Interaction of diet, access to food, and age on the susceptibility of the buff-tailed bumblebee (*Bombus terrestris*) to neonicotinoid pesticides

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Abstract

The pollination service provided by bees helps to maintain plant species in natural ecosystems and is worth billions of dollars annually to agriculture. The development of agricultural activities coincides with the increase of pesticide use to control pests. How harmful these pesticides are to non-target insects such as bees is still debated. Neonicotinoids are world widely used in insect control programs because of their unique neurotoxic and systemic properties. Consequently, trace residues of neonicotinoids can appear in nectar and pollen; thus bees are exposed in their dietary by foraging from treated crops.

Surprisingly, little is known about nutritional needs of wild pollinators and whether or not neonicotinoids can affect the nutrient requirements and survival of wild pollinators. For a better understanding how sub-lethal doses of neonicotinoids affect bees, I examined the impact of the three most commonly used neonicotinoids (clothianidin, imidacloprid and thiamethoxam) on nutrient balancing using the Geometric Framework for nutrition on Buff-tailed bumblebee (*Bombus terrestris* L.) were fed diets composed of protein and carbohydrates (in Protein:Carbohydrate ratios). I also examined the influence of the age on feeding choice of bumblebees given a choice of sucrose or sucrose containing a low sub-lethal dose of imidacloprid. In each experiment, all diets were provided *ad libitium*.

In chapter 1, I tested how the P:C ratio (in P:C ratios for Casein 1:x, 400, 250, 180, 100, 75, 50 and 25) affected food consumption and survival of adult worker bumblebees fed with different sub-lethal doses of imidacloprid (0, 1, 10 and 100nM). These experiment provided clear evidences that imidacloprid has reduces food intake and influences nutrient balancing. Imidacloprid also shifted the intake target (IT) of adult worker bumblebees for a higher protein diet and this was dose-dependent. I also observed that the mortality significantly increased with the concentration of protein in diets and the dose of imidacloprid in food. Bumblebees were showing signs of malaise, more likely to be less active and spent more days lying on their back with increasing doses of imidacloprid.

In the second chapter, I examined how the ease of access to food influenced the IT, the survival and the behavior of bees exposed to imidacloprid in food. I used an increasing

concentration in protein (in P:C ratios for Casein 1:x, 250, 100, 75, 50 and 25), a sub-lethal dose of imidacloprid (0 and 10nM) and two different position of tubes (LT for low tubes and HT for high tubes). Keys results emerging from my work are that bumblebees that have to work less to access to food (LT) significantly eat more even if they are exposed to imidacloprid (10nM) in their diets, live longer in the control (no imidacloprid) and higher protein diets (no imidacloprid and 10nM), are less active and lost less weight. The most striking result is that bumblebees in LT initially increased their protein intakes before switching for carbohydrates after day 3.

The third chapter compared the impact of a sub-lethal dose (10nM) of thiamethoxam, clothianidin, and imidacloprid on nutrient balancing, behavior, weigh loss and survival of bees fed diets containing only sucrose (0.5M), a dietary source of protein (in P:C ratios for Casein 1:50) and free amino acids (in EAA:C ratios for EAA 1:50). My results show that the neonicotinoids have different effects on the food IT, survival, physiology and behavior. Thiamethoxam seems to have the most critical impact on bumblebees.

In chapter 4, I compared the attraction or repellent property of a sucrose solution (0.5M) in a two choice assay with different sub-lethal doses of imidacloprid (0, 1 and 10nM). I also examined whether this effect was age-dependent (adult forager *vs.* newly-emerged). My results show that bumblebees are able to detect imidacloprid in their diet. Adult forager bumblebees were significantly more attracted by contaminated food and the lower dose of imidacloprid (1nM). Bumblebees were returning to feed according to their food preference over the three days.

My results provide valuable data that shows that neonicotinoids have different toxic properties on bumblebees and emphasize the difficulty of understanding the complexity of their impacts. I also highlighted that residues traces in the diet can strongly influence the foraging behavior and thus explain possible mechanisms involved in the bee decline due to exposure to neonicotinoids. My work shows that bumblebees cannot control their exposure to neonicotinoids in food, but can reduce their impacts by adapting their intake in protein and implies that treated crops with neonicotinoids presents a sizeable hazard to foraging bees.

Key words: bumblebees, *Bombus terrestris* L., Geometric Framework, food intake target, neonicotinoids, caste, repellent effect

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Table of Contents

List of tables

List of figures

General introduction

I. Context of the thesis

1. Biodiversity and ecosystem services in agroecosystems

Biodiversity is a term coined the 1980's from concerns about a sixth extinction crisis caused by human activity (Barbault, 2006); an extinction crisis later confirmed for numerous taxa (Millennium Ecosystem Assessment, 2005). The term biodiversity immediately found wide use following its invention and become one of the main keywords in biology (Sarkar, 2002). Biodiversity is now a central concept in agronomical research (CBD, 1992) and there is global consciousness of the importance of the biodiversity protection for sustainable development (Brundtland, 1987). The central tenant of conservation biology is the management of the crisis faced by declining species or biological populations. Conservation is important because without it humans risk using ecosystems to a point they no longer sustain human (Barbault, 2006). Thus, the conservation of biodiversity is motivated by practical reasons such as the preservation of the human natural resources which we do not know the potential future value of, such as genetic reserves of interesting genes for plant breeding or services for agriculture (Paoletti *et al.*, 1992; Peeters & Janssens, 1995; Cairns, 1997; Altieri, 1999; Duelli & Obrist, 2003).

The needs for conservation are particularly relevant to ecological services that are defined as the profits that we gain from the functions of natural ecosystems (de Groot, 1994; Boyd & Banzhaf, 1997; Costanza *et al.*, 1997), that contribute to human well-being as determined by the Millennium Ecosystem Assessment (2005). Ecosystem services are categorized as follows: provisioning services, regulating services, supporting services and cultural services –each which has several sub-categories that include pollination services in the regulating services (Millennium Ecosystem Assessment, 2005).

In the European Union, agricultural areas are significantly more of the land area (44%) than protected areas (<5%) (Piorr, 2003). Furthermore, mosaic landscapes based on a mixture of agricultural and semi-natural areas can represent particular kind of biodiversity reserves (Clergue *et al.*, 2005). Biodiversity conservation in agroecosystems is ultimately the result of the interaction between production supplies with respect to those environmental conditions, which are regarded as sustainable (Peeters & Janssens, 1995; Vereijken *et al.*, 1997; Altieri, 1999; Büchs, 2003). Agroecosystems are principally driven to optimize crop yield, but these services essentially depend on regulation services such as pollination (Zhang

et al., 2007). Pollination services can marginally increase crop production but also be responsible for crop productivity, and hence pollination is important for agricultural productivity (Klein *et al.*, 2007; Gallai *et al.*, 2009; Garibaldi *et al.*, 2011; Lautenbach *et al.*, 2012). Pollinators are also important for sexual reproduction of plant species in natural ecosystems that play a crucial role in ecosystem functioning (Bascompte *et al.*, 2006; Fontaine *et al.*, 2006; Kremen *et al.*, 2007; Williams & Osborne, 2009; Winfree, 2010).

According to the FAO (Food and Agriculture Organization), contemporary agriculture has to deal with two conflicting objectives: the production of food to meet the demands of the world population increases (FAOSTAT 2007) while simultaneously preserving the biodiversity and ecosystem services on the other side.

2. The pollination service provided by pollinators

Pollination is the sexual reproduction of angiosperms that maintains genetic diversity that vegetative reproduction cannot (Clergue *et al.*, 2005; Nabors, 2008). It is the process by which the transportation of pollen from the anthers to the stigma occurs (Dumas & Zandonella, 1984; Pesson & Louveaux, 1984). Entomophilous pollination can be done by a large diversity of insects that include honeybees (*Apis mellifera*), bumblebees and wild bees (Clergue *et al.*, 2005), as well as other insects including Coleoptera, Lepidoptera or Diptera including hoverflies (Buchmann & Nabham, 1996; Chittka & Thompson, 2001). In Europe, bees (apiform Apoidea) are crucial pollinators playing a central role in the maintenance of flora and fauna (Kevan, 1999).

Most of pollination service conservation studies are focused on bees (Steffan-Derwenter & Tscharntke, 1999; Kremen *et al.*, 2002, 2004; Farhig, 2003; Steffan-Derwenter, 2003). Bees have morphological features that enhance their efficiency to be a pollen vector, such as branched body hairs densely packed (Linsley *et al*., 1963; Thorp, 1979), electrostatic surface potential and specialized hair groups for the acquisition of pollen (Thorp, 1979). Social bees that live in colonies have different foraging groups and target different sites (Beekman & Ratnieks, 2000; Steffan-Dewenter & Kuhn, 2003). Bees are foraging only a small fraction of flowers available in their habitat, and visit the same flower patches inside their foraging area and have a strong area loyalty to them (Robinson, 1989; Dramstad & Fry

1995). Moreover, bees always use the same patch to visit flower patches to which they are faithful (Teodorović & Dell'Orco, 2008).

The plant-pollinator relation is a mutualistic relation that started throughout the Cretaceous and was fertile as it led to the evolutionary explosion of diversity of flowering plants (Pesson & Louveaux, 1984). The links between species within this mutualistic web of relations are resulting from both complementarity and competition between individuals that create a canvas of specific and generalist interactions (Olesen & Jordano, 2002; Olesen *et al.*, 2007; Santamaria *et al.*, 2007). The complex interactions between the components of the web may even maintain the importance of pollination, but one perturbation such as the loss of one habitat can deeply affect the pollinator community and consequently has an impact on the overall pollination service (Meynié *et al.*, 1997; Fortuna & Bascompte, 2006; Memmott *et al.*, 2004; Fontaine *et al.*, 2008).

In European agroecosystems, pollination is provided for 84% of plant species by pollinators of the leading global food crops. Furthermore, 65% of flowering crops benefit from insects and more specifically from bees in the World (Williams *et al.*, 1994; Buchman & Nabhan, 1996; Allen-Wardell *et al.*, 1998; Klein *et al.*, 2007). Moreover, the pollination service provided by bees has an impact on the yields, the quality and the measured gauge of fruit and vegetable (Allen-Wardell *et al.*, 1998; Pouvreau, 2004). The complete vanishing of bees would not completely endanger food production, but it would cause a massive alteration of food diversity and a deep crisis of agriculture sector would result (Gallai *et al.*, 2008). All economic studies of the importance of the pollination service underestimate the crucial value of the pollination service as the pollination service provided by insects is probably immeasurable and consequently justify debates about its conservation (McCauley, 2006). The pollination service provided by insects is both an ecosystem service and agricultural input, analogous to water and nutrients (Free, 1993; Delaplanne & Mayer, 2000; Kremen *et al.*, 2007; Zhang *et al.*, 2007). The needs for pollination services in agricultural systems is exemplified by the intentional introduction of honeybee colonies to crops to increase the fruit set in flowering crops (Free, 1993; Delaplanne & Mayer, 2000) and the production of million colonies of bumblebees every year for greenhouse plant pollination (Velthuis & van Doorn, 2006).

3. Bee decline

Since the *Convention on Biological Diversity* in 1992, the conservation and sustainable use of pollinators has highlighted the potential impact of the bee population decline. The *International Pollinator Initiative* (IPI) in 2001 led by the FAO wanted to estimate the economic value of the pollination service and the economic impact of pollinator decline. More recently, the ALARM project (Assessing Large-scale environmental Risks for Biodiversity with tested Methods; Settele *et al.*, 2005) investigated the biotic and abiotic factors that can explain pollinator decline in Europe and estimate its economic impact. The recent *Grenelle de l'Environnement* (Grenelle Environment Round Table) in summer 2007 in France opened a multi-party debate about how to develop new ecology policies and sustainable development issues, particularly in agronomy, by bringing together the different actors involved (Politicians, Industry, Labors, Associations, Non-governmental organizations etc.). One of the main plans subsequently developed was *Ecophyto 2018* – a policy that aims to reduce pesticide use in French agriculture by 50% before 2018. Policies of this kind show that the question of the bee population decline is not recent and is the subject of investigations at different levels. It also shows that some factors remain hard to understand for the moment due to gaps in knowledge (Brown & Paxton, 2009). The relative lack of knowledge about the bee fauna and the lack of good data remains the biggest problem for understanding bee population decline (Eardley *et al.*, 2009).

In natural ecosystems, the mutualistic relation between plants and pollinators offers a theoretical strong resilience capacity to the pollinator network, as the replacement of specialist pollinators by more generalist species does not have much of an impact on the pollinator network (Memmott *et al.*, 2004). Only 15% of pollinator species (principally generalist) play a major role in crop pollination (Olesen *et al.*, 2007). These observations show that agroecosystems are potentially vulnerable to deficits in only a small sub-group of pollinators. Several crops can lose more than 70% of production due to lack of pollinators (Reddi, 1987). For example, the pollination crisis of almond tree in California in 2006 revealed that honeybees play a crucial role for the pollination of some plants, as the vanishing of 90% of colonies forced producers to introduce thousands of colonies to counteract the withdrawal of pollinators (Holden, 2006; Stokstad, 2007a,b). The introduction of Africanized honeybees in Central America coincided with the increase by 50% in the

coffee production in this region (Roubik, 2002). Moreover, the pollinator diversity can also be a crucial factor that enhances crop productivity (Klein *et al.*, 2002; Steffan-Derwenter, 2003). Some studies have also compared differences in pollinator efficiency and effectiveness that depend on species (Kremen *et al.*, 2002; Ricketts *et al.*, 2004) and suggest that the competition between at least two generalist species can significantly increase the production of entomophilous crops (Chagon *et al.*, 1993; Degrandi-Hoffman & Watkins, 2000; Klein *et al.*, 2003; Dag *et al.*, 2006; Greenleaf & Kremen, 2006).

The increasing needs for bees in crop pollination in worldwide food production have occurred at the same time that there is emerging evidence that bee populations are declining (Biesmeijer *et al.*, 2006; Fitzpatrick *et al.*, 2007). The documented declines in pollinators (Biesmeijer *et al*., 2006; Potts *et al.*, 2010; Carvalheiro *et al.*, 2013) may therefore threaten food security, which in return may lead to an increasing demand for agricultural land (Aizen *et al.*, 2009). In United Kingdom and Netherlands, the bee population declined by 20 to 60% after 1980 in most studied areas, but surprisingly the hoverfly population did not drop over that period (Biesmeijer *et al*., 2006). It remains difficult to clearly estimate and understand the bee population variations from one year to another (Roubik, 2001). Thus the crisis of the pollination service and bee decline remains controversial (Ghazoul, 2005 *vs.* Steffan-Derwenter *et al.*, 2005; Aizen *et al.*, 2008), as some controversial studies did not observe significant decline of bee diversity in some areas in North America (Cane, 2001; Cane & Tepedino, 2001).

This decline appears to be worldwide and is likely to be anthropogenically driven. Many factors could be responsible and each may affect domesticated and wild pollinators in different ways (Table 1). Habitat loss and fragmentation appear to be the main universal changes that influences wild bee population, bee genetic diversity and bee diversity in general (Farhig, 2003; Foley *et al.*, 2005; Brown & Paxton, 2009; Zayed, 2009; Syndenham *et al.*, 2014). Other biotic factors such as invasive species (plants, new competitors or predators), parasites and pathogens also play a significant role in this decline (Stout & Morales, 2009), but may be more relevant for honeybee populations (Downey & Winston, 2001; Evans *et al.*, 2003; Cox-Foster *et al.*, 2007). Moreover, honeybees and wild bees have parasites and pathogens in common that enhance the decline (Genersch *et al.*, 2006; Hoffman *et al.*, 2008). Agricultural practice (Banaszak, 1995), pollution (O'Toole, 1993) and competition with honeybees introduced by beekeepers (Steffan-Derwenter & Tscharntke,

1999; Roubik & Wolda, 2001) might also be important factors that can explain the decline of wild bee populations. Climate change is likely to drive down the pollinator diversity and increase the risk of pollination deficits (Parmesan *et al.*, 1999; Polce *et al.*, 2014). These factors could also give rise to *Colony Collapse Disorder* (CCD) reported in USA (Stokstad, 2007a,b; Cox-Foster *et al.*, 2007). All these factors are not independent and interact with each other, and thus their impact on bee population is unlikely to be simple to predict (Brook *et al.*, 2008).

	Solitary bees				Bumblebees	Honeybees		
	Australia	**Central and South America**	**Europe, Mediterranean and North Africa**	**Sub-Saharan Africa and Madagascar**	**Worldwide**	**Asia**	**Europe**	**Africa**
Habitat loss, fragmentation and degradation	X	X	X	X	X	X	X	X
Invasive species[a]	?	X			X	X	X[d]	
Parasites and disease[b]			?		X	X	X	X
Exploitation						X		X
Extinctions cascades[c]	?	?	?	?	?			
Climate change	?	?	?		?	?	?	?

Table 1: Causes of bee decline: the geographic and taxonomic impact of factors as reviewed by Brown & Paxton (2009, Apidologie vol.40, issue 3).

X: strong effect and ?: suspected effect. [a] Includes only plants and animals, [b] includes native and invasive parasites and pathogens, [c] e.g. when a plant species may have a knock-on effect for a specialist pollinator and [d] hybridization with non-native subspecies.

II. The role played by pesticides & neonicotinoids on bee decline

1. General information about neonicotinoids

a. Agronomical context and ecological bottleneck

Pesticide use increased throughout the world after the Second World War and has been driven by greater demands for food production by an increasing world population.

Pesticides are toxic compounds widely used in agriculture. Due to the large number of pesticides used, there is an increased interest that they are the cause of toxicological and environmental issues (Younes & Galal-Gorchev, 2000) including pollinators. Killing pest insects without harming useful pollinators is an intrinsically difficult task and all pesticides used on crops represent some risk to non-target organisms (Gross, 2013). Honeybees may have an even greater risk than pest insects like aphids as the honeybee has fewer genes encoding xenobiotic detoxifying enzymes compared to other insects (Claudianos *et al.*, 2006). The decline of bees observed in the countryside with a possible forthcoming pollination crisis and subsequent irremediable consequences could be a direct result of pesticide use in combination with poor nutrition caused by disappearing food resources for bees caused by human land use.

b. Neonicotinoids: a new generation of pesticides

One class of pesticides, the neonicotinoids, was developed during the 1970's by the chemical industry (Maienfisch *et al.*, 2001). Since 1994, they have been the most commonly used class of insecticides used throughout the world (Elbert *et al.*, 2008). The discovery of neonicotinoids as important novel pesticides was a milestone in insecticide research over the past three decades (Nauen & Denholm, 2005). Neonicotinoids supplanted older pesticide classes such as pyrethoids, chlorinated hydrocarbons, organophosphates, and carbamates on many major crops (Denholm *et al.*, 2002). The invention and the subsequent commercial development of neonicotinoids have provided farmers with an effective new tool for managing some of the world's most destructive crop pests (Nauen & Denholm, 2005) and may explain their increasing use.

The term neonicotinoids has been chosen because the molecular target of this class of pesticides is the nicotinergic acetylcholine receptor (nAChRs). Neonicotinoids are widely

used in agriculture against sucking insects, such as aphids, whiteflies, leafhoppers, planthoppers, thrips, some micro Lepidoptera, and several coleopteran pest species (Jeschke & Nauen, 2005; El Hassani *et al.,* 2008). Moreover, neonicotinoids constitute an effective tool for controlling parasites presents on pets and cattle, and for hygiene pest such as cockroaches, houseflies and termites (Jeschke & Nauen, 2005). Furthermore, insects are consistently considered as the most sensitive taxa to neonicotinoids, whether they are topically or orally in contact with the pesticide (Matsuda *et al.*, 2001; Goulson, 2013). In contrast, neonicotinoids display the advantages of a significant lower toxicity towards vertebrates, including mammals (Tomizawa *et al.*, 2000; Matsuda *et al.*, 2001; Tomizawa & Casida, 2003; Goulson, 2013). Neonicotinoids also have a faster penetration into the insect central nervous system over vertebrates, but it is more related to the target site interactions that are different between insects and vertebrates (Methfessel, 1992; Matsuda *et al.*, 2001). Sub-lethal doses of neonicotinoids can have a strong impact on beneficial insects that are natural enemies of crop pests. For example, the consumption of aphids that were in contact with dietary neonicotinoids in sap by ladybugs (*Harmonia axyridis* and *Hippodomia undecimnotata*: Coleoptera: Coccinellidae) causes higher mortality and reduction in fertility (Vincent *et al.*, 2000; Papachristos & Milonas, 2008; Moser & Obrycki, 2009). Predatory mites like *Neoseiulus californicus* and *Phytoseiulus macropilis* are less effective predators when exposed to neonicotinoids; they show reduced capacity to attack other mites such as *Tetranychus urticae* (Poletti *et al.*, 2007). Sub-lethal doses of neonicotinoids can also impair the infestation success of parasitoid wasps (Hymenoptera: *Anagrus nilaparvatae*) that commonly infest the eggs of the brown planthopper (Homoptera: *Nilaparvata lugens*) and green leafhopper (Homoptera: *Nephotettix* spp.) (Xiang *et al.*, 2008; Liu *et al.*, 2010, 2012).

c. Neonicotinoids uses and relative benefits

Neonicotinoids are mostly used as systemic pesticides. Exposures to neonicotinoids are more likely to occur because massive crop flowering attracts bees (Blacquière *et al*., 2012). As systemic pesticides, neonicotinoids are distributed throughout plant tissues to control sucking insect pests (Elbert *et al*., 2008). Consequently, trace residues can appear in nectar and pollen (Blacquière *et al*., 2012), although concentrations of neonicotinoids found in nectar are generally lower than those in pollen (reviewed in EFSA, 2012 and USEPA, 2012;

Stoner & Eitzer, 2012). Consequently, neonicotinoids can be harmful for non-target insects such as bees (El Hassani *et al*., 2008), as bees are exposed to dietary neonicotinoids by foraging flowers from treated crops (Elbert *et al*., 2008). Furthermore, neonicotinoids do not readily penetrate the insect cuticle, and consistent results are obtained in assays that have measured the contact LD_{50} of bees (Stark *et al*., 1995). Instead, bees are more likely to come into contact with neonicotinoids when they eat substances containing them, and for this reason, neonicotinoids are currently one of the potential factors leading to the decline of wild bee populations (Laycock *et al*., 2014).

In developed countries, the neonicotinoids, imidacloprid, clothianidin and thiamethoxam, are mainly used for seed dressing for a broad diversity of crops, like oilseed rape, sunflower, cereals, beets and potatoes, (Goulson, 2013). Seed coating is usually described as the solution that minimizes the pesticide use and consequently reduces the development of pest resistance, and also mitigates the impact on non-target organisms (Goulson, 2013). The great success of seed coatings is in part due to the fact that they do not require any action from the farmer, as all the parts of the crop are prophylactically protected for several months following sowing, and moreover, they provide better targeting for the crop than spray applications (Jeschke *et al.*, 2011).

In addition to their systemic properties, neonicotinoids are water-soluble (Obana *et al.*, 2003) and thus absorbed by plants by either roots or leaves, then transported throughout all the tissues of the plants (Di Muccio *et al.*, 2006). These properties provide many advantages in pest control, as the whole plant would be protected such as from boring insect and root-feeding insects, that both cannot be effectively done by non-systemic compounds applied using foliar spray for example (Castle *et al.*, 2005; Byrne & Toscano; 2006). Likewise, neonicotinoids are routinely used as foliar sprays on fruit crops that are obviously visited by managed and wild pollinators (Lye *et al.*, 2011; Defra, 2012). Moreover, neonicotinoids are used as foliar sprays in gardens, where they are recommended and used for both vegetables and flowers, offering another route of exposure for pollinators (Goulson, 2013).

Concentrations in plants tissues and sap between 5 and 10ppb are commonly regarded to be sufficient to provide protection against pest insects (Castle *et al.*, 2005; Byrne & Toscano, 2006). When neonicotinoids are applied as seed dressing, their concentrations are between <1 and 8.6ppb in nectar and between <1 to 51ppb in pollen (Table 2; USEPA,

2012). Moreover, direct soil applications of neonicotinoids induce concentrations between 1 to 23ppb in nectar and 9 to 66ppb in pollen (Table 2; USEPA, 2012). The highest concentrations of neonicotinoids in nectar and pollen appear to result from foliar applications (Table 2; Dively & Kamel, 2012).

	Plant	Details		Concentration (nM)			References
				Nectar	Pollen	Guttation	
IMIDACLOPRID	Maize (*Zea mais*)	Seed coated	Field	.	7.8nM	.	Bonmatin *et al.,* 2003
		Seed coated		.	11.7nM	.	Charvet *et al.,* 2004
		Seed coated	Greenhouse	.	.	183.8-327.8nM	Girolami *et al.,* 2009
		Seed coated	Greenhouse	.	.	66.5-312.9nM	Tapparo *et al.,* 2011
		Seed coated	Field	.	<19.56nM	.	Sur & Strok, 2005
		Seed coated	Field	.	.	250.3nM	Reetz *et al* ., 2011
	Oil Seed Rape (*Brassica napus*)	Seed coated	Field	3.9-19.6nM	7.8nM	.	Pohorecka *et al*., 2012
		Seed coated	Field	<19.56nM	<19.56nM	.	Sur & Strok, 2005
	Pumpkin (*Cucurbita pepo* L.)	Sprayed		23.5-35.2nM	238.6-496.8nM	.	Dively & Kamel , 2012
		Sprayed or drip irrigation		<40.2nM	<86.1nM	.	Stoner & Eitzer, 2012
	Sunflower (*Helianthus annuus*)	Seed coated	Greenhouse	7.8nM	15.7nM	.	Schmuck *et al.,* 2001
				.	<2.0–140.1nM	.	Laurent & Rathahao, 2003
		Seed coated	Field	19.6-39.1nM	19.6-39.1nM	.	Bonmatin *et al.,* 2003
		Seed coated	Field	<19.56nM	<19.56nM	.	Sur & Strok, 2003
CLOTHIANIDIN	Maize (*Zea mais*)	Seed coated	Greenhouse	.	.	108.1nM	Girolami *et al.* ,2009
		Seed coated	Greenhouse	.	.	29.2–408.5nM	Tapparo *et al.,* 2011
		Seed coated	Field	.	>16.0nM	.	Pistorius *et al.,* 2009
		Seed coated	Field	.	.	32.0nM	Reetz *et al* ., 2011
		Seed coated	Field	.	4.0-16.0nM	.	Pilling *et al* ., 2013
	Oil Seed Rape (*Brassica napus*)	Seed coated	Field	2.0-8.0nM	4.0-12.1nM	.	Pohorecka *et al* .,2012
		Seed coated		14.8nM	12nM	.	Cutler & Scott-Dupree, 2007
THIAMETHOXAM	Maize (*Zea mais*)	Seed coated	Greenhouse	.	.	51.4nM	Girolami *et al.,* 2009
		Seed coated	Greenhouse	.	.	3.5–531.3nM	Tapparo *et al.,* 2011
		Seed coated	Field	.	3.4-24.4nM	.	Pilling *et al*., 2013
	Oil Seed Rape (*Brassica napus*)	Seed coated	Field	3.4-17.1nM	10.3-51.4nM	.	Pohorecka *et al*., 2012
		Seed coated	Field	2.2-8.2nM	<3.4-12.0nM	.	Pilling *et al.,* 2013
	Pumpkin (*Cucurbita pepo* L.)	Sprayed		20.6-30.9nM	209.1-435.4nM	.	Dively & Kamel, 2012
		Sprayed or drip irrigation		58.3nM	72.0nM	.	Stoner & Eitzer, 2012

Table 2: Concentrations of neonicotinoids in pollen, nectar and guttation fluids of crops.

d. Economic benefits of neonicotinoids

The relative higher toxicity of neonicotinoids for insects over vertebrates, the flexibility of use and the systemic properties described previously explain the worldwide success of neonicotinoids. The unique success of neonicotinoids is reflected in their sales and percentage market share in 1990 in comparison with 2005. In 1990, the agrochemical market was dominated by organophosphates (43%) before the launch of imidacloprid in 1991. Imidacloprid was representing 41% of the pesticide market in 2011 (Elbert *et al.*, 2008; Jeschke *et al.*, 2011; Pollack, 2011). The market of neonicotinoids is now extended to 120 countries and has a global value of $2.6 billion (Jeschke *et al.*, 2011; Pollack, 2011). In United Kingdom, the use of seed dressings accounted for 91% of all neonicotinoids in farming in 2011 (Defra, 2012) and 60% of neonicotinoids are used this way globally (Jeschke *et al.*, 2011). Neonicotinoid use significantly increased between 2000 and 2012 in United Kingdom by spraying crops, through the irrigation system, granule application, crop dusting or seed coating (Table 3). Neonicotinoid use as a seed treatments are becoming increasingly important in agricultural crops as they target organisms that are not controlled by Bt δ-endotoxins (Tomizawa & Casida, 2005). For example, in United Kingdom, the use of neonicotinoids has not substantially improved the yields of oilseed rape when data obtained before-1994 is compared to today's yields (Parry & Hawkesford, 2010: Defra, 2012). The use of the type of neonicotinoids has also changed in United Kingdom from 1994 to 2012 (Figure 1). After a sharp increase in imidacloprid use, its utilization sharply dropped with the arrival of clothianidin and thiamethoxam on the market. Clothianidin was the most used neonicotinoid in 2012.

	2000		2012	
	Area treated (ha)	Weight applied (t)	Area treated (ha)	Weight applied (t)
Insecticides				
Carbamates	251,534	25	125,037	89
Organochlorines	3,482	3	.	.
Organophosphates	252,000	94	102,853	78
Pyrethroids	3,088,801	56	4,349,631	68
Others (e.g. Neonicotinoids)	63,914	7	223,439	18
Total insecticides	**3,659,731**	**185**	**4,800,960**	**252**
Fungicides	14,428,727	4,072	20,252,722	5,061
Growth regulators	3,994,784	3,134	5,517,515	2,804
Herbicides & dessicants	13,513,475	8,123	14,940,062	6,619
Molluscides & repellents	1,267,729	387	877,965	126
Nematicides	34,517	254	6,232	14
Seed treatments*	4,234,967	345	4,744,969	192
Total pesticides	**41,178,451**	**28,289**	**51,174,157**	**15,187**

Table 3: Comparison of pesticide usage in United Kingdom between 2000 and 2012. Data for Northern Ireland were not available in 2000. *: Including neonicotinoids. Data includes oxamyl and ethoprophos, which have both insecticidal and nematicidal properties (Fera Stats, 2013 https://secure.fera.defra.gov.uk/).

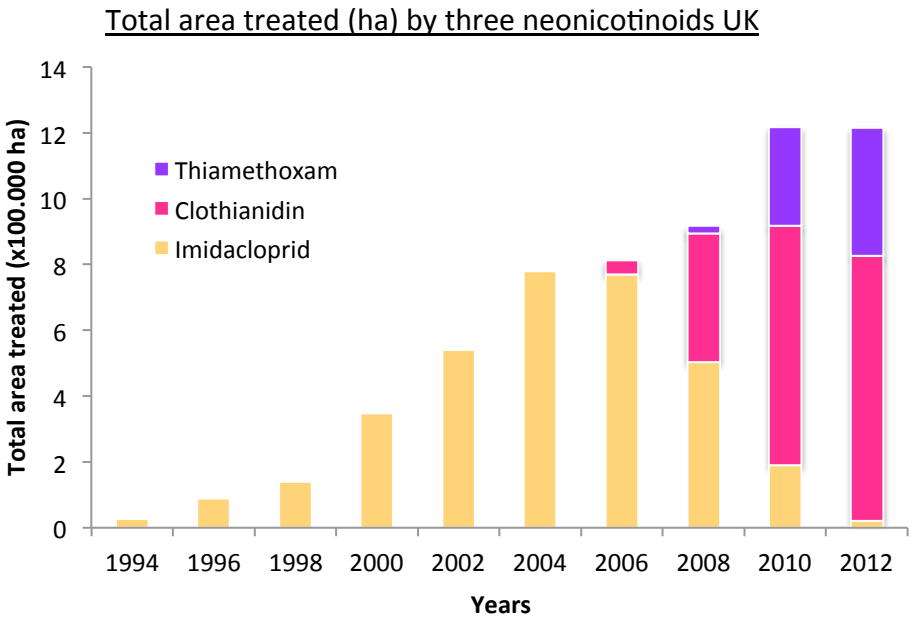

Figure 1: Comparison of three neonicotinoids usage in United Kingdom between 1994 and 2012 for all crops including seed treatments. Data for Northern Ireland were not available in 2000. (Fera Stats, 2013 https://secure.fera.defra.gov.uk/).

Mixtures containing insecticides also increase the toxicity of neonicotinoids by factor from 1.5 to 1141 (Iwasa *et al.,* 2004). All elements were topically applied to bees at low sub-lethal doses. A wide range of pesticides could be found in combinations in pollen, honey and beewax of honeybee colonies foraging on agricultural land (Bernal *et al.*, 2010; Gernersch *et al.*, 2010; Mullin *et al.*, 2010). In this context, no larval mortality is observed in honeybee colonies but a delayed worker development when the brood was exposed to highly contaminated food with low residue concentrations in neonicotinoids was found (Wu *et al*., 2001).

The potential side effects of long-term exposure to neonicotinoids need to be evaluated as adult bees consume more nectar than pollen while larvae consume more pollen than nectar (Rortais *et al.*, 2005). Moreover, it is important to note that different results can be also explained by a lack of standardization of tests. The risk assessment of neonicotinoids is not the same for winter bees, nectar foragers and nurses which are more sensitive to neonicotinoids than for workers and drone larvae, wax-producing bees and pollen foragers (Halm *et al.*, 2006). Those effects have to include the impact of social interactions.

Neonicotinoids have particularly been singled out for blame (Shardlow, 2013), which has led to call for restrictions on their use (EFSA, 2013; Maxim & van der Sluijs, 2013) that has recently been implemented across European Union and include imidacloprid, thiamethoxam and clothianidin (European Union, 2013).

e. Neonicotinoids sub-lethal effects on pollinators

Most of the arguments against the use of neonicotinoids are focused on their effects at sub-lethal doses on beneficial insects such as bees. Sub-lethal effects are defined as effects on physiology and behavior of an individual that has been exposed to a pesticide without directly causing death (Desneux *et al.*, 2007).

As previously explained, neonicotinoid insecticides act as neurotoxic agents and the large range of intoxication symptoms reported show that they can strongly affect the pollinator community. It is well reported that sub-lethal doses of neonicotinoids can reduce learning, foraging and homing abilities for both honeybees and bumblebees (Yang *et al.*, 2008; Decourtye & Devillers, 2010; Han *et al.*, 2010, Mommaerst *et al.*, 2010; Gill *et al.*,

2012; Henry *et al.*, 2012; Yang *et al.*, 2012), and consequently have a strong impact on the colony fitness by reducing queen production, colony growth and health (Gill *et al.*, 2012; Whitehorn *et al.*, 2012). Furthermore, the exposure through the diet to sub-lethal doses of neonicotinoids increases the number of larvae ejected from their cocoons inside bumblebee colonies with the wastes of the colony (Tasei *et al.*, 2000). Moreover, a large range of symptoms that impair bees at an individual level such as knockdown, trembling, uncoordinated movements, hyperactivity can be observed (Lambin *et al*., 2001; Nauen *et al*., 2001; Suchail *et al*., 2001; Medrzycki *et al*., 2003; Colin *et al*., 2004). Such sub-lethal effects are not revealed or considered in standard safety-testing protocols.

Furthermore, the majority of studies are solely focused on imidacloprid, which is historically relevant because it was the first neonicotinoid extensively use because it appeared on pesticide market in 1991 (Elbert *et al.*, 2008) and was identified publicly as a potential hazard to bee health in 1999 (Maxim & van der Sluijs, 2013). The rise in reliance on development neonicotinoid insecticides for crop protection, consumer/professional products and animal health reflects the unique success of this chemical class (Elbert *et al.*, 2008). Though, newer neonicotinoid generation such as thiamethoxam that arrived in 1998 and its toxic metabolite clothianidin in 2001 are now preferred for crop protection. Concurrently, imidacloprid use is declining cause of bad adverts on its impact on beneficial insects (Figure 1).

2. Nicotinic Acetylcholine Receptors (nAChRs) in insects

Acetylcholine (ACh) is the main neurotransmitter in the central nervous system of insects (Breer, 1987; Bicker, 1999). This neurotransmitter plays a crucial role in insect synaptic transmission (Gerschenfeld, 1973; Callec & Sattelle, 1973; Gundelfinger & Hess, 1992; Thany *et al.*, 2007). Three main types of acetylcholine receptors have been identified: muscarinic receptors for *e.g*. that regulate the liberation of ACh pre-synaptically (Hue *et al.*, 1989; Trimmer & Qazi, 1996), nicotinic receptors (nAChRs) that are fast acting ligand-gated ion channel (Hue & Callec, 1990; Lapied *et al.*, 1990; Changeux & Edelstein, 1998; Thany *et al.*, 2007), and receptors with dual characteristics that pharmacologically present muscarinic and nitotinic properties and play an important role in calcium and neurosecretion modulation (Lapied *et al.*, 1990; Benson, 1992; David & Pitman, 1993).

Cholinergic neurotransmission is used for many functions in insects (Dacher *et al.*, 2005; Thany & Gauthier, 2005); it is the main neurotransmitter used in sensory and motor function. For example, the application of cholinergic agonists on the ventral nerve of the tobacco hornworm larva (Lepidoptera: *Manduca sexta*) induced muscle contraction. This stimulation showed that a synapse exists between the interneuron and the motor neuron innervating the main muscle of the abdominal retraction (Trimmer & Weeks, 1989). Cholinergic transmission is also important in sensory processing; the stimulation of the antennal lobe induces the post-synaptic depolarization of the mushroom body in honeybees that is blocked by the application of an antagonist of nACh receptors such as curare. This demonstrates that nAChRs are involved in synaptic transmission between antennal lobe and mushroom bodies of honeybees (Oleskevich, 1999), and that ACh-binding sites are present in the honeybee brain (Kreissl & Bicker, 1989; Scheidler *et al.*, 1990). Furthermore, the injection of nicotinic antagonists in honeybee brain reduces the proboscis extension reflex and causes learning impairments implying that nAChRs are involved in these processes (Dacher *et al.*, 2005; de Brito Sanchez *et al.*, 2005, Dacher & Gauthier, 2008).

The discovery of the important role played by nAChRs has called the attention on pentameric membrane proteins, and ligands able to modulate their functions. These transmembrane allosteric proteins may spontaneously exist under several discrete interconvertible conformational states: basal or resting (close), active (open) or desensitized (closed) (Edelstein *et al.*, 1996). Moreover, the existence of different subtypes has complicated the understanding of the role of this receptor type in the insect nervous system (Romanelli & Gualtieri, 2003; Taillebois *et al.*, 2014). These receptors are all members of the Cys-loop ligand-gated ion channel (LGIC) superfamily and contribute to a wide range of nervous activities and influence numerous physiological functions (Gotti & Clementi, 2004; Wang *et al*., 2007).

Neonicotinoids belong to the group of neurotoxic pesticides and there is considerable evidence that the target of the neonicotinoid compounds is nAChRs, where they act as partial or almost-full agonists (Déglise *et al.*, 2002; Tomizawa & Casida, 2003, 2005; Nauen *et al.*, 2003). Like nicotine, neonicotinoids show strong binding affinity for nAChRs of insects, which may be due to their hydrophobic properties, but nicotine and neonicotinoids differ in insecticidal activity (Yamamoto *et al.*, 1998). Neonicotinoids are broad-spectrum neurotoxins that disrupt the action of the insect nervous system by inducing

membrane depolarization at nerve synapses (Matsuda *et al.*, 2001; Thany, 2010). The binding site of neonicotinoids on nAChRs is not consistent between insect species (Honda *et al.*, 2006). All neonicotinoids insecticides act with nanomolar affinity against insect nAChRs (Wiesner & Kayser, 2000). Consequently, even sub-lethal doses of neonicotinoids can affect insects, including bees.

3. Imidacloprid

One of the most commonly used systemic neonicotinoids is imidacloprid (Figure 2), known under a large number of trade names: Admire, Confidor, Conguard, Gaucho, Intercept, Kohinor, Mallet, Turfthor, Winner etc. Imidacloprid was the first neonicotinoid commercialized in 1991 for seed coating and is still one of the most commonly used chemicals on crops (Elbert *et al.*, 2008; Decourtye and Devillers, 2010).

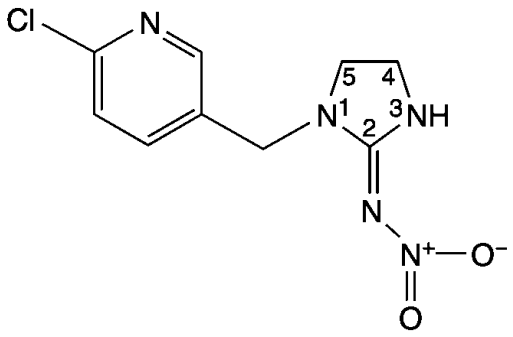

Figure 2: Molecular structure of imidacloprid (Chemical formula: $C_9H_{10}ClN_5O_2$).

Numerous feeding test studies on honeybees proved that imidacloprid is highly toxic and presents an oral LD_{50} 22 times higher than contact LD_{50} (Schmidt, 1996). Honeybees show symptoms of poisoning after oral ingestion of sub-lethal doses of imidacloprid such as stationary and inactive behavior, movement coordination disorders, trembling, hyperactivity and tremors. However, bees presenting hyperactivity gradually become hypoactive. It is also important to consider that imidacloprid can induce opposite effects on activity depending on the dose (Lambin *et al.*, 2001; Suchail *et al.*, 2001; Medrzycki *et al.*, 2003; Colin *et al.*, 2004).

Sublethal effects of imidacloprid are well related to have a strong influence on bee social behavior. Sub-lethal doses have been shown to change foraging behavior, and presumably may also influence social colony life and pollination efficiency (Thompson and

Maus, 2007; Desneux *et al.*, 2007; Mommaerts & Smagghe, 2011). On the other hand, studies on whole colonies of the buff-tailed bumblebee, *Bombus terrestris,* exposed to sublethal doses did not show any side effects that influenced foraging behavior on imidacloprid and thiamethoxam treated plants; however, effects have been observed inside the colony that include a reduction in brood size and higher mortality (Columbo & Buonocore, 1997; Taséi *et al.*, 2001; Alarcòn *et al.*, 2004).

Contradictory observations might be due by a discrepancy between field and laboratory tests for sub-lethal effects. In the field, bees have the ability to change their behavior in response to pesticide perception (Decourtye & Devillers, 2010) and may have access to non-contaminated food. In the field, avoidance behavior can be observed in honeybees for high doses of contaminated food. This protective avoidance behavior of bees is only possible in the field towards that high sub-lethal dose and might reduce the risk of pesticide exposure and side effects but may also reduce their foraging activity. The reduction of foraging activity in reaction to imidacloprid presence in the countryside decreases the general fitness of bees (Cresswell, 2011). Also, little is known about the capacity of bees to detect imidacloprid or other neonicotinoids in nectar.

4. Thiamethoxam

Thiamethoxam is a 'second-generation' neonicotinoid (Figure 3) and was the first commercial neonicotinoid from the thianicotinyl class (Senn *et al.*, 1998; Maienfisch *et al.*, 2001). Thiamethoxam appeared in 1998 on the market under the name Cruiser for seed coating for a large range of crops or for foliar and soil treatments under the name Actaral (Maienfisch *et al.*, 1999). Like imidacloprid, thiamethoxam is currently one of the most widely use insecticides in crop protection in the world (Elbert *et al.*, 2008). Nitro-substituted neonicotinoids such as imidacloprid and thiamethoxam applied topically are the most toxic to the honeybee, with LD_{50} values in the nanograms per bee range (Iwasa *et al.*, 2004).

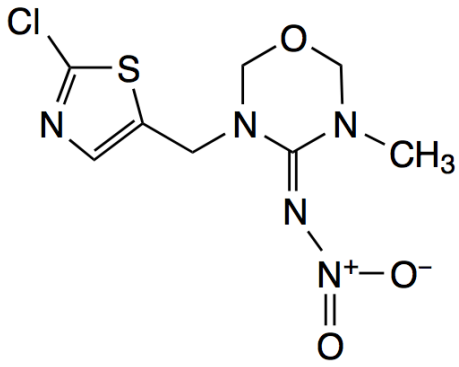

Figure 3: Molecular structure of thiamethoxam (Chemical formula: $C_8H_{10}ClN_5O_3S$).

Unlike imidacloprid and acetamiprid, thiamethoxam does not exhibit competitive interaction with other neonicotinoids on nAChRs (Tan *et al.*, 2007). The conversion of thiamethoxam into the toxic metabolite clothianidin has been proposed as the cause of its biological effect (Nauen *et al.*, 2003; Iwasa *et al.*, 2004).

The same debated observations about thiamethoxam toxicity at sub-lethal doses can be applied as for imidacloprid. Some studies concluded that regardless of the exposure mode (spray, intake or residue on the surface of the culture), thiamethoxam is extremely toxic for bees and sub-lethal doses can cause the death to up to 80% of bees after 3 days (Antunes-Kenyon & Kennedy, 2001; Carvalho *et al.*, 2009). Additionally, the exposure to thiamethoxam of newly emerged Africanized honeybees reduces their lifespan by 41.2% (Oliveira *et al.*, 2013). But for tomatoes grown in greenhouses, the utilization of thiamethoxam in drip irrigation system does not affect the pollinating rate of *B. terrestris* (Alarcón *et al.*, 2005) as noticed for imidacloprid in similar conditions (Colombo, 1997). Moreover, the orientation capacities of honeybees in complex maze (Iwasa *et al.*, 2004) or on the field (Henry *et al.*, 2012) are affected by thiamethoxam. As well, chronic exposure to thiamethoxam (0.1µg/bee) can induce a decrease of memory capacities 24 hours after learning and can be followed by a recovery at 48 hours and rules out long term memory impairments (Aliouane *et al.*, 2009). Statistical models suggest that dietary thiamethoxam would not precipitate collapse in healthy honeybees colonies in spring, but might have effects later in the year when the capacity of colonies to replace lost workers has diminished (Cresswell & Thompson, 2012).

The chronic intoxication of young honeybees with sub-lethal doses of thiamethoxam has an impact on the mushroom bodies of Africanized honeybees (Oliveria *et al.*, 2013).

Likewise, the sensory perception of sugar can be reduced. The responsiveness to antennal sucrose stimulation decreases for high sucrose concentration when honeybees are orally treated with sub-lethal doses of thiamethoxam (1ng/bee) (Aliouane *et al.*, 2009).

The main target of thiamethoxam is nAChRs that are present in the central nervous system of bees (Tan *et al.*, 2007; Tomizawa & Casida, 2003), but a secondary target may also be affected, such as the organs involved in the metabolism of the compound (Catae *et al.*, 2014) and finally explain divergent observations about its toxicity. Thiamethoxam has a deep impact on bee metabolism. It has been reported that thiamethoxam has cytotoxic properties in the midgut that impair the digestion capacities and on Malpighian tubules that prejudice excretory abilities (Oliveira *et al.*, 2013; Catae *et al.*, 2014).

5. Clothianidin

Clothianidin is considered as the third member of the chloronicotinyl class that also includes imidacloprid and thiamethoxam (Jeschke *et al.*, 2003) and entered on the worldwide market of pesticide in 2001 (Figure 4) (Nauen *et al.*, 2003).

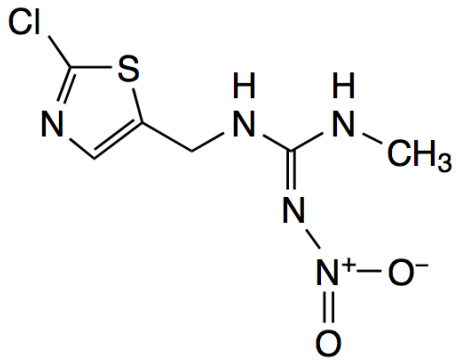

Figure 4: Molecular structure of clothianidin (Chemical formula: $C_6H_8ClN_5O_2S$).

The toxic action of thiamethoxam is related to its easy and quick conversion to clothianidin that is a highly active open-chain neonicotinoid (Ohkawara *et al.*, 2002; Schwarz *et al.*, 2002; Nauen *et al.*, 2003). Clothianidin is a metabolite compound that binds with high affinity and is considered as a full agonist of nAChRs and act on the same site as imidacloprid; consequently, agronomists have concluded that thiamethoxam should not be used alone and should instead be used with other neonicotinoids in resistance management strategies (Nauen *et al.*, 2003).

As pointed for the previous neonicotinoids, clothianidin has a high activity against a broad range of insects, including sucking and chewing insects, and some lepidopterans (Jeschke *et al.*, 2003). In developed countries, clothianidin is mainly used as seed dressings for a broad variety of crops (Goulson, 2013) and displays on the field excellent control properties for insect pests when it is used by foliar, paddy water and soil applications (Ohkawara *et al.*, 2002). Because of its broad spectrum of insectidal activity, good systemic properties and a relative low toxicity on mammals, clothianidin is the most compatible neonicotinoid for use in integrated pest management strategies (Ohkawara *et al.*, 2002). Nonetheless, the good systemic property of clothianidin could represent a hazard for pollinators that feed on pollen and nectar. For example, the residues of clothianidin found in pollen and nectar of seed dressed sunflowers (*Helianthus annuus* L.) and oilseed rape clearly ranged at doses that can have sub-lethal effects on bees (Schmuck *et al.*, 2001; Schmuck & Keppler, 2003; Franklin *et al.*, 2004). Likewise, similar residues level of clothianidin can be found in corn guttation fluid (Girolami *et al*., 2009).

Concerns of adverse effects of imidacloprid and thiamethoxam sub-lethal dose on pollinators have been subject of debate, but there is agreement on the relative small impact of clothianidin on pollinators such as honeybees, bumblebees and solitary bees (*Osmia lignaria* and *Megachile rotundata*) (Bailey *et al.*, 2005). These authors suggested that clothianidin offers an increased margin of safety for bees compared with the other neonicotinoids for both orally and topically contact with sub-lethal doses commonly found on the field (Schmuck & Keppler, 2003; Franklin *et al.*, 2004; Cutler & Scott-Dupree, 2007; Abbott *et al.*, 2008; Girolami *et al.*, 2012; Goulson, 2013). However, mortality with clothianidin can be twice more important at very high air humidity (Girolami *et al.*, 2012). For example, no sides effects have been observed on honeybees that forage on clothianidin seed treated oilseed rape (Cutler & Scott-Dupree, 2007), as for *Bombus impatiens* foraging ability and colony health (Franklin *et al.*, 2004). *Bombus impatiens* is 1.3 times more tolerant of clothianidin and imidacloprid sub-lethal doses in their diet than solitary bees (*Osmia lignaria* and *Megachile rotundata*) (Scott-Dupree *et al*., 2009).

III. Bumblebees

According to Michener (2007), wild bees including bumblebees may now become more important as pollinators than in the past, because of the dramatic decline of honeybee populations in north-temperate climates. Moreover, Michener (2007) highlights that honeybees are poor pollinators of various crops in comparison with wild bees.

Bumblebees (Super-family Apoidea) are social Hymenoptera and are often described as primitively eusocial (Goulson, 2003). The genus *Bombus* includes ubiquitous species with a very high adaptation potential, is very resistant under various environments (Dramstad & Fry, 1995; Hingston & McQuillan, 1998; Dafni & Giurfa, 1999), and is naturally globally distributed, except for Australia and New Zealand where it has been introduced (Sakagami, 1976; Rasmont, 1983). According to the *Bumblebee Conservation Trust*, there are 24 species of bumblebees in United Kingdom but only 8 can be commonly found in most places and the others are confined to a handful of sites and have uncertain futures. Morphologically, all bumblebee species looks similar and the only obvious difference is the tongue length. *Bombus* have broadly similar annual life cycle (few species are partially can have two generations per year like *B. terrestris*), depend only on nectar and pollen for food, and most species do not have precise habitat requirement (Williams, 1986; Rasmont *et al.*, 2012). Our model in this thesis is the buff-tailed bumblebee (*B. terrestris* L.) that is one of the most abundant wild pollinators across Europe, including the United Kingdom (Goulson *et al.*, 2008).

1. Foraging behavior

Bumblebees are social bees and rely on the cooperation of many individuals carrying out multitudes of tasks to ensure the colony functions efficiently. Foraging is a fundamental task as the colony growth and health depends on a continuous food supply. Consequently many factors that can impair the foraging behavior may have serious consequences for the colony survival (Gill *et al.*, 2012; Bryden *et al.*, 2013) and reproduction (Whitehorn *et al*., 2012).

B. terrestris belong to the group of the highly polylectic bees that can collect pollen and nectar from hundreds of different flowers species (Fussell & Corbet, 1992; Goulson *et*

al., 2005; Rasmont *et al.*, 2013). In contrast with long tongue species, *B. terrestris* shows several types of adaptations, which render it as a generalist able to harvest floral resources from a large spectrum of plant species in various habitats. These adaptations include early seasonal emergence, specifically of queens (Sladen, 1912; Prys-Jones & Corbet, 1991), longer mean foraging distances (Walther-Hellwig & Frankl, 2000; Ne'eman *et al.*, 2000; Kreyer *et al*., 2004; Greenleaf *et al.*, 2007), and a variety of efficient behavioral skills for nectar and pollen gathering that include nectar robbing and buzz pollination (sonication) to collect pollen (Prys-Jones & Corbet, 1991; Proctor *et al.*, 1996).

Another important aspect that influences the foraging behavior of *B. terrestris* is the alloethism that is the different sizes of workers that perform different tasks (Goulson *et al.*, 2012). The size polymorphism is an important life history trait of *B. terrestris* that has a strong impact on individual behavior and the organization of the colony. Inside the colony, the larger workers tend to serve as foragers while the smaller workers fulfill in hive tasks (Spaethe & Weidenmüller, 2002; Goulson *et al.*, 2012). However, the number, the duration and the proportion of nectar trips, and the nectar foraging rates are affected by worker size. According to these factors, large foragers appear to contribute disproportionately more to the current nectar influx of their colony (Spaethe & Weidenmüller, 2002). Besides, larger foragers are able to forage in cooler conditions, over long distance and are maybe less susceptible to predation. Conversely, small workers are presumably cheaper to produce and may be more dexterous at within-nest tasks (Goulson *et al.*, 2012).

Additionally, honeybees do not usually forage for temperature below 16°C, whereas bumblebees are still foraging actively at temperature down to 10°C (Heinrich, 1979). But bumblebees stop foraging when the temperature rises above 32°C (Kwon & Saeed, 2003) and are able to fly at temperature up to 35°C, but instead stay at the nest to ventilate the brood (Heinrich, 1979; Vogt, 1986).

2. Bumblebees as pollinators

The potential value of bumblebees as pollinators in agriculture has been recognized for a long time. Bumblebees have longer tongues than honeybees and are consequently reported by some authors for being much better at pollinating flowers with deep corollas (Hobbs *et al*, 1962; Holm *et al*., 1966). Because of these observations, hundreds of

bumblebee queens from United Kingdom were deliberately introduced in New Zealand in 1885 and 1906 to improve seed set of red clover and then four of new species became established (Hopkins, 1941). The same arguments and ascertainments have been done for *Bombus ruderatus* more recently 'successfully' introduced in Chile from the New Zealand established population (Arretz & MacFarlane, 1986).

Contrary to honeybees, bumblebees are hardy and will forage under poor weather conditions like rain and cold temperatures (Corbet *et al.*, 1993). Moreover, bumblebees tend to forage faster and thus visit more flowers than honeybee in similar conditions (Poulsen, 1973; Free, 1993; Stanghellini *et al.*, 2002; Fuchs & Müller, 2004). Consequently, bumblebees such as *B. terrestris* have been reported for being particular good pollinator of oilseed rape (Delbrassinne & Rasmont, 1988) and apple (Goulson, 2010) that flower in early season under possible poor weather conditions. Furthermore, bumblebees are bigger and hairier than honeybees that may contribute to higher pollination efficiency in transferring pollen (Willmer *et al.*, 1994).

Before the domestication and the commercial production of bumblebees for greenhouse pollination, diverse techniques were used to pollinate flowers manually or by vibration without the efficiency success expected (Free, 1993; Straver & Plowright, 1991; Pressman *et al.*, 1999; Vicherat, 2003; Hanna, 2004). Consequently, bumblebee domestication (genus *Bombus*) started to become an interesting model (Pouvreau & Marilleau, 1979) and manual pollination has been abandoned progressively (Dafni, 1998). Even in early day of *B. terrestris* domestication, bumblebee pollination was cheaper (9,100€ per ha per year; van den Bogaard, 1991) than mechanical pollination (10,000€ per ha per year; van Ravestijn & Nederpel, 1988). Another striking example of the evolution of the bumblebee market is the evolution of the price of one colony: tomato growers used to pay 200€ per colony between 1988-1990 and around 50-60€ per colony today (Velthuis & van Doorn, 2006).

Within the *Bombus* genus, the buff-tailed bumblebee (*B. terrestris*) rapidly became the center of researches for its domestication as it presents numerous advantages as colonies between 300 and 400 foragers and the flexibility of foraging easily in restricted spaces (Pouvreau & Marilleau, 1977; Pouvreau, 1984). Consequently, *B. terrestris* become the most produced bumblebee species for commercial uses in Europe, while *Bombus impatiens* is bred for North and South America (Straver & Plowright, 1991) and *Bombus*

hypocrite & *Bombus ignites* for Japan (Asada & Ono, 2000). This specific regionalization of bumblebee specific species use is mainly due to the cost of delivery (Dafni, 1998; Hingston & McQuillan, 1998; Goulson, 2000; Hogendoorn *et al.*, 2000). Bumblebees are also preferred when the temperature and light intensity are low, and in both greenhouse and open field (Velthuis & van Doorn, 2004).

Since 1988, *B. terrestris* has been used commercially in many countries beyond its natural range to improve the pollination in greenhouses, particularly for tomatoes (*Lycopersicon esculentum* Mill.) (de Ruijter, 1997; Velthuis & van Doorn, 2006). The tomato pollination involves about 95% of all bumblebee sales and comprises a total over 40,000ha of greenhouse culture (Velthuis & van Doorn, 2006).

In this thesis, we decided to use commercial bumblebee colonies of *B. terrestris terrestris* from Koppert B.V. (The Netherlands) that contains a colony (Queen, workers and brood) and a bag of sugar solution as commonly found in European greenhouses for fruitset of tomato crops and many other crops pollination. Bumblebees were fed *ad libitium* with the sugar bag and additional pollen. Bumblebees were maintained in a calm place in continuous darkness at 22°C. Owing to their abilities to forage under variety of weather conditions and temperatures, bumblebees are reliable pollinators in a large range of habitats. As they also able to have large ranges and thus having easier access to small and fragmented plant communities commonly found in Europe (Steffan-Dewenter & Tscharntke, 1999). Unfortunately, we know little about the pollination requirements of a great number of plant species and families. Despite this lack of fundamental knowledge, highly specific plant-pollinator relations are well known, as example, the alpine flower called Sky pilot (*Polemonium viscosum*) coevolved with their bumblebee pollinators (Galen, 1989). On the other hand, some plant families are largely dependent of bumblebees for their pollination such as Boraginaceae, Ericaceae, Lamiaceae, Fabaceae, Orchidaceae, Solenaceae etc. (Corbet *et al.*, 1991).

3. Bumblebee conservation

Several stressors are identified for being implicated in bee decline (Vanbergen & The Insect Pollinators Initiative, 2013), that include pesticides (Desneux *et al.*, 2007), disease and parasites (Brown & Paxton, 2009), and habitat change, fragmentation and loss (Kremen *et*

al., 2007; Potts *et al.*, 2010). There is still a debate over which stressors are most harmful, meanwhile no single factor has emerged as an overall principal cause (Ratnieks & Carreck, 2010; Vanbergen & The Insect Pollinators Initiative, 2013). There is a consensus around the decline in numbers of bumblebees that highly suggest the intensification of farming practices as the principal cause (Williams, 1986; Osborne & Corbet, 1994; Goulson, 2003).

One of the consequences of the intensification of farming practices is the increasing use of neonicotinoid pesticides, which can act as a source of stress without causing direct mortality. Bumblebees exposed to field realistic sub-lethal doses of neonicotinoids have been reported to have less motor function, poor memory, orientation and foraging performances (Desneux *et al*., 2007; Gill *et al.*, 2012; Schneider *et al.*, 2012; Bryden *et al.*, 2013). While these sub-lethal effects do not kill bumblebees, they may have deep effects on the dynamics and functioning of the whole colony. Despite this, very little is known about the chronic stress experienced by individuals, although bumblebees have a very high positive density dependence and a critical stress level can make the difference between failure and success for the colony survival (Bryden *et al.*, 2013).

Indeed, there is also evidence that neonicotinoids can detrimentally feedback to colony hatching and death rate (Gill *et al.*, 2012), the production of sexuals (Whitehorn *et al.*, 2012) and increase the prevalence of disease (Brown *et al.*, 2000). The plight of bumblebee fauna justifies particular care, as the loss of one species would almost certainly have consequences for other wildlife. A large number of wild plants are mainly pollinated by bumblebees (Corbet *et al.*, 1991; Osborne *et al.*, 1991). Consequently, the reduction of the abundance and species richness of bumblebees may lead to widespread changes in plant communities (Corbet *et al.*, 1991). These changes will have further knock-on effects for associated herbivores and other animal that depend of plant resources (Goulson *et al.*, 2005). Besides, it is of concern that autumn and winter populations of *B. terrestris* rely on a narrow choice of flower and may be locally endangered by scrub clearance and their favorite autumn flowers (Rasmont *et al.*, 2013). Finally, the decline of some wild plant species is likely to have severe repercussions for British bumblebee species diversity (Goulson *et al.*, 2005).

IV. Insect nutrition regulation

Nutritional ecology is based on the representation of the organism, the ecological environment and the nutritional relationship between organism and environment. Very few studies have examined the nutritional ecology of bumblebees, and fewer still have examined how forms of stress such as exposure to pesticides influence the need for nutrients in bees.

1. The Geometric Framework

The Geometric Framework (GF) for nutrition was developed by David Raubenheimer and Stephen Simpson at the University of Oxford. The GF is a theoretical framework based on the multiple interactions among the mechanisms that are regulating the intake of different classes of nutrients in animals. It is based on the premise that foraging behavior is a process that has been optimized by natural selection to maximize fitness and reflects physiological demands for specific nutrients (Raubenheimer & Simpson, 1993; Simpson & Raubenheimer, 1993). This method of nutritional modeling has been developed to enable the question to be focused: (i) which nutrient and other food components are important to an animal in a given situation, (ii) how does each nutrient influence the responses of animals, and (iii) what are the performance and ecological consequences to the animal of responding in the way that it does (Raubenheimer *et al.*, 2009). Thus, the GF is a method for predicting nutrient regulation of animals in a population and can be applied to specific conditions found in an animal's environment (Raubenheimer & Simpson, 1993, 1994, 1997; Simpson & Raubenheimer, 1993, 1995, 1999). In the GF, the relevant variables are expressed and linked to each other within a geometric space defined by two or more relevant food components and can be used to theorize problems that involve these components and others such as toxins (Raubenheimer *et al.*, 2009).

2. Estimating the food intake target

The GF treats any given animal within a multidimensional nutrient space where axes represent functionally studied nutrients. Nutrient-centred approaches can be developed in a chart (Fig. 5A) in which abscissa and ordinate represent the two nutrients studied. The food Intake Target (IT) is the amount of nutrients that any given animal needs to ingest to reach the optimal amount and blend of each nutrient (Raubenheimer & Simpson, 1993, 1995, 1999; Simpson & Raubenheimer, 1993; Behmer, 2009). By eating, the animal evolves suite of behavioral and physiological mechanisms its nutritional state along the vector of the chosen food rail (Raubenheimer & Simpson, 1993; Simpson & Raubenheimer, 1999). A food rail is a trajectory that represents a food's balance of nutrients (Behmer, 2009).

A nutritionally imbalanced diet (Fig. 5B) forces the animal into a compromise between eating an excess of some nutrients and under-eating others. This rail does not pass through the IT and the animal would not be able to satisfy its optimal food requirements, unless it regulates its intake such that the deficit incurred in one nutrient exactly matches the excess in the other one (Raubenheimer & Simpson, 1999). In this extreme case, the animal would encounter serious fitness associated costs to reach its macronutrient nutritional optima (Behmer, 2009; Simpson *et al.*, 2010).

Animals, and especially herbivores, rarely encounter foods that are perfectly balanced according to their nutritional needs (Raubenheimer, 1995). Animals are forced to 'move' (Fig. 5C) through the nutrient space design by the GF if, in addition to one source of food, a second nutritionally imbalanced food provide the second food lay on the opposite side of the IT of the first one. The animal would be switching between the foods and regulating the selection and intake of nutrients to minimize any discrepancy between current nutritional state and the IT (Simpson & Raubenheimer, 1993; Raubenheimer & Simpson, 1997). The combinations of nutritionally imbalanced food that allow the animal to 'zigzag' its way between foods in this manner are termed *complementary* foods (Raubenheimer & Simpson, 1999).

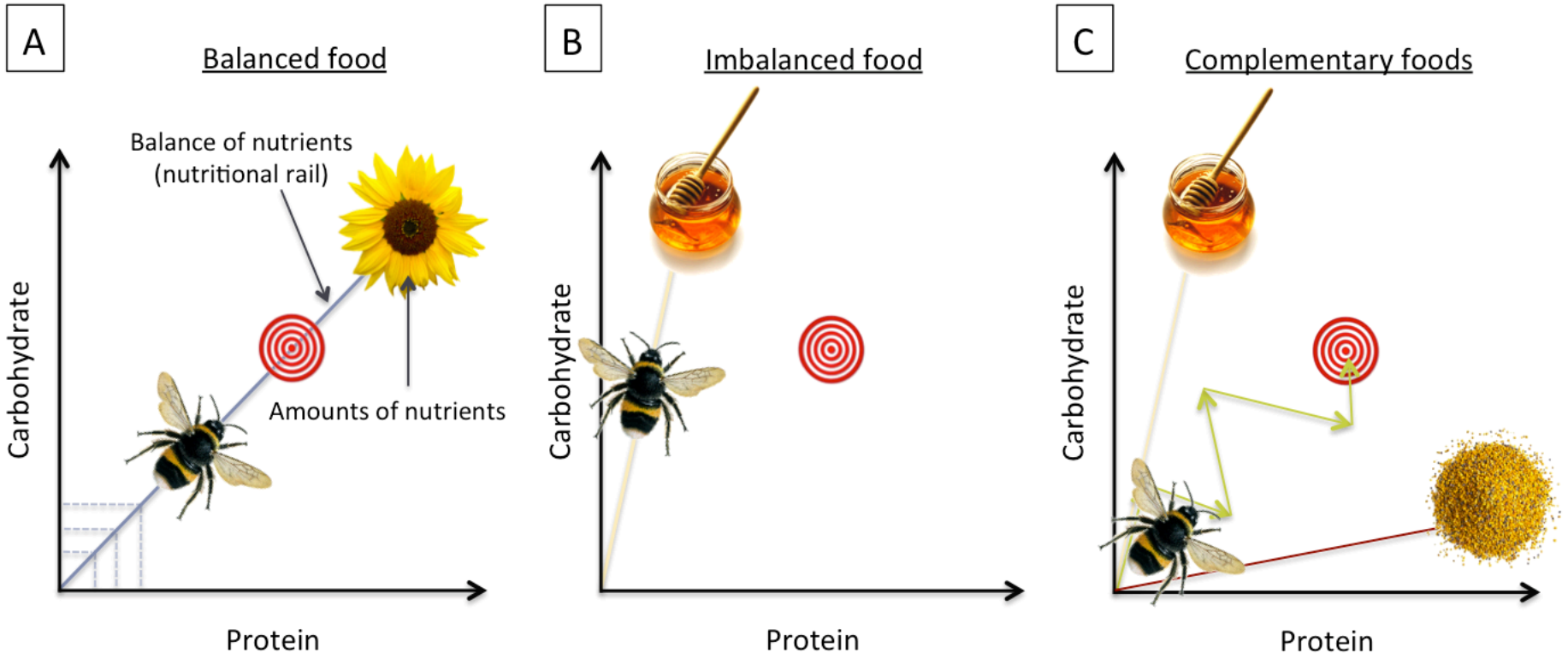

Fig. 5: Geometric framework approach of the food intake target (Adapted from Raubenheimer & Simpson, 1999). (A) The bumblebee has in this example a Protein:Carbohydrate intake target ratio of 1:1 (red target) and that amount of the two nutrients is obtained from a single source of food. (B) The bumblebee cannot reach its intake target as it is following high carbohydrate nutritional rail and has no access to pollen. (C) But if a second source of food is present, the bumblebee can switch for a suboptimal but complementary food containing a large amount of proteins. In that case, each arrow represents a meal with length correlated to the meal size.

Consequently, the IT would be expected to reflect the relative functional importance for nutrients involved in relation with the animal regulatory systems and physiological needs (Raubenheimer & Simpson, 1997). Points of compromise where animals cannot obtain their optimum macronutrient values are measured across a range of suboptimal food rails and form intake arrays that define a more overall rule of compromise. The analysis of these arrays allows the manner in which regulatory systems weight under- and over-eating of different nutrients to be quantified (Raubenheimer & Simpson, 1997, 1999). This probability should be high for generalist insect herbivores, as they tend to be mobile and switch between food sources. They should eat a large amount of imbalanced food when encountered (Behmer, 2009).

3. Impact of allelochemicals and other stressors on nutrient balance

Aside from the variation of nutrients in plant feeding insects (Slansky & Scriber, 1985), animals encounter non-nutritive compounds in food that can have an impact on their physiology and survival. Animals like plant-feeding insects commonly encounter non-nutritive or toxic chemical compounds in plants; such compounds can induce a trade-off in the regulation of food intake because the insect is forced to alter its ingestion of food to avoid the potentially harmful compounds (Bernays & Chapman, 1994; Bernays *et al.*, 1994; Hagele & Rowell-Rahier, 1999; Singer *et al.*, 2002). Thus, food mixing by herbivores is thought to balance nutrient intake and possibly dilute secondary metabolites characteristic of different host plant species (Singer *et al.*, 2002). Both nutrients and allelochemicals can be included as dimensions within the GF, but they may result in contradictory IT coordinates and costs, facilitating investigation and modeling of their influences and interactive effects (Behmer *et al.*, 2002).

Other forms of stress, including pathogens can also affect the intake of macronutrients. For example, the African armyworm (*Spodoptera exempta*) increases its protein intake relative to control larvae when it is exposed to an opportunist bacterium (*Bacillus subtilis*) (Povey *et al.*, 2009). Increased ingestion of protein is associated with the cost of the compensation to the virulence resistance. It can be more strikingly observed with the African cotton leafworm (Spodoptera littoralis) with a highly virulent entomopathogen (nucleopolyhedrovirus) that stimulate the protein intake to survive (Lee *et al.*, 2006).

Likewise, exposure to allelochemicals present in insect diets can affect nutrient diet reduce their protein intake or their rate of intake for diets rich in protein, while tannic acids in foods containing excess protein also reduces nitrogen utilization efficiency (Simpson & Raubenheimer, 2001).

Nicotine is an alkaloid commonly found in *Solanaceae* that contributes miticide, insecticide and fungicide natural properties to plants (Palazón *et al.*, 1998). The presence of nicotine in food of Tobacco hornworm larvae (*Manducta sexta*, Lepidoptera) reduces its food consumption. Moreover, *M. sexta* larvae parasitized by *Cotesia congregata* (Hymenoptera) and exposed to nicotine in diet reduce their food consumption to minimize their exposure to nicotine and consequently fail to regulate their nutrient intake (Thompson & Redak, 2007).

Thus, it appears difficult to separate herbivore responses to secondary metabolites and other stressors from those to other phytochemicals that include primary nutrients because the interactions among such chemicals that directly influence the physiology of the herbivore. It is possible that chemical compounds in food such as neonicotinoids could affect the nutrient intake and thus other life history traits such as food preference, metabolism, behavior and survival. The development of agricultural activities coincides with the increase of pesticide use to control pests. How harmful these pesticides are to non-target insects such as bees is still debated, but neonicotinoids are used in insect control programs worldwide because of their unique neurotoxic and systemic properties. Consequently, bees are chronically exposed to pesticides residues in the food stored inside the nest (Schmuck *et al.*, 2001; Bonmatin *et al.*, 2003; Chauzat *et al.*, 2006). In the context of a stronger legislation on neonicotinoids use to limit environmental, health and pollinator population concerns, approaches to describe and understand different aspect of action of neonicotinoids at sub-lethal doses on bumblebees are needed.

Surprisingly, little is known about nutritional needs of wild pollinators and whether or not neonicotinoids can affect their nutrient requirements and their survival. For a better understanding of contrasting previously published results on the toxicity of sub-lethal doses of neonicotinoids on colonies of Buff-tailed bumblebee (*B. terrestris*), I examined the impact of the three most commonly used neonicotinoids (clothianidin, imidacloprid and thiamethoxam) on the nutrient balancing behavior and survival of the bumblebee to

determine the impact of these pesticides on bee nutrition. Using the Geometric Framework for nutrition, I studied the behavior and survival of adult worker bumblebees fed diets composed of protein and carbohydrates. In the first experiment, each diet had different sub-lethal doses of imidacloprid (0, 1, 10 and 100nM). The second set of experiments examined how the position of the food in the experimental arena (food access) influenced survival and nutrient balancing. The third set of experiments compared the impact of thiamethoxam, clothianidin, and imidacloprid on nutrient balancing and survival of bees fed diets containing a dietary source of protein or free amino acids. The fourth set of experiments highlighted the difference of attractiveness of food containing different doses of imidacloprid (0, 1 and 10nM) to two ages of worker bumblebees.

Chapter 1: Bumblebees (*B. terrestris*) exposed to imidacloprid need higher protein diet, but are less active and are more likely to die quickly

I. Introduction

Animals, including insect herbivores, eat to obtain a mix of nutrients needed for growth, development and reproduction. Most herbivore insects strongly regulate their nutrient intake when given the opportunity and their foraging decisions underpin many aspect of their fitness (Behmer, 2009). Foraging by insect pollinators such as bumblebees can be considered as an exercise in acquiring the correct blend and balance in nutrients as amino acids, carbohydrates, fatty acids, vitamins, minerals, water from floral nectar and pollen (Chapman, 1998; Schoonhoven *et al.*, 2005). Variation in food quality provided by plant tissues arises from genotypic differences and environmental conditions (Behmer & Nes, 2003). The different plant species vary considerably in the quality rewards they offer to pollinators although the exact effects of this variation on pollinator foraging behavior are less understood (Hanley *et al*., 2008). The major components of pollen include lipids, carbohydrates, proteins, amino acids, vitamins, etc. (Hügel, 1962). The nutritive value of protein in pollen can be roughly estimated to be within the range 2.5-61% (Roulston *et al.*, 2000). Pollen chemical content depends on the species and is closely related to the floral species can offer similar chemical compositions (Roulston *et al.*, 2000; Vanderplanck *et al.*, 2014b). Nectaris primarily a source of energy from carbohydrates like sucrose, glucose, and fructose, but in addition to sugars contains various minor constituents that may have nutritional significance (Corbet, 2003; Nicolson, 2011). Moreover, the composition of sugars in nectar can be determined by plant phylogeny (Nicolson, 2007) and the water content can vary greatly according to environmental conditions (Nicolson, 2011).

For those reasons, all animals including bumblebees that eat plant material experience a heterogeneous nutritional landscape when they attempt to acquire a balanced nutritional intake (Behmer, 2009). However, within zoophilous plants there are considerable variation in the quality of pollen offered and plants that are most frequently visited by bumblebees produce the highest-quality pollen (Roulston *et al.*, 2000). Bumblebees also respond to highly rewarding flowers in terms of sugar content by developing learned association between the reward and flower scent, shape or color so that they preferentially search out for them (Goulson, 1999; Stout & Goulson, 2002). The process of nutritional regulation is itself a complex challenge and may be affected by the recent nutritional history, developmental stage and levels of activity (Simpson *et al.*, 1995).

Interspecific variability in essential nutrients such as proteins in pollen might be a constraint for polylectic bees (Praz *et al.*, 2008; Haider *et al.*, 2013) like bumblebees (*Bombus terrestris*). Previous studies on generalist bees showed that some pollen diets are inadequate for bees (Sedivy *et al.*, 2011; Tasei & Aupinel, 2008) and social bees such as bumblebees and honeybees increase their foraging in response to food deprivation (Cartar, 1992; Fewel & Winston, 1992; Plowright *et al.*, 1993). The chemical composition and the quality of the pollen are commonly assessed to explain the foraging behavior of polylectic species (Hanley *et al.*, 2008; Leonhardt & Blüthgen, 2012). The significance of the range of nutrients in pollen has not previously been evaluated on the feeding behavior (nectar and pollen collection) of polylectic bees (Vanderplanck *et al.*, 2014a). Moreover, polylectic bees fed with monofloral pollen or with pollen containing low-protein concentrations have a shorter life than bees fed with pollen blends (Schmidt *et al.*, 1987) whilst feeding honeybees with pollen blends or some protein-rich pollens enhances bee life-spans (Schmidt *et al.*, 1987). The nutritive value of the pollen is considered as a key factor of mass bumblebee rearing for commercial use (Velthuis & Van Doorn, 2006). Bumblebees are commercially used for greenhouse pollination and were domesticated primarily for tomatoes pollination (Morandin *et al.*, 2001). Tomato pollen is reported as having high protein content (Roulston *et al.*, 2000), and is considered as adequate for bumblebee diet, for colony growth and also brood production (Whittington & Winston, 2003).

The Geometric Framework (GF) of Raubenheimer and Simpson (1999) is a method of studying how animals balance their nutritional intake when they are faced with variation in food quality. The GF is a state-space modeling approach that explores how an animal simultaneously regulates the intake of multiple nutrients and takes into account the multiple interactions among mechanisms to regulate the intake of those different nutrients (Behmer, 2009). Bumblebees can be considered as living in a multidimensional nutrient space where there are as many axes as there are functionally relevant nutrients and that affect the fitness of bumblebees. The nutrient space is the area defined by the nutrient axes (in most instances) and reduces to two dimensions. The “nutritional rail” is the trajectory that starts at the origin in nutrient space and represents a food’s balance of nutrients (Behmer, 2009).

The food intake target (IT) is the amount of nutrients that an animal needs to ingest to reach the optimal amount and blend of nutrients for any given animal (Behmer, 2009).

The IT can be defined by the intersection of the two axes that define the minimum of protein and carbohydrates required over a set period (Raubenheimer & Simpson, 1999). The GF provides a clear description of the trade-off reached by animals in regulating their nutritional balance (Raubenheimer & Simpson, 1993, 1994, 1997; Simpson & Raubenheimer, 1993a, 1997; Simpson *et al.*, 1995).

The IT is the amount and balance of the nutrients that bumblebees need to eat to achieve their maximal fitness and influence their foraging choice. The amount of nutrients can be represented by a flower (Fig. 5 in General Introduction), and the balance of nutrients is described by the foraging activity on flowers that projects the ratio of protein and carbohydrates depending of the pollen and nectar collected on each flower. In the case of a nutritionally balanced food, the bumblebee has access to a ratio of nutrients that is reached it to its intake target by being fed only by the flower (Fig. 5A in General Introduction). The balance of nutrients is only used to describe the food until the amount of nutrients in each is assumed to exceed the scale of each nutrient axis (Fig. 5B&C in General Introduction; Raubenheimer & Simpson, 1999). The IT cannot be reached by feeding on an imbalanced food but in that case the bumblebee can eat until it meets its requirement for carbohydrates but suffers of a deficit in proteins (Fig. 5B in General Introduction). The optimum can be often indirect and be regulated by driving to the IT when the animal has access between complementary but unbalanced foods (Fig. 5C in General Introduction; Altaye *et al.*, 2010). The benefits of reaching that target are inversely proportional to the distance that it needs to travel through nutrient space in order to reach the intake target (Kearney *et al.*, 2010). This depends on the type of pollen and nectar collected by bees; if no floral species provided optimal nutrition, then bumblebees would have to forage on different types of flowering plant species to reach the IT by balancing between different sources of nutrients that would be complementary. The foraging challenge is in consequence to reach the food intake target, even if ecological or other factors constrain the animal.

Fluctuations in a large range of environmental parameters, such as temperatures, rate of predation or amount of food available, can provoke stress (Buchanan, 2000). Pronounced or prolonged exposures to stressful conditions induce costs that can disrupt individual homeostasis and increase mortality in the extreme (Marshall & Sinclair, 2009). Physiological or behavioral responses can be elicited by a moderate stress and can finally

increase survival, but resources are required to produce or maintain those responses (Kourtis & Tavernarakis, 2011). Energy or specific nutrients can be required to synthesize detoxification enzymes, fat, or carbohydrate to fuel thermoregulation (Simpson & Raubenheimer, 2012). The feeding behavior can change when an animal has to overcome illness due to parasitism, toxin, mineral or secondary compounds. Animals can adapt their feeding behavior to seek out substances for "self-medication" by choosing a diet rich in protein and low in carbohydrates, or exhibiting illness-induced anorexia (Povey *et al.*, 2014).

Bees could be potentially exposed to a wide range of insecticide traces in their diet from crop seed dressing (Cresswell, 2011). Neonicotinoids such as imidacloprid are commonly used chemicals in agro-ecosystems (Elbert *et al.*, 2008) and are often systemically applied via seed dressing (Sur & Stork, 2003). Sub-lethal doses of neonicotinoids have a large number of impacts on bees that include reducing learning ability (Decourtye *et al.*, 2003; Williamson *et al*., 2013; Williamson & Wright, 2013), foraging success (Henry *et al.*, 2012), modifying the rate of food uptake (Ramirez-Romero *et al.*, 2008), increasing their susceptibility to pathogens (Alaux *et al*., 2010; Di Prisco, 2013) and the locomotory activity (Lambin *et al.*, 2001). Nonetheless, uncertainties about the magnitudes of lethal and sub-lethal effect of doses of neonicotinoids on bees have been identified (Cresswell, 2011). However, thinking that bees are exposed to single doses may not be realistic, because mass flowering crops (e.g. oil seed rape) bloom over several weeks and foraging bees may ingest these nectar repeatedly (Kearney *et al.*, 2010). Therefore, chronic exposure to imidacloprid can be considered as one of those stresses and can have an impact on the diet of bumblebees thus on food intake target.

Numerous studies have assessed the individual or the colony effects of diet quality or the impact of neonicotinoids like imidacloprid on feeding behavior and performances of bumblebees. In the present study, I applied the GF to define the Protein:Carbohydrate (P:C on a molar-molar basis) intake target of bumblebees. However, little is known about how the diet quality and the chronic exposure to sub-lethal doses of imidacloprid affect the feeding behavior and performance of individual bumblebee workers Bumblebees are exposed in their environment to a large range of doses of imidacloprid from crops or residues (Table 2 in General Introduction) and the consequences of these chronic exposures on their food requirement, survival and behavior are not known. The purpose of this study

was to show how a large range of sub-lethal doses of imidacloprid commonly found in the countryside could modify the amount of nutrients required. The interaction of diet and imidacloprid ingestion was examined on nutrition, survival and behavior. Based on previous studies, I predicted that sub-lethal chronic exposure to imidacloprid would change the bumblebee's feeding requirement in favor of protein. I expected that diets high in protein would improve survival. I also predicted that imidacloprid concentration should influence dietary regulation and survival in a dose-dependent manner.

II. Material and Methods

1. Animals

All experiments were performed with worker bumblebee colonies (*Bombus terrestris terrestris* L.) provided for commercial uses (Koppert B.V., AD Berkel en Rodenrijs, Netherland) and conducted in laboratory conditions (22-25°C and 65-80% RH) and continuous darkness from September 2012 to March 2013 in Newcastle University bee lab. Bumblebees were provided *ad libitum* with commercial sugar water and pollen (Pollen mix, Koppert B.V., AD Berkel en Rodenrijs, Netherland) until the beginning of each experiment.

Worker bumblebees – defined as foragers - were collected at the exit of the colony and weighed. They were housed in individual plastic boxes (17x12x7cm) with access to 3 liquid diets provided in 2ml microcentrifuge tubes (4 holes were drilled on the top side of the tube, Fig.1.1) until the end of the experiment. The boxes were placed on the bench at a temperature of 22-23°C and 65-80% RH or in the incubator 21-22.8°C and 40-60%RH in continuous darkness. Experiments were conducted for 7 consecutive days.

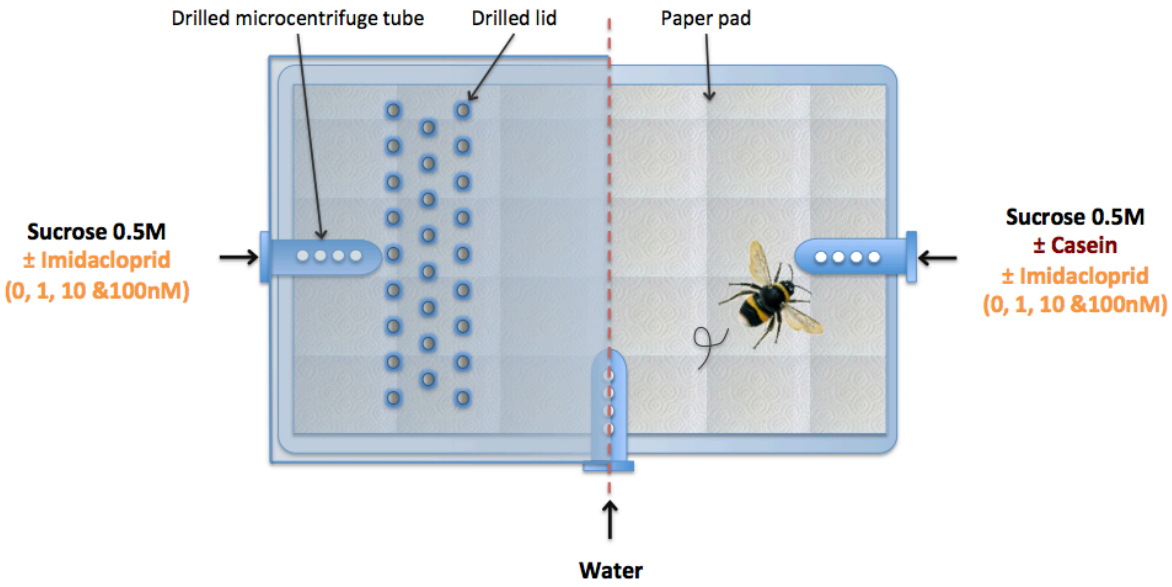

Figure 1.1: Individual bumblebee in feeding box with the 3 different liquid diets provided in drilled Eppendorf tubes at 2cm high.

2. Diets

To assess if bumblebees change their food intake when they are exposed to imidacloprid, I applied the Geometric Framework model of nutrition (Raubenheimer & Simpson, 1997, 1999).

Casein (Casein sodium salt from bovine milk, Sigma-Aldrich) was the protein source added to sucrose solution (0.5M, Sigma-Aldrich) in one tube; a second tube only had 0.5M sucrose solution and in a third tube had only water. Different treatments were tested in a protein to 0.5M sucrose solution ratio on a molar-molar basis to the P:C ratio (Protein : Carbohydrate; Table 1.1). Imidacloprid was provided in sucrose and sucrose with casein solutions. Imidacloprid was diluted at 1, 10 and 100nM in both solutions. A control with no casein supplied in food was done in the same conditions (called 0:1x in the following table and results).

	Solution compositions							
Sucrose	**0.5M**							
	Increasing concentration in protein →							
Casein	**0:1x**	**1:400**	**1:250**	**1:180**	**1:100**	**1:75**	**1:50**	**1:25**
Control	35	20	35	20	35	15	35	35
Imidacloprid 1nM	15	.	.	.	.	.	15	.
Imidacloprid 10nM	35	.	15	20	15	15	15	15
Imidacloprid 100nM	20	20	20	20	20	.	20	20

Table 1.1: Sample size for each liquid diet treatments. The experiment was done by run of 5 bumblebees in parallel per treatment and repeated (. = no bees in this set).

All tubes were weighed and replaced every 24 hours for 7 consecutive days. To measure food consumption, the difference between day *t* and day *t+1* was calculated. At the end of the experiments, bumblebees were weighed to estimate weigh loss or gain during the experiment to compare the impact of the different diets. Different parameters of the body were measured: body length, thorax width and length, abdomen width and length, and head width and length to study the homogeneity of the different population used in each trial. Bumblebees were placed in a freezer at -80°C in a labeled pocket for further investigations.

A control for the evaporation rate of each diet was performed for 3 days in empty boxes places in the same conditions as the trial boxes to measure the weight loss in feeding tubes. An average of evaporation rate was calculated and subtracted from the value obtained during the experiment. Negative and null values obtained by the subtraction of the evaporation rate were replaced by a null value and consequently considered because there was no food consumption from the tube. The total food consumption was calculated by multiplying the amount consumed by the weight of sucrose and casein in 1ml of solution for each diet.

3. Bumblebee activity

To determine if bumblebee behavior changed with imidacloprid exposure, bumblebee activity was observed each day for 10 min after taking them out of the incubator. Activity observed was defined as displayed in the following table (Table 1.2).

Behavior	Description of the behavior
Flying	Bumblebee moving in the air with its wings
Climbing	Bumblebee going up and using legs to catch up the tube that was containing the food or along the walls of the box
Running	Bumblebee moving very fast on the surface of the paper pad
Walking	Bumblebee moving slowly on the surface of the pad
Grooming	Bumblebee brushing its legs, body, head or wings
Eating	Bumblebee drinking at one of the tubes displayed in the box
Sitting	Bumblebee still on the paper pad
Lie on the back	Bumblebee upside down, slowly moving legs in the air or still on the back.

Table 1.2: Descriptions of the bumblebee behaviors observed during the experiment.

4. Statistics

The normality of each sample of data was tested. The average daily food consumption was analyzed using repeated-measures ANOVA. To measure the effect of imidacloprid on the average daily food in the subset of data 'sucrose only' and 'casein 1:50' average daily consumption, a one-way ANOVA and Tukey's post-hoc analysis were performed. The impact of imidacloprid dose within casein 1:50 subset was measured with a two-way ANOVA and LSD post-hoc tests. To test the effects of casein and carbohydrates at different ratios in diets on the cumulative consumption, repeated-measures ANOVA was performed with LSD post-hoc comparisons. The average imidacloprid daily intake was analyzed using one-way ANOVA and Tukey's post-hoc comparisons. Survival was analyzed using Kaplan-Meier analysis with censoring (Kaplan and Meier, 1958) to measure how each treatment influenced survival. To identify the correlations in the behaviors depending on the

diet and the dose of imidacloprid, a principal components method for factor analysis (Johnson and Winchern, 1992) was performed. The resulting factor scores generated for the factors with eigenvalues greater than 1.0 were then entered into a repeated-measure ANOVA and LSD post-hoc analysis. The impact of the diets and imidacloprid doses were analyzed with repeated-measures ANOVA. All non-significant interactions in models performed during the statistical analysis were removed from the previous one; the new model was rejected and not presented in the following results section. All analyses were performed using IBM SPSS v15.0 software (SPSS Inc., Chicago, IL).

III. Results

1. Imidacloprid reduced bumblebee feeding

The bumblebees in the control treatment (no imidacloprid) regulated their intake of sucrose and sucrose containing casein in a way that was markedly different to that of bees exposed to 10nM or 100nM imidacloprid in their food (Figure 1). One of the most striking differences was the fact that the average daily consumption of diet by the bumblebees exposed to imidacloprid was half the amount eaten by bees not exposed to imidacloprid (Figure 1.2). Specifically, bumblebees exposed to imidacloprid had a significantly lower daily sucrose consumption (Table 1.3a &1.4a). Bees also ate less sucrose-casein solution (within-subjects, repeated measures ANOVA $F_{(1,168)}$=10.1, P<0.001; day; Table 1.3b). The amount of food eaten in each tube depended on the concentration of casein in the diet and the dose of imidacloprid in food (Table 1.4b).

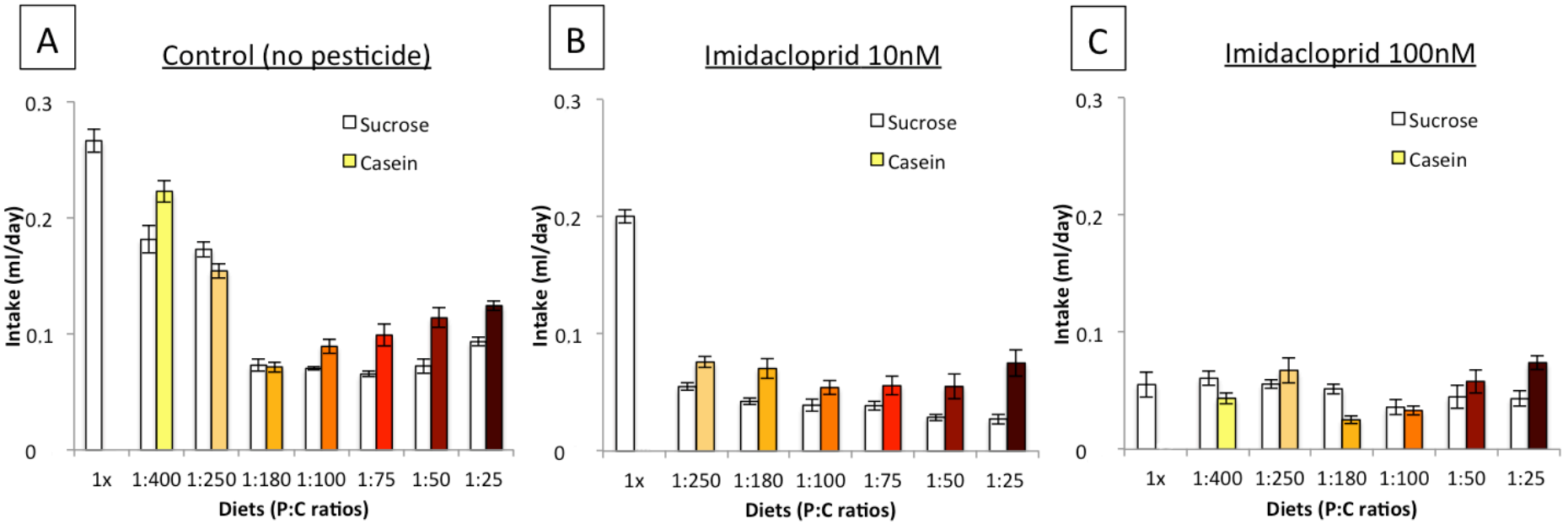

Figure 1.2: Comparison of the average daily food intake (± standard error) for different dose of imidacloprid (0, 10 and 100nM). For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

Dependant variables	Type III Sum of Squares	Mean Square	F	df	P-value
Sucrose intake	17107.362	17107.362	20.130	1	<0.001
Sucrose intake x Diet	**18633.144**	**2661.878**	**3.132**	**7**	**0.003**
Sucrose intake x Dose	263.534	87.845	0.103	3	0.958
Sucrose intake x Diet x Dose	7112.683	592.724	0.697	12	0.753
Error(Sucrose intake)	203113.183	849.846		239	

Table 1.3a: **Results of linear tests of within-subjects contrasts of repeated-measures ANOVA to test the daily sucrose intakes with different dose of imidacloprid (0, 1, 10 and 100nM) and different diets (in P:C ratios for Casein 1:x, 400, 250, 180, 100, 75, 50 and 25).** For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

Dependant variables	Type III Sum of Squares	Mean Square	F	df	P-value
Casein intake	2.030	2.030	28.805	1	<0.001
Casein intake x Diet	4.865	0.811	11.507	6	<0.001
Casein intake x Dose	0.379	0.126	1.793	3	0.150
Casein intake x Diet x Dose	**1.377**	**0.153**	**2.172**	**9**	**0.026**
Error(Casein consumption)	11.837	0.070		168	

Table 1.3b: Results of linear tests of within-subjects contrasts of repeated-measures ANOVA to test the daily casein intakes with different dose of imidacloprid (0, 1, 10 and 100nM) and different diets (in P:C ratios for Casein 1:x, 400, 250, 180, 100, 75, 50 and 25). For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

Source of variation	Type III Sum of Squares	Mean Square	F	df	P-value
Intercept	1025091.68	1025091.68	399.088	1	<0.001
Diet	68440.400	9777.200	3.806	7	<0.001
Dose	207869.362	69289.787	26.976	3	<0.001
Diet x Dose	**52793.043**	**4399.420**	**1.713**	**12**	**0.065**
Error	613892.102	2568.586		239	

Table 1.4a: **Results of tests of between-subjects effects of repeated-measures ANOVA with variable transformed in averages to test the daily sucrose consumption with different dose of imidacloprid (0, 1, 10 and 100nM) and different diets (in P:C ratios for Casein 1:x, 400, 250, 180, 100, 75, 50 and 25).** For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

Source of variation	Type III Sum of Squares	Mean Square	F	df	P-value
Intercept	59.452	59.452	172.452	1	0.001
Diet	**34.369**	**5.728**	**16.615**	**6**	**0.001**
Dose	**8.935**	**2.978**	**8.639**	**3**	**0.001**
Diet x Dose	1.280	0.142	0.413	9	0.927
Error	57.918	0.345		168	

Table 1.4b: **Results of tests of between-subjects effects of repeated-measures ANOVA with variable transformed in averages to test the daily casein consumption with different dose of imidacloprid (0, 1, 10 and 100nM) and different diets (in P:C ratios for Casein 1:x, 400, 250, 180, 100, 75, 50 and 25).** For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

The effect of imidacloprid on food consumption, however, depended on the concentration of imidacloprid. In a separate analysis, the consumption of food by bees given 1nM, 10nM, or 100nM in their food was compared. Bumblebees fed only sucrose and a subset of the nutritional rails experiments that included only the 1:50 dietary ratio pair were examined. In contrast to the 10nM and 100nM concentrations shown above, bees fed the 1nM treatment with imidacloprid ate *more* of the sucrose and casein (Figure 1.3). Bees fed 1nM imidacloprid in sucrose alone ate more sucrose, but bees fed food containing 10nM or 100nM imidacloprid ate less (Figure 1.3A, one -way ANOVA $F_{(3,101)}$=7.28, P<0.001). A Tukey's *post-hoc* test revealed that the average daily sucrose consumption of bees exposed to 1 nM imidacloprid was significantly more than bees exposed to the 10nM imidacloprid (0.200±0.035; P=0.001) or the 100nM imidacloprid (0.055±0.048; P<0.001). For bees fed sucrose alone, the average daily sucrose consumption of the control bees (no imidacloprid; 0.266±0.058) was not significantly different to the bees exposed to 1 nM imidacloprid (0.306±0.078; P=0.567). When fed the 1:50 diet pairs including casein, bees always ate more of the 1:50 diet with casein than sucrose (Figure 1.3B). Bees fed with both the sucrose and the 1:50 diet solutions ate different quantities of food depending of the imidacoprid dose (Figure 1.3B, two-way ANOVA $F_{(3,85)}$=4.16, P=0.009, sucrose; $F_{(3,85)}$=4.06, P=0.010, casein). Likewise, the LSD *post-hoc* analysis uncovered that bees exposed to 1nM imidacloprid ate more of both the sucrose and the 1:50 diet solutions than the control (sucrose: P=0.038; casein: P=0.013), but the 10nM (sucrose: P=0.001; casein: P=0.002) and 100nM (sucrose: P=0.014; casein: P=0.008) bees ate less.

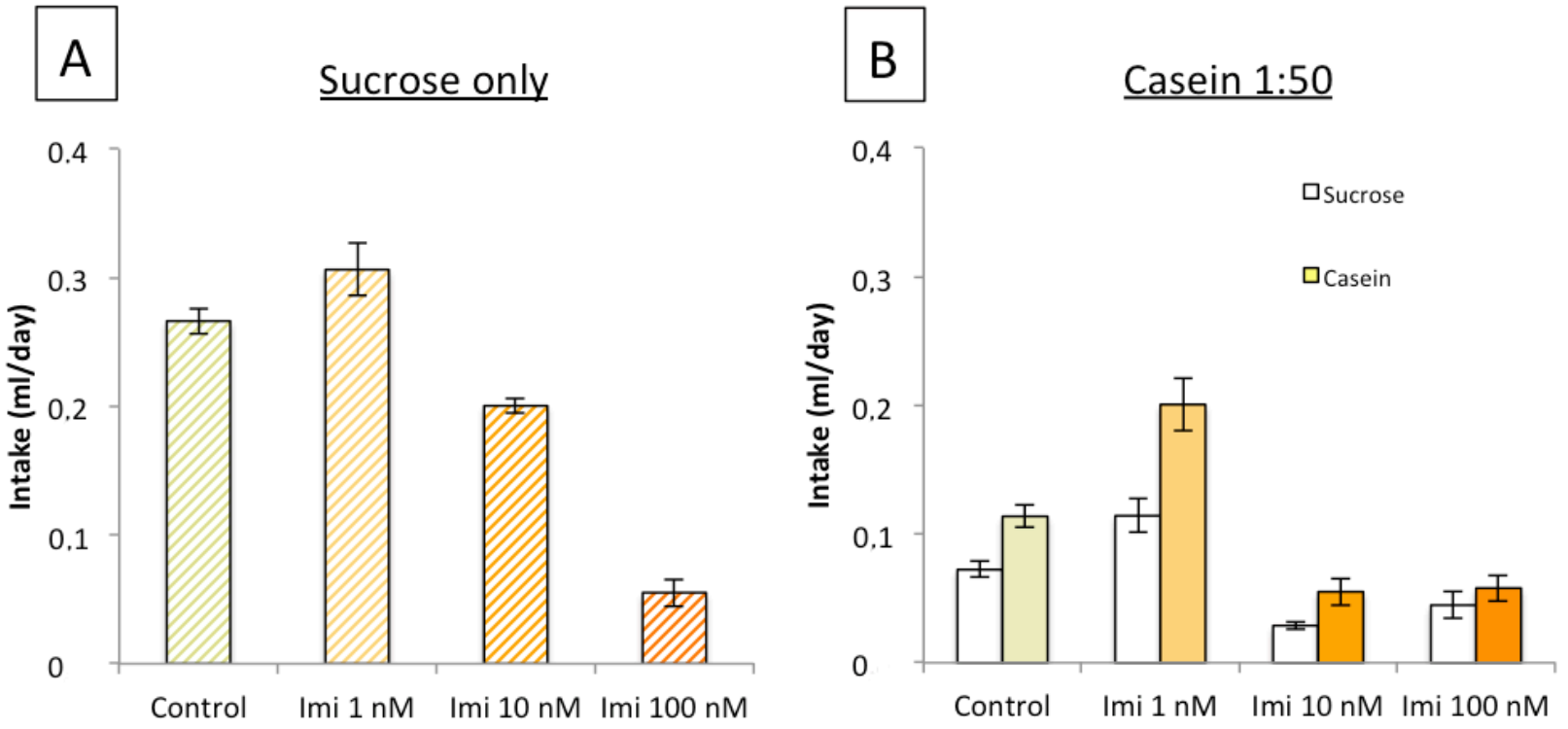

Figure 1.3: Comparison of the impact of different doses of imidacloprid (0, 1, 10 and 100nM) on the average daily food consumption (± standard error) for bumblebees eating sucrose only and sucrose with casein 1:50 diet. "Control" means that there is 'no pesticide' in the diet. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

2. Bumblebees exposed to imidacloprid switch to higher protein diet

The Geometric Framework model predicted that animals exposed to unbalanced diets make "rules of compromise" to reach their optimal dietary ratio of protein and carbohydrates (P:C) (Simpson *et al.*, 2004). The bumblebee workers were given diet pairs with one diet containing a specific ratio of P:C and the other containing 0.5 M sucrose alone. Using this approach, how bumblebees achieved their optimal dietary ratio and their food intake target over the 7 days was investigated. Figure 1.4 represents a cumulative plot of the mean protein and carbohydrate consumption for each dietary pairing. The influence of imidacloprid on daily volume of food intake depended on the day and the dietary ratio (sucrose or sucrose with casein) (Annex A).

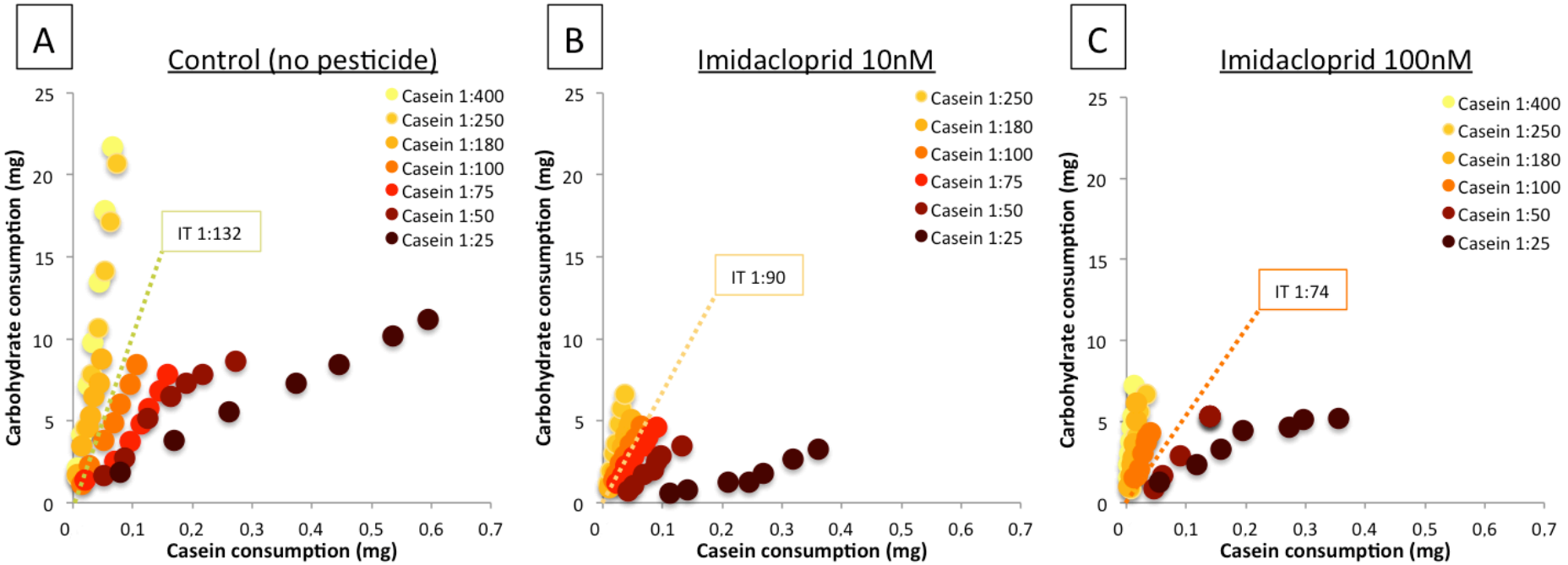

Figure 1.4: **Cumulative average daily food consumption on protein and carbohydrate intakes for individual bumblebees confined to one of the different diets tested varying in both ratio and the total amount of protein and carbohydrate (in P:C ratios for Casein 1:400, 250, 180, 100, 75, 50 and 25) and over different doses of imidacloprid (0, 10 and 100nM) over the 7-days.** For each diet, one dot represent one day. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

The intake target (IT) was the P:C ratio calculated from the average intake of carbohydrates and protein made by the treatments and were found to be not significantly different in the MANOVA (Annexe A). Using this method, the IT of the P:C was determined to be 1:132 for the control subjects. When bees were exposed to imidacloprid, the IT shifted towards higher protein diet (Figure 1.5). Bees exposed to 10nM imidacloprid shifted their IT toward 1:90 (P:C). The few bees that survived the exposure to 100nM imidacloprid shifted their IT even further towards protein – to obtain an IT of 1:74 (P:C).

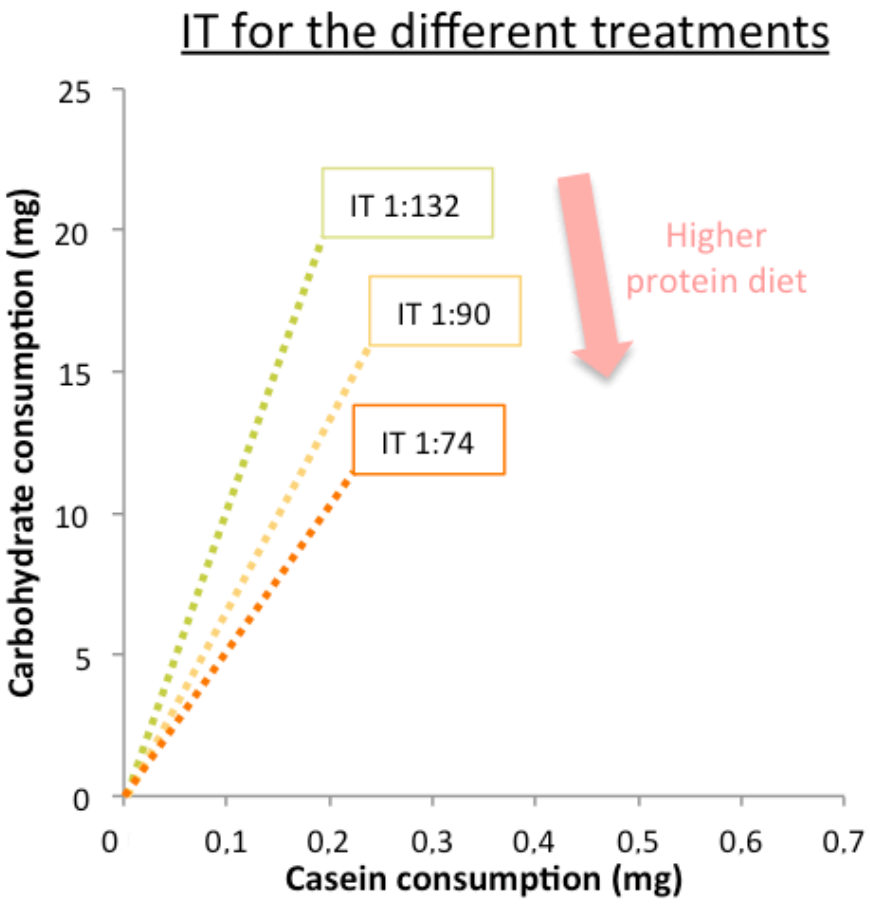

Figure 1.5: **Comparison of the food intake target (IT) for individual bumblebees over the different diets of protein and carbohydrate (in P:C ratios for Casein 1:400, 250, 180, 100, 75, 50 and 25) and over different doses of imidacloprid (0, 10 and 100nM).** For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

3. Average daily imidacloprid intake

To compare our results with published studies, the average daily dose the worker bumblebees experienced was also calculated in our experiments (Table 1.5). As expected, the mean daily dose of imidacloprid consumed by bumblebees in these experiments depended on the concentration in the food (one-way ANOVA ($F_{(3,297)}$ = 47.000.6, P <0.001). The dose consumed by the bees fed the 1nM imidacloprid was lower (71.8±8.28pg, Tukey's *post-hoc, P*<0.001; Table 1.5) when compared to the 10nM concentration (313.6±22.2pg, Tukey's *post-hoc*, *P*<0.001) and the 100nM concentration (2524.5±168.9pg, Tukey's *post-hoc, P*<0.001).

Dose (I)	(Dose (J)	Average imidacloprid daily intake (pg)	STD	Mean difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
							Lower Bound	Upper Bound
1 nM	10nM	71.821	8.277	-241.7960	278.92443	0.662	-898.8094	415.2174
	100nM			-2452.6370*	277.05034	**<0.001**	-3105.2359	-1800.0380
10 nM	1nM	313.617	22.204	241.7960	278.92443	0.662	-415.2174	898.8094
	100nM			-2210.8410*	167.72780	**<0.001**	-2605.9280	-1815.7540
100 nM	1nM	2524.458	168.862	2452.6370*	277.05034	**<0.001**	1800.0380	3105.2359
	10nM			2210.8410*	167.72780	**<0.001**	1815.7540	2605.9280

Table 1.5: **Mean (± standard deviation) of the daily intake of imidacloprid (pg) and results of multiple comparisons Tukey's post-hoc test between the average daily intakes depending of the average doses (1, 10 and 100nM) to which bumblebees were exposed**. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

4. Survival

Bumblebees exposed to imidacloprid died sooner (Figure 1.6; Table 1.6). For the control group, bees fed diets high in protein also had reduced survival (Figure 1.6A). For example, survival was significantly reduced with casein 1:50 compared to the control diet of sucrose alone (Table 1.6) and casein 1:25 (Table 1.6). When bumblebees were exposed to a 10nM concentration of imidacloprid, they were more likely to die when fed diets high in protein (Table 1.6). In fact, dietary ratios of 1:75, 1:50, or 1:25 all had a significantly greater risk of dying when exposed to 10nM imidacloprid (Figure 1.6B). For bees fed the 100nM imidacloprid, this was even more striking. Bees fed dietary P:C ratios of 1:250-1:25 all had a greater probability of dying when exposed to >10nM imidacloprid than the control bees fed sucrose alone (Figure 1.6; Table 1.6). There was one exception: bees fed the1:180 diet did not have a significantly greater of risk of dying. This was also the dietary pairing that was easiest for bumblebees to achieve their IT in Figure 1.4A.

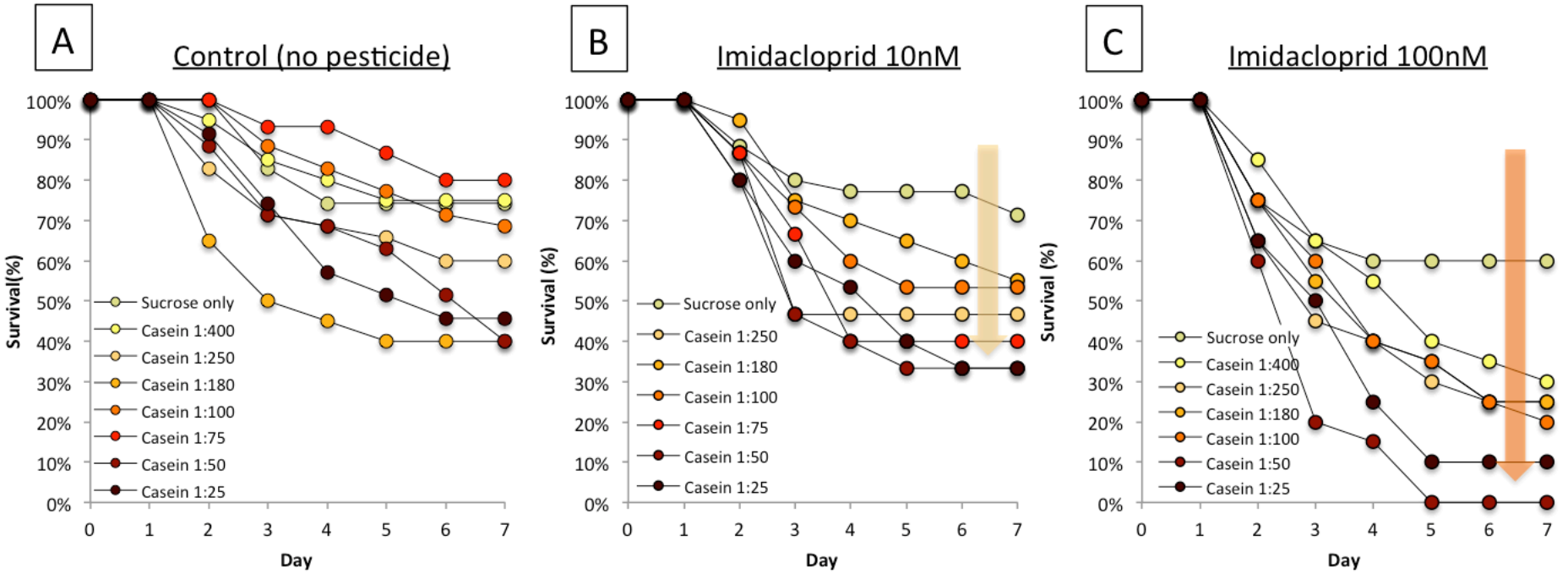

Figure 1.6: **Comparison of the mortality of bumblebees exposed to different protein diets (sucrose only and in P:C ratios for Casein 1:400, 250, 180, 100, 75, 50 and 25) and sublethal doses of imidacloprid (0, 1, 10 and 100nM).** For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

Dose of imidacloprid	Diet	N	Sucrose only		Casein 1:400		Casein 1:250		Casein 1:180		Casein 1:100		Casein 1:75		Casein 1:50		Casein 1:25	
			χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.
No pesticide	Sucrose only	35	.	.	0.000	0.982	1.970	0.160	8.635	**0.003**	0.159	0.690	0.244	0.621	7.490	**0.006**	5.577	**0.018**
	Casein 1:400	20	0.000	0.982	.	.	1.304	0.253	5.674	**0.017**	0.139	0.710	0.189	0.664	5.010	**0.025**	3.877	**0.049**
	Casein 1:250	35	1.970	0.160	1.304	0.253	.	.	2.530	0.112	0.983	0.321	2.066	0.151	1.631	0.202	0.906	0.341
	Casein 1:180	20	8.635	**0.003**	5.674	**0.017**	2.530	0.112	.	.	6.969	**0.008**	6.446	**0.011**	0.456	0.500	0.921	0.337
	Casein 1:100	35	0.159	0.690	0.139	0.710	0.983	0.321	6.969	**0.008**	.	.	0.685	0.408	5.642	**0.018**	4.464	**0.035**
	Casein 1:75	15	0.244	0.621	0.189	0.664	2.066	0.151	6.446	**0.011**	0.685	0.408	.	.	5.956	**0.015**	4.987	**0.026**
	Casein 1:50	35	7.490	**0.006**	5.010	**0.025**	1.631	0.202	0.456	0.500	5.642	**0.018**	5.956	**0.015**	.	.	0.036	0.848
	Casein 1:25	35	5.577	0.018	3.877	**0.049**	0.906	0.341	0.921	0.337	4.464	**0.035**	4.987	**0.026**	0.036	0.848	.	.
1nM	Sucrose only	15	.	.	.	.	.	.	.	.	.	.	.	.	0.194	0.660	.	.
	Casein 1:50	15	0.194	0.660	.	.	.	.	.	.	.	.	.	.	.	.	.	.
10nM	Sucrose only	35	.	.	.	.	2.738	0.098	1.245	0.265	1.480	0.224	4.146	**0.042**	6.809	**0.009**	6.629	**0.010**
	Casein 1:250	15	2.738	0.098	.	.	.	.	0.498	0.481	0.203	0.652	0.007	0.935	0.498	0.480	0.336	0.562
	Casein 1:180	20	1.245	0.265	.	.	0.498	0.481	.	.	0.056	0.813	0.997	0.318	2.436	0.119	2.017	0.156
	Casein 1:100	15	1.480	0.224	.	.	0.203	0.652	0.056	0.813	.	.	0.467	0.494	1.364	0.243	0.991	0.320
	Casein 1:75	15	4.146	**0.042**	.	.	0.007	0.935	0.997	0.318	0.467	0.494	.	.	0.332	0.564	0.094	0.759
	Casein 1:50	15	6.809	**0.009**	.	.	0.498	0.480	2.436	0.119	1.364	0.243	0.332	0.564	.	.	0.037	0.847
	Casein 1:25	15	6.629	**0.010**	.	.	0.336	0.562	2.017	0.156	0.991	0.320	0.094	0.759	0.037	0.847	.	.
100nM	Sucrose only	20	.	.	2.041	0.153	4.032	0.045	3.450	0.063	4.424	0.035	.	.	13.821	**0.000**	7.817	**0.005**
	Casein 1:400	20	2.041	0.153	.	.	0.650	0.420	0.360	0.549	0.602	0.438	.	.	11.597	**0.001**	3.735	0.053
	Casein 1:250	20	4.032	**0.045**	0.650	0.420	.	.	0.063	0.802	0.008	0.928	.	.	4.523	**0.033**	0.822	0.364
	Casein 1:180	20	3.450	0.063	0.360	0.549	0.063	0.802	.	.	0.020	0.888	.	.	7.040	**0.008**	1.504	0.220
	Casein 1:100	20	4.424	**0.035**	0.602	0.438	0.008	0.928	0.020	0.888	.	.	.	.	7.613	**0.006**	1.366	0.243
	Casein 1:50	20	13.821	**0.000**	11.597	**0.001**	4.523	**0.033**	7.040	**0.008**	7.613	**0.006**	.	.	.	.	2.054	0.152
	Casein 1:25	20	7.817	**0.005**	3.735	0.053	0.822	0.364	1.504	0.220	1.366	0.243	.	.	2.054	0.152	.	.

Table 1.6: **Results of Kaplan-Meier analysis (Log rank Mantel Cox) with different dose of imidacloprid (0, 1, 10 and 100nM) and different diets (Sucrose only and Casein 1:400, 250, 180, 100, 75, 50 and 25).** The Kaplan-Meier analysis measures the fraction of subjects living for a certain amount of time after treatment. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

The comparison of the survival between sucrose only diet and casein 1:50 showed that bees fed with sucrose only had a similar mortality rate (Figure 1.7A) but bees exposed to imidacloprid 1nM doses had a different rate of death than the others (Kaplan-Meier: χ^2=0.194, df=3, *P*=0.660; Table 1.7). Bees fed with a high protein diet were more likely to die quickly with higher imidacloprid doses (Figure 1.7B).

Sucrose only									
Dose	N	No pesticide		1nM		10nM		100nM	
		χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.
No pesticide	35	.	.	0.138	0.711	0.143	0.706	1.959	0.162
1nM	15	0.138	0.711	.	.	0.384	0.535	1.699	0.192
10nM	35	0.143	0.706	0.384	0.535	.	.	0.945	0.331
100nM	20	1.959	0.162	1.699	0.192	0.945	0.331	.	.
Casein 1:50									
Dose	N	No pesticide		1nM		10nM		100nM	
		χ^2	Sig.	χ^2	Sig.	χ^2	Sig.	χ^2	Sig.
No pesticide	35	.	.	**7.215**	**0.007**	0.977	0.323	**24.726**	**<0.001**
1nM	15	**7.215**	**0.007**	.	.	**7.677**	**0.006**	**24.553**	**<0.001**
10nM	15	0.977	0.323	**7.677**	**0.006**	.	.	5.998	0.014
100nM	20	**24.726**	**<0.001**	**24.553**	**<0.001**	5.998	0.014	.	.

Table 1.7: **Results of Kaplan-Meier analysis (Log rank Mantel Cox) with different dose of imidacloprid (0, 1, 10 and 100nM) and two different diets (Surcose only and Casein 1:50 in P:C ratio).** The Kaplan-Meier analysis measures the fraction of subjects living for a certain amount of time after treatment. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

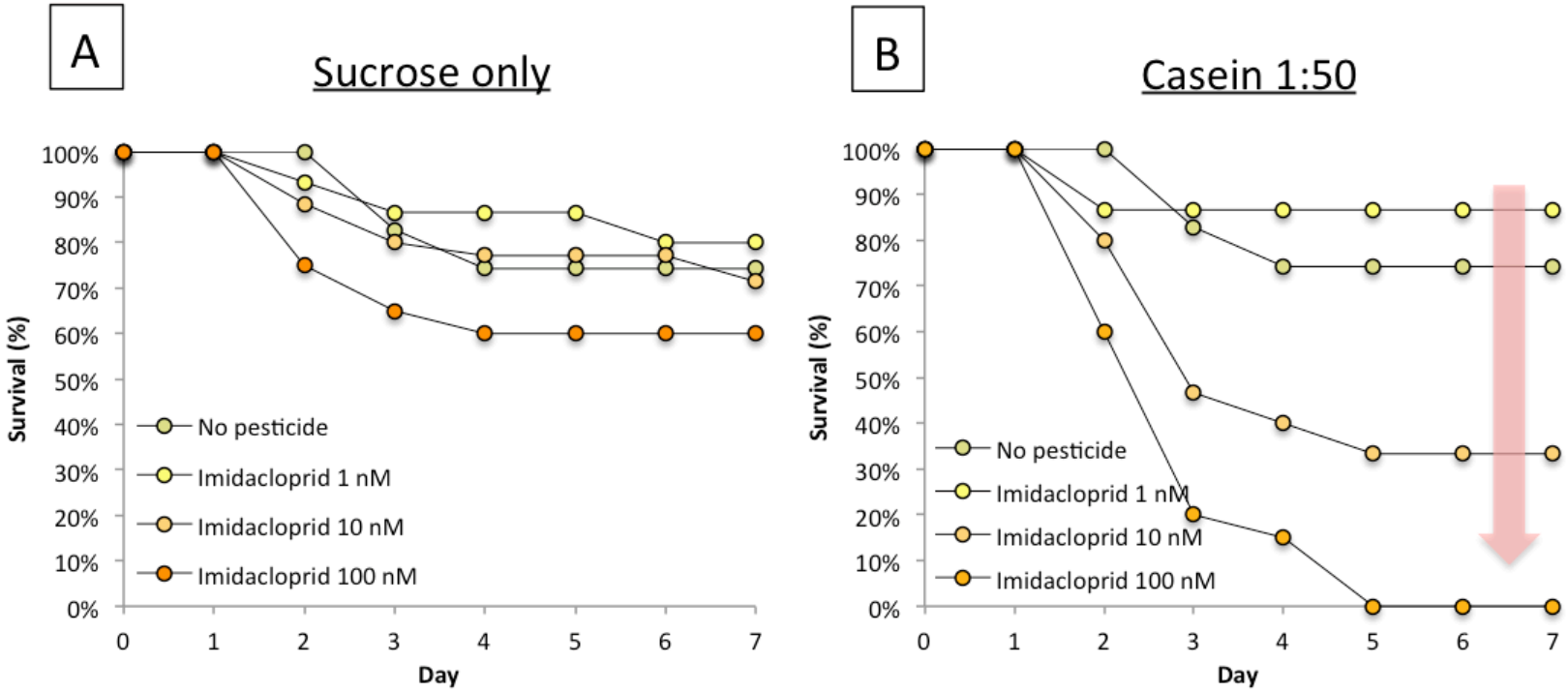

Figure 1.7: Comparison of the mortality of bumblebees exposed to different diets (sucrose only and casein 1:50 in P:C ratio) and sublethal doses of imidacloprid (0, 1, 10 and 100nM). For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

5. Imidacloprid influences bumblebee behavior

In addition to measuring food consumption, the behavior was also observed of each bee on each day. The behaviors recorded were plotted to show the proportion of each behavior depending of the imidacloprid dose. In the control (no imidacloprid), bumblebees were showing a very large panel of behaviors and were active in 79% of the observations (Figure 1.8A). In contrast, bumblebees exposed to imidacloprid exhibited less activity and a greater diversity of behaviors that depended on the dose (Figure 1.8B, C & D). The frequency of active behaviors like running, walking and flying decreased as a function of imidacloprid dose (Figure 1.9). Bees spent more time sitting or lying on their back when they were exposed to imidacloprid, and the prevalence of these behaviors in the population was exacerbated by higher doses of imidacloprid (Figure 1.9). In contrast, the lowest dose of imidacloprid (1nM) increased the frequency of the climbing behavior in comparison with the control (no imidacloprid). However, high concentrations of imidacloprid reduced the frequency of observed climbing behavior. There was no effect of imidacloprid on grooming and eating behavior (Figure 1.8). When bees consumed diets containing imidacloprid, they were more likely to spend time lying on their back (Kruskal-Wallis, χ^2_1=30.9, P<0.001; Figure 1.10). The number of days spent on the back also significantly increased with the imidacloprid dose (Kruskal-Wallis, χ^2_2=11.3, P=0.003; Figure 1.10) and during the experiment, bees were able to survive up to 6 days in this position.

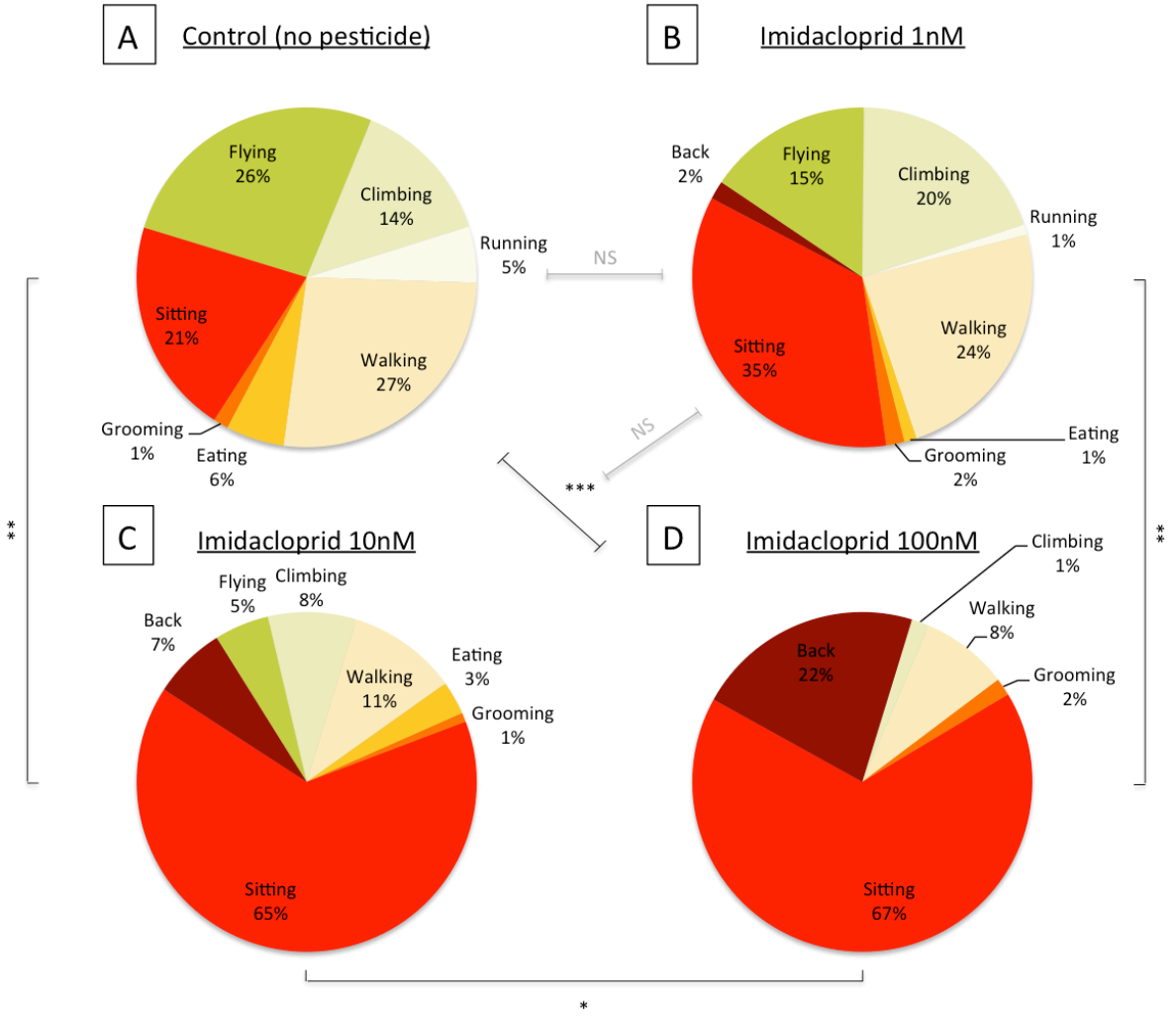

Figure 1.8: **Comparison of the behavior displayed by bumblebees over different diets (sucrose only and in P:C ratios for Casein 1:400, 250, 180, 100, 75, 50 and 25), sublethal doses of imidacloprid (0, 1, 10 & 100nM) and over 7 days.** (LSD *post-hoc* comparison after repeated-measures ANOVA: '*': 0.005<*P*<0.010; '**': 0.001<*P*<0.005; '***': *P*<0.001). For the number of specimen per run, please refer to Table 1.1 in Materials and Methods.

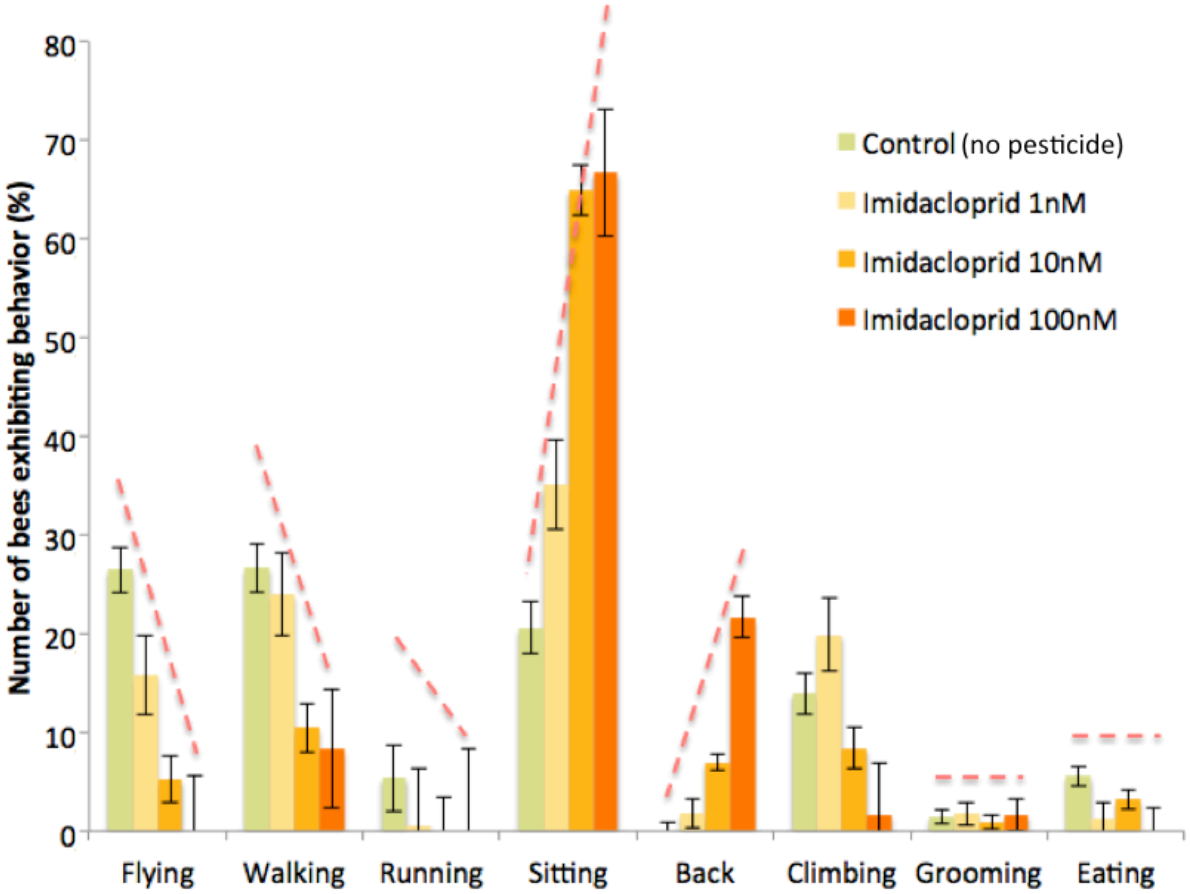

Figure 1.9: Frequency of bumblebee's behavior observed (± standard error) over different diets (sucrose only and in P:C ratios for Casein 1:400, 250, 180, 100, 75, 50 and 25), sublethal doses of imidacloprid (0, 1, 10 & 100nM) and over 7 days. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

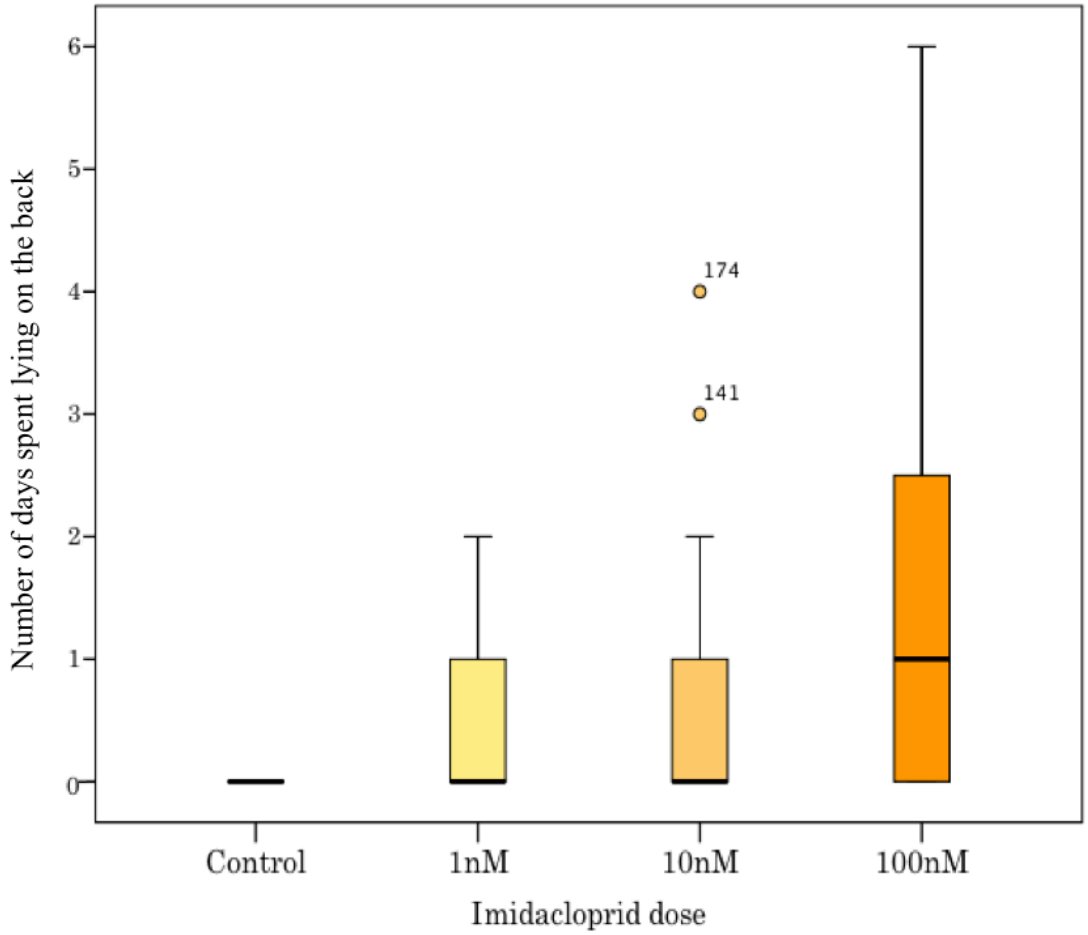

Figure 1.10: **Average number of days spent on the back by bumblebees for different doses of imidacloprid (0, 1, 10 & 100nM) over diets (in P:C ratios) and days.** "Control" means 'no pesticide'. Lines in bold represent medians; boxes represent 1st and 3rd interquartile ranges, bars represent the minimum and maximum range of the data and open circles represent outliers. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

To compare how diet and exposure to imidacloprid affected behavior, a factor analysis was performed on the behavior observed for day 3. The factor analysis reduced 8 variables to three variables represented in three factors that accounted for 75.0% of the variation in the data (Table 1.8). The first factor, which accounted for 41.5% of the variation in behavior, was mainly representative of the inverse relationship between active behaviors such as flying, walking and eating in contrast to sitting behavior. The second factor accounted for 17.1% of the variation in the behavior and represented the time spent grooming and running. The third factor characterized 16.5% of the variation in the behavior and represented the inverse relationship between climbing and the exhibition of bees lying on their backs that are mutually exclusive.

	Principal components		
	1	2	3
Eigenvalue	3.316	1.366	1.318
Percent variance	41.455	17.074	16.478
Factor loading			
Flying	**0.651**	0.489	0.274
Climbing	-0.189	-0.064	**0.754**
Running	0.247	**0.934**	0.080
Walking	**0.684**	0.207	0.352
Eating	**0.823**	0.258	-0.100
Grooming	0.082	**0.965**	-0.104
Sitting	**-0.882**	0.048	0.204
Back	-0.167	-0.052	**-0.724**

Table 1.8: Factor analysis of bumblebee's behavior using principle components method of factor extraction with varimax rotation. Factor loading in bold indicate which behavioral variables made the largest contribution to each factor. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

The factor scores obtained by the factor analysis were used to test how the chronic exposure to different doses of imidacloprid affected the expression of behavior. As represented by factor 3 (climbing and laying on the back), the exposure to imidacloprid induced an opposition between active behavior such as climbing, and inactivity like lying on the back largely correlated to bumblebees exposed to imidacloprid (between-subjects, repeated measures ANOVA $F_{(3,15)}$=3.38, P=0.048; dose x factor 3; Table 1.9). The ability to be less or more active was dependent on the dose of imidacloprid and exposed bees spent significantly more time lying on the back than flying or grooming (Table 1.9) and the diet had an increasing significant impact on the grooming behavior (Table 1.9).

To highlight this phenomenon of the number of day spent lying on the back that increase with the dose of imidacloprid, a subset of the data was extracted. The behavior was compared for two different diets (sucrose only and casein 1:50) and three doses of imidacloprid (0, 1 and 10nM). Bumblebees were not acting the same when they were exposed to different imidacloprid doses (within-subjects, repeated measures ANOVA $F_{(2,84)}$=9.96, P<0.001; behavior x dose), but no interaction of the behavior with the diet (within-subjects, repeated measures ANOVA $F_{(1,84)}$=0.81, P=0.371; behavior x diet) and with the diet and the imidacloprid dose (within-subjects, repeated measures ANOVA $F_{(2,84)}$=0.05, P=0.953; behavior x diet x dose) were significant.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	factor 1	5.554	3	1.851	2.412	0.122
	factor 2	0.461	3	0.154	0.125	0.944
	factor 3	6.710	3	2.237	3.375	0.058
Intercept	factor 1	0.701	1	0.701	0.913	0.360
	factor 2	0.009	1	0.009	0.008	0.932
	factor 3	0.062	1	0.062	0.094	0.765
Dose	factor 1	5.554	3	1.851	2.412	0.122
	factor 2	0.461	3	0.154	0.125	0.944
	factor 3	**6.710**	**3**	**2.237**	**3.375**	**0.048**
Error	factor 1	8.446	11	0.768		
	factor 2	13.539	11	1.231		
	factor 3	7.290	11	0.663		
Total	factor 1	14.000	15			
	factor 2	14.000	15			
	factor 3	14.000	15			
Corrected Total	factor 1	14.000	14			
	factor 2	14.000	14			
	factor 3	14.000	14			

Table 1.9: Results of tests of between-subjects effects of repeated-measures ANOVA comparing the doses of imidacloprid (0, 1, 10 and 100nM) using scores generated by the factor analysis of the behavior with the three factors produced by the analysis of principal component. For the number of specimen per run, please refer to Table 1.1 in Material and Methods.

IV. Discussion

Key results emerging from my work are that chronic exposure to sub-lethal field relevant doses (1-100nM) of imidacloprid reduced food intake, shifted the nutrient balance towards a higher protein diet, reduced survival and depressed the activity of bumblebees. These results are consistent with Cresswell *et al.* (2012) who observed that bumblebees exposed to doses of imidacloprid doses up to ~40nM have a reduction in feeding rate of 10-30% and reduced locomotor activity.

1. Field relevant doses

The amount of imidacloprid daily ingested by bumblebees was field relevant. Gaucho® seed-dressed plants provide 4.75pg of imidacloprid per mg of sugar in nectar or honey and 3.4pg of imidacloprid per mg of pollen (Rortais *et al.*, 2005). The concentrations of imidacloprid in sunflowers that have been seed treated are reportedly not greater than 10mg.kg^{-1} (~40nM) in honey or pollen collected by honeybees on those flowers (Taséi *et al*., 2000; Table 2 in the General Introduction).

In my experiments, bumblebees were ingesting between 71.8 ±8.3 to 2524.5 ±168.9 pg of imidacloprid per day depending of the dose provided. In an extensive study, Rortais *et al.* (2005) estimated that forager honeybees experienced doses of imidacloprid in the range of 152-609.9pg in contaminated nectar and 49.4-74.1pg in contaminated pollen per day of foraging activity. They also estimated that worker honeybees have a daily sugar intake between 1.2-4.4mg and forager honeybees have a daily sugar intake between 10.4-128.4mg. Honeybee foragers require about 8-12mg of sugar per hour of flight (Balderrama *et al.*, 1992) and undertake 7-13 foraging trips per day (Crane, 1990), whereas bumblebees undertake 17-27 per day foraging trips (Alford, 1975). For this reason, Thompson and Hunt (1999) estimated that bumblebees potentially take up to 5 times more the level of contaminated nectar in a day than honeybees. In addition, they also estimated that bumblebees forage on ~2.5 times more flowers per minute than honeybees and carry up to 112µl per trip compared to 50µl for honeybees. For these reasons, wild bees like bumblebees might be exposed to imidacloprid residues lower than 10ppb (≈40nM) of nectar and pollen from treated crops (Bonmatin *et al*., 2003, 2005; Chauzat *et al*., 2006). In

addition, my data indicate that bumblebees seem to be physiologically more vulnerable to imidacloprid and are perhaps more sensitive to its effects than honeybees

2. Direct interaction of imidacloprid and diet

a. Exposure to imidacloprid reduced the bumblebee's food intake.

Bumblebees exposed to imidacloprid even at very low sub-lethal doses exhibited a significant reduced food intake. Laycock *et al.* (2012) also observed that queenless microcolonies exposed to similar imidacloprid doses in food reduced feeding on both syrup and pollen. This effect was dose-dependent as shown in our results. Moreover, Cresswell *et al.* (2012) did not observe any changes in the feeding response of honeybees, contrary to bumblebees that progressively showed over time a dose-dependent reduction in feeding rate decline of 10-30% with field relevant doses of imidacloprid. They suggested that honeybees were better pre-adapted than bumblebees to feed on nectar containing allelochemicals such as alkaloids, such as imidacloprid, as a benefit of their ancestral adaptation to tropical nectars that contain common natural alkaloids. These results and ours were consistent with previous studies that showed that bumblebees were affected by similar imidacloprid doses (Tasei *et al*., 2000; Mommaerts *et al*., 2010). The higher sensitivity of bumblebees to chemicals such as imidacloprid and the antifeedant property of imidacloprid estimated above ~150nM (40µg.l^{-1}) for honeybees (DEFRA, 2012) can explain that in our experiment bumblebees showed a lower food intake at a lower dose than honeybees. Imidacloprid antifeedant property does not alone explain the lower food intake of bumblebees, as physiological impacts can also explain the lower food intake; see Sections 3 and 4 where digestive issues and signs of malaise are investigated.

b. The food intake target switched to a higher protein diet

The experimental doses of imidacloprid used impaired bumblebee food nutrient balance shifting it toward a higher protein diet. In our experiment, the intake target switched as a consequence of imidacloprid dose. Bumblebees that were not exposed to imidacloprid maintained a strict P:C ratio like honeybees separated from the queen and without any contact with the brood (Altaye *et al.*, 2010) and both bumblebees and

honeybees reduced feeding when they are exposed to imidacloprid due to its toxicity rather than an aversion (Laycock *et al.*, 2012). It is possible that the detoxification process induced by a higher protein diet can be an adaptive response. Honeybees have substantial capacity to detoxify diets containing imidacloprid (Suchail *et al.*, 2004; Puinean *et al.*, 2010), but showed detrimental effects at lower doses than at higher (Cresswell, 2011). The differential sensitivity of bumblebees and honeybees could be explained by differences in imidacloprid target site sensitivity (Liu *et al.*, 2005), although, honeybees are not particularly likely to exhibit high levels of insensitivity to imidacloprid as their nAChR ligand binding domain interacts with neonicotinoids (Matsuda *et al.*, 2009).

A plethora of different cytochrome P450 genes are described that may affect insecticide resistance in many pest insect studies (Feyereisen, 1999). Cytochrome P450 is part of the monooxygenase superfamily involved in the oxidation or activation of organic substances and in imidacloprid metabolism (Guengerich & Isin, 2008; Karunker *et al.*, 2009) and may show some cross-resistance to other neonicotinoids (Mota-Sanchez *et al.*, 2006; Rauch and Nauen *et al.*, 2003). The activity of cytochrome P450 is thermally rate-limited (Puntarulo & Cederbaum, 1989). Cresswell *et al.* (2012) observed that the cytochrome P450 activity at 25°C was not efficient enough to help immobilized harnessed honeybees to detoxify their body. Harnessed honeybees cannot thermally regulate their body. Whereas their body temperature is above 30°C inside the nest in a social interaction context (Coehlo, 1991) or at 30-35°C during flight (Cooper *et al.*, 1985). Bumblebees can be active at very low temperature in comparison with honeybees, smaller workers can fly at temperatures below 10°C (Heinrich, 1975) and the nest temperature can be maintained at around 30°C (Goulson, 2003). That relative capacity to live at lower temperatures can impair the bumblebee's capacity to maintain the detoxification process (Cresswell *et al.*, 2012) and switch to a higher protein diet to compensate this process.

Other enzymes present in the digestive tract can be involved in a detoxification process in addition of cytochrome P450. Stygar *et al.* (2013) suggested that β- glucosidase, α-galactosidase and β-galactosidases might be a part of detoxification processes in the digestive tract as their activity significantely increased with the presence of imidacloprid in a caterpillar diet (Lepidoptera *: Cameria ohridella*).

The increasing protein need due to exposure to imidacloprid may also be explained by an immune system that is consequently challenged (Halm *et al.*, 2006) and thus change the nutrient requirements. This has been observed in other species, for example, the African armyworm (*Spodoptera exempta*) increases its protein intake relative to control larvae when it is exposed to an opportunist bacterium (*Bacillus subtilis*) (Povey *et al.*, 2009). Increased ingestion of protein is associated with the cost of the compensation to the virulence resistance. It can be more strikingly observed with the African cotton leafworm (*Spodoptera littoralis*) with a highly virulent entomopathogen (nucleopolyhedrovirus) that stimulates the protein intake to survive to the pathogen (Lee *et al.*, 2006). In the long run, repeated ingestion of sub-lethal doses of imidacloprid could cause immunodeficiency and diseases in bumblebees, and this impairment of the immune system is non-specific (Glinsky & Kauko, 2000).

c. Bumblebees might have digestive issues due to imidacloprid

The lower food intake and the switch towards a higher protein diet may reflect the adaptation to different energy needs caused by stress. During the daily behavior observations, bumblebees exposed to diets contaminated by imidacloprid were presenting symptoms such as abdomen spasms and brown defecations on the paper pad displayed in the box. It is clear that imidacloprid and its metabolites might have an effect on the physiology of the digestive tract and on digestive enzymes.

The abdominal spasms observed in bumblebees might be due to slower peristaltic movements and chyme passage lead to the apparent higher activity of glycosidase or the result of a lower or higher digestive enzymes secretion in midgut as observed in insects exposed to pesticides (Lehane *et al.*, 1996). Imidacloprid may not directly disturb digestive processes but initiate a constant depolarization of the nerves that can influence the peristalsis and the passage of chyme in the intestine. The constant depolarization of neuron inervating the digestive system is followed by a constant activation of the muscles until the cellular energy systems are depleted and the motile proteins are destroyed (Mehlhorn *et al.*, 1999).

Deshmukh & Tembhare (1998) and Deshmukh *et al.* (2009) observed that sublethal doses of organophosphate pesticides induced an enzyme hypersecretory activity in the

midgut of *Othreis maternal* larva (Lepidoptera: Noctuidae) and stimulated the activity of amylase, invertase, lipase and proteinase. Furthermore, Stygar *et al.* (2013) observed that imidacloprid diet presence changed the digestive enzymes profile of a caterpillar *Cameria ohridella* (Lepidoptera: Gracillariidae). They observed a significant increase of the activity of sucrose and lactase with imidacloprid exposition and through generations. The most striking observation was made on the activity of proteolytic enzymes. Exposure to imidacloprid at low doses induced a significant reduction of trypsin, chymotrypsin and aminopeptase activity in the caterpillar digestive tract. The reduction of the activity of enzymes that are involved in the digestion and assimilation of protein might explain why our bumblebees exposed to imidacloprid switch to higher protein diet to compensate the lower activity of those enzymes and achieved their food intake target. The lower activity of proteolytic enzymes could be the consequence of the phosphorous liberation for energy metabolism, and decreased metabolism rate or transport of nutrients rate, the gut activity and the heart rate could be lower (Senthil-Nathan *et al.*, 2006).

d. Bumblebees exposed to imidacloprid were presenting signs of malaise leading them to death

Bumblebees exposed to imidacloprid and having a higher protein diet were more likely to die quickly than bumblebees in the control group. Through scientific literature, survival of bumblebees to sub-lethal doses of imidacloprid shows disparity, probably arising because the severity of the toxic effect of imidacloprid depends of test conditions. Tasei *et al.* (2000) observed similar elevated rates of mortality for imidacloprid doses above 10µg.kg^{-1} (>40nM) in queenless microcolonies studies. Their study showed that the first two weeks of imidacloprid exposure affects bumblebee survival rate without any dose effect relationship and then mortality evolved at the same rate until the end of brood rearing. Moammerts *et al.* (2010) observed that foraging bumblebees fed with contaminated food in the same range of imidacloprid dose were 3 to 10 times more sensitive than bumblebees that did not forage. The exposure to sub-lethal doses of imidacloprid might drive the colony to collapse more quickly when bumblebees are foraging in the field as they are exposed to extra factors in the countryside that can enhance the effect of imidacloprid in a synergic way (Cresswell, 2011).

Moreover, bumblebees were artificially maintained alive by laboratory conditions and this might explain a higher survival rate in our experiment than in a field experiment. The mortality rate can increase when bumblebees are forced to fly 6m trips to have access to syrup contaminated by imidacloprid (≈40nM) under glasshouse conditions (Mommaerts *et al.*, 2010). The underlying physiological basis of this locomotion-dependent toxicity remains unknown. According to Laycock *et al.* (2012), the effects of a dietary toxin can be manifold as physiological functions of individual bees are tightly integrated with their nervous systems. A significant reduction of activity was observed for bumblebees exposed to imidacloprid. Bumblebees under 10nM and 100nM imidacloprid doses spent days upside down before dying or changing activity. Such behavior in the field would expose bees to predation and quicker death, as they might not be able to survive environmental conditions or being rejected by colony. Likewise, mobility ability failures were observed as uncoordinated leg movements impairing walk capacities, incapacity of flying due to uncoordinated or immobile wings. Bumblebees were also presenting abdominal spasms and heavy tremors. Similar symptoms due to imidacloprid intoxication (intake <0.5ng/beetle/day) were observed in the beetle *Adelges tsugae* (Hemiptera: Adelgidae) and enhanced mortality resulting for starvation or dehydratation due to the paralysis that impaired access to food capacities (Eisenback *et al.*, 2010).

The most important behavior failure seems to be the appearance of tarsi paralysis starting to present after 3 days on 1 and 10nM diets and after one day on 100nM imidacloprid diet. The tarsi paralysis prejudiced tube-handling capacities and by consequence the eating capacity, ability to taste food capacity and mobility. These observations motivated the investigations related in the next chapter (Chapter 02).

3. Colony and field level implications

Bumblebees live in colonies in which individuals depend on the collective performance of many individuals. Therefore, bumblebees exposed to field-relevant sublethal doses of imidacloprid can present signs of intoxication on individuals (Gill *et al.*, 2012). It is not known how the colony can buffer such effects or the results of cumulative effects of imidacloprid at the colony level.

For most of animals, it is vital to eat a variety of foods to achieve an optimal balance of nutrition and their decisions about the nutrients collected are based on their current nutritional state. Social insects are subject to more complex nutrient regulation, as foraging is restricted to a subset of individuals, whose requirements can differ from the other members of the colony as they can be at different stages of their life (Atlaye *et al.*, 2010).

Individual bumblebees exposed or not to imidacloprid were able to balance their food intake to balance their nutrient needs. Exposure to very low sublethal doses of imidacloprid modified both the food intake and the food balance of individuals. The lower appetite and the higher protein needs can affect the foraging behavior and the whole colony. In colonies of ants (Heminoptera), foragers react to food encountered according to their individual needs and to internal demands for nutrients in the nest. Like bumblebees and honeybees, the food collected by forager ants is also brought back to the colony to be stored (Dussutour & Simpson, 2009). Forager honeybees are able to modulate the intensity of foraging for nectar (Seeley, 1986) and pollen (Camazine *et al.*, 1998; Dreller *et al.*, 1999; Pernal and Currie, 2001) according to the nutritional status and needs of the whole colony. The age also has an impact on the ability of individuals inside the colony on the nutrient regulation. Honeybee and ant workers are able to select from a choice of diets to regulate their own protein and carbohydrates intakes independently (Dussutour & Simpson, 2009; Archer *et al.*, 2014).

As imidacloprid sublethal doses have a very rapid impact on bees. In such situations, the altruistic behavior of food foraging for the colony can be dramatically affected by the sudden new individual behaviors and needs. The importance of protein inside the colony can be illustrated by the behaviors observed in honeybee colonies when the pollen stores drop below a finely tuned homeostatic set point, workers cannibalize brood (Schmickl & Crailsheim, 2001) and start foraging earlier (Janmaat & Winston, 2000). The higher needs in protein and the lower appetite can suddenly drive the bumblebee colony to collapse. The demographic consequences of these and their ecological impact on pollination services are uncertain.

Chapter 2: Ease access to food changes nutritional needs, behavior and survival of bumblebees (*B. terrestris*)

I. Introduction

Acquiring food requires energy. The distances over which animals have to travel to access food can strongly affect their population dynamics, genetic structure and life history. It can also affect the traits of all other organisms with which they interact (Greenleaf et al., 2007). Most animal habitats contain food resources that are crucial for an animal species survival, but that are unlikely to be located close together. Consequently, any energy budget required by animals contains an investment in resources used to locate food, as well as build or find nesting sites, mates, and reproduce (Gathmann & Tscharntke, 2002).

Bees are the most pollinator taxon because of their importance to pollination services and their domestication by humans, and consequently their energy requirements have been the best studied of all pollinators. Most of the models used in the study of bee foraging behavior hypothesize about the ways that bees maximize their rate of their net energy intake (Schoener, 1971; Pyke et al., 1977). In the case of bumblebees, the energetic calculations of time-energy budget have been made in term of rates of flower visitation that a bumblebee must achieve to make an energetic profit rather than in terms of energy intake (Heinrich, 1975, 1976, 1977, 1983). For this reason, bumblebee foraging behavior has been compared to the way that predators hunt (Hodges, 1981, 1985). Moreover, Heinrich (1981) estimated and detailed the energetic cost of foraging depending on many variables including: the flower community (i.e. rate of flower visitation, volume of nectar obtained, energy obtained per flower, distance between flower patches), bumblebee physical activity (i.e energy expenditure on physical activity, proportion of time spent in flight), bumblebee metabolism (i.e. passive heat loss from thorax, energy expenditure on thermoregulation) and abiotic factors (i.e. ambient temperature).

Honeybee foragers exhibit a metabolic rate during flight that is the highest of any animal recorded; it was estimated to be 20 to 100-fold above that of bees at rest (Joos et al., 1997; Suarez et al., 1998). A previous study of honeybee nutrition found that honeybee foragers were strongly biased towards consuming large amounts of carbohydrates (Paoli et al., 2014). Honeybees fuel their flight with hexose sugars and have high enzymatic flux capacities (Suarez et al., 1996; Suarez, 2000). Suarez et al. (2005) observed that carbohydrate oxidation predominated as the major source of energy for flight in bees. They also emphasized that proline is crucial in fueling flight as it stimulates the oxidation of

pyruvate (a key metabolite at the intersection of several metabolic pathways that produce energy).

It is possible that neonicotinoids could influence bees by impairing their motor function required to find and obtain food (Williamson et al., 2014). Imidacloprid impairs the ability of honeybees to perform the waggle dance (Eiri & Nieh, 2012; Lambin et al., 2001) and their motor functions, as for example honeybees would lose the righting reflex that leads them to spend more time lying on their back, spent more time immobile and less time walking or running (Williamson et al., 2014). The lower activity observed with sub-lethal doses of imidacloprid can be explained by the impact of imidacloprid on metabolism and gene transcription. Derecka et al. (2013) concluded that exposure to sub-lethal doses of imidacloprid (8nM) led to a downregulation of genes in glycolytic and sugar metabolism pathways. During flight, muscles have to work at very high glycolytic rates (Suarez, 2000) and that suggests that flight performance could be impacted as imidacloprid also induces downregulation of energy metabolizing genes in adult pollinators too (Derecka et al., 2013). Derecka et al. (2013) also observed an increase in the RNA levels of a cluster of genes encoding for detoxifying Cytochrome P450 enzymes. Cytochrome P450 enzymes play a fundamental role in the metabolism of xenobiotic compounds by catalyzing the breakdown of a wide range of different toxins and synthetic insecticides that contribute greatly to insecticide resistance (Giraudo et al., 2010).

Bumblebees visit more flowers (Goulson et al., 2001), carry more nectar and pollen per unit time than honeybees (Goulson et al., 2002) and can consequently be more exposed to xenobiotic compounds as imidacloprid. Bumblebee workers exhibit a large range of sizes that corresponds closely with tongue length of workers inside the colony (Peat et al., 2005). Greenleaf et al. (2007) suggested that the foraging distance of bees increases with their body size and their intertegular span. The intertegular span is the distance measured with a micrometer between the points of wing insertion called tegulae (Cane, 1987). Due to their large body size and their wide foraging capacities, larger bumblebees might have different nutritional needs and high basal metabolic needs.

Nutritional models rarely include details about how the energetic demands of finding food affects nutrient balancing. In most studies, animals are confined to a housing cage or a box under constant conditions and provided with ad libitum food. (i.e. Simpson &

Raubenheimer, 1993, 2001; Lee et al., 2002; Raubenheimer & Simpson, 2003; Clissold et al., 2006; Clissold et al., 2009; Paoli et al., 2014). Insect nutrition studies are mainly focused on nutritional conflicts when the IT (Intake Target) cannot be reached, the dynamics of IT depending of new physiological demands, post-ingestive regulations, animal performances (i.e. survival, growth, larval development) and the impact of allelochemicals on the IT under ad-libitum conditions.

In the research described in the previous chapter, bumblebees fed on neonicotinoids were much less mobile and were often found lying on their backs. In the previous chapter, the food tubes were provided at a height of 2cm above the bottom of the plastic housing box. If bees had difficulty accessing the food because imidacloprid impaired motor function, this might have contributed to the accelerated mortality observed previously and potentially also affected their intake target. Here I report a method for estimating how the access to food and a sub-lethal dose of imidacloprid impact on bumblebees. The average daily food consumption, the food intake target, the survival, the average body mass loss and the behavior were observed in ad-libitum food choice conditions with an easy and a little challenging access to food.

II. Material and Methods

5. Animals

All experiments were performed with worker bumblebee colonies (*Bombus terrestris terrestris* L.) provided for commercial uses (Koppert B.V., AD Berkel en Rodenrijs, Netherland) and conduced in laboratory conditions (22-25°C and 65-80% RH) and continuous darkness from June 2013 to August 2013 in Newcastle University bee lab. Bumblebees were provided *ad libitum* with commercial sugar water and pollen (Pollen mix, Koppert B.V., AD Berkel en Rodenrijs, Netherland) until the beginning of each experiment.

Worker bumblebees – defined as foragers - were collected at the exit of the colony and weighed. They were housed in individual plastic boxes (17x12x7cm) with access to 3 liquid diets until the end of the experiment provided in 2ml microcentrifuge tubes (4 holes were drilled on the topside of the tube, Figure 2.1) until the end of the experiment. The

boxes were placed in the incubator at a temperature of 21-22.8°C and 40-60%RH in continuous darkness. Experiments were conducted for 7 consecutive days.

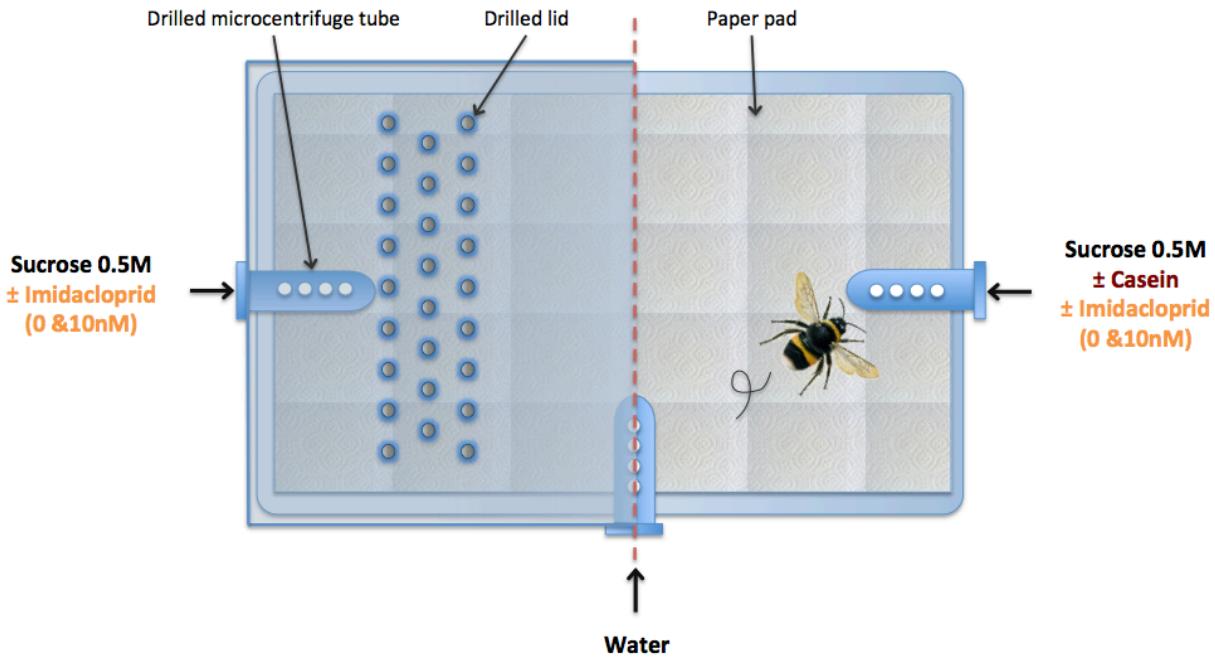

Figure 2.1: Individual bumblebee in feeding box with the 3 different liquid diets provided in drilled Eppendorf tubes.

To assess if the ease access to food influenced the regulation of protein intake target by bumblebees with and without sub-lethal exposure to imidacloprid (Sigma-Aldrich), two tube positions were tested in the rearing boxes. The tubes were positioned at 2cm high for the high tube groups (HT) and on the bottom of the box (0.5 cm high) for the low tubes groups (LT) (Figure 2.2).

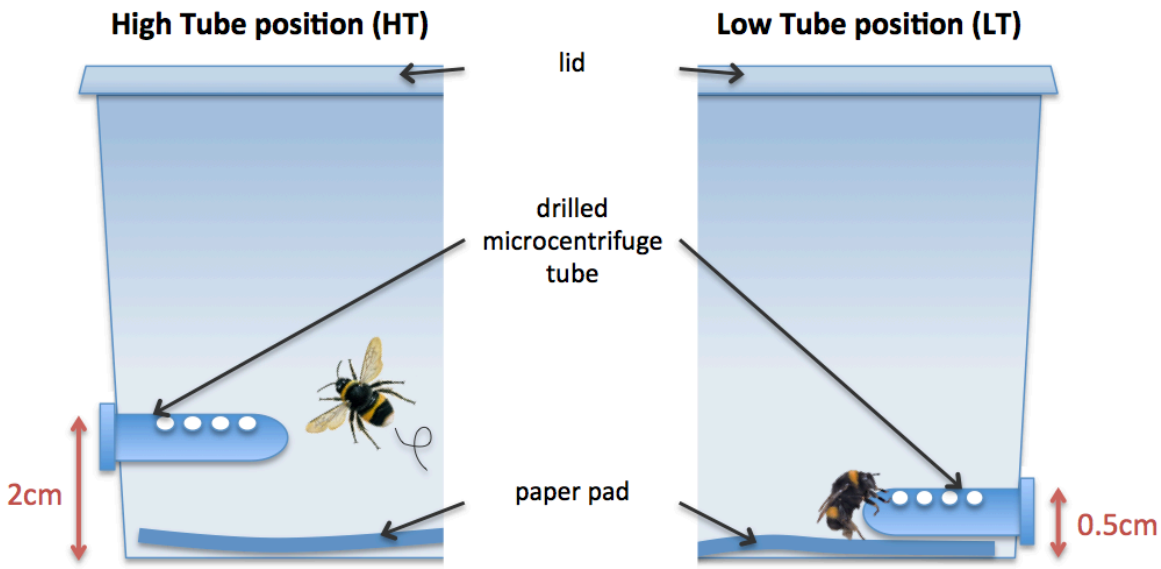

Figure 2.2: **Representation of the position of the drilled tubes that provided the 3 liquid diets in individual boxes.**

6. Diets

To assess if bumblebees changed their food intake depending on the ease access to food and the exposure to sub-lethal doses of imidacloprid, the Geometric Framework (GF) model of nutrition was applied (Raubenheimer & Simpson, 1997, 1999).

Casein (Casein sodium salt from bovine milk, Sigma-Aldrich) was the protein source added to sucrose solution (0.5M, Sigma-Aldrich) in one tube; a second tube had 0.5M sucrose only and water in a third tube. Different treatments were tested in a protein to 0.5M sucrose solution ratio on a molar-molar basis to the P:C ratio (Protein : Carbohydrate; Table 2.1). Imidacloprid was provided in sucrose and sucrose with casein solutions. Imidacloprid was diluted at 10mM in both solutions. A control with no casein supplied in food was done in the same conditions (called 0:1x in the following table and results).

	Solution compositions					
Sucrose	**0.5M**					
	Increasing concentration in protein →					
Casein	**0:1x**	**1:250**	**1:100**	**1:75**	**1:50**	**1:25**
Control HT	35	35	35	15	35	35
Imidacloprid 10nM HT	35	15	15	15	15	15
Control LT	10	10	10	10	10	10
Imidacloprid 10nM LT	10	10	10	10	10	10

Table 2.1: Sample size for each liquid diet treatments. The experiment was done by run of 5 bumblebees in parallel per treatment and repeated. Part of the sampling used for this experiment comes from the sampling from Chapter 1.

All tubes were weighed and replaced every 24 h for 7 consecutive days. To measure food consumption, the difference between day *t* and day *t+1* was calculated. At the end of experiments, bumblebees were weighed to estimate weigh loss or gain during the experiment to compare the impact of the different diets. Different parameters of the body were measured: body length, thorax width and length, abdomen width and length, and head width and length to study the homogeneity of the different population used in each trial. Bumblebees were placed in a freezer at -80°C in a labeled pocket for further investigations.

A control for the evaporation rate of each diet was performed for 3 days in empty boxes places in the same conditions as the trial boxes to measure the weight loss in feeding tubes. An average of evaporation rate was calculated and subtracted from the value obtained during the experiment. Negative and null values obtained by the subtraction of the evaporation rate were replaced by a null value and consequently considered as there was no food consumption from the tube. The total food consumption was calculated by multiplying the amount consumed by the weight of sucrose and casein in 1ml of solution for each diet.

7. Bumblebee activity

To determine if bumblebee behavior changed with imidacloprid exposure, bumblebee activity was observed each day for 10 min after taking them out of the incubator. Activity observed was defined as displayed on the following table (Table 2.2).

Behavior	Description of the behavior
Flying	Bumblebee moving in the air with its wings
Climbing	Bumblebee going up and using legs to catch up the tube that was containing the food or along the walls of the box
Running	Bumblebee moving very fast on the surface of the paper pad
Walking	Bumblebee moving slowly on the surface of the pad
Grooming	Bumblebee brushing its legs, body, head or wings
Eating	Bumblebee drinking at one of the tubes displayed in the box
Sitting	Bumblebee still on the paper pad
Lie on the back	Bumblebee upside down, slowly moving legs in the air or still on the back.

Table 2.2: Descriptions of the bumblebee behaviors observed during the experiment.

8. Statistics

The normality of each sample of data was tested. To study the daily average food consumption, the average daily food consumption was analyzed using repeated-measures ANOVA. To measure the effect of imidacloprid on the average daily food consumption in the subset of data containing sucrose only average daily consumption, a one-way ANOVA and a Tukey's post-hoc analysis were performed. According to these results repeated-measures ANOVA was performed to test the effects of casein and carbohydrates at different ratios in diets on the cumulative consumption and define the food intake target of bees at day 4 and day 7. Survival was analyzed using Kaplan-Meier analysis (Kaplan and Meier, 1958) to measure how each treatment influenced survival. To identify the correlations in the behaviors depending of the diet and the dose of imidacloprid, a principal components method for factor analysis (Johnson and Winchern, 1992) was performed. The resulting factor scores generated for the factors with eigenvalues greater than 1.0 were then entered into a repeated-measure ANOVA and LSD post-hoc analysis. The average body mass loss depending of the treatment was analyzed using two-way ANOVA and Tukey's *post-hoc* test. All analyses were performed using IBM SPSS v15.0 software (SPSS Inc., Chicago, IL).

III. Results

1. Bumblebees with easy access to food eat more

Bumblebees forced to eat sucrose liquid diet only from tubes in the 'high' position (HT) ate less food on average than those fed with the food tubes in the 'low' position (LT) (Figure 2.3; between-subjects, two-way ANOVA, $F_{(1,90)}$=15.6, P<0.001; tube main effect). There was no significant effect of imidacloprid dose (0 and 10nM) observed (Figure 2.3, between-subjects, two-way ANOVA, dose main effect, $F_{(1,90)}$=4.13, P=0.197). A Tukey's *post-hoc* test revealed that the average daily food intake was significantly different between control (no imidacloprid) HT and LT as bees ate significantly more sucrose when they had easy access (LT) to food (Figure 2.3; P=0.016). Imidacloprid 10nM HT bees ate less than the LT bees (Figure 2.3). Bees in the control LT group also had significantly greater daily food intake than bees in imidacloprid 10nM HT.

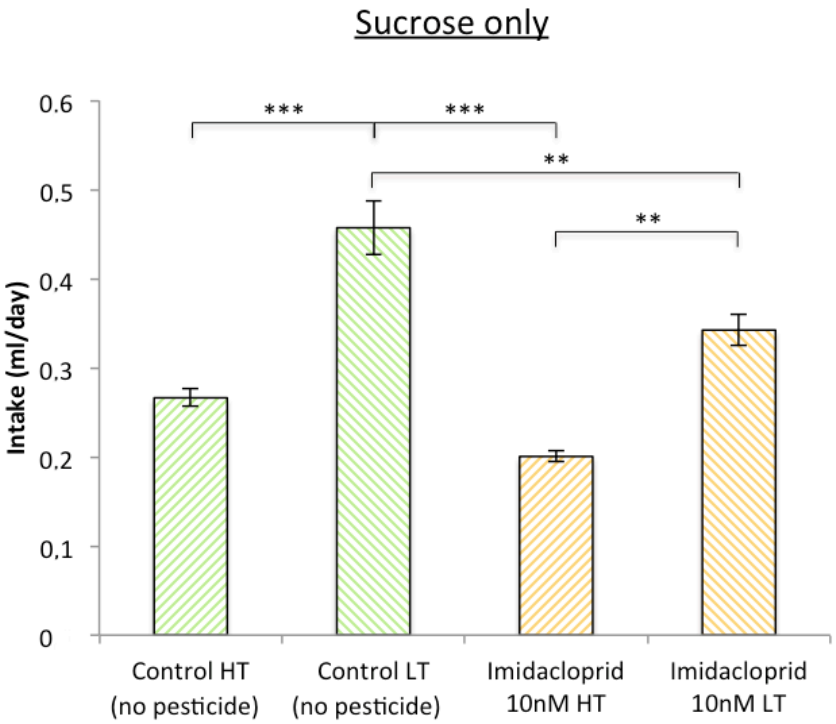

Figure 2.3: **Comparison of the impact of imidacloprid (no pesticide and 10nM) and the position of tubes (HT: High Tubes or LT: Low Tubes) on the average daily sucrose consumption (± standard error) for bumblebees eating sucrose only.** (Tukey's *post-hoc* comparison: '*': 0.005<P<0.010; '**': 0.001<P<0.005; '***': P<0.001; only significant

interactions are displayed on the graph). For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

In addition to consuming more food on average, bees in the LT control group ate proportionally more protein per day (Figure 2.4). This is in contrast to bees that consumed food containing imidacloprid: these bees, when fed with tubes in the HT position, ate more protein per day than bees fed with the LT position (Figure 2.4). The average daily sucrose consumption was affected by the dose of imidacloprid in the diet, the position of the tube (HT and LT) and the diet provided (Table 2.3a). Thus, the between the dose of imidacloprid and the diet had an impact on the average daily casein consumption that were significantly different (Table 2.3b).

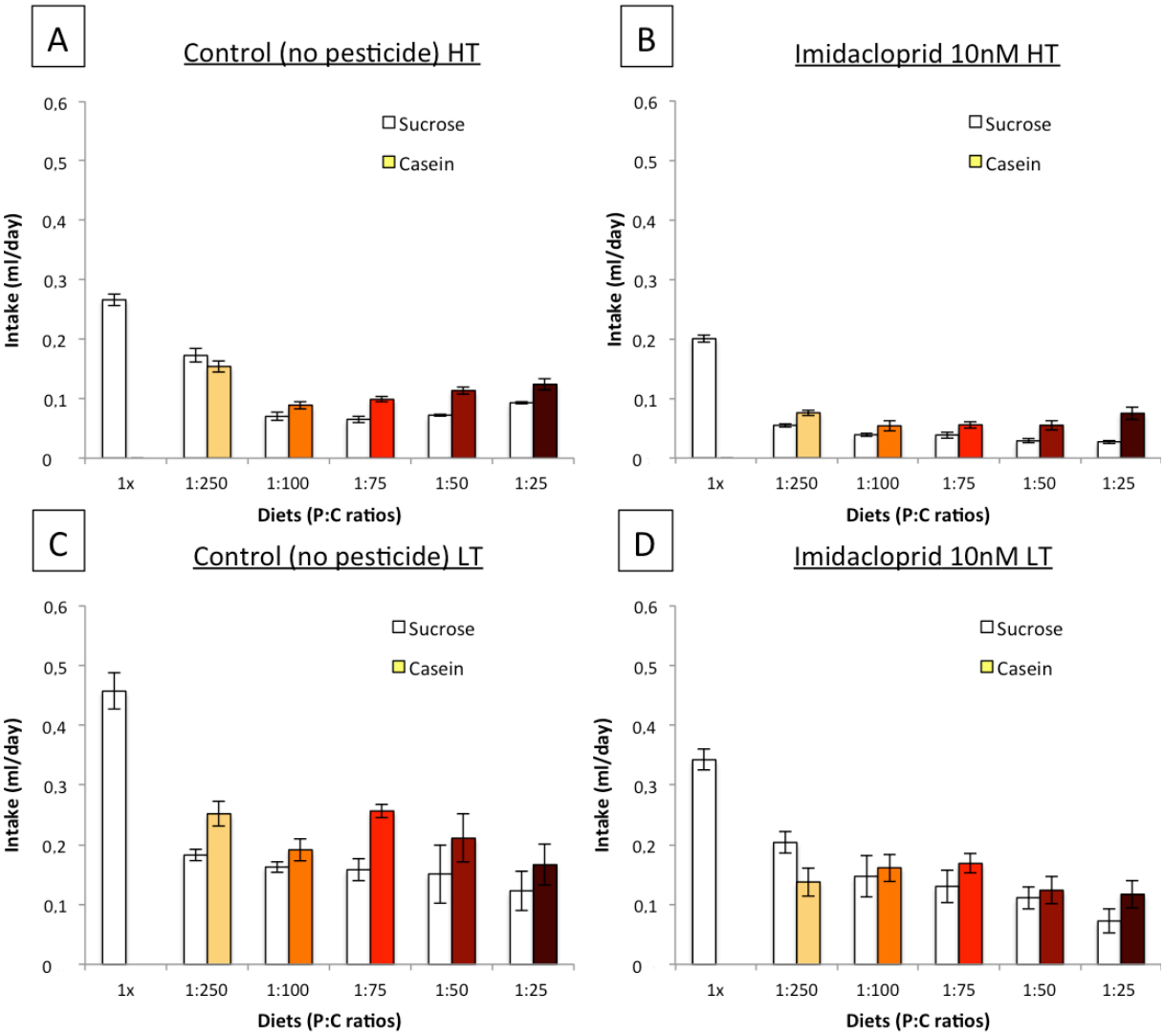

Figure 2.4: The average daily food consumption (± standard error) for different dose of imidacloprid (0 and 100nM), diets (Sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25) and the position of the tubes (HT and LT). For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

Source	Type III Sum of Squares	Mean Square	F	df	Sig.
Corrected Model	232.474	33.211	16.931	7	<0.001
Intercept	1139.453	1139.453	580.894	1	<0.001
Dose	**37.471**	**37.471**	**19.103**	**1**	**<0.001**
Position	**149.515**	**149.515**	**76.223**	**1**	**<0.001**
Diet	**77.030**	**15.406**	**7.854**	**5**	**<0.001**
Error	808.159	1.962		412	
Total	2199.238			420	
Corrected Total	1040.633			419	

Table 2.3a: Results of tests of between-subjects effects of repeated-measures ANOVA with the daily sucrose consumption variables transformed in to averages to test their interactions with different doses of imidacloprid (no pesticide and 10nM), different position of the tubes (HT and LT) and different diets (Sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25). For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

Source	Type III Sum of Squares	Mean Square	F	df	Sig.
Corrected Model	0.183	0.018	19.789	10	<0.001
Intercept	0.252	0.252	272.306	1	<0.001
Dose	0.049	0.049	52.598	1	<0.001
Position	0.016	0.016	16.775	1	<0.001
Diet	0.083	0.021	22.546	4	<0.001
Dose * Diet	**0.013**	**0.003**	**3.384**	**4**	**<0.001**
Error	0.294	0.001		318	
Total	0.804			329	
Corrected Total	0.477			328	

Table 2.3b: Results of tests of between-subjects effects of repeated-measures ANOVA with the daily casein consumption variables transformed in to averages to test their interactions with different doses of imidacloprid (no pesticide and 10nM), different position of the tubes (HT and LT) and different diets (Sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25). For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

2. Bumblebees with ease of access to food switch their food intake after 4 days

The bumblebees were given access to diet pairs where one diet contained a specific ratio of P:C and the other contained 0.5M sucrose alone. Using this approach, the way that bumblebees achieved the intake target was measured over the 7-day experiment. The influence of the tube position and imidacloprid on daily volume of food intake depended on the day and the dietary ratio (various ratio of sucrose or sucrose with casein). Figure 2.5 represents a cumulative plot of the mean protein and carbohydrate consumption for each dietary pairing. The influence of the tube position and imidacloprid on daily volume of food intake depended on the day and the dietary ratio (sucrose or sucrose with casein).

It was clear from the plot that the bees changed their intake trajectory half way through the course of the experiment (Figure 2.5). Again, a three-way interaction was observed between diets, the position of the tube (HT and LT) and the dose of imidacloprid had on the average daily intake of carbohydrates and protein (Table 2.4). For this reason, the intake target (IT) was calculated from the average intake of carbohydrates and protein made by the treatments that were not significantly different in the repeated-measures ANOVA (Annex B: Table 1, 2, 3 & 4). Using this method, the IT was determined at day 4 and day 7 (Table 2.5). In control HT group, the IT was 1:82 at day 4 and shifted to 1:88 at day 7. For bees fed diets containing imidacloprid in the HT position, the IT was 1:53 at day 4 and 1:50 at day 7. In contrast, bees in the LT group exhibited a significant change in the trajectory of their cumulative food consumption after day 4, which shifted the IT towards carbohydrates. Bees in the control LT shifted their IT from 1:56 at day 4 toward 1:130 at day 7. When bees were exposed to 10nM of imidacloprid with in the LT group, the IT shifted from 1:64 at day 4 even further towards carbohydrates 1:142 at day 7.

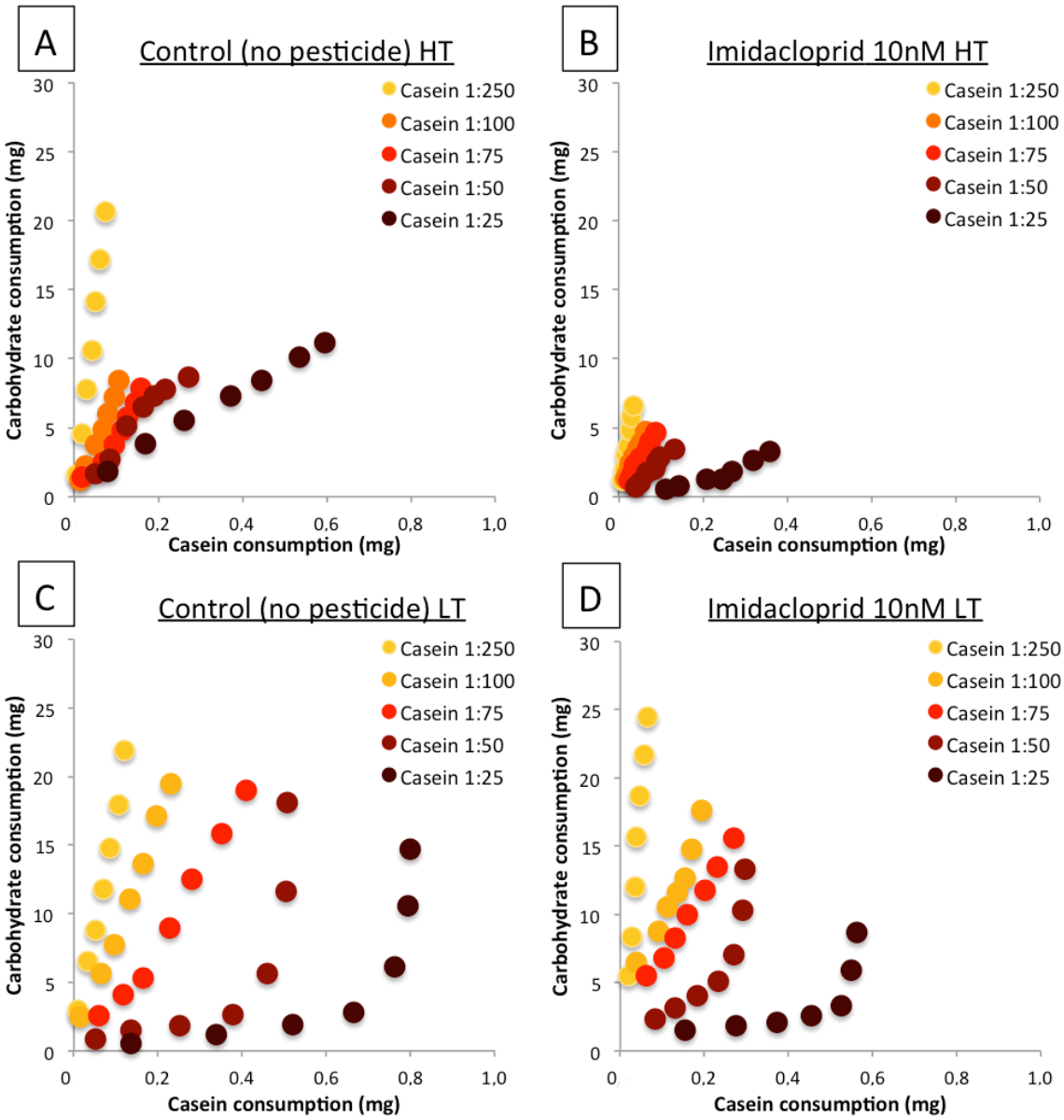

Figure 2.5: **Cumulative average daily food consumption on protein and carbohydrate intakes for individual bumblebees confined to one of the different diets (in P:C ratios Casein 1:250, 100, 75, 50 and 25)tested varying in both ratio and the total amount of protein and carbohydrate, different position of the tubes (HT and LT) and over different doses of imidacloprid (no pesticide and 10nM).** For the number of specimens per run, please refer to Table 2.1 in Materials and Methods.

Source	Type III Sum of Squares	Mean Square	F	df	Sig.
Corrected Model	259.992	11.304	5.734	23	<0.001
Intercept	2082.536	2082.536	1693.414	1	<0.001
Diet	52.057	10.411	83.457	5	<0.001
Dose	24.376	24.376	195.394	1	<0.001
Position	8.519	8.519	68.284	1	<0.001
Day	26.802	4.467	35.807	6	<0.001
Diet * Dose	30.004	6.001	48.101	5	<0.001
Diet * Position	54.397	10.879	87.208	5	<0.001
Dose * Position	35.685	35.685	286.050	1	<0.001
Dose * Day	0.546	0.091	0.730	6	0.627
Position* Day	14.362	2.394	19.187	6	<0.001
Diet * Dose * Position	**37.801**	**7.560**	**60.602**	**5**	**<0.001**
Dose * Position* Day	0.820	0.137	1.095	6	0.369
Error	780.641	1.971		396	
Total	2199.238			420	
Corrected total	1040.633			419	

Table 2.4: Results of tests of between-subjects effects of repeated-measures ANOVA depending of imidacloprid dose (no pesticide and 10nM), position of the tubes (HT and LT) and different diets (Sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25) of the bumblebees observed over the 7 days. For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

		Food Intake Target	
Tube position		**Day 4**	**Day 7**
HT	Control (no pesticide)	1:84	1:88
	Imidacloprid 10nM	1:53	1:50
LT	Control (no pesticide)	1:56	1:130
	Imidacloprid 10nM	1:64	1:147

Table 2.5: Comparison of the food intake target between day 4 and day 7 depending of imidacloprid dose (no pesticide and 10nM), position of the tubes (HT and LT) and over the different diets (in P:C ratios Casein 1:250, 100, 75, 50 and 25). For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

3. Survival

Tube position had a strong influence on survival. The survival of bees fed with the tubes in the HT was also influenced by treatments. The lifespan of bumblebees depended on the HT group depended on diet and dose (Kaplan-Meier: χ^2=6.87, df=3, *P*=0.009; Figure 2.6) and tube position (Kaplan-Meier: χ^2=20.4, df=3, *P*<0.001; Figure 2.6). Bees fed with the LT were more likely to die in the control than those fed with imidacloprid 10nM (Figure 2.6; Table 2.6).

Bees fed with high protein diets (Casein 1:50 and 1:25) without imidacloprid having tubes in the high position exhibited a significant shorter lifespans than bees fed with sucrose only and lower protein diets (Figure 2.6; Table 2.6). The comparison of the survival in control (no imidacloprid) LT did not show any significant differences between the diets (Figure 4; Table 3). Bees exposed to imidacloprid 10nM in the HT and fed with a high protein diets (Casein 1:75, 1:50 and 1:25) had a greater risk of mortality than in sucrose only and lower protein diets (Figure 2.6; Table 2.6). Bees in control (no casein) imidacloprid 10nM LT exhibited greater mortality than when fed diets containing protein. Bees fed low protein diets (Casein 1:250 and 1:100) were more likely to die earlier than bees fed on higher protein diets when the tube was in the low position (Figure 2.6; Table 2.6).

Diets	No pesticide				Imidacloprid 10 nM			
	HT		LT		HT		LT	
	X^2	Sig.	X^2	Sig.	X^2	Sig.	X^2	Sig.
Casein 1:250	3.123	0.077	0.001	1.000	2.738	0.098	2.110	0.146
Casein 1:100	0.562	0.454	1.000	0.317	1.480	0.224	2.149	0.143
Casein 1:75	0.068	0.795	0.001	1.000	**4.146**	**0.042**	**4.431**	**0.035**
Casein 1:50	10.120	**0.001**	0.001	1.000	**6.809**	**0.009**	**5.795**	**0.016**
Casein 1:25	7.734	**0.005**	1.000	0.317	**6.629**	**0.010**	**5.032**	**0.025**

Table 2.6: **Results of Kaplan-Meier analysis (Log rank Mantel Cox) with different dose of imidacloprid (no pesticide and 10nM), position of the tubes (HT and LT) and different diets (in P:C ratios Casein 250, 100, 75, 50 and 25) against sucrose only.** The Kaplan-Meier analysis measures the fraction of subjects living for a certain amount of time after treatment. For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

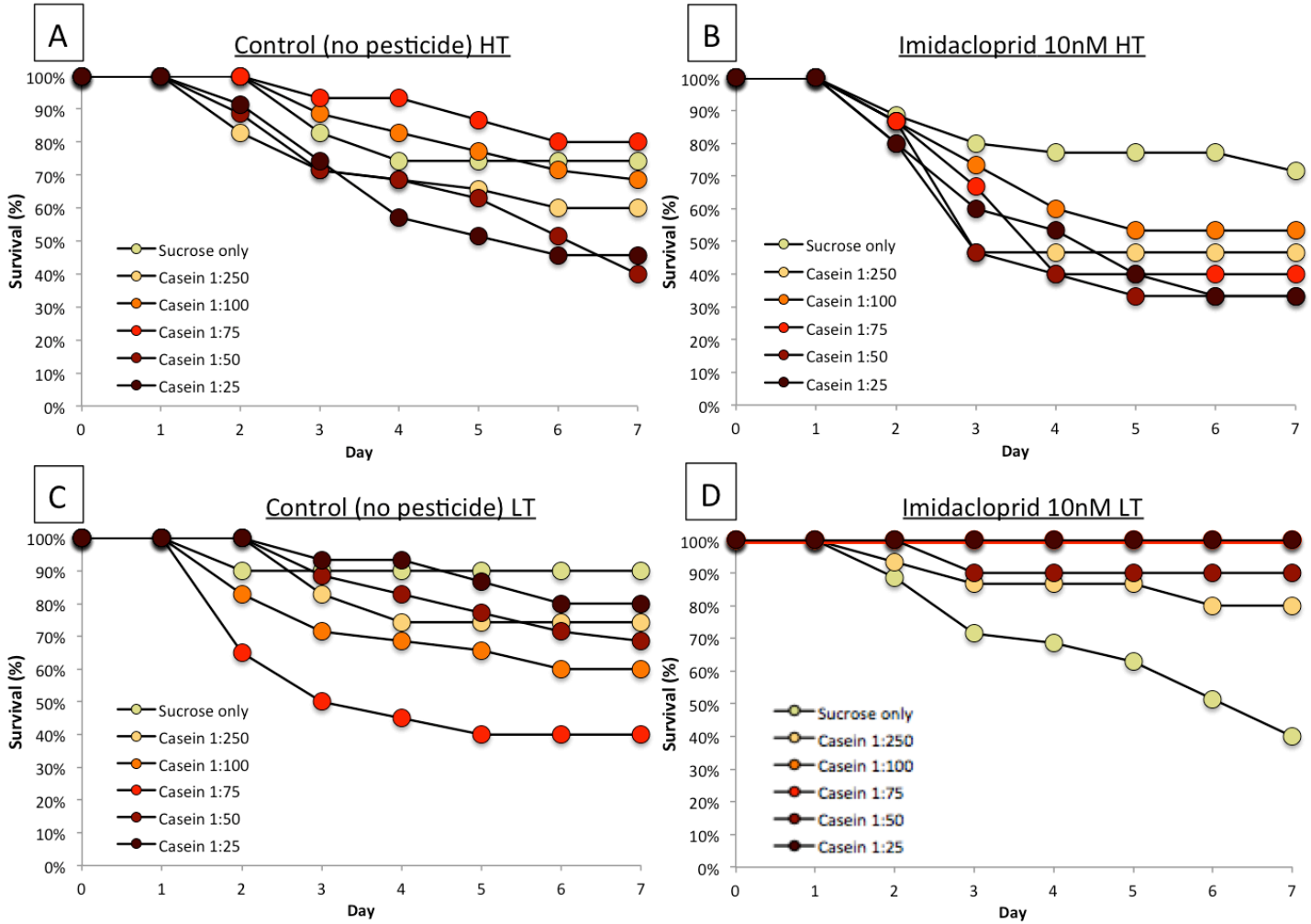

Figure 2.6: Comparison of the mortality of bumblebees exposed to different protein diets (sucrose only and in P:C ratios for Casein 1:250, 100, 75, 50 and 25), sublethal doses of imidacloprid (no pesticide and 10nM) and the position of the tubes (HT and LT). For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

The comparison of the no protein groups with and without imidacloprid 10nM showed that the tube position did not have any significant impact on survival between the control (no pesticide) HT and LT (Figure 2.7; Table 2.7) and between control (no pesticide) HT with imidacloprid 10nM LT groups (Figure 2.7; Table 2.7).

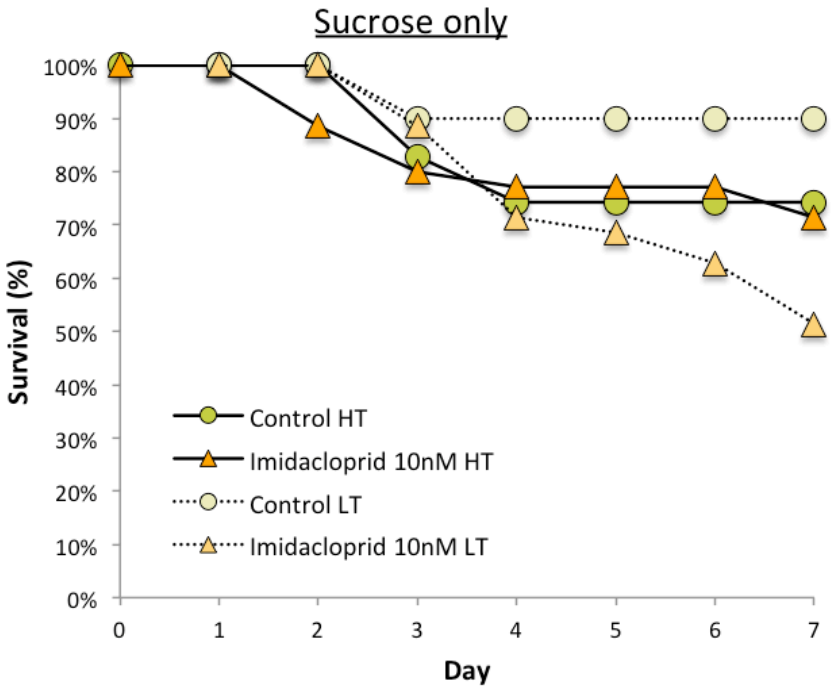

Figure 2.7: **Comparison of the mortality of bumblebees fed with sucrose only, sub-lethal doses of imidacloprid (no pesticide and 10nM) and with the position of the tubes (HT and LT).** For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

Dose	Tube	HT	
		χ^2	Sig.
No pesticide	HT	.	.
	LT	0.869	0.351
Imidacloprid 10nM	HT	.	.
	LT	3.364	0.067

Table 2.7: **Results of Kaplan-Meier analysis (Log rank Mantel Cox) with different dose of imidacloprid (no pesticide and 10nM), position of the tubes (HT and LT) and sucrose only diet.** The Kaplan-Meier analysis measures the fraction of subjects living for a certain amount of time after treatment. For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

4. Influence of the tube position on the bumblebee behavior

The behavior of the bees was measured in each treatment group each day. The behavior of the bees was affected by the tube position (Figure 2.8A&C). Bees in control HT group were active in 77% of the observations (Figure 2.8A) in contrast with bees in control LT that were active in only 56% of the observations (Figure 2.8C). Moreover, bees in control HT were showing a large range of active behaviors and spending large proportions of time flying, walking, climbing and running. The less active bees were bees fed with food containing imidacloprid 10nM in HT group with only 27% active. This group was the only one where bees were recorded to be 'laying on their backs' (Figure 2.8B). The most outstanding difference was observed in the LT set where bees fed with imidacloprid 10nM were more active than in the control with 58% of active behaviors observed (Figure 2.8D). Bees in imidacloprid 10nM LT group spent more time flying, climbing and walking; they also exhibited more grooming behavior.

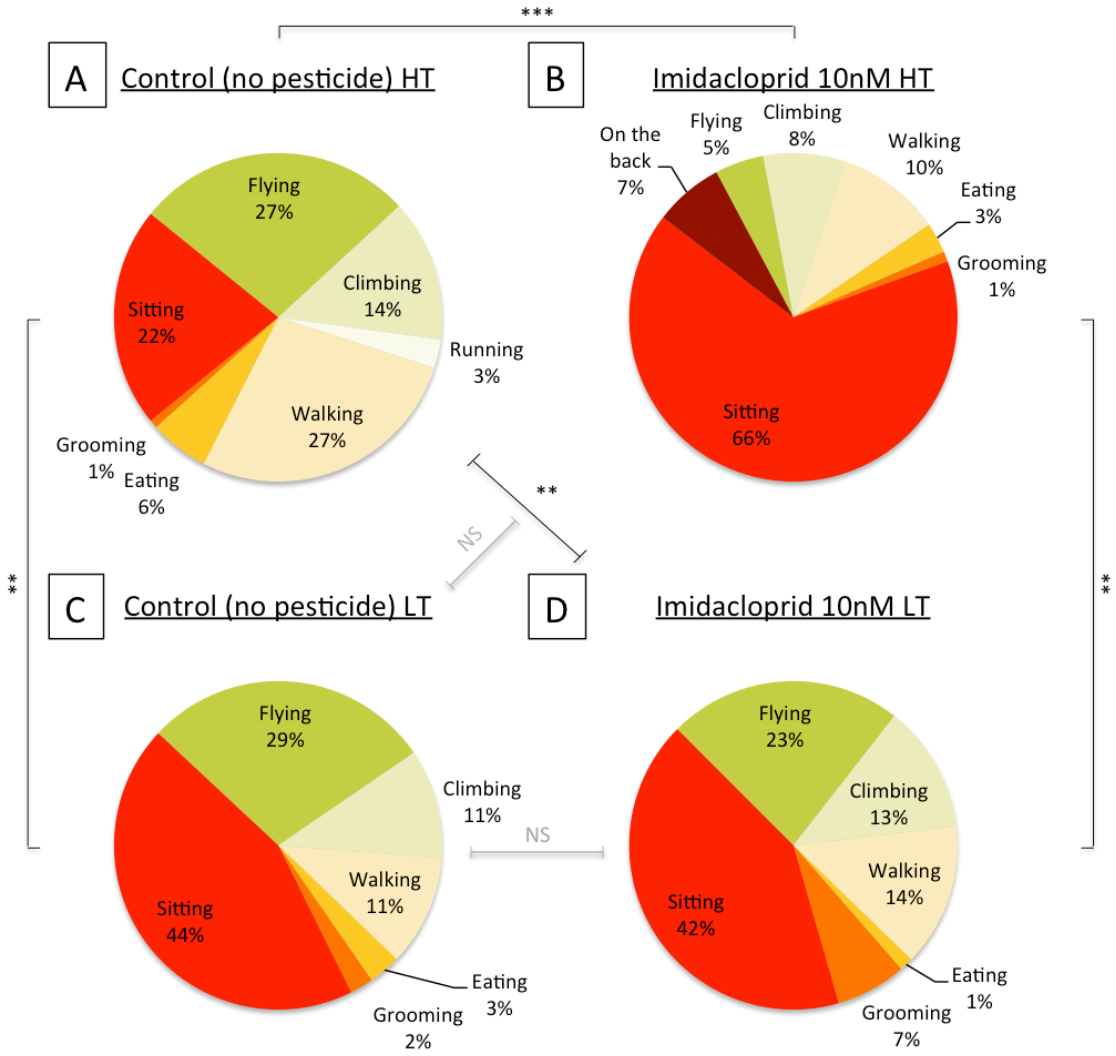

Figure 2.8: **Comparison of the behavior displayed by bumblebees over different diets (sucrose only and in P:C ratios for Casein 1:250, 100, 75, 50 and 25), sublethal doses of imidacloprid (no pesticide and 10nM), the position of the tubes (HT and LT) and over 7 days.** (LSD *post-hoc* comparison after repeated-measures ANOVA: '*': 0.005<*P*<0.010; '**': 0.001<*P*<0.005; '***': *P*<0.001). For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

To compare how diet, the tube position and exposure to imidacloprid affected behavior, a factor analysis was performed on the behaviors observed over the 7 days. The factor scores obtained by the factor analysis were used to test how the chronic exposure to different doses of imidacloprid affected the expression of behavior.

The factor analysis for the behavior observed over the 7 days reduced 8 variables to three variables represented in three factors that accounted for 81.4% of the variation in the data (Table 2.8). The three factors extracted had eigenvalues greater than 1.0 (all others factors were not included as they explained less than 10% of the variation in the data). The first factor, which accounted for 49.0% of the variation in behavior, was mainly representative of the 'active' behaviors such as flying, climbing, running, walking and eating. The second factor was accounting for 17.6% of the variation in the behavior and represented the time spent sitting and bees laying on their backs. The third factor characterized 14.8% of the variation in the behavior and characterized time spent grooming. Exposure to imidacloprid in food affected the expression of 'active' behaviours represented in factor 1 (Table 2.9). The expression of these behaviors was also influenced by tube position (Table 2.9) and the interaction between imidacloprid with the tube position (Table 2.9). Moreover, factor 2 that mainly represented sitting and laying on the back (inactive behaviors) was affected by the imidacloprid dose (Table 2.9) and the position of the tube (Table 2.9). In contrast, observation of grooming behavior depended mainly on the position of the tube (Table 2.9).

	Principal component		
	1	2	3
Eigenvalue	3.918	1.409	1.183
Percent variance	48.977	17.615	14.785
Factor loading			
Flying	**0.648**	-0.645	0.184
Climbing	**0.818**	-0.329	0.063
Running	**0.866**	0.145	0.389
Walking	**0.856**	-0.320	-0.064
Eating	**0.623**	-0.181	-0.486
Grooming	0.166	-0.138	**0.887**
Sitting	-0.278	**0.799**	0.333
Back	-0.060	**0.838**	-0.273

Table 2.8: Factor analysis of bumblebee's behavior using principle components method of factor extraction with varimax rotation over different diets (sucrose only and in P:C ratios for Casein 1:250, 100, 75, 50 and 25), sublethal doses of imidacloprid (no pesticide and 10nM), the position of the tubes (HT and LT) and over 7 days. Factor loading in bold indicate which behavioral variables made the largest contribution to each factor. For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

Source	Dependent Variable	Type III Sum of Squares	Mean Square	F	df	Sig.
Corrected Model	factor score 1	18.211	1.401	2.925	13	0.048
	factor score 2	18.981	1.460	3.633	13	0.024
	factor score 3	15.157	1.166	1.486	13	0.268
Intercept	factor score 1	0.000	0.000	.000	1	1.000
	factor score 2	0.000	0.000	.000	1	1.000
	factor score 3	0.000	0.000	.000	1	1.000
Diet	factor score 1	3.484	0.697	1.455	5	0.287
	factor score 2	3.379	0.676	1.681	5	0.226
	factor score 3	3.242	0.648	.827	5	0.559
Dose	**factor score 1**	**2.964**	**2.964**	**6.189**	**1**	**0.032**
	factor score 2	**5.307**	**5.307**	**13.204**	**1**	**0.005**
	factor score 3	1.908	1.908	2.433	1	0.150
Position	factor score 1	4.253	4.253	8.880	1	0.014
	factor score 2	3.773	3.773	9.387	1	0.012
	factor score 3	**7.743**	**7.743**	**9.872**	**1**	**0.010**
Diet * Dose	factor score 1	4.559	0.912	1.904	5	0.181
	factor score 2	0.764	0.153	.380	5	0.851
	factor score 3	0.962	0.192	.245	5	0.933
Dose * Position	**factor score 1**	**2.950**	**2.950**	**6.160**	**1**	**0.032**
	factor score 2	**5.758**	**5.758**	**14.327**	**1**	**0.004**
	factor score 3	1.301	1.301	1.659	1	0.227
Error	factor score 1	4.789	0.479		10	
	factor score 2	4.019	0.402		10	
	factor score 3	7.843	0.784		10	
Total	factor score 1	23.000			24	
	factor score 2	23.000			24	
	factor score 3	23.000			24	
Corrected Total	factor score 1	23.000			23	
	factor score 2	23.000			23	
	factor score 3	23.000			23	

Table 2.9: Results of tests of between-subjects effects of MANOVA comparing the doses of imidacloprid (no pesticide and 10nM), the position of the tubes (HT and LT) and different diets (sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25) using scores generated by the factor analysis of the behavior over the 7 days with the three factors produced by the analysis of principal component. For the number of specimen per run, please refer to Table 2.1 in Materials and Methods.

5. Body mass loss depending of the treatment

In addition, the average percentage of body mass loss over the course of the experiment was measured for each treatment. In the control (no imidacloprid), the average percentage of body mass loss was 13.7%±16.8 lower for bees fed with the HT position and 5.80%±15.1 lower for bees fed with the LT position (Figure 2.9). In the imidacloprid 10nM groups, the average percentage of body mass loss was 16.2%±13.4 lower for the HT group and 12.5%±11.9 lower for LT group (Figure 2.9). Diet, dose, and tube position also influenced the change in body mass from day 1 to day 7 (Table 2.10). A LSD *post-hoc* test revealed that average percentage of body mass loss were highly significantly different between control (no imidacloprid) HT and LT, between imidacloprid 10nM HT and LT, and also between control (no imidacloprid) LT and imidacloprid 10nM HT (Table 2.11). It also showed a significant difference between control (no imidacloprid) LT and imidacloprid 10nM LT (Table 2.11).

Source	Type III Sum of Squares	Mean Square	F	df	Sig.
Corrected Model	13957.425	606.845	2.824	23	<0.001
Intercept	45021.047	45021.047	209.506	1	<0.001
Diet	884.886	176.977	0.824	5	0.533
Dose	1844.102	1844.102	8.582	1	0.004
Position	2221.622	2221.622	10.338	1	0.001
Diet * Dose	3102.762	620.552	2.888	5	0.014
Diet * Position	1198.775	239.755	1.116	5	0.351
Diet * Dose * Position	**2433.541**	**405.590**	**1.887**	**6**	**0.082**
Error	85096.919	214.891		396	
Total	170536.115			420	
Corrected Total	99054.345			419	

Table 2.10: Results of tests of MANOVA comparing the average mass loss over the 7 days depending of imidacloprid dose (no pesticide and 10nM), the position of the tubes (HT and LT) and different diets (Sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25). For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

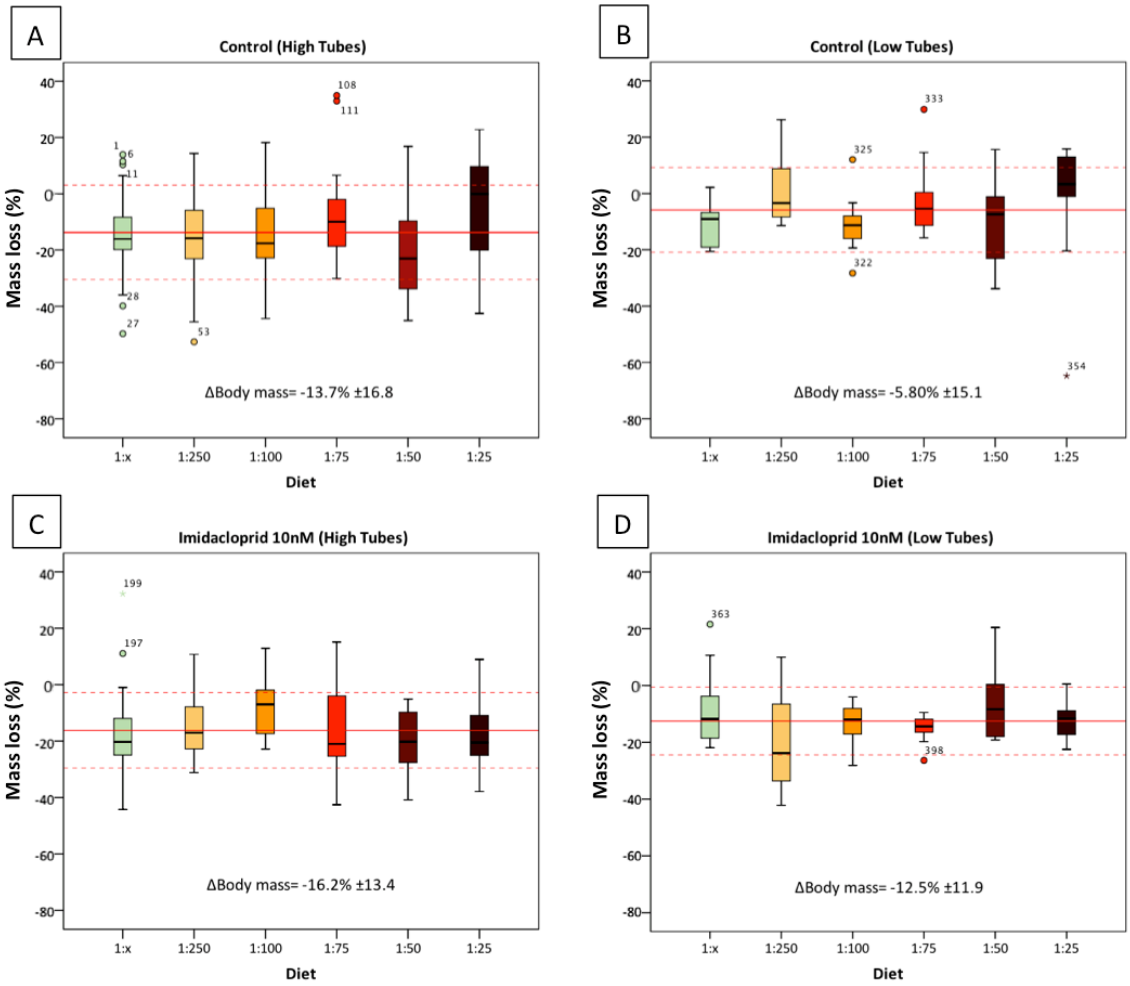

Figure 2.9: Comparison of the average mass loss of bumblebees over the 7 days depending of the different diets (sucrose only and in P:C ratios for Casein 1:250, 100, 75, 50 and 25), sublethal doses of imidacloprid (no pesticide and 10nM) and the position of the tubes (HT and LT). Lines in bold represent medians; boxes represent 1st and 3rd interquartile ranges, bars represent the minimum and maximum range of the data and open circles represent outliers. The average body mass loss is represented by — and the STDEV of the average body mass loss is represented by - - - . For the number of specimen per run, please refer to Table 2.1 in Materials and Methods

Imidacloprid dose (I)	Imidacloprid dose (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control HT	Imidacloprid 10nM HT	2.5294	1.75629	0.475	-2.0018	7.0606
	Control LT	**-7.8715**	**2.17084**	**0.002**	**-13.4722**	**-2.2708**
	Imidacloprid 10nM LT	-1.1738	2.17084	0.949	-6.7745	4.4269
Imidacloprid 10nM HT	Control HT	-2.5294	1.75629	0.475	-7.0606	2.0018
	Control LT	**-10.4009**	**2.35267**	**<0.001**	**-16.4708**	**-4.3311**
	Imidacloprid 10nM LT	-3.7032	2.35267	0.395	-9.7731	2.3666
Control LT	**Control HT**	**7.8715**	**2.17084**	**0.002**	**2.2708**	**13.4722**
	Imidacloprid 10nM HT	**10.4009**	**2.35267**	**0.000**	**4.3311**	**16.4708**
	Imidacloprid 10nM LT	**6.6977**	**2.67639**	**0.061**	**-.2073**	**13.6027**
Imidacloprid 10nM LT	Control HT	1.1738	2.17084	0.949	-4.4269	6.7745
	Imidacloprid 10nM HT	3.7032	2.35267	0.395	-2.3666	9.7731
	Control LT	**-6.6977**	**2.67639**	**0.061**	**-13.6027**	**0.2073**

Table 2.11: Results of multiple comparison of LSD post-hoc test comparing the average mass loss (± standard error) over the 7 days depending of imidacloprid dose (no pesticide and 10nM), the position of the tubes (HT and LT) and different diets (Sucrose only and in P:C ratios Casein 1:250, 100, 75, 50 and 25). For the number of specimen per run, please refer to Table 2.1 in Material and Methods.

IV. Discussion

The key results emerging from my work are that bumblebees that had easy access to food eat more even if they are exposed to sub-lethal doses of imidacloprid (10nM) and initially increased the proteins in their diets before switching to carbohydrates after day 3. The easy access to food by the low tubes allowed these bees to survive during the experiment in the control (no imidacloprid) and higher protein diets when they were exposed to imidacloprid 10nM. Bees were less active and losing less weight when they had to work less to obtain food from the tubes in the boxes.

Similar results have been reported from other insect species. Carroll (1999) made analogous observations with larvae of the Hemlock looper (*Lambdina fiscellaria fiscellaria*; Lepidoptera: Geometridae) that consume both new and old foliage of Balsam fir (*Abies balsamea* L.; Pinaceae) despite the poor nutritional quality of old tissues. The old foliage can be compared to the sucrose tube and the new foliage to the sucrose with protein tube in our experiments. The easy access to the new foliage was the critical aspect that influenced the survival of larvae and an early emergence in comparison with larvae that had a restricted access to old foliage. Despite the reliance of the Hemlock looper for the new foliage for the survival, larvae that had easy access to both new and old foliage were heavier and survived better, just as my bees that had easy access to both kind of food. The author suggested that larvae that had easy access to food that provide different quality of nutrients can more easily circumvent the limited availability of food and gaining access to more nutrients to achieve their needs.

Imidacloprid's sub-lethal effects did not have a direct impact on the mortality over the days of the bees in these experiments. Instead, I observed that imidacloprid impaired their abilities to obtain food by impacting their activity. This has also been observed by others in bumblebees and honeybees including impacts on their fecundity, neurophysiology, learning performance or other aspects of their behavior (Desneux *et al.*, 2007; Laycock *et al.*, 2012). During their foraging trips, bumblebee foragers spend their time (a) travelling between their nest and foraging patches, (b) searching rewarding patches, (c) flying between flowers within a patch, (d) handling flowers, and finally (e) removing nectar and pollen from flowers (Pyke, 1979, 1980). All these steps to investigate, access and finally bring food back to the colony could be the reason that confounding results have been reported for the

impact of imidacloprid on bees. It is essential, for these reasons, to identify how bees obtain food in experiments that test their impacts on bees.

1. How can we define an easy access to food in the field?

In my experiment, easy access to food was via free access to food *ad libitum* without any effort of the bumblebee. Bees just had to put their front legs on the tube and deployed their proboscis in the holes on the tube. Defining easy access to food in the field for bumblebees is more difficult, unless bees are fed within the colony.

Corbet (2000) identified from the plant side, that the attractiveness of nectar sources could be determined by nectar quality, availability to pollinators (i.e. corolla depth, flower morphology) and quantity (i.e. nectar supply, flower clustering). Nectar feeding on plants also depends of the synchronization between timing of flowering and pollinator emergence (Porter *et al.*, 1992). Consequently, Tudor *et al.* (2004) observed that a wide diversity of flowers that are contrasting in color, structure, density and growth forms are necessary to attract different butterfly taxa.

A parallel can be drawn between the challenge to access food resources in my experiment and bee morphology that can have an impact on their capacities to access resources provided by flowers. There is a clear match between floral morphology and insect feeding structures that can be done and pollinators have certainly driven selection for nectary sequestration and specialization in higher plants (Heinrich, 1976; Nilsson *et al.*, 1985; Johnson & Steiner, 1997; Temeles and Kress, 2003). In nature, to have an easier access to food and to facilitate exploitation of nectar sources, pollinators such as honeybees and bumblebees use plant cues between morphologically identical plants (Kunze & Gumbert, 2001). The body size of bees can vary from millimeters to centimeters; this variation can restrict exploitation of flower resources and consequently have an impact on their foraging behavior (van Nieuwstadt & Iraheta, 1996; Gathmann & Tscharntke, 2002, Greenleaf *et al.*, 2007, Kuhn-Neto *et al.*, 2009). Michener (2000) emphasized that bees have different physical adaptations (i.e. *Colletes nasutus*, *Andreana nasuta* and *Cubitalia parvicornis* that have hooked haired on the face and forelegs to grip *Boraginaceae* flowers), tongue lengths and structures that define the range of flowers on which they can forage according to their

degree of floral specialization (oligoleptic *vs.* polylectic). Michener (2000) also highlighted that polylectic bees like bumblebees were showing flower preferences according to their experience to handle a flower type that could give a competitive advantage in resource exploitation. All of these traits that make floral foraging for bumblebees on flowers with specialized structures for pollen or nectar sequestration could then be a disadvantage when bees were influenced by neonicotinoids.

2. Impact of ad libitum carbohydrates and protein contaminated with a sub-lethal dose of imidacloprid on bumblebees

a. Survival increase when food was ad libitum

Bees exposed to imidacloprid survived by feeding on wild flowers growing in field margins and patches of seminatural habitats to supplement mass flowering crops (Diekötter *et al*., 2010). In comparison with honeybees, bumblebees have smaller colonies. The smaller size of colonies makes it more difficult to monitor the survival of wild bumblebee colonies (Osborne, 2012). For that reason, European regulatory authorities are now studying how to integrate data on bumblebees into pesticide risk assessments (Blacquière *et al.*, 2012; Osborne, 2012). One of the consequence of having a large number of workers able to forage is it increase the colony workforce going outside to undertake hazardous tasks (Molet *et al.*, 2008). The survival of workers looks more crucial for the survival and fitness of the whole colony.

My results showed that survival is linked to the diet, the challenge that represents the access to food and the exposure to imidacloprid (10nM). Bumblebees exposed to imidacloprid and that have difficulty accessing food were surviving better to imidacloprid when they were feeding on low protein diets, unlike bees that were having an easy access to food that were surviving better on higher protein diets. This is consistent with Gill *et al.* (2012) who observed that exposure to sub-lethal doses of imidacloprid was significantly increased the mortality of workers inside the colonies. In most of these studies, having access *ad libitum* to food was a crucial point according to my results. These bees also had access to sucrose solution *ad libitum* as my bumblebees in LT position.

In the lab, Taséi *et al.* (2000) did not observe an impact of contaminated sunflower nectar and pollen by imidacloprid (up to 40nM) on bumblebee survival. The bumblebees were having access to all flowers blooming around the microcolony on the field and unlimited access to contaminated syrup and pollen inside the microcolony. In these conditions, bees were able to manage to find all nutrients they needed that contain lower quantities of pesticide and a large range of nutrients to complete their individual diets and the overall colony diet. However, these authors estimated survival by the weight of the whole colony, and this measure is not precise.

Whitehorn *et al.* (2012) observed that bumblebee colonies that received *ad libitum* contaminated pollen and sugar water displayed an initial weight gain over 5 weeks followed by a decline as the colony switched from a growing phase to producing new reproductives. This result implies that even though the imidacloprid dose in food and was not reducing the survival of the workers, on a long-term basis, the chronic exposure was a source of stress that induced an early decline phase which might compromise producing reproductive for the following year. It is also important to note that the number of queens produced in contaminated colonies dropped by 85% in comparison with the control (no imidacloprid) and only the largest colonies succeed in producing queens. Similar results have been reported by Laycock *et al.* (2012) who observed that microcolonies of bumblebees were showing a dose-dependent decline in fecundity with environmentally realistic dosages of imidacloprid and a brood production reduction by one third. Contaminated food could thus seriously impair the survival of the bumblebee population in the area as they have an annual life cycle and only new queens survive to winter to found colonies in the spring (Whitehorn *et al.*, 2012). Bumblebees have an annual life cycle and new queens would have to found their colonies single-handedly the next generation of colonies (Goulson, 2010). Only the most successful nests would produce new queens and participate to the next generation (Muller & Schmid-Hempel, 1992) and reductions of queens produced one year will likely have knock on effects for the number of colonies initiated the following year (Feltham *et al.*, 2014).

b. Impact of imidacloprid on bumblebee behavior depended on food accessibility

The presented results highlight the nutritional needs of bumblebees according to their physical capacities, as well as their lower activity and mobility capacities and the necessity of an easy access to a larger amount of high nutrient quality foods to counteract the effect of sub-lethal doses of imidacloprid.

i. Foraging to access to food resources

For most animals, food resources are patchily distributed in space and time (Hansson *et al.*, 1995). To optimize foraging success in a contrasted environment, animals should relate the choice of a foraging patch to the spatial distribution as well as to resource quality (Cresswell *et al.,* 2000; Hill *et al.*, 2001). The spatial and temporal distribution of resources is particularly important in social insect such as bumblebees as they are central-place foragers and thus concentrate their food investigations in a restricted area around the nest (Dukas & Edelstein-Keshet, 1998). According to Goulson *et al.* (2002), in modern agricultural landscapes, the bumblebee colony health (i.e. survival, growth, brood production) seems to be limited by the availability of food resources. In parallel, Westphal *et al.* (2006) highlighted that the immediate access to food and quality of resources at landscape level affected the duration of foraging trips and the colony growth. Furthermore, once bumblebees have discovered a rewarding foraging site, they will memorize the location and exploit the site as long as it is rewarding (Osborne & Williams, 2001).

Bumblebee colony fitness signs of decline observed can be partially explained by a diminution of the foraging efficiency of workers due to imidacloprid sub-lethal exposure with observed knock-on effects on forager recruitment, worker losses and overall worker productivity (Gill *et al.*, 2012; Whitehorn *et al.,* 2012). By the observations of my individually housed bumblebees, a large panel of impacts of sub-lethal doses of imidacloprid on the behavior can explain at an individual level the lower foraging efficiency. In my experiment, bumblebees that were exposed to imidacloprid were less active, presenting disrupted wings movements that impaired their flying capacities, spending days still or upside down and also presenting signs of malaise such as grooming or abdomen spasms.

At the colony level, chronic exposure to imidacloprid can have a deeper impact than expected. Feltham *et al.* (2014) observed a persistence of foraging impairment on

bumblebee colonies for at least 4 weeks after the source of exposure is removed. They suggest that bees were continuously exposed to imidacloprid in food stored within the nest and new foragers might have been exposed as larvae. Moreover, Yang *et al.* (2012) observed that residues of imidacloprid not only affect honeybees but also their larvae and impair their learning capacities when adult. This suggests that the persistence of imidacloprid within the nest can affect generations of workers and have a long-term effect on the capacities of workers to forage for the entire colony.

ii. Handling flowers and accessing the food reward

The petal epidermis has been found to be essential in facilitating flower-pollinator interactions (Rands *et al.*, 2011). To grip a flower, bumblebees use the conical (or papillate) cells in the petal epidermis that are a common trait present in the majority of extent flowering plants (Kay *et al.*, 1981; Christensen & Hansen, 1998). These conical cells provide tactile properties that benefit the plant by influencing pollinator grip and thus preference (Whitney *et al.*, 2009; 2011). Alcorn *et al.* (2012) observed that bumblebees always learn to favor conical-celled flowers that offer a better grip than flat-celled flowers.

In the field, flowers are in movement in the wind and the bumblebee capacity to grip petals is very important. The tarsal paralysis and uncoordinated movement of wings observed in my experiments might seriously impact the capacity of bumblebees to grip flowers. Consequently, bumblebees would have to adapt their foraging choice to conical-celled flowers available in the countryside to increase their chance to access to food. Furthermore, bumblebees with imidacloprid-induced tarsal paralysis were sometimes totally stuck on the paper pad on the bottom of the box as their claws stayed stuck to it in a way that made it impossible for them to free themselves. In such conditions, it would be really difficult for bumblebees to grip even on conical-celled flowers.

The complexity of the social life of bees suggests that taste might have a more important role for inter individual recognition than expected (de Brito Sanchez *et al.*, 2007). The main chemosensory appendages of bees are antennae, mouthparts and tarsi of forelegs (Goodman, 2003). De Brito Sanchez *et al.* (2008) showed that honeybees can taste sucrose using their tarsi and have greater sensitivity to low concentrations on the tarsi than on the

antennae. They also showed that starvation influenced this response. For this reason, tarsal paralysis could influence the bee's sense of taste and could even potentially modify bee food preferences. In the LT position grouping my experiments, bees had direct access to food by putting their tarsi of their forelegs on the tube. This could have allowed them to easily determine the contents of the tube and therefore change their diet. The easy contact between tube and bumblebee tarsi might suggest that the sucrose taste was favored in LT position experiment.

iii. Bringing food back to the colony

In both case, LT and HT position, bumblebees exposed to imidacloprid ate less food than in the control bees (no imidacloprid). The LT (Low Tube) position facilitated easy access to food to bumblebees that were significantly eating more food and promoting proteins for the first three day of the experiment before switching for carbohydrates. Laycock *et al.* (2012) observed that colonies of bumblebees exposed to sub-lethal dose of imidacloprid reduced feeding on syrup and suggest that imidacloprid reduced the ability or need to feed. They also observed that the initial reduction in feeding rate due to imidacloprid intensifies over days and they suggest that it is due to toxicity rather than aversion as bees were also reducing their pollen intake (non treated pollen). Eating less food could be an adaptive response by bumblebee workers that are struggling to maintain a constant protein to carbohydrates ratio (P:C), because queenless honeybees fed on a choice of diets conserve strict P:C ratios (Altaye *et al.*, 2010; Paoli et al., 2014).

Raine and Chittka (2007) spotted that for bees foraging for pollen is more challenging than foraging for nectar. Foraging for pollen is usually restricted to dry and sunny weather, whereas nectar can be collected in most conditions except heavy rain (Peat & Goulson, 2005). Pollen rather than nectar shortages appear to be more likely to reduce the colony success (Goulson, 2010). Feltham *et al.* (2014) observed that bumblebees exposed to field realistic doses of imidacloprid were not changing their nectar feeding behavior but were bringing back pollen less often (40 of trips for treated colonies *vs* 63 trips in control). Treated colonies were bringing back 31% less pollen per hours than control (no imidacloprid). This is consistent with Gill *et al.* (2012) who ranked pollen loads of bumblebees returning to the nest as small, medium or large. They found that bees exposed to imidacloprid were bringing

back proportionally more small loads than unexposed bees and reducing by 28% pollen collecting trips compared to control (23% of reduction for Feltham *et al.*, 2014).

Imidacloprid impairs bee motor functions (Williamson *et al.,* 2014) and might have an impact on bee dusting body abilities, the capacities of bees to make a pollen charge and hence could have serious impacts on the bee's ability to fix pollen properly to the pollen baskets. This might explain why the exposure to imidacloprid reduces the efficiency of bees for pollen foraging rather than limiting this to aversion for protein. Bee manipulating and packing the pollen for transport to their brood requires a complex series of behaviors. Casteel (1912) described and figured in details the full process of the body dusting in behavior patterns including grooming, adding nectar to moisten the pollen load, preimaginal conditioning and compacting the charge on the pollen baskets during a stationary flight. The dusting of the body is done by complex series of movements of legs to brush the body and then the pollen load is secured on a single hair for *Apis* (Casteel, 1912; Hodges, 1952) and two or three hairs for *Bombus* (Sladen, 1912). The fixation of the pollen load on the pollen basket needs considerable flexibility with bee specialization at the individual level (Thorp, 1979) and different pollen morphologies may require different levels of effor to collect (Vaissière &Vinson, 1994).

Bee weight appears to be another critical parameter that can explain bumblebee foraging behavior and impact their faculties to handle flowers. An increase of the body size of bumblebees from a colony has been observed as a result of colonies foraging on some plant species as the seasons (Knee & Medler, 1965; Plowright & Jay, 1968, 1977). It has been noticed that larger bumblebees that visited flowers commonly visited by smaller specimen had to be more skillful as the stalk of the inflorescence was unable to support the weight of the bee and collapsed to the ground, making flower handling more difficult (Peat *et al.*, 2005).

3. Conclusions

These experiments provide insight into the reasons that many papers report contradictory results regarding the impact of imidacloprid on bees in lab and field conditions.

In the future, aassessing the bumblebee's ability to recover from short-term exposure to imidacloprid is likely to be crucial because their exposure is greatest during synchronous mass-flowering contaminated crops. For example, the most widespread exposure for bees to imidacloprid and neonicotinoïds in their food in Europe would probably be treated oilseed rape (Feltham *et al.*, 2014). In the UK, a field of winter oilseed raped would bloom for approximatively 28 days with a peak of 75% of flowering occurring for about 18 days in April and May (Hoyle *et al.*, 2007). Colonies of bumblebees are initiated in spring and developed over several months before inducing the production of queens and drone in later stages (Goulson, 2003). Queens and drones would consequently emerge after oilseed rape blooming. Aphids, whiteflies and midges are able to recover after an exposure in their diets to neonitcotinoïds (Nauen, 1995; Azevedo-Pereira *et al.*, 2011; He *et al.*, 2011). In such conditions, Laycock *et al.* (2012) suggest that bumblebee colonies would be able to recover, as the exposure in diets would lessen after the rape flowering period and the impact on reproduction and colony growth might be less severe than otherwise.

The presence of imidacloprid in pollen and nectar during mass flowering would dramatically impair the whole colony health more quickly as bees would collect these resources and succumb to poisoning. One conservation schemes could be to improve easy access to uncontaminated rewarding food sources to counteract ongoing decline of bumblebees.

Chapter 3: Neonicotinoids have different effects on the nutritional needs, behavior and survival of bumblebees (*B. terrestris*)

I. Introduction

The invention and the subsequent commercial development of neonicotinoids have provided to farmers new tools for managing some of the most destructive pests of the world (Nauen & Denholm, 2005). Neonicotinoids have established themselves as key components in insect control as their unique chemical and biological properties offer a broad spectrum of insecticidal activity, low application rates, excellent uptake and translocation in plants and new modes of action (Maienfisch *et al.*, 2001). The insect groups primarily targeted by neonicotinoids are Hemiptera (aphids, whiteflies and planthoppers) and Coleoptera (beetles), which include species with a long history of resistance to previous products (Nauen & Denholm, 2005). All neonicotinoids act like nicotine on the insect central nervous system as agonists of nAChRs with a potent insecticidal activity but low toxicity to vertebrates (Bai *et al.*, 1991; Chao *et al.*, 1997; Zhang *et al.*, 2000; Nauen *et al.*, 2001; Matsuda *et al.*, 2001, 2005; Liu *et al.*, 2005; Tomizawa & Casida, 2005).

The first neonicotinoid, imidacloprid was introduced to the market as a systemic and a contact pesticide with a primary activity on sucking insects, some Coleoptera, Diptera and Lepidoptera. Thiamethoxam and clothianidin represent the second generation of systemic neonicotinoids developed for a wide range of applications as for foliar, soil, drench and seed treatments on the same targets (Senn *et al.*, 1998; Maienfisch *et al.*, 1999). Imidacloprid, thiamethoxam and clothianidin are all used as systemic pesticides and can be found in nectar and pollen (Tan *et al.*, 2007). However, their relative toxicity to insects is not the same. For example, thiamethoxam has a 100-fold higher affinity in the locust and 5-fold higher in the aphids than nicotine for nAChRs, while this affinity of imidacloprid is 160-fold lower in the locust and 300-fold lower in the aphids than nicotine (Maienfisch *et al.*, 2001).

For these reasons, the impacts of these insecticides on non-target organisms such as pollinators could be expected to depend on the type of neonicotinoid they are exposed to. In fact, the variable effects of neonicotinoids could account for the debate over their impact on bees (Cutler & Scott-Dupree, 2007). A recent study in honeybees showed that motor function was more impaired by sub-lethal doses of thiamethoxam than by clothianidin or imidacloprid (Williamson et al., 2014). Whether or not bees can detect these pesticides in nectar and pollen has not been carefully tested. This inhibition of the foraging activity varies according to the concentration of imidacloprid tested (Schmuck, 1999). Thiamethoxam does

not display any clear anti-feedant properties, but a lower responsiveness to antennal stimulation with high sucrose concentrations has been observed for honeybees (Aliouane *et al.*, 2009). Clothianidin appears to be the least dangerous of these three neonicotinoids even at higher concentrations in food (Franklin *et al.*, 2004; Cutler & Scott-Dupree, 2007).

The nutritional composition of food rarely matches the nutritional requirements of an organism (Mattson, 1980; Modi *et al.*, 2007). Most animals, forage for complex resources that include proteins, vitamins, minerals, carbohydrates and a range of other food components (Simpson and Raubenheimer, 2012). Macronutrients are not the only functionally important nutritional components of foods: the constituent molecules that make up macronutrients such as amino acids and micronutrients play a critical role in nutritional strategies and physiology of animals (Simpson & Raubenheimer, 2012). From bacteria to mammals, all organisms are able to detect key nutrients such as amino acids with specialized receptors that give to the central nervous system information about nutritional composition of the food before, during and after digestion (Dethier, 1976; Yarmolinsky *et al.*, 2009). Amino acids can induce the storage of energy into the fat body (fat and glycogen) and prevent a decrease of sucrose in hemolymph that contributes to satiation (Arrese & Soulages, 2010). Studies on *Drosophila melanogaster* (Diptera) and more recently, mosquitoes (Diptera) have shown that the fat body specifically expresses amino acids transporters that function as nutrient sensors (Attardo *et al.*, 2005; Hansen *et al*., 2005). On the other hand, some amino acids can reduce lifespan if present in the diet in high concentrations; the lifespan of *Drosophila* (Diptera) was decreased by the addition of amino acids in the diet, with an interaction between methionine and other essential amino acids having a key role (Attardo *et al.*, 2005; Hansen *et al*., 2005). These observations highlight the complicated role of amino acids in the balance of the diet depending of the metabolic routes allocated by the organism and the conditions of the experiment.

The consumption of plants or animal proteins is the principal source of essential amino acids for animals. Proteins are digested and absorbed across the gut, basic amino acids take different metabolic routes throughout the lifespan of the animal. Protein intake is actively regulated around a nutritional optimum depending on the age, the physiological needs and reproductive capacity of an individual (Simpson and Raubenheimer, 2012). The existence of a specific appetite for essential amino acids is strongly suggested, as

experiments on rats fed with diets lacking in one essential amino acid (e.g. tryptophan) are able to identify its presence in food when offered a choice between diets with or without these essential amino acids according to different patterns (Feurte *et al.*, 1999, 2002; Gietzen & Magrum, 2001). However, the regulation of protein intake mechanisms by the body to reach the needs in essential amino acids is mainly unidentified (Morrison *et al.*, 2012). These can be explained by the fact that animals have to face complex challenges in satisfying their multiple nutrient needs and that involve choosing the right food and how much of each to eat to satisfy demands for energy metabolism, energy and nutrient storage, tissue growth, secretions, etc. (Simpson and Raubenheimer, 2012).

Eusocial insects such as honeybees and ants have unusually low protein dietary requirements even as adults; for example forager honeybees need 250 times less dietary essential amino acids than bee larvae (Paoli *et al.*, 2014). The protein needs of eusocial insect workers are mainly subservient to somatic maintenance, nonetheless, bees offer a specific model of dietary regulation of essential amino acids intake as it is regulated in absence of reproduction (Stabler *et al.*, *in review*). Petanidou *et al.* (2006) 's work on amino acids in nectar observed that all amino acids can be found in the floral nectar and that phenylalanine was the most abundant one, especially in keystone plant species visited by foragers. Moreover, they observed that amino acid content of the nectar is positively related to the number of species of long tongues bees, whilst the nectar volume was negatively related to flies and the sugar content was not significant for any guild. Hermosín *et al.* (2003) highlighted that pollen is the major source of proteins and amino acids in honeys and that the determination of free amino acids composition in honey can provide an approximation between different botanical origins. They also noticed that sulphur-containing amino acids (methionine and cysteine) were in the minority and not found in some samples. This suggests that amino acids from both nectar and pollen play a critical role in food foraging choice and in bees diet, consequently in links in the plant-pollinator web and plant pollination.

Few studies have reported on comparative effects of the diet and how these nutrients might interact with exposure to toxins. Here, I examined how dietary protein or essential amino acids interacted with the three most commonly used neonicotinoids (imidacloprid, thiamethoxam and clothianidin) to affect the food intake, nutrient balance,

survival, behavior and physiological parameters of bumblebees after a chronic exposure to a sub-lethal dose over 7 consecutive days. The purpose of this experiment is to determine whether different neonicotinoids had similar impacts on the nutrient balancing of adult worker bumblebees and whether dietary composition affected nutrient balancing and the apparent toxicity of the neonicotinoids.

II. Material and Methods

1. Animals

All experiments were performed with worker bumblebee colonies (*Bombus terrestris terrestris* L.) provided for commercial uses (Koppert B.V., AD Berkel en Rodenrijs, Netherland) and performed in laboratory conditions (22-25°C and 65-80% RH) and continuous darkness from September 2012 to August 2013 in Newcastle University bee lab. Bumblebees were provided *ad libitum* with commercial sugar water and pollen (Pollen mix, Koppert B.V., AD Berkel en Rodenrijs, Netherland) until the beginning of each experiment.

Worker bumblebees – defined as foragers - were collected at the exit of the colony and weighed. They were housed in individual plastic boxes (17x12x7cm) with access to 3 liquid diets until the end of the experiment provided in 2ml microcentrifuge tubes (4 holes were drilled on the topside of the tube, Figure 3.1) until the end of the experiment. The boxes were placed in the incubator 21-22.8°C and 40-60%RH in continuous darkness. Experiments were conducted for 7 consecutive days.

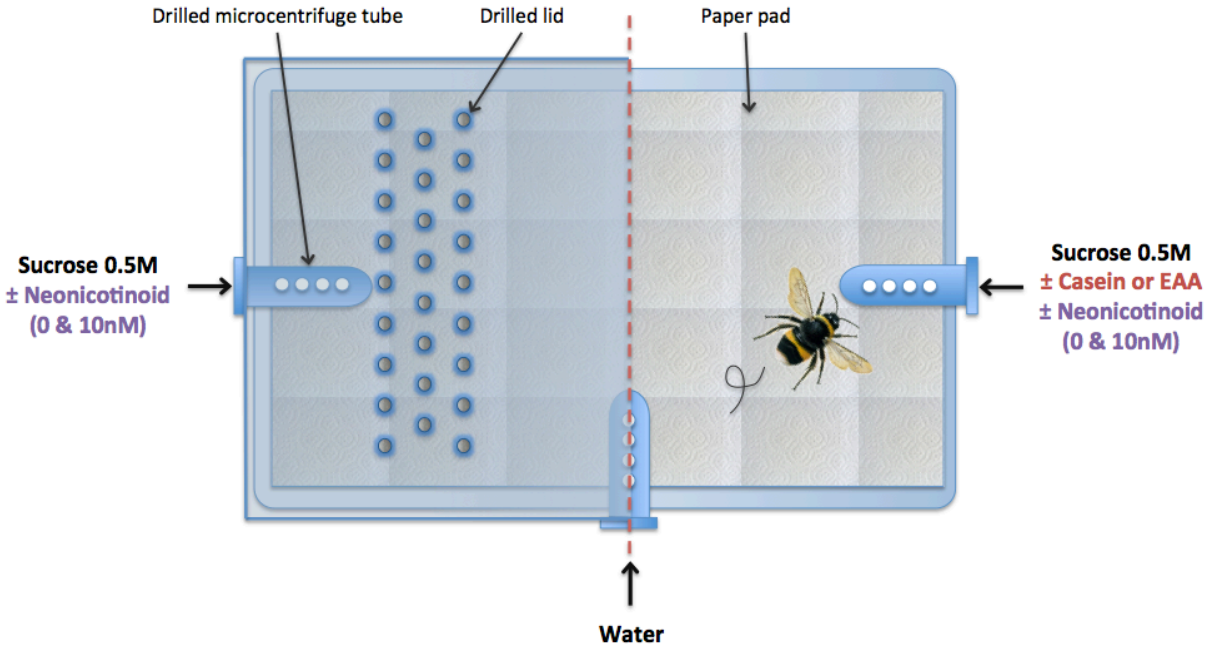

Figure 3.1: Individual bumblebee in feeding box with the 3 different liquid diets provided in drilled Eppendorf tubes (EAA: essential amino-acids).

2. Diets

To assess if bumblebees change their food intake when they are exposed to different neonicotinoids (imidacloprid, thiamethoxam and clothianidin), the Geometric Framework model of nutrition was applied (Raubenheimer & Simpson, 1997, 1999).

Casein (casein sodium salt from bovine milk, Sigma-Aldrich) was the protein source added to sucrose (0.5M, Sigma-Aldrich) in one tube, sucrose (0.5M, Sucrose Grade II, Sigma-Aldrich) only in a second tube and water in a third one. Different treatments were tested with a protein or essential amino acids (EAA) to 0.5M sucrose solution ratios on a molar-molar basis to the P:C ratio (Protein : Carbohydrate; Table 3.1). Casein 1:50 (Casein sodium salt from bovine milk, Sigma-Aldrich) and essential amino acids were the protein source added to sucrose (0.5M, Sigma-Aldrich) in one tube, sucrose (0.5M, Sucrose Grade II, Sigma-Aldrich) only in a second tube and water in a third one. The EAA solution has been developed after the determination by HPLC of the composition in free amino acids of the casein 1:50 solution. The EAA solution was made by the dilution in sucrose 0.5M liquid solution of the different amino acids in the proportions define by the HPLC similar to a

casein 1:50 solution (Table 3.2). Imidacloprid, clothianidin and thiametoxam were provided in sucrose and sucrose with casein or free amino acids solutions. The three pesticides were diluted at 10mM in both solutions. A control with no casein supplied in food was done in the same conditions (called 0:1x in the following table and results).

	Solution compositions		
Sucrose		**0.5M**	
Protein	**0:1x**	**Casein 1:50**	**EAA 1:50**
Control	35	35	15
Clothianidin 10nM	15	15	15
Imidacloprid 10nM	35	15	15
Thiamethoxam 10nM	15	15	15

Table 3.1: Sample size for each liquid diet treatments. The experiment was done by run of 5 bumblebees in parallel per treatment and repeated. Part of the sampling used for this experiment comes from the sampling from Chapter 1.

Amino acids	% Average
Arginine	3.50
Histidine	2.15
Isoleucine	7.48
Leucine	9.42
Lysine	0.95
Methionine	0.18
Phenyalanine	4.70
Threonine	3.63
Tryptophan	1.60
Valine	12.52

Table 3.2: **Average composition in free essential amino acids of the casein 1:50 solution**. Essential amino acids were used to do the free amino acids solution in those proportions. The composition has been determined by HPLC and the percentage of tryptophan was determined by UV spectrometry in Silvestre *et al.* (1994).

All tubes were weighed and replaced every 24 h for 7 consecutive days. To measure food consumption, the difference between day *t* and day *t+1* was calculated. At the end of experiments, bumblebees were weighed to estimate weigh loss or gain during the

experiment to compare the impact of the different diets. Different parameters of the body were measured: body length, thorax width and length, abdomen width and length, and head width and length to study the homogeneity of the different population used in each trial. Bumblebees were placed in a freezer at -80°C in a labeled envelop for further investigations.

A control for the evaporation rate of each diet was performed for 3 days in empty boxes places in the same conditions as the trial boxes to measure the weight loss in feeding tubes. An average of evaporation rate was calculated and subtracted from the value obtained during the experiment. Negative and null values obtained by the subtraction of the evaporation rate were replaced by a null value and consequently considered as there was no food consumption from the tube. The total food consumption was calculated by multiplying the amount consumed by the weight of sucrose and casein in 1ml of solution for each diet.

3. Bumblebee activity

To determine if bumblebee behavior changed with imidacloprid, clothianidin and thiamethoxam exposure, bumblebee's activity was observed each day for 10 minutes after taking them out of the incubator. Activity observed was defined as displayed on the following table (Table 2).

Behavior	Description of the behavior
Flying	Bumblebee moving in the air with its wings
Climbing	Bumblebee going up and using legs to catch up the tube that was containing the food or along the walls of the box
Running	Bumblebee moving very fast on the surface of the paper pad
Walking	Bumblebee moving slowly on the surface of the pad
Grooming	Bumblebee brushing its legs, body, head or wings
Eating	Bumblebee drinking at one of the tubes displayed in the box
Sitting	Bumblebee still on the paper pad
Lie on the back	Bumblebee upside down, slowly moving legs in the air or still on the back.

Table 3.3: Descriptions of the bumblebee behaviors observed during the experiment.

4. Statistics

The normality of each sample of data was tested. To study the daily average food consumption, the average daily food consumption was analyzed using two-way ANOVA for each diet and a Tukey's *post-hoc* analysis were performed. To measure the effect of pesticides on the average daily protein/amino-acids consumption in the subset of data of bumblebees fed with casein and amino-acids diets, a two-way ANOVA and a Tukey's *post-hoc* analysis were performed. Survival was analyzed using Kaplan-Meier analysis (Kaplan and Meier, 1958) to measure how each treatment influenced survival. To identify the correlations in the behaviors depending of the diet and the dose of pesticides, a principal components method for factor analysis (Johnson and Winchern, 1992) was performed. The impact of each diet and each pesticide has been tested by a repeated measure ANOVA, and then all diets were compiled together to test the effect of the pesticides on the behavior by a MANOVA. The resulting factor scores generated for the factors with eigenvalues greater than 1.5 (10% or more of the overall variation per factor) were then entered into a repeated-measure ANOVA and LSD post-hoc analysis. The average body mass loss depending of the treatment was analyzed using two-way ANOVA and Tukey's *post-hoc* test. To compare the effect of each diet on the average body mass loss, a one-way ANOVA was performed and Tukey's *post-hoc* test. All non-significant interactions in models performed during the statistical analysis were removed and the new models were compared to the previous one. If the new model was significantly different of the previous one, the new model was rejected and not presented in the following result section. All analyses were performed using IBM SPSS v15.0 software (SPSS Inc., Chicago, IL).

III. Results

1. Dietary source of essential amino acids and exposure to pesticides affected food consumption

In these experiments, the susceptibility of adult worker bumblebees of being influenced by the dietary source of essential amino acids to pesticides was examined. The dietary source of EAAs affected the average sucrose daily consumption (between-subjects, two-way ANOVA, F (2,240)=3.73, *P*=0.025, diet main effect). The pesticide also influenced the amount of daily sucrose consumption (between-subjects, two-way ANOVA, F(3,240)=8.86, *P*<0.001, pesticide main effect). There was no interaction between the diet type and the pesticides on the average sucrose daily consumption (between-subjects, two-way ANOVA, F (6,240)=1.02, *P*=0.415, pesticide x diet). Dietary source of EAA influenced how much of the sucrose-EAA/casein diet was consumed each day (between-subjects, two-way ANOVA, F (1,133)=12.6, *P*=0.001, diet main effect). The pesticides also influenced how much of the sucrose-EAA/casein diet was consumed each day (between-subjects, two-way ANOVA, F (2,133)=8.64, *P*=0.015, pesticide main effect). No significant interaction was observed between the diets and pesticides on the average EAA/casein daily intake (between-subjects, two-way ANOVA, F (3,133)=0.20, *P*=0.912, pesticide x diet).

Bumblebees fed with sucrose solution ate less food on average when the food contained 10nM imidacloprid compared to the control (no pesticide), thiamethoxam 10nM and clothianidin 10nM (Figure 3.2A; between-subjects, two-way ANOVA, F (3,99)=2.75, *P*=0.047, pesticide main effect). Pesticides in the diet solution had a significant impact on the relative proportion of the sucrose and sucrose-casein diet eaten by adult worker bumblebees (Figure 3.2B; between-subjects two-way ANOVA, F (3,79)=5.20, *P*=0.003, pesticide main effect) (Figure 3.2B; between-subjects, two-way ANOVA, F (3,78)=3.04, *P*=0.034, pesticide main effect). Moreover, pesticide influenced the relative proportion of sucrose and the sucrose-EAA diet each day (Figure 3.2C; between-subjects, two-way ANOVA, F (3,59)=3.57, *P*=0.020, pesticide main effect).

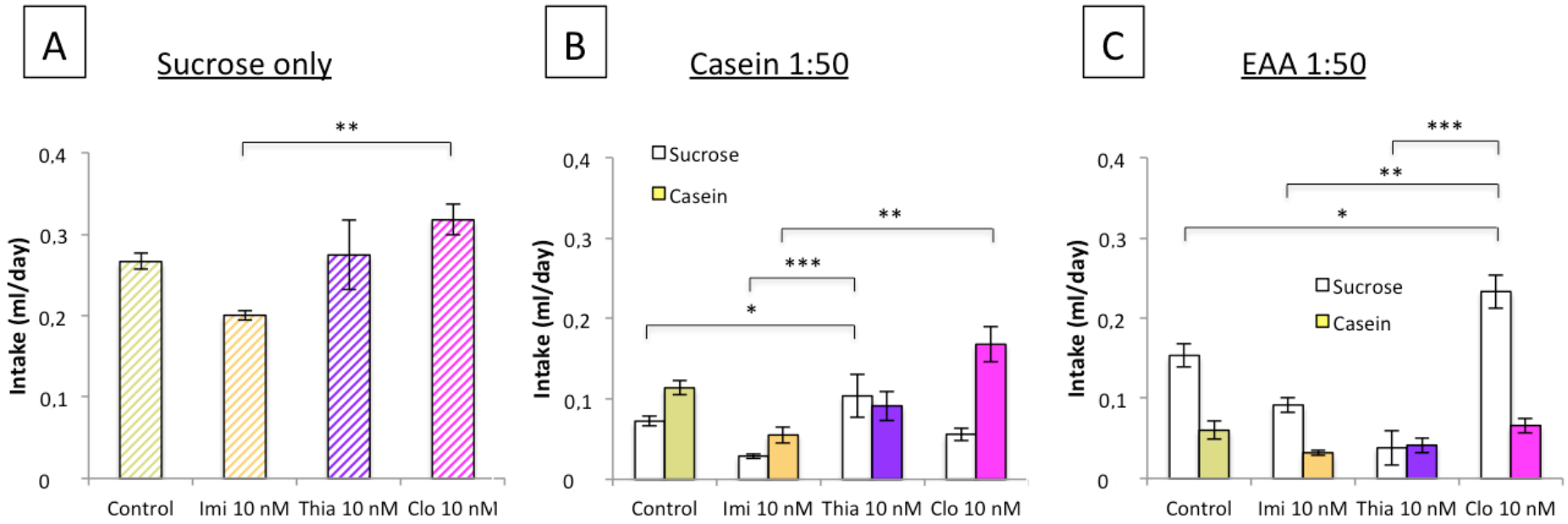

Figure 3.2: Comparison of the impact of a sub-lethal dose of neonicotinoids ("control" means no pesticide, imidacloprid, thiamethoxam and clothianidin) and the diet (sucrose only, casein 1:50 and EAA 1:50) on the average daily food consumption (± standard error) by bumblebees. (Tukey's *post-hoc* comparison: '*': 0.005<*P*<0.010; '**': 0.001<*P*<0.005; '***': *P*<0.001). For the number of specimen per run, please refer to Table 3.1 in Material and Methods.

2. The intake target depended on diet type when bees are fed thiamethoxam and clothianidin

As in the previous chapters, the bumblebees were given access to diet pairs where one diet contained casein 1:50 or free amino-acids 1:50 with sucrose 0.5M and the other contained sucrose 0.5M alone. Using this approach, the way that bumblebees achieved the intake target was measured over the 7-day experiment by calculating the P:C ratio. The influence of the neonicotinoids on daily volume of food intake depended on the day and the dietary ratio (sucrose or sucrose with casein or free amino-acids). Figure 3.3 represents a cumulative plot of the mean protein and carbohydrate consumption for each dietary pairing. The influence of the neonicotinoids on daily volume of food intake depended on the day and the dietary ratio (sucrose or sucrose with casein or free amino-acids).

It was clear from the plot that dietary source of EAAs influenced the intake target (IT) (Figure 3.3). The intake target of bumblebees fed with the 1:50 casein diet depended on the pesticide treatment (Figure 3.3A). Bees from the control (no pesticide) and imidacloprid 10nM had similar ITs (Table 3.4). The presence of thiamethoxam and clothianidin sub-lethal dose (10nM) in food was displayed the daily cumulative food intake on each side of the control (no pesticide) and showing an opposed impact of these pesticides on the food balance (Figure 3.3A). The cumulative food consumption showed that bees exposed to imidacloprid ate half as much sucrose and casein over the 7 days. Bees fed with thiamethoxam were balancing their diet for carbohydrates while bees fed with clothianidin exhibited a large preference for protein in their diet (Table 3.4).

Bumblebees fed with EAA 1:50 diet were not displaying the same IT as for casein 1:50 (Figure 3.3B). The influence of the pesticides on the IT trajectories of bees fed with the EAA diets were the opposite of those observed when bees were fed on casein. Bees fed with no pesticide, imidacloprid or clothianidin were biased towards carbohydrates (Table 3.4) whereas bees fed with thiamethoxam skewed their food intake towards EAAs (Table 3.4). Bees fed with no pesticide or with clothianidin had a similar cumulative EAA intake over the 7 days, but bees fed with clothianidin ate a larger amount of carbohydrates in total. As observed for casein 1:50 cumulative food consumption, bees ate twice more carbohydrates and EAA in the control (no pesticide) than when they were having imidacloprid in their food.

The presence of thiamethoxam dramatically reduced the carbohydrate consumption and biased the bees towards consuming EAAs.

The variation of the food intake target between bees fed with casein and EAA can be observed (Table 3.4). The carbohydrate intake variation depended on the diet (casein or EAA) (between-subjects, repeated-measures ANOVA, $F(1,6)=20.2$, $P=0.002$, diet main effect) and the pesticide (between-subjects, repeated-measures ANOVA, $F(3,7)=21.8$, $P<0.001$, pesticide main effect), likewise the protein intake depended on the diet (casein or EAA) (between-subjects, repeated-measures ANOVA, $F(1,6)=1.87$, $P=0.023$, diet main effect) and the pesticide (between-subjects, repeated-measures ANOVA, $F(3,4)=1.04$, $P=0.036$, pesticide main effect).

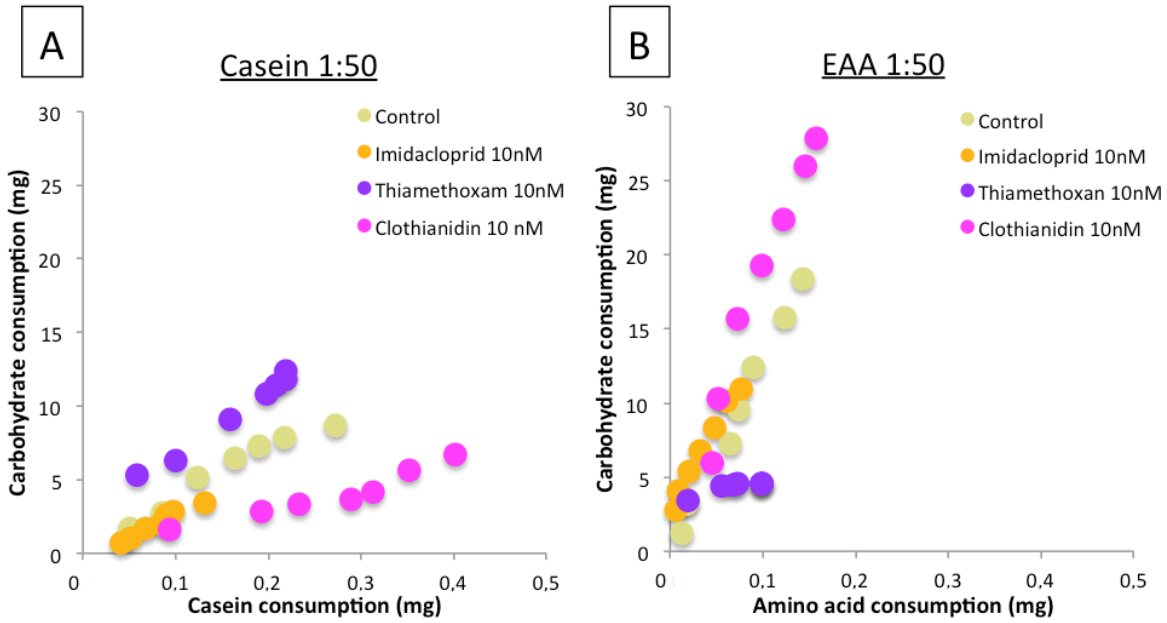

Figure 3.3: **Cumulative average daily food consumption on protein and carbohydrate intakes for individual bumblebees confined to one of the different pesticide ("control" means no pesticide, imidacloprid, thiamethoxam and clothianidin) and diets (casein 1:50 and EAA 1:50) tested.** For the number of specimen per run, please refer to Table 3.1 in Material and Methods.

P:C ratio	Casein 1:50	EAA 1:50
Control (no pesticide)	1:82	1:132
Imidacloprid 10nM	1:76	1:193
Thiamethoxam 10nM	1:107	1:96
Clothianidin 10nM	1:67	1:227

Table 3.4: Comparison of the food intake target calculating on P:C ratio over the 7-days for the different neonicotinoids ("control" means no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Casein 1:50 or EAA 1:50). For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

3. The impact of thiamethoxam sub-lethal dose on bumblebee survival

The identity of the neoniconinoid pesticide ingested by bumblebees strongly impacted survival (Figure 3.4). Bees in the control group (no pesticide) and exposed to clothianidin 10nM did not exhibit significantly different mortality over the diets (Table 3.5) and showed similar mortality rates over the 7 days (Figure 3A&D). Overall mortality rates were significantly higher in bees exposed to sub-lethal doses of thiamethoxam, but there were no significant differences in these mortality rates between the diets (Table 3.5, Figure 3.4C). Only bees fed with 10 nM imidacloprid exhibited a diet-dependent mortality rate (Table 3.5). Bees fed with casein 1:50 were more likely to die quickly whereas there was no difference in the mortality of bees fed with sucrose only and EAA diets over the 7 days (Figure 3.4B).

	χ^2	df	*P*
Control (no pesticide)	1.738	1	0.187
Imidacloprid 10 nM	**4.391**	**1**	**0.036**
Thiamethoxam 10nM	0.114	1	0.736
Clothianidin 10nM	0.185	1	0.667

Table 3.5: Results of Kaplan-Meier analysis (Log rank Mantel Cox) with different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) comparing the different diets (Sucrose only, Casein 1:50 or EAA 1:50). For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

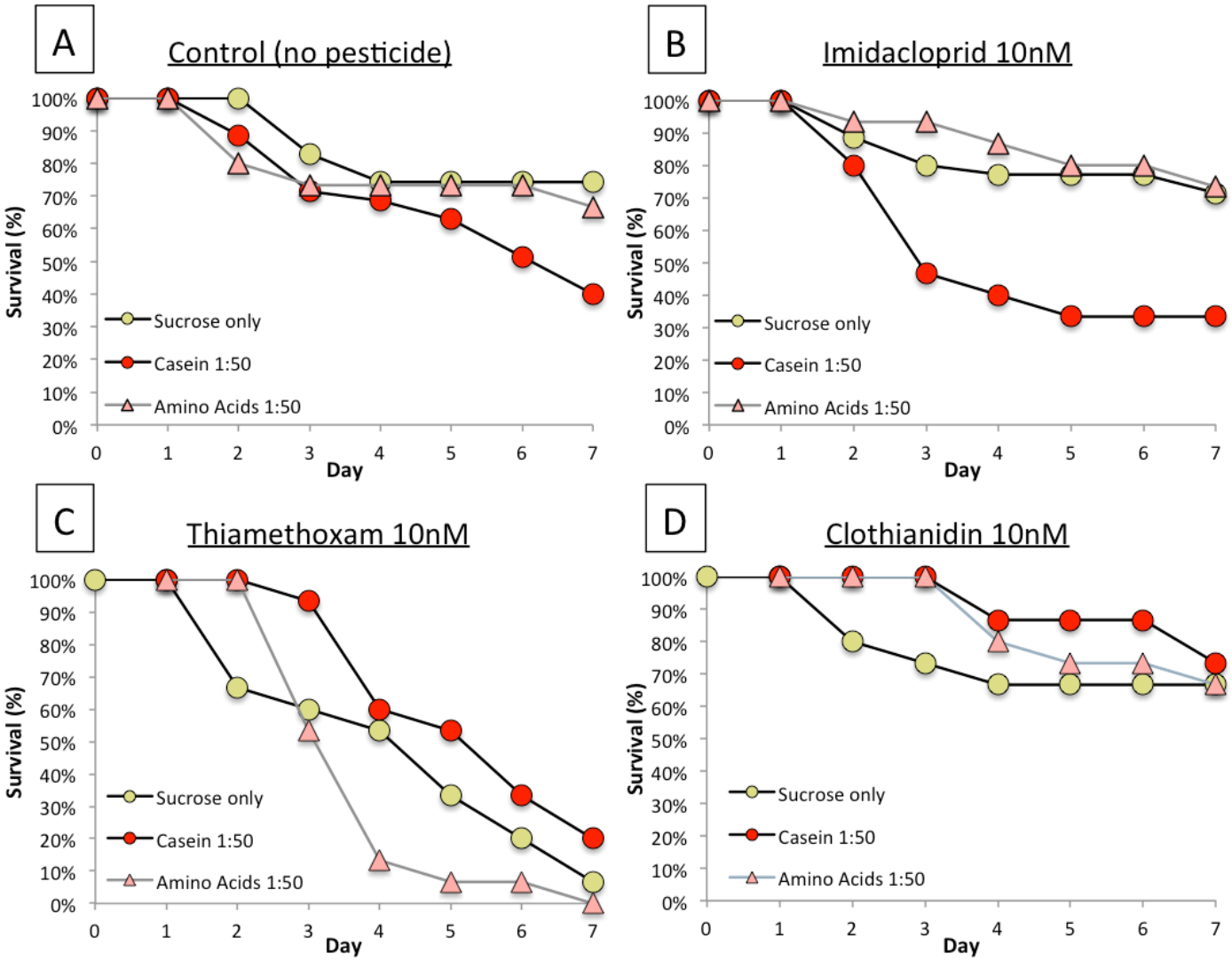

Figure 3.4: **Comparison of the mortality of bumblebees exposed to different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and comparing different diets (Sucrose only, Casein 1:50 or EAA 1:50).** For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

Moreover, the comparison of diets showed that the survival rates can change depending of the pesticide to which bees were exposed. Bees fed with sucrose only had similar mortality rates in the control (no pesticide), imidacloprid 10nM and clothianidin 10nM, but significantly higher mortality rates when exposed to sub-lethal dose of thiamethoxam (Figure 3.5A; Table 3.6). There were no significant differences in overall survivorship with exposure to the different neonicotinoids for bees on the casein 1:50 diet (Figure 3.5B; Table 3.6). In contrast, bees fed with free amino-acids 1:50 had a significantly higher overall mortality rate when they were fed with thiamethoxam 10nM in comparison with bees without pesticide in the diet or exposed to imidacloprid 10nM and clothianidin 10nM (Figure 2.5C; Table 3.6).

Diet	Pesticide	Control	
		χ^2	Sig.
Sucrose	Control	.	.
	Imi10	0.143	0.706
	Thia10	21.201	**<0.001**
	Clo10	0.589	0.443
Casein 1:50	Control	.	.
	Imi10	0.977	0.323
	Thia10	2.550	0.110
	Clo10	1.380	0.240
EAA 1:50	Control	.	.
	Imi10	0.221	0.638
	Thia10	15.740	**<0.001**
	Clo10	0.249	0.617

Table 3.6: Results of Kaplan-Meier analysis (Log rank Mantel Cox) with different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or free amino-acids 1:50). The Kaplan-Meier analysis measures the fraction of subjects living for a certain amount of time after treatment. For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

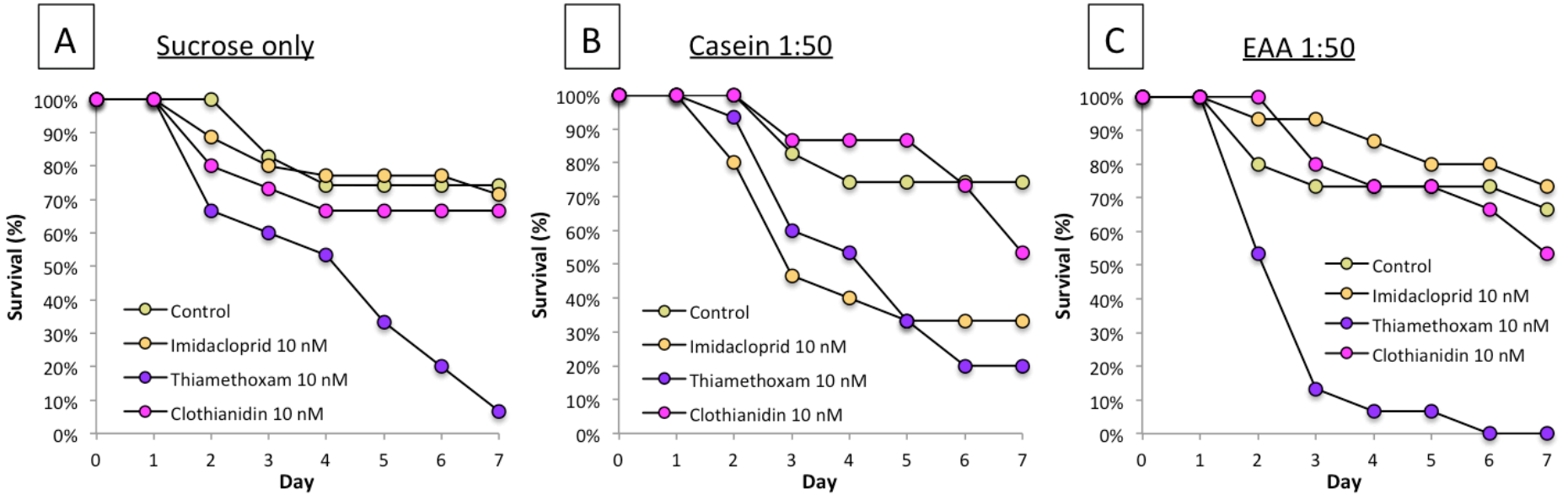

Figure 3.5: **Comparison of the mortality of bumblebees exposed to different diets (Sucrose only, Casein 1:50 or EAA 1:50) and comparing different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin).** For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

4. Contrasting effects of sub-lethal dose of neonicotinoids on the behavior of bumblebees

The behavior of the bumblebees was measured in each treatment group each day. There was a significant interaction between the behavior and the pesticide (Figure 3.6; Table 3.7). As the diet did not have a significant interaction with the pesticide treatments on the behavior (Table 3.7), the behavior observed was compiled for each pesticide for the 7 days of the experiment in Figure 3.6. There were significant effects of the pesticides on the following behaviors: flying, running, eating, grooming and sitting (Table 3.8). Bees in the control (no pesticide) were active in 76% of the observations and presented a large panel of active behavior such as flying, climbing to access to the food, running, walking and eating (Figure 3.6A). No bees were observed lying upside down in the control (no pesticide; Figure 3.6A). In contrast, bees exposed to 10 nM imidacloprid were active in 45% of the observations over all diets (Figure 3.6B). Moreover, bees exposed to clothianidin 10nM spent most of their time inactive (36%) or exhibiting signs of malaise such as grooming (Figure 3.6D). The most outstanding difference was observed for bees exposed to 10nM thiamethoxam. These bees were active in 87% of the observations, but also spent 4% of their time grooming (Figure 3.6C).

Source of variation	Type III Sum of Squares	df	Mean Square	F	Sig.
Factor	0.393	1	0.393	0.310	0.578
Factor x Pesticide	**31.913**	**3**	**10.638**	**8.392**	**<0.001**
Factor x Diet	1.875	2	0.938	0.740	0.479
Factor x Pesticide x Diet	12.056	6	2.009	1.585	0.154
Error (Behavior)	212.971	168	1.268		

Table 3.7: **Results of linear tests of within-subject contrasts of repeated-measures ANOVA to test the behavior with different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or EAA 1:50).** For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

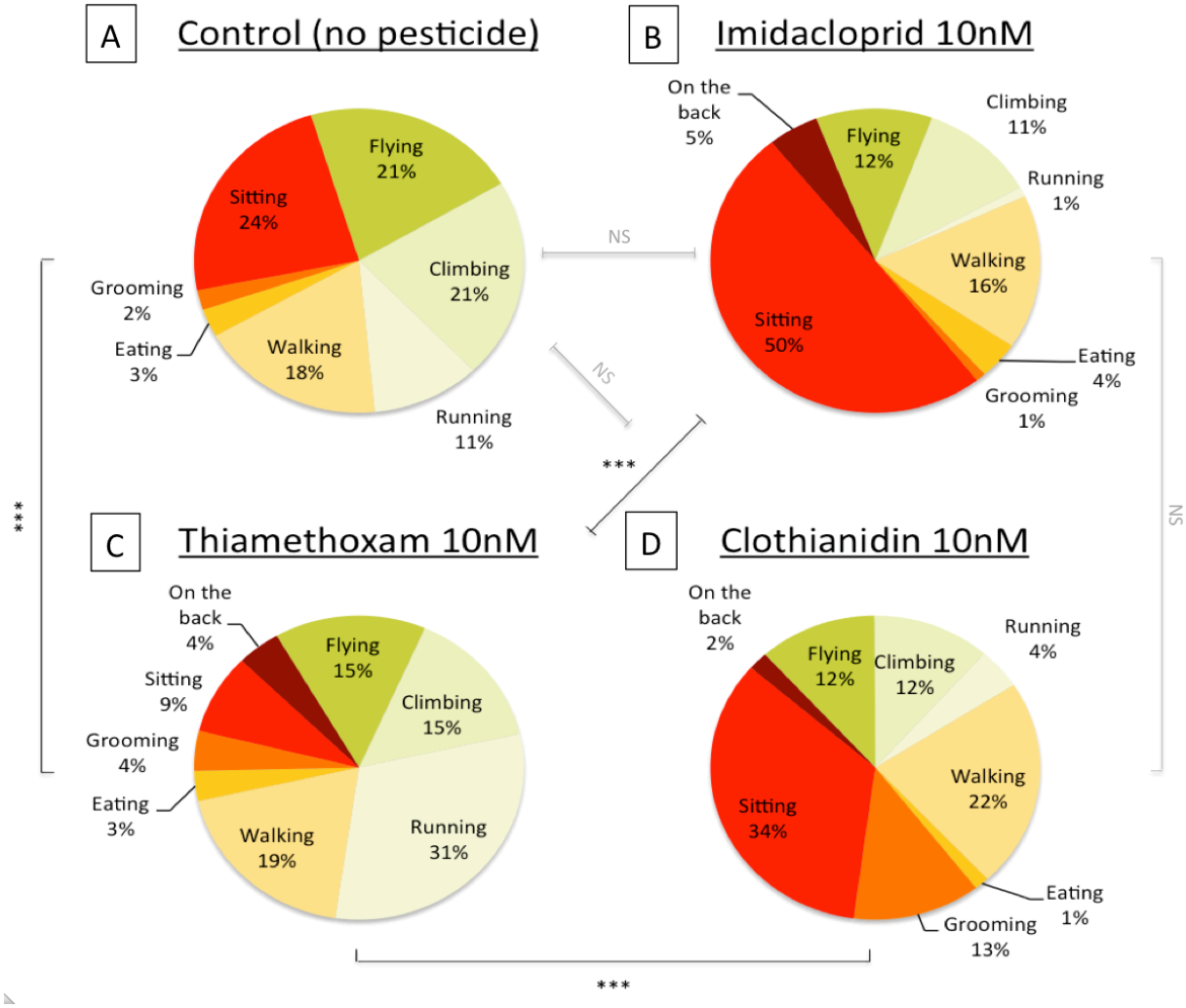

Figure 3.6: **Comparison of the behavior displayed by bumblebees over different diets (Sucrose only, Casein 1:50 or free amino-acids 1:50), for different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and over 7 days.** (LSD *post-hoc* comparison: '***': *P*<0.001) For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

To compare how pesticides and diets affected behavior, a factor analysis was performed on the behaviors observed over the 7 days. The factor scores obtained by the factor analysis were used to test how the chronic exposure to different doses of imidacloprid affected the expression of behavior. The factor analysis for the behavior observed over the 7 days reduced 8 variables to three variables represented in three factors that accounted for 80.3% of the variation in the data (Table 3.9). The three factors extracted had eigenvalues greater than 1.5 (all others factors were not included as they explained less than 10% of the variation per factor). The first factor, which accounted for 31.9% of the variation in behavior, was mainly representative of the 'active' behaviors such as flying, walking and climbing to access to food. The second factor was accounting for 26.3% of the variation in the behavior and represented the time spent inactive as sitting and bees lying on their backs. The third factor characterized 22.1% of the variation in the behavior and characterized time spent eating versus the time spent exhibiting signs of malaise as grooming. The factor scores extracted were significantly explained by the pesticide action on the behavior of the bumblebees (Table 3.10).

Effect	N obs.	Value	F	Hypothesis df	Error df	Sig.
Intercept		0.808	69.866	2	173	<0.001
Flying	**106**	**0.081**	**2.810**	**5**	**174**	**0.018**
Intercept		1.536	132.876	2	173	<0.001
Climbing	107	0.041	1.432	5	174	0.215
Intercept		0.819	389.269	2	172	<0.001
Running	**180**	**0.141**	**4.090**	**6**	**173**	**<0.001**
Intercept		1.812	155.872	2	172	<0.001
Walking	73	0.040	1.147	6	173	0.337
Intercept		1.543	135.818	2	176	<0.001
Eating	**163**	**0.086**	**7.593**	**2**	**177**	**<0.001**
Intercept		2.068	180.961	2	175	<0.001
Grooming	**152**	**0.139**	**8.131**	**3**	**176**	**<0.001**
Intercept		4.526	389.269	2	172	<0.001
Sitting	**79**	**0.079**	**2.271**	**6**	**173**	**0.039**
Intercept		0.520	45.757	2	176	<0.001
On the back	163	0.032	2.871	2	177	0.059

Table 3.8: Results of the multivariate tests of between-subject effects MANOVA with the pesticide as the dependent variable to test the behavior with different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or EAA 1:50) compile in one table. For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

	Principal components		
	1	2	3
Eigenvalue	2.231	1.844	1.548
Percent variance	31.871	26.340	22.121
Factor loading			
Flying	**0.892**	0.056	-0.293
Climbing	**0.666**	0.559	0.396
Running	**0.877**	-0.257	0.109
Walking	0.237	0.195	**-0.729**
Eating	0.157	0.054	**0.877**
Grooming	0.088	**0.933**	0.020
Sitting	-0.241	**0.758**	-0.218
Back	**0.892**	0.056	-0.293

Table 3.9: Factor analysis of bumblebee's behavior using principle components method of factor extraction with varimax rotation and Kaiser normalization. Factor loading in bold indicate which behavioral variables made the largest contribution to each factor. For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	factor 1	8.012	5	1.602	3.217	0.094
	factor 2	8.224	5	1.645	3.556	0.077
	factor 3	8.641	5	1.728	4.397	0.050
Intercept	factor 1	0.000	1	0.000	0.000	1.000
	factor 2	0.000	1	0.000	0.000	1.000
	factor 3	0.000	1	0.000	0.000	1.000
Diet	factor 1	0.852	2	0.426	0.856	0.471
	factor 2	0.006	2	0.003	0.006	0.994
	factor 3	2.994	2	1.497	3.808	0.086
Pesticide	**factor 1**	**7.159**	**3**	**2.386**	**4.792**	**0.049**
	factor 2	**8.219**	**3**	**2.740**	**5.922**	**0.032**
	factor 3	**5.647**	**3**	**1.882**	**4.789**	**0.049**
Total	factor 1	2.988	6	0.498		
	factor 2	2.776	6	0.463		
	factor 3	2.359	6	0.393		
Corrected Total	factor 1	11.000	12			
	factor 2	11.000	12			
	factor 3	11.000	12			

Table 3.10: Results of tests of between-subjects effects of MANOVA comparing the different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or free amino-acids 1:50) using scores generated by the factor analysis of the behavior with the three factors produced by the analysis of principal component. For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

5. Bumblebee body mass loss depended on the pesticide

In addition, the average percentage of body mass over the course of the experiment was measured for each diet and pesticide treatments. The pesticide only influenced the average body mass loss and there was no interaction of the diets with pesticides (Table 3.11; Annex C: Figure 1). The average percentage of body mass in the control (no pesticide) was 15.9%±14.3, while bees fed imidacloprid lost slightly more body mass than the control (17.2%±15.6) (Figure 3.7). Bees fed clothianidin lost ~15.5%±15.4 of their body mass, whereas the bees fed thiamethoxam lost (12.8%±16.6) (Figure 3.7).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4007.165	11	364.288	1.587	0.104
Intercept	45893.397	1	45893.397	199.919	<0.001
Diet	405.783	2	202.891	0.884	0.415
Pesticide	**1867.130**	**3**	**622.377**	**2.711**	**0.046**
Diet * Pesticide	1320.349	6	220.058	0.959	0.454
Error	52339.587	228	229.560		
Total	114231.353	240			
Corrected Total	56346.752	239			

Table 3.11: Results of tests of between-subject effects of two-way ANOVA to test the average body mass lost with different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or EAA 1:50). For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

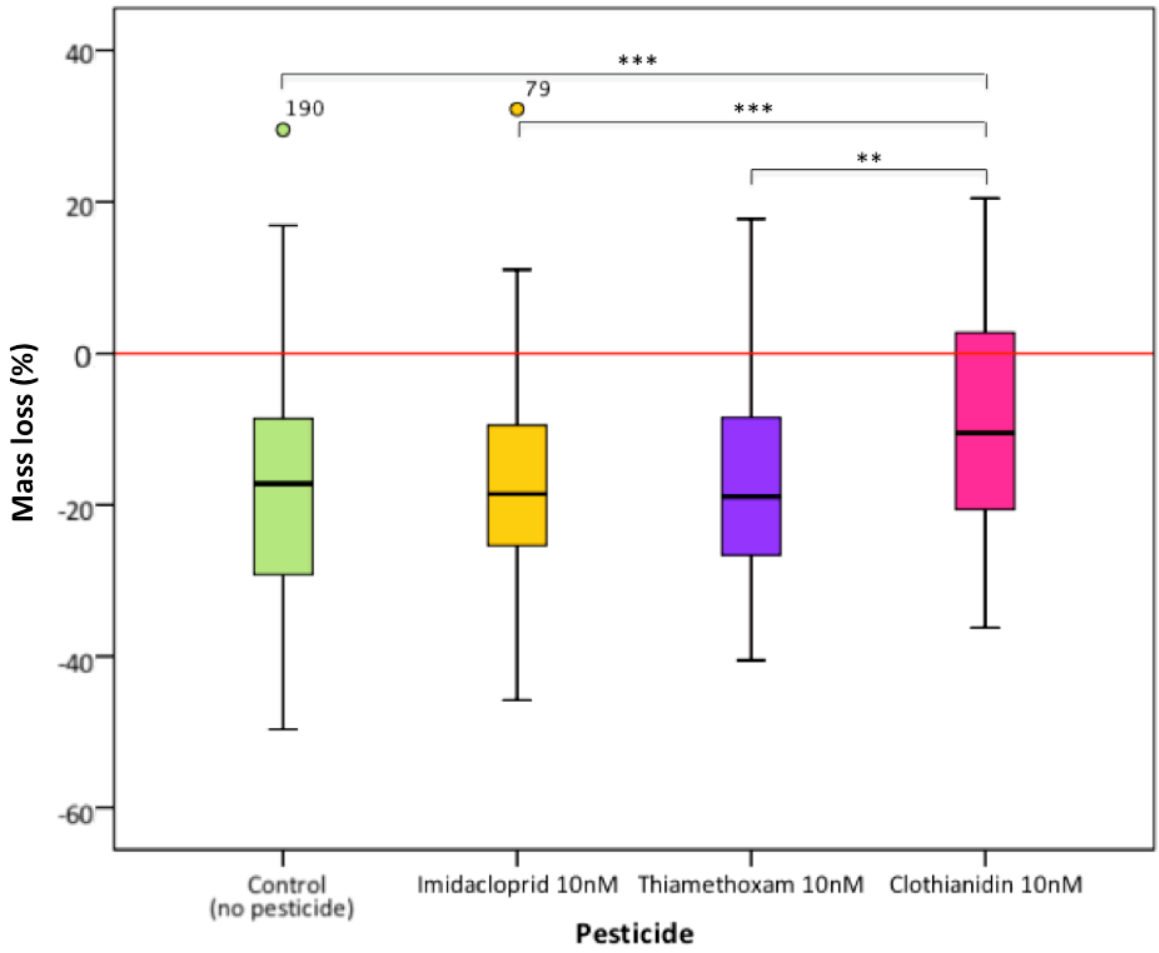

Figure 3.7: **Comparison of the average mass loss of bumblebees over the 7 days depending of different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and over the diets (Sucrose only, Casein 1:50 or EAA 1:50**). Lines in bold represent medians; boxes represent 1st and 3rd interquartile ranges, bars represent the minimum and maximum range of the data and open circles represent outliers. The zero is represented by —. (LSD *post-hoc* comparison: '**': 0.001<*P*<0.005; '***': *P*<0.001). The zero is represented by —. For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

6. The average daily intake of pesticide per bumblebee

To compare our results with field relevant data of the possible daily intake of pesticide in bumblebees, the average daily intake of each neonicotinoid for each diet has been calculated. The type of neonicotinoid had a significant impact on the average daily intake, but no interaction between the pesticide and the diet was identified (Table 3.12). Bumblebees exposed to imidacloprid 10nM experienced a lower dose (106.5±11.7pg/day/bee), while bees exposed to 10nM clothianidin (234.9±31.8pg/day/bee) and 10nM thiamethoxam (278.3±26.9pg/day/bee) consumed higher doses (Figure 3.8).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	652881.655	6	108813.609	3.516	0.003
Intercept	4920864.224	1	4920864.224	159.009	<0.001
Diet	55773.688	2	27886.844	0.901	0.409
Pesticide	**460679.731**	**2**	**230339.865**	**7.443**	**0.001**
Diet x Pesticide	901.357	2	450.678	0.015	0.986
Error	3497015.128	113	30947.037		
Total	10197270.299	120			
Corrected Total	4149896.783	119			

Table 3.12: Results of tests of between-subjects effects of two-way ANOVA to test the average daily intake of pesticide per bumblebee with different neonicotinoids (imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or EAA 1:50). For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

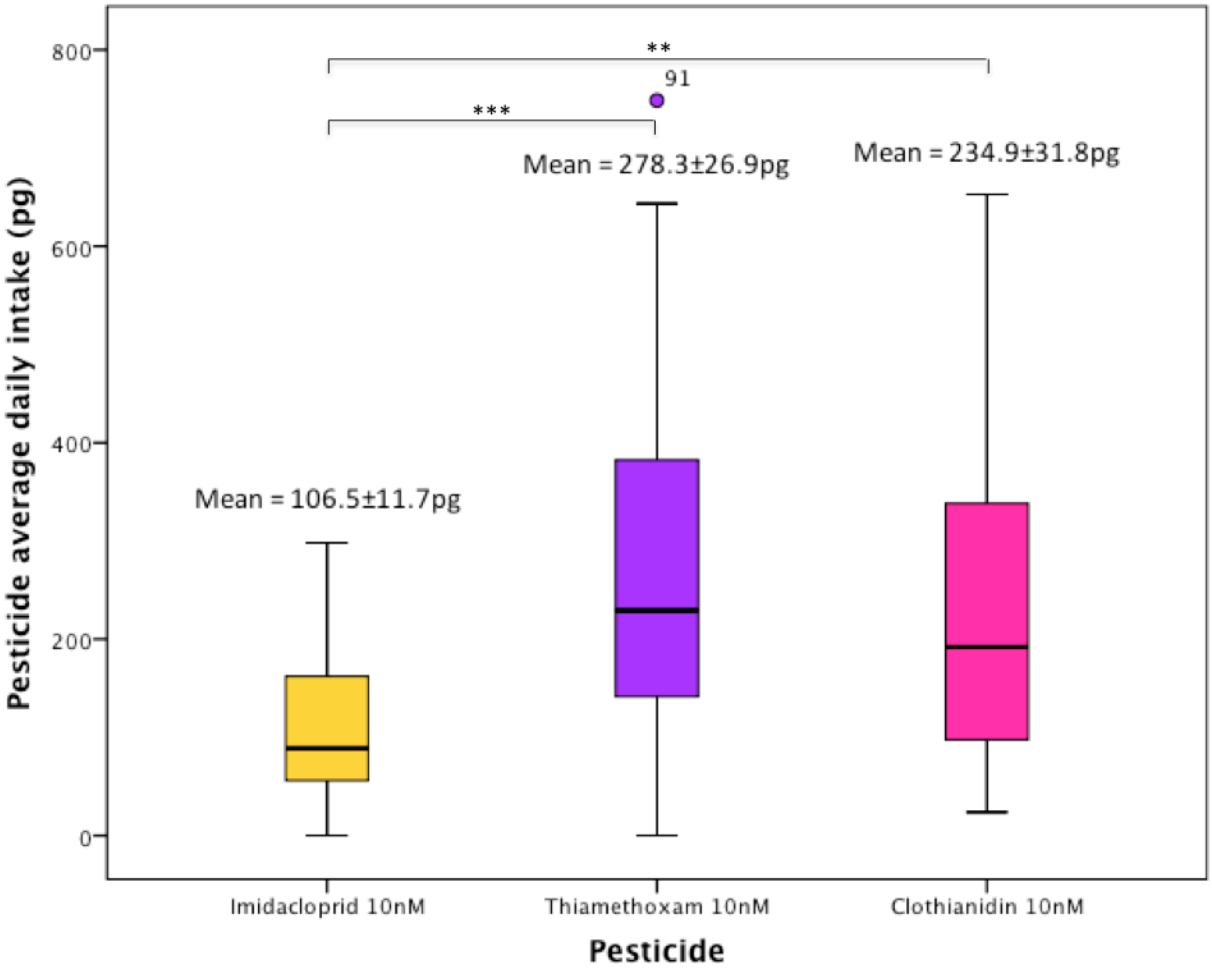

Figure 3.8: **Comparison of the average daily intake of pesticide per bumblebee over the 7 days depending of different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and over the diets (Sucrose only, Casein 1:50 or EAA 1:50).** Lines in bold represent medians; boxes represent 1st and 3rd interquartile ranges, bars represent the minimum and maximum range of the data and open circles represent outliers. (Tukey's *post-hoc* comparison: '**': 0.001<*P*<0.005; '***': *P*<0.001). For the number of specimens per run, please refer to Table 3.1 in Material and Methods.

IV. Discussion

My results show that sub-lethal doses of the three most common neonicotinoids have different effects on the food consumption and behavior of adult worker bumblebees. These effects include changes in food intake target, survival, physiology and behavior. The fact that the neonicotinoids did not all have the same effects on bees could explain the conflicting results observed in the literature and emphasizes the difficulty of understanding the complexity of the different observed impacts of pesticides on bees.

1. Neonicotinoids had a variable effect on feeding depending of the diet

a. The anti-feedant aspect of imidacloprid

As previously developed in chapter 1 and 2, a 10nM dose of imidacloprid reduced bumblebee appetite and changed their food intake. In comparison, few studies focus on feeding behavior of a single bee. Most have studied the food collection of whole colonies or micro-colonies that have access to pesticide treated nectar and pollen. Yang *et al.* (2008) observed no significant differences in the frequency of feeder visitations between their control and the low contaminated sucrose solution (≈20nM), but noticed that the foraging frequency was abnormal for doses over 195nM in the sucrose solution. They also noticed that honeybees were not affected at the same time by exposure to imidacloprid. In my experiments, the sub-lethal dose was lower than the lowest dose used by Yang et al. (2008) and yet significant differences in feeding behavior of individual bumblebees were observed. My results are consistent with Schmuck (1999) and Decourtye *et al.* (2004) who observed a rapid decrease of the foraging activity of honeybees after they consumed imidacloprid and suggest that it might be due to the anti-feedant character of the compound. Additionally, the anti-feedant effect of imidacloprid due to illness caused by its consumption appears to be dose dependent.

Thompson *et al.* (2014) studied the impact of different doses of imidacloprid (between 3.9 and 390nM) in 0.7M sucrose solutions on single housed bumblebees (*B. terrestris*) and observed that the overall sucrose intake was significantly lower for doses over 39nM, but not for the lower dose of imidacloprid (3.9nM). Moreover, my results show that imidacloprid significantly reduced the average daily food intake of bees over diets. Bees

were showing similar food intake target when they were fed with protein (casein 1:50) between the control (no pesticide) and imidacloprid 10nM even if the total amount of food consumption was 2x less for each of the types of diets laced with imidacloprid. The nutrient balance shifted toward carbohydrates when bees were offered amino acid in their diet in the same proportion as in the control (no pesticide) and the total of nutrient intake was 2x less for bees exposed to imidacloprid suggesting that imidacloprid reduced the total intake of food but did not change nutrient requirements.

b. Thiamethoxam variable impact on food intake

The effects of thiamethoxam on feeding behaviour were distinct from those caused by imidacloprid and clothianidin. Bumblebees exposed to sub-lethal doses of thiamethoxam increased their food intake when fed sucrose and avoided diets containing free amino acids, but did not avoid solutions containing protein. This is somewhat consistent with Thompson *et al.* (2014) observations who found that bumblebees (*Bombus terrestris*) did not avoid thiamethoxam in sucrose solution when it contained less than 34nM of thiamethoxam. But their study was limited to carbohydrate intake and did not provide any information about protein intake.

Neonicotinoids have known effects on the nervous system but they could also influence metabolism. This would be revealed in my experiments with diets with different sources of EAAs. For example, Yee (2008) reported in a comparative study that thiamethoxam in a sugar and yeast diet and found it had a strong impact on insect mortality of the western cherry fruit fly (*Rhagoletis indifferens*, Diptera: Tephriditae). In my experiment, the intake target of bumblebees was influenced both by the diet and the presence of thiamethoxam. This suggests that thiamethoxam could interact with the mechanisms of digestion, or with metabolism after the nutrients had been absorbed. Oliveira *et al.* (2014) observed on Africanized honeybee (*Apis mellifera*) that the digestive and regenerative cells of the midgut of exposed bees display morphological and histochemical alterations. They suggested that sublethal doses of thiamethoxam could cause impairment in the brain and the gut that contribute to honeybee lifespan reduction.

c. Clothianidin somewhat increase food intake

The exposure to sub-lethal doses of clothianidin increased the appetite for sucrose solutions and bumblebees avoided diets containing free amino acids. Limited evidence was recorded that exposure to clothianidin may induce reduced nectar consumption. Although, Thompson *et al.* (2014) had contrasted observations and concluded that 40 nM clothianidin significantly reduced the total sucrose intake over the 4 days of their experiment, the 4nM dose of clothianidin did not have a substantial impact on the total sucrose intake.

Bumblebees fed with 10 nM clothianidin had a food intake target that shifted towards protein in comparison with the control (no pesticide). This is contrary to Franklin *et al.* (2004) who noticed that clothianidin did not affect the pollen consumption of *Bombus impatiens* colonies when the consumption was estimated for an average weekly intake per bee. Sandrock *et al.* (2014) did not observe a significant impact of sub-lethal doses of clothianidin. Furthermore, chronic exposure to clothianidin did not influence pollen consumption and storage by honeybee colonies, suggesting that the needs for protein did not change for the whole colony despite the exposure to clothianidin. These studies suggest that at the colony level, bees might have different nutrients requirement depending of their age (larvae, newly emerged and workers) or activity (foraging or not) inside the colony that can be compensated at the colony level when the colony is exposed to clothianidin.

My results also show that bumblebees exposed to clothianidin dramatically switched their food intake toward carbohydrates when they had access to free amino-acids (EAA) diet suggesting that the needs and may be the metabolism of EAA changed. Organophosphorus pesticide can induce strong disturbance in the metabolism of amino acids, with significantly increased amounts of amino acids recorded in mice serum with pesticide exposure (Gomes *et al.*, 1999). Casida (2011) proposed that neonicotinoids as clothianidin can have an impact on phase II metabolism that include the production of amino acids and may explain the aversion for EAA.

2. Neonicotinoids cause diverse locomotion impairments and metabolic issues

The impacts of neonicotinoids on many aspects of bee behavior are widely documented to explain the pollinator decline. Imidacloprid, thiamethoxam and clothianidin are known for affecting bee performance by reducing the ability to forage and homing flights in field situations (Bortolotti *et al.*, 2003; Mommaerst *et al.*, 2010; Schneider *et al.*, 2012; Fisher *et al.*, 2014). In comparison, few studies focused on more refined effect of neonicotinoids on motor function or on metabolic issues; neither has any previous study highlighted the contrasting effects of three different neonicotinoids, compared them on bumblebee behavior and locomotion abilities at field realistic doses and might confront them to metabolic topics involved.

a. Imidacloprid

The exposure to sub-lethal doses of imidacloprid had a severe impact on the behavior observed and on the locomotion of bumblebees. In my experiment, bees spent most of their time inactive, having disrupted movement of wings, staying stuck on the pad of their box and trembling. Moreover bees displayed the highest average body mass loss observed over the 7 days suggesting that the nutrient intake might not be enough to preserve bee activity. The impact of imidacloprid on the central nervous system could play a considerable role as well as its impact on the lack of nutrients when less food is consumed. This might explain the wide range of observations of most of studies about the impact of sub-lethal doses of imidacloprid that report foraging capacity deficiency and locomotion impairment. Imidacloprid can endanger single bee survival at very low doses but the impairment of individual can be counteracted at the colony level and explain contrasting results about sub-lethal impact of imidacloprid depending on the experimental context.

Bortolotti *et al.* (2003) demonstrated that honeybees exposed to sub-lethal doses of imidacloprid (<400nM) in sucrose solution were confused, disoriented and failed to return to the hive or feeding site for up to 24 h. Besides, Kirchner (1999) and Schmuck (1999) found a 78nM dose of imidacloprid affected the waggle dance, communication inside the colony, the recruitment of foragers and the foraging activity. Moreover, sub-lethal doses of imidacloprid

prejudice bee movement as Suchail *et al.* (2000) observed that imidacloprid induce trembling honeybees and Franklin *et al.* (2004) made later the same observation on *Bombus impatiens*.

Yang *et al.* (2008) also noticed that imidacloprid impaired behavior on long term after exposure up to 90 min and suggested that the colony did not completely recover after a single exposure. This suggests that the long-term impact observed on a single bee can be correlated to the contaminated food brought back to the colony to feed the other workers and the brood and suggests a longer exposure inside the colony.

b. The major negative effect of thiamethoxam on bumblebee locomotion and unexpected metabolic issues

The impact of thiamethoxam on bees reflects what has been observed at the colony level. Thiamethoxam is known for reducing the abilities of bees to forage and perform homing flights on the field (Bortolotti *et al.*, 2003; Mommaerst *et al.*, 2010; Schneider *et al.*, 2012; Fisher *et al.*, 2014). In my experiment, bumblebees exposed to thiamethoxam were hyperactive in comparison with the control (no pesticide) and the other neonicotinoids tested and were presenting signs of malaise as grooming and trembling. Williamson *et al.* (2014) compared the impact of four neonicotinoids (imidacloprid, thiamethoxam, clothianidin and dinotefuran) at field realistic dose (10nM) and a plant toxin, nicotine, on honeybee behavior. They observed that bees exposed to thiamethoxam significantly increased the frequency and duration of grooming bouts. In my experiment, the frequency of grooming bouts did not increase in the same proportions but bumblebees were presenting the same loss of postural control and spending significant time lying on their back.

Bumblebees exposed to thiamethoxam also exhibited hyperactivity signs with a loss of coordination in the movements of wings and frenetic movements alternating with moment still and lying on the back unable to right themselves as observed by Williamson *et al.* (2014). On the other hand, El Hassani *et al.* (2008) did not observe any particular vulnerability of honeybees to a gradient of sub-lethal doses of thiamethoxam (below 34nM) after 60 min in their experiment. Thiamethoxam did not impair the responsiveness of bees

to sucrose and water after oral or topical exposure whatever the dose and no extra mortality. But they observed that the impact of thiamethoxam on locomotor activity was more important when it was orally applied than topically. They suggest that thiamethoxam is not a direct-acting agonist or antagonist of nAChRs. The conversion of thiamethoxam into the toxic metabolite clothianidin has been proposed as the cause of its biological effect (Nauen *et al.*, 2003). Moreover, these authors suggest that thiamethoxam has a long-term impact on bee metabolism and behavior. Williamson *et al.* (2014) hypothesized that the loss of coordination when exposed bees are performing motor behavior might be the cause of the foraging and homing impairment and a dose of 1ng of thiamethoxam per bee would be enough to cause these losses (Henry *et al.*, 2012).

Oddly, the average body mass loss observed in my experiment was the lowest for bumblebees exposed to thiamethoxam 10nM. It is possible that these bees died prior to experiencing the weight loss accompanying being fed the diets in the boxes over 7 days.

c. Clothianidin contrasted toxicity

Bumblebees fed with clothianidin were less active as for imidacloprid. This is consistent with observations made on *Bombus impatiens* colonies exposed to highly contaminated pollen (Franklin *et al.*, 2004) and on colonies of honeybees foraging on flowering oil seed rape from treated seeds (Cutler & Scott-Dupree, 2007).

At the level of single bumblebee, clothianidin can induce malaise signs as grooming and trembling (also perceived by Franklin *et al.*, 2004). Besides, the increasing of grooming behavior has been reported for honeybees exposed to other neurotoxic substances that use the same motor function assay, substances such as ethanol and an organophosphate acaricide called coumaphos (Maze *et al.*, 2006; Williamson *et al.*, 2013). Resulting as a sign of malaise, Schneider *et al.*, 2012 also observed that sub-lethal dose of clothianidin can induced arched abdomen that did not reduce the mobility of bees like with imidacloprid and do not reduce the flying capacities (Girolami *et al.*, 2009).

Clothianidin can consequently alter the ability of foraging and collecting food of a single bee at a lower scale than imidacloprid. Schneider *et al.*, (2012) reported that at field relevant doses, clothianidin did not have adverse effects but reduce foraging activity and

increase flight times during both foraging and homing during the first three hours after the exposition. The alteration of the behavior was reversible when the pesticide was orally ingested, Clothianidin elicited detrimental sub-lethal effects at somewhat lower doses than imidacloprid with both oral and topical exposure (Girolami *et al.*, 2009; Bailey *et al.*, 2005; Schneider *et al.*, 2012). Franklin *et al.* (2004) also concluded that clothianidin may have less potential impact on bumblebees compared with imidacloprid as in my experiment on single foragers, but they reported that clothianidin did not have detrimental sub-lethal effects on the foraging ability of workers bees.

Bumblebees exposed to clothianidin were losing less body mass than in the control (no pesticide) and the other neonicotinoids. Franklin *et al.* (2004) did not observe an effect on *Bombus impatiens* worker newly emerged mean weight exposed to different doses of clothianidin (1, 24 and 146nM). They concluded that clothianidin might have less potential impact on bumblebees compared with imidacloprid as in my experiment on single foragers. Additionally, Cutler & Scott-Dupree (2007) did not notice a significant difference on weight gain between honeybee colonies exposed to sub-lethal dose of clothianidin with the control (no pesticide) and the treatment groups. The lower activity combined to the high food intake can explain this lower body mass loss. The lower activity rate observed in my experiment might be due to the low challenge represented by the access to food in the box in comparison to foraging in the field and the communication between bees inside the colony that can stimulate activity of individuals.

3. The miscellaneous consequences of neonicotinoids on survival

a. Contrasted effect of imidacloprid on the mortality rate over diets

Bumblebees fed with imidacloprid 10nM exhibited low mortality rates when they were fed with sucrose only and with EAA. Bees fed with the casein diet were more likely to die than in the control (no pesticide) and the bees fed the other diets. This suggests that imidacloprid influences protein digestion/absorption in a way that causes greater mortality. My experiment also shows that the intake of protein had a critical impact on the first three days suggesting that the metabolic issues that increase the toxicity of imidacloprid can be counteract by bees that survived after these critical days.

Thompson *et al.* (2014) only observed 5% mortality over three day for an exposure to 20nM of imidacloprid in sucrose diet (0.7M). My bumblebees exposed to 10nM imidacloprid and having the sucrose diet were exhibiting a higher mortality at the same stage. Suchail *et al.* (2001) found a substantial mortality at a lower imidacloprid doses on honeybees. This suggests that the experimental time frame affects the sub-lethal impact measured due to imidacloprid on a single or a small group of bees.

b. Dramatic impact of thiamethoxam on survival

In my experiments, thiamethoxam had the most dramatic negative impact on bumblebee survival. Bee survival quickly plummeted, especially when they were fed solutions containing free amino acids, such that most bees were dead within 3 days when fed this diet.

Williamson *et al.* (2014) observed a similar mortality rate for honeybees fed with sucrose and thiamethoxam 10nM as bumblebees in my experiment at day 1. They also indicated that the overnight mortality due to thiamethoxam exposure is significantly dose-dependant and that thiamethoxam might not be distasteful. Likewise, Thompson *et al.* (2014) noticed a 4-fold lower mortality rate over three days for similar thiamethoxam doses. Moreover, they did not observe a significant dose dependence effect on the mortality rate on bumblebees, which may be due to the fact that they chose to exclude all the mortality data for the highest thiamthoxam dose that exhibited 100% mortality after day 3 from statistical analysis.

Besides these observations on single and small group of bees, Cresswell & Thompson (2012) suggested that at the colony level, thiamethoxam would not precipitate collapse in healthy colonies in spring. They suggested that the colonies would be more vulnerable later in the year when the ability to replace lost worker has diminished.

c. Clothianidin low influence on bumblebee mortality

The survival was similar to the control (no pesticide) and slightly decreased when bees had access to protein or amino acids in their diet.

Thompson *et al.* (2014) observed the same mortality rate as in my experiment for the lower doses of clothianidin (<40nM) on single housed bumblebees, but noticed greater mortality (up to 100%) at day 3 for the highest clothianidin dose (400nM). They also decided to not include the data of the highest clothianidin dose and concluded that the mortality due to clothianidin exposure is not dose and day dependent.

Additionally, Cutler & Scott-Dupree (2007) did not notice at the colony level significant differences on honeybee mortality, worker longetivity, brood development and colony weight gain between control (no clothianidin) and treatment groups. They concluded that honeybee colonies will be unaffected on long-term by exposure to clothianidin seed-treated oil seed rape and that clothianidin offers a margin of safety to bees compared to imidacloprid. As observed on bumblebees in my experiment, Iwasa *et al.* (2004) found in their comparative study on several neonicotinoids on a small group of honeybees (n=10-15 per boxes) that clothianidin had the lowest impact on honeybees. These studies suggest that exposed bees would be able to counteract the relative lower toxicity of clothianidin by the compensation of the toxicity on individual within the colony or the group of the effect of the pesticide.

4. Bumblebees daily pesticide intake in my experiment was field realistic

One of the most important aspects of research on the impact of pesticides on pollinators is testing concentrations that are experienced by bees in the field (Williamson *et al.*, 2014). The concentration of 10nM of neonicotinoids used in my experiments is within the range of reported concentrations from nectar and pollen of treated seed (Schmuck *et al.*, 2001; Rortais *et al.*, 2005; Blacquière *et al.*, 2012). Of the three neonicotinoids tested in my experiments, imidacloprid is commonly found at very low concentration (from 0.8 to 15nM), clothianidin is usually present at higher concentration (from 7 to 10nM) and thiamethoxam is frequently found at very high concentration in nectar and pollen in the countryside (from 22 to 34nM) (Schmuck *et al.*, 2001; Bonmatin *et al.*, 2003; Rortais *et al.*, 2005; Cutler & Scott Dupree, 2007; Blacquière *et al.*, 2012).

A neonicotinoid dose ranging from 0.45 to 0.54ng/honeybee can affect bee motor function mainly by disruption of the righting reflex and causing more grooming bouts

(Williamson *et al.*, 2014). Few studies have investigated about the direct effects of neonicotinoids on bee motor function, most of them are focused on foraging impairment and homing abilities. Such intoxication signs were spotted for clothianidin (dose ≥0.5ng/bee), thiamethoxam (dose of 1ng/bee) and imidacloprid (dose exceeding 1.5ng/bee) (Henry *et al.*, 2012; Schneider *et al.*, 2012). These are higher doses of neonicotinoids than the average daily dose ingested by bumblebees in my experiment. My bumblebees were exhibiting different signs of intoxication such as loss of co-ordination, trembling, signs of malaise and days spent lying on the back. The intoxication signs such as foraging impairment and homing failure might be due to the loss of motor function capacities observed for lower doses of neonicotinoids ingested.

To explain this difference in sensitivity observed between honeybee and bumblebee, Cresswell *et al.* (2012) proposed that honeybees are better pre-adapted than bumblebees to feed on nectars that contain synthetic alkaloids, such as imidacloprid, by virtue of their ancestral adaptation to tropical nectars in which natural alkaloids are prevalent. Conversely, Hardston & Scott (2010) compared the toxicity of several pesticides on bees and suggested that bumblebees tend to be less sensitive than the honeybee to various compounds that include neonicotinoids. They suggest that other parameters might be involved and explained this difference of toxicity between honeybees and bumblebees.

5. To conclude

My data support the idea that clothianidin is less toxic and has a lower risk of affecting wild bee pollinator communities. My results showed the impact of one of three neonicotinoids on a single housed bumblebee and also highlighted the impact on bees of chronic exposure. The interpretation of the impact of neonicotinoid sub-lethal doses on colonies and micro-colonies are complicated by the potential storage of non contaminated collected food within the nest and the possibility given to bees to access to non contaminated food too in the countryside. This might explain contrasting results between my experiments on individuals and field experiments. However, wild pollinators in the field may experience contact with pesticides in a way that contributes to their decline (Allen-Wardell *et al.*, 1998) by the exposure to contaminated pollen and nectar. Previously, the

impact of the three neonicotinoids may have been underestimated because multiple pesticides are used, and they may have synergistic effects on survival and behavior with other chemical agents and pathogens. A better understanding of how these pesticides impact other stages of the life cycle is also necessary. More knowledge about the impact of early exposure would provide more information of the evolution of potential more acute impact for bee community.

Chapter 4: Forager bumblebees (B. terrestris) are attracted by low doses of imidacloprid in their food

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Available in Annex D.

I. Introduction

Floral nectar is a rich source of nutrients for bees, which provides them with sugars, organic acids, lipids, minerals, vitamins and aromatic compounds at different concentrations. Moreover, pollen contains proteins but also lipids, mineral salts, albumin, vitamins, amino acids, growth regulators factors, folic acids and enzymes (Harborne, 1993). However both nectar and pollen can contain toxins, pesticides and other chemical compounds, which are not profitable for bees at high concentrations (Sánchez-Bayo, 2011). Many studies have been done to try to explain the presence of secondary compounds in nectar and pollen, but their presence is still not understood. It has been suggested that these compounds protect plants from nectar robbers (Baker *et al.*, 1978; Janzen, 1977), providing antimicrobial properties (Hagler and Buchmann, 1993; Manson *et al.,* 2010), and altering pollinator behavior by reinforcing their fidelity to a flower species or avoiding contact with these flowers (Baker and Baker, 1975; Ehlers and Olesen, 1997; Rhoades and Bergdahl, 1981; Wright *et al*., 2013; Tiedeken *et al.*, 2014).

Taste is a crucial sense for bees that help them to choose profitable edible food sources, recognize their nestmate and for others different aspect of their life. Taste stimuli may play further vital roles in the life of bees (Sánchez-Bayo, 2011). Nevertheless, it seems that generalist bee species have poor acuity for the detection of nectar toxins (Tiedeken *et al*., 2014) Bee are able to detect toxins in food preingestively and the hunger state can influence the sensitivity of bees to toxins. The bee response to toxins may also depend of the concentration of toxins in food and too high concentrations would be rejected (Wu *et al.*, 2005; Wright *et al.*, 2010; Sánchez-Bayo, 2011). In the wild, it has been observed that honeybees can avoid nectar, which contains nicotine, and wild bee species like *Bombus impatiens*, which can avoid foraging flowers in which nectar contains high concentrations of the alkaloid gelsemine (Detzel and Wink, 1993; Hagler and Buchmann, 1993 Adler and Irwin, 2005; Manson and Thomson, 2009). It also has been confirmed in several studies, which have shown that bumblebees and honeybees detect toxins which have a bitter taste and learn to avoid floral traits associated with the compounds in the sucrose rewards (Chittka *et al*., 2003; Mustard *et al*., 2012; Wright *et al*., 2010). However, antennal and tarsal gustation sensitivities can also be increased by starvation time and decreased if one of these gustation receptors is damaged (de Brito Sanchez *et al.*, 2008). Sánchez-Bayo (2011) also suggested

that considering bitter solutions as deterrent is incorrect and that aversion or preference may also depend of resources available for bees. Honeybees do not present an avoidance behavior when different concentrations of quinine are associated with sucrose 1M. Bitter taste is not represented as a separate perceptual quality in food and no cell receptors exist on antennae tips of honeybees (de Brito Sanchez *et al.*, 2005).

Pesticides are toxic compounds widely used in agriculture to protect them from pests. Foraging social bees are directly exposed to pesticides in their food, which is collected and then shared and stored within the colony (Rortais *et al.*, 2005). For example, in an extensive study on pesticide residue on North American honeybee apiaries, Mullin *et al.* (2010) found that 87 pesticides and metabolites were found on 259 wax samples (with an average of 8 pesticides and a maximum of 39), 98 pesticides and degrade residues on 350 pollen samples (with an average of 7 pesticides and up to 31 different pesticides) and 49.9% of those samples contained systemic pesticides. Pesticide presence in pollen and nectar might change bee behavior in response to their sensory perception by reducing bee foraging activity or feeding stimulation (Haynes, 1988). Those avoidance behaviors are commonly considered as a protective behavior to reduce the risk associated with these potentially dangerous chemicals.

Neonicotinoids are commonly used as systemic pesticides in plants visited by bees such as oilseed rape (OSR), corn cotton, sunflower and sugar beets (Elbert and Haas, 2008). Exposure to neonicotinoids is more likely to occur because bees focus their visits on the most important sources of pollen and nectar that represent flowering crop, which appear throughout seasons (Westerkamp, 1991 ; Forup & Memmott, 2005). In case of seed-dressing treated plants, the pesticide becomes systemic and is translocated to nectar and pollen (Blacquière *et al.*, 2012). Overall, a higher toxicity is observed by oral route than contact mode, which can be explained by the weak of hydrophobicity of neonicotinoids.

One of this systemic neonicotinoids is imidacloprid, which is commonly known as under a large number of trade names (Admire, Confidor, Conguard, Gaucho, Intercept, Kohinor, Mallet, Turfthor, Winner). Imidacloprid was the first neonicotinoid commercialized and is still one of the most commonly used chemical on crops (Decourtye and Devillers, 2010). Numerous feeding test studies on honeybees have shown that imidacloprid is toxic and presents an oral LD_{50} 22 times higher than contact LD_{50} (Schmidt, 1996). Honeybees exhibit

symptoms of poisoning after oral ingestion of sublethal doses of imidacloprid such as stationary and inactive behavior, movement coordination disorders, tumbling, hyperactivity and tremors (Suchail *et al.*, 2001; Medrzycki *et al.*, 2003; Colin *et al.*, 2004). It is also important to note that imidacloprid can also induce opposite effects on activity depending on the dose (Lambin *et al.*, 2001).

Imidacloprid can influence subsequent social colony life and foraging (Thompson and Maus, 2007; Desneux *et al.*, 2007; Mommaerts and Smagghe, 2011) but also affects whole colonies, as colonies exposed to thiamethoxam and imidacloprid have been reported to have less brood and higher worker mortality (Columbo and Buonocore, 1997; Taséi *et al.*, 2001; Alarcòn *et al.*, 2004). Bees can change their foraging behavior in response to pesticide presence (Haynes, 1988). This response can be considered as a protective behavior to reduce the risk associated with these potentially dangerous chemicals (Decourtye and Devillers, 2010). On the other side, Tasei *et al.* (2001) did not observed any differences regarding the presence of *Bombus terrestris* on blooming sunflower heads and their visit durations between imidacloprid treated and control flowers. Similar observations have been done with *Bombus impatiens*, which did not show any deterrent behavior for imidacloprid 7ppb, but foragers were less active when they were exposed to imidacloprid 30ppb (Morandin and Winston, 2003). A delay of the inhibition of the foraging activity can appear depending of the concentration of imidacloprid tested, as for example a quick decrease of honeybee activity can be observed at about imidacloprid 20ppb (Schmuck, 1999; Decourtye *et al.*, 2004). This observation has been confirmed by different studies that suggest that the honeybee foraging activity decreased when they were exposed to concentrations above 20ppb (Mayer and Lunden, 1997; Kirchner, 1999) and might be due to the anti-feedant character of imidacloprid (Decourtye and Devillier, 2010). It also has been observed that honeybees have a lower foraging activity when they are exposed to imidacloprid doses between 20-100ppb due to induction of trembling dances to prevent other bees and reduce the recruitment for this source of food (Kirchner, 1999).

The impact of neonicotinoids in the wild are unclear and depend on whether or not bees can avoid foraging on crops with pesticides in nectar and pollen. Many lethal and sublethal effects have been described in laboratory studies, but field studies tend to report fewer impacts on bees when they are exposed to the doses found in nectar. Among the

various approaches in laboratory studies tested the toxicity of imidacloprid on bees, most methods used in previous experiments were forced choice assays. The purpose of the experiment was to study the impact of low sublethal doses of imidacloprid on the food choice depending of the caste of bumblebees. My assay will determine whether bees can detect and avoid pesticides in sugar solutions at pesticide levels found in nectar in the field. I am also testing whether foragers and newly emerged bumblebees have the same ability to detect imidacloprid.

II. Material and Methods

1. Animals

All experiments were performed with worker bumblebee colonies (*B. terrestris terrestris* L.) provided for commercial uses (Koppert B.V., AD Berkel en Rodenrijs, Netherland) and performed in laboratory conditions (22-25°C and 65-80% RH) and continuous darkness in August 2013 in Newcastle University bee lab. Bumblebees were provided *ad libitum* with commercial sugar water and pollen (Pollen mix, Koppert B.V., AD Berkel en Rodenrijs, Netherland) until the beginning of each experiment.

Bumblebees were collected from colonies under red light with rubber forceps. Newly-ecclosed workers were collected based on visual inspection: when they are newly emerged, they are pale in color and have a greyish-yellow stripe on the abdomen (this stripe is bright yellow in older workers; Figure 4.1) (Pouvreau, 1984). Newly-ecclosed workers are often smaller than foragers and tend to stay on the nest their entire life as nurses (Goulson *et al.*, 2002). To avoid any food consumption differences cause of the difference of body size, we collected workers of a range of body length (12-14 mm) and width (4-6 mm).

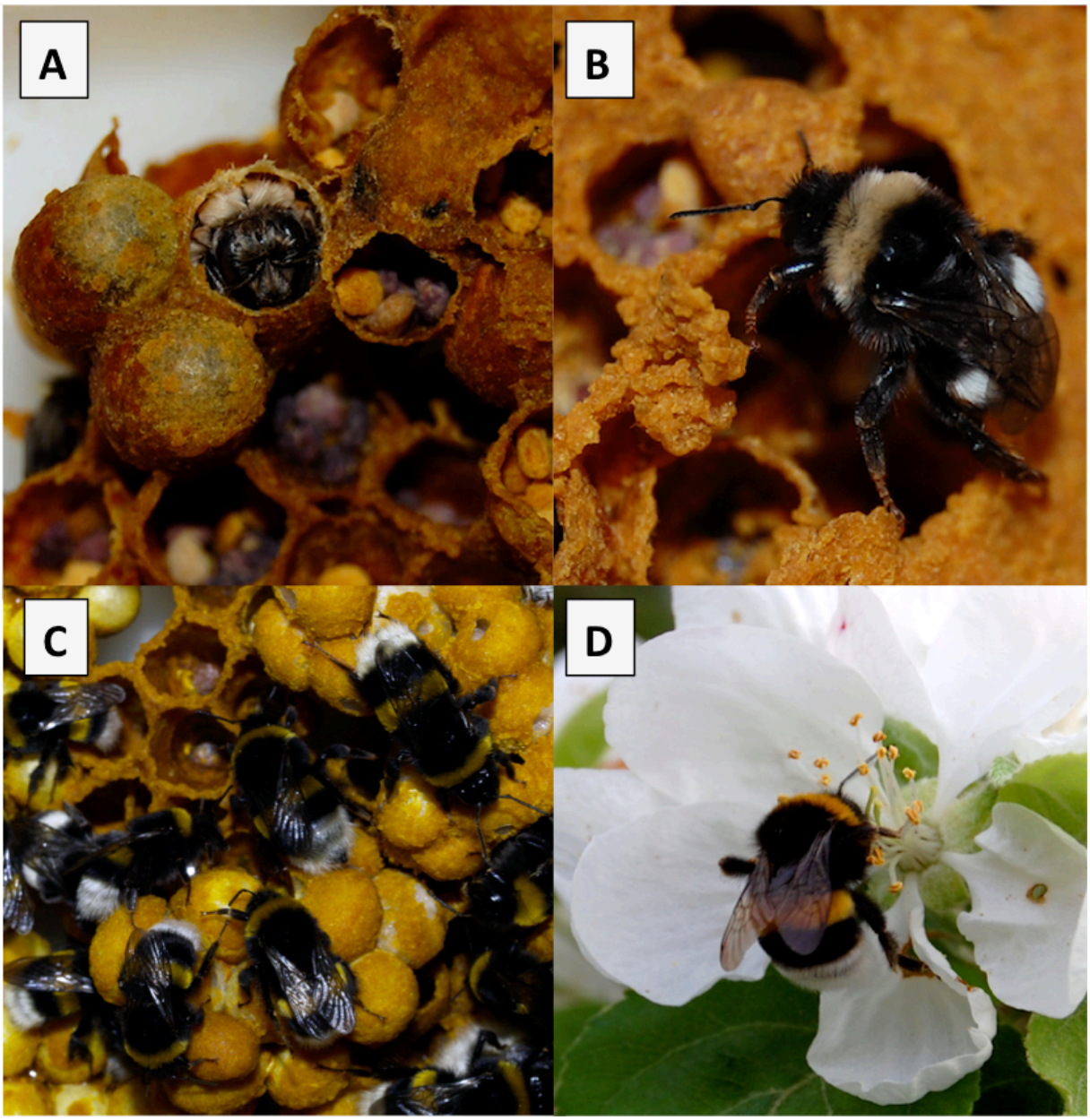

Figure 4.1: Distinguishing newly emerged bumblebees form adult foragers. (A) Emerging bumblebee with greyish thorax stripe; (B) Newly emerged bumblebee with a greyish thorax stripe and a white stripe on the 2nd abdominal segment; (C) Adult bumblebees with bright yellow thorax and 2nd abdominal segment stripes; and (D) Adult forager with bright yellow stripes foraging on an apple flower (© Sophie Derveau).

Bumblebees were housed housed in individual plastic boxes (17x12x7cm) with access to 3 liquid diets until the end of the experiment provided in 2ml microcentrifuge tubes (4 holes were drilled on the top side of the tube, Figure 4.2). The boxes were placed in the incubator 21-22.8°C and 40-60%RH in continuous darkness. Experiments were conducted for 3 consecutive days.

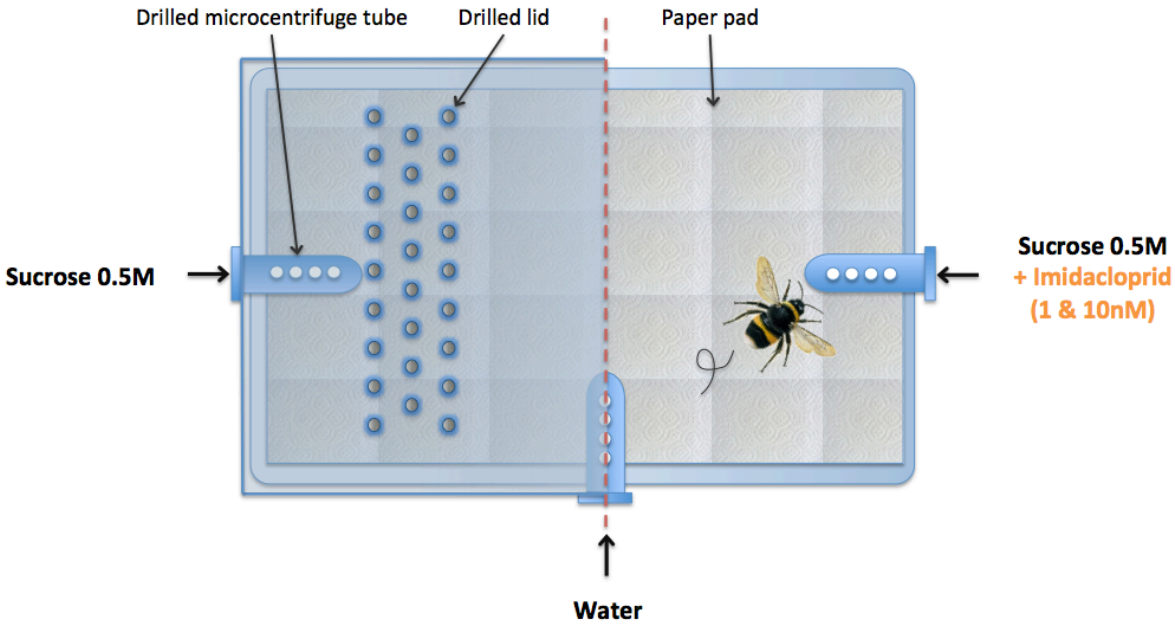

Figure 4.2: Individual bumblebee in feeding box with the 3 different liquid diets provided in drilled Eppendorf tubes.

2. Diets

To assess if bumblebees have a preference for food that contain or no imidacloprid sub-lethal doses depending of their age, bees were having access to three 2 ml microcentrifuge tubes: one containing water, one containing 0.5 M sucrose solution (Sucrose Grade II, Sigma-Aldrich), and another containing 0.5 M sucrose solution and a dose of imidacloprid (>99% purity, Sigma- Aldrich). Solutions of imidacloprid were made to concentrations 1nM and 10nM directly dissolved in 0.5 M sucrose solution (Table 4.1). Fresh solutions were prepared for each 3 days run. Each microcentrifuge tube had 4 holes drilled

on the topside of the tube for the bees to access the solution. Two concentrations of imidacloprid (1 nM & 10 nM) were used in the test solutions.

	Solution compositions	
Sucrose	**0.5M**	
Imidacloprid	**1nM**	**10nM**
Newly emerged	27	27
Adult forager	31	32

Table 4.1: Sample size for each liquid diet treatments. The experiment was done in two runs that included the two concentrations of imidacloprid (1 and 10nM) for the two different ages of bumblebees tested (newly emerged and adult forager).

Boxes were removed from the incubator daily to weigh each tube containing liquid diets and replaced them by new solutions. The amount of food consumed was measured by the weight difference on a 24-hour period. Different parameters of the body were measured: body length, thorax width and length, abdomen width and length, and head width and length to study the homogeneity of the different population used in each trial. Bumblebees were placed in a freezer at -80°C in a labeled envelop for further investigations.

A control for the evaporation rate of each diet was performed for 3 days in empty boxes placed in the same conditions as the trial boxes to measure the weight loss in feeding tubes. An average of evaporation rate has been done and subtracting to the value obtained during the experiment. Negative and null values were replaced by a null value and considered as there was no food consumption in the tube. The total of food consumption was calculated by multiplying the amount consumed by the weight of sucrose in 1ml of solution.

3. Statistics

To study the interaction of the age, the dose of imidacloprid (0, 1 & 10nM) and the food preference (no pesticide or imidacloprid) on the average food daily intake, a repeated-measures ANOVA was performed. To measure the effect of imidacloprid dose on the average daily intake a one-way ANOVA was performed for each diet (no pesticide and

imidacloprid) on the subset of data of newly-emerged bumblebees and adult worker bumblebees. The daily feeding behavior depending of the age and the dose of imidacloprid was analyzed by repeated-measures ANOVA. All non-significant interactions in models performed during the statistical analysis were removed and the new models were compared to the previous one. If the new model was significantly different of the previous one, the new model was rejected and not presented in the following result section. All analyses were performed using IBM SPSS v15.0 software (SPSS Inc., Chicago, IL).

4. Why running the experiment on 3 days?

The experiment has been conducted for 3 consecutive days as nurses can start to express genes to become foragers 2 days after emergence depending of the needs and the food stress in the colony (Pouvreau, 1989; Yerushalmi *et al*., 2006). Those genes increase their odor, light and visual resolution sensitivity (Kapusjanskij *et al*., 2007; Spaethe & Chittka, 2003) and can bias the results.

III. Results

1. Bumblebees can detect imidacloprid within food

My results suggest that bumblebees were able to detect imidacloprid in the food provided (Figure 4.3). A three-way interaction showed that the feeding behavior was significantly influenced by the interaction of the age of the bee, the type of food chosen (no pesticide or imidacloprid) and the dose of imidacloprid in the food (Table 4.2). When bumblebees were offered the choice between sucrose and sucrose containing imidacloprid food, adult foragers chose the imidacloprid solution with a clear preference for the lower dose (One-way ANOVA, dose main effect, $F_{(1,55)}$=13.9, P<0.001). Unlike newly emerged bumblebees that avoided both doses of imidacloprid within the food equally (One-way ANOVA, dose, $F_{(1,62)}$=1.86, P=0.177). The average daily sucrose consumption was the same for both newly-emerged (One-way ANOVA, dose main effect, $F_{(1,62)}$=2.77, P=0.101) and adult foragers (One-way ANOVA, dose main effect, $F_{(1,55)}$=1.94, P=0.170).

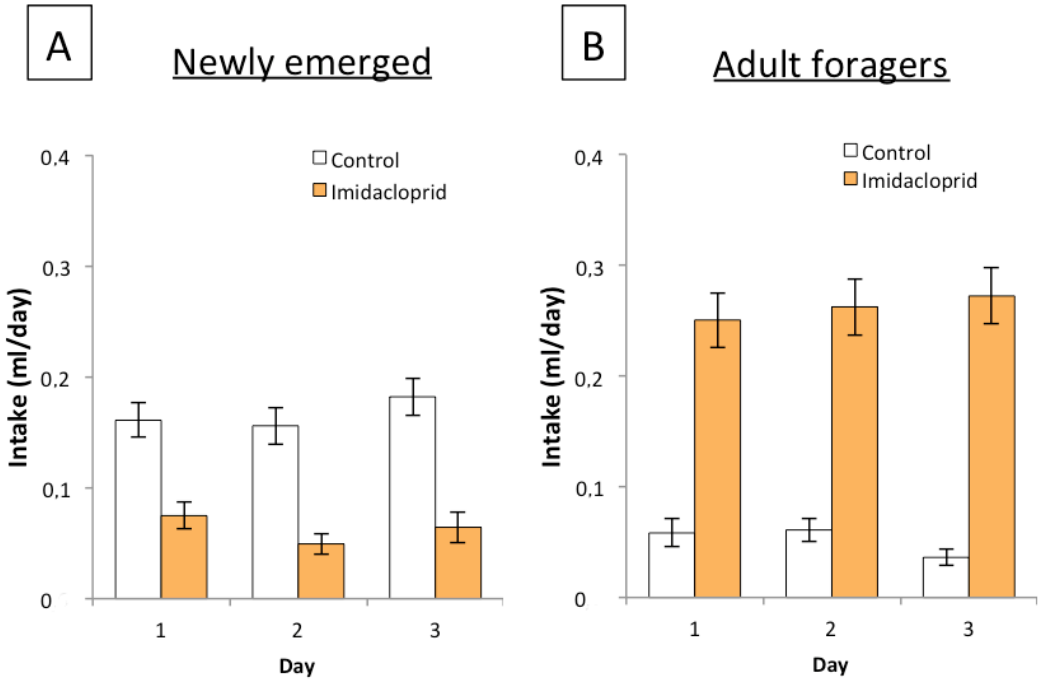

Figure 4.3: Comparison of the average daily intake (± standard error) between two sucrose diets (no pesticide *vs.* imidacloprid 1nM or 10nM) depending of the age of bumblebees for the three days. For the number of specimens per run, please refer to Table 4.1 in Material and Methods.

Source of variation	Type III Sum of Squares	Mean Square	F	df	P-value
Intercept	11.454	11.454	1143.033	1	<0.001
Age	0.277	0.277	27.620	1	<0.001
Dose	0.207	0.207	20.639	1	<0.001
Choice	0.488	0.488	48.705	1	<0.001
Age x Dose	0.054	0.054	5.359	1	0.022
Age x Choice	3.910	3.910	390.162	1	<0.001
Dose x Choice	0.068	0.068	6.749	1	0.010
Age x Dose x Choice	**0.145**	**0.145**	**14.447**	**1**	**<0.001**
Error	1.994	0.010		199	

Table 4.2: Results of tests of between-subject contrasts of repeated-measures ANOVA with variable transformed in averages to test the daily food consumption of bumblebees depending of their age (newly emerged and adult foragers) and depending of their feeding choice for one of the two diets offered (no pesticide *vs.* imidacloprid 1 & 10nM). For the number of specimens per run, please refer to Table 4.1 in Material and Methods.

2. Bumblebees were consistent in their food preferences over the 3 days

The variation of the average daily food consumption was also analyzed in the 3-day experiment. The average daily intakes of both sucrose solution and sucrose with imidacloprid solution were significantly different for newly emerged bumblebees and for adult foragers, and moreover bumblebees returned to feed according to their food preferences over the three days. A three-way interaction showed that the feeding behavior over the three days was significantly influenced by the day, the food preferred (no pesticide or imidacloprid) and the age of the bumblebee (Table 4.3). Newly-emerged bumblebees were significantly more attracted by the sucrose tube and avoided consuming contaminated food, and the average daily volume of each diet was constant over the three days (Figure 4.4). The opposite behavior was observed for adult foragers, which were significantly more attracted by diets containing imidacloprid with the same constancy of average daily intake of both sucrose and sucrose containing imidacloprid food (Figure 4.4).

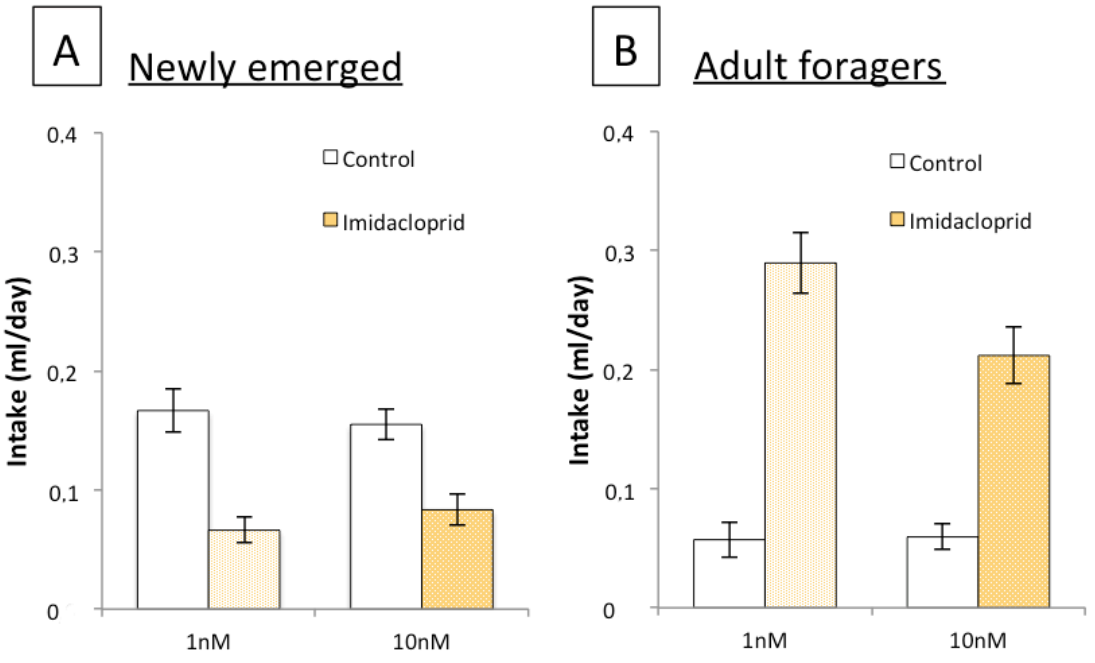

Figure 4.4: Comparison of the average intake (± standard error) over three days between two sucrose diets (no pesticide *vs.* imidacloprid 1 & 10nM) depending of the age of bumblebees. For the number of specimens per run, please refer to Table 4.1 in Material and Methods.

Source of variation	Type III Sum of Squares	Mean Square	F	df	P-value
Day	8.886E-5	8.886E-5	0.014	1	0.907
Day x Age	6.854E-6	6.854E-6	0.001	1	0.974
Day x Dose	0.025	0.025	3.842	1	0.051
Day x Choice	0.000	0.000	0.029	1	0.864
Day x Age x Dose	0.003	0.003	0.516	1	0.474
Day x Age x Choice	**0.033**	**0.033**	**4.998**	**1**	**0.026**
Day x Dose x Choice	0.015	0.015	2.288	1	0.132
Day x Age x Dose x Choice	0.007	0.007	1.139	1	0.287
Error(day)	1.300	0.007		199	

Table 4.3: Results of tests of within-subject contrasts of repeated-measures ANOVA with variable transformed in averages to test the daily food consumption of bumblebees depending of their age (newly emerged and adult forager) and depending of their feeding choice for one of the two diets offered (no pesticide *vs.* imidacloprid 1 & 10nM). For the number of specimens per run, please refer to Table 4.1 in Material and Methods.

IV. Discussion

My results suggest that regardless of the concentration of imidacloprid, newly-emerged bumblebees and adult foragers did not have the same feeding behavior as expected in our hypothesis. This finding first proved that bumblebees were able to detect imidacloprid in the sucrose solutions and were able to choose which one eating depending of their age. Newly-emerged bumblebees avoided food containing imidacloprid, when adult foragers were attracted by food containing imidacloprid, especially by low concentration of imidacloprid. Those feeding behaviors were constant on the 3 days of the experiment.

1. Foragers attraction to sub-lethal imidacloprid doses

As suggested in our hypothesis, foragers were able to detect imidacloprid in sucrose solutions and were also significantly more attracted by the low imidacloprid dose. This behavior was constant on the 3 days of the experiment. This behavior can be compared to nicotine addiction studies on mammals. Neonicotinoids like imidacloprid are targeting cholinergic signals to compromise the majority of neurotransmissions in insect central nervous system (Millar & Denholm, 2007) and have a similar effect as nicotine on receptors

in the brains of mammals. Nicotine acts as an agonist on nAChRs, which are found at the neuromuscular junction, in the peripheral and central nervous systems. It has psychoactive effects by binding with those specific nicotine binding sites (Steinbach, 1990; Luetje & Patrick, 1991).

Cigarettes and other form of tobacco consumption cause addiction. Nicotine is a substance found in tobacco and acts as a psychoactive drug classified as a drug of abuse (US Department of Health and Human Services, 1988). Humans and as well a large number of species including rats, squirrel monkeys can become addictedto nicotine and can self-administer intravenous nicotine at dose level comparable to those taken by a human smokers (Benowitz, 1996). This self-administration behavior has been also observed for other addicting drugs and provided methods for studies about exploring mechanisms of reinforcement (Corrigall *et al.*, 1994).

It has been demonstrated that neonicotinoids act as nAChRs agonists;imidacloprid has been shown to be a partial agonist of nAChRs in dissociated honeybee Kenyon cells (KCs) in culture (Déglise *et al.*, 2002; Dupuis *et al.*, 2011). The KCs are the major neuronal component of the mushroom body and represent more than 40% of neurons in the brain of honeybees (Rössler & Groh, 2012). The mushroom body is the higher brain structure of insects and mediates multisensory integration, learning and memory (Zars, 2000; Heisenberg, 2003). It has been proved that neonicotinoids can disrupt cognitive functions managed by the mushroom body (Belzunces *et al.*, 2012; Blacquière *et al.*, 2012). Palmer *et al.* (2013) observed that imidacloprid inhibited action potential that impaired mushroom body function by affecting the neurophysiological properties of KCs and they also assumed the exposure to multiple pesticide sources that target cholinergic signaling would increase the toxicity of those compounds on pollinators.

2. Newly-emerged avoided imidacloprid

As previously said, imidacloprid is an acetylcholine mimic that activates nAChRs hereby interfering in neuronal synapses. In case of overstimulation of those neuronal pathways, a paralysis following by the death of the insect is observed (Matsuda *et al.*, 2001).

Imidacloprid is expected to negatively affect the behavior of bumblebees and the avoiding behavior of newly-emerged can be interpreted in many different ways.

The newly-emerged bumblebees avoidance behavior can be a protective behavior toward larvae to protect them from pesticide during their development as bumblebees act as nurses the first few days after emergence (Pouvreau, 1989; O'Donnell *et al.*, 2000) and can start to forage for pollen as early as two days after emergence for the larger workers (Pouvreau, 1989; Yerushalmi *et al.*, 2006). The age of foraging depends of the needs of the colony (Robinson, 1992), as smaller bees can forage to comply the nutritional needs of the colony (Free, 1955; Goulson, 2010) and the low expression of *Btfor* genes in nurse caste (Brothers, 1999; Tobback *et al.*, 2011).

Imidacloprid sensitivity might not be the same between the newly emerged and foragers and might act differently on nAChRs of young bumblebees than on adults. Brelau *et al.* (1991) observed that in the case of nicotine, the dependence is 20% more important in young adults. They described a stronger impact on young adults as prevalence of psychiatric disorders like major depression and anxiety disorders for young adult smokers and a prevalence of associated dependence to other substance as alcohol, cannabis and cocaine. They also observed that young nonsmoker adults had a higher rate of other substance dependencies without the negative major side effects observed for the young smoker adults. Age dependence is also observed for rats, which have a differentially sensitivity to both acute and repeated nicotine injection on adolescent bran relative to adult brain. A single nicotine injection during adolescence can induce a significant conditioned place preference and a substantial inhibitory response of the locomotion activity that is not observed in later ages (Belluzzi *et al.*, 2004). Nicotine also affects more young rats capacities to answer to acoustic stimulation than older rats and makes them less reactive to their environment as a repulse inhibition is observed (Acri *et al.*, 1995).

Nicotine solutions are reported to be bitter and can elicit more ingestive responses than tap water by an immediate higher palatability of nicotine (Flynn *et al.*, 1989). Adriani *et al.* (2002) tested the vulnerability to nicotine solution and the evolution of the taste depending of mice age. Mice were free to drink tap water or nicotine solution (10mg/l) and a lower concentration of nicotine was offered in the following trials to test any compensation behavior of nicotine intake. They observed that young adolescent mice had a significant

preference for nicotine solution, increased their nicotine solution consumption when the nicotine concentration was reduced to compensate their nicotine intake and that nicotine produced a prominent hyperactivity. This behavior has not been observed for middle adolescent, which did not show any preferences for any solution, and late adolescent mice were avoiding nicotine solutions, which suggested avoidance for the bitter taste of nicotine solution.

3. Consequences for the bumblebee life circle and colony health

Foragers are more attracted by imidacloprid-contaminated food but workers avoided that food and can have a strong impact on the brood production, quality and survival. Tasei *et al.* (2000) observed a significant lower number of adults produced per colony when colonies were exposed to sub-lethal doses of imidacloprid in pollen and nectar. The impact of sub-lethal doses on the production of adults was the same for the two doses tested (imidacloprid 40nM and 100nM in nectar and pollen). This study also suggested that the new workers fed during their larval stage with contaminated food were more likely to die young and that the mortality observed in the study was restricted to newly emerged workers. Those newly emerged workers were significantly smaller that the adults present at the beginning of the experiment which may be due to a lack of care and food providing by workers exposed to contaminated food and their contaminated food avoiding behavior. Thus, despite of impact on adult bumblebees, the impact of imidacloprid on brood may be more damaging for the colony health if flexibility in labor division is no longer possible due to the decline in the number of adult bumblebees in the colony. The process of trophallaxis is an important factor to understand the impact of food containing imidacloprid on larvae future. An accumulation of insecticides is observed among the worker bumblebees and high imidacloprid doses may cause a reduction of sugar water consumption (Nauen *et al.*, 2001).

The presence of imidacloprid in pollen and nectar inside the nest can have a strong impact on the future newly emerged as their nurses would avoid to consume that food and might not share it with larvae in development. Bumblebees belonging to a same colony have different sizes. This size difference can be due to the position of larvae inside the nest and well-fed larvae might become larger adult than less-fed larvae (Couvillon & Dornhaus, 2009).

The size of the bumblebee also has an incidence to its future cast: larger bumblebees would forage to provide the food for the colony survival (Spaethe & Weidenmüller, 2002; Goulson *et al.*, 2012), exhibit an increased odor and light sensitivity (Kapustjanskij *et al*., 2007; Spaethe *et al.*, 2007), have a better visual resolution (Spaethe & Chittka, 2003) and a faster learning capacities (Worden & Papaj, 2005). A reduction of the size of bumblebees can have in impact on the cohesion of the colony. If the size of adult bumblebee decreases, a tenfold difference in the colony biomass can be observed (Alford, 1978; Goulson *et al.*, 2002).

Fruit fly *Drosophila melanogaster* presents an allelic variation of the *foraging* (*for*) gene, which is responsible of behavioral polymorphism. In the presence of food, both larvae and adult of rover phenotype are more active than sitter phenotype (Sokolowski, 1980; Pereira & Sokolowski, 1993). This active food foraging behavior can be linked to higher PKG activities in the brain for rover phenotype (Osborne *et al.*, 1997) and to the *for* gene expression, which depends of food deprivation (Kaun *et al.*, 2007). Honeybee foragers have a higher level of *Apis mellifera foraging* (*Amfor*) mRNA and higher PKG enzymatic activities than honeybee nurses (Ben Shahar *et al*., 2002). The transition from nurses to forager for honeybees is aged dependent and is coupled to an increase of *Amfor* gene expression around the onset of foraging initiation (Heylen *et al*., 2008). The higher expression of *Amfor* genes in honeybees is related to the changes in phototactic behavior, which is modulated by cGMP second messager activity and therefor PKG enzyme activities (Ben Shahar *et al*., 2002). Tobback *et al*. (2011) observed that PKGs enzymes have the same role in labor division for bumblebees and honeybees. They noticed that *Bombus terrestris foraging* (*Btfor*) gene expression is age dependent like honeybees and is higher in the larger foragers in comparison with smaller sized nurses. They also observed that exposure to sub-lethal doses of imidacloprid (≈80nM) have for consequences a lower *Btfor* gene expression with a stimulation of ovarian growth and a shift towards nest-related tasks. The synergy of food depravation due to newly emerged avoidance to imidacloprid and an exposure to sub-lethal dose of imidacloprid can force the colony to produce smaller bumblebees, which would massively become nurses.

An increasing number of studies on the field and in laboratory show that the exposure to sublethal doses of neonicotinoids have an impact on bee survival, learning and memory capacities, navigation abilities and significantly reduced foraging activity (Belzunces

et al., 2012; Gill *et al.*, 2012). The effects of cholinergic pesticides on KCs can cause significant impairment on cognitive functions including multisensory integration (Zars, 2000; Heisenberg, 2003) and preserving the integrity of nAChRs is important to maintain optimal memory performance (Felix & Levin, 1997). Saldago and Saar (2004) suggest that exposure to neonicotinoids causes nAChRs desensitization and disruption of KC functions.

Moreover, imidacloprid can cause an immunosupression to honeybees. The occurrence of sub-lethal doses of imidacloprid on the field suggests that it might have a negative effect in wild conditions. It also suggests more appropriate guidelines for testing chronic and sub-lethal effects of pesticides used in agriculture (Di Prisco *et al.*, 2013).

4. Conclusion

Forager attraction for food contaminated by neonicotinoids as imidacloprid can have a chain of consequences inside the colony, which can rapidly lead the colony to a quick decline. The foraging activity of *Bombus terrestris* did not show any difference of the presence of workers on blooming heads and in the visit duration of workers between imidacloprid-treated and control sunflowers on the field (Tasei *et al.*, 2001) that show that the food quality provided by foragers is versatile. The co-exposure to other pesticides can also exacerbate the effects of neonicotinoids (Belzunces *et al.*, 2012; Iwasa *et al.*, 2004). The newly-emerged bees could avoid the food provided by foragers and can change their behavior inside the colony. As previously exposed, newly-emerged are nurses for at least two days or the rest of their life. Nurses are in charge of providing the food collecting by foragers to larvae. The avoidance behavior of newly emerged bumblebees in case of traces of imidacloprid in pollen and nectar can deprive larvae of food. Food deprived larvae are more likely to be smaller, yet smaller individuals inside the colony are intended to become nurses. Even if nurses can become foragers in case of colony needs, the process would take couple of days and would upset the organization inside the colony and the stock of food would continue to severely impact the colony survival. To finish, Whitehorn *et al.* (2012) explained that neonicotinoids are implicated in bees decline as they occur at trace levels in nectar and pollen of crop plants. After exposing bumblebees to field relevant concentration of imidacloprid in laboratory conditions, they placed them on the field and observed a

significant reduction of the growth rate of colonies and a reduction of 85% of new queen production in comparison with control colonies. Authors suggested that their results can give an estimation of the negative impact of imidacloprid on wild bumblebees populations across developed countries. Our results suggest that exposure to sub-lethal doses of imidacloprid can have a quick impact on the whole colony and might lead a decline in short terms.

Synthesis and final discussion

I. Synthesis of the results

The research presented in this thesis has made a significant contribution to our knowledge of some of the likely impacts of neonicotinoid pesticides on bumblebees. By quantifying the responses of different life-traits to three neonicotinoids of bumblebees under different experimental conditions, new evidence of the role of neonicotinoids was provided at the scale of the individual worker bee that may explain some of the likely causes of the decline of bumblebee colonies and populations. The severity and consistency of the sub-lethal effects observed over the three neonicotinoids and parameters (nutrient intake, behavior, survival and weight loss) tested are of concern not only from the perspective of the sustainability of the bee community, but also from the perspective of the sustainability of the pollination service in natural and agricultural systems. As neonicotinoids are currently the most common insecticides used to protect crops, it stands to reason that their systemic and remarkable neurotoxic properties towards insects at very low dose make them one of the main factors that explain the decline of bees. The exposure to field realistic sub-lethal doses showed that a single bumblebee had the ability to adapt its nutrient intake to enhance its survival and behaviors, the specific toxicity and the sub-lethal dose of the neonicotinoid.

The exposure to different sub-lethal doses of imidacloprid described in Chapter 1 provided the opportunity to observe how different doses of imidacloprid (0, 1, 10 and 100nM) can affect individual bumblebees and emphasized the dose dependent response of bumblebees to the different parameters considered (nutrient intake, survival, survival, behavior and body mass loss). The results were predictable in that higher doses of imidacloprid exacerbated effects observed for the lower doses. Importantly though, the study showed that even if bumblebees shifted towards a higher protein diet in response to the exposure to sub-lethal doses of imidacloprid, they surprisingly had a greater mortality when they had access to high protein diets. It also confirmed observations previously done at the colony level that imidacloprid can reduce appetite of bees for all but the lowest dose of imidacloprid (1nM) I used. I will return on this observation later in this discussion when I will examine the results of Chapter 4.

Findings from the experiment that assessed the impact of the ease of access to food (Chapter 2), demonstrated that the pesticide (imidacloprid 10nM) can affect the nutrient needs in relation to the behavioral impairment observed in the Chapter 1 (Figure 1.4). As

formerly observed in Chapter 1 and confirmed in Chapter 2, imidacloprid sub-lethal doses induced trembling, discoordination of the movements of wings and legs, and a distal paralysis of tarsi has been observed. It had become clear that these impairments may affect the capacity of bees to access to food and can consequently change their nutritional needs, metabolism and survival. It was confirmed by the results that show striking differences within both controls (LT *vs.* HT) and pesticides (LT *vs.* HT), and furthermore between both controls (LT *vs.* HT) and pesticides (LT *vs.* HT).

The comparison of the three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) 10nM in Chapter 3 demonstrated that they do not all have the same toxic impacts on bumblebees. This suggests that neonicotinoids do not only target the nAChRs, they also may target separate nAChRs and have different metabolic effects on bees. Imidacloprid reduced the activity of bumblebees in general in a way that was consistent with the lower appetite and the impairments previously described. Clothianidin had lower related toxicity in comparison with the other two neonicotinoids, but bees were exhibiting low activity and largest signs of malaise affecting important behaviors such as the proportion of time spent grooming. Thiamethoxam had the highest toxicity and was also associated with hyperactivity, with lower appetite and a substantial premature mortality (Figure 3.4).

The novelty of the experiment in Chapter 4 was in the choice given to a single worker bumblebee to choose between diets that contained imidacloprid (0 *vs.* 1 and 10nM) and considering the age of the worker bumblebee (newly emerged *vs.* older) to explain its food preferences. The diets containing sub-lethal doses of imidacloprid were deterrent for newly emerged bumblebees, while they were attractive for older bees. These results were also dose dependent as the lower dose (1nM) was more attractive for older bees and less deterrent for newly emerged. The concomitant observations of the higher average daily intake of bees exposed to imidacloprid 1nM than for the other higher doses of pesticide in Chapter 1 can be considered as an unexpected confirmation of this phenomenon.

II. Originality and limitations of my experiments

While significant progress in understanding the interaction of bumblebee life-traits (morphological, physiological and phenological characteristics that affect individual performance) and diet with sub-lethal of neonicotinoids has been made in achieving the aim of the thesis, there are a few caveats connected to the methods used.

By using the Geometric Framework (GF) for nutrition to categorize the optimal nutrition of bumblebees, a paired-diet design provided a clear opportunity to let the worker bumblebees balance their intake for each nutrient class (carbohydrates and protein/EAAs) and achieve their intake target. As worker bumblebees mainly require protein for somatic maintenance, they represented an ideal model to test how the dietary requirements interact with neonicotinoid pesticides and the consequences on the life-traits of bumblebees in absence of sexual reproduction.

Nonetheless, bumblebees are social organisms that live in a colony of hundreds of individuals that achieve different tasks and interact with the other individuals inside the nest. In my experiments, bumblebees were single housed without any possibility of any social interactions. This has allowed studying all parameters at the scale of a single bee but can be altered by the stress of the lack of interactions and the enclosed life inside the box.

The difference of my study with field relevant exposure of forager bumblebees is that my bees did not have to fly and forage to have access to food. This in turn suggests that bumblebees that have to forage would require more nutrients and have greater pesticide intake that can consequently have greater impacts on their motor functions. Such kind of detail of the behavioral observations should be taken into consideration when pesticides are tested for ecotoxicity. The observation of the fluctuating behavior after the exposure to pesticide would rapidly affect the long-term impact on bee motor function and could be used as a reliable bioassay for sub-lethal effects on pollinators.

These may also help to explain the different observations about the sub-lethal toxicity of neonicotinoids at the colony level where the other individual can have access to non-contaminated food in the field that can help the whole colony to counteract the effect of contaminated food encounter on the field or displayed inside the colony.

III. Discussion of the results

In considering whether the quality of diet provided and the exposure to neonicotinoid pesticides can play a significant role in their nutritional requirements, behavior and survival, a number of questions need to be addressed for further studies on the impact of neonicotinoids and the conservation of bumblebees. This fall into 5 areas, some of which have been considered directly in this thesis and others will be discussed in the context of requirements for further research:

i. Can bumblebees reach their own nutritional requirements and efficiently assess the needs of their colonies in agroecosystems?

Even if bumblebees are generalist pollinators, their nutritional requirements are specific and change with the exposure to neonicotinoids. Stabler *et al.* (*in review*) demonstrated that bumblebees are able to balance their intake target of carbohydrates and protein (casein) in a range of diets between 1:250 and 1:25 (P:C ratio) but are unable to regulate both carbohydrates and protein/EAAs simultaneously outside this range. The different plant species vary considerably in the quality rewards they offer to pollinators and very little is known about the way that influence pollinator foraging behavior (Hanley *et al*., 2008). Pollen mainly contains lipids, proteins and amino acids that vary in nutritive value from one species to another one (Hügel, 1962; Roulston *et al.*, 2000; Vanderplanck *et al.*, 2014), whilst nectar principally contains sugars and free amino acids in different proportions (Corbet, 2003; Petanidou *et al.* 2006; Nicolson, 2011).

There are strong relations between bee community and habitat. Populations of polylectic bees such as bumblebees require access to various types of habitats and flower foraging spots to survive (Westrich, 1996; Steffan-Derwenter *et al.*, 2002; Potts *et al.*, 2003, 2005). Thus, bee species have multi-habitat structuring of their community and species seek for complementarity in their foraging area (Tscharntke & Brandl, 2004). Many human actions can directly and indirectly affect the bee community. A mixed diet may therefore be essential for harvesting sufficient quantities of nutrients required as well as polylectic bees may need pollen from several sources (Herbert *et al.*, 1970; Sigsgaard *et al.*, 2001; Patt *et al.*, 2003), even if bees have a preference for visiting common forms of flower in the

environment through flower constancy mechanisms (Waser, 1986; Goulson & Wright, 1998; Chittka *et al.*, 1999). Pollinator services are required for many agricultural monocultures, where pollinators receive pollen and nectar rewards for their services (Rands & Whitney, 2010). These monocultures may only be present in the environment of pollinators for a small portion of the year, as for example about 4 weeks for oilseed rape flowers (Diepenbrock, 2000; Rands & Whitney, 2010). Moreover, bumblebees are reported as showing 'neophobia' to novel flowers that they have not encountered before (Forrest & Thompson, 2009). This suggests that bumblebees are likely to show density dependence (the positive correlation between population density and individual fitness) for common flowers within their environment (Smithson & MacNair, 1996, 1997) and that the end of flowering of a crop can be a stressor for them. The agroecosystems represent large homogenized spaces in which monoculture effects could be magnified if flowering period ties in with a critical developmental phase in the life history of pollinators (Diepenbrock, 2000; Rands & Whitney, 2010).

Among mass flowering crops, bees face floral emptiness that can severely affect their fitness or their diversity (Potts *et al.*, 2010). It is well established that the foraging behavior of polyleptic bees is affected by the density of flower types and show some sort of density-dependence choice behavior (Greenwood & Elton, 1979; Smithson & MacHair, 1997). Moreover, the lower density cover and diversity of flowering plant is positively related to bee diversity (Hole *et al.*, 2005). The different species of *Bombus* forage at different scale (Westphal *et al.*, 2006) and many bee species forage over small distances up to several hundred meters from their nest (Walter-Hellwig & Frankl, 2000; Knight *et al.*, 2005; Greenleaf *et al.*, 2007), which could potentially be affected by the resource availability within foraging range from the nest (Knight *et al.*, 2009). Consequently, a careful consideration can be given to the organization of fields margin to maintain wild flower and pollinator population within landscape (Rands & Whitney, 2010) as it can remain to be the only nutrient source available in agroecosytems after the end of a massive crop flowering.

The managing of field margin can offer a means of reducing the impact of agricultural monocultures within intensively managed environments. Fields margins provide a separation in agroecosystems by providing semi-managed area of uncultivated land around field edges (Marshall & Moonen, 2002), and can provide a refuge for wild plants and

pollinators and enhance the pollination service within the monoculture (Rands & Whitney, 2010). The foraging behavior of bumblebees can be affected by the potential floral resource quality (Osborne *et al.*, 2008) and field margins can represent a significant source of nutrients among massive crops or a refuge in lack flowering periods.

ii. Would bumblebees change their foraging behavior to satisfy only their nutrient needs and consequently provide a different food quality to the colony?

Bumblebees are exposed to neonicotinoids by foraging contaminated nectar and pollen sources in agroecosystems (Elbert et al., 2008; Blacquière et al., 2012; Laycock et al., 2014). I demonstrated that bumblebee nutrient requirements changed with the exposure with neonicotinoids and that the increasing age of the worker can induce a higher attraction for contaminated food whereas newly emerged bumblebees were deterred.

The preference of adult foragers for low doses of imidacloprid (Chapter 4) gives rise to concerns about any foraging behavior alteration possibility. Adult foragers might change their foraging behavior to favor contaminated food sources and their own new nutritional requirements. Moreover, the exposure to sub-lethal doses of neonicotinoids has also an impact on bee learning and memory capacities, navigation abilities and a significantly reduced foraging activity (Belzunces *et al.*, 2012; Gill *et al.*, 2012). Thus, the presence of a large amount of contaminated food source and a change in foraging behavior can have a deep impact on the whole colony.

iii. Synergy of neonicotinoids with the other allelochemicals encountered in the countryside

The assessment of the toxicity of individual pesticides on bees is routinely considered. However, few data have been generated for realistic mixtures of neonicotinoids and fungicides or other pesticides with regard to exposure levels used. The potential exposure to multiple pesticide sources is not limited to combinations of sprayed products, but also to systemic seed treatments that lead to residues in pollen, nectar and guttation fluids (Thompson *et al.*, 2014). Direct or residual contacts to these substances have been

reported to act synergistically on the metabolism of bees and may explain the annual colony loss observed (Smith *et al.*, 2013).

Triazole fungicides are some of the most widely used in the world (Fishel, 2005). The exposure to triazole fungicides has been shown to increase the toxicity of some neonicotinoids several hundred fold and inhibit the P450s involved in bee resistance to pesticides (Iwasa *et al.*, 2004). Further studies showed that the scale of increase toxicity was fungicide dose dependent with a greater synergy of oral toxicity of neonicotinoids such as thiamethoxam (Iwasa *et al.*, 2004; Thompson *et al.*, 2014). This underlines the needs for the use of field realistic exposure levels and routes in studies (Thompson *et al.*, 2014).

iv. Are tests used for pesticide approval fit for purpose?

Pesticides are worldwide used on a large scale in agriculture and some concerns increased for last decades about possible side effects and many countries are implementing the legislation to control the registration of pesticides. This legislation requires risk assessments on human health and environmental impact to be conduced before registration.

In both Europe and USA, the evaluation of side effects on non-target organisms is required for the registration of plant protection products. In the case of pollinators, official guidelines recommend a series of laboratory, semi-field and field studies on honeybees (OECDE guideline 401, 1987; USEPA, 2012). Thus, many insecticides are hazardous to bees and special restrictions or recommendations, which differ among countries, limit their use on crops during bloom. With few exceptions, other pesticides such as herbicides, plant growth regulators and fungicides are considered relatively safe to bees (Atkins *et al.*, 1981; Fell *et al.*, 1983; Mayer & Lunden, 1986; Johansen & Mayer, 1990; Bohmont, 1990; Devilliers, 2002) and their use during bloom is not restricted. Nevertheless, bee losses after fungicides treatments have been reported for honeybees (Brasse, 2001; Oomen, 2001; Fletcher & Barnett, 2003; Rivera *et al.*, 2003) and some larval mortality and malformations in adults were described (Atkins & Kellum, 1986; Mussen, 2003; Thompson, 2003).

The behavior of bumblebees is severely impacted by the exposure to neonicotinoids and their survival to chronic exposure is impaired. These impairments make them more

vulnerable to biotic (predation, disease, parasitism, etc.) and abiotic (climate, other pesticides, etc.) factors. The existing process of homologation has limitations in that it fails to investigate the real risks to the pollinator community, and should therefore be modified in the context of new scientific knowledge. This process should include more parameters such as chronic sub-lethal exposure impacts on life-traits of wild pollinators, restriction of use during crop bloom and daylight and synergic effect with other pesticides present in the countryside.

v. How could we improve agriculture to a more sustainable way?

There are clear evidence to suggest that pollinators are in decline (Biesmeijer *et al.*, 2006, Fitzpatrick *et al.*, 2007; Memmott *et al.*, 2007), and blame for this decline has been laid on a wide range of possible causal factors (Goulson *et al.*, 2008), most of them are associated with intensive agricultural practices. However, it has been argued that we need a greater understanding of how pollinator behavior is affected by agricultural practices in order to counteract some of the underlying problems faced by pollinators (Aizen & Feinsinger, 2003).

As shown in section (i), landscape fragmentation (Rathcke, 1993; Aizen & Feinsinger, 2003), the presence of margins (Rands & Whitney, 2010), and facing floral emptiness (Potts *et al.*, 2010) can affect the pollinator diversity and consequently the pollination service (Kremen *et al.*, 2007). Moreover, the field margins can play a central role in the countryside as they are less disturbed and conserve natural enemies of arthropod pests (Landis *et al.*, 2000; Marshall & Moonen, 2002).

Between 80 and 98% of the active ingredient of seed dressings are lost after sowing and not absorbed by the crop (Sur & Stork, 2003; Tapparo *et al.*, 2012). The comparison of the benefits of integrated pest management (IPM) and seed coating use might be contrasted by other factors. For example, crop yields in soya were indistinguishable between IPM and seed coating, but costs and use of pesticides were much lower in the IPM and the populations of beneficial natural enemies were depressed in treated plots (McCornack & Ragsdale, 2006; Cox *et al.*, 2008; Ohnesorg *et al.*, 2009; Seagraves & Lundgren, 2012). Moreover, yield benefits could be achieved more economically by using foliar insecticides

when it is appropriate (McCornack & Ragsdale, 2006; Johnson *et al*., 2009). These demonstrate that IPM can still represent a good alternative to neonicotinoid seed coating use.

Promoting shorter loops between agricultural production and community- supported agriculture can help to enhance local crop diversity, favor reasoned agriculture, involve people with environment quality and sustainability. Hinrichs (2000) described this system as "a form of resistance and mobilization against socially and environmentally destructive conventional agricultural paradigm" and "where consumers have access to fresh, local produce (usually, but not exclusively organic), while supporting environmentally sound agricultural practices and land use".

IV. Conclusion and recommendations

In conclusion, I refer to an example that reviews potential devices for conserving bumblebees and pollinators in relation with neonicotinoids uses. As suggested by Holzschuh *et al.* (2007 & 2008), the reduction of pesticide use should have a positive impact on density and diversity of bee populations. Urgent action to reverse pollinator decline is being called for (Brown & Paxton, 2009), as pollinator extinctions could have very noticeable effects upon intensive agricultural practices (Klein *et al.*, 2007; Aizen *et al.*, 2009), and could in turn lead to further increase in land use thus putting additional pressures on already fragile ecosystems.

The recommendations of this thesis can be summarized as follows:

- Bumblebees displayed a large range of symptoms that reduced their fitness with chronic exposure to sub-lethal doses of neonicotinoids. Developing new homologation tests that include potential impairments with sub-lethal doses and considering them for the validation of use on the countryside require further investigations to design them for the benefit of broader biodiversity.
- The age-dependent attractiveness of imidacloprid can have a strong impact on the whole colony fitness. Knowledge of potential change about underlying metabolic mechanisms such as taste perception or different nAchRs affinity also requires additional investigations.

- The exposure to neonicotinoids is mostly done through nectar, pollen and exudation of drops of xylem sap. The usage of such systemic pesticides that cannot target specific pests and can be consequently potentially harmful for beneficial insects should be restricted in crops where beneficial insects might be harmed. Moreover, the toxicity of neonicotinoids is well related in numerous studies. Recent discoveries should be taken in consideration to review periodically the appropriate usage of a pesticide.
- In parallel with the reduction in use of such kinds of pesticide, methods to promote bumblebee conservation can be done through alternative farming practice that enhance globally the pollinator diversity and general biodiversity, such as conservation of field margins.

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Appendices

Appendix A

Diet	Diet	Mean Sucrose					Mean Casein				
		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound				Lower Bound	Upper Bound
Casein 1 :400	Casein 1:250	15.361	5.814	0.009	3.892	26.830	0.002	0.075	**0.975**	-0.146	0.151
	Casein 1:180	39.815	6.559	0.000	26.876	52.754	0.033	0.085	**0.696**	-0.134	0.201
	Casein 1:100	31.121	5.814	0.000	19.652	42.590	-0.060	0.075	**0.427**	-0.209	0.088
	Casein 1:75	29.212	7.084	0.000	15.236	43.188	-0.136	0.092	**0.139**	-0.318	0.044
	Casein 1:50	27.691	5.814	0.000	16.222	39.160	-.0275*	0.075	0.000	-0.424	-0.126
	Casein 1:25	24.186	5.814	0.000	12.716	35.655	-.0602*	0.075	0.000	-0.751	-0.453
Casein 1:250	Casein 1:400	-15.361	5.814	0.009	-26.830	-3.892	-0.002	0.075	**0.975**	-0.151	0.146
	Casein 1:180	24.453	5.814	0.000	12.984	35.922	0.031	0.075	**0.682**	-0.118	0.180
	Casein 1:100	15.759	4.958	0.002	5.978	25.540	-0.062	0.064	**0.333**	-0.189	0.064
	Casein 1:75	13.850	6.401	0.032	1.223	26.478	-0.139	0.083	**0.096**	-0.303	0.025
	Casein 1:50	12.329	4.958	0.014	2.548	22.110	-0.278*	0.064	0.000	-0.405	-0.150
	Casein 1:25	8.824	4.958	0.077	-.956	18.605	-0.605*	0.064	0.000	-0.732	-0.477
Casein 1 :180	Casein 1:400	-39.815	6.559	0.000	-52.754	-26.876	-0.033	0.085	**0.696**	-0.201	0.134
	Casein 1:250	-24.453	5.814	0.000	-35.922	-12.984	-0.031	0.075	**0.682**	-0.180	0.118
	Casein 1:100	-8.693	5.814	**0.137**	-20.162	2.775	-0.093	0.075	**0.217**	-0.242	0.055
	Casein 1:75	-10.602	7.084	**0.136**	-24.578	3.373	-0.170	0.092	**0.066**	-0.351	0.011
	Casein 1:50	-12.123	5.814	0.038	-23.593	-0.654	-0.309*	0.075	0.000	-0.458	-0.159
	Casein 1:25	-15.629	5.814	0.008	-27.098	-4.160	-0.636*	0.075	0.000	-0.785	-0.486
Casein 1:100	Casein 1:400	-31.121	5.814	0.000	-42.590	-19.652	0.060	0.075	**0.427**	-0.088	0.209
	Casein 1:250	-15.759	4.958	0.002	-25.540	-5.978	0.062	0.064	**0.333**	-0.064	0.189
	Casein 1:180	8.693	5.814	**0.137**	-2.775	20.162	0.093	0.075	**0.217**	-0.055	0.242
	Casein 1:75	-1.908	6.401	**0.766**	-14.535	10.718	-0.076	0.083	**0.359**	-0.240	0.087
	Casein 1:50	-3.430	4.958	**0.490**	-13.211	6.350	-0.215*	0.064	0.001	-0.342	-0.088
	Casein 1:25	-6.935	4.958	**0.164**	-16.716	2.845	-0.542*	0.064	0.000	-0.669	-0.415
Casein 1:75	Casein 1:400	-29.212	7.084	0.000	-43.188	-15.236	0.136	0.092	**0.139**	-0.044	0.318
	Casein 1:250	-13.850	6.401	0.032	-26.478	-1.223	0.139	0.083	**0.096**	-0.025	0.303
	Casein 1:180	10.602	7.084	**0.136**	-3.373	24.578	0.170	0.092	**0.066**	-0.011	0.351
	Casein 1:100	1.908	6.401	**0.766**	-10.718	14.535	0.076	0.083	**0.359**	-0.087	0.240
	Casein 1:50	-1.521	6.401	**0.812**	-14.148	11.105	-.0138	0.083	**0.097**	-0.303	0.025
	Casein 1:25	-5.026	6.401	**0.433**	-17.653	7.600	-0.465*	0.083	0.000	-0.630	-0.301
Casein 1:50	Casein 1:400	-27.691	5.814	0.000	-39.160	-16.222	0.275*	0.075	0.000	0.126	0.424
	Casein 1:250	-12.329	4.958	0.014	-22.110	-2.548	0.278*	0.064	0.000	0.150	0.405
	Casein 1:180	12.123	5.814	0.038	0.654	23.593	0.309*	0.075	0.000	0.159	0.458
	Casein 1:100	3.430	4.958	**0.490**	-6.350	13.211	0.215*	0.064	0.001	0.088	0.342
	Casein 1:75	1.521	6.401	**0.812**	-11.105	14.148	0.138	0.083	**0.097**	-0.025	0.303
	Casein 1:25	-3.505	4.958	**0.480**	-13.286	6.275	-0.327*	0.064	0.000	-0.454	-0.199
Casein 1:25	Casein 1:400	-24.186	5.814	0.000	-35.655	-12.716	0.602*	0.075	0.000	0.453	0.751
	Casein 1:250	-8.824	4.958	**0.077**	-18.605	0.956	0.605*	0.064	0.000	0.477	0.732
	Casein 1:180	15.629	5.814	0.008	4.160	27.098	0.636*	0.075	0.000	0.486	0.785
	Casein 1:100	6.935	4.958	**0.164**	-2.845	16.716	0.542*	0.064	0.000	0.415	0.669
	Casein 1:75	5.026	6.401	**0.433**	-7.600	17.653	0.465*	0.083	0.000	0.301	0.630
	Casein 1:50	3.505	4.958	**0.480**	-6.275	13.286	0.327*	0.064	0.000	0.199	0.454

Table: Results of pairwise comparisons of repeated-measures ANOVA (LSD) with variable transformed in averages to test the daily casein consumption with different dose of imidacloprid (0, 1, 10 and 100nM) and different diets (Sucrose only and in P:C ratios Casein 1 :400, 250, 180, 100, 75, 50 and 25). The Kaplan-Meier analysis measures the fraction of subjects living for a certain amount of time after treatment. For the number of specimen per run, please refer to Table 1.1 in Material and Methods of Chapter 1.

Appendix B

(I) diet	(J) diet	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Casein 1:250	Casein 1:100	-0.03382	0.008461	<0.001	-0.05065	-0.01699
	Casein 1:75	-0.01116	0.010247	**0.279**	-0.03154	0.00922
	Casein 1:50	-0.02788	0.009770	0.005	-0.04732	-0.00845
	Casein 1:25	-0.08432	0.009397	<0.001	-0.10302	-0.06563
Casein 1:100	Casein 1:250	0.03382	0.008461	<0.001	0.01699	0.05065
	Casein 1:75	0.02266	0.010012	0.026	0.00274	0.04258
	Casein 1:50	0.00594	0.009523	**0.535**	-0.01301	0.02488
	Casein 1:25	-0.05050	0.009139	0.000	-0.06868	-0.03232
Casein 1:75	Casein 1:250	0.01116	0.010247	**0.279**	-0.00922	0.03154
	Casein 1:100	-0.02266	0.010012	0.026	-0.04258	-0.00274
	Casein 1:50	-0.01672	0.011140	**0.137**	-0.03888	0.00544
	Casein 1:25	-0.07316	0.010814	<0.001	-0.09468	-0.05165
Casein 1:50	Casein 1:250	0.02788	0.009770	0.005	0.00845	0.04732
	Casein 1:100	-0.00594	0.009523	**0.535**	-0.02488	0.01301
	Casein 1:75	0.01672	0.011140	**0.137**	-0.00544	0.03888
	Casein 1:25	-0.05644	0.010363	<0.001	-0.07706	-0.03582
Casein 1:25	Casein 1:250	0.08432	0.009397	<0.001	0.06563	0.10302
	Casein 1:100	0.05050	0.009139	<0.001	0.03232	0.06868
	Casein 1:75	0.07316	0.010814	<0.001	0.05165	0.09468
	Casein 1:50	0.05644	0.010363	<0.001	0.03582	0.07706

Table 1: **Results of pairwise comparisons of repeated-measures ANOVA (LSD) with variable transformed in averages to test the daily casein consumption with in the control (no imidacloprid), different diets (in P:C ratios Casein 250, 100, 75, 50 and 25) and HT position of the tubes.** For the number of specimen per run, please refer to Table 2.1 in Material and Methods of Chapter 2.

(I) diet	(J) diet	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Casein 1:250	Casein 1:100	-0.00300	0.003545	**0.406**	-0.01028	0.00429
	Casein 1:75	-0.00635	0.003811	**0.108**	-0.01419	0.00148
	Casein 1:50	-0.00847	0.004011	0.044	-0.01672	-0.00023
	Casein 1:25	-0.03701	0.004011	0.000	-0.04526	-0.02877
Casein 1:100	Casein 1:250	0.00300	0.003545	**0.406**	-0.00429	0.01028
	Casein 1:75	-0.00336	0.003700	**0.373**	-0.01096	0.00425
	Casein 1:50	-0.00548	0.003905	**0.173**	-0.01350	0.00255
	Casein 1:25	-0.03402	0.003905	<0.001	-0.04205	-0.02599
Casein 1:75	Casein 1:250	0.00635	0.003811	**0.108**	-0.00148	0.01419
	Casein 1:100	0.00336	0.003700	**0.373**	-0.00425	0.01096
	Casein 1:50	-0.00212	0.004148	**0.614**	-0.01065	0.00641
	Casein 1:25	-0.03066	0.004148	<0.001	-0.03919	-0.02214
Casein 1:50	Casein 1:250	0.00847	0.004011	0.044	0.00023	0.01672
	Casein 1:100	0.00548	0.003905	**0.173**	-0.00255	0.01350
	Casein 1:75	0.00212	0.004148	**0.614**	-0.00641	0.01065
	Casein 1:25	-0.02854	0.004332	<0.001	-0.03745	-0.01964
Casein 1:25	Casein 1:250	0.03701	0.004011	<0.001	0.02877	0.04526
	Casein 1:100	0.03402	0.003905	<0.001	0.02599	0.04205
	Casein 1:75	0.03066	0.004148	<0.001	0.02214	0.03919
	Casein 1:50	0.02854	0.004332	<0.001	0.01964	0.03745

Table 2: **Results of pairwise comparisons of repeated-measures ANOVA (LSD) with variable transformed in averages to test the daily casein consumption with 10nM of imidacloprid, different diets (in P:C ratios Casein 250, 100, 75, 50 and 25) and HT position of the tubes.** For the number of specimen per run, please refer to Table 2.1 in Material and Methods of Chapter 2.

(I) diet	(J) diet	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Casein 1:250	Casein 1:100	-0.02040	0.017207	**0.243**	-0.05518	0.01438
	Casein 1:75	-0.03638	0.017207	0.041	-0.07116	-0.00160
	Casein 1:50	-0.05152	0.017207	0.005	-0.08629	-0.01674
	Casein 1:25	-0.08113	0.016797	<0.001	-0.11508	-0.04718
Casein 1:100	Casein 1:250	0.02040	0.017207	**0.243**	-0.01438	0.05518
	Casein 1:75	-0.01598	0.016693	**0.344**	-0.04972	0.01776
	Casein 1:50	-0.03112	0.016693	**0.070**	-0.06486	0.00262
	Casein 1:25	-0.06073	0.016271	0.001	-0.09361	-0.02784
Casein 1:75	Casein 1:250	0.03638	0.017207	0.041	0.00160	0.07116
	Casein 1:100	0.01598	0.016693	**0.344**	-0.01776	0.04972
	Casein 1:50	-0.01514	0.016693	**0.370**	-0.04888	0.01860
	Casein 1:25	-0.04475	0.016271	0.009	-0.07763	-0.01186
Casein 1:50	Casein 1:250	0.05152	0.017207	0.005	0.01674	0.08629
	Casein 1:100	0.03112	0.016693	**0.070**	-0.00262	0.06486
	Casein 1:75	0.01514	0.016693	**0.370**	-0.01860	0.04888
	Casein 1:25	-0.02961	0.016271	**0.076**	-0.06249	0.00328
Casein 1:25	Casein 1:250	0.08113	0.016797	<0.001	0.04718	0.11508
	Casein 1:100	0.06073	0.016271	0.001	0.02784	0.09361
	Casein 1:75	0.04475	0.016271	0.009	0.01186	0.07763
	Casein 1:50	0.02961	0.016271	**0.076**	-0.00328	0.06249

Table 3: **Results of pairwise comparisons of repeated-measures ANOVA (LSD) with variable transformed in averages to test the daily casein consumption with the control (no imidacloprid), different diets (in P:C ratios Casein 250, 100, 75, 50 and 25) and LT position of the tubes.** For the number of specimen per run, please refer to Table 2.1 in Material and Methods of Chapter 2.

(I) diet	(J) diet	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Casein 1:250	Casein 1:100	-0.01492	0.019164	**0.441**	-0.05362	0.02378
	Casein 1:75	-0.02269	0.019164	**0.243**	-0.06139	0.01601
	Casein 1:50	-0.02425	0.019597	**0.223**	-0.06383	0.01533
	Casein 1:25	-0.05091	0.019164	0.011	-0.08961	-0.01221
Casein 1:100	Casein 1:250	0.01492	0.019164	**0.441**	-0.02378	0.05362
	Casein 1:75	-0.00777	0.017391	**0.657**	-0.04289	0.02735
	Casein 1:50	-0.00933	0.017867	**0.604**	-0.04541	0.02675
	Casein 1:25	-0.03599	0.017391	0.045	-0.07111	-0.00087
Casein 1:75	Casein 1:250	0.02269	0.019164	**0.243**	-0.01601	0.06139
	Casein 1:100	0.00777	0.017391	**0.657**	-0.02735	0.04289
	Casein 1:50	-0.00156	0.017867	**0.931**	-0.03764	0.03452
	Casein 1:25	-0.02822	0.017391	**0.112**	-0.06334	0.00690
Casein 1:50	Casein 1:250	0.02425	0.019597	**0.223**	-0.01533	0.06383
	Casein 1:100	0.00933	0.017867	**0.604**	-0.02675	0.04541
	Casein 1:75	0.00156	0.017867	**0.931**	-0.03452	0.03764
	Casein 1:25	-0.02666	0.017867	**0.143**	-0.06274	0.00943
Casein 1:25	Casein 1:250	0.05091	0.019164	0.011	0.01221	0.08961
	Casein 1:100	0.03599	0.017391	0.045	0.00087	0.07111
	Casein 1:75	0.02822	0.017391	**0.112**	-0.00690	0.06334
	Casein 1:50	0.02666	0.017867	**0.143**	-0.00943	0.06274

Table 4: **Results of pairwise comparisons of repeated-measures ANOVA (LSD) with variable transformed in averages to test the daily casein consumption with 10nM of imidacloprid, different diets (in P:C ratios Casein 250, 100, 75, 50 and 25) and LT position of the tubes.** For the number of specimen per run, please refer to Table 2.1 in Material and Methods of Chapter 2.

Appendix C

		Mean Sucrose					Mean Casein				
Pesticide	Pesticide	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Control	Imidacloprid	10.7873	5.418	0.052	-0.0857	21.6602	0.18	0.071	**0.013**	0.04	0.33
	Thiamethoxam	-1.3241	10.28	0.898	-21.9541	19.3059	-0.01	0.130	0.937	-0.27	0.25
	Clothianidin	-16.4302	5.418	**0.004**	-27.3032	-5.5573	-0.10	0.071	0.159	-0.24	0.04
Imidacloprid 10nM	Control	-10.7873	5.418	0.052	-21.6602	.0857	-0.18	0.071	**0.013**	-0.33	-0.04
	Thiamethoxam	-12.1114	10.56	0.257	-33.3067	9.0840	-0.19	0.131	0.146	-0.46	0.07
	Clothianidin	-27.2175	5.935	**<0.001**	-39.1282	-15.3067	-0.28	0.074	**<0.001**	-0.43	-0.14
Thiamethoxam 10nM	Control	1.3241	10.28	0.898	-19.3059	21.9541	0.01	0.130	0.937	-0.25	0.27
	Imidacloprid	12.1114	10.56	0.257	-9.0840	33.3067	0.19	0.131	0.146	-0.07	0.46
	Clothianidin	-15.1061	10.56	0.159	-36.3014	6.0892	-0.09	0.131	0.492	-0.35	0.17
Clothianidin 10nM	Control	16.4302	5.418	**0.004**	5.5573	27.3032	0.10	0.071	0.159	-0.04	0.24
	Imidacloprid	27.2175	5.935	**<0.001**	15.3067	39.1282	0.28	0.074	**<0.001**	0.14	0.43
	Thiamethoxam	15.1061	10.56	0.159	-6.0892	36.3014	0.09	0.131	0.492	-0.17	0.35

Table 1: **Results of pairwise comparisons of repeated-measures ANOVA (LSD) with variable transformed in averages to test the daily food consumption (± standard error) with different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Casein 1:50 or EAA 1:50).** For the number of specimens per run, please refer to Table 3.1 in Material and Methods in Chapter 3.

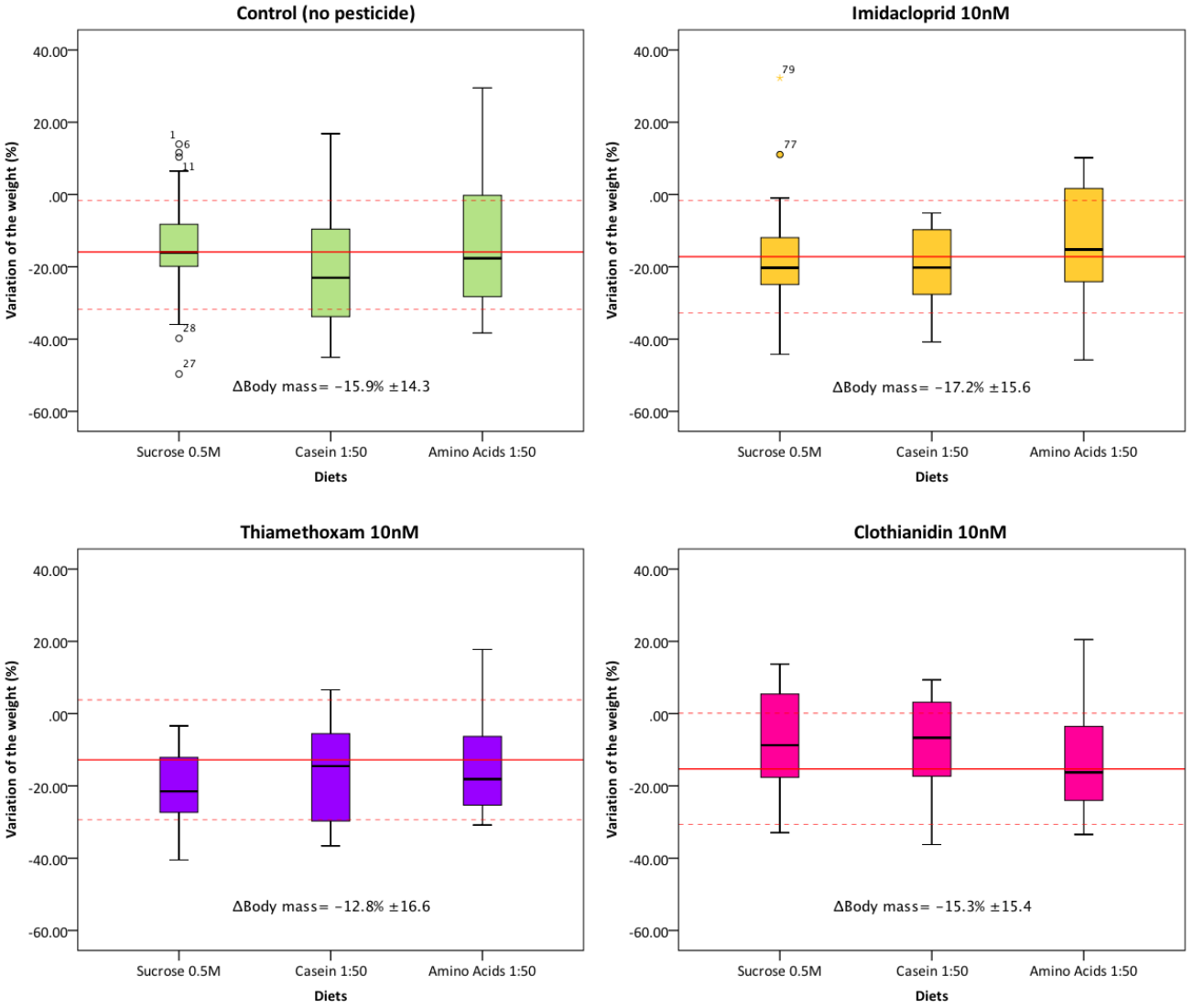

Figure 1: Comparison of the average mass loss of bumblebees over the 7 days depending of different neonicotinoids (no pesticide, imidacloprid, thiamethoxam and clothianidin) and different diets (Sucrose only, Casein 1:50 or EAA 1:50). Lines in bold represent medians; boxes represent 1st and 3rd interquartile ranges, bars represent the minimum and maximum range of the data and open circles represent outliers. The average body mass loss is represented by — and the STDEV of the average body mass loss is represented by - - - . For the number of specimens per run, please refer to Table 3.1 in Material and Methods in Chapter 3.

Appendix D

Article published:

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LETTER

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Bees prefer foods containing neonicotinoid pesticides

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The impact of neonicotinoid insecticides on insect pollinators is highly controversial. Sublethal concentrations alter the behaviour of social bees and reduce survival of entire colonies[1–3]. However, critics argue that the reported negative effects only arise from neonicotinoid concentrations that are greater than those found in the nectar and pollen of pesticide-treated plants[4]. Furthermore, it has been suggested that bees could choose to forage on other available flowers and hence avoid or dilute exposure[4,5]. Here, using a two-choice feeding assay, we show that the honeybee, *Apis mellifera*, and the buff-tailed bumblebee, *Bombus terrestris*, do not avoid nectar-relevant concentrations of three of the most commonly used neonicotinoids, imidacloprid (IMD), thiamethoxam (TMX), and clothianidin (CLO), in food. Moreover, bees of both species prefer to eat more of sucrose solutions laced with IMD or TMX than sucrose alone. Stimulation with IMD, TMX and CLO neither elicited spiking responses from gustatory neurons in the bees' mouthparts, nor inhibited the responses of sucrose-sensitive neurons. Our data indicate that bees cannot taste neonicotinoids and are not repelled by them. Instead, bees preferred solutions containing IMD or TMX, even though the consumption of these pesticides caused them to eat less food overall. This work shows that bees cannot control their exposure to neonicotinoids in food and implies that treating flowering crops with IMD and TMX presents a sizeable hazard to foraging bees.

Determining the impacts of pesticides on pollinators is important to resolve for the future of world food security. Pollinating insects like bees increase the yields of human crops, but in doing so, are inadvertently exposed to pesticides in floral nectar and pollen[6,7]. Several studies have concluded that bees exposed to sublethal doses of neonicotinoid pesticides in food have difficulty learning floral traits, feeding, navigating and foraging[2,3,8–11], and have impaired motor function[12]. These changes in behaviour often lead to colony failure[2,3]. This body of work has galvanized public concern over bee welfare, and in 2013, led to a two-year ban on the use of the three most common neonicotinoids (IMD, TMX, CLO) on flowering crops by the European Union. The agricultural importance of these pesticides has motivated agrochemical producers and government scientists to challenge this ban. Critics of laboratory-based experiments contend that such studies use food laced with neonicotinoid concentrations that exceed the levels found in nectar and pollen[13], or give bees no choice of food solutions[4,5]. They propose that free-living bees and other insect pollinators could choose to avoid the nectar and pollen of pesticide-treated crops[4] if pollinators are repelled by neonicotinoids[14,15], and if alternative sources were provided such as field margins in agricultural settings.

These arguments require that pollinators are able to detect neonicotinoids in food in order to avoid exposure. We tested whether bees avoid sucrose solutions (that is, nectar) containing neonicotinoids using a two-choice test designed to identify the bumblebee's gustatory detection thresholds for nectar toxins[16]. Individual foraging-age worker bumblebees or cohorts of 25 forager honeybees were housed in plastic boxes for 24 h and given access to two types of food tubes: one containing sucrose solution and one containing sucrose solution laced with a specific concentration of the IMD, TMX or CLO. The concentrations used included values in the range reported from nectar and pollen (0.5–150 nM, Extended Data Table 1). Neither bumblebees nor honeybees avoided concentrations found within the naturally occurring range (Fig. 1a, b), even though high concentrations of TMX and CLO reduced their survival (Extended Data Fig. 1). We also tested whether these pesticides inhibited the honeybee's feeding reflex (proboscis extension) or caused honeybees to retract the proboscis once extended[17]. None of the sucrose solutions containing IMD, TMX or CLO affected proboscis extension or retraction (Extended Data Fig. 2).

Unexpectedly, we observed that both bumblebees and honeybees showed a preference for solutions containing IMD or TMX over sucrose alone (Fig. 1, Extended Data Tables 2, 3). Concentrations of IMD and TMX proximate to those found in nectar (1–10 nM, Extended Data Table 1) were most attractive to bumblebees (Fig. 1a), whereas honeybees preferred to consume IMD and TMX across a broader range of concentrations (Fig. 1b). The 'attractive' effect of IMD also depended on bee age: newly emerged adult worker bumblebees and honeybees largely avoided 1–10 nM IMD (Extended Data Fig. 3a). In addition, the presence of neonicotinoids influenced the total amount of food consumed from both tubes during 24 h (Fig. 1c, d). Bumblebees fed with IMD or CLO consumed less total food on average than those fed TMX or the sucrose control (Fig. 1c, Extended Data Table 2); this effect has also been observed by others[11,15]. In contrast, the total food consumption of forager honeybees was reduced only when bees fed from solutions containing 100 nM or 1 μM TMX or CLO (Fig. 2d, Extended Data Table 2). Thus, even in treatments where bees ate considerably less food in 24 h, they still preferred to consume solutions containing IMD over sucrose alone. Bumblebees also consumed 1.5–10-fold more of the neonicotinoid-laced food than honeybees and were, therefore, exposed to higher pesticide doses (Extended Data Table 4).

Insects detect nutrients and toxins in food via gustatory neurons in hair-like sensilla on the proboscis (mouthparts)[18]. Toxic, non-nutritious compounds elicit spikes in 'bitter'-sensing neurons[19,20], but can also be detected via suppression of the responses of sugar-sensing neurons[21,22]. Previous research has established that gustatory neurons located in sensilla on the honeybee's mouthparts are more sensitive to toxins in food[17] than its antennae[21] or tarsi[23]. If bees have mechanisms for detecting neonicotinoids, sensilla on the mouthparts should respond to these substances in the same way they respond to other toxins[17]. To test this, we recorded from gustatory neurons in sensilla on the galea (part of the proboscis) of bumblebees and honeybees using the tip recording technique (Fig. 2a, b). Stimulation with IMD, TMX or CLO in water did not elicit spikes from any of the neurons in the galeal sensilla of either bumblebees (Fig. 2c) or honeybees (Fig. 2d), whereas

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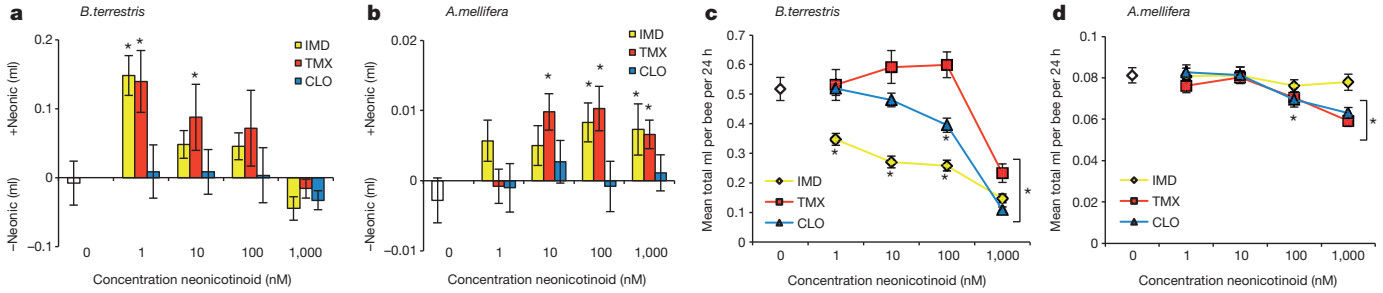

Figure 1 | Foraging-age bees prefer to eat food containing neonicotinoids. **a**, **b**, Bumblebees (**a**) and honeybees (**b**) given a choice of sucrose or sucrose containing a neonicotinoid pesticide chose to eat solutions containing IMD and TMX (Extended Data Table 2, bumblebees: generalized linear model (GLM): $\chi_2^2 = 12.1$, $P = 0.002$; honeybees: GLM, $\chi_2^2 = 11.1$, $P = 0.004$). Data represent the mean difference in the amount consumed over 24 h; positive values indicate a preference for solutions containing neonicotinoids. White bars indicate the sucrose control. Asterisks indicate $P \leq 0.002$ (Bonferroni-adjusted critical value) for one-sample *t*-tests against the '0' value (indicating no preference, see Extended Data Table 3). Sample sizes: bumblebees: IMD: 1 nM = 57, 10 nM = 66, 100 nM = 65, 1 μM = 66; TMX: 1 nM = 38, 10 nM = 39, 100 nM = 36, 1 μM = 40; CLO: 1 nM = 57, 10 nM = 59, 100 nM = 48, 1 μM = 62. Honeybees: $n = 40$ cohorts of 25 bees per treatment. Experiments were replicated with individuals taken from over 20 different bumblebee colonies and 4 honeybee colonies. **c**, The total amount of food eaten from both tubes by bumblebees was affected by the concentration and the presence of a neonicotinoid pesticide (GLM: $\chi_6^2 = 47.7$, $P < 0.001$, Extended Data Table 2) in one of the food tubes. **d**, Honeybees ate less total food only when it contained 1,000 nM TMX or CLO (GLM: $\chi_2^2 = 10.5$, $P = 0.005$, Extended Data Table 2). White diamonds indicate amount eaten by sucrose control group. $^*P < 0.05$ in post hoc comparisons against sucrose. Error bars represent ± s.e.m.

stimulation with nicotine hydrogen tartrate (NHT), KCl and sucrose did (Fig. 2c–f). This effect was the same for all three neonicotinoids in both bee species (Extended Data Table 5). To test whether neonicotinoids are detected via suppression of the neurons' responses to sugars, we applied sucrose solution laced with IMD, TMX and CLO in an ascending series of concentrations from 1 nM to 1 μM (Fig. 2g, h). None of the concentrations we tested altered the spiking activity of sucrose-sensitive gustatory neurons in the bumblebees' or the honeybees' sensilla (Fig. 2g, h, Extended Data Table 5). (Note: we confirmed that the mean spike rates reported in Fig. 2h were not a result of simultaneous excitation of bitter neurons and inhibition of sucrose-sensing neurons by manually spike sorting the records for IMD, Extended Data Fig. 4.) Furthermore, we found that both forager and newly emerged honeybees lack taste neurons that respond to these compounds (Extended Data Fig. 3b). Therefore, the behavioural data and electrophysiological recordings from mouthparts' gustatory neurons lead us to conclude that bumblebees and honeybees cannot taste neonicotinoids in nectar.

The preference of the bees in our assays for solutions containing IMD or TMX probably arises from the pharmacological action of these compounds on nicotinic acetylcholine receptors (nAChRs) in the bees' brains. It does not reflect a generalized enhancement of feeding

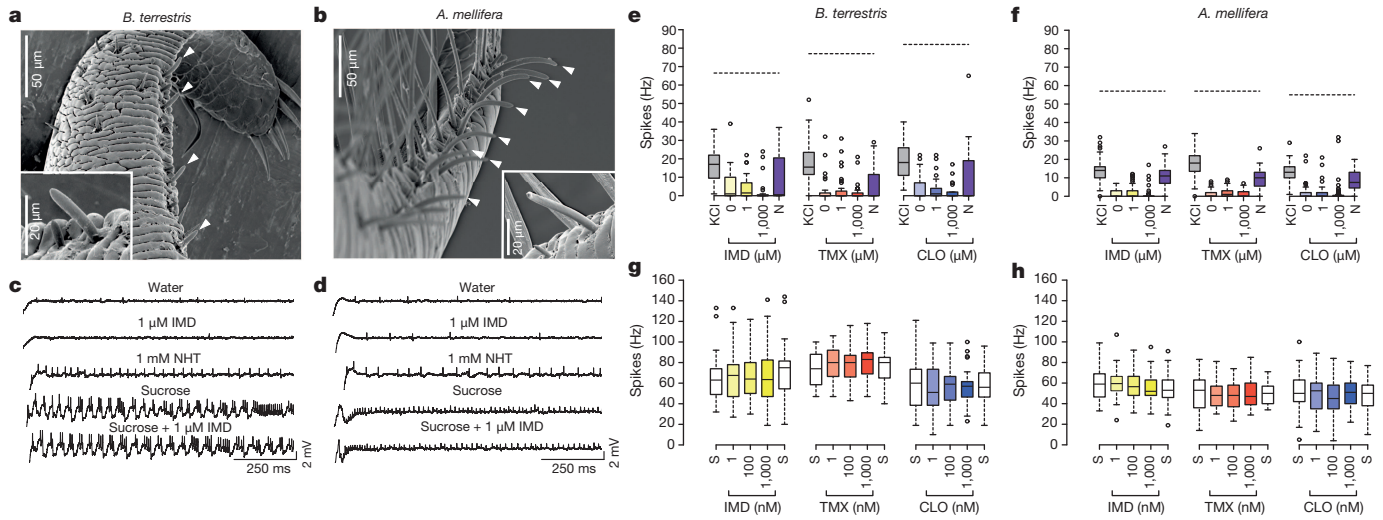

Figure 2 | Electrophysiological recordings of the gustatory receptor neurons from the mouthparts of bumblebees and honeybees during stimulation with neonicotinoids. **a**, **b**, Scanning electron micrographs (SEM) of the galea of bumblebees (**a**) and honeybees (**b**). Recordings were made from the basiconic sensilla of the galea (white arrows); inserts are higher resolution SEM of individual sensilla. **c**, **d**, Spike trains recorded from both species reveal responses to NHT and to sucrose, but not to IMD. **e**, **f**, Boxplots of the spiking responses of gustatory neurons of the mouthparts of bumblebees (**e**) and honeybees (**f**) to KCl, NHT and two concentrations of each of the neonicotinoids. Dashed lines represent the median response to 50 mM sucrose. Solutions of the three neonicotinoids did not elicit activity from gustatory neurons greater than the response to water (indicated as '0' on *x* axis) (Extended Data Table 5, ANOVA: bumblebees: $F_{2,77} = 0.935$, $P = 0.397$; honeybees: $F_{2,144} = 2.38$, $P = 0.096$). (Note: NHT elicited spike frequencies in gustatory neurons greater than those elicited by water in only 11/17 of the bumblebees we tested, whereas NHT elicited spike frequencies greater than water in all of the honeybees tested). Sample sizes: bumblebees: $n_{IMD} = 5$; $n_{TMX} = 7$; $n_{CLO} = 5$. Honeybees: $n_{IMD} = 5$; $n_{TMX} = 5$; $n_{CLO} = 6$. **g**, **h**, The spiking response to sucrose was not reduced by the presence of the neonicotinoids at concentrations in the nectar-relevant range (Extended Data Table 5, ANOVA: bumblebees: $F_{1,86} = 0.579$, $P = 0.449$; honeybees: $F_{1,127} = 2.00$, $P = 0.053$). Bumblebees: $n_{IMD} = 8$; $n_{TMX} = 5$; $n_{CLO} = 6$. Honeybees: $n_{IMD} = 6$; $n_{TMX} = 5$; $n_{CLO} = 6$. Boxplots represent the median (black bars), the 1.5 interquartile range (whiskers) and outliers (circles). Stimuli on *x* axes of **e**–**h** are in order of presentation during the experiment. Bumblebees in both experiments were randomly selected from 8 colonies; honeybees in both experiments were randomly selected from 4 colonies. N, NHT; S, sucrose.

because bees consuming these pesticides ate less food overall. Remarkably, the preference occurred even when bees consuming these solutions were more likely to die. Our data may indicate, therefore, that IMD and TMX affect the neural mechanisms involved in learning about the location of rewarding food. Previous studies have demonstrated that free-flying honeybees prefer to collect sucrose solutions containing low concentrations of nicotine[24]. Nicotine also activates nAChRs[25] expressed throughout the bee brain, including the mushroom bodies required for learning and memory[26,27]. It is notable that several studies have shown that chronic neonicotinoid administration impairs olfactory learning and memory in honeybees[1,8,28,29]. Our finding that bees acquire a preference for food laced with IMD or TMX could be explained by shorter neonicotinoid exposure in our experiments or by differential sensitivity of the nAChRs in the relevant brain regions necessary for each task[26]. It is also plausible that differential sensitivity of nAChRs accounts for our observed avoidance of newly emerged bees towards solutions containing IMD.

Consumption of neonicotinoid-laced nectar by foraging bees could lead to higher attrition in this behavioural caste as well as reducing their foraging efficiency for pollen[2,30]. This would have a greater impact on solitary bee species and on wild bee colonies with relatively few foragers than on honeybee colonies. If foragers prefer to collect nectar containing IMD and TMX, they will also bring more neonicotinoid-laced food back to the colony. For these reasons, whole colonies could be exposed to higher levels of these pesticides in the field than had been predicted previously. Mitigation strategies that rely on planting alternative sources of nectar and pollen, therefore, might not be enough to decrease the risk of poisoning pollinators with pesticides. Instead, long-term changes to policy that include reducing their use may be the only certain means of halting pollinator population decline.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to G.A.W. (jeri.wright@ncl.ac.uk).

METHODS

Behavioural two-choice assays. Experiments were performed at Trinity College, Dublin with *Bombus terrestris dalmatinus* (Unichem Ltd, Co. Dublin, Irish distributor for Koppert). Colonies were maintained at 25–30 °C in 24 h darkness and fed commercial pollen and Biogluc (Agralan Ltd, Swindon) bee food *ad libitum*. Experiments were also performed at Newcastle University, Newcastle upon Tyne with *Bombus terrestris audax* (Biobest, Belgium) and *Bombus terrestris terrestris* (Koppert Biological Systems, NATURPOL, Netherlands). Bees from 3–5 different colonies were used for each neonicotinoid. Individual worker bumblebees were collected as they tried to exit the colony. For the experiments with newly emerged bumblebees, colonies were monitored for newly emerged bees daily; newly emerged adults were identified by their pale colour. These bees were extracted using forceps from within the colony. As previously described in Tiedeken *et al.* (2014)[16], individual bumblebees were cold anaesthetized, weighed and sex-determined, and transferred to individual 650 ml plastic containers (160 × 110 × 45 mm). Containers were fitted with three 3 ml feeding tubes, inserted horizontally. Feeding tubes had four 2 mm holes so bees could alight on the tubes and feed from the openings. The feeding tubes contained one of three solutions: (1) deionized water; (1) 0.5 M sucrose; or (3) 0.5 M sucrose with a specific concentration of a neonicotinoid compound. Whether or not the bee was alive was noted 24 h after the start of the experiment. Bees that did not drink from either tube were excluded from the final analysis; the total number of these subjects was never greater than 3 per treatment (note: these subjects were always dead and likely to have died from stress or other causes).

Experiments with honeybees (*Apis mellifera* var. Buckfast) were performed at Newcastle University during the summer months using 2 free-flying outdoor colonies originally obtained from the UK's National Bee Unit (Sand Hutton, Yorkshire). Foraging adult worker honeybees were collected at the colony entrance as they returned from foraging; newly emerged adult workers were collected from brood comb as they emerged in a purpose-built box kept in an incubator at 34 °C. Bees were cold anaesthetized before placing in rearing boxes. Cohorts of 25 bees were placed in rearing boxes as previously described in Paoli *et al.* (2014)[31]. Five food tubes (as described above) were provided: (1) one with deionized water; (2) two with 1 M sucrose; (3) two with 1 M sucrose containing a specific concentration of a neonicotinoid. The number of bees alive in each cohort was counted at the time of measurement of the food consumption (24 h later).

All of the two-choice experiments were performed experimenter-blind (except IMD with bumblebees). Three neonicotinoid pesticides, imidacloprid (IMD), thiamethoxam (TMX) and clothianidin (CLO), were used in the experiments (Pestanal, Sigma-Aldrich). The neonicotinoid concentrations used were 1 nM, 10 nM, 100 nM, 1 μM (see Extended Data Table 4 for conversions to ppb and ng per bee). Bees were kept in continuous darkness for 24 h at constant temperature and 60% RH (bumblebees: 28 °C; honeybees: 34 °C). Control boxes identical to the experimental boxes (without bees) for each neonicotinoid treatment were placed in the incubator simultaneously with the experiments to measure the rate of evaporation from the food solutions. Feeding tubes were weighed, placed in the experimental boxes with the bees for 24 h, and then removed and weighed a second time. The position of the treatment tubes was randomized across subjects. The amount of solution consumed was determined as the difference in the weight of each tube after 24 h; the average value for the evaporation control for each treatment was subtracted from this final value for each tube. For bumblebees, sample sizes were: IMD: 1 nM = 57, 10 nM = 66, 100 nM = 65, 1 μM = 66; TMX: 1 nM = 38, 10 nM = 39, 100 nM = 36, 1 μM = 40; CLO: 1 nM = 57, 10 nM = 59, 100 nM = 48, 1 μM = 62. For honeybees, $n = 40$ cohorts of 25 bees per treatment. Sample size was chosen as $n \geq 40$ based on previous work[16]; sample size varied because some individuals died from unknown causes at the start of the experiments. No statistical methods were used to predetermine sample size.

Honeybee antennal and mouthparts assays. Honeybees were collected at the entrance of an outdoor colony as they returned from foraging, cold-anaesthetized, and harnessed as described in Bitterman *et al.* (1983)[32]. Each was fed 1 M sucrose to satiety and left overnight in a humidified plastic box and assayed ~ 18 h later. Briefly, two assays were employed: one in which individual honeybees were lightly tapped on the antenna with a stimulating solution (for example, sucrose) to elicit the feeding reflex (that is, proboscis extension reflex, or PER) and a second assay in which a droplet of stimulating solution was placed at the end of the extended proboscis to test whether bees would consume it (further details described in Wright *et al.* 2010[17]). Stimulating solutions were 1 M sucrose containing one of the following concentrations (1 nM, 10 nM, 100 nM, 1 μM, 10 μM) of one of three neonicotinoids (IMD, TMX, CLO).

Electrophysiology. Individual bumblebees (*B. terrestris audax* and *B. terrestris terrestris*) and honeybees were cold-anaesthetized on ice for 3–5 min, and then restrained in a metallic restraining harness as described in Bitterman *et al.* (1983)[32]. To avoid any movements of the mouthparts during recordings, muscles that trigger proboscis retraction were cut by making an incision at the level of the proboscis fossa. Each galea was fixed with a curved metallic wire pinned into dental wax.

Electrophysiological recordings were made from taste neurons located in the first 11 sensilla chaetica[33] located at the tip of the galea on the honeybee's proboscis as in Wright *et al.* (2010)[17] and in the first 6 sensilla in bumblebees. Bees were electrically grounded via a chlorinated silver wire inserted into the head. Sensilla were visualized under a microscope (M205C, Leica, Germany) at a magnification of ×256. To record from gustatory neurons, we used a method first described by Hodgson *et al.* (1955)[34]. Sensilla were stimulated with a recording borosilicate electrode (50 mm long, 20 μm diameter) containing the test compounds diluted in demineralized water. The recording electrode was connected via a chlorinated sliver wire to a high impedance 'non-blocking' pre-amplifier (TastePROBE, Syntech, Germany)[35] mounted on a motorized micromanipulator (MPC-200, Sutter Instrument, USA). The signal was further amplified and filtered with an AC amplifier (model 1800, gain: 100×, band-pass filter: 10–1,000 Hz, A-M Systems, USA). Each stimulus trial was digitized (sampling rate 10 kHz, 16 bits; DT9803 Data Translation), stored on a computer with dbWave software (version 4.2014.3.22) and analysed with Matlab R2012b (version 8.0.0.783) using PeakFinder with fixed thresholds as the peak detection algorithm (PeakFinder.m., Mathworks file ID: 25500). Recordings were made for 2 s, but only data for the first second were included in the analysis. The first 100 ms were removed to avoid the contact artefact. For bumblebees, 2–6 sensilla were sampled per bee; for honeybees, 6–10 sensilla were sampled per bee.

Recording started when the open end of the electrode was placed over the tip of the sensillum. Individuals were repeatedly sampled in one of two protocols: (1) 50 mM sucrose, 100 mM KCl, water, 1 μM neonicotinoid, 1 mM neonicotinoid, 1 mM NHT, 100 mM KCl, 50 mM sucrose; or (2) 50 mM sucrose, 50 mM sucrose + neonicotinoid in one of the following concentrations (1 nM, 10 nM, 1 μM), 50 mM sucrose. The neonicotinoids IMD, TMX, or CLO were used in each protocol. Neonicotinoid (Pestanal, Sigma-Aldrich) solutions were prepared as serial dilutions starting with 1 mM concentration. Sucrose and nicotine tartrate were purchased from Sigma-Aldrich and KCl from Fisher Scientific at purity ≥ 98%. Demineralized water was used to prepare all solutions. Intervals between stimuli were 2–5 min.

Recordings with IMD diluted in sucrose (Extended Data Fig. 4) were further analysed using dbWave (http://perso.numericable.fr/frederic.marion-poll/deterrents/tk/dbwave/index.htm). Predicted spiking neurons or 'units' were sorted from the digitally filtered signals according to their amplitude with the help of interactive software procedures. Electrophysiological recordings were then visually inspected to search for spike doublets, that is, two spikes separated by an interspike interval shorter than the silent period[36,37]. Spike trains were analysed over 1 s following the first 100 ms removed to avoid the contact artefact.

Electron microscopy. Scanning electron microscopy was performed using a Cambridge Stereoscan 240 on samples that had been fixed with glutaraldehyde, washed in phosphate buffer then dehydrated through an ethanol gradient followed by critical point drying. Specimens were then mounted on an aluminium stub with Acheson's silver dag before gold coating with a Polaron SEM coating unit.

Statistics. All analyses were performed using IBM SPSS v 19. The mean total number of spikes in the electrophysiological recordings was analysed using repeated-measures analysis of variance (ANOVA) for each species with neonicotinoid as a main effect, sensillum number and bee as covariates, and stimulus as a repeated measure; a Levene's test was employed to test for equality of variance. Post hoc comparisons were pairwise *t*-tests with a Bonferroni adjustment for experiment-wise error rate. A two-way generalized linear model (GLM) was used to compare the behaviour of bees fed each of the neonicotinoid treatments for each bee species with least squares post hoc comparisons (Note: the sucrose-sucrose choice data were not included because of the requirements of GLM for factorial design). The difference in the amount eaten between the 2 food tubes in the behavioural choice assays was also analysed using a one-sample *t*-test against zero for each treatment; critical values were Bonferroni-adjusted. The proportion of bees alive after 24 h was analysed using logistic regression (lreg). Each individual bee was entered in the analysis for the experiments with bumblebees and with honeybees. For the analysis with honeybees, 'cohort' was entered as a covariate. No statistical methods were used to predetermine sample size.

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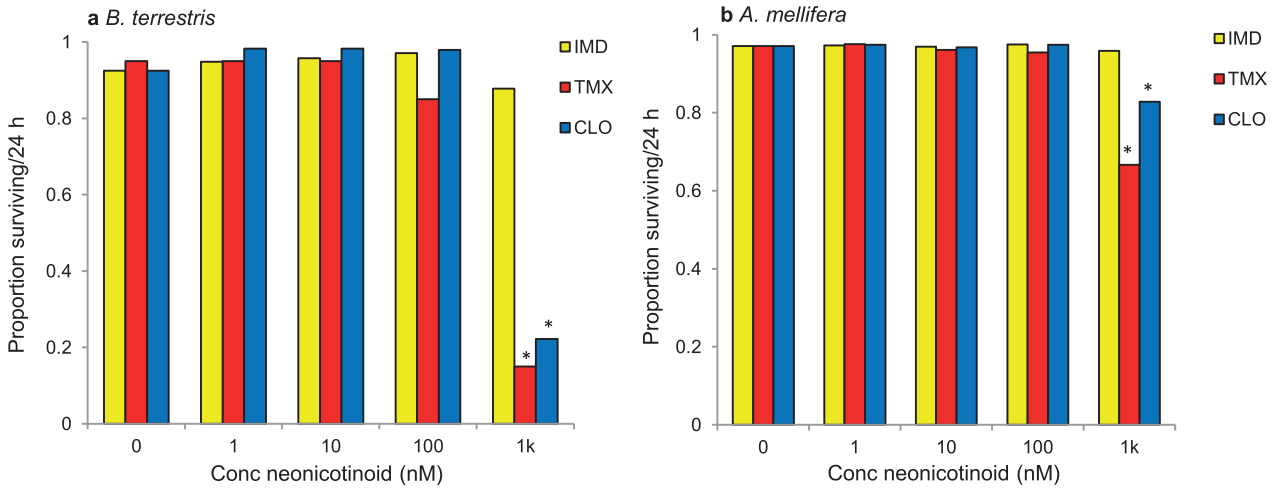
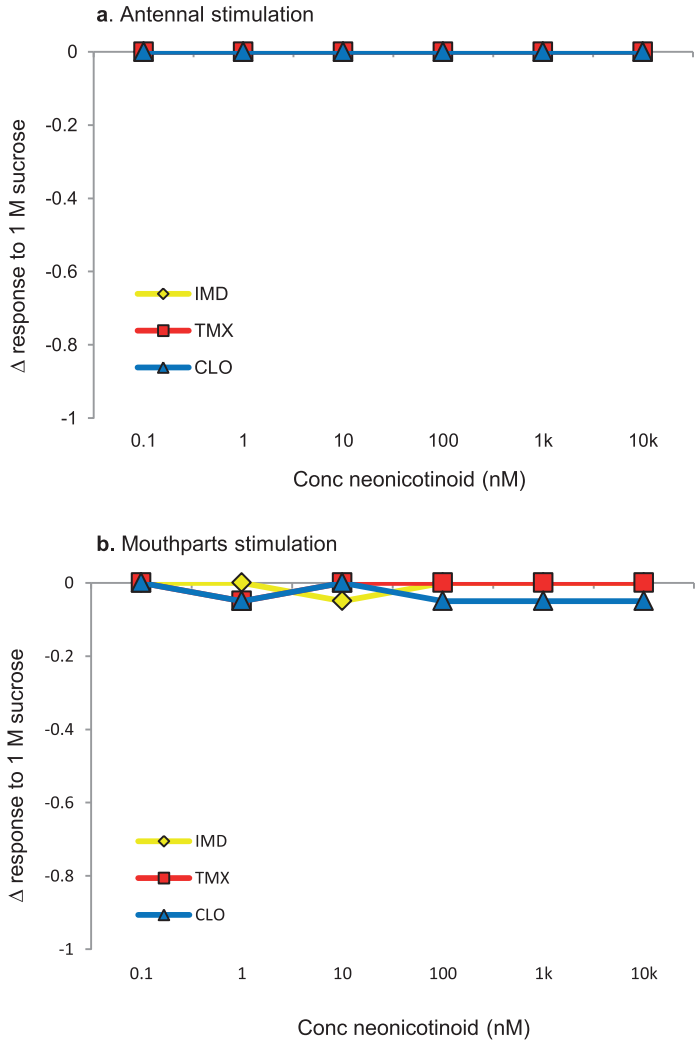
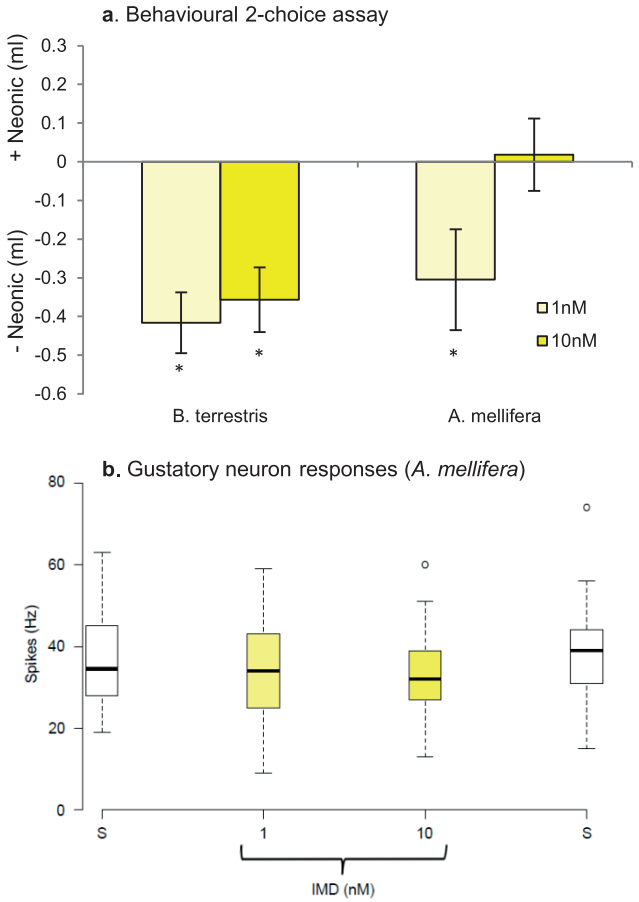


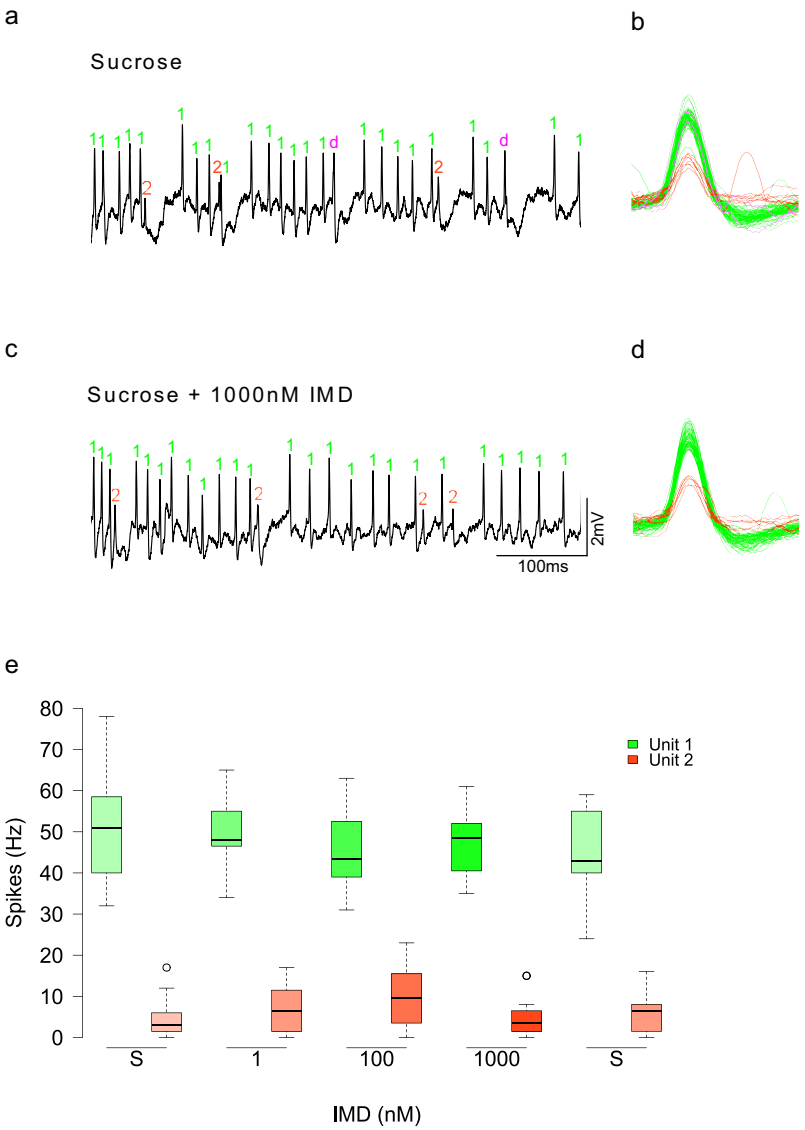
Extended Data Figure 1 | The proportion of bees surviving after 24 h in the two-choice assay. Data from Fig. 1. **a**, Bumblebees given a choice between sucrose and sucrose laced with 1,000 nM TMX or CLO were less likely to survive after 24 h (lreg: IMD: $\chi_4^2 = 4.36$, $P = 0.359$; TMX: $\chi_4^2 = 62.3$, $P < 0.001$; CLO: $\chi_4^2 = 79.7$, $P < 0.001$). **b**, Honeybees given a choice between sucrose and sucrose laced with 1,000 nM TMX or CLO were less likely to survive after 24 h (lreg: IMD: $\chi_4^2 = 5.18$, $P = 0.269$; TMX: $\chi_4^2 = 577$, $P < 0.001$; CLO: $\chi_4^2 = 243$, $P < 0.001$). Cohort (cov) accounted for a significant portion of the variance in survival for all three treatment groups (lreg: IMD: $\chi_1^2 = 22.0$, $P < 0.001$; TMX: $\chi_1^2 = 32.4$, $P < 0.001$; CLO: $\chi_1^2 = 70.2$, $P < 0.001$). Sample sizes are the same as in Fig. 1. $^*P < 0.05$ in least squares post hoc comparisons against sucrose in each treatment



Extended Data Figure 2 | Antennal proboscis extension response (PER) and mouthparts assay of honeybees to solutions containing neonicotinoids. **a**, Stimulation of the antennae with 1 M sucrose solutions containing neonicotinoids did not affect the elicitation of PER. **b**, Honeybees did not refuse to consume solutions containing neonicotinoids; only one bee in the CLO treatments failed to drink the solutions. $n = 40$ per neonicotinoid treatment for antennal stimuli and $n = 10$ for each concentration of each neonicotinoid for the mouthparts taste assay. Bees were randomly selected from 2 colonies.



Extended Data Figure 3 | Young bees avoid solutions containing neonicotinoids. **a**, Newly emerged worker bumblebees ($n = 30$ bees per treatment) and honeybees ($n = 20$ boxes per treatment) were tested in the behavioural choice assay with 1 nM and 10 nM IMD in sucrose solution as in Fig. 1. Bumblebees avoided consuming both solutions containing IMD (one-sample t-test against 0, 1 nM: $P < 0.001$, 10 nM: $P = 0.001$), whereas honeybees avoided only the 1 nM concentration (one-sample t-test against 0, 1 nM: $P = 0.003$, 10 nM: $P = 0.773$). Error bars represent ± s.e.m. **b**, The presence of IMD did not alter the spike frequency of gustatory neurons in the galeal sensilla of newly emerged honeybees (repeated-measures ANOVA, stimulus: $F_{1,47} = 0.207$, $P = 0.653$). Recordings were made from the basiconic sensilla on the galea as in Fig. 2. Boxplots represent the frequencies of responses to 50 mM sucrose or to 50 mM sucrose solutions containing 1 nM or 10 nM IMD. $n = 5$ bees, 10 sensilla per bee. Boxplots represent the median (black bars), the 1.5 interquartile range (whiskers) and outliers (circles). Stimuli on x axis are in order of presentation during the experiment.



Extended Data Figure 4 | Spike-sorted recordings. Data from four of the honeybees in Fig. 2h. **a**, To verify that the spike rates we observed in Fig. 2h were not a result in changes in the rates of firing of individual neurons, we spike-sorted recordings from four honeybees stimulated with sucrose and IMD. **b**, Spike sorting revealed two potential spiking neurons (units) characterized by different spike amplitudes; both units spiked in response to sucrose stimulation. (This was also observed previously by Wright *et al.* 2010[17]). One neuron is labelled in green, the other in red. Spike doublets (indicated in pink as 'd') where both neurons spiked nearly simultaneously were also observed. **c**, **d**, These same two spiking neurons continued to respond when stimulated with sucrose containing 1 μM IMD. **e**, Boxplots reveal that the rate of spiking was lower on average for one of the neurons (repeated-measures ANOVA, unit: $F_{1,36} = 596$, $P < 0.001$). The rate of firing of both neurons was not affected by IMD concentration (repeated-measures ANOVA, unit: $F_{1,36} = 0.369$, $P = 0.547$). Spikes from additional neurons (units) were not detected, and so we concluded that no other neurons were recruited during stimulation with IMD. 'S' indicates stimulation with sucrose. Boxplots represent the median (black bars), the 1.5 interquartile range (whiskers) and outliers (circles). Stimuli on *x* axis are in order of presentation during the experiment.

Extended Data Table 1 | Concentrations of neonicotinoids reported in floral nectar

Source	Imidacloprid			Thiamethoxam			Clothianidin		
	ng/g	PPB	nM	ng/g	PPB	nM	ng/g	PPB	nM
Schmuck et al. 2001[7]	1.9	1.9	7.43	-	-	-	-	-	-
Pohorecka et al. 2012[38]	0.6	0.6	2.34	4.2	4.2	14	2.3	2.3	9.2
Dively and Kamel 2012[6]	0.4-11	0.4-11	1.5-43	8.2-9.5	8.2-9.5	28-37	-	-	-
Stoner and Eitzer 2012[39]	10	10	39	11	11	37	-	-	-
Byrne et al. 2013[40]	2.9-39	2.9-39	11-154	-	-	-	-	-	-
Larson et al. 2013[41]	-	-	-	-	-	-	171	171	684
Pilling et al. 2013[42]	-	-	-	0.65-2.4	0.65-2.4	2.2-8.2	-	-	-
Defra 2013[43]	0.13	-	0.5	1-3.9	1-3.9	3.4-13	0.18-4	0.18-4	0.7-16

References 38–43 are cited in this table.

Extended Data Table 2 | Generalized linear models for the neonicotinoid choice experiment and total food consumption

B. terrestris	**Choice test**			**Total food consumption**		
Between-subjects contrasts	**df**	**χ^2**	**P-value**	**df**	**χ^2**	**P-value**
Concentration	3	27.9	**<0.001**	3	263	<0.001
Neonicotinoid	2	12.1	**0.002**	2	150	<0.001
Neonic x Conc	6	7.97	0.240	6	47.7	**<0.001**
A. mellifera	**Choice test**			**Total food consumption**		
Between-subjects contrasts	**df**	**χ^2**	**P-value**	**df**	**χ^2**	**P-value**
Concentration	3	4.93	0.176	3	37.1	**<0.001**
Neonicotinoid	2	11.1	**0.004**	2	10.5	**0.005**
Neonic x Conc	6	5.89	0.435	6	11.4	0.076

Data from Fig. 1. Values in bold indicate interpreted model parameters. Note: sucrose–sucrose (control) data were not included.

Extended Data Table 3 | One-sample *t*-tests against '0' for each treatment of the 24 h behavioural assay

B. terrestris									
		IMD			TMX			CLO	
	N	t(df)	P-value	N	t(df)	P-value	N	t(df)	P-value
Sucrose	55	-0.24(54)	0.402						
1nM	57	5.13(56)	**<0.001***	38	3.11(38)	**0.002***	57	0.22(56)	0.246
10nM	66	2.39(65)	**0.010**	39	3.11(37)	**0.002***	59	0.26(58)	0.183
100nM	65	2.33(64)	**0.012**	36	1.31(35)	0.099	48	0.09(47)	0.465
1µM	66	-2.6(65)	**0.005**	40	-1.15(39)	0.128	62	-2.36(61)	**0.021**
A. mellifera									
		IMD			TMX			CLO	
	N	t(df)	P-value	N	t(df)	P-value	N	t(df)	P-value
Sucrose	40	-0.85(39)	0.199						
1nM	40	1.93(39)	**0.031**	40	-0.32(39)	0.376	40	-0.288	0.387
10nM	40	1.75(39)	**0.044**	40	3.80(39)	**<0.001***	40	0.882	0.191
100nM	40	2.97(39)	**0.002***	40	3.23(39)	**0.001***	40	-0.221	0.414
1µM	40	2.00(39)	**0.026**	40	3.25(39)	**0.001***	40	0.423	0.337

Data from Fig. 1. *P* values are for 1-tailed tests. *P* values in bold are below $P = 0.05$. *Application of a Bonferroni adjustment criterion alters the *P* value threshold from $P = 0.05$ to $P = 0.002$.

Extended Data Table 4 | Comparison of doses consumed by each bee species for each treatment

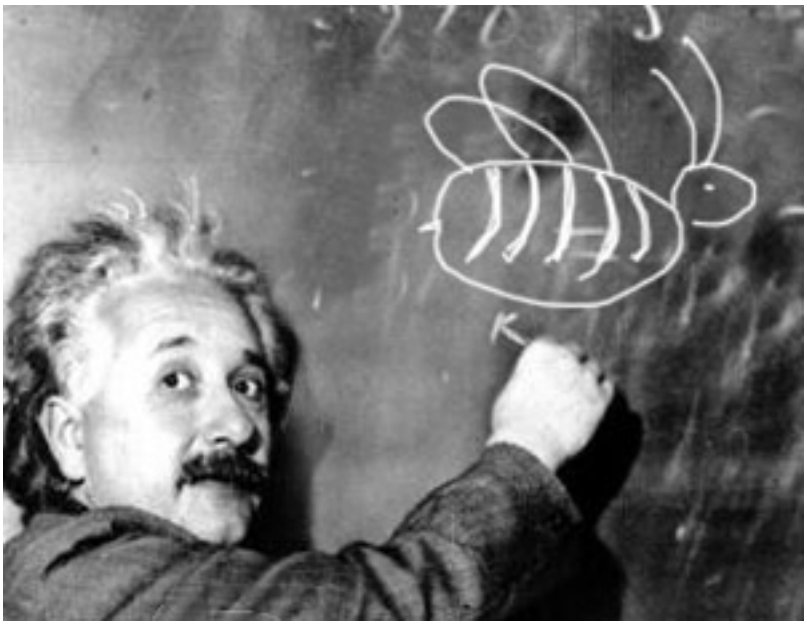
B. terrestris												
	1nM			10 nM			100 nM			1μM		
	ml/bee	PPB	ng/bee/24 h	ml/bee	PPB	ng/bee/24 h	ml/bee	PPB	ng/bee/24 h	ml/bee	PPB	ng/bee/24 h
IMD	0.257	0.256	0.064(0.043)	0.167	2.56	0.418(0.337)	0.159	25.6	3.98(3.22)	0.055	256	13.9(18.4)
TMX	0.360	0.292	0.105(0.077)	0.357	2.92	1.05(0.862)	0.354	29.2	10.3(8.74)	0.115	292	33.6(33.9)
CLO	0.279	0.250	0.070(0.065)	0.259	2.50	0.647(0.600)	0.211	25.0	5.28(4.93)	0.041	250	10.3(13.6)
A. mellifera												
	1nM			10 nM			100 nM			1μM		
	ml/bee	PPB	ng/bee/24 h	ml/bee	PPB	ng/bee/24 h	ml/bee	PPB	ng/bee/24 h	ml/bee	PPB	ng/bee/24 h
IMD	0.046	0.256	0.012(0.010)	0.046	2.56	0.118(0.103)	0.045	25.6	1.16(0.974)	0.045	256	11.7(9.95)
TMX	0.040	0.292	0.012(0.011)	0.048	2.92	0.141(0.117)	0.036	29.2	1.07(1.02)	0.035	292	10.3(8.63)
CLO	0.043	0.250	0.011(0.010)	0.044	2.50	0.112(0.101)	0.043	25.0	1.08(0.868)	0.034	250	8.51(7.86)

Data from Fig. 1. Note: ng/bee values were calculated based on the mean values consumed from the neonicotinoid-containing food tubes for each treatment (ml/bee). This calculation is the product of the ng/μl of neonicotinoid in the food solution and the amount of solution eaten (μl) per bee in 24 h. The values in parentheses in the ng/bee/24 h column are the expected values if bees had eaten from both tubes equally. This value was calculated by dividing the total amount eaten for each treatment in Fig. 1c and d by 2 and using this quantity to estimate the dose.

Extended Data Table 5 | Repeated-measures ANOVA

B. terrestris	Water			Sucrose solution		
Within subjects contrasts	**df**	**F**	**P-value**	**df**	**F**	**P-value**
Stimulus	1	8.60	0.004	1	0.579	0.449
Stimulus x bee (cov)	1	4.45	0.038	1	1.23	0.271
Stimulus x sensillum (cov)	1	0.038	0.846	1	0.558	0.458
Stimulus x neonicotinoid	2	0.935	0.397	2	0.287	0.752
Error(stim)	77			86		
Between subjects contrasts	**df**	**F**	**P-value**	**df**	**F**	**P-value**
Neonicotinoid	2	10.2	0.937	2	0.004	0.996
Bee (cov)	1	0.164	0.686	1	0.871	0.354
Sensillum (cov)	1	5.63	0.020	1	3.35	0.071
Error	77			86		
A. mellifera	Water			Sucrose solution		
Within subjects contrasts	**df**	**F**	**P-value**	**df**	**F**	**P-value**
Stimulus	1	95.6	<0.001	1	7.47	0.007
Stimulus x bee (cov)	1	4.20	0.042	1	5.31	0.023
Stimulus x sensillum (cov)	1	0.303	0.583	1	0.142	0.707
Stimulus x neonicotinoid	2	2.38	0.096	2	3.00	0.053
Error(stim)	144			127		
Between subjects contrasts	**df**	**F**	**P-value**	**df**	**F**	**P-value**
Neonicotinoid	2	1.23	0.295	2	6.70	0.002
Bee (cov)	1	0.335	0.563	1	1.67	0.198
Sensillum (cov)	1	1.37	0.244	1	12.6	0.001
Error	144			127		

Data from Fig. 2. Note: for 'Water' model, the stimulus variable included: sucrose, KCl, nicotine, water, 1 μM, and 1 mM neonicotinoid. For the 'sucrose solution' model, the stimulus variable included: sucrose, 1 nM, 100 nM, and 1 μM neonicotinoid. The significant 'stimulus × neonicotinoid' term in the sucrose solution experiment for honeybees reflects a slight adaptive effect that occurred in the experiments with IMD, but not with TMX or CLO. Pairwise comparisons of each stimulus applied in the IMD experiment revealed that the 1 μM IMD and the final sucrose control stimulus produced fewer spikes than the first sucrose stimulus ($P = 0.024$ and $P = 0.002$). However, the 1 μM IMD and the final sucrose stimulus were not significantly different ($P = 0.546$) indicating either that the neurons in these experiments exhibited a slight adaptation effect or that the 1 μM IMD concentration had a toxic effect that influenced the integrity of their responses to sucrose.



Prof. Albert Einstein during an epic Pictionary game at the 1st Bee Symposium of Newcastle University