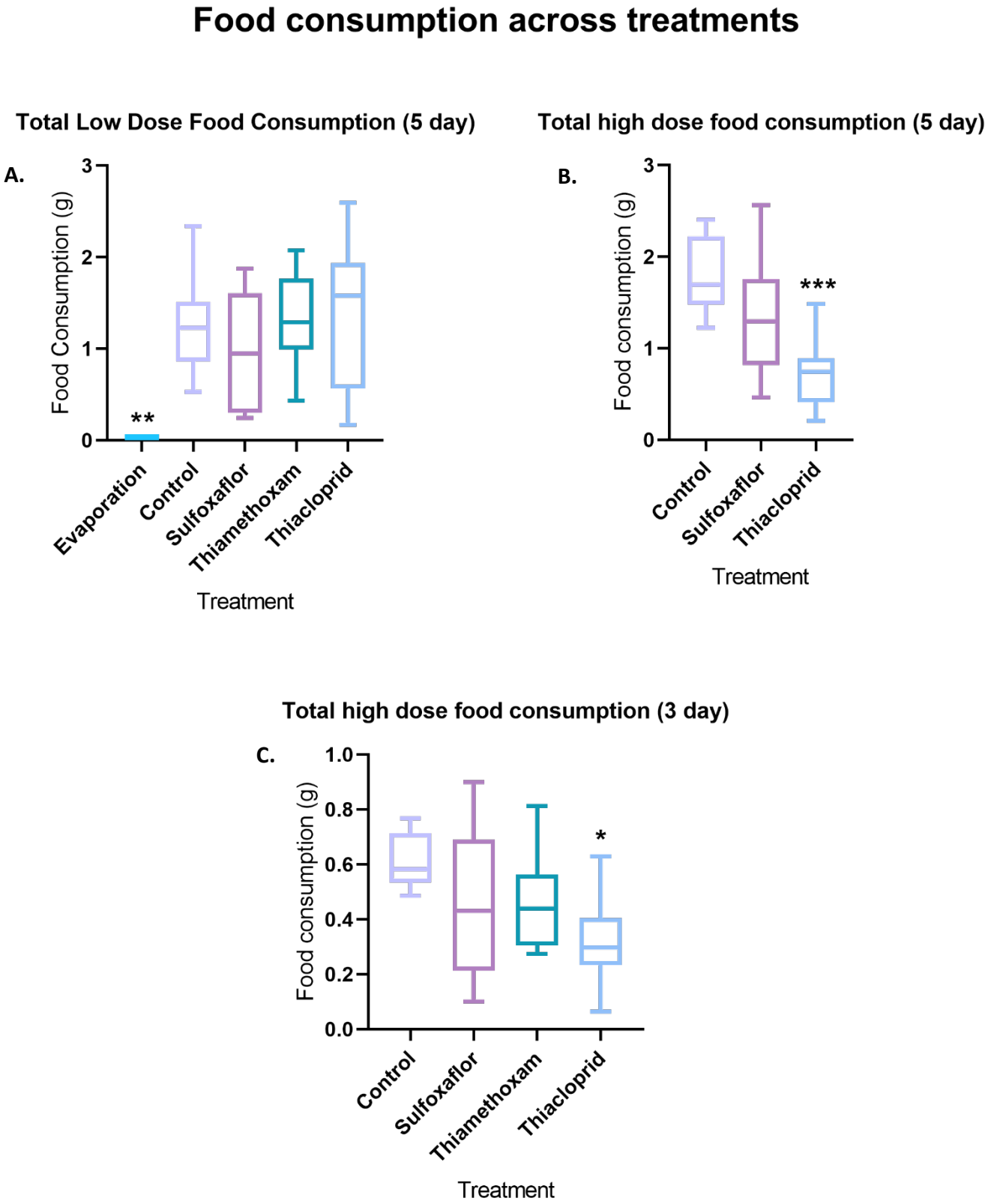

Figure 5.3 (A-C): Food consumption across treatments. A) Total 5-day food consumption of bees in the low dose trials compared evaporation feeders. B) Total 5-day food consumption of bees in the high dose trials. The Thiamethoxam treatment was removed from this 5-day analysis as several thiamethoxam bees died by day four. C) Initial 3-day food consumption of bees in the high dose trials (including thiamethoxam).

5.3.3 Pesticide impacts on training parameters

Trajectory graphs

Figures 5.4.A to 5.4.G give example training parameter graphs pre- and post- training for one bee in each treatment group for both low and high dose trials. All bees were subjected to aversive conditioning and therefore the "control" bees here demonstrate similar trajectories to the "aversive" bees from Chapter 4.

Control condition

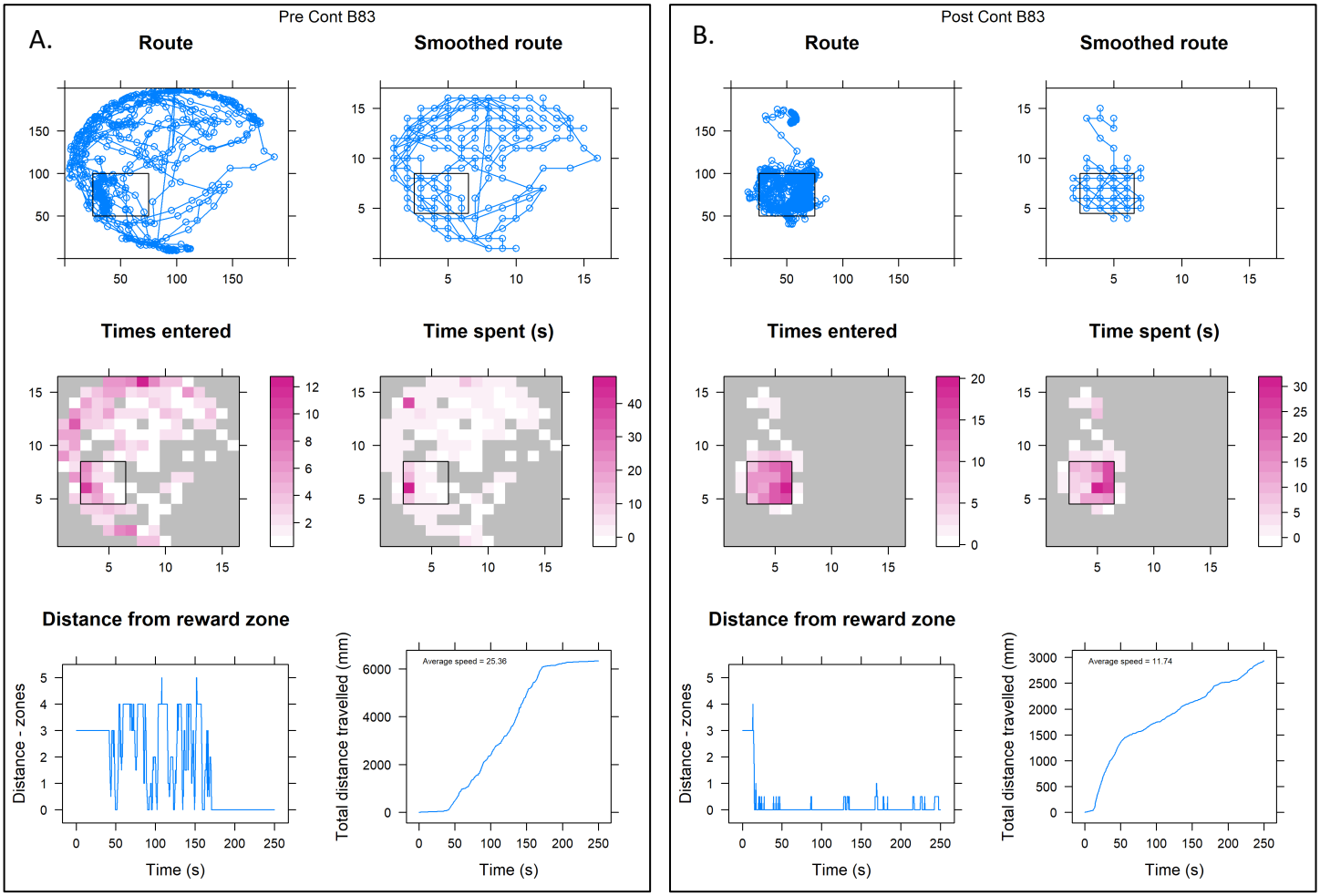

Figure 5.4.A: Training parameters from a control bee (B83) pre- A) and post- B) training. Control condition identical between low and high dose trials. The black square indicates the reward zone location within the arena.

Low dose trials

Sulfoxaflor condition (low dose)

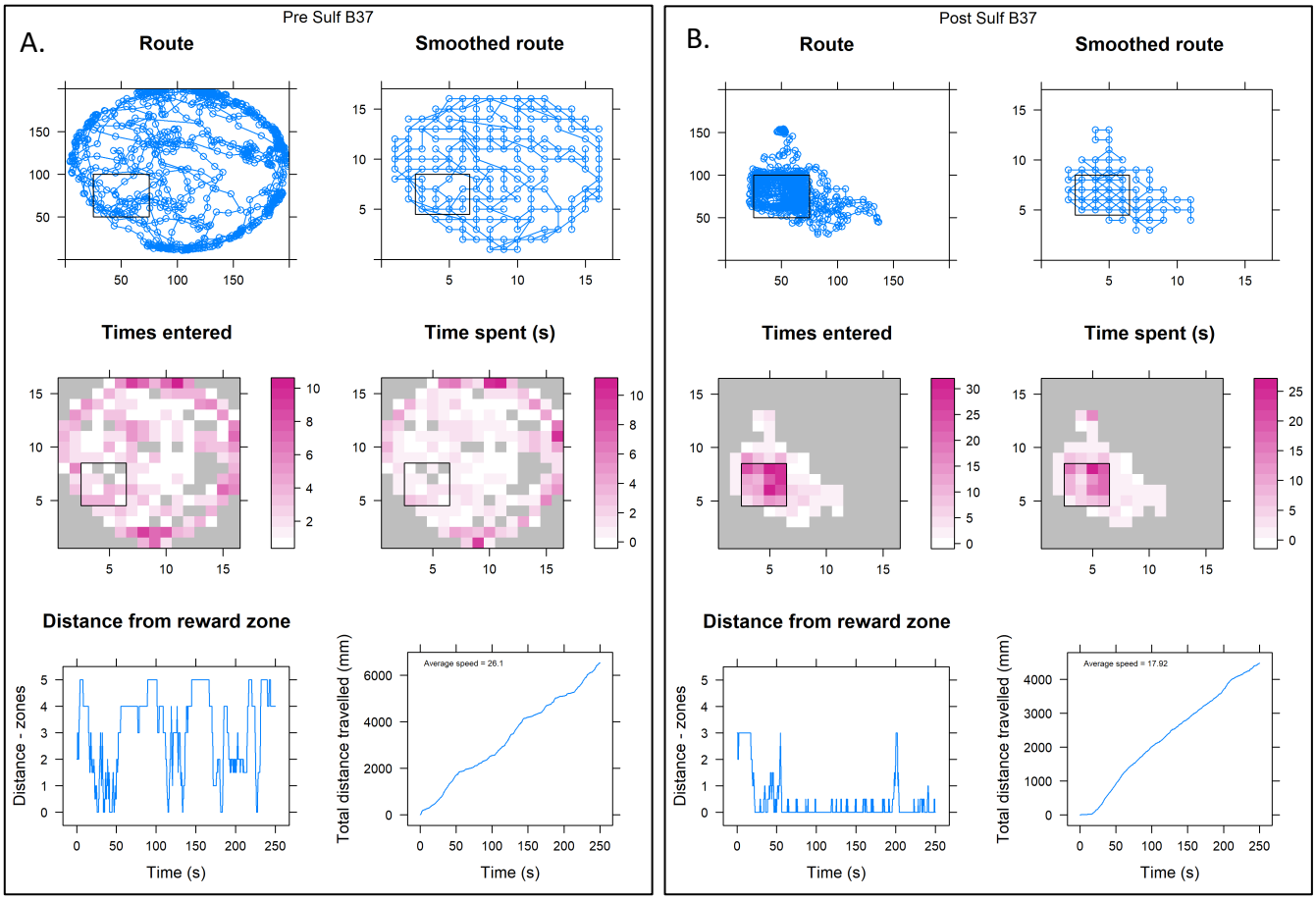

Figure 5.4.B: Training parameters from a low dose sulfoxaflor bee (B37) pre- A) and post- B) training. The black square indicates the reward zone location within the arena.

Thiacloprid condition (low dose)

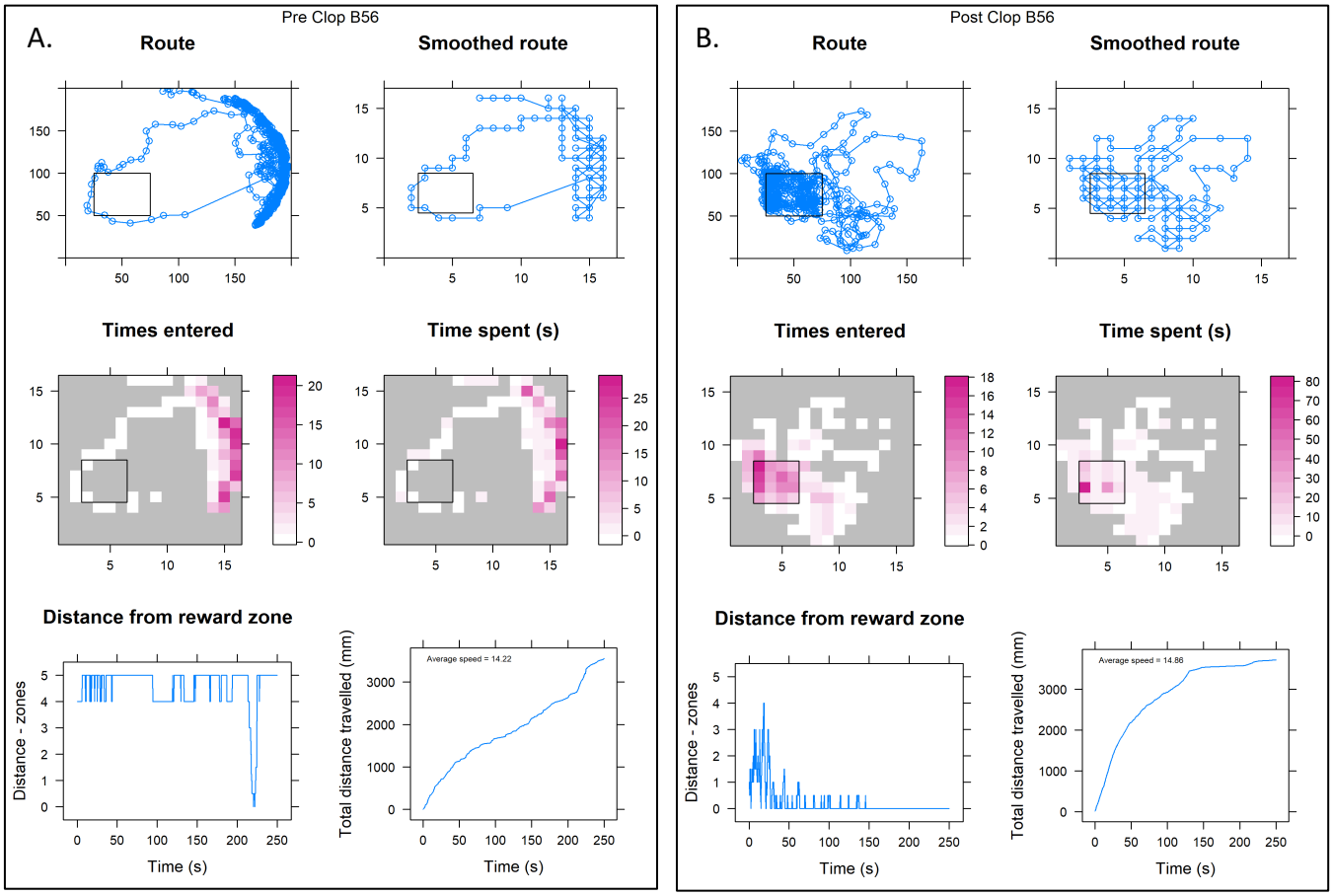

Figure 5.4.C: Training parameters from a low dose thiacloprid bee (B56) pre- A) and post- B) training. The black square indicates the reward zone location within the arena.

Thiamethoxam condition (low dose)

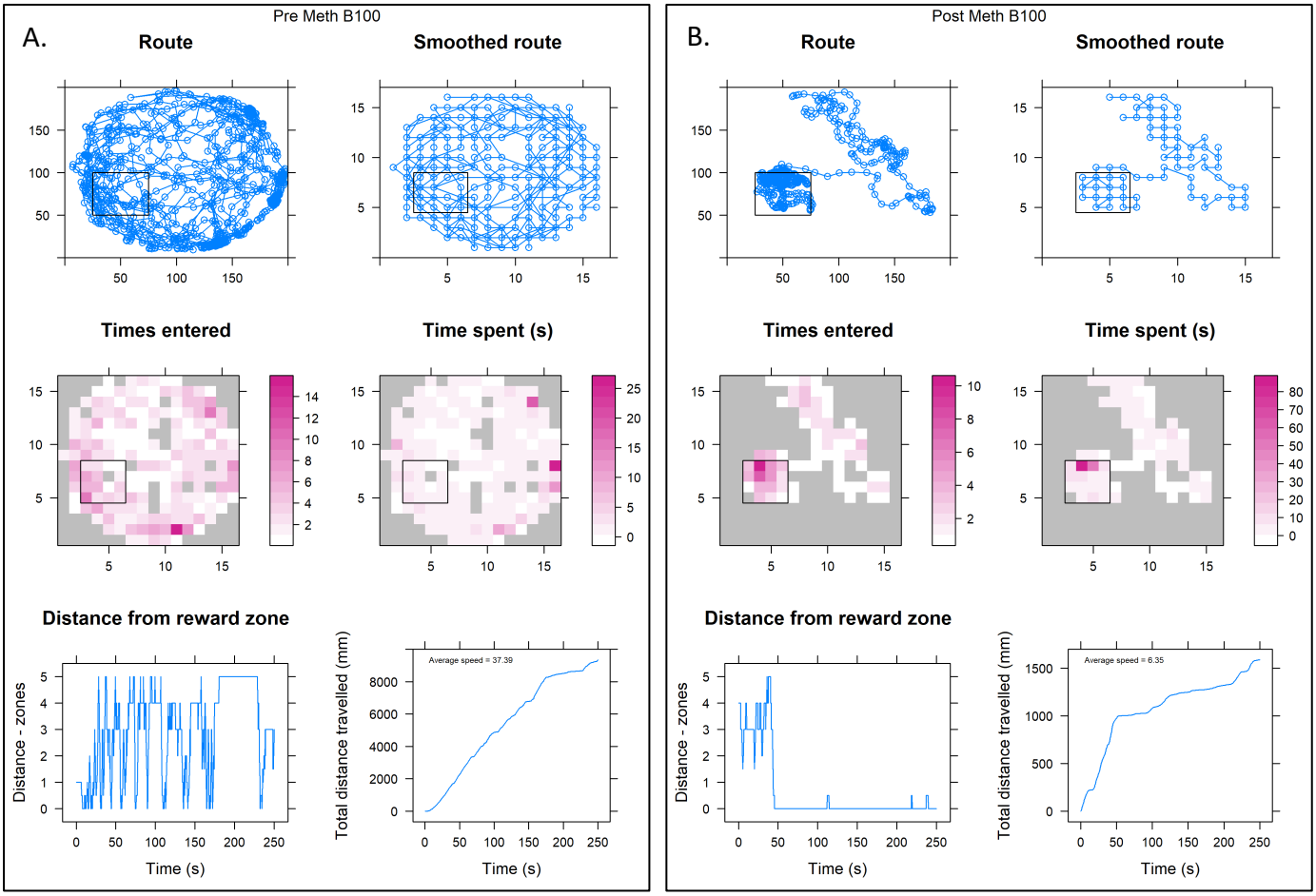

Figure 5.4.D: Training parameters from a low dose thiamethoxam bee (B100) pre- A) and post- B) training. The black square indicates the reward zone location within the arena.

High dose trials

Sulfoxaflor condition (high dose)

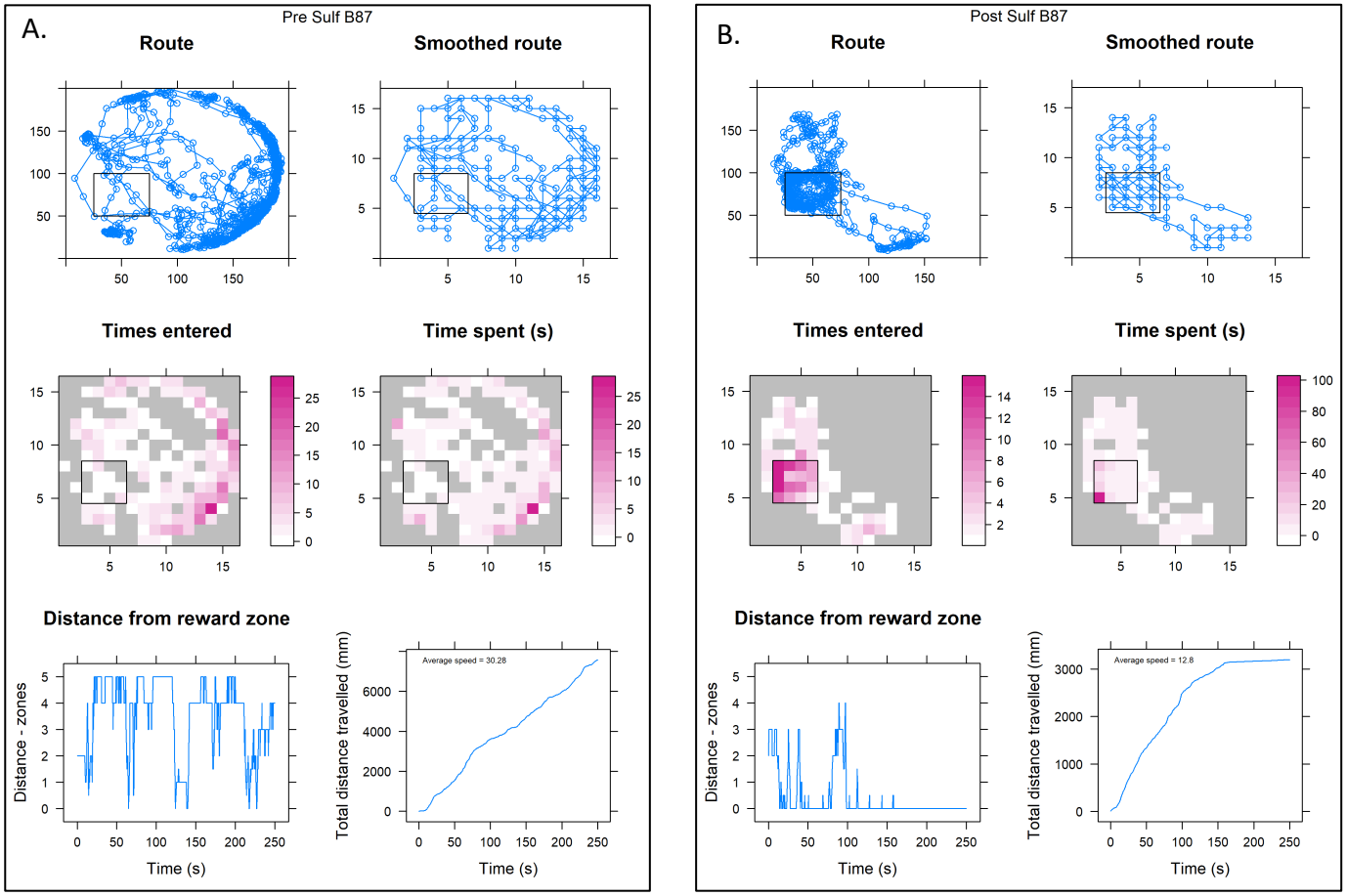

Figure 5.4.E: Training parameters from a high dose sulfoxaflor bee (B87) pre- A) and post- B) training. The black square indicates the reward zone location within the arena.

Thiacloprid condition (high dose)

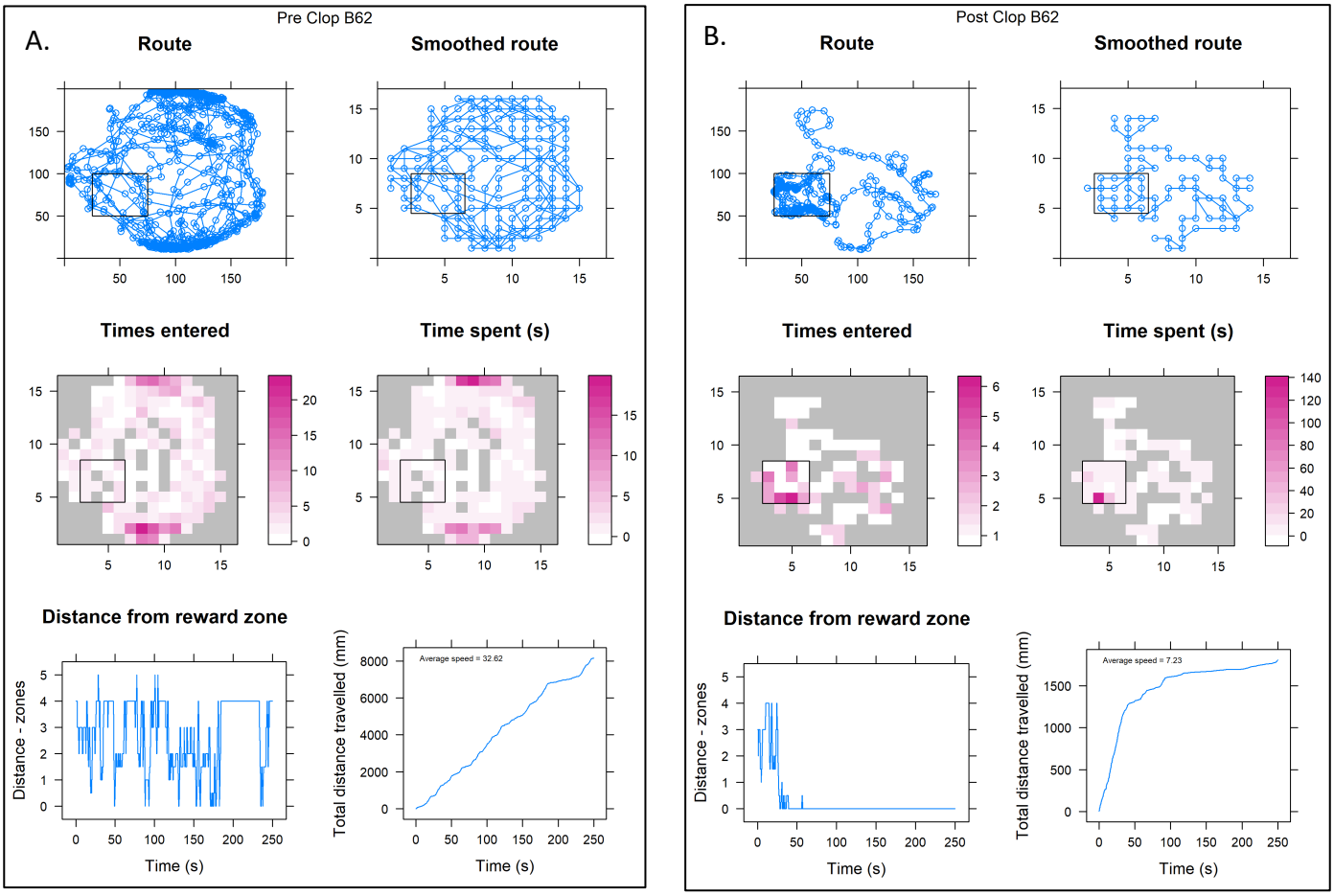

Figure 5.4.F: Training parameters from a high dose thiacloprid bee (B62) pre- A) and post- B) training. The black square indicates the reward zone location within the arena.

Thiamethoxam condition (high dose)

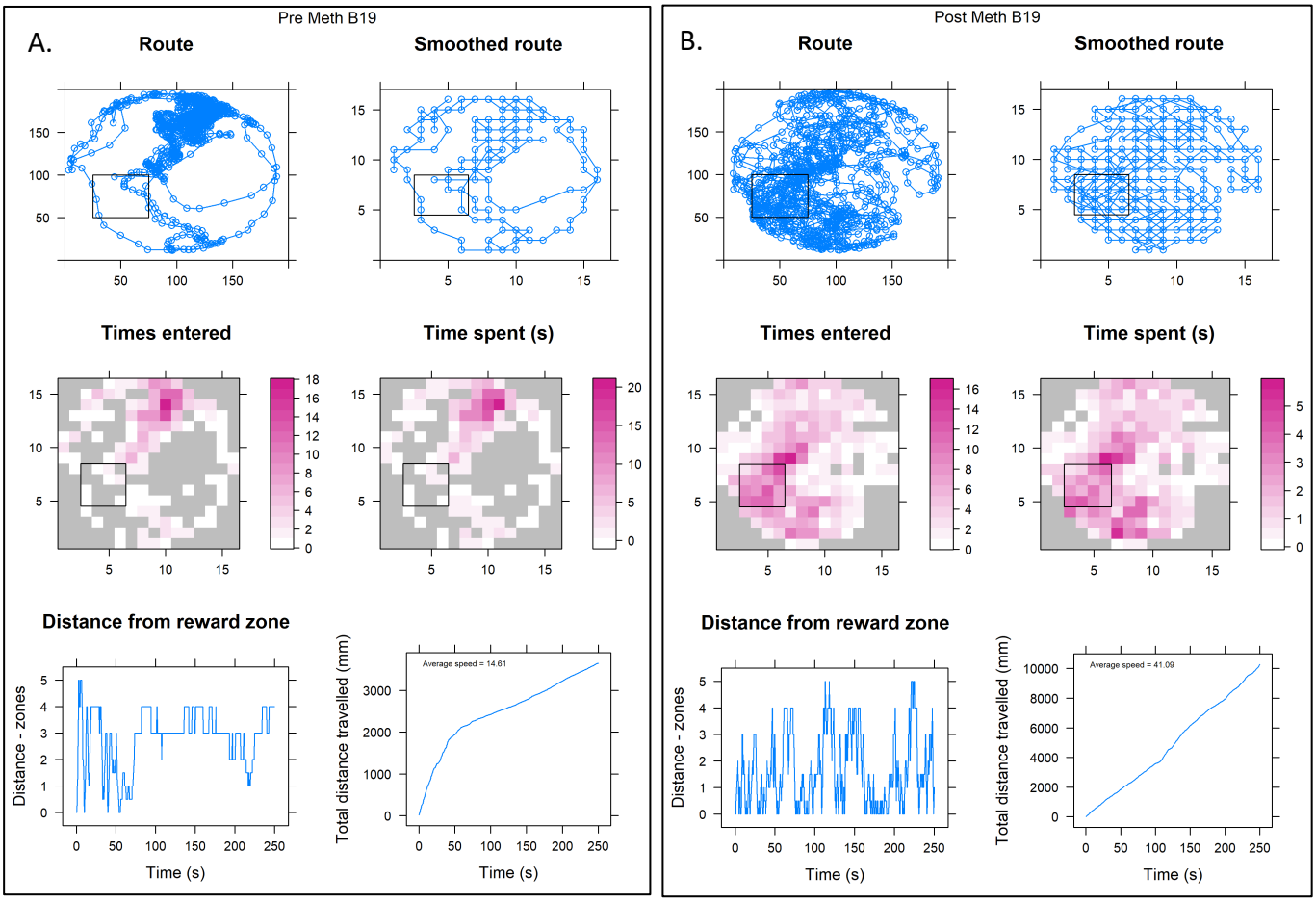

Figure 5.4.G. Training parameters from a high dose thiamethoxam bee (B19) pre- A) and post- B) training. The black square indicates the reward zone location within the arena.

Low dose trials

Time spent in the reward zone

(i) Pre-training - there was no significant difference in the time spent in the reward zone by any of the treatments (P = >0.99 for all comparisons, Kruskal-Wallis test with multiple comparisons) (Figure 5.5.A).

(ii) Post-training - there was no significant difference in the time spent in the reward zone by any of the treatments (P values range from 0.21 to 0.99, One-way ANOVA with Tukey correction for multiple comparisons) (Figure 5.5.B).

(iii) Pre- versus post-training - paired t-tests or Wilcoxon matched-pairs signed rank tests (dependent on whether data was normal or non-normal) were conducted to assess whether bees within each treatment had significantly increased their time spent in the reward zone post-training, compared to their own pre-training values. All treatments, apart from the thiamethoxam bees, spent significantly more time in the reward zone post-training compared to their pre-training values (Control (paired t-test, P = 0.0004***, t = 5.901, df = 8), thiacloprid (paired t-test, P = 0.0002***, t = 6.594, df = 8), sulfoxaflor (Wilcoxon matched-pairs signed rank test, P = 0.0195*) (Figure 5.5.C). Bees in the thiamethoxam treatment did not significantly increase the time they spent in the reward zone post training (versus pre-training) (Wilcoxon matched pairs signed rank test, P = 0.1289), but this is likely because one individual (B14) spent a large amount of time in the reward zone pre-training (216.8 seconds), as when this individual is removed the comparison becomes significant for all other bees in the thiamethoxam treatment (P = 0.0391*) (Figure 5.5.C).

(iv) Difference in time spent pre- and post-training was calculated for each bee (post-training – pre-training value). A positive value indicates a bee improved, spending more time in the reward zone post-training whereas a negative value indicates the bee got worse at the task, decreasing the time spent in the reward zone post-training. There were no significant disparities between the differences in time spent in the reward zone pre- and post-training for any of the treatment groups. This supports the notion that aversive training is still highly effective across all treatment groups, regardless of pesticide exposure at the low dose values (Figure 5.5.D).

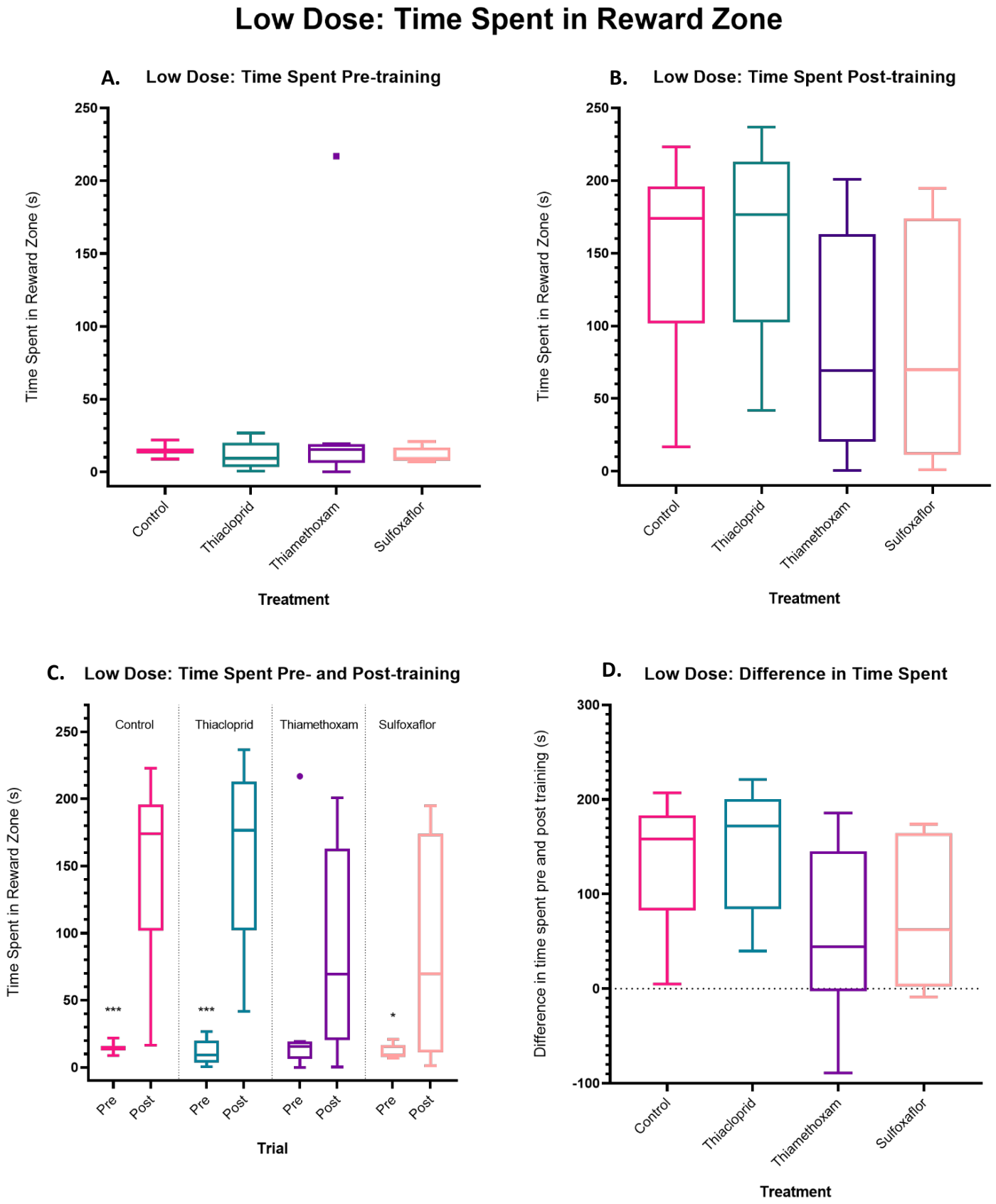

Figure 5.5 (A-D): Time spent in the reward zone by bees in the low dose trials. A. Time spent in the reward zone pre-training. B. Time spent in the reward zone post-training. C. Time spent in reward zone pre- versus post-training. D. Difference in time spent (post-training – pre-training value). (Control n = 9, thiacloprid n = 9, thiamethoxam n = 9, sulfoxaflor n = 9).

Low dose trials

Total distance travelled

i) Pre-training - thiamethoxam bees travelled significantly less distance than control bees (one-way ANOVA with Tukey correction for multiple comparisons, P = 0.027*). Neither the thiacloprid (P = 0.66) or sulfoxaflor (P = 0.72) groups travelled significantly different distances to the controls or each other (P = 0.99) and the thiamethoxam group was not significantly different from the sulfoxaflor (P = 0.24) or thiacloprid (P = 0.28) groups (Figure 5.6.A).

ii) Post-training - there were no significant treatment comparisons (one-way ANOVAs with Tukey correction for multiple comparisons), with none of the groups travelling significantly different distances to each other. Control vs. thiacloprid (P = 0.86), control vs. thiamethoxam (P = 0.99), control vs. sulfoxaflor (P = 0.99), thiacloprid vs. thiamethoxam (P = 0.67), thiacloprid vs. sulfoxaflor (P = 0.75) and thiamethoxam vs. sulfoxaflor (P = 0.99) (Figure 5.6.B).

iii) Pre- vs. post-training - paired t-tests or Wilcoxon matched-pairs signed rank tests (dependent on whether data was normal or non-normal) were conducted to assess whether bees within each treatment had significantly altered the distanced travelled within the post-training trial versus the pre-training trial. Control (P = 0.0022**) and thiacloprid (P = 0.0092**) bees significantly reduced the distance they travelled in the post-training trial versus the pre-training trial. However, the sulfoxaflor (P = 0.06) and thiamethoxam (P = 0.817) bees did not significantly alter the distance travelled post-training (all tests were two-tailed paired t-tests). However, it should be noted that the sulfoxaflor group (P = 0.06) was just 0.01 away from the significance threshold of 0.05, suggesting a general reduction in distanced travelled, although not significant. The thiamethoxam bees had a much higher significance level of P = 0.8, implying very little change between pre- and post-training (Figure 5.6.C).

iv) Difference in distance travelled pre- and post-training - as for 'time spent in the reward zone', the difference in distance travelled was calculated for each bee pre- and post-training. A negative value indicates a bee travelled less distance post training vs. pre-training. There were no significant differences between the differences in distance travelled pre- and post-training for any of the treatment groups. However, as Table

5.1 shows, there is a large difference between the treatment means here, suggesting that thiamethoxam bees improved the least (the least negative value) between pre- and post-training trials (Figure 5.6.D).

Table 5.1: Descriptive statistics for low dose difference in distance travelled pre vs. post training across treatments.

Treatment:	**Control**	**Thiacloprid**	**Thiamethoxam**	**Sulfoxaflor**
Mean	-3741	-3648	-334.4	-2422
Std. Deviation	2539	3209	4204	3328
Std. Error of Mean	846.5	1070	1401	1109

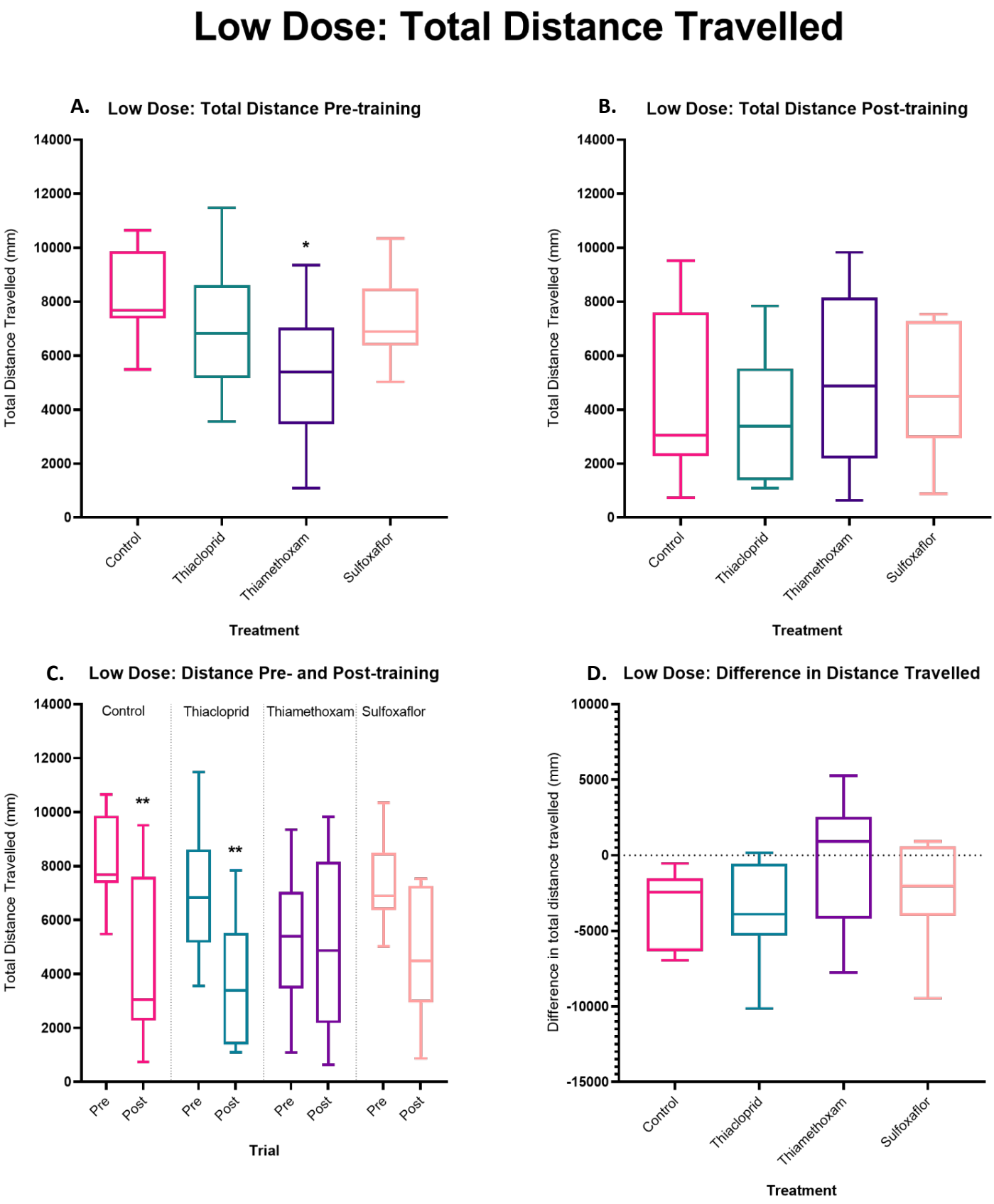

Figure 5.6 (A-D): Distance travelled by bees in the low dose trials. A. Total distance travelled pre-training. B. Total distance travelled post-training. C. Total distance travelled pre- versus post-training. D. Difference in distance travelled (post-training – pre-training value). (Control n = 9, thiacloprid n = 9, thiamethoxam n = 9, sulfoxaflor n = 9).

Low dose trials

Average speed

Speed was looked at in terms of fold change pre- versus post-training for each bee in each treatment. Fold change was calculated by dividing speed post-training by speed pre-training for individual bees. A fold change of 1 indicates a bee did not change its speed, a positive value would indicate speed increased post-training and a negative change would indicate a bee decreased its speed post-training. There was a mean fold change in speed of 0.53 for the control group, 0.53 for thiacloprid, 1.36 for thiamethoxam and 0.71 for sulfoxaflor between pre- and post-training trials (Figure 5.7). This indicates that control and thiacloprid bees virtually halved their speed by the post-training trial. Sulfoxaflor bees also decreased their speed, whereas thiamethoxam bees, on average, increased their speed post-training.

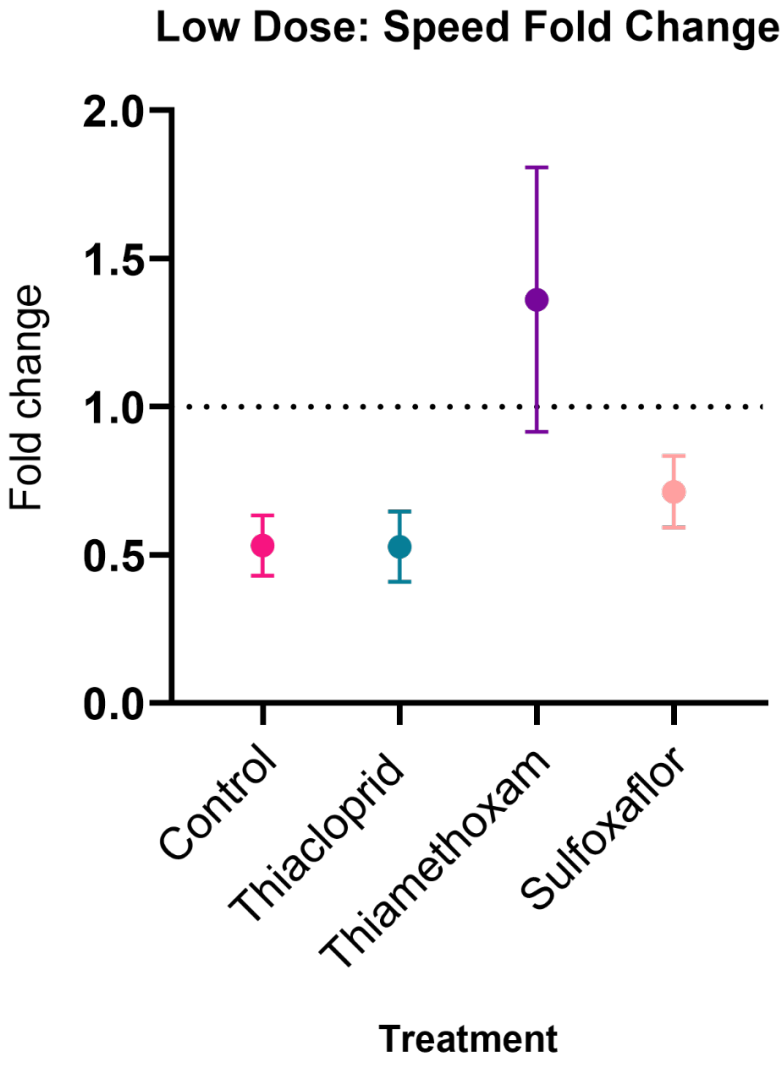

Figure 5.7: Bee speed fold change pre- versus post-training in the low dose trials (± SEM). (Control n = 9, thiacloprid n = 9, thiamethoxam n = 9, sulfoxaflor n = 9).

High dose trials

Time spent in the reward zone

(i) Pre-training, there were no significant differences in the time spent in the reward zone by any of the treatments (P = >0.99 for all comparisons, Kruskal-Wallis test with multiple comparisons) (Figure 5.8.A).

(ii) Post-training, there was a significant difference in the time spent in the reward zone between the thiamethoxam and sulfoxaflor treatment bees (P = 0.01*). There were no other significant treatment comparisons (Kruskal-Wallis test with multiple comparisons, control vs. thiacloprid (P = >0.99), control vs. thiamethoxam (P = 0.27), control vs. sulfoxaflor (P = >0.99), thiacloprid vs. thiamethoxam (P = 0.33) and thiacloprid vs. sulfoxaflor (P = >0.99)) (Figure 5.8.B).

(iii) Pre- versus post-training - paired t-tests or Wilcoxon matched-pairs signed rank tests (dependent on whether data was normal or non-normal) were conducted to assess whether bees within each treatment had significantly increased their time spent in the reward zone post-training, compared to their own pre-training values. Control bees spent significantly more time in the reward zone post-training compared to their pre-training values (P = 0.004**), as did thiacloprid bees (P = 0.01*) and sulfoxaflor bees (P = 0.004**) (Figure 5.8.C). Thiamethoxam bees were the only group which did not significantly improve the time spent in reward zone post-training in comparison to pre-training values (P = 0.22) (Figure 5.8.C).

(iv) Difference in time spent pre- and post-training - there was a significant difference in differences in time spent in the reward zone pre- and post-training between the thiamethoxam and sulfoxaflor treatment bees (P = 0.007**). There were no other significant discrepancies between the differences in time spent in the reward zone pre- and post-training for any of the other treatment groups (control vs. thiacloprid (P = >0.99), control vs. thiamethoxam (P = 0.14), control vs. sulfoxaflor (P = >0.99), thiacloprid vs. thiamethoxam (P = 0.09) and thiacloprid vs. sulfoxaflor (P = >0.99). The lowest non-significant P values were also seen in the other thiamethoxam comparisons. This supports the notion that thiamethoxam bees were not improving as much in the 'time spent' training measure pre= vs. post-training (Figure 5.8.D).

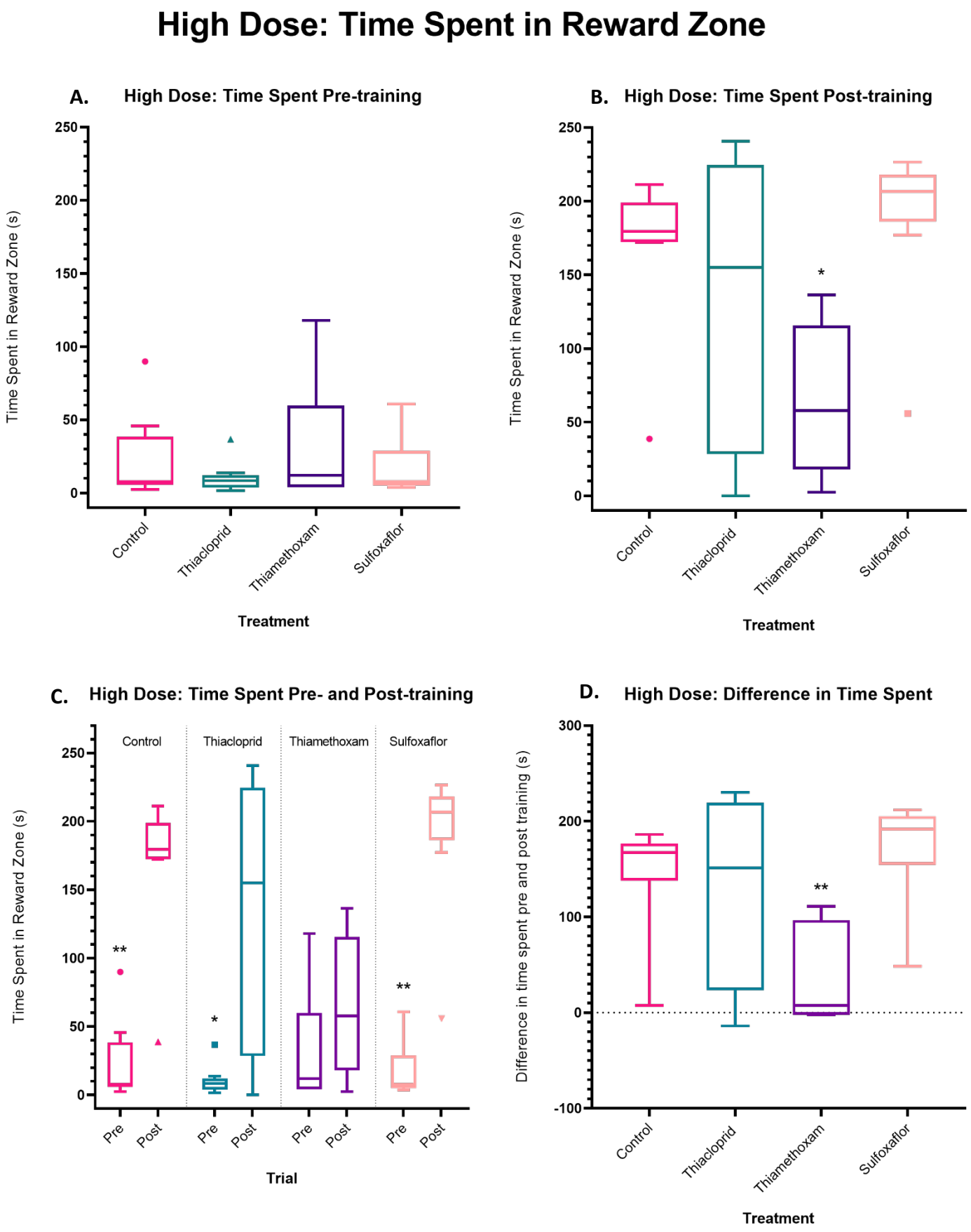

Figure 5.8 (A-D): Time spent in the reward zone pre- and post- training by bees in the high dose trials. A. Time spent pre-training. B. Time spent post-training. C. Time spent pre- versus post-training. D. Difference in time spent (post-training – pre-training value). (Control n = 9, thiacloprid n = 9, thiamethoxam n = 7, sulfoxaflor n = 9).

High dose trials

Total distance travelled

i) Pre-training - there were no significant differences in the distances travelled between bees in any of the treatments (all P values >0.7, Kruskal-Wallis test with multiple comparisons) (Figure 5.9.A).

ii) Post-training - there were no significant treatment comparisons (Kruskal-Wallis test with multiple comparisons), with none of the groups travelling significantly different distances to each other. Control vs. thiacloprid (P = >0.99), control vs. thiamethoxam (P = >0.99), control vs. sulfoxaflor (P = 0.93), thiacloprid vs. thiamethoxam (P = 0.78), thiacloprid vs. sulfoxaflor (P = >0.99) and thiamethoxam vs. sulfoxaflor (P = 0.37) (Figure 5.9.B).

iii) Pre- versus post-training - paired t-tests or Wilcoxon matched-pairs signed rank tests (dependent on whether data was normal or non-normal) were conducted to assess whether bees within each treatment had significantly altered the distanced travelled within the post-training trial versus the pre-training trial. Control (pre vs post; P = 0.0039**), thiacloprid (pre vs post; P = 0.0049**) and sulfoxaflor (pre vs post; P = 0.0039**) bees all significantly reduced the distance they travelled in the post-training trial versus the pre-training trial. However, the thiamethoxam (pre vs post; P = 0.1563) bees did not significantly alter the distance they travelled post-training (Figure 5.9.C).

iv) Difference in distance travelled pre- and post-training - there were no significant differences between the differences in distance travelled pre- and post-training for any of the treatment groups (One-way ANOVA with multiple comparisons) (Figure 5.9.D).

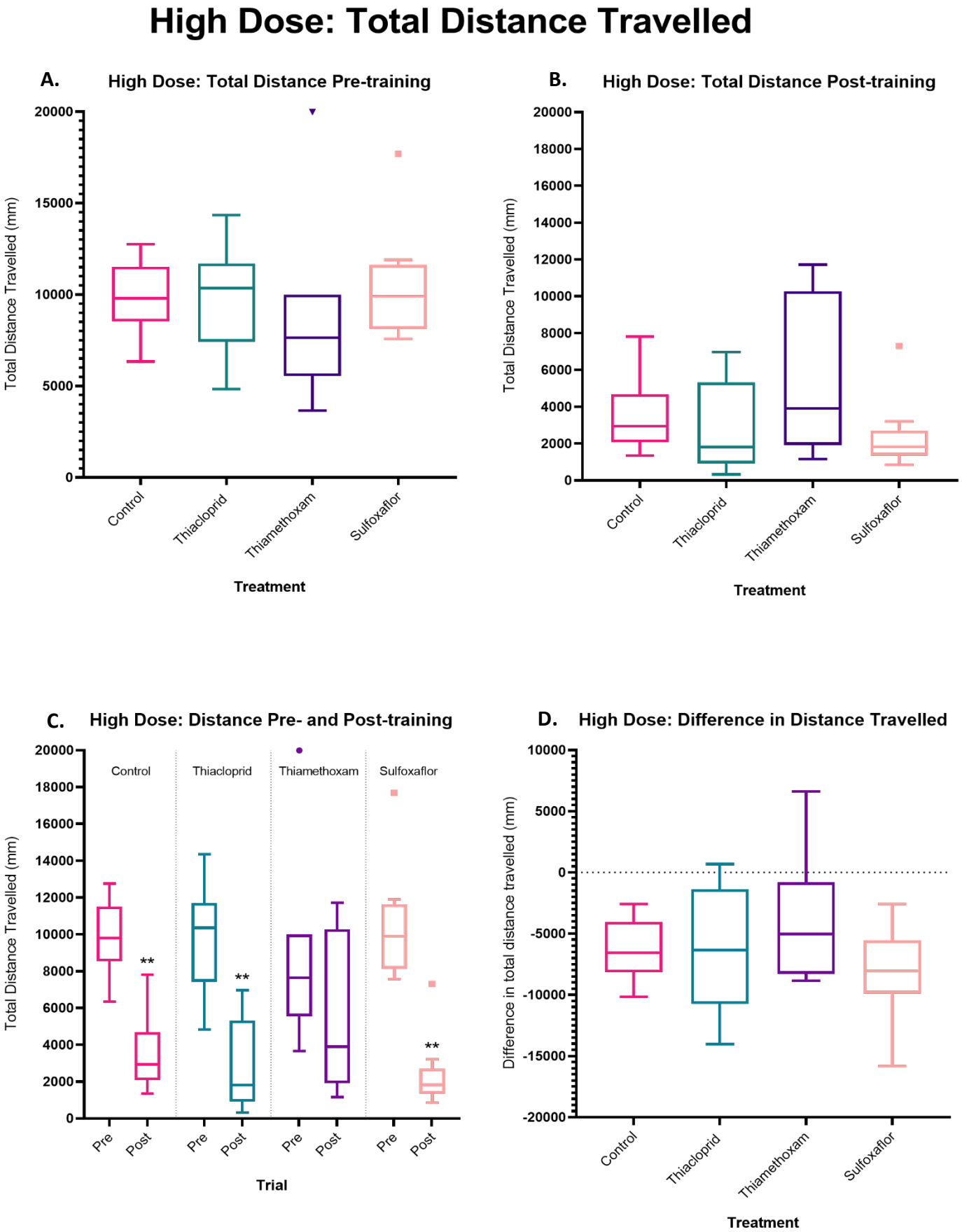

Figure 5.9 (A-D): Total distance travelled pre- and post- training by bees in the high dose trials. A. Total Distance travelled pre-training. B. Total distance travelled post-training. C. Total distance travelled pre- versus post-training. D. Difference in distance travelled (post-training – pre-training value). (Control n = 9, thiacloprid n = 9, thiamethoxam n = 7, sulfoxaflor n = 9).

High dose trials

Average speed

There was a mean fold change in speed of 0.36 for the control group, 0.42 for thiacloprid, 0.82 for thiamethoxam and 0.24 for sulfoxaflor between pre- and post-training trials (Figure 5.10). This indicates that control, thiacloprid and sulfoxaflor bees more than halved their speed by the post-training trial. Although thiamethoxam bees, on average, also decreased their speed post-training, this was by a much smaller fold change than the other groups.

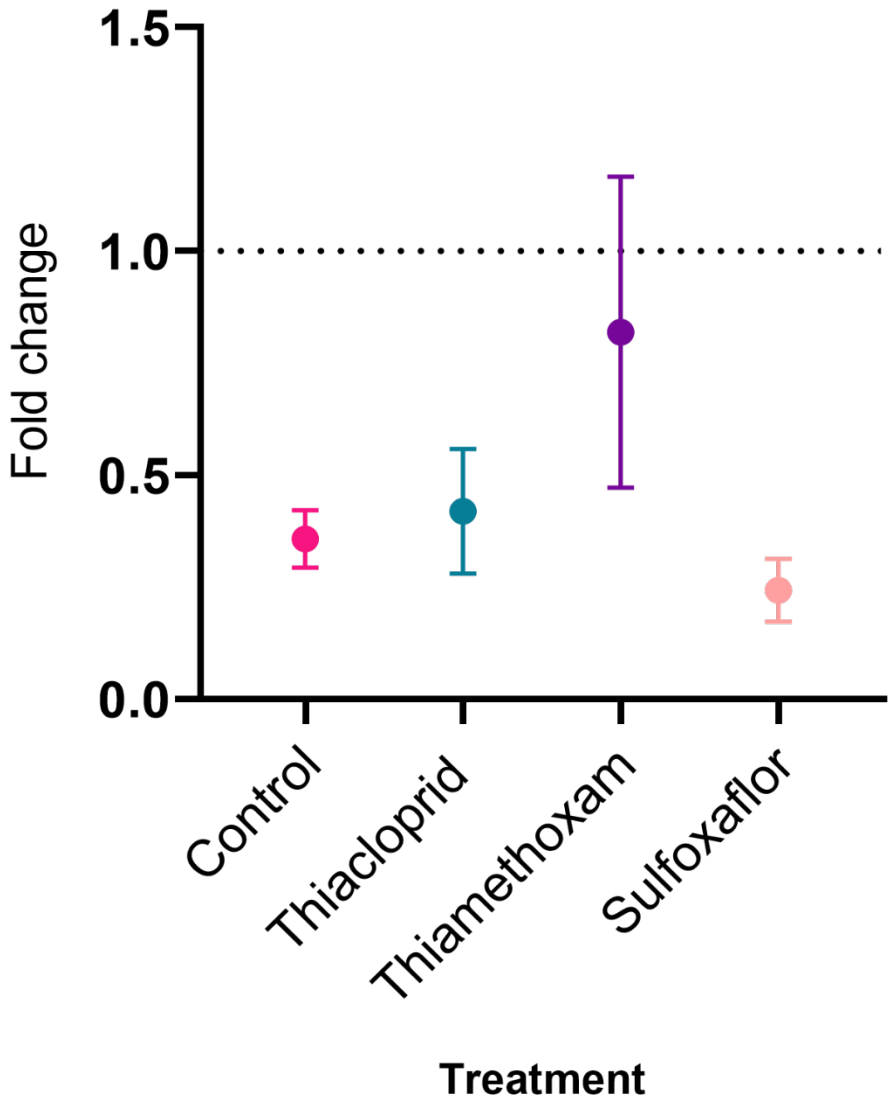

Figure 5.10: Bee speed fold change pre- versus post-training across treatments in the high dose trials (± SEM). (Control n = 9, thiacloprid n = 9, thiamethoxam n = 7, sulfoxaflor n = 9).

5.4 Discussion

Several key findings emerged from this study, all of which have potentially large-scale implications for wild bumblebee foragers.

5.4.1 Dietary consumption of pesticides

Perhaps the most surprising discovery was that dietary thiacloprid has the potential to reduce food consumption by *B. terrestris* foragers. Bees exposed to dietary thiacloprid consumed significantly less syrup in the chronic high dose trials (5000ppb thiacloprid) at both the 3-day and 5-day markers (Figure 5.3). However, in the low dose trials no significant differences in syrup consumption were observed between any of the treatment groups. The maximum field recommended concentration (MFRC) for thiacloprid is reported at 120 ppm (120,000 ppb) (Mommaerts et al., 2010a); here, just 5ppm (5000ppb) of thiacloprid was used in the high dose trials, 1/24 of the MFRC. Although the high-dose exposure rate used is well below the MFRC, it is unlikely that bees would regularly come into contact with a 5ppm dosage of thiacloprid in contaminated pollen and nectar, as residue reports are usually much closer to the low dose trial value of 0.5ppm (Ellis et al., 2017; Ketola et al., 2015; Sanchez-Bayo and Goka, 2014). Nonetheless, we have demonstrated that dietary traces of thiacloprid, at 'worst case field scenario' levels lead to reduced syrup consumption. This could have potentially large-scale detrimental impacts if bumblebee foragers were foraging in a homogenous landscape in which a large area of crop was treated with thiacloprid, perhaps resulting on an individual level, in less food consumption per forager and energetic needs not being met, and on a whole hive level this could result in reduced hive resource collection by foragers. However, it is important to note that agricultural landscapes will vary in their level of heterogeneity dependent on agricultural setting and country. The observed decrease in food consumption by thiacloprid bees within the higher dose trials could represent a repellency effect of the compound, deterring its consumption by foragers. In a heterogenous landscape where alternative foraging options are present (in this experiment foragers were only given the choice of their treatment food and not clean syrup alternatives) this may be beneficial, as it could mean that foragers choose alternative, non-treated food sources. However, repellency seems unlikely as research has suggested zero repellency effects of bumblebees to thiacloprid in field settings (Havstad et al., 2019).

Although the thiamethoxam dose used in the high dose trials here was 1/1000 of the reported MFRC (100ppm), notable mortality was seen in this treatment, with seven of the nine thiamethoxam bees dying prior to day 6 of the trials (one bee died prior to day three, four bees on day five and two on day 6 of the trials. The bumblebee LD_{50} for thiamethoxam is reported as 120ppb and the median sublethal effect concentration (EC_{50}) as 35ppb (Mommaerts et al., 2010), meaning that the 100ppm dose used in these trials should have been sublethal for foragers. However, the fact that so many of the thiamethoxam bees died prior to trial 10 suggests that it was not a sublethal dose for the foragers tested here. We should also not overstate the thiamethoxam findings as it is likely that although appearing visually unaffected and still able to complete trials prior to their death, bees were most likely highly physiologically affected and 'sick', meaning that findings may be as a result of these effects and not necessarily learning and memory deficits. Nonetheless, these findings confirm previous findings that thiamethoxam has potent toxicity at levels far below the recommended MFRC (maximum 100 ppb used in this study). Interestingly, we did not find that thiamethoxam bees consumed significantly less food than any of the other treatments in either the low or high dose trials, even for bees which later died (Figure 5.3). These observations are contradictory to findings reported by Laycock *et al*. (Laycock et al., 2013), who found a significant reduction in consumption of thiamethoxam contaminated syrup and pollen at concentrations between 39 - 98 µg kg^{-1} (equivalent to 98 ppb). Perhaps this is because Laycock *et al.* studied consumption over a longer time period (17 days) and measured average colony, rather than individual forager consumption, not considering significant worker mortality over the 17 days.

5.4.2 Sublethal effects of pesticides

Clear sublethal effects were observed in the foragers exposed to dietary thiamethoxam in both the low and high dose trials. Time spent in reward zone was identified as a key indicator of training in Chapter 4. Although there were no significant inter-treatment comparisons pre or post training for the low dose trials, in the high dose trials thiamethoxam bees spent significantly less time in the reward zone than the sulfoxaflor bees (Figure 5.8). Even more interesting, is when we look at the difference (improvement) between pre- and post-training parameters for individual bees, which is arguably an even better indicator of training. When we compare pre- and post-training 'time spent in the reward zone' for each bee, we see that

in the low dose trials all treatment groups apart from the thiamethoxam bees improved significantly in the time they spent in the reward zone post training (Figure 5.5), indicating that control, sulfoxaflor and thiacloprid bees remained highly capable of responding to the aversive training task. In the high dose trials, this became even more pronounced, with all groups but thiamethoxam treated bees improving in the time spent parameter (Figure 5.8). An inability to respond to aversive conditioning, implied by the lack of improvement in the 'time spent in the reward zone' parameter could have detrimental implications for bees in the field, for example, an inability to make aversive associations.

We examined a further proxy of learning in the thermal-visual arena; total distance travelled by bees within a trial. We would expect that as bees learn a reward location (in this instance the cool reward zone), their routes to and from this reward become optimised, resulting in minimising travelling distances (Lihoreau et al., 2012a, 2012b, 2011; Woodgate et al., 2017). In the low dose trials, as expected, the control and thiacloprid bees significantly reduce the distance they travel pre- versus post-training (Figure 5.6). The sulfoxaflor treatment bees were also very close to the significance threshold at $P = 0.06$, indicating that, although not significant, these bees also reduced the distance they are travelling post-training. The low dose thiamethoxam bees did not significantly reduce the distance they travelled pre- versus post-training (Figure 5.6). Again, this difference becomes even more pronounced in the high dose trials, where all treatment groups (control, thiacloprid and sulfoxaflor) other than thiamethoxam significantly decreased the distance they travelled post-training.

Developing an efficient route between destinations is a common occurrence for foraging bees in the wild, allowing them to minimise travelling costs between foraging locations and nest sites (Lihoreau et al., 2012b, 2012a; Woodgate et al., 2017) and relies on spatial learning and memory (Lihoreau et al., 2012a; Ohashi et al., 2007; Saleh and Chittka, 2007). The suggested inability of the thiamethoxam bees in these trials to develop an efficient route by reducing the distance they travelled to and from the cool reward zone, and thus reduce the overall distance travelled in the post training trials, has potentially concerning field implications. Foraging bees which are unable to streamline their routes presumably have greater energy expenditures and feeding requirements than bees which can minimise route travel. Previous studies in honeybees have observed hyperactivity in response to acute thiamethoxam exposure (Tosi and Nieh, 2017). Jacob *et al.* (2019) found that stingless bees (*Tetregonisca*

angustula) increased the distance they travelled by fivefold and Tosi *et al.* (2017) noted that honeybees increased flight duration (+78%) and distance (+72%) (on a flight mill) in response to acute thiamethoxam exposure. However, different effects are seen under chronic exposure, with Tosi *et al.* (2017) finding that honeybees significantly decreased their flight duration (-54%) and distance (-56%). We can equate the chronic exposure period in the Tosi et al. (2017) study (1-2 days of continual exposure) to the pre-training trials of our study (after 3 days of exposure). Pre-training, in the low dose trials, we also see a reduction in distance travelled (walking not flight), with thiamethoxam bees travelling significantly less distance than control bees (P = 0.027*). This finding, paired with the thiamethoxam bees' inability to streamline their navigational routes (minimise distance travelled pre- versus post- training), suggests that chronic thiamethoxam exposure could drastically affect bumblebee foraging behaviours in the field.

One way to further examine this notion of hyperactivity under thiamethoxam exposure is to consider the speed fold change bees exhibit pre- versus post-training. In the low dose trials the control and thiacloprid bees almost halve their speed between pre- and post-training trials, and sulfoxaflor treated bees also reduce their post-training speed by around 25% (Figure 5.7). However, the thiamethoxam bees increased their speed post-training by over 25% (Figure 5.7). These results support previous findings of hyper-activity induced by thiamethoxam exposure (Jacob et al., 2019), suggesting they may be maintained in the longer term. In the high dose trials, we see a similar pattern, with control, thiacloprid and sulfoxaflor bees more than halving their speed post-training compared to their pre-training values (Figure 5.10). On average, in the higher dose trials thiamethoxam bees did decrease their speed post-training (possibly indicating that they are less fit and healthy), however, this was by a much smaller amount than the other treatment groups (Figure 5.10). The high dose trajectory graphs (Figure 5.4 E-G) provide a visual representation of this hyper-activity in the thiamethoxam bees compared to the other treatment groups, with bees following comparably widely varied routes. As with increased distance travelled, there are potentially costly implications to this hyper-activity for forager energy expenditure.

The dosages selected for each pesticide compound were, in the case of the low-dose trials, highly field realistic and for the high-dose trials, still possible 'worst-case scenario' concentrations, making these thiamethoxam sublethal effect findings concerning, but not

unsurprising given existing literature (Baron et al., 2017; Friol et al., 2017; Grillone et al., 2017; Henry et al., 2012; Laycock et al., 2014; Tavares et al., 2017, 2015; Tesovnik et al., 2020, 2017; Tosi et al., 2017; Tosi and Nieh, 2017). However, no significant differences were noted for sulfoxaflor across any of the parameters we studied. This is promising for the potential future use of sulfoxaflor as a replacement compound to the neonicotinoids. Sulfoxaflor bees demonstrated marked improvement in the time spent, distance and speed parameters studied, demonstrating very similar patterns to control bees across all areas. This suggests that the sulfoxaflor bees were highly capable of completing the aversive conditioning task presented by the thermal-visual arena. This is perhaps supported by previous findings of no detrimental effects of sulfoxaflor on olfactory learning or memory (Siviter et al., 2019). Dietary exposure to sulfoxaflor in this study also did not alter feeding regimes. However, it should be noted that no reproductive effects were measured here, and it is these which have previously had detrimental effects reported (Siviter et al., 2018). There is clearly a lack of research into potential sublethal effects of sulfoxaflor exposure, as bee studies to date can be counted on one hand. There is a need to urgently rectify this deficit if this compound has the potential to become as widespread in its usage as the neonicotinoids.

As of the beginning of this research, the neonicotinoids acetamiprid and thiacloprid were still licensed for usage by home gardeners (acetamiprid) and growers (thiacloprid) in the EU. However, the EU renewal license for thiacloprid has now not been approved, meaning that the license will expire in August 2020 (European Commission, 2019). The grounds for this non-renewal were stated as “risk assessments for…birds and mammals…potential human metabolites…residues in food…residues in surface water…aquatic organisms…bees (and) terrestrial non-target plants could not be finalized” (European Commission, 2019). Although this cautious approach to non-target effects should be praised, this is clear testament to the lack of evidence of non-targets effects of thiacloprid. The EU’s caution on neonicotinoids is certainly not however being matched on a global scale where, outside of Europe, the three banned neonicotinoid compounds (thiamethoxam, clothianidin, imidacloprid) remain the world’s most widely used pesticides (Rowe, 2019). In 2018 the Trump administration in the USA overturned a previous ban on neonicotinoids, allowing the continued use of clothianidin, thiamethoxam, acetamiprid, dinotefuran and imidacloprid (Environmental Protection Agency, 2020). Outside of the EU, the attitude to neonicotinoid usage very much remains a

free for all, with 35% of the world having no pesticide legislation at all (Rowe, 2019). It is important not to take a European-centric view, that these compounds are banned here and therefore no longer an issue. Most of the world is still using these chemistries and we cannot forget that over 50% of the UK's food supply is imported, with 11% coming from non-EU countries (House of Lords, 2018). This is an issue which is still affecting large portions of the world and, despite the EU bans, Europe is certainly not exempt from that.

Here we have developed further "tier 1" (laboratory tests on individual insects) and "tier 2" (involving extended laboratory tests which include the evaluation of pesticides impacts on key processes such as worker survival, reproduction and behaviour) studies as suggested by Mommaerts and Smagghe (2011), to improve the gaps in the assessment of sublethal pesticide impacts on bumblebee species. However, there is a clear, continuing need for further research into sublethal effects of neonicotinoids, as well as newly emerging replacement compounds, across a wide variety of bee species, in order that the evidence can be fully presented, and accurate risk assessments finalized.

Chapter 6

Demonstrating the existence of a speed-curvature power law in *Bombus terrestris* locomotion patterns

Chapter 6: Demonstrating the existence of a speed-curvature power law in *Bombus terrestris* locomotion patterns.

Preliminary introduction

The following Chapter is based on a paper submitted to *PLoS ONE* in June 2019 for which I was the first author. The paper was accepted in November 2019 and published in January 2020. The final publication is available at: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0226393 (manuscript).

The Chapter is presented in the style of *PLoS ONE* and is identical to the published manuscript with the following exceptions. A broader general introduction has been added to the front of the manuscript to improve the clarity of the work and sections, figures and tables have been renumbered in accordance with their position in the final thesis.

Statement of contribution

As primary author I was responsible for the experimentation, methodologies, data acquisition, data analyses and writing of the original draft of the manuscript. My supervisors, Dr T. G. Emyr Davies and Dr Ka S. Lim conceived the concept of the thermal-visual arena, which was then built by Dr Ka S. Lim. Co-author Dr Andrew Reynolds conducted the power law analyses, validation and visualisation and assisted with the writing of the corresponding methods. Three anonymous reviewers provided comments on the manuscript during the *PLoS ONE* review process. Rebecca Reid provided advice and assistance with experimental design and testing.

Abstract

We report the discovery that *Bombus terrestris audax* (Buff-tailed bumblebee) locomotor trajectories adhere to a speed-curvature power law relationship which has previously been found in humans, non-human primates and *Drosophila* larval trajectories. No previous study has reported such a finding in adult insect locomotion. We used behavioural tracking to study walking *B. terrestris* in an arena under different training environments. Trajectories analysed from this tracking show the speed-curvature power law holds robustly at the population level, displaying an exponent close to two-thirds. This exponent corroborates previous findings in human movement patterns but differs from the three-quarter exponent reported for *Drosophila* larval locomotion. There are conflicting hypotheses for the principal origin of these speed-curvature laws, ranging from the role of central planning to kinematic and muscular skeletal constraints. Our findings substantiate the latter idea that dynamic power-law effects are robust, differing only through kinematic constraints due to locomotive method. Our research supports the notion that these laws are present in a greater range of species than previously thought, even in the bumblebee. Such power laws may provide optimal behavioural templates for organisms, delivering a potential analytical tool to study deviations from this template. Our results suggest that curvature and angular speed are constrained geometrically, and independently of the muscles and nerves of the performing body.

6.1 Introduction

6.1.1 General Introduction

Animal movements are being studied in novel and exciting ways in the emerging field of movement ecology (reviewed in Nathan & Giuggioli, 2013). Automated tracking of animal movements has made a large contribution to this field and reduced the need for continual, direct observation of subjects over long time periods (Block et al., 2011; Costa et al., 2012). Movement data can be used to garner information about a wide array of species traits, such as behaviour, interactions with individuals (conspecifics and other animals) and landscapes, migration and dispersal (Breed et al., 2015, 2013; Fagan et al., 2013; Potts et al., 2013). A better understanding of animal movement patterns over a range of spatial scales can allow a better understanding of complex ecological systems and will only increase in importance in conservation strategies as human populations expand and new environmental stressors emerge (Reynolds & Rhodes, 2009).

The characterisation of animal movement patterns remains contentious. Historically, Brownian motion was the primary model used to describe stochastic animal movements (Kareiva, 1983; Kareiva and Shigesada, 1983; Knight, 1962; Skellam, 1951). However, this conceptual model has been challenged in recent years, by a conflicting model which suggests animals follow alternative movement patterns; Lévy flights or Lévy walks (Edwards et al., 2007; Reynolds & Rhodes, 2009; Reynolds & Ouellette, 2016; Reynolds, 2018; Viswanathan et al., 1996). It is posited that these Lévy movements may optimise search efficiency for animals (Viswanathan et al., 2000) and may therefore have evolved as the result of natural selection processes (de Jager et al., 2011). These Lévy flight models use statistical distributions with power-law tails and have been shown to be superior to the model of Brownian motion in their description of animal searching patterns (Viswanathan et al., 1999).

Power laws are said to be ‘scale-free’ i.e. both short and long values can occur, and no scale is more frequent (dominant) than another (Mashanova et al., 2010). Increasingly, power law distributions have been found in the movement patterns of a wide range of animals (Breed et al., 2015; Gomez-Marin et al., 2016; Mashanova et al., 2010; Sims et al., 2008; Zago et al., 2017), further supporting the presence of Lévy movements. Understanding the presence of power law distributions in a wider range of species will be beneficial to conservation and

prediction efforts, allowing the study of how a species' movement patterns may be altered in response to stressors or environmental changes.

6.1.2 Manuscript Introduction

At any point along a curve there is a unique circle or line which most closely approximates the curve near that location. The radius of that circle defines the 'radius of curvature', R, whilst curvature, C, is defined to be its reciprocal, 1/R. According to this definition, it can be expected that straight lines will have zero curvature, and for a given observer at a fixed scale large circles will have small curvature and small circles will have high curvature. Curvature along with angular speed, A, has been used to quantify human writing signatures (Lacquaniti et al., 1983).

Remarkably the human signature, a powerful individual identifier, adheres to a speed-curvature power law (Lacquaniti et al., 1983). The speed-curvature, or two-thirds, power law dictates that the instantaneous angular speed of movements vary proportionally to two-thirds power of their curvature (Lacquaniti et al., 1983). According to the law, movements under high curvature tend to slow down, whereas movements under low curvature speed up (Gribble and Ostry, 1996). The law is given by:

$$A = kC^{2/3} \quad (1)$$

where k is a constant of proportionality.

Maximally-smooth movements, which minimize rates of change of acceleration (i.e., jerks and jolts), are generated under the two-thirds power law (Flash and Hochner, 2005; Gomez-Marin et al., 2016; Wann et al., 1988), which holds true across a range of voluntary human movements, including drawing, walking and pursuit eye movements (de'Sperati and Viviani, 1997; Ivanenko et al., 2002; Lacquaniti et al., 1983; Wann et al., 1988). The law also holds true across a diverse range of taxa. The law has been observed in the motor cortical control of Rhesus monkey hand movements whilst drawing (Schwartz, 1994), and even in the larval movement of the fruit fly (*Drosophila melanogaster*)(Gomez-Marin et al., 2016) albeit with a marginally different power-law exponent, three quarters rather than two thirds.

The principal origins of this speed-curvature power law are contentious. One hypothesis suggests that the law results from central planning constraints imposed by the nervous system (Schwartz, 1994; Viviani and Flash, 1995). Another, that the law arises due to physiological constraints conferred by muscular properties and kinematics (Gomez-Marin et al., 2016; Paul L Gribble and Ostry, 1996; Viviani and Cenzato, 1985). A further view, that the law exists to maximize movement smoothness and minimize jerk (Viviani and Flash, 1995; Wann et al., 1988). Identifying the generative mechanism holds the key to understanding the statistical law, the occurrence of which is remarkable given that behaviours are shaped by individual psyches and by complex social and environmental interactions. It's identification may help to elucidate how other statistical regularities can occur within the complex movement patterns that arise in nature (Barabasi, 2005; González et al., 2008; Nakamura et al., 2007; Proekt et al., 2012; Song et al., 2010; Viswanathan, 2011). Progress towards identifying the underlying mechanism can be made by determining the pervasiveness of the two-thirds law, and by establishing whether or not it occurs in other modes of locomotion.

Given that the locomotive patterns of *Bombus terrestris*, and indeed animal organisms, are probably shaped by their motivational states and by environmental factors, a seemingly natural null hypothesis would be that individuals have unique locomotive patterns and that statistical regularities are absent or trivial (for example, a tendency to move forwards with near constant speed). Therefore, to determine the pervasiveness of the law, we must first determine whether the speed-curvature power law persists in the walking trajectories of the bumblebee at all and, if it does, whether the law differs depending on a bee's environment. We must then determine whether the exponent of the law adheres closely to the two thirds exponent. Finally, it is necessary to also assess whether the power law is the best mathematical descriptor of walking bumblebee trajectories or whether an alternative better describes the relationship.

Walking is distinctly different from the crawling movements made by limbless larvae (Tanaka et al., 2012). Therefore, we might predict that walking bee trajectories would adhere more closely to the two-thirds power law exponent reported for unconstrained movements such as human drawing and walking (Ivanenko et al., 2002; Lacquaniti et al., 1983), than the three-quarters exponent reported for the mechanically constrained movements of larvae (Gomez-Marin et al., 2016).

To the best of our knowledge the speed-curvature power law has not been studied in any other invertebrate other than *Drosophila melanogaster* larvae (Gomez-Marin et al., 2016) and never in the final, adult stage of an insect. Here, we report that *B. terrestris audax*, a social bumblebee species with a complex behavioural repertoire, displays a two-thirds speed-curvature power law whilst walking in an arena, under differing environments.

6.2 Methods

6.2.1 Bee subjects

All subjects were *B. terrestris audax* from research hives obtained from Biobest Belgium NV (Westerlo, Belgium). Colonies were settled in wooden nest boxes (29 x 21 x 16 cm) and provided with Biogluc (Biobest Belgium NV, Westerlo, Belgium) in two gravity feeders in a Perspex foraging tunnel (26 ×4×4 cm) connected to the nest box. Pollen was also provided in baskets in the Perspex tunnel. Gravity feeders and pollen were replenished, as necessary, to ensure a consistent supply of food to the colony. Newly emerged individuals were marked in colour groups by age cohort with coloured plastic bee marking tags (EH Thorne Ltd, Market Rasen, UK) superglued to the top of the thorax. This allows tracking of an individual's age. All individuals used in a single trial were one-week post-emergence (to allow bees to begin foraging and to be monitored) and of the same age cohort. The hive was observed each day and foragers of each age cohort were identified in the foraging tube by their colour and number. From the foragers recorded in each age cohort ten individuals were randomly selected to be tested per trial. The selected individuals are then randomly allocated to either the treatment or control groups for each trial. Trials were replicated three times; all treatments replicated three times across three different hives.

6.2.2 The experimental arena

Experiments were conducted within a thermal-visual arena (Figure 6.1 a-d), similar to a platform previously used for *Drosophila* tracking (Ofstad et al., 2011). The arena enables the creation of controlled, but naturalistic, environments. A Peltier array of 64 2.5x2.5 cm individually controllable thermoelectric Peltier elements, arranged in an 8x8 grid, facilitates control of the arena's floor temperature. The arena's floor is covered in white masking tape to create an inconspicuous, featureless surface which can be easily cleaned and replaced

between trials to prevent the use of scent marks by foragers to locate arena rewards. In the training trials, visual patterns were adhered to the surface of the arena's walls to create a visual landscape consisting of repeating patterns of stars, dots, horizontal and vertical bars, denoting the four quadrants of the arena's circumference. Light-emitting diodes (LEDs) (colour temperature 6500K) around the top edge of the arena were used to light the arena consistently above the bee flicker fusion frequency (Inger et al., 2014) (Figure 6.1c). The arena was kept in a controlled environment room at 22^0 C with a day: night cycle of 16:8 hr.

6.2.3 Training environments

The task required forager bees to use visual landscape patterns to locate a reward zone within the arena, in response to four training environments: 1) control environment with no reward or punishment, 2) appetitive reward environment (0.02ml 50% sucrose solution in reward zone), 3) aversive punishment environment (heated arena floor (45°C), cool (25°C) reward zone) and 4) combined aversive and appetitive environment (heated arena floor (45°C), 0.02ml 50% sucrose solution in cool (25°C) reward zone). All rewards (cool zone or sucrose) were inconspicuous and not visually distinguishable from any other tiles on the arena floor.

6.2.4 Training regime

None of the test subjects had experience of the thermal-visual arena prior to the training trials. Each bee was given ten trials in the arena (each trial was of three minutes duration) spaced across three days. Spaced conditioning, in which temporal spacing exists between successive conditioning trials, has been shown to lead to higher memory consolidation in bees, especially at long intervals (Menzel et al., 2001). When placed into the thermal-visual arena, bees were confined under a clear plastic tube for one minute prior to the trial start, to allow orientation within the arena. The tube was then removed, and the three-minute trial started. All bees were starved for one hour prior to trial start to motivate individuals in the appetitive condition and to remove starvation as a confounding variable between treatments. Bees were confined to individual cages in-between trials to prevent further foraging experience not in the arena and standardise the amount of foraging experience in the arena each bee received. Cages were placed next to each other and adjacent to the hive to allow visual and olfactory communication between hive members.

6.2.5 Trajectory tracking

To facilitate 2D trajectory tracking, foragers were confined to walking on the test platform by wing clipping. Selected foragers' wings were clipped using a queen marking cage and dissection scissors (EH Thorne Ltd, Market Rasen, UK).

Individual bee trajectories were filmed using a camera (FLIR C2 Infrared Camera) attached to a tripod above the arena (Fig 1b). Video recording was at four frames per second for ten, three-minute trials per bee. Video files were tracked using CTRAX: the Caltech Multiple Walking Fly Tracker software (Branson et al., 2009). The raw centroid tracking data files outputted by CTRAX were then used for speed-curvature power law calculation.

6.2.6 Speed-curvature power law calculation

For the data analysis, the x, y coordinates and corresponding timestamps for whole trajectories, for individual bees, from the centroid tracking were used to compute angular speed A(t) and curvature C(t) using standard differential geometry(Wikipedia, n.d.). Velocities were calculated from consecutive, regularly timed, positional fixes, $\dot{x} = \frac{x(t+\Delta t) - x(t)}{\Delta t}$and $\dot{y} = \frac{y(t+\Delta t) - y(t)}{\Delta t}$where $\Delta t = 0.2\ s$is the time interval between consecutive recordings. Accelerations $\ddot{x}$and $\ddot{y}$ were calculated in a directly analogous way from consecutive velocities. Together these quantities determine the 'radius of curvature'[36],

$$R = \left| \frac{\left(\dot{x}^2 + \dot{y}^2\right)^{3/2}}{\dot{x}\ddot{y} - \dot{y}\ddot{x}} \right| \quad (2)$$

which in turn gives the angular speed,

$$A = \left(\dot{x}^2 + \dot{y}^2\right)^{1/2} / R \quad (3)$$

and the curvature,

$$C = 1/R \quad (4)$$

6.2.7 Data selection

Whole trajectories were analysed, with data selected so that only individual bee tracks which had greater than 50 data points (n = >50) were used for analyses (for all other tracks n = between 66 and 1047). Excluded bees: n = 14. Bees used for analysis, n = 45. When we removed all bees with under 100 data points the outcomes of our analyses did not change and therefore, we can consider selection at 50 data points to be robust and there was no need to exclude further bees. Data were not filtered (smoothed) prior to processing. Filtering does not affect the outcomes of our analyses (see Chapter 6 Appendix).

6.2.8 Statistical analysis

The hallmark of a power-law relationship between curvature, C, and angular speed, A, is a straight-line relationship between log(C) and log (A). Taking the logarithm of both sides of the two-thirds power-law rule gives the linear relationship log A = log K + beta log C, with β=2/3. Here, following Zago et al. (2017) we looked for such relationships by least squares linear regression of log(C) and log(A). Using this method, we estimated the exponent, β, and the variance, r^2, accounted for by the power-law.

The power-law scaling demonstrated by our analysis extends over two or more scales of magnitude. This fulfils Stumpf and Porter's (2012) 'rule of thumb'; after critically appraising power laws identified in biological systems, they suggested that a candidate power law probability frequency distribution should apply over at least two orders of magnitude along both axes and should be explainable by a viable mechanism.

We then went beyond previous analyses (Gomez-Marin et al., 2016; Huh and Sejnowski, 2015) by comparing our observations with strongly competing functions that resemble power-laws but are not underpinned mechanistically. The power-law relationship between curvature and angular speed cannot, of course, extend to arbitrarily large curvatures and angular speeds because of physiological constraints that place limits on the tightness of turning and on the speed that can be attained by an individual. Departures from power-law are expected when the maximum curvatures and speeds are approached by an individual. Here we examine this by fitting our data to two functions that resemble power-laws over a range of scales, but

which depart from power-laws when curvatures and speeds are sufficiently high. These functions are stretched exponentials (which include exponentials as a special case),

$$A = a.\exp\left(bC^{p}\right)$$

and log-normal like functions,

$$A = a.\exp\left(b\left(\ln C - \ln d\right)^{2}\right)$$

where a, b, p and d are free parameters that are determined by fitting the functions to our data. The relative merits of the power-law, stretched exponential and log-normal functions as representations of our data were determined using the Akaike information criterion (Spanos, 2007).

The stretched exponential and the log-normal like functions can be considered as strongly competing descriptions of our data that contain three rather than two free parameters. This extra flexibility could result in better fits to our data. Functions were fitted to individuals' movement patterns, rather than to pooled data as we sought to capture an individual's constraints. We then compared the pooled data with functions parameterized in terms of the average best fit parameters.

Stretched exponentials (typically with p~0.007) provided good fits to our data, but better fits are obtained with power-laws. Even better fits were obtained with the log-normal like functions which is not surprising given that they are more flexible than simple power-laws (Fig 3a-d). In all cases, the Akaike weights for the log-normal like functions are 1.00 which indicates that the log-normal like functions are convincingly favoured over the power-law and stretched exponential functions. However, as is often the case, the better fit of the complex model (the log-normal like function) trades off with the elegance and clarity of the simpler model (the power-law function). The log-normal functions are, however, convex with maxima at lnC=lnd. Such maxima are not evident in our observations and consequently the estimates for lnd (approximately 35) were much larger than $\ln C_{max}$ (approximately ten). This implies that the fitted log-normal like functions are effectively fits to power-laws because when lnd are much larger than $\ln C_{max}$

$$A \approx a.\exp\left(-2b\ln(d)\ln(C) + b\ln(d)^2\right)$$
$$= kC^{\beta}$$

where $k = a.\exp\left(b\ln(d)^2\right)$ and $\beta = -2b\ln(d)$.

Our mean estimates for the power-law exponents; 0.59 (controls, n = 14, range 0.42 – 0.87), 0.61 (appetitive + aversive, n = 12, range 0.43 – 0.87), 0.60 (aversive, n = 7, range 0.49 – 0.94) and 0.57 (appetitive, n = 12, range 0.44 – 0.8) are broadly consistent with the two-thirds power-law rule. We have therefore arrived at this law using two different approaches; by fitting our data to power-laws and by fitting our data to log-normal functions.

Statistically significant differences between the power exponents (β) of treatment groups and expected exponent values of two thirds (0.66) and three quarters (0.75) were calculated using non-parametric tests (Kruskal-Wallis ANOVA by ranks), as data were not normally distributed (Shapiro-Wilk test, p value = 0.000587518***). Kruskal-Wallis tests were conducted in RStudio (Version 1.0.44 – © 2009-2016 RStudio, Inc.). Summary boxplot, Figure 6.4 was produced in RStudio using the 'ggplot' package.

6.3 Results

6.3.1 Varying exploratory strategies

To facilitate the creation of different walking trajectories, bees were tested across differing training environments within a thermal-visual arena (Fig 6.1). Training environments differed in the reward or incentive provided to foragers, providing either no reward or punishment (control), an appetitive sucrose reward, an aversive punishment (heated arena floor) or a combined aversive punishment and appetitive reward environment. Each bee was given ten training trials, experiencing only one of the training environments across all ten trials. In each training trial bees were required to use visual landscape patterns, around the circumference of the arena, to locate the appropriate reward zone (refer to 'training environments' in methods section for further details).

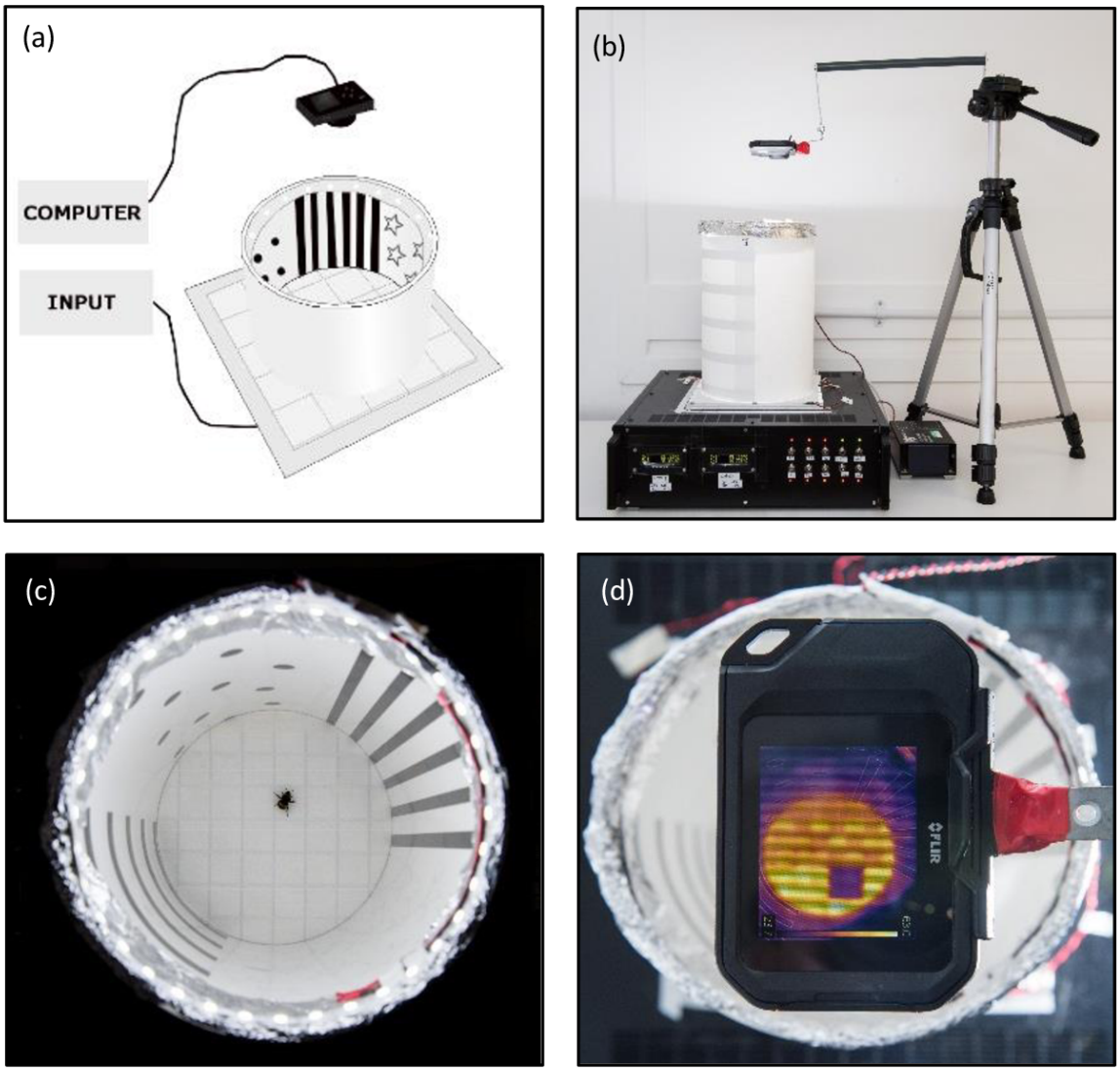

Figure 6.1 (a-d) The thermal-visual arena: (a) Diagrammatic representation of the thermal-visual arena. (b) The arena in-situ in the lab. (c) A Bombus terrestris forager completing a training trial. (d) Thermal positioned above the arena showing the inconspicuous cool reward zone.

In all environmental conditions, bees traced complex trajectories (Figure 6.2 panels a, b, c, d). In each case curvature is seen to occur across a broad range of scales, as evidenced by the presence of nearly straight-line movements with low curvature and the presence of tight turns with high curvature. Across differing environments bees appeared to display varying exploratory trajectories. Individuals tested in the control condition often traced concentric paths, delineating the boundary of the arena (Figure 6.2). Individuals in the aversive condition located and remained in the cool reward zone for extended periods, making directed

exploratory trajectories to a section of the arena's edge (Figure 6.2b). Similar trajectories were seen for individuals in the combined aversive and appetitive environment where both a sucrose and cool zone reward were given in the same location (Figure 6.2c). In the appetitive reward environment individual's trajectories were more varied, not being constrained to particular routes (Figure 6.2d).

Individual bees' trajectories may be governed in part by differing motivations in response to differing training stimuli. When provided with no training stimuli there is no motivation for foragers to complete any task other than escape, resulting in delineating pathways (control group, Figure 6.2a). Training appears to be most effective in the aversive (Figure 6.2b) and combined aversive and appetitive (Figure 6.2c) conditions as foragers are increasingly motivated to take direct paths to and from the reward zone. Nonetheless, these complex, highly unique pathways all have statistical regularities characterised by a simple power law, which holds true irrespective of motivational environment or training regime.

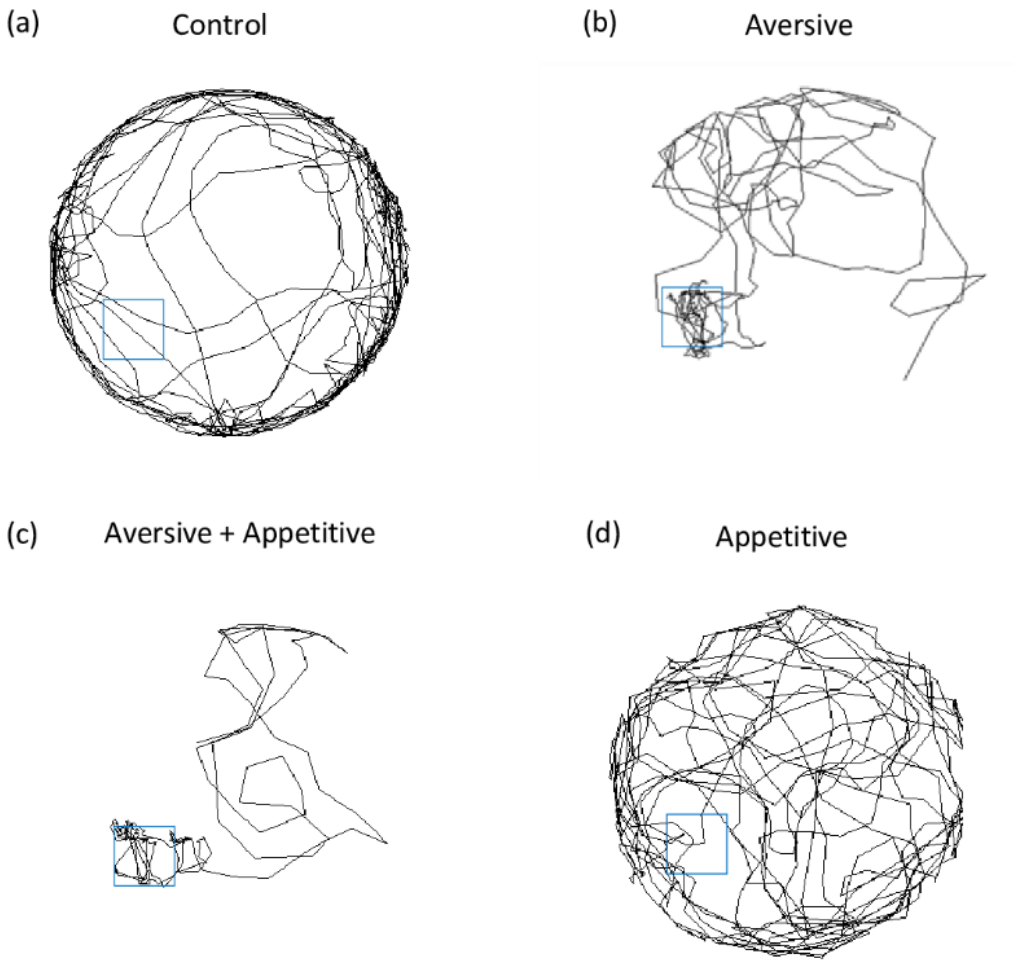

Figure 6.2 (a-d). Trajectories of representative bees from the control (a), aversive (b), appetitive (c) and combined aversive and appetitive conditions (d). The blue squares indicate the location of the reward zone (specific to condition) in the arena environment. Bees appear to implement differing exploratory strategies, dependent on the reward or punishment environment they are in. In the control condition (a), individuals often trace concentric paths which delineate the arena boundary. In the aversive condition (b), with a heated floor, individuals were motivated to locate and remain in the cool reward zone. Therefore, trajectories often showed directed exploratory paths out from the reward zone to a facet of the arena. Similar directed trajectories are seen for individuals in the combined aversive and appetitive condition (d). This is not surprising as this is the condition which should provide foragers with the most motivation to remain in the reward zone, with two rewards (sucrose and cool zone) and a punishment in the form of the heated arena floor. Individuals in the appetitive reward environment (c) often tracked more varied paths, not constrained to set routes or areas of the arena.

6.3.2 The speed-curvature relationship

A power-law relationship between curvature, C, and angular speed, S, (C=aS^b) will manifest itself as a straight-line (log A = log K + beta log C) on a log-log plot. We tested for such a straight-line relationship by linearly regressing log C on log S for each bee within each environmental condition (Figure 6.3 a, b, c, d). The average (mean) estimates for the power-law exponents for bees in trial 1 (pre-training) are 0.59 (controls, n = 14, range 0.42 – 0.87), 0.61 (appetitive + aversive, n = 12, range 0.43 – 0.87), 0.60 (aversive, n = 7, range 0.49 – 0.94) and 0.57 (appetitive, n = 12, range 0.44 – 0.8).

The suitability of the power law to describe our data was tested against two competing statistical relationships; stretched exponentials and log-normal like functions (Figure 6.3 a, b, c, d) (see 'statistical analysis' methods section for further details). Power laws provide better fits than stretched exponentials, and although good fits are obtained with log-normal functions, they are consistent with the two-thirds power law rule, making the simpler, more elegant power law model the best choice.

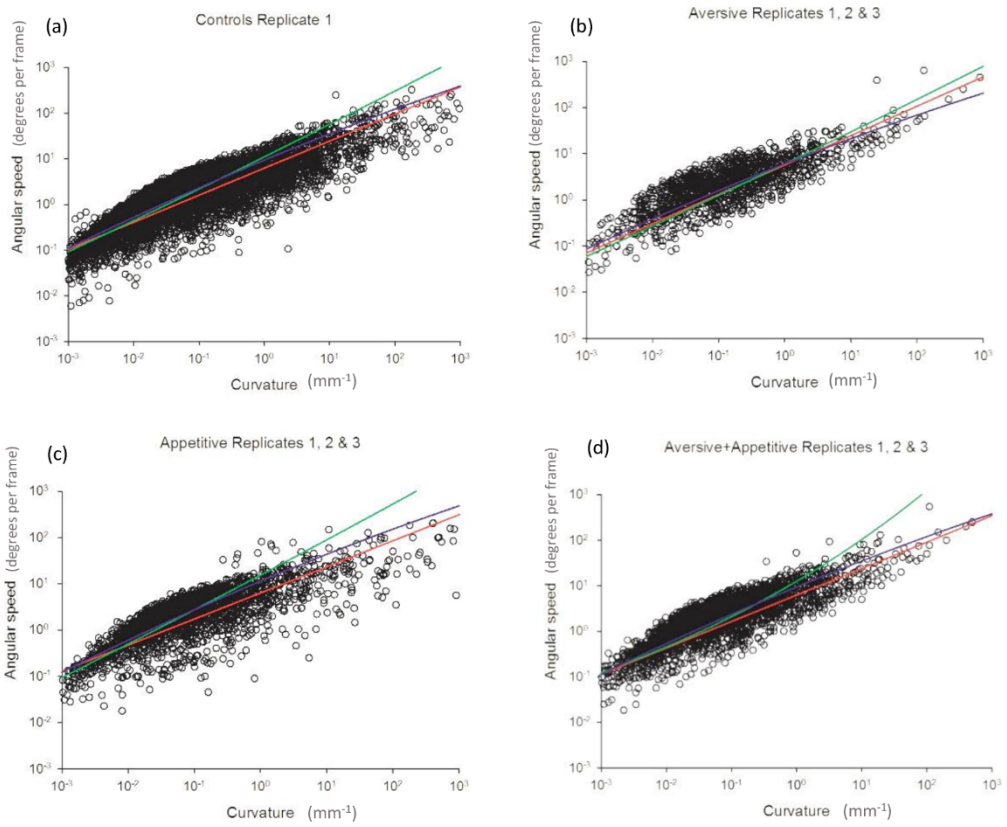

Figure 6.3 (a-d). The relationship between angular speed and curvature in walking bee trajectories. The two-thirds power law holds true in walking bees across differing environments (control (a), aversive (b), appetitive (c) and combined aversive+ appetitive (d). (a) Scatter plot of instantaneous angular speed plotted against local path curvature at a population level on a log-log scale, for all individuals in the control group. All data points (n = 12224) were sampled at equal time intervals along the trajectories of 14 individual bees. Data was fitted to the power function A(t) = kC(t)2/3 (red line), to stretched exponentials (green line) and log-normal (blue line) functions. Stretched exponentials and log-normals can resemble power-laws and are strongly competing models of the data. (b) Log-log plot of angular speed versus curvature for 7 bees in the aversive group (n = 1081). (c) Log-log plot of angular speed versus curvature for 12 bees in the appetitive group (n = 1835). (d) Log-log plot of angular speed versus curvature for 12 bees in the combined aversive + appetitive group (n = 2309).

6.3.3 Adherence to a power law across environments

Adherence to the law did not depend on the environment an individual forager was exposed to (see Fig 3 a-d) and the distribution of power exponents did not differ significantly between treatments (including controls) (Kruskal-Wallis ANOVA by ranks, chi-squared = 0.62489, df = 3, p-value = 0.8907 (>0.05)). As would be expected, all treatment exponents were significantly different from zero (Kruskal-Wallis ANOVA by ranks, chi-squared = 32.321, df = 4, p = 1.645e-06**** (<0.00001)).

6.3.4 Two-thirds or three-quarters?

To determine whether bees' trajectories adhered more closely to the two-thirds or the three-quarters power law exponent, treatments were tested for significance against populations with assumed power exponents of 0.66 and 0.75.

Treatment populations were highly significantly different from the three-quarters power law exponent (0.75) (Kruskal-Wallis ANOVA by ranks, chi-squared = 17.79, df = 4, p-value = 0.001356** (<0.05)).

However, treatment populations were not found to be significantly different from the two-thirds power law (0.66) (Kruskal-Wallis ANOVA by ranks, chi-squared = 6.0816, df = 4, p-value = 0.1931 (>0.05)). However, Figure 6.4 shows that although treatment groups did not differ significantly from 0.66, the medians of treatment groups vary around a 0.55 power exponent line. Populations were found to not significantly differ from this 0.55 power exponent either (Kruskal-Wallis ANOVA by ranks, chi-squared = 1.7447, df = 4, p-value = 0.7826 (>0.7826).

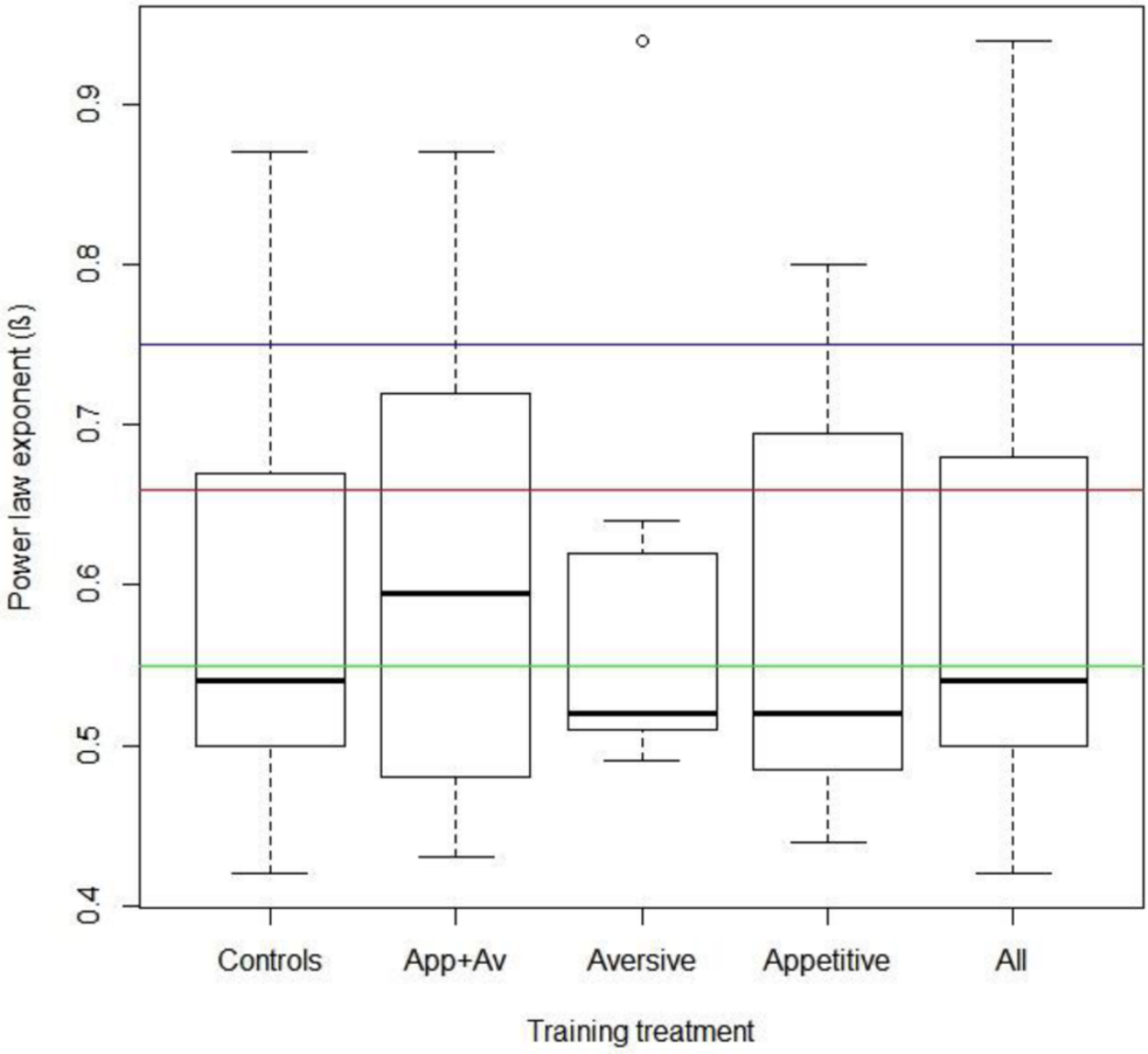

Figure 6.4: ß-exponent of bees in the control (n=14), aversive (n=7), appetitive (n=12) and aversive + appetitive (n=12) (post data filtering) and individuals from all conditions combined. 99% of all data lies within the boxplot whiskers (outliers represented as dots). The two-thirds power exponent (0.66) is represented by the red line. The three-quarters exponent (0.75) by the blue line and a new predicted exponent of 0.55 by the green line. Although treatment groups did not differ significantly from the two thirds exponent (Kruskal-Wallis analysis), when visualised, it is clear that median ß-exponent values vary around a 0.55 power exponent value, suggesting that an exponent range of 0.5 to 0.66 best describes the exponents of our walking bees.

6.4 Discussion

Locomotive patterns are frequently complex but do, nonetheless, have surprising regularities (primitives) that may provide insights into the underlying generative mechanisms for movement and into motor planning. These regularities take the form of power-laws that have been shown to characterise not only curvature (Lacquaniti et al., 1983), but also the duration of movement bouts and pauses (Reynolds et al., 2015).

Our work in *B. terrestris* supports previous findings in *Drosophila* larvae (Gomez-Marin et al., 2016) that the power laws which govern voluntary human behaviours (Flash and Hochner, 2005; Ivanenko et al., 2002; Lacquaniti et al., 1983; Wann et al., 1988) also govern the behaviours of less complex organisms. Remarkably, this law holds, not just across vastly different locomotive methods and speeds (walking (Ivanenko et al., 2002), drawing (Lacquaniti et al., 1983), crawling (Gomez-Marin et al., 2016)), but also across greatly differing organisms (human (Flash and Hochner, 2005; Ivanenko et al., 2002; Lacquaniti et al., 1983; Wann et al., 1988) and non-human primates (Schwartz, 1994), Diptera (Gomez-Marin et al., 2016), and now Hymenoptera).

The explanations for these power laws within movement patterns are contentious with contrasting hypotheses for their existence. Originally ascribed to central motion planning by the nervous system (Schwartz, 1994; Viviani and Flash, 1995) it was thought that the existence of the relationship between speed and curvature could not be a result of muscular properties and limb dynamics (Viviani and Cenzato, 1985). This is supported by the observation that the law holds true for human drawing under isometric conditions (Massey et al., 1992). Notably, the speed-curvature power law is also corroborated across widely diverse taxa. Evidence that the law originates as a result of decoding complex cortical processes is apparent in the motor cortical control of Rhesus monkey hand movements, as population vectors in the motor cortex obey the power law during drawing (Schwartz, 1994), adding weight to the central planning origin hypothesis.

Drosophila Larval locomotion power exponents have been recorded to deviate from the two-thirds exponent reported for human voluntary movements (Flash and Hochner, 2005; Ivanenko et al., 2002; Lacquaniti et al., 1983; Wann et al., 1988), at closer to three-quarters (Gomez-Marin et al., 2016). The researchers suggest that these findings prove a role for

dynamic effects adding on purely kinematic constraints (Gomez-Marin et al., 2016). In support of this notion, the power exponent recorded for human drawing shifts closer to this value of three-quarters (0.73) when drawing underwater (Catavitello et al., 2016), suggesting that power laws can indeed be governed by kinematic constraints. Our analyses suggest that, in walking bumblebees, a power law exponent between 0.55 and 0.66 (two-thirds) better defines movements than the near 0.75 exponents previously reported for *Drosophila* (Gomez-Marin et al., 2016) and constrained human movements (Catavitello et al., 2016). Our evidence further supports the idea that exponents are forced closer to the three-quarters value when kinematic constraints are present, as our constraint-free bees have a generally much lower exponent at closer to two thirds.

However, other studies take a less definitive approach, suggesting that biomechanical factors and central planning may interact to constrain kinematic movement aspects, limiting the degrees of freedom which they can take (Gribble & Ostry, 1996). An extension of this, the minimum jerk hypothesis (Viviani and Flash, 1995; Wann et al., 1988) states that the law exists to maximize smoothness, selecting for jerk-free, stable, controllable movements. The occurrence of these laws across organisms could be seen to support a convergent evolution theory of a jerk-free movement mode which remains behaviourally efficient across organisms of different size, complexity, and phyla. Maximally smooth movements may seem to be without biological significance for grounded invertebrates, like crawling *Drosophila* larvae (Gomez-Marin et al., 2016) and walking bumblebees. However, they could, nonetheless, be adaptive for airborne invertebrates, allowing for downwind flights in the absence of visual cues for orientation. Such common orientation has been widely documented since the advent of entomological radar, and allows noctuid fliers to add their flight speed to the wind speed, so maximizing their dispersal (Reynolds et al., 2016). Our analysis suggests that this ability is a spandrel that predates flight, lying dormant in terrestrial movements.

Contrarily, the pervasiveness of the law may be an inconsequential by-product of the noise inherent to central pattern generators (CPGs)(Maoz et al., 2006). Or more positively, an accidentally advantageous property of noise, as somewhat paradoxically, noise may result in maximally smooth, controllable movement. Possibly, the law may stem from simple harmonic motions (Schaal and Sternad, 2001), such as those outputted by CPGs when combined with

muscular viscoelastic properties (Gribble & Ostry, 1996). However, this hypothesis seems unrealistic when considering the power law in walking bees as we report here.

Our findings, together with those of Gomez-Marin *et al*. (2016) for *Drosophila* larvae, are suggestive of common mechanics of model switching in the locomotion of limbless and legged animals. As first suggested by Kuroda *et al.* (2014) who noted similarities between leg-density waves of centipedes and millipedes and the locomotive waves of limbless animals. Our findings hint at a deeper analogy. Marken & Shaffer (2017) have argued that these power laws are artefacts of the calculations themselves. However, this seems improbable, as the law is shown to persist regardless of its calculation methodology (Zago et al., 2017).

Any tendency to walk around the perimeter of the circular arena (of radius r=10 cm) either in part or wholly will be associated with a curvature of radius R = r. Our data for this curvature is consistent with the overall power-law scaling seen across all radii and is not anomalous. This suggests that the circular geometry of the arena is not impacting on the speed-curvature power law. This may not be true of other geometries, such as squares, who's corners might be associated with high curvatures.

In our analyses, individual bee's tracking data were pooled within each learning environment. This allowed us to collectively compare each training group to differing statistical models and to examine a potential training environment impact on power law exponents. We acknowledge that this approach minimises the role of intra-individual behavioural variation often seen in bees (Muller and Chittka, 2012). Although we have not examined it here, future studies could examine the impact of this intra-individual variation on power law exponents between bees and across learning experience.

The multitude of evidence for varying originating mechanisms suggests that the origins of such power laws are most likely pluralistic in nature and potentially constraints vary across organisms. Nonetheless, the pervasiveness of these multiple scaling laws, across both taxa and locomotive mode, could imply an underlying driver. The notion that scale-free movements are intrinsic (Barabasi, 2005) suggests universal scaling laws could present an optimal behavioural template which may then be favoured by natural selection.

Nonetheless, this might be overemphasizing the role of evolution as the fundamental determinate of behaviour, and underemphasizing the role of physical laws and mechanical

limitations, as exemplified by the minimum jerk hypothesis (Viviani and Flash, 1995; Wann et al., 1988). As animals, may simply be predisposed to have jerk-free movements due to physical constraints. The argument for process structuralism (Thompson, 1992), in which mathematical laws supersede natural selection as a "shaping agency" (Ball, 2013) may therefore be more applicable. This resonates with the occurrence of Levy walks; movement patterns that are characterised by power-laws and seen across taxa from single cells to humans. In many cases these appear to be shaped by physical constraints rather than by natural selection (Reynolds, 2015).

Understanding the basal behavioural templates behind organisms' locomotive trajectories may provide a tool for behavioural study. Biological stressors, such as disease, have been shown to cause deviations from this optimal behavioural template (Viswanathan et al., 1997). Power laws may therefore provide a diagnostic tool for the sublethal impact of such stressors at a finer scale.

Our work with *B. terrestris* is one of the few examples of the speed curvature power law outside human movements. Supporting the notion of an optimal behavioural template which is pervasive across movement modes and organisms as a result of kinematic constraints. The discovery of this null template in *B. terrestris* may add a tool to the arsenal of scientists, allowing us to better study potential sublethal disruptors of optimal behaviour.

6.4.1 Supporting information

S1: Raw centroid tracking data: this data was used to calculate speed-curvature power laws from bee trajectories. [Not included with thesis submission but available upon request].

S2: Data filtering and pre-processing: additional information is provided on the processing and filtering of the raw centroid tracking data prior to analyses [available in Chapter 6 Appendix].

Chapter 7

The speed curvature power law: a new tool to assess sublethal pesticides effects in *Bombus terrestris*

Chapter 7: The speed curvature power law: a new tool to assess sublethal pesticides effects in *Bombus terrestris*

7.1 Introduction

Understanding animal movement patterns is vital for conservation practices and will be key in the prediction of how future landscape changes and agricultural practices may impact on animal foraging and searching, particularly of beneficial organisms such as pollinators and pest predators. Lévy walks and the power law distributions they display are one such way in which animal movements can be characterised and have been observed across astonishing scales, from the cellular to the landscape level (Reviewed in Reynolds, 2018). In Chapter 6 we demonstrated for the first time the existence of a speed-curvature power law, previously found in human, non-human primate and *Drosophila* larval trajectories, in the walking locomotor trajectories of *B. terrestris* foragers. This is the first time this law has been studied in adult insect locomotion and corroborated previous findings of a speed-curvature power law exponent close to two thirds in human movement patterns. The discovery that these laws pervade the movements of a greater range of species than previously thought may provide a novel way of looking at animal movement templates, providing a new tool to study the impacts of environmental stressors on such templates. This may be particularly applicable to vitally important pollinators, such as the bumblebees, which regularly come in to contact with stressors in the agricultural environment. One such stressor may be exposure to agrochemicals, particularly in the form of insecticides.

Currently, it remains unclear if and how stressors such as agrochemicals may affect the spatial movement patterns of animals and what consequences a potential loss of behavioural complexity may entail for organisms (Macintosh et al., 2013). Research to date suggests that physiological stressors such as pregnancy, intoxication, social disharmony and pathogen load, can lead to a reduction in overall behavioural complexity resulting in more stereotypical behaviours being observed (Alados et al., 1996; Escós et al., 1995; Seuront and Cribb, 2011). However, this has never been studied in relation to pesticides as potential physiological stressors to pollinators. Based on the detrimental effect pathogen-infections have been shown to have on the complexity of the temporal structure of behavioural sequences (Alados

et al., 1996; MacIntosh et al., 2011), we can hypothesize that similar effects may be observed on the movement patterns of bees under pesticide exposure regimes.

As is highlighted throughout this thesis, sublethal pesticide effects, which do not cause outright mortality, are nuanced and more problematic to study than traditional toxicological measures. Bees are active, mobile foragers, required to have adept movements and behaviours to navigate to and from floral patches and to manoeuvre when collecting floral nectar and pollen rewards. Bee mobility has been shown to affect cross-pollination effectiveness when foraging (Ish-Am and Eisikowitch, 1998). Assessment of pesticide impacts on bee movement patterns and locomotion is therefore highly relevant, and it is important that a wide array of realistic behaviours are examined when sublethal pesticide effects are quantified, as it is clear that pesticide impacts can vary widely across bee species and pesticide compounds (Brandt et al., 2017; Ellis et al., 2017; Iwasa et al., 2004; Manjon et al., 2018; Sandrock et al., 2014a; Sgolastra et al., 2019; Tison et al., 2017).

Neonicotinoid insecticides are known to affect bee mobility, manifesting in symptoms such as tremors, incoordination, hyperactivity and shaking (Blacquière et al., 2012; Lambin et al., 2001; Nauen et al., 2001; Suchail et al., 2001). It is relatively easy to determine these effects on bees at high levels of neonicotinoid exposure. However, at lower, more field-realistic exposure levels it can be increasingly difficult to observe sublethal effects. Studying bee movement patterns and developing 'normal' movement templates (as we have in Chapter 6), is one way in which we can better understand how fine scale sublethal effects may be manifesting in bee mobility and locomotion. In Chapter 6, we discovered a speed-curvature power law as a baseline template of bee movements under aversive conditioning in the thermal-visual arena. Here, this speed-curvature law is revisited to examine whether the power law exponent is altered under pesticide exposure, making this law a potential tool for identifying fine-scale sublethal effects on mobility and locomotion in *B. terrestris*.

Being able to better understand basal behavioural templates behind bees' locomotive trajectories may provide a critical tool for the study of fine scale sublethal pesticide effects. Biotic stressors, such as disease load, have been demonstrated to lead to deviations from optimal behavioural templates in primates, seabirds and humans (Macintosh et al., 2013; MacIntosh et al., 2011; Viswanathan et al., 1997). Nonetheless, in honeybees optimal Lévy flight characteristics are not disrupted by infection with either *Nosema* sp. or Deformed Wing

Virus (DWV) (Wolf et al., 2016). Wolf *et al.* (2016) suggest that the robustness of the Lévy search patterns observed in honeybees may be due to Lévy flights being fundamental characteristics of neuronal processes, which are therefore unaffected by the physiological impacts of stressors such as disease. However, as discussed extensively in Chapter 6, it is likely that the speed curvature power law is governed by biomechanical constraints (Viviani and Flash, 1995; Wann et al., 1988) and may therefore be responsive to physiological stressors. Further study of the speed curvature power law in relation to bee movement patterns may therefore provide a diagnostic tool for the sublethal impact of agricultural stressors at a finer physiological scale.

We predict that pesticide exposure (thiamethoxam, thiacloprid or sulfoxaflor) may lead to non-optimal movements in *B. terrestris*. This may be reflected in a change in the optimal power law relationships observed as a template in Chapter 6 and here in untreated control bees.

7.2 Methods

7.2.1 Data origin

The data analysed in this Chapter is derived from the tracking data produced in Chapter 5 pesticide trials in the thermal-visual arena. The full experimental procedures for the trials are detailed in the methods section of Chapter 5.

7.2.2 Video processing and tracking

As detailed in Chapter 5, all trials were recorded using a FLIR C2 thermal camera (FLIR Systems UK, West Malling, Kent, UK) situated above the arena. Recorded video files were then tracked with idTracker using custom parameters (Pérez-Escudero et al., 2014). The raw trajectory data outputted by idTracker was then used for power law calculation.

It should be noted that in Chapter 6, in which we first studied the speed curvature power law in *B. terrestris*, power law exponents were calculated from video tracking of trial 1 (pre-training) for each bee. Here we decided to calculate the exponents based on individual bee's post- training trial (trial 10), to see the effect that pesticide exposure regimes were having on the most trained phenotype (as bees will have learnt the task to the best of their ability by trial 10). This also allows us to examine the effect of training on the power law exponent and whether this changes over time. For bees which died prior to trial 10 in the high dose trials, the last available trial prior to death was tracked and used as the "post training" trial (e.g. bee 19 (trial 4), bee 80 (trial 4), bee 42 (trial 5), bee 17 (trial 4), bee 78 (trial 7).

7.2.3 Speed-curvature power law calculation

To assess whether the exponent (β) of the speed curvature power law relationship changes under different pesticide exposure regimes, exponents of bees in each treatment during the post training trial (in most instances trial 10) were calculated. Speed-curvature power law calculation was identical to the method used in Chapter 6 and was conducted by Andy Reynolds (Computational and Analytical Sciences Department, Rothamsted Research).

7.2.4 Speed-curvature power law statistical analyses

All data passed normality testing (Shapiro-Wilk test, D'Agostino & Pearson test, P = >0.05) and therefore parametric statistical tests were used to assess whether there were treatment differences in speed curvature power law exponents (β). ANOVAs with multiple comparisons were used to compare power law exponents across treatment groups.

7.3 Results

7.3.1 Low dose experiments

The low dose experimental data analysed here was taken from the experiments conducted in Chapter 5, where bees were orally exposed to either 10 ppb thiamethoxam, 500 ppb thiacloprid or 5 ppb sulfoxaflor over a series of days (mimicking realistic nectar exposure levels in the field). Further information on dosages and the exposure regime can be found in the Methods section of Chapter 5.

The mean speed curvature power law exponents for the low dose treatments were; 0.51 (control, n = 9, range = 0.39 : 0.63), 0.49 (sulfoxaflor, n = 9, range = 0.36 : 0.57), 0.49 (thiacloprid, n = 9, range = 0.4 : 0.56) and 0.59 (thiamethoxam, n= 9, range = 0.45 : 0.74). The thiamethoxam treatment group had the highest mean power law exponent and the largest range of any of the groups.

There is a significant difference in the observed speed curvature power law exponents between the thiamethoxam and the sulfoxaflor treatments (ANOVA with multiple comparisons, P = 0.036*), and the thiamethoxam and the thiacloprid treatments (P = 0.022*) (Figure 7.1). There were no other significant comparisons.

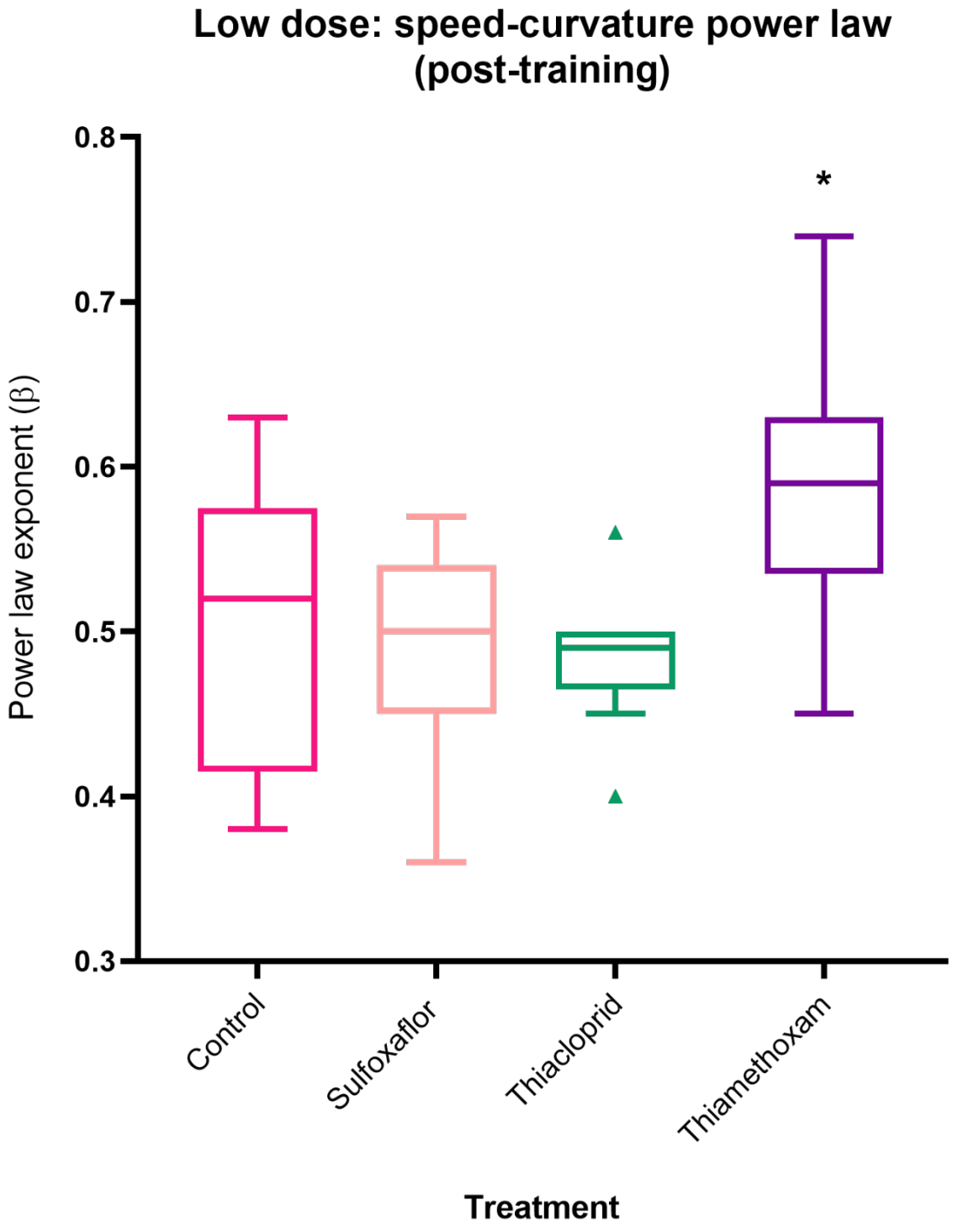

Figure 7.1: β-exponent of bees in the low dose pesticide experiments post-training. Control (n = 9), sulfoxaflor (n = 9), thiacloprid (n = 9) and thiamethoxam (n = 9).

7.3.2 High dose experiments:

The high dose experimental data analysed here was also taken from the experiments conducted in Chapter 5. High dose concentrations were calculated as 10x that of the field-realistic low dose concentrations, equating to 100 ppb thiamethoxam, 5000 ppb thiacloprid or 50 ppb sulfoxaflor.

The mean power law exponents for the high dose treatments were 0.44 (control, n = 9, range = 0.36-0.55), 0.44 (sulfoxaflor, n = 9, range = 0.36 : 0.51), 0.48 (thiacloprid, n = 9, range = 0.38-0.68) and 0.63 (thiamethoxam, n = 7, range = 0.52-0.73). As in the low dose experiment, the thiamethoxam group had the highest average speed curvature exponent.

There was a highly significant difference in the post-training power law exponents of the control and thiamethoxam groups (ANOVA with multiple comparisons, P = 0.0002***), the sulfoxaflor and the thiamethoxam groups (ANOVA with multiple comparisons, P = 0.0002***) and the thiacloprid and thiamethoxam groups (ANOVA with multiple comparisons, P = 0.005**). There were no other significant treatment comparisons (Figure 7.2).

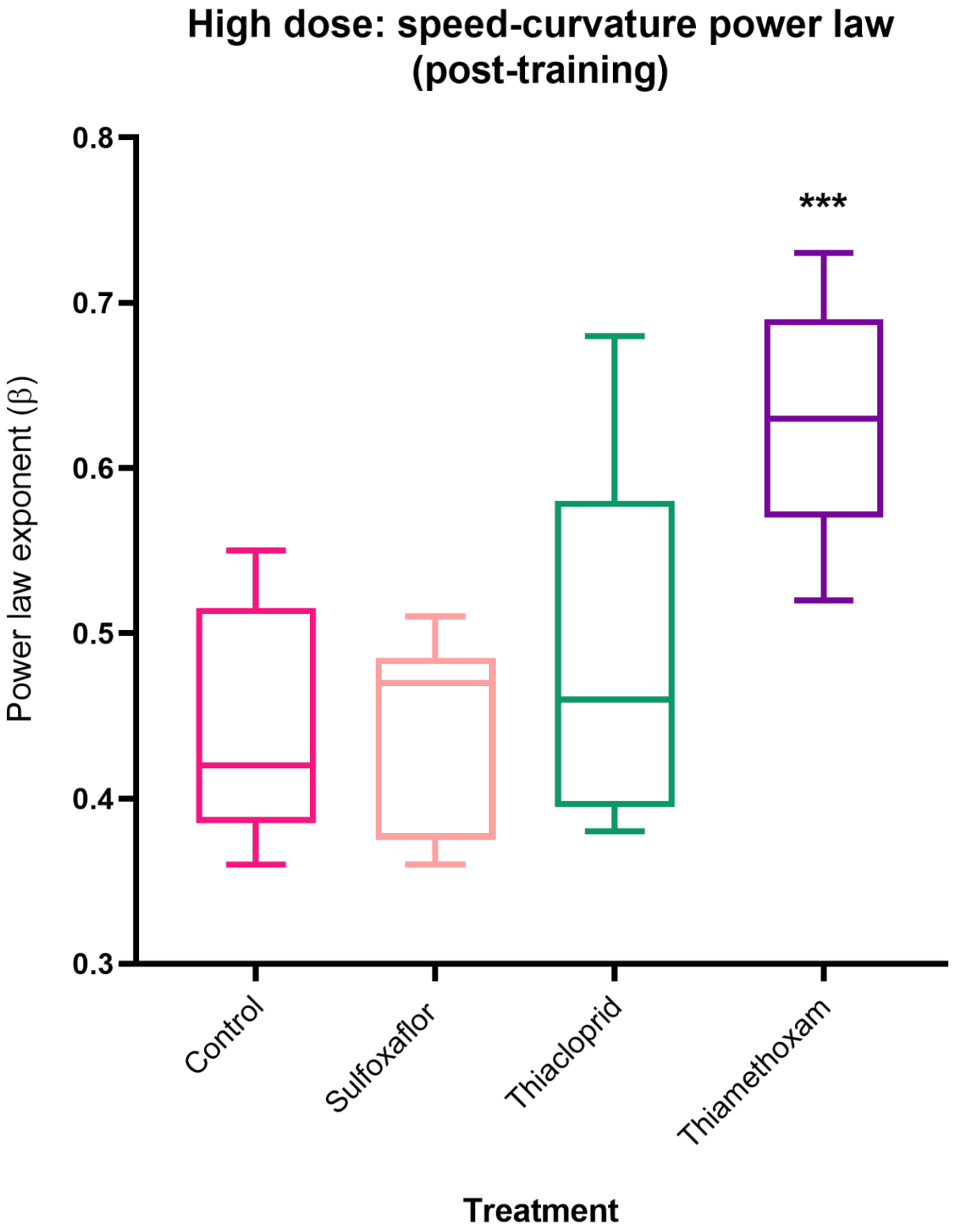

Figure 7.2: β-exponent of bees in the high dose pesticide experiments post-training. Control (n = 9), sulfoxaflor (n = 9), thiacloprid (n = 9) and thiamethoxam (n = 7).

7.3.3 Comparing the control groups

The T1 control exponents reported here are in line with the T1 control exponents reported in Chapter 6 (no significant difference, ANOVA with multiple comparisons, P = 0.58) (Figure 7.3). The mean power law exponent for the control bees in T1 was 0.52 (n = 9, range = 0.42-0.59). The mean power law exponent for the Chapter 6 T1 control bees was 0.60 (n = 7, range = 0.49-0.94). The 'Aversive' group in Chapter 6 is equivalent to the control group here and so is referred to as 'Chapter 6 T1 Controls' (both groups were not treated with pesticides, exposed to an aversive training arena with a heated floor and a cool reward zone).

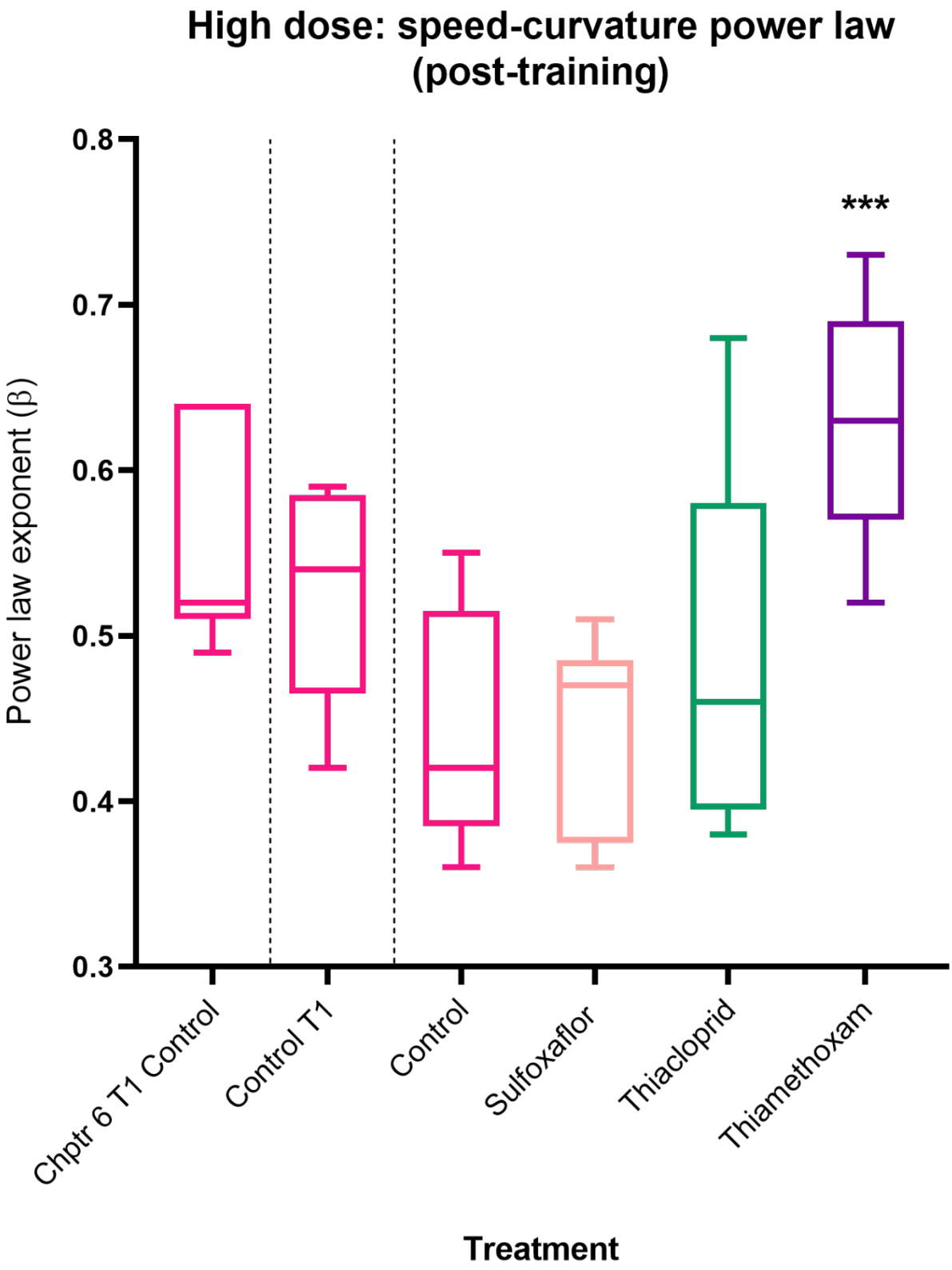

Figure 7.3: β-exponent of bees in the high dose pesticide experiments post-training, compared to pre-training (T1) control bees from this Chapter and Chapter 6. Chapter 6 T1 control (n = 7), control T1 (n = 9), control (n = 9), sulfoxaflor (n = 9), thiacloprid (n = 9) and thiamethoxam (n = 9).

7.3.4 Visualising the power law relationship

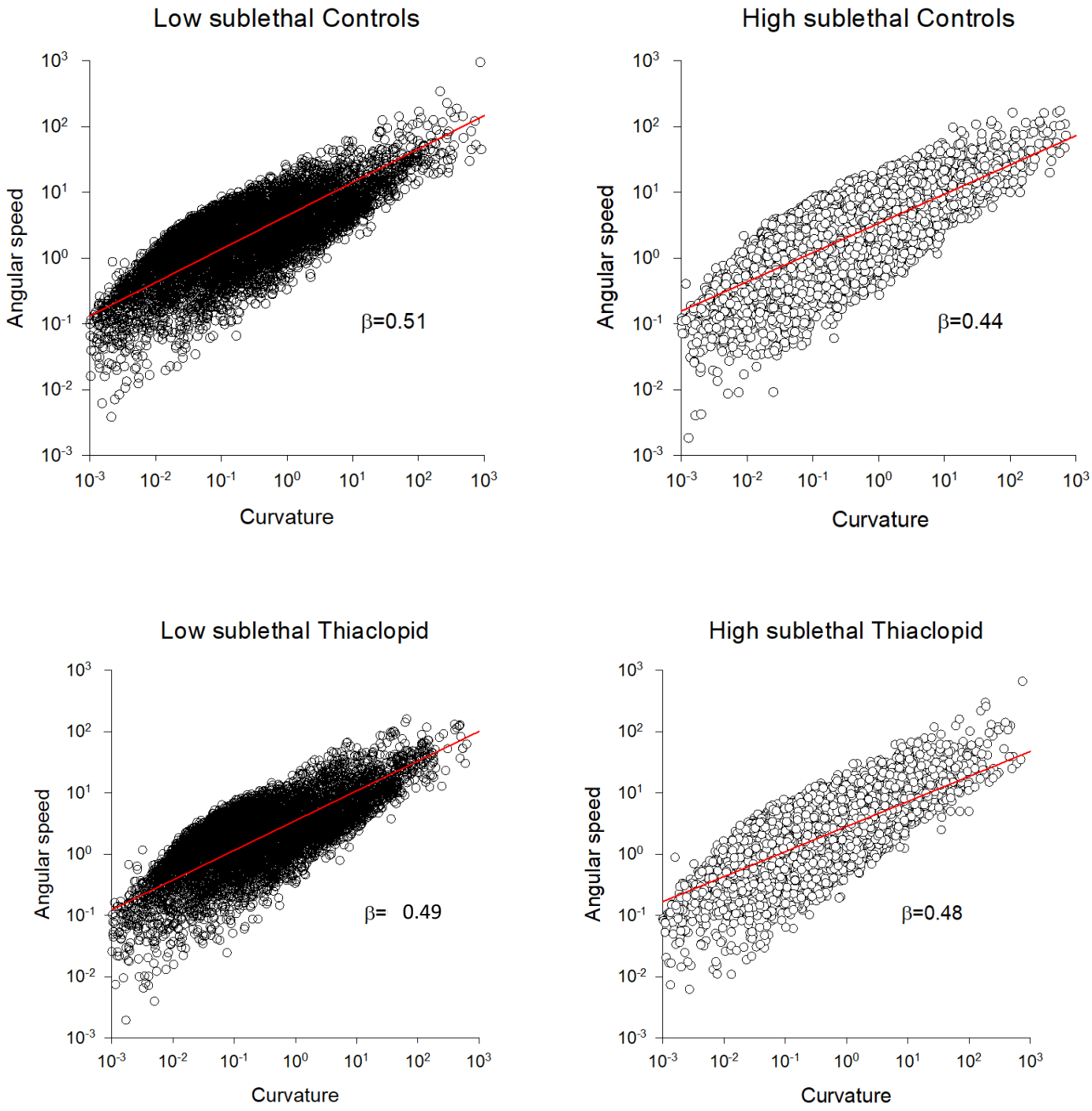

***Figure 7.4.A:** Scatter plots of the relationship between angular speed and curvature in the walking trajectories of control and thiacloprid bees in the low and high dose pesticide experiments (post-training). Scatter plots are of the whole bee population for that treatment and on a log-log scale. Low dose control (n = 9), high dose control (n = 9), low dose thiacloprid (n = 9), high dose thiacloprid (n = 9).*

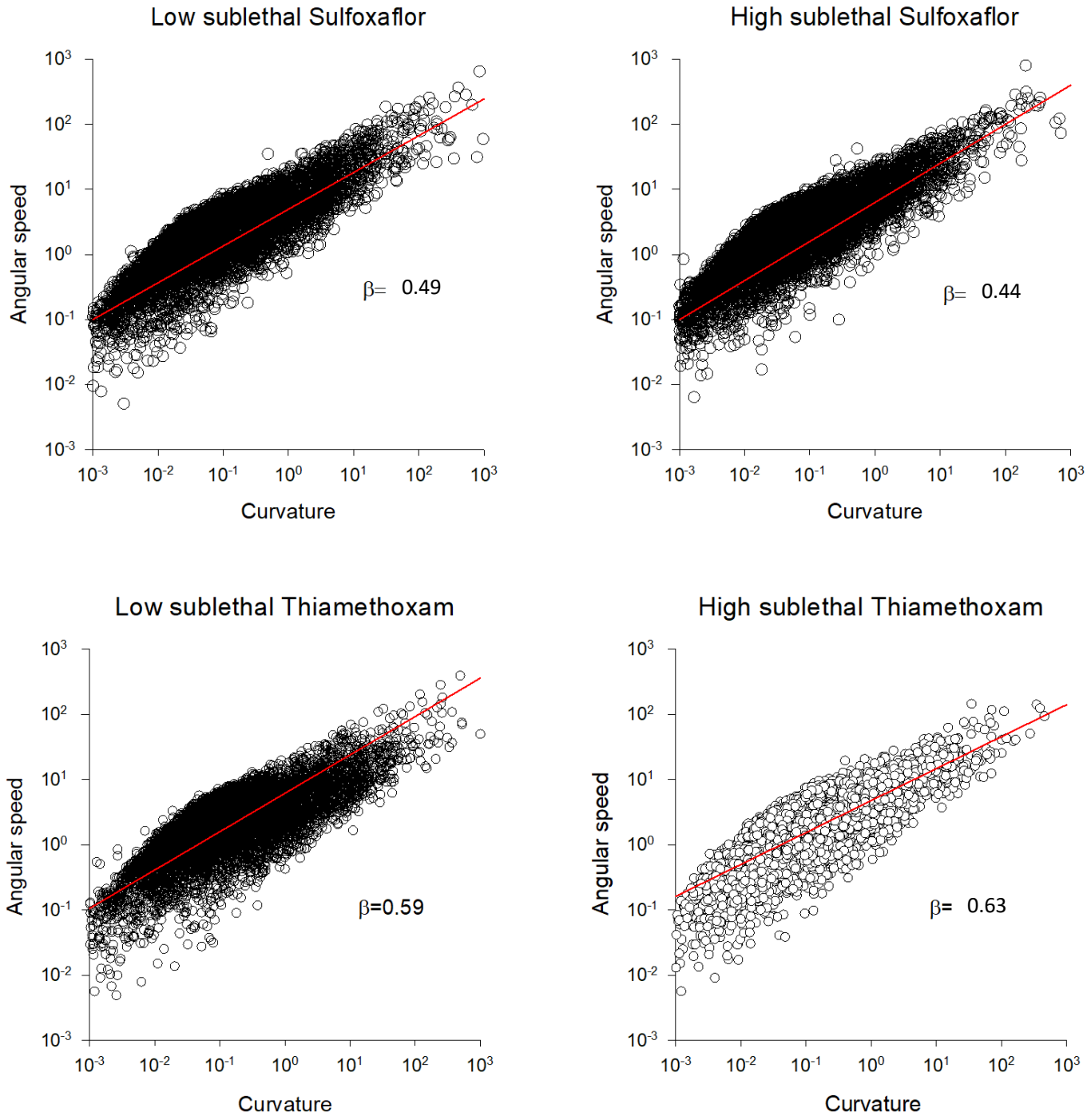

Figure 7.4.B: Scatter plots of the relationship between angular speed and curvature in the walking trajectories of sulfoxaflor and thiamethoxam bees in the low and high dose pesticide experiments (post-training). Scatter plots are of the whole bee population for that treatment and on a log-log scale. Low dose sulfoxaflor (n = 9), high dose sulfoxaflor (n = 9), low dose thiamethoxam (n = 9), high dose thiamethoxam (n = 7).

7.4 Discussion

A better understanding of the basal behavioural templates of bees has the potential to provide a critical tool to study fine scale sublethal pesticide effects. Fractal analyses (such as power law analyses) have emerged as a key tool to distinguish between systems which are operating in a normal versus pathological state (Goldberger et al., 1990; MacIntosh et al., 2011; West and Goldberger, 1987). In wider biological systems, stress has been demonstrated to lead to a reduction in both temporal and structural complexity e.g. in heart rate fluctuations (West and Goldberger, 1987), lung geometry (Mishima et al., 1999) and plant branching architecture (Escós et al., 1995). Stressors such as disease have been demonstrated to cause variations from optimal behavioural templates in animals (Macintosh et al., 2013; MacIntosh et al., 2011; Viswanathan et al., 1997). Similarly, stressors such as disease load and pesticide exposure are prevalent in agricultural landscapes, and yet, relatively little is known as to how they may impact animal movement patterns, particularly of less well studied pollinator species such as the bumblebees. Monitoring of pollinator health is vital to accurately assess the impacts of agricultural management practices on key ecosystem service providers. Being able to detect subtle sublethal pesticide effects could add power to the toxicological assessment tools currently available.

Here, we aimed to further study the speed curvature power law discovered in the walking trajectories of *B. terrestris* foragers in Chapter 6 (James et al., 2020), this time in foragers exposed to different sublethal pesticide regimes, to determine whether power laws have the potential to be used as diagnostic tools for the sublethal impact of pesticides on pollinators. We predicted that pesticide exposure may lead to non-optimal movements in *B. terrestris*, which would be reflected in a change in the power law relationships observed as a movement template in Chapter 6, and in the untreated control bees of this study.

As predicted, we see a disruption to the power law exponent template under certain exposure regimes. All the bee trajectories analysed in these pesticide experiments adhere to the speed curvature power law we discovered in the walking trajectories of untreated bees in Chapter 6. However, under certain pesticide exposure regimes we see a very different power law relationship. In both the low and high dose experiments, the speed curvature power law exponent for the thiamethoxam bees is significantly higher than the sulfoxaflor and the

thiacloprid groups. In the high dose trials this comparison was also highly significant between the thiamethoxam and control groups. By T10 the mean exponents for bees in the low dose treatments were; control (0.51), sulfoxaflor (0.49) and thiacloprid (0.49), whereas the mean exponent for thiamethoxam bees was significantly higher at 0.59. We see the same pattern in the high dose experiment where the mean speed curvature exponents were; control (0.44), sulfoxaflor (0.44) and thiacloprid (0.48), but again the mean for the thiamethoxam bees was significantly higher at 0.63. It appears that the control, sulfoxaflor and thiacloprid groups are characterised by a speed curvature power law relationship of approximately a half, whereas thiamethoxam bees' speed curvature relationship is characterised by an exponent closer to two thirds, demonstrating that sublethal doses of thiamethoxam, in both the low and high dose experiments, led to a change in the underlying movement patterns of the bees.

These changes in the movement patterns of the thiamethoxam exposed bees are remarkable, as we can demonstrate that the walking trajectories of treated bees have changed in subtle, yet detectable ways. These changes are consistent with wider conclusions, for example, Macintosh *et al.*'s (2011) findings that physiological stressors (e.g. parasitism) affect the locomotion behaviour of wild Japanese Macaques (MacIntosh et al., 2011).

The lack of significant difference in power law exponents of the sulfoxaflor and thiacloprid bees (relative to controls) is largely reflective of the lack of sublethal effects reported in Chapter 5 for these compounds. In Chapter 5, no sublethal effects were reported for thiacloprid across any of the assessed training parameters in the high or low dose trials. However, sulfoxaflor bees (unlike thiacloprid and control bees) did not significantly reduce the distance they travelled (pre- versus post-training) in the low dose trials (but this was very close to the significance threshold and bees showed a general reduction in distance compared to thiamethoxam bees). Nonetheless, sulfoxaflor bees showed no sublethal effects in other observed behavioural parameters (speed travelled, or time spent in the reward zone) in either the low or high dose trials, and did decrease their distance travelled post-training in the high dose trials, suggesting that the level of sublethal effects observed in Chapter 5 is well matched by the power law parameter here. Nonetheless, as a tool, the power law exponent may miss non-movement based behavioural changes under pesticide exposure e.g. the reduced feeding behaviour observed in high dose thiacloprid bees in Chapter 5.

The significant effects of thiamethoxam on bee movement patterns found here mirror the sublethal findings of Chapter 5. In the high dose pesticide trials (Chapter 5), thiamethoxam bees did not significantly improve their training parameters post-training (time spent in the reward zone, distance travelled, speed). Similarly, in the low dose trials (Chapter 5) thiamethoxam bees travelled significantly less distance in the pre-training trial (versus controls) and did not decrease their distance travelled post-training, potentially suggesting physical impairment preventing them from travelling as far in the initial trials and then further inability to learn or streamline the route in later trials. It therefore appears that power laws can provide a robust estimate of the presence of underlying physiological or biomechanical disruption in response to sublethal pesticide exposure.

Movement analyses, such as those conducted here, are clearly effective in detecting subtle changes to bee movement patterns. These changes may otherwise have been overlooked under other assessment paradigms which do not pick up such fine-scale changes, and yet such changes could still have very real-world implications for foraging bees in the wild. Power law analyses may therefore be an effective way to assess the general state of pollinator health under sublethal pesticide exposure regimes.

The T1 control bees assessed here were under the same training regime as the “aversive” bees analysed in Chapter 6 and therefore we can compare these two “control” treatments in T1 to check whether the power law exponent is consistent across experiments. There was no significant difference in the speed curvature power law exponents of the T1 control bees from Chapter 6 or the T1 control bees from this study (ANOVA with multiple comparisons, $P = 0.58$) (Figure 7.3). Therefore, we can see that the method used to analyse power law exponents used in Chapter 6 and here is a reliable and reproducible way to study *B. terrestris* trajectories.

Power law analyses have clear weight when it comes to assessing sublethal effects of pesticide exposure on bee movements. It is clear that the thiamethoxam exposed bees, in both the low dose and high dose experiments, were affected far more than bees in any other treatment. Power law analyses could be used to determine sublethal pesticide levels at which exponents, and therefore movement relationships, are not disrupted, and optimal behavioural templates are maintained.

Disruptions to simple movement patterns (e.g. power laws) can be used to elucidate underlying stressors and potential sublethal effects in bees, but this tool could be far wider reaching. The power law approach has not yet been extended to further agricultural stressors or to other beneficial invertebrates. Power laws could be used in further pollinator assessments, for example, in examining the physiological or behavioural stresses of bee virus infections or varroa infestations, or of poor diet and nutritional stress. Equally, power law analyses could be used to assess other beneficials' (e.g. pest predators and parasitoids) responses to pesticide exposure. Currently, power law analyses remain vastly underutilised and have the potential to allow us to detect a range of subtle changes in our native pollinators.

Chapter 8

Differential learning performance of *Bombus terrestris* foragers: a genetic basis for learning?

Chapter 8: Differential learning performance of *Bombus terrestris* foragers: a genetic basis for learning?

8.1 Introduction

8.1.1 Inter-individual differences in learning ability

Animals differ in their cognitive abilities even within species and the origins and phenotypic influence of such variation have long been sought and debated. A myriad of scientific approaches have been employed to investigate animal intelligence, both in the wild and in the laboratory. Unlike in the study of human cognition, animals cannot be directly asked as to their psychological state, meaning that the study of animal intelligence often relies upon external behavioural observations to infer internal mechanisms.

As Stevens (2010) notes, a hallmark of the study of comparative animal psychology throughout history is an acknowledgement of the existence of individual differences, but just as marked is an absence of understanding of the sources of these variations. Traditional comparative cognition methods have been criticised for their heavy 'top down' approach, which attempts to map human-like traits and behaviours onto other animals with proximate and ultimate perspectives, leading to potentially restrictive, biased experimentation (Chittka et al., 2012). The 1970's and 80's saw a notable shift from rigid, theoretical behaviour studies into a new era of experimental exploration through comparative cognition and cognitive ethology (Hulse et al., 1978). The continuing development of scientific approaches and techniques, particularly those in the laboratory, has facilitated new ways of studying animal cognition. Increasingly, 'bottom-up' approaches, which aim to discover underlying neural cognitive features and the genes which influence them, are becoming well utilised and look to elucidate the mechanisms which are the basis of interindividual variation (Chittka et al., 2012; de Waal and Ferrari, 2010; Willems, 2011).

Many studies have demonstrated that learning and cognition have a genetic basis (reviewed in Wahlsten, 1972), with forward or reverse genetics and transgenic approaches being used to study areas such as intelligence, spatial learning and memory, categorization, problem solving and perception in animals (Matynia et al., 2002; Vonk, 2016; Wasserman and Zentall,

2006). Examples of forward genetics approaches utilised to study the relationship between genetic variation within a species and observed differences in phenotype include the use of Quantitative Trait Loci (QTL) mapping or large scale Genome Wide Association Studies (GWAS), and have been applied in both humans and other animals (Bovo et al., 2019; Davies et al., 2011; Flint and Eskin, 2012; Sharmaa et al., 2015). Transcriptomics provides further forward genetics techniques which can be used to link genotypic and phenotypic traits in individuals (Konopka, 2017; Miller et al., 2013; Naumova et al., 2012). However, traits such as cognition and intelligence are often highly polygenic and complex, making it difficult to make causal inferences between singular genetic polymorphisms and end phenotypes.

Animal behavioural flexibility can represent a conditional response to environmental stimuli, or to the behaviour of others in the vicinity (Bergmüller, 2010). There are clear advantages to an ability to adapt behaviours in response to the world in which an animal lives. However, individual differences in behaviour are often observed between members of the same species in response to the same stimuli. This behavioural individuality is commonly observed within the vertebrates and often remains temporally and spatially consistent, suggesting an element of individual 'personality' or a 'behavioural syndrome', when this variation remains consistent across two or more scenarios (Bergmüller, 2010; Dall et al., 2004; Dingemanse et al., 2007; Garamszegi and Herczeg, 2012; Gosling, 2001; Pervin and John, 1999; Sih et al., 2004a) (see Table 8.1 for definitions). The limited plasticity of a behavioural syndrome is contrary to the assumed advantages of behavioural flexibility. Hence, the basis and advantage of these variations in animal behaviour has received substantial research attention (Clark and Ehlinger, 1987; Dingemanse et al., 2004; Sih et al., 2004a; Sinn et al., 2008), with consistent individual behaviours being reported across a wide variety of animal taxa, from blue tits (*Cyanistes caeruleus*) (Dingemanse et al., 2004) to squid (*Euprymna tasmanica*) (Sinn et al., 2008) and minke whales (*Balaenoptera acutorostrata*) (Hoelzel et al., 1989). These interindividual differences can be displayed in various forms, for example, a differing in willingness to take risk (boldness), increased levels of aggression or shyness, differential learning ability, or variations in activity level. Consistency in individual behaviours or behavioural 'syndromes' across time may suggest limitations on phenotypic variability as a result of an individual's developmental system e.g. pleiotropic genetic effects (Maynard Smith et al., 1985). Individual

differences may also represent life history trade-offs as individuals display sub-optimal behavioural or learning plasticity (Sih et al., 2004a).

Learning, through the nervous system, contributes to an animal's phenotypic plasticity, facilitating adaption of the phenotype to varying environments (Riveros and Gronenberg, 2009). Dukas (2008) suggests that there are three simple attributes of learning which vary within and among species: the initial ability to learn a task, the rate at which the task can be learnt, and the best result which can be learnt after practice. Individuality is a rarer concept within the invertebrate group (versus the vertebrates) and the notion of individual differences between insects may seem strange. However, intraspecific variation is increasingly well observed across insect and spider species (Jandt et al., 2014; Keiser et al., 2018; Pruitt and Riechert, 2011; Segev et al., 2017), and particularly within the social insects (Bengston and Dornhaus, 2014; Jandt et al., 2014; Weller, 2015).

Table 8.1 Comparative behavioural terminologies used across the fields of behavioural and social insect sciences. Sourced from Jandt et al. (2014).

Animal behaviour terms	Social insect biology terms
Behavioural syndrome *Individuals within a population exhibit consistency in two or more functionally different behaviours.*	
Animal personality *Individuals within a population exhibit consistency in a single behaviour.*	**Morphological/reproductive caste** *Physiological constraints (genetics, morphology, hormones, etc.) predispose individuals to perform specific behaviours (e.g. queens and workers).*
Behavioural type *Individual exhibits consistent behaviour across contexts and/or over time. Repeatability or consistency of the magnitude of behaviour often observed.*	**Behavioural specialisation** *Individual exhibits consistent task performance (that is not necessarily determined by its morphology).*
Episodic personality *Response to one set of stimuli predicts the response to other sets of stimuli over a short time scale. Responses are inconsistent over longer time scales.*	**Temporal polyethism** *In a predictable order, individuals switch among tasks as they develop. Task switching individuals switch among tasks throughout their lifetime.*
Keystone individuals *An individual that significantly alters the behaviour of other individuals within the group.*	**Elite workers/activators** *Individuals that stimulate others, perform multiple tasks, or perform a disproportionate amount of work in the colony.*

Bees have been demonstrated to have extraordinary cognitive abilities considering the small size of their brains (Cartwright and Collett, 1982; Giurfa, 2007; Giurfa and Giurfa, 2003; Menzel and Giurfa, 2006; Muth et al., 2015). Previous studies have demonstrated the existence of inter-colony variation in bumblebee learning ability, particularly in response to their handling of novel stimuli and their learning speed (Evans and Raine, 2014; Muller et al., 2010; Raine et al., 2006).

Traditionally research has associated these intraspecific animal 'syndromes' with phenotypic polymorphisms, such as between males and females or between different mating morphs e.g. smaller sneaky males versus larger aggressive, territorial males (Gross and Charnov, 1980). However, of increasing interest is the intraspecific variation observed between conspecifics (i.e. members of the same species) with no marked phenotypic polymorphisms (Sih et al., 2004b). Research is increasingly focused on elucidating the driving mechanisms of this variation between individuals, as this variation within a species is the raw material upon which natural selection may act and individual variation clearly plays a key role in evolution (Darwin, 1859).

Bees make good model organisms for the study of this interindividual variation, as they face complex learning tasks in their everyday environments during foraging. Learning to navigate complex environments, handle a large variety of flowers and monitor floral rewards is clearly integral to foraging success. We would therefore predict little genetic variation in learning ability, both between individuals and between hives, as this variation should be eliminated by strong selective pressures. And yet the existence of large variation in bees' individual learning ability is documented (Evans and Raine, 2014; Muller et al., 2010; Raine et al., 2006), bringing in to question how such varying cognitive capabilities can evolve.

The fact that inter-individuality exists between individuals' learning performance, even within the same environment, could suggest that there is a fitness cost to higher cognitive capabilities. Otherwise, as predicted above, we would expect this variation to be eliminated by selection. Indeed, trade-offs to being smart have been seen in other animals. For example, Great tits (*Parus major*) which were able to solve a complex food finding task in the lab laid more eggs in their nests (Healy, 2012). However, these problem-solving birds were more likely to go on to abandon their nests, resulting in lower fitness versus birds which were unable to

solve the task (Healy, 2012). Evans, Smith and Raine (2017) suggest that similarly, bees with enhanced learning capabilities may display a life history trade-off. Their study showed that bumblebees which were faster at learning a visual task in the lab, did not in fact benefit from this enhanced skill when in the field, collecting food at comparable rates to their slower learning counterparts, completing a similar number of foraging bouts per day but foraging for fewer days in total. This resulted in the slower learners actually collecting more resources for their hive over their foraging life span and suggests some energetic detriment to higher learning performance in a natural environment (Evans, Smith and Raine, 2017). However, that does not mean that enhanced learning would not be beneficial under a different set of environmental conditions (e.g. urban areas). Goulson (2010) postulates that individuals which 'know too much' may be disadvantaged as, although fast learning individuals quickly accumulate information in their long-term memory, this may interfere with long-term memory retrieval, making it slower and less accurate (Chittka, 1998; Chittka and Thomson, 1997). Again, this suggests a potential detriment to being a quick learner and optimum forager.

8.1.2 A genetic basis for individual differences?

The underlying structural and genetic bases for individual cognitive differences in bees remain largely a mystery. Some previous studies have attempted to elucidate a link between structural brain anatomy and learning ability in bumblebees. Li *et al.* (2017) discovered that bumblebees which were better at visual tasks (i.e. made fewer errors) and had better memory retention, had a higher microglomerular density (an area of the bee mushroom bodies linked to visual associative learning). Genetic studies, utilizing high-throughput sequencing to examine differences between individuals given a visual learning task and controls who were not, identified candidate genes which are potentially crucial to the learning and memory formation process at different time points i.e. short-term learning and memory vs long-term memory (Li et al., 2018). But no studies, to our knowledge, have examined genetic differences in the cognitive abilities of individual forager bumblebees when given the same learning task. Genetic analyses and transcriptomics provide tools with which to study interindividual learning ability both between and within hives, to elucidate potential differentially expressed genes (DEGs) which could predetermine an individual bumblebee's cognitive capability.

If significant interindividual differences naturally exist within and between hives, then it is essential that this is understood and studied in more depth. A failure to do so could potentially lead to a miss-diagnosis of the factors responsible for apparent differences in bee learning ability e.g. an over estimation of the detrimental effects of pesticides or parasite load. The existence of both spatial and temporal consistency in interindividual differences is commonly accepted to have a substantial genetic basis (Dingemanse et al., 2004). Therefore, in bees, interindividual differences between sister members of the same hive are of great interest. Bumblebee (*B. terrestris*) nests are typically founded by a lone, single-mated queen (Schmid-Hempel and Schmid-Hempel, 2000). Queens mate with a single haploid male to produce a colony of highly related diploid sister workers (relatedness, r = 0.75) (Porath et al., 2019). The haplodiploid sex determination system of the bumblebee means that all of the females in a hive are produced by a single queen (females are diploid, fertilised eggs), and most of the males too (males result from unfertilised haploid eggs and so also have the potential to be laid by workers) (Dreier et al., 2014; Evans et al., 2004). Therefore, genetic variation amongst sister hive members is expected to be low. However, hive members still demonstrate incredible diversity in factors such as learning capacity, behaviour, body size and physiology, among others (Spaethe and Chittka, 2003; Yerushalmi, Bodenhaimer and Bloch, 2006; Jeanson and Weidenmüller, 2014).

The question therefore becomes, how does this variation arise and how is this variation maintained in natural populations and what role does it serve? The existence of differences in learning performance between and within hives is fascinating and, to the best of our knowledge, has never been studied at the genetic level in *B. terrestris*. Controlled learning environments in the lab provide an ideal basis in which to study the possible genetic mechanisms underlying intraspecific variation.

8.1.3 The thermal-visual arena: discovering the existence of individual differences

The development of the thermal-visual arena as a novel bee aversive training tool (see Chapter 3 and 4) has allowed us to study and identify differences in individual bee's learning and navigation abilities both across and within hives. These observations prompted us to want

to better understand the genetic causes underlying these individual's differing learning abilities (identified and discussed in Chapter 4).

The genetic determinants of associative learning in the bee are well understood in terms of appetitive learning ability (Bhagavan et al., 1994; Brandes, 1991; Brandes et al., 1988). However, until fairly recently, genetic influences on aversive learning ability had not been studied in bees. Junca *et al.* (Junca et al., 2014) demonstrated for the first time that genotype has a strong influence on aversive learning capacity in honeybees, suggesting that genetic determinism of aversive and appetitive abilities across a hive's population may play a role in efficient task partitioning amongst individuals. It has since been suggested that honeybees display a trade-off between appetitive and aversive abilities, for example, if an individual is better at appetitive learning, they are generally a less efficient aversive learner, creating a bias of individuals towards either appetitive or aversive learning (Junca et al., 2019).

8.1.4 Aims of this study

The thermal visual arena (Chapters 3 and 4) permits the study of differences in bee learning ability and behaviour when bees are subjected to the same task and the same training motivation over ten learning trials. During Chapter 4 trials it was observed that some individuals are more adept at completing the learning task (to identify and learn the location of the cool reward zone), both between and within hives. I therefore became interested in why these inter-individual differences persisted and specifically, what makes one individual in a hive smarter than another in the same hive? And what would make one hive smarter than another?

To explore this further I sought to establish what the genetic determinants of the observed differences might be. It was therefore not the existence of the genetic variation, but specifically what this variation between individuals may be that was of most interest here. Therefore, in these analyses, I focused predominantly on the differences between those individuals identified as "good" and "bad" learners, across all bees in the experiment, and not specifically the hive from which they came, as this allowed me to potentially identify the most differentially expressed genes (DEGs) involved in differences in learning ability. I did however

construct the bioinformatics analyses in such a manner as to take into account the influence of the hive a bee came from, to better understand this variable within the samples.

To answer these questions, an RNA-seq pilot study was conducted to compare genome wide differences in expression between bumblebees which are highly capable and not capable of learning the same aversive learning task in the thermal-visual arena, to better understand potential genetic causes for the observed differences in individual's aversive learning performance**.**

8.2 Materials and methods

The bees used for these analyses were all untreated bees in the aversive training group taken from the thermal-visual arena experiments conducted in Chapter 4. Immediately after bees had completed trial 10 of the Chapter 4 experiments, they were placed in a 1.5 ml Eppendorf and snap frozen in liquid nitrogen. Samples were then stored at -80˚C to await further processing. Full details of the experimental arena and process can be found in Chapter 4.

8.2.2 Sample Preparation

To select bees which were 'good' learners and bees which were 'bad' learners under the same training regimes (aversive conditioning to find the cool reward zone within the thermal-visual arena), the following protocols were employed.

8.2.3 Bee selection

This study examined one of Dukas' (Dukas, 2008) three purported ways in which learning can differ between individuals; the best result which can be learnt after practice, in our forager bees. We did this through comparison of trained (trial 10) and untrained (trial 1) parameters for each bee.

Based on the videos processed in idTracker for each bee, for trial 1 (pre-training) and trial 10 (post-training) a number of training parameters were calculated (full details given in Chapter 4).

Eight bees (the capacity of one RNA seq run, as dictated by budget constraints) were selected as either 'bad' or 'good' learners, based on the Chapter 4 parameters, to capture the most

individual variation possible between 'good' and 'bad' learners, whilst also trying to get a spread of bees from each of the three hives where possible (Table 8.2).

Bad bees were bees which displayed either no change or a slight negative or positive change (up to -5 or +5) in the 'time spent in the reward zone parameter' between trial 1 and trial 10 and, as observed from the visual trajectory maps had clearly not been able to find and remain in the reward zone by trial 10.

Good bees were bees which improved most in the 'time spent in the reward zone parameter' between trial 1 and trial 10 and, as observed from the visual trajectory maps, had clearly identified the reward zone by trial 10. The time a bee spent in the reward zone was used as the primary determinant of 'good' or 'bad' learning, as Chapter 4 trials found this to be the most effective parameter to assess learning.

Table 8.2: Bee selection from each hive based on training parameters. RNA sample preparation details are given for each bee.

Learner type	BEE ID	Hive	Head diameter (mm)	NanoDrop RNA conc. ng/µl	QuBit RNA conc. µg/µl	GENEWIZ ID
BAD	54	H2	4	261.2	1.72	LJ1
BAD	19	H2	4	177.7	1.56	LJ2
BAD	94	H2	4	213.2	1.89	LJ3
BAD	82	H2	4	166.9	1.46	LJ4
GOOD	50	H3	4	199.1	1.68	LJ6
GOOD	62	H3	5	118.2	1.17	LJ7
GOOD	98	H1	5	191.6	1.75	LJ8
GOOD	38	H2	3.5	230.8	1.62	LJ9

8.2.4 Total RNA extraction, RNA-seq library construction and high-throughput sequencing

RNA was extracted from individual bee heads. Previously snap frozen bee samples were taken from the -80˚C freezer and individually placed in a weigh boat of liquid nitrogen, resting on dry ice. Heads were removed using a razor blade to separate the head from the neck joint and the head diameter recorded (See Table 8.2). Heads were then placed in individual 1.5ml

Eppendorf tubes where they were ground to a fine dust using a plastic pestle. A bath of liquid nitrogen was used to keep the Eppendorf and pestle frozen at all times during this process. RNA extraction from bee heads was then conducted using the ISOLATE II RNA mini kit from Bioline (Tennessee, USA).

A NanoDrop™ 2000/2000c spectrophotometer (Thermo Fisher Scientific) and a Qubit Fluorometer (Thermo Fisher Scientific) were used to quantify the RNA (recorded in Table 8.2). The overall quality of the RNA was assessed using gel electrophoresis, to substantiate the NanoDrop readings. All samples met GENEWIZ quality requirements and were sent for standard RNA-Seq next generation sequencing at GENEWIZ. Libraries were constructed and paired-end sequenced by GENEWIZ (Leipzig, Germany) using Illumina HiSeqTM 2000, generating approximately 50 million paired-end 150 bp raw reads per sample (total of 8 samples).

8.2.5 Head diameter across hives and learner type

Worker bumblebees exhibit large intraspecific size variation even within a hive, as adult bee size is dependent on larval food consumption. Larger worker bees have been attributed with increased foraging success (Willmer and Finlayson, 2014). It was therefore decided to measure the head diameter (mm) of the selected bumblebee foragers (recorded in Table 8.2) to determine whether a) head diameter differed significantly between hives or b) whether head diameter was a signifier for learning type e.g., whether a bee was a good or bad.

As the sample size from each hive was small (e.g., only one bee for hive 1), it was determined that a statistical test could not be conducted for this comparison. However, an independent t-test was conducted between the two learner types (good vs. bad) to determine if head diameter was significant between learning groups. the independent t-test was not significant ($P = 0.3559$, $n = 8$) therefore we accept the null hypothesis; that there is no apparent significant difference in head diameter between learning type (good vs. bad) in this study. However, it should be noted that as the sample size is still relatively small, we cannot assume normal distribution of the populations and this could affect how robust the t-test is.

8.3 Bioinformatics

Galaxy (Afgan et al., 2018) and RStudio (R Core Team, 2017) platforms were used for all bioinformatics analyses. The Galaxy workflow used is shown in Figure 8.1. All tool parameters were default unless otherwise specified.

8.3.1 Reference Genome and Annotations

The *B. terrestris* reference genome (key features summarized in Table 8.3), DNA, cDNA, CDS, protein, GTF and GFF3 files were downloaded from Ensembl Metazoa (http://metazoa.ensembl.org/Bombus_terrestris/Info/Index).

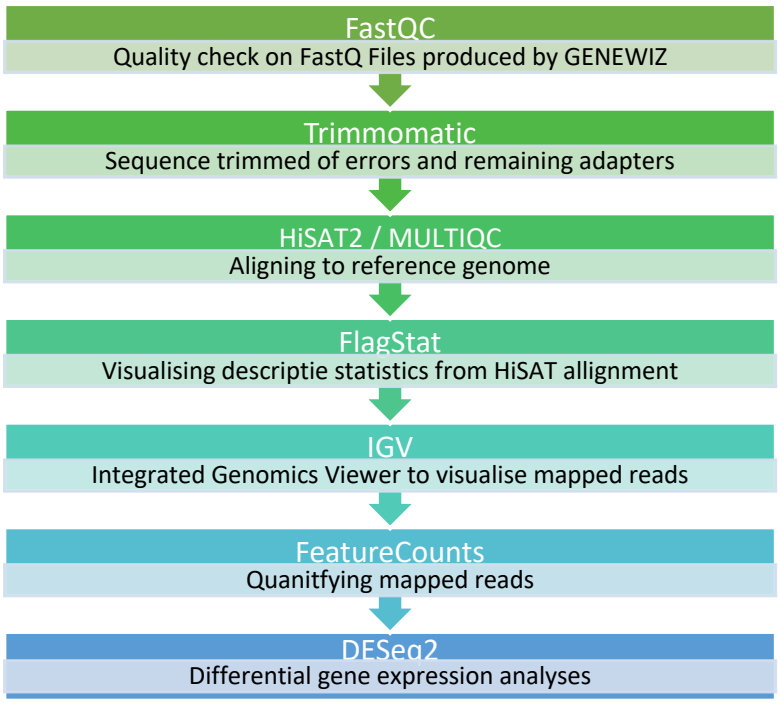

Figure 8.1: Bioinformatics analyses pipeline in Galaxy.

Table 8.3: Summary table for the NCBI Bombus terrestris reference genome.

Statistics

Summary

Assembly	Bter_1.0, INSDC Assembly GCA_000214255.1,
Database version	97.1
Base Pairs	236,382,229
Golden Path Length	248,654,244
Genebuild by	NCBI
Genebuild method	Import
Data source	Baylor College of Medicine - HGSC

Gene counts

Coding genes	10,587
Non coding genes	1,347
Small non coding genes	324
Long non coding genes	1,023
Pseudogenes	74
Gene transcripts	24,601

8.3.2 Sample quality checking

FastQC (Andrews, 2010) was used to quality control all samples. The adapter content analysis identified the presence of the Illumina universal adapter in our sequences from around 90-137bp (example adapter content graph shown in Figure 8.2). Trimmomatic (Bolger et al., 2014) was therefore used to remove adapter sequences and it was confirmed with FastQC that the adapter had successfully been removed (Figure 8.3).

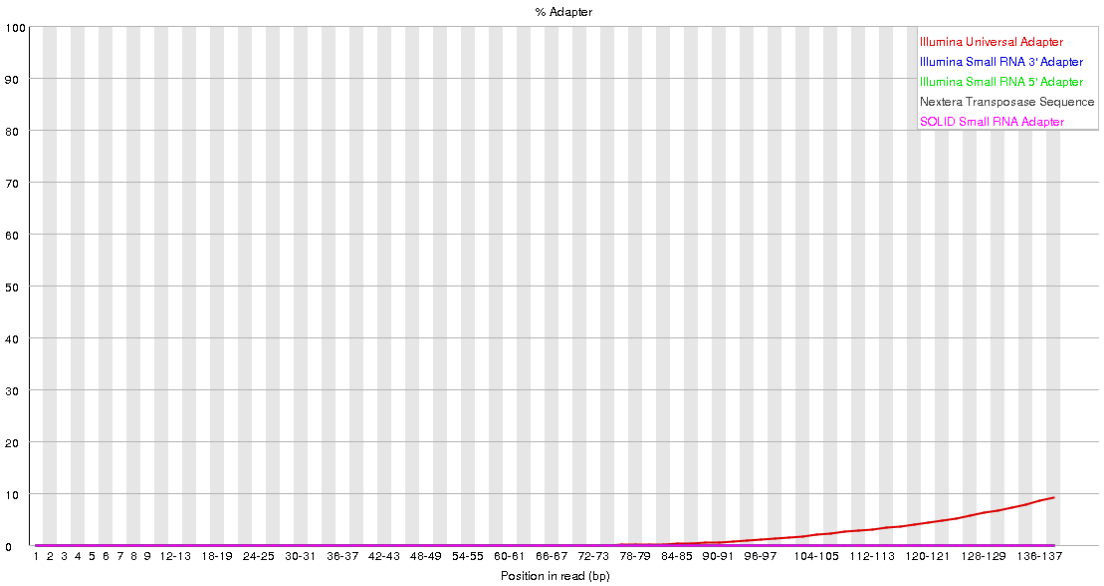

Figure 8.2: Adapter content (raw sample LJ1) identifying the presence of the Illumina Universal Adapter.

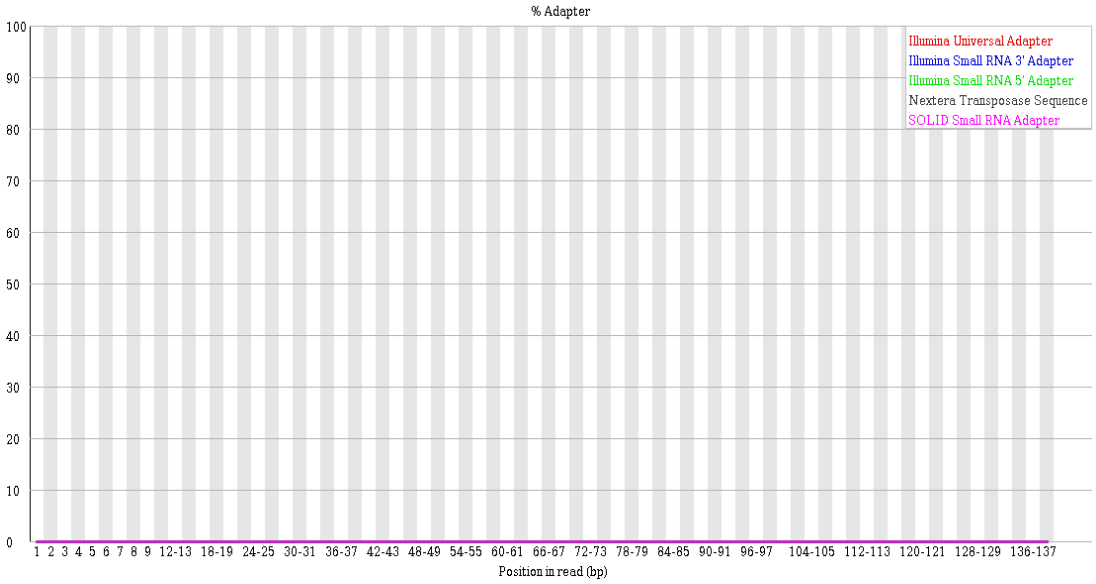

Figure 8.3: Adapter content (sample LJ1) post Trimmomatic showing absence of the Illumina Universal Adapter.

8.3.4 Read mapping and gene expression calculation

Genome alignment: HiSat2 and MULTIQC

Cleaned reads were then aligned to the *B. terrestris* genome and reference genes. HiSat2 (Kim et al., 2015) was used to map the reads to the genome. MULTIQC was used on the HiSAT2 reports to assess mapping rates of each sample to the genome. All samples had an >90% alignment to the genome (Table 8.4) and >20 million uniquely mapped reads (Figure 8.4).

Table 8.4: Alignment rates of each sample to the B. terrestris reference genome.

Sample Name	% Aligned
HISAT2: LJ1 [BAD, HIVE 2]	92.6%
HISAT2: LJ2 [BAD, HIVE 2]	92.2%
HISAT2: LJ3 [BAD, HIVE 2]	92.3%
HISAT2: LJ4 [BAD, HIVE 2]	92.3%
HISAT2: LJ6 [GOOD, HIVE 3]	91.8%
HISAT2: LJ7 [GOOD, HIVE 3]	91.7%
HISAT2: LJ8 [GOOD, HIVE 1]	92.0%
HISAT2: LJ9 [GOOD, HIVE 2]	92.7%

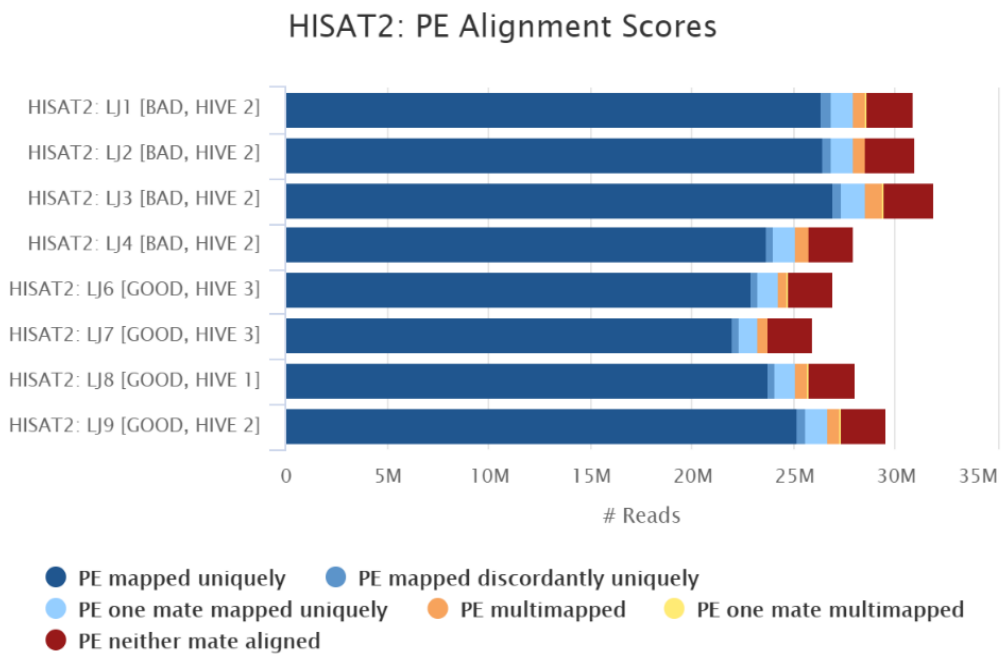

Figure 8.4: Number of reads mapped to the genome for each bee sample.

Differential Gene Expression Analysis

Gene expression levels were quantified using FeatureCounts (Liao et al., 2014) on the HiSat2 output. DESeq2 (Love et al., 2014) was used to analyse the quantified reads data outputted by Galaxy's 'featurecounts' function. DESeq2 provides differential gene expression analyses based upon the negative binomial distribution. DESeq2 P-values were adjusted using the Benjamini Hochberg Procedure (Haynes, 2013) to decrease the false discovery rate (also known as type I errors), where small P-values occur by chance, leading to an incorrect rejection of the null hypothesis. These adjusted P-values were then used to filter differentially expressed genes (DEGs) with a P-value of <0.05 post adjustment. Principle Component Analysis (PCA) and heatmap plots were produced with DESeq2.

8.3.5 Over Representation Analyses of GO and KEGG terms

The online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa, 2000) was used to search for the identified DEG gene IDs. The database links higher order function information with genomic information. Searches were conducted by entering the identified gene ID e.g. LOC100648451, without the 'LOC' precursor, into the KEGG search bar at https://www.genome.jp/kegg/. This search returns KEGG Orthology (KO) definitions and higher order associated functions e.g. 'tyrosine metabolism', 'aromatic-L-amino-acid-decarboxylase'. An example search for the *Bombus* LOC100648451 gene can be found here: https://www.genome.jp/dbget-bin/www_bget?bter:100648451 .

8.4 Results

8.4.1 Principal Component Analyses

The PCA (Figure 8.5) shows the variation in the transcription profiles of the samples analysed. The 'PC1' is the axis which spans the most variation in gene expression of the samples.

Therefore, samples with similar transcription profiles should cluster together. PCA analysis also takes in to account the 'influence' of genes e.g. genes with the largest variation between samples (bees) will have the most influence on the principle components.

There appears to be some variation based upon which hive bees were from (Figure 8.5.A). However, there is only one bee from hive 1 (the red data point on Figure 8.5.A) and the sample size across hives was low, and so it is difficult to draw any firm conclusions here.

A significant separation exists between the good and bad learning groups (Figure 8.5.B), as delineated by the diagonal yellow line. This split suggests there could be an underlying genetic signal responsible for the differences between good and bad learner groups which can be explored further through more bioinformatic analyses.

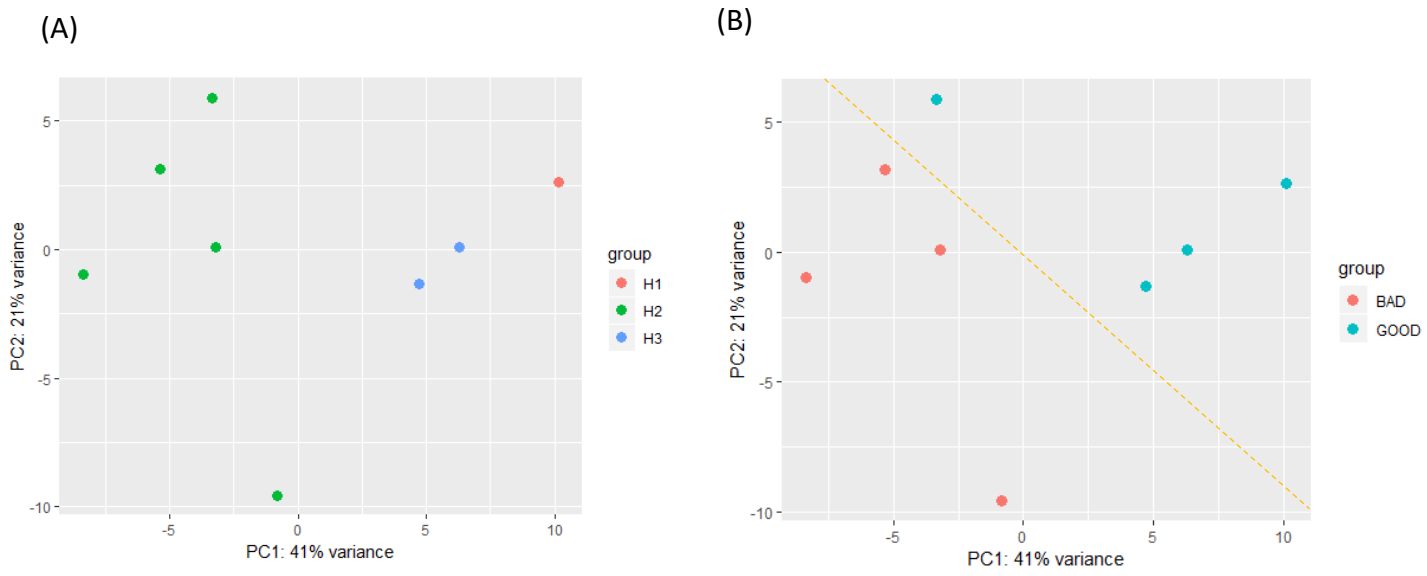

Figure 8.5: Principal Component Analyses of (A) samples by hive and (B) samples by learning type (good or bad).

8.4.2 Identification of differentially expressed genes and cluster analysis

DESeq2 was run with treatment (GOOD or BAD) and hive (H1, H2, H3) as factors which allowed for three comparisons: 1) Treatment: Good vs. Bad, 2) Hive: H2 vs. H1 and 3) Hive: H3 vs. H1. A normalised counts file of DEGs by bee was produced (filtered by adjusted P value <0.05). This resulted in 98 significant* (<0.05) DEGs, 83 of which were unique to the Good versus Bad

treatment comparison (Chapter 8 Appendix Table A8.1) (Figure 8.6) and 35 of which were highly significant** (<0.01).

8.4.3 DEGs in relation to Bombus terrestris genome

B. terrestris contains 10,587 protein coding genes. The DESeq2 analysis, taking account hive as a confounding factor, produced 98 DEGs between good and bad learners**,** which represents 0.93% of the genome. 83 of these DEGs are unique to this comparison, which represents 0.78% of the genome. Comparisons between hive 2 and hive 1 result in 33 DEGs, representing 0.3117% of the genome. And comparisons between hive 3 and hive 1 produced 67 DEGs, representing 0.633% of the genome. Overlap of DEGs between treatment and hive comparisons is small. As there is scarcely any overlap with the hive variation genes (9, 2 and 4 genes) (Figure 8.6), we can be fairly certain that these 83 genes are capturing differences in learning between good and bad groups and not the background hive variation.

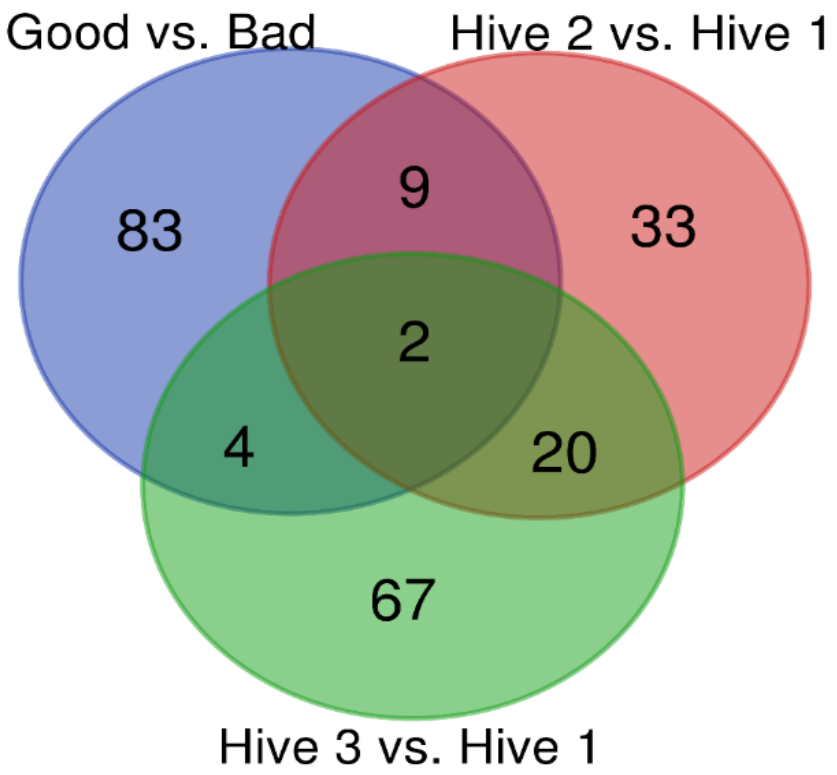

Figure 8.6: Venn diagram detailing DEGs between all analysis comparisons.

Table 8.6: DEGs from multiple comparisons and the percentage of the B. terrestris coding genome which these represent.

Comparisons	**Number of DEGs**	**Number of unique DEGs**	**Percentage of the *B. terrestris* coding genome (10,587)**
Good vs. Bad Learners	98	83	0.784%
Hive 2 vs. Hive 1	64	33	0.312%
Hive 3 vs. Hive 1	93	67	0.633%

8.4.4 Heat Map

A heat map, produced using the pheatmap R package (Kolde, 2019), displays the 100 top up or down regulated genes (Figure 8.7) and separates them by treatment [in this comparison only 98 significant DEGs were identified and so all are shown]. Visually, there is a distinct demarcation in expression levels between the top up and down regulated genes between the two treatments.

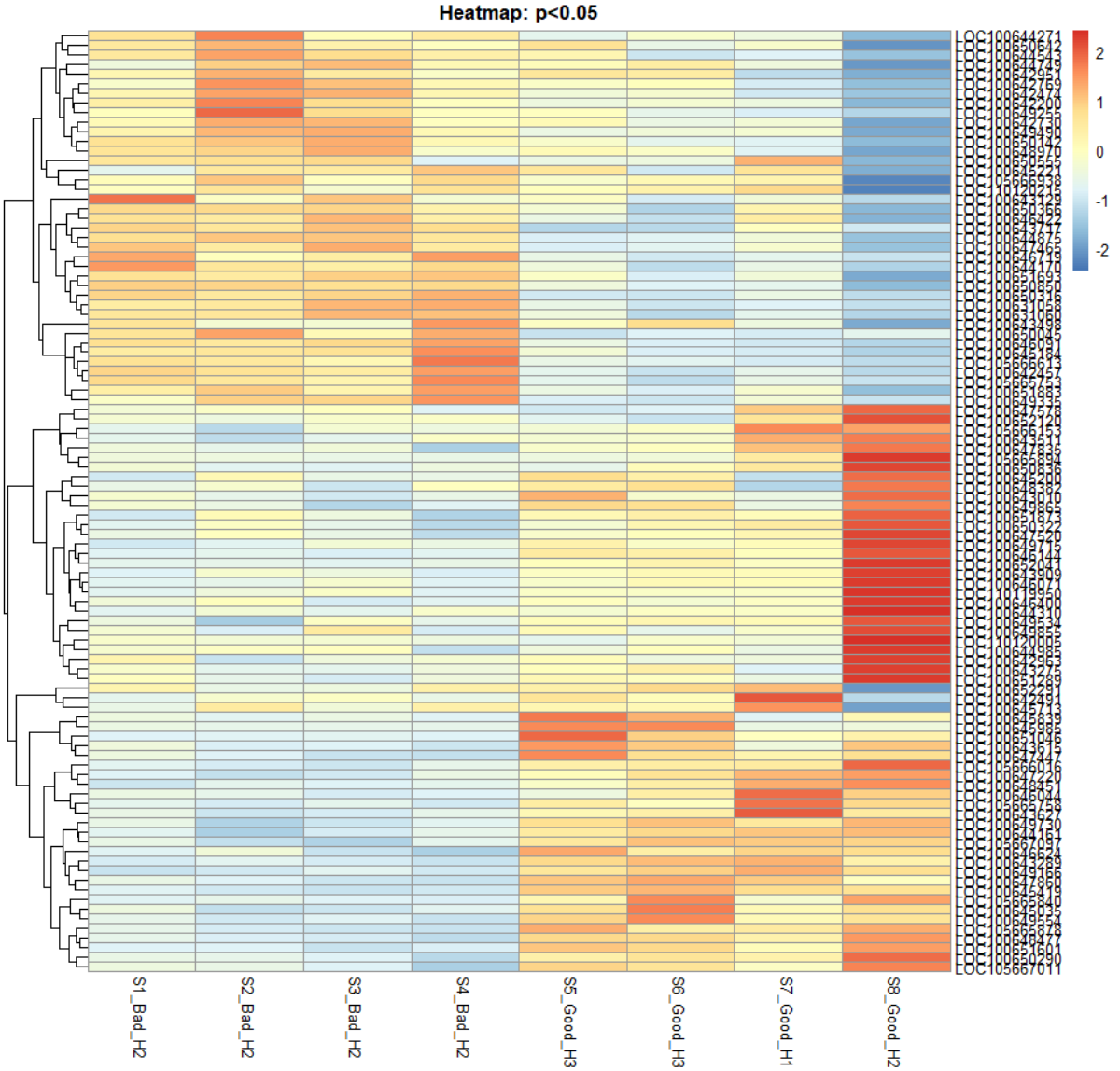

Figure 8.7: Heat map of top 100 up and down regulated genes between good and bad learners. Colour indicates Log2fold change. On the left are expression levels for the bad learners and on the right the good learners.

8.4.5 Gene ontology over representation analyses of DEGs

BLAST2GO was used to blast the whole *B. terrestris* genome against NCBI NR to identify all associated gene ontology (GO) terms. BLAST2GO was then used to conduct a Fisher's Exact Test, an over representation analysis (ORA) to identify GO terms which are over-represented in the list of 83 identified DEGs versus the whole *B. terrestris* genome. A p-value threshold of 0.05 was applied to detect significantly enriched GO terms and pathways. In total, 62 GO terms were over-represented with p-value<0.05 and 22 GO terms with p-value<0.01.

The 62 over-represented GO terms associated with the 83 (<0.05 adjusted p-value filtered) DEGs identified between good and bad learners are summarized in Figure 8.8 (see Chapter 8 Appendix Table A8.2 for full list of the 62 GO terms). The DEGs are annotated into three main categories; 'Molecular Function', 'Cellular Component' and 'Biological Process' and further grouped into smaller sub-categories dependent on function (Figure 8.8). The number of genes in each GO term sub-category is displayed above the bars. Of the 62 over-represented GO terms, 26 were classified as 'Biological Process', 6 as 'Cellular Component' and 30 as 'Molecular Function'. The ORA is useful in a broad sense, but the enriched terms are too high level and unspecific to be useful alone.

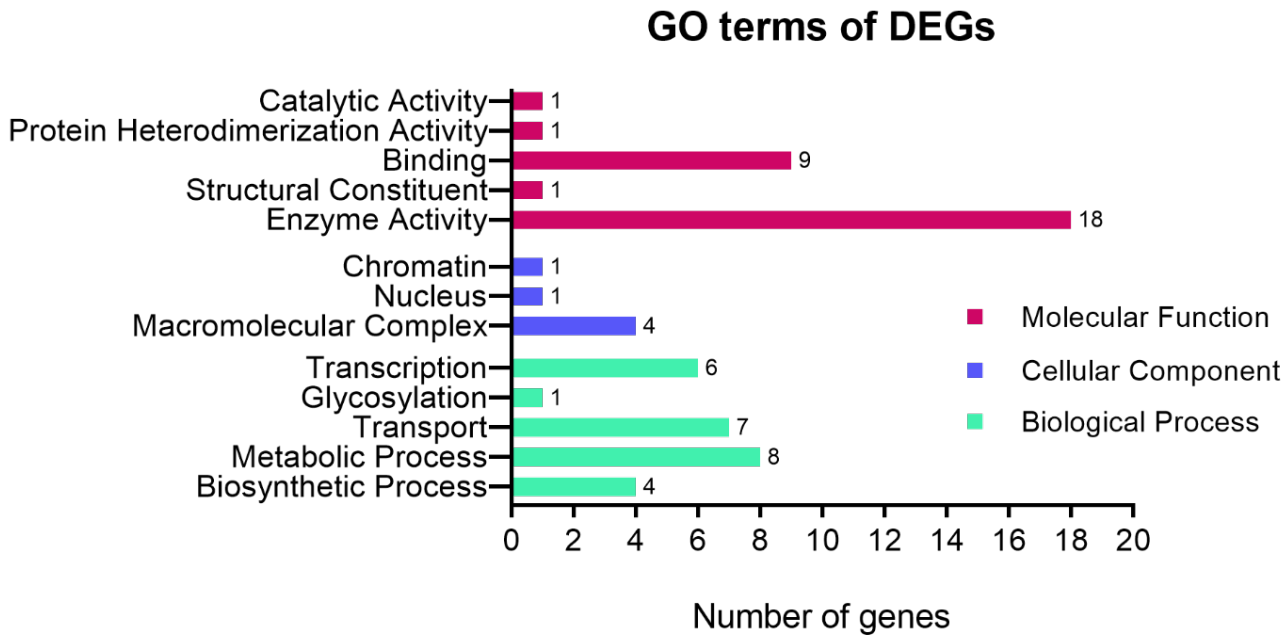

Figure 8.8: Summary of the 62 over-represented GO terms associated with the 83 DEGs between good and bad learning groups.

8.4.6 Further identifying DEG function with the Kyoto Encyclopaedia of Genes and Genomes (KEGG)

All KO identifiers and KEGG definitions linked to the 83 DEGs can be found in full in supplementary material S2. KEGG terms which stood out for their potential relevance for learning and memory are listed below.

Aromatic-L-amino-acid decarboxylase

Gene LOC100648451, associated with aromatic-L-amino-acid decarboxylase, was significantly downregulated ($p = 0.0000001$, log2 fold change = -2.45) in the bad bee learner group versus the good learner group (Figure 8.9.A). Aromatic-L-amino-acid decarboxylase, also referred to as L-dopa decarboxylase or tryptophan decarboxylase, catalyses several important decarboxylation reactions including; tryptophan to tryptamine (Ishii et al., 1996), L-DOPA to dopamine, L-Phenylalanine to phenethylamine, L-Tyrosine to tyramine, L-Histidine to histamine and 5-HTP to serotonin. Perhaps most relevant of these reactions for learning and memory is the decarboxylation of L-DOPA to dopamine and 5-HTP to serotonin, both biogenic amines important for associative learning reinforcement (Sitaraman et al., 2008). Dopamine neurons play a crucial role in insect learning and memory systems and have a well-documented role in insect aversive learning performance in the fruit fly (Kim et al., 2007), honeybees (Blenau and Baumann, 2001; Blenau and Erber, 1998) and crickets (Mizunami et al., 2009; Terao and Mizunami, 2017). Significantly, dopaminergic neurons have terminals in the mushroom bodies (known for their key role in insect learning and memory) of the insect brain, which have been shown to be activated by aversive stimuli in both honeybee (Jarriault et al., 2018) and *Drosophila melanogaster* (Riemensperger et al., 2005). Inhibition of dopamine receptors has also been shown to impair aversive memory in *Drosophila* (Kim et al., 2007; Qin et al., 2012), further supporting the role of dopamine in insect aversive learning and memory. Conversely, serotonin has been shown to be vital for place learning and memory in *Drosophila* (Sitaraman et al., 2008). Given that the arena used in this study was an aversive, place learning assay, it seems highly relevant that bumblebees which were good at this task have significantly higher expression of a gene involved in serotonin and dopamine production, biogenic amines which are vital for place and aversive learning respectively.

Histone-lysine N-methyltransferase

Gene LOC100649554, associated with histone-lysine N-methyltransferase, was significantly downregulated (p = 0.001, log2 fold change = -1.07) in the bad learner group versus the good learner group (Figure 8.9.B). Histone methyltransferases are histone modification enzymes which catalyses the transfer of methyl groups to, in this case, the lysine residues of histones. Histone methylation is important for epigenetic activation, and epigenetic mechanisms are good candidates for the regulation of learning and memory related genes. The Western honeybee (*A. mellifera*) displays differential methylation of histones within its genome (Lyko et al., 2010) and distinctive histone modifications have been shown to play a role in memory formation and synaptic plasticity in rats (Miller et al., 2008). A previous study (Li et al., 2017), on the impact of DNA methylation on honeybee learning and memory, demonstrated that as well as differentially methylated specific learning and memory genes, key enzymes for histone methylation were also differentially methylated, suggesting a role of these enzymes in learning and memory of honeybees via regulation of other epigenetic modification processes. In honeybees methyltransferase function mediates the discriminatory power of associative long-term memory and adjusts the specificity of memories according to the learning context (Biergans et al., 2016, 2012), suggesting a potential role of methyltransferase upregulation in increased long-term memory abilities in our study's good bees.

Neurochondrin

Gene LOC105665758 which was significantly downregulated in the bad bees versus the good bees (p = 0.000006, log2 fold change = -1.29) (Figure 8.9.C), is suggested to be a neurochondrin homolog, a signal transduction protein involved in the regulation of neuronal synaptic plasticity, reportedly related to learning and memory (Zhang et al., 2015) and required for spatial learning in humans and mice (UniProt, 2020a, 2020b). The significantly large difference in expression of this neurochondrin homolog in the good bees of this study suggests a potential role for this gene in enhanced aversive learning ability in the bumblebee.

Neuropeptide CCHamide-2 receptor

The LOC105667011 gene is associated with the neuropeptide CCHamide-2 receptor and is significantly downregulated in the bad bee group (p = 0.005, log2 fold change = -1.2) (Figure

8.9.D). CCHamides are arthropod neuropeptides found in insects. CCHamide-1 and -2 are neuroendocrine peptides produced in the neurons (Ren et al., 2015), which activate G-protein coupled receptors in *Drosophila* (Hansen et al., 2011)

Differential expression of genes involved in non-learning and memory functions

Other genes may be of potential interest for their role in non-learning and memory functions, for example a gene involved in DNA repair (LOC100631060) is significantly upregulated ($p = 0.0003$, log2 fold change = +1.07) in the bad bee group (Figure 8.9.E). This could suggest that these bees are experiencing a higher physiological stress level under the aversive heat stimuli than the good bee group, and as a result are upregulating repair proteins to attempt to deal with their stressful environment. There is also differential expression of a gene involved in the production of cuticle protein (LOC100647447) ($p = 0.003$, log2 fold change = -1.3) (Figure 8.9.F), the significantly higher expression of this gene in the good bee group signifying that perhaps these bees are better able to deal with the stressful heat stimulus by increasing the protective properties of their exoskeleton. Although seemingly irrelevant, these differential expressions may demonstrate a genome response to the heat stress of the aversive learning environment, and an attempt to mediate stress through physiological responses, allowing bees which are better able to deal with stress to be more effective learners.

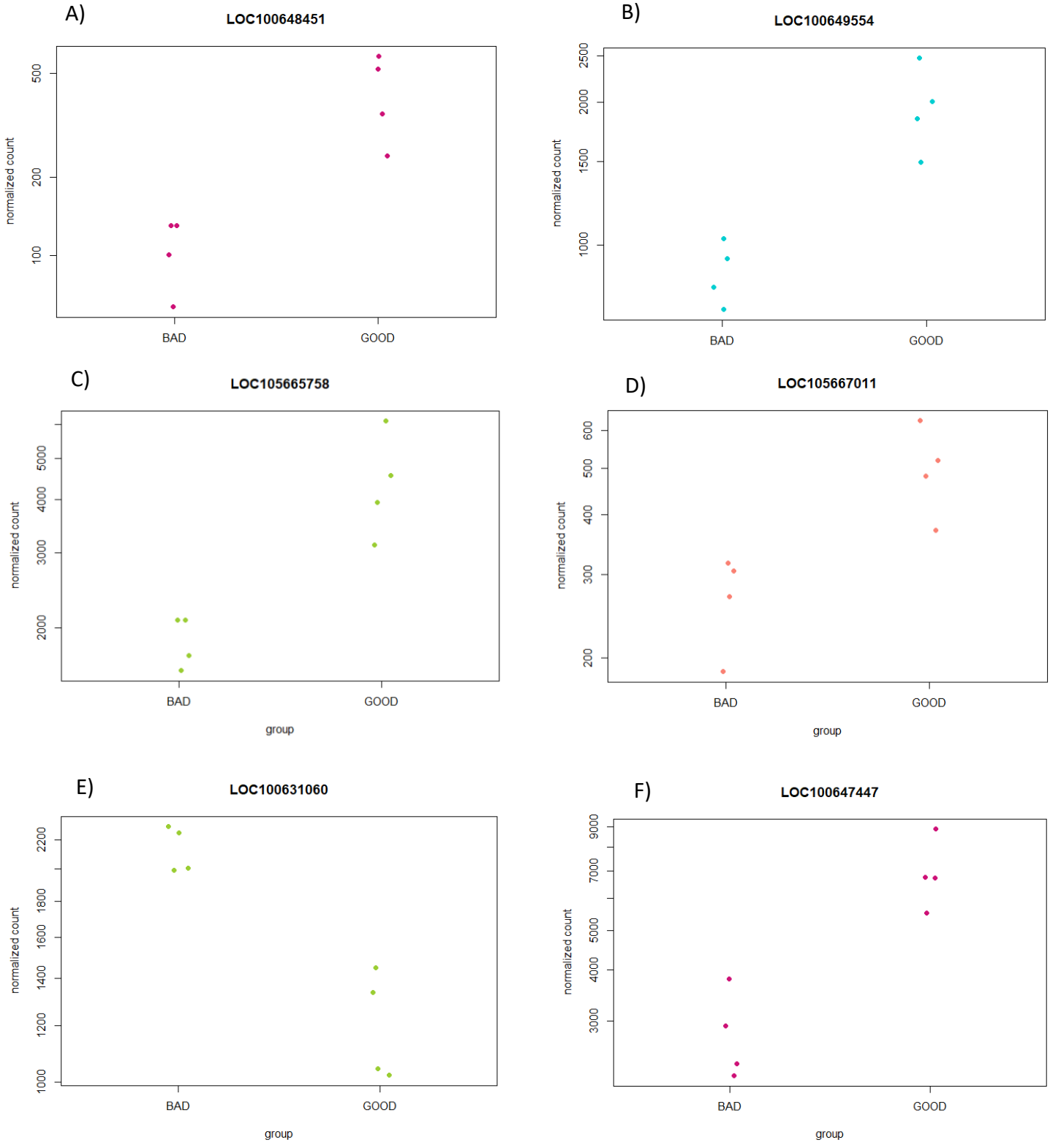

Figure 8.9 A-F: Gene expression counts of key genes between good and bad learner groups: A) Gene LOC100648451, associated with aromatic-L-amino-acid decarboxylase.

B) Gene LOC100649554, associated with histone-lysine N-methyltransferase. C) Gene LOC105665758, a suggested neurochondrin homolog. D) Gene LOC105667011, associated with the neuropeptide CCHamide-2 receptor. E) Gene LOC100631060, associated with DNA repair and F) Gene LOC100647447, associated with cuticle protein production.

8.5 Discussion

In this study gene expression changes after aversive place learning were compared between bees which were highly adept at the task ('good' bees) and those which were not ('bad' bees), from which 83 unique DEGs were identified. It must be stated that the observed gene expression changes are a comparative analysis between two sample types (good vs. bad bees), displaying different responses to the same aversive conditioning task; however, it is impossible to clearly separate the effects of aversive place learning from other sources and therefore it is important not to overstate the significance of the 83 DEGs identified, other than in relation to the good vs. bad bee comparison.

Bee behavioural traits have been linked to transcriptomic expression patterns in previous studies which have examined learning and memory related expression patterns including; at key time points after associative colour learning (111 genes) (Li et al., 2018), in bees which received and did not receive a visual learning task (388 genes) (Qin *et al.*, 2014), and olfactory learning (259 and 77 genes) (Cristino et al., 2014; Wang et al., 2013). As in this study (83 genes), these studies found relatively small numbers of DEGs between key groups and, as Li *et al*. (Li et al., 2018) note, this suggests that specific memory formation involves a moderate numbers of genes in comparison to the large numbers of genes associated with transitions from one behavioural or physiological life stage to another e.g. from a honeybee nurse to a forager (Whitfield et al., 2003) or environmental influences experienced whilst foraging (Lutz et al., 2012).

A study aiming to elucidate individual differences in cognition of *B. terrestris* foragers found that variation in learning and foraging performance could not be predicted by colony membership and that faster learners did not actually provide additional resource collection benefit over their lifetime (Evans, Smith and Raine, 2017). Fast learners collected food at comparable rates and conducted similar numbers of foraging bouts to slower learners (Evans, Smith and Raine, 2017). The study also found that fast learners foraged for fewer days, suggesting a potential fitness cost to their enhanced learning abilities. If, as Evans, Smith and Raine (2017) suggest, it is not beneficial to a hive's resource collection to have individuals with enhanced learning traits as hive members, then we would expect natural selection to act in favour of minimising such traits, and yet, large variation persists between individuals, both

across and within hives. Could it be that the existence of variation is adaptive in another way? Could there be alternative benefits, besides resource collection, to having a few particularly fast learners in a hive? Bees rely heavily on social learning and cues from other foragers when entering the foraging environment, particularly in the process of 'local enhancement' in which bumblebees are attracted to areas where others are already foraging (Leadbeater and Chittka, 2007). Furthermore, when bees forage with more experienced conspecifics they can more quickly learn which floral species are most rewarding (Leadbeater and Chittka, 2007). Therefore, these 'fast learners' may be acting as 'keystone' or 'activator' hive members (see Table 8.1 for definitions) by providing good foraging templates to other 'slower learning' nestmates whom are not as good at learning initial cues individually. It makes sense for a hive to produce individuals which may, alone, be slower learners, but that in a social learning setting can still learn the task and collect higher rewards over their lifetime as well as fast learning individuals which can grasp tasks quickly and act as learning templates. Hence, although fast learner lifetime resource collection is lower, these bees may afford essential learning prompts to other hive members, which could not forage as effectively without such cues. This could explain why such variation persists within hives, as both learning types could be essential to optimum hive foraging.

It is simplistic to assume that genetic variation is the only cause of interindividual differences in learning and behaviour. For example, the role of nutrition (de Brito Sanchez et al., 2008) or environmental factors such as pre-larval emergence conditions (e.g. temperature) (Jones et al., 2005) have been shown to play a role in individual behaviour and phenotypes. Nonetheless, the existence of 83 significantly different DEGs between our good and bad learner groups, suggests that there is clearly a strong transcriptomic profile to individual differences in cognition and learning ability. The fact that genetic variation exists to some extent, even between sisters of the same hive, is not wholly surprising as proximate causes such as recombination will introduce some genetic changes between individuals. Although, recombination rates have been shown to be relatively low in less social taxa such as *B. terrestris* (versus highly eusocial taxa such as honeybees) (Jeanson and Weidenmüller, 2014) and other proximate factors, such as polyandry (queen multiple matings) and polygyny (multiple queens), are unlikely to be causal in our bumblebee colonies.

It is therefore not the existence of such genetic variation, but specifically what this variation between individuals may be, that was of most interest in this study. Hence, in these analyses, we focused predominantly on the differences between those individuals identified as the best and the worst learners across the whole experiment, and not specifically the hive from which they came. This allowed us to identify the DEGs which were potentially involved in differential learning ability, regardless of the hive from which the bees came. Future avenues of analyses would be to further unpick the differences between individuals within the same hive, where we see some nest mates which outperform others in the learning task or some hives which outperform others. For example, bees from hive 2 in this experiment were, observationally, much poorer learners than hive 1 or 3 and thus made up the majority of the "bad" learners selected for analyses as we wished to capture the largest variation in learning ability between all individuals rather than specifically within hives (see Table 8.2).

The gene ontology results from our study may misleadingly infer the involvement of genes which are not solely brain derived, since the RNA used for transcriptomic analysis was obtained from bee heads rather than dissected bee brains. For example, the identification of actin related genes which are associated with muscle fibres in the head rather than the brain itself. This makes it tricky to unpick which DEGs are brain specific. However, as it is genes predominantly involved in learning and memory which are of most interest here, and good model insects with well-defined learning and memory pathways exist, candidate genes of interest are relatively straight forward to identify.

Epigenetic mechanisms have the potential to result in large individual changes and could therefore account for a large portion of the intra-hive variation we see between bees' learning ability. However, the tools used for the molecular analyses (RNA-seq) will probably not pick up these epigenetic changes, which are predominantly driven by small/micro RNAs which are too short to be identified by the RNA seq. This suggests that the majority of the DEGs we see between good and bad learners within a hive are not epigenetic in nature.

Studies, such as this one, which examine the underlying drivers of individual variation within bee populations are crucial to our understanding of the social organisation of hives and how variation can persist in traits such as learning which we would expect to be optimised. RNA seq and bioinformatic analyses provide crucial tools to allow us to begin to unpick the role of genetic variation in observed learning phenotypes. However, how underlying genetic

variation, such as that which we have begun to identify here, interacts with other causes of phenotypic variation, such as nutrition and environment, in bumblebees is still not wholly understood and presents an avenue for further research.

Junca *et al.* (2019) noted a trade-off between appetitive and aversive learning abilities in bees, suggesting that the "good" bees identified in this study were indeed good aversive learners. However, it does not necessarily mean that the "bad" bees were bad learners; instead, these bees may simply be biased towards appetitive learning, demonstrating a trade-off, resulting in poor aversive learning ability. I must therefore clarify here that we can only be certain that the "good" bees are good at this aversive task and may in fact have been poor learners if given an alternative, appetitive paradigm.

Further to this, we cannot necessarily determine that bees which were better at the aversive task were always better at aversive "learning" per say. It may in fact be that these bees are simply better at coping with the stressful heat environment e.g. they are more resistant to heat, they have thicker exoskeleton etc. We see some suggestion of this in non-learning and memory related DEGs between the groups. For example, gene LOC100631060, involved in DNA repair, is significantly upregulated ($p = 0.0003$) in the bad bee group (Figure 8.9.E), perhaps suggesting that these "bad" bees are experiencing a higher level of DNA damage or physiological stress from the heat stimuli than the good bee group. The cuticle protein gene LOC100647447 is significantly higher expressed ($p = 0.003$) (Figure 8.9.F) in the good bee group, suggesting perhaps that these bees are better able to respond to the stressful heat stimulus. The observed genetic differences between the 'good' and the 'bad' groups could also be a result of the observed differences in behaviour between bees, e.g. bad learners travelled more/ covered a greater distance and expressed a different set of genes as a result.

This pilot study has certain flaws, primarily due to the small sample size used. However, it has been a highly useful exercise in identifying potential candidate genes with roles in learning and memory between our "good" and "bad" aversive learner groups. Also, due to the nature of the experiment, there were issues in the allocation of a realistic control condition. Nominal 'controls' were taken from each hive (samples LJ10, LJ11 and LJ12), and placed in the thermal-visual arena with no training stimuli i.e. no heated floor or cool reward zone and ten trials were conducted, as for the aversive treatment. This meant that the control bees were not required to learn a task but were still placed in the arena to observe if bees showed any bias

to certain movement patterns or trajectories in the arena alone. These controls were selected from each hive and age cohort to match the bees in the aversive training condition. However, these control bees in reality do not provide a good genetic base line to the bees which were 'good' or 'bad' learners from the same hive, as only one bee from each hive was taken and there is no way of telling whether these bees would have been good or bad learners themselves. It was therefore decided to remove these control bees from the bioinformatics analyses early on, as they would skew the true signal of interest; the genetic contrast between good and bad learners.

Future follow up studies to this pilot could include, verifying candidate genes identified from this bioinformatic analysis using quantitative polymerase chain reaction (qPCR) or knock outs of genes involved in potential enhanced aversive and place learning abilities, e.g. Gene LOC100648451 associated with aromatic-L-amino-acid decarboxylase and serotonin and dopamine production. The role of dopamine and serotonin in learning is well documented in honeybees and drosophila (Agarwal et al., 2011; Blenau and Erber, 1998; Kim et al., 2007; Riemensperger et al., 2005; Sitaraman et al., 2008; Terao and Mizunami, 2017), but has never been shown in bumblebees, although we would expect similar results due to shared neural circuitry, it would be apt to test the role of such genes through knockouts in the bumblebee. The bee selection process (Table 8.2) should also be revisited, as I believe the selection criteria for each bee can be more succinctly developed into a 'bee learning score' which accumulates individual's scores across the different behavioural parameters into a singular value, allowing for easy visualisation of a bee's overall ability in the thermal-visual arena.

If this pilot study could be scaled up into a larger design with a bigger resource allocation (staff and consumables) it would be ideal to properly run the three conditions, 1) Good bees, 2) Bad bees and 3) Control bees, across three hives again but with six bees in each condition for each hive (a total of 54 bees). In this context, a larger subset of each hive would be taken as controls (6 bees) and instead of placing these controls in the thermal-visual arena where they are exposed to foraging stimuli such as light, colour and pattern, they should be taken directly from the hive where they have had very limited external-hive stimuli exposure. In this way, these control bees could provide an in-hive genetic base line with limited learning experience. There is still no way to determine whether these bees would be 'good' or 'bad' at the aversive learning task if given the chance, but it would allow determination of the DEGs of good vs.

bad bees exposed to a task vs. bees of the same hive with highly limited stimuli exposure – an improvement on the previous controls design. Assuming an approximate estimate of £200 per RNA sequencing sample, this theoretical study would cost approximately £10,000, a consumables budget not achievable within this PhD's scope. Furthermore, in this type of experiment we can never truly state causation between the identified DEGs and differential bee learning ability. Therefore, it must be considered whether such an experiment be worth the investment, if one could never definitively link these genes to bee IQ. It can also be questioned whether you can ever really achieve full biological replicates due to epigenetic mechanisms and alternative genetic variation outside of our control.

Chapter 9

A neonicotinoid pesticide, imidacloprid, impacts foraging behaviour, but not associative or reversal learning in *Bombus terrestris*

Chapter 9: A neonicotinoid pesticide, imidacloprid, impacts foraging behaviour, but not associative or reversal learning in *Bombus terrestris*.

9.1 Introduction

Many managed and wild bee populations are reported to be in decline worldwide (Ellis et al., 2010; Goulson et al., 2008; Potts et al., 2010) and it has been suggested that up to a third of bumblebee species may be affected (Arbetman et al., 2017; Colla and Packer, 2008; Williams and Osborne, 2009). This is highly concerning given that bumblebees are vital crop and wildflower pollinators (Cock et al., 2012; Garratt et al., 2014; Goulson, 2010; Potts et al., 2016; Sowmya et al., 2015).

Neonicotinoids have been implicated in global bee declines (Banks et al., 2020; Bryden et al., 2013; Tomé et al., 2020) and a diverse range of sublethal effects on bees have been documented at both acute and chronic exposure levels (reviewed in Blacquière et al., 2012; Lundin et al., 2015). Bees typically become exposed to neonicotinoids through contaminated nectar and pollen collected by foragers (Codling et al., 2016; Rortais et al., 2005) and exposure can have diverse and wide reaching individual and hive effects. For individual bees there are effects on behaviour, foraging, motivation, thermal regulation, gene expression, motor function or learning ability (Charreton et al., 2015; Colgan et al., 2019; Jacob et al., 2019; Lämsä et al., 2018; Dara A. Stanley et al., 2015; Stanley et al., 2016; Tison et al., 2016; Tosi et al., 2016; Whitehorn et al., 2017; Williamson et al., 2014; Wright et al., 2015) and at a hive level, reports include effects on queen reproduction, genetic diversity, colony development and survival (Brandt et al., 2017; Doublet et al., 2015; Ellis et al., 2017; Forfert et al., 2017; Laycock et al., 2012; Lu et al., 2014; Samson-Robert et al., 2017; Sandrock et al., 2014b, 2014a; Whitehorn et al., 2012; Williams et al., 2015; Wood and Goulson, 2017).

The neonicotinoid imidacloprid is effective against a wide range of crop pests, including, whiteflies, aphids, lepidopterans, coleopterans and heteropterans (Elbert et al., 1991). The LD_{50} (median lethal dose) of imidacloprid varies considerably, depending on the method of exposure and the species tested, Oral LD_{50} values have been reported at between 184–

6000ppb for *A. mellifera* when ingested orally (Fairbrother et al., 2014) and a similar LD_{50} value has been reported for bumblebee species (*Bombus impatiens*) (Scott-Dupree et al., 2009). This highly variable range makes pesticide management decisions extremely difficult and there is a need to look at further, non-mortality-based assessments. It is unlikely that imidacloprid poses a lethal threat to bees in the field, as it is commonly found at levels around 10 ppb in agricultural settings (Cresswell, 2011), far below the reported LD_{50} levels for honeybees and bumblebees. However, there is increasing evidence of deleterious sublethal and chronic effects in honeybees, bumblebees and solitary bees, including on mobility (Moffat et al., 2016), feeding (Laycock et al., 2012), body mass (Anderson and Harmon-Threatt, 2019), sonication or 'buzz pollination' (Switzer and Combes, 2016), gene expression and physiology (De Smet et al., 2017), resource collection and visual and olfactory learning (Decourtye et al., 2003; Phelps et al., 2018). However, these findings are often diverse and sometimes contradictory. For example, one set of studies found that imidacloprid exposure negatively impacts olfactory associative learning (Williamson and Wright, 2013) and medium-term memory retention (Decourtye et al., 2004), whereas other studies have found that exposure can actually enhance olfactory learning and memory (Lambin et al., 2001; Williamson et al., 2013). Such effects are inherently dose dependent and what is considered a field realistic dose varies across studies, as does the methodology and outcomes reported for the same pesticides. In view of such discrepancies, there is an increasing need for assessment paradigms which closely mimic field exposure levels and allow bees to behave in natural ways to build a realistic picture of likely wild-bee impacts.

Whilst collecting pollen and nectar, foraging bees rely on a multitude of cues (e.g. colour, odour, pattern) to learn reward associations (Leonard and Masek, 2014; Muth et al., 2016; Nicholls and De Ibarra, 2014), among which colour plays a central role in flower recognition during foraging trips (Backhaus, 1993; de Camargo et al., 2019; Giurfa et al., 1995; Lunau et al., 1996; Lunau and Maier, 1995; Raine and Chittka, 2007) and is the most important floral attractant signal even under differing environmental conditions (Dyer and Chittka, 2004). Therefore, the study of potential effects of neonicotinoids on colour association learning and memory performance as an endpoint in ecotoxicology tests is ecologically relevant. Especially since individual learning and memory disruption may indirectly account for changes in colony

survival, particularly in bumblebee species with much smaller colony sizes compared to honeybees.

Bee brain function, has been shown to be subject to 'extinction', a process whereby a conditioned behaviour decreases upon failure of reinforcement (withdrawal of the US) (Eisenhardt, 2012, 2014; Myers and Davis, 2002). Such extinction or 'reversal learning' enables animals to rapidly and appropriately respond to change within their environments and is considered to require complex brain functioning to be able to acquire new memories when previously learnt associations are no longer valid (Myers and Davis, 2002). Extinction learning is highly relevant to bees, playing a role in the adaptive behaviour necessary to forage on variable floral resources within a landscape. Successful foraging requires that bees are able to exploit profitable food sources in a landscape where food availability (nectar and pollen) varies tremendously across the foraging season, and sucrose concentration and volume varies across and within floral patches (Núñez, 1977; Percival, 1946; Rathcke, 1992). To maximize food collection, foraging bees must continually adapt to these changes. Foragers tend to choose food sources with the best reward/cost ratio (termed maximization) and when the reward decreases they switch to an alternative food source (Greggers and Menzel, 1993). Bees therefore adjust their foraging strategies based on previously experienced rewards, and to do this they must form short-term memories (STM) of the most recently experienced reward, which they then match with the actually occurring reward (Greggers and Menzel, 1993; Waddington and Holden, 1979). Based on this comparison the bee makes a decision to stay at their current food source or shift to a new one (Greggers and Menzel, 1993; Visscher and Seeley, 1982; Waddington and Holden, 1979).

Studies have used extinction learning paradigms to test an individual's ability to extinguish an acquired learnt association when it is no longer rewarded (Bevilaqua et al., 2008; Münch et al., 2010; Schulz et al., 2007). In honeybees, it has been shown that extinction does not lead to the forgetting or deletion of the memory, but rather to the consolidation of an opposing memory that the conditioned stimulus (CS) now does not equal a unconditioned stimulus (US) reward (Bouton, 2004) and the transient suppression of the previously learnt memory (Myers and Davis, 2002). This allows a 'renewal effect', whereby, once the learning context changes again, a robust return to the original conditioned response is seen (Bouton and Bolles, 1979;

Bouton and King, 1983). As for the study of pesticide impacts on bees, the study of extinction learning has largely focused on harnessed honeybees (reviewed in Eisenhardt, 2012).

9.1.1 Research gaps

88% of the studies identified in Muth and Leonard's (2019) review of pesticide impacts on bee learning and memory, used the PER protocol and over 90% of the studies used olfactory conditioning to assess pesticide impacts on cognition (Muth & Leonard, 2019) (Figure 9.1). The PER has been invaluable in assessing many aspects of bee learning and memory (reviewed in Giurfa & Sandoz, 2012). However, a potential drawback of PER is that bees are harnessed throughout the experiments, only allowing the bee's antennae and proboscis to move. This restricted movement is not representative of a realistic foraging and learning scenario for bees and studies have reported clear differences in several responses. For example a willingness to accept toxic substances (Ayestaran et al., 2010), or different sucrose concentrations (Mujagic and Erber, 2009) varies between bees which are harnessed or free moving. There is a need for more holistic pesticide assessments involving free moving (e.g. flying, or walking) bees to ensure findings are more field representative. There is clearly also a need for further non-*Apis* pesticide assessments on bee learning and memory (discussed in Chapter 2) (Figure 9.1). Non-olfactory based learning paradigms need to be developed in order to fully assess whether pesticides are impacting learning in the field.

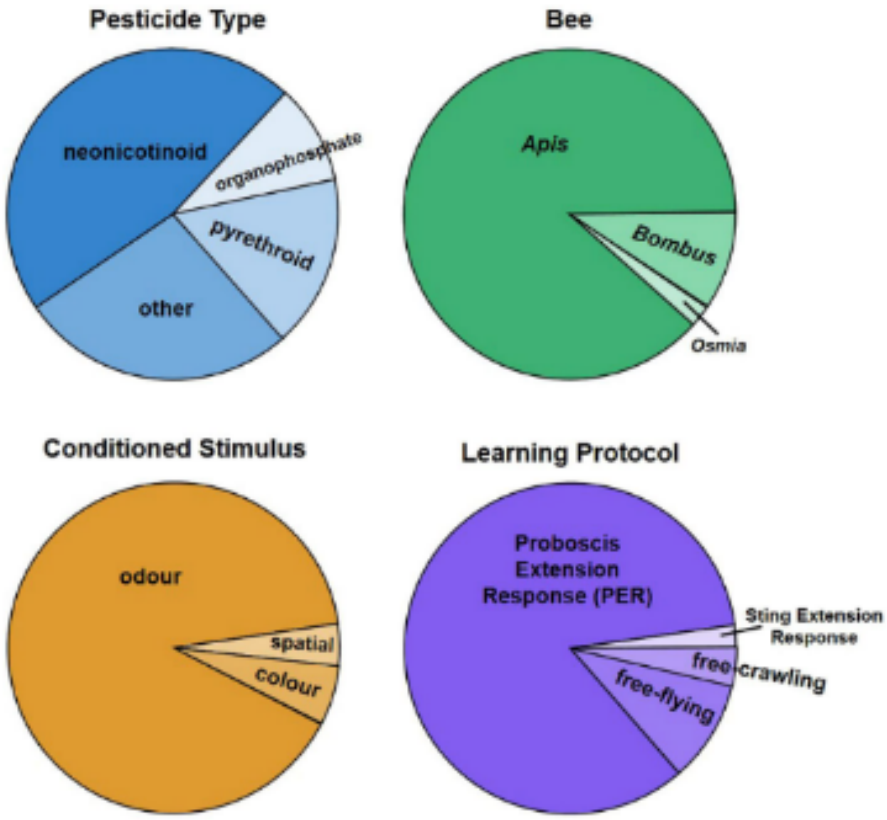

Figure 9.1: Summary of methodologies and study species used in 50 studies addressing the impact of pesticide exposure on bee learning. Taken from Muth and Leonard (2019).

9.1.2 Study aims

Previous studies have demonstrated that acute imidacloprid exposure affects foraging motivation but not visual association ability in the bumblebee *Bombus impatients* (Muth and Leonard, 2019). Here, we take this further and examine the effect of a chronic, realistic imidacloprid dietary exposure, on bumblebee (*B. terrestris*) foragers' ability to both colour association and extinction learn. We examine the 'renewal effect' (Bouton and Bolles, 1979; Bouton and King, 1983) over a series of training trials, in which the original association is presented, extinguished and then presented again in an 'A, B, A' fashion. We further conducted the first free-flying study into the impacts of chronic imidacloprid exposure on the ability of *B. terrestris* to first learn and then extinction learn visual colour associations.

9.2 Methods

9.2.1 Bee colonies and test subjects

Queen right colonies (n = 4) of *Bombus terrestris audax,* with approximately 80 workers, were obtained from Biobest (Biobest, Westerlo, Belgium). Colonies were split into queen-less microcolonies of 20 workers following the protocol described in Mommaerts *et al.* (2010). To allow the randomisation of treatments across the microcolonies. Only one bee from each microcolony is then used for the imidacloprid treatment, preventing bees taking imidacloprid contaminated food back to the hive and potentially exposing the entire microcolony to the compound. When not being used for testing, microcolonies were maintained on 30% sucrose solution provided in a gravity feeder in the hive's foraging tunnel and honeybee-collected pollen placed directly into the hive (both purchased from Biobest, Westerlo, Belgium). The colonies were maintained at 22°C on a day: night cycle of 16:8 hrs with fluorescent lighting.

9.2.2 Arena design

When a microcolony was selected for testing, the hive's foraging tunnel was connected to a large flight arena (12mm plywood, L x W x H: 65 x 45 x 25 cm) where all testing took place. The arena was lit from above with LED light strips (2100 lumens, colour temperature 6500K) and the floor was covered in white paper, which was replaced between trials to prevent scent marking by bees and to keep the arena clean. The walls of the arena were covered in a red tinted optic flow pattern (Figure 9.2) to provide optic flow stimulation for the foraging bees, necessary for positioning and speed control (Linander et al., 2015). The roof of the arena consisted of two clear Perspex sheets to facilitate filming of the trials from above, and to accommodate easy topping up of the reward solutions during trials. One side of the arena had a hinged trapdoor with spring clips to allow for the wall to be removed to configure the colour array between trials. An entrance hole cut into one of the end walls allowed easy connection to microcolonies via a foraging tunnel.

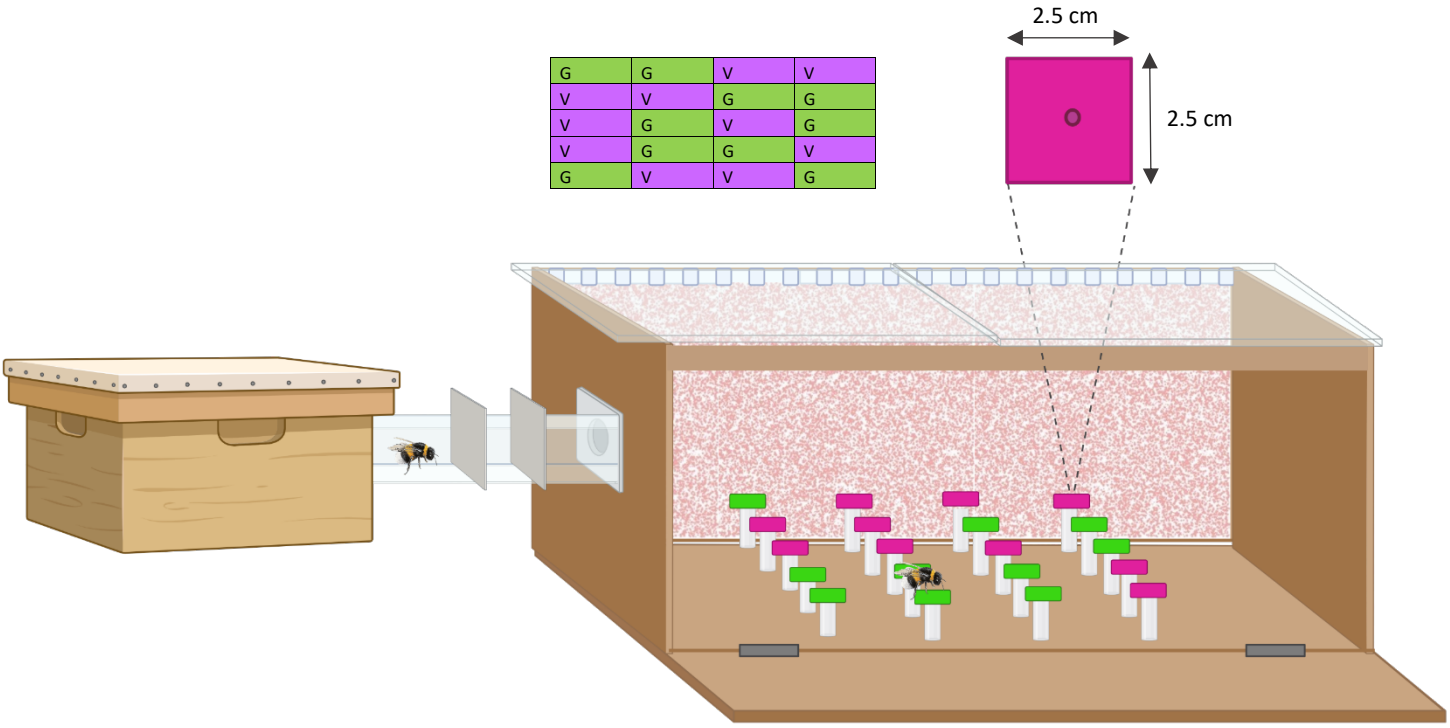

Figure 9.2: Arena design. Example random colour array and a schematic of a flower chip are given above the arena.

Colour selection and generation of random colour arrays

The Floral Reflectance Database (FRED) (Armold et al., 2010) was used to select three colours which were spatially distant on the loci of chip colour in the bee visual space, these were human-violet (colour reference 8T01), green (colour reference 6205) and yellow (colour reference 250) (www.perspex.co.uk). 3mm thick Perspex samples of these three colours were obtained from perspex.co.uk.

The three coloured samples were sent to the Chittka lab at Queen Mary University, London for spectral testing (under standardised conditions as described in Chittka & Kevan, 2005) to select the two colours which were a) most easily visible and b) most easily distinguishable in bumblebee colour vision, using the hexagonal model of bee colour space (Chittka, 1992) (Figure 9.3). This showed that although bees could differentiate between any of the three colours, the green and the yellow would be harder to tell apart and appeared most visually

similar, therefore violet and green were selected as the two most differentiated colours to ensure easy colour-reward association learning.

A range of random arrays of the two colours were then generated in Microsoft excel by creating a randomly ordered list of ten violet and ten green options (Figure 9.2). One of these arrays was selected at random using a random generator to select from 12 arrays, prior to the start of each foraging bout (defined as when a bee makes one or more choices and then returns to the hive to deposit the sucrose solution collected). Arrays were recreated with coloured artificial 'flowers' ((2.5 x 2.5 cm squares with a 4mm well drilled in the centre, capable of holding a volume of 10 µl) created from the Perspex chips in a 5x4 grid within the arena (Figure 9.2). The flowers were adhered to the top of small glass vials (positioned within the arena) using white tac to allow easy removal, cleaning and rearrangement between foraging bouts. The glass vials were also white tacked to the arena's floor to provide stability when the bees landed. This gave random colour locations within the array between each foraging bout but with the number of unrewarding and rewarding chips remaining constant (ten of each colour). A new array was used each time a bee started a new foraging bout to ensure that bees were learning the rewarding colour and not the rewarding locations within the array.

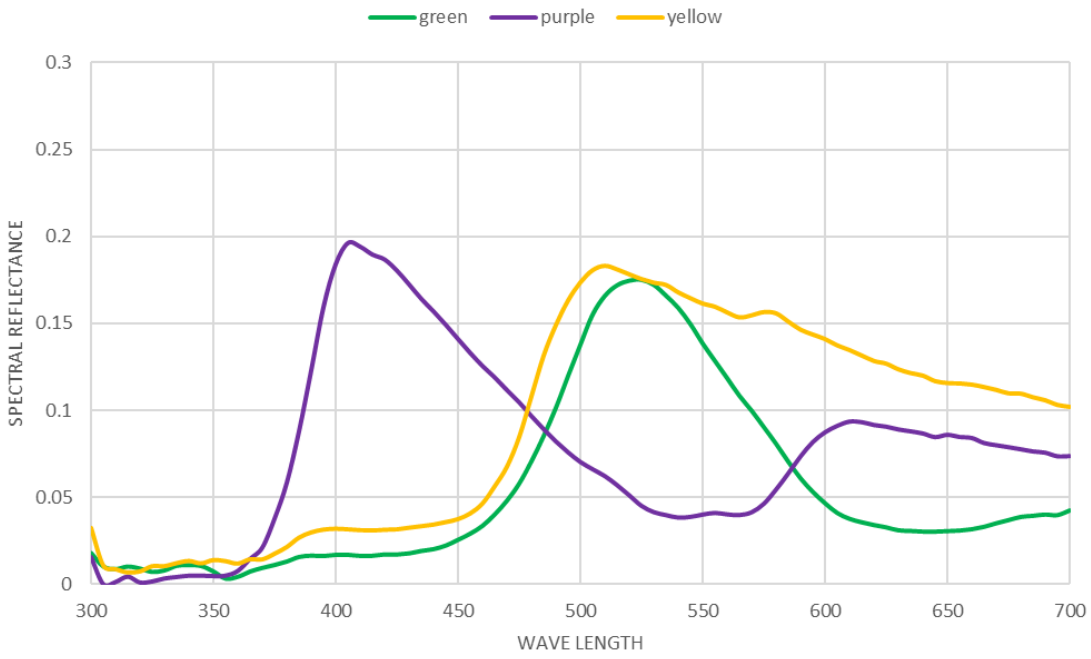

Figure 9.3: Spectral reflectance of violet, green and yellow demonstrating that in bee-vision, green and violet are the most distinct.

Trial recording

All trials were filmed using a Sony handycam (CX450 Handycam®, SONY) mounted on a cross bar above the arena. This allowed visualisation of the full flight arena during recording.

9.2.3 Training and testing

An outline of the pre-training, probe, testing and re-testing process is shown in Figure 9.4.

Mass feeder pre-training

Once a microcolony was selected for testing and connected to the arena, all hive members were given access to a colourless mass feeder placed in the centre of the arena, and bees which successfully foraged from it were tagged with coloured bee marking discs (EH Thorne, Market Rasen, UK) (Figures 9.4.1 and 9.5.A).

Clear chip pre-training

Bees which were successful at feeding from the mass feeder in the arena were then given access to the arena, equipped with an array of clear artificial flowers, each containing a 10µl 50% sucrose reward (Figures 9.4.1 and 9.5.A), the flowers were continually replenished with sucrose as they became depleted by the foraging bees. This pre-training stage was designed to select foragers which were successfully able to fly to and forage from the artificial flowers, whilst not being exposed to colour stimuli. Foragers were observed and those which could forage successfully from the clear chips had their tag numbers recorded, along with how many artificial flowers they foraged from in each foraging bout and their inter-trip intervals (time remained in the hive between foraging bouts). The bees which were the most reliable foragers (small inter-trip intervals and multiple flower choices) from each microcolony were then taken forward to the testing trial.

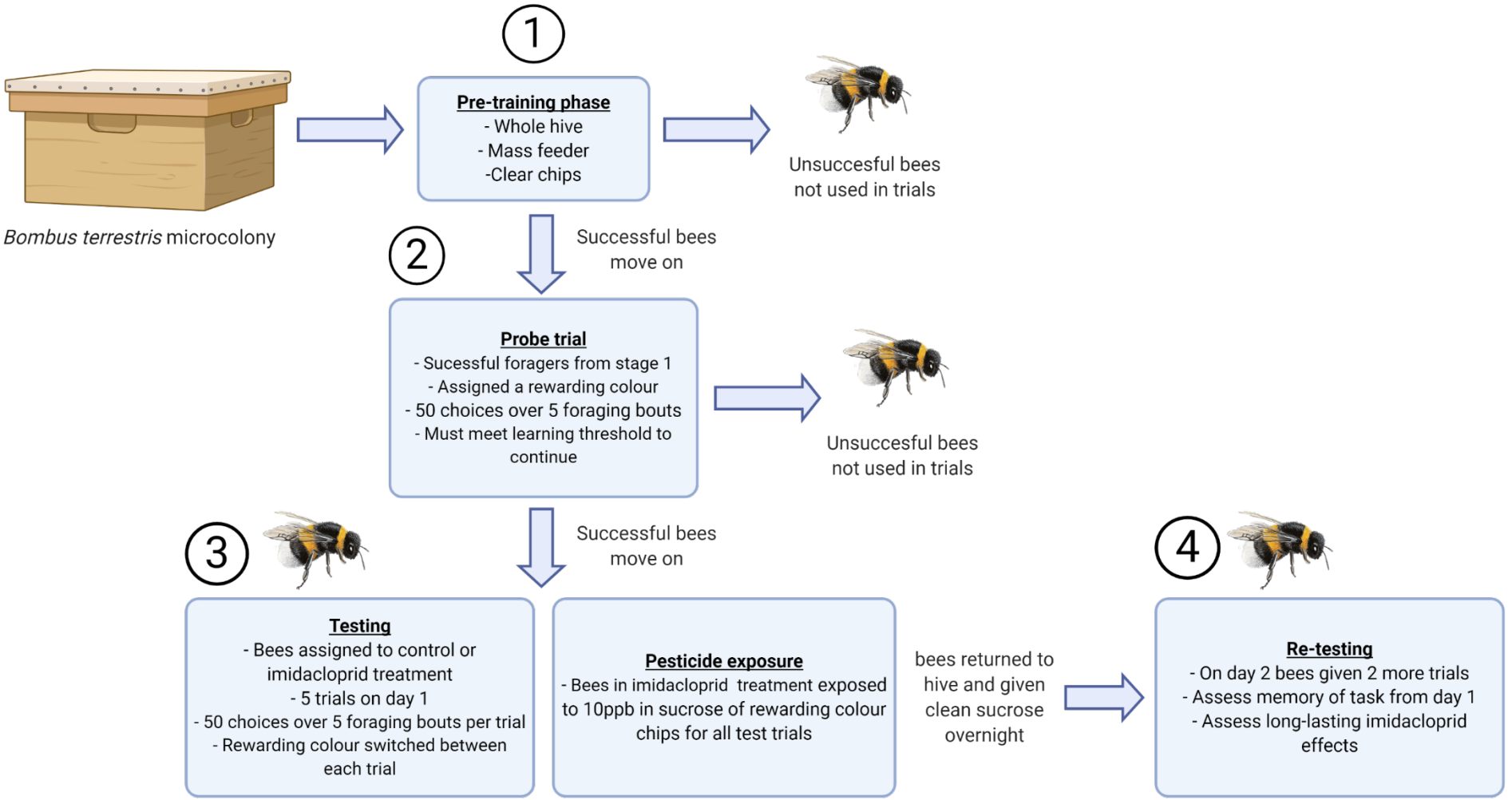

Figure 9.4 (1-4): Outline of the pre-training, probe, testing and re-testing process for bees involved in the colour trials. 1) The pre-training phase. 2) The probe trial phase. 3) The Testing and pesticide exposure phase. 4) The re-testing phase.

Figure 9.5 (A and B): A) Mass feeder placed in the arena showing tagged bees. B) Clear chips used for pre-training of foragers.

Probe trial

Bees which were deemed to be successful foragers at the pre-training stage were introduced into the arena individually to prevent any social learning of colour-reward associations. Each bee was randomly assigned either green or violet as the rewarding colour for trial 1 (the probe trial) (Figure 9.4.2). The wells in the designated rewarding flowers were filled with a 10µl 30% (w/w) sucrose reward, a concentration close to that of nectar and often used in bee behavioural studies (Pyke and Waser, 1981; Scheiner et al., 2013; Tiedge and Lohaus, 2017). The non-rewarding flower colour wells contained 10µl water. A trial consisted of a minimum of 50 choices over five foraging bouts. No pesticide solutions were used in the probe trial, even for bees which went on to be exposed in the later test trials. To progress to the test trials, bees had to get to a minimum 80% learning threshold of correct choices across the probe trial.

Training: associative and extinction learning

Colour-reward associations were presented across a series of trials in an A, B, A, B fashion for 6 trials (including probe) and re-trialled (two trials) on the following day, as studies have demonstrated that the more extinction trials are applied, the more extinction is observed and that after 2+ trials a long term extinction memory is formed, remaining behaviourally visible

one day later (Stollhoff et al., 2005). Rewarding colour flowers were filled with a 10µl 30% (w/w) sucrose reward. Non-rewarding ones were filled with 10µl of water. The probe trial plus five additional test trials were conducted on day 1 of testing. All flowers were cleaned in 70% ethanol and left to dry between each foraging bout and between each trial. The rewarding colour of the artificial flowers was switched between each trial so that bees had to reverse learn the previously learnt colour association (e.g. that violet was previously the rewarding colour, but green is now rewarding and violet no longer so). This rewarding colour switching continued from the probe trial all the way through to retesting (a total of 8 trials).

Pesticide solutions and exposure

Bees which were assigned to the pesticide treatment were exposed from trial 2 (not in trial 1 – probe trial) to 10ppb imidacloprid in the 10µl sucrose rewards in the wells of the rewarding flower colour of each trial. 10ppb was chosen because it is within the concentration range commonly found in the pollen and nectar of crops i.e. 1-50ppb (Anderson and Harmon-Threatt, 2019; Cresswell, 2011; Goulson, 2013; Phelps et al., 2018) This concentration is significantly below the doses previously used in some bee behaviour imidacloprid exposure studies (Decourtye et al., 2004; Eiri and Nieh, 2012; Mengoni Goñalons et al., 2015; Tan et al., 2015; Williamson et al., 2013). Imidacloprid dilutions were prepared by dissolving analytical standard imidacloprid powder in acetone. Aliquots of this solution were then added to 30% (w/w) sucrose solution for use in experimental trials. Solutions were stored in a refrigerator at 4°C and fresh solutions were made up daily for testing. Bees in the control group were given 10µl clean 30% (w/w) sucrose rewards. The same amount of acetone was added to the clean sucrose solution as to the imidacloprid solution. The pesticide treated bees were exposed to the imidacloprid over the course of 5 trials on day 1, designed to mimic the way in which foraging bumblebees would be exposed to pesticides in the field whilst foraging on the nectar of treated crop plants. Two sets of each coloured flower were used, so that each flower colour had an imidacloprid set and a clean set, so that no control bees were accidentally exposed to imidacloprid residues. Chips were cleaned in 70% ethanol between all foraging bouts and trials.

Retesting

Retesting was conducted on the day after initial testing (day 2). Bees were given two more trials, one of each colour as the rewarding colour to test the memory of the task and a continuing ability to associatively and reversal learn colour associations. This also allowed us to study whether there were any longer-lasting effects of the imidacloprid exposure that may not have been observed during day 1. After the final retest trial, bees were flash frozen in liquid nitrogen and stored in a -80°C freezer.

9.2.4 Behavioural data recording and analyses

For each individual bee, for each trial, the bees' colour choice and whether this was the correct (rewarding colour) choice was recorded for a minimum of 50 choices over a minimum of five foraging bouts. Both of these conditions had to be met for a bee to move on to the next colour learning trial. The time at which bees made each choice was also recorded. A choice was defined as a bee landing on an artificial flower and extending its proboscis (as we assumed this to be when a bee considered that artificial flower rewarding and was searching for food).

From this data bee learning accuracy (% of correct choices within a trial), bee Learning Performance Index (LPI) scores (see below for calculation), time taken for a bee to make the first correct choice in a trial, and time taken for a bee to learn the colour association (considered to be when a bee made a minimum of five consecutive correct colour choices) were calculated.

Learning Performance Index score

The LPI scores were calculated for each bee for each trial, to give an indication of the rate of change of learning performance and variation in performance saturation. Scores were generated using an altered version of the method of Evans & Raine (2014). The score was the sum of the number of errors made by a bee in the first 5, 25 and 50 choices after probing a rewarding flower for the first time in a given trial. If there were not 50 choices made post probe trial, then the final choice was used as the 50^{th} reading. If no correct choice was made in the first 10 choices, then the 10^{th} choice was considered the probe trial. This produced a

maximum score of 50. A low LPI value suggested rapid learning, whereas higher values indicate slower learners. Imidacloprid-treated bees all ceased foraging at some point during the trials on day 1, making it impossible to calculate LPIs for these trials. Therefore, scores were only calculated for completed trials.

9.3 Results

9.3.1 Bee learning accuracy

Assessing learning accuracy by colour in the probe trial

No bees in the imidacloprid treatment were exposed to pesticides in the first, 'probe' trial (trial 1) as this trial was designed to screen for bees capable of getting > 80% correct colour choices. Only bees which achieved this threshold continued on to trial 2.

The accuracy of each bee (% correct choices) was assessed in relation to the rewarding colour (green or violet) which the bee was randomly assigned to determine whether the colour of the rewarding artificial flower had a significant influence on the accuracy of the bee's choices due to innate colour preferences. All bees which achieved > 80% correct choices were judged as able to perform this test even if they did not complete all trials through to fruition (e.g. died or ceased foraging).

This showed that regardless of which colour (green or violet) was assigned as a bee's first rewarding colour association, bees could readily make colour associations to either colour and learning accuracy did not differ significantly (unpaired t-test, violet n = 10, green n = 5, t = 0.04889, df = 13, P = 0.9618) (Figure 9.6). This was despite the fact that bees have been shown to have an innate preference for certain colours (e.g. blue and violet) (Gumbert, 2000; Lunau et al., 1996; Lunau and Maier, 1995), suggesting that innate colour preference did not significantly affect bee learning ability in this trial.

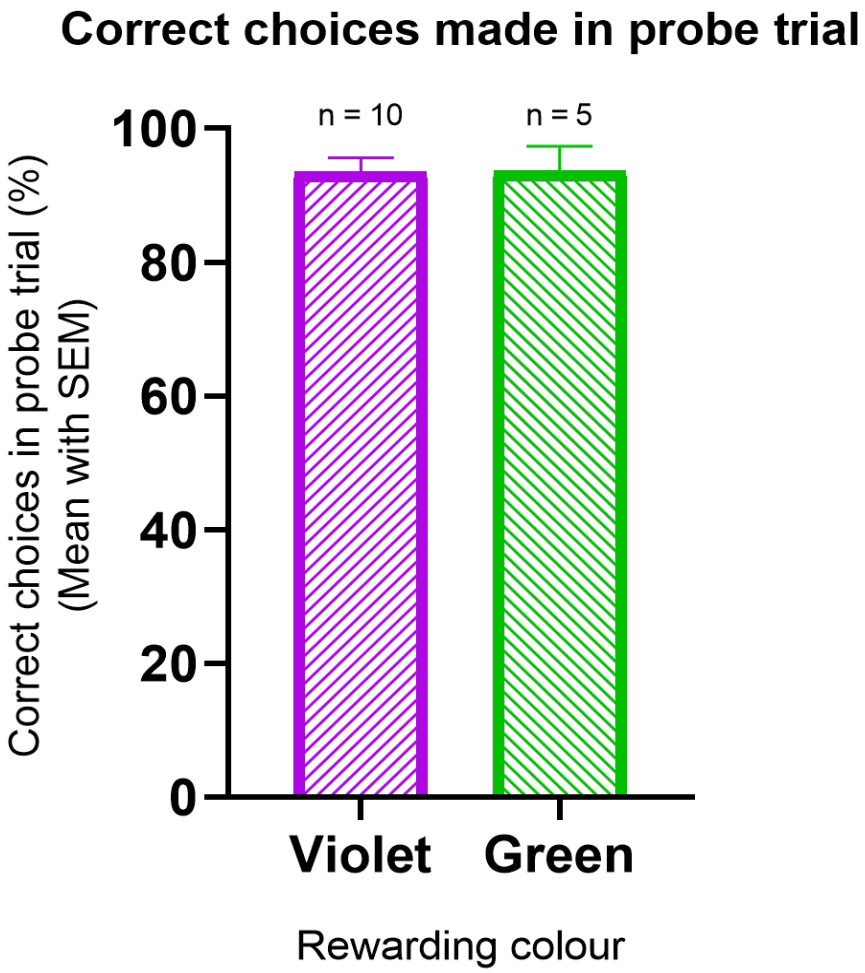

Figure 9.6: Percentage of correct choices made by bees in the probe trial, dependent on colour of rewarding chips assigned.

Bee learning accuracy by trial

Imidacloprid treated bees' and control bees' learning accuracy across each trial was assessed. Learning accuracy was calculated as the percentage of correct choices of all the choices the bee made in that trial. Figure 9.7 shows learning accuracy graphs for the control bees (n = 4) and Figure 9.8 shows graphs for the imidacloprid-treated bees (n = 5).

The control bees generally maintained a high level of learning accuracy across the trials (Figure 9.7). The exception being trial 2, and in the case of bee number 28 (B28) also in trial 3. Trial 2 is the first reversal trial in which the rewarding colour is switched and learning accuracy would be predicted to decrease when bees are exposed to novel learning stimuli

(new rewarding colour) prior to learning the new colour-reward association as their knowledge of the current association is being challenged. Once the new association has been learnt (after trial 2) there was a return to a high learning accuracy, and this continued in subsequent trials when the rewarding colour was switched again. This supports Bouton's (2004) assertion that in extinction learning memories are not extinguished, but instead opposing reward memories are consolidated, allowing a robust return to the original conditioned response when the rewarding colour is switched back.

The data for the imidacloprid-treated bees is harder to interpret, as they all ceased foraging at some point on day 1, making the calculation of learning accuracy for ceased trials impossible (ceased trials are indicated by a red hashed circle in Figure 9.8). There were varying degrees of learning accuracy post exposure (trial 2 onwards) and, as for the control bees, there was a decrease in learning accuracy for all bees in trial 2 (Figure 9.8). For three of the five bees (B2, B93 and B22) there was then an increase in learning accuracy post-trial 2, up until the trial at which the bee ceased foraging on day 1. This suggests that imidacloprid exposure is not affecting bees' ability to make colour associations, as if it were, we would expect this learning accuracy to either plateau or decrease as trials and therefore the consumption of imidacloprid progressed. Similarly, to control bee B28, imidacloprid-treated B80 also decreased learning accuracy in trial 3. However, by trial 4, B80's learning accuracy increased to a level even higher than in the probe trial, again suggesting that imidacloprid is not preventing the learning of colour associations in this context. Imidacloprid-treated B56 ceased foraging prior to trial 3 and did not forage again even on day 2. It is therefore impossible to assess learning accuracy for any trials post trial 2, but in trial 2 the bee did display the same decrease in learning accuracy as the controls and other imidacloprid-treated bees.

Perhaps the most interesting data for the imidacloprid exposed bees is the retesting on day 2 (for those bees which completed trials) (Figure 9.8), when B2, B93 and B80 (all of the bees which completed day 2 trials) showed a level of learning accuracy similar to day 1, suggesting that they have made long term memories of the colour associations made on day 1. This further supports the view that imidacloprid exposure is not affecting bees' ability to fix visual colour-reward associations into long term memory.

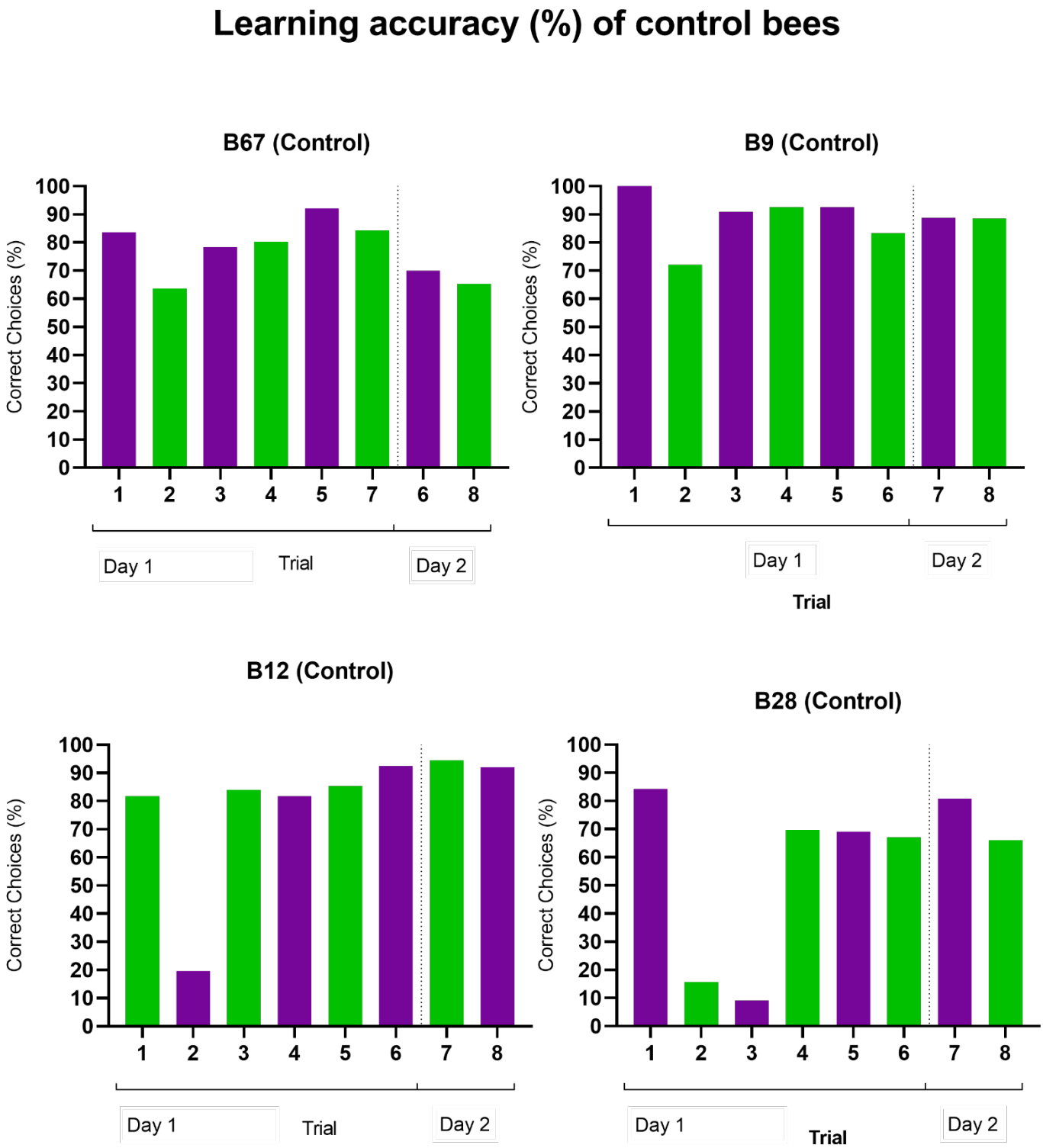

Figure 9.7: Learning accuracy (% of correct choices) graphs for bees in the control group (B67, B9, B12 and B28).

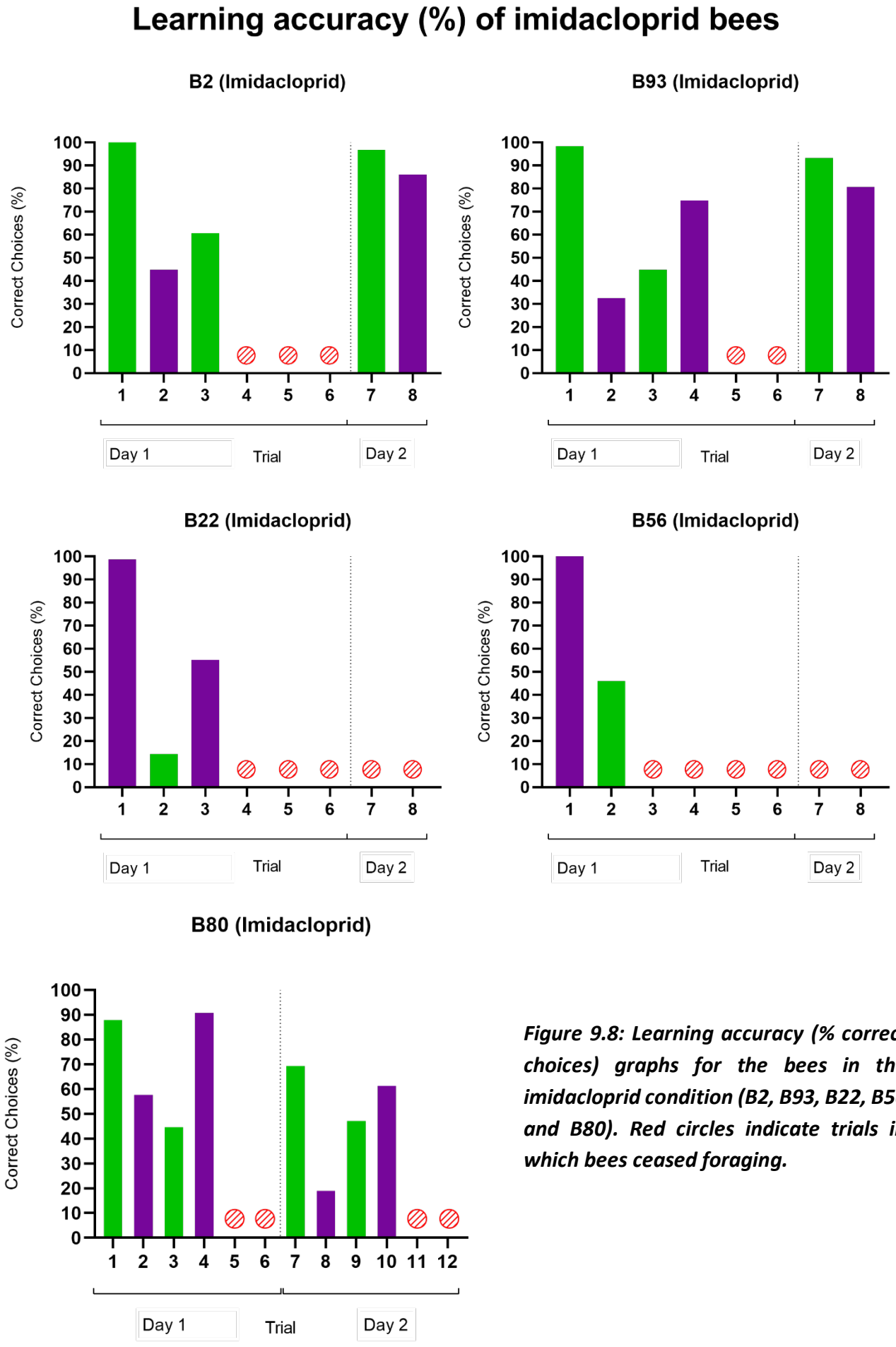

Figure 9.8: Learning accuracy (% correct choices) graphs for the bees in the imidacloprid condition (B2, B93, B22, B56 and B80). Red circles indicate trials in which bees ceased foraging.

9.3.2 LPI scores

LPI scores could be a maximum of 50 for each bee for each trial. The score gives an indication of how quickly bees learnt the association within the first 50 choices of the trial. A low LPI score indicates that a bee learnt quickly with a low error rate.

Three quarters of the control bees (B67, B9 and B12) demonstrate low LPI scores (< 21) across all trials (Figure 9.9). However, for control bee B28 we see higher LPI scores for trials T2 and T3, suggesting that this bee was slower to learn the colour association, making more errors than the other control bees when the rewarding colour was switched in trial 2, and again in trial 3. It is clear from the control bee data that there is an inherent level of variation in error and learning rates between bees, when not exposed to any pesticide. It is important to note that day 2 LPI scores for the control bees remained low, suggesting that bees had committed the colour-reward associations to long term memory and were able to efficiently recall these to complete the tasks with a low error rate.

In the imidacloprid-treated bees the LPI scores were generally higher than the control bee scores in trial 2 (Figure 9.10). The previous learning accuracy scores tell us that all bees (control and imidacloprid) decreased the overall percentage of correct choices they made in trial 2 compared to the probe trial (trial 1). The LPI scores suggest that although all bees decreased their overall learning accuracy, the treated bees made more errors in the first 50 choices of trial 2, than the control bees did. However, for three (B2, B93 and B22) of the four imidacloprid-treated bees, who completed any of the testing on day 2, there were low LPI scores on day 2, suggesting, that like the control bees, these bees were able to learn and remember colour-reward associations. Imidacloprid-treated bee, B80, displayed a lower LPI score for trial 7 on day 2 but then a high LPI score of 49/50 on trial 8 of day 2, displaying a similar pattern to trial 2 where the colour is first reversed on day 1. This suggests that bees may also vary in their ability to extinction learn and remember the opposing memory created on day 1. Since this only applied to one of the treated bees which completed trials on day 2, it is unlikely that this variation can be attributed to imidacloprid exposure.

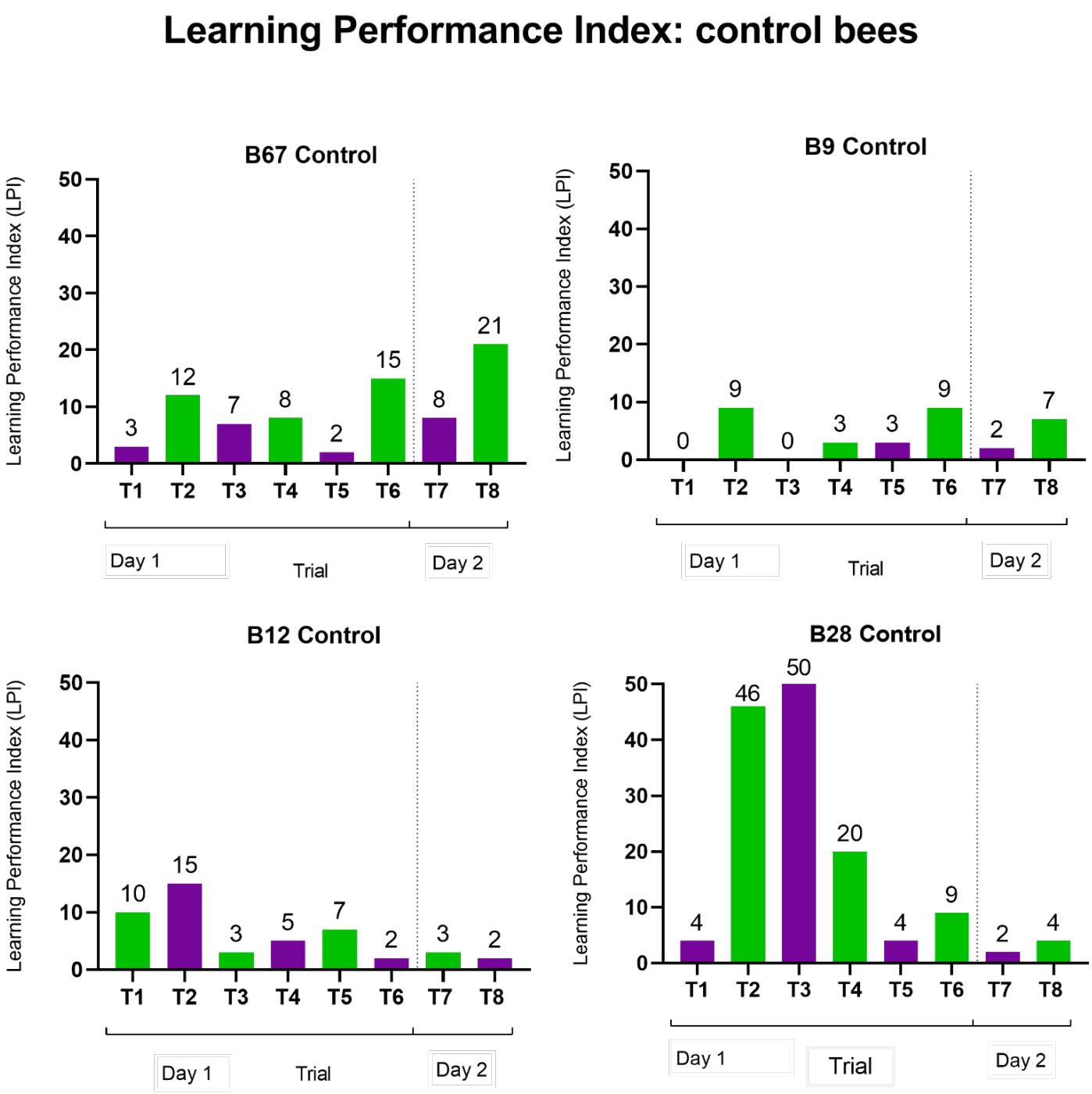

Figure 9.9: LPI scores for bees in the control condition (B67, B9, B12 and B28). The rewarding colour for each trial is indicated by the bar colour. Individual trial scores given above bars.

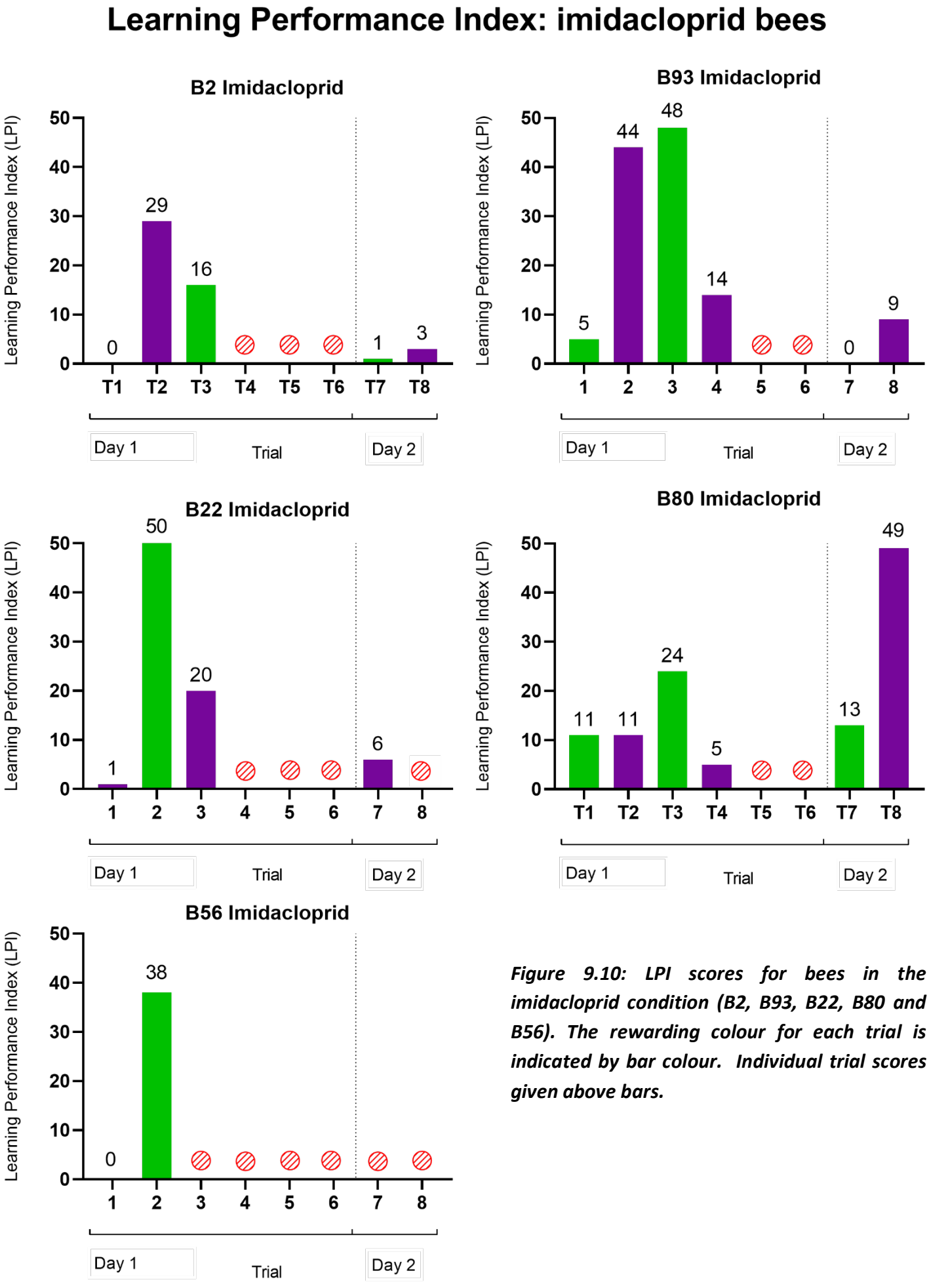

Figure 9.10: LPI scores for bees in the imidacloprid condition (B2, B93, B22, B80 and B56). The rewarding colour for each trial is indicated by bar colour. Individual trial scores given above bars.

Differences in LPI scores between day 1 and day 2

Each bee's average LPI score on day 1 was subtracted from the bee's average LPI score on day 2 to produce the difference between the scores. This was done for the whole day (across all trials regardless of colour) for each bee and also specifically by colour on each day for each bee (e.g. violet day 2 LPI score – violet day 1 LPI score) as scores may have varied depending on which colour was being learnt as rewarding. A positive difference indicates that a bee declined in its performance between day 1 and 2, whereas a negative score indicates that a bee improved (decreasing its LPI). Bee B56 in the imidacloprid treatment was excluded from the average daily calculations as it did not complete any trials from T3 onwards. For the daily colour calculations, B56 and B80 were excluded for both colours, and B22 for the green calculations as they did not complete the colour trials needed to compare LPI scores on the second day (T7 or T8).

75% of both the control and the imidacloprid-treated bees improved or maintained their average LPI scores between days 1 and 2 (Figure 9.11) and the difference in LPI scores by colour, showed that the only bees which declined in performance on day 2 were control bees (Figure 9.12). However, it should be noted that two imidacloprid-treated bees (B56 and B80) were excluded from the analyses as they did not complete day 2 trials, and B22 only completed the first trial on day 2 (trial 7), therefore only purple flower data was available for this day. All of the treated bees which completed the trials on day 2 improved their LPI scores for each individual colour in comparison to day 1, suggesting their colour learning ability was not impaired by the imidacloprid exposure.

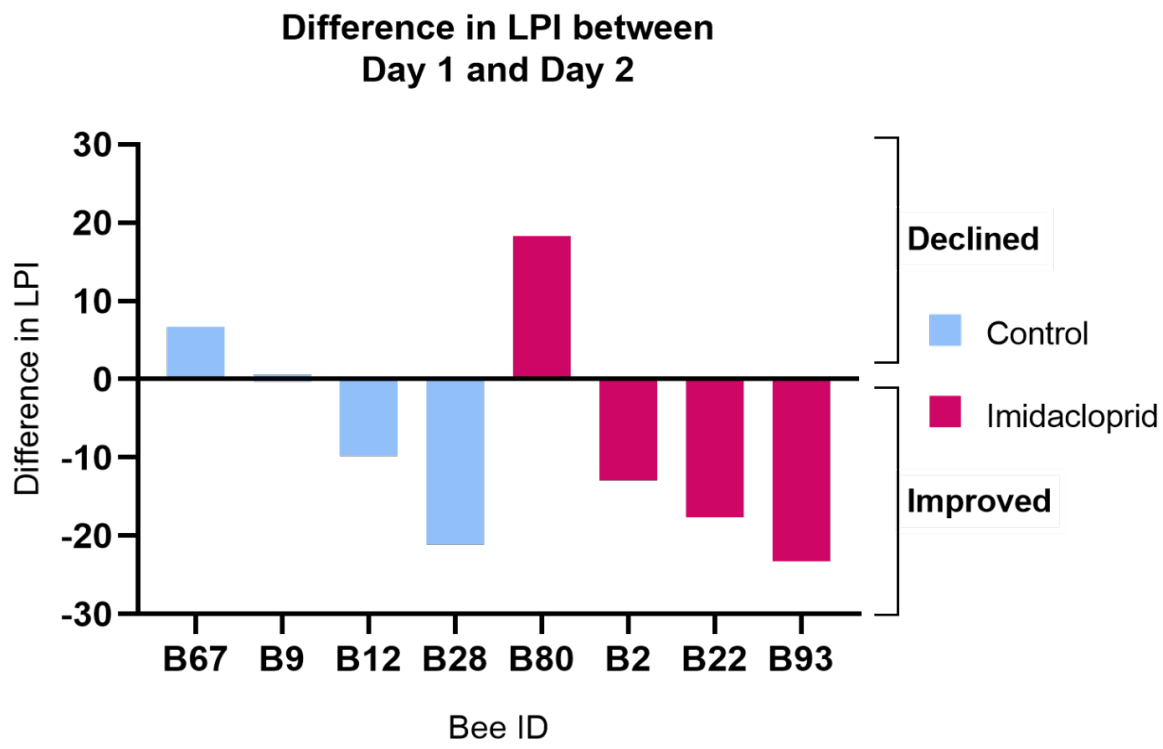

Figure 9.11: Difference in LPI scores between day 1 and day 2 for individual bees in the control (blue) and imidacloprid (pink) groups.

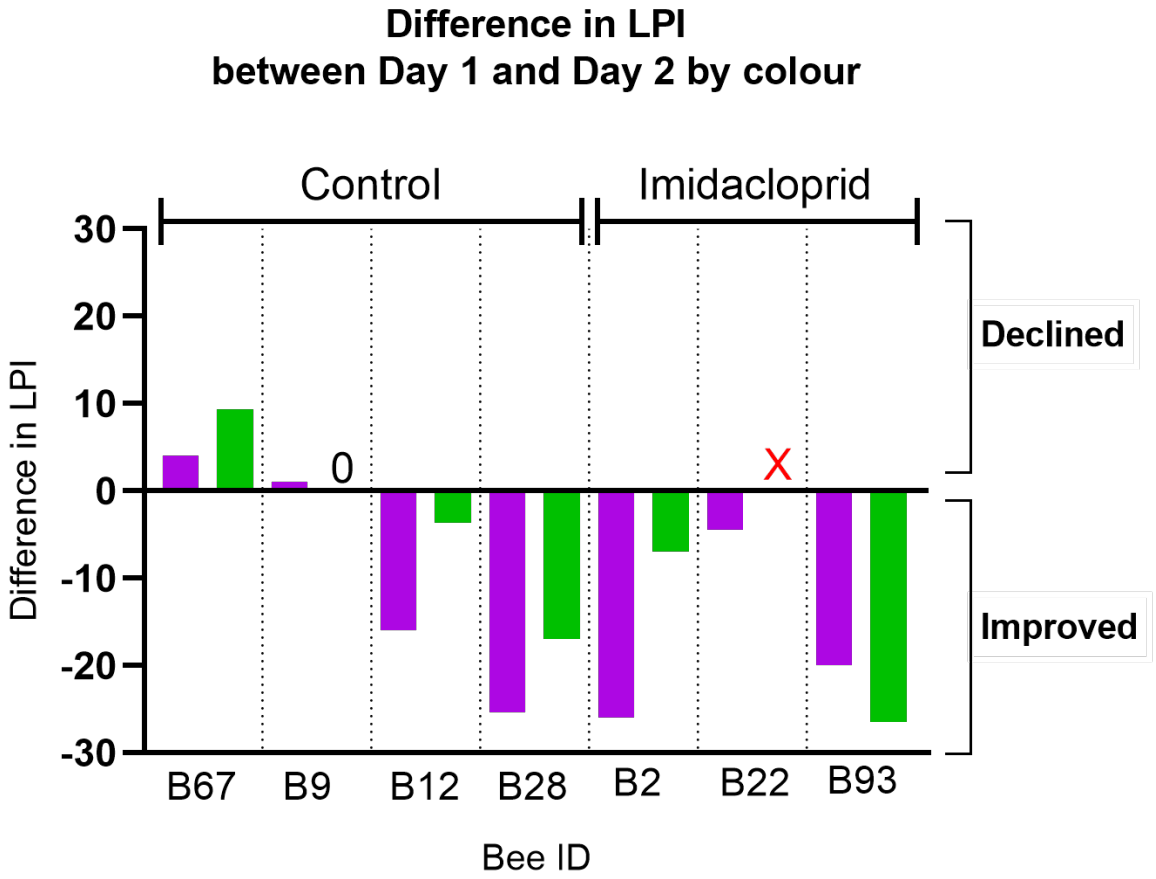

Figure 9.12: Difference in LPI scores between day 1 and day 2 for individual bees separated by rewarding chip colour. Control bees are on the left-hand side and imidacloprid bees on the right.

9.3.3 Time from first imidacloprid exposure to ceasing foraging

The imidacloprid-treated bees all stopped foraging at some point on day 1. The time from first imidacloprid exposure until treated bees ceased foraging was calculated to see whether there was a time dependent effect of exposure on willingness to forage. This 'time to cease foraging' was calculated as the time at which a bee first made a correct choice in trial 2 (the first time a 10µl imidacloprid laced reward was consumed) until the time at which trials were ceased due to impairment on day 1 (in minutes). This tells us the amount of time, post first imidacloprid exposure, which bees ceased foraging (Figure 9.13). There is a large variation in the amount of time treated bees took to cease foraging (132-349 minutes), but none of the bees ceased foraging prior to 2 hours post exposure. This may be due to bees consuming different amount of the pesticide compound across the trials, it was therefore important to also look at the number of choices and the amount of imidacloprid consumed by each bee, as bees which made more correct choices across the trials would have been exposed to more

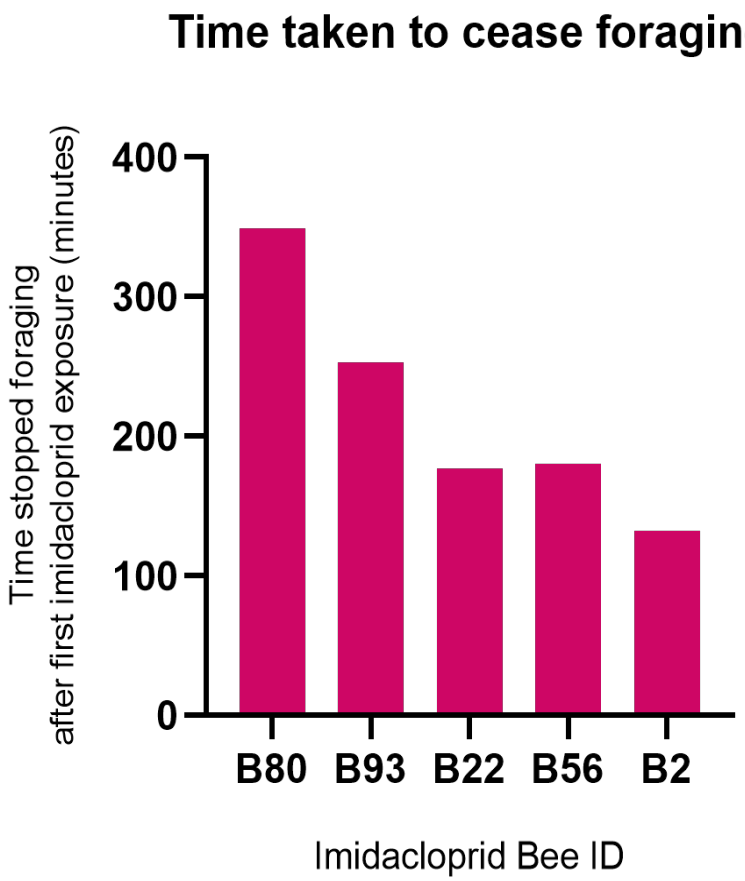

Figure 9.13: Time taken by imidacloprid bees to cease foraging (minutes) after first exposure.

imidacloprid and therefore may have taken a shorter amount of time to cease foraging due to impairment because of this higher consumption.

9.3.4 Pesticide consumption prior to ceasing foraging

The number of correct choices made from trial 2 until the bees stopped foraging due to impairment on day 1 was calculated (Figure 9.14) and used to estimate how many µl of 10ppb imidacloprid solution each individual bee consumed, as each correct choice represents the consumption of 10µl. This will be an upper estimate of exposure, as foragers will not consume all the reward which they collect and take back to the hive.

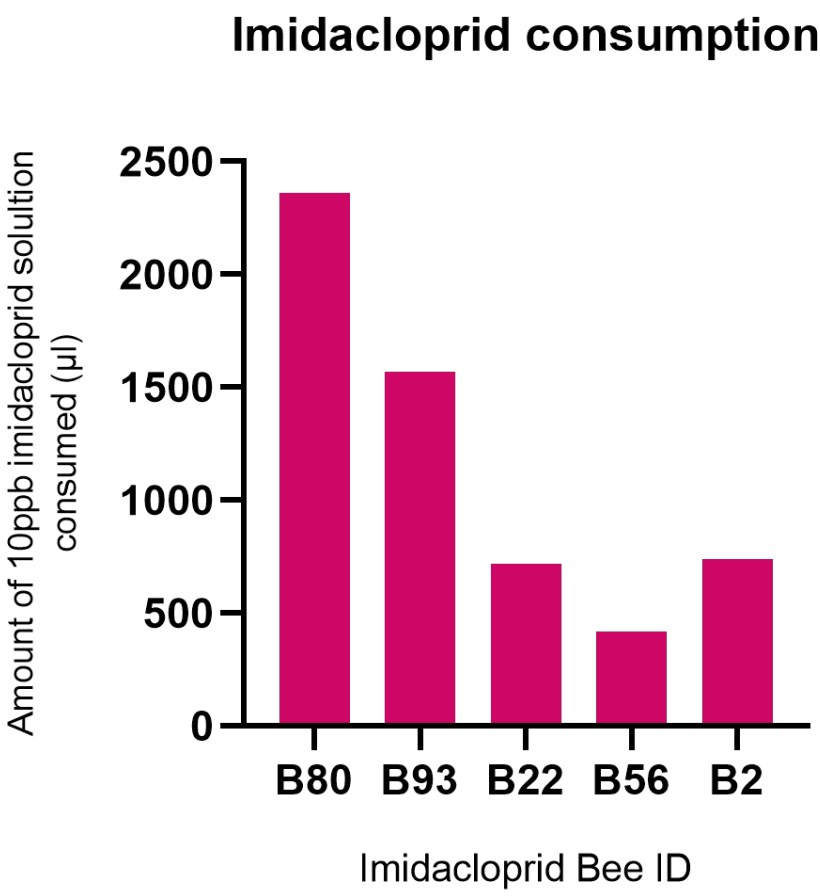

Figure 9.14: Imidacloprid solution consumption (µl) by bees in the imidacloprid treatment.

9.3.5 Time taken to make first correct choice

The time taken from the start of the trial for bees to make their first correct colour choice was calculated for each trial. Bees in the control group increased the time taken to make a correct choice in trial 2 when the rewarding colour was first reversed, and again in trial 3 when the colour is reversed again, but post trial 3 the bees began to quickly find the rewarding colour (Figure 9.15). By trial 5, the imidacloprid-treated group took longer to make the first correct choice (Figure 9.15). Mann-Whitney tests (non-normal data) were used to analyse the time taken to make the first rewarding choice between control and treated bees for each trial. The n value for the imidacloprid treatment varied dependent on whether the trial was ceased due to impairment (also there are no trial 6 data for the imidacloprid-treated bees as all bees in the treatment ceased foraging prior to this trial on day 1). We would not expect any significant difference between treatments in trial 1 (probe) as the imidacloprid treatment hadn't started ($P = 0.29$, control n = 4, imidacloprid n = 5). There was also no significant differences in trial 2 ($P = 0.68$, control n = 4, imidacloprid n = 5), trial 3 ($P = 0.70$, control n = 4, imidacloprid n = 5) or trial 4 ($P = 0.46$, control n = 4, imidacloprid n = 3 (one of the imidacloprid bee values is 0 here and consequently does not show on Figure 9.15)). However, by trial 5 there was a significant difference, ($P = 0.0007$***, control n = 4, imidacloprid n = 2) with the imidacloprid-treated bees taking significantly more time to make a correct colour choice. By day 2, neither treatment was significantly different from each other in trial 7 ($P = 0.66$, control n = 4, imidacloprid n = 4) or trial 8 ($P = 0.4$, control n = 4, imidacloprid n = 3) It should however be noted that the sample sizes for these tests were small and variation in response is large, therefore although statistical testing is possible the data should be interpreted with caution.

9.3.6 Time taken to learn the colour association:

Time taken to learn the colour association was calculated as the time taken from the start of the trial until a bee made five consecutive correct colour choices. On the first colour reversal (trial 2), bees in both the control and imidacloprid treatments increased the time they took to make a correct association (Figure 9.16). Following trial 2, control bees decreased the time they took to make the colour association in each trial, even when the rewarding colour was alternated between trials. Mann-Whitney tests (non-parametric data) were used to analyse the difference in time taken to make the colour association between control and imidacloprid bees for each trial. The n value for the imidacloprid treatment varied per trial, dependent on whether the trial was ceased due to impairment (and that there are no trial 6 data for the imidacloprid bees as all bees in the treatment ceased foraging prior to this trial on day 1). As expected, in trial 1, before imidacloprid treatment there was no significant difference in the time taken to make the association between control and imidacloprid-treated bees ($P = 0.73$, control n = 4, imidacloprid n = 5). In trial 2, there was again no significant difference ($P = 0.68$, control n = 4, imidacloprid n = 5) and none in trial 3($P = 0.69$, control n = 4, imidacloprid n = 4). In trial 4, there was a low significant difference ($P = 0.049$*, control n = 4, imidacloprid n = 3) but it was not possible to test trial 5 data, as there was only one bee which completed this trial. This bee took 32 minutes to make the association, in comparison to the control bee values of 3, 0, 6 and 8 minutes, again suggesting that imidacloprid-treated bees take longer to make colour associations. By day 2, there were no significant differences between control and imidacloprid groups for trial 7 ($P = 0.74$, control n = 4, imidacloprid n = 4) or 8 ($P = 0.46$, control n = 4, imidacloprid n = 3), suggesting that long term memory of the colour association task was not affected by the imidacloprid exposure on the previous day.

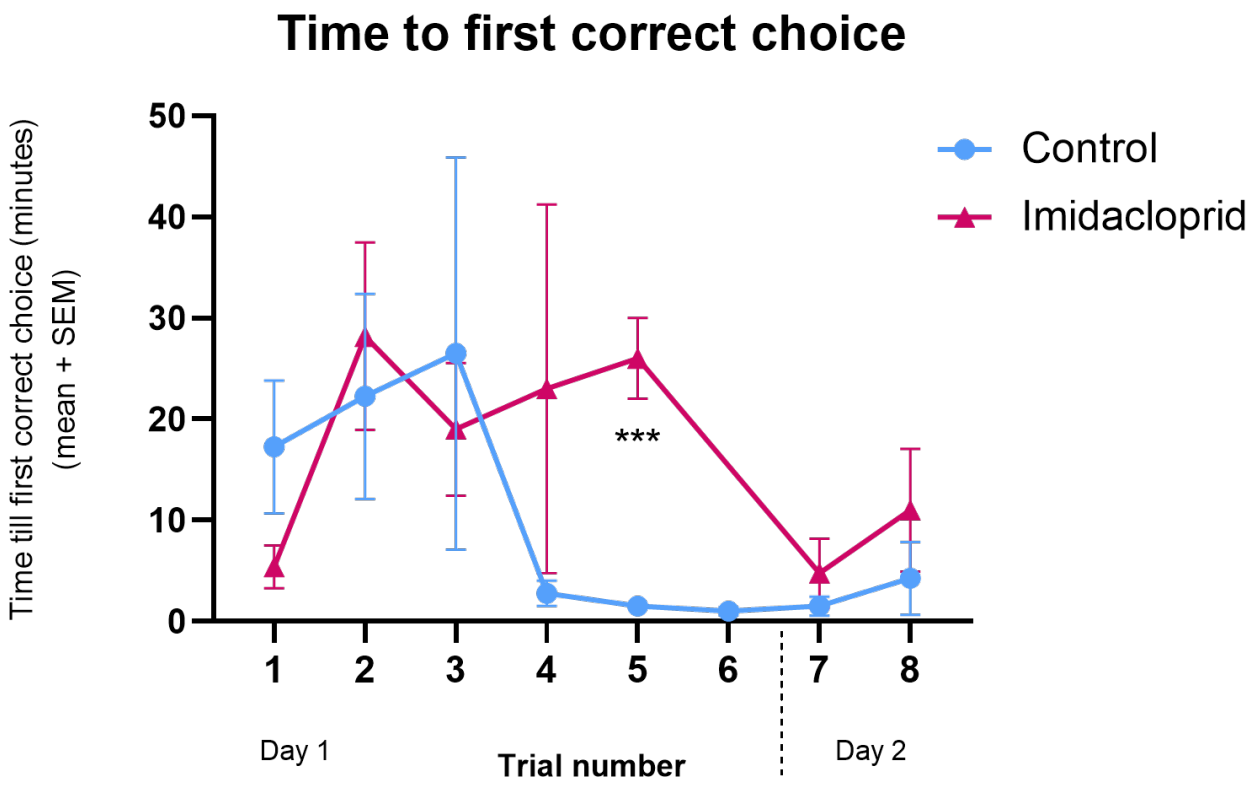

Figure 9.15: Time taken by bees in the control (blue) and imidacloprid (pink) conditions to make a first correct choice in each trial. (Mean + SEM).

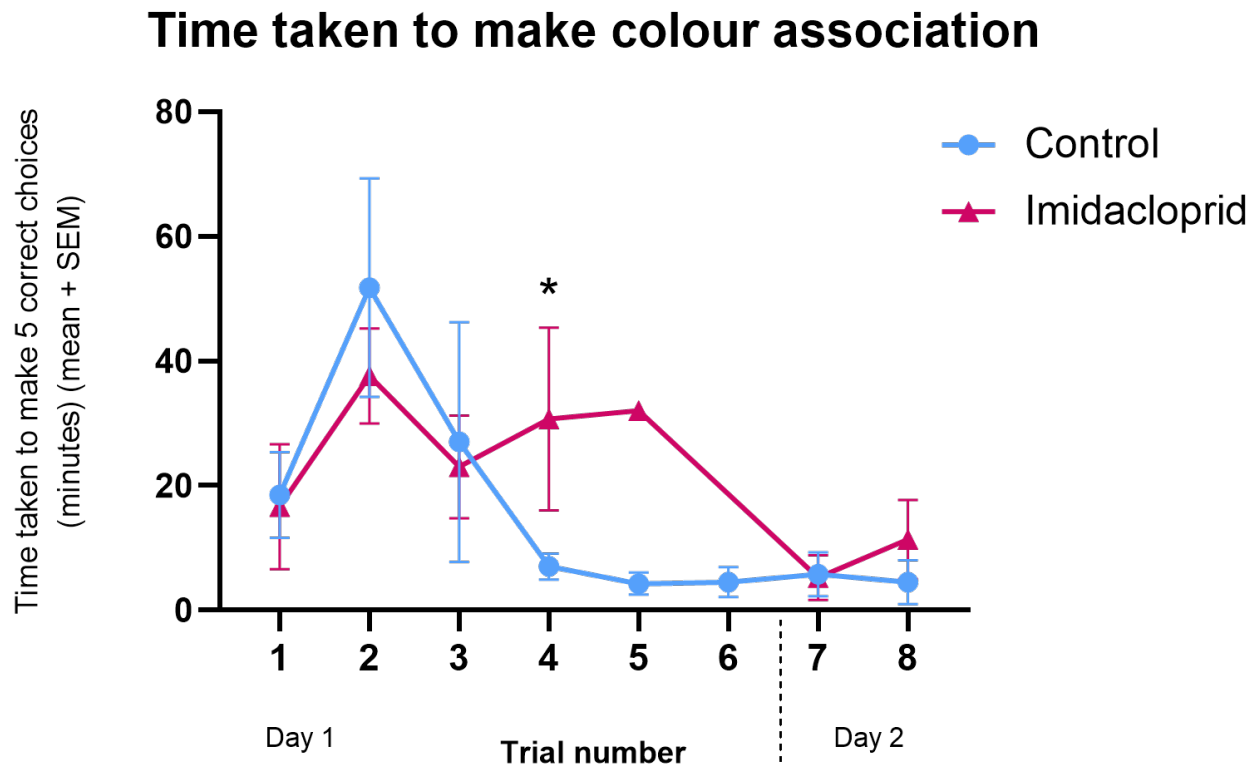

Figure 9.16: Time taken by bees in the control (blue) and imidacloprid (pink) conditions to make the colour association in each trial. (Mean + SEM).

9.4 Discussion

Understanding the proximate causes of sublethal pesticide effects on pollinators is key to being able to implement accurate mitigation strategies. Here we assessed the effect of chronic, sublethal imidacloprid exposure in a realistic feeding scenario on bumblebee foragers' ability to learn and extinction learn colour associations. A previous study (Muth and Leonard, 2019) examined the effect of acute imidacloprid exposure on *Bombus impatiens* ability to learn visual associations, finding that acute exposure did not appear to impair visual association learning. However, no study to date had examined the impact of chronic, field realistic imidacloprid exposure on visual learning in a free-flying scenario or looked at effects on both associative and extinction learning.

Overall, bumblebee foragers' ability to learn and extinguish visual associations between floral colour and a sucrose reward does not appear to be affected by chronic imidacloprid exposure. This is evidenced by the treated bees' ability to maintain high learning accuracy and low LPI scores across completed trials (Figures 9.8 and 9.10), as well as the ability to improve their by-colour LPI performance in comparison to the previous day (Figure 9.11). Notably, all imidacloprid bees ceased foraging at some point on day 1 (Figure 9.8). However, this refusal to forage appears to be due to an apparent lack of motivation or motor impairment, as opposed to an inability to learn. Once bees had metabolised the pesticide overnight, consuming clean sugar, learning accuracy was again high the next day (Figure 9.8) and colour LPI performance improved for all imidacloprid exposed bees (Figure 9.10). These findings agree with previous studies which have implicated imidacloprid in the reduction of feeding motivation (Cresswell et al., 2014a, 2012; Thompson et al., 2015) and foraging (Feltham et al., 2014) in bumblebees, but there are no reported effects on visual associative learning (Muth and Leonard, 2019). However, this reduction in feeding motivation appears to be highly species dependent and has been observed in bumblebees (Cresswell et al., 2014a, 2012; Thompson et al., 2015) but not in honeybees (Cresswell et al., 2014a, 2012). Perhaps due to Honeybees being able to maintain much lower levels of bodily imidacloprid (Cresswell et al., 2014a), further highlighting the importance of studying individual species and not using honeybees as a model for all bee species. Honeybees appear to have enhanced imidacloprid metabolism (Suchail et al., 2004b) and bee cytochrome P450s have been shown to have a role

in this enhanced ability of honeybees to metabolise imidacloprid and could explain the differential species sensitivity between honeybees and bumblebees (Manjon et al., 2018).

However, the time which bees took to make colour associations by trial 5 was significantly affected by imidacloprid exposure (Figures 9.15 and 9.16). This could be due to cognitive impairment, but more likely seems to be due to physical or motor impairments, which later caused bees to cease foraging on day 1, and on day 2 when B80's trials were extended (Figure 9.8). Phelps *et al*. (2018) reported similar findings in another bumblebee species (*B. impatiens*), finding that bees exposed to 10ppb imidacloprid were much slower to gain a preference for feeding on rewarding flower colours. The reason for these slower preference acquisitions, like we see here (Figures 9.15 and 9.16), are tricky to determine in a purely behavioural setting. Slower acquisition could be due to a lack of motivation, leading to less flower sampling or reduced duration of foraging trips or due to a degree of motor impairment. Although the mechanism cannot be elucidated here, a reduction in association speed could clearly have significant impacts on wild foragers and could explain previous findings which show imidacloprid exposure leads to reduced foraging efficiency (Feltham et al., 2014).

As individuals were only exposed to imidacloprid in the test solutions of the artificial flowers and not in the hive overnight, the tests may be under estimating effects that could occur if bumblebees were continually exposed to imidacloprid contaminated nectar in their diet. It is also unlikely that foragers would be exposed to just one pesticide, it would more likely be a cocktail of chemistries and stressors, which have been suggested to have potentially synergistic effects (Azpiazu et al., 2019; Grassl et al., 2018; Iverson et al., 2019; Nazzi et al., 2012; Sgolastra et al., 2017).

Our findings, and those reported by Muth and Leonard (2019), highlight the importance of utilising non-PER and non-odour-based learning assays (Figure 9.1). Previous findings have shown that imidacloprid has a detrimental impact on long term odour memory formation and the ability to differentiate odours (Williamson and Wright, 2013). It may therefore have been presumed that visual associative learning would also be disrupted, but this was not the case. It is key that a variety of learning assays and modalities are used to study sublethal effects on behaviour in ecotoxicological testing as the utilisation of only one method (e.g. the PER or visual learning assays), could result in either an over or under estimation of detrimental effects.

The switching of the rewarding colour between trials was not only to test bees' ability to associatively learn visual colour and reward associations, but to also see whether their ability to colour switch or 'extinction' learn a rewarding colour was affected by chronic imidacloprid exposure. To extinction learn, bees had to acquire new, opposing colour-reward association when the previously learnt colour association was no longer rewarding. Studies have shown that the prior rewarding memory is not extinguished but that instead a new, opposing memory is created (Bouton, 2004), allowing the bee to robustly return to the original learnt response once the context changes again (Bouton and Bolles, 1979; Bouton and King, 1983). This was supported by our data, where control bees, when first presented with a colour reversal in trial 2, decreased their learning accuracy but on the second colour reversal in trial 3 (back to the original rewarding colour of trial 1) the bees were generally able to maintain a high level of learning accuracy, shifting back to the original learning context and visual association. This 'renewal effect' was also maintained on day 2 of testing (Figure 9.7). There was some evidence of the same renewal effect for the imidacloprid treated bees, particularly on day 2 for bees 2 and 93 (Figure 9.8), however, the data is more variable as the bees all ceased foraging at some point on day 1 and so determining this renewal effect is tricky without a larger sample size.

Muth *et al.* (2015) demonstrated that bees are capable of simultaneously learning two rewarding colours at once, even when the colours represent different rewards (e.g. pollen or nectar) and that bees 'generalise' based on similar colours e.g. they treated purple flowers as if they were blue and orange flowers like yellow, despite the fact that bee colour space firmly predicts that bees can readily distinguish between these colours (Dyer and Chittka, 2004). This suggests that if the tests reported here were repeated using a harder colour learning task (e.g. colours which were less visually distinguishable from each other in bee colour space) the bees may have found the task of reversal learning and switching between colours more difficult. This provides an avenue for future research in this arena, whereby various less distinguishable colour pairs could be tested to determine whether imidacloprid exposure has a finer effect on the skills needed for these harder visual tasks.

We could also question whether the bees in our trials were 'extinction learning' or whether they were simply learning a rule e.g. that after x amount of choices the rewarding colour switched. Bees have been shown to be highly capable of rule learning (Giurfa, 2007; Zhang et

al., 1996, 1999). To avoid this each bee had to meet a set of criteria before the trial was deemed complete and the rewarding colour was switched for the next trial. Bees had to complete a minimum of 50 flower choices over a minimum of 5 foraging bouts. Furthermore, the location of the rewarding flowers was randomised between each foraging bout within a trial. It was therefore unlikely that bees were learning a rule e.g. 'when the bee comes out for a new foraging bout the rewarding colour will have changed' or 'that after x number of choices it changes' or that bees were learning rewarding locations.

It is important to note that there was a marked difference in the consumption level of 10ppb imidacloprid laced sucrose between foragers prior to ceasing foraging on day 1 (Figure 9.14). Foragers were selected for trials based on a series of pre-training steps and a probe trial (Figure 9.4) which meant that only bees who were highly capable of completing the learning task (80% + learning accuracy in the probe trial) were taken on to further trials. This selection for learning ability did not take account of forager size which may affect food consumption capacity and thus affect the differences in imidacloprid consumption. In future trials measurement of bee head or thorax size, as a proxy for forager size (as in Chapter 8), should be conducted so that this data can be further considered. Despite there being possible, unrecorded variation in forager size, it is still remarkable that forager imidacloprid consumption prior to ceasing foraging (day 1) varied so widely (range 420-2360 µl) (Figure 9.14), suggesting that individuals may have very different pesticide tolerances even within the same species and the same pesticide compound.

The original plan for this research was to conduct a further suite of pesticide testing in the summer of 2020. However, all further experiments were cancelled due to the COVID-19 pandemic. Our preliminary analysis here confirms that this system has a clear application for assessment of pesticide impacts on associative and reversal learning, and the research could now expand to a wider range of pesticides and bee species. The benefit of this experimental set-up is it provides a free-flying foraging environment with easy manipulation of colour stimuli to test both associative and extinction learning. It also facilitates chronic exposure studies which can be run over a series of trials and days to mimic field-realistic exposure regimes. Studies such as this, which provide detailed behavioural observations under controlled, field-realistic, chronic exposure levels are vital if we are to understand pesticide impacts on bee cognition and behaviour.

Chapter 10

General Discussion

Chapter 10: General Discussion

10.1 Project summary

Wild pollinators remain less well studied than their managed counterparts. This is predominantly due to the economic importance of crop pollinating species such *A. mellifera*, but also due to the difficultly of rearing non-commercial, particularly solitary species, in the lab for toxicological testing. Nonetheless, wild bee species are vital pollinators which play a crucial role in the productivity of agricultural and ecological systems worldwide (Garibaldi et al., 2013) and provide the bulk of global bee biodiversity. There are approximately 20,000 known bee species globally, of these, 250 are bumblebees, nine are honeybees and a small number are social stingless bees (Clark, 2018). In the UK there are approximately 270 bee species, of which around 250 are solitary and all but one (*A. mellifera*) are wild species (Clark, 2018).

Toxicological studies have shown that responses of bees to pesticides can vary widely, between individuals (Gill et al., 2012) and species (Arena and Sgolastra, 2014; Cresswell et al., 2012; Piiroinen and Goulson, 2016) and can depend on the study level i.e. individuals or colonies/populations. An increased understanding of how individuals and species respond to realistic pesticide exposure is essential to guide pesticide regulation and minimise impacts on (non-target) pollinators. The current deficit of toxicological studies for non-*Apis* bee species prompts the need for appropriate laboratory and field-based toxicology testing for wild bee species.

In this thesis, *B. terrestris* was used as a model for wild bee species, aiming to build on previous studies, which have looked at visual indicators of sublethal pesticide effects, and delve further by developing new tools and methods to assess novel sublethal effects on bumblebee mobility, navigation, memory and learning.

10.2 Thesis objectives and original contributions

In the introduction to this thesis, four key knowledge gaps were identified to guide the research and produce clear outcomes which would enhance current understanding of

sublethal pesticide effects on wild pollinators and could benefit future policy and best practice. These were:

1. Studies examining sublethal effects on non-*Apis* bee species (Siviter et al., 2018b)
2. Studies utilising non-olfactory learning paradigms (particularly non-PER (Proboscis Extension Response) paradigms) to examine cognitive sublethal effects (Muth and Leonard, 2019; Siviter et al., 2018b)
3. Chronic studies for bumblebee adults and larvae (Abdourahime et al., 2019)
4. Comparative testing platforms which facilitate across species and across compound testing (my own conclusion drawn from the current literature)

The core original findings of this work and the key contributions made to current understanding of the sublethal effects of pesticides on *B. terrestris* can be summarised thus:

a. The thermal-visual arena was developed and optimised as a novel *B. terrestris* aversive assay. Environmental temperature (in the form of a heated arena floor) was effectively perceived as a strongly aversive stimulus and bees in treatments containing aversive stimuli spent significantly more time in the reward zone. A visual pattern around the arena's circumference allowed the bees to learn the reward zone location sooner than bees without the visual cues (pre-trained bees with pattern aid spent more time in the reward zone and entered the reward zone more times). However, by trial 10 (post-training) these visual aids did not provide any additional benefit, as evidenced by the lack of significant difference in time spent in, or times entered, the reward zone between bees in the dark and light treatments post-training. Bees subjected to aversive treatment with (light) and without (dark) visual aids all spent significantly more time in the reward zone post-training (versus pre-training), further supporting the notion that aversive conditioning is a highly effective training tool in *B. terrestris*.
b. Oral treatment (chronic) with thiacloprid led to significantly reduced food consumption in *B. terrestris* at 5000ppb, but not at 500ppb. Thiamethoxam treatment at 100ppb led to increased bee mortality.
c. Low dose thiamethoxam exposure at 10ppb prevented bees from improving training parameters in the thermal-visual arena, and the bees displayed increased speed of movement post-training, whereas all other treatments decreased post-training speed.

Whereas thiacloprid and control bees significantly reduced the distance travelled post training, thiamethoxam and sulfoxaflor treated bees did not (difference more pronounced in thiamethoxam bees). These differences became more marked at the high dose exposures, with thiamethoxam bees spending less time in the reward zone, not decreasing the distance travelled and only slightly decreasing speed post-training.

d. Walking bumblebee trajectories in the thermal-visual arena adhered to a speed-curvature power law which has previously been observed in humans, other primates and *Drosophila* larval trajectories. No previous study has reported such a finding in adult insect locomotion. This power law relationship has the potential to elucidate 'optimal', 'non-stressed' behavioural templates in insects and therefore provides a powerful tool for assessing the impact of stressors (e.g. pesticides) on underlying movement patterns. Thiamethoxam treated bees (low dose, 10ppb) had a significantly higher speed curvature power law exponent than the low dose sulfoxaflor and thiacloprid treated bees. This thiamethoxam power law disruption was even more evident at the higher dose (100ppb), where treated bees had a significantly higher exponent than all other treatment groups (control, thiacloprid, sulfoxaflor). These subtle, yet detectable changes in *B. terrestris* movement patterns in response to thiamethoxam are remarkable, suggesting a whole new way of studying sublethal effects of pesticide exposure.
e. Individual variation was seen in forager performance in the thermal-visual arena, both within and between hives. RNA-seq and bioinformatic analyses show that there were 83 significant (<0.05) and 35 highly significant (<0.01) unique differentially expressed genes identified between the "good" and the "bad" learner groups. This suggests that there may be some hive benefit to having a variation in learning ability between workers and that learning ability may have a trade-off with other fitness measures e.g. lifespan/resource collection.
f. Chronic imidacloprid exposure led to foraging behaviour disruption but did not affect long-term colour association or extinction learning in a colour-reward association assay. Imidacloprid treated bees also took longer to make correct colour choices (trial 5) and made more errors (trial 2) than control bees.

10.3 Implications of the observed sublethal effects of the pesticides on bees in a wider study context

Thiamethoxam is known to have detrimental sublethal effects at field realistic concentrations on both honeybees and bumblebees (Baron et al., 2017; Friol et al., 2017; Grillone et al., 2017; Henry et al., 2012; Laycock et al., 2014; Mommaerts et al., 2010; Dara A. Stanley et al., 2015; Tavares et al., 2017, 2015; Tesovnik et al., 2017, 2020; Tosi et al., 2017; Williamson et al., 2014) and is currently banned for outdoor use in the EU (European Commission, 2018b, 2018a, 2018c). Indeed the nitroguanidine neonicotinoids in general (imidacloprid, dinotefuran, clothianidin, and thiamethoxam) are more toxic to bees (Blacquière et al., 2012; Iwasa et al., 2004; Laurino et al., 2011; Mommaerts et al., 2010) compared to their cyano-neonicotinoid counterparts (thiacloprid and acetamiprid). However, recent work has elucidated the genetic determinants of differential neonicotinoid sensitivity in bees (Beadle et al., 2019; Manjon et al., 2018), which is thought to be mediated by divergent metabolism of CYP9Q subfamily Cytochrome P450s, specifically, CYP9Q3 in honeybees and CYP9Q4, CYP9Q5 and CYP9Q6 in *Bombus* (Manjon et al., 2018; Troczka et al., 2019). These P450s effectively detoxify cyano-neonicotinoid compounds e.g. thiacloprid, but have little effect on the nitroguanidines e.g. imidacloprid (Manjon et al., 2018). The testing of both thiacloprid (a cyano-neonicotinoid) and thiamethoxam (a nitroguanidine neonicotinoid) in Chapter 5 therefore provides a further interesting comparison of sublethal effects of different neonicotinoids subclasses on *Bombus spp.* It has also been discovered that some species, such as the leafcutter bee *Megachile rotundata*, do not possess P450s, making them >2,500-fold more sensitive N-cyanoamidine neonicotinoids (Hayward et al., 2019) and further highlighting the importance of not generalising sensitivity responses across species.

Indeed, the work presented here finds that thiamethoxam (a nitroguanidine neonicotinoid) has pronounced sublethal effects on *B. terrestris*, in agreement with existing literature, which demonstrates reduced learning speed and short term memory impairment, and additionally highlights reduced brood production and worker survival at field realistic concentrations of <10ppb (Laycock et al., 2014; Dara A. Stanley et al., 2015). However, recent contemporary studies have shown no-effect of field realistic thiamethoxam exposure on reproductive

parameters such as colony growth or number of sexual offspring produced (Stanley & Raine, 2017).

Contrarily, a meta analyses of bee sensitivity to pesticides found that the most toxic neonicotinoids to non-*A. mellifera* bees were the cyano-neonicotinoids (acetamiprid and thiacloprid), which have a relative lower toxicity to *A. mellifera* (Arena and Sgolastra, 2014). This supports the need for further testing of wild bee species, outside of the *B. terrestris* model, in toxicological assays.

It is possible that the detrimental sublethal effects of the cyano-neonicotinoid, thiacloprid, reported in this study have been overlooked in traditional toxicology testing, as the reduced feeding effects observed for *B. terrestris* (Chapter 5) are finer-scale, more nuanced impacts on food consumption. Thiacloprid has previously been reported to increase homing flight duration, reduce homing success and memory retrieval, disrupt foraging behaviour, learning acquisition and motor functions and social communication in honeybees (Fischer et al., 2014; Tison et al., 2017, 2016). Tison *et al.* (2016) observed a similar reduction in feeding in honeybee foragers in response to thiacloprid exposure and suggest that reduced sugar consumption may be the causal factor behind these other observed impairments e.g. feeder visitation rates, foraging motivation and prolonged stays inside the hive, due to reduced availability of energy resources (Tison et al., 2016).

There are few reports on the potential impact of sulfoxaflor on bees, but those available suggest a negative impact on bumblebee worker survival (Tizi Taning et al., 2019) and reproductive success (Siviter et al., 2018a) at field realistic levels. However, there is no reported effect on bumblebee larval mortality (Siviter et al., 2020), olfactory conditioning or working memory (Siviter et al., 2019). These studies (Siviter et al., 2020, 2019) suggest that although sulfoxaflor shares a mode of action with the neonicotinoid group, the detrimental cognitive effects reported for the neonicotinoids may not be seen for sulfoxaflor exposure. This may be because although both compounds bind to the same receptor (nAChR) they have different binding sites on the receptor (Beck et al., 2015; Sparks et al., 2013; Wang et al., 2016; Watson et al., 2011; Zhu et al., 2011). Furthermore, these studies only examine very low-level acute exposure, perhaps underrepresenting likely exposure events in the field. It is vital that multiple compounds are tested in sublethal assays, as there is a need to highlight the comparison between compounds. It may be the case that newer, more selective pesticide

compounds are found to have sublethal effects in laboratory assays but not in the field and would be a better alternative to alternative compounds that might be used if the newer class is removed from use.

The colour-association arena described in Chapter 9 was also designed as a comparative pesticide testing platform. However, due to the impact of COVID-19, further trials which were due to take place in the summer of 2020 could not be completed. Nonetheless, the initial testing of imidacloprid in the arena shows a promising methodology for assessing not just colour-association learning but also extinction learning through the introduction of conflicting colour associations trial by trial. To the best of our knowledge, the impact of pesticide exposure on extinction learning has not been studied previously in *B. terrestris*. Extinction learning is a highly ecologically relevant process, used by foragers to process a continually changing floral reward landscape, in which floral associations are switched as rewards are depleted by foragers or rivals. Considering different forms of learning e.g. extinction, as well as the development of novel learning assays, has the potential to provide unique insights into previously understudied sublethal effects. For example, it is possible that compounds could affect one type of learning and not the other, necessitating the study of broader sublethal effects when studying pollinator impacts.

The power law studies reported in Chapters 6 and 7 demonstrate that sublethal pesticide exposure can lead to behaviours being compromised on a minute scale, so it is no longer enough to simply observe macro-scale behavioural effects to assess impairment. Power laws and movement tracking could provide a new avenue for analysing impacts on animal biomechanics and increasing behavioural complexity. The development of 'normal' movement templates can be used to determine when behavioural complexity is lost. Movement template analyses has the potential to be used on a wide range of species and stressors (e.g. disease, parasite load), adding a vital tool to the assessment of detrimental pollinator impacts.

10.4 Underlying genetic determinants of differences in individual learning performance in B. terrestris.

It is likely that foraging bees are under a significant selective pressure to maximise learning ability, as learning to navigate complex environments, handle a large variety of flowers and

monitor floral rewards is clearly integral to foraging success. We might therefore predict little genetic variation in learning ability, both between individual foragers and between hives, as this variation should be eliminated by such strong selective pressure. And yet, large variation was observed in the learning ability of individual foragers in the thermal-visual arena trials (Chapter 4). Genome wide differences in expression between the best and worst aversive learners revealed 83 significant* (<0.05) and 35 highly significant** (<0.01) DEGs between the learner groups. The most notable DEGs associated with learning and memory functions included; gene LOC100648451 (associated with aromatic-L-amino-acid decarboxylase), gene LOC100649554 (associated with histone-lysine N-methyltransferase), gene LOC105665758 (associated with neurochondrin) and gene LOC105667011 (associated with the neuropeptide CCHamide-2 receptor). In addition, genes which are typically associated with stress responses were also flagged up in the significant DEGs. As all the bees were exposed to the same heat stress during the aversive learning task, this implies that there may be a differential ability to deal with heat stress between the learner groups. For example, the 'bad' learner group significantly upregulated a gene involved in DNA repair (LOC100631060), perhaps suggesting these bees are experiencing higher levels of physiological stress in response to the heat stimuli, whereas three of the DEGs significantly upregulated by the 'good' learner group are associated with the mitochondria (gene IDs: LOC105667097, LOC100650290 and LOC100646071). External stressors e.g. physical stress (Margotta et al., 2018) and pesticides (Martelli et al., 2020) have been linked to disrupted mitochondrial functioning and elevated oxidative DNA damage in honeybees (Margotta et al., 2018) and other insects (Nareshkumar et al., 2018).

A similar high-throughput sequencing approach was used by Li et al. (2018) to study transcriptomic changes during long term visual memory formation in *B. terrestris* workers which were either given an appetitive visual colour learning task or no task. When we compare the 83 DEGs identified in our study to the 111 DEGs identified by Li et al. (2018) no matching DEGs are identified. This is perhaps unsurprising as both studies utilised different bee learning paradigms, here we used an aversive visual learning task, whereas in the Li et al study an appetitive colour learning task was used, which, is known to elicit different neural pathways (Vergoz et al., 2007a).

The changes in the expression of the genes identified in Chapter 8, between the learner groups, suggests potential genetic determinants of the observed differences in individual learning performance in *B. terrestris.* However, due to the small sample sizes of the pilot study, these results must be interpreted with caution. As detailed in Chapter 8, further full-scale trials could be conducted as well as the verification of candidate genes using quantitative polymerase chain reaction (qPCR) or gene knock outs to determine involvement in learning and memory function.

10.5 Effects of pesticide compounds on B. terrestris performance

Toxicological research into non-target effects of plant protection products has focused its efforts on the Western honeybee, *A. mellifera*. There is therefore a large body of evidence and comprehensive datasets available for the evaluation of sublethal pesticide impacts on *A. mellifera* (highlighted in Ippolito et al., 2020). However, as the EFSA note, "the dataset for bumblebees and solitary bees is smaller and too scattered to be able to draw robust conclusions" (Ippolito et al., 2020). This is especially concerning, given the observed differences in response to pesticide compounds across bee species and within castes of the same species (Arena and Sgolastra, 2014; Cresswell et al., 2012; Poquet et al., 2016). This historical approach to testing assumes likeness across an extremely biodiverse group (Apoidea: Anthophila) which contains over 17,000 known species (Michener, 2007) and ignores potential pesticide impacts to other, wild pollinators known to be important crop pollination service providers (Garibaldi et al., 2013).

Chapters 5 and 7 describe work that elucidated some surprising pesticide impacts. For example, the finding that chronic thiacloprid exposure (5ppm) significantly decreased *B. terrestris* food consumption over a three- and five-day period. This was not expected given that thiacloprid is still marketed under its commercial names Calypso and Biscaya as 'safe to bees' and 'can even be applied during flowering' (Bayer Crop Science UK, 2020b, 2020c; Klatt et al., 2016). The exposure level which had feeding effects (5ppm), is 1/24 of the Maximum Field Recommended Concentration (MFRC) (Mommaerts et al., 2010) and therefore, although seemingly a high sublethal dose, represents a possible "worst case field scenario" for potential detrimental impacts in extreme exposure cases. In January 2020 the European commission voted not to renew the EU licence for thiacloprid (European Commission, 2019),

reflecting growing concerns, and evidence against this neonicotinoid's impact on native pollinators (Blacquière et al., 2012; Brandt et al., 2017; Godfray et al., 2014). The work reported here would support that decision. However, it is also important to note what was not found in the current study. Sulfoxaflor did not affect the foragers' ability to complete the aversive learning task in the thermal-visual arena, nor did it impact their ability to improve the task over time (decreasing speed and distance travelled, spending more time in the reward zone) or their food consumption. Thiamethoxam exposure did not lead to reduced food consumption at either the low (10 ppb) or high (100ppb) dose (prior to death). This agrees with Laycock et al.'s (2014) previous findings of no detectable reduced consumption effect for *B. terrestris* with oral exposure of 1 and 11 µg kg−1 (equivalent to ppb) thiamethoxam, but contradicts their findings of a reduced syrup consumption at 39 and 98 µg kg^{-1}. These diverse findings highlight the importance of comparative pesticide testing. New or alternative compounds will often replace those which are removed by pesticide restrictions and it is vital that compounds can be assessed across the same species and assays to inform better policy making decisions.

10.6 Applications of this work and environmental relevance

This body of work has begun to fill several key gaps in the existing study of pesticide impacts on bees and provides environmentally relevant findings which can be applied to real world scenarios. Specifically, it addresses a lack of non-PER, and free moving training paradigms through the creation of two novel testing platforms; the thermal-visual arena (Chapters 3, 4 and 5) and the colour reward association arena (Chapter 9), which provide new tools to examine novel sublethal effects on bees in the laboratory. The thermal-visual arena clearly demonstrates the success of aversive conditioning as a bee training tool, particularly the use of environmental temperature. This opens up a new avenue for bee training assays, facilitating the use of free-moving arenas where bees can display more natural behaviours (and new sublethal effects can be studied) whilst still receiving highly effective, and ecologically relevant, training stimuli. The colour-reward association arena is a highly flexible colour learning assay which allows the study of both associative and extinction/reversal learning. This arena is again a free moving assay which allows foragers to fly and behave in a more natural manner. The free-moving nature of the two assays facilitates the study of new, subtle sublethal effects in the movements of the bees e.g. the speed curvature power law,

which may never have been examined in tethered test subjects. The discovery of these new, subtle sublethal effects in the laboratory could now be transitioned into field studies, in which wild bees are tracked and pesticide impacts on real-world movement trajectories observed. These findings have a wide potential to be used in the field in combination with new developing technologies e.g. radar tracking and tagging (Fischer et al., 2014; Schneider et al., 2012; Woodgate et al., 2017) and would not require laboratory reared insects. These assays can also facilitate comparative testing across species and pesticide compounds, and both platforms enable either acute or chronic testing.

All pesticide testing reported in this thesis was chronic (defined as more than one acute exposure and over several days) and at field realistic or 'worst-case' field scenario pesticide concentrations. This is important as it allows us to simulate impacts based on realistic bee foraging scenarios, in which a forager is continually exposed to low-level pesticide residues in the pollen and nectar of forage flowers. Under acute exposure bees may have metabolised bodily pesticide residues prior to learning and memory trials, down -playing potential detrimental impacts. Therefore, chronic exposure represents a more likely scenario for a foraging bee during learning and memory trials. It has been suggested that chronic exposure is not necessarily inevitable, due to pesticide-free floral resources in the landscape e.g. wildflowers (Garbuzov et al., 2015; Godfray et al., 2014). However, chronic exposure is increasingly likely when we consider the regular usage of pesticides on plants in garden centres and their more or less unrestricted availability for home-use (Lentola et al., 2017), as well as the continued escalation in the adoption of systemic pesticides which are predominantly water soluble and therefore may be transported to wild flowers in field margins (Botías et al., 2015; Siviter et al., 2018b).

The finding that chronic exposure to dietary thiacloprid can lead to a significant reduction in food consumption (Chapter 5) is particularly environmentally relevant. Nectar is essential to the functioning and success of a social bee colony, as it is both consumed by the workers and fed to the queen and the larvae, which derive all their nutrients from a rich pollen-nectar mixture (Heinrich, 2004). If field realistic residues of thiacloprid have a detrimental effect on food consumption in wild bumblebees to the same extent that has been observed here in *B. terrestris* in the laboratory, then the success of wild bee colonies may be placed at risk.

In general, the results presented in this thesis are consistent with other laboratory trials which suggest that thiamethoxam exposure causes hyperactivity (as demonstrated by the trajectory graphs and increase in speed fold change of thiamethoxam bees in Chapter 5), motor function, learning and decision making impairment (perhaps evidenced by thiamethoxam bees' inability to improve in training parameters post-training) (Blacquière et al., 2012; Ludicke and Nieh, 2020; Tosi and Nieh, 2017). The finding that imidacloprid negatively impacts foraging behaviour and motivation, but not learning and memory function is consistent with Muth & Leonard's findings (2019).

Although two of the pesticides tested here; thiamethoxam and thiacloprid are unlikely to be licensed for usage in the EU again, it remains apt that they were tested alongside the newly emerging compound sulfoxaflor. Furthermore, these studies are still relevant in a broader worldwide context, as many countries still prophylactically use these neonicotinoids (and also are often still dependent on much older groups of chemistries which are even more detrimental to the environment and human health). Comparative testing platforms, such as those developed here, can be easily recreated for testing with multiple species and compounds across multiple countries.

10.7 Limitations and caveats of this work

There are many limitations and caveats to conducting environmental research in the laboratory, the majority of which stem from the inability to recreate field-realistic scenarios in an artificial environment (Walters, 2013). Although criticised for being unrealistic, laboratory experiments allow the tight control of pesticide concentrations and accurate measurements of their effects, something which is unachievable in a multi-facetted field environment. In the realm of pesticide studies, perhaps these criticisms stem from the often acute, short-term exposures used in laboratory studies, which are certainly unrealistic representations of field scenarios. It is essential that chronic exposure studies, like the ones conducted in this work, continue to be carried out if we are to mimic real-world exposure windows. However, laboratory studies alone will never be sufficient, as Mommaerts & Smagghe (2011) put forward, a multi-tiered approach which consists firstly of laboratory tests on individuals, secondly of laboratory tests on key processes e.g. worker survival,

reproduction and thirdly semi-field trials to ascertain whether laboratory findings are supported in the field, provides a solid framework for pesticide impact testing.

Further to this, laboratory studies usually examine only one chemical or stressor impact at a time. In the field, bees will be potentially simultaneously exposed to a complex mix of agrochemical combinations, as well as additional stressors such as disease and parasites and adverse climate. The effects produced by cocktails of chemicals and stressors in the wild may lead to synergistic sublethal effects, enhancing those predicted in single stressor laboratory tests. For example, isolated treatment with sulfoxaflor or the fungal parasite *Nosema bombi* has no effect on *B. terrestris* larval mortality (Siviter et al., 2020). However, when larvae were exposed to the two stressors in combination, there was an additive negative effect (Siviter et al., 2020).

During the pesticide studies in this thesis, only the dietary syrup of the bees was dosed with the relevant pesticide compounds, and not the pollen which was fed to the hive. This was predominantly due to the ease of diluting pesticide compounds into the feeding syrup and the ability to track syrup consumption per bee (see Chapter 5). This is however an unrealistic field scenario as in reality bees would be exposed to pesticide compounds in both pollen and nectar and there are reports of much higher pesticide concentrations in the pollen of treated plants (Bonmatin et al., 2005; Dively and Kamel, 2012). It may therefore be the case that effect estimates on wild bumblebees are underestimated in these studies and that further impacts may be seen when both pollen and nectar are dosed. Future studies could consider the relative importance, and therefore most likely routes of exposure, of each nutrient source for different hive members e.g. queens and the brood eat large volumes of pollen, whereas worker subsist largely on nectar (Heinrich, 2004). A further caveat is that bees in the studies were not given alternative pesticide-free foraging choices. This is unlikely to be the case in the wild as bumblebees are known to be generalist foragers, selecting both flowering crops and wild flower species as foraging sources (Stanley, 2013).

It is possible that in the field, the observed negative effects of dietary thiacloprid exposure on food consumption would not be replicated, as bees would avoid thiacloprid-treated crops. However, Havstad et al. (2019) and Tison et al. (2016) reported no repellency effects for thiacloprid for bumblebees or honeybees in the field, making this deliberate avoidance

unlikely, and supporting the more concerning notion that thiacloprid could cause reduced resource consumption in wild populations. Nonetheless, it is improbable that foraging bees would forage continually on only pesticide-treated crops and therefore consumption of wild flower nectar and pollen would dilute the exposure received from treated crops, consequently, the extended exposure periods of multiple days with only pesticide treated syrup used in these studies could be overestimating sublethal effects.

The bees used in these studies (*Bombus terrestris audax*) were obtained from Biobest (Belgium), where colonies are commercially produced for pollination and very few details are available about the production methods. Hence, we do not know the degree of inbreeding or relatedness between the hives used in these experiments. This has potential detrimental impacts for Chapter 8 of this thesis, in which transcriptomic analyses were undertaken to examine underlying genetic differences of the best and the worst learners between and within hives. This uncertainty was one of the reasons for the decision to predominantly examine the biggest difference in learning ability i.e., best and worst learners from all hives, rather than the best and the worst learners from each hive. This has further relevance for the pesticide studies and the potential influence of inbreeding on responses to stress and pesticides exposure. Studies in butterflies have demonstrated that genetic variation plays a role in neonicotinoid exposure tolerance (Kobiela and Snell-Rood, 2020) and similarly, studies in honeybee populations demonstrate variable pesticide tolerances across different breeding stocks (Milone et al., 2020). This highlights the importance of considering the impacts of genetic variation in laboratory pesticide testing.

10.8 Future research directions

1) A move away from testing the three neonicotinoids associated with detrimental bee impacts; clothianidin, thiamethoxam and imidacloprid. There is now a large body of evidence against their usage and focus should be shifted to assessing impacts of novel compounds, new to the market.
2) Studies which focus on non-commercial wild bee species should be prioritised in both the field and the laboratory.
3) Bee learning and memory studies, which use non-appetitive and free moving paradigms. Non-PER paradigms, such as aversive conditioning, are ecologically

relevant and can facilitate the study of subtle sublethal effects, particularly when it comes to bee movement, flight or tracking.

4) Pre-licensing studies typically do not look at subtle, fine scale sublethal effects. However, the findings of this work highlight the existence of subtle effects (e.g., on movement patterns, food consumption, learning improvement over trials) at field realistic exposure methods and concentrations. It is therefore likely that there is a suite of sublethal effects which are being missed when it comes to pesticide registration decisions but are likely to have significant effects when pesticides are applied at landscape scales.

10.9 Take home message

The findings of the work reported in this thesis demonstrate the remarkably subtle sublethal effects that field realistic pesticide dosages can have on wild bees, whilst also providing new tools and assays with which to study them. The consequences of these subtle effects may seem virtually undetectable but are likely to have profound impacts on wild bees at the landscape scale.

The farmers and growers I have met care more about the environment and the protection of biodiversity than anyone. However, the pesticide debate has become divisive, often pitting farmers and the agricultural industry as profiteering against environmental organisations that want to 'save the pollinators at all costs. The reality is far more nuanced. Farmers are facing more challenges than ever, on maintaining crop production, on lower prices, on pesticide bans and on environmental standards. Equally, the environment is being pushed further than ever before by human actions, with increased biotic (e.g. parasites, diseases and invasive species) and abiotic (e.g. habitat loss and fragmentation, chemical usage and climate change) stressors and ever increasing species extinction rates (Brondizio et al., 2019). We need to create a sustainable agricultural system that works for all parties; maintaining and even increasing food production, protecting wildlife and our native pollinators and giving farmers flexible pest control options which allow both agriculture and nature to flourish.

“Live as though you’ll die tomorrow, but farm as though you’ll live forever”

John Marsden

Appendix Chapter 2

Aversive conditioning in bees: are we missing a trick?

Table A2.1 Final papers screened by title (n = 44) from original web of science literature search results (n = 135) and reasons for further study exclusion.

Study number	Reference	Included (Y or N)	Why was the study excluded?
1	(Howard et al., 2019)	Y	
2	(Marchal et al., 2019)	Y	
3	(Fouks et al., 2019)	N	No aversive learning task given
4	(Junca et al., 2019)	Y	
5	(Nouvian and Galizia, 2019)	Y	
6	(de Camargo et al., 2019)	N	No aversive learning task given
7	(Jarriault et al., 2018)	N	No aversive learning task given
8	(Black et al., 2018)	Y	
9	(Guiraud et al., 2018)	Y	
10	(Plath et al., 2017)	Y	
11	(Kirkerud et al., 2017)	Y	
12	(Avalos et al., 2017)	Y	
13	(Park et al., 2016)	N	Cannot access proceedings
14	(Cholé et al., 2015)	Y	
15	(Junca and Sandoz, 2015)	Y	
16	(Zhang and Nieh, 2015)	Y	
17	(Plascencia et al., 2015)	N	Cannot access proceedings and abstract did not include an aversive learning task.
18	(de Brito Sanchez et al., 2015)	Y	
19	(McQuillan et al., 2014)	Y	
20	(Tan et al., 2014)	N	No aversive learning task given
21	(Giannoni-Guzmán et al., 2014)	Y	
22	(Junca et al., 2014)	Y	
23	(Bos et al., 2014)	Y	
24	(Tedjakumala et al., 2014)	Y	
25	(Dinges et al., 2013)	Y	

26	(Dinges et al., 2013)	N	Replication of study number 25, returned in duplicate by Web of Science.
27	(Rodríguez-Gironés et al., 2013)	Y	
28	(Geddes et al., 2013)	Y	
29	(Tedjakumala & Giurfa, 2013)	N	Review not a primary study
30	(Roussel et al., 2012)	Y	
31	(Fouks and Lattorff, 2011)	N	No aversive learning task given
32	(Agarwal et al., 2011)	Y	
33	(Ayestaran et al., 2010)	Y	
34	(Avarguès-Weber et al., 2010)	Y	
35	(Roussel et al., 2010)	Y	
36	(Vergoz et al., 2007b)	Y	
37	(Vergoz et al., 2007a)	Y	
38	(Sandoz et al., 2006)	N	Cannot access proceedings – text requested but not returned
39	(Roussel et al., 2009)	Y	
40	(Walker, 2004)	N	Text not available and excluded based on title
41	(Smith et al., 1991)	Y	
42	(Abramson and Smith, 1991)	N	Appears to be a published meeting abstract, cannot find access.
43	(Abramson, 1986)	Y	
44	(Rodríguez-Gironés and Jiménez, 2019)	N	No aversive learning task given

Appendix Chapter 5

Assessing the impacts of two neonicotinoids and a sulfoximine pesticide on *Bombus terrestris* performance in the thermal-visual arena.

Food consumption data and pesticide dosage calculation

Pesticide solutions were made fresh daily, individual feeders replenished and food consumption recorded. The pesticide concentrations of the stock solutions were not verified with additional chemical analyses. Three evaporation test feeders were set up in empty cages to gage sucrose solution lost to evaporation over the experimental period. The amount lost (g) due to evaporation was averaged across the three feeders and subtracted from all bee food consumption values.

Weight of Biogluc calculations:

Biogluc was used for all pesticide dilutions and in-hive feeding. The density of a 50% Biogluc ®/ 50% water solution is approximately 1.15 kg/L^{-1} (Crall et al., 2018). The density of a 100% Biogluc solution (used for dilutions in this study) is therefore estimated at 1.3 kg/L^{-1} (density of water = 1 kg/L^{-1}).

Pesticide consumption calculations:

Biogluc consumed (g) / 1000 = (biogluc consumed Kg)

Biogluc (kg) / Density (kgL-1) = Biogluc L

Biogluc (L) x thiamethoxam (µg/L) = thiamethoxam (µg)

x 1000 = thiamethoxam (ng)

Simplified to: Biogluc consumed (g) * µgL / density

Low dose five-day food consumption:

Table A5.1: Total food consumption per bee in the low dose treatments over the course of the full five days of the experiment. Evaporation is taken into account in calculations. Pesticide dosage consumed per bee (ng) calculated.

Evaporation feeders (g)	**Control solution consumption (g)**	**Control - evaporation (g)**
0.0213	0.5292	0.506866667
0.0308	0.871	0.848666667
0.0149	1.2303	1.207966667
	2.3411	2.318766667
	0.8505	0.828166667
Average evaporation (g):	1.1087	1.086366667
0.022333333	1.3813	1.358966667
	1.6002	1.577866667
	1.4223	1.399966667
Thiacloprid solution consumption (500 ppb) (g)	**Thiacloprid - evaporation (g)**	**Thiacloprid consumed (ng)**
0.4285	0.406166667	156.2179487
0.7849	0.762566667	293.2948719
2.5935	2.571166667	988.9102565
2.0486	2.026266667	779.3333335
1.5811	1.558766667	599.5256412
0.1671	0.144766667	55.67948731
1.7003	1.677966667	645.371795
1.8261	1.803766667	693.7564104
0.6984	0.676066667	260.0256412
Sulfoxaflor solution consumption (5ppb) (g)	**Sulfoxaflor - evaporation (g)**	**Sulfoxaflor consumed (ng)**
1.8727	1.850366667	7.116794873
0.2587	0.236366667	0.909102565
0.3447	0.322366667	1.239871796
0.2441	0.221766667	0.852948719
0.9514	0.929066667	3.573333335
0.6664	0.644066667	2.477179488

1.6421	1.619766667	6.229871796
1.1803	1.157966667	4.45371795
1.5689	1.546566667	5.948333335
Thiamethoxam solution consumption (10ppb) (g)	**Thiamethoxam - evaporation (g)**	**Thiamethoxam consumed (ng)**
2.0728	2.050466667	15.77282052
1.1128	1.090466667	8.388205131
1.5311	1.508766667	11.60589744
1.0132	0.990866667	7.622051285
2.0031	1.980766667	15.23666667
0.4304	0.408066667	3.138974362
0.967	0.944666667	7.266666669
1.4318	1.409466667	10.84205128
1.2923	1.269966667	9.768974362

High dose five-day food consumption:

Table A5.2: Total food consumption per bee in the high dose treatments over the course of the full five days of the experiment. Thiamethoxam bees are omitted as seven of the nine bees died prior to day five food consumption measurements.

Control solution consumption (g)	**Control - evaporation (g)**	
1.5433	1.520966667	
2.4051	2.382766667	
1.6933	1.670966667	
2.0987	2.076366667	
2.3487	2.326366667	
1.2267	1.204366667	
1.4829	1.460566667	
1.6991	1.676766667	
1.4758	1.453466667	
Thiacloprid solution consumed (5000ppb) (g)	**Thiacloprid - evaporation (g)**	**Thiacloprid consumed (ng)**
0.907	0.884666667	3402.564104
0.682	0.659666667	2537.179488
1.485	1.462666667	5625.641027

0.84	0.817666667	3144.871796
0.7634	0.741066667	2850.256412
0.206	0.183666667	706.4102577
0.7274	0.705066667	2711.794873
0.3218	0.299466667	1151.794873
Sulfoxaflor solution (50ppb) (g)	**Sulfoxaflor - evaporation (g)**	**Sulfoxaflor consumed (ng)**
1.6498	1.627466667	62.59487181
2.5661	2.543766667	97.8371795
1.0842	1.061866667	40.84102565
1.3657	1.343366667	51.66794873
0.4603	0.437966667	16.84487181
1.8664	1.844066667	70.92564104
0.7626	0.740266667	28.47179488
1.2966	1.274266667	49.01025642
0.8758	0.853466667	32.82564104

High dose three-day food consumption:

Table A5.3: Total food consumption per bee in the high dose treatments over the course of the three days prior to thiamethoxam bee deaths.

Control solution consumption (g)	**Control - evaporation (g)**
0.5716	0.549266667
0.7675	0.745166667
0.6157	0.593366667
0.4966	0.474266667
0.6632	0.640866667
0.7641	0.741766667
0.5823	0.559966667
0.5805	0.558166667
0.4854	0.463066667

Thiacloprid solution (5000ppb) (g)	Thiacloprid - evaporation (g)	Thiacloprid consumed (ng)
0.4251	0.402766667	1549.102565
0.2877	0.265366667	1020.641027
0.6296	0.607266667	2335.641027
0.3187	0.296366667	1139.871796
0.278	0.255666667	983.3333346
0.0638	0.041466667	159.4871808
0.3861	0.363766667	1399.102565
0.19	0.167666667	644.8717962
0.2979	0.275566667	1059.871796
Sulfoxaflor (50ppb)	**Sulfoxaflor - evaporation (g)**	**Sulfoxaflor consumed (ng)**
0.6743	0.651966667	25.07564104
0.8996	0.877266667	33.74102565
0.4256	0.403266667	15.51025642
0.6311	0.608766667	23.41410258
0.1014	0.079066667	3.041025654
0.7044	0.682066667	26.23333335
0.2811	0.258766667	9.952564115
0.4316	0.409266667	15.74102565
0.1465	0.124166667	4.775641038
Thiamethoxam solution (100ppb) (g)	**Thiamethoxam - evaporation (g)**	**Thiamethoxam consumed (ng)**
0.5874	0.565066667	43.46666669
0.4617	0.439366667	33.79743592
0.4152	0.392866667	30.22051285
0.2735	0.251166667	19.32051285
0.8123	0.789966667	60.76666669
0.4879	0.465566667	35.81282054
0.2879	0.265566667	20.42820515
0.3588	0.336466667	25.88205131

Appendix Chapter 6

Demonstrating the existence of a speed-curvature power law in *Bombus terrestris* locomotion patterns.

Data filtering and pre-processing:

For the data analysis, the x, y coordinates and corresponding timestamps for whole trajectories from the centroid tracking were used to compute angular speed A(t) and curvature C(t) using standard differential geometry. Positional data were not filtered to reduce any noise or cusps that may be present before computing speed and curvature. To assess the impact of filtering we compared analyses based on the raw, consecutive positional fixes (x_1,y_1), (x_2,y_2), (x_3,y_4) with those based on filtered positional fixes ½ (x_1+x_2,y_1+y_2), ½(x_3+x_4, y_3+y_4),…, obtained by averaging over pairs of consecutive positional fixes. Such filtering did not affect the outcomes of the analysis. This is illustrated in Fig A. The effects of this filtering on our trajectory data are shown in Fig B. To further assess the impact of any noise and cusps we carried out conditional analyses on speed-curvature data associated exclusively with relatively low velocities and low accelerations. We found that such conditioning does not impact significantly on our estimates for the power-law exponents characterizing the speed-curvature power-law relationship. This is illustrated in Fig C. The results suggest that the high inter-quartile range of the β fitting shown in Fig 4 are not related to the absence of any filtering or interpolation before the differentiation of positional data used to compute angular speed and curvature. Data filtering was conducted by Andrew Reynolds (Rothamsted Research).

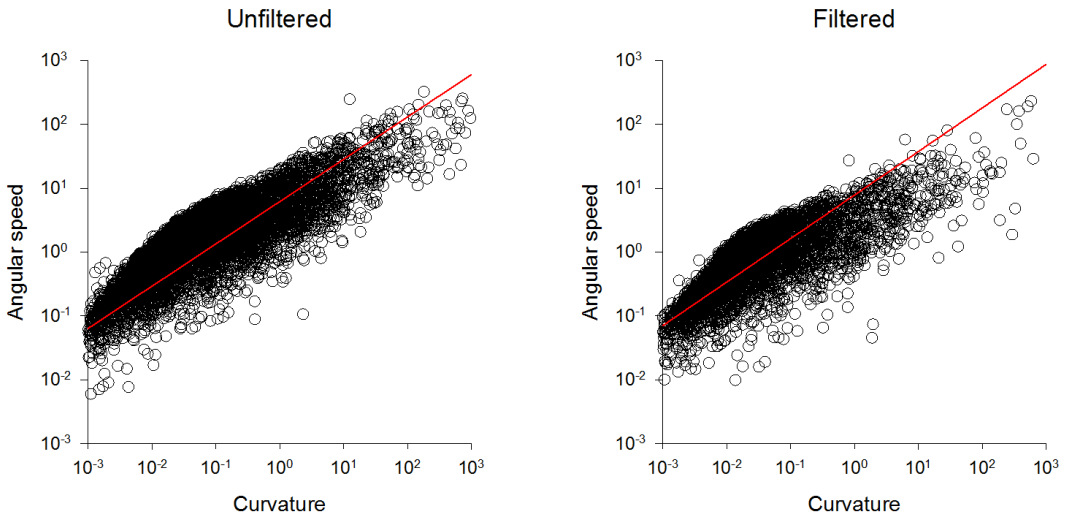

Figure A6.1: This figure demonstrates that filtering implemented prior to data analyses does not affect the outcome of the analyses. Analyses are presented here for the Control Replicate 1 dataset with and without filtering (smoothing). In both cases the best fit power-law exponent is 0.68.

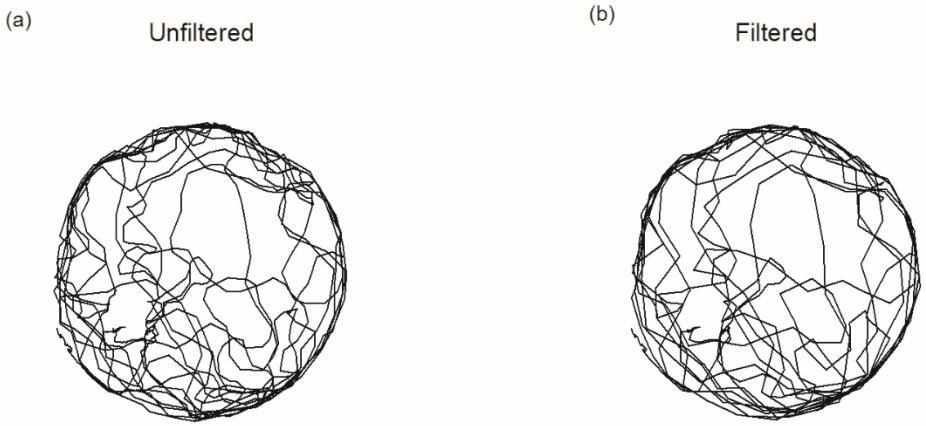

Figure A6.2: This figure demonstrates the effect of filtering on our trajectory data. (a) Demonstrates an example of a single bee's unfiltered trajectory. (b) Demonstrates the same bee's post-filtered trajectory.

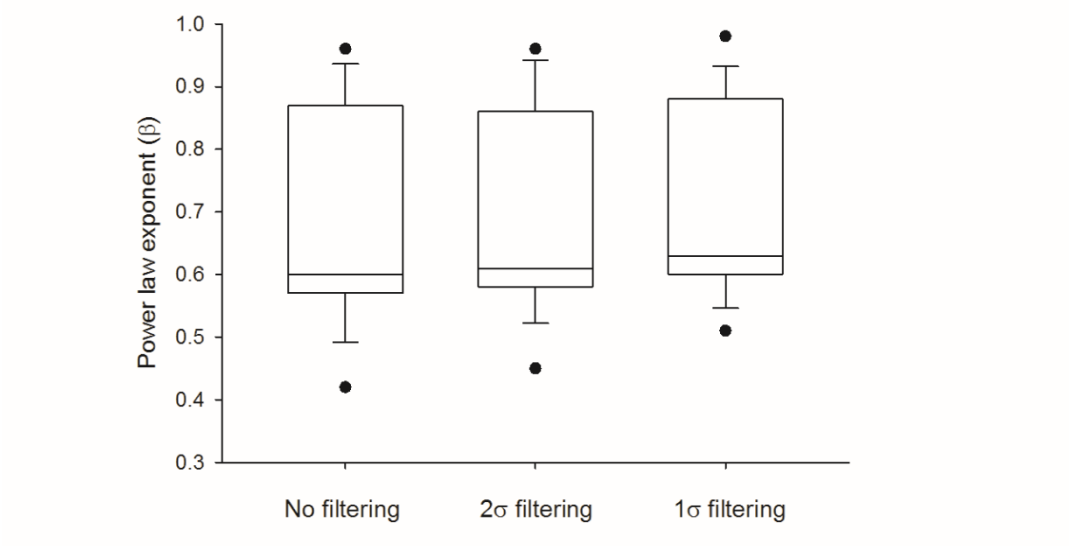

Figure A6.3: This figure demonstrates that our estimates for the power-law exponents, *β*, are not unduly influenced by the presence of noise and cusps, that are expected to be associated with high-velocities and/or high-accelerations. Results are shown for Replicate 1 Controls (15 individuals): full dataset with no filtering (left); a reduced dataset excluding speeds $v = \sqrt{\dot{x}^2 + \dot{y}^2} > 2\left\langle v^2 \right\rangle^{1/2}$ and accelerations $a = \sqrt{\ddot{x}^2 + \ddot{y}^2} > 2\left\langle a^2 \right\rangle^{1/2}$(middle) and; a reduced dataset excluding speeds $v > \left\langle v^2 \right\rangle^{1/2}$ and accelerations $a > \left\langle a^2 \right\rangle^{1/2}$(right). The conditioning removes around 10% and 50% of the data from the dataset.

Speed curvature power law exponents

Table A6.1: Raw speed curvature power law exponents for individual bees in each treatment (pre- training, T1), calculated from bee tracking data (exponents calculated by Andy Reynolds, Rothamsted research).

Control	Appetitive + Aversive	Aversive	Appetitive
0.60	0.87	0.37	0.44
0.42	0.71	0.94	0.5
0.89	0.59	0.49	0.85
0.59	0.73	0.52	0.45
0.57	0.60	0.51	0.7
0.92	0.48	0.6	0.52
0.56	0.48	1.14	0.43
0.66	0.58	0.64	0.35
0.55	0.45	0.1	0.52
0.84	0.43	0.38	0.53
0.96	0.44	0.87	0.5
0.58	0.64	0.51	0.71
0.66	0.76	0.55	0.47
0.56	0.45	0.45	0.8
0.86	0.51		0.69

Appendix Chapter 7

The speed curvature power law: a new tool to assess sublethal pesticides effects in *Bombus terrestris*.

Speed curvature power law exponents

Table A7.1: Raw speed curvature power law exponents for individual bees in the low dose pesticide experiments (post-training), calculated from bee tracking data (exponents calculated by Andy Reynolds, Rothamsted research). (Control; n = 9, Sulfoxaflor; n = 9, Thiacloprid; n = 9, Thiamethoxam; n = 9).

Control	Sulfoxaflor	Thiacloprid	Thiamethoxam
0.58	0.57	0.56	0.58
0.57	0.48	0.49	0.74
0.52	0.55	0.4	0.62
0.5	0.5	0.45	0.57
0.38	0.53	0.49	0.64
0.43	0.36	0.5	0.45
0.63	0.5	0.5	0.5
0.4	0.42	0.48	0.61
0.56	0.52	0.5	0.59

Table A7.2: Raw speed curvature power law exponents for individual bees in the high dose pesticide experiments (post training), calculated from bee tracking data (exponents calculated by Andy Reynolds, Rothamsted research). Exponents are also given for T1 for the controls from this Chapter (Chapter 7) and from Chapter 6 experiments to facilitate comparison. (Control (Chapter 6, T1); n = 7, Control (Chapter 7, T1); n = 9, Control (Chapter 7, T10); n = 9, Sulfoxaflor; n = 9, Thiacloprid; n = 9, Thiamethoxam; n = 9).

Control (Chapter 6, T1)	**Control** (Chapter 7, T1)	**Control** (Chapter 7, T10)	**Sulfoxaflor**	**Thiacloprid**	**Thiamethoxam**
0.94	0.54	0.4	0.47	0.55	0.73
0.49	0.51	0.51	0.49	0.46	0.69
0.52	0.59	0.42	0.51	0.38	0.52
0.51	0.59	0.47	0.47	0.61	0.59
0.6	0.58	0.4	0.36	0.43	0.57
0.64	0.57	0.36	0.46	0.46	0.63
0.51	0.5	0.37	0.48	0.38	0.68
	0.42	0.55	0.39	0.41	
	0.43	0.52	0.36	0.68	

Appendix Chapter 8

Differential learning performance of *Bombus terrestris* foragers: a genetic basis for learning?

Expression data

Table A8.1: Expression data for the 83 significant (P<0.05) differentially expressed genes between good and bad learner groups.

Gene ID	Log2 Fold Change	P Value	Adjusted P Value
LOC100642951	1.428827858	0.000142	0.022556
LOC110120005	-1.655150939	1.06E-05	0.003273
LOC100648970	1.775087974	5.61E-07	0.000316
LOC100642730	0.662992466	0.000469	0.049575
LOC100643717	0.855729059	0.000148	0.023116
LOC100643498	1.195404992	0.000407	0.048102
LOC100649554	-1.072265678	2.4E-06	0.001013
LOC100642769	1.895832086	2.94E-06	0.001194
LOC100643129	2.271515616	3.65E-05	0.008613
LOC100649490	0.748674279	1.16E-05	0.003457
LOC100644749	0.734673162	9.21E-05	0.017309
LOC105666016	-1.335608538	4.58E-08	5.81E-05
LOC100652041	-1.959968978	2.49E-07	0.00022
LOC100647465	0.800443067	2.6E-07	0.00022
LOC100649715	-0.92733395	0.000285	0.038009
LOC100644875	0.605797795	0.000107	0.019023
LOC100650142	0.655929484	0.00037	0.045746
LOC100644170	0.925988832	8.97E-06	0.002936
LOC100651046	-1.204375803	3.9E-05	0.008998
LOC105667097	-0.680453577	0.000315	0.04046
LOC100642200	0.800181451	3.53E-05	0.008518
LOC110119950	-2.069135986	3.2E-05	0.008109
LOC100647220	-1.165522571	3.23E-07	0.000234
LOC100644310	-0.773090381	4.82E-05	0.010634
LOC105665753	0.706388294	0.000246	0.03379
LOC100645419	-0.557780533	6.76E-05	0.014285

LOC100631058	0.617931355	0.00013	0.021749
LOC100642963	-1.295501202	0.000383	0.046778
LOC100650366	0.604539329	0.000483	0.04998
LOC100650836	-0.871077378	0.000366	0.045746
LOC100650642	0.768491216	0.000217	0.030188
LOC100648477	-0.643824922	0.000126	0.021717
LOC100649534	-0.729155261	0.000466	0.049575
LOC105666153	-0.788004888	0.000151	0.023116
LOC100631060	1.065101402	6.4E-07	0.000342
LOC100649865	-1.784608748	0.000142	0.022556
LOC100647447	-1.262546854	1.02E-05	0.003227
LOC105665878	-1.846649682	1.78E-09	4.52E-06
LOC100644543	0.650835873	0.000422	0.048102
LOC100647860	-1.027627051	0.000289	0.038009
LOC100643511	-0.750052695	0.00044	0.048102
LOC100642474	0.813283234	7.5E-05	0.015219
LOC100649335	2.11959681	5E-06	0.00195
LOC100646071	-0.987302516	1.18E-07	0.000119
LOC100649730	-0.716497143	0.000409	0.048102
LOC100643627	-0.922338682	1.7E-05	0.004926
LOC100644271	0.767880145	6.23E-05	0.013454
LOC100647835	-0.783922242	0.000206	0.029467
LOC100644161	-0.666959317	8.48E-05	0.016229
LOC100647520	-1.11253304	0.000133	0.021835
LOC100645221	1.311026414	3.46E-05	0.008518
LOC100646044	-1.238406189	2.19E-06	0.00101
LOC100649255	0.959997939	0.000262	0.035515
LOC100645035	-1.085104641	3.14E-05	0.008109
LOC100649855	-0.966853282	0.000436	0.048102
LOC100646144	-2.016520046	7.95E-15	8.07E-11
LOC100650850	0.488601514	0.000422	0.048102
LOC105666938	0.662927299	0.000474	0.049575
LOC105665758	-1.285897405	2.94E-09	5.98E-06
LOC100644985	-0.983980948	0.000166	0.024448
LOC100646624	-1.226374318	0.000441	0.048102
LOC100646400	-0.683314065	0.000217	0.030188
LOC100648451	-2.453583449	3.35E-11	1.13E-07

LOC100646719	0.852642626	0.0001	0.018495
LOC105665840	-0.79909926	0.000124	0.021674
LOC100651693	0.737264575	8.05E-05	0.016024
LOC100651873	-0.755431351	0.000426	0.048102
LOC100651883	0.74855756	0.000438	0.048102
LOC105666613	1.185991642	0.000131	0.021749
LOC100646091	0.772001292	2.39E-06	0.001013
LOC105667011	-1.224586439	1.85E-05	0.005189
LOC100650290	-0.893882817	5.81E-06	0.002107
LOC100649166	-0.915442142	2.67E-08	4.51E-05
LOC100650322	-0.764339046	4.34E-05	0.009792
LOC100645184	1.07530395	5.28E-08	5.95E-05
LOC105665894	-0.734967678	3.93E-08	5.7E-05
LOC100650316	0.582362259	8.44E-05	0.016229
LOC100651601	-1.389767757	0.000104	0.018841
LOC100643010	-0.792952937	0.000396	0.047835
LOC100642457	0.616236015	0.000159	0.023747
LOC100643289	-1.37244535	4.42E-07	0.000264
LOC100650045	1.454762047	5.24E-06	0.00197

Overrepresented GO terms

Table A8.2: the 62 overrepresented GO terms from the 83 significant (<0.05) differentially expressed genes between the good and the bad learner groups.*

GO ID	GO Name	GO Category
GO:0016887	ATPase activity	MOLECULAR_FUNCTION
GO:0016861	intramolecular oxidoreductase activity, interconverting aldoses and ketoses	MOLECULAR_FUNCTION
GO:0009713	catechol-containing compound biosynthetic process	BIOLOGICAL_PROCESS
GO:0009712	catechol-containing compound metabolic process	BIOLOGICAL_PROCESS
GO:0042302	structural constituent of cuticle	MOLECULAR_FUNCTION
GO:0032993	protein-DNA complex	CELLULAR_COMPONENT
GO:0043546	molybdopterin cofactor binding	MOLECULAR_FUNCTION
GO:1903825	organic acid transmembrane transport	BIOLOGICAL_PROCESS

GO:0004807	triose-phosphate isomerase activity	MOLECULAR_FUNCTION
GO:0004854	xanthine dehydrogenase activity	MOLECULAR_FUNCTION
GO:0004855	xanthine oxidase activity	MOLECULAR_FUNCTION
GO:0016810	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds	MOLECULAR_FUNCTION
GO:0001046	core promoter sequence-specific DNA binding	MOLECULAR_FUNCTION
GO:0001013	RNA polymerase I regulatory region DNA binding	MOLECULAR_FUNCTION
GO:0001047	core promoter binding	MOLECULAR_FUNCTION
GO:0001067	regulatory region nucleic acid binding	MOLECULAR_FUNCTION
GO:0042423	catecholamine biosynthetic process	BIOLOGICAL_PROCESS
GO:0004647	phosphoserine phosphatase activity	MOLECULAR_FUNCTION
GO:0019752	carboxylic acid metabolic process	BIOLOGICAL_PROCESS
GO:0098533	ATPase dependent transmembrane transport complex	CELLULAR_COMPONENT
GO:0006493	protein O-linked glycosylation	BIOLOGICAL_PROCESS
GO:1990837	sequence-specific double-stranded DNA binding	MOLECULAR_FUNCTION
GO:0006520	cellular amino acid metabolic process	BIOLOGICAL_PROCESS
GO:0016787	hydrolase activity	MOLECULAR_FUNCTION
GO:0006563	L-serine metabolic process	BIOLOGICAL_PROCESS
GO:0006564	L-serine biosynthetic process	BIOLOGICAL_PROCESS
GO:0044815	DNA packaging complex	CELLULAR_COMPONENT
GO:0006584	catecholamine metabolic process	BIOLOGICAL_PROCESS
GO:0043436	oxoacid metabolic process	BIOLOGICAL_PROCESS
GO:0016725	oxidoreductase activity, acting on CH or CH2 groups	MOLECULAR_FUNCTION
GO:0016726	oxidoreductase activity, acting on CH or CH2 groups, NAD or NADP as acceptor	MOLECULAR_FUNCTION
GO:0016727	oxidoreductase activity, acting on CH or CH2 groups, oxygen as acceptor	MOLECULAR_FUNCTION
GO:0016714	oxidoreductase activity, acting on paired donors	MOLECULAR_FUNCTION
GO:0004497	monooxygenase activity	MOLECULAR_FUNCTION
GO:0043190	ATP-binding cassette (ABC) transporter complex	CELLULAR_COMPONENT
GO:0004525	ribonuclease III activity	MOLECULAR_FUNCTION
GO:0006361	transcription initiation from RNA polymerase I promoter	BIOLOGICAL_PROCESS
GO:0006360	transcription by RNA polymerase I	BIOLOGICAL_PROCESS
GO:0000976	transcription regulatory region sequence-specific DNA binding	MOLECULAR_FUNCTION
GO:1905039	carboxylic acid transmembrane transport	BIOLOGICAL_PROCESS
GO:0070860	RNA polymerase I core factor complex	CELLULAR_COMPONENT
GO:0004511	tyrosine 3-monooxygenase activity	MOLECULAR_FUNCTION

GO:0000120	RNA polymerase I transcription factor complex	CELLULAR_COMPONENT
GO:0070897	transcription preinitiation complex assembly	BIOLOGICAL_PROCESS
GO:0005506	iron ion binding	MOLECULAR_FUNCTION
GO:1901475	pyruvate transmembrane transport	BIOLOGICAL_PROCESS
GO:0006850	mitochondrial pyruvate transmembrane transport	BIOLOGICAL_PROCESS
GO:0001164	RNA polymerase I core promoter sequence-specific DNA binding	MOLECULAR_FUNCTION
GO:0001163	RNA polymerase I regulatory region sequence-specific DNA binding	MOLECULAR_FUNCTION
GO:0006848	pyruvate transport	BIOLOGICAL_PROCESS
GO:0001188	RNA polymerase I preinitiation complex assembly	BIOLOGICAL_PROCESS
GO:0044212	transcription regulatory region DNA binding	MOLECULAR_FUNCTION
GO:0032296	double-stranded RNA-specific ribonuclease activity	MOLECULAR_FUNCTION
GO:0009070	serine family amino acid biosynthetic process	BIOLOGICAL_PROCESS
GO:0046189	phenol-containing compound biosynthetic process	BIOLOGICAL_PROCESS
GO:0018958	phenol-containing compound metabolic process	BIOLOGICAL_PROCESS
GO:0046982	protein heterodimerization activity	MOLECULAR_FUNCTION
GO:0006082	organic acid metabolic process	BIOLOGICAL_PROCESS
GO:0042578	phosphoric ester hydrolase activity	MOLECULAR_FUNCTION
GO:0000786	nucleosome	CELLULAR_COMPONENT
GO:0000785	chromatin	CELLULAR_COMPONENT
GO:0003824	catalytic activity	MOLECULAR_FUNCTION

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